Microbial Biotechnology

Volume 2
Application in Food and Pharmacology

Jayanta Kumar Patra
Gitishree Das
Han-Seung Shin *Editors*

Springer

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Preface

Microbial biotechnology is an interdisciplinary field, and successful development in this field requires major contributions in a broad range of disciplines encompassing specialties as diverse as biochemistry, cell biology, genetics, taxonomy, pathogenic bacteriology, pharmaceutical microbiology, food and industrial microbiology, and ecology. The book *Microbial Biotechnology: Application in Food and Pharmacology – Volume 2* is a collection of articles highlighting the interdisciplinary scope and recent developments of microbial biotechnology in the areas of food and pharmacology. The book envisages the recent ideas of novel findings in microbiology and provides insights into the various interdisciplinary research avenues.

The book contains a total of 24 chapters and is divided into two major sections: (a) application of microbial technology in food and (b) application of microbial technology in pharmacology. These sections cover the recent developments in the applications of microorganism in various fields like food and pharmacology. Section (a) covers some of the emerging areas like the recent advanced food preservation techniques, metagenomic approaches in food and pharmaceutical industries, uses of probiotics, chitinous bioresources, seaweed fertilizers, green algae as food, enzymatic abatement of urethane in fermentation of beverages, bioethanol production, pesticides, microbes in drought tolerance, agrochemicals, etc. Similarly Section (b) covers some of the interesting applications of microbial technology in pharmaceutical industries. Chapters like application of oncolytic viruses in cancer treatment, microbe-based metallic nanoparticles, etc., are based on more advanced technologies used in microbial technology. Besides, there are also chapters on endophytes, probiotic biosurfactants, antibiotics in dental managements, etc.

The book is written in a simple and clear text with a number of figures, tables, and illustrations that will help the reader to better understand the subject matter. This volume is the collective efforts of several researchers, scientists, graduate students, and postdoctoral fellows across the world who are well known in their respective areas of specialization. Overall, this book will serve as a suitable reference for students, researchers, scientists, industries, and government agencies interested in the field of microbial biotechnology, pharmacology, food technology, and all disciplines related to microbial biotechnology.

We express our gratitude to all of the contributing authors who helped us enormously with their contributions in this volume. The editors thank all of them for their time, critical thoughts, and suggestions that enabled us to put together this peer-reviewed edited volume. The editors are also thankful to Springer Nature Singapore Pte Ltd., Singapore, and their team members particularly Dr. Sue Lee, Associate Editor Biomedicine, Springer Nature, South Korea, for giving us the opportunity to publish this volume. Lastly, we thank our family members for their love, support, encouragement, and patience during the entire period of this work.

Goyang-si, South Korea

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Part I
Application of Microbial Technology in Food

Chapter 1
Recent Food Preservation Techniques Employed in the Food Industry

Shiny Shajil, Andrea Mary, and C. Elizabeth Rani Juneius

1.1 Introduction

Food is an organic substance when consumed provides human body with the required energy. Food contains carbon and hydrogen molecules and degenerates over time and is considered mostly organic. Food once exposed to air undergoes reaction which is normally referred to as degeneration.

Researchers have witnessed that, if left unattended, food provides an impeccable breeding site for micro-organisms. These micro-organisms include bacteria and fungi produce toxins as they multiply while feeding on the food. This, if consumed can lead to fatal cases of diarrhea and food poisoning. It is due to this degeneration that people have come up with means of food preservation to make sure food lasts longer. Food preservation involves the delaying of the onset of multiplication of these micro-organisms. Food preservation can be defined as a process through which food is prevented from getting spoilt for a long time. The nutritive value, taste and color of the food are preserved during this process. (The National Institute of Open Schooling (NIOS)).

Throughout the ages, man has been faced with the problem of preserving food so as to carry over supplies from times of plenty to those of want (Table 1.1). As conditions of living changed, the driving force prompting them to seek out new methods of preservation was to have the ability to transport food unimpaired from country to town and finally to present it in a form that could be prepared with ease and eaten with safety, benefit and enjoyment (Ananou et al. 2007).

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Table 1.1 Major sources of food spoilage and poisoning that are targets for the use of food preservatives

Causes of quality loss of food	
Physical	Change in the texture, evaporation, induction of damage by repeated freezing and thawing
Enzymatic	Proteolysis, lipolytic rancidity, enzymatic browning reaction
Chemical reactions	Oxidative rancidity,
	Loss of colour, non-enzymatic non maillard browning reactions
Microbiological	Food poisoning and spoilage

Table 1.2 Major methods of food preservation

Method	Mode of action
Refrigeration	Retardation of growth by low temperature
Freezing	Reduction of water activity preventing microbial growth, slowing of oxidation reactions
Drying, curing and conserving	Reduction in water activity to prevent microbial growth
Smoking	Anti-oxidant and anti-microbial
Salting and pickling	Salt kills and inhibits growth of microorganisms at 20% concentration
Lye/sodium hydroxide	Increasing the intracellular pH of micro-organisms
Sugar preservation	Osmotic pressure
Ethanol preservation	Toxic inhibition of microbes.
Emulsification	Compartmentalization/nutrient limitation within the aqueous droplets

People over time have leant on better ways of keeping their food safe. The historical perspective of food preservation can be led back to the early human development. When humans were hunters and gatherers, there was no need to preserve any food and early human could gather what they needed for the day. However, with the onset of agricultural production, farmers were faced with a challenge of making sure the harvest lasts until the next and they had to devise a way of preserving food.

Early methods of preserving food were by smoking and drying. Later on salt was discovered and used for preservation. Over time, these methods have been improved to the modern ones including food additives and refrigeration (Table 1.2).

All foods tend to lose quality at a varied rate depending on the type of food, the composition, the formulation, packaging and storage conditions. The chance of food spoilage may happen any time during the storage of raw material, formulation, processing, food packaging, storage in factories, food distribution to shops, stores, retail outlets, while selling to consumer, transport and consumption (Gould 1989).

The quality of food is lost due to a wide variety of reasons. The quality of food may be lost due to some physical factors, chemical reactions and enzymatic action.

1.1.1 Basis of Food Preservation

The basis of preservation is the control or delay and prevention of microbial growth. The methods of preservation must target the factors that plays an effective role in the growth and survival of microorganisms. The major factors are separated as follows which is the most often quoted categorization (Mossel and Ingram 1955; Mossel et al. 1983; HuisInt Veld 1996) which are

Intrinsic factors: The factors include the chemical and physical nature of the food with which the microorganism exhibit contact

Processing factors: These factors are applicable to foods inorder to improve preservation.

Extrinsic factors: These factors act on the microorganism in food are applied from outside.

Implicit factors: These factor is associated to the nature of the microorganism and the interactions between them and the external environment with which they exhibit contact during growth.

Net effects: These account for the effect of combining many factors together the combined net effect would be greater than the effect caused by single factor alone. (Leistner and Gould 2002).

1.1.2 Major Food Preserving Technologies

The word "Food Preservation" is referred to as any one of the technique employed to prevent food from spoiling (Okeke 2009).

Chilling and freezing – Retard the growth and reduction in water activity to prevent the growth of microbes.

Drying curing and conserving – reduces the water activity to prevent and delay microbial growth.

Oxygen free packing – Reduced oxygen tension prevents and delays the growth of aerobes.

Modified atmosphere packaging – This includes the use of carbon dioxide in combination with other gases inhibits the growth.

Reduction in PH-Results in additional inhibition of growth.

Acetic and Lactic fermentation – Reduction in pH by microbial action.

Alcoholic fermentation – Increase in the concentration of ethanol.

Addition of preservatives, Pasteurization and Sterilization (Gould 1989).

1.1.2.1 Low Temperature

Low temperature is the most common method of preserving food, refrigerators and freezers are used commonly. The most universal method of preserving almost any type of foodstuff is by lowering temperature, wherever refrigerators and freezers are commonplace.

1.1.2.2 Reduction of Water Activity

The most extensively used method of preservation is the practice of lowering the water activity of the food product. Reduction in water activity can be achieved by addition of salt, Nacl, sugar or by drying either partially or completely. The stability of the foods with regard to microbial growth, the loss of quality of food due to physical, chemical and enzymatic changes are predicted by the (aw) water activity values. (Christian 2000).

1.1.2.3 Vacuum and Modified-Atmosphere Packaging

There has been a swift development of vacuum and modified atmosphere packaging since recent times. During the past 15 years there has been a rapid expansion of vacuum and modified-atmosphere packaging of foodstuffs (Parry 1993; Davies 1995).The technique is very effective as it removes oxygen as in vacuum resulting in the inhibition of growth of microorganism that need oxidative metabolism for growth. Many consumers do not object for the storage of food in chilled gas or vacuum, as raw meat deteriorate through slow growth of lactic acid bacteria and other related microorganisms. Where as in air, extracellular polysaccharides, amines, and other unpleasant metabolites are produced by oxidative bacteria. The use of carbon dioxide modified atmosphere packs has an advantage of not only replacing oxygen but also having the property of antimicrobial action. Thereby providing safe, high quality, and long shelf life for food (Faber 1991; Davies 1995). However, it should be noted that *Yersinia enterocolitica* is resistant to the exhibition of Co_2 whereas, sensitivity is observed in *Bacillus cereus* (Ronner 1994). Packing material with specific permeability and selective gas exchange has been developed (Rooney 1995). Controlled atmospheric packing technique is practiced on large scale where bulk material needs to be stored.

1.1.2.4 Acidification

Below pH value of 4.5, *Clostridium botulinum* is found as being not able to grow. Below pH 4.2. Other microorganism is found to be well controlled for growth (Corlett and Brown 1980).

1.1.2.5 Uses of Food Preservatives

The effective way of preservation is the use acids as preservatives as the nature of the acids used, to lower pH is an important factor influencing preservation. The most commonly used preservatives are Sorbate, Propionate, Lipophilic Organic acids, sulfide, Nitric acid, etc., which are effective at pH value lower than 5.5.

1.1.2.6 Heat

Pasteurization and sterilization at appropriate temperature inactivates vegetative microorganism and bacterial spores, form the basics of industrial application. However, with the irradiation procedures the application of heat remains substantial in killing the microorganisms.

1.1.2.7 Physical Preservation Technologies

Non thermal physical technologies include ultrasound, irradiation, hydrostatic pressure, pulsed electric fields have the potential in preserving food that is minimally processed (Hendrickx and Knorr 2001). The major advantage of non-thermal physical method is, it causes less loss of nutrients and vitamins and flavor of the food during processing (Gould 2000). Irradiation has been researched in several countries and it is now approved in about 30 countries. However only limited countries have actually employed irradiation technique for certain applications, decontamination spices. Microwave processing exerts its action through heating but has only few application in the field of food processing (Mullin 1995). Japan has won the 1st place in the development of high pressure treated foods in Jam and fruit juices (Palau et al. 1999) and other food product guacamole. Chill stored sliced meats (Hendrickx and Knorr 2001), 100–600 Mpa pressure are used. Than spores vegetative cells are more susceptible but yeast and moulds are vulnerable to death. The other application of this technology is the change in the property of gelling, induction of structural alterations in foods, such as dairy product and yogurts, tenderization of meat (Knorr 2001).

Ultrasound is generally considered in combination with heat treatment referred as thermo sonication or with heat combined with pressure treatment referred as mano thermo sonication both increases the effect of ultrasound at elevated temperature. Ultrasound is applied in food industry for mixing, cleaning, emulsifying but now it is explored for preservation in the form of mano thermo sonication (Ercan and Soysal 2013). Several ways of electrical treatment has been studied for its microbial effect and for its potential in food preservation. Methods of low voltage cause heating of food (Ohmic heating) and they exert thermal effects. Whereas high voltage pulsed electric field cause little heating. Electric pulse technology is receiving wide spread attention now a days (Barbosa et al. 1999).

1.1.3 Novel and Traditional Food Preserving Technology

Techniques are not just working towards the increase of the shelf-life of the food. But also striving towards the maintenances of the same quality of the food, with no compromise on the chemical composition as well as the physical characteristics of the food (Table 1.3).

Focus on Combinations

Pulsed electric fields, Radiofrequency and high pressure heating are examples of novel major technologies which are currently developed and researched for food preservation. The inactivation capacity of the above methods are specific towards spores and enzyme inactivation hence using these techniques alone will not bring out its full effectiveness. For extensive application of non-thermal processing combination of traditional practice and emerging non thermal techniques are needed. Non thermal technology stands upper hand to heat treatment in maintaining the nutritional value of the food. The conventional heat treatments holds high stand for having a broad range of microbial lethality (Gould and Russell 2003). The combination treatment may yield the desired enhancement in shelf life along with good preservation of product quality.

The first great discovery was made in the art of food preservation, when a method of heating food in sealed glass containers was introduced, to be followed very shortly by the use of the tinplate container. Canning is the oldest as well as the most widely used of the present methods of long-term preservation of foods. Following it, many other preservation techniques were explored (Leistner 1992) (Table 1.4).

All foods in their natural state tend to deteriorate on storage, the chief agents of decomposition are yeasts, moulds and bacteria, and the primary aim of most methods of preservation is to destroy these micro-organisms or to produce conditions under which they cannot grow.

Table 1.3 Outline on the principle, advantages and limitations of food preserving methodologies

S.No	Technique	Advantages	Limitations
Latest preservation technology			
1	**High pressure processing:**	Less process timing	High capital expenditure spores are not sensitive
	Non thermal pasteurizing technique the products are held at 300–600 Mpa pressure for 10 mins	Reduced heat damage	Resistant bacteria
		Retaining product flavor, texture, colour	
2	**Pulsed electric field:**	Better retention and storage stability, lower operationql cost	High conductivity foods are Not suitable for treatment
	Food is exposed to a high voltage filed for less than 1 s		Liquid product only
			Spores are not sensitive

(continued)

Table 1.3 (continued)

S.No	Technique	Advantages	Limitations
3	**Ultrasound:**	Improves stability of dispersion reduce settling	The generation of free radicals in *food* (which causes poor taste)
	Energy generated by sound waves of 20,000 or more vibration per sec.		
4	**Irradiation:**	No nutritional loss	Poor taste and nutrient content
	Treatment of food with x-rays or gamma radiation	Reduction in the microbial risk to consumer	
5	**Electron beam irradiation:**	There is no radioactive waste.	High electric power consumption, complexity and high maintenance, Shallow depth of penetration
	High energy electron beams are produced in an electron gun and directed by magnetic field to target food	They do not require replenishment of the source	
6	**Ohmic energy**	Ability to heat materials rapidly and uniformly Enhancement food quality by limiting heat damage	
7	**Ozone in food industry**	Food contamination can be reduced	Harmful
		Increases shelf-life	
8	**Nanotechnology in Food Industry**	Forms a criss cross net over the container that makes the air to take a long time to enter	Health effects yet to be studied
Older traditional methods of food preservation			
9	Refrigeration and freezing	Slows the bacterial contamination	Changes the texture of fruits
10	Canning and bottling:	The food edible for many years	Foods shows susceptibility to the growth of anaerobic
	Food is boiled in the can to kill the bacteria and it is sealed	Foods can be stored for a longer periods of time	Organisms
11	Pasteurizing	Kill the bacteria and make the food sterile	It affects the nutritional value of food
12	Dehydrating food	Kills bacteria	Changes chemical property of food
	Freeze drying, salting and pickling, Drying and fermentation, use of antioxidants		

Table 1.4 Research development in food preservation techniques

S.no	Method	Experimental details
1	Canning and quick freezing	1795 – Preserving of food in sealed containers by means of heat
		1809 – Peter Durand used tinplate container for the preservation of food (Adam 1960)
2	Refrigerated storage and gas storage	Control of temperature and humidity
3	Dehydration	1795 – Preservation of foods by means of heat
		Drying of vegetables in hot air (105 °C) (Adam 1960)
4	Irradiation	Use of Gamma rays from radioisotope or high energy electrons discharges as a beam from a linear accelerator as a source
5	Antibiotics	Example: Use of Nisin in some cheese to prevent bacterial growth
		Experiments on spraying or injection of meats with antibiotics have been successful in prolonging storage life
6	Hurdle technology	Deliberate combination of existing and novel presentation procedures aiming to establish many preservative factors that several microorganisms would not be able to overcome. Like temperature, water activity, pH, redox potential, preservatives

1.2 Recent Food Preservation Techniques Employed

The Food preservation techniques employed in recent times are advanced adaptations of ancient age old techniques (Lado and Yousef 2002). Hence the methodology involved to overcome microbial contamination or oxidative rancidity is the same however, the technical procedure is highly advanced and innovative, exploiting recent experimental findings.

The following are some of the recent food preservation techniques used based on the two major age old technique of food preservation freezing and heating.

1.2.1 Novel Techniques of Food Preservation Based on Freezing

Freezing is usually considered the finest for long lived preservation of foods with high quality. However, various physio-chemical and biochemical developments take place during freezing of food, storage in frozen conditions and thawing that are of great concern to producers and consumers. Hence, Food engineers are fascinated in crafting efficient equipment and procedures that will gratify the necessities at marginal cost while elevating product value. The following are the new advance techniques employed for food preservation that are based on freezing.

1.2.1.1 High Pressure Freezing and Thawing

Pressure shift freezing (PSF) is based on Le Chatelier's principle (an upsurge in pressure lead to a fall in the freezing point) (Otero et al. 1998). In PSF pressure can be released very quickly, thus we get from fluid to ice in a short time, before there is time for nucleation to occur. A large amount of super cooling can henceforth be acquired, leading to synchronized and even nucleation, and the development of a huge number of tiny crystals. This, can give a much higher product quality.

Pressure assisted thawing (PAT) involves subjecting Frozen food to pressure until it reaches thawing point (Zhao et al. 1998). Heat is later applied under pressure to thaw the ice. When the food has been entirely defrosted, pressure is freed. The benefit of PAT is that there is a reduction of freezing point, hence the temperature motivating force (difference between the air temperature and product temperature) can be amplified numerous stretches, but keeping the temperature small to prevent the microbial growth. Usually thawing occurs at an air temperature of about 5 °C. Thus, by declining the freezing point to about −5 °C, with the help of pressure, the temperature motivating force and henceforth there is a double in the thawing rate.

1.2.1.2 Progressive Freeze Concentration (PFC)

The concentration or amount of a solution left out by chilling out the ice and eradicating it is referred to as Freeze concentration. PFC has formerly been used for methodical purpose but recently it has been applied (Miyawaki et al.) for food processing. Here, as an alternative of forming crystals in a suspension within a stirred tank, the liquid is propelled past a cold surface (mostly a cold tube) and thereby forming ice on that surface. The relaxed rate of growth causes a better parting of solute and water.

1.2.1.3 Osmo-Dehydrofreezing

Dehydrofreezing involves dehydration of food and then freezing. The condensed amount of water reduces the amount of ice crystals, its size (because of increased viscosity) and henceforth the freezing expansion, and thus can reduce tissue damage while freezing, especially in foods that are fragile like strawberries. The freezing time will be less because there is little amount of water to freeze. Adding to this the cost of transport and packaging is decreased due to decreased weight. The Dehydration process can be done by air dehydrating or osmotic desiccation (osmo-dehydrofreezing). The latter involves the immersing of food in a concentrated solution containing some solute. As the water is slurped out of the fruit, some of the solute will diffuse into food, consequently this will change the foods taste. For fruits, sucrose is the most popular while, lactose, glucose, malto-dextrin, fructose, corn syrup can also be utilized. Sodium Chloride is utilized for vegetables sodium

chloride can be used. Recently oligofructose, high-DE maltodextrin and trehaloseare being used (Dermesonlouoglou and Taoukis 2006). It is observed that osmodehydrofrozen food samples are considerably enhanced, when paralleled to the respective value features of conventionally cold samples.

1.2.1.4 Freezing in Ice Slurry and Immersion Freezing

Orthodox immersion freezing involved the use of brines to reduce the freezing point, or any other type of refrigerant (Miyawaki et al. 2005). The food product is usually wrapped in order to prevent absorption of the added refrigerant. However, in case of some food products like deserts absorption may be an advantage. Thus, ice slurries (made up of sugar-ethanol aqueous solutions) are employed to freeze fruit for dessert.

1.2.1.5 Use of Antifreeze Proteins (AFP)

Mammals that has the ability to endure subfreezing body temperatures either:

- By carefully controlling the freezing process in body (freeze tolerance): Seen in certain reptiles and amphibians: they produce ice nucleating proteins that recruit ice nucleation as soon as the body temperature touches −2 or −3 °C, nonetheless also yields cryoprotectants (glycerol or glucose) that reduces the freezing point of the very sensitive organs, postponing or diminishing freezing there. Freezing therefore takes place in a highly controlled manner.
- By maintaining body fluids in a super-cooled state (freeze avoidance): Seen in certain insects and fish, which synthesizes antifreeze proteins to avoid their blood from icing at −1.9 °C (sea waters freezing point), even though their bloods freezing point is around −0.8 °C (and there are ice crystals that float about that may cause nucleation). This is done by the production of antifreeze proteins that bind to the exterior of ice crystals and thereby avert their growth.

The use of these antifreeze proteins in foods (ice-cream) is a potential technique employed to avoid crystal progression during storage, specifically during temperature fluctuation (Warren et al. 1992). However due to high cost this has been unexploited. But, Genetic engineered AFP or synthetic APC (Liu et al. 2007) has helped overcome this.

1.2.2 Novel Techniques of Thermal Food Preservation

Thermal food preservation techniques aims at inactivation of microorganisms using heat. However, the quality of food is lost during this process. And hence new advances in thermal food preservation involves two often complementary ways. First is the use of high temperature- Short time treatment of food followed by

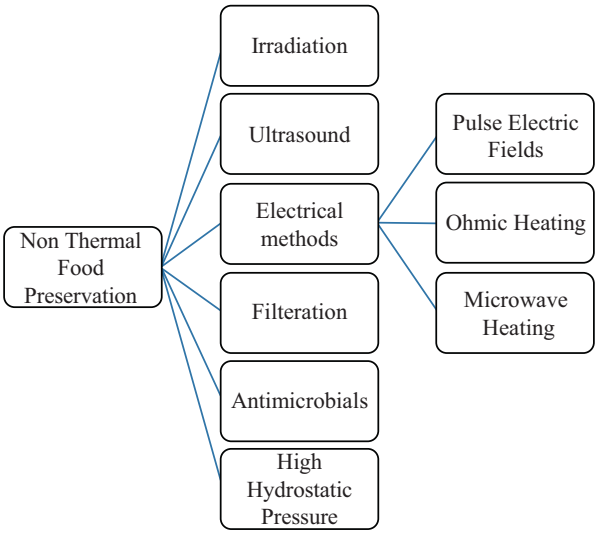

Fig. 1.1 Non thermal food preservation techniques

aseptic methods of packaging. Second, is the use of alternative sources of heat (delivering heat by new ways). This is referred to as Non-Thermal food preservation techniques.

1.2.2.1 High Temperature: Short Time (HTST) Pasteurization

The introduction of high-temperature short-time (HTST) pasteurization occurred in 1933. The methodology involved heat treatment of milk at 71.7 °C for 15 s, (Based on survivability of such pathogens as *M. tuberculosis*, *Mycobacterium bovis*, *Brucella*, and *Streptococcus*). HTST pasteurization currently is the primary method for heat treatment of milk in dairy processing plants as it has helped in the decline of human borne illness. And at the same time helped retain the fresh characteristic of the food thereby meeting the consumer's demands.

1.2.2.2 Non Thermal Food Preservation Techniques

Novel non-thermal food processing technologies target to provide safe, high value foods with necessary nutritive, physico-chemical and sensorial properties. Non-thermal approaches permits the handling of foods at lower temperatures utilized during the process of thermal pasteurization, so essential nutrients, flavors of the food and vitamins undergo negligible or absolutely zero changes. Hence, meeting the consumers demand for high-quality foods accompanying "fresh-like" characteristics. Foods can be non-thermally processed as shown in Fig. 1.1.

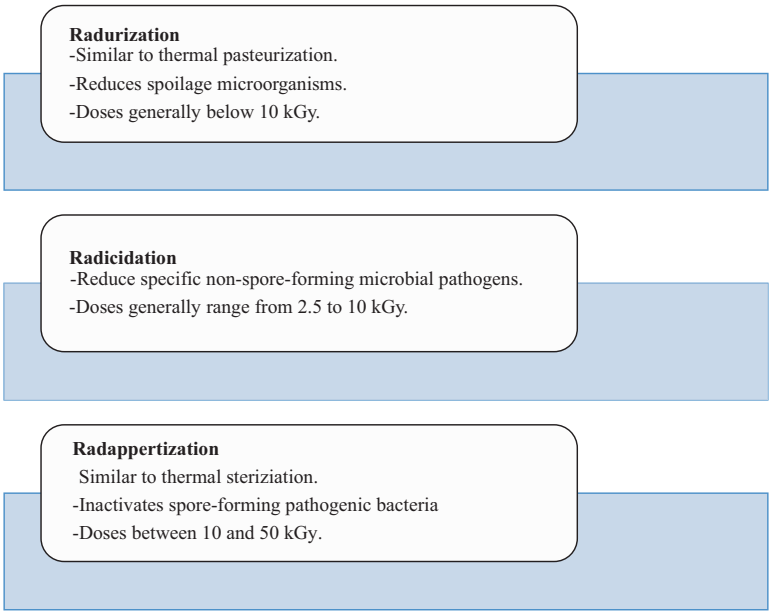

Fig. 1.2 Irradiation types

1.2.2.2.1 Irradiation

Sterilization of food by irradiation for destruction of (virtually) some or all micro-organism organisms (Loaharanu 1995). Ionizing irradiation is referred to as the energy utilized in food irradiation technology. Irradiation is known to control senescence or ripening of certain raw fruitlets as well as vegetables and is effective in inactivating spoilage causing and disease causing micro-organisms (Beatrice et al. 2002). Irradiation causes microbial death by:

- Inhibition of DNA synthesis process
- Inactivating the cellular membrane alterations,
- Denaturation enzymes,
- Alternating ribonucleic acid (RNA) production,
- Effecting phosphorylation
- Changing the composition of DNA.

According to the amount of dosage utilized and the objective of treatment, there are three categories of food radiation (Fig. 1.2).

Nonetheless, Irradiation might cause certain variations in the sensory features of food and in the functional assets of food constituents. Irradiation initiates the auto-oxidation of fats, which causes rancid off flavors.

Ultrasonication (US)

Application of ultrasound at low temperature.

Used for the heat sensible products.

Requires long treatment time to inactivate stable enzymes and/or microorganisms which may cause high energy requirement. (Zheng, L et al, 2006).

Thermosonication (TS)

The product is subjected to ultrasound and moderate heat simultaneously.

This method produces a greater effect on inactivation of microorganisms than heat alone. (Mason et al, 1996).

Manosonication (MS)

Combined method in which ultrasound and pressure are applied together.

Inactivate enzymes and/or microorganisms by combining ultrasound with moderate pressures at low temperatures

Manothermosonication (MTS)

Combined method of heat, ultrasound and pressure.

Inactivate several enzymes at lower temperatures and/or in a shorter time than thermal treatments at the same temperatures (Chemat, F. et al, 2011).

Fig. 1.3 Ultrasound types

1.2.2.2.2 Ultrasound

Ultrasound is utilized to inactivate microorganisms and overcome the objectionable outcomes of thermal processing. Microbial inactivation mechanisms of ultrasound is by cavitation's phenomena that can be caused by the alterations in pressure (Piyasena et al. 2003). The extremely quick formation and breakdown of bubbles produced by ultrasonic waves in the medium generates the antimicrobial influence of ultrasound. In the course of cavitation process, contained variations in pressure and temperature leads to the collapse of cell wall, disorder and dilution of cell membranes, and Deoxyribose nucleic acid damage due to free radical production.

Hence ultrasound can be applied with either pressure or temperature (Sala et al. 1995), else with both pressure and temperature. And it can be classified into four types (Fig. 1.3) (Songül and Çiğdem 2013).

However, Ultrasound mode of food preservation has the following limitations:

- It has a very Complex approach of action
- The Depth of infiltration is affected by the solids and air inside the product
- There is a Possible harm due to free radicals
- Unnecessary alteration of texture and food structure
- Must be utilized along with additional process (e.g. heating)
- Impending issues with scaling-up plant

1.2.2.2.3 Electrical Methods

Heating via the usage of electric fields and/or elevated pressure changes from conservative thermal handling because it can evenly infiltrate numerous centimeters into food. Heat is produced rapidly and uniformly all through the mass. Hence the electrical methods include –

- Electrical Microwave heating for fruit and vegetable products.
- Ohmic processing applied to particulates in a solution that conducts electricity by passing them a series of low-frequency discontinuous electric currents of about 50 or 60 Hz.
- Pulsed electric field processing, which is presently used in juices, involves the use of very short pulses of a very strong electric field on a fluid that flows in order to kill the vegetative cells of microorganisms.

(a) **Microwave heating:** Microwave heating denotes the generation of heat by utilizing electromagnetic waves of particular frequencies. When the food is placed within a microwavable container inside a microwave oven and activated, the food present in the edge of the vessel heats up quicker and there is a temperature gradient that develops between the middle and the edges. Most regularly utilized microwave frequencies are observed as 2450 MHz and 915 MHz. Food stuffs and feed stuffs have been processed using microwaves for pasteurization or sterilization or simply to improve their digestibility. The mechanism involved in microwave heating is dipolar interaction and ionic interaction (Mathavi et al. 2013).

(b) **Ohmic Heating:** It involves the methodology of passing electric current over an electrically conducting food product, similar to microwave energy, the electric energy is then transformed into heat. Ohmic heating is a resourceful method of treating foods encompassing solid large particulates (Mathavi et al. 2013). After heating the products can be cooled uninterruptedly by heat exchangers and then can be aseptically filled into sterilized containers in a way comparable to orthodox aseptic packing. High as well as low acid products can be treated by using this method. The critical parameters affecting ohmic heating include –

- Conductivity of electricity by the food
- electrical conductivity is depended on temperature
- The type of design of heating device
- Residence time
- Time required for distribution
- Physical as well as Thermo assets of the foods
- Electric field strength

(c) **Pulsed Electric Field Technology:** High voltage electric pulses (of about 70 kV/cm) are discharged into the food products, and for doing so the food product is placed in between two electrodes for a little microseconds (Heinz et al. 2002). PEF is known to replace the traditional method of thermal pasteurization (Heinz et al. 2002). An exterior electric field is utilized to surpass

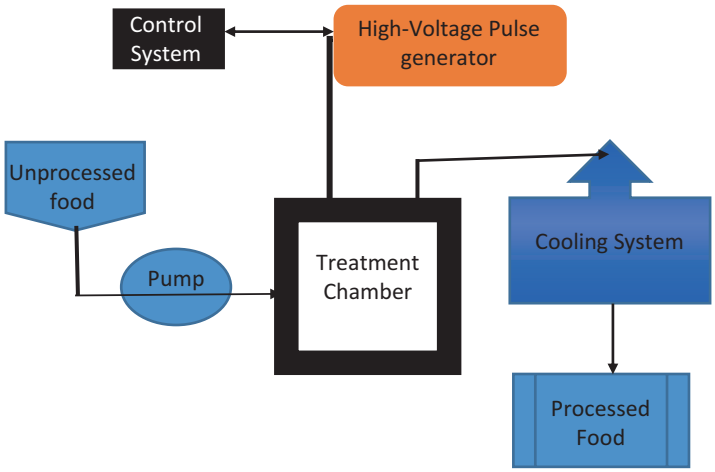

Fig. 1.4 PEF setup

a precarious trans membrane potential of 1 volt. PEF results in a very rapid electric collapse as well as conformational variations of cellular membranes, which hints to the discharge of intracellular fluid, and cellular death of the microorganisms (Hamilton and Sale 1967).However, treatment temperature must be very low in order to dodge heat impairment to the preserved product and to avoid off-flavors.

The components of a standard pulse electric field processing apparatus includes (Fig. 1.4)

- A proper power supply
- An energy storage element- can be either electric (capacitive) or magnetic (inductive).
- A switch which may be closing or opening.
- A pulse shaping and triggering circuit (some cases).
- A treatment chamber
- A pump in order to supply products to the treatment chamber
- A cooling system for controlling the temperature of feed as well as the output material

PEF disrupts microorganism by electroporation and electrical breakdown. Hence PEF has the following advantage. Some include:

- It kills vegetative cells.
- Colors, flavors and nutrients are preserved.
- There is no indication of toxicity formation and at the same time it requires short treatment time.
- Mild preservation of fluid foods at ambient or somewhat raised temperature.

1.2.2.2.4 Ultrafiltration

Ultrafiltration is employed to pass liquid food comprising of flavor in addition to aroma constituents while holding spoilage causing microorganisms in an UF retentate. The UF retentate is later on treated and processed to deactivate an adequate amount of degrading microorganisms to prevent spoilage of the juice in storing conditions. A UF permeate (the Initial fluid) containing flavor and aroma components is then recombined with the treated UF retentate to provide juice suitable for storage while avoiding the loss or alteration of the desirable flavor and aroma components of fresh juice encountered in conventional juice processing. Reverse osmosis can be employed further to treat the UF permeate.

1.2.2.2.5 Anti-Microbials

Anti-Microbials is a part of Hurdle technology of food preservation. The microbial derived hurdles include – Competitive flora, protective culture, bacteriocins and antibiotics. Among them Bacteriocins is the recently advanced methods employed. Bacteriocins are produced by different groups of bacteria and are known to be peptides or proteins with antimicrobial action that are ribosomally synthesized. Bacteriocins with relatively broad spectra of inhibition is synthesized by many lactic acid bacteria (LAB). It is used as an alternative to chemical preservatives thus results in foods that are more naturally preserved and that are richer in nutritional as well as organoleptic properties. Some commercially available preparations are nisin and pediocin PA1/AcH. Numerous bacteriocins display additive or synergistic properties when used in a mixture with additional antimicrobial agents, along with chemical preservatives, natural phenolic compounds, including other antimicrobial proteins. The amalgamation of bacteriocins and physical processing's like pulsed electric fields or high pressure processing can also offer good opportunities for more effective conservation of foods, providing other barrier to more retractile forms of microbes such as bacterial endospores (Galvez et al. 2007). Natural phytochemicals of anti-microbial activity from plants and animals can also be used for food preservation and shows potential benefits compared to synthetic anti- microbial food preservatives.

1.2.2.2.6 High Hydrostatic Pressure

High Hydrostatic Pressure/High Hydrostatic Processing/Ultra High Pressure processing utilizes about 900 MPa to eradicate numerous amount of micro-organisms found in food items, at room temperature lacking the degradation of essential vitamins, flavor and colour molecules. And when high pressures to about 1000 MPa are exposed to packs of food that are flooded in a fluid, the pressure is circulated promptly and regularly throughout the food (which is referred to as isostatic). It has also been observed that typically a pressure of about 350 MPa when exposed for

around 30 min or a pressure of about 400 MPa is exposed for 5 min it will cause a tenfold reduction in the vegetative cells of bacteria, moulds and yeasts. The processing time is reduced compared to thermal processing because high pressure processing has no heating and cooling phases and there is a hasty pressurization/depressurization cycle (Mathavi et al. 2013).

The components of HPP include:

- A pressure vessel along with its closure
- A pressure generator system
- A temperature controller device
- A food materials handling system

The food to be preserved are loaded into the pressure vessel and the top is closed. Usually water is used as a pressure medium and is pumped into the food vessel from the base by the pressure generator system. Once the desired pressure is reached, the pumps are stopped and the pressure is maintained. It is to be noted that the high pressure applied is in an isotonic manner that is the food experiences uniform pressure unlike the gradient generated during heating.

Advantages of HPP are as follows:

- At higher temperatures it helps in killing vegetative bacteria and spores
- No evidence of toxicity is observed
- The colors, flavors and nutrients of the food are preserved
- There is a reduction in the processing time
- Isotonic treatment of food
- It helps attain desirable texture changes
- In-package processing of food is possible
- It has a high prospective for lessening or eradicating chemical preservatives
- Meets consumer's demands.

Limitations seen in HPP are as follows:

- HPP has a very little consequence on foods enzyme activity
- Some microbes tend to survive post HPP treatment
- The equipment is expensive
- Only foods with 40% water content can be treated
- It is a type of batch processing
- There are limited packaging options
- There are many regulatory issues to be resolved

1.3 Conclusion

Food preservation based on combined methods involving the improvement of traditional methods by innovative advances has helped in meeting the demands of consumers for fresh and stable foods. Hence this has resulted in products with high sensory and nutritive properties thereby increasing the value of the food material.

These new advance techniques have been well-thought-out to be enhanced in terms of energy efficiency and to preserve better value qualities in food. These procedures also meet industrial needs by contributing value-added products, novel market prospects and additional safety boundaries. Hence, it is observed that there are a surprisingly large and growing number of improved technologies that are being researched or are in the early stage of applications. A number of ways are available in order to maintain the quality of food during the various stages right from food manufacture, storage, and distribution to retail shops and sale to consumer. These methods vary in usage and in effectiveness as they depend upon the food type, susceptibility to deterioration. The quality loss of food is associated with the type of growth of microorganism. The growth of microorganism can be related to the composition, extent of preservation, their actual shelf-life and storage conditions. Currently the use of natural, addictive free or addictive reduced food is in demand from the consumer side. It is essential to create the best utilization of the available preservatives and use them effectively and efficiently by combining them with other methodologies and to possibly make an effort to understand the mechanism of action. Interest in non-thermal technique of food processing has increased over the past decade. The drawback laid by the emerging novel techniques can be overcome by combining the latest technology with traditional practices to increase the effectiveness of the technology in full fledge.

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Chapter 2
Metagenomic Insights into Environmental Microbiome and Their Application in Food/ Pharmaceutical Industry

Ramya Sree Boddu and K. Divakar

2.1 Introduction

2.1.1 Metagenomics

Our environment is a vast reservoir of a large number of microbes and only a minor share of them are culturable. Metagenomics is a culture- independent technique to extract and analyse the genomic information of unculturable microbiota (Rinkoo and Rakesh 2011). It is defined as a direct genetic analysis of all the genome contained within an environmental sample (Torsten et al. 2012). The word 'metagenomics' is derived from two words, meta- which is a method for statistically relating separate analyses and genomics- a complete analysis of chosen genetic material (Rondon et al. 2000; Handelsman 2004; Rinkoo and Rakesh 2011). When compared to traditional microbial and genomic techniques, metagenomics has advantage of exploring more genomic data from chosen environment (Fig. 2.1).

2.1.2 Metagenomics in Food and Pharmaceutical Industries

Since many decades, biotechnology in food and pharmaceutical industry has been the key theam of various scientific research/reviews. Crucial business confederations are formed on a worldwide scale, associating advanced biotechnological research skills with industrial scale producers/marketers of food and pharmaceutical products across the globe, chiefly in the US, Japan, the UK and Europe. These coalitions include Amgen/Kodak, Molecular Genetics/Upjohn, CalBio/American

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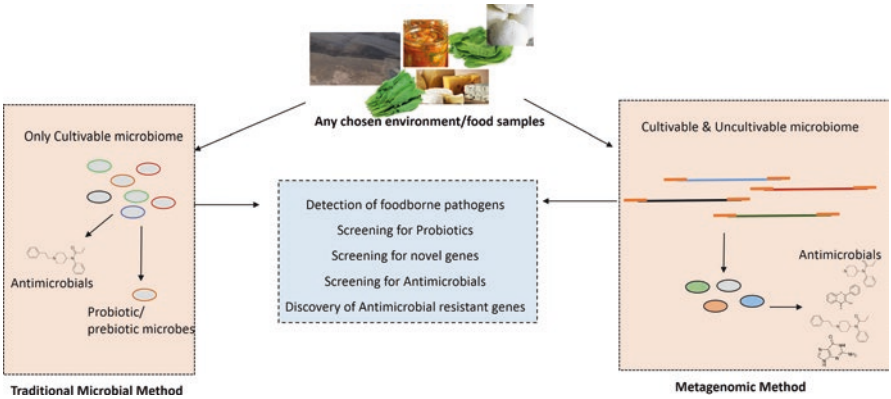

Fig. 2.1 Microbiology and metagenomics in food and pharmaceutical applications**.** Quantity of and quality of information/knowledge obtained through metagenomics and traditional microbial method was compared

Home Products, Synergen/Procter and Gamble and the list goes on (Lawrence 1988).

Of the various classes of enzymes employed by the food industry, the proteases and carbohydrases occupy a major share in the market. The most common protease sold is chymosin (rennin), used in cheese manufacture in coagulating milk to produce curds. Among carbohydrases, cornstarch processing ones (starch hydrolysers like α-amylases, glucose isomerases and glucoamylases) account for about 85% of sales. The other enzymes have applications in flavor/aroma development (e.g., lipases in cheese making) (Arbige et al. 1986), hydrolysis of pectin (e.g., pectinases in juice processing) (Kilara 1982), and manufacturing of functional foods (e.g., α-amylases in retarding bread staling) (Boyce 1986; Lawrence 1988). A wide range of fermented foods and drinks (beer, wine, olives, cheese, bread, salami, etc.) are available in the market. Many beneficial microorganisms play very important role in production of processed foods, in the other end the spoilage microorganisms causes foodborne infections in humans and cattle.

Microbiology and genomics has evidenced to be a boon particularly in the manufacturing of pharmaceutical ingredients, vaccines, fermented foods, prebiotics and probiotics. In addition to this microbial and omics (genomics and proteomics) techniques has been applied broadly in monitoring clinical pathogens, genetic testing and foodborne infections. The foodborne illness rate has been constantly increasing in many developing and developed countries (Flint et al. 2005). The traditional microbial and genomic methods widely depend on culturing microbes under laboratory conditions. The significant advancement in genomic methods can be applied to detect food pathogens for improving food safety. Metagenomics has become powerful tool to screen pharmaceutically important biocatalysts/enzymes and already contributing to detect foodborne pathogens. Role of metagenomics in food and pharmaceutical industries has been discussed intensely in this chapter.

2.2 Metagenomics in Food Industry

2.2.1 Metagenomics of Probiotic and Prebiotic Foods

Probiotics are extracts of fairly large numbers of live useful gut microbes, devised into several beverages that, when consumed in sufficient amounts, bestows health benefit on the host (Joint 2001). A variety of microbes have been used as probiotics, the ones used most often being *Lactobacillus* and *Bifidobacterium* species. Most of the strains employed currently have been isolated from the intestinal microbiome of healthy humans, thereby establishing a firm relationship between probiotics and research on gut microbiota. Also, they are thought to effect human health by modulating intestinal microbiome composition/activity (Gueimonde and Collado 2012). Some of the important probiotic foods/food products available in the market are yogurt (fermented milk), kefir (fermented milk with kefir grains), sauerkraut (fermented shredded cabbage), tempeh (fermented soybean), etc. Few examples of discoveries/products used in food industries which are obtained through metagenomic approach is given in Table 2.1

As probiotics/products are generally ingested orally, they face troubles in efficacy due to deficient cell count that reaches the intestine, on account of the requirement of passing through most of the gastro intestinal tract to reach the bowel, their site of action. The harsh conditions (pH and osmolarity) of the upper gastro intestinal tract can ruin a large dimension of the ingested cells. Functional metagenomic studies were successful in identifying an acid resistant metagenomic clone from the surroundings of Tinto River by Guazzaroni et al. (2013) and discovering a gene exhibiting salt tolerance in an *E.coli* host from the gut microbiota library of humans by Culligan et al. (2013). Genomic data on vital intestinal microbiota such as

Table 2.1 Examples of products used in food industries, discovered through metagenomic studies

Inventions/products through metagenomic studies	Applications	References
Acid resistant metagenomic clone	Probiotics	Guazzaroni et al. (2013)
Genome sequencing of specific probiotic strains	Mechanism of action is studied	Salminen et al. (2005)
Novel prebiotic degradation pathways	Food supplements	Cecchini et al. (2013)
Bas-Congo virus	Disease eruption	Grard et al. (2012)
Hybrid circo/parvovirus (NIH-CQV)	Hepatitis disease mechanism studies	Xu et al. (2013) and Miller et al. (2013)
Presence of sapovirus, norovirus, and Hepatitis A viruses in oysters, clams, and cockles	Viral infections and sources	Benabbes et al. (2013)
Studies on retail spinach	Outbreaks of bacterial infections	Lynch et al. (2009) and Wendel et al. (2009)
Protease that hydrolyses casein, gelatin and leather powder at alkaline pH	Meat tenderizers, protein hydrolysate producers	Ogino et al. (2008)

Bifidobacterium or *Bacteroides* have portrayed the genetic basis of acclimatization of these organisms to the intestinal environment. Genome sequencing of particular probiotic strains has also furnished data on interesting attributes of probiotics like their mechanism of adherence to the intestinal mucosa, interaction with the immune system, etc. (Salminen et al. 2005).

Due to limitations of culture dependent techniques in detecting gut microbial composition and detection of food prone pathogens, DNA sequencing methods were applied extensively for these applications. Since last two decades, various qualitative and quantitative culture- independent techniques have been formulated and applied to the assessment of gut microbiome. These techniques, based on extensive DNA sequencing, have exceedingly enhanced our knowledge on the gut microbiota, their composition and activity (Gueimonde and Collado 2012). More than three million genes have been sequenced from the intestinal microbiome (Qin et al. 2010) and different human enterotypes have been identified (Arumugam et al. 2011). Recently Patro et al. (2016) applied NGS methods for analysing microbial viability, detection of pathogens, qualitative analysis of batch to batch variation and lot-to-lot differences in the packed probiotic products. However, as yet, very limited metagenomic data of probiotics is available from human intervention studies. The results obtained through these direct sequencing methods would be influenced by certain biases with the metagenomic techniques, like the sampling, storage of samples, DNA extraction methods, incomplete bacterial genome databases/sequence information, sequence annotation methods etc.,. The limitations in identification of food pathogens through culture independent techniques makes these metagenomic methods not yet ready for regulatory purpose (Yang et al. 2016), thus comparative studies in this area are always important (Gueimonde and Collado 2012). Further improvement in molecular techniques and sequence analysis tools will help to overcome the metagenomical biases and improve the quality/reliability of the results.

To maintain human gut health by promoting growth of useful bacteria already surviving in one's lower gastro-intestinal tract, another line of approach is the use of prebiotics. They are non-digestible oligosaccharides degraded in the gut by beneficial microbes to produce fatty acids with short acyl chain and other organic acids that render nutritional benefit to the host (Gibson and Roberfroid 1995). A functional metagenomics approach was used by Cecchini et al. (2013) to discover novel prebiotic degradation pathways. Numerous unknown gut microbiome capable of hydrolyzing prebiotics were identified.

2.2.2 Metagenomics for Detection of Foodborne Pathogens

Foodborne illnesses are a national and world-wide health concern. Estimates of the Centers for Disease Control and Prevention (CDC) state that, foodborne pathogens are responsible for more than 48 million illnesses, 1,28,000 hospitalizations, and nearly 3000 deaths in the US every year (Scallan et al. 2011). Recently 350,000 cases with foodborne infections were reported from European Union (EFSA and

ECDC 2013). Foodborne illnesses have a substantial impact on global economy. The tolls of these illnesses stretch from the direct medical costs of the illness to the prices incurred by the food industry in terms of product recalls, judicial issues and loss of consumer confidence. Scharff (2012) approximated that the combined annual costs of the foodborne illnesses in the US surpass $77 million. It is therefore crucial to study the spread of foodborne pathogens in the food production plants and develop authentic and rapid techniques for their detection (Yang et al. 2016).

Traditional assays for the detection of pathogen like microscopy and culture-based methods, whilst useful, are predetermined. Modern approaches like immuno-assays and nucleic acid amplification only permit detection of one or a few specific pathogens at once. Hence, these advanced approaches are also confined in investigating interactions between the pathogens and the environment (Miller et al. 2013; Yang et al. 2016). Therefore, metagenomics emerged as a promising field in foodborne pathogen detection and their surveillance to trace disease outbreaks and novel unknown virus identification (Nieuwenhuijse and Koopmans 2017).

Shotgun metagenomics studies aided in the recent discovery of the Bas- Congo virus, a rhabdovirus linked with a hemorrhagic fever eruption in 2009 in the African Congo (Grard et al. 2012). Other examples of new pathogens discovered using metagenomics include novel cycloviruses recovered from the cerebrospinal fluid of paraplegia patients of unidentified etiology (Smits et al. 2013) and a distinct hybrid circo/parvovirus (NIH-CQV) from sero-negative hepatitis patients (Miller et al. 2013; Xu et al. 2013). Reoviridae and Picobirnaviridae from field-grown lettuce have been identified through metagenomics by Aw et al. (2014). Another study by Benabbes et al. (2013), on oysters, clams, and cockles has proven to hoard sapovirus, norovirus, and Hepatitis A virus in them. Recent studies have demonstrated potential in the metagenomic detection of chikungunya, Ebola, hepatitis C and Zika virus in hospital backdrops by Greninger et al. (2015), Sardi et al. (2016), and Nieuwenhuijse and Koopmans (2017).

2.2.3 Antimicrobial Resistant Genes from Food and Food Products

Diverse metagenomic studies have been executed to discover antimicrobial resistance genes in fresh packed food products. According to Hawkey, antibiotic treatments for the intervention of bacterial infections in the farm animals pick out for resistant microorganisms in food production plants (Hawkey 2008). Though majority of the microbes in fresh/processed foods are usually non-pathogenic, resistant bacteria found in the food products may transmit their resistance to the opportunistic pathogens or to the human microbiome. Therefore, if the foods carrying resistance genes are eaten raw then the human gut microbiome themselves may possibility become a source for the antibiotic resistance genes. Outbreaks of many

reported bacterial infections have been linked to retail spinach which is usually eaten raw (Lynch et al. 2009; Wendel et al. 2009).

2.3 Metagenomics for Pharmaceutical Application

2.3.1 Predicting Novel Antimicrobial Resistant Genes

Functional metagenomics may be used to fight antimicrobial resistance through two strategies; (1) discovery of novel anti-infectives/antibiotics and (2) recognition of resistance genes in the microbiome. It can be employed to discover novel resistance mechanisms exploited by bacteria naturally which might not have evidenced in the clinical context yet and therefore allow us to anticipate possible paths through which resistance to the present antibiotic treatments could arise (Coughlan et al. 2015). Metagenomic screening of the DNA fragments obtained from an apple orchard soil library confabbed antibiotic resistance to the *E.coli* host, after screening clones for resistance to ten antibiotics. Two novel enzymes were discovered by them, one resistance to kanamycin and the other to ceftazidime (Donato et al. 2010). TLC-based protocol to screen chloramphenicol resistant genes from alluvial soil-derived metagenomic library was successful in identifying a clone conferring resistance to chloramphenicol and florfenicol to the host (Tao et al. 2012).

Functional screening of two metagenomic libraries derived from spinach found to have resistance to 16 different antimicrobials and commonly used antibiotics. Many new genes conferring resistance to aztreonam, ampicillin, trimethoprim, ciprofloxacin and trimethoprim- sulfamethoxazole has been identified from spinach (Berman and Riley 2013). It suggests that microbes intimate with fresh foods/products may serve as reservoirs of antimicrobial resistance genes. Similarly, a metagenomic library constructed from Italian Mozzarella cheese is found to display ampicillin and kanamycin resistance. At last, these studies also prove that food products can potentially nurse bacterial species having clinically relevant antimicrobial resistance which may be transferred horizontally to pathogens, either directly or by a secondary track through the human microbiota (Devirgiliis et al. 2014).

Environments naive to antibiotics and microbes which are not confronted selectivity to develop antibiotic resistance, are found to possess unanticipated resistance and antibiotic resistance genes with them. A metagenomic library constructed from the fecal samples of 22 six-month old naive infants revealed resistance to clinical antibiotics in a naïve environment (Fouhy et al. 2014). Other studies of microbiome from distant zone where lesser or no antibiotic therapy has been practiced have also been identified with unexpected resistance (Pallecchi et al. 2008; Bartoloni et al. 2009). Very recently, the bacterial microbiome of 34 Yanomami individuals from an isolated Amerindian village in South America revealed resistance genes to clinical antibiotics. These studies underline the fact that genes imparting resistance to the

present antibiotic therapies can be encountered in environments void of such selective pressure (Clemente et al. 2015). Many antibiotic resistance determinant genes were discovered in the recent years using metagenomic studies. Thirty nine clones conferring resistance to kanamycin, gentamicin, chloramphenicol, rifampin, trimethoprim, and tetracycline were identified by McGarvey et al. (2012) via activity-based screening of *E. coli* plasmid library obtained from urban soils of Seattle, WA, USA. A novel kanamycin resistance gene fused to a hypothetical protein domain was identified by screening *E. coli* fosmid library from four human fecal samples by Cheng et al. (2012). More recently, Su et al. (2014) isolated 45 clones resistant to tetracycline, chloramphenicol, kanamycin, minocycline, gentamicin, amikacin, aminoglycosides, streptomycin and rifampicin through activity-based screening of *E. coli* plasmid library from four agricultural soil samples from China. Few examples of pharmaceutically important genes/gene products obtained through metagenomic approach is given in Table 2.2

Table 2.2 Examples of products used in pharmaceutical industries, discovered through metagenomic studies

Inventions/products through metagenomic studies	Applications	References
Discovery of novel bacterial resistance mechanisms	Anticipate possible antibiotic resistance pathways	Coughlan et al. (2015)
Two novel enzymes resistant to kanamycin and ceftazidime	Better disease control	Donato et al. (2010)
Genes resistant to aztreonam, ampicillin, trimethoprim, ciprofloxacin and trimethoprim-sulfamethoxazole	Antibiotic resistance studies	Berman and Riley (2013)
Turbomicin B, patellamide D, terragine D, psymberin, onnamide A, erdacin, borregomcin A	Pharmaceutically important secondary metabolites	Novakova and Marian Farkasovsky (2013)
SIGEX	Discovery of novel genes	Uchiyama et al. (2005)
Lipolytic clones	Lipid hydrolysis	Peng et al. (2014)
Two novel esterases	Ester hydrolysis in aqueous solutions	Ouyang et al. (2013)
14 clones having genes encoding all four classes of β-lactamases	Antibiotic- resistance against β-lactam antibiotics	Allen et al. (2009)
Novel nitrilases	Nitrile bioconversion to carboxylic acids and ammonia	DeSantis et al. (2002), Podar et al. (2005), and Robertson et al. (2004)
Indirubin	Treatment of chronic human myelocytic leukemia	Hoessel et al. (1999) and Marko et al. (2001)

2.3.2 *Screening Pharmaceutically Important Novel Compounds*

Screening novel natural products of microbial origin through traditional microbial techniques has been in practice for several decades (Berdy 2012). In last two decades, there has been increasing trend in screening natural products through high-throughput screening methods involving microbiology and genomics (microarray and metagenomics). This leads to discovery of novel compounds like platensimycin, a completely novel antibiotics produced by *Streptomyces platensis.* Several pharmaceutically important secondary metabolites turbomicin B, patellamide D, terragine D, psymberin, onnamide A, erdacin, borregomcin A and several other compounds has been discovered through metagenomic screening (Novakova and Marian 2013). In 2004, Brady et al. discovered long-chain *N*-acyl tyrosine synthase genes by functional screening of cosmid library constructed from different soil samples obtained from Ithaca, NY Boston, MA Costa Rica. Functional metagenomic screening of an uncharacterized protein with antimicrobial activity was obtained from the soil sample of a deciduous forest in Belgium using *E. coli, Bacillus subtilis* shuttle vector library (Biver et al. 2013a).

2.4 Overview of Metagenome Derived Enzymes for Food and Pharmaceutical Application

Enzymes are noteworthy catalysts, capable of having a plethora of complex molecules as substrates. The reactions catalyzed by them are highly chiral/enentio selective and regio/positional specific, which is not always possible through usual chemical synthesis (Ferrer et al. 2005a). Amongst the most important biocatalysts obtained from metagenomes, esterases/lipases, proteases, β-lactamases, polysaccharide modifiying enzymes, nitrilases, dehydrogenases, oxidoreductases, enzymes involved in the biosynthesis of vitamins and antibiotics plays key role in the biocatalytic conversion/biotransformation processes in food and pharmaceutical industries. More recently, enzymes catalyzing the degradation of aromatic hydrocarbons were also discovered using metagenomics (Knietsch et al. 2003; Voget et al. 2003; Daniel 2004; Gabor et al. 2004;Streit and Schmitz 2004;Yun et al. 2004; Erwin et al. 2005; Ferrer et al. 2005a; Solbak et al. 2005; Song et al. 2005; Walter et al. 2005; Uchiyama et al. 2005).

Many of these enzymes become essential ingredient/catalyst for processed food industries, for tenderization of meat (papain, microbial proteases), manufacturing of dairy products (Chymosin/renin, lipases), enhancing flavors of dairy products (lipases, esterases, brewing (glucoamylase, β-glucanase) etc., Development of process in food and pharmaceutical industries, that fits available enzymes from isolated microbial cell has been in practice for last many decades. In recent years researchers started to screen enzymes based on requirement from the industrial sector.

Metagenomic mining for industrially important enzyme has become current trend to explore novel enzymes from uncultivable/unexplored environment.

Functional metagenomics is a straightforward approach for finding genes with desired function/enzymatic activity, where environmental genome is directly cloned into the fosmid/plasmid vectors and expressed in suitable host systems. Substrate-Induced Gene EXpression screening (SIGEX), unconventional metagenomic method for discovery of novel genes was developed by Uchiyama et al. (2005). The basic principle of this technique was influence of specific substrates/metabolites, regulatory elements on expression of catabolic genes. Metagenomic DNA inserts are fused with vector carrying green fluorescent protein and induced with the required substrate. For high-throughput screening, the cells which constitutively expressing functional gene by substrate induction can be selected using fluorescent-activated cell sorting (FACS).

2.4.1 Lipases

Lipases are enzymes that hydrolyse acylglycerides to fatty acids and glycerols. Various novel lipases have been isolated from different microbial metagenomes. A metagenomic library was screened for clones exhibiting lipolytic activity in an *E. coli* host by Peng et al. (2014) from a Chinese marine sediment to discover a highly alkaline-stable novel lipase with greater specificity for buttermilk fat esters. Cold-active lipases were discovered from metagenomic libraries from psychrophilic environmental sample. One of them has been found to exhibit the highest activity at 10 °C and pH 7.5 (Roh and Villatee 2008). Another cold-active lipase obtained from a deep-sea sediment metagenome displayed optimum lipolytic activity at pH 8.0 and 25 °C, and retained 50% activity even at 5 °C. It was also found to be resistant to detergents like Tween and Triton (Jeon et al. 2009). Cold activated lipases with selectivity towards primary alcohols and optically pure enentiomeric (R-) form of ibuprofen, a non-steroidal antiinflammatory drug (Elend et al. 2007).

2.4.2 Esterases

Esterases hydrolyse esters into their respective alcohols and produce acids in aqueous solution. Two novel esterases were isolated using activity based screening of plasmid and fosmid libraries transformed into *E. coli* from soil and water samples (Ouyang et al. 2013). In 2011, Kang et al. discovered a thermostable family VII esterase with high stability in organic solvents using activity based screening of *E. coli* fosmid library from compost sample. Another thermostable esterase was also unravelled by the activity based screening of *E. coli* fosmid library from a mud sediment-rich water (Rhee et al. 2005). Esterases also play a vital role in the

synthesis of chiral drugs including pain relievers and inflammation reducers (Bornscheuer 2002; Shen et al. 2002; Panda and Gowrishankar 2005).

2.4.3 Proteases

Proteases degrade proteins into their constituent aminoacids. They make- up about two-thirds of the total enzyme usage in the world. Papaya proteases (papain) are widely used in food industry as meat tenderizers, protein hydrolysate producers to manufacture formulated or texturized food products, in brewing and baking (Wong 2010). Chymosin (rennin), an acidic protease, is required for the manufacture of cheese. Ogino et al. 2008 isolated a *Bacillus* sp. from soil metagenome to produce a alkaline protease which can hydrolyse casein, gelatin and leather powder. Lee et al. 2007 identified a novel metalloprotease gene that hydrolysed azocasein as well as fibrin from a metagenomic library from deep-sea sediments. Fibrinolytic enzymes have been used as thrombolytic drugs. They are mostly serine proteases that dissolve the clot by breaking down the fibrinogen and fibrin in a clot. Recently, Biver et al. (2013b) isolated a novel alkaline serine protease from the forest soil using IPTG- inducible vector library expressed in *E.coli* based on their activity.

2.4.4 β-Lactamases

The presence of β-lactamase gene confers antibiotic-resistance to the host against β-lactam ring containing antibiotics. A functional metagenomic analysis of an Alaskan soil sample by Allen et al. (2009) produced 14 clones, possessing genes encoding all four classes of β-lactamases, bestowing resistance to the *E. coli* host. In 2009, Sommer et al., identified ten novel β-lactamase gene families using functional metagenomic library obtained from human saliva and fecal samples. Numerous β-lactamase genes were also discovered by Forsberg et al. (2012) from different soil samples of USA by functional metagenomic library expressed in *E.coli* coupled with PARFuMS.

2.4.5 Other Pharmaceutically Important Enzymes

Organonitriles, amides and carboxylic acids, are extensively used in industrial applications in the synthesis of plastics, synthetic fibres, dyes, agrochemicals, fine chemicals and pharmaceuticals. The enzyme nitrilase converts naturally occurring as well as xenobiotic-derived nitriles to their corresponding carboxylic acid and ammonia (Pace and Brenner 2001). Mandelic acid is synthesized from mandelonitrile (an antibacterial agent) by nitrilase in acquous solution without the requirement

of any cofactors or harsh conditions, making it the most cost- effective method of production. Also, nitrile bioconversion can be achieved using nitrile hydratase and amidase that hydrolyse nitriles to their acids in two steps. Functional screening of vast environmental DNA libraries from different sources yielded about 137 novel nitrilases by the scientist communities around the globe (DeSantis et al. 2002; Robertson et al. 2004; Podar et al. 2005). They were found to have stereoselective activity on industrially important nitrile substrates. Liebeton and Eck (2004) isolated Nitrile hydratase genes from the metagenomic libraries of soil samples and evinced them in the active E. *coli* clones. Indirubin, a cyclin- dependent kinase (CDK) inhibitor, used in treating chronic human myelocytic leukemia, was also isolated from metagenomic inserts of active clones by Hoessel et al. (1999) and Marko et al. (2001). Lim et al. (2005) identified another indirubin compound with antimicrobial activity from a forest soil metagenomic library via activity-based screening.

2.5 Conclusion

The application of metagenomics in food and pharmaceutical industry has been witnessed from recent trends in increasing number of publications and patents in this research area. Recent developments in genomic techniques for mining of genetic resources from unculturable microbiome lead to the discovery of new genes, enzymes, natural compounds and bioproducts. More light should be shed on the progress for the detection of foodborne pathogens in trace amounts before it gets epidemic and to pool large number of pharmaceutically important novel genes, natural compounds. Further to make it ease, metagenomics needs improvements in molecular screening strategies, post-sequencing methods, sequence annotation and analysis tools need to be developed. Advancements in high-throughput molecular techniques and sequence analysis tools will overcome the metagenomical biases and improves the quality and reliability of the results. In near future, metagenomics, together with further improvements in high-throughput screening methods, gene synthesis, developing host expression system(s) for metagenome derived genes and systems biology techniques will opens a gateway to reveal useful information from unexplored genetic resources.

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Chapter 3
RETRACTED CHAPTER: Changing Paradigm of Probiotics from Functional Foods to Biotherapeutic Agents

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The authors have retracted this chapter [1] because of overlap with previously published articles [2], [3], [4]. All authors agree to this retraction.

[1] Johnson E.M., Jung YG., Jin YY., Yang S.H., Jayabalan R., Suh J.W. (2018) *Changing Paradigm of Probiotics from Functional Foods to Biotherapeutic Agents*. In: Patra J., Das G., Shin HS. (eds) Microbial Biotechnology. Springer, Singapore.

[2] Daniela Elena Serban *Gastrointestinal cancers: Influence of gut microbiota, probiotics and prebiotics*, Cancer Letters (2014) Volume 345, Issue 2, Pages 258–270, https://doi.org/10.1016/j.canlet.2013.08.013.

[3] Di Cerbo A, Palmieri B, Aponte M, et al *Mechanisms and therapeutic effectiveness of lactobacilli* Journal of Clinical Pathology 2016; 69: 187–203. http://jcp.bmj.com/content/69/3/187.

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Chapter 4
Microbial Valorization of Chitinous Bioresources for Chitin Extraction and Production of Chito-Oligomers and N-Acetylglucosamine: Trends, Perspectives and Prospects

Suman Kumar Halder and Keshab Chandra Mondal

4.1 Introduction

Carbohydrates, often called saccharides, are compounds principally made with three elements; carbon, hydrogen and oxygen having general formula $C_n(H_2O)_n$ and are the utmost abundant organic compounds in the biosphere. Monosaccharides are the smallest carbohydrates called sugars are the building blocks for polysaccharides. Oligosaccharides are the short chain of monosaccharides often produced as intermediate either in anabolic and catabolic process. Polysaccharides have versatile involvement in biological system; they give mechanical support, involves in multiple regulatory processes or interactions between molecules, cells, or extracellular space etc. Metabolism of polysaccharide is ancient and widespread biological activity found in all types of organisms, and due to its central role in energy flux, and as an obvious phenomenon a wide range of enzymes have evolved over time to catalyze these reactions (Jollès and Muzzarelli 1999). Monosaccharides have the key role in generation of energy for metabolism. They, in their native or derivatized form are found in DNA, RNA, ATP and also important components of coenzymes. Moreover as monomeric, oligomer and polymeric form, carbohydrates plays vital roles in an array of biological processes like cell interactions, signal transduction, antibody recognition, tumor metastasis, hormones and musculoskeletal physiology.

N-acetylglucosamine (GlcNAc or simply NAG), 2-(acetylamino)-2-deoxy-D-glucose or 2-acetamino-2-deoxy-β-D-glucose, an amino sugar and is a natural monosaccharide derivative of glucose. GlcNAc have cosmopolitan distribution are synthesized in nearly all creatures, including bacteria, yeast, fungi, plants and animals in different form and plays different role (Chen et al. 2010; Jung and Park

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2014). However, unlike that simple sugar, N-acetylglucosamine is not really a sugar but belongs to a class of compounds called amides although it is commonly described as a carbohydrate. In human, N-acetylglucosamine is found in hyaluronan, mucosal layer of the gastrointestinal tract, keratan sulfate, heparan sulfate, H-antigen of ABO blood groups and also is the key components of glycoproteins, proteoglycans and glycosaminoglycans (Jung and Park 2014). N-acetylglucosamine and its deacetylated form, glucosamine have vital biochemical functions within the physiological system which reflect their mechanism of benefit in various disease processes in human. In bacteria, GlcNAc is polymerized with N-acetylmuramic acid to form peptidoglycan layer of the cell wall. Some independent studies attested that GlcNAc is also a component of teichoic acid (of some Gram positive bacteria) as well as capsular polysaccharide-adhesin.

N-acetylglucosamine polymerizes linearly with β-(1,4)-glycosidic linkages to form chitin which is the plenteous nitrogen-bearing organic compounds and second most abundant carbohydrate found in nature (after cellulose) (Halder et al. 2013a, b). Chitin is obtained as a polymer of molecular weight (MW) ranging from 30 to 3000 kDa with degree of acetylation (DA) more than 90% (Mourya et al. 2011). It is white, hard, inelastic, insoluble and is the principal component of cell walls of fungi, exoskeletons, peritrophic membrane and cocoons of insect, shells of crustaceans, radulae of mollusks, the beaks and internal shells of cephalopods and parts of invertebrates and in the microfilarial sheath of nematodes (Kurita 2006; Gohel et al. 2006; Khoushab and Yamabhai 2010). Throughout evolution, these organisms utilize chitin as structural component (Jollès and Muzzarelli 1999). Based on X-ray diffraction and infrared spectroscopic analysis it was suggested that chitin occurs in three different polymorphic forms, viz., α-, β- and γ-chitins (Fig. 4.1). In α-chitin, anti-parallel monomeric chains oriented with strong intermolecular hydrogen bonding. It is the most abundant form of chitin in nature (shrimps, crabs, lobsters and krill) having crystallinity higher than 80%. The β-chitin has chitin chains in parallel pattern and occurs in squid pens and tube worms, and in γ-chitin, chains are arranged in both parallel and anti-parallel manner (Dahiya et al. 2006; Khoushab and Yamabhai 2010; Mincea et al. 2012). Owing its ubiquitous distribution, it was estimated that annual turnover of chitin is ~10^{11} metric tons in the aquatic biosphere (Lien et al. 2007; Zaku et al. 2011).

Recently, the commercial exploitation of chitin has greatly increased because of the valuable and advantageous properties of its soluble derivatives. When the DA of chitin is about 50% or less, it becomes soluble in acidic aqueous solution, and is called as chitosan (Fig. 4.1). Chitosan can be described and classified on the basis of several indices viz. degree of acetylation (DA) or fraction of N-acetylation (F_A) [or degree of deacetylation (DD)], degree of polymerization (DP) or molecular weight (MW), and the pattern of acetylation (PA). Most commercial chitosan are of molecular weights ranging from 1500 Da to 50–2000 kDa, with DA of 50–100% (commonly 80–90%) (Aam et al. 2010). In acidic condition, protonation of the –NH_2 at the C-2 site of the D-glucosamine (GlcN) repeat unit converted the polymer to a polyelectrolyte which leads to its solubilization. Being soluble, it is impressively used for diverse applications as solutions, chelates, gels or films and fibers.

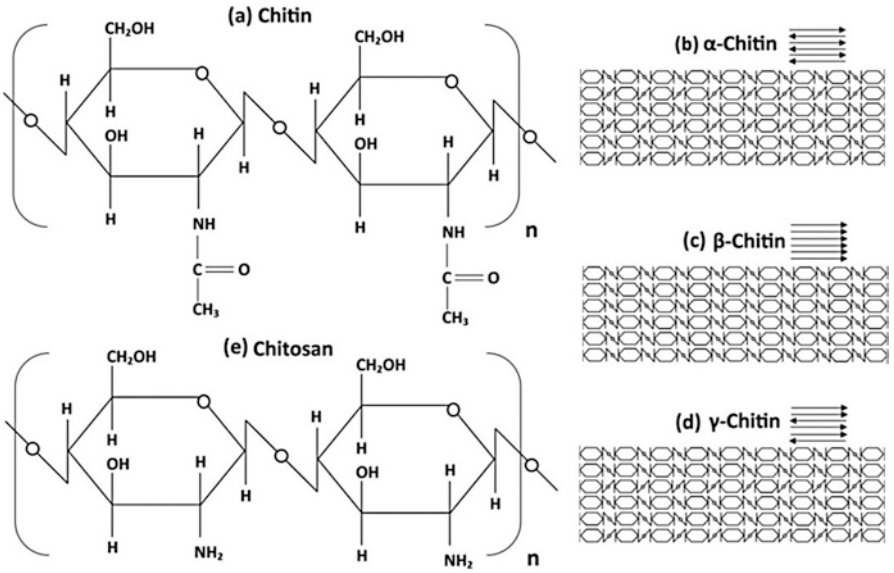

Fig. 4.1 Structure of (**a**) chitin and (**e**) chitosan, (**b**–**d**) pattern of arrangement of chitin fiber in α, β and γ-chitin

Chitosan also occurs naturally in some fungi (e.g. *Mucoraceae*) but its occurrence is limited and less widespread than chitin. Chitin and chitosan are non-toxic, biocompatible and biodegradable. Unlike cellulose, they are amino polysaccharides and show interesting biological, physiological and pharmacological properties (Kurita 2006).

A polymer of chitin/chitosan is being hydrolyzed (hydrolytic degradation) at its glycoside bonds when treated with hydrolyzing agents. Chitosaccharides (CS) like chtin/chitosan oligomer (homooligomer: oligomer of GlcNAc or GlcN, heterooligomers: oligomer contain both GlcNAc and GlcN), N-acetylglucosamine and D-glucosamine, can be produced from chitin or chitosan through chemical, enzymatic and fermentative methods or combinations thereof.

4.2 Chitin Extraction and Subsequent Generation of Chitosan, Oligosaccharides and Monosaccharides

4.2.1 Chitin Extraction from Crustacean Shells

Crustaceans, insects and microorganisms are the three major sources of chitin extraction. So far, the major source of industrial chitin comes from sea-food processing wastes, mainly crustacean shells (e.g. prawn, shrimp, crab, krill shells), in

which chitin is found incrusted with proteins onto which calcium carbonate ($CaCO_3$) deposits to form the rigid, recalcitrant and crystalline shell, and the relative percentage of chitin, protein and minerals varies species to species and even season to season (Pachapur et al. 2016). Therefore, chitin extraction from shellfish involves the removal of proteins by deproteinization and inorganic $CaCO_3$ by demineralization in association with small extents of lipids and pigments that are often removed during deproteinization and demineralization or by additional bleaching step (Younes and Rinaudo 2015). The extracted chitin is subsequently used as the raw material to generate chitin-derived products, viz. chitosans, oligosaccharides, GlcNAc and GlcN, which collectively have paramount and versatile appliances in different sectors. Since beginning, many methods have been adopted to prepare pure chitin by various researcher(s), however, there are some relative advantages and lacuna of each process and hence neither one is sane percent foolproof and ultimate, and therefore continuous effort is always given to optimize the process and to overcome the challenges. Both deproteinization and or demineralization can be carried out either chemically or biologically, or by combining both (Fig. 4.2). During chemical treatment, any of the two processes can be carried out firstly. The order of two steps may be reversed especially when enzymatic treatment is considered, whereas, during microbial fermentation, deproteinization and demineralization may occur simultaneously depending on the efficacy of the strain.

4.2.1.1 Chemical Extraction

Traditionally, in commercial preparation of chitin from crustacean shell alkali and acidic treatment was carried out for deproteinization and demineralization respectively, followed by (occasional) bleaching step to obtain a colourless chitin. An array of chemicals have been tested as for their efficacy of deproteinization including NaOH, $NaHCO_3$, Na_2CO_3, Na_2SO_3, $NaHSO_3$, Na_3PO_4, Na_2S, KOH, K_2CO_3, $Ca(OH)_2$ and $CaHSO_3$, and many study advocating NaOH as preferential reagent. Likewise, demineralization is usually achieved by acid treatment using HCl, HNO_3, H_2SO_4, HCOOH and CH_3COOH, and the efficacy of HCl was found pronounced (No and Hur 1998; Percot et al. 2003; Younes and Rinaudo 2015). The concentration of acid and alkali, treatment temperature, time depends on the nature of chitin and rigidness. During demineralization, calcium carbonate is decomposed into the water-soluble calcium salts ($CaCl_2$) and carbon dioxide, where two molecules of HCl required converting one molecule of $CaCO_3$ into $CaCl_2$ and it is difficult to remove all minerals practically due to heterogeneity of the solid (Younes and Rinaudo 2015). To ease the reaction, demineralization reaction at elevated temperature was also reported (Truong et al. 2007).

In chemical chitin extraction process, harsh acid treatments may lead polymer hydrolysis with inconsistent physico-chemical properties as well as source of environmental pollution, whereas high NaOH concentrations and high deproteinization

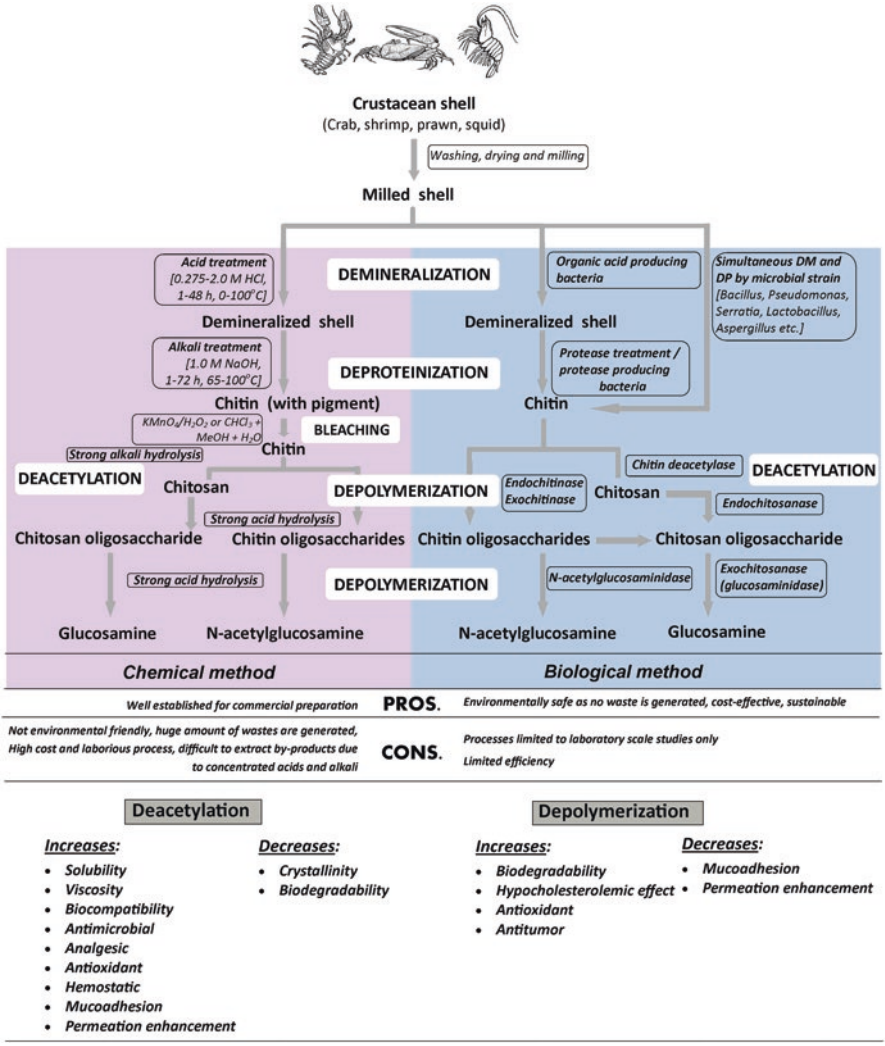

Fig. 4.2 Flow chart for the chemical and bioproduction of chitin, chitosan, their oligosaccharides and monosaccharides from crustacean shells, and influence of degree of deacetylation and depolymerization on different properties of chitin/chitosan (Adopted from Kumirska et al. 2011; Arbia et al. 2013; Jung and Park 2014)

temperatures may cause undesirable deacetylation and depolymerisation of chitin fiber (Arbia et al. 2013). Neutralization and detoxification of the discharged effluent is necessary which creates disposal problem (Khanafari et al. 2008). Moreover, in chemical extraction it is impossible to use the protein components of shells as feed for domestic animal which has large potential for commerce.

4.2.1.2 Biological Reclamation

In contrast to chemical extraction, biological methods was found comparatively safe, low-cost, environment friendly green technology favoring the application of enzymes as well as microorganisms in chitin extraction. Biological extraction of chitin from crustacean shells can be achieved either by enzymatic digestion or microbial fermentation, where the latter may leads to co-digestion of proteins and minerals. In these processes, the liquid waste fraction rich in proteins, minerals and astaxanthin can be used either as a food/feed supplement for human or animal (Arbia et al. 2013; Chandrasekaran 2013). Due to superiority over chemical process, biological methods are currently being employed for chitin extraction. However, research efforts are required for downstream processing of potent biomolecules of nutraceutical and pharmaceutical importance (Khanafari et al. 2008; Chandrasekaran 2013).

4.2.1.2.1 Enzymatic Deprotenization

Proteolytic enzymes such as alcalase, chymotrypsin, papain, pepsin, proteinase, pancreatin, and trypsin in single or in combination, of different biological origin, removes proteins from crustacean shells and minimizes the chance of deacetylation and depolymerization during chitin extraction which is a big burden of chemical extraction (Gagne and Simpson 1993; Jo et al. 2008; Kim 2010). Both crude and purified form of the enzymes are reported to deproteinize chitin, however, the use of crude preparation is not only less expensive but also contain several other coexisting proteases which acts on crustacean shells simultaneously. Recently, our group reported the recovery of chitin and bioactive materials from shells of *Penaeus monodon* (black tiger shrimp) by treating with crude protease cocktail of *Paenibacillus woosongensis* TKB2 (Paul et al. 2015). Thus, application of these crude enzyme cocktail in the chitin extraction could be thought-provoking in economic and environmental perspective. In addition demineralization must be performed before enzymatic deproteinization which may increase proteases accessibility and subsequent deproteinization efficiency.

4.2.1.2.2 Fermentative Deprotenization

Deproteinization of the crustacean shell waste and concurrent liquefaction of the protein fraction is takes place essentially by proteolytic enzymes biosynthesized by endogeneous microbes through auto-fermentation or by inoculating selected microbial strains. The latter can be achieved by single-stage, two-stage, co- or successive fermentation (Younes and Rinaudo 2015). An array of microbial species was proposed for fermentation of crustacean shells for chitin recovery. On the basis of kind of organism, fermentative method can be categorized into two broad categories: lactic acid and non-lactic acid fermentation. In fermentative extraction, various

physico-chemical and process parameter have great impact on the demineralization and/or deprotenization efficiency which are optimized by either classical one-variable-at-a-time or statistical response surface methodology based approach.

4.2.1.2.2.1 Lactic Acid Fermentation

Fermentation of crustacean shells was performed by selected *Lactobacillus* strains. Different lactic acid bacteria were employed for chitin recovery from shrimp waste, crayfish exoskeleton, scampi waste and prawn waste (Arbia et al. 2013; Younes and Rinaudo 2015). Due to lack of proficiency, lactic acid fermentation combined with dilute chemical treatments has been employed as an alternative of concentrated chemical based extraction of chitin (Cira et al. 2002), whereas, in many instance, demineralization efficiency was much higher after addition of commercial proteases (Younes and Rinaudo 2015). However, simultaneous demineralization and deprotenization was also recorded during fermentation by many studied *Lactobacillus* sp. which produces proteases as well as organic acid and hence ideal for single-step fermentative extraction of chitin from the biomaterials (Kim 2010). In this bioprocess, *Lactobacillus* strains produces lactic acid along with proteases; the former suppressing the growth of spoilage microorganisms (due to low pH) and the latter which degrade the proteins.

4.2.1.2.2.2 Non-lactic Acid Fermentation

Several bacteria and fungi were reported to carried out non-lactic acid fermentation of crustacean shells for chitin recovery, and among them *Bacillus* sp. (Yang et al. 2000; Sini et al. 2007; Ghorbel-Bellaaj et al. 2012), *Pseudomonas* sp. (Oh et al. 2007; Ghorbel-Bellaaj et al. 2011; Wang and Chio 1998), *Serratia* sp. (Jo et al. 2008) and *Aspergillus* sp. (Mahmoud et al. 2007) are pioneer. Such as, fermentation of shrimp (*Metapenaeopsis dobsoni*) shell was carried out by *Bacillus subtilis* in jaggery broth and 84% and 72% deprotenization and demineralization was noticed respectively (Sini et al. 2007). Likewise, protease, chitinase/lysozyme producing *Pseudomonas aeruginosa* K-187 strain employed for shell waste deproteinization in solid-state, liquid-solid or liquid fermentation with higher performance in solid-state fermentation (82% deproteinization after 5 days) (Wang and Chio 1998). *Aspergillus niger* grown in shrimp shell powder containing medium released proteases which facilitated the deproteinization of shrimp-shell powder and subsequent release of hydrolyzed proteins. The latter was utilized by fungi as nitrogen source for their growth, which consequently decreased the pH of the fermentation medium, resulting in further enhancement of demineralization process (Teng et al. 2001).

4.2.1.2.2.3 Co-fermentation

The concept of co-fermentation was found fruitful in single step extraction of chitin from crustacean shells. For chitin extraction, red crab shell was co-fermented with *Lactobacillus paracasei* ssp. *tolerans* KCTC-3074 with protease producing *Serratia*

marcescens FS-3 resulted in the highest level of demineralization (97.2%) and moderate deproteinisation (52.6%) after 7 days (Jung et al. 2006). Two proteolytic-chitinase-deficient *Bacillus licheniformis* strains were employed for fermentation of shrimp shells. After 48 h of fermentation, the shrimp shells were harvested, washed, demineralized with lactic acid (0.9% for 3 h) and thereafter oven dried, resulted in 99% deprotenization and 98.8% demineralization (Waldeck et al. 2006).

As stated earlier, biological methods of chitin extraction are simple, more productive and eco-friendly in comparison with chemical processes. However longer processing time, poorer accessibility of proteases are the two major negatives which restricts the yield of pure chitin. Pure form of chitin is very much desirable owing their biomedical applications, and getting of the same can be ameliorated by using simultaneous or sequential processes viz. two-step fermentations, co-fermentation of microorganisms or sometime treatment with mild chemical in order to remove the residual protein and minerals.

4.2.2 Preparation of Chitosan from Chitin

Despite of occurrence in *Mucorales*, in particular *Mucor*, *Rhizopus* and *Absidia* species, chitosan is not extensively present in nature (de Queiroz Antonino et al. 2017). The commercial chitosan is nothing but the extensive deacetylated form of its parent polymer chitin derived from crustacean shells by sequential deproteinization, demineralization and deacetylation. Both chemical and biological route of production of chitosan were well-documented. As the initial part of chitin extraction was aforementioned, the following section therefore concerning about the deacetylation of chitin to yield chitosan.

4.2.2.1 Chemical Deacetylation

Though concentrated acid and alkali have the potential to deacetylate chitin, due to vulnerability of glycosidic bond in concentrated acid, alkali mediated deacetylation is used more frequently. Chitin is treated with concentrated alkaline solution such as 45–50% sodium hydroxide (NaOH) to remove acetyl group from chitin polymer to obtain chitosan of varied DD depending on type of crustacean shells (de Queiroz Antonino et al. 2017). Reports indicate that MW and DD of chitosan are mainly affected by alkali concentration, reaction time, temperature, repetition of alkaline step, atmosphere, particle size, chitin to solvent ratio, source of raw material etc. which were optimized through both classical and statistical approach (Younes and Rinaudo 2015).

4.2.2.2 Biological Deacetylation

Chemical deacetylation has also different shortcomings like energy consumption, environmental pollution (due to strong alkali), heterogeneous range of soluble and insoluble products thus become a challenge to many researchers to accomplish a good chitosan. As a remedy, an alternative enzymatic deacetylation is now possible after harnessing chitin deacetylase (EC 3.5.1.41) which was firstly extracted from *Mucor rouxii* (Araki and Ito 1974). Chitin deacetylase (CDA) catalyzes the production of chitosan by hydrolyzing the N-acetamido bonds in chitin. At present, CDA is reported in several fungi and insect species, however, most of studies were deals with CDA extracted from *Mucor rouxii*, *Absidia coerulea*, *Aspergillus nidulans*, *Colletotrichum lindemuthianum*, *Rhizpus* sp., *Saccharomyces* sp., *Cryptococcus neoformans* etc. either from periplasm or culture medium (Zhao et al. 2010; Younes and Rinaudo 2015). Catalytic elucidation stated that the CDA recognize a sequence of four GlcNAc units in chitin chain followed by deacetylation in any one unit, and hence the resulting chitosan has a more regular pattern of deacetylation than chitosan generated through alkali treatment (Zhao et al. 2010). These findings suggest enzymatic deacetylation on chitin is an attractive process that ensures production of novel chitosan in green route. However, paucity of well characterized CDAs and poor accessibility of CDAs in crystalline chitin are the main obstacles for its industrial exlploitation. Exploration of novel CDAs exhibiting unique properties is of prime importance which is believed to overcome the first limitation, whereas, mild pretreatment of chitin substrates before enzyme addition may enhance the deacetylation efficiency.

4.2.3 Production of Chitin/Chitosan Oligosaccharides (COS) and N-acetylglucosamine

Poor water solubility of high molecular weight chitin and chitosan at neutral pH values is a limitation for wide range of applicability, which can be resolved by using their oligomers or monomers. As like above, this conversion is also possible by chemical and biological route.

4.2.3.1 Chemical Production of COS and N-acetylglucosamine

Production of chitin/chitosan oligosaccharides (COS) from polymeric precursors have been conventionally achieved by digestion with strong acid such as HCl, and the acid-catalyzed degradation rate was depend on DD. Acid hydrolysis was highly specific to cleavage of glycosidic linkage between N-acetylglucosamine-N-acetylglucosamine (GlcNAc-GlcNAc) and N-acetylglucosamine-Glucosamine (GlcNAc-GlcN) with two to threefold higher rates than the Glucosamine- Glucosamine (GlcN-GlcN) and

Table 4.1 Few application of production of NAG and chitooligosaccharides by enzymatic treatment (by crude/purified chitinase) or by microbial fermentation

Microorganism	Raw material(s)	Product(s)	References
Enzymatic production			
Vibrio alginolyticus	Colloidal chitin	(N-acetyl glucosamine)$_{2,3,5}$	Murao et al. (1992)
Streptomyces griseus	Colloidal chitin	Hexamer	Terayama et al. (1993)
Burkholderia cepacia TU09	α-chitin of crab shells	N-acetyl glucosamine	Pichyanagkura et al. (2002)
Bacillus licheniformis SK-1	β-chitin of squid pens	N-acetyl glucosamine	Pichyangkura et al. (2002)
Aeromonas hydrophila H-2330	α-chitin	N-acetyl glucosamine	Sashiwa et al. (2002)
Bacillus subtilis W-118	Colloidal chitin	(N-acetyl glucosamine)$_{1–6}$	Wang et al. (2006a)
Paenibacillus illioisensis KJA-424	Colloidal chitin	(N-acetyl glucosamine)$_{1,2,3,7,8}$	Jung et al. (2007b)
Bacillus cereus TKU027	Chitosan	(N-acetyl glucosamine)$_{4–9}$	Wang et al. (2012)
Aeromonas sp. GJ-18	Swollen chitin	(N-acetyl glucosamine)$_2$ at 55 °C	Kuk et al. (2005a)
		(N-acetyl glucosamine) at 45 °C	
Vibrio anguillarum E-383a	Chitin	(N-acetyl glucosamine)$_2$	Lan et al. (2004)
Lecanicillium lecanii	Steam explored chitin	(N-acetyl glucosamine)$_{1–9}$	Makino et al. (2006)
Fermentative production			
Pseudomonas aeruginosa K187	Squid pen	(N-acetyl glucosamine)$_{1–5}$	Wang et al. (2010a)
Bacillus cereus TKU022	Shrimp head	(N-acetyl glucosamine)$_{2,4–6}$	Liang et al. (2012)
Bacillus cereus TKU027	Shrimp head	(N-acetyl glucosamine)$_{2–5}$	Wang et al. (2012)
Serratia sp. TKU020	Squid pen	(N-acetyl glucosamine)$_{1,2}$	Wang et al. (2009a)
Serratia ureilytica TKU013	Squid pen	(N-acetyl glucosamine)$_{1,2,5}$	Wang et al. (2009b)
Aeromonas hydrophila SBK1	Shrimp shell	(N-acetyl glucosamine)$_{1,2}$	Halder et al. (2013b)

Glucosamine-N-acetylglucosamine (GlcN-GlcNAc) linkages (Jung and Park 2014). The rate of depolymerization and deacetylation was depends on the concentration of acid and treatment temperature. Anciently, several companies prepared GlcNAc in commercial scale by a process based on the acid hydrolysis (15–36% HCl and at 40–80 °C) of crude chitin (Chen et al. 2010). Though acid hydrolysis was commonly adopted due to simplicity, factors like high cost and generation of waste (due to use of concentrated HCl) are the burning problems, and thus, alternative bioproduction of COS and N-acetylglucosamine is desirable.

4.2.3.2 Biological Production of COS and N-acetylglucosamine

Production of COS and N-acetylglucosamine either by enzymatic degradation of chitin/chitosan or microbial fermentation were theorized and practically realized.

4.2.3.2.1 Enzymatic Production

In the last few decades, enzymatic hydrolysis of chitin and chitosan for production of COS and GlcNAc has been proposed as a sustainable alternative. Enzymatic hydrolysis seems to be generally preferable over chemical methods due to mild reaction condition and controlled generation of COS of specific DP (Chen et al. 2010). Till date, various chitin/chitosan specific enzymes were reported to produce their oligo- and monomeric products, maximum of which are of microbial origin (Table 4.1). Chitinases and chitosanases, are two different group of glycoside hydrolase (GH). As per Carbohydrate-Active enZYmes (CAZy) database, chitinases belongs to GH18 and GH19 families, whereas chitosanases belongs to GH 5, 7, 8, 46, 75 and 80 families (Jung and Park 2014). Both chitinases and chitosanases are a group of enzymes takes part in several step of biodegradation of the polymer, and often called and chitinolytic and chitosanolytic enzymes, respectively. Analysis of the hydrolytic products suggested the different hydrolytic specificity of chitinases and chitosanases. The ensemble of chitinolytic enzymes comprises endochitinases (EC 3.2.1.14), exochitinases (EC 3.2.1.52), chitobiosidases (EC 3.2.1.30) and N-acetylglucosaminidases (EC 3.2.1.96) biosynthesize and secreted from both prokaryotes and eukaryotes. Usually, the endochitinases cleave chitin in random manner in internal sites of the polymer at the β-(1,4)-glycosidic bonds in between GlcNAc-GlcNAc, GlcN-GlcNAc, and GlcNAc-GlcN to release smaller and more soluble chitin oligomers of variable size. Exochitinases or chitobiosidases catalyze the progressive release of dimers from the non-reducing end of chitin. Finally, N-acetylglucosaminidases (NAGases) cleave the oligomeric and dimeric products of the two aforementioned enzymes to generate GlcNAc (Cohen-Kupiec and Chet 1998; Chen et al. 2010; Jung and Park 2014). On the basis of cleavage site, chitosanases can also be classified into endochitosanases and exochitosanases. Endochitosanases (EC 3.2.1.132) cleave a partly acetylated chitosan in random manner to produces COS, whereas exochitosanases (GlcNase, EC 3.2.1.165), releases GlcN residues from the non-reducing termini of the substrate. Though both of the group of enzyme capable to hydrolyze the glycosidic bond between GlcN-GlcNAc and GlcNAc-GlcN, chitinases have the unique ability to hydrolyze glycosidic bond between GlcNAc-GlcNAc (Fig. 4.3) (Uragami and Tokura 2006; Jung and Park 2014).

The presence, relative efficiency of chitinolytic/chitosanolytic enzymes in reaction mixture and hydrolyzing time are the important determinants which confirm the end product(s) of the chitinolysis/chitosanolysis. For instance, the production of chitooligosaccharides requires high levels of endochitinase and low levels of N-acetylglucosaminidase and exochitinase, whereas the production of GlcNAc

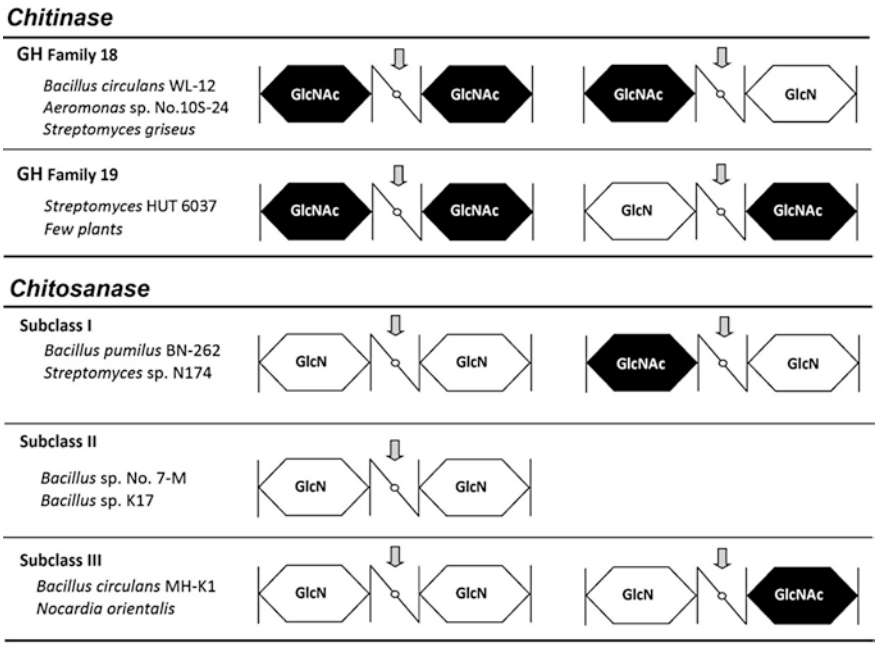

Fig. 4.3 Hydrolytic specificities of chitinase (GH 18 and 19) and chitosanase (subclass I–III). *GlcNAc* N-acetylglucosamine, *GlcN* Glucosamine (Adopted from Uragami and Tokura 2006; Thadathil and Velappan 2014 with modification)

requires higher proportion of exochitinase and N-acetylglucosaminidase. For example, ~100% of pure GlcNAc was produced by hydrolyzing with crude enzymes from *Aeromonas hydrophila* H2330 (Sashiwa et al. 2002). On the contrary, variation in thermostability of NAGase (inactive above 50 °C) and chitobiase (active above 50 °C) of *Aeromonas* sp. GJ-18 helps in selective preparation of GlcNAc and $(GlcNAc)_2$ from α-chitin (Kuk et al. 2005a, b). Likewise, purified exochitosanase of *Aspergillus oryzae* IAM2660 produced GlcN as a final product, whereas chitosan oligosaccharides over DP 7 were produced by endochitosanase purified from same organism (Zhang et al. 2000). Moreover, partial degradation of chitin/chitosan may generate COS with higher DP. Apart from the two, some non-specific enzymes viz. lysozyme, cellulase, pectinase, protease, lipase, pepsin etc. were also reported to generate COS and GlcNAc from chitin/chitosan or take accessory role (Jung and Park 2014). Another possibility of production of chitin oligosaccharides to chitosan oligosaccharides is by the treatment of chitin deacetylase. However, in some cases pre-treatment of chitin to convert crystalline form to colloidal or swollen form by chemical means was carried out before enzymatic treatment which increases the accessibility of the substrate towards the enzyme (Jung et al. 2007a, b; Thamthiankul et al. 2001; Jung and Park 2014).

4.2.3.2.2 Fermentative Production

When production of COS and GlcNAc are concern, microbial biotransformation is well suited as it minimizes the cost of enzymes production and subsequent downstream processing of the enzymes. Chitinase-producing microorganisms are found promising for bioconversion of chitinous substrate and concomitant production of enzymes and bioactive chitosaccharides, which in turn made the process favorable in terms of commerce and environment. To date, various bacterial and fungal strains were reported to produce chitinolytic enzymes belongs to the genera of *Aeromonas, Bacillus, Paenibacillus, Serratia, Enterobacter, Vibrio, Aspergillus, Trichoderma* etc. (Dahiya et al. 2006; Chen et al. 2010; Jung and Park 2014) (Table 4.1), from which few are practically exploited for COS and GlcNAc production. The sugars can be purified from the ferments by several well-known technique like chromatography, solvent extraction etc.

A flow chart of the production of chitin, chitosan, their oligosaccharides, momosaccharides from crustacean shells by chemical, enzymatic and by microbial activity is shown in Fig. 4.2 which summarize the aforementioned different perspectives. In the following section, cutting edge strategies for advancement of the biological process is discussed.

4.3 Advancement of Production Strategies

4.3.1 Transglycosylation

Specifically COS shows some unique property and bioactivity which neither be shown by its polymeric precursor or monomeric end products, and hence often desirable to produce in large extent for application. Through transglycosylation, COS with higher DP can be synthesize with starting materials of low DP, and the property is often found associated with some chitinolytic enzymes. For example, through transglycosylation property, purified chitinase from *Trichoderma reesei* KDR-11 was reported to convert $(GlcNAc)_4$ into $(GlcNAc)_2$ and $(GlcNAc)_6$ (Usui et al. 1990). Similar result was also reported by others.

4.3.2 Direct Enzymatic Production of COS and GlcNAc from Crustacean Shell

Owing their rigid crystal structure, direct degradation of chitin from crab and shrimp shells poses a substantial challenge due to high degree of crystallinity. In this context, chitinous crustacean shells can be pretreated by mechanical, mild chemical or mechanochemical means are reported fruitful in many cases. Steam explosion,

mechanochemical grinding, ultrasonication prior to enzymatic reaction are often employed. For instance, 11.28% reduction of the crystallinity index of native chitinous substrate was observed through steam explosion resulting in yield of chitin oligosaccharides (23.6%) having DP up to 5 during subsequent enzymatic treatment (Villa-Lerma et al. 2013). Direct production of GlcNAc and $(GlcNAc)_2$ from crab shell was reported by sequential execution of mechanochemical grinding and enzymatic hydrolysis (Nakagawa et al. 2011). However, extensive study is needed for its commercial large scale exploitation.

4.3.3 Exploration of New Enzymes with Novel Properties

In nature, crystalline chitinous crustacean shells are continuously generated through biological process, but all of them are mineralized by indigenous microorganisms that take part in chitin cycle. There are some factors which ease the degradation of the crystalline chitin. Therefore, finding of the factors will helps to understand the real facts involved in degradation of crystalline chitin to maintain the chitin cycle in nature and in turn provides knowledge which helps in precise handling of the process of COS and GlcNAc production. Some ancient study giving hints about the existence of substrate-disrupting factor which enhances the accessibility of crystalline substrate for hydrolytic enzymes (Reese et al. 1950), and in case of chitin metabolism, a chitin binding protein was reported to produce by chitinolytic bacterium *Serratia marcescens*. It acts as enzyme and catalyses the glycosidic bonds in crystalline chitin which in turn loosen the structure and ease the hydrolysis carried out by normal glycoside hydrolases (Vaaje-Kolstad et al. 2010). Now, it was well documented that chitinases often work synergistically with chitin-binding proteins (CBPs). Bacterial CBPs contain carbohydrate-binding modules of family 33 (CBM33) and sometimes family 5/12 (CBM5/12). CBM33s are thought to facilitate chitinase accessibility to crystalline chitinous matrices by introducing breaks in the chitin chains (Frederiksen et al. 2013).

Recently chitinolytic enzymes having stability in wide range of extreme condition like high salt, high temperature, acidic/alkaline condition, polar/non-polar solvent, surfactant were reported. These properties may accelerate the hydrolysis rate to produce COS and GlcNAc. A cocktail of several nonspecific hydrolytic enzymes like lysozyme, protease, cellulase, papain, pectinases, and hemicellulase with chitinase, chitosanase, CDA were reported to catalyze the breakdown of the glycosidic bond in chitin/chitosan not only pure form but also in native crustacean shells (Jung and Park 2014). However, compatibility of the enzymes with each other is the matter of major concern. To avoid the deadlock, sequential treatment of shrimp shell powder by immobilized crude proteases and chitinases were performed by us for generation of COS and GlcNAc (Halder et al. 2014).

4.3.4 Direct Biotransformation of Crustacean Shells to COS and GlcNAc

Perusal of literature attested that native crustacean shells can be utilized by several microorganisms like *Pseudomonas, Bacillus, Serratia, Aeromonas* as sole C/N sources and reasonable amount of GlcNAc and COS was accumulated in the production medium. For instance, *Serratia* sp. TKU020 and *Aeromonas hydrophila* SBK1 reported to produce GlcNAc and its dimer when squid pen and shrimp shell was supplemented in the production medium, respectively (Wang et al. 2009a; Halder et al. 2013b). Chitin oligosaccharides of DP 2, 4–6 were directly obtained after 2 days of fermentation of 1.5% shrimp head powder with proteo-chitosanolytic *Bacillus cereus* TKU022 (Liang et al. 2012). Due to differential activity of different chitin degrading enzymes, co-fermentation of crustacean shells with two or more chitinolytic/proteo-chitinolytic strains may be feasible for COS and GlcNAc biosynthesis.

4.3.5 Genetic and Metabolic Engineering

4.3.5.1 Overproduction of Chitinolytic Enzymes

Chitinases are inducible enzymes, and produces by microorganisms when chitinous sources are abundant in the environment. Hence, for microbial production of chitinase in the laboratory microenvironment, supplementation of chitin or chitin rich substrate is needed. Moreover, amount of the enzyme liberated by the microorganisms is not sufficient to fulfill the demand of chitinase for generation of COS and GlcNAc. On the contrary, majority of the microorganism reported to produce chitinases in large magnitudes are human pathogenic like *Vibrio, Aeromonas, Bacillus, Pseudomonas, Serratia* etc. To overcome the shortcoming, number of attempts has been made to clone and express chitinase genes from several organisms into *E. coli* (Dahiya et al. 2006; Yan and Fong 2015). During cloning, the chitinase genes are inserted into the expression-vector under the control of a strong (highly expressive) promoter, and apart from chitin, promoter-specific inducer (e.g. IPTG+X-gal for lacZ promoter) need to supplement in the medium to upregulate the expression of chitinase gene. Due to overwhelming production rate, recombinant chitinase production are now promising platforms for commercialization (Yan and Fong 2015).

4.3.5.2 Production of GlcNAc from Glucose

Upto that point, it is crystal clear that microbial production of GlcNAc from crustacean shells has several constains, and the Lion's share of that is the recalcitrant crystalline nature of crustacean shells. With the advent and advancement of

molecular biotechnology, genetic modification of microorganisms for production of GlcNAc and GlcN from glucose became possible. Specifically, the change in the metabolic route can be done by genetic engineering techniques in order to increase enzyme activities, reduce product inhibition, overexpress proteins and increase the substrate affinity (Chen et al. 2010). By overexpressing the GlcN-6-phosphate acetyltransferase, GlcN-6-phosphate synthase and GlcN-1-phosphate acetyltransferase gene and concomitant suppressing of the GlcN-6-phosphate and GlcNAc-1-phosphate uridyltransferase gene, a recombinant *E.coli* strain was produced which capable to biosynthesize high concentration of GlcNAc (exceeding 120 g/L) after 60 h of fermentation. Additional genetic modifications to increase glutamine synthetase, phosphoglucoisomerase and glucose 6-phosphate dehydrogenase activities and inactivation of phosphofructokinase and genes for glycogen synthesis enzymes was also made in the strain to control the flux of metabolites. The strain produces GlcNAc from glucose whereas fructose is supplemented in the medium acts as energy source for bacterial growth (Deng et al. 2005; Chen et al. 2010). However, this approach is completely divergent from the theme of valorization of crustacean shell.

4.3.5.3 Improving Chitinase Property by Directed Evolution

From industrial perspectives, naturally occurring enzymes are not well-suited due to the difference between the magnitude of chitinase production in by the microorganisms in cellular microenvironment and market demand. Proficient and real-world utilization of chitinases for degradation of crustacean shells can be achieved after improving the yield and catalytic activity (Rathore and Gupta 2015). The yield enhancement approaches are already discussed. To improve the catalytic activity, protein engineering of enzyme is now in the limelight. Protein engineering can be done either by site directed mutagenesis or by directed evolution. In case of site directed mutagenesis, amino acid residues residing specifically in the chitin binding and catalytic sites are mutated. Whereas, in directed evolution approach, chitinase can be randomly mutated via error-prone polymerase chain reaction to generate a mutant library, identification of variants having improved target properties, and repeating the whole process until accomplishing the desired function (Rathore and Gupta 2015; Yan and Fong 2015). The technology is just in the embryonic stage; both the approaches are very much promising and effective in few cases. Researchers are very much optimistic about comprehensive acceptability of the approaches for improvement of catalytic activity of different protein and enzymes including chitinolytic enzymes.

4.3.6 Reactor Systems

Commercialization of any microbial enzyme or products needs the optimization of all physico-chemical condition in laboratory scale followed by its scale up. In this regard, the reactor systems are considered to support high yield of the desired

product(s). Employment of different types of reactor system for production of chitin, chitinase, COS and GlcNAc are documented. For instance, chitin production by *Lactobacillus plantarum* 541 through fermentation of shrimp shell waste in a drum reactor was reported (Rao and Stevens 2005). Production of chitinase by continuous mode of fermentation by using *Paenibacillus* sp. CHE-N1 in a membrane bioreactor was studied utilizing crab shell chitin powder as substrate (Kao et al. 2007). Production of chitinase by *Verticillium lecanii* in 5 l stirred tank and 30 l airlift bioreactor was also investigated (Liu et al. 2003). Enzymatic production of chitooligosaccharides (predominantly DP 3–6) from chitosan using an ultrafiltration membrane reactor (Jeon and Kim 2000a) or combination of ultrafiltration membrane reactor and column reactor packed with immobilized enzyme (Jeon and Kim 2000b; Santos-Moriano et al. 2016) were carried out. Similarly, continuous production of GlcNAc from chitin by *Serratia marcescens* QM B1466 was described involves five essential unit operations: the fermentation unit, the enzyme recovery unit, the pretreatment of the chitin substrate, the two-stage chitin-hydrolysis reactor and the final purification unit (Louise et al. 1999). We also demonstrated concomitant production of chitinolytic enzymes, GlcNAc monomer and dimer by potent chitinolytic *Aeromonas hydrophila* SBK1 in jar bioreactor utilizing shrimp shell powder as substrate (Halder et al. 2013b).

4.4 Applications of Chitin, Chitosan, Chitooligosaccharides and N-acetylglucosamine

Since after Muzzarelli's description in 1977 regarding the properties of chitin in wounds of human, a lot of research has been commenced throughout the globe. Due to their unique properties chitin, chitosan, chitooligosaccharides and N-acetylglucosamine have multifaceted dynamic applicabilities, such as in tissue engineering, food processing, nutrition, water detoxification, paper finishing, drug delivery, growth promotion for plants and animal, fat and cholesterol trapping, haemostasis, wound and bone healing, antibacterial therapy, immobilization (as matrix), anti-inflamation, immune enhancement and in lots of other biomedical issues (Ravi Kumar 1999; Guibal 2004; Rinaudo 2006; Kurita 2006; Xia et al. 2011; Kumirska et al. 2011; Karagozlu and Kim 2014). The rationale of some potent bioactivities with regards to the degree of acetylation and molecular weight was represented in Fig. 4.2. In the following sections biological and biomedical implications, applications and prospects of chitin, chitosan, chitooligosaccharides and N-acetylglucosamine are discussed:

4.4.1 Biological Activities

4.4.1.1 Antimicrobial

Independent studies by various researchers attested that chitin, chitosan and chitooligosaccharides are potential natural antimicrobials and the antimicrobial activity depends on various intrinsic and extrinsic factors viz. amino contents at C-2 position, degree of polymerization, degree of deacetylation, and the effective dose varied probably due to alterations in the experimental methods, structural complexity, pH and type of organism like bacteria, yeasts and fungi (Kurita 2006; Aam et al. 2010; Xia et al. 2011; Kim 2010). Due to better solubility and free amine group chitosan imparts better antimicrobial activity than chitin. It was also established that lower molecular weight chitosan (<10 kDa) has a greater antimicrobial activity. On the contrary, to impart antimicrobial activity of chitooligosaccharides, minimum DP of 7 is required (Kim 2010).

The antimicrobial activity of the oligomeric or polymeric aminosugar has been considered to be one of the utmost important properties corresponding to their possible biological applications. There are various postulates regarding the mechanism of antimicrobial activity of chitin/chitosan or COS which are supported experimentally. Being aminosugar they electrostatically interact with membrane, altered the permeability of the same leads to internal osmotic imbalances, and as a consequence of which microbial growth inhibited. Alternatively, they may hydrolyze the murine layer of cell wall of bacteria leading to the outflow of intracellular electrolytes and low molecular weight constituents. Another proposed mechanism suggested that the aminosugar may get entry into the cell and made complex with DNA by binding at major/minor grooves of microbial genomic DNA leads to the inhibition of transcription and translation process. In addition, the chitin/chitosan is an effective chelator which binds with essential metals and nutrients (Kim 2010; Halder et al. 2014). The amine groups are responsible for the chelation with metal cations at pH >6 when the electron pair of unprotonated amine groups is available for binding with positive ions.

4.4.1.2 Anti-inflammation and Immunostimulation

Perusal of literature regarding the bioactivity of chitin, chitosan and their derivatives advocating their immuno-enhancing anti-inflammatory potential which will be thought-provoking to improve health status and in developing remedies for preventing or treating several chronic diseases such as periodontal disease, asthma, arthritis, hepatitis, colitis, gastritis, atherosclerosis etc. Different study separately advocating the immunostimulatory and anti-inflammatory activity of chitin/chitosan and COS in induced animal models. For instance, chitosan (70%-deacetylated) activate macrophages and natural killer cells and improve the delayed-type hypersensitivity, increase cytotoxicity and induce mitosis of interleukins producing cells,

breeding factors and interferon (Nishimura et al. 1984). Chitin was reported to up-regulate the expression or production of TNF, TLR2, dectin-1, mannose receptor, inflammatory cytokines, differentially activated NF-κB, spleen tyrosine kinase, IFN-γ (Da Silva et al. 2009; Shibata et al. 1997a, b), whereas chitosan partially attenuate the secretion of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) from mast cells helps to minimize the allergic inflammatory response (Kim et al. 2005). Alongside, the efficacy of chitosan as a novel adjuvant for an inactivated influenza vaccine was documented where the antibody content in serum and the antiviral defense remarkably increased (Chang et al. 2004).

Besides the polymeric precursors, chitin/chitosan oligosaccharides also play a pivotal role in the said purpose. Chitooligosaccharides are reported to encourage the migration of macrophage at the inflammation site (Okamoto et al. 2003a, b; Mori et al. 2005; Moon et al. 2007), inhibits the secretion of lipopolysaccharide (LPS)-stimulated IL-6 and TNF-α in macrophage (RAW 264.7) cells (Yoon et al. 2007), whereas chitin oligosaccharide (MW 229.21–593.12 Da) of crustacean (crab) origin inhibit the activity of myeloperoxidase in human myeloid cells and the oxidation of DNA and protein (Ngo et al. 2009).

4.4.1.3 Antioxidant

Generation of reactive oxygen species (ROS) viz. superoxide anion radicals, hydroxyl radicals, hydrogen peroxide etc. is an unavoidable consequence during aerobic respiration, metabolic processes or resulting from stress imposed by a range of diseases. Increasing evidence advocating their involvement in various diseases and aging processes due to their detrimental consequence on bio-macromolecules like DNA, protein and lipid (Kim and Rajapakse 2005; Uttara et al. 2009; Halder et al. 2014). Free radical scavengers are preventive antioxidants which can break the oxidative sequence either by delaying or preventing oxidant generation, competing for pre-existing radicals and eliminating them from the reaction, and therefore protecting biomolecules from oxidative damage (Halder et al. 2014). Despite the availability of synthetic antioxidant, there has been an increasing interest in exploring natural antioxidants from different sources for dietary, pharmacological and cosmetic uses. In this context, chitin, chitosan, chitooligosaccharides as well as N-acetylglucosamine were separately proven to have antioxidant potentiality both in *in vitro* and *in vitro*. The *in vitro* antioxidant activity was evaluation in terms of scavenging of radical of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, peroxyl, superoxide as well as singlet oxygen-quenching, ferrous ion-chelating, reducing, lipid peroxidation inhibiting properties, either in single or combination by various researchers. Moreover, *in vitro* experiments with various biomolecules (like DNA, protein, RBC etc.) pre-exposed with potent oxidant confirmed their preventive role against oxidative stress (Halder et al. 2014; Qingming et al. 2010).

Chitosan with varied DD (50, 75, 90%) generated from crab chitin showed antioxidative activity revealed through electron spin resonance (ESR) spectroscopy

(Park et al. 2004a, b). Yen et al. (2008) reported paramount antioxidant activity of chitosan prepared by alkaline N-deacetylation of crab chitin. Chitosan recovered from shrimp waste exhibited antioxidant potentiality in *in vitro* systems including DPPH radical scavenging, total reducing and lipid peroxidation inhibition property (Trung and Duy Bao 2015). Various *Serratia* strains while growing by utilizing crustacean wastes produces antioxidant compounds in the medium which is correlated with the generation of chitooligosaccharides and N-acetylglucosamine (Wang et al. 2009a, b, 2010a, b). Chitooligosaccharides (MW 229.21–593.12 Da) produced from crab shell showed cellular antioxidant effects by inhibiting myeloperoxidase activity, decreased free-radical mediated oxidation of DNA and membrane proteins and also uphold the intracellular GSH levels (Ngo et al. 2008). N-acetylglucosamine and chitobiose produced in shrimp shell waste containing medium of *Bacillus cereus* EW5 after 4 days of incubation which contribute antioxidant activity of the culture supernatant (Azam et al. 2014). N-acetylglucosamine and chitooligosaccharides of varied DP generated either by enzymatic treatment or fermenting shrimp shells showed paramount antioxidant activity and also helps to upregulate the indigenous antioxidant defense system (catalase and superoxide dismutase level) of rats *in vitro* (Halder et al. 2013a, b, 2014).

4.4.1.4 Human Blood Hemostasis

Recently, hemostatic effects of chitin, chitosan, and their hydrolytic/derivative products have been reported with their interactions with the key hemostatic factors, platelet aggregation and blood coagulation factors (Zhang et al. 2010; Kim 2010). Chitosan (MW = 50 kDa, DD >90%) dose-dependent increment of platelet aggregation with raised intracellular Ca^{2+} level and upregulation of expression of glycoprotein IIb/IIIa complex on platelet surface was validated through flow cytometry (Chou et al. 2003). In a dose-dependent manner, chitin (MW = 300 kDa, DD <10%) and chitosan (MW = 80 kDa, DD >80%) reduced blood coagulation time significantly with enhanced secretion of the transforming growth factor-β1 (TGF-β1) as well as the platelet-derived growth factor-AB (PDGF-AB) from the platelets, predominantly with chitosan (Okamoto et al. 2003b). Even, chitosan treatment is proved to be effective to bring the bleeding time in normal range in therapeutically anticoagulated (heparinized) rabbit model (Ishihara et al. 2002). Based on the outstanding hemostatic activity, lots of chitin and chitosan based derivatives with different physical, chemical or spatial structures have also been explored (Zhang et al. 2010).

4.4.1.5 Antihypertensive Actions

Hypertension is also known as high blood pressure (>140/90 mmHg over a number of weeks) is one of the commonest cardiovascular diseases and has become a worldwide problem as like as epidemic (Zhang et al. 2006; Kim 2010). Unwarranted activity of renin–angiotensin system is believed to increase blood pressure by the

overproduction of angiotensin II in which angiotensin-converting enzyme (ACE) plays pivotal role. Experiment on animal and human model governed that diet containing high level of chlorine salts (e.g. NaCl) responsible for hypertension (Boon and Aronson 1985). Supplementation of chitosan with high-NaCl diet for 'normotensive and stroke-prone spontaneously hypertensive' rats significantly lowered systolic blood pressure, the level of serum ACE in 'stroke-prone spontaneously hypertensive' group as well as and serum chloride ion in the 'normotensive' group (Okuda et al. 1997). Likewise, oral administration of chitosan normalizes the increment of both systolic blood pressure and ACE in human. One chitin is derivative namely aminoethyl-chitin (DD 50%) efficiently minimizes systolic blood pressure of 'spontaneously-hypertensive' rats in concentration-dependent manner (Je et al. 2006). Owing their better solubility, attempts were made to evaluate the same activity using chitooligosaccharides. Oral administration of COS of varied DP (1–6) in spontaneously hypertensive rats suggested that trimer have comparatively better potential to inhibit the level of ACE (Hong et al. 1998). These effects may be due to cationic nature of the polymer/oligomer which forms complexes with chloride ions consequently reduces ACE activity (Kim et al. 2010). Degree of deacetylation and molecular weight are the two vital determinant of that activity (Park et al. 2003).

4.4.1.6 Antitumor and Anticancer Activity

The uncontrolled cell proliferation due to physical and environmental factors, microbial interaction and genetic defects can leads to onset of cancer. Antitumor activity of COS was known since 1970s and the evidence of their positive effect on reducing metastasis from tumors was established thereafter and now well-documented. They were found to induce acquired immune response by accelerating T-cell differentiation to escalation cytotoxicity as well as maintain T-cell activity in cancerous mice, and thus exert antitumor activity (Suzuki et al. 1986). COS also found to enhanced the natural killer activity in intestinal intra-epitelial lymphocytes as well as reduced tumor growth in mice (Maeda and Kimura 2004). Highly charged COS derivative reported to reduced cancer-cell viability significantly through necrosis in cancerous cell lines (Huang et al. 2006). In order to establish the relation of MW on antitumor/anticancer activity, chitosan oligosaccharides with different MW was studied and oligosaccharide of 1.5–5.5 kDa effectively suppress the growth of Uterine cervix carcinoma No. 14 (U14) or Sarcoma 180 solid (S180) tumor in BALB/c mice (Jeon and Kim 2002), however high molecular weight COS was found to induce apoptosis of human hepatocellular carcinoma cell by stimulating the production of pro-apoptotic protein Bax (Xu et al. 2008) and also inhibiting angiogenesis (Wang et al. 2007; Xiong et al. 2009). On the other hand, chitosan was found to trigger lymphokines production resulting in cytolytic T-lymphocytes proliferation (Dass and Choong 2008), inhibit tumor cell proliferation by inducing apoptosis via caspase-3 activation (Hasegawa et al. 2001) and decrease glucose uptake and ATP pool in the tumor cells (Guminska et al. 1996).

Comparative analysis of antitumor activity of chitin (338,000 Da), chitosan (12,000 Da) and low molecular weight chitin (2480 Da) was performed and the latter was found most promising to exert antitumor activity in tumor cell line (Salah et al. 2013). Apart from natural form of chitin, chitosan and chitooligosaccharides many derivatives and nanoparticle of them are made which impart significant antitumor or anticancer potential. Injectable *In Situ* chitosan based hydrogels for lung cancer (azide–chitosan–lactose), breast cancer (chitosan/β-glycerophosphate), fibrosarcoma (chitosan/β-glycerophosphate), cervical cancer (chitosan/β-glycerophosphate), mucin-production associated cancers (chitosan/glyceryl monooleate), osteosarcoma (chitosan/dibasic orthophosphate) were already documented (Obara et al. 2005; Ruel-Gariepy et al. 2004; Berrada et al. 2005; Han et al. 2007; Jauhari and Dash 2006; Ta et al. 2009). Study on effect of chitin, chitosan and irradiated chitin originated from *Parapenaeus longirotris* shell showed dose-dependent cytotoxic effect against Human larynx carcinoma and Human embryo rhabdomyosarcoma cell lines indicating the electrostatic interactions between the negative charges of tumor cells groups and the positive charges of the material tested and interactions between hydrophobic groups and tumor cells derived molecules (Bouhenna et al. 2015). Though the antitumor activity was believed to be attributable to the cationic properties and MW, the exact molecular mechanism is still under investigation.

4.4.1.7 Antidiabetic Effect

Obesity and diabetes are two foremost important correlated health status increasing morbidity and mortality rate all over the world. Diabetes in clinical diagnosis usually accompanies the symptom of hypercholesterolemia with hyperglycemia increases the threat of stroke, heart attack and renal failure (Dieterle et al. 2006). Due to global burden, many antidiabetic medicines from natural origin have been developed/formulated. Despite the potential in the treatment of diabetes most of these natural antidiabetic agents not suited for large scale production to meet the demand and henceforth cannot be considered as pharmaceutical agent (Karadeniz and Kim 2014). In this context, chitin, chitosan, chitooligosaccharides and their derivatives shown reasonable antidiabetic and hypocholesterolemic activity either directly or indirectly along with the possibility of huge turnover from natural low cost crustacean waste made them potential candidate for the same. Chitosan was found effective against both diabetes models: type 1 (insulin-dependent or childhood-onset diabetes) and type 2 (noninsulin-dependent or adult-onset diabetes). Food containing 5% chitosan responsible for maintaining consistent blood glucose level as well as lower the lipid level in both normal and neonatal streptozotocin (STZ)-induced diabetic mice (Miura et al. 1995).

The results of Ito et al. (2000) and Kondo et al. (2000) is also in confirmed that low molecular weight (LMW) chitosan prevent the progression of low-dose STZ-

induced diabetes. In a separate study, Hayashi and Ito (2002) proved that LMW chitosan lowered the serum glucose levels and improved overdrinking and polyuria observed in genetically obese diabetic male KK-A^y mice. Whereas, administration of high molecular weight (HMW) chitosan (0.8%) in daily basis reduced the consumption of drinking water, non-fasting serum glucose, urine glucose, urine volume and serum triglyceride levels in STZ-induced type-1 diabetic mice model (Do et al. 2008). In a related study, 3-month administration of chitosan significantly increased insulin sensitivity in obese patients as well as decreased body weight and triglyceride levels (Hernandez-Gonzalez et al. 2010). Recent study revealed that anti-obesity activity of chitosan is associated with PPAR-γ pathway of adipogenic differentiation (Karadeniz and Kim 2014). As like as chitosan, COS also inhibit the fat accumulation and adipogenesis in 3T3-L1 cell line (Cho et al. 2008). Enough proof is available regarding the protective effect of COS for pancreatic beta-cells against high glucose-dependent cell deterioration and as well as upregulate the proliferation of pancreatic islet cells with higher insulin secretion to lower blood glucose levels (Karadeniz et al. 2010).

4.4.1.8 Hypocholesterolemic Effect

Many independent line of evidence suggested that chitosan and chitooligosaccharides can lower plasma and liver triacylglycerol (TG), total cholesterol (TC) levels and increases fecal neutral steroid and bile-acid excretion endorsing hypocholesterolemic and hypolipidemic effects (Cho et al. 1998; Fukada et al. 1991; Sugano et al. 1980; Razdan and Pettersson 1996; Xia et al. 2011; Halder et al. 2013a). *In vitro* study shown that fat-binding activity is concomitantly increase with DD and MW, bile-salt-binding capacity was greatly influenced by the viscosity-average MW. No such correlation is found regarding cholesterol-binding capacity rather affected by the particle size (Liu et al. 2008a, b; Zhou et al. 2006; Xia et al. 2011). Chitosan supplemented diet lowered the level of plasma triglyceride (PTG), total cholesterol (TC) and low-density-lipoprotein cholesterol (LDL-C) as well as elevated high-density-lipoprotein cholesterol (HDL-C) levels, and the activity is inversely proportional with increasing DD (Li et al. 2007; Xia et al. 2011). We established that indices like triglyceride (serum and liver), total cholesterol (serum and liver), serum LDL-cholesterol are significantly decreased in rat feed with cholesterol rich diet supplemented with 10% deacetylated chitin or 10% shrimp shell hydrolysate containing huge amount of chitooligosaccharides (DP 1–30) with respect to cholesterol rich diet group (Halder et al. 2013a). The hypocholesterolemic activity of chitosan and COS may be owing to the electrostatic attraction between cationic amino group of chitosan and anionic elements such as fatty acids or bile acids (Xia et al. 2011). Altogether, the hypocholesterolemic effect is more pronounced with finer particle size as well as relatively high DD.

4.4.2 Biomedical Applications

4.4.2.1 Drug Delivery

Chitosan is natural cationic polymer derived from chitin has received growing attention mainly due to their biodegradable, biocompatible, non-toxic, mucoadhesive nature and ability of target specific delivery (Patel et al. 2010; Saikia et al. 2015). Since last 25 years, substantial amount of work has been documented on potential of chitosan in drug delivery systems. The primary amine groups and hydroxyl of chitosan allows controlled drug release, mucoadhesion, *in situ* gelation, enhanced permeation, transfection, and efflux pump inhibition (Bernkop-Schnürch and Dünnhaupt 2012). They are frequently used as matrix making material in both nano and micron-sized particles that improve the performance and effectiveness of encapsulated macromolecules or low molecular weight drugs. Water-soluble chitosan found much more effective than water insoluble chitosan as the latter one required acidic solution to dissolve which may be a great obstacle for application of DNA, protein/peptide based drug, and anticancer drugs due to their instability in acidic solution (Kim 2010). In order to improve the aforementioned properties, several modifications (viz. pegylation, carboxymethylation) of chitin or chitosan was performed (Jeong et al. 2008; Chen et al. 2004). It was well-documented that chitosan as solution and carrier, can transiently open epithelial tight junctions, resulting in the enhancement of permeation of macromolecules through well-organized epithelia like the nasal, ocular, intestinal, buccal and pulmonary (Azuma et al. 2015). The transfection efficiency of low molecular weight chitosan is increased with increasing molecular weight due to high positive charge (Jang et al. 2007).

4.4.2.2 Vaccine Delivery

Most of the vaccination strategies suffer from the problem of low antibody titer due to weak immune response. Hence these strategies require the coadministration of either some immunoadjuvants or antigen encapsulated within a carrier system, which may serve as an adjuvant as well as helps in controlled delivery of antigen to the cells involved in immune responses (Kim 2010). As like as its immense applicability in drug delivery, chitosan is also notable for its pragmatic applications as immunoadjuvant in the field of vaccine delivery owing its intrinsic immune stimulating activity and bioadhesive properties and assist in the permeation of the antigen by opening tight junctions (Kim 2010). Evidence of encapsulation of antigen like ovalbumin, diphtheria toxoid, tetanus toxoid in chitosan micro/nanoparticles in recent times ensure controlled vaccine delivery through all major routes of administration viz. pulmonary, subcutaneous, oral and nasal, etc., and trigger the establishment of both humoral and cellular immunity (Jain et al. 2006; Kim 2010). Application of chitosan and chitooligomer as potential adjuvants for influenza vaccine is also documented (Hiep et al. 2008; Illium et al. 2001; Read et al. 2005). An

array of related study showed that chitosan in both soluble and particulate system improves antigen uptake by mucosal epithelial cells, thereby permitting vaccine admittance to subepithelial antigen-presenting cells (APC) and as a consequence increasing local immune responses (Esmaeili et al. 2010). As the polymer displaying adjuvant properties also, chitosan and various chitosan based derivatives are used for vaccine delivery, singly or in amalgamation with other polymers. Ionic interactions between positively charged primary amine of chitosan and negatively charged phosphate backbone of DNA protects the latter from nuclease degradation and hence found effective in DNA based vaccine delivery (Bivas-Benita et al. 2004; Esmaeili et al. 2010). Similar interaction between chitooligosaccharides and DNA was also revealed from our previous study (Halder et al. 2014).

4.4.2.3 Gene Therapy and Gene Silencing

Through gene therapy, human diseases or disorders can be treated by transferring the therapeutic genes into specific cells/tissues of patients in order to correct/supplement defective, causative genes. As obvious, the success is depends on the efficiency of delivery of the therapeutic gene at the target site. Several shortfalls of viral based vectors (safety, non-tissue-specific transfection) as well as non-viral gene transfer system (low tissue specificity, transient gene expression) are the current huddle of the expansion the scope of gene therapy. In this context, chitosan and chitooligosaccharides were proved as efficient DNA/small interfering RNA (siRNA) delivery system (Shi et al. 2011). Stable complex between chitosan and nucleic acid materials helps in the administration of genes to mucosal cells and tissues such as the lungs (Köping-Hoggard et al. 2001) and intestinal epithelium (MacLaughlin et al. 1998; Roy et al. 1999), as aforementioned. Due to partial solubility of high MW chitosan, water soluble chitooligosaccharides was used in the said purpose and found promising (Köping-Höggård et al. 2003, 2004). Employment of chitosan and its derivatives have attracted attention in the field of gene silencing through siRNA delivery. For instance, inhibition of respiratory syncytial virus (RSV) replication in A549 cells (Zhang et al. 2005), silencing of EGFP expression in H1299 human lung carcinoma cells (Howard and Kjems 2006) were reported through chitosan based siRNA delivery. Lots of *in vivo* trial was already carried out successfully which may open a new era of therapeutics.

4.4.2.4 Wound Healing

Chitin, chitosan and their derivatives with biological, antimicrobial, hemostatic, anti-inflamatory, non-toxic properties are found to accelerate the healing processes at molecular, cellular and systemic levels. Chitin and chitosan based gels and films and their role as wound-dressing/healing material in cartilage repair, nerve regeneration was well-documented (Yusof et al. 2003; Muzzarelli 2009). As like chitosan, similar positive effects have been documented for chitooligosaccharides. For

instance, enhanced migration of polymorphonuclear cells and induce persistent release of IL-8 was noticed by chito-hexamer treatment (Aam et al. 2010). In this context, it was reported that monomers, oligomers, chitin (GlcNAc/N-acetylated COS/chitin) and chitosan (GlcN/fully deacetylated COS/chitosan) enhances the wound break strength and the increases the collagenase activity in wound (Minagawa et al. 2007). It was also believed that, apart from the unique and versatile biocompatible properties, wound healing ability of these compounds is highly encouraged by its ordered tissue-like structures (Muzzarelli 2009; Aam et al. 2010).

4.4.2.5 Tissue Engineering, Joint Damage and Bone Regeneration

Polymeric nanofibers that have the ability to mimic the natural extracellular matrix both structurally and functionally are of prime importance in tissue engineering as scaffold (Jayakumar et al. 2010). Several injectable preparations based on chitosan and its derivatives were employed in osteogenic bone substitution and tissue engineering. Surgically produced in sheep and rabbit having bone defects have been treated with modified chitosans in freeze-dried form. Being cytocompatible, chitin nanofibers endorsed attachment and spreading of human fibroblasts and keratinocytes (Noh et al. 2006). For instance, the chitin/PGA fibers with bovine serum albumin coating was considered as a noble tissue engineering scaffold, whereas chitin/SF fibrous mats encourages highest spreading of normal human epidermal fibroblasts and normal human epidermal keratinocytes (Jayakumar et al. 2010). Similarly, chitosan/PEO nanofibrous scaffolds endorsed the attachment of human osteoblasts and chondrocytes consequently maintained characteristic cell morphology and viability (Bhattarai et al. 2005). The chitosan sponges incorporating PDGF encouraged new bone formation in rat with calvarial defect (Park et al. 2000). Chitosan has the potentiality to encourage osteogenic progenitor cell recruitment and attachment consequently facilitating bone formation (Kim et al. 2002). The chitosan oligomer and the monomer were reported to enhance the expression of the BMP-2 mRNA (Kim 2010).

After 9 months of implantation of fully deacetylated cotton-like chitosan into the alveolar bone cavities, the same almost completely filled with the newly formed bone tissue which recognized the merit of chitosan in wound healing (Kim 2010). Preparation of bioactive chitosan-hydroxyapatite nanofibrous scaffolds significantly stimulated bone formation capability (Jayakumar et al. 2010). On the other hand, GlcNAc containing preparations delivered by parenteral, oral, transmucosal and topical route significantly enhances the prevention of joint damage (Talent and Gracy 1996; Tamai et al. 2003; Chen et al. 2010). In the perspective of bone damage repair, bone regeneration was reported when chitin–hydroxyapatite composite loaded with mesenchymal stem cell-induced osteoblasts implanted into bone defects of rabbit femur (Ge et al. 2004). Chitin hexamers or $(GlcNAc)_6$ reported to promote

osteogenesis in mesenchymal stem cells (Lieder et al. 2012). All the above examples authorize the application of chitin based materials in tissue engineering, wound healing and bone regeneration.

4.4.2.6 Stem Cell Technology

For therapeutic purpose large numbers of stem cells are required, and it is believed that chitin may encourage the self-renewal as well as differentiation of the stem cells (Wan and Tai 2013). *In vitro* expansion of pluripotent stem cells by application of chitin was documented (Lu et al. 2012). Besides stem cells expansion, chitin-based microfibrous scaffolds can be used effectively for stem cell differentiation when all the necessary signals are provided (Wan and Tai 2013). Many other applications of chitin based materials in stem cell research are in pipeline, which may open new vista in the arena of regenerative medicine.

4.4.2.7 Dental Applications and Hair Care

Several studies have been conducted to ascertain the application of chitin based materials in dentistry. Owing their incredible bioactivities, chitin-based materials have been explored extensively for widespread dental applications like oral drug delivery, tissue regeneration, as dentifrices, enamel repair, adhesion and dentine bonding etc. (Husain et al. 2017). Being positively charged, chitosan prevent plaque formation. Recent studies have demonstrated that chewing the chitosan oligomer-containing gum effectively inhibited the growth of the cariogenic and periodontopathic bacteria (*Porphyromonas gingivalis*) in saliva (Hayashi et al. 2007). These findings strongly suggested that the application of chitosan is useful for oral hygiene. Chitosan based polyherbal toothpastes found promising in oral care as chitosan inhibits the growth of *Streptococcus mutans* and *Porphyromonas gingivalis*; microorganisms responsible for caries and gingivitis, respectively (Mohire and Yadav 2010). Alongside, due to several taught bioactivities, chitosan based preparation is also used in hair care (Rinaudo 2006).

4.4.2.8 Treatment of Inflammatory Bowel Disease

Due to colossal anti-inflammatory activity, GlcNAc was reported to restoring the protective structure of gastrointestinal tract through enhancement of the release of acid mucopolysaccharides by fibroblasts (Karzed and Domenjoz 1971; Chen et al. 2010). GlcNAc was also helps to increases increase in arterio-capillary blood flow (by enhancing the elasticity of perivascular tissue) and also imparted cytoprotective role for restoring the integrity and normal physiological function of the mucous

membrane in humans and thus preventing individuals from inflammatory bowel disease (Burtan 1998; Burtan and Freeman 1993; Chen et al. 2010). Through a pilot scale clinical trial recognizable improvement of children with symptomatic Crohn's disease was documented by administration of GlcNAc through oral or rectal route (Salvatore et al. 2000).

4.4.2.9 Other Applications

Apart from biological and biomedical appliances, chitin, chitosan, chitooligosaccharides and N-acetylglucosamine have other diversified employment in industrial sectors. For instance, in food and beverage industries, they are used as fruit juice clarifier, stabilizer, antioxidant and also for preservation of foods from microbial deterioration. In agriculture, chitin and chitosan derivatives reported to induces the production of secondary metabolites, enhancing crop production, enhancement of fruit shelf life etc. (Kim 2010). Highlights of other notable applications are given in Table 4.2.

Table 4.2 Some applications of chitin, chitosan, chitooligosaccharides and N-acetylglucosamine

Field	Specific use/role	References
Agriculture	Plant defense, enhancement of plant growth and crop production, seed coating, anti-freeze activity	Arbia et al. (2013); Kim (2010); Muzzarelli (1997); Rinaudo (2006); Wang et al. (2008); Younes and Rinaudo (2015)
	Plant elicitor	
	Upregulation of chitinase and glucanase production (increased response against pathogen attack as pathogenesis related protein)	
	Stimulation of chitinolytic activity in compost	
	Antifungal agent and biopesticide	
	Improving plant vitality and ability to digest fungal cell wall	
	Fertilizer	
	Growth promoter of *Rhizobium* sp. (nodulation factor)	
	Enhancing biocontrol efficiency of plant growth-promoting rhizobacteria	
	Production of secondary metabolites	
	Enhancement of fruit shelf life	
Water and wastewater treatment	Flocculant to clarify water (drinking water, pools)	Arbia et al. (2013); Felse and Panda (1999); No et al. (1996); Rhazi et al. (2002); Rinaudo (2006); Shahidi et al. (1999)
	Removal of metal ions including heavy metals, radioactive waste	
	Treatment of sludge and biological denitrification	
	Antimicrobial agent, reduce odors	

(continued)

Table 4.2 (continued)

Field	Specific use/role	References
Food and beverages	Bind lipids (reduce cholesterol), acts as hypolipidemic and hypocholesterolimic agent (slimming agent)	Arbia et al. (2013); Kim (2010); Rinaudo (2006); Yen et al. (2009)
	Filtration, fruit juice/beverage clarification	
	Antioxidant (scavenger of free radicals) and nutraceuticals	
	Preservative, thickener and stabilizer for sauces/fruit juice	
	Protective against fungal infection, antibacterial coating/wrapping	
	Bioconversion for the generation of value-added food stuffs	
	Phenolic compound adsorption	
	Chitosan hydrogels for cell immobilization and for pigment encapsulation	
	Iron extractor to prevent bad odors in cooked meat	
	Prebiotics (dietary fibers) ingredients	
Cosmeceuticals	Skin rejuvenating agent	Arbia et al. (2013); Felse and Panda (1999); Kim (2010); Rinaudo (2006)
	Ingredients for hair and skin care (moisturizer)	
	Skin toning, acne treatment	
	Improve suppleness and reduce static electricity of hair	
	Oral care (ingredient of toothpaste, chewing gum)	
Paper	Confer strength to paper against moisture	No et al. (1996)
	Biodegradable packaging for food wrapping	
	As antimicrobial agent, preservation of historical books	
Enzymology	Chitin/chitosan-based matrix for enzyme immobilization	Shahidi et al. (1999); Fernández-Saiz and Lagaron (2011)
	Biosensors manufacturing for in situ measurements of environmental contaminants and metabolite control in artificial organs	

4.5 Conclusion

Large amounts of crustacean shells are generated by the sea-food processing industries and considered as recalcitrant waste. At the outset, the above discussion has endeavored to provide an explanation of valorization of the ‘zero-valued’ crustacean

shell waste into ‘value added’ biopolymer chitin/chitosan and their subsequent degradation to form oligomeric and monomeric products. Extensive bibliography of past and recent studies related to cutting edge strategies of resurgence of chitin and its oligo and monomeric derivatives by basic and applied approaches are presented. In this perspective, the pros and cons of both chemical and microbial process (enzymatic and fermentative) for their generation are thoroughly discussed. Due to some major offense and environment impairment issue, chemical treatments need to be masked by alternative biological methods in upcoming eons, which not only overcome the shortcoming but also sustainable in terms of commerce and value-addition. With the advancement of biotechnology, genetic and metabolic engineering are considered as most auspicious and fascinated technology for enhancement of yield of the products. Taking into account its numerous benefits microbial extraction/production is predicted to be a promising technology for commercialization in near future. Versatile and unique properties of chitin and its oligo and monomeric form offer their potential applications in different sectors. This seems that the present deliberation may be considered as innovative approach for bioresource utilization, value added product preparation and for sustainable development of mankind. In spite of information regarding biological activities of chitin, chitosan, COS and GlcNAc, we are still far from understanding their hidden mechanisms, and exploration of that shall offer new outstanding applications in near future.

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Chapter 5
Seaweed Liquid Fertilizers: A Novel Strategy for the Biofortification of VegeTables and Crops

C. Elizabeth Rani Juneius, M. Sundari, R. Eswaralakshmi, and S. Elumalai

5.1 Introduction

Utilization of seaweed sap has been gained the attention of scientists to explore its impact on edible plants. There are varieties of seaweed in the marine environment have been identified to be valuable additions to the organic garden and can be plentifully accessible for the costal populations. But there is even a threat about pollutants because of the absorptive nature of these macro algae. If the area is polluted with harmful chemicals, which may be absorbed by these seaweeds and proper precautionary measures must be there while collecting it, particularly from areas that are prone to pollution. There are also legal effects relating to gathering seaweed, and concerns about exploiting the sea macro algae (Seaweed products 2009). The presence of alginate content in the seaweed and its jelly like nature improves the soil structure and it contains all soil nutrients including the sufficient ratio of NPK and full range. A commercial sea weed fertilizer is available in a dried form or as a concentrated seaweed liquid fertilizer form and it is active in considerably lesser concentrations. While dried powder forms are inadequate to soil requirements owing to their impenetrability whereas fluid formula is chosen for the reason that foliar spraying or root region additions over drip routes are potential with soluble forms.

Seaweed can be either applied to the soil as a protective layer like a mulching process or can be added to the compost, where it can act an excellent activator (Coleby-Williams and Jerry 2009). Another important factor must be considered to accept sea weed fertilizer is that is less salt content. The farmer's friend earth worm's presence must be favoured but vermicompost production may be affected if excess

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amount of seaweed containing higher salt content is added. Seaweeds can be washed to remove excess salt content in advance to decrease its adverse effect to soil's buffering capacity.

5.2 Experimental Study

5.2.1 Collection of Seaweed Material

The seaweed sample *Kappaphucus alverizii* was collected from Kanniyakumari Sea shore. The finest quality sea weeds were collected and washed thoroughly with fresh water to remove all the unwanted impurities, attached soil particles, epiphytic symbiotic organisms etc. The collected samples were packed in sealable bags and transferred to the lab.

5.2.2 Preparation of Seaweed Extract

Washed seaweeds were chopped and distilled water was added in the ratio of 1:1. The mixture was pulverized until smooth, and filtered using filter paper. This seaweed extract was treated as 100% concentration. From the 100% seaweed extract, different concentrations (ranging from 25% to 100%) of seaweed liquid fertilizer (SLF) were prepared using distilled water. Since the seaweed liquid fertilizer contained rich nutrients and more prone to contamination, they were stored at 4 °C. The edible plant, selected for the present research was *Ablemoschus esculentus* belonging to the family Malvaceae (Pise and Sabale 2010).

5.2.3 Biochemical Analysis

The plant growth parameters such as, Total plant height, Shoot height, Root height, Total fresh vegeTable weight, Shoot fresh weight, Number of vegeTables in plant and Average weight/vegeTable was determined and the results were recorded. The effects of treated and control plants were compared. Analysis of Nutritive values and phytochemicals of *Abelmoschus esculents* were done. Estimation of protein was by Lowry's method, estimation of carbohydrates was by Anthrone method, Amino acid was estimated by Ninhydrin method and Test for tannins, Test for phlobatannins, glycosides, anthraquinone, terpenes, steroids and Test for Flavanoids were also carried out by standard qualitative methods (Parthiban et al. 2013).

5.2.4 Extraction and Estimation of Astaxanthin from Seaweed

Astxanthin was extracted from the seaweed sap by solvent extraction procedure using the solvent hexane: acetone (3:1) in a laboratory mixer, and then filtered using Whatmann filter paper and it was collected in a conical flask. About 12 ml of petroleum ether and 0.73% of NaCl was added. Then the epiphase was collected by using separating funnel. Finally washed with water by mixing equal amount of distilled water into the epiphase then the water is separated at the bottom and the above phase was collected, then the petroleum ether is evaporated by kept it in a water bath at 50 °C (Thirumaran et al. 2009). Lambda max was found out for the extracted Astaxanthin using UV- Visible spectrophometer by measuring the absorbance between 190 and 1000 nm. (Divya et al. 2015).

5.2.5 Antibacterial Test

Antibacterial sensitivity test was carried out by Modified Kirby Bauer method. Muller Hinton agar was prepared and sterilized by autoclaving. The sterilized media were poured into petri dishes and wells were cut using sterile well puncher. Different concentration ranging from 25 µl, 50 µl, 75 µl, and 100 µl ethanolic root extract of treated and control plants were tested against bacterial pathogen *Shigella dysentriae.* Zone of inhibition was observed after incubation period.

5.2.6 Gas Chromatography–Mass Spectrometry (GC/MS)

Various phytochemicals present in SLF treated and untreated plants were analysed by GC-MS. The samples were injected into a VF-5 mass spectroscopy column (30 m×0.25 mm with 0.25 µm film width), Agilent equipment GC-MS-QP 2010 SHIMADZU model. Following chromatographic conditions were used:

Carrier gas – Helium
Flow rate – 1 mL/min
Injector operation temperature – 240 °C
Column oven temperature – 70 °C at a rate of 10 °C/min injection mode.

Following MS conditions were used:

Ionization voltage of EI (8–70 ev)
Ion source temperature of 200 °C
Interface temperature – 240 °C
Mass range – 50–600 mass units

5.3 Outcome of the Study

Seaweed extract as a natural product containing rich nutrients which can support the growth of plants, in addition to that it also contain a substantial quantity of organic and inorganic elements and plant growth regulators which induces the plant to growth, faster than the regular growth rate and promotes maturity leading to high plant Yield. Especially rapid maturity in pea plant may prevent the crop from diseases, which was consider the main hurdle facing crop cultivation in the domain. Seaweed extract has a significant influence to make the plant immune system stronger, that may lead to give disease resistant plant with high product yield. Colapietra and Alexander (2006) and Sivasankari et al. (2006) stated that there was a specified improvement in plant harvest and health in diverse crops following administration of the seaweed *Ascophyllum nodosum*, while the mechanism of action have not yet found out. Another promising sea weed fertilizer was determined as Kelp 40. The researcher has recommended using Kelp 40 as organic manure for the good fortune of human wellbeing and ecosystem.

Kappaphycus alvarezii is one of the highly valuable marine red macroalge, which was used to produce carageenan, for the diverse applications such as gelling, solidifying and stabilizing agents, particularly in food manufacturing. Apart from these claims, carrageenans are utilized in pharmacological preparations, cosmetics and manufacturing applications such as mining. The current study has utilized these *Kappaphycus alvarezii* sap as fertilizer with different concentrations for the foliar applications on the chosen plants *Abelmoschus esculentus*.

The foliar shower was applied two times at four different concentrations of seaweed extract. The supreme growth was recorded at 1 in 5 concentrations. The plant parameters such as seed sprouting, young branch size, root measurement, number of floras, quantity of berries, fresh mass and dry weight were significantly improved when compared to control (Divya et al. 2015).

Leindah Devi and Mani (2015) has reported that the amount of chemical composition present in in the sap of *Kappphycus alvarezii* was determined as total nitrogen 0.103%, total phosphorus 0.007%, total potassium 11.099%, and total calcium 13.473% and total magnesium 9.288. Our study results were little different from the previous work and our study showed the presence of other essential chemical compositions required for the fertilizers also. Chemical compounds present in the sap of *Kappaphycus alvarezii* contains all the essential nutrients for the plants including Amino acids + Fulvic acid + Humic acid – 1.88%, and Cytokinins + Alginic Acid + Mannitol + Gibberellins – 0.5% (Table 5.1). **S**ince these compounds are present in the sea weed liquid fertilizer, these formulation can be used to grow plants without any other synthetic fertilizers.

Table 5.1 Composition of seaweed liquid fertilizer from *Kappphycus alvarezii*

S.no	Compounds	%
1.	Nitrogen(N) + Phosphorus (P2O5) + potassium (K2O	3
2.	Calcium(Ca) + Magnesium(Mg) + Zinc(Zn)	0.9
3.	%, Amino acids + Fulvic acid + Humic acid	1.88
4.	Cytokinins + Alginic Acid + Mannitol + Gibberellins	0.5

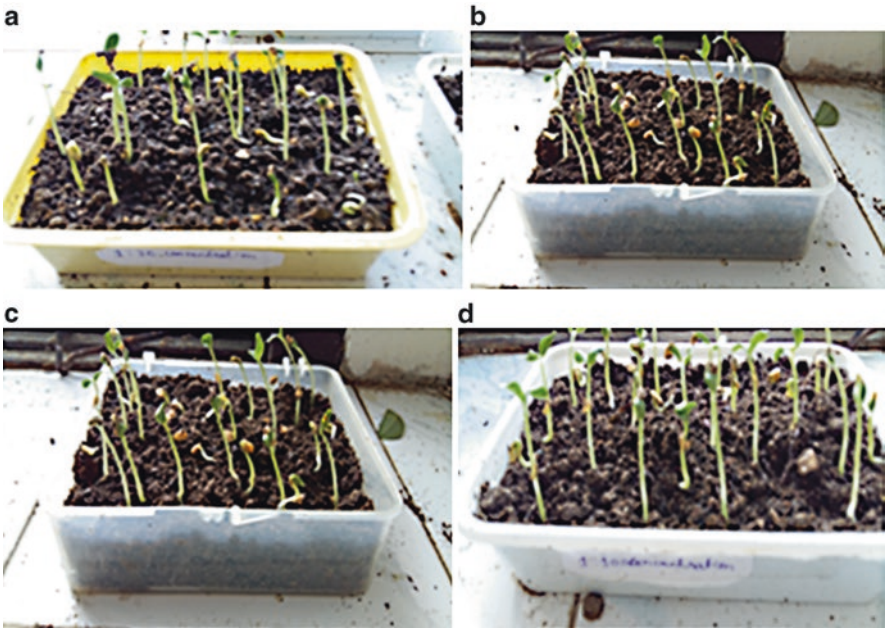

Fig. 5.1 Fengreek seed germination on the influence of SLF from Kappaphycus alvarezi-(**a**) 1:25, (**b**) 1:50, (**c**) 1:75 and (**d**) 1:100

5.3.1 Seed Germination

The effect of sea weed liquid fertilizer (SLF) extracted from *Kappphycus alvarezii* was used to grow *Abelmuscus esculentus*. The seeds germination in the control were compared with a seeds treated with SLF. The seed germination was studied using fenugreek seeds and the germination was good in 1 in 100 dilutions (Fig. 5.1). In contrast to the previous results, the higher growth parameters were noted in *Abelmoschus esculentis* plants treated with one in ten diluted SLF. Yield in relations to number and weight of the ladies finger were 30% and 32% higher than the control plants respectively (Table 5.2.). If the yield is higher, the profit for the farmers will

Table 5.2 Growth and nutritive parameters of test and control plants (4 weeks)

S.no	Parameters	Test plants	Control plant	% increase
1.	Total plant height in cm	38.2	20.3	53
2.	Shoot height in cm	28	10.6	37.8
3.	Root height in cm	10	9.5	1
4.	Number of vegetable per plant	20	6	30
5.	Vegetable fresh weight in grams	5.241	1.704	32
6.	Number of branches per plant	23	12	52
7.	Carbohydrate	3.46 mg/ml	1.92 mg/ml	55.49
8.	Amino acid	0.8 mg/ml	0.352 mg/ml	44
9.	Protein	1.8 mg/ml	0.852 mg/ml	47.3

also be higher. Seaweed extract has been accepted as a product comprising organic matter, and other essential macro and micro nutrients and some plant development regulators which promotes and encourages the plant to nurture well, quicker and timely ripeness leading to high plant yield. The properties of SLF and its influence in timely maturity in pea plant and the influence are very significant because it might inhibit the crop from infections, which is the foremost offending facing crop agronomy. Seaweed extract has an important role to maintain the plant protection system stronger, so that may lead to give healthy and high yield. Colapietra and Alexander (2006) as well as another author named Sivasankari et al. (2006), reported that there was an improved plant yield and disease resistance in different crops following application of the seaweed fertilizers extracted from *Ascophyllum nodosum*.

The nutritive values were increased in multiple folds in application of sea weed liquid fertilizer extracted from *Kappaphycus alvarezii* on *Abelmoschus esculentus* (Table 5.2). Pise and Sabale (2010) had described that the liquid fertilizer extracted from *Ulva fasciata, Sargassum ilicifolium* and *Gracilaria corticata* influenced the carbohydrates, protein and free amino acids content of *Trigonella foenum*. Our results substantiate with the previous results and there were an increase in the nutritive parameters such as carbohydrates (55.49%), amino acids (44%) and protein (47.3%) in the study plants *Abelmoschus esculentus* in application of sap from *Kappaphycus alvarezii* (Table 5.2).

As per the report of Senthamil and Kumaresan (2015), the absorbance maxima for astaxanthin will be at 466 nm. We have found out that the absorbance maxima at 450 nm, which is almost closer to the previous study. Antibacterial activity of the crude extract of plant roots were determined against *Shigella dysentriae* and the result revealed the moderate sensitivity of the treated root extract against the bacteria whereas there is no effect for the control plant root extract (Fig. 5.2). The plant root extracts contain Tannin, Glycosides, Flavonoids, Anthraquinone and Resin. These phytochemicals were almost same for both control and treated plants except the presence of glycosides in treated plants.

GC/MS results for treated and control plant root extracts were remarkable. Common compounds present in treated and control plants were 9,12-Octadecadienoic acid(z,z)methyl ester, Hexadeconic acid methyl ester and Stearic acid methyl ester.

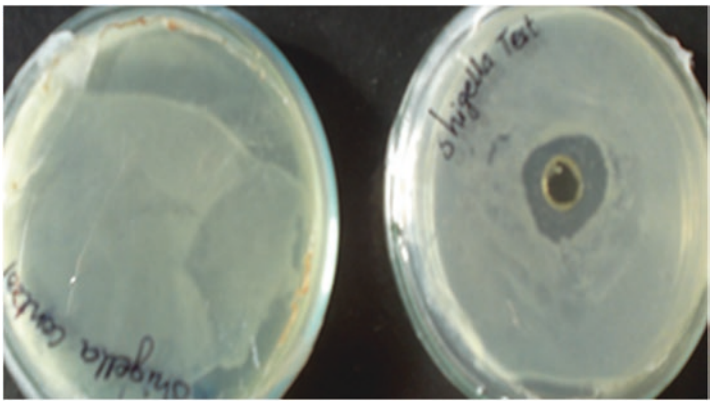

Fig. 5.2 Antibacterial effects of methanolic root extract of treated plants

Compounds exclusively present in treated plant root extract were 13-Hexyl oxacyclotridec-10-en-2 one, Methyl cis-7 octadecanoate, Butyl-2-ethyl hexyl phthalate, 2-Hydroxy-3(9E)-9-Octadecenoyloxy) propyl (9E)9-octadecenoate and (3R,3′S) and astaxanthin (58%) (Fig. 5.3) and it's a peak compound. It is a deep red, fat-soluble pigment that is found widely throughout nature. As one of the world's most commanding carotenoids, astaxanthin is considered an excellent antioxidant; it is more powerful than many other carotenoids, including beta-carotene, lutein, and zeaxanthin, and a hundred-times more powerful than alpha-tocopherol. There are several natural sources of astaxanthin available right in your own grocery store. Adding certain types of proteins that are rich in this pigment will help you increase your intake of this beneficial antioxidant. Proteins that are high in astaxanthin include sockeye salmon, red trout, red seabream, lobster, shrimp, crawfish, crabs, and salmon roe. (http://www.nutrex-hawaii.com/natural-sources-of-astaxanthin) mainly present in the sea weed *Kappaphycus alvarezii, a source for* SLF. Astaxanthin offers prevailing free radical hunting properties and defends the body against lipid peroxidation and oxidative impairment cell membranes, cells, and tissues.

Astaxanthin has been described in algae such as *Chlorella vulgaris*, *Chlorococcum* sp. *Haematococcus pluvialis* up to 3% (Suganuma et al. 2010; Yoshihisa et al. 2014; Hama et al. 2012; Maoka et al. 2012; Tominaga et al. 2012; Rao et al. 2013) but in our study, *Kappaphcus alvarezii* sap treated *Abelmoschus esculentus* has shown more that 85% of the compound. Mahfuzur et al. (2016)*, stated that Haematococcus pluvialis* is one of the significant source of astaxanthin and it is considered as "brilliant anti-oxidant but there were certain evidence of *Kappaphycus alvarezii* having high levels of astaxanthin. There were several therapeutic properties reported about astaxanthin and some are Antioxidant property, treatment for Carpal tunnel syndrome, Dyspepsia**,** upsurges Exercise capacity, controls High cholesterol, inhibits Macular degeneration, therapies for Male infertility, resolves Menopausal symptoms, controls Muscle soreness, cures Rheumatoid arthritis and solves problems associated with Skin (http://www.naturalmedicinejournal.com/journal/2012-02/astaxanthin-review-literature).

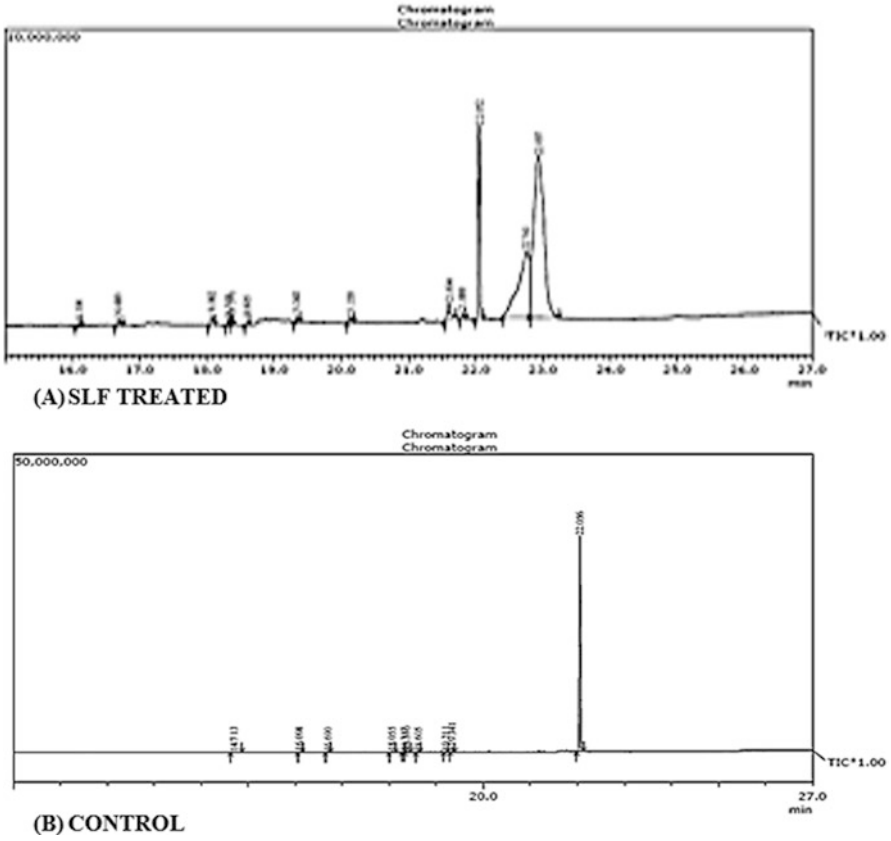

Fig. 5.3 GC/MS analysis of SLF treated and control *Abelmoschus esculentis* root extract

5.4 Conclusion

The seaweed liquid fertilizer extracted from *Kappaphycus alvarezii* could serve as a best choice of bio-fertilizer for organic farming which increases yield, nutritive value and therapeutic values of the edible plants. Fortification of such kind of organic fertilizer could rejuvenate soil health as well as imparts various beneficial effects in the crops and vegetables which in turn improve human livelihoods.

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Chapter 6
Green Algae Biomass Cultivation, Harvesting and Genetic Modifications for Enhanced Cellular Lipids

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6.1 Introduction

Biofuels are not a new idea. Actually, biofuels have been as old as the invention auto vehicles. The first model of vehicle Model T was to run on ethanol. Primarily, it was in trend to run diesel engine on vegetable oil. Rudolf Diesel invented the diesel engine originally to run on vegetable oil. Although due to cost petroleum fuels won out over biofuel. During the energy crisis of the 1970s demand for biofuels increased. In 1990s maximum biofuel demand increased due to increased emissions of GHG standards and increasing prices of fossil fuels. Basically biofuels are derived from biological material. The major difference between biofuels and fossil fuels is the difference of time period in which the fixation takes place. In case of biofuels carbon fixation takes place within months or within years. While in fossil fuels, carbon fixation takes a long time of thousands or millions of years. Additionally, chemical composition of both type of fuels are also different. Chemically fossil fuels are derived from hydrogen and carbon atoms only, while biofuels are made up of carbon (C), hydrogen (H), and oxygen (O) atoms.

Biofuels are mainly divided into three generations. First generation biofuels are also called conventional biofuels. They include sugar, starch, or vegetable oil. It is notable that these all are food products. Biofuel in which human food is used as feedstock is considered as first generation biofuel. Sustainable feedstock is used for the production of second generation biofuel. The sustainability of a feedstock is

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measured in terms of reduction in Green House Gases as well as use of crop land, Although second generation biofuel is produced from food crops, when they are not used for human consumption. Second generation biofuels are generally called as advanced biofuels. In general, third generation biofuels are algae fuels. Algae derived biofuel has distinguished class of fuel energy due the capability of separate production mechanism and removal of some drawbacks of other fuel classes. When we talk about the energy content of biofuel, it is lesser than energy content of petroleum fuels with the advantage of lesser CO_2 emissions. Percent wise it is 90% for biodiesel. Other than energy content there are a number of advantages of biofuels such as biofuels burn with less smoke than fossil fuels, resulting in fewer emissions of greenhouse gases, particulate emissions, and acid rain causing substances such as sulfur to increase energy supplies, reduce emissions, and improve operational efficiencies.

Throughout the world war 2nd there was large portfolio of biofuel developmental programs. It is believed that fundamental technology improvements and scientific approaches are still necessary for biomass optimization as well as processing of biomass into biofuels. More specifically, scientific approaches are needed to certain that advanced biofuels can be upgraded economically and produced with the desired environmental benefits like lowered life cycle greenhouse gas (GHG) emissions. In this regard approachable research with clear understanding of challenges and breaking it down into manageable questions which can be addressed through science are the first crucial steps to find out solutions. A number of Scientists and engineers at various universities of world, government laboratories, and companies are examining a wide range of feedstocks and processes to develop advanced biofuels. Microalgae biofuel production research is in positive direction with some leading researchers to develop the science which is required to deliver advanced biofuels with environmental benefits.

6.2 Algae Biofuel

Third generation biofuel is developed from algae. Algae are low-intake, more-yielding source biofuels development. Microalgae generate many times more energy as compared to the land crops. With uprising cost of petroleum products, there is need to go towards algal culture (farming algae). One of the advantages with algae farming is its renewability and nonpolluting due to which relatively more harmless to the environment of spilled. Third-generation biofuels is mainly defined as the biofuel derived from algae feedstock, which has very high biomass productivity relative to the crop plant biomass (Brennan and Owende 2010). Microalgae include a vast group of photosynthetic organisms which are good for biomass production. Microalgae is thought to be most promising renewable biofuel feedstock capable of producing maximum biomass for the substitute of fossil fuels without

sacrificing other edible products. Microalgae produces biofuels like other land crop plants as well as microalgae are greenhouse gas removers. Biomass cultivation of microalgal biomass can be achieved by various developed methods, which are the indicators of high productivity of microalgal lipids. Various microalgae are with good percentage of lipid containing and analytical approach which makes the quality of lipids produced by many microalgal species. In brief, many parameters like percentage lipid productivity, biomass production rate, composition of fat molecules, methods of biomass cultivation should be taken into account for maximum biofuel production. Green microalgae namely *Chlorella* sp. and *Scenedesmus* sp. showed tremendous growth in culture condition in BG-11 medium, when grown for lipid accumulation strategy (Kumar et al. 2015). Many types of fuels are the sources of fuels from ancient time. Due to more disadvantages of these fuel sources, and limited reservoirs, they are not promising candidate for future. Therefore, third generation biofuels derived from microalgae can be thought as better substitute as compared to the other alternatives of fossil fuels. (Nigam and Singh 2010; Chisti 2007; Li et al. 2008). Algae are capable of generating 15–30 times biofuel than traditional crop plants on an area basis. Furthermore compared with the food crops which are generally harvested seasonally, microalgae have very short duration of near about 1–10 days for harvesting with significantly increased yields (Schenk et al. 2008).

6.2.1 Algal Fuel Versus Other Biofuels

Microalgae are widely diversified group of photosynthetic microorganisms having simplest cell structure either unicellular or multicellular. They are capable of growing where water and sunlight both are present, including soils, ice, lakes, rivers, hot springs and ocean etc. (Parker et al. 2008). Algae are capable of capturing carbon dioxide and change sunlight energy to chemical energy. Algae lipids can be used to produce biodiesel after processing, which are present in cells in the form of some membrane parts and other storage forms. Nowadays microalgae come into light due to some advantages with this non competing and good percentage of lipid inside their cells and rapid biomass growth, feedstock e.g. *Scenedesmus, Nannochloropsis, Chlorella, Schizochytrium, Chlamydomonas*, and some other eukaryotic algae, etc. Thus microalgae biomass can be considered as good feedstock for more energy generation and, fungible liquid fuel for transportation. On the basis of some combined advantages of algae biomass feedstock, researchers are paying attention in algae biofuel development.

- Algae biomass production can be more per acre of cultivation.
- Algae cultivation methods can inhibit competition with crop land and nutrients which are used for agricultural purpose.

- Algae can use wastewater as well as saline water, in order to reducing competition for controlled supply of freshwater.
- Algae are capable of recycling carbon di oxide rich polluting gases emitting from stationary sources like thermal power plants.
- Algae biomass feedstock is also useful for the production of several types of fuel as well as important co-products.

6.2.2 *Advantages of Microalgae Biofuel Over First and Second Generation Biofuel*

A number of biofuel sources are used for generation of biofuels like Jatropha, corn, repeseed/canola, palm oil etc. But with some disadvantages of need of more land area and less lipid %for their proper cultivation, with some other disadvantages like slow growth, more nutrients intake and environmental incompatibility, they are not able to complete the dependence of world on fuels. Algae were once thought to be 'aquatic plants' as they lack characteristics of plants like true roots, stems, leaves, and embryos, these are considered as separate group of organisms. Algae biomass feedstock includes both types of algae i.e. eukaryotic as well as prokaryotic algae. The main advantages of microalgae biofuel derived biofuels over the first and second generation biofuels are listed as. First of all, the microalgae can be grown throughout the year and therefore, quantity of bio-oil produced from algae is much greater than oilseed crops, e.g. biodiesel yield of 58,700 l/ha for microalgae containing only 30% oil by weight., compared with 1190 l/ha for rapeseed or Canola (Schenk et al. 2008), 1890 l/ha for Jatropha (Chisti 2007), and 2592 l/ha for Karanj (*Pongamia pinnata)* (Lele 2009).

Fast rate of biomass growth of algae with oil content in the range of 20–50% dry weight of biomass is main benefit in favor of algae to be chosen as biofuel candidate. Microalgae grow rapidly as compared to terrestrial crops. They commonly double every 24 h. The exponential growth rates can double their cellular biomass in a period of as short as 3.5 h (Chisti 2007; Lele 2009; Metting 1996; Spolaore et al. 2006). Secondly, despite of their growth in aqueous medium, the algae needs less water than terrestrial crops thus the stress on freshwater sources is also reduced (Dismukes et al. 2008). This is the reason behind that microalgae can also be grown on arable land and under useless water which minimize environmental impacts (Searchinger et al. 2008), without competing with human food and animal fodder (Chisti 2007). According to Chisti (2007), 1 kg of dry algae biomass uses near about 1.83 kg of CO_2, thus the microalgae biomass production can help in bio-fixation of waste carbon dioxide relative to quality (Fig. 6.1).

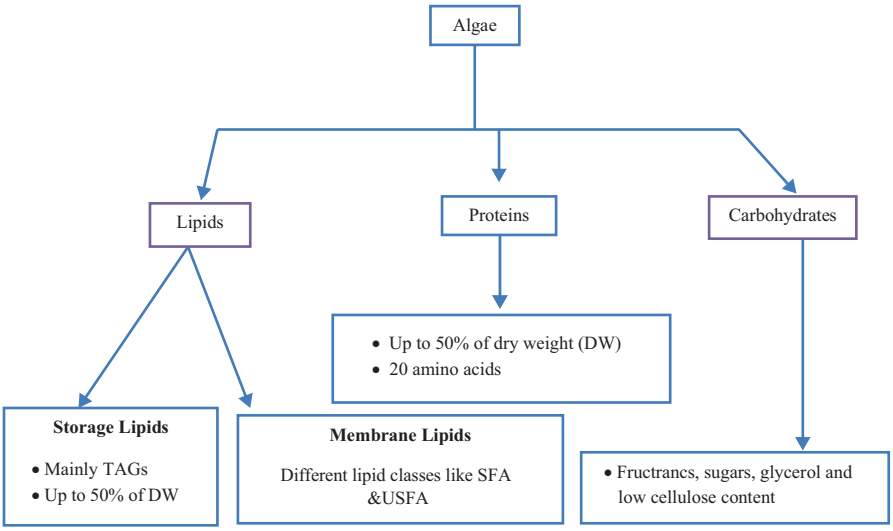

Fig. 6.1 Cellular components of microalgae

6.2.3 Potent Feedstock for Microalgae Biofuels

It is clear that microalgae are potent feedstock for biofuel development. Microalgae are biodegradable source that has capacity to replace fossil fuels. Most algae species are potent feedstock for biofuel development out of them *Botryococcus braunii* (25–75% dry wt. biomass), *Dunaliella* sp. (17–67% dry wt. biomass) and *Schizochytrium sp.* (50–77% dry wt. biomass) are best lipid accumulating algae which are capable of replacing all fossil fuels (Table 6.1).

6.2.3.1 Collection and Screening of Microalgae Strains for Biofuel Production

Looking for the microalgae species having good lipid percentage as well as rapid growth rate is the key of biofuel development. Worldwide, over 50,000 microalgae species are available, in aquatic and terrestrial environment (Richmond 2004). A few number, about 4000 algae are till identified and grouped into cyanobacteria, diatoms, yellow-green algae, green algae, golden algae, brown algae, red algae, dinoflagellates and 'picoplankton' (Hu et al. 2008). Out of these all groups green algae and diatoms are better feedstock for biodiesel development (Khan et al. 2009). *Botryococcus*, *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Neochloris* are good lipid accumulator with 20–75% by weight of dry biomass.

Table 6.1 Lipid % in various microalgae species (Chisti 2007; Li et al. 2008; Mata et al. 2009; Um and Kim 2009; Sialve et al. 2009)

Microalgae species	Lipid content (% dry wt. biomass)
Ankistrodesmus sp.	24–41
Botryococcus braunii	25–75
Chlorella emersonii	14–57
Chlorella protothecoides	14–57
Chlorella vulgaris	5–58
Dunaliella sp.	17–67
Zitzschia sp.	45–47
Isochrysis sp.	7–40
Nannochloropsis sp.	20–56
Neochloris oleoabundans	29–65
Crypthecodinium cohnii	20–51
Chlorella minutissima	57
Phaeodactylum tricornutum	18–57
Scenedesmus obliquus	11–55
Scenedesmus dimorphus	16–40
Schizochytrium sp.	50–77
Isochrysis galbana	7–40

These all are good feedstocks for biofuel development. NREL has near about 300 algae strains which are costly diatoms and green algae, as oil producing candidates via screening over 3000 microalgae strains. Nowadays, the collection of screened strains of algae is later on shifted to University of Hawaii which is available for research purpose. Other than oil percentage of an algal strain biomass is also another thing for keeping high for most suitable algae strain for biofuel development. In general, less lipid accumulating algae grow rapidly than more lipid accumulating strains (Vasudevan and Birggs 2008). Low percentage algae grow faster than high oil percentage algae species (Becker 1994). It is also difficulty that under stress conditions oil accumulation occurs with slow rate. Hu et al. (2008) observed in his study that is increased under stressed conditions up to two or three-folds. Deprived nitrogen leads to increased lipid percentage in *Neochloris oleoabundans* (Li et al. 2008) *Chlorella vulgaris, Chlorella sorokiniana, Chlorella emersonii, Chlorella protothecoides,* and *Chlorella minutissima* (Illman et al. 2000). So, the ability of microalgae to prosper in extreme environmental and physical parameters should be considered for improved biofuel production.

6.3 Transesterification

When lipids are extracted from algae cells, green crude oil comes out in the form of vegetable oil. More oxygen is present in green crude oil due to the reason behind, it cannot be blended directly with petroleum (Neenan et al. 1986). Transesterification

process and catalytic conversion process are the two methods used for conversion of lipids extracted from algae. In transesterification method glycerol is obtained as by product which can be substituted by ethanol. Ethanol is thought as most appropriate substitute used. The triglycerides (TAG) obtained from algae oil reacts with ethanol and produces biodiesel similar to diesel. Transesterification process was useful for the development of biodiesel from vegetable sources till Second World War and continued after that period.

Up to 98% product recovery has been measured from transesterification reaction of algae lipids at 60 °C temperature. On temperature fluctuations efficiency may decrease Energy efficiency of biodiesel is less than diesel oil, while boiling point and viscosity are closer to diesel oil. Solar Energy Research Institute (SERI) reported that use of biodiesel near freezing point of water creates problem, because at this temperature it becomes cloudy and starts to freeze. Therefore, slightly high temperature maintenance is needed for its operation. Microalgae lipids contain more PUFA with up to four double bonds as compared to vegetable oils (Chisti 2008). Catalytic reaction of crude oil needs more extensive study, which can convert the lipids to a mixture of hydrocarbons very similar to that of gasoline with ethanol as a byproduct. Major reaction of microalgae FAME is derived by transesterification in which glycerol is obtained as byproduct. Currently biodiesel is prepared by some feedstocks like waste cooking oil, animal fat and some oleaginous species like, corn, rapeseed, sunflower, peanut, Jatropha and oil palm on commercial scale (Barnwal and Sharma 2005; Felizardo et al. 2006; Vasudevan and Birggs 2008). In European countries rapeseed oil is dominantly used as feedstock for biofuel production while soybean oil is mainly used in USA. However, these food-based raw materials have resulted in the debate "food vs. fuels". Large increase in food prices was seen in the world due to use of vegetable oils as biofuel feedstock (Fig. 6.2) (Chakravorty et al. 2009).

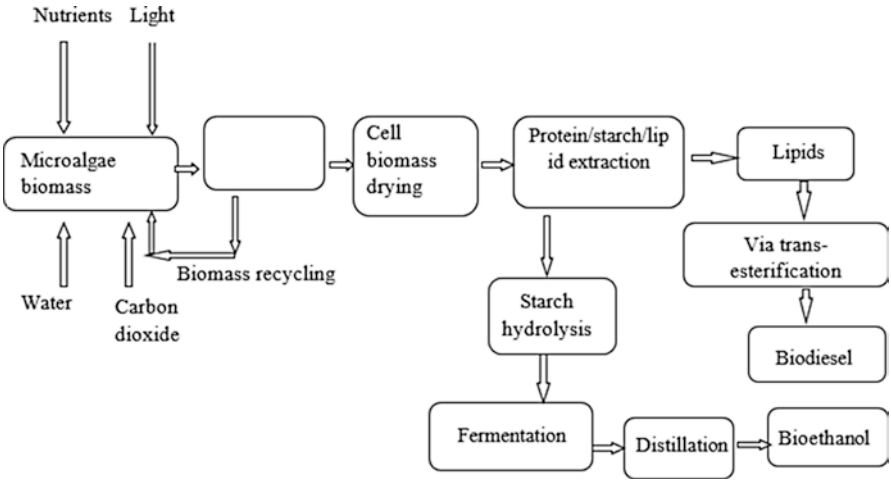

Fig. 6.2 Biodiesel and bioethanol production from microalgae

6.4 Fatty Acid Analysis of Microalgae

Lipid and fatty acids are the primary metabolites of microalgae to produce biodiesel. *Chlorella vulgaris* (Gouveia and Oliveira 2009; *Scenedesmus obliquus* (Da-Silva et al. 2009; Gouveia and Oliveira 2009) Sahu et al. 2013), *Spirulina maxima* (Gouveia and Oliveira 2009), *Nannochloropsis sp*. (Gouveia and Oliveira 2009), *Neochloris oleabundans* (da-Silva et al. 2009; Gouveia and Oliveira 2009), *Botryococcus braunii* (Yoo et al. 2010), *Scenedesmus sp*. (Sahu et al. 2013), *Nannochloropsis* sp. (Bondioli et al. 2012), *Tetraselmis suecica* (Bondioli et al. 2012) and D*unaliella tertiolecta* (Gouveia and Oliveira 2009) have been extensively studied. *Neochloris oleabundans* (freshwater microalgae) and *Nannochloropsis* sp. (marine microalgae) proved to be much suitable as feedstock for biofuel production (da-Silva et al. 2009; Gouveia and Oliveira 2009). The conditions of cultivation influence lipid and fatty acids production in microalgae. *B. braunii*, cultivated in 10% CO_2, contains high lipid and oleic acid (Yoo et al. 2010) and suitable for biodiesel production. Fatty acids are the major components in forming biodiesel. Kumar et al. (2017) studied approximately 53.31% lipids in indigenous *Scenedesmus* strain, when grown under various physical and cultural conditions of growth. Previous research has done to observe fatty acids in some microalgae. *Desmodesmus* sp., *Desmodesmus elegans*, *Scenedesmus* sp., *Chlorella* sp. and *Chlorococcum macrostigmatum* all contain similar type of fatty acids. The major fatty acids in some microalgae as observed were C16:0, C16:4, C18:1, C18:2 and C18:3 (Abou-Shanab et al. 2011; Kaur et al. 2012; Sahu et al. 2013).

6.5 Microalgae Biomass Cultivation

Microalgae biomass cultivation for biofuel production can be achieved by two major phases which involve upstream and downstream processes. Biomass quantity and quality can be enhanced by upstream phase while downstream phase is important for harvesting technology. The upstream phase is useful for sustainable production of biofuel. These two main phases of microalgae cultivation are described as-

- ***Upstream phase***

6.5.1 Microalgae Cultivation Technologies

Cultivation of microalgae biomass can be achieved by three different types of culture systems like batch system, semi-batch system and continuous systems. The growth rate and maximum biomass cultivation of microalgae in these culture systems are significantly affected by abiotic factors such as light, temperature, pH, salinity, O_2, CO_2, nutrient stress, and toxic chemicals, similarly biotic factors like pathogens and antagonistic effect of other algae and operational techniques like

shear, depth, frequency of harvest, and addition of bicarbonate ions are also responsible. Various cultivation technologies have been studied for bulk production of microalgae biomass (Spolaore et al. 2006; Huesemann and Benemann 2009). Generally four major techniques are useful for microalgae cultivation (Wang et al. 2014).

- Phototrophic cultivation method
- Heterotrophic cultivation method
- Mixotrophic cultivation method
- Photoheterotrophic cultivation method

Among these all, only phototrophic cultivation is commercially achievable for large scale microalgae biomass cultivation (Borowitzka 1999). Additionally, phototrophic microalgae can also utilize CO_2 from atmosphere as carbon dumping site.

6.5.1.1 Phototrophic Cultivation Method

Microalgae have high photosynthetic efficiency as well as high growth rates when compared to higher plants (Chisti 2007). In phototrophic cultivation technique algae can be grown and biomass can be produced by- -

- Open ponds
- Closed photobioreactors

6.5.1.1.1 Open Pond Biomass Production

These are oldest and simplest systems commonly used for large scale microalgae biomass production. Open ponds microalgae biomass cultivation systems have been practiced since the 1950s (Borowitzka 1999). Nowadays, near about 98% of commercial algae are produced by these cultivation systems (Sheehan et al. 1998). There are a variety of open pond cultivation on the basis of their size, shape, construction material, agitation type, and inclination (Borowitzka 2005). Some of them are raceways, thin layer cascade system, shallow unmixed ponds. Out of all listed here raceways cultivation systems are the most suitable artificial cultivation system (Jiménez et al. 2003). Open pond system is the cheapest method for large scale cultivation of microalgae compared to close photobioreactors. Open pond systems has more advantages as these systems are free from the crises of use of agricultural lands as they are settled in waste land areas (Chisti 2008). There is no problem of construction, regular maintenance, and cleaning of systems because it is so easy and consume lesser energy (Rodolfi et al. 2009). Open pond systems are lesser technical in design. Although open ponds have some drawbacks of abiotic factors like light intensity, pH, and temperature and dissolved oxygen also with the risk of contamination (Harun et al. 2010). Air and soil contamination is often a serious problem and drawback for open cultivation system, the species which are cultured in open pond

systems are grown under specific environments like high alkalinity and high salinity (Belay 1997; Cysewski and Lorenz 2004; Moheimani and Borowitzka 2006; Borowitzka 2010).

6.5.1.1.2 Closed Photobioreactors (PBR)

Closed photobioreactors design are like tubes, bags or plates made by transparent material such as glass, plastic, or other materials. In such cultivation system adequate supply of light, nutrients, and carbon dioxide are also available (Pulz 2001; Carvalho et al. 2006). Out of most photobioreactors rare design is suitable for commercial biomass production (Ugwu and Aoyagi 2012). Commonly used PBR designs are in the form of annular, tubular, and flat-panel reactors design, with large surface areas (Pulz 2001; Chisti 2006).

6.5.1.1.3 Annular Photobioreactors

These photobioreactors design are prominently used as bubble columns or airlift reactors (Lee et al. 2006). But sometimes they are used as stirred tank reactors (Sobczuk et al. 2006). Column photobioreactors are arranged with vertical columns where aeration is provided from below, and transparent walls are meant for light illumination. Column photobioreactors are featured with some benefits of regulatory growths and better culture mixing and maximum volumetric gas transfer rates (Eriksen et al. 2007).

6.5.1.1.4 Tubular Photobioreactors

In tubular phobioreactors, algae biomass is forcefully thrown via transparent tubes. The mechanical pumps or airlifts are used to create pumping force simultaneously airlift also allows the gaseous exchange for oxygen and carbon dioxide in liquid medium and aeration gas (Travieso et al. 2001; Molina et al. 2001; Hall et al. 2003; Converti et al. 2006).

6.5.1.1.5 Flat-Panel Photobioreactor

Flat-panel photobioreactors faccilitates more growth densities and increase photosynthetic efficiency (Rodolfi et al. 2009; Eriksen 2008). In such type of systems, a thin layer of dense culture is spread on a flat panel and light is made absorbed within the first few millimetres (mm) on the top of the culture (Hu et al. 1998; Degen et al. 2001; Richmond et al. 2003).

Photobioreactors are advantageous in comparison to open pond cultivation system likewise controlled growth, efficient system, and contamination free algae cul-

tures. However, there are some disadvantages like high construction cost, heavy operation, and high maintenance. Though such disadvantages can be overcome up to a limit by more production capability, they still limit the economical biomass production of at commercial scale.

6.5.1.1.6 Hybrid Production Systems

Hybrid systems constituted by both type of cultivation system can be used together for better outcomes (Table 6.2). In such systems, the required pure inoculum from photobioreactors can be poured into open ponds or raceways to get best biomass yield (Grobbelaar 2000; Greenwell et al. 2010). Astaxanthin can be produced from *Haematococcus pluvialis* Olaizola (Olaizola 2000) Huntley and Redalje (Huntley and Redalje 2007) used these hybrid systems. However, this hybrid system is not better for biofuel development due to costly system. It is also a batch culture system not a continuous culture system.

6.5.1.2 Heterotrophic Cultivation

In heterotrophic mode of algae cultivation, organic carbon is utilized as a source for their growth and development other than photosynthesis. As microalgae are photosynthetic organisms, they shows light deficiency due to more requirement of light photons for proper photosynthesis of large scale green cells for biomass cultivation

Table 6.2 Comparision of open and closed cultivation system (Borowitzka 2010; Pulz 2001)

S.No.	Factor	Open system	Closed system
1.	Cultivation time	Less	Prolonged
2.	Water evaporation	More	Least
3.	Sterilization	Low	High
4.	Temperature	Not fixed	Fixed
5.	Light utilization	Poor	Good
6.	Cleaning	Not mandatory	Mandatory
7.	Contamination prevention	None	Contamination free
8.	Weather dependence	High	Low
9.	Reproducibility	Limited	Possible
10.	Harvesting capacity	Low	High
11.	CO_2 transfer rate	Not good	Very good
12.	Water loss	Very high	Very low
14.	O_2 concentration	Low	Continuous exchange
15.	Investment	Low	High
16.	Maintenance	Low	High
17.	Operating cost	Less	More
18.	Harvesting cost	High	Low

(Wen and Chen 2003) or at high light intensity they experience photo inhibition, thus a balance of light intensity is necessary for more biomass accumulation (Myers and Burr 1940). As disadvantages of phototrophic cultivation, heterotrophic cultivation of microalgae can be considered as more suitable cultivation system (Chen 1996). In case of heterotrophic algae cultivation, the cultivation procedure can be controlled in better way as compared to phototrophic algae cultivation system (Chen and Johns 1991). However, there are some limitations associated with heterotrophic cultivation system.

1. There are few species which are capable of growing under heterotrophic mode of cultivation. Still only four species with high lipid accumulation are identified for heterotrophically growing microalgae which are *Chlorella. protothecoides* (Xu et al. 2006; Xiong et al. 2008; Cheng et al. 2009), *Chlorella vulgaris* (Liang et al. 2009), *Crypthecodinium cohnii* (Couto et al. 2010), and *Schizochytrium limacinum* (Johnson and Wen 2009).
2. Presence of organic substrate can cause contamination from other organisms (Chen and Oswald 1998).
3. Glucose can be considered as preferred organic substrate in case of heterotrophic system. However it may lead to food versus fuel crises because this is also used for human food (Perez-Garcia et al. 2011). Therefore, glucose can be technologically replaced with lignocellulose and glycerol derived glucose.
4. Generally microalgae release the CO_2 through respiration but in heterotrophic cultivation it cannot isolate the CO_2 from atmosphere (Li et al. 2008). Therefore, more detailed studies of life cycle assessment (LCA) of microalgae and proactive research for heterotrophic cultivation are highly required.

6.5.1.3 Mixotrophic Cultivation Method

Most of the species of microalgae utilize both type of cultivation system i.e. autotrophic as well as heterotrophic cultivation system for algae biomass production, showing that algae are capable to photosynthesize and can use organic substrate (Zhang et al. 1999; Graham et al. 2009). In mixotrophic growth system, microalgae are not completely dependent on photosynthesis because there is a choice in between light and organic substrate whether can be used for growth and development (Chen 1996; Andrade and Costa 2007). Microalgae which show mixotrophic cultivation system are *Spirulina platensis* (cyanobacteria) and *Chlamydomonas reinhardtii* (green algae) (Chen 1996). In such organisms, photosynthesis occurs with the utilization of light while aerobic respiration takes place under organic carbon source for biomass growth and development (Zhang et al. 1996). In this system growth of the algae is stimulated by the medium supplemented with glucose (as carbon source) during the light and dark phases. Hence, reduction in biomass is more in light phase in comparison to dark phase (Andrade and Costa 2007). Another term for mixotrophy is called amphitrophy where an organism has choice to survive as autotrophically or heterotrophically according to nutritional availability of organic carbon

source and light intensity (Chojnacka and Marquez-rocha 2004). Growth of *Spirulina* sp. in photoautotrophic, heterotrophic, and mixotrophic cultures were studied by Chojncka and Noworyta (Radakovits et al. 2010). Their study showed that cultures grown in mixotrophic conditions resulted in less inhibition of light and growth rate in comparison to autotrophic and heterotrophic cultivation system. Therefore, mixotrophic cultivation system of microalgae for biomass production allows the incorporation of photosynthetic as well as heterotrophic substrate during light phase life cycle. Mixotrophic cultivation is useful for growth and development of microalgae as it reduces biomass loss as well as use of organic substrate during dark respiration. On the basis of these advantages mixotrophic system is much important for biomass cultivation for biofuel production.

6.5.1.4 Photoheterotrophic Cultivation Method

Photoheterotrophy is also called as photometabolism or photoorganotrophy or photoassimilation. In this biomass cultivation system, organic substrate is used as carbon source in presence of light. There is very minor distinction between these two cultivation systems, most importantly both requires energy for survival and production of any metabolite (Chojnacka and Marquez-rocha 2004).

- ***Downstream phase***

6.6 Harvesting of Algal Biomass

Harvesting of microalgae biomass involves mechanical, chemical, biological and, sometimes electrical methods. Generally a set of methods can be constituted to obtain a greater separation rate at lower costs (Barros 2015). Microalgae biomass recovery from cultivation pond is necessary step for maximum biomass production. Most of the methods utilized nowadays for microalgae harvesting are centrifugation, filtration and screening, gravity sedimentation, flotation, flocculation and electrophoresis techniques (Uduman et al. 2010). Biomass harvesting technique should be effective for wide variety of microalgae strains and should produce high biomass concentrations, with low costs of operation, energy and maintenance.

6.6.1 Centrifugation

Centrifugation is the fastest method of algae biomass harvesting among all. Centrifugation is based on the centripetal force for the sedimentation of heterogeneous mixtures with a centrifuge, and it is used in industrial and laboratory settings.

A centrifuge is a very useful multipurpose device for bio-lipid extraction from microalgae and chemical separation in biodiesel. Coupled with A homogenizer is attached with centrifuge which is capable of separation of other useful materials from microalgae (Grima et al. 2003). During the centrifugation process, at first the semi concentrated microalgae biomass is obtained from the culture tank using a ball valve. Flow regulator, rotary van pump and centrifuge, helps in concentrating algae biomass in order to flow outward. Centrifugation is really a preferred method for harvesting of microalgae biomass, as it increases shelf life but time taking and costly (Spilling et al. 2011). Due to its high energy consumption it is an expensive process, it is applicable only to high valued products, like unsaturated fatty acids, pharmaceuticals and other commodities (Rawat et al. 2011).

The harvesting of microalgae biomass is the next step after cultivation which is the key part of biofuel development processing. Microalgae biomass recovery from cultivation system is much difficult as compared to macroalgae. It is due to microscopic cell size of tiny cells of microalgae. Cell size of unicellular algae are in the range of 3–30 μm (Grima et al. 2003), and the range of cyanobacteria (prokaryotic cell) is 0.2–3 μm (Chorus and Bartram 1999). The betterment and commercial scale application of advanced technologies for cultivation, harvesting and lipid extraction for biofuel commercialization is currently a heavy task to the scientists and the technologists of active research society. Ordinarily it is believed that a number of properties and active transformation of biomass are the main bases of biofuel energy (McKendry 2002; Goyal et al. 2008). Chemical methods as well as mechanical methods are used for the purpose. These methods include centrifugation, flocculation, flotation, screening, gravity sedimentation, filtration, and electrophoresis for harvesting of algal biomass (Uduman et al. 2010). Harvesting methods of microalgae biomass can be selected by considering many critical parameters. Major parameters are density, size, and value of the required products. Generally two-step processes are used for the harvesting of microalgae-.

6.6.1.1 Commercial Scale Harvesting of Biomass

In this step separation of microalgae is caused for biomass separation from the bulk suspension with the help of flocculation, flotation, or gravity sedimentation.

6.6.1.2 Thickening of Biomass

Next to the commercial scale harvesting, thickening is caused to thicken the slurry by filtration or centrifugation (Brennan and Owende 2010). Centrifugation is the most operative method used for the separation of algal biomass, but it is a costly process, so it is done on high-valued products only (Grima et al. 2003).

6.6.2 Flocculation

In this process circulated microalgae cells are combined together to form bulky biomass collection of cells for settling. Various methods are used for microalgae biomass harvesting such as auto flocculation, chemical coagulation or electrolytic processes. Chemical coagulation can be divided into organic and inorganic coagulation results in auto flocculation (Grima et al. 2003).

6.6.2.1 Chemical Coagulation

Initiation of chemical flocculation can be performed by making the mixture of chemicals for the fusion of microalgae. The mixture of chemicals may be inorganic flocculants and organic flocculants or polyelectrolyte flocculants. Godos et al. (2011) studied the activity of two predictable chemical coagulants ($FeCl_3$) and $Fe_2(SO_4)_3$ and five commercial polymeric flocculants namely Drewfloc 447, Flocudex CS/5000, Flocusol CM/78, Chitosan, and Chemifloc CV/300 in order to study the ability to remove bacterial biomass in microalgae from the discharge of an oxygenated wastewater biodegradation procedure. With the highest absorption rate of 150–250 mg/L ferric salts eliminated uppermost biomass up to 66–98%.

6.6.3 Combined Flocculation of Biomass

A multi-step process was studied by few researchers in which more than one flocculants and electroflocculation or electrocoagulation is done (Chen et al. 2011). Electrocoagulation–flocculation method has also been studied by Vandamme et al. (2011) for harvesting of fresh water and marine microalgae. Algae separation from industrial wastewater was examined by Azarian et al. (2007) by continuous flow electrocoagulation. It is observed that other than centrifugation process, power consumption is less in electrocoagulation–flocculation and no sulphate and chloride anions was recorded (Vandamme et al. 2011). Due to these advantages, electrocoagulation–flocculation is counted as a convenient procedure, which can be used for biomass harvesting of microalgae. There are some failure with this method like inconsistency of metal hydroxides, pH and conductivity of water etc.

6.6.4 Gravity Sedimentation of Biomass

With the method of gravity sedimentation, microalgae can be simply separated in water and wastewater treatment in which flocculation process is followed to upsurge the effectiveness of gravity sedimentation (Chen et al. 2011). Although flotation

procedure can also be used for gravity sedimentation which is more effective in comparison to sedimentation and can pick thin bits of less than 500 μm (Yoon and Luttrell 1989). Chemical precipitation and centrifugation methods are thought most efficient methods (Chen et al. 2011). The procedures of chemical flocculation and centrifugation are very costly for the production of biogas. Similarly, algae biomass condensation methods include mainly filtration but with integration of some other methods such as gravity sedimentation, flocculation and flotation can also be implemented. Anaerobic digestion can occur in slurry efficiently for the production of biogas (Prajapati et al. 2012, 2013). Conventional algae biomass digestion requires more water as compared to the wet algae biomass for the production of biogas.

6.6.5 Flotation

In this method microalgae are removed from aqueous solutions. It can be said more advance and effective method than sedimentation (Brennan and Owende 2010). Flotation, a gravity separation method, in which air or gas is bubbled via solid-liquid suspension and the gaseous molecules bind with the solid particles. Later on floating particles can be removed from liquid surface (Brennan and Owende 2010). There are three flotation techniques with reference to the bubble generation.

- Dispersed air flotation
- Dissolved air flotation
- Electrolytic flotation

6.7 Molecular Techniques to Enhance Microalgae Biomass for Biofuel Development

Metabolic pathways of microalgae are easier due to its unicellular cell structure. So it will be convenient to manipulate it. The real target of genetic engineering to be implemented on microalgae metabolism is to enhance the lipid accumulation via biomass for biodiesel development. Although, biomass and lipid increase ratio is not in linear proportion. The progress in genetic engineering research of microalgae was extremely slow up to the recent time. Genome sequences of microalgae will greatly facilitate the genetic engineering technology for enhanced biodiesel production. Genome sequencing projects of many lipid accumulating species has been completed (Radakovits et al. 2010). During the last 15 years genetic engineering methods developed significantly. Thirty microalgae strains which includes green algae, red algae, brown algae, diatoms, euglenoids have been successfully transformed (Radakovits et al. 2010). A model green alga *Chlamydomonas reinhardtii* has been gone under transformation experiments at both nuclear and chloroplast levels (Lumbreras et al. 1998; Fuhrmann et al. 2005).

6.7.1 *Genetic Modifications of Microalgae for Increased Biomass Development*

Microalgae growth is generally stimulated by some physical conditions like temperature, salt concentration, pH and light. Such parameters can be regulated by genetic engineering and manipulations of some growth characteristics, but these all environmental manipulations are costly to manage. Genetic manipulations can overcome such environmental stress conditions. It will be more successful strategy for enhanced lipid accumulation. The average light intensity is around 200–400 μM photons $m^{-2}s^{-1}$ under which best biomass accumulation occurs in most microalgae. Light intensity more than this range may decrease the microalgae growth and development. During mid-day, the light intensity is very high, maximum up to 2000 μM photons $m^{-2}s^{-1}$ (Melis 2009).

Due to this, microalgae growth efficiency during day time is lesser. In order to reduce the effect of photoinhibition and to enhance photosynthetic efficiency of microalgae many studies were carried out. Out of these, most were taken as decreasing number of light harvesting complexes to reduce light absorbing capacity of each chloroplast (Mussgnug et al. 2007). In an experiment, LHC expression in transgenic *C. reinhardtii* was down regulated to increase the resistance to photo oxidative damage and to increase photosynthesis up to 50% (Mussgnug et al. 2007; Beckmann et al. 2009). This alteration permitted *C. reinhardtii* to tolerate photoinhibition. In another study taken by Huesemann et al. (2009), no growth increment was recorded in algae antenna mutants grown in outdoor ponds and also at lab scale. Genes, which are capable to hold some other physical parameters like pH, temperature, salt concentration etc., have been recognized.

6.8 Conclusion

Microalgae are the potential source of lipids, which can be converted into biodiesel via processing. There are many advantages of algae to choose as feedstock for biodiesel production. In this chapter, we described various efficient methods to cultivate potential microalgae to recover microalgae biomass from culture medium. However, there is no perfect and universal method for cultivation and harvesting of microalgae biomass. An efficient method can be designed according to properties of a particular microalgae species, like cell morphology, cell size and cell surface structure and features, on the basis of the culture medium and on the basis of quality of end product and value of the end product. Additionally, combinations of processes can be implemented to improve harvesting efficiencies and costs. Regarding biofuel applications from microalgae, bioflocculation followed by gravity sedimentation seems to be a cost-effective method of microalgae biomass harvesting. However, due to the possibility of microbiological contaminations application of bioflocculation may be limited. Genetic engineering is the most advance and fruitful

strategy for enhanced biodiesel production from microalgae. Similarly multi gene approach can be effective for achieving the desired results. A number of microalgae have been sequenced till now. Genome sequencing of microalgae will facilitate to study more extensively. Unicellular machinery of unicellular microalgae facilitate metabolic pathway of microalgae. Thus several challenges are still in the way to achieve fruitful and desired product from microalgae on commercial scale, it is hopeful if combination of several strategy will be developed then microalgae can be a feedstock for biofuel at commercial level.

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Chapter 7
Probiotics: The Ultimate Nutritional Supplement

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7.1 Introduction

"For life", it is a simplified version of the term probiotic which includes a group of microorganisms specifically bacteria that exudes various lucrative impact on human and animal health. The first record of intake of probiotic drinks by humans was over 2000 years ago. However in the present century probiotics were first put onto a scientific basis by the sensational work of a Russian Zoologist Elie Metchnikoff in Paris at the Pasteur Institute. According to his suggestion the ingested diet has a significant impact on host microbiota and their ability to replace pathogenic flora (Metchnikoff and Mitchell 1907). In a different observation it was found that infants infected with diarrhea had a decreased number of a particular "bifid" bacteria in their stools in contradiction to the healthy children where the number was abundant. It was therefore speculated that these bacteria orchestrates a significant role in improving the health of intestinal microflora if administered by the patient suffering from diarrhea (Tissier 1906).

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Though the term “probiotics” was not coined until 1960 the independent astonishing scientific observation of changed the course of the concept of using bacteria as probiotics and its definition (Lilly and Stillwell 1965). Though the observations of Metchnikoff and Tissier in the year 1906 and 1907 respectively were much more enthusiastic and though their scientific works were commercial exploited but still then the results were not up to the mark or in other words the results were capricious. Thus the concept was regarded as scientifically ambiguous along with minor research in the field of animal feeding and certain other substitutes for growth promoting agents for decades. Recently in these two decades there has been conspicuous advancement in the field of selecting and characterizing specific probiotic strains and validating their consumption for health benefits.

The serviceable role of the microbiota in gut, lucratively elucidated as “colonization resistance” or “barrier effect” which simply means a mechanism or a process by which gut microflora predominantly barricade its inhabited gut environment from the freshly ingested pathogenic microbes (Perez-Cobas et al. 2015; Lewis et al. 2015). Moreover, it could be presumed that manipulation of gut microflora, in order to elevate the number of propitious microbiota plays an indispensible role in modulation of heterogeneous immune, digestion and metabolic function, which directly or indirectly affects and influences brain-gut communication. It was found that these modern approaches appear to be cost-effective, highly sophisticated and inconvenient for the use of common man. Hence, a simple, cheaper, receptive and intrinsic means for health benefactor became a vital compulsion of the present era. The properties which are shown by probiotics a groups of bacteria serve as a supplementation for host and against various enteric pathogens through their unique ability to compete with pathogenic microbiota for adhesion to the gut and improve their colonization (Rao et al. 2016).

Probiotics also stimulates, modulate and regulate the host’s immune response by initiating the activation of specific genes of localized host cells. It can even modulate the gastrointestinal hormone release, and regulation of brain behavior through bidirectional neuronal signaling as part of the gut-brain axis (Kristensen et al. 2016). Probiotics also plays a significant role in inducing intestinal angiogenesis by VEGFR signaling that in turn regulate acute and chronic inflammation in intestinal mucosal tissue caused due to the progression of inflammatory bowel disease (IBD) (Bakirtzi et al. 2016; Chen et al. 2013). Sudden demand for energy due to overweight or obesity resulting from abnormal fat accumulation poses an indirect influence on entire-body respiration by directly influencing the gut microflora. The probiotics assist in various physiological and intrinsic biochemical functions essential for the maintenance of steady state of the host gut regulating microbes (Kobyliak et al. 2016). Though probiotics have much greater nutritional and clinical application but still research regarding their application in field of medical and nutrition is very limited. Thus, the chapter aims to underline the possible approval of probiotics for betterment of human health and nutrition.

7.2 Properties of Probiotics and Its Related Terms

There might be many definitions of probiotics and the scientific terms related to it, but the following definition are more convincing and simple to understand. "Food supplements consisting of live microbial organisms which also impart certain beneficiary affects within the host by improving intestinal balance" is one of these simple definitions for probiotics given by Fuller in 1989. Similarly, certain non-digestible food particles that helps the host by selectively accelerating the growth and development of colon microbiota is defined as prebiotics by Gibson and Roberfroid (1995) (Yoo and Kim 2016). Lastly, when probiotics and prebiotics together improve nutritional supplementation of the host then this mutuality can be defined as synbiotics (Hamasalim 2016).

Currently, the Food and Agricultural Organization (FAO; Rome) World Health Organization (WHO), the International Life Science Institute (ILSI) and the European Food and Feed Cultures Association (EFFCA) all have included in their definition of probiotics as living microflora that confer beneficial roles within the host. They are commonly consumed as preparations with active live cultures and contain bacteria, such as *lactobacilli*, *lactococci* or *bifidobacteria*, isolated from natural environments (Bongaerts and Severijnen 2016; World Health Organization 2017).

7.3 Probiotic Genera and Species

A number of bacterial species have been used as probiotics (Fig. 7.1). Recent findings suggest that bacterial products, in the absence of viable organisms, may have similar impact on signaling pathways and different barrier function and are broadly regarded as postbiotics. In a simpler way these groups of products can be defined as a part of the probiotic bacteria such as metabolic byproducts or any other non-viable bacterial products which showed similar biochemical functions that of the bacteria (Patel and Denning 2013). General postbiotic include bacterial metabolic byproducts such as bacteriocins, organic acids, ethanol, diacetyl, acetaldehydes and hydrogen peroxide etc. but it is also found that certain heat-killed probiotics could able to exert biologic activity in the host because there are certain important bacterial structures that still remain intact (Islam 2016). Research shows that these metabolic products have a broad inhibitory property toward pathogenic microbes therefore they can also be used as an alternative to antibiotics (Ooi et al. 2015).

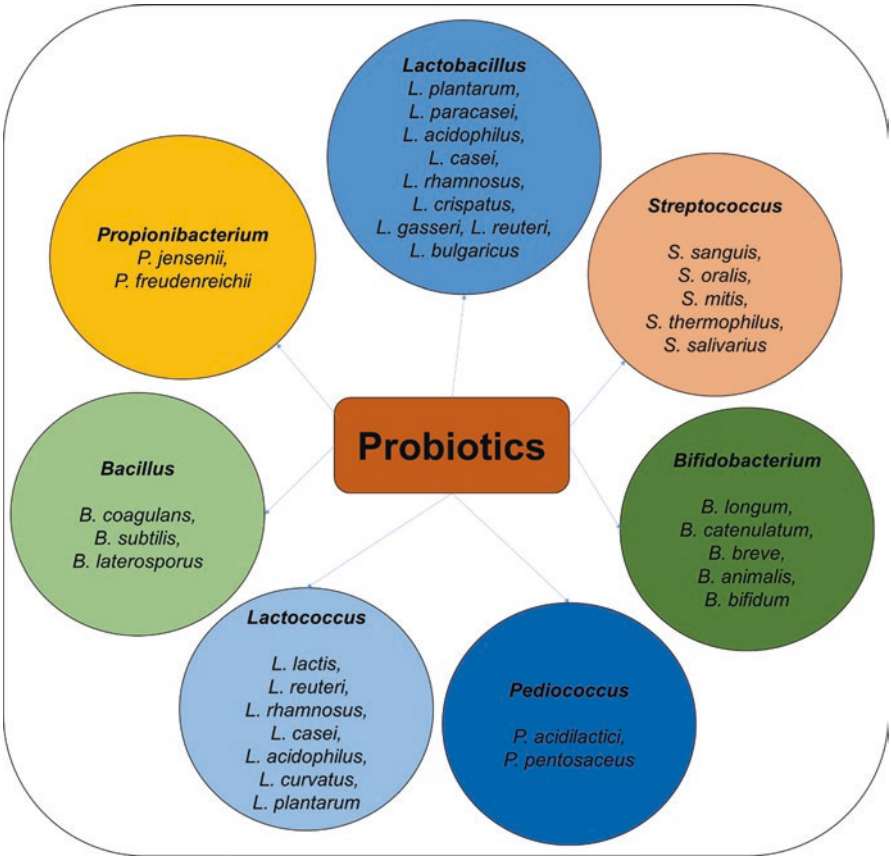

Fig. 7.1 Different species of microorganisms used as probiotics

7.4 Significance of Probiotics

Some commercial strains currently sold as probiotics and their sources are given in Table 7.1. According to the manufactures reported the species are listed, furthermore it might not reflect the current taxonomy.

7.4.1 Probiotics in Human Health

Use of probiotics for clinical health benefits is one of fascinating area of research that the present era yet has not completely explored. Except for few of its elite properties such as anti-pathogenicity, anti-diabetic activity, anti-obesity activity, anti-inflammatory activity, anti-cancer activity, Anti-allergic activity, angiogenic activity

Table 7.1 Some of the commercially available probiotics

Commercial products	Strain	Source
Align	*Bifidobacterium infantis* 35264	Procter and Gamble (Mason, OH)
BioGaia probiotic chewable tablets or drops	*Lactobacillus reuteri* ATCC 55730 ("*L. reuteri* Protectis")	Biogaia (Stockholm, Sweden)
Culturelle; Dannon Danimals	*Lactobacillus rhamnosus* GG ("LGG")	Valio Dairy (Helsinki, Finland)
DanActive fermented milk	*Lactobacillus casei* DN-114001 ("*L. casei* Immunitas")	Danone (Paris, France)
EcoVag	*Lactobacilli rhamnosus* PBO1	Bifodan (Denmark), www.ecovag.com
	Lactobacilli gasseri EB01	
Florastor	*Saccharomyces cerevisiae boulardii*	Biocodex (Creswell, OR)
ProBiora3	*Streptococcus oralis* KJ3	Oragenics Inc. (Alachua, FL)
EvoraPlus	*Streptococcus uberis* KJ2	
Sold as ingredient	*Lactobacillus acidophilus* NCFM	Danisco (Madison, WI)
	Bifidobacterium lactis HN019 (DR10)	
	Lactobacillus fermentum VRI003 (PCC)	Probiomics (Eveleigh, Australia)
	Bifidobacterium lactis Bb-12	Chr. Hansen (Milwaukee, WI)
	Lactobacillus rhamnosus 271	Probi AB (Lund, Sweden)
	Lactobacillus plantarum OM	Bio-Energy Systems, Inc. (Kalispell, MT)
Sustenex, digestive advantage and sold as ingredient	*Bacillus coagulans* BC30	Ganeden Biotech Inc. (Cleveland, Ohio)
Yakult	*Lactobacillus casei* strain Shirota	Yakult (Tokyo, Japan)
	Bifidobacterium breve strain Yakult	

of probiotics and their effect on brain and central nervous system. Some of these properties have been explained briefly below.

7.4.2 Anti-allergic Activity of Probiotics

The prevalence of allergic diseases caused by immune disorders has been increasing worldwide and has raised a serious economic and social burden. Comprehending the basic molecular mechanism that contributes to the etiology of allergic disease as well as new treatment approaches are some of the primary follow-up and prevention of these diseases (Akelma and Topcu 2016). Recent years the beneficial role of probiotics in the protection and management of these allergic diseases has taken a

new root in understanding their cause and prevention. Probiotics such as *Lactobacillus plantarum* L67 could able to induce the allergic inflammatory response by the production of interleukin-12 and interferon-γ (IFN- γ) in the host (Song et al. 2016). In another study, a different strain of *L. plantarum* 06CC2 could able to significantly alleviated allergic symptoms and reduce the levels of total IgE, ovalbumin-specific Ig E, and histamine in the sera of ovalbumin-sensitized mice. And the *in vitro* studies revealed that this strain 06CC2 significantly enhanced the secretions of interferon-γ and interleukin-4 spleen cells of that mouse which are responsible for alleviating allergic symptoms (Takeda et al. 2016). Probiotics are also help in reduction of liver tumor growth by inhibiting angiogenesis. There is very less research has been conducted regarding the anti-allergic activity of probiotics. Therefore further exploration should be made in order to evaluate the anti-allergic activity and its mechanism mode of action.

7.4.3 Anti-pathogenic Activity of Probiotics

Anti-pathogenic activity is regarded as one of the beneficial effect of probiotics, because unlike classic antibiotics, disturbance or reformation in the composition of the intricate population of the gut microbiota is inhibited. Currently there has been considerable research regarding the utilization of probiotics or a probiotic mixture on the human colonic microbiota and how this influences *S. Typhimurium* and *C. difficile* challenge in an *in vitro* model and it was postulated that, by the possible production of short-chain fatty acids (SCFAs) specifically acetic, propionic and butyric acids and lactic acid, probiotics microflora could able to vanquish these challenges (Tejero-Sarinena et al. 2013). Whereas many produce a wide verity of antipathogenic bioactive compounds like bacteriocins, ethanol, organic acids, diacetyl, acetaldehydes and hydrogen peroxide and peptides (Islam 2016). Out of these bioactive compounds specifically peptides and bacteriocins are mostly involved in increasing membrane permeability of the target cells, which leads to the loss of polarization of the ionic strength of the membrane which eventually leads to cell death (Simova et al. 2009). Similarly the production of H_2O_2 by these groups of bacteria causes the oxidation of sulfhydryl groups resulting in the denaturation of a number of enzymes that gives rise to the peroxidation of membrane lipids thus increasing membrane permeability of the pathogenic microorganism which ultimately kills these pathogens (Ammor et al. 2006). Some act by lowering pH by organic acids like lactic and acetic acids (Kareem et al. 2014). In addition to producing anti-pathogenic bioactive compounds that affects directly on pathogens, probiotics may stimulate host anti-pathogenic defense pathways such as stimulating or activating the pathway of producing defensins that are cationic antimicrobial peptides that are produced in a number of cell types including Panetch cells in the crypts of the small intestine and intestinal epithelial cells (Figueroa-Gonzalez et al. 2011).

7.4.4 Anti-diabetic Activities of Probiotics

According to IDF SEA, 415 million people have diabetes in the world and 78 million people in the SEA Region; by 2040 this will rise to 140 million. The management of this metabolic disorder through conventional therapy of medication up to certain limit is unsatisfactory. Current research is slowly and steadily is diverted towards a much cheaper and convenient remedy at the bimolecular and pharmacological level to evaluate the efficacy of synbiotics in combating this dreadful disorder (Iqbal et al. 2014). It is a common fact that nourishment the intestinal microflora by probiotics helps significantly in the neutralization or amelioration of some of the metabolic disorders. Basing on some of the advanced and sophisticated instrumentation and technology such as large-scale 16S rRNA gene sequencing, quantitative real-time PCR (qPCR) and fluorescent in situ hybridization (FISH) a relation between intestinal microbiota and metabolic disorders like diabetes and obesity are being researched and is slowly fascinating the current scientific world (Larsen et al. 2010). More specifically patients with type-2 diabetes have significantly reduced numbers of Firmicutes species in such a way that the ratio of Bacteroidetes to Firmicutes species increases which positively correlates with plasma glucose concentration (Barrett et al. 2012). Alteration in microbiome also paves the path for a larger number of opportunistic pathogens, which are resistant to oxidative stress and simultaneously capable of reducing sulfates, inhibiting the growth of butyrate-producing bacteria (Hartstra et al. 2015). In most of the cases, this alteration has also implicated in the development of autoimmune disease such as type-1 diabetes which is directly associated with increased Bacteroidetes, with a concurrent abatement in the number of Firmicutes, similar in pattern to that of type-2 diabetes (Ljungberg et al. 2006; Barrett et al. 2012; Hu et al. 2015).

7.4.5 Urogenital Heath Care

Over one billion and above women throughout the globe suffer from infections such as non-sexually transmitted urogenital infections, such as bacterial vaginosis (BV) urinary tract infection (UTI) and some of the other infections caused by yeasts (Waigankar and Patel 2011). BV is the result of a shift to a mixture of primarily anaerobic bacteria species. *Gardnerella vaginalis*, *Ureaplasma*, and *Mycoplasma* are some of the concomitant species of BV (Hanson et al. 2016). Not leaving behind sexually transmitted diseases (STDs) which are also a significant cause of morbidity in most of the country. The two most common reportable bacterial STDs in some developed counties are gonorrhea and Chlamydia caused by the bacterium *Neisseria gonorrhoeae* and *Chlamydia trachomatis* respectively (Chan et al. 2016). The major problem of current decade is not that we do not have better medicine, but the actual problem is that these pathogenic microbes are concurrently becoming resistance to

the present medicine. Therefore instead of developing new medicine our present focus should be in developing new live supplements like these microbes but non-pathogenic and acts against the pathogenic microbes.

7.4.6 Anti-obesity Activity of Probiotics

Sudden demand for energy, sedentariness and controlled temperature up to some extent is linked to overweight/corpulence/obesity resulting from abnormal fat accumulation (Kobyliak et al. 2016). This poses indirect influences on whole-body metabolism by directly influencing the gut microbiota, the link which was previously unexplored. And later was first demonstrated by transplanting gut microbe from obese mice which could exhibit the obese phenotype in aseptic mice. Recent research also proves that over weight and obesity is confederated with increased Bacteroidetes over time concurrent (Barz et al. 2015; Kobyliak et al. 2016).

In most of the cases it is found that the deduction in weight is facilitated through stimulating the sympathetic nervous system by thermogenic and lipolytic responses (Karimi et al. 2015). Moreover, probiotics also play a major role in inhibition of the growth of subcutaneous adipose tissue, which is regarded as the main source of leptin and adiponectin hormones that controls body weight by regulating food intake and energy expenditure. For example a probiotic strain namely *Lactobacillus gasseri* BNR17 was successfully evaluated for its efficiency to inhibit the growth of adipose tissue thereby limiting leptin secretion (Kang et al. 2013).

7.4.7 Anti-inflammatory Activity of Probiotics

Chronic inflammatory diseases of the gastrointestinal system such as Crohn's disease (CD) and ulcerative colitis (UC) are collectively known as inflammatory bowel disease (IBD) (Iqbal et al. 2014). The heterogeneous impacts of CD on gastrointestinal system involves mucosa, submucosa, and serosa inflammation which if unnoticed would spread throughout the gastrointestinal track. On the other hand in UC the colon mucosa, submucosa is generally inflamed (Palumbo et al. 2016). Currently it is understood that the disorder could be possibly altered by the supplementation of probiotics, prebiotics, and synbiotics (Cammarota et al. 2015, 2016; Spiller 2016). This supplementation enhances the health of the host gut environment regulating the well being of colonic bacteria that extracts its required nutrients by fermenting the fiber (prebiotics) resulting in the exudation of SCFAs which have anti-inflammatory effects and could improve the propulsive colonic function (Curro et al. 2016). Presently research is mostly concerned with developing genetically engineered probiotic strains which could directly effuse immunomodulators such as interleukin-10, trefoil factors, or lipoteichoic acid that can affect the immune system's anticipation in the restoration of the level of protective commensal bacterial

species (Shahverdi 2016). Most widely used probiotic organisms used with dairy and non- dairy products are *Lactobacillus*, *Bifidobacterium*, *Enterobacter* and *E. coli* apart from these organisms new or genetically modified should be developed to counteract the inflammatory disorder (Gowri et al. 2016).

7.4.8 Anti-cancer Activity of Probiotics

According to the data given in WHO fact sheet (2014), "cancer is a generic term for a large group of diseases that can affect any part of body", which makes it a serious global health problem (Gayathri and Rashmi 2016). It is also regarded as the foremost causes of infirmity and lethality around the globe. More than 70% of the deaths due to cancer are from Asia, Africa and Central and South America (Vidya and Thiruneelakandan 2015). Currently intensified research on finding a suitable therapy for cancer by concatenation of diversified, high throughput tools of genomics, proteomics and sophisticated instrumentation and technologies such as nanobiotechnology and molecular biology have broaden the perception of understanding the nature of this life threatening disease. Despite such scientific advancement actually finding a cure for this deadly disease is far from meeting the present requirements of present needs. Many new drugs using nanotechnology and biotechnology such as nanocapsule with fascinating properties of luminescent have been discovered but still than tolerance to their burden and side effect are really challenging tasks. Therefore in this case prevention is better than finding a cure, could be an alternative to these risky sophisticated and costly drugs, by using natural food supplements that endows anti-carcinogenic effects (Gayathri and Rashmi 2016). Therefore dietary intercession for prevention of this deadly disease has received a tremendous consideration from clinical nutritionists, scientists, and pharmacologists (Vafaeie 2016). Certain specific probacterial strain such as *Lactobacillus fermentum* NCIMB 5221 has proven to be more potent in suppressing colorectal cancer (CRC) cells and promoting normal epithelial colon cell growth through the production of SCFAs (ferulic acid), this ability was also compared with other probiotics such as *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 51303 both of which were previously characterized anti-tumorigenic activity (Kahouli et al. 2015).

7.4.9 Effect of Probiotics in Brain and Central Nervous System

The colonization of microbiota in the gastrointestinal tract is associated with both gastrointestinal tract and gastrointestinal diseases. Many studies in recent years are devoted towards finding the effect of gut microbiota on Central Nervous System (CNS), the so-called "microbiota-gut-brain axis". An interactive, bi-directional

communication can be established by the exchange of regulatory signals between the gastrointestinal tract and CNS represents the gut-brain axis (Mayer et al. 2015). The study of probiotics effect on CNS is mainly done in pre-clinically where the evidence show that gut microbiota influence human brain development function (Tillisch 2014). There are certain cases in children having Autism Spectrum Disorder (ASD) if fed with *Lactobacillus plantarum* WCFS1 (daily dose 4.5×1010 CFU) shows an improved result in their school records and attitude towards food (Umbrello and Esposito 2016). A randomized trial in healthy volunteers treated with the oral administration of *L. helveticus* R0052 and *B. longum* R0175 give a result as reduced psychological distress (Messaoudi et al. 2010). Another clinical trial shows a decrease in anxiety symptoms by application of *Lactobacillus casei* strain Shirota to patients suffering from Chronic Fatigue Syndrome (Rao et al. 2009).

7.5 Conclusion

Form the above critical description it can be concluded that probiotic plays functional selective sources which could fulfill most of our basic nutritional and clinical supplements requisites. Generally these probiotics are supplemented through dairy products which are also their sources but a non-dairy fermented food product presents an alternative and advantageous source in the process of evaluating new probiotic strains. Screening for non-pathogenic microbes along with their evaluation of basic characteristic such as acid and bile tolerance, ability to adhere to gut epithelial cells, to combat against pathogens in the GI tract, and the safety-enhancing property which includes inability to transfer any antibiotic resistance genes to pathogens would be an efficient measure for identification of probiotics. Though massive progress has been made in the field of science and technology, and multidisciplinary fields have been evolved including proteomics along with its derivatives such as metabolomics, transcriptomics, and genomics, nanotechnology, not forgetting biotechnology the field which pioneered this multidisciplinary era, are all marching united towards a common goal. Unfortunately the goal it is still out of reach in the present scenario. The problem is not the technique, infect it's our idea of emphasizing or prioritizing this natural food supplement probiotics for the betterment of mankind.

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Chapter 8
An Insight into the Prevalence and Enzymatic Abatement of Urethane in Fermented Beverages

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8.1 Introduction

Urethane is the ethyl ester of carbamic acid (ethyl carbamate, $NH_2COOC_2H_5$), which had been used extensively in the past as a chemical intermediate for the production of pharmaceuticals, as a food flavoring agent, as a cross-linker in the textile industry, as a diluent in hair conditioner, as a hydrocarbon extractant from crude oil, as a food flavorant, and as a co-solvent and solubilizer in the synthesis of pesticides, cosmetics and fumigants (Adams and Baron 1965; Nomura 1975; Zhao et al. 2013). It was also used as a drug for the treatments of neoplastic and sclerosis of varicose vein diseases (Adams and Baron 1965), as a hypnotic (Salmon and Zeise 1991), as a topical bactericidal (NTP 2016) and as a tranquillizer for animals (Hara and Harris 2002). In 1976, U.S. Food and Drug Administration prohibited urethane as a therapeutic agent for humans owing to its genotoxic, mutagenic and clastogenic effects on mice, rats, hamsters and monkeys (Salmon and Zeise 1991; Thorgeirsson et al. 1994). The carcinogenic and teratogenic effects of urethane are primarily attributed to the generation of the metabolic by-products, such as vinyl carbamate and N-hydroxy-urethane during the metabolic processing of urethane (Guengerich and Kim 1991; Barbin 2000). This metabolic processing is mediated by a bioactivation pathway involving two oxidation steps. Both the oxidation steps are catalyzed by the cytochrome P450 2E1 (CYP2E1). The first oxidation step is the desaturation of urethane to vinyl carbamate, while in the second step the double bond of vinyl carbamate undergoes epoxidation to form vinyl carbamate epoxide

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Table 8.1 Prevalence of urethane in fermented food products and alcoholic beverages

Fermented products	Range of urethane concentration (μg/L)	References
Food		
Bread	1.6–4.8 (μg/kg)	Sen et al. (1993)
Citrus juice	ND-0.1	IARC (2010)
Grape juice	ND-0.2	IARC (2010)
Kimchi	ND-16.2 (μg/kg)	Kim et al. (2000)
Soy sauce (regular)	ND-19.5	IARC (2010)
Soybean paste	0–1.18 (μg/kg)	Ryu et al. (2015)
Vinegar	0.3–2.5	IARC (2010)
Yogurt and acidified milk	0.1–0.3 (μg/kg)	Vahl (1993)
Alcoholic beverage		
Beer	0.5–0.8	Ha et al. (2006)
Korean raspberry wine (Bokbunjaju)	0–6.3 (μg/kg)	Ryu et al. (2015)
Sake	10–904	Salmon and Zeise (1991)
Sherry	32–242	NTP (2016)
Stone fruits brandies	100–20,000	NTP (2016)
Table wine	1.7–117	NTP (2016)
Whisky	68–389	NTP (2016)

ND not detected

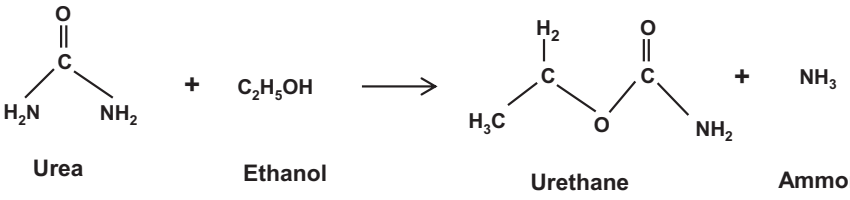
(VCE). The resulting VCE reacts with DNA to form etheno-adducts that induce the carcinogenesis (Park et al. 1993; Barbin 2000). Additionally, N-hydroxy-urethane also induces carcinogenesis by Cu(II)-mediated DNA damage specifically at thymine and cytosine residues (Sakano et al. 2002).

Despite the regulatory prohibition of the therapeutic usage of urethane, humans have been exposed to urethane via fermented food products and alcoholic beverages (EFSA 2007; Hasnip et al. 2007; Lachenmeier et al. 2009; Ryu et al. 2015). The concentration of urethane in different food products and beverages are listed in Table 8.1. The prevalence of higher concentrations of the carcinogenic urethane in alcoholic beverages has been a concern to the public health (NTP 2016; Health Canada 2013a). Although there is no harmonized standard available as yet for the maximal allowable limit of urethane, the regulatory agency of Canada has established a standard for maximum level of urethane in distilled spirit (150 μg/L), fortified wine (100 μg/L), fruit brandy (400 μg/L), sake (200 μg/L) and table wines (30 μg/L) (Health Canada 2013a). The European Union has recommended that the target limit of urethane in stone fruits distillates should not exceed 1 mg/L (EFSA 2007).

8.2 Urethane Synthesis in Alcoholic Beverages

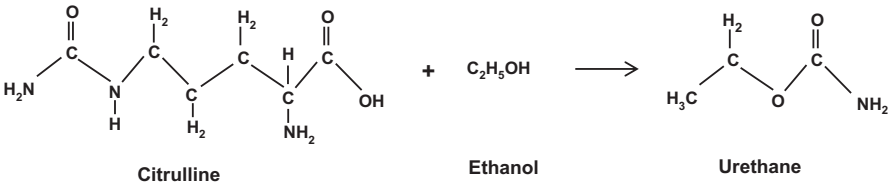
The formation of urethane in alcoholic beverages is a complex mechanism, and stoichiometrically depends on the precursor molecule, pH, temperature, ethanol concentration, oxygen and the type of fermenting microorganisms (Ough et al. 1988; An and Ough 1993). Five compounds (urea, citrulline, carbamyl phosphate, hydrocyanic acid and diethyl pyrocarbonate) have been identified as precursor molecules for the synthesis of urethane (Zhao et al. 2013; Jiao et al. 2014). Among these five precursors, urea, citrulline and carbamyl phosphate are produced via the metabolic pathways of yeast and/or lactic acid bacteria. Of the other two precursors, hydrocyanic acid is formed as a result of enzymatic reactions, and diethyl pyrocarbonate is added as a preservative.

Urea has been reported as the principal precursor in the majority of alcoholic beverages, including table wine, sake and whisky, because a copious amount of urea is secreted by yeast during the L-arginine metabolism (An and Ough 1993; IARC 2010). L-Arginine is the most abundant amino acid available in grape juice for yeast metabolism. Arginine hydrolase converts L-arginine into L-ornithine and urea (Schehl et al. 2007). The resulting urea, released by the yeast, reacts with ethanol in the fermentation medium to form the carcinogenic urethane (Eq. 8.1) (Ough et al., 1988; Hara et al. 1988; Kitamoto et al. 1991).



(8.1)

Citrulline, the second important urethane precursor, is produced in grape must, wine, and fruit brandies during the arginine metabolism of lactic acid bacteria via the arginine deiminase (ADI) pathway of malolactic fermentation (Azevedo et al. 2002). Additionally, the ADI pathway is also responsible for the generation of carbamyl phosphate (third urethane precursor) during the transformation of L-citrulline to L-ornithine using ornithine transcarbamylase (EC 2.1.3.3) (Ough 1976a). The excreted citrulline and carbamyl phosphate spontaneously react with ethanol to synthesize urethane (Eqs. 8.2 and 8.3, respectively) (Arena and Manca de Nadra 2005).



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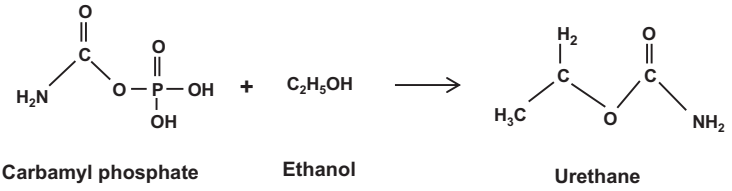
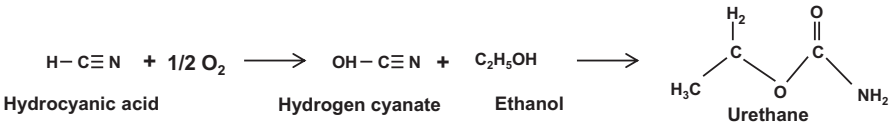


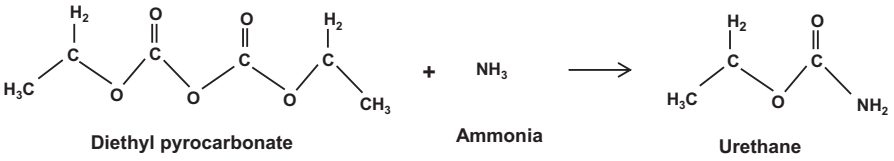
(8.3)

Hydrocyanic acid, the fourth precursor, is mostly responsible for the generation of urethane in spirits prepared from stone fruits (plums, apricots, cherries and mirabelles). These stone fruits contain cyanogenic glycosides, which are ultimately removed by the enzymatic action of β-glucosidase (Lachenmeier et al. 2005). This enzymatic removal process produces hydrocyanic acid, which oxidizes to hydrogen cyanate and subsequently reacts with ethanol to form urethane (Eq. 8.4) (Taki et al. 1992; Aresta et al. 2001). The accumulation of urethane in stone fruit spirits has been reported to show dependence not only on pH and ethanol concentration, but also on light intensity, temperature and time of storage (Suzuki et al. 2001).



(8.4)

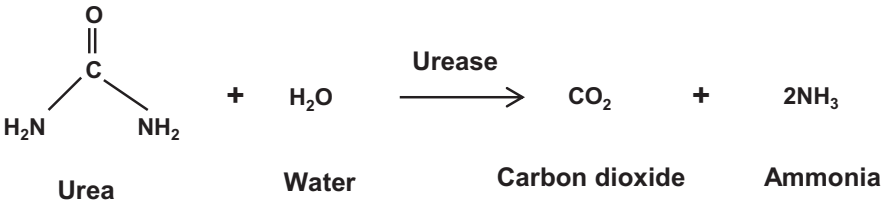
Diethyl pyrocarbonate, the fifth precursor, is incorporated into wine as an additive to inhibit the growth of wine spoilage microorganisms (Ough 1976b; Solymosy et al. 1978). This compound reacts with ammonia to produce urethane (Eq. 8.5). The use of diethyl pyrocarbonate as an additive has been restricted owing to its toxicity (Polychroniadou et al. 2003; Jiao et al. 2014).



(8.5)

8.3 Enzymatic Abatement of Urethane

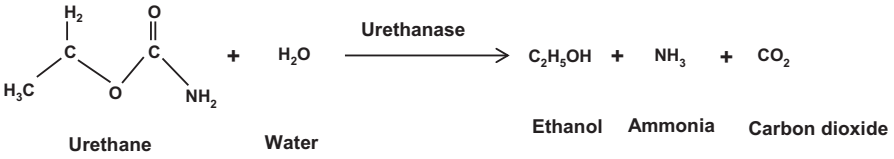
In recent years, the global regulatory agencies have been developing strategies to reduce the concentration of urethane accumulated in fermented beverages due to the upgradation of the carcinogenic effect of urethane from Group 2B (possibly carcinogenic to humans) to Group 2A (probably carcinogenic to humans) by IARC (IARC 2010). Several plans to prevent the accumulation of urethane in alcoholic beverages have been proposed by the US Food and Drug Administration (Butzke and Bisson 1997). These plans include the vineyard management with low-nitrogen fertilizers, selection of vine grape cultivar with low-arginine content, adjustment of nutrients in grape must and juices, selection of yeast and lactic acid bacterial strains with low-urea and low-citrulline producing abilities, modification of distillation and fortification processes and control of temperature during storage and transport. In addition, the potential of chemical (addition of copper salt and potassium bisulfite) and enzymatic (addition of acidic urease) methods have also been reported (Zhao et al. 2013; Jiao et al. 2014). Among all the urethane elimination methods, enzymatic treatment with acidic urease (EC 3.5.1.5) has been considered as a convenient way to inhibit urethane synthesis by scavenging its primary precursor urea (Eq. 8.6) (Kobashi et al. 1988; Andrich et al. 2009; Health Canada 2013b; Cerreti et al. 2016).



(8.6)

Additionally, the enzymatic bioprocesses have been getting substantial industrial attention because of their environmental friendliness via operation of the processes under mild conditions, recording of higher catalysis rate and substrate affinity, and production of nontoxic by-products. Acidic urease has been documented from the bacterial genera *Arthrobacter*, *Bifidobacterium*, *Enterobacter*, *Escherichia*, *Lactobacillus*, *Morganella*, *Staphylococcus* and *Streptococcus* (Kakimoto et al. 1989; Matsumoto 1993; Miyagawa et al. 1999; Yang et al. 2010).

Despite the importance of enzymatic abatement processes, the effectiveness of acidic urease is primarily restricted to the hydrolysis of urea, the key precursor of urethane. This treatment is resistant to urethane that has already been accumulated in alcoholic beverages (Kobashi et al. 1990; Zhao and Kobashi 1994; Zhao et al. 2013). Therefore, there is a considerable interest to search for enzymes that can directly decompose urethane. Kobashi et al. (1990) reported an enzyme "urethanase (EC 3.5.1.75)" from *Citrobacter* sp. that can catalyze the hydrolysis of urethane into ethanol, carbon dioxide and ammonia (Eq. 8.7).



(8.7)

Later, urethanase activity has been reported from bacterial strains of the genera *Bacillus* (Zhao et al. 1991; Zhao and Kobashi 1994), *Micrococcus* (Mohapatra and Bapuji 1997) and *Providencia* (Zhang et al. 2016), from yeast strain *Rhodotorula mucilaginosa* (Wu et al. 2013) and from fungal strain *Penicillium variabile* (Zhou et al. 2014).

Owing to the unavailability of commercial urethanase preparations, research efforts have been devoted to screen out urethanase-producing microorganisms from different environmental niches for their potential application as microbial biocatalysts. The advantage of microbial biocatalyst is the cost-effectiveness of the bioprocesses by direct utilization of the microbial whole-cells and/or their crude enzymes (Schmid et al. 2001; de Carvalho 2011). In order to utilize the microbial biocatalysts, it is essential to design methods for sustaining their catalytic efficacy in harsh industrial processes (Sheldon and van Pelt 2013; de Carvalho 2017). Immobilization of microbial whole-cells and/or enzymes on inexpensive natural nontoxic polymeric substances, especially on chitosan support, has been reported as a robust means to improve the versatility of the biocatalyst towards harsh conditions, efficient recovery and reusability, lower chance of product contamination with enzyme residues and lower allergenic potential (Sheldon and van Pelt 2013).

Chitosan is a polymer of D-glucosamine and N-acetyl-D-glucosamine with β (1→4) linkages, and is documented as nontoxic, biodegradable and biocompatible

(Chen et al. 2006). It is commercially prepared by N-deacetylation of chitinous wastes of the crustaceans shells (crab, shrimp, lobster, krill and other shellfish). Immobilization of microbial enzymes on chitosan can be accomplished by chemical modification with bifunctional glutaraldehyde (a cross-linker), where the – CHO functional groups of glutaraldehyde react with the amino groups present in chitosan as well as in the terminal end of the enzyme (Krajewska 2004).

Chitosan particles of different size, such as macro-, micro- and nanoparticles have been fabricated for immobilization of microbial biocatalysts (Agnihotri et al. 2004). In recent years, chitosan nanoparticles have been received significant attention as immobilizing matrix because of increased surface to volume ratio and thereby facilitating higher enzyme loading and reaction rate, reduction in diffusion hindrance and fouling effect (Shukla et al. 2013). In the following sections, the comparative biocatalytic efficacy of free and chitosan nanoparticles immobilized urethanase from a newly isolated strain of *Exiguobacterium* species Alg-S5 is reported.

8.4 Methods to Evaluate the Comparative Biocatalytic Efficacy of Free and Immobilized Urethanase from *Exiguobacterium* sp. Alg-S5

Sixty-seven bacterial strains were isolated from the invasive floating *Sargassum* seaweed off the coast of Barbados and screened for their potential in producing industrially-important enzymes. During this enzymatic screening, a potent urethanase-producing bacterium was isolated. This bacterium was identified as *Exiguobacterium* sp. Alg-S5 using 16S rRNA gene sequencing approach (Mohapatra 2017). The GenBank accession number of this bacterium is KY009691.

8.4.1 Batch Cultivation of Exiguobacterium sp. Alg-S5 and Urethanase Extraction

Batch cultivation of *Exiguobacterium* sp. Alg-S5 was performed in 20×250 mL Erlenmeyer's flasks containing 50 mL of medium (1% tryptone, 0.5% yeast extract and 2% NaCl) in each flask. The cultures were grown at 35 °C by shaking (150 rpm) in an orbital shaker for 48 h. Bacteria cells were harvested by centrifugation at 13,000 × *g* (4 °C) for 30 min. The resulting pellets were washed thrice with 50 mM Trizma™ buffer (pH 7.5) to remove the residual medium and then resuspended in 15 mL of 50 mM citrate-phosphate buffer (pH 5). The cell resuspension was passed thrice through a French pressure cell press (SLM Aminco; SLM Instruments, Rochester, NY, USA) at 10,000 psi (69 Mpa), followed by centrifugation for 30 min at 13,000 × *g*

(4 °C). Afterward, the resulting filtrate was desalted and concentrated through a Vivaspin20™ 10 kDa cut off ultrafiltration unit (Sartorius Inc., Gloucestershire, UK). Concentrated fraction was diluted to 40 mL with 50 mM citrate-phosphate buffer (pH 5) and used for the immobilization as well as characterization studies.

8.4.2 Preparation of Immobilized Urethanase Biocatalyst on Chitosan Nanoparticles

Chitosan nanoparticles were prepared by the ionotropic gelation method as described previously (Klein et al. 2012) with some modifications. Briefly, 1 mL of 1.4 M aqueous Na_2SO_4 solution was added dropwise to 19 mL stirred solution of chitosan (0.25% chitosan dissolved in 0.35 M acetic acid). After further stirring of the suspension for 4 h at 500 rpm (25 °C), the resulting nanoparticles were harvested via centrifugation at 13,000 × *g* (4 °C) for 30 min. The nanoparticles were washed thrice with 50 mM Trizma™ buffer (pH 7.5) and resuspended in 20 mL of the same buffer supplemented with 2% glutraldehyde. This resuspension was agitated at 50 rpm in a rotary shaker at 30 °C for 2 h. After cross linking, the chitosan nanoparticles were washed six times with 50 mM Trizma™ buffer (pH 7.5) to remove the glutaraldehyde.

The immobilized biocatalyst of the extracted urethanase from *Exiguobacterium* sp. Alg-S5 was prepared by adding 20 mL of enzyme solution to the glutraldehyde-treated chitosan nanoparticles under gentle stirring at 4 °C for 24 h. Then the resuspension was centrifuged at 13,000 × *g* (4 °C) for 30 min to remove the supernatant containing unbound enzyme, followed by repeated washing of the pellet with the 50 mM citrate-phosphate buffer (pH 5) until there was no urethanase activity detected in the supernatant. The immobilized biocatalyst was resuspended in 15 mL of the same buffer and stored at 4 °C until further use. Prior to using the immobilized biocatalyst for enzyme assay, the resuspension was mixed at moderate speed in a magnetic stirrer to keep the nanoparticles in suspension. The enzyme activity retention (ER) on chitosan nanoparticles was calculated using Eq. 8.8.

$$\mathrm{ER}(\%) = \left[\left\{ E_a - \left(E_u + E_w \right) \right\} / E_a \right] \times 100 \tag{8.8}$$

Where E_a = Urethanase activity added, E_u = Unbound urethanase activity in the supernatant and E_w = Unbound urethanase activity in the pooled washing fractions.

8.4.3 Assay of Urethanase Activity

The standard reaction mixture for the assay of urethanase was comprised of 50 µL free or immobilized biocatalyst, 150 µL of phosphate-citrate buffer (pH 5) and 800 µL of 45 mM urethane prepared in the same buffer. The reaction was incubated at 35 °C for 1 h, followed by centrifugation at 10,000 × *g* for 5 min. Ammonia content in the supernatant due to the enzymatic hydrolysis of urethane was measured spectrophotometrically at 690 nm using indophenol method (Bower and Holm-Hansen 1980). One unit (U) of urethane activity was defined as the release of 1 nmol of ammonia per min per mg-protein under the standard assay conditions. Appropriate blanks were also tested by using heat-inactivated biocatalyst (boiled at 100 °C for 30 min) in the standard reaction mixture. All the measurements were performed in triplicate and the data were represented as mean ± standard deviation.

Protein concentration in the enzyme solution was measured by Bradford method using bovine serum albumin as standard. Amount of protein in chitosan nanoparticles was estimated by subtracting the unbound protein content in supernatant and in pooled washing fractions from the protein content initially added to the chitosan nanoparticles.

8.4.4 Effect of pH and Temperature on Free and Immobilized Urethanase Activity

The influence of pH on free and immobilized enzyme was evaluated by assaying the enzyme activity (Sect. 8.4.3) of the standard reaction mixture at 35 °C and 1 h incubation with buffers of different pH: 50 mM phosphate-citrate buffer (pH 3–6), 50 mM Trizma™ (pH 7–8) and 50 mM borate (pH 9–10). To study the effect of temperature, the enzyme activity of the reaction mixtures was analyzed (Sect. 8.4.3) at pH 5 with 1 h incubation at different temperatures (20–60 °C, with 5 °C increments).

8.4.5 Thermostability of Free and Immobilized Urethanase

Both free and immobilized enzymes were pre-incubated at different temperatures ranging from 20 to 60 °C in 5 °C increments for 30 min, followed by rapid cooling on ice to avoid spontaneous thermal inactivation and measurement of the residual activity as mentioned in Sect. 8.4.3.

8.4.6 Kinetics of Free and Immobilized Urethanase

Free and immobilized enzyme activities were assayed as described in Sect. 8.4.3 with the addition of different concentrations (0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 60 mM) of urethane. The values of Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver-Burk plot.

8.4.7 Effect of Ethanol on Free and Immobilized Urethanase

To test the effect of ethanol on urethanase activity, the standard reaction mixture consisting of free or immobilized enzyme (50 µL), 150 µL of phosphate-citrate buffer (pH 5) containing different concentrations (5–40%, in steps of 5%) of ethanol and 800 µL of 45 mM urethane in the same buffer, were incubated at 35 °C for 1 h. After that, the residual enzyme activity was determined as described in Sect. 8.4.3.

8.4.8 Reusability of Immobilized Urethanase

The operational stability of immobilized enzyme was examined in batches for urethane hydrolysis as described in Sect. 8.4.3. After the first reaction cycle, the immobilized urethanase was recovered by centrifugation at 10,000 × *g* for 5 min, followed by washing thrice with the 50 mM citrate-phosphate buffer to remove the substrate (pH 5) and used in subsequent reaction cycles as the biocatalyst. Urethanase activity in the first cycle was considered as 100%.

8.5 Characteristics of Free and Immobilized Urethanase from *Exiguobacterium* sp. Alg-S5

The free and chitosan nanoparticles immobilized urethanase were characterized with respect to their maximal activity against pH and temperatures. Additionally, the comparative substrate-specific kinetics and the effect of ethanol on free and immobilized activities were also assessed. The ER (enzyme activity retention) value was recorded as 75.1 ± 1.53% for the extracted urethanase immobilized on glutaraldehyde cross-linked chitosan nanoparticles. The pH-activity studies revealed that both the free and immobilized urethanase had maximal activity at pH 5 (Fig. 8.1). At pH 4 and pH 7, the enzyme activity was recorded as 76.4% ± 2.61 and 68.5 ± 2.23% for free enzyme and 79.2 ± 2.43% and 70.3 ± 2.32% for immobilized enzyme. Above pH 7, the activity of both the free and immobilized enzyme declined drastically. Previous studies have documented the optimal pH of 4.5, 5 and 6 for the free

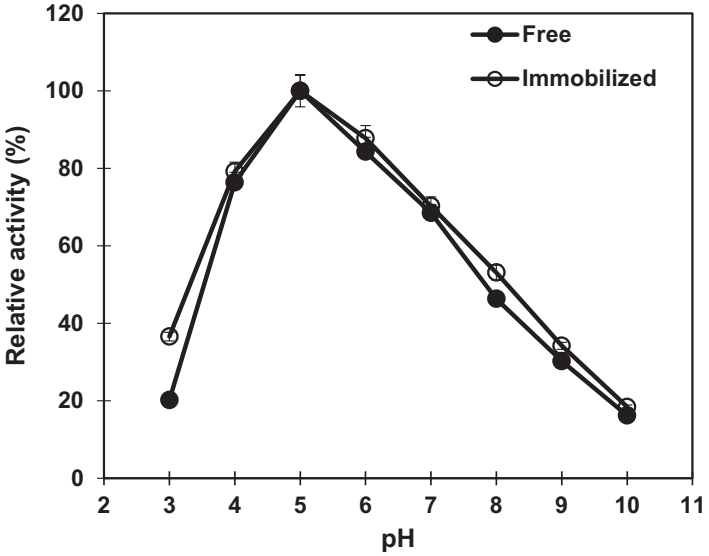


Fig. 8.1 Effect of pH on free and immobilized urethanase activity. Data points are the mean values of triplicate measurements. Error bar is visible when it exceeds the size of the data point

urethanase from *Bacillus licheniformis* 1013 (Zhao et al. 1991), *Micrococcus* sp. (Mohapatra and Bapuji 1997) and *Penicillium variable* JN-A525 (Zhou et al. 2014), respectively.

The temperature-activity profile of free and immobilized urethanase at different temperatures (20–60 °C) exhibited the highest enzyme activity at 35 °C (Fig. 8.2). Both the free and immobilized enzyme lost more than 51% activity at 50 °C. The optimal temperature recorded here was found to be within the range reported for the free urethanase from *Bacillus licheniformis*: 37 °C (Zhao et al. 1991; Zhao and Kobashi 1994) and lower than *Micrococcus* sp.: 45 °C (Mohapatra and Bapuji 1997) and *Penicillium variable* JN-A525: 50 °C (Zhou et al. 2014).

Thermal denaturation studies at different temperatures (20–60 °C) indicated that both the free and immobilized enzyme were quite stable at 40 °C by retaining more than 86% of enzyme activity after 30 min incubation (Fig. 8.3). The half-life of the free and immobilized urethanase was noted *ca.* 30 min at 50 °C and 55 °C, respectively. At 60 °C, the free and immobilized enzyme lost 84.9% and 63.6% activity, respectively, suggesting that the immobilization increased the conformational flexibility of urethanase at higher temperature. This urethanase was found to be more thermostable compared to the urethanase isolated from *Bacillus licheniformis* sp. 1013 (Zhao et al. 1991) and *Bacillus licheniformis* sp. IFO 12107 (Zhao and Kobashi 1994).

The substrate-specific kinetics of free and immobilized urethanase were examined using different concentrations of urethane (0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 60 mM). At the optimal pH (5.0) and temperature (35 °C), both the free and immobilized enzyme exhibited Michaelis-Menten kinetics. The K_m and V_{max} values were

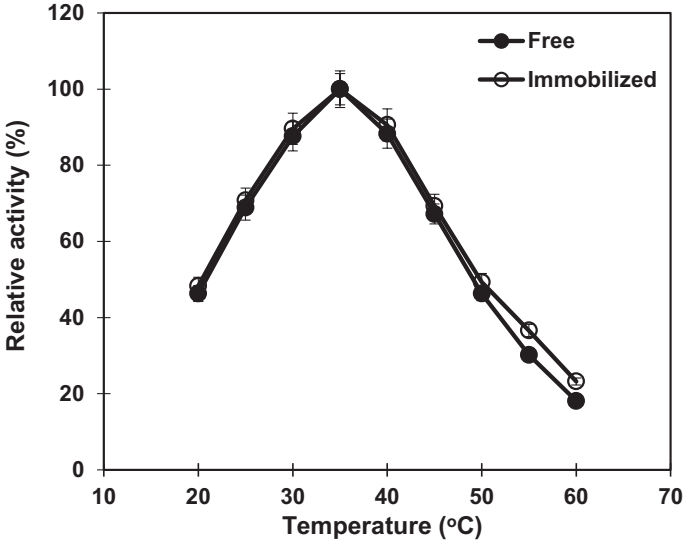


Fig. 8.2 Effect of temperature on free and immobilized urethanase activity. Data points are the mean values of triplicate measurements. Error bar is visible when it exceeds the size of the data point

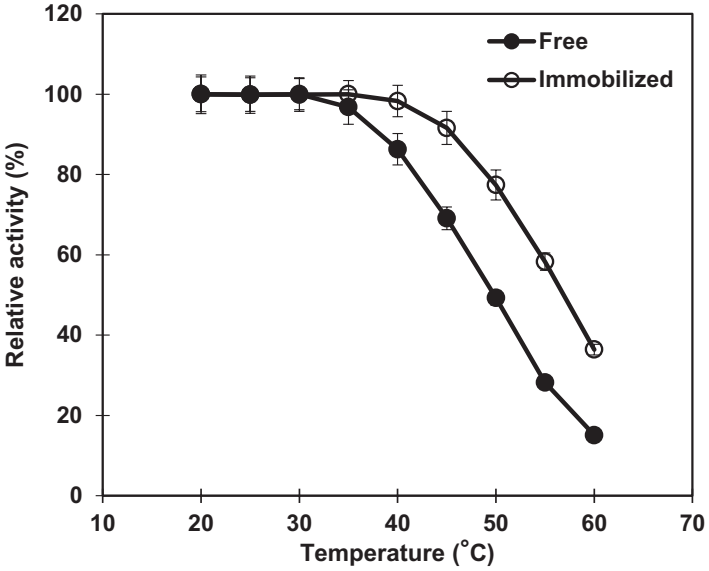


Fig. 8.3 Thermal denaturation of free and immobilized urethanase. Data points are the mean values of triplicate measurements. Error bar is visible when it exceeds the size of the data point

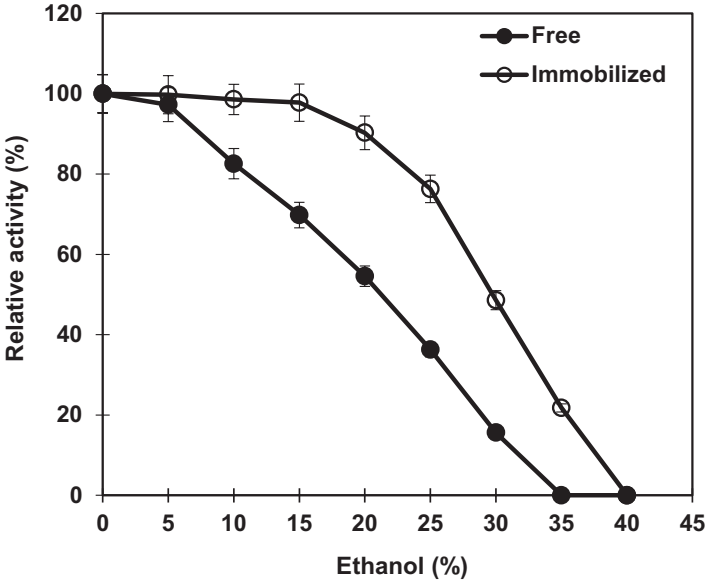


Fig. 8.4 Effect of ethanol on free and immobilized urethanase activity. Data points are the mean values of triplicate measurements. Error bar is visible when it exceeds the size of the data point

estimated using Lineweaver-Burk plot as 3.25 ± 0.14 mM and 15.7 ± 0.74 nmol/min/mg-protein for free urethanase and 3.43 ± 0.16 mM and 11.4 ± 0.56 nmol/min/mg-protein for immobilized urethanase. Restriction by the immobilizing matrix for the substrate to get access to the enzyme active site might lead to the observance of higher K_m value in immobilized enzyme (Kumar et al. 2009). The free urethanase of *Bacillus licheniformis* IFO 12107 (Zhao and Kobashi 1994), *Citrobacter* sp. (Kobashi et al. 1990), *Penicillium variable* (Zhou et al. 2014), and *Bacillus licheniformis* 1013 (Zhao et al. 1991) had displayed a K_m value of 0.17 mM, 1.6 mM, 27.2 mM and 42 mM, respectively.

The effect of ethanol on the activities of free and immobilized urethanase was tested by incubating the standard reaction mixture of enzyme with various concentrations of ethanol (5–40%) (Fig. 8.4). It was observed that both the free and immobilized urethanase could function well at 5% ethanol by retaining 97.3 ± 4.21% and 99.8 ± 4.75% of activity, respectively. Above 10% ethanol concentration, the free enzyme was susceptible to denaturation by exhibiting 69.8 ± 3.15% relative activity at 15% ethanol concentration (similar to the ethanol content in rice and table wine). In contrast, the immobilized enzyme showed a relative activity of 97.8 ± 4.66% and 90.3 ± 4.17% at 15% and 20% ethanol, respectively, suggesting the protective action of immobilizing matrix on sustaining the conformation of urethanase. A total loss of free and immobilized urethanase activity was noticed at 40% ethanol. The free urethanase of different bacteria, *Bacillus licheniformis* IFO 12107 (Zhao and Kobashi 1994), *Bacillus licheniformis* 1013 (Zhao et al. 1991) and *Citrobacter* sp. (Kobashi

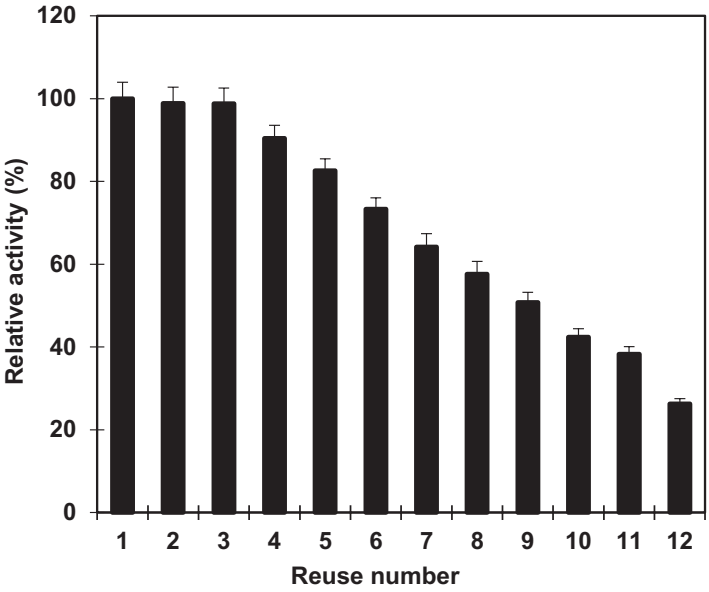


Fig. 8.5 Reusability of immobilized urethanase. Data points are the mean values of triplicate measurements

et al. 1990) retained 64%, 54% and 8.9% enzyme activity at 20% ethanol, whereas free urethanase of the fungus, *Penicillium variable* retained 65% activity at 15% ethanol (Zhou et al. 2014).

Reusability of immobilized enzyme, which is an important factor in reducing the cost of a bioprocess by utilizing the enzyme repeatedly, was assessed for the immobilized urethanase of Alg-S5 for 12 consecutive reaction cycles (Fig. 8.5). The catalytic activity of immobilized urethanase was unaffected until three successive cycles. After the third cycle, the enzyme activity gradually decreased which might be ascribed to the leaching of proteins during the washing steps. The recording of more than 70% of activity of immobilized urethanase at the sixth cycle indicates the potential of chitosan nanomaterials for efficient recycling of immobilized biocatalyst during the enzymatic hydrolysis of urethane.

8.6 Conclusion

The accumulation of urethane in alcoholic beverages has been a major concern for the regulatory agencies after the reclassification of urethane as a Group 2A carcinogen. Substantial efforts have been devoted to the development of cost-effective and environmentally sustainable processes to eliminate urethane. Several techniques, including vineyard management, physicochemical, enzymatic and metabolic

engineering methods have been proposed to reduce the synthesis of urethane in fermented beverages by inhibiting the formation of its precursor molecule urea. However, once urethane is formed, these indirect methods are not effective for its degradation. Urethanase, which hydrolyzes carcinogenic urethane into nontoxic products, has substantial industrial potential for direct abatement of urethane. Therefore, efforts have been directed to identify urethanase-producing microorganisms from various environmental sources. The immobilized urethanase of *Exiguobacterium* sp. Alg-S5 on chitosan nanoparticles displayed a pH optimum at 5.0 and resistance to 15% ethanol. In addition, the reuse of immobilized urethanase for three consecutive batches indicates the biocatalytic potential of microbial urethanase for scavenging carcinogenic urethane in fermented beverages.

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Chapter 9
Sea Water as a Reaction Medium for Bioethanol Production

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9.1 Introduction

Due to the depletion of fossil resources, it is expected that biorefineries will be preferred for the production of biofuels. Efficient biocatalytic systems are being made to produce biofuels with reduced ecological footprints. Among the challenges in biofuel industries, fresh water consumption is an important challenge which has become the global concern. Fresh water shortage which is already existing in our overpopulated world has also brought more pressure and uncertainities in this regard. Several research groups around the world have reported some enzymatic, fermentative, and chemocatalytic applications using sea water as a reaction medium for large scale biorefineries. The use of nonpotable water resources as reaction medium is promising. This chapter contextualises in detail about seawater-based applications for biorefineries.

9.2 Ethanol Production

Two types of ethanol production process are generally in practice: wet mill and dry grind. Around 80% of corn ethanol is produced through the dry grinding process. Corn is milled, and then the slurry is prepared with water. Either enzymes or chemical saccharification is performed to hydrolyze the starch to glucose. Yeast is then

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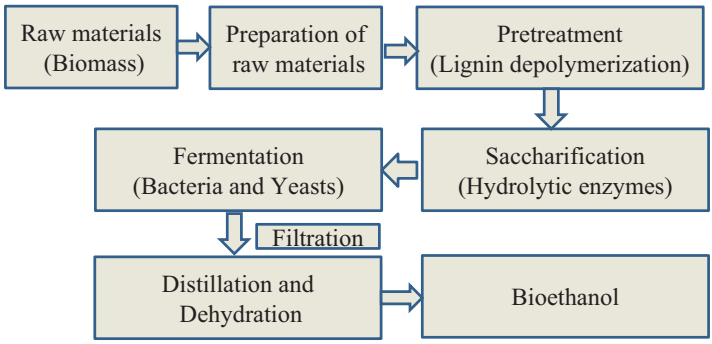

Fig. 9.1 Production processes of the bioethanol

added to the hydrolyzed biomass to ferment sugars into ethanol, which is then purified by distillation and molecular sieve dehydration to create the fuel. The processes involved in production of ethanol from biomass are given in Fig. 9.1. These ethanol plants have either a very little or almost no waste water discharge. They recycle a significant portion of industrial waste water through centrifuges, evaporation, and anaerobic digestion. The majority of water demand is primarily associated with cooling units or the boilers. As calculated by IATP (2006) an estimated water usage per gallon of ethanol production ranges from 3 to 4 gal. Few other reports calculated it to be in 3:1 ratio. Thus, 50 million gal of ethanol facility per year is expected to consume 15–200 million gal of water per year, i.e. 438 acre-feet.

9.3 Cellulosic Ethanol

Sugar and starch rich biomass gained popularity for fuel production which increased the competition with their use as food (Pimentel et al. 2008). The supply of sugar crops for ethanol industry was insufficient. Lignocellulosic biomasses are the most abundant renewable source, which can be used as an attractive alternative biomass for biofuel production. Lignocellulosic biomasses can be obtained from agricultural, municipal, forestry, and industrial source. The security of supply of biomass is ascertained by the widespread geographical distribution of lignocelluloses sources, which is not the case with fossil fuel reserves. Lignocellulose sources also put an end to the food vs. fuel conflict. Lignocellulosic biomasses are less expensive, can be produced with the low input of fertilizers, energy, and pesticides. The major cost involved in biomass production is mainly the purchase and processing of feedstock. Lignocellulosic feedstocks are the least expensive when compared to the recent prices of sugarcane and corn (Lynd et al. 2009). The conversion cost of feedstocks using current technology is, however, opposite to that of the purchase cost of feedstocks:

Cellulosic biomass > Corn > Sugarcane

Both, the feedstock purchase price and the conversion technology accounts for the fuel cost.

Sugarcane < Corn ethanol < Cellulosic ethanol

The growing technological advancement has the potential to reduce the selling price of cellulosic ethanol which is cheaper than the purchase cost of feedstocks (Lynd et al. 2009). Cellulosic ethanol is beneficial to the environment, can be produced in large-scale, and has a potential at a low fuel-production price, and thus cellulosic biomass is considered as the best candidate for energy production in the long run. However, they have high processing cost and not produced at a competition level (Lynd et al. 2008).

9.4 Cellulosic Ethanol Plants

Cellulosic ethanol production at different development stage is a part of many public and private international projects in few countries in the bio-renewable energy sector. Iogen's demonstration plant, located in Ottawa, Canada is operational since 2004 and is designed to process 20–30 t of feedstock per day and to produce approximately 5000–6000 L of cellulosic ethanol per day. It achieved a production capacity of 1,464,978 L/year in 2009 from wheat straw. Sekab's plant in Ornskolsvik, Sweden started producing cellulosic ethanol from sawdust in 2005. Currently, Sekab sells ethanol to 1400 E85 petrol pumps in Sweden, and the number of flex-fuel cars is about 147,000. In 2003, the U.S Department of Energy and Abengoa Bioenergy New Technologies, signed a 4-year contract, $35.5 million to develop technology for "Advanced Biorefining of Distillers Grain and Corn Stover Blend: Pre-Commercialization of Biomass-derived Process Technologies". In agreement to the contract, Abengoa Bioenergy is developing pilot scale processes integrating both lignocellulosic and cereal ethanol production to achieve best economic results. The major objective of the project is the conversion of residual starch, cellulose, and hemicelluloses, mainly corn stover to ethanol. The first phase of the project is completed and is in the testing phase with successful conversion of residual starch to bioethanol and improved co-product production. Inbicon has installed biorefineries for cellulosic bioethanol production at different sites. At Kalundborg, Denmark, 1.4 million gal of cellulosic ethanol per year is produced from bagasse, miscanthus grass, and fruit bushes. In Malaysia, projects with production estimated between 5 and 10 million gal of cellulosic ethanol per year are in the plan. In the United States, the first commercialisation produced 20 million gal/year. POET has 27 plants in seven states in the United States, which produces more than 1.6 billion gal of ethanol annually. For commercial cellulosic ethanol plant construction at Emmetsburg, Iowa with a capacity of 25 million gal/year using corn cobs, over $40 million had been invested.

NREL is working hard to develop and standardize technology for production of ethanol from agriculture residue, woody feedstocks, and switchgrass. Aden et al.

(2002) report demonstrates a detailed process design and economy of conversion of corn stover to ethanol. This design can be used to produce 69.3 million gal of ethanol per year at a consumption rate of 6 gal of water per gallon of ethanol produced. This process being non-optimized has the further scope of improvement. Another report by Aden et al. (2005) documents a detailed process design and economy for conversion of wood chips to ethanol by the thermochemical approach. This report suggests a minimal usage of 1.9 gal of water per gallon of ethanol produced. In this process in place of cooling water, forced air cooling was used to minimize water usage.

9.5 Water Consumption in Ethanol Production and Issues

Growing demands and growth of biofuel industry has triggered concern over many issues other than food security. One of the major concerns is water availability and its utilization for biofuel production. In the Midwest, the growing conflict over water use in agriculture facilities, livestock management units, and urban areas raised many eyebrows. Institute for Agriculture and Trade Policy (IATP) has warned in 2006 that the utilization of fresh water in biofuel industries is a serious concern. Life Cycle Inventory and Assessment (LCA) method is being used to analyse and quantify the consumption of water. It involves the quantification of material and energy flow during crop production, harvesting, transportation, ethanol production, and its final utilization in engines. Major areas of focus are crop production and ethanol production.

9.6 Freshwater Usage in Energy Industries

Unlike fossil resources, the sources for the production of biofuels are widely available. Amount of water consumed in biofuel industries and in irrigating the feedstocks vary significantly based on the processing technology and growing regions, respectively. For each liter of ethanol produced from switchgrass, 1.9–9.8 l of water is consumed (Wu et al. 2009). On average, corn ethanol production tends to consume more water than cellulosic ethanol on a life-cycle basis. Net water use for cellulosic ethanol production is comparable to that of gasoline from conventional crude or oil sands. Water use is declining because of rapidly evolving technologies for second-generation biofuel (cellulosic ethanol) and steady improvement of existing first-generation corn ethanol production. This is also true for crude oil recovery.

Crop sector leads in the consumption of freshwater accounting for 91.85% (1237 $km^3 year^{-1}$) of the 1314 $km^3 year^{-1}$ of global annual freshwater consumption. Agricultural production is considered as the principal driver of pressures on freshwater resources globally. Industrial and domestic demand accounts for 5.88% (77 $km^3 year^{-1}$) of the remaining fresh water consumption. In 5.88% of industrial

and domestic utilization, 23.78% utilized directly by energy sectors. Although this figure is comparatively small, the importance of considering freshwater consumption associated with energy sectors arises for two reasons (Holland et al. 2015).

9.7 Water Footprint of Bioethanol

Water footprints (WFs) refer to the volume of water directly and indirectly used by producers or consumers. Blue, green, and grey are the three key water elements suggested. The blue WF refers to the amount of fresh water (surface and ground) consumed in producing goods and services. The green WF is the consumption index of green water resources, which refers to rain water that falls and remains on the ground without flowing away or becoming part of ground water. The grey WF is a measure of pollution and is expressed as the volume of water required to assimilate the pollutant to load to meet ambient water quality standards (Hoekstra 2002). According to WF assessment manual published by the Water Footprint Network (WFN) (Hoekstra et al. 2011), a WF is an indicator used to measure water use based on the perspectives of freshwater resource use and pollution. Table 9.1 lists out the WF of bioethanol produced from various biomass.

Table 9.1 Water footprint of bioethanol from various raw materials (Chiu et al. 2016)

Raw materials	Country	Volume of fresh water (L) per L ethanol
Sugarcane	Brazil	2450
	US	2775
	India	2995
	Thailand	1396–2196
	Global average	2855
Sugar beet	France	790
	Germany	845
	US	1290
	Russian Federation	2075
	Ukraine	2780
	Global average	1355
Maize	US	1220
	Global average	1335
Cassava	Thailand	2374–2841
Sugarcane molasses	Thailand	1976–3105

9.8 Importance of Fresh Water

Water is the driving force of all nature with a little quantum of 3% as fresh water that has been intensively consumed in agriculture, industrial sectors and domestic purposes. Today's freshwater scarcity at alarming rate sensitized the global researchers and environmentalists that could trigger the next great global crisis given third world war. World Water Development Report for the year 2014 by United Nations has estimated that global industrial sectors consume 19–23% of the fresh water available in the world. The recent climb in oil prices and consumer demand for environmentally friendly products has now opened new windows of opportunity for bio-based chemicals. In the establishment of biomass-based industries, consideration should be given to possible unintended consequences such as the competition for food and biomass resource, the impact on water use and quality, changes in land-use, soil carbon stocks and long-term fertility, the net balance of greenhouse gasses and impacts on biodiversity. With the increase in the production through biomass-based industries, there is a need to discuss the impact of these industries on the usage of water. Water is the main component of all the biomass-based industries irrespective of the products produced. With the continued threat of the depletion of fresh water sources, the rapid growth of biomass-based industries adds more threats on the usage of fresh water shortly.

Also, very few or no research studies are being done to address this issue. The recent National Water Policy (NWP) of India strongly advocated the implementation of new technology to minimize the fresh water consumption in industries. This critical situation warrants the design and development of economically, environmentally, and socially sustainable practices for accomplishing sustainability in the global biofuel industries. It is necessary to find processes that are not only efficient but also sustainable. The biorefinery of lignocelluloses has drawn the central attention of researchers as it could meet the global energy and fine biochemical demands sustainably due to much better economics and ecological footprint. The conversion of lignocellulosic biomass to value-added chemicals such as bioethanol, furfural, and vanillin are gaining popular globally. Recent advancements in life cycle assessment and water footprint analysis revealed that around 6–10 L of fresh water are being consumed to produce 1 L of bioethanol. Utilization of sea water in industries to produce the biofuels could reduce the dependence of fresh water significantly as the sea water contains 97% fresh water and rest as minerals. Though desalination technology to generate fresh water has been explored in the recent decades as an excellent option, it has been reported as expensive and unsustainable. Although scientific literature on hydrolytic enzymes derived from marine microbes is available in plenty, only very few studies targeted the utilization of sea water as a nutrient medium.

9.9 Government Policies

Several Governments around the world have started to reduce the usage of fossil fuels through several policies. National Alcohol Program was launched by Brazilian Government in the 1970s after the oil crisis to utilize sugarcane as the primary energy crop for bioethanol research, development, and production. Brazil has produced approximately 32.5 billion litres of bioethanol in 2011 due to the encouragement of research on improvement in cultivation technologies of sugarcane species by the Brazilian Government (Azadi et al. 2012). The United States announced the implementation of the Energy Independence and Security Act (EISA 2007) to augment the standards of fuel efficiency and to decrease the crude oil dependency. EISA 2007 also involved a motto to increase the usage of bioethanol produced from maize by more than six times with an annual use of 36 billion gal in 2022 (Wallner and Mike 2011). The European Union has mandatory volumes of using renewable energy and biofuels in road transportation to 10% in 2020 (Gerbens-Leenes et al. 2012). Thailand Government has proposed a strategy to inspire the use of bioethanol produced from sugarcane molasses and cassava as transport fuels (Silalertruksa and Gheewala 2009). The Vietnam Government has declared to use rice straw as raw material for bioethanol production. In 2011, the global ethanol production volume was 22,742 million gal, 87.4% of which was produced only by US and Brazil. From 2007 to 2012, the global bioethanol production volume was tremendously increased from 13,089 to 22,715 million gal, indicating a 74% increase in 5 years. This increase suggested the importance of bioethanol as renewable energy in transportation sector which indirectly increased the competition over freshwater resources (Gerbens-Leenes et al. 2012). In 2030, the global annual WF of biofuels will be ten times that in 2005 (IEA). There is a huge threat imposed on fresh water resources due to a global increase in water consumption for the production of bioethanol. Along with energy problems, the water-resource related problems are also gradually increasing which is significantly critical and worthy of exploration (Earth Policy Institute 2012).

9.10 Opportunities for Saving Water

From various reports it is clear that energy demands and water consumption are interrelated, reduced water demands can be achieved through reduced energy consumption. Various options are being considered for reducing water utility in ethanol production. Production of broth with higher ethanol concentration reduces the energy required for distillation. One of the methods to potentially decrease water consumption involves pervaporation instead of distillation. Replacing the use of water in heat transfer process by forced air fans for cooling will cut short water loss due to evaporation and blowdown losses. Owens (2007) demonstrated a patented water conservation technology to be used in cooling towers to reduce 20% of water

consumption and also proposed a new high-efficiency dryer design. These technologies are modelled in NREL laboratory and are under review. DOE is examining the water use associated with the cultivation of fuel crops as most of them use ground water for the same. Optimization of cultivation condition is necessary to cut down the water consumption and to meet the renewable fuel goals. Apart from this, replacing fresh water with sea water in all bioprocessing steps in ethanol industries will save the fresh water to a larger extent.

9.11 Sea Water as Production Medium

The assessment of nonpotable water resources as a reaction medium for lignocellulosic biorefineries has been recently started. Despite its great potential, very few investigations of the use of seawater in fermentations have been reported to date with its utilization been strictly limited to seafood culture, the production of salts, and vegetable pickling (Al-Hotti and Kamel 1981; Sabu et al. 2000; Komives et al. 2005). The major concern in its use is its high salinity. In general, the salinity of seawater is around 25–35 practical salinity units (PSU), depending on the oceanographic conditions. Chemical composition of sea water is given in Table 9.2. These values are three times higher than those normally employed in conventional bacterial fermentation media (e.g. ten PSU for 10 g L^{-1} NaCl in the Luria-Bertani (LB) broth). Figure 9.2 explains the production of bioethanol using sea water based approach. Some proof-of-concept application has been developed by using sea water in succinic acid fermentation, chemoenzymatic, and chemocatalytic processes (Dominguez de Maria 2013; Lehmann et al. 2012; Grande et al. 2012; Lin et al. 2011). No significant inhibition of cell growth of *Actinobacillus succinogens* and succinic acid production was observed, even if fresh water was replaced by 100% synthetic sea water (Lin et al. 2011). Vom Stein et al. (2010) studied the depolymerisation of cellulose using (bio-based and biodegradable) organic dicarboxylic acids in concentrated sea water. The use of seawater-derived media as fermentative broth has received very little attention, with only some examples to produce

Table 9.2 Composition of sea water (Indira et al. 2016)

Elemental composition	Concentration ($\times 10^3$ ppm)
Na	5.5
Mg	3.8
Ca	3.7
Mn	1.2
Cu	1.2
K	<0.2
Co	<0.1
Fe	<0.1

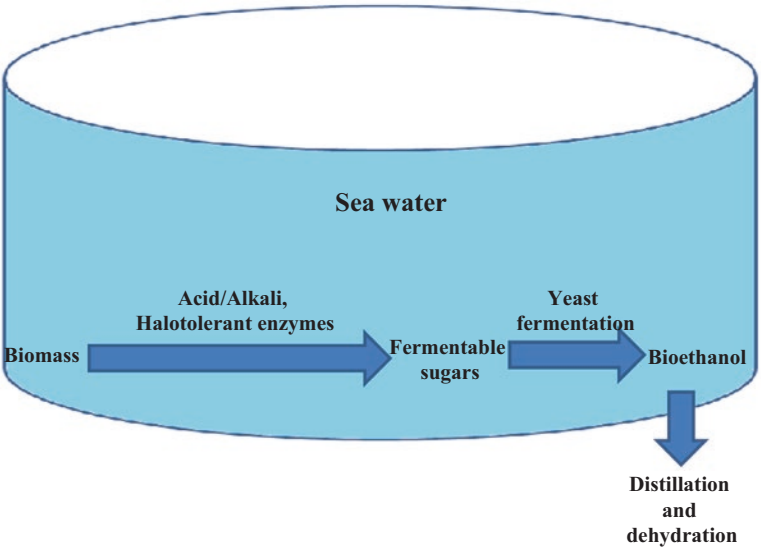

Fig. 9.2 Sea water approach for the production of bioethanol

carboxylic acids like lactic acid (Al-Hotti and Kamel 1981) and succinic acid (Lin et al. 2011), for the biosynthesis of enzymes (L-Glutaminase) (Sabu et al. 2000), or for growing *Bacillus methanolicus* with methanol as the sole source of carbon (Komives et al. 2005). Lignocellulosic (LC) biomass has to be pretreated to overcome its recalcitrant nature and to increase the yield of hydrolysis. Various methods using different physical treatments by mechanical utilities, chemical (acids, alkali, ionic liquids, oxidizing agents, organic solvents, steam explosion, ammonia, supercritical CO_2 explosion, etc.) and biological (enzymes like laccase and peroxidase) methods have been proposed for depolymerization of lignin. However, these technologies suffer from relatively low sugar yields, the formation of compounds inhibiting subsequent fermentation, severe reaction conditions, and high processing costs (Kumar et al. 2009). Nevertheless, to our knowledge, there is very less number of a report on the utilization of seawater for pretreatment of lignocellulosic biomass in the literature, where seawater based hydrothermal pretreatment (Fang et al. 2015) and ionic liquid pretreatment (Ren et al. 2016) of biomass were conducted. Apart from this, few saccharification studies have been conducted using seawater as the reaction medium. In the process of depolymerization of several amorphous and crystalline cellulose by commercially available enzyme cocktails (Accellerase 1500), only slightly lower production rates (~90%) were observed in seawater media about those in pure citrate buffer (Grande and de Maria 2012). The C5 and C6 sugars derived from the lignin depolymerization can be converted to various platform chemicals like 1,4-diacids (succinic acid, fumaric acid, malic acid), 2,5-furan dicarboxylic acid (2,5-FDCA), 3-hydroxy propionic acid (3-HPA), aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxy

butyrolactone (3-HBL), glycerol, sorbitol, and xylitol/arabinitol (Werpy et al. 2004). Though there are well-established procedures available for the conversion of sugars to value-added chemicals, none of these reactions was performed using seawater.

9.12 Development of Seawater Based System for Saccharification

The increase in the proportion of water for human use due to changing lifestyle and increasing population coupled with spatial and temporal variation in water availability denotes that the water for agriculture, industrial usage, and other human usage is becoming scarce. By decreasing fresh water usage in industries, fresh water usage can be cut short manifolds. Around 70% of world fresh water is used in agriculture, by using marine algae as a substrate for biofuel production total water intake for energy crop production can be reduced. Use of saline water in the cultivation of marine algal biomass as a substrate for biofuel production and use of the saline system in pre-treatment (saccharification) and fermentation process will result in water management. However, the substrate, i.e. marine algal biomass and the saline system for saccharification demands halotolerant enzymes.

Cellulases and other hydrolytic enzymes are inhibited at higher salt concentration. Reports suggest the isolation of cellulases from halotolerant organisms with optimal activity in between 6% and 22% of NaCl. Xylanolytic and ligninolytic activity have been reported in several marine fungal isolates. Bonugli-Santos et al. (2010) reported the production of laccase, manganese peroxidase, and lignin peroxidase from Brazilian marine-derived fungi. Wejse et al. (2003) reported purification and characterization of two extremely halotolerant xylanases from a novel halophilic bacterium strain, CL8 with optimal activity at 1 M NaCl concentration. Khandeparker et al. (2011) reported a novel halotolerant xylanase from marine isolate *Bacillus subtilis* cho40. Salt tolerant cellulases can tolerate high salt levels and ionic liquids better than normal cellulases. Use of salt tolerant cellulase in biofuel industry for pre-treatment of biomass shall lead to advancement in the usage of seawater/brackish water. Grant et al. (2004) reported the presence of cellulase-producing microorganisms in Wadi al Natrum Soda Lake that are capable of depolymerisation of polysaccharides in ionic solvents or a saline solvent (4 M NaCl) (Liang et al. 2011; Pottkämper et al. 2009). The commercially available Accellerase-1500 is capable of depolymerising amorphous and microcrystalline cellulose with 90% of the original rate in a seawater-based media (Grande and De Maria 2012). The other reports involves utilization of seawater for bioprocessing of carboxylic acids like lactic acid (Al-Hotti and Kamel 1981) or succinic acid (Lin et al. 2011), for the biosynthesis of enzymes (L-Glutaminase) (Sabu et al. 2000), or for growing *Bacillus methanolicus* with methanol as the sole carbon source (Komives et al. 2005). Despite such promising and immense prospect, use of seawater for biomass processing is still in infancy. The present chapter is aimed to

minimize the fresh water consumption in biomass-based industries by developing seawater based systems to convert agricultural biomass into fermentable sugars. Enzymatic conversion of biomass to bioethanol in seawater would be done using halotolerant hydrolytic enzymes produced by the potent isolates. The envisaged outcome could open up a door to utilize the seawater based system in industries to make it sustainable and assist the Mother Nature to solve freshwater scarcity problems of the world.

Grande and De Maria (2012) assessed the cellulolytic activity of commercially available Accellerase-1500, an enzymatic cocktail of different glycosidases (cellulase, hemicellulase, and a higher level of β-glucosidase, derived from *Trichoderma reseii*) in different concentrated seawater systems. This was the first report published regarding the enzymatic hydrolysis of cellulose in seawater. Cellulose molecule with varying degrees of crystallinity was assessed for hydrolysis in seawater, and the reports proved that hydrolysis is possible with slight diminishing rates (~90%) as compared to that of the reaction carried out in a controlled buffer system. The enzyme effectively hydrolyzed both amorphous and microcrystalline cellulose in seawater. Hydrolysis of amorphous cellulose Sigmacell-101 using Accellerase-1500 remained same in buffer and in seawater, which may be due to low crystallinity of the samples. For (Sigmacell-20 and Avicel) the hydrolytic activity of enzyme in seawater was 90% to that of the activity achieved with buffer.

9.13 Production of Bioethanol in Sea Water Based Medium

Water plays a major role in the production of energy, both in the process of extraction of sugars as well in the processing of fuels. Growing demands of an alternative source of energy from conventional and non-conventional renewable resources are going to increase the demand for water (Wu et al. 2009). According to the Energy Independence and Security Act (EISA 2007), U.S. is committed to producing 36 billion gal of biofuel by 2022 and for which the production rate is increasing at an unprecedented rate crossing the record of 9.0 billion gal of ethanol in 2008. Total water consumption in biofuel production can be divided under two heads, one being the production of energy crops and the other is processing of biofuel. Ethanol has been produced from sugar or starch rich crops since ancient times, and the water for the growth of energy crops was supplied through either precipitation or irrigation. Replacement of sugar-rich crops with lignocellulosic feedstock has subtracted the water consumption in this regard. Studies conducted by the U.S. Department of Energy (DOE) and USDA reported the availability of more than a billion tonnes of biomass for fuel production (Perlack et al. 2005). The biochemical process for pre-treatment of biomass requires additional water that sums up the total water consumption for cellulosic ethanol-equivalent to the water use for corn ethanol production. The current technology of biochemical pre-treatment process of biomass for ethanol production consumes 9.8 l of water for producing one litre of ethanol, which is estimated to be reduced to 5.9 l with an increase in ethanol yield

(Aden et al. 2002). Increasing demand for water and global climate change is going to pose a serious threat to the availability of freshwater (WWAP 2012). It is estimated that requirement of water for biofuel production may arise by 5.5% by 2030 exerting an extra load on scanty freshwater resources (Gerbens-Leenes et al. 2012).

Development of a seawater-based biorefinery strategy could make a strong impact in these areas with a holistic utilisation of seawater, aiming at more efficient, low cost, and small carbon footprint processes. There are reports for the use of seawater in enzymatic hydrolysis of lignocellulosic biomass (Grande and de María 2012; Klement et al. 2012; Vom Stein et al. 2010), fermentation process using halotolerant yeasts (Senthilraja et al. 2011) also, few marine yeasts were isolated and tested for their fermentation capacity in seawater (Urano et al. 2001).Utilization of seawater for biofuel production reduces stress on freshwater resources while enabling cultivation of biomass, saccharification, and processing of biofuel over a common platform (Goncalves et al. 2015). Fermentation of ethanol in seawater using *S. cerevisiae* has reported production of 0.5 g ethanol per gram of glucose (Goncalves et al. 2015). To utilize sea water as a reaction medium for saccharification, the enzymes must be salt tolerant and can saccharify in the presence of salt. Hence, halotolerant cellulase enzymes and microorganisms producing halotolerant cellulase are needed to develop sea water based system to produce ethanol.

9.14 Halotolerant Cellulase Producers

Strong acids and alkali solutions are used at a higher temperature during the pretreatment process, the neutralisation of these acids and bases results in the formation of salts. Removal of salt is done using tonnes of water and energy to facilitate further downstream processing. Also, ionic liquids (ILs), i.e. a liquid form of salts at room temperature have found recognition as an alternative to chemical solutions as a green solvent for lignocellulosic pretreatment (Mäki-Arvela et al. 2010; Gunny and Arbain 2013). One of the major shortcomings of ILs is their ability to inhibit cellulase enzyme in subsequent saccharification process, rendering the whole process ineffective. The high salinity of ILs may be the reason behind enzyme inhibition (Turner et al. 2003; Zhao et al. 2009; Salvador et al. 2010). To overcome the risk of enzyme inhibition, the residual ILs are washed after the pretreatment process, which again adds up to the energy consumption and processing cost (Engel et al. 2010; Zhang et al. 2011). This issue was addressed by proposing ideas like utilisation of one-pot process for pretreatment and saccharification of biomass by utilising salt tolerant cellulases (Kamiya et al. 2008). Given the above discussion, identification and utilisation of halotolerant cellulase, which are stable at saline conditions of neutralised acids, bases, and compatible with salts of ILs, is important. Thus, halotolerant enzymes with stability and good catalytic activity are in vigorous search, which will be suitable for industrial production and consumer affordability (Xing et al. 2012; Kuhad et al. 2011; Garg et al. 2016).

Hydrolysis of cellulose is a common ability found in many bacterial and fungal species within the Eucarya domain, although from the domain Archaea cellulolytic organism is yet to be identified (Holt et al. 1994). Many aerobic and anaerobic cellulolytic bacteria have been isolated from diverse environments (Sukumaran et al. 2005). There are a good number of reports over alkaliphilic cellulase-producing microorganism but limited reports on haloalkaliphilic cellulase producers (Aygan et al. 2011; Sukumaran et al. 2005; Zhang et al. 2012). *Bacillus sp. BG-GS10* isolated from Zabuye Salt Lake (Tibet) was reported to have high cellulolytic activity in 0–18% NaCl concentrations (Zhang et al. 2012). *Aspergillus terreus* UniMAP AA-6 showed halotolerant cellulolytic ability accompanied with thermotolerance (Gunny and Arbain 2013). The cellulase stability and relative activity was more with less viscous solvents and decreased with increase in viscosity, which affects the enzyme-substrate interaction (Romero et al. 2008; Samayam and Schall 2010).

The genus *Streptomyces* is an attractive industrial organism due to the growth rate, its extracellular secretion and biosafety reasons. *Streptomyces roseosporus* and *S. griseus* showed CMCase activity at pH 8 and in the presence of 5% NaCl and 2% NaCl, respectively, at 37 °C. Highest enzyme activity was detected after 90 min incubation under shaking at 37 °C (Hakobyan et al. 2013). Dasilva et al. (1993) reported *Streptomyces sp*. S36-2 to produce alkaliphilic cellulase at optimum pH and temperature of 6.0–7.0 and 55 °C, respectively.

Halophilic microorganisms can produce halotolerant enzymes that are active in a high saline environment (Oren 2010). There are reports suggesting stability of halotolerant enzymes at a wide range of temperature, i.e. from 40 to 80 °C, though a gradual decrease in enzyme activity was noticed. The enzyme displayed 55% residual activity at 70 °C, which is a thermophilic characteristic (Oren 2006; Mesbah and Wiegel 2005). The stability of halophilic cellulase at a higher temperature may be due to increasing in surface charge of the microbial cell membrane, ionic interaction and change in the cytoplasmic membrane to adapt and acclimatise to high-temperature conditions (Karan et al. 2012). Few halotolerant cellulases have been described from metagenomic studies; *Thermotoga maritima,* Archaea and *Pseudoalteromonas* sp. (Datta et al. 2010; Ilmberger et al. 2012; Trivedi et al. 2013; Raddadi et al. 2013).

Paenibacillus tarimensis was characterised for CMCase activity over a wide range of pH (3.0–10.5) and salt concentration (9 mM–5 M NaCl). At high salt concentrations, i.e. 20% of ILs at 80 °C, >76% of relative activity was retained, and at 40% concentration of ILs, >40% relative activity was detected (Raddadi et al. 2013). Stability at such high concentration of salts makes these enzymes a promising candidate for application in detergents, textiles, paper/pulp industry; and ILs treatment-saccharification of lignocellulose. *Paenibacillus tarimensis* L88 showed highest CMCase activity at 72 h after incubation with a decrease in viscosity of the growth medium suggesting that cellulase(s) to be β-endoglucanase(s) (Percival et al. 2006). Earlier reports of CMCase from other *Paenibacillus* strains include *Paenibacillus curdlanolyticus* B-6 and *Paenibacillus campinasensis* BL11 with enzyme activity of 0.05 U/ml and 0.1 U/ml, respectively (Ko et al. 2007; Pason et al. 2006). Highest endoglucanase activity was obtained by the recombinant enzyme purified from

Paenibacillus barcinonensis expressed in the yeast *Saccharomyces cerevisiae* with 1.2 U/ml (Mormeneo et al. 2012) or the cellulase from *Paenibacillus cookie* expressed in *Escherichia coli* and that exhibited 39.1 U/ml CMCase activity (Shinoda et al. 2012). Multiple cellulases have been reported from *Paenibacillus* genus. *P. campinasensis* BL11 (Ko et al. 2007) was reported with three different cellulases, zymogram analysis of cellulolytic system from *P. curdlanolyticus* strain B-6 showed the presence of 12 xylanases, and 9 CMCases (Pason et al. 2006). Liang et al. (2011) reported that Cel5A to be stable at pH range 4.5–10; Hirasawa et al. (2006) showed a cellulase from *Bacillus agaradhaerens* was active in a pH range 5–11.5 and Trivedi et al. (2010) reported cellulase activity from *Bacillus flexus* having optimal activity at pH 10 with stability in the pH range 8–12.

Haloarcula G10 strain isolated from the saline soil of Yuncheng Salt Lake, China showed endoglucanase activity at an optimal temperature of 60 °C, and pH of 9.0 at 17.5% NaCl. Endoglucanase activity and stability was reported over broad ranges of temperature (40–80 °C) with 60% residual enzyme activity at 80 °C, pH (7.0–10.0) and NaCl concentration (12.5–27.5%), showing its thermostable, alkali-stable and halostable properties (Li and Yu 2013). A similar range of optimal temperatures has been reported for halophilic endoglucanases from *Thalassobacillus* sp.LY18 and *Bacillus* sp. L1 (Li and Yu 2012). EDTA, phenylmethylsulfonyl fluoride (PMSF) and diethyl pyrocarbonate (DEPC) lead to some significant inhibition of enzyme activity revealing it as a metalloenzyme with serine and histidine residues essential for enzyme catalysis. The surfactants tested had little effects on the enzyme activity. The *Haloarcula* G10 endoglucanase was active and stable in the nonpolar hydrophobic organic solvents with log $P_{ow} \geq 0.88$. All the characteristics mentioned above make this endoglucanase an ideal choice for applications in the industrial process under harsh conditions.

Bacillus vallismortis RG-07 was reported for maximum cellulase production from sugarcane bagasse (4105 U ml^{-1}) with optimum temperature and pH of 65 °C and 7.0, respectively. The enzyme retained its residual activity of 95% and 75% of activity even at a temperature of 95 °C, and pH 9.0, respectively. The presence of organic solvents like (30%) n-dodecane, iso-octane, n-decane, xylene, toluene, n-hexane, n-butanol, and cyclohexane enhanced enzyme activity on prolonged incubation (7 days). Ca^{2+}, mercaptoethanol, Tween-60, and sodium hypochlorite promoted whereas Hg^{2+} strongly inhibited the enzyme activity. Kinetic analysis of purified enzyme showed the *Km* and *Vmax* to be 1.923 mg ml^{-1} and 769.230 μg ml^{-1} min-, respectively (Singh and Kumar 1998).

Bacillus, Clostridium, Cellulomonas, Rummminococcus, Alteromonas, Acetivibrio, Bacteroides, etc. are among the most commonly reported bacterial genera for endo-cellulase activity. Among *Bacillus* sp., *B. brevis* (Singh and Kumar 1998), *B. pumilus* (Gachomo 2003), *B. amyoliquefaciens* DL-3 (Lee et al. 2008), and *B. subtilis* YJ1 (Yin et al. 2010) are well studied for cellulase production under submerged conditions (Jo et al. 2008; Mayende et al. 2006).

The Cel5R (belong to GH5 family) identified by soil metagenomic approach shows thermostability up to 58 °C and pH stability from 5 to 9 with halotolerance and extreme halostability in 4M NaCl, 3M LiCl and 2M KCl (Garg et al. 2016)

which is higher than other known halostable cellulases (Zhou et al. 2016; Zarafeta et al. 2016). The halostability of Cel5R may be due to the presence of acidic residues (Asp and Glu) on the surface of the protein, which is supported by the crystal structure analysis of Cel5R with acidic residues (16.7% with 52 residues) present on the surface of the protein. The mutation of surface residues in malate dehydrogenase from *H. marismortui* (Madern et al. 1995) and glucose dehydrogenase from *H. mediterranei* (Esclapez et al. 2007) altered only the halophilic properties of mutant without affecting the kinetic parameters and enzyme activity of the protein.

Two strategies generally opted to obtain better biocatalysts are either protein engineering through rational design or directed evolution (Dalby 2011; Bornscheuer et al. 2012; Bornscheuer and Pohl 2001) or mining nature's genetic reservoir for genes that encode enzymes with novel properties from previously uncharacterized organisms either bioinformatically or by functional screening (Lorenz et al. 2002). Extremophiles are a rich source of such enzymes, as they have evolved to thrive in extreme environments. Culturing or culture-independent approaches are applied to retrieve genomic or metagenomic material from extreme habitats followed by functional or bioinformatics screening to reveal novel enzymes with the desired properties (Kim and Peeples 2006; Demirjian et al. 2001).

The cellulolytic enzyme, CelDZ1 which is predicted to be located on the outside of the cell membrane, requires halotolerant features in order to maintain the enzyme activity, which could be achieved by lowering the affinity of the active site of the enzyme to the chloride and potassium/sodium ions, to avoid competitive inhibition with the substrate. The surface potential of CelDZ1 clearly shows an overall negative charge in the active site channel which does not favour binding of chloride ions. Monovalent cation binding sites are usually formed by a carboxylic side chain and at least one carbonyl from main protein chain. CelDZ1 ligand groove revealed no carbonyls exposed to solvent near carboxyl side chains which would form an alkaline ion-binding site (Zarafeta et al. 2016).

High extracellular CMCase activity was reported in *Marinimicrobium* sp. LS-A18 is grown on mineral salt medium with CMC as the sole carbon source (Zhao et al. 2012). Maximum CMCase activity was recorded at 55 °C and pH 7.0 in the absence of NaCl. Optimised fermentation conditions yielded CMCase up to 2.5 U/ml, which was 3.1-fold higher than non-optimized process. Eighty-four percent of enzyme activity was retained after incubation at 60 °C for 1 h and more than 88% of enzyme activity was retained after incubation for 72 h at a pH range between (5–11) and NaCl concentrations (0–25%, w/v), indicating it was halotolerant, thermostable and alkali-stable.

9.15 Indian Scenario

India has a vast variety of agricultural and forest bioresources, which can be efficiently used for the sustainable biorefinery industries. An estimated 50 MMT (million metric tons) of liquid fuels are consumed annually in India, but with the actual

biomass potential and its full utilization, India is capable of generating almost double that amount per annum. Several studies have been carried out using various treatment methods for the depolymerization of lignin available in different biomass and subsequent saccharification and fermentation of the hydrolyzed products to value added products (biodiesel, biogas, bioethanol, acetic acid, etc.) for many decades. Singh et al. (2014a, b) studied the enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by *Saccharomyces cerevisiae, Scheffersomyces stipitis,* and their co-culture. Separate hydrolysis and fermentation methods are adopted to produce bioethanol from dilute acid pretreated Indian bamboo variety (*Dendrocalamus* sp.) (Sindhu et al. 2014). Other researchers also actively contributed to bioethanol production from lignocellulosic materials (Behera et al. 2014; Singh et al. 2014a, b, 2015a, b). Besides such studies, Biswas et al. (2014) developed the methods for efficient enzymatic hydrolysis by wet explosion pretreatment of sugarcane bagasse. Sharma et al. (2015) carried out a pilot-scale study on the steam explosion and mass balance for higher sugar recovery from rice straw. Microwave and ultrasonic waves assisted depolymerization of biomass are being studied with or without alkali treatment by several India researchers on green coconut fiber (Jeyanthi and Subramanian 2011), rice straw and hulls (Singh et al. 2011), rice straw (Singh et al. 2014a, b), paddy straw (Kaur and Phutela 2016), and waste newspaper (Subhedar et al. 2015). Many researchers in India have performed chemical transformation of sugars to various value added chemicals, but none of these works was attempted in sea water-based reaction media. Recently in India, €110 million joint venture cellulosic ethanol project of Chempolis Ltd. and Numaligarh Refinery Limited (NRL) is moving towards construction after getting approval from NRL's board. By 'Hydrocarbon Vision 2030 for Northeast", the big project will be started to produce bioethanol from bamboo with the co-production of furfural and acetic acid. Although many of researchers are potentially contributed, the methods and strategies to adopt different methods for biorefineries of biomass favourably, there is no one till now subject the water issue during the production process. It will be a significant problem in India also if fresh water usage in such biomass-based industries continues. Therefore, it is the time of using some alternative source of water like sea water for the use in such industries.

9.16 Conclusion

Water is precious, and depletion of water may lead to the unfavourable situation between countries. Several initiatives have been taken by international agencies to save the fresh water. It has been already recommended to reduce the usage of freshwater in industries for bioprocessing. Sea water would be an excellent alternative to replace the fresh water in industries as it has 97% fresh water and 3% salts. Halotolerant microorganisms and their enzymes can tolerate the salts in sea water and saccharify the biomass in sea water. Halotolerant yeasts can ferment the sugars in sea water to produce ethanol. However, there must be a suitable technology to

measure the ethanol present in sea water and to concentrate the ethanol from sea water. Sea water based systems will tremendously reduce the requirement of fresh water in industries not only in saccharification processes but also in several other bioprocesses.

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Chapter 10
Bacterial Mediated Plant Protection: Induced Systemic Resistance in Soybean

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10.1 Introduction

Agricultural products are the primary dietary source of protein, carbohydrate, fat, vitamin and other nutrients, and soybean (*Glycine max* L. Merrill) is one of the best source of 'all in one' with a very high nutritional value. Its nutrient composition includes mainly protein, saponnins, fibers, phytosterols, and important minerals that includes iron (Fe), calcium (Ca), potassium (K), phosphorus (P) and magnesium (Mg). Along with these, it also possesses good sum of different vitamins like A, E, K and B complex, lecithin, large amount of linoleic acid and powerful isoflavonoids, and valuable vitamin C is also found in its sprouting grains. It contains about 20% oil rich in poly unsaturated fatty acids, specially omega-6 and omega-3 fatty acid and 40% high quality protein which is rich in valuable amino acid lysine (5%). Due to presence of Ca and Fe, it is very helpful for the women who suffer from osteoporosis and anemia. It is the only vegetarian food with such a balanced nutrient

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profile and that's why called by different name such as miracle bean, golden bean, cow of the field, meat of the field, gold from the soil, pearl of the orient, Cinderella crop etc. (Chauhan et al. 2002; Khan and Tyagi 2013).

With respect to the nutritional and economical importance of soybean, worldwide, its production has increased dramatically in different parts of the world (Ruiz Diaz et al. 2009). It is produced world-wide and the leading five producers of soybean are the United States (35.02%), Brazil (28.13%), Argentina (17.31%), China (3.96%) and India (3.41%) (FAOSTAT 2014). So as per the FAOSTAT India rank 5th in the world-wide soybean production. In India, Soybean production has been increased dramatically over the past four decades. In 1970, the area sown and production was reported around 30,000 ha and 14,000 ton respectively that reached 9.95 million ha and 12.57 million ton respectively in 2011. Soybeans occupied 42% and 25% of India's total oilseeds and edible oil production respectively (Sharma et al. 2014). Statistical data of the Soybean Processor Association of India (SOPA) for the year 2014 shows that Madhya Pradesh (57.73%) is the leading producer of soybean with more than half of the total country production followed by Maharashtra (29.44%) and Rajasthan (5.4%) (Fig. 10.1). In M.P., majorly soybean growing regions are Central Highland, Malwa plateau and part of Deccan plateau, and that's why due to such a large production it is known as 'Soybean bowl of India' (Tiwari et al. 2002).

Although, it is a well adapted crop but still some changes in the environmental conditions that leads to different abiotic stress and severe diseases that cause biotic stress are major threat to soybean production in India and worldwide. Different phytopathogen that includes fungi, bacteria, viruses and nematodes not only reduce the quantity of soybean but also decreases seed quality. With increasing population and demand of food, use of chemicals to ward off pathogens has been increasing day-by-day. Even though reducing diseases, these chemicals are further more harmful for the ecosystem and lead to create toxic environment and affect human health. So in the form of environment friendly strategy, one of the alternatives and emerging technologies used to solve this problem is naturally occurring plant growth promoting rhizobacteria (PGPR). Different PGPR such as *Agrobacterium, Acinetobacter, Bacillus, Azospirillum, Rhizobium, Thiobacillus, Bradyrhizobium, Arthrobacter, Serratia, Pseudomonas, Frankia* provide enhanced level of resistance by eliciting induced resistance in plant that is known as 'Induced Systemic

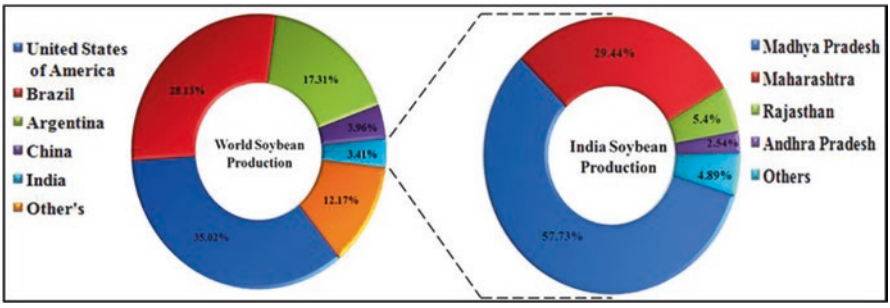

Fig. 10.1 Soybean productions across the World and India (*Source*: FAOSTAT 2014; SOPA 2014)

Resistance' (ISR) to protect plant against invading pathogens. Along with this, they also promote plant growth and increase the availability of essential nutrients through nutrient cycling activities (Mendes et al. 2011; Wahyudi et al. 2011; Berendsen et al. 2012; Bulgarelli et al. 2013). The use of this environment friendly approach could be among the most efficient methods for minimizing the use of chemicals, which can adversely impact human health directly and indirectly. This review chapter will focus on different biotic stress with special concern on fungal pathogens of soybean and different research study done on soybean plant protection concerning interaction between PGPR and plants under biotic stress condition.

10.2 Biotic Stresses in Soybean

Soybean, being a nutrient rich plant by way of an extensive range of advantageous effects on human health and by product, also had a wide range of enemy in the form of different pathogens that affect it in different manner to reduce its quality as well as quantity. Several pathogens like fungi, bacteria, viruses and nematodes attack on the soybean plant and cause different diseases leading to the great loss in the yield (Fig. 10.2). Whatever the type, biotrophic/hemi-biotrophic/necrotrophic, all the successful pathogen have a specific strategy that facilitate them to proficiently attack, live and reproduce within their host plant and cause a disease. Disease

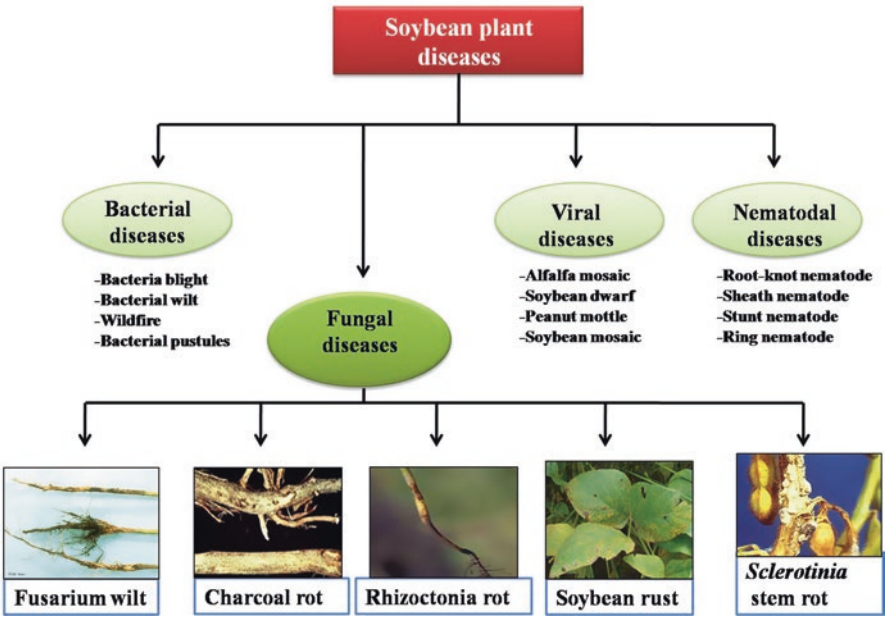

Fig. 10.2 Soybean plant diseases caused by different phytopathogens

situation is also vary from severe, in which plants die soon after infection, to chronic, in which plants survive but with retarded growth and development due to pathogen interference with plant metabolism leading to yield loss. The range of diseases affecting soybean crops is rather extensive, and those caused by fungi are considered of major importance; not only by their higher number, but also by the loss caused to the quality of the seeds and yield and can infect at any stage of soybean life cycle, starting from the seed germination to seed production (Diniz et al. 2013).

A wide range of diseases are caused by different fungal phytopathogens in the soybean, few of the most notables are; Alternaria leaf spot caused by *Alternaria* sp., Charcoal rot caused by *Macrophomina phaseolina*, Damping-off caused by *Pythium* sp., Fusarium root rot caused by *Fusarium oxysporum*, *Pythium* rot caused by *Pythium* sp., *Sclerotinia* stem rot caused by *Sclerotinia sclerotiorum*, Rhizoctonia root rot caused by *Rhizoctonia solani,* Anthracnose stem blight caused by *Colletotrichum truncatum,* Stem canker caused by *Diaporthe phaseolorum* and many more others affects soybean production on a large scale (Inam-Ul-Haq et al. 2012). Among all the phytopathogens, *S. sclerotiorum*, *M. phaseolina, Fusarium*, *P. pachyrhizi* and *R. solani* cause major losses in soy production (Fig. 10.2). Distribution of the diseases in the country is random where few diseases were distributed throughout the country while others were restricted to specific regions.

10.2.1 Soybean Rust

Soybean rust disease is caused by phytopathogenic basidiomycetes fungi *P. pachyrhizi* originated in Asia–Australia (Goellner et al. 2010). It is an aggressive disease capable of causing defoliation and significant yield loss. *P. pachyrhizi* can survive only on the green tissue and cause great yield loss by rapid deterioration of the leaf tissue that leads to premature leaf fall and hence early maturity and smaller grain formation. Usually early symptoms of rust infection appears after flowering, begin on lower leaves surfaces starting as small, gray spots that further changed into tan or reddish-brown. Due to rapid life cycle and production of large numbers of infectious urediospores under optimal environmental conditions it causes potentially high yield losses by high inoculum load (Goellner et al. 2010). Soybean rust is listed in the soybean diseases in India which can severely affect yield depending on the time of infection, genotype planted, and climate (Wrather et al. 2010). Historically, it was first confirmed in Paraguay, with yield high losses 60% in some fields and even the resistant cultivar also get affected due to the variability of pathogens. Environmental condition is too much important for causing disease as late-planted soybean was found more affected then the early planted one because of the suitable temperature and humidity for the rust at the time of seed development (Wrather and Koenning 2006).

10.2.2 Sclerotinia Stem Rot

Soybean Sclerotinia stem rot, also known as white mold, is caused by *S. sclerotiorum* (Lib.) de Bary fungi belong to Ascomycetes and was first reported in 1951 (Angelique et al. 2012). It is pathogenic to a broad range of hosts around more than 370 plant species worldwide including soybean (Bolton et al. 2006, Sumida et al. 2015). The weather and soil condition required for the soybean development also favorable for the *S. sclerotiorum* growth resulting in formation of sclerotium and production of apothecia that release large amount of ascospores, which can spread over a long distance and period, and alight on plant canopies (Abawi and Grogan 1979). Due to capability to survive for several years in the soil by forming soil-hardy sclerotia, it is very difficult to manage this disease (Coley-Smith and Cooke 1971). New sclerotia develop within infected plants, leaving plant debris full of sclerotia at the end of the season. *Sclerotinia* stem rot results in decreased seed number and weight, and hence significant yield losses (Danielson et al. 2004; Hoffman et al. 1998). In addition to causing yield loss, Sclerotinia stem rot can lessen seed quality by means of decreasing protein and oil concentrations in infected seeds (Danielson et al. 2004). *S. sclerotiorum* can also infect even unaffected field by air-borne spores (sclerotia) and fungal mycelium produced by these can grow in and outside stems inhibiting the growth of the infected plants and can be severe during an extended period of cool and moist weather (Angelique et al. 2012; Mueller et al. 1999; Yang et al. 1999; Grau and Hartman 1999).

10.2.3 Charcoal Rot

Charcoal rot disease is caused by the ascomycete fungus *M. phaseolina* and can notably decrease the yield of oilseed crops soybean. *M. phaseolina* (Tassi) Goidanich, is among the most important soil-borne pathogens having a wide host range that includes around 500 plant species form more than 100 plant families worldwide (Smith and Wyllie 1999). Disease symptoms can be observed after flowering in the form of light gray or silver color tap root and lower stem. *M. phaseolina* form microsclerotia produced in the xylem and pith tissues of the stem that may block water flow in the shoot and can be diagnose as numerous small, black bodies of microsclerotia upon peeling of epidermis from the stem (Mengistu et al. 2013). Furthermore, it can infect plant throughout the season, may alter soybean seed composition and influence nitrogen fixation activity, resulting in progressive weakness of the host. Charcoal rot, which is also referred as dry weather wilt or summer wilt due to symptoms appearance under heat and drought stresses, resulted in higher yield loss in India since 2004 because of unequal rainfall and greater drought periods (Smith and Wyllie 1999). According to a survey, maximum damage to soybean has occurred in the major soybean-producing states of Maharashtra, Rajasthan, Karnataka, Chhattisgarh and Andhra Pradesh and yield loss have been reported as high as 77% in some fields (Wrather et al. 2010).

10.2.4 Fusarium Wilt and Root Rot

Fusarium is a very common and threatened soil fungus which belongs to Class-*Sordariomycetes* and Family-*Nectriaceae* under the Division-*Ascomycota* and more than ten different species were identified to cause root rot by infecting belowground part of the soybean. *F. oxysporum* is mainly responsible for the Fusarium wilt. It's a cosmopolitan soil borne fungi found in every type of soil throughout the world and can survive for the long time due to its saprophytic nature and ability to grow on rhizosphere of broad range of host plants. Furthermore, as it occurs in blend with other pathogens and belowground symptoms also not differentiable from other root rots, it is hard to estimate its effect on the soybean yield (Arias 2012). Symptomatically, brown vascular tissue in the roots and stems and wilting of the stem tips can be seen in infected plants. The symptoms are more noticeable if subjected to reduced moisture and hot conditions. Foliar symptoms include scorching of the upper leaves, while middle and lower leaves may turn yellow or pale yellow spots may appear. Soybean seedlings are more vulnerable for the disease and due to poor root system and poor nodulation infected plants get wilted and die (Naito et al. 1993). The Fusarium wilt of soybean is an important disease around the world that can cause huge damage by decreasing up to 59% average yield of soybean (Sinclair and Backman 1989).

10.2.5 Rhizoctonia Root Rot

Rhizoctonia root rot of soybean is caused by *R. solani* that belongs to Division-*Basidiomycota*, Class-*Agaricomycetes* and Family-*Ciratobasidiaceae*. This is also a soil borne, saprophytic fungal phytopathogen with a vast range of host plants and worldwide distribution. It's a very versatile pathogen causing different diseases including collar rot, root rot, damping off, wire stem; and different strains of *R. solani* varies in the host selection, the severity of infection, optimum temperature for infection, the capability to form sclerotia and develop in lower soil levels, the growth rate and the survival in a certain area. *Rhizoctonia* infects young seedlings, causing pre- and post-emergence damping off. Reddish-brown symptomatic lesions were found on the hypocotyls at the soil line in the infected seedlings. These lesions are sunken, remain firm and dry and are limited to the outer layer of tissue. Overcoming of damping phase by the seedling can leads to root rot in later stage which can be persevere into late vegetative to early reproductive growth stages. Poor root system, stunned and yellow plants are the characteristics of older infected plants. It does not produce any asexual spores/conidia and very rarely produced sexual spores. Due to lack of conidia/spores and the scarcity of the sexual spore it only exists as vegetative hypae and sclerotia in nature. In the form of sclerotia it can stay alive in the soil for an extended period. Sclerotia are nothing but encapsulated compact mass of hardened fungal mycelium that contain reserve food, have the capability to survive in extreme environment and can give rise to a new individual in favorable condition (García et al. 2006).

10.3 Indigenous Defence System and Systemic Acquired Resistance

Like animals, plants does not produce antibody but possess an indigenous defence system to respond pathogens by activating a complex signaling events and that's why, often termed as plant immune system. Depending upon the type of molecule recognized plants have two types immune system from basal to higher level (Malinovsky et al. 2014). First, PAMP-triggered immunity (PTI) which is a basal level defence where pattern recognition receptor (PRR) present on the plasma membrane of cell recognize microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) of the invading pathogen and activate a complex cascade of signaling events, including ion fluxes that leads to depolarization of plasma membrane, reactive oxygen species (ROS) production, nitric oxide (NO) production and activation of calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) leading to change in transcription factor (TF) activities to activate defense genes (Boller and Felix 2009; Mariutto and Ongena 2015; Burketová et al. 2015; Choudhary et al. 2016).

Secondly, Effector-triggered immunity (ETI) which is a higher level of defence by which plant respond to some potent pathogens that possess virulence effectors molecules and hence, can successfully overcome first line of defense either by suppressing PTI signaling or preventing detection by the host (Borges and Sandalio 2015). Like PRRs in PTI, plant produces resistance (R) NB-LRR (nucleotide-binding–leucine rich repeat) receptor proteins in ETI which recognize attacker-specific effector molecules and activate a strong local defence hypersensitive response that end up with programmed cell death at the site of infection and hence avoid further infection nearby the infected cells (Dodds and Rathjen 2010; Cui et al. 2015).

Further, the commencement of PTI and ETI often triggers an induced resistance in tissues distal from the site of infection through long-distance signals in the form of salicylic acid (SA) accumulation that propagate an enhanced defensive capacity in still undamaged plant parts (Shah and Zeier 2013). This well-characterized form of pathogen-induced resistance is commonly known as systemic acquired resistance (SAR) and confers enhanced resistance against a broad spectrum of pathogens (Spoel and Dong 2012). In the SAR, increased level of SA upon pathogen priming is followed by coordinated activation of a specific set of pathogenesis related (PR) genes, numerous of which encode PR proteins having antimicrobial activity and only expressed upon subsequent pathogen challenge (Van Loon et al. 2006). Methyl salicylate (MeSA), volatile form of SA can also act as a long-distance mobile signal for the establishment of SAR. Sandhu et al. (2009) have shown upon infection with *Phytophthora sojae,* SAR get induced in soybean plant results in induced expression of PR gene *GmPR1,* and also found that *GmNPR1* genes orthologous to *Arabidopsis NPR1* (Nonexpressor of PR genes 1) gene is responsible for the regulation of SAR pathway in soybean. In another study it was sown that *RAR1, SGT1* genes are vital components of effector-triggered immunity (ETI) and SAR in soybean (Fu et al. 2009).

10.4 Bacterial Mediated Protection: Induced Systemic Resistance

To increase the yield of soybean by means of growth promotion and plant protection, a lot of research have been done till now that includes use of PGPR inoculation, plant growth promoting fungus (PGPF) inoculation, co-inoculation of two or more PGPR or fungus (Dashti et al. 1998; Han and Lee 2005; Dardanelli et al. 2010; Juge et al. 2012; Kumar et al. 2015). Apart from plants indigenous defence system and SAR where pathogen infection is required means plant have to suffer before getting protection, induced systemic resistance (ISR) is a heightened level protection elicited by beneficial bacteria to uplift the level of disease resistance in plants before pathogen attack. ISR is initiated in roots by PGPR priming and leads to resistance not only in distant parts of roots but in the aerial parts of the plant also. Signaling events of the ISR were well studied in *Arabidopsis* which shows that jasmonic acid (JA) and ethylene (ET) dependent mechanism is responsible for the ISR (Pieterse et al. 2014). Different studies show the role of JA in tandem with the ET hormone for the defense against wounds, necrotrophs and the herbivore attacks (Robert-Seilaniantz et al. 2011). A heightened level of resistance was provided by ISR-triggering bacteria through root colonization against a diverse set of intruders. After getting elicitation from PGPR, transient synthesis of JA and ET take place in root and formation of phloem-mobile signal move these signal toward distal part of the plant and after challenge inoculation JA and ET response activate *npr-1* gene expression, which encode NPR-1 protein followed by activation of defense related gene. NPR-1 protein are known as master regulator of both defense pathway, as upon getting preceding signal it activate expression of either PR gene or defense related gene for the establishment of SAR and ISR respectively. Like MeSA, methyl jasmonate (MeJA) also work as a volatile signal for the distal part of the plant (Jain et al. 2016).

Fernando et al. (2004) have reported that bacterial biocontrol agents *Bacillus amyloliquefaciens, Pseudomonas chlororaphis*, and *Pantoea agglomerans* produce volatile and nonvolatile antimicrobial antibiotics to suppress carpogenic germination and mycelial growth of *S. sclerotiorum.* Recently, Lee et al. (2015) have done comparative study of seeds of healthy soybean plants and soybeans infected by *Cercospora kikuchii* and *Phomopsis longicolla,* and revealed significant increase in three isoflavones namely, daidzein, glycitein, and genistein in diseased seeds. Earlier, Araujo et al. (2005) have reported the biocontrol role of *B. subtilis* PRBS-1 against seed pathogen *R. solani, Colletotrichum truncatum, S. sclerotiorum, M. phaseolina* and *Phomopsis* sp. and found it soybean plant growth promoting also. In another study, *P. aeruginosa* (PGPR-104) isolated from the root nodules showed significant control on the infection caused by root rotting fungi *M. phaseolina, R. solani* and *F. solani* on soybean (Batool et al. 2013). Jung et al. (2007) have reported *P. aureofaciens* 63–28 mediated induction of PR proteins in soybean plants upon challenge inoculation with *R. solani.* Vasebi et al. (2013) reported reduction of microsclerotia coverage of *M. phaseolina* on soybean root and stem by *P. agglomerans*, *Bacillus* sp. BIN and *T. harzianum* T100 treatment.

Sharma et al. (2013) isolated *B. amyloliquefaciens* form diseased root of soybean and found it plant growth promoting. PR protein GmPRP, isolated by Jiang et al. (2015) from highly resistant soybean infected with *P. sojae* and found with antagonistic activity against *P. sojae* 1 in '*in vitro'* study. Dalal and Kulkarni (2015) have treated soybean plants with endophytic bacteria *Pseudomonas* sp. JDB3 and *Bacillus* sp. JDB9, and found to be elicited induced resistance against *R. solani.* Arfaoui et al. (2016) treated soybean with calcium prior to inoculate fungus pathogen *S. sclerotiorum* and found enhanced level of defense related genes expression involved in the isoflavones pathway. Simonetti et al. (2015) have applied two bacterial strains *P. fluorescens* 9 and *B. subtilis* 54 individually as well as in combined treatment with manganese phosphate to treat soybean seeds and found it effective in controlling *M. phaseolina.* Gao et al. (2015) indicated that inoculation with rhizobia and arbuscular mycorrhizal fungi can keep pathogen in check by direct inhibition and further activation of the plants overall defense system by enhancing PR gene expressions to control soybean red crown rot. Al-Ani et al. (2012) evaluated the antagonistic activity of *Rhizobium japonicum* against *F. solani* and *M. phaseolina*, causative pathogens of root rot disease in soybean. In an earlier study, Vyas (1994) described that co-inoculation of biocontrol bacteria *B. subtilis, P. fluorescens* and *fungus T. viride, T. harzianum,* being quite effective for controlling root-rot fungus *M. phaseolina* in soybean. In addition to these, Ebtehag et al. (2009) have also reported the biocontrol potential of certain PGPR like *Azospirillum brasilense, Azotobacter chroococcum, B. megaterium, B. cereus, B. japonicum* and *Pseudomonas* against *M. phaseolina* for soybean plants while Tewari and Arora (2014) reported the similar role of fluorescent pseudomonads even under saline stress conditions in fields. The biocontrol agent *Clonostachys rosea* strain ACM941 was identified as an antagonist against a number of soil-borne and seed-borne pathogens, including *F. solani*, *F. oxysporum*, and *F. graminearum* and its potential was checked in field experiments taking soybean as a test crop (Xue et al. 2011).

In our recent study, we found elevated level of different defence related proteins namely, lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), chitinase, β-glucanase and phenolics in the *Bacillus* sp. SJ-5 treated soybean plant against the challenge inoculation of *F. oxysporum* and *R. solani* (Jain et al. 2017). Peroxidases are expressed to restrain cell spreading of disease through generation of highly toxic environments by enormously producing ROS and reactive nitrogen species (RNS) or foundation of basic obstructions, for example, lignin and suberin deposition (Cavalcanti et al. 2004; Passardi et al. 2005) while PPO also plays an important role in defense against plant pathogens due to its reaction products and wound inducibility property (Mayer and Harel 1979). PPOs catalyze oxidation of hydroxy phenols present in the plants to their quinone derivatives, which possess antimicrobial activity to combat against pathogens (Chunhua et al. 2001). LOX catalyzes the formation of unsaturated fatty acid hydroperoxide through dioxygenation of (*Z*, *Z*)-1, 4-pentadiene system containing polyunsaturated fatty acids, that leads to commencement of the synthesis of oxylipins (a group of acyclic or cyclic compounds), which are involved in defense responses to pathogen, wounding and stress (Feussner and Wasternack 2002; La

Camera et al. 2004; Shah 2005; Baysal and Demirdöven 2007). PAL is the key enzyme in phenylpropanoid metabolism and responsible for the biosynthesis of the polyphenol compounds such as phenylpropanoids, lignin and flavonoids in plants which play important role in protection against pathogen attack (Liang et al. 1989). Fungal phytopathogens, major threat for the plant world, possess β-1,3 glucan and chitin, polymer of N-acetylglucosamine (NAG) , as their cell wall components. In addition to described inducible defence enzymes, chitinase and β-1,3 glucanase are the major enzymes play a direct role in plant protection by defending against pathogen via direct pathogens cell wall degradation. The phenolic compounds produced by the plants improve the defence system by reinforcement of host cell wall and due to its fungitoxic nature inhibit the growth of fungal phytopathogens.

10.5 Conclusion

Soybean is one of the very important crops regarding nutritional and economical value. Biotic stresses imposed by different phytopathogen in the form of disease is a great concern for the decreasing quality and quantity of soybean, and another important thing about it is the increased use of chemicals to ameliorate biotic stress that further threatened world with its toxic nature and environmental pollution. Even though, worldwide several research has been done to develop stress resistant soybean cultivars for conquering the losses, but further, development of such genetic breeds of soybean is not an easy, recommendable, and economical approach for sustainable agriculture. Furthermore, high variability of most of the pathogens make difficult to resist disease even in resistant variety. Hence, the use of potential beneficial microbes to overcome the effect of such stresses is a better strategy to increase the production of soybean in terms of good quality and high yield. This chapter has focused on the major fungal diseases that affect soybean production globally and need to be control. Further, various research done to ameliorate these diseases have been also discussed with special reference to use of different PGPR. As a future prospective, it is a strong need to apply already scrutinized potent PGPR in the field and discover the new stains. Further, to develop deep understanding of complex plant-microbe interaction also need to be improved to get best from microbes. Improvement in the yield of such a commercially important oil seed crop by using eco-friendly microbe will be really a boot for the economies of developing countries.

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Chapter 11
Mycotoxins and Pesticides: Toxicity and Applications in Food and Feed

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11.1 Introduction

11.1.1 Mycotoxins

As a result of a veterinary crisis in England in 1961 due to the mycotoxins came in to prominance where thousands of animal died for the first time. This crisis was due to toxins, produced by the *Aspergillus flavus* in peanut contaminated meal (Bennett and Klich 2003; Richard 2007). The chemical structure of mycotoxins ranged from C4 compound to complex substances are characterized as low molecular weight compounds produced by secondary metabolism (Paterson and Lima 2010). For example honey, eggs or milk are reported to contain the staphylococcal toxin produced by bacteria and the toxins pyrrolizidine the alkaloids produced by plants. Though the contamination of bacteria/fungi can be eliminated by the heat treatment, however the toxins remain in the food products even after heat treatments. The amount and prevalence of mycotoxins are variable in different growing and storage conditions for different climates. Different mycotoxins including aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2), fumonisins (FB1, FB2, FB3), ochratoxins

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(OTA, OTB, OTC), zearalenone (ZON), trichothecenes (T-2 and HT-2 toxin, diacetoxyscirpenol patulin, nivalenol, neosolaniol, deoxynivalenol, 3-acetyl DON, 15-acetyl DON, fusarenon B1,B2) are tabulated as Table 11.1 these are reported to contaminate various food stuff such as cereals, dry fruits, nuts, infant preparation and beverages as coffee, wine, fruit juice.

The vertebrates and animals are affected by very low concentrations of mycotoxins, some of these mycotoxins can cause autoimmune illnesses, allergy, while others are carcinogenic and mutagenic (Bosco and Mollea 2012). Though mycotoxins have no biochemical significance on fungal growth however they may act as a defense against microorganisms, insects, animals or human (Bosco and Mollea 2012; Etzel 2002). Around 25% crops are affected by fungal diseases and mycotoxins produced in pre and post-harvest stages (Bryden 2007). At present known mycotoxins are more than 400 in number but, the carcinogenic and/or toxicogenic mycotoxins to humans and animals need more scientific attention. The mycotoxins are largely derived from polyketide (PK) metabolism in agriculture and food industry *via* biosynthetic pathway known as terpenoid origin. The repetitive acetate units or other short carboxylic acid produces the PKs metabolites *via* enzymatic mechanisms similar to fatty acid synthesis (Huffman et al. 2010).

Higher amount of mycotoxins are needed in food and feed to produce adverse health symptoms in human and animals. It damages the immune system. These mycotoxins can be limited by limiting the consumption of contaminated food and feeds. Availability of mycotoxins data in different commodities are essential for the adoption of safety against mycotoxins and pesticides. Standard sampling methods availability and analysis of mycotoxin and pesticide in different commodities are necessary for food supply in inter-country trades. Based on the data available from scientific publications and other sources on mycotoxins permissible limit in different countries for foodstuffs, dairy products and animal feeds are summarized country wise in Table 11.1. The rapid onset of an adverse effect from a single exposure (acute) and slow onset owing to long term exposure (chronic) may be caused due to the negative effects of mycotoxins. Based on efficacy of toxin and the dose, mycotoxins can be acute or chronic or both. Therefore, mycotoxins are classified into neurotoxins, hepatotoxins, nephrotoxins, immunotoxins and so on. However, the cell biologists added it into generic group's like- allergens, carcinogens, mutagens and teratogens (Bennett and Klich 2003).

In most of the cases mycotoxicoses (harm to the animals and human) resulting from eating contaminated food. Human can be directly affected by mycotoxin contaminated cereals or indirectly by feedings animal products (CAST 1989, 2003). Complete destruction of mycotoxins does not occur under normal cooking conditions of boiling, frying or even pasteurization as they are generally heat-stable (80–121 °C) (Bosco and Mollea 2012) however, its concentration may be reduced significantly during food processing by cleaning and milling (physical treatments) and by thermal processes (e.g. cooking, baking, frying, roasting and extrusion) (Bullerman and Bianchini 2007). The complete elimination of mycotoxins are not achieved till date during food processing (Venkataramana et al. 2015).

Table 11.1 Major food borne mycotoxins, producing fungal species, commodities most frequently contaminated and major health effects on animals and humans. Limits for Mycotoxins in foodstuffs, dairy products and animal feedstuff

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
Aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2) Aflatoxins (AFs) are highly toxic, mutagenic, and carcinogenic compounds (Wogan 1999). They are secondary metabolites The name "aflatoxin" is derived from the first letter in Aspergillus, and the first three letters in flavus	*Aspergillus flavus, A. nomius, A. parasiticus, A. arachidicola, A.bombycis, A. pseudotamarii, A. inisclerotigenes, A. rambellii, A. ochraceoroseus, Emericella astellata, E. venezuelensis, E. olivicola*	Argentina	Groundnut, maize and by-products	B1	5	Birds: Duckling, turkey, poultry pheasant chick, mature chicken, quail Mammals: Young pigs, pregnant sows, dog, calf, mature cattle, sheep, cat, monkey, human Fish: Laboratory animals	Carcinogenic, mutagenic, teratogenic, hepatotoxic, nephrotoxic, immune suppressive, hemorrhage of intestinal tract and kidneys, liver disease
				B1,B2,G1,G2	20		
			Infant food	B1	0		
			Infant food based on cereals	B1,B2,G1,G2	N.D		
			Infant food based on milk products	M1	N.D		
			Liquid milk, powdered milk	M1	0.05		
			Milk products	M1	0.5		
			Soya meal	B1	30		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		Australia	Peanut, peanut butter, nuts	B1,B2,G1,G2	15		
			And nut products -all other foods	B1,B2,G1,G2	5		
		Austria	All foods except meal and	B1	1		
			Husked products	B1,B2,G1,G2	6		
			Meal and husked products	B1	2		
			Milling, shelled and derived products	B1,B2,G1,G2	6		
				B1	2		
			Pasteurized fresh milk, baby and children's milk, reconstituted milk food for children	B2,G1,G2	5		
				M1	0.01		
			Other milk and it's products	M1	005		
			Whey, liquid products	M1	0.025		
			Whey powder and whey paste	M1	0.4		
			Butter -cheese	M1	0.25		
			Dried milk and it's products,	M1	0.4		
			Condensed milk	B1,B2,G1,G2			
			Other reconstituted children's foods	M1	0.02		

			Feed (see European Union)				
		Bahamas	All food and grains	B1,B2,G1,G2	20		
		Brabados	Milk	M1	0.05		
			All other foods	B1,B2,G1,G2	20		
			All feedstuffs	B1,B2,G1,G2	50		
		Belgium	Peanuts	B1	5		
			Milk, powdered milk and condensed milk (reconstituted)	M1	0.1		
			Feed (see Europen Union)				
		Belie	Corn peanuts, maize, groundnuts	B1,B2,G1,G2	20		
		Bosnia	Wheat, maize, rice, cereals	B1,G1	1		
		Herzegovina	Beans	B1,G1	5		
		Brazil	All foodstuffs	B1	15		
			Imported foodstuffs	B1,B2,G1,G2	30		
			Industrially prepd	B1	5		
			Foodstuffs for school meals for children 0–2 yrs. age	B1,B2,G1,G2	10		
			Maize groundnut	B1,B2,G1,G2	3		
			Milk (products)	B1,G1	30		
			Imported milk (products)	M1	0.5		
			Peanut meal (feed)	M1, B1,B2,G1,G2	0.1–50		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		Bulgaria	Peanuts, kernels and their products, coca beans, cocoa butter, cocoa powder	B1,B2,G1,G2	5		
			Grains, cereals and their products	B1,B2,G1,G2	2.5		
			Liquid milk products, cheese and similar products	M1	0.5		
			Powdered milk	M1	0.1		
			Powdered milk for dietetics and infant feeding	M1	0		
		Canada	Nut and it's products	B1,B2,G1,G2	15		
			Animal feedstuffs	All aflatoxis	20		
		Chile	Feedstuffs	B1,B2,G1,G2	5–50		
		China	Rice, edible oils	B1	10		
			Maize, peanut products	B1	20		
			Wheat, barley, oats, grains,	B1	5		
			Fermented foodstuffs				
			Cow's milk and it's products	B1	0.5		
			Compound feed for laying	B1	20		
			Hens and fattening pigs				
			Maize, peanut cake, peanut residue	B1	50		

		Colombia	Foods	B1,B2,G1,G2	20		
			Cereals,grains, (sorghum,millets)	B1,B2,G1,G2	30		
			Oil seeds (peanuts)	B1,B2,G1,G2	10		
			Cattle feed	B1,B2,G1,G2	50		
			Sesame seeds, poultry feedstuffs	B1,B2,G1,G2	20		
		Costa Rica	Corn for human consumption	B1,B2,G1,G2	35		
			Maize (feed)	B1,B2,G1,G2	50		
		Coted' lvoire	Straight feedstuffs	B1,B2,G1,G2	100		
			Complete feedstuffs	B1,B2,G1,G2	10		
			Complete feedstuffs pigs/poultry	B1,B2,G1,G2	38		
			Complete feedstuffs cattle/sheep/goats	B1,B2,G1,G2	75		
			Complete feedstuffs for dairy cattle	B1,B2,G1,G2	50		
		Cuba	Foods, cereals, peanuts, groundnuts	B1,B2,G1,G2	5		
			Feedstuffs, raw materials for feedstuffs	B1,B2,G1,G2	5		
		Cyprus	Cereals, pulses, oilseeds, dried fruit, sesame and foods produced exclusively these, caraway seed, poppy seeds and other seeds used in baker products confectionery	B1,B2,G1,G2	5		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		Czech Slovak republics	All foods except reconstituted baby milk	B1	5		
			Infant food, children's food and other baby foods and milk	B1,B2,G1,G2, M1	15		
			Reconstituted baby milk		5		
			Other baby food	B1	0.1		
				B1,B2,G1,G2,	0.3		
			Liquid milk	M1	0.1		
				B1	1		
				B1,B2,G1,G2	3		
		Denmark	Foods including peanuts & it's products, nuts, dried figs	B1	2		
			Raw materials for manufacturing foods before cleaning read for processing	B1,B2,G1,G2	4		
			Feed (see European Union)	B1,B2,G1,G2	10		
		Dominican Republic	Imported corn	B1,B2,G1,G2	20		
			Corn, tomatoes and their products, peanut, soya, groundnut	B1,G1	0		

		Egypt	Peanuts, oil seeds, cereals and their products	B1	5		
				B1,B2,G1,G2	10		
				B1	10		
			Maize	B1,B2,G1,G2	20		
				B1,B2,G1,G2	0		
			Starch and it's derivatives	B1	0		
				M1	0		
			Milk and dairy products	G1,B2,G1,G2	0		
				B1	10		
			Animal and poultry fodder	B1,B2,G1,G2	20		
		El Salvador	All foods	B1,B2,G1,G2	20		
			Feedstuffs	B1	10–20		
		European Union	Cereals, nut, nut products and dried fruits	B1	2–4		
			Milk and it's products	M1	0.05		
			Baby and infant food	B1	1–2		
			Straight feedstuffs like groundnut, copra, palmunt, cottonseed, babassu, maize and their products	B1,B2,G1,G2, M1	0.05		
					20–50		
			Complete feedstuffs for cattle and sheep excerpt their young ones	B1	20		
			Complete feedstuffs for calves, lambs	B1	50		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
			Complete feedstuffs for others	B1	10		
			Complementary feed for pigs, poultry except their young ones	B1	10		
			Complementary feed for cattle, sheep, goat except their young ones	B1	30		
			Other complementary deed	B1	50		
			Raw materials like groundnut, copra, maize, palmunt, cottonseed babassu and their products	B1	5		
				B1	200		
		Finland	All foods except meat, orgn, milk or cream	B1,B2,G1,G2	5		
			Feed (see European Union)				
		France	Any food for which level not been fixed	B1	10		
			Peanuts, pistachios almonds, oilseeds, other than those used for preparation of vegetable oils, children food	B1	1		
			All cereals	B1	5		
			White wheat flour	B1	3		

			Wheat bran (raw)	B1	10		
			Whole wheat flour obtained from all the components of grain	B1	5		
			All vegetable oils, virgin or mixed with refined oil	B1	5		
			Food products readily consumed by children	B1	1		
			Milk for direct consumption or for prepn. Of dairy products	M1	0.05		
			Liquid milk for infant food (under 3 yrs)	M1	0.03		
			Milk powder	M1	0.5		
			Milk powder for infantsbelow 3 years (for feed see E. Union)	M1	0.3		
		German	All foods except enzymes and it's prepns. Intended for the production of food stuffs	B1,B2,G1,G2	4		
				B1	2		
			Enzymes and it's prepns intended for the preparation of food stuffs	B1,B2,G1,G2	0.05		
			Milk	M1	0.05		
			Dietetic foods for babies and infants	B1,B2,G1,G2	0.05		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
			Feed (see European Union)	M1	001		
		Greece	Dried fruits like peanuts, hazelnuts, walnuts, cashew nuts, pistachios, almond, pumpkin seeds, sunflower seeds, pine seeds, apricot seeds, maize, dried figs, dried apricots, dried prunes, dates, resins, plums, pears, grapes	B1	5		
			Feed (see European Union)	B1,B2,G1,G2	10		
		Guatemala	Corn, kidney beans, rice, sorghm, peanuts and peannut butter	B1,B2,G1,G2	20		
			Feed concentrate	B1,B2,G1,G2	20		
		Honduras	Grounded or whole corn	B1	1		
			Baby foods	B1,B2,G1,G2	001		
				M1	0.02		
			Milk and it's products	M1	0.05		
			Cheeses	M1	0.25		
			All other foods	B1,B2,G1,G2	1		

		Hong Kong	Peanut and it's products	B1,B2,G1,G2	20		
				M1,M2,P1,			
			Any food other than peanuts	B1,B2,G1,G2	15		
				M1,M2,P1,			
		Hungary	All foods	B1,B2,G1,G2	10		
			Groundnut kernels	B1	30		
			Groundnuts	B1,B2,G1,G2	5		
			Milk products	M1	0.5		
		India	All foods	B1	30		
			peanut meal (export)	B1	120		
		Indonesia	Copra in cow pig, duck, sheep feed	B1,B2,G1,G2	10,000		
			Groundnut, sesame seed, rapeseed meal	B1,B2,G1,G2	200		
			Cassava in chicken feed	B1,B2,G1,G2	120		
			Capok seed, coconut meal in chicken feed and cow/pig duck/sheep feed	B1,B2,G1,G2	100		
			Sunflower seed meal in chicken feed	B1,B2,G1,G2	90		
			Others	B1,B2,G1,G2	20–50		
		Iran	All foods	B1,B2,G1,G2	30		
		Ireland	All foods	B1	5		
			Feed (see European Union)	B1,B2,G1,G2	30		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		Israel	Nuts, peanuts, fig and their products and corn meal	B1,B2,G1,G2	20		
			Milk and milk powder	M1	0.05		
		Italy	All foods including dried	B1,B2,G1,G2	10		
			Figs	B1	5		
			Spices	B1,B2,G1,G2	40		
			Fed (see EUROPEAN Union)	B1	20		
		Jamaica	Foods and grains	B1,B2,G1,G2	20		
		Japan	All foods	B1	10		
			Imported peanut meal feed	B1	1000		
		Jordan	Almonds, cereals, maize, peanuts, pistachios, pine nuts, rice, allepo and cedar nuts, walnuts	B1	15		
				B1,B2,G1,G2	30		
			All feedstuffs	B1,B2,G1,G2	15–30		
		Kenya	Peanut and it's products and vegetable oils	B1,B2,G1,G2	20		
		Luxembourg	Peanut and it's products	B1	5		
			Feed (see European Union)				
		Macedonia	Wheat, maize, rice, cereals beans	B1,G1	1		
				B1,G1	5		

		Malawi	Peanuts for export	B1	5		
		Malaysia	All foods	B1,B2,G1,G2	10		
		Mauritius	Peanuts, groundnuts	B1,B2,G1,G2	5–15		
			Other foods	B1,B2,G1,G2 and M1,M2	5		
					10		
		Mercosur (Argentina, Uruguay, Brazil and Paraguay)	Maize kernels, maize flour meal, peanuts it's products	B1,B2,G1,G2	20		
			Fluid milk	M1	0.5		
			Milk powder	M1	5		
		Mexico	All foods	B1,B2,G1,G2	20		
			Flour	All aflatoxin	20		
			Feedstuffs for bovine and porcine fattening	B1,B2,G1,G2	200		
			Feedstuffs for dairy cattle/poultry	B1,B2,G1,G2	0		
		Netherlands	All food and drink products and raw produce (except peanuts kept in stock for the prepn. of peanut oil	B1	5		
			Infant food and milk for infants ilk and other milk products	M1	0.05		
				M1	0.05		
			Butter and cheese				
			Feed (see European Union)	M1	0.02		
				M1	0.2		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		New Zealand	Peanut butter, shelled nuts and the nut portion of products containing nuts	B1,B2,G1,G2	15		
			All other foods	B1,B2,G1,G2	5		
		Nigeria	Infant foods	B1	0		
			All other foods	B1	20		
			Fluid milk	M1	1		
			Feedstuffs	B1	50		
		Norway	All foodstuffs including	B1,B2,G1,G2	5		
			Brail nuts, buck wheat missed feedstuffs	B1	10–50		
		Oman	Complete feedstuffs for poultry	B1	20		
			Other feedstuffs	B1	10		
		Peru	All foods	B1,B2,G1,G2	10		
			All foods	B1,B2,G1,G2	10		
			Complementary feedstuffs	B1	10		
			Cereals for porcine growing feedstuffs	B1,B2,G1,G2	100		
		Philippines	Nuts and its products and all foods	B1,B2,G1,G2	20		
			Poultry feedstuffs foods	B1,B2,G1,G2	20		
			Poultry feedstuffs	B1,B2,G1,G2	20		
			Livestock feedstuffs	B1,B2,G1,G2	50		

		Poland	All foods	B1	0		
			Feedstuffs and their ingredients, complete feedstuffs for cattle/ sheep/goats	B1	50		
			Complete feedstuffs for pigs, poultry and dairy cows	B1	0		
		Portugal	Peanuts	B1	25		
			Infant foods	B1	5		
			Other foodstuffs	B1	20		
		Romania	All foods	B1,B2,G1,G2	0		
			Milk and diary products	M1	0		
			All feedstuffs	B1,B2,G1,G2	50		
		Russia	Animals fats	B1	0		
			Bottled fruits and	M1	0.5		
			Vegetables	B1	5		
			All others foods	B1	5		
			Milk and milk products	B1, M1	0, 0.5		
		Saudi Aribia	Infants and children foods	M1	0.05		
			Dried milk not for infants	M1	0.5		
			Liquid milk and its products	M1	0.2		
			Grains, nuts and their products	B1,B2,G1,G2	20		
			Other foods	B1,B2,G1,G2	20		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		Senegal	Peanut products as straight feedstuffs	B1	50		
			Peanut products as feedstuff	B1	300		
		Serbia	Wheat, maize, rice cereals	B1,G1	1		
			Beans	B1,G1	15		
		Singapore	All foods	All mycotoxins	n.d		
		South Africa	All foods	B1	5		
			Milk and milk products	B1,B2,G1,G2	10		
				M1	0.05		
		South Korea	Grains, beans, peanuts and their simple processed foods	B1	10		
		Spain	All foods for human consumption	B1	5		
			Feeds (see European Union)	B1,B2,G1,G2	10		
		Sri lanka	Groundnuts and other oilseeds, flour and cereals	B1,B2,G1,G2	30		
			Baby foods till 3 years	All aflatoxins	1		
			Milk and its products	All aflatoxins	1		
		St. Vincent	Imported corn	B1,B2,G1,G2	20		
			Corn and its products, peanuts, soya, tomatoes and its products	B1,G1	0		

		Surinam	Peanuts, peanut products, pulses, groundnuts and their products, legumes	B1	5		
			Corn	B1,B2,G1,G2	30		
			Feedstuffs	B1,B2,G1,G2	30		
		Sweden	All foods	B1,B2,G1,G2	5		
			Liquid milk products	M1	0.05		
			Feedstuff ingredients	B1	1–50		
			Mixed feed	B1	3		
			Complete feedstuffs	B1	1.5–50		
		Switzerland	All foodstuff except maize and cereals	B1	1		
				B1,B2,G1,G2	6		
			Maize and cereals (in grains or ground)	B1	2		
				B1,B2,G1,G2	6		
			Herbs	B1	5		
			Baby/infant food	B2,G1,G2	5		
				M1	0.02		
			Milk and dairy products	B1,B2,G1,G2			
				M1	0.025–0.05		
			Cheese	M1	0.25		
		Taiwan	Peanuts and maize	B1,B2,G1,G2	20		
			Rice, sorghum, legumes, cereals and nuts	B1,B2,G1,G2	15		
			Feeds, oilseed meals	B1,B2,G1,G2	1000		
		Thailand	All foods including oils and fats	B1,B2,G1,G2	20		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
		Trinidad and	All foods	B1,B2,G1,G2	10		
		Tobago	Ice cream	All mycotoxins	0		
			Feedstuffs	B1,B2,G1,G2	10		
		Turkey	All foodstuffs except infant foods	B1	5		
				B1,B2,G1,G2	20		
			Infant foods	B1,B2,G1,G2	2		
			Milk and milk products	M1	0.5		
		United States of America	All foods	B1,B2,G1,G2	20		
			Whole milk, low fat milk ad skim milk	M1	0.5		
			Feedstuff ingredients	B1,B2,G1,G2	20		
			Cottonseed meal intended for beef cattle swine, poultry feedstuffs	B1,B2,G1,G2	300		
			Maize and peanut product for beef cattle swine or poultry	B1,B2,G1,G2	100–300		

		Uruguay	Butter	B1	0		
			Butter, milk and milk products	M1	0.5		
			Foods and spices	B1,B2,G1,G2	20		
			Peanuts, dried fruits and products, texturized soyprotein products (flour, starch concentrate)	B1,B2,G1,G2	30		
			Cocoa beans	B1,B2,G1,G2	10		
			Infant foods	B1,B2,G1,G2	3		
		Venezuela	Rice flour	B1,B2,G1,G2	5		
			Feed stuffs	B1,B2,G1,G2	20		
		Zimbabwe	Foods	B1	5		
			Groundnuts, Maize,	G1	4		
			Sorghum	B1	5		
			Peanut butter, cereals, flour	G1	4		
			and bread	B1,B2,G1,G2	20		
			Poultry feed	B1,G1	10		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
Ochratoxins (OTA, OTB, OTC): It was discovered in 1965 in South Africa (Van der Merwe et al., 1965): it was isolated as a toxic metabolite of *Aspergillus ochraceus* from corn meal artificially inoculated with the fungus	*A. alutaceus, A. alliaceus, A. auricomus, A. glaucus, A. niger, A. carbonarius, A. melleus, A. albertensis, A. citricus, A. flocculosus, A. fonsecaeus, A. lanosus, A. ochraceus, A. ostianus, A. petrakii, A. sulphureus, A. pseudoelegans, A. Roseoglobulosus, A. sclerotiorum, A. steynii, A. westerdijkiae, Neopetromyces muricatus, Penicillium viridicatum, P. verrucosum, P. cyclopium, P. carbonarius*	Austria	Wheat, rice, durum	OTA	5	Swine, dog, duckling, chicken, rat, human	Carcinogenic, mutagenic, nephrotoxic, hepatotoxic, teratogenic, immunodepressants, carcinogenic (urinary tract tumors), inhibition of protein synthesis

		Brazil	Rice, barley, beans, maize	OTA	50		
		Czech republic	All foods	OTA	20		
			Infant foods	OTA	5		
		Denmark	Pig kidneys	OTB	10–25		
			Cereal products	OTA	5		
		France	Cereals	OTC	5		
		Greece	Raw coffee beans, apple juice, apple products	OTA,OTB	20		
		Hungary	All foods	OTA, OTB, OTC	20		
		Israel	Cereal products, pulse products	OTA, OTB, OTC	50		
			Grain for feed	OTA	300		
		Natherlands	Cereals	OTA	3		
		Romania	All foods	OTC	5		
			Feed	OTB	5		
		Sweden	Feedstuffs for poultry	OTA, OTC	200		
			Feedstuffs for pigs	OTC	100		
		Switzerland	Cereal products	OTB	2		
		Uruguay	Rice, barley, beans, coffee, maize	OTC	50		
		United kingdom	Cereals	OTA, OTB, OOTC	10		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
Trichothecenes (T-2 and HT-2 toxin, diacetoxyscirpenol, Neosolaniol, nivalenol, deoxynivalenol, 3-acetyl DON, 15-acetyl DON, fusarenon B1,B2): Trichothecin was first isolated from *Trichothecium roseum* and described by Freeman and Morrison in 1949.	*Fusarium culmorum; F. graminearum; F. sporotrichioides; Fusarium sporotrichioides, F. poae, F. acuminatum, F. culmorum, F. quiseti, F. graminearum, F. cerealis, F. moniliforme, F. myrothecium, F. lunulosporum, Cephalosporium sp., Myrothecium sp., Trichoderma sp., Trichothecium sp., Phomopsis sp., Stachybotrys sp.,*	Austria	Wheat, rye durum wheat	DON	500	Swine, cattle, chicken, turkey, horse, rat, dog,mouse, cat, human	Immuno-depressants, mutagenic, gastrointestinal haemorrhaging, neurotoxic;
		Canada	Diets for cattle, poultry, swine, young calves	HT-2 toxin	100		
			Lactating dairy animals		25		
		Israel	Grain for feed	T-2 toxin	100		
		Russia	Cereals, flour, wheat bran	T-2 toxin	100		
		Canada	Uncleaned soft wheat	DON	2000		
			Diets for cattle/poultry		5000		
			Diets for swine calves/ lactating		1000		
			Dairy animals				
		Israel	Grains for feed	DON	1000		
		Romania	All feedstuffs	DON	5		
		Russia	Cereals, flour, wheat bran		1000		
		USA	Finished wheat product	DON	1000		
			Grains and grain by product		10,000		
			Grains and grain by products for swine		5000		

Fumonisins (FB1, FB2, FB3): Fumonisins are a group of non-fluorescent mycotoxins. They were discovered and characterized in 1988	*Alternaria alternata, Fusarium anthophilum, F. moniliforme, F. dlamini, F. napiforme, F. proliferatum, F. nygamai, F. verticillioides;*	USA	Feed for horses	FB1	500	Horses, swine, rats, humans	Hepatotoxic, cerebral edema, cause necrosis, immunotoxic
			Feed for swinr	FB2	10,000		
			Feed for beef cattle and poultry	FB3	50,000		
		South Africa	Corns and corn products	FB2	100–200		
Zearalenone (ZON)	*F. graminearum, F. culmorum, F. crookwellense, F. equiseti, F. sporotrichioides; F. culmorum; F. graminearum; F. sporotrichioides*	Austria	Wheat re, durum wheat	ZON	60	Swine, dairy cattle, chicken, turkey, lamb, rat, mouse, guinea pig	Estrogenic activity (infertility, vulval oedema, vaginal prolapse, mammary hypertrophy in females, feminisation of males)
		Brail france	Maize	ZON	200		
			Cereals, vegetable oils	ZON	200		
		Hungary	All foods	ZON	50		
		Romania	All foods	ZON	30		
		Russia	Cereals, flour wheat bran, leguminous protein, isolates, Vegetable oils, nuts (kernel)	ZON	1000		
		Uruguay	Maize, barley	ZON	200		

(continued)

Table 11.1 (continued)

Mycotoxins groups	Fungal species	Suffering countries	Food commodities	Mycotoxins affected	Maximum limits ($\mu g.kg^{-1}$ or $\mu g.l^{-1}$)	Affected species	Pathological effects
Patulin: which are derived from polyketide (PK) metabolism; Patulin (PAT) was discovered in 1943 in relation to *P. griseofulvum* and *P. expansum*. The molecule was first studied as a potential antibiotic, but the subsequent research demonstrated its toxicological properties	*A. clavatus, A. longivesica, A. terreus, P. expansum, P. griseofulvum, Byssochlamys sp.*	Austria	Fruit juice	Patulin	50		Immuno-depressant, pulmonary and cerebral oedema, nausea, gastritis, paralysis, convulsions, capillary damage, carcinogenic
		Belgium	Apple juice	Patulin	50		
		Czech and Slovak	All foods	Patulin	50		
		Republic	Infant foods	Patulin	20		
			Children food		20		
		Finland	All foods	Patulin	50		
		France	Apple juice	Patulin	50		
		Greece	Raw coffee beans, apple juice, apple products		50		
		Hungary	All foods	Patulin	50		
		Israel	Apple juice	Patulin	50		
		Norway	Apple juice	Patulin	50		
		Romania	All foods	Patulin	30		
			All feedstuffs		30		
		Russia	Bottled/canned/potted fruits and berries and canned	Patulin	50		
			Vegetables	Patulin			
		South Africa	All foods	Patulin	50		
		Sweden	Barries, fruits, juices	Patulin	50		
		Switzerland	Fruit juice	Patulin	50		
		Uruguay	Fruit juice	Patulin	50		

Phomopsin, Diacetoxyscirpenol, Stachybotryotoxin, chetomin		Australia	All foods	Phomopsin	5		
		Israel	Grain for feed	Diacetoxy-scirpenol	100–1000		
		Romania	All feedstuffs	Stachybotryotoxin, chetomin	0		
Ergot alkaloids	*Claviceps africanana, C. purpurea, C. fusiformis, C. paspali, Neotyphodium coenophialum; Acremonium coenophialum*		Wheat, rye, hay, barley, millet, oats, sorghum, triticale			Fish larvae, pigs, cattle, swine, horses, swine, human	Vaso constrictive activity (oedema of the legs, paraesthesias, gangrene at the tendons) convulsive form: gastrointestinal symptoms (nausea, vomiting), effects on the central nervous system (drowsiness, ataxia, convulsions, blindness, paralysis)
Cyclopiazonic acid	*A. flavus*						

Source: JECFA 1998, Mazumder and Sasmal (2001), Reports on Carcinogens [ROC] 2003, Greco et al. 2012, CAST 2003, and Koppen et al. 2010

11.1.1.1 Mycotoxins Induced Oxidative Stress and Disease

For detoxification of mycotoxins in feedstuffs using microorganisms or enzymes are the best way. In contrast to physicochemical techniques for detoxification microbial and enzymatic approach is environment friendly. The microbes or enzymes are potential source for mycotoxin biodegradation in ruminants (Upadhaya et al. 2010). In vertebrate mycotoxins can be metabolized by the conversion of cytochrome P450 enzymes into guanine-N7 adduct. Mycotoxins, generally affect to the DNA, RNA, protein synthesis and metabolic reactions (Fig. 11.1) and that alters the growth, development and reproduction (Surai et al. 2008).

The mycotoxin/pesticide can pass through food in the stomach *via* cell-lining in the intestinal walls, some of the harmful toxins are absorbed in bloodstream and partly prolonged stay by the intestinal microbes. Moreover, mycotoxins directly absorbed into the bloodstream may invade deeper body tissues and create oxidative stress. Thus the reactive oxygen species (ROS) and mycotoxins mediated toxicity causes cytotoxicity. ROS are the chemically reactive molecules due to presence of unpaired electrons. An important role of ROS is known in cell signalling and homeostasis. Environmental stress due to elevated levels of mycotoxins lead to increased ROS levels causing oxidative stress (Devasagayam et al. 2004; Shah et al. 2001). The oxidative stress occurs, when ROS level increased beyond antioxidant capability of the cell (Sies 1991). However, under normal condition enzymes superoxide dismutase (SOD) or glutathione peroxidase (GPx) together with enzyme catalase (CAT) scavenge increased level of ROS (Shah and Nahakpam 2012). The ROS-induced alteration of macromolecules causes the damage to membrane lipids, proteins and DNA. The antioxidants can nullify the effect by oxidizing xenobiotics (Antibiotics, carcinogens, drugs, environmental pollutants, hydrocarbons, food additives, mycotoxins and pesticides) that generate the free radical intermediates causing oxidative stress by bio activation of lipoxygenases (LPOs) or microsomal P450s and prostaglandin H synthase (PHS) (Tafazoli 2008) (Fig. 11.2). In vivo studies by Eaton and Groopman (1994) reveal that the carcinogenic potency of mycotoxins has significant correlation with the total DNA adducts. Primarily xenobiotics

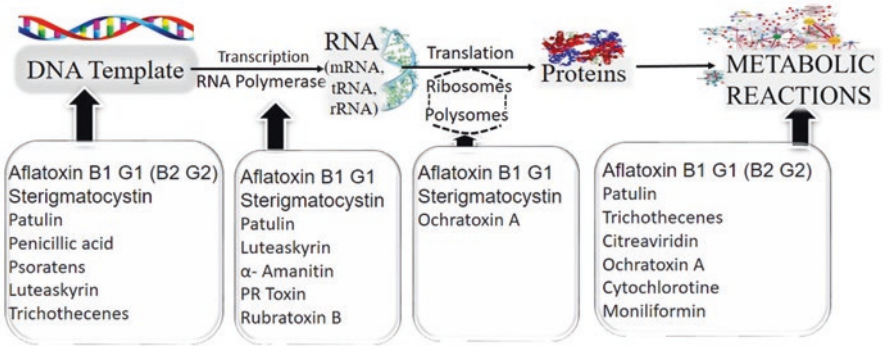

Fig. 11.1 Mycotoxins affecting major sites in DNA, RNA and protein synthesis

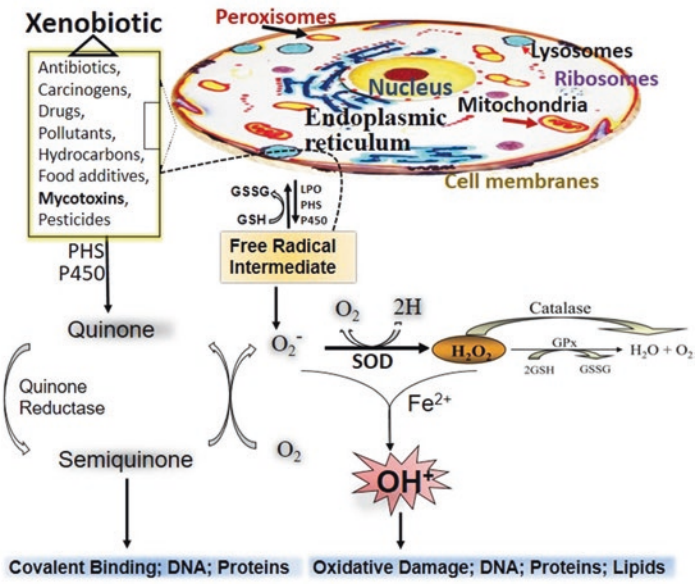

Fig. 11.2 General pathways for the effects of Xenobiotics on ROS production and clearance by antioxidants

attack the fatty acids in the membrane, remove hydrogen atoms and thereby convert the fatty acids into free radicals themselves to counteract the excess oxidants (Halliwell and Gutteridge 2007). A correlation between ROS generation and malondialdehyde (MDA) formation in higher concentration in a time dependent manner and the effect of mycotoxins on cell viability were also observed by Ferrer et al. (2009). The protective effects of natural antioxidants play an important role in oxidative stress due to the toxicity of mycotoxins (Surai et al. 2008).

After the pre and post-harvest practices of agricultural produce harms, it must be assured that reducing the mycotoxin concentrations of aflatoxins, deoxynivalenol or others might be a critically important factor (Scott and Zumno 1995; Reid et al. 1996; Desjardin et al. 1998). Now a days numerous techniques are available for the management of harmful mycotoxin contaminated food stuffs that help to reduce or eliminate or to reject the infected agricultural produce. Some of the practical methods as cleaning of grain bins, storage under low moisture levels (14%), use of dry ingredients for feed that are stored in oxygen-free environment, fermented or treated with mould growth inhibitors) are reported by Harris (1997) for preventing mycotoxin contamination in foodstuffs. Mycotoxins of fungal origin produced after colonization of fungal pathogens are harmful for the crops, food and food products. So, for the enhancement in the productivity of crops and their preservation is the main target and now a days various types of chemicals and pesticides are used by the farmers. Mycotoxin contamination of agricultural products is generally prevented by (1) Pre-harvest methods as (i) using biological and chemical agents; (ii) using resistant varieties; (iii) proper field management (2) Post-harvest methods as

(i) drying; (ii) storage conditions; (iii) use of natural and chemicals agents and (iv) irradiation. Food spoilage by fungi can be a real problem for food security and may become contaminated with toxic secondary metabolites or mycotoxins. Mycotoxins are of concern to international trade of agricultural products and falls under phytosanitory regulation of WTO that cannot be compromised as they are harmful to the human and animal health bearing thereby economic and international trade implications globally (Bryden 2007, 2009; Wild and Gong 2010).

11.1.2 Pesticides in Farming Fields

All fungicides, herbicides, insecticides, rodenticides, molluscicides, nematicides and plant growth regulators are commonly known as pesticide (Aktar et al. 2009). These can bear in pest prevention destruction or control including human or animal disease vectors. It also includes chemicals used as growth regulators, defoliants, desiccants, fruit thinning agents or agents applied to preventing the premature fruits from deterioration of crops during storage or transport. The chemicals used to control the insects, weeds, fungi, rodents and microbes known as pesticides are harmful to the environment, human and animal health for they are being used in many different sectors e.g. agriculture, forestry, food industry etc. (Damalas and Eleftherohorinos 2011). Based on their purpose pesticides are classified in different classes as given in Table 11.2. The historical background of pesticides used in agriculture is dated back to the beginning of agriculture itself and it became more pronounced with time due to increased pest population paralleled with decreasing soil fertility (Muir 2002). In 1860s the First generation pesticides involved highly toxic calcium and lead salts of arsenate and fumigant hydrogen cyanide for the control of fungi, insects and bacteria. They were abandoned because of their toxicity and ineffectiveness. The second generation in 1870s comprised of synthetic organic compounds and dichlorodiphenyltrichloroethane (DDT) (Othmer 1996). DDT was also used for many non-agricultural applications such as in medicine. “Green revolution” witnessed intensive use of pesticides in agriculture wherein use of pesticides was an integral part (Zacharia and Tano 2011). A further illustration to the problem of pests in the world is shown as Fig. 11.3 (Hellar 2002).

Pesticide use can be checked and reduced substantially as pest control research focuses upon pest ecology and on the agro-ecosystem as a whole in different regions of the world. The integrated management of pest and diseases emerged as a better option than the pesticide alone. The need based application and new generation pesticides are also likely to reduce the load of pesticide from food and feed as well. Plants do contain many chemicals and mycotoxins however their role in influencing the incidence of certain types of human cancer and the exact proportion of cancers caused due to “natural” versus synthetic carcinogens is yet uncertain (Perera et al. 1991; Wild and Gong 2010).

Table 11.2 List of pesticide groups based on their potential use and purposes

S. No	Group of pesticide	Purposes
1.	Algaecides	Kill and prevent growth of algae (e.g. swimming pools)
2.	Antimicrobes	Kill the microorganisms that produce disease
3.	Attractants	Attaract specific pests using natural insect chemicals called pheromones that confuse the mating behaviour of insects
4.	Avicides	Control pest birds
5.	Biopesticides	Naturally occurring substances with pesticidal properties
6.	Defoliants	Causes foliage to drop from a plants or insects usually for laboratory purposes. Promote drying of living tissues such as unvanted plant tops
7.	Fumigants	Produce vapours or gasses to control air or soil borne insects and disease
8.	Herbicides	Kill weeds and other plants that are growing or competing with a desired species
9.	Insect growth regulator (IGRs)	Accelerate or retard the growth of insects
10.	Insecticides	Control or aliminate insect that affect plants, animals or people
11.	Miticides (Acaricide)	Kill mites that live on plants, livestocks and people
12.	Molluscides	Kill snails and slugs
13.	Nematicides	Kill the nimatodes which are microscopic warm like organisms that live in the soil and causes damage to food crops
14.	Ovicides	Control insect eggs
15.	Piscicides	Control pest fish
16.	Plant growth regulator (PGPR)	Accelerate or retard the rate of growth of plant substances (excluding fertilizers or other plant nutrients) that alter the expected growth flowering or reproduction rate of plants
17.	Predacides	Control vertebrate pest
18.	Repellents	Repel pests such as mosquitoes, flies, tickes and flea
19.	Rodenticides	Kill mice, rats and other rodents

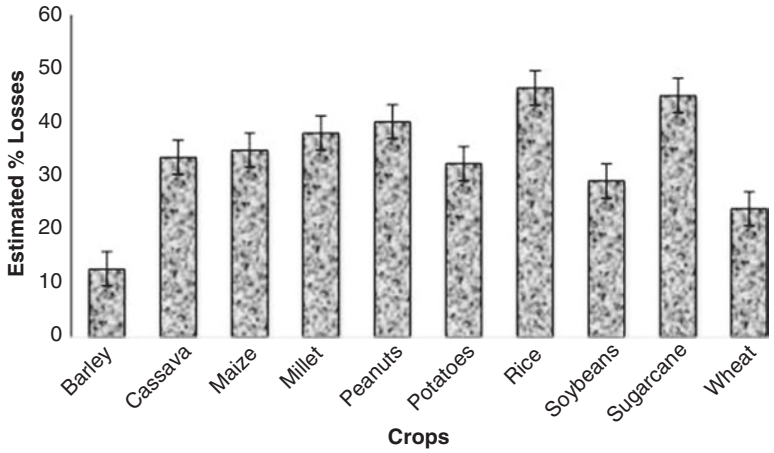

Fig. 11.3 Estimated % losses caused by pests in major crops per year

The causes of chronic illnesses, including cancers are extremely complex. Agro chemical contaminants can be present in foods mainly as residues of pesticides from environmental sources (water, air or soil pollution), due to their high biological activity and toxicity (acute and chronic), presence or contamination by natural toxins, or by cross-contamination or formation during food processing or by migration of toxic chemicals from food packaging materials or use of unapproved food additives and adulterants.

Approximately 80% of the pesticides produced annually in the world are used in developed countries (Pimentel et al. 2000; WRI/UNEP/UNDP 1994), but less than 50% of all pesticide are fatal in these countries (Pimentel and Greiner 1996). The importance of a nutritious food supply to human health has been emphasized in reports and Dietary guidelines for Americans (2005) which recommend a diet high in complete carbohydrates, i.e. fruits and vegetables particularly those vegetables high in carotene like- cabbage, broccoli and other brassicas that are likely to reduce the risk of cancer, diabetes, blood pressure and chronic diseases.

Due to more use of pesticides and their absorption in fruits and vegetables more contaminations in foodstuffs is noted. Maximum pesticides exposure globally is reported in people associated with farm work who apply pesticides or those residing near heavily treated agricultural farms. Since agriculturists and people engage in farming directly use pesticides ~70–80% and they are at highest risk of exposure (McDuffie 1994). Pattern of pesticide usage in India is 76% of insecticide as against 44% globally (Mathur 1999), herbicides and fungicides is correspondingly less (Aktar et al. 2009).

Pesticides can be toxic to humans over a wide range from acute, sub-chronic to chronic (Damalas and Eleftherohorinos 2011; Wikipedia 2015). World Health Organisation (WHO 2015) classified the hazardous level of pesticides into different classes based upon lethal doses ranging from LD50 class I to IV as former being extremely hazardous to latter (IV) representing negligible chances to cause acute hazard under normal use (International Agency for Research on Cancer 2015). A pesticide ability to cause bad health effects after long-term or repeated exposure to a sub-chronic toxicity (term from few weeks to few months) or chronic toxicity (term from few months to years). Pesticides which tend to accumulate or break down slowly in human organ usually are of the greatest chronic exposure hazard (Crop Protection Association 2007; Damalas and Eleftherohorinos 2011; Wikipedia 2015). As chemicals, pesticides are main source of air, water and soil pollution. They are also significant risk factors on human life with not only health effects as a result of misuse or accident, but also by introduction of long lasting harmful chemicals into the environment.

According to Dietary Guidelines for Indians by National Institute of Nutrition (NIN), it is recommended that the proper consumptions of daily amounts of pulses, cereals, fruits/oils, vegetables, milk and sugar must be there. However, the limiting pesticide residues in food products must be maintained if they are known for being and in the crops. International organization as Codex Alimentarius Commission have taken initiation to rely to independent scientific advice provided by expert bodies organized by FAO/WHO to harmonize chemical residues in food to be establish-

ing statutory limitations Maximum Residue Limits (MRLs) Acceptable Daily Intake (ADI) levels (FAO; Food and Agriculture Organization of the United Nations, 2003), No Observed Adverse Effect Levels (NOAEL), Acute reference dose (ARfD) etc. Overall the risk assessment is governed by three committees of FAO/WHO experts: the Joint FAO/WHO Expert Committee on Food Additives (JECFA); the Joint FAO/WHO Meeting on Pesticide Residues (JMPR); and the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA); and on other scientific advice provided by FAO/WHO. Up to 2016, the Codex Alimentarius Commission has adopted 4844 MRLs (CXLs) for different combinations of pesticide/commodity (ies). The numerical Codex standards for food additives, veterinary drugs maximum residue levels and pesticide maximum residue levels, can also be accessed via databases listed in Table 11.3 that facilitate their use (Bustamante et al.

Table 11.3 Updated list of Codex standards recommended by Codex Alimentarius Commission for the period 1976–2016 in chronological order

S. No	Titles	Committee	Year of modification	Code references
1.	Statement on infant feeding	CCNFSDU	1976	CAC/MISC 2-1976
2.	Classification of foods and animal feeds	CCPR	1993	CAC/MISC 4-1989
3.	Recommended methods of sampling for the determination of pesticide residues for compliance with MRLs	CCPR	1999	CAC/GL 33-1999
4.	Guidelines on the use of mass spectrometry (MS) for identification, confirmation and quantative determination of residues	CCPR	2005	CAC/GL 56-2005
5.	Working principles for risk analysis for food safety for application by governments	CCGP	2007	CAC/GL 62-2007
6.	Guidelines on good laboratory practice in pesticide residue analysis	CCPR	2010	CAC/GL 40-1993
7.	Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods	CCMAS	2010	CAC/GL 74-2010
8.	Guidelines for risk analysis of foodborne antimicrobial resistance	TFAMR	2011	CAC/GL 77-2011
9.	Principles and guidance on the selection of representative commodities for the extrapolation of maximum residue limits for pesticides to commodity groups	CCPR	2012	CAC/GL 84-2012
10.	Principles and guidelines for the establishment and application of microbiological criteria related to foods	CCFH	2013	CAC/GL 21-1997

(continued)

Table 11.3 (continued)

S. No	Titles	Committee	Year of modification	Code references
11.	Guidelines on the application of risk assessment for feed	TFAF	2013	CAC/GL 80-2013
12.	Guideline for the validation of food safety control measures	CCFH	2013	CAC/GL 69-2008
13.	Principles and guidelines for the conduct of microbiological risk assessment	CCFH	2014	CAC/GL 30-1999
14.	Maximum residue limits (MRLs) and risk management recommendations (RMRs) for residues of veterinary drugs in foods	CCRVDF	2015	CAC/MRL 2
15.	Code of practice for the prevention and reduction of mycotoxin contamination in cereals	CCCF	2016	CAC/RCP 51-2003

CCFH Codex Committee on Food Hygiene, *CCGP* Codex Committee on General Principles, *CCMAS* Codex Committee on Methods of Analysis and Sampling, *CCPR* Codex Committee on Pesticide Residues, *TFAMR* Task Force on Antimicrobial Resistance, *TFAF* Trade Facilitation Agreement Facility, *CCCF* Codex Committee on Contaminants in Foods, *CCNFSDU* Codex Committee on Nutrition and Foods for Special Dietary Uses, *CCRVDF* Codex Committee on Residues of Veterinary Drugs in Foods

2017). WHO recommendations for predicting the dietary intake of pesticide residues. For example for every 100 g/day intake of leafy vegetables by an adult or 50 g/day by a child (1–3 years) the MRL (mg/kg) for Methyl Parathion and Monocrotophos present is 1 and 0.2 respectively, whereas the pesticidal residue intake (mg) is 0.1 and 0.02 respectively. Similarly different food commodities have varied MRLs and pesticide residue intake in adult and children.

Central Insecticides Board and Registration Committee (CIBRC) release the pesticides for agricultural uses. However, at second level, the State Agricultural Universities and Departments of agriculture make their own recommendations for different crop commodities for the pesticide has been cleared by CIBRCs. These recommendations are mostly in the form of good package of practices for crops significant to respective states. For the recommendation of pesticides it is mandatory to provide the crop pest combination data for any manufacturer or importer seeking registration. However, a number of pesticides recommended for different crops by almost all of agricultural universities and departments that were not registered by CIBRC for these crops. The 20 pesticides that have been considered are most used and widely recommended pesticides in India (Table 11.4).

Recommendations of the committee based on data taking into account the food habits, consumption of raw and processed food, contaminant and pesticide level and acceptable limits, use of pesticides in agriculture and other public health programs be evolved by institutions like Indian Council of Medical Research (ICMR) and National Institute of Nutrition and Central Food Technological Research Institute

Table 11.4 The 20 pesticides that have been considered are some of the most used and widely recommended pesticides in India

S.No	Name of pesticide	Number of crops registered (crop name)	Number of crops for which MRLs are set (crop name)
1.	Phorate	23 (bajra, barley, maize, paddy, sorghum, wheat, black gram, green gram, pigeon pea, soybean, sugarcane, cotton, groundnut, mustard, sesamum, brinjal, cauliflower, chilies, potato, tomato, apple, banana and citrus fruits)	23 (bajra, barley, maize, paddy, sorghum, wheat, black gram, green gram, pigeon pea, soybean, sugarcane, cotton, groundnut, mustard, sesamum, brinjal, cauliflower, chilies, potato, tomato, apple, banana and citrus fruits)
2.	Mancozeb	23 (potato, tomato, wheat, maize, paddy, jowar, chilies, onions, tapioca, ginger, sugarbeet, cauliflower, groundnut, grapes, guava, banana, apple, cumin, tobacco, mustard, black pepper, pearl millet and cucumber)	14 (potato, tomato, wheat, maize, paddy, jowar, chilies, tapioca, groundnut, grapes, guava, banana, apple, pearl millet)
3.	Methyl parathion	7 (Paddy, cotton, black gram, green gram, soybean, mustard, groundnut)	None
4.	Cypermethrin	8 (brinjal, cotton, cabbage okra, sugarcane, wheat sunflower, rice)	6 (brinjal, cotton, cabbage okra, wheat sunflower)
5.	Carbendazim	18 (paddy, wheat, barley, tapioca, cotton, jute, groundnut, sugarbeet, peas cluster, beans, cucurbits, brinjal, apples, grapes, walnut, rose, ber mango)	14 (paddy, wheat, barley, cotton, groundnut, sugarbeet, peas cluster, beans, cucurbits, brinjal, apples, grapes, ber mango)
6.	Monocrotophos	14 (paddy, maize, bengal gram, green gram, pea, red gram, sugarcane, cotton, castor, mustard, citrus fruits, mango, coffee, cardamom)	11 (paddy, maize, bengal gram, green gram, pea, red gram, cotton, citrus fruits, mango, coffee, cardamom)
7.	Malathion	16 (paddy, sorghum, soybean, cotton, castor, groundnut, mustard, sunflower, okra, cauliflower, radish, turnip, tomato, apple, grape, mango)	11 (paddy, sorghum, groundnut, mustard, okra, cauliflower, radish, turnip, tomato, apple, grape, mango)
8.	Quinalphos	32 (chilies, paddy, sugarcane, sorghum, okra, cotton, brinjal, tomato, tea, tur, groundnut, wheat, bengal gram, black gram, red gram, French bean, soybean, jute, mustard, sesamum, cabbage, cauliflower, onion, apple, banana, citrus fruits, mango, pomegranate, cardamom, coffee, gram, safflower)	4 (chilies, rice, tea, cardamom)
9.	Acephate	3 (cotton, safflower, rice)	2 (cotton, safflower)

(continued)

Table 11.4 (continued)

S.No	Name of pesticide	Number of crops registered (crop name)	Number of crops for which MRLs are set (crop name)
10.	Triazophos	4 (cotton, rice, soybean, brinjal)	3 (cotton, rice, soybean)
11.	Dichlorvos	10 (paddy, wheat, soybean, sugarcane, castor, groundnut, mustard, sunflower, cucurbits, cashew)	3 (paddy, wheat, cucurbits)
12.	Fenvalerate	4 (cotton, cauliflower, brinjal, okra)	4 (cotton, cauliflower, brinjal, okra)
13.	2,4 – D	8 (paddy, maize, wheat, sorghum, potato, sugarcane, citrus fruits, grapes)	7 (paddy, maize, wheat, sorghum, potato, citrus fruits, grapes)
14.	Dimethoate	24 (bajra, maize, sorghum, red gram, cotton, castor, groundnut, mustard, safflower, bhindi, brinjal, cabbage, cauliflower, chilies, onion, potato, tomato, apple, apricot, banana, citrus fruits, fig, mango, rose)	14 (bhindi, brinjal, cabbage, cauliflower, chilies, onion, potato, tomato, apple, apricot, banana, citrus fruits, fig, mango)
15.	Captan	14 (paddy, maize, bengal gram, green gram, pea, red gram, sugarcane, cotton, castor, mustard, citrus fruits, mango, coffee, cardamom)	11 (paddy, maize, bengal gram, green gram, pea, red gram, cotton, citrus fruits, mango, coffee, cardamom)
16.	Zineb	18 (jowar, paddy, wheat, ragi, tobacco, onion, potato, tomato, chilies, brinjal, cucurbits, cauliflower, cumin, apple, citrus fruits, cherries, grapes, guava)	13 (jowar, paddy, wheat, ragi, potato, tomato, chilies, cucurbits, apple, citrus fruits, cherries, grapes, guava)
17.	Paraquat dichloride	10 (tea, cotton, potato, rubber, rice, wheat, maize, grapes, apple, Aquaticweeds)	7 (potato, cotton, rice, wheat, maize, grapes, apple)
18.	Chlorpyrifos	13 (rice, beans, gram, sugarcane, cotton, groundnut, mustard, brinjal, cabbage, onion, apple, ber, citrus fruits)	10 (rice, beans, gram, cotton, brinjal, cabbage, onion, apple, ber, citrus fruits)
19.	Phosaloe	13 (barley, paddy, sorghum, cotton, jute, groundnut, bhindi, brinjal, cabbage, chilies, tomato, tea, mustard)	5 (bhindi, brinjal, cabbage, chilies and tomato)
20.	Carbofuran	27 (barley, bajra, sorghum, jute, groundnut, French bean, potato, tomato, apple, citrus fruits, maize, paddy, mustard, soybean, sugarcane, bhindi, chilies, cabbage, wheat, brinjal, banana, peach, mandarins, cotton, pea, tea, sweet pepper)	22 (barley, bajra, sorghum, groundnut, French bean, potato, tomato, apple, citrus fruits, maize, paddy, mustard, soybean, sugarcane, bhindi, chilies, cabbage, wheat, brinjal, banana, peach, mandarins, pea)

(CFTRI). Pesticides are registered and recommended for crops by the Central Insecticides Board and Registration Committee (CIBRC) in India for various crops. However, the responsibility of setting MRLs for pesticides rests with Food Safety and Standard Authority of India (FSSAI). There are top 20 pesticides that have been most used and widely recommended pesticides in India. The main criticism for setting MRLs are according to the Good Agricultural Practices and are checked for compliance for safety limits for intake of pesticides. For a particular diet the Theoretical Maximum Daily Intake (TMDI) is estimated maximum pesticide intake from all sources for a person and is calculated from the MRLs of a pesticide in different food products and the average daily intake of those products. The MRLs prescribed by the FSSAI in the Food Safety and Standards Regulations, 2011 are used for the calculation (Table 11.5).

Many fungal pathogens produce melanin to protect themselves from the various environmental stress and fungicide applications (Kumar et al. 2016). Melanins are resilient bio-pigments resulting from oxidative polymerization of indolic or phenolic precursors. The protection by melanin depends on its subcellular location, thus subcellular compartmentalization of the melanin biosynthetic machinery occurs in cells. Fungal melanin biosynthesis appears to be initiated in endosomes with exocytosis leading to melanin extracellular deposition. The melanin biosynthesis pathway is composed of the initial step to fuse five isoprenyl units, two sets of alternating reduction and dehydration steps, and the final step to polymerize 1,8-di-hydroxynaphtalene into melanin (Bell and Wheeler 1986; Chand et al. 2014). To date, two types of melanin biosynthesis inhibitors (MBI) or chemical fungicides have been developed and used in the field: Tricyclazole (Tokousbalides and Sisler 1979), pyroquilon (Schwinn et al. 1979) and phthalide inhibit the enzymes in the reduction step (MBI-Reductase MBI-R) (Nambu 1972) and carpropamid diclocymet and fenoxanil (MBI-Dehydratase, MBI-D) interfere with the enzymes in the dehydration step (Yamada et al. 2004). Among them, tricyclazole and pthalide lead to inhibition of NADPH-dependent reduction of 1,3,6,8-tetrahydroxynaphthaline preventing formation of scytolone and vermelone (Wheeler 1982). However, Carpropamid inhibits the scytalone dehydratase, an essential enzyme for synthesis of melanin precursors 1, 3, 8-trihydroxynaphthaline and 1, 8-dihydroxynaphthaline.

Melanin also acts as pathogenicity factor and is involved in the penetration of host cell wall by parasitic fungi. In such cases fungal melanin is positively correlated to virulence of fungal pathogens (Chand et al. 2014). Even though it is not directly involved in pathogenesis, likely it is required for survival in harsh environment and lytic enzymes (Scharf et al. 2014). However, they defines response of plants and animals are also enhanced by occurrence of melanin against fungal infection (Nosanchuk and Casadevall 2003). A specialized cellular apparatus causes the penetration of host epidermis by few plant-pathogenic fungi known as appresorium, which develops at the tip of germ tubes and adjacent to host epidermis. The appresorium turns dark brown as they mature by accumulating melanin at the inner layer of the appresorial cell wall (Jacobson 2000).

Table 11.5 An update on the consumption of 20 most widely used pesticides in the world

Name of pesticide	Consumption (MT)	(MRLs) set	ADI (mg/kg BW)	ADI (adult) (mg/day)	ADI (child) (mg/day)	(TMDI) (adult) (mg/day)	TMDI (child) (mg/day)	(RfD)	RfD (adult)	RfD (child)	JMPR ADI (per day per kg body weight)	EPA RfD (per day per kg body weight)
Phorate	3284	23	0.0007	0.042	0.00903	0.0564	0.0196	0.0005	0.03	0.00645	0–0.0007 mg/kg, 2004	0.0005 mg/kg, 1993
Mancozeb	3118	14	0.03	1.8	0.387	0.565	0.354	0.05	3	0.645	0.03 mg/kg, 1993	0.05 mg/kg, 2005
Methyl Parathion	2739.32	0	0.003	0.18	0.0387	0.52	0.17	0.00025	0.015	0.003225	0.003 mg/kg, 1995	0.00025 mg/kg, 1991
Cypermethrin	2473	6	0.02	1.2	0.258	0.0885	0.044	0.01	0.6	0.129	0–0.02 mg/kg, JECFA 2002	0.01 mg/kg, 1990
Carbendazim	1992	14	0.03	1.8	0.387	0.71	0.355	0.025	1.5	0.3225	0.03 mg/kg, 1995	0.025 mg/kg, 2005
Monocrotophos	1815	11	0.0006	0.036	0.00774	0.17	0.122		0	0	0.0006 mg/kg, 1996	
Malathion	1739.39	11	0.3	18	3.87	3.756	1.266	0.02	1.2	0.258	0–0.3 mg/kg, 1997	0.02 mg/kg, 1992
Quinalphos	1595	4		0	0	0.00176	0.00028	0.0005	0.03	0.00645		0.0005 mg/kg body weight
Acephate	1513	2	0.03	1.8	0.387	0.025	0.025	0.003	0.18	0.0387	0–0.03 mg/kg, 2011	0.003 mg/kg, 1993
Triazophos		3	0.001	0.06	0.0129	0.0096	0.002		0	0	0–0.001 mg/kg (1993; confirmed 2002)	

Dichlorvos	960	3	0.004	0.24	0.0516	0.535	0.1225	0.0005	0.03	0.00645	0.004 mg/kg, 2011	0.0005 mg/kg, 1993
Fenvalerate	776	4	0.02	1.2	0.258	0.15	0.0384	0.025	1.5	0.3225	0.02 mg/kg −1986	0.025 mg/kg, 1992
2,4 – D	662	7	0.01	0.6	0.129	0.243	0.232	0.01	0.6	0.129	0.01 mg/kg for sum of 2,4-D and its salts esters expressed as 2,4-D, 1996	0.01 mg/kg, 1988
Dimethoate	636	14	0.002	0.12	0.0258	1.2	0.5	0.0002	0.012	0.00258	0.002 mg/kg, 1996	0.0002 mg/kg, 1990
Captan	471	11	0.1	6	1.29	9	3.75	0.13	7.8	1.677	0–0.1 mg/kg 1984; confirmed 1990, 1995	0.13 mg/kg, 1989
Zineb	462	13	0.03	1.8	0.387	0.565	0.354	0.05	3	0.645	0.03 mg/kg, 1993	0.05 mg/kg, 1988
Paraquat dichloride	NA	7	0.006	0.36	0.0774	0.0924	0.0258	0.0045	0.27	0.05805	0.006 mg/kg, 1986	0.0045 mg/kg,1991
Chlorpyrifos	NA	10	0.01	0.6	0.129	0.13	0.07		0	0	0.01 mg/kg, 1999	
Phosalone	NA	5	0.02	1.2	0.258	0.864	0.59		0	0	0.02 mg/kg, 1997	
Carbofuran	NA	22	0.001	0.06	0.0129	0.122	0.03	0.005	0.3	0.0645	0.001 mg/kg, 1996	0.005 mg/kg, 1987

MT Metric ton, *MRL* Maximum residue limits, *ADI* Acceptable Daily Intake, *TMDI* Theoretical Maximum Daily Intakes, *RfD* Reference doses

11.1.3 Natural Toxins in Foods

The chemicals like- carbohydrates, amino acids, fats, oils and vitamins are found in a complex mixtures in all foodstuffs, and if consumed in large quantities it may be toxic (Strong 1974). Plants contain some toxic chemicals and that protect them from insects, pathogens and other soil and plant organisms (Pimentel 1988). For example, the chemical hydrazine present in some mushrooms are carcinogenic. Toxic chemicals interfere with metabolic processes, nutrient availability, detoxification mechanisms and allergic reactions in animals and humans causing adverse effects. Lectin proteins (phytohemagglutinins) are present in varying amounts in legumes as chickpea and vetch the lathyrogens (derivatives of amino acids) act as metabolic antagonists of a neurotransmitter in the brain, the glutamic acid (NAS 1973).

The protease inhibitors that inhibit the digestive enzymes trypsin are ubiquitously present throughout the plant kingdom and in the Leguminosae in particular. (Bender 1987). A type of proteins found in raw soybean that inactivates to the trypsin resulted in enlargement of pancreas and an increase in its secretory activity. Potatoes which contain two major glycol alkaloid fractions, alpha-solanine and alpha-chaconine that exhibit a significant increased alkaloid content when exposed to sunlight (NAS 1973). Cyanogenic glycosides of plants like cassava, lima beans and the seeds of some fruit species when ingested may create problems due to their cyanide content (Strong 1974). Cassava toxicity is much reduced by peeling, washing in running water to remove the cyanogen. Goitrogens (glucosinolates) inhibit the uptake of iodine by thyroid, and are present in commonly consumed plants like- Cabbage, cauliflower, broccoli, kohlrabi, turnips, radish, mustard and oil seed meals from rape and turnip (Coon 1975; Liener 1986; Heaney and Fenwick 1987).

11.1.4 Modern Analysis of Contaminants in Food

The determination of mycotoxin occurrence in foodstuffs can be frequently underlined to the importance of multi-mycotoxins by a number of analytes. The evolution of methods for mycotoxin determination from HPLC-UV to state of the art LC-MS/MS was reported by Berthiller et al. (2007). A number of efforts has been applied to prevent mycotoxin formation, however contaminations of those secondary fungal metabolites still occurs (Cole et al. 2003). Therefore, the mycotoxin as well as strategies like- adsorption, biotransformation, biodegradation and bioprotection for the addition of mycotoxin deactivating products should be considered. To obtain a more detailed picture, the development and application of multi-mycotoxin LC-MS/MS methods is encouraged in order to get a more accurate picture of the multi-mycotoxin contamination (Streit et al. 2012).

Essentially, the term refers to conjugates of mycotoxins from fungus itself (3-AcDON, 15-AcDON) or formed during defence reactions in plant subjected to infection (DON-3-Glucoside, ZEN-4-Glucoside) (Vendl et al. 2009). Apart from

exerting toxic effects few of these conjugates may be converted into parent toxin during digestive process, further adding to the feed toxicity (Garais et al. 1990; Vendl et al. 2009). Consequently, pesticides can be present in food via contamination caused during applications, storage or transportation or from environmental sources (water or soil) (http://www.foodsafetymagazine.com/magazine-archive1/februarymarch-2013/modern-analysis-of-chemical-contaminants-in-food). Similar to pesticides, veterinary drugs as agrochemicals also have to undergo a mandatory registration process, followed by setting of their maximum residue limits/tolerances in animal-derived foods.

The technique of mass spectrometry (MS) has revolutionized the analysis of chemical contaminants in food. The combination of MS with gas chromatography (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) has opened the door to the direct analysis of many more contaminants, including modern, new-generation pesticides and the majority of veterinary drugs and toxins such as mycotoxins. Many of the emerging and recently identified contaminants, including acrylamide, melamine or dyes are analysed preferably by LC-MS (Rahmani et al. 2009). In addition, tandem MS should be employed to help elucidate the structure of unknown contaminants.

The moisture content is an important consideration during sampling procedures, as it affects the extent of sample heterogeneity. It may be necessary to determine the moisture content through sample drying for expressing analytical results on a uniform scale. Drying should be rapid and at a low temperature as possible. In addition vacuum methods and freeze-drying or lyophilisation or microwave drying can be used. By using the proper sample preparation and methodology one can reduce analytical error and costly detection mistakes that could jeopardize the safety of the food produced as well as lead to an even more costly food safety-related recall.

11.2 Concluding Remarks

Mycotoxins plays a significant risk and food safety issue to the health and wellbeing of human and animals. Therefore, though, the mycotoxin concentrations may significantly reduce during food processing by physical treatment as cleaning and milling or by cooking, frying, baking, roasting and extrusion i.e. thermal processing (Bullerman and Bianchini 2007) yet it cannot be completely eliminated. Research efforts to delineate the multiple aspects of mycotoxin contamination in the food and feed supply chains, still have many questions which remain unanswered. Scientists and food technologists have achieved an understanding of the chemistry, production and biological effects of these natural feed contaminants however, with time, newer strategies and agronomic practices need to be developed involving plant breeding, transgenic research, toxin binding and deactivation of feed additives in addition the education about feed to reduce mycotoxin contamination and exposure is also required. This is significant for security of both food and feed at the global level. Further complications arise when many thousands of secondary fungal metabolites

that have not been tested for toxicity are to be considered. Developing regulations for the risk management of mycotoxins seek to balance the need to protect human health with economic concerns and in doing so a thorough detailed risk assessment process is essentially required.

It is concluded that mycotoxins constitute a significant problem for the animal feed industry and ongoing risk to the security of the feed supply. Thus, due to the increasing public pressure it is important to provide a more safe and eco-friendly way to control mycotoxin contamination. Use of microbial antagonists as bacteria, yeast and fungi are suitable to reduce the chemical use and help as biocontrol agents. Recent approach for protecting animals from harmful effects of mycotoxin contaminated feed is to use substances as mycotoxin binders to reduce the mycotoxin contamination. These binders when added to the diet reduce the absorption of mycotoxins from the gastrointestinal tract and their subsequent distribution to blood and target organs, thus preventing or reducing mycotoxicosis in livestock. Cytotoxicity and reactive oxygen species (ROS) generation are mechanisms of mycotoxins mediated toxicity that affects the genetic material and metabolic reactions leading to altered physiological functions of growth, development and reproduction. However, the direct consumption of mycotoxin contaminated foodstuffs are major problems of the human and animal feeds.

To overcome the problem of fungal pathogen and production of mycotoxins in food various types of pesticides are also being used. In food chain the residue of pesticides contamination in food stuffs are also sowing a problem as chemical contamination in foodstuffs. **Codex Alimentarius Commission initiation organized by FAO/WHO established the statutory limitations viz MRLs, ADI levels, ARfD, NOAEL etc.** The risk assessment governed by the Joint FAO/WHO Expert Committee on Food Additives, the Joint FAO/WHO Meeting on Pesticide Residues and the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment supported by the analytical techniques established for detection and quantification of mycotoxin and pesticide contamination in foodstuff. The numerical Codex standards for food additives, veterinary drugs maximum residue levels and pesticide maximum residue levels, can also be accessed *via* databases. To fully understand and overcome the problems of mycotoxin contamination in food and feed more detailed studies are required. This will help address food security in light of toxicity of mycotoxins and known and unknown pesticides being used in farming practices.

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Chapter 12
Microbes and Their Role in Drought Tolerance of Agricultural Food Crops

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12.1 Introduction

In 2016 around 11% of the total world population (7 billion) were suffering from undernourishment, 800 million people had deficiency of food and the number people facing crisis level food insecurity were about 108 million. It is about 35% increments in comparison to 80 million people back in 2015 (IFPRI 2017; FSIN 2017). The world population would increase almost by 30% over next 35 years and would reach to 9 billion by the year 2050 and 11 billion by 2100 and simultaneously the millions of people facing crisis level food insecurity will also increase drastically and will be a nightmare by then. If the situation of food insecurity will not considered by 2030 about 653 million people will be undernourished (Fita et al. 2015; FAO 2017). Under such draconian conditions, natural hazards and swinging weather will posse additional burden on the growing food demand and substantially imparts food insecurity in the countries that are adequate or inadequate to respond to shocks (FSIN 2017).

Among all natural disaster that occurs directly or indirectly due to global warming, drought is one of the most significant abiotic stress limiting biomass which consequently reduces crop yield and productivity globally (Singh and Laxmi 2015; Garcia et al. 2017). Drought occurs slowly and silently, without causing any prior short-term impact, which makes it harder for advance or timely detection or

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identification and preparation (Sena et al. 2017). Thus, it is high time for mankind to awake and come out of their "sleeping shell of ignorance" to face the most impelling global challenge of the present and the future; which is to be prepared for the chronic impact of the food scarcity. According to Food and Agricultural Organization (FAO) the demand and need for food will dramatically increase by 2050 or the further years to come. To combat this challenge the sustainable agricultural developments act as shielding agent against these natural calamities such as drought that have the capacity to hinders the agricultural productivity to an extensive limit (Landi et al. 2017). Moreover, this situation is briskly deteriorating further by the extensive use of non-degrading chemical fertilizers, overgrazing, overuse of land and unsustainable exploitation of natural resources (Ruggiero et al. 2017).

Mother Nature has its own way to deal with its problem. In order to cope up with stresses like drought plants have evolved with heterogeneous approaches such as stress escape, stress avoidance and stress tolerance (Sourour et al. 2017). These approach involves various aggregated intricate mechanisms, including modulation of transcriptional and expressible genes, epigenetic plasticity, metabolic reprogramming, and other such phenomenon that are being studied and evaluated constantly at the molecular level in the present (Miao et al. 2017). Secondly, at the cellular level the staggering synergetic relationship of endophytic microorganism and their contribution to the drought tolerance capacity of the agricultural crop plants have shifted the scientific understanding of benefaction microbes and host interaction to the next possible level (Govindasamy et al. 2017).

Such interactions are most commonly seen between host plants (*Phaseolus vulgaris* L., *Arabidopsis thaliana*, *Simmondsia chinensis*, *Alstonia boonei*, *Phaseolus vulgaris*, *Brassica campestris* etc.) and their endophytes mycorrhizal fungi (*Penicillium citrinum*, *Trichoderma atroviridae*, *Mucor* sp.) or rizosphere microbes (*Aeromonas caviae*, *Kosakonia radicincitans*, *Trichoderma polysporum*, *Oceanobacillus kimchi*) are widely being studied in the present decade for their efficacy and importance in rendering the host the ability to withstand drought directly or indirectly (Table 12.1) (Tolulope et al. 2015; Pierre et al. 2016; Witzel et al. 2017; Perez-Rosales et al. 2017; Zahoor et al. 2017). The ubiquitous roles orchestrated by the endophytes in regulating plant homeostasis under abiotic stress by supplying essential nutrients, minerals, sequestering of complex compounds, neutralizing reactive oxygen species (Vurukonda et al. 2016; Santoyo et al. 2016; Waqas et al. 2017). These intrigued attributions of plant homeostasis and tolerance to drought is achieved by some of the interrelated microbial mediated phenomenon such as bioremediation, bioaugmentation, bioaccumulation, biotransformation, phytostimulation and biofertilization (Conesa et al. 2012).

Bioremediation is a process commonly employed with an intension to upraise metal speciation which in turn reduces bioaccumulation (Ayangbenro Babalola 2017). To accelerating the process of bioremediation certain other endophytic microorganisms (hyperaccumulator-endophyte symbiotic system) are further added to the soil where host plant is cultivated (Ma et al. 2016). The cultivated land sometimes is contaminated with chains of hydrocarbons which through chemical modifications by living microorganisms are transformed into simpler or neutralized form

in a process called biotransformation (Anitha et al. 2013; Perez-Rosales et al. 2017). Efficient remediation will be hard to achieve without systematic excretion or extraction in to atmosphere by the process called biovolatilization (Limmer and Burken 2016). In most of the cases microbes promote plant growth through the up/down-regulation of specific phytohormones directly or indirectly by phytostimulation (Hussain et al. 2013). Biofertilazation on the other hand is process were microbes effectively enhances the nutrients and mineral uptake of the plants by means of establishing a synergetic relationship with the plant and simultaneously assist in the

Table 12.1 Microbial associated function in the plants

Host plant	Associated/endophyte	Function	References
Alium macrostemon	*Enterobacter* sp.	Enhanced pyrenes tolerance	Sheng et al. (2008)
Lolium multiflorum	*Psudomonas* sp.	Enhanced diesel tolerance	Andria et al. (2009)
Arabidopsis	*Piriformospora indica*	Drought tolerance	Sherameti et al. (2008)
Triticum aestivum	*Azospirillum brasilense*, *A. halopreaferanse*, *A. irakense*, *A. lipoferum*	Drought tolerance	Arzanesh et al. (2009), Gałązka et al. (2012)
Helianthus annuus	*Micrococcus* MU1, *Klebsiella* BAM1	Bioaugmentation, cadmium-tolerance and plant growth promotion	Prapagdee et al. (2013)
Poplar	*Psudomonas putida*	Tolerance and degredation of trichloroethylene in conjugation with nickel	Weyens et al. (2015)
Lycopersicon esculentum	*Azospirillum brasilense*	Stimulates the root hair development	Mangmang et al. (2015)
Achillea millefolium, *Dactylis glomerata*, *Solidag ocanadensis* and *Trifolium aureum*	*Microbacterium foliorum*, *Plantibacter flavus*	Petroleum tolerance	Lumactud et al. (2016)
Populus deltoids	*Rhodotorula graminis*, *Burkholderia vietnamiensis*, *Rhizobium tropici*, *Acinetobacter calcoaceticus*, *Sphingomonas yanoikuyae*	Plant growth promotion, tolerance and reduction of oxidative damages	Khan et al. (2016)
Sedum plumbizincicola	*Achromobacter piechaudii*	Bioaugmentation, hyperaccumulation growth promotion and translocation under stress	Ma et al. (2016)

(continued)

Table 12.1 (continued)

Host plant	Associated/endophyte	Function	References
Agrostis, *Ammophilia*, *Bromus*, *Descampsia*, *Festuca*, *Hordeum*, *Lolium*, *Phalaris*	*Agreia* sp., *Achromobacter* sp., *Actinomycetales bacterium*, *Aeromicrobium* sp., α *proteobacterium* sp., *Alter erythrobacter*, *Agrobacterium* sp., *Bacillus* sp., *Betaproteobacteria bacterium*, *Bordetella* sp., *Brachybacterium tyrofermentans*, *Brevundimonas* sp., *Caryophanon* sp., *Chryseobacterium* sp., *Clavibacter michiganensis*, Curtobacterium sp., *Enterobacteriaceae bacterium*, *Exiguobacterium* sp., *Flavobacterium* sp., *Frigoribacterium faeni*, *Luteimonas aestuarii*, *Lysobacter* sp., *Kocuria* sp., *Microbacterium* sp., *Oerskovia turbata*, *Pantoea* sp., *Plantibacter* sp., *Pseudomonas* sp., *Ralstonia* sp., *Rhizobium* sp., *Rhodococcus* sp., *Roseomonas* sp., *Sphingomonas* sp., *Stenotrophomonas* sp., *Uncultured bacterium*, *Xanthomonas* sp.	Tolerance to abiotic stress, increased growth, yield and biomass	Dombrowski et al. (2017)
Oryza sativa	*Piriformospora indica*	Enhance plant's tolerance to abiotic stress and bioaccumulation	Mohd et al. (2017)
Citrullus lanatus	*Paenibacillus polymyxa*	Plant growth promotion	Yaoyao et al. (2017)
Glycine max	*Bacillus aryabhattai*	Enhanced tolerance towards oxidative, nitrosative stress and promotes plant growth	Park et al. (2017)

maintenance of dynamic nature of the soil (Roychowdhury et al. 2017). All of these processes contribute to the plants ability to tolerate abiotic stress such as drought. Presently, these processes are further improved by certain sophisticated molecular tools and technologies, the depth of which is presented in this chapter along with conventional processes.

12.2 Abiotic Stress

Abiotic stress is a type of environmental stress which causes immense depletion to the growth, development, production and seed quality of plants. It includes extreme levels of light (low or high), radiation, temperature, water (drought or flooding), chemical factors (heavy metals or pH), salinity due to excessive Na^+, deficient or in excess of essential nutrients, gaseous pollutants (ozone, sulphur oxide) and among these drought and heat are frequent one (Suzuki et al. 2014). According to estimation, more than 50% loss of crop yield results because of the abiotic stresses. Multigene responses are known to be excited which results in the changes in various proteins and primary and secondary metabolite accumulation. Four major multisubunit protein complexes, photosystem I, PSII, the ATP synthase complex and cytochrome b6 or cytochrome f complexes that control the photosynthesis process in plants are largely affected by such stress. Under stress, the different plant parts and tissues are influenced and their intensity of impact depends upon the level of stress.

Salinity hugely affects the plants and osmotic stress, ion toxicity (which triggers conformational changes in proteins) and nutrient deficiency such as decreased uptake of phosphorus, iron, nitrogen, calcium, potassium and zinc are the out-turns of saline stress (Shrivastava et al. 2015). Saline refers to the excess amount of Na^+ and Cl^- ions and extreme accumulation of these ions in cell walls can ultimately lead to cell death. It has been analyzed that tomato plant height was reduced to 15.8% under high salinity condition, which denotes that plant is restricted to undergo photosynthesis through which it produces energy for growth (Han and Wang 2016). It is estimated that the seed germination is completely inhibited by the salinity levels greater than 20 g sea salt/L (Houle et al. 2001).

Under radiation stress various abnormalities can be seen in the plants as the genetic material (DNA) is immensely affected. In this case, chromosomal aberrations and deletion of a section of DNA sequence occurs which results in the reduction in viability of offspring (Cavinato et al. 2017).

Nutrients are so crucial for plants that they have evolved a number of signalling cascades for the uptake of the elements. There are 16 such mineral nutrients and predominantly nitrogen, phosphorus, potassium, calcium, magnesium, sulphur, boron, chlorine, iron, manganese and zinc are the vital ones. These elements are desired to carry out several plant processes particularly in photosynthesis, cell division, protein synthesis, disease resistance and for the primary and secondary growth of the plants. All the important physiological functions are disrupted in the deficiency or the excess of such requisite elements, for example, Fe deficiency leads to leaf chlorosis which results in not only inefficient photosynthesis and electron transport but also disregulation of the function of certain enzymes such as Rubisco, phosphoribulokinase, FBPase etc. whereas its increased amount causes necrotic spots in leaves (Shi et al. 2017). Another such crucial environmental condition is temperature, which is also an indispensible requirement of plant growth and development but elevated level of temperature could also be the main hindrance to plant productivity. It is predicted that over the next 30–50 years the change in the temperature

will be drastic (Intergovernmental Panel Climate Change 2007). Disrupted pollination process followed by infertility of flowers resulting in limitation in the ability to produce fruits is of the ultimate impacts on plant physiology under such stress (Hatfield and Prueger 2015; Singh and Laxmi 2015). The loss in plant productivity can be caused due to a reduction in the conductance of stomata which in turn occurs due to extreme heat stress (Irmak 2016).

It is known fact that water is mandatory for plants and that is why drought is one of the major restraint which limit the vegetation production worldwide (Nelson and Melvin 2017). Increased inundation or inadequate rain fall for a prolonged period of time severely affects the plants and decreases soil moisture. When the rate of transpiration exceeds the rate of water absorption from the soil, drought stress appears which interfere with the normal plant processes. Drought impairs the rate of photosynthesis that is limited due to membrane damage, stomatal closure and distressed activity of various enzymes (Sousa et al. 2017). Furthermore reduction in the rate of cell division and morphological or cellular expansion in the plants are regarded as the primary affect of drought. Water deficit leads to the biosynthesis of abscisic acid (ABA), which restricts the transpiration through stomata.

12.3 Morphological, Cellular and Physiological Responses of Drought on Plant

As previously known drought stresses are considered as one of the most hazardous abiotic stress apart from salinity, which bestows a series negative impact on plant growth, development, associated physiochemical processes and molecular responses (Muthamilarasan and Prasad 2016). These impacts could be best understood when viewed at the morphological, cellular and physiochemical level.

12.3.1 Morphological Responses

Plant growth and development is severely affected by drought at the juvenile stage, which begins from seed germination: A stage highly sensitive to water deficit. Vigour and coleoptiles are also significant aside from germination of seed for the proper establishment of plant. In the vegetative phase under water deficient condition the visible morphological aberrations expressed by the plant includes leaf wilting, decreased plant height, area of leaves, number and impediment in the formation of bud and flowers (Sourour et al. 2017). The main photosynthetic organ leaves is primarily affected and is expressed through the reduction in its size extension which is an adaption in response to drought stress. Fundamentally in this manner plant could attain a balance between the water retention capacity of the plant tissue and the extent of root to absorption from soil and moreover to avoid hydratation (Comas

et al. 2013; Sourour et al. 2017). Though roots are sometime capable of tolerating drought but still then water deficiency to an extended period of time affect shoot length and root growth. In return most of the plant response to drought includes sub-root architecture or anatomical alterations, adjustment of root biomass, and physiological acclimations (Brunner et al. 2015).

12.3.2 Cellular Responses

Adaptations at cellular level have a direct and tremendous influence on plant growth, tolerance or survivable and biomass production. Most of these adaptations could be briefly understood if viewed at tissue level though it is a multiple cellular function that together gives rise to or contribute to a crucial tissue function. One of such examples includes closing of stomata which is a direct result of some subtle biochemical and molecular changes in the guard cell on the leaf (Kar 2011). These cellular responses are governed by diversified signaling networks, which overall provides or results in cellular responses. The CO_2 absorption influenced by environmental stress (drought) also affects the activity of stomata. In a defensive response to such stress ion- and water-transport systems intervening the membranes acts as modulator of turgor pressure in guard cells resulting in closing of the stomata (Osakabe et al. 2014). This response characterized by endogenous ABA production which further triggers a cascade of such physiological responses that in turn regulated by a set of single transduction pathways. It is found that in ABA biosynthesis 9-*cis*-epoxycarotenoid dioxygenase 3 catalyzes significant step and its expression could induce drought tolerance in plants like *Nicotiana* and *Arabidopsis* (Pedrosa et al. 2017). Similarly there are many other mechanistic approaches where the stem hydraulic capacitance in improved in addition to cellular and molecular responses in *Quercus ilex* for the betterment of plant growth and development under abiotic stress like drought (Salomon et al. 2017).

12.3.3 Physiological Responses

The most fundamental physiological process in plant system is photosynthesis which is also affected by drought to a greater extent due to reduction in CO_2 assimilation rate, malfunctioning of primary photosynthetic reaction and disruption of pigments. On the other hand, drought also primarily induces, the generation of reactive oxygen species (ROS) thus stimulating concatenation antioxidative defense mechanisms, affecting many important metabolic process and gene expression (Rao and Chaitanya 2016). In addition the physiological responses manifested within the cell are closely connected to alteration of whole plant transcriptome and metabolome ultimately leading to major modification in chemical composition of the plant. These alterations also include accumulation of ubiquitous chemicals such as sugars,

salt ions and certain specific osmotica such as betaine, glycinebetaine, and quercitol constituents which also temporarily enhances plants tolerance to drought stress (Niinemets 2016). Presently studies are being conducted regarding the impact of drought on different plant species. One of such study was conducted on evaluating the effect of water deficit on physiochemical responses of *Pachira aquatica* and *Sterculia foetida* showed that drought severely affects pigmentation, represented by strict decrease in gas exchange and chlorophyll fluorescence (Frosi et al. 2017).

12.4 Microbial Mediated Drought Tolerance

These devastating impacts soothing responses of agronomically important crop plants could be further improved to a greater extent naturally by microbial mediated phenomenon or processes such as bioremediation, bioaugmentation, biotransformation, phytostimulation and biofertilization (Conesa et al. 2012). The microbial communities mostly contributing these processes are rhizobium and endophytic bacteria or funguses.

12.4.1 Bioremediation

Billions of microorganisms symbiotically associated with animal and plant species and support the growth and survivability. One of such intrigue interaction is bioremediation, a process where microorganisms are used to sequester xenobiotics pollutants into simpler subunits for their bio-absorption (Mushtaq et al. 2017). Bioremediation bacteria with drought-resistance activity such as *Rhodococcus erythropolis*, *Acinetobacte johnsonii*, *Micrococcus luteus*, *Methylobacterium extorquens* were mostly evaluated in the past (Weekers et al. 1998). Presently the research is more focused towards development of different bioremediation strategies for improvement in plant tolerance to drought stress (Reuben et al. 2013). The microorganisms widely explored for this purpose endophytes or more specifically plant growth promoting bacterial endophytes (PGPRE) a group of endophytes that plays a significant role in promotion and maintenance of plant health, growth and biomass (Mushtaq et al. 2017). One of such species is *Azospirillum*, an efficient bioremediating rhizosphere also favors drought tolerance in host plant. A study conducted in this regard proves that *Azospirillum* sp. effectively supports plant growth, production and survivability under different physiological and ecological condition such as drought. Here *Triticum aestivum* L. seedlings were planted in vitro under different drought intensities. Further 25 different *Azospirillum* sp. such as *A. brasilense*, *A. halopreaferanse*, *A. irakense*, and *A. lipoferum* were also incubated along the seedlings (Arzanesh et al. 2009; Gałązka et al. 2012). In a different study a total of ten endophytes were selected on the basis of previous evaluations for determining the impact of stress on *Populus deltoids*. It was found that inoculated endophytes

Rhodotorula graminis, *Burkholderia vietnamiensis*, *Rhizobium tropici*, *Acinetobacter calcoaceticus*, *Sphingomonas yanoikuyae* etc. could able to promote plant growth, tolerance, reduction of oxidative damages and production of significant phytohormones (Khan et al. 2016). Apart from *Azospirillum* sp. there are many other PGPRE that promote plant growth and tolerance, namely *Acinetobacter* sp., *Arthrobacter* sp., *Alcaligenes* sp., *Azotobacter* sp., *Azomonas* sp., *Bacillus megaterium*, *Beijerinckia* sp., *Burkholderia* sp., *Enterobacter sakazakiia*, *Erwinia* sp., *Flavobacterium* sp., *Gluconacetobacter diazotrophicus*, *Herbaspirillium seropedicae*, *Klebsiella* sp., *Methylobacterium mesophilicuma*, *Paenibacillus odorifer*, *Pseudomonas synxantha*, *Rhizobium leguminosarum* and *Serratia* sp. (Mushtaq et al. 2017).

12.4.2 Bioaugmentation

A typical bioaugmentation product actually comprises of a mixed multiple strains of microorganisms, most commonly bacteria or fungi isolated from nature without any genetic alteration within them. The selection of these microbes depends on their vigorous rate of reproduction or the ability to degrade certain bioactive compounds etc. (Shah 2017). Bioaugmentation could also be in between microorganisms and plants where the microbe in association with plant is capable of directly degrading contaminants at the site. Some such example could be seen in between *Pseudomonas putida* PD1 and *Salix alba*, *Burkholderia* sp. HU001; *Pseudomonas* sp. HU002 and *Salix viminalis* cv Tora, *Burkholderia cepacia* G4, BU0072 and VM1330 and *Lupinus leteus* etc. (Redfern and Gunsch 2016). Bioaugmented endophytic species of *Micrococcus* MU1 and *Klebsiella* BAM1 are cadmium-tolerating plant growth promoting Rhizobacteria (PGPR) were successfully evaluated for their efficacy to induce the production of elevated level of indole-3-acetic acid (IAA) in *Helianthus annuus* towards the end of stationary phase. Almost all of the beneficiary roles of these endophytes like plant growth promotion by phytostimulation, tolerance to metal stress and root elongation could also be highly beneficial to the plant under abiotic stress like drought (Prapagdee et al. 2013). This asymptomatic or mutualistic relationship between microbes and the host plant is actually a beneficial tool in the hand of present researchers for exploration to the maximum possibility and to find the most convenient contribution and role in drought tolerance. The findings on which is limited in this sector.

12.4.3 Bioaccumulation

The process where absorption rate of a particular substance within the organism surpasses the rate of catabolism of that substance which in turn results in the accumulation, then the process can be regarded as bioaccumulation. (Gupta et al. 2017a, b).

This process is highly exploited by endophytic microbes for the improvement of phytoremediation. A bioaugmentated hyperaccumulating-endophytic bacterium identified as *Achromobacter piechaudii* homologous could efficiently improve phytoremediation in *Sedum plumbizincicola*. Moreover the endophyte could able to enhance plant growth and bioaccumulation/translocation under stress (Ma et al. 2016). Current research is concerned in evaluating these endophytes and their hosts. In the coast of Oregon 34 plants consisting of about 133 bacterial endophytes of which 94 were unique representing 36 different taxonomic groups. The common taxonomic groups include *Pseudomonads*, *Curtobacterium*, *Microbacterium*, *Bacillus*, and *Xanthomomas* species that enhanced tolerance to abiotic stress to plant and increased yield and biomass (Dombrowski et al. 2017). Furthermore there are many funguses that enhance plants tolerance to abiotic stress by the process of bioaccumulation. For example *Piriformospora indica* an endophytic fungus which hypre-colonizes on the root of *Oryza sativa* and amazingly immobilized and transformed arsenic salt in to insoluble particulate matter (Mohd et al. 2017). Similarly bioaccumulation could also be useful tool in rendering plants tolerance to drought. Bioaccumulation of ABA, proline, ajmalicine, internal tissue lipid, cuticular wax, epidermal wax, the common adaptation acquired by the plants under drought stress (Broun et al. 2004; Jaleel et al. 2008; Yu et al. 2017; Xue et al. 2017; Sheikh et al. 2017; Gupta et al. 2017a, b). Microorganisms could be further used to induce or promote the accumulation of the drought tolerating bioactive compounds but the research in this regard is scanty.

12.4.4 Biotransformation

Biotransformation is a generalized biochemical transformation of foreign compounds/contaminants without complete alteration of their stereochemistry or stability by living organisms. Plants efficiently perform this phenomenon where they neutralize xenobiotics. It is evidenced form the studies that this property could be further enhanced by the help of certain plant associated microbes. Presently such plant associated microbes or previously known endophytic bacteria, fungus, actinomycites and up to some extent algae portray their significant usability in naturally or artificially by bioaugmentated form to promote biotransformation (Redfern and Gunsch 2016; Shakoor et al. 2017). The dramatic change in climate and increase in global warming is a direct result of extensive use of automobiles and refrigerators which radiate greenhouse gasses. Moreover, this impact is accelerated by rapid industrialization, over exploitation of land by contaminating it with pesticides, explosives, agrochemicals, and other xenobiotics contribute immensely to the destabilization of the agricultural sustainability. The removal of these agrochemical pollutants would be a tedious job, therefore instead of removal, it is much convenient to transform or biotransform these pollutants into simple stable form.

There are many different bacterial and fungal species that promote plant growth and enhances plants tolerance to various abiotic stresses. Presently, studies are being conducted on evaluating these species that promote biotransform. In one of such studies, about 190 endophytic bacterial species most of them belonging to two taxonomic group namely *Microbacterium*, and *Plantibacter* in four different plants, *Achillea millefolium*, *Dactylis glomerata*, *Solidag ocanadensis* and *Trifolium aureum* growing well under petroleum soil contaminated were identified (Lumactud et al. 2016). *Porostereum spadiceum*, *Penicillium minioluteum*, *P. funiculosum*, *Metarhizium anisopliae*, *Beauveria bassiana*, *Mucor* sp. are some of such endophytic funguses that promote biotransformation and help plant to grow and survive (Greenfield et al. 2016; Zahoor et al. 2017; Hamayun et al. 2017). Despite the efficacy of these amalgamated co-evolutionary synergetic interaction between plants and microbial species is quite been known but still than the current research is focused towards the sophisticated techniques like proteomics and genetically modified organisms (GMOs) (Wang et al. 2016a, b, c).

12.4.5 Phytostimulation

The areas encircling the roots of a plant, which is more prone to release compounds which gradually enhances the microbial activity, is referred as phytostimulation. From promoting plant growth, triggering of phytohormones, till development of root hairs, rhizosphere supports microbial culture (Vacheron et al. 2013). This process involves a symbiotic co-existence, which undeniably benefits both the microbes as well the plant. From fixing of atmospheric nitrogen till production of phytohormones, everything is accompanied (Hussian et al. 2015). Rhizospheric bacteria or endophytic bacteria both of these not only secrete ethylene but also modify the levels of ethylene in plants, and this helps the plant to fight against conditions like drought (Gamalero and Glick 2015). Ethylene produced by *Azospirillum brasilense* stimulates the root hair development in *Lycopersicon esculentum* plants (Mangmang et al. 2015). Some PGPRs like *Arthrobacter brasilense, Bacillus licheniformis, Paenibacillus polymyxa* etc. produce cytokinins which promote the plant cell divison, root hairs proliferation, and root meristem differentiation. It was shown that endophyte stimulate the growth of plants by increasing the biomass accumulation of host plants (Rho et al. 2017). It has been hypothesized that aflatoxin produced by *Aspergillus* sp. may provide resistance to the plants under drought stress, reducing the level of ROS and oxylipins accumulated in the tissues of the plants (Yang et al. 2016). Microorganisms present in the rhizosphere helps in dealing with stress conditions when they are inoculated in the plant tissues and favour development and function (Souza et al. 2015).

12.4.6 Biofertilization

Drought adversely affects the environment which prohibits the normal plant growth and productivity and is manifested by reduced photosynthesis, respiration, iron uptake and nutrient metabolism. To facilitate the plant growth and productivity under such stress, biofertilizers can be used in the soil (Sahoo et al. 2014). Biofertilizers are nonpathogenic microbial inoculants consisting of living cells of micro- organism like bacteria, fungi, and algae. It intensifies the soil fertility by proffering all kinds of requisite nutrients via nitrogen fixation, solubilization or mineralization of phosphate and potassium, antibiotic production, biodegradation of organic matter and release of plant growth regulating substances in the soil (Sinha et al. 2014).

The beneficial microbes like *Azotobacter* sp., *Azospirillum* sp., *Rhizobium* sp., endo and ectomycorrhizal fungi, cyanobacteria are some of the PGPRs used as biofertilizers for the standard growth of the plants, uptake of nutrients and tolerance to various stresses (Aziz et al. 2012). Some of the common crop plants such as *L. esculentum*, *Capsicum annum*, *Triticum aestivum*, and *Z. Mays* could tolerate when augmented with these fungal and bacterial endophytes specifically *Achromobacter piechaudii*, *Proteus vulgaris*, *Azospirillum* sp. etc. It is estimated that during drought condition, the plant photosynthetic process is mostly affected and synthesis of abscisic acid increases while the use of these PGPRs in stressed plants, enhance the photosynthetic efficiency by increasing the IAA content in it. Furthermore *Bacillus subtilis* can furnish endurance of the stress by augmenting the photosynthetic activity and depleting the ABA and ACC (1-amino cyclopropane-1-carboxylic acid) contents (Bhardwaj et al. 2014). These physiological criterions are marked by elevated expression of TaCTR1 gene in the wheat seedlings treated with the biofertilizer (Barnawal et al. 2017). The efficacy of these microbial community consisting different strains of microbes specifically *Gammaproteobacteria*, *Bacilli* and *Actinobacteria* were studied successfully in imparting *C. annuum* resistance under drought (Marasco et al. 2012). Hence, it could be said that there are many of microorganisms which could be used as biofertilizers to enhance the plant productivity under stress.

12.5 Microbial Technologies for the Enhancement of Drought Tolerance

Microbial technologies involved in rendering tolerance to drought in crop plants could be briefly understood if ventured into the micro or molecular world. The world which could only be quantified and analyzed by the use of most advanced techniques and instrumentation of the present era such as genomics, proteomics, transcriptomics, metabolomics, and other heterogeneous human and machine interfacing tools of molecular biology and rDNA technology (Manavalan et al. 2009; Hocquette et al. 2009; Balammal and Reddy 2012; Komatsu and Hossain 2017). In

enhancing the efficacy of plant responses towards various stress as well as improving their performance by modulating and discovering diversified genetic variations genomics or more specifically plant genomics has played the central role (Bevan et al. 2017). The research focus of the present generation is towards developing and understanding the genetic basis of plant which could be achieved through certain advanced marker-assisted selection (MAS) like genomic selection (GS) (Shikha et al. 2017). Though these advanced technologies have revolutionized the current scientific community in its complex way of manipulation and assembly, but the origin of this idea was from a simple root nodulating bacteria. The present era advancement where on one hand superior breeds are selected by effective markers and genome is modified by the rDNA technology in highly sophisticated laboratories there on the other hand nature dose these all in its own simple way.

Naturally there are many endophytic microbes that contribute to plant growth and development by conferring the induction of up-regulation of certain essential genes in varity of plant such as *Gossypium hirsutum* L., *G. max* L., *Z. mays* L. etc. under abiotic stress like drought (Ngumbi and Kloepper 2016). *Pirifomospora indica* a mycorrhiza-like endophytic Agricomycetes fungus is an example of such organism which colonizes in the roots of plants including *A. thaliana* and stimulates the expression of many genes related to drought stress in leaves by manipulating phytohormonal signal transduction pathway (Sherameti et al. 2008; Gill et al. 2016). *Epichloe* an interspecific hybrid endophytic genus when inoculated in the woodland grass *Hordelymus europaeus*, it was found that the biomass and the tiller production was increased by 10–15% in both the plants under drought stress and the plant which are not (Oberhofer et al. 2014). Another synergetic nitrogen-fixing bacteria *Gluconacetobacter diazotrophicus* in *Saccharum officinarum* was evaluated for its efficiency to enhance the production of biomass and plant growth hormones under water deficit condition via differential transcriptome profile characterization (Vargas et al. 2014). In a different study *P. indica*'s ability to enhance antioxidant activity and drought related gene expression in *Z. mays* L. was evaluated. It was found that the antioxidant enzyme such as catalase and superoxide dismutase were accelerated as well as some drought related genes such as DREB2A, CBL1, ANAC072, and RD29A (Xu et al. 2017).

The integrate information on plant- microbe interactions and their relation with the external environments, are offered by proteomic approaches as all the plant stress responses are expressed by proteins and proteomics. Moreover this approach also provides a deeper insight to protein-protein interactions, physiological metabolism in microbes and plants (Meena et al. 2017). Proteomics has recognized so many microbe mediated plant responses and the stress responses have been studied in various plants including Arabidopsis, wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*), maize (*Z. maize*), rice (*Oryza sativa*), soybean (*Soybean max*), common bean (*Phaseolus vulgaris*), pea (*P. sativum*), oilseed rape (*Brassica napus*), potato (*Solanum tuberosum*) and tomato (*L. esculentum*) (Liu et al. 2015; Kosova et al. 2015; Wang et al. 2016a, b, c). The Rhizobacteria make plants drought tolerant by enhancing the levels of antioxidants, defense related proteins and enzymes, phytohormones and epoxypolysaccharide in them. The drought

stress in wheat and maize is mitigated by the *Burkholderia phytofirmans* strain PsJN (Naveed et al. 2014). The root fungal endophyte *Piriformospora indica* increases the levels of antioxidants in Chinese cabbage under the stress (Franken 2012). On the basis of proteome profile, it is estimated that *P. indica* suppresses drought stress in barley by enhancing photosynthetic activity and production of antioxidant. The treatment of mycorrhiza and rhizobia results in the synthesis of stress related proteins which increase the immune system of *P. sativum* (Desalegn et al. 2016).

12.6 Future Prospects

It is estimated that the national cereal production has been dropped by 9–10% due to drought stress resulting in the scarcity in food through the world (Lesk et al. 2016). In future by 2050, crops cultivated in more than 50% of the arable land on earth will face serious plant growth and survivable issues due to drought (Ngumbi and Kloepper 2016). Therefore the present and future prospect should be to develop diversified ways and means to modulate or prepare/enhance plant's capability to tolerate the most devastating abiotic stress like drought. The present status for combating drought was described above, but the future prospective must be more effective, persuasive and cheap.

There are many microbial mediated interdisciplinary fields of science that could be highly exploited for the upliftment of the current technologies in plant science. Example of such technologies includes bio-volatilization, microbial-biosensors and GMOs. Though these technologies have recently being developed, their efficient utilization in the path of developing or rendering tolerance to plant for various abiotic stress has not yet been properly evaluated or studied. Furthermore to completely understand these technologies and their application in improvement of plant responses towards drought and its associated impacts more systematic studies and efficient screening protocols should be devised which would help the current researchers to obtain precise data and manifest it in to reasonable output with great precision.

The efficacy of these current microbial based technologies in the future can be glimpsed if their function is explained. For instance bio-volatilization is the process where an organism through certain metabolic phenomenon could able to remove contaminants from the site and release them into atmosphere in a volatile form. This phenomenon is highly exploited in phytotechnology programs to enhance plant growth and survivability under certain abiotic stress (Schiavon and Pilon-Smits 2017). The studies show that there are many endophytic microbes that perform this spectacular function. Example of such symbiotic or plant associated bacteria includes *P. aeruginosa*, *P. putida*, *P. stutzeri*, *Rhodococcus wratislaviensis*, *Acinetobacter* sp., *Burkholderia* sp., *Gordonia* sp., *Dietzia* sp., *Gordonia* sp., *Mycobacterium* sp., *Nocardioides* sp., *Novosphingobium* sp. *Ochrobactrum* sp.,

Polaromonas sp., *Rhodococcus* sp., *Sphingomonas* sp. etc. and funguses includes *Alternaria alternate*, *Cladosporium cladosporioides*, *Cochliobolus sativus*, *Fusarium oxysporum*, *Muscodor yucatanensis*, *Talaromyces wortmannii*, *Trichoderma viride* etc. (Ningxiao et al. 2016; Gkorezis et al. 2017).

Microbial-biosensors are the plant associated or endophytic microbes or their secretions acts as a bioreporter to detect differential metabolomic, transcriptomic, proteomic and genomic alteration within the plant. Experimentations of the present are given much emphasis on evaluating such microbial species. One such example is *Rhizobium leguminosarum* a rhizosphere of *P. sativum* which by combining transcriptomics and metabolomics analysis 14 bacterial *lux*-fusion bioreporters for different biomolecules such as sugars, amino acids, organic acids and polyols have been validated in vivo and in vitro (Pini et al. 2017). Plants are genetically modified to give better harvest, improved tolerance, resistance to biotic and abiotic stress, improved nutritional values etc. where the modification is irreversible. But if the same values in the plant could be induced without altering the genetic makeup of the plants would be more appreciable. This could be achieved if the plant associated or symbiotic microbes are genetically modified and then bio-augmented to the targeted plant for the production of such values.

12.7 Conclusion

It is obvious that crop plants are hugely affected by drought stress and for mitigating its impact on their productivity and development; plants need to cope up with the adverse conditions. Moreover it is also well known that microorganisms have the ability to survive in such diverse and edaphic conditions. Microbes are synergistically linked to plants, through differential mode of biological interactions and provide defense under such stress directly or indirectly. Bioremediation, bioaugmentation, biotransformation, phytostimulation and biofertilization are some such interface between microbes and plants that are being used or could be used for ameliorating devastating impacts soothing responses of agronomically important crop plants (Conesa et al. 2012). These multifunctional symbiotic benefits bestrode by the bacteria on plant life could be further improved by genetic modification in plants or the associated microbes. The series negative effects of hazardous abiotic stress imparted on plant could be effectively remaindered by these microbe-based processes which could be quantified and analyzed through estimating the degree of associated morphological, physiochemical processes and molecular responses (Muthamilarasan and Prasad 2016). Finally it could be concluded that direct research in relation to genetic modification in plant associated microbes for enhancing host plant's tolerability towards drought stress is lagging behind. Therefore instead of developing genetic modified plant and altering the God gifted genetic makeup of the plant completely, it is better to modify the genetic makeup of the already mutating associated microbe which could bring about the same change in its host plants character from outside.

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Chapter 13
Microbial Remediation of Persistent Agro-chemicals by Soil Bacteria: An Overview

Suraja Kumar Nayak, Byomkesh Dash, and Bighneswar Baliyarsingh

13.1 Introduction

Agricultural innovations during 'Green Revolution' (GR) period of 1966–1985 had transformed agricultural practice and productivity. By virtue of 'Green Revolution' the world has witnessed a significant growth of crop productivity over the past five decades, which is primarily due to rapid increase in the use of fertilizer, pesticides, new crop strains as well as developments in irrigation and other technologies. This improved modern agriculture feeds around six billion people globally. Despite the success, rising world population and change in diets demands to increase food production by 70% (FAO 2009), which is also challenged by limited provisions for additional agricultural lands and protection of crops from various pests. During pre- and post-harvest period an average 35% of global yield is decreased due to diverse pests (Oerke 2006). In the wake of these new and old challenges, use of agrochemicals for sustainable production of food or feed on existing land is by far became the better choice.

13.2 Prevalence of Agro-chemicals in Agriculture and Environment

The beneficial outcome of pesticide usage provides an easy and economic way of maintaining and improving living standards of the people. Mostly in many developing countries, there has been rigorous practice of employing various agrochemicals as fertilizers and pesticides (Carvalho 2006). More specifically the production of

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various pesticides dramatically increased after the 1960s that resulted in doubling of the average yield of wheat, rice and maize per year. Per annum the worldwide market capital of chemical-pesticide is worth of USD 40 billion with total consumption 3 million tones (Popp 2011), of which 24% is consumed alone in the USA and 45% in Europe. Currently, the application of chemical pesticides covers 25% of the cultivated area. The estimated consumption of pesticides is about 5–7 kg/ha in UK, 13 kg/ha in China, 6.6 kg/ha in Korea and 12.0 kg/ha in Japan (Gurusubramanian et al. 2008). Moreover, the three common pesticides namely DDT, HCH and malathion shared 70% of the total pesticide consumption in developing countries.

Similarly the dependence of India on agro-chemicals has increased immensely since independence. This is evident from the fact that India had produced over 5000 mt of basic pesticides in the year 1958, mainly DDT and benzene hexachloride (BHC), whose productions were subsequently increased approximately to 85,000 mt in mid-1990s (Gupta 2004). Since then India has become the number one manufacturer of basic pesticides in Asia and fourth largest global manufacturer after USA, Japan and China. Approximately the demand for pesticides by the domestic consumers is accounted to be 50% which is expected to increase from 6.5% per annum in 2015 to 9% by 2020. Among these agrochemicals, 75% of India's total pesticide consumption is accounted by insecticides followed by 12% of fungicides and 10% of herbicides.

Various entomologists termed the vicious cycle of dependence on chemical pesticides as "*pesticide treadmill*" (Bosch 1989). The "pesticide treadmill" has two major aspects. The first one is the enhancement of the dosage and frequency of less effective pesticide which is due to the development of resistance in pests as well as destruction of the natural enemies of pests and the second one is the formulation and commercialization of new pesticide. Despite the popularity of various agrochemicals or pesticides, the harmful effects of pesticides during occupational exposure of farmers have raised worldwide concern. To that extent, the general population is also affected from the residues in food and drinking water (van der Werf 1996; Pimentel 2005).

13.3 Impact of Agro-chemicals

13.3.1 Direct and Indirect Effects on Humans and Environment

The worldwide human morbidity and mortality is the result of considerable acute human poisonings due to various pesticides. In developing countries, about 25 million farm workers are exposed to synthetic pesticides (Jeyaratnam 1990). The World Health Organization (WHO) indicated that at least in a year three million people are affected severely by pesticide poisoning. Even though developing countries use only 20% of the World's agrochemicals, 99% of 3 lakh deaths from pesticide

poisoning are from low and middle income countries (Gunnell and Eddleston 2003). Various incidences of accidental poisoning by pesticides have been reported in India since its production. In 1958, the first pesticide poisoning case was reported in Kerala state of India where over 100 people died after ingesting wheat flour contaminated with parathion pesticide, introduced by Bayer (Karunakaran 1958). Similar poisoning cases of consuming contaminated wheat with aldrin dust and gammexane in Madhya Pradesh of India, hexachlorocyclohexane (HCH) in Uttar Pradesh of India (1977) and deaths due to aluminum phosphide (1992) have been reported (Gupta 2004). Not to forget the painful tragedy of '*Bhopal gas leakage*' of methyl isocyanate pesticide. Most of these poisoning cases had developed symptoms like myoclonic jerks, generalized colonic convulsions, weakness in the extremities and grand mal seizures (Nag et al. 1977).

In addition, agricultural intensification and widespread use of pesticides have caused the extinction of many local wild plant and animal species and this has a profound effect on functioning the agro-ecosystems. Long after their use, these persistent and recalcitrant organic compounds such as heterocyclics, polynuclear aromatics, chlorinated aromatics and nitroaromatics, either remain in soils-sediments or percolate down to ground water. Thereby contaminating and dispersing to other land or aquatic ecosystem. So, indirect accumulation or biomagnifications has serious impact on the health of higher tropic level organisms and biodiversity of the ecosystem. Even when organic pollutants are present in minute quantities, their variety, toxicity and persistence capability have an adverse effect on the equilibrium of ecosystems to which welfare of humans, birds, fish and trees are associated (Gupta 1986).

The revised classification of pesticides based on their toxicity introduced by the World Health Organisation (WHO) in 2009 has five groups. The following groups (i) extremely hazardous (Ia), (ii) highly hazardous (Ib), (iii) moderately hazardous (II), (iv) slightly hazardous (III), (v) unlikely to present acute hazard (U) are categorized on the basis of LD_{50} for the rat (mg/kg body weight). Food and Agriculture Organization and WHO recommends of pesticides based on the toxicity Ia and Ib pesticides should be avoided in developing countries (PAN-UK 2003) and that of class II pesticides be avoided. However, aggressive marketing strategies of large chemical industries and ignorance of farmers allows the use of these toxic agrochemicals extensively.

It has been reported that organophosphorus based pesticides such as malathion, parathion, dimethoate, chloropyrophos and monochrotophos are the prime cause of deaths in certain developing countries. The mortality rate observed following poisoning by organophosphate (OP) pesticides varies between 4% and 30% (Yamashita et al. 1997). Organophosphate and lipophilic OPs compounds get easily absorbed by the mucosa linings of respiratory or gastrointestinal tracts and through the skin, respectively (Karalliedde 1999). Similarly, accumulation of non-degradable, chlorinated pesticides in various living systems for extended period induces a variety of toxic symptoms (Gupta and Salunkhe 1985). Scientific studies reported that even if persons are exposed to little amount of persistent

chemicals, the combined effect these chemicals manifests various clinical conditions like suppression of immune response and hypersensitivity to other chemical antigens, reduction in sperm count or male sterility and development of breast cancer (Carvalho 2006).

13.3.2 Persistent Organic Pollutants (POPs)

Persistent organic pollutants (POPs) are groups of semi-volatile, synthetic chemicals produced/released intentionally or non-intentionally that has harmful effects on humans and wildlife. These toxic chemicals are characterized as resistant to degradation as they may take decennia or centuries to degrade substantially. This enables them either to get transported to a long distance leading to global pollution or to enter into food cycles affecting mostly to humans. Being fat-loving chemicals, they particularly partitioned into solid organic tissues or matter avoiding the aqueous phases in the ecosystems or sediments. Thus, these chemicals eventually accumulated in fatty tissue without entering into an aqueous milieu of cells and the persistence in biota is more augmentation by slow metabolism of cells. Though, it is most difficult to establish a direct relationship between the illness and exposure to specific persistent organic pollutants, sharp effects after a high-level of exposure have been described for some of the organochloride and organophosphate pesticides. For example, pregnant women exposed to persistent pesticides may have no or minor disease manifestations, but their children acquired developmental disorders due to disruption in the endocrine system.

There are many thousands of POP chemicals that are grouped into series or families of chemicals. For instance, there are theoretically 209 different polychlorinated biphenyls, which is grouped under ogranochlorides, differing from each other by the number of chlorination and position of substitution. Most of the major POPs fall under families of chlorinated (and brominated) aromatics that includes polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and -furans (PCDD/Fs), polybrominated diphenyl ethers (PBDEs) and organochloride pesticides (eg. DDT and its metabolites, toxaphene, chlordane etc.). At Stockholm Convention, a global treaty was ratified under the United Nations Environment Programme (UNEP) that motivates to eliminate 12 designated POPs, called the "*dirty dozen*". Interestingly the "dirty dozen" comprise of 8 pesticides, two industrial chemicals and two unintended industrial by-products. Although these eight persistent toxic pesticides are banned by many developed countries but are still in use in many developing countries because of its production cheapness.

13.3.3 Fate of Agro-chemicals in Agricultural Soil

Long after their use, these agrochemicals stay behind in soil and sediments. Once the pesticide finds its way into the soil environment, many and varied of factors act upon it. They either enter into food chain or subjected to diverse natural degradation process. This can happen in several ways like photo-decomposition, volatilization, adsorption, leaching, and diverse microbial activity. Each pesticide reacts differently to each of these factors and often several of these processes are occurring simultaneously.

Photo-decomposition (light breakdown) and volatility are influenced by the climatic conditions where as soil texture and quality has larger impact on adsorption, leaching and microbial activity. Adsorption is the accumulation of a component of a mixture at an interface. The Leaching ability of a pesticide depends on (1) the entrance of the compound into solution, (2) adsorption pops to soil particles and (3) evapotranspiration. More likely the pesticides leach or follow the free water, when heavy rain or instant irrigation is occurred after the pesticide application. If the soil surface is dry, the pesticide will most likely move in with the water i.e. percolated to ground water. If there is a high evapo-transpiration ratio, there is more water being utilized by the plant, thus more pesticide is being moved into the plant via the roots. Broadly the fate of pesticides depends on three major process, i.e. transport, transfer and transformation. During transport process the POPs move away from its point of application into the environment and spread throughout surface-water system. The transfer process is more of controlling phenomena involving various factors in distribution in soil and environment. Finally the transformation process refers to the biological and chemical processes that converts the POPs into simpler compounds or completely degrade it. In general, the fates of pesticides in surface water system are affected by the structural, chemical and biological attributes of the pesticides.

13.4 Soil Microbes and Pesticide Degradation

Biologically catalytic curtailment of complex, noxious organic molecules such as pesticides or agrochemicals to its simpler, smaller and non-toxic (iso)forms coined as biodegradation. Soil microbes possess some inherent mechanisms to carry out the biodegradation process. So, to understand the process of biodegradation by the microbes needs a better understanding about the microbes (Alexander 1994). However, microbial degradation of the majority of recalcitrant compounds is restricted due to anionic species in the structure. The anions like Cl^{-}, SO_4^{2-} etc. are strongly bonded to the close-ring and prevents the common microbes from attacking the compound and this may be due to increased toxicity of anionic group (Julia et al. 2001). Apart from nutrient recycling (Van Der Heijden et al. 2008), plant growth promotion, biological nitrification and denitrification occurring in dynamic soil ecosystems, soil microbes collectively involved in extensive degradation of pesticides

or agrochemicals (xenobiotics) (Schneider et al. 2010; Pino and Penuela 2011; Zhao et al. 2009).

Bacteria exhibit diverse physiological metabolism of degrading many varieties of natural and synthetic organic compounds, including pesticides in soils. Reports suggest that consortia soil microbes can more efficiently degrade pesticides than single non-indigenous strains. Even if the degrading metabolic processes are longer, it's been more viable and suitable option for eliminating the xenobiotic compounds from soil (Finley et al. 2010). The continued exposure of contaminated environment makes the soil bacteria to evolve and develop a modified genetic system against various toxicants (Parsek et al. 1995). Bacterial degradation of pesticides is basically higher on the surface than subsurface soils which has been influenced by spatial variability and heterogeneity soil (Linn et al. 1993). In rhizosphere, the efficient degradation of these xenobiotic compounds results from co-metabolism which is further augmented by roots exudates generated due to gross microbial activity (Fang et al. 2001).

Pesticides have reduced bioavailability due to efficient absorption of pesticides in soil organic matter. Consequently pesticide absorption affects the soil microbial community structures, metabolisms and more specifically bacterial multiplication and growth (Johnsen et al. 2001). In situ removal of and detoxification of agrochemicals from the soil ecosystem by the soil microbes, more specifically by the bacteria, have exceptional significance (Wang et al. 2005). A large pool of soil bacterial isolates including *Bacillus* sp., *Pseudomonas* sp., *Arthobacter* sp., *Ralstonia* sp. (erstwhile in *Pseudomonas*), *Rhodococcus* sp., *Acetobacter* sp., *Alcaligenes* sp., *Flavobacterium* sp., *Burkholderia* sp., *Yersinia* sp., *Stenotrophomonas* sp., and *Weeksella virosa* (Padmanabhan et al. 2003) from different soils environments have been discovered with pesticide degrading ability.

13.4.1 Microbes Involved in Degradation of Persistent Agro-chemicals

As the name bioremediation suggests, it is the detoxification or reclamation of the POP contaminated environment by the biological system, more specifically with the indigenous or genetically modified bacteria. This inclusive potential of bacteria facilitates efficient and economical viable way of removing contaminants. Wide varieties of bacteria are involved in degradation of different persistent pesticides (Table 13.1).

In most instances aerobic remediation favours much faster than anaerobic decomposition. On the contrary, anaerobic conditions favour the degradation of DDT (dichloro-diphenyl-trichloro-ethane) ten times faster than aerobic conditions (Scott 2000). DDT and HCH (Hexachlorohexane) degrading potential of the species of *Micrococcus* and *Lactobacillus* (Azizi 2011) have been established. Different strains of ubiquitously available *Pseudomonas* genus are actively involved pesticide

Table 13.1 Soil bacteria involved in degradation of the most persistent agrochemicals/pesticides

Sl.	Pesticides group/ classes	Representatives	Degrading bacteria	References
High persistence agrochemicals				
1.	Organochlorates or chlorinated hydrocarbons	Alachlor	*Pseudomonas* sp. strain ADP, *Ancylobacter* sp. S15, *Agrobacterium* sp. CZBSA1	Katz et al. (2001) and Ewida (2014)
		Aldrin	*Pseudomonas* sp., *Bacillus* sp., *Micrococcus* sp	Sharma et al. (2016)
		DDT	*Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumonia*, *Bacillus* sp., *Pseudomonas putida*, *E. coli*, *Hydrogenomonas* sp.	Patil et al. (1970) and Sharma et al. (2016)
		Dieldrin	*Pseudomonas* sp.	Sharma et al. (2016)
		1,4- dichlorobenzene	*Pseudomonas* sp.	Spain and Nishino (1987)
		Endosulfan	*Pseudomonas* sp., *Bacillus* sp., *Flavobacterium* sp.	Karpouzas et al. (2005)
		Heptachlor	*Clostridium* sp.	Sethunathan (1973)
		Lindane (γ-HCCH, α-BHC, β-BHC)	*Bosea thiooxidans*, *Sphingomonas paucimobilis*	Karpouzas et al. (2005)
		Methoxychlor	*Enterobacter aerogenes*	Fogel et al. (1982)
		Pentachloronitrobenzene	*Cupriavidus* sp. strain BIS7	Teng et al. (2017)
		Pentachlorophenol (PCP)	*Arthrobacter* sp., *Flavobacterium* sp., *Sphingobium chlorophenolicum*	Sharma et al. (2016)

(continued)

Table 13.1 (continued)

Sl.	Pesticides group/ classes	Representatives	Degrading bacteria	References
2.	Organo-phosphate	Cadusafos	*Pseudomonas putida*, *Flavobacterium* sp.	Karpouzas et al. (2005)
		Chlorpyrifos	*Achromobacter xylosoxidans* (JCp4), *Ochrobactrum* sp. (FCp1)	Akbar and Sultan (2016)
		Diazinon	*Pseudomonas capaciea*	Tewari et al. (2012)
		Dimethoate	*Bacillus cereus*, *B. subtilis*, *B. safensis*	Ishag et al. (2016)
		Ethoprophos	*Sphingomonas paucimoblis*	Karpouzas (2005)
		Glyphosate, acephate	*Clostridium* sp., *Arthrobacter* sp	Tewari et al. (2012)
		Malathion	*Pseudomonas aeruginosa* AA112	Abo-Amer (2007)
		Monocrotophos	*Rhodococcus* sp.	Tewari et al. (2012)
		Tetrachlorvinphos	*Stenotrophomonas malthophilia, Proteus vulgaris, Vibrio metschinkouii, Serratia ficaria, Serratia* sp., *Yersinia enterocolitica*	Ortiz-Hernández and Sánchez-Salinas (2010)
3.	Carbamates	Aldicarb	*Arthrobacter* sp., *Rhodococcus* sp.	Behki and Khan (1994)
		Carbayl (1-naphthalenyl methyl carbamate)	*Pseudomonas* sp., *Achromobacter* sp., *Arthrobacter* sp., *Xanthomonas* sp. and *P. cepacia*	Chapalamadugu (1991) and Gunasekara et al. (2008)
		Carbofuran	*Achromobacter* sp., *Pseudomonas* sp., *Flavobacterium* sp.; Pseudomonas sp., *Flavobacterium* sp. *Achromobacterium*, *Sphingomonas* sp. Arthrobacter sp.,	Chaudhry and Ali (1988) and Sharma et al. (2016)

(continued)

Table 13.1 (continued)

Sl.	Pesticides group/ classes	Representatives	Degrading bacteria	References
4.	Thiocarbamates	Butylate (S-ethyldiiso butylthio carbamate)	*Rhodococcus* TE1	Behki and Khan (1991)
		Dietholate	*Pseudomanas putida*, *Azotobacter chroococcum* (NRC 18002), *B. cereus Rhodococcus erythropolis,*	Tam et al. (1988)
		EPTC (S-ethyl dipropyl carbamothioate)	*Rhodococcus* sp., *Arthrobacter* sp.,	Nagy et al. (1995) and Tam et al. (1987)
		Molinate (S-ethyl-N-hexahydro-1 H-azepine thiocarbamate)	*Achromobacter xylosoxidans* ssp. *denitrificans*, *Stenotrophomonas maltophilia*, *P. chlororaphis* IFO 3904, *P. nitroreducens* IAM 143, *Curtobacterium flaccumfaciens* var. *flaccumfaciens* LMG 3645	Barreiros et al. (2003)
5.	Organotins	Tributyltin (IV) TBT	*Desulfovibrio* sp., *Pseudomonas fluorescens*	Gajda and Jancso (2010)
		Tributyltin chloride (TBTCl)	*Pseudomonas aeruginosa*, *P. fluorescens*	Ebah et al. (2016)
		Triphenyltin acetate	*Nitrosomonas* sp., *Nirrosococcus* sp., *Nitrospira* sp., *Nitrosolobus* sp., *Nitrobacter* sp.	Kuthubutheen et al. (1989)
6.	Organosulfur	Rizolex	*Bradyrhizobium* sp.	Moawad et al. (2014)
		TMTD	*Pseudomonas aeruginosa*	Ray and Mondal (2017)
		Thiram (tetramethyl thiuram disulfide)	*Pseudomonas aeruginosa*	Shirkot and Gupta (1985)
		VitavaX (37.5% thiram)	*Rhizobium leguminosarum*	Moawad et al. (2014)

(continued)

Table 13.1 (continued)

Sl.	Pesticides group/ classes	Representatives	Degrading bacteria	References
7.	Dinitrophenols	Nitrobenzene	*Pseudomonas pseudoalcaligenes* JS45, *P.* putida HS12, *P.* mendocina KR-1, *P. pickettii* PKO1,*Comamonas* sp. JS765, *Arthrobacter* sp. NB1, *Serratia* sp. NB2, *Stenotrophomonas* sp. NB3, *Clostridium* sp. and *Comamonadaceae* sp.	Sharma et al. (2016)
		Pendimethalin	*P. aeruginosa*, *B. mycoides, B. cereus*	Sharef Ibrahim et al. (2013)
8.	Urea derivatives	Chlorsulfuron, rimsulfuro, imazosulfuron,	Aerobic cellulolytic bacteria	Gigliotti et al. (1998)
		Isouron	*Pseudomanas putida*	Ozaki et al. (1986)
		Sulfometuron, thifensulfuro, metsulfuron, bensulfuron-methyl	*Pseudomonas striata* Chester	Kaufman and Blake (1973)
9.	Triazine (s)	Atrazine	*Arthrobacter* sp., *Clavibacter* sp.	Sharma et al. (2016)
		Methylthio-s-triazines	*Rhodococcus* sp. strain FJ1117YT	Fujii et al. (2007)
10.	Trichloro-picolinic acid complexes or Pyridine family	Isonicotinic acid (4-pyridine carboxylic acid)	*Acinetobacter* sp. and *Bacillus brevis*	Sims and O'Loughlin (1989)
		Monohydroxy pyridines, 2,3-dihydroxy pyridine, amino- and methyl pyridines	*Micrococcus luteus, Brevibacterium* sp. and *Corynebacterium* sp.	Sims et al. (1986)
		Pyridine	*Paracoccus* sp.	Qiao and Wang (2010)
		2-pyridine carboxylic acid	*Bacillus* sp.	Sims and O'Loughlin (1989)
		2,6-pyridinedicarboxylic acid (2,6-dipicolinate)	*Bacillus* sp. and *Agrobacterium* sp.	Sims and O'Loughlin (1989)
		2-pyridone	*Arthrobacter crystallopoietes*	Sims and O'Loughlin (1989)
		Strobilurin	*Stenotrophomonas maltophilia*, *Bacillus amyloliquefaciens*, *B. flexus*, *Arthrobacter oxydans*	Clinton et al. (2011)

(continued)

Table 13.1 (continued)

Sl.	Pesticides group/ classes	Representatives	Degrading bacteria	References
11.	Naphthoquinones	Juglone	*Pseudomonas* sp.	Schmidt (1988)
		1,4-naphthoquinone	*Bacillus* sp.	Sethunathan (1989)
Moderate persistence agrochemicals				
1.	Imidazolinone	Carbendazim	*Pseudomonas* sp., *Brevibacillus borstelensis*	Arya et al. (2017)
		Iprodione	*Pseudomonas fluorescens*, *P. paucimobilis, Arthrobacter* sp. (strain C1), *Achromobacter* sp. (strain C2)	Mercadier et al. (1997) and Campos et al. (2015)
2.	(Nitro) guanidines	Thiamethoxam, clothianidin, dinotefuran	*Leifsonia* sp.	Sabourmoghaddm et al. (2015)
Less persistence agrochemicals				
1.	Neonicotinoids	Acetamiprid	*Ochrobactrum* sp. strain D-12	Wang et al. (2013)
		Imidacloprid	*Achromobacter* sp., *Pseudoxanthomonas* sp., *Sinorhizobium* sp., *Mesorhizobium* sp., *Microbacterium* sp.,	Sharma et al. (2016)
2.	Pyrethroids	Cyhalothrin	*Klebsiella* sp., *Pseudomonas oleovorans*	Thatheyus and Selvam (2013)
		Cypermethrin	*E. coli*, *S. aureus*, *P. aeruginosa, B. subtilis, Enterobacter asuburiae*, *Pseudomonas stutzeri*	Thatheyus and Selvam (2013)
		Fenvalerate	*Bacillus cereus* and *Pseudomonas viridiflava*	Thatheyus and Selvam (2013)

degradation. Such as, soil isolates of *Pseudomonas putida* degrade organophosphates, including fenamiphos, oxamyl and carbofuran carbamates (Chanika et al. 2011). Gigliotti et al. (1998) reported efficient endurance of soil bacteria under sulfonylureas pesticides in alkaline conditions. Even highly persistent ring substituents like pyridine and pyridine derivatives, including picloram, nitrapyrin, flouridone, 4-amino pyridine and triclopyridine are degraded aerobically by *Bacillus* sp. and *Pseudomonas* sp. in soil sediments (Sims and O'Loughlin 1989). Degradation of polychlorinated biphenyls (PCBs) by *Pseudomonas* sp. are guided by chromosomal genes and naphthalene, salicylate, toluene, xylene, parathion, and quinoline through plasmid-associated genes (Chapalamadugu and Chaudhry 1991). *Klebsiella pneu-*

moniae and *Pseudomonas* sp. excretes excellular hydrolytic enzymes and breaks down atrazine, a member of s-triazine herbicides (Baishya and Sarma 2015). Broad spectrum phenylurea herbicides, including N-methoxy-N-methyl and N,N-dimethyl-substituted phenylureas are degraded by *Arthrobacter globiformis* (Tixier et al. 2001; Cullington and Walker 1999).

The bacterial degradation of pesticides is fundamentally based on two processes i.e. growth and co-metabolism. Organic pollutants are consumed as the source of carbon and energy for bacterial growth and metabolism and thereby facilitating conversion or mineralization of organic pollutants (Fritsche and Hofrichter 2008). Degradation ability of the soil bacteria depends on various physio-chemical parameters such as surrounding temperature, environmental pH, water holding potential (Ψ) of soil, available nutrients, soil texture and porosity (Singh 2008). Chlorpyrifos, malathion, parathion, fonofos, diazinon, ethion, and gusathion are some of the persistent organophosphorous insecticides that serve as carbon sources for the growth of *Pseudomonas* sp., *Flavobacterium* sp. and *Arthrobacter* sp. either in pure or in mixed cultures (Digrak et al. 1995; Ghisalba et al. 1987).

Species of *Rhodococcus* isolated from soil degrade metamitron (Parekh et al. 1994) while chlorpyrifos is degraded by *Flavobacterium* sp. (ATCC 27551), *Pseudomonas diminuta* strain (Gm) and *P. putida* (Rani and Kumari 1994). *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Klebsiella oxytoca* are able to degrade phenylephrine in mixed consortia in cultivable land, enriched with PAHs and other metals (Kim et al. 2009). Few species of *Sphingomonas* (Ye et al. 1996; Van Herwijnen et al. 2003), *Burkholderia* (Kang et al. 2003), *Stenotrophomonas*, *Mycobacterium*, and *Rhodococcus* are also characterized as biodegrading microbes for various PAHs starting from bi-ring to penta-ring structures (Beate et al. 1993; Moody et al. 2004). Group bacteria able to degrade collectively and sequentially the carbamate pesticides such as carbofuran (a member of *N*-methylcarbamate class) are *Arthrobacter* sp., Achromobacterium sp., Flavobacterium sp., *Pseudomonas* sp. and *Sphingomonas* sp. (Porto et al. 2011).

In ideal environmental condition, the microorganisms degrade or modify persistent compounds by the help of a variety of enzymes. Extracellular enzymes predigest poorly transportable pesticides to facilitate transportation these compounds into the cell. Once inside of an organism, organic compounds are metabolized by internal enzyme systems. Maximal degradation of the pesticides are achieved with the interaction between pollutant and the enzymes (intracellular or extracellular) and sufficient biomass (Baishya and Sarma 2015).

13.4.2 Basic Mechanism of Degradation

The biodegradation of persistent organic compounds like synthetic agrochemicals is often complex, involving vast array of biochemical reactions. The organic compound ranges from simple carbon compounds to more complex structures which may be broken down by enzymes (both extracellular and intracellular) before

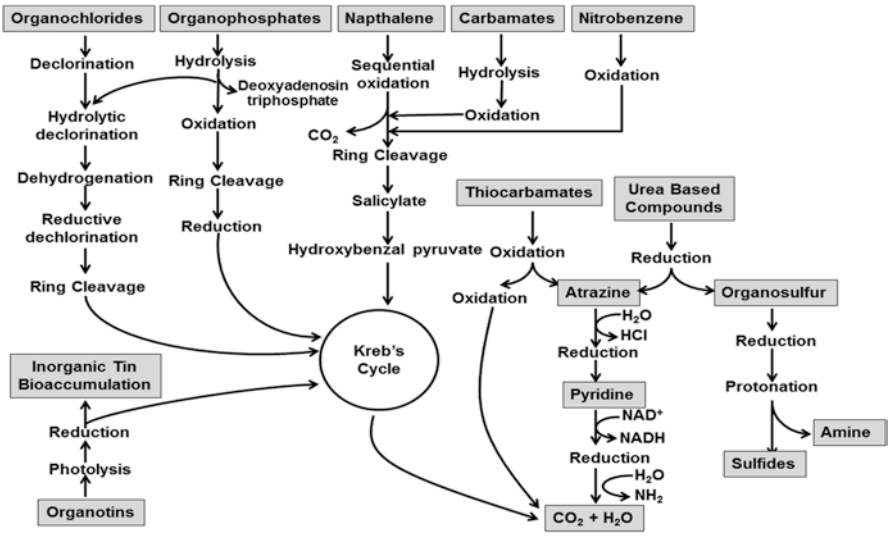

Fig. 13.1 Schematic representation of various biochemical pathways followed by microbes in remediation of complex and persistent agro-chemicals into simpler utilizable forms

cellular uptake and metabolism (Gadd 2010). Thus detoxification or degradation of pesticides is co-ordinated by various enzyme-controlled pathways (Fig. 13.1). According to Abraham, metabolic cooperation can be interpreted as interchanging of the organic molecules, substrates and products of the enzymatic reactions, within a microbial community (Abraham et al. 2002). Understanding the metabolic pathways involved in bacterial degradation of the agrochemicals (Somasundaram and Coats 1990) will widen our capability enhance the remediation process by modifying soil microbes.

Enzyme based pesticide biodegradation is a timely advanced treatment method for removal of pesticide pollutants from environment. Various classes of transferase, isomerase, hydrolase and other enzymes catalyze reactions, including hydrolysis, oxidation/reduction, addition of oxygen to a double bound, oxidation of an amino group (-NH_2) to a nitro group, addition of a hydroxyl group to a benzene ring, dehalogenation, reduction of a nitro group (NO_2), replacement of a sulfur with oxygen, metabolism of side chains, ring cleavage etc. (Megharaj et al. 2011). For example, the degradation of carbofuran is carried out via single-step hydrolysis which is sufficient enough to inactivate carbamate group of insecticides. The initial step of dechlorination of organochlorides like DDT to DDD was carried out by *Pseudomonas* species under aerobic condition. Then it is further oxidized by enzyme dioxygenases to produce a dihydroxy derivative for meta-cleavage reaction, finally yielding a degraded product of 4-chlorobenzoic acid (Nadeau et al. 1994).

In aerobic condition *Alcaligenes* sp., *Arthrobacter* sp., *Flavobacterium* sp. and *Pseudomonas* sp. metabolize 2, 4-D (2,4-dichlorophenoxyacetic acid) through a ortho-cleavage pathway, converting ultimately into chloromaleylacetic acid with the

intermediates of 2,4-dichlorophenol and 3,5-dichlorocatechol (Gibson and Sulflita 1990). Likewise *Flavobacterium* sp. (ATCC 27551) isolated from paddy field (Sethunathan and Yoshida 1973) are able to fulfil the carbon requirement by metabolizing the organophosphate compounds, parathion, diazinon. Enzyme parathion hydrolase (elsewise phosphotriesterase) inactivates parathion along with diazinon and other organophosphates by a single catalysed reaction (Brown 1980). Some soil bacteria biodegrade atrazine through N-dealkylation of the side ethyl and isopropyl chains to de-ethylatrazine and de-isopropyl atrazine. Even if, the methylthioether and N-alkyl substituents on the s-triazine ring in herbicides deter the direct metabolism by bacteria (Wackett et al. 2002), but few researchers have reported that soil bacteria are capable of degrading atrazine partially or fully with end products as CO_2 and NH_3 (Rousseaux et al. 2003; Singh et al. 2004). *Pseudomonas sp.* ADP isolated from atrazine contaminated soil was able to biomineralize the triazinic ring completely (Mandelbaum et al. 1995).

In the case of pyridine carboxylic acids biodegradation, the ring is usually hydroxylated via nucleophilic attack of OH^- group. But in case of hydroxyl pyridines, a second hydroxyl is generally introduced by nucleophilic addition at electron deficient sites. However, the fission of pyridine and some alkyl pyridines can occur by a mechanism which apparently does not involve any hydroxylated intermediates. The suggested mechanism of initial reduction to form dihydropyridine and subsequent break down into saturated aliphatic intermediate compounds is observed in soil *Bacillus* sp. and *Pseudomonas* sp. (Sims and O'Loughlin 1989). Bacterial species like *Burkholderia* sp., *Ralstonia* sp. and *Rhodanobacter* sp. involvement in degradation of aromatic hydrocarbons (Bacosa et al. 2010), remediation of n-hexadecanoic acid by intracellular β-oxidation pathways (Yuan et al. 2013), has also been reported.

13.4.3 Microbial Genes Involved in Degradation

Soil microorganisms are highly adaptable to polluted environment and produce various enzymes for the mineralisation of persistent agrochemicals. The biochemical reactions of degrading persistent chemicals are carried out by variety of microbial enzymes. The productions of such enzymes are controlled by a special kind of genes which gives them the ability to consume these compounds as their growth substrates (Suenaga et al. 2001). Moreover, studies on the distribution of catabolic genes in different bacteria can significantly contribute to our understanding of how bacteria evolve new metabolic functions. The evolution of microbial genes guiding biodegradation of xenobiotic molecules is a powerful development in mitigating challenges of environmental pollution. Anti-catabolic plasmids with pesticide degrading genes and transposons encoding the pollutant-degrading enzymes (Laemmli et al. 2000) are present in soil bacteria *viz. Pseudomonas sp.*, *Alcaligenes sp.*, *Actinobacter sp*., *Cytophaga sp*., *Moraxella sp*. and *Klebsiella* sp. (Sayler et al. 1990).

In polluted or contaminated environments bacteria carry out genetic events, such as transformation, transduction or conjugation, which equip them in acquiring improved abilities to degrade contaminants (Stoodley et al. 2002). The atrazine degrading soil bacteria generally initiate degradation through dechlorination and hydrolysis. *Pseudomonas* sp. ADP was studied extensively to comprehend the response and interaction between the catabolic genes and stress response of atrazine. The carbamate degradation by soil microbes is carried out by enzymes atrazine chlorohydrolase, hydroxy-atrazine ethylamino-hydrolase and N-isopropyl-ammelide isopropyl-amino-hydrolase, encoded by *atzA, atzB (trzB) and atzC (trzC)* gene respectively (De Souza et al. 1996; Sadowski et al. 1998). These gene clusters convert atrazine successively to cyanuric acid and then mineralizing completely into CO_2 and NH_3 (Sene et al. 2010).

Plesiomonas sp. strain M6 carry gene *mpd* which encodes methyl parathion hydrolase (MPH) enzyme, therby hydrolyzing series of organophosphorus compounds. The encoded enzymes from *mcd* (methyl carbamate degradation) gene of these species have broad spectrum activity against carbamates. Similarly, the cluster of genes managing the degradation of EPTC by aldehyde dehydrogenase enzymes and thiocarbamate degradation by P-450 have been identified and cloned from different bacteria like *Rhodococcus* sp. NI86/21, *Achromobacter* sp. WMll (Tomasek and Karns 1989) and *Rhodococcus* sp. strain NI86/21 (Nagy et al. 1995). The degradation of 2,4-D (2,4-dichlorophenoxyacetic acid) by *Alcaligenes eutrophus* JMP134 is controlled by the plasmid pJP4. The genes involved in degradation of 2,4-D are designated as *tfd* that are arranged in three operons as *tfdA* encoding 2,4-dichlorophenoxyacetate monooxygenase (Streber et al. 1987), *tfdB* encoding 2,4-dichlorophenol hydroxylase (Kaphammer and Olsen 1990) and *tfdCDEF* encoding chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, chlorodiene lactone isomerise and chlorodienelactone hydrolase, respectively (Kaphammer et al. 1990). In addition to this *Alcaligenes* sp. (Tn5271), *Burkholderia cepacia* (Tn5530), *P. putida* (Tn4654) and *Ralstonia eutropha* (Tn4371) bears various transposons that facilitates the degradation of carbofuran, 2,4-DToluene, 3-chlorobenzoate and biphenyl 4-chlorobi-phenyl molecules, respectively (Verma et al. 2014). An enzyme, carbofuran hydrolase, capable of hydrolyzing several N-methyl carbamate insecticides, including carbaryl, carbofuran, and aldicarb was purified (Derbyshire et al. 1987), and the gene *mcd* responsible for expression these has been cloned (Tomasek and Karns 1989).

13.5 Strategies to Enhance the Efficiency of Pesticide Degradation

Pesticide degradation in soils provides an important type of information for predicting the end result of pesticides in the ecosystem. For enhanced biodegradation, direct application of bacterial decontaminants without proper knowledge on

application area, may result in minimal success. These may be due to several reasons such as overgrowth of autochthonic microflora (Slater et al. 1983) and sometimes because of the inoculated microorganisms have been partly degenerated by in vitro cultivation, shifting of metabolism for degradation. Not adapted to the conditions in the soil and need a quite long time for adaptation. To overcome such types of limitations few biotechnological tools and techniques are discussed.

13.5.1 Whole Cell Immobilization

Cell immobilization has been a favourable choice of biodegradation strategy for persistent pesticides due to the ease of maintaining catalytic activities for a long period of time (Richins et al. 2000; Martin et al. 2000; Chen and Georgiou 2002). The enhanced degradation through immobilized cells is primarily because of restriction of direct contact with inhibitory substances in the surroundings. The advantages of whole-cell immobilization technique over traditional biological methods of employing free cells directly for efficient biodegradation of agro-pollutants, are involvement of large number of cell, limited cell washout, smooth separation of cells from the reaction system, repeatability in cell use and better protection of cells from adverse environmental conditions. Moreover, immobilized cells are much more tolerant and resistant to fluctuations during the reaction and less susceptible to toxins, which makes immobilized cells, potentially suitable for the treatment of pesticides toxic substances (Ha et al. 2008). The degradation rates for repeated operations in batch cultures were successfully increased for successive batches, indicating that cells became efficiently adapted to the reaction conditions over the time period (Ha et al. 2009).

Based on methods of physical processes involved, the cell immobilization can be grouped into two categories; one as retention type (entrapment and inclusion membrane) and other involving chemical bond formation as in biofilms (Kennedy and Cabral 1983). In cell immobilization methods may use various inorganic and organic materials or substrates such as clays, silicates, glass and ceramics (inorganics) and cellulose, starch, dextran, agarose, alginate, chitin, collagen, keratin, etc. (organics) (Arroyo 1998). Mostly, the immobilized cells entrapped in polymeric gels were particularly successful against xeniobiotic comunds (Lusta et al. 1990). Furthermore, several reports suggested the use of variety of materials for immobilization of microorganisms such as plant fibres used as the supporting material for immobilizing bacterial consortium are more efficient in degrading persistent or recalcitrant pesticides. The use of materials such as petiolar felt sheath of *Aracaceae* sp. (palm tree) for entrapment method is an addendum to natural matrices for immobilization. The advantages accrued from likewise bioforms are repeatability in use, toxin free structures, support through mechanical strength and ample spaces for entrapped cell for growth thus eliminating cell rupture and diffusion. The *Luffa cylindrica* (the loofa sponge) have been used as natural support for immobilization of *Porphyridium cruentrum,* for chlorinated substances treatment (Iqbal and Edyvean 2004; Mazmanci and Unyayara 2005).

Removal of chlorpyrifos increased from 40% to 71% with the application of immobilized cells of *Streptomyces* sp., while 14% increased from 5% in case of pentachlorophenol. These in situ remediation results confirm the successful cleaning of mixtures of xenobiotics through free or immobilized cell cultures (Fuentes et al. 2013). Profenofos, an organophosphate pesticide efficiently degraded by *Pseudoxanthomonas suwonensis* strain HNM isolated from pesticide-contaminated soil. Common immobilization matrices are sodium alginates (SA), sodium alginate-polyvinyl alcohol (SA-PVA) and SA-bentonite clay (Talwar and Ninnekar 2015). Propachlor an organochlorine persistent herbicide degraded through the immobilized cells of *Pseudomonas* strain GCH1. The cells use ceramic support to be used as an immobilized cell system which reaches upto 98% with increased in cell viability (Martin et al. 2000). Immobilized cells of *Pseudomonas putida* on calcium alginate beads or on granular activated carbon have been used successfully in vitro to degrade phenols with concentrations ranging from 100 to 1200 ppm (Mordocco et al. 1999; El-Naas et al. 2009). Few *Rhodococcus* species P1 are also involved in phenol degradation via suspended cells of immobilization (Basha et al. 2010).

13.5.2 Enzyme Application

Microorganisms have elaborate enzymatic systems for the breakdown of xenobiotic chemicals. Biodegradation of various organic pollutants involves diverse enzymes such as phosphatase, esterase, hydrolase and oxygenase etc. Solubility of the enzymes influences their performance. The xenobiotics are commonly catalysed by extracellular enzymes, which are deliberately released by the cells into their nearby environment.

The utilisation of cell free enzymes extracted from the cells has been always an advantageous one. The cell-free enzymes have unique catalytic sites for the specific reaction and have the ability to bio-remediate recalcitrants or substances toxic to active bacterial cells. Extracellular enzymes from the class of oxidoreductases and hydrolases may exhibit degradation of complex structures to simpler forms or oxidizing products which can easily be absorbed by bacteria. Incomplete oxidation of PAHs by extra cellular oxidative enzymes gives rise to ionic products with increased polarity and solubility (Meulenberg et al. 1997). Various iso-forms of oxidoreductases and hydrolases, extracted from cells of soil *Pseudomonas* sp. and *Bacillus* sp. have significant role in the biotransformation of xenobiotic molecules (Tabatabai and Fu 1992). Modern molecular techniques are helpful in monitoring the health of environment, reducing various pollutants into nonhazardous forms and establishing protocols for disposal of contaminants from industries. Genetic modification of indigenous bacteria or engineering catalytic proteins facilitates removal of contaminants (Fig. 13.2).

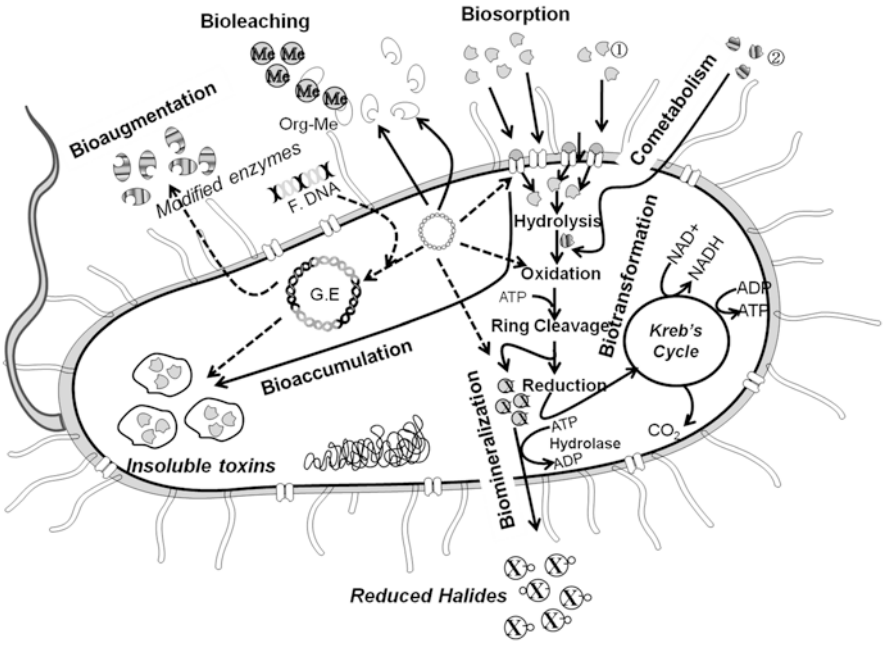

Fig. 13.2 Different processes exhibited by diverse soil bacteria in remediating persistent agrochemicals. (Insoluble Metal, Organo Metal, Geneticaly engineered plasmid, Pesticide 1, Pesticide 2, Non reduced Halides, Reduced Halides, Cell surface proteins, Modified enzymes, Foreign DNA)

13.5.3 Genetically Modified Organisms

Microbial biodegradation is an ecofriendly, nontoxic, economical method of removing pollutants (Amarger 2002). Due to developments in microbial genetics, construction of new strains with pollutant eradiating capabilities have been made possible and are grouped as Genetically Modified Microorganisms (GMMs). In comparison to indigenous or natural strains these genetically modified organisms are more efficient in bioremediating in-situ and can mineralize various organic pollutants (Cases and de Lorenzo 2005). The *E. Coli* bacteria are genetically modified for degrading organophosphate pesticides by successfully inserting genes for secretory organophosphorus hydrolase enzyme (Mulbry and Kearney 1991; Chen and Mulchandani 1998). Similarly, consortia of *E. coli* microbes with plasmids carrying 2,4-D degrading genes accelerates degradation of 2,4-D by native soil bacteria (Top et al. 1998).

The *P. fluorescens* HK44 with a combined gene of *lux* to naphthalene-degradative proteins were the first GMMs that are approved for field trials in the United States (Ripp et al. 2000; Strong et al. 2000). Even the *P. putida* KT2442 is genetically modified to carry three different properties; *E. coli* S17-1 (pTn*Mod*-OTc) provides tetracycline resistance gene, *Pseudomonas* sp. 142NF (pNF142) enables it to degrade naphthalene and *P. putida* KT2442 provides *gfp* gene acting as a bioreporter. In 2001, Lipthay et al. (2001) reported the degradation of 2,4-D by *Ralstonia eutropha* and *E. coli* HB101 carrying pRO103 plasmid which bear genes for 2,4-dichlorophenoxyacetic acid/2-oxoglutaric dioxygenase. Rodrigues et al. (2006) have successfully developed two GMMs strains, *Rhodococcus* sp. RHA1 (pRHD34: *fcb*) and *Burkholderia xenovorans* LB400, to degrade mixture of PCBs and Aroclor 1242 in soil. In some cases the GMMs can also be applied in promoting plant growth. Thus, these GMMs are enabled with multiple functions from degrading soil pollutants to promoting agriculture production.

13.6 Benefits and Limitations of Microbial Remediation

Microbial degradation is the major process involved in the disappearance or detoxification of pesticides. During *in situ* bioremediation, the nutrients or substrates added to the contaminated site or environment modify or stimulate the growth of indigenous or GMMs to increase the rate of degradation. Most of the cases *in situ* bioremediation being most cost effective as it minimizes disruption of remediation site. Non-disturbance of the soil will insure soil integrity and fertility. In addition, the *in situ* bioremediation has other advantages like contaminants are usually converted to innocuous products or destroyed i.e. not just simply transferred to different environmental site, nonintrusive thereby potentially allowing continued use of site and relatively easy to implement. The technology required to multiply microorganisms may be ideally suited for small business development. While in *ex situ* bioremediation, the polluted materials are collected from polluted sites and treated with requisite microorganisms (a consortium of microorganisms) at a designed place/site. This has been most successful in bio-remediating water. Most instances, it is more controlled compared to *in situ*, and the process can be improved with enrichment of microbes.

Bio-remediation, although considered advantageous in mitigating present day environmental pollution situations, has some limitations and can be considered problematic to use. As the bioremediation is carried out by microbes, the conditions must be ambient for microbial activities which make the process venerable to physiochemical changes. If the process is not controlled, there is possibility that the partially broken down contaminants may become more toxic and mobile than the original pollutants. Continuous site monitoring is needed to track the speed and status of biodegradation of the organic contaminants. In ex situ process, volatile organic compounds (VOCs) are difficult to control. Being dependent on biological activity the treatment time is generally longer than other less effective physical pro-

cess. Evaluating performance efficiency is difficult because there are no universal guidelines defining the level of a "clean" site and therefore there is variation in performance regulations. Even after certain time recombinant microbes are difficult to remove from the site of application and always there is apprehension about potential damage may caused by the modified organism than the pollutant itself.

13.7 Future Prospects

The bioremediation biochemistry of detoxifying agrochemicals or pesticides is more fascinating, multi disciplinary, active and challenging area of scientific research. The microbial remediation to improve the fertility of soil as well as removing the soil contaminants extensively used in developed countries. India is also progressing towards the application of microorganisms for the restoration of POP contaminated soil. Moreover, developing countries need extensive research programs to increase the capabilities of bioremediation. The present chapter has discussed and highlighted key bacteria that are involved in degrading key groups of pesticides or agrochemicals and underline the role of biotechnology in remediation process. However, much of the research has to be done to improve microbial inventories and biochemical pathways involved in degrading persistent agrochemicals. A key aspect, in bioremediation process is to understand and use of chiral compounds in these techniques. In addition, developments of sophisticated physico-chemical property based models and creation of database for physicochemical property will be highly beneficial. Explicit studies are needed to quantify the effects of POPs on humans and bigger animals of the food chain. Apart from this research on molecular modeling of biodegradation, transformation and mechanisms of toxicity have to hasten. The on-going microbial genomics studies will guide development of newer technologies for the remediation of contaminated water and soil.

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Part II
Application of Microbial Technology in Pharmacology

Chapter 14
Insectivorous Plants of India: Sources of Bioactive Compounds to Fight Against Antimicrobial Resistance

Sanjeet Kumar, Sunil S. Thorat, Rajendra K. Labala, and Jayanta Kumar Patra

14.1 Introduction

Biodiversity has always been an interesting and untamed parts of the earth for human beings even they are much closer to each other. Many of its parts have unexplored and from primitive to modern advance era, human beings are trying to unfold the rational of its magic in the form of literature, traditional, cultural, folk and science. Among the magical parts of biodiversity, plants play the role of a leader. Diversity of plant species creates a rainbow in our mind having millions of colour compositions. Human beings gave the name of a plant species as per their behavior, utilization, habitat and distinct features. Commonly herbivorous consume the plant species but some group of plant species consume the small animals/insects and play the role of predators in living kingdom of biodiversity.

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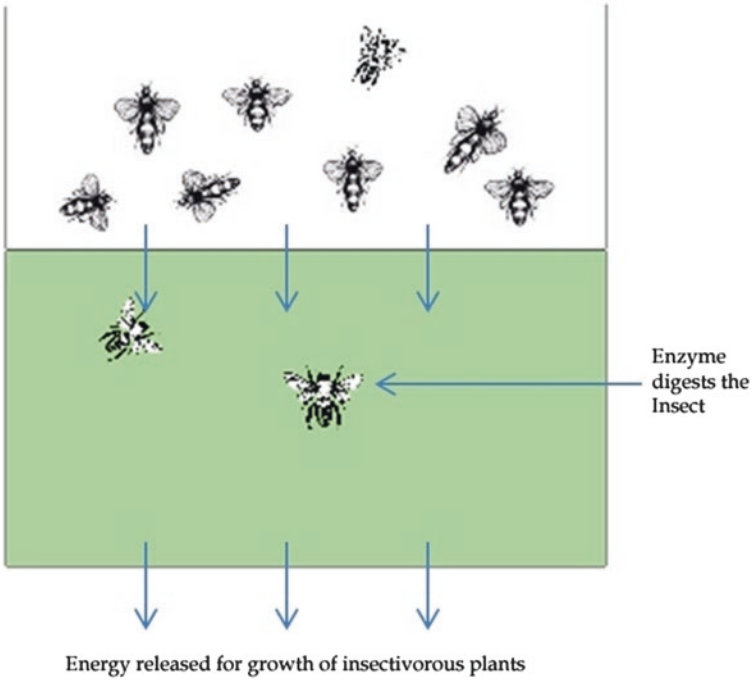

Fig. 14.1 Enzymatic activity gives nutrient to insectivorous plants for growth

Such plants are not killer but they use some bio-molecules who will help to get nutrient from the animals for appropriate growth. In the mentioned rainbow of the plant diversity, these are known as insectivorous plant species. They do not only consume the insects but some plant species like *Nepenthes* species also consume spiders, amphibian, critters, small reptiles and small mammals for proper growth (Fig. 14.1). For the above behavior, they have modified their parts as a trapper during the evolution. They also show the mutualism behavior as larva of mosquito species develop in the pool of trapping parts of these plant species. They are usually found in the regions which are low in nitrogen, phosphorus and potassium concentrations. For the above information, human beings took decades to gather the behavior of these plant species. The scientific concept and mechanisms of the insectivorous plant species started from the work of Auslasser in the year 1479. He reported about the *Pinguicula* then De Lobel reported *Sarracenia* in the year 1576 and *Utricularia* in the year 1591. Many other researcher given a look on such plants like Ellis on *Dionaea* in the year 1769, Roth on *Drocera* in 1780, Bartram on *Sarracenia* in 1791, Korthals on *Nepenthes* in 1839, Hooker on digestive enzymes of *Nepenthes* and finally in detail on insectivorous plants by Darwin in the year 1875 (Wolf and Heinz 2004; Dash 2016). India enjoys the rich floral diversity and about 40 plants species are reported as insectivorous or carnivorous plants. They belong to mainly three families, Droseraceae, Nepenthaceae and Lentibulariaceae. There are 3 species

Fig. 14.2 Some common insectivorous plant of India, (**a**) *Drosera indica* (photo credit: Shashikant S. Naik, Goa), (**b**) *Drosera burmannii* (photo credit: Rakesh Kumar Mohalik), (**c**) *Drosera peltata* (photo credit: Jesuraja Bakthan Daniel), (**d**) *Nepenthes khasiyana*, (**e**) *Utricularia aurea*

which belongs to Droseracea family, 1 species belongs to Nepenthaceae and 36 species belongs to Lentibulariaceae. The most common insectivorous plants are *Drocera burmanii* (Fig. 14.2b), *Drocera indica* (Fig. 14.2a), *Drocera peltata* (Fig. 14.2c), *Utricularia aurea* (Fig. 14.2e), *Utricularia bifida, Utricularia hirta, Utricularia scandens, Utricularia pubescens, Utricularia reticulata, Nepenthes khasiana* (Fig. 14.2d) etc.

They are not only the part of biodiversity but also protect human beings from the lethal insects like mosquito, midges, deerflies, horseflies etc. They have sound medicinal values. In India, *D. indica, D. burmannii* and *D. peltata* are used as main components in the Ayurvedic preparation Swarnabhasma which is used in diverse clinical manifestations including the memory loss, eye problems, infertility, weakness and incidence ageing (Shaikh et al. 2016). In the year 2008, Etkin reported that all insectivorous plants are used against skin infections caused by fungus. *Sarracenia* species are used to cure muscular pain and Nepenthes species are used to treat

gastrointestinal problems while *Pinguicula vulgaris* are used to treat sores (Etkin 2008). *Drocera anglica* and *Drocera intermedia* are used against cough and to decelerate ageing. *Drocera peltata* is popular medicinal herb to cure dental caries (Didry et al. 1998). The above reports reveal that insectivorous plants have sound ethnobotanical and medicinal values.

The era of antibiotics during the twentieth century has substantially reduced the peril of infectious diseases. Nevertheless, over the years, there has been abate in microbial susceptibility to existing antimicrobial agents responsible for critical point drug resistance in healthcare departments and in communities. In fact the theme of the World Health Day 2011 was "Antimicrobial resistance: no action today, no cure tomorrow". It is a big problem in pharmaceutical industry and for future health care. Recently different researchers have reported the urgent need for novel antimicrobial agents to replenish the arsenal of anti-infective agents or drugs. It is reported that, average two or three antibiotics are launched each year. After a downturn in recent decades, the pace is again quickening as scientists realize that the effective life span of any antibiotics is limited. Therefore there is a need to know more about antimicrobial resistance (AMR), its action mechanisms and screening of secondary metabolites from biowealth. Plant materials are of wide use in traditional systems of medicine, and in several local communities throughout the world (Kumar et al. 2013, 2017; Kumar and Jena 2014).

They are the only resources available for the treatment of different microbial infections among the tribal communities of wild. Most of the common plants are reported for their antimicrobial activity, still numbers of unexploited wild plants are available in forest having good ethnic values as traditional medicine. The medicinal potential of insectivorous plants indicates that these plants could be a good sources of bioactive compounds to fight against the antimicrobial resistance (Kumar et al. 2017). Hence they are sound for the human health and therefore, there is an urgent need to explore such plant species, documentation of their pharmacological properties, bioactive compounds present in the parts of insectivorous plants is a need of the hour. Keeping this in mind an attempt has been made to gather all medicinal values and the associated bioactive compounds present in insectivorous plants of India.

14.2 Insectivorous Plants

The plant species which consume the small insects or small animals for their growth are called Insectivorous plants or Carnivorous plants. They are mostly known as moist habitat and nutrient –poor soil habitat plants. Except some genera, they are annual to perennial herbaceous with tuberous roots. They also show the presence of fibrous roots and sometime vertical stolon attached with roots. They are terrestrial, epiphytic, saprophytic, aquatic and climbing shrubs. The genera, *Genlisea* and *Utricularia* are rootless. Genera *Pinguicula* produce single, ephemeral root. The leaves of insectivorous plants may be whorled, heterophyllous, scattered on stolons and alternate. The genus Lentibulariaceae show scapose raceme having one – many

Table 14.1 Most common insectivorous/carnivorous plant of India

Family	Genus	Species	Habitat
Droseraceae	*Drosera*	*Drosera burmannii*	Herb
		Drosera indica	Herb
		Drosera peltata	Climbing herb
Nepenthaceae	*Nepenthes*	*Nepenthes khasiana*	Climber
	Pinguicula	*Pinguicula alpina*	Herb
	Utricularia	*Utricularia aurea*	Floating herb
		Utricularia bifida	Herb
		Utricularia exoleta	Herb
		Utricularia hirta	Herb
		Utricularia minutissima	Herb
		Utricularia polygaloides	Herb
		Utricularia praeterita	Herb (near threatened)
		Utricularia pubescens	Herb
		Utricularia scandens	Herb
		Utricularia stellaris	Large sized herb
		Utricularia striatula	Herb
		Utricularia uliginosa	Herb

flowers, Droceraceae show scapose, axillary or terminal and Nepenthaceae show racemes or panicles. The flowers of insectivorous plants are bisexual, actinomorphic, nectarines glands present, diverse colour with aromatic secretions. They are found in damp heaths, swamps, muddy, sandy shores and bogs. Their distributions are Canadian arctic, Scandinvia, Greenland, Malaysia, Australia, Brazil, India etc. They are not found in Antarctica (Barthlott et al. 2007; Dash 2016). Approximately 700 species of insectivorous plants under 20 genera of 12 families are reported (Fleischmann 2012; Dash 2016).

14.2.1 Insectivorous Plants of India

India is rich with diverse floral species. Among them insectivorous plants are quite interesting which are belongs to three families (Droseraceae, Nepenthaceae and Lentibulariaceae). In the year of 2012, Kamble et al. reported 44 species of insectivorous plants under 5 genera available in India (Table 14.1). They are found in different ranges like *Drosera* species are found in crop fields, plains, near water bodies; *Pinguicula* species in the altitudes of the Himalayas; *Nephenthes* (*Nephenthes khasiana*) found in North-Eastern Part of India which is also endemic to India; *Utricularia* species mostly found in water bodies. Many researchers reported the insectivorous plants of Indian regions such as in 1965, Saxena reported that *Utricularia pubescens* from Orissa, in 1994, Saxena and Brahmam reported 14 species of insectivorous/carnivorous plants of Orissa, again Dash reported two more

species of *Utricularia* (*U. reticulate* & *U. praeterita*) in the year 2009 and 2013 (Dash 2016) from Orissa. In the year 2008, Natarajan et al. reported six insectivorous plants (*D. burmanii, D. indica, U. bifida, U. hirta, U. minutissima and U. polygaloides*) from the Tamil Nadu while in the year 2009, Barbhuiya et al. reported *Drosera* species from Barak valley of Assam. Mohapatra et al. (2009) reported *Utricularia* species at Yellagiri of Tamil Nadu state whereas in the same year Madhavan et al. *Drosera* species from Karnataka. Mitra and Mukherjee (2010) also reported *Drosera* species from West Bengal. Gokhale et al. (2011) reported *Drosera* species from Maharashtra. Shaikh et al. (2016) reported *D. indica* from the state of Rajasthan.

14.3 Trapping Action and Chemistry of Insectivorous Plants

Insectivorous plants show diverse action and mechanisms to trap the prey. As per the effectiveness to prey species, two types of trapping systems are observed. These are:

(a) Active traps
(b) Passive traps

Active trap: in this trap system, plant species do not move physically during the trapping of prey. It has two actions, Pitfall trap (trapping prey in a leaf containing a pool of enzymes) and Flypaper or Sticky trap (trapping prey using sticky mucilage).

Passive trap: in this trap system, plant species move their trapping parts. It has three actions, Snap trap (rapid leaves movement), Bladder trap (trapping insects or microorganisms) and Lobster pot trap (force to prey to move).

Above mentioned, trapping behavior, insectivorous plant species consume the prey for the proper growth (Figs. 14.3, 14.4, and 14.5). Rational behind the consumptions is the chemistry associated with these plant species. They possess some unique enzymes and biomolecules which break the prey into nutrients and do easy digestion of prey. The most common biochemical agents are acetic acid, propionic acid, butyric acid and valerianic acid (Dash 2016). Some insectivorous plant species have both trapping organ to trap the prey and do photosynthesis with photosynthetic organs simultaneously (Adamec 2010). In the year 1972, Amagase et al. demonstrated the role of chitinases as a killer component for the prey of *Drosera* and *Nepenthes* species whereas in the year 1974, Zoltan et al. reported that an enzyme nepenthesin which is similar to pepsin is responsible for the digestion of prey in *Nepenthes macferilanei*.

In the year 2006, Eilenberg et al. showed that Ia and Ib chitinases are up regulated in response to prey and secreted into the pitcher fluid to digest the prey in *Nepenthes khasiana*. Recently Linda et al. (2016) reported the most effective enzymes in the *Nepenthes* species are proteases, nucleases, peroxidases, chitinases, phosphatase, glucanase, aspartic proteases, nepenthensis, prolyl endopeptidases, neprosins, nephthoquinones (Fig. 14.6) and serine carboxypeptidas.

Fig. 14.3 Trapping parts and flower of *Drosera peltata*, (**a**) Prey trapped, (**b, c**) Tentacles or trapping organ, (**d**) Flower (Collected from Chakrata, Uttarakhand, dt. Aug. 2013 by Rajesh Sachdev)

Fig. 14.4 Collection and morphological characterization of *Utricularia aurea*, (**a**) Collection from Odisha, (**b**) Vegetative parts, (**c**) Roots, fruits and Flowers

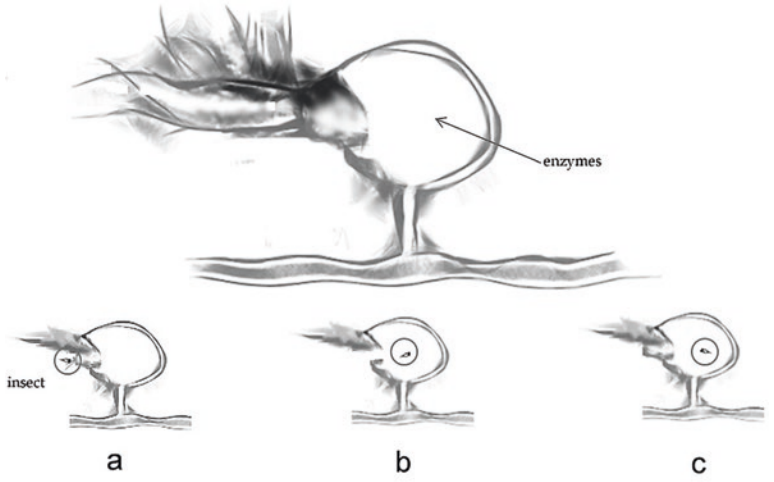

Fig. 14.5 Trapping behavior in *Utricularia* species

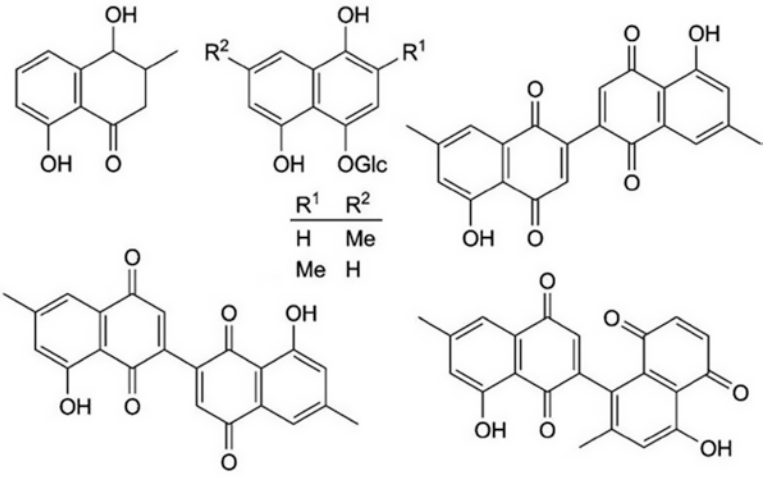

Fig. 14.6 Some common chemical compounds found in insectivorous plant species

14.4 Medicinal Values of Insectivorous Plants

Human beings developed skill to screen the plants for certain medicinal uses from primitive. The skills are called traditional therapeutic systems or ethnobotany. One question arise in our mind that how a primitive human beings screened the plants as per their utilization in various diseases and disorders. The answer we authors of this chapter got from the literature and first author got from the survey too that before the screening they had been observing the visible plant defense mechanisms. Now, it is scientifically proved that rational of medicinal properties of any taxa is defense mechanisms. Keeping the above texts, Insectivorous plants should have the medicinal values because it traps the prey for their growth. Literature has many reports on the medicinal values of insectivorous. Details are discussed below.

14.5 Ethnobotany of Insectivorous Plants

Ethnobotanical values of plant species is the base of modern clinical biology and drug development. India is rich country in terms of plant diversity which gives sound ethnomedicinal plants and their formulations to cure various diseases and disorders. Among the floral wealth of the country, Insectivorous plant species show unique ethnobotanical values practiced by the various local communities. *Drosera burmunnii* is reported to use for curing loss of memory (Raju and Christina 2013). Authors found that tribal communities of Jharkhand use *Drosera burmunnii* against the cough and same claims were found from the tribal communities of Sundargarh, Odisha. Mitra and Mukherjee (2010) also reported that whole plant paste of *Drosera burmannii* is used to cure blood dysentery among the local of North Bengal of the state West Bengal and same claim was reported by Vaidyanathan et al. (2013) that Malayali tribal communities of Koli Hills of Eastern Ghats, Tamil Nadu used *Drosera burmannii* against blood dysentery. Singh et al. (2011) reported that Garo tribe of Nokrek Biosphere Reserve, Meghalaya use *Nepenthes khasiana* for the treatment of urinary problems, itching of the eye, night blindness and cholera while Ramashankar et al. (2015) reported that *Nepenthes khasiana* is used to cure cuts in Nagaland. Rao et al. (2011) also reported that Gadabas tribe of Visakhapatnam, Andhra Pradesh used the *Drosera burmannii* against stomach problems. Divakar et al. (2013) reported that Madayipara hillock tribals of Kerala use the *Utricularia reticulata* against urinary problems. In the year 2015, Deka and Devi reported that *Utricularia aurea* (Fig. 14.4) is used to cure diseases caused by mosquito. All above reports reveal that, an insectivorous plant found in India has sound ethnobotanical values.

14.6 Antimicrobial Activity of Insectivorous Plants

The ethnomedicinal values of insectivorous/carnivorous plants indicate that plant parts posses antibacterial and antifungal potentials. Didry et al. (1998) showed that *Drosera peltata* extracts inhibited the growth of oral bacteria like *Streptococcus*. Ferreira et al. (2004) reported the antimicrobial activity of *Drosera* species against *Staphylococcus aureus, Enterococcus faecium, Pseudomonas aeruginosa, Escherichia coli, Salmonella choleraesuis, Klebsiella pneumonia* and *Candida albicans*. In the year 2007, Shin et al. reported the antifungal activity of *Nepenthes* species against *Alternaria alternate, Aspergillus niger, Bipolaris oryzae, Fusarium oxysporum, Phytophthors capsici, Rhizoctonia solani, Rhizopus stolonifer* var. *stolonifer* and *Sclerotinia sclerotiorum*. In the year 2012, Taraszkieewicz et al. reported the antibacterial activity of *Drosera* species against *Pseudomonas syringae*. Buch et al. (2013) reported that the trap fluid of *Nepenthes* species inhibit the growth of bacteria. In the year 2014, Gwee et al. reported the antifungal activity of *Nepenthes* species against *Candida albicans, Issatchenkia orientalis* and *Trichophyton mentagrophytes*. Krychowiak et al. (2014) reported the antibacterial activity of *Drosera* species against *Staphylococcus aureus*. Bhau et al. (2015) reported the antimicrobial activity of leaf extracts of *Nepenthes khasiana* against *Escherichia coli, Candida albicans* and *Aspergillus niger*. In the same year, Ismail et al. (2015) showed the antimicrobial activity of *Nepenthes* species against *Staphylococcus aureus, Bacillus subtilis, Bacillus spizizenii, Candida albicans* and *Saccharomyces cerevisiae*. Kumar et al. (2016) reported the antimicrobial activity of *Drosera* species against *Staphylococcus aureus, Klebsiella pneumonia, Staphalococcus pneumoniae* and *Aspergillus niger*. Majeed et al. (2016) reported the bacterial activity of *Nepenthes* species against *Escherichia coli, Salmonella enterica* and *Staphylococcus aureus*. All above reports and studies revealed that the insectivorous/carnivorous plants have sound antifungal activity which could help to fight against the resistance problems.

14.7 Other Pharmacological Values of Insectivorous Plants

The ethnobotany and their antimicrobial activity show that these plants have other pharmacological values. Many reports are cited in literature. Recently, Thanh et al. (2015b) reported that *Nephenthes* species show the antioxidant activity and it also showed the anti-osteoporotic activities using murine osteoclastic RAW 264.7 cells. Again Thanh et al. (2015a) reported that *Nephenthes* contain a flavonoid which is responsible for anti-oxidant activity. Ismail et al. (2015) also showed that *Nepenthes* plant parts extracts have antioxidant activity whereas Uriah et al. (2015) showed the antioxidant and hepatoprotective potential of *Nepenthes khasiana* against ethanol induced liver injury in rats. In the year 2012, Banasiuk et al. documented that *Drosera* species is good against cancer cells. Recently Singh and Bajwa (2017) reported that *Drosera* species showed antispasmodic effect and anticancer activity. Choosawad et al. (2005) reported the antioxidant activity of *Utricularia aurea*.

Table 14.2 Some insectivorous plants of India and their uses and co-relation with bioactive compounds for future drug development to fight against antimicrobial resistance

Plant name	Traditional claim	Responsible bioactive compounds	Probable activity	Future drugs
Drosera burmannii	Blood dysentery	Saponin, tannin	Antibacterial	Antibacterial agents for *E. coli*
Drosera indica	Cough	Terpenoids	Analgesic	Against oral bacterial infections
Drosera peltada	Rheumatism	Flavonoids, steroids	Antibacterial	Antibacterial agent
Nepenthes khasiana	Cure cuts	Flavonoids, saponin	Anti-infection	Antimicrobial agent
Pinguicula alpina	Swelling	Flavonoids	Antibacterial, anticancer	Antibacterial agent
Utricularia aurea	Malaria	Digestive enzymes	Stomach problems	Digestive agent
Utricularia bifida	Inflammation	Flavonoids	Anti inflammatory	Antibacterial agent
Utricularia exoleta	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia hirta	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia minutissima	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia polygaloides	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia praeterita	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia pubescens	Diuretic	Lack of work	Lack of work	Lack of work
Utricularia scandens	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia stellaris	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia striatula	Lack of claim	Lack of work	Lack of work	Lack of work
Utricularia uliginosa	Lack of claim	Lack of work	Lack of work	Lack of work

14.7.1 Medicinal Properties vs Bioactive Compounds

The above all reports and claims indicate that the insectivorous plants posses certain compounds or group of compounds which are responsible for their bioactivity and medicinal values. Their medicinal values revealed the presence of probable compounds are presented in the Table 14.2.

14.8 Recommendation for Research and Conservation of Insectivorous Plants of India

The above all reports, study, experiments revealed that there is an urgent need to do population inventory of the carnivorous plants found in different regions of India and have to categorized under different threats defined by the IUCN. This step will help to give attention on the particular species region wise. As they show unique characters, hence need advanced studies on the trapping biology, physio-kinetics and chemo-kinetics. Such study could provide us beneficial information for further advanced research on insectivorous/carnivorous plants. As the present study indicated that they are used as therapeutic medicines and posses antimicrobial activities, it needs proper screening in terms of reverse pharmacology for the development of new antimicrobial drugs to fight against the Antimicrobial Resistance.

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Chapter 15
Exploring the Multifaceted Role of Microbes in Pharmacology

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15.1 Introduction

The accidental invention of penicillin in 1929 by A. Fleming has provided a pave to explore the pharmaceutical potential of microbes. The economic utility of microbes has gained attention of different pharmaceutical industries as unique features of microbes make them inimitable, industrially compatible hosts and real workhorses for large-scale production of structurally diverse and valuable products. Such products include antibiotics, enzyme inhibitors, enzymes, vitamins, peptides, organic acids, vaccines, polysaccharides having applications in the field of medicines to improve health. Moreover, in order to achieve efficient and biologically compatible delivery of chemotherapeutic agents, antibiotics, therapeutic proteins, and biomolecules, the biofilm producing drug-carrying microbes have been utilized these days to deliver antibiotics/drugs to the specific site (Martin and Averous 2001; Sinclair and Elliott 2005; Walsh and Jefferis 2006; Jenkins 2007). Microorganisms can detect a wide range of chemical analytes, having acquiescence for genetic modifications and wide operating range of pH and temperature, which make them ideal for bio-sensing (Kim et al. 2001). These features of microbes have been exploited in developing microbial biosensor having widespread applications in biomedical

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research, health care and pharmaceuticals. The advancements in metabolomics, bioinformatics, genetic engineering, proteomics technologies have driven microbial genome mining field to discover novel products. With this background, the present chapter aims to provide an insight into the various applications of microbes in pharmaceutical industries, their present status and future prospects.

15.2 Microbial Cell Biofactories for Value Added Products

Microbes epitomize diverse source of different metabolites possessing bioactivity and have been utilized in production of various important products useful in pharmaceutical industry including antibacterial agents, aminoglycosides, cephalosporins, immunosuppressive agents, tetracyclines, and antiparasitic drugs etc. (Buss and Waigh 1995; Hollister and Jarvis 2001; Hollister et al. 2002; Aumiller et al. 2003).

Marine microbes are another rich source for metabolites. The sustainable and cost-effective availability of the active pharmaceutical ingredient (API) can be easily attained through microbial fermentation techniques as compared to production by slower growing macroorganisms (Lueschet al. 2001; Harrigan et al. 1998). For example, Salinosporamide A can be synthesized using the marine actinomycete *Salinispora tropica* (Feling et al. 2003). However, bryostatin was originally analyzed from the bryozoans *Bugula neritina*, but it is also observed to be synthesized by the uncultured symbiotic bacteria (Davidson et al. 2001; Trindade-Silva et al. 2010). Trabectedin, a secondary metabolite, which has been approved for treating cancer in European Union (EU), is synthesized by symbiotic bacteria. Trabectedin and Zalypsis have ring systems similar to saframycin synthesized by bacteria, and the partial synthesis of Trabectedin is based on cyanosafracin B produced by *Pseudomonas fluorescens*. Marine microbes have a capability to provide a balanced supply of starting materials for the synthesis of distinctive class of drugs.

U.S. Department of Agriculture, Rural Business Development states that changing the physical state of a product to the other, results in the production of value added products. This process enhances the value as well as the quality of the product. This concept is exploited in the marketing approach of the product enabling the consumer to select the product of interest from a wider range of improved quality and thus generating large revenues for the producer.

Pharmacists have integrated the fundamentals of microbiology and human cell mechanisms, which allowed them to form the interlinking discipline between human cell mechanisms and microbiology. It has led to the development of various value added products such as antibiotics, anti-cancerous drugs, and immunosuppressive agents, and nutraceuticals (Ghosh et al. 2008). The resultant products are site-specific and are eventually biocompatible. Further, metabolic engineering provide the ways to enhance activity and yield of the target compound along with novel activities (Du et al. 2011). Some of the products are discussed in this section below.

15.2.1 Ecomycins

Ecomycins are majorly produced by *Pseudomonas viridiflava* (EB273) which is an endophytic bacterium comprised of amino acids such as homoserine and β-hydroxy aspartic acid. *P. viridiflava* strains EB274 and EB277 found in USA and Israel respectively are also known to produce antifungal lipopeptides (Miller et al. 1998; Harrison et al. 1991). Ecomycin A, B and C are varieties of antifungal lipopeptides that are produced by this organism (Christina et al. 2013). Out of which, Ecomycin A has been reported as an antibiotic syringotoxin (Ballio et al. 1990).

15.2.2 Munumbicins

Munumbicins exhibit a wide range of activity against pathogenic organisms like bacteria, fungi and few plasmodium species. They are extracted from *Streptomyces* NRRL 30562 (USDA national Laboratory), which is an endophytic bacteria found in *Kennedia nigricans* (Christina et al. 2013). They are known to affect many Gram-positive bacteria and some drug resistant strains such as *E. faecalis* and *S. aureus* (Castillo et al. 2002). Munumbicins E-4 and E-5, extracted from Streptomyces NRRL 30562, are active against both Gram-positive and Gram-negative bacteria (Christina et al. 2013; Castillo et al. 2006).Munumbicins are divided into a category of four bioactive substances namely A, B, C and D. Among four bioactive substances, B, C and D are less sensitive against human pathogenic fungi (Christina et al. 2013).

15.2.3 Alkaloids and Terpenoids

Nutaceuticals can be derived from plants in the form of phytochemicals, which includes compounds like alkaloids, terpenoids, polyphenolic compounds and their derivatives. Alkaloid, a phytochemical used to produce nutraceuticals is an important anti-cancerous agent and possess anti-malarial effects. The engineering of Benzylisoquinoline alkaloids biochemical pathways in *E. coli* and *S. cerevisiae*, has enabled its pure synthesis in a cost-effective manner with better yields (Wang et al. 2016; Nakagawa et al. 2011). Terpenoids possess anti-cancerous and anti-inflammatory properties and tetra- terpene carotenoids, for example, a-carotene, b-carotene and lutein serve feed supplements in nutraceutical industries (Wang et al. 2016; Marienhagen and Bott 2013). Its biosynthesis demands the metabolic engineering of respective biochemical pathways in *E. coli* and *S. cerevisiae*. When metabolic routes of *E.coli* are engineered to enhance the levels of ATP and NADPH, b-carotene production increased to 2.1 g/L with a yield of 60 mg/g DCW (Zhao et al. 2013). In addition, *E.coli* and *S. cerevisiae* serve as the basic organisms for the

biosynthesis of many polyphenolic compound such as flavonoids and isoflavonoids (Misra et al. 2006; Wang et al. 2016).

15.2.4 Acremonin and Xanthone

Reaction of free radicals such as hydroxyl and peroxyl species as well as nitrogen and oxygen species results in diseases like atherosclerosis and cancer. Compounds such as Acremonin A derived from marine fungi *Acremonium sp*. and Xanthone from *Wardomyces anomalus* serves as naturally occurring anti-oxidants used in therapy and food supplements (Abdel-Lateff et al. 2003, 2005).

15.2.5 Dietary Supplements of Lactobacilli *sp.*

The dietary supplements of *Lactobacillus sp*. are known to evade colon cancer by activating anti-inflammatory cytokines, for example IL-10 and thereby modulating the mucosal immune system. This influences dendritic cell maturation in addition to cell proliferation and apoptosis (Devine and Marsh 2009).

15.2.6 Recombinant Proteins

Proteins act as catalysts of metabolic changes, structural components and are involved in different cellular interactions and cell signaling events acute for life. Hence, decrease in the expression of specific polypeptides or production of mutants result in mild to severe disorders in the body. These disorders can be treated through clinical administration of specific proteins produced using external sources, (Manning et al. 1989). Recombinant DNA (rDNA) technology represent a technical platform for the controlled production of polypeptides of interest. In early 1980s, FDA approved the utilization of human insulin produced from recombinant *E. coli* for diabetes treatment (Fig. 15.1) (i.e. Humulin-US/Humuline-EU) (Redwan 2007). In the past decade, several other sources of recombinant polypeptides have been developed and are being approved for their clinical applications.

15.3 Microbial Assisted Biosensors for Diagnosis

Biosensor is a device that can detect biochemical changes and transmit it in the form of electrical signals. Fundamentally, biosensor is a probe that assimilates a biological component with a transducer that converts biochemical signals into optical,

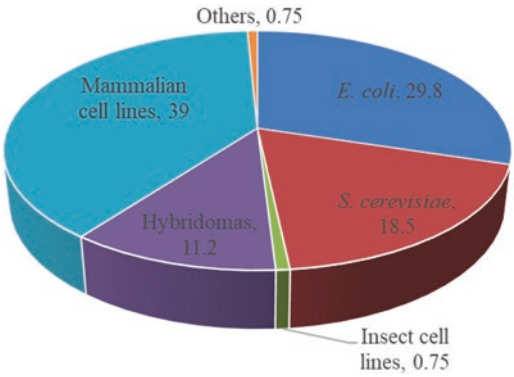

Fig. 15.1 Percentage of recombinant proteins approve for use as pharmaceutical applications with their production systems (Modified from Redwan 2007)

electrochemical, electronic and acoustic signals. The commonly used biological sensing elements in a biosensor are enzyme, DNA, antibody, organelles receptor, and microorganism. Biosensors can further be classified based on the utilization of biological sensing element or the transducer showing broad variety of applications in research, medical diagnostics and industry (Rogers and Gerlach 1999; Nissim and Chernajovsky 2008; Mohan et al. 2008).

15.3.1 Whole Cell Biosensor

Bioreceptors denote viable genetically modified microbial cells for production of measurable signals in response to chemical signals. Bioreporters consist of two important genetic elements, promoter and reporter genes. The promoter gene is transcribed in the presence of target stimulant in the environment and is interconnected to other genes that are transcribed and then translated into proteins (Struss et al. 2010). In bioreporters, these genes are replaced by a reporter gene and activation of reporter gene results in production proteins that further produces a detectable signal. They have a number of applications including contaminant detection in environment, medical diagnostics, food safety assurance, process monitoring control and bio-microelectronic computing (Ivnitski et al. 1999; Hoffmann et al. 2015; Wang et al. 2013).

In the past decade use of intact cells as recognition element in biosensors is continuously increasing (Kintzios 2007; Su et al. 2011) and such devices are termed as whole cell or microbial biosensors. The merit associated with microbial biosensors is that, whole cell immobilization enhances the stability of the recognition element by retaining it in its natural environment. Microbial biosensors are reported to have sensitivity and selectivity because of the present cell wall and membrane acting as a permeability barrier to unwanted interfering substances. Genetically engineered microorganisms provide a way for altering the selectivity and sensitivity of microbial biosensors (Rechnitz et al. 1977).

15.4 Microbial Polymers and Their Role in Drug Delivery

An enormous diversity of biopolymers, like polyamides, polysaccharides, polyesters, are produced by microbes (Mihai et al. 2001). They vary from different forms and their physical properties are hooked on the mass and constituents of the polymer. The recombinant DNA technology opens the door for innovating methods for gene manipulation of these microbes. Eventually, it leads to production of high valuable products for drug delivery. In recent times, application of microbial biopolymer has gained tremendous importance in many fields. These are macromolecules produced from polymerization of monomers derived from microorganisms. They are complex, large molecular entities, which differ by subunit of monomers. As an alternative source, they present the solution for many problems. As they are biodegradable, they tend to be alternative for different plastics. Many useful properties make them eco-friendly and user friendly. Some of their properties are biocompatible and are easily used in tissue engineering, and drug delivery (Hidalgo-Bastida et al. 2007). Due to their biodegradable nature, they can be certainly decomposed in the environment. Hence, their uses range from packaging industry, chemical industry, agriculture and medicine. Such biodegradable polymers can be grouped into two different types i.e. natural and synthetic polymers. Natural polymers are synthesized from natural processes whereas synthetic polymers are produced by chemical synthesis. Some of the natural polymers include collagen, which is a chief natural protein in animals made-up of repeats like proline, glycine to form a helical structure. As of now, only 19 types of collagen molecules are being used in various medical and pharma industry. Due to wide properties like biocompatibility and low antigenicity, collagen is preferred for drug delivery system. Some of the gels formed from collagen as a matrix are used for tissue engineering. Furthermore, these gels form 3D scaffold for gene therapy, cell culture, and persistence of transfected fibroblasts (Bleckwenn and Shiloach 2004). They help in formation of matrix by crosslinking of different agents like formaldehyde, carbodiimde etc. and under external treatments like heating. During the process, drugs are encapsulated in the liposomes first and then inserted in a collagen gels which increases storage stability, enhances the drug release and efficiency.

15.4.1 Gelatin

It is a type of biomolecule formed by uncoiling complex collagen protein. Gelatin has varied usage in different biomedical industries as it is environmental friendly, easily degraded in soil and does not cause harm to the humans. Further, due to its flexible isoelectric point, it is used in different environmental conditions by medical practitioners. It is utilized as a carrier material for gene delivery, some macromolecules and as matrix for scaffold structure. Due to its unreactive nature, being used as liposome mediated biomolecule delivery into PEGylated gel for sustained drug

delivery at target sites. However, due to some of the drawbacks like weak structure and inability to supervise the affected areas, its applications are still restricted in medical field. In one of the examples, protein serum albumin was attached to PEG and formed complex structure as hydrogels (Masci et al. 2002). These hydrogels are being used for drug transportation in tissue engineering, based on the affinity of these biomolecules to protein albumin in a complex state.

15.4.2 Chitosan

It is a polymer formed by polymerization of monomer N-acetyl glucosamine and glucosamine and produced by removal of acetyl group of chitin molecule from external covering of arthropods. It can also be utilized in pharmaceutical applications for delivering drugs, covering of tablets, and as an agent in tablets. Due to its important properties like nontoxicity, safe to use and lightweight, it has been used on coca-2 cell lines. During the removal of capsule using polymers, these biomolecules are prone to loss of activity and structure. Hence, in that case, the chitosan biopolymer is the best choice. Some of the gels based on chitosan and ovalbumin proteins are being used in biomedical applications. Some emulsions like creams or solutions can be prepared by mixing neutral surfactant like sorbitan ester with chitosan biopolymer. It can be used as bioadhesive at different pH values (Kurkuri and Aminabhavi 2004). Due to presence of amide and hydroxyl group, it forms hydrogen bonding. Because of these properties, chitosan can be used as mucoadhesive. Further, by addition of thiol groups in chitosan, the properties can be enhanced but its biodegradable nature is maintained. So, the biopolymer property of chitosan was changed by joining to thioglycolic acid (TGA) to the amino-group of chitosan by NH_2 bond. Although, the better property of bioadhesion increase the time of stay of these drugs at the target area. It also shows antimicrobial property by formation of complex between chitosan-TGA which could be used for antimycotics to treat mycotic problems in the vagina, for example, some microbial infections in the vulvovaginal tract by *C. albicans*. Most of the women have vaginal problems with Candida during their lifetime. Hence by addition of thiol groups, the affinity properties of this biopolymer would be enhanced. For instance, molecule of clotrimazole was added which allowed the interactive properties of biopolymer on to the surface.

15.4.3 Alginate

This is one of the other naturally occurring linear polymer that is produced from algae (seaweed), and bacteria (Borowitzka and Borowitzka 1988). Although, it seems to have complex chemical structure made up of small subunits to form both homo polymeric and hetero polymeric sequences. They are also frequently used by

researchers for biomolecules transportation and as matrix to scaffold. Due to some of its useful properties like low cost value, easily soluble in water, stable in nature it is used in various biomedical applications.

15.4.4 Cellulose Derivatives

Cellulose tend to be the widely available natural biopolymer. It is present in mostly all natural fibers and plants holds the major share in its abundance. Cellulose is made up of monomer anhydro-D-glucopyranose (AGU) having 3 OH groups per AGU units but not present at the ends. It is not soluble in water, some solvents due to the presence of inter molecular, and intra molecular hydrogen bonding between each chain. Despite its low solubility, it can be applied in various areas like packing, covering, printing paper, netting etc. However, some of the changes made to cellulose derivatives in order to enhance its characteristics and improve its applications in many industries. Some of the important cellulosic ethers are as follows: hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC), methyl hydroxyethyl cellulose (MHEC), ethyl hydroxyethyl cellulose (EHEC), methyl cellulose (MC), and hydroxylpropyl cellulose (HFC).

15.4.5 Starch

Plants have the ability to form starch and save it as energy source for future use. This can be stored in different forms as cells and small structures with different sizes between 1 and 100 μm. As cellulose, even starch is an important raw material in plant. Total world production of starch accounts to 58 million tonnes, where (46 million), comes from maize, (4.6 million) is extracted from wheat, (3.5 million) potatoes and further from cassava roots (tapioca) and rice. It holds to be the major carbohydrates in plants and energy source during time of development. As it is environmental friendly having efficient chemical properties, it holds great renewable source for food and other industries. The properties of starch have been largely studied and these are depended on different plant sources. It is defined as diverse and complex biopolymer made up α-D-glucose monomers. These anhydrous glucose units (AGUs) are formed by α-(1,4)-bonds and to lesser extent by α-(1,6)-bonds. It has two different forms: amylose and amylopectin.

15.4.5.1 Amylose

It is formed as linear biopolymer by joining of many (around 700) α-(1,4)-bonds to glucose units (up to 6000 AGUs), having 105–106 g mol^{-1} molecular weight. Initially these structures form single and double helices.

15.4.5.2 Amylopectin

Further, this is a branched molecule having 107–109 g mol^{-1} molecular weight. The structured polymer contains α-(1,4)-bonded glucose units and also α-(1,6)-bonds spreading to different points (i.e. after 10–60 glucose monomers) which means only 5% of the glucose subunits are branched. Also the starch is altered for its usage as pregelatinized starch bioproduct for simple sustained release matrix system. This process involves break down of potato starch by enzymes then precipitation, filtration and finally washing the ethanol.

They show various advantages for tablet preparation, sustained release (zero-order) for continuous time period and its property to incorporate many drugs with various physical chemical properties. These sustain rates of pre gelatinized starch tablets can be further improved or decreased to particular conditions by altering different conditions like tablet structures, compaction force and preparing different formulations.

15.5 Conclusion and Future Prospects

Under biopharmaceuticals, the most preferred source for its production include microorganisms; developing strain by using recombinant DNA technology, mutagenesis or metabolic pathway engineering. There is continuous rise in repository of therapeutic molecules involving the use of microbes, plants and animals from fresh source of water. Furthermore, by analyzing the specific data having whole-cell sensor properties, it might be plausible to mention the merits and demerits of microbial biosensors. Amalgamation of synthetic biology and metabolic engineering would add in the augmentation of drug discovery and biopharama production for treatment of disease efficiently in near future.

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Chapter 16
Pharmacological Applications of Metabolites of Mangrove Endophytes: A Review

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16.1 Introduction

Endophytes are represented by a diverse group of prokaryotic or eukaryotic organisms that form lifelong associations within tissues of plants without causing any apparent harm to the host plant. The association between microbe and the host plant can be explained under different symbiotic relationships ranging from facultative saprobiotic, parasitic, exploitive, mutualistic and commensalistic symbioses (Molina et al. 2012). The microbes function as the biological defence for the plant against different pathogens. The endophytes may attack any antagonists or lyse affected cells by releasing metabolites directly or by either inducing host defence mechanisms or promoting growth indirectly (Stone et al. 2000). In most of the cases bacteria and fungi are involved as endophytes, but in some cases microbes like mycoplasmas and archaebacteria are also involved as endophytes. It has also been cited elsewhere that blue-green algae can also be a potential endophytes, though no evidence has been reported yet (Mcinroy and Kloepper 1995; Fisher et al. 1992). It has been suggested that nearly 3 lakhs plant species that inhabiting the earth has been associated with one or more endophytes. It is being said that about one million of endophytes species are there but only few of them have been characterized till date (Strobel and Daisy 2003; Andrew and Hirano 1991).

Recently, studies on endophytes isolated from different medicinal plants have received attention because it is thought that plant with medicinal potentials may provide a good niche for the discovery of novel microbes which could produce new secondary metabolites (Strobel et al. 2004). Further, the secondary metabolites of

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endophytic origin extracted from medicinal plants have revealed that a wide variety of new and useful compounds can be exploited for various pharmaceutical uses such as antimicrobial, anticancer, antimalarial and antioxidant activities (Huang et al. 2007; Wiyakrutta et al. 2004). Endophytes have been isolated from all terrestrial plants from both temperate and tropical regions demonstrating their ubiquitous distribution throughout the world (Freeman 1904; Arnold et al. 2001; Frohlich et al. 2000). Since marine and coastal environments are being reported to produce greatly diversified secondary metabolites, they can serve as potential candidates for drug discovery. Therefore, it might be assumed that mangroves that inhabit marine ecosystems and produce outstanding natural products on their own can exploited pharmaceutically for development of drugs for treating a number of ailments. Mangrove derived endophytes are known to produce metabolites such as anthraquinones, eniatin G, and xyloketals, alkaloids, terpenoids, flavonoids etc. Furthermore, mangrove fungi constitute the second largest ecological group of the marine fungi, and many of them are new or inadequately described species and may produce extracellular polysaccharides with novel functions and structures that could be explored as a source of useful compounds. But, isolation effort of endophytes from mangrove plants is limited.

In this review we highlight the pharmacological potential of endophytes from mangroves and their contribution to drug discovery. Selected examples of new bioactive metabolites and known compounds with their biological activities are summarized here.

16.2 Phytochemistry of Metabolites Isolated from Mangrove Endophytes

Endophytes can produce the same or similar class of secondary metabolites in the host they reside. Since mangrove plants produce secondary metabolites which are chemically unique yet diverse in nature, it is expected that the mangrove derived endophytes would produce a wide variety of metabolites that are structurally unique and pharmacologically active. In fact, the chemical investigation of mangrove derived endophytes resulted in the discovery of various bioactive secondary metabolites including alkaloids, terpenoids, chromones, coumarins, polyketides, and peptides with diverse structural features. Few important phytochemicals derived from mangrove derived endophytes are discussed below.

16.2.1 Alkaloids

Alkaloids are one of the important class of secondary metabolites and it inhibit the herbivores and insects. These alkaloids can be exploited pharmaceutically for treatment of various ailments. In mangrove derived endophytes alkaloids of

different classes have been reported (Table 16.1). Several alkaloids viz. (5R) 5-Hydroxy-3-[[2-(4-hydroxy phenyl) ethyl]amino]-5-vinyl-2-cyclopenten-1-one, 3-Isobutylpropanamide-2-cyclopenten-1-one, (5**R**) 3-Amino-5-hydroxy-5-vinyl-2-cyclopenten-1-one, (5R) 5-Hydroxy -3-[(methoxycarbonyl) amino]-5-vinyl-2-cyclopenten-1-one have been reported in mangrove endophyte *Streptomyces* sp. derived from *Aegiceras corniculatum* (Wenhan et al. 2005). Similarly, presence of alkaloids have also been other mangrove derived endophytes from other mangrove species like *Acanthus ilicifolius, Rhizophora mucronata, Hibiscus tiliaceus* (Shao et al. 2010; Xu et al. 2009a; Yan et al. 2012).

16.2.2 Coumarins

Coumarins and their derivatives were reported in few mangrove plants (Table 16.1). Coumarin derivatives like 1,10-Dihydroxy-8-methyldibenz [b,e]oxepin-6,11-dione, 6-hydroxy-4-hydroxymethyl-8-methoxy-3-methylisocoumarin,3-hydroxymethyl-6,8-dimethoxycoumarin, Aspergillumarins A, B have been reported in fungal endophytes like GX4-1B and *Aspergillus* sp. from the leaves and barks of *Bruguiera gymnorrhiza* (Huang et al. 2010; Li et al. 2012). Similarly, acremonones A-H are reported from metabolites of *Acremonium sp*. PSU-MA70, an endophyte reported in *Rhizophora apiculata* (Rukachaisirikul et al. 2012).

16.2.3 Terpenoids

Terpenoids of different types have been isolated from the metabolites of mangrove derived endophytes (Table 16.1). Different classes of terpenoids metabolites that belong to different like drimane sesquiterpenes, norsesquiterpene peroxides; talaperoxides A-D; steperoxide B, expansols A and B; (S)-(+)-11-dehydrosydonic acid andguignardones F-I are isolated from endophytes derived from leaves and barks of mangrove plants *Acrostichum aureum, Pongamia pinnata* (Zhou et al. 2011; Huang et al. 2011a, b), *Sonneratia apetala* (Li et al. 2011), *Excoecaria agallocha* (Lu et al. 2010), *Scyphiphora hydrophyllacea* (Mei et al. 2012).

16.2.4 Flavonoids

Flavonoids have also been isolated from metabolites of endophytes derived from mangrove plants (Table 16.1). Few flavonoids like 5-Hydroxy-7-methoxy-4′-O-(3-methylbut-2-enyl);3,6,7-Trihydroxy-1-methoxyxanthone;1,3,6-Trihydroxy-8-methylxanthone, 1,8-Dihydroxy-4-methyl-7-(3-methyl-2-butenyl)-1,2,3,3a,4,9b-hexahydro cyclopenta[e]chromene-9-carbaldehyde); 2- (2′,3-Epoxy-1′-

heptenyl)- 6-hydroxy-5-(3″-methyl-2″-butenyl)benzaldehyde; (E)-6-hydroxy-7-(3-Methyl-2-butenyl)-2-(3-oxobut-1-enyl)chroman-5-carbaldehyde; 2-(1′,5′-Heptadienyl)-3,6-dihydroxy-5-(3″-methyl-2″-butenyl) benzaldehyde, 5-hydroxy-6,8-dimethoxy-2- benzyl-4H-naphtho[2,3-b]-pyran-4-one have been isolated from mangrove endophytes derived different from *Kandelia candel, Hibiscus tiliaceus, Excoecaria agallocha* plants (Huang et al. 2010, 2012; Li et al. 2008).

16.2.5 Polyketides

Polyketides like -(7′-hydroxyoxooctyl)- 3-hydroxy-5-methoxybenzeneacetic acid ethyl ester; dothiorelones A-C, penicillenone, arugosin I; 9-demethyl FR- 901235; pestalotiopyrones A–H; pestalotiopisorin A; pestalotiollides A-B, pestalotiopin A, leptosphaerone C, penicillenone, bacillosporin A&C, sequoiamonascin D, sequoiatone A and sequoiatone B have been isolated from *Excoecaria agallocha* and *Aegiceras corniculatum* derived endophytes Phomopsis sp. ZSU-H76 (Huang et al. 2009) and *Penicillium* sp. (Lin et al. 2008b).

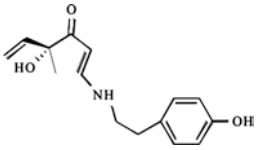
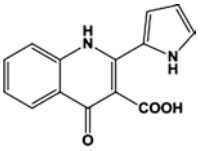
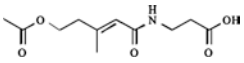
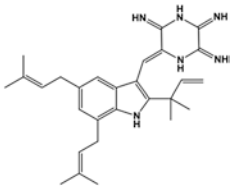
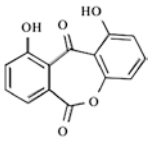
16.2.6 Miscellaneous Compounds

Few other metabolites that of anthraquinone, lactone, cytochalasin, indolic enamides, vermistatin have also been isolated from different mangrove derived endophytes (Table 16.1).

16.3 Biopharmaceutical Potential of Mangrove Endophytes

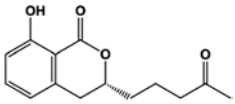
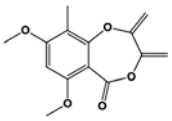
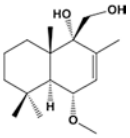
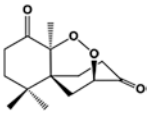
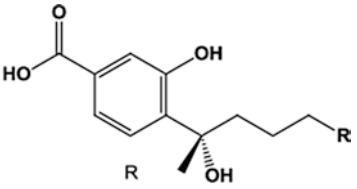
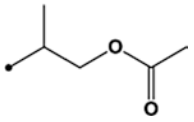
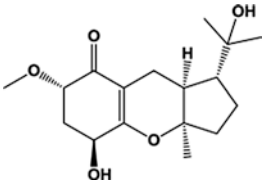
It is the need of hour to search for new antimicrobial agents because infectious diseases are still a global problem because of the development and spread of drug resistant pathogens. Major active compounds used in medicines are obtained from microbes. The endophytic microbes were well studied in terrestrial plants which are found to possess antibacterial, antifungal, anticancer, antimalarial, antiviral, antioxidant and antidiabetic activities. Medicinal properties of different parts of mangrove plant may be fully or partially dependent on the endophytes (Table 16.1).

Table 16.1 Major metabolites isolated from mangrove derived endophytes

Endophytes	Mangrove plant	Important metabolites and their Structures	References
Alkaloids			
Streptomyces sp.	*Aegiceras corniculatum*	 {(5R) 5-Hydroxy-3-[[2-(4-hydroxy phenyl) ethyl]amino]-5-vinyl-2-cyclopenten-1-one} 3-Isobutylpropanamide-2-cyclopenten-1-one (5**R**) 3-Amino-5-hydroxy-5-vinyl-2-cyclopenten-1-one;(5R) 5-Hydroxy -3-[(methoxycarbonyl) amino]-5-vinyl-2-cyclopenten-1-one	Lin et al. (2005)
Penicillium sp.	*Acanthus ilicifolius*	 Penicinoline	Shao et al. (2010)
Pestalotiopsis sp.	*Rhizophora mucronata*	 Pestalotiopamide E	Xu et al. (2009a)
Eurotium rubrum	*Hibiscus tiliaceus*	 12-demethyl-12-oxoeurotechinulin B	Yan et al. (2012)
Coumarins			
Endophytic fungus (No. GX4-1B)	*Bruguiera gymnorrhiza*	 (1,10-Dihydroxy-8-methyldibenz [b,e] oxepin-6,11-dione) 6-Hydroxy-4-hydroxymethyl-8-methoxy-3-methylisocoumarin; 3-Hydroxymethyl-6,8-dimethoxycoumarin	Huang et al. (2010)

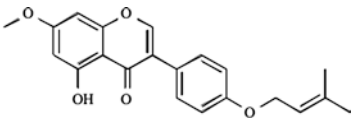
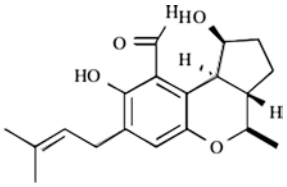
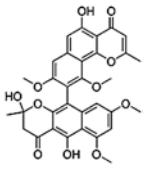
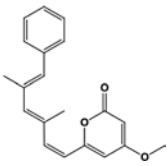
(continued)

Table 16.1 (continued)

Endophytes	Mangrove plant	Important metabolites and their Structures	References
Aspergillus sp.	*Bruguiera gymnorrhiza*	 (Aspergillumarins A) Aspergillumarins B	Li et al. (2012)
Acremonium sp. PSU-MA70	*Rhizophora apiculata*	 (Acremonones A) acremonones A-H	Rukachaisirikul et al. (2012)
Terpenoids			
Aspergillus ustus.	*Acrostichum aureum* *Pongamia pinnata*	 Drimane sesquiterpenes; atropisomers	Zhou et al. (2011) and Huang et al. (2011a)
Talaromyces flavus	*Sonneratia apetala*	 (Talaperoxides C) norsesquiterpene peroxides; talaperoxides A-D; steperoxide B	Li et al. (2011)
Penicillium expansum 091006	*Excoecaria agallocha*	 (7S,11S)-(+)-12- acetoxysydonic acid  (R= Expansols A and B; (S)-(+)-11-dehydrosydonic acid; (Phenolic bisabolane sesquiterpenoids)	Lu et al. (2010)
Endophytic fungus Guignardia sp.	*Scyphiphora hydrophyllacea*	 (Guignardones I) guignardones F-I (meroterpenes)	Mei et al. (2012)

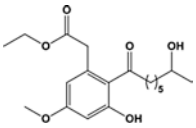
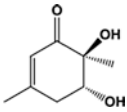
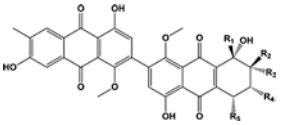
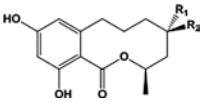
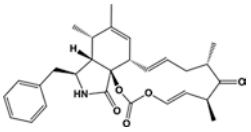
(continued)

Table 16.1 (continued)

Endophytes	Mangrove plant	Important metabolites and their Structures	References
Flavonoids			
Endophytic fungus, *Fusarium* sp. ZZF60	*Kandelia candel*	 [5-Hydroxy-7-methoxy-4'-O-(3-methylbut-2-enyl)] 3,6,7-Trihydroxy-1-methoxyxanthone; 1,3,6-Trihydroxy-8-methylxanthone	Huang et al. (2012)
Endophytic fungus *Eurotium rubrum,*	*Hibiscus tiliaceus*	 (1,8-Dihydroxy-4-methyl-7-(3-methyl-2-butenyl)-1 ,2,3,3a,4,9b-hexahydro cyclopenta[e]chromene-9-carbaldehyde) 2-(2′ ,3-Epoxy-1′-heptenyl)- 6-hydroxy-5-(3″-methyl-2″-butenyl)benzaldehyde; (E)-6-hydroxy-7- (3-Methyl-2-butenyl)-2-(3-oxobut-1-enyl)chroman-5-carbaldehyde; 2-(1′,5′-Heptadienyl)-3,6-dihydroxy-5-(3″-methyl-2″-butenyl)benzaldehyde;	Li et al. (2008)
Phomopsis sp.	*Excoecaria agallocha*	 5-hydroxy-6,8-dimethoxy-2- benzyl-4H-naphtho[2,3-b]-pyran-4-one (naphtho-α-pyrone)	Huang et al. (2010)
Aspergillus niger	*Avicennia marina*	 (Nigerapyrones E) nigerapyrones A-E; nigerapyrones F-H (α-pyrone derivatives); asnipyrones A and B	Liu et al. (2011)

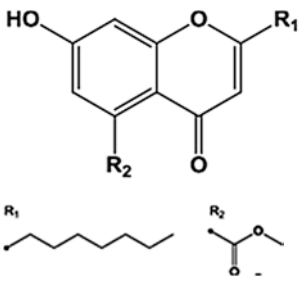
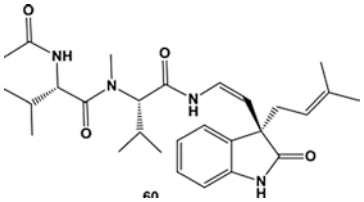
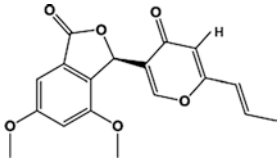
(continued)

Table 16.1 (continued)

Endophytes	Mangrove plant	Important metabolites and their Structures	References
Polyketide			
Phomopsis sp. ZSU-H76	*Excoecaria agallocha*	 [2-(7′-hydroxyoxooctyl)-3-hydroxy-5-methoxybenzeneacetic acid ethyl ester] Dothiorelones A-C	Huang et al. (2009)
Penicillium sp.	*Aegiceras corniculatum*	 (Eptosphaerone C) penicillenone, arugosin I; 9-demethyl FR- 901235; pestalotiopyrones A–H; pestalotiopisorin A; pestalotiollides A-B, pestalotiopin A, leptosphaerone C, penicillenone, bacillosporin A&C, sequoiamonascin D, sequoiatone A and sequoiatone B	Lin et al. (2008)
Miscellaneous			
Alternaria sp. ZJ9-6B	*Aegiceras corniculatum*	 Alterporriol K-M (Bianthraquinone derivatives)	Huang et al. (2011a)
Fungal strain Zh6-B1	*Sonneratia apetala*	 3R,5R-sonnerlactone (R1=H, R2=OH); 3R,5S-sonnerlactone (R1=OH, R2=H)	Li et al. (2010)
Aspergillus flavipes	*Acanthus ilicifolius*	 (Cytochalasin derivatives Z16) Cytochalasin derivatives Z16-Z20	Lin et al. (2009)

(continued)

Table 16.1 (continued)

Endophytes	Mangrove plant	Important metabolites and their Structures	References
Endophytic fungus Pestalotiopsis sp.	Rhizophora mucronata	 (Pestalotiopsones A) Pestalotiopsones A-F	Xu et al. (2009)
Aspergillus sp. (W-6)	*Acanthus ilicifolius*	 (terpeptins A) terpeptins B (indolic enamides)	Lin et al. (2008)
Guignardia sp. No. 4382	*Kandelia candel*	 (Methoxyver mistatin) hydroxyvermistatin (vermistatin derivatives)	Xia et al. (2007)

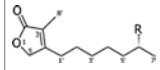
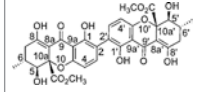
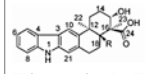
16.3.1 Antimicrobial

It has been observed that the endophyes possess resistance mechanism against many pathogenic organisms which may be attributed to their ability to produce secondary metabolites (Tan and Zou 2001). Earlier studies have shown that endophytes contain several classes of antimicrobial compounds such as alkaloids, peptides, steroids, terpenoids, phenols, flavonoids etc.Mangrove endophytes, have been recognized as rich sources of natural products with unique structures having antimicrobial activity. Both fungal and bacterial endophytes of mangrove origin were reported for their antimicrobial potential (Table 16.2).

Different levels of antimicrobial activities were recorded for the 227 marine isolated endophytic fungi against the pathogenic bacteria *Staphylococcus aureus* and *Vibrio parahaemolyticus, Salmonella typhi* (Christophersen et al. 1998). Similar findings were also reported by Maria et al. (2005), who reported antimicrobial potential of 14 endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum*

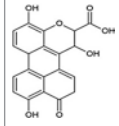
Table 16.2 Pharmacological activities of mangrove endophytes

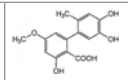
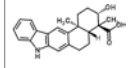
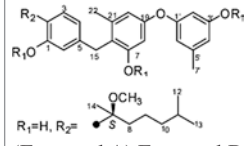
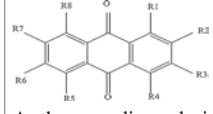
Endophytes	Mangrove species	Isolated bioactive compound/class of compound	Biological activity	References
Antimicrobial				
Endophytic fungi: *Cumulospora marina; Pestalotiopsis* sp.; *Aspergillus* sp.	*Acanthus ilicifolius Acrostichum aureum*	–	Antibacterial, antifungal	Maria et al. (2005)
Colletotrichum, Alternaria, Phomopsis, Pestalotiopsis, Guignardia, Cladosporium	*Aegiceras corniculatum*	–	Antimicrobial	Bin et al. (2014)
Xylaria sp. *Hypocrea lixii*, *Bacillus* sp. *Enterobacter* sp.	*Avicennia marina*	n-hexadecanoic acid, cyclic peptides: xyloketals, xyloallenolides	Antibacterial Antifungal	Zhu et al. (2009), sona Janarthine et al. (2011) and Norhayati et al. (2009)
Endophytic fungi *Acremonium, Cladosporium, Curvularia* and *Saccharomyces*	*Avicennia officinalis*	–	Antifungal	Job et al. (2015)
Endophytic fungi Guignardia sp. Neusartoya sp.	Avicennia sp.	Structures similar to trimeric catechin and Helenalin	Antibacterial activities: *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* Antifungal activities: *Candida albicans* and *Aspergillus niger*	Ling et al. (2016)
Nigrospora sp.	*Bruguiera gymnorrhiza*	Compounds indole-3-carbaldehyde;	Antibacterial Antifungal	Kjer (2009)

Phomopsis sp	*Excoecaria agallocha*	Phomopsin A , B, C and , cytosporon B and C.	Antibacterial Antifungal	Huang et al. (2008)
Endophytic fungi *Phomopsis amygdale, Trichodermasp* and *Alternaria sp*	*Rhizophora mucronata Excoecaria agallocha*	–	Antibacterial	Salini et al. (2015)
Endophytic fungi *Pestalotiopsis microspora* VB5	*Rhizophora mucronata Avicennia officinalis*	–	Antimicrobial	Joel and Bhimba (2012)
Endophytic fungi Pestalotiopsis sp.	*Rhizophora mucronata*	 Pestalolide (R=H)	Antifungal activity	Klaiklay et al. (2012)
Endophytic fungus Talaromyces sp.	*Kandelia candel* (L.) Druce	 (Secalonic acid A) 7-epiaustdiol, 8-O-methylepiaustdiol, stemphyperylenol, skyrin, , emodin and norlichexanthone	Antimicrobial	Liu et al. (2010)
Streptomyces sp. HKI0595	*Kandelia candel*	 Xiamycin A (R=H) xiamycin B (R=OH), indosespene, sespenine,	Antimicrobial	Ding et al. (2011)
Endophytic fungi such as *Fusarium* sp., *Penicillium* sp., *Alternaria* sp., *Nigrospora* sp., *Rhizopus* sp.	*Rhizosporia annamalayana*	5-Eicosene, (E) – and 1Dodecanol, 2-methyl (S)	Antimicrobial	Elavarasi et al. (2014)

(continued)

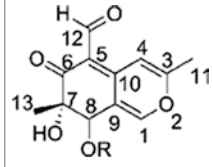
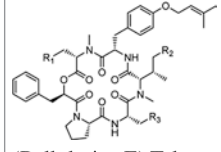
Table 16.2 (continued)

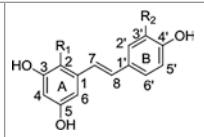
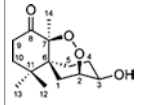
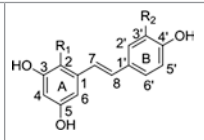
Endophytes	Mangrove species	Isolated bioactive compound/class of compound	Biological activity	References
Endophytic bacteria Genus: *Serratia, Bacillus, Pseudomonas, Micrococcus Enterobacter*	*Rhizophora mucronata*	–	Antibacterial and antifungal	Jose and Christy (2013)
Bionectria ochroleuca	*Sonneratia caseolaris*	Peptide pullularins A,C,E and F and verticilli D,flavonoids, triterpenoids, cardiac glycosides and alkaloids.	Antibacterial Antifungal	Ebrahim et al. (2012); Jiny et al. (2010)
Alternaria sp	*Sonneratia alba*	 (Xanalteric acids I) Alternarian acid, altenusin, altenuene , 4'- epialtenuene, alternarienonic acid, alternariol and altertoxin. xanalteric acids I and II	Antibacterial Antifungal	Saad et al. (2012)
Endophytic bacteria *viz. Staphylococcus sciuri, Staphylococcus intermedius, Bacillus pumilus, Staphylococcus lentus, Bacillus licheniformis, Bacillus coagulans, Bacillus pumilus,*	*Avicennia lanata, Rhizophora mucronata, Rhizophora apiculata, Sonneratia caseolaris, Xylocarpus moluccensis*	Bacteriocins	Antibacterial	Eldeen (2014)
Antiviral				
Endophytic fungus *Nigrospora sp.*	*Bruguiera sexangula*	Anthraquinones	Anti- human rhino viruses	Kjer (2009)

Alternaria sp.,	*Sonneratia alba*	 Altenusin	Anti- human rhino viruses	Kjer (2009)
Streptomyces sp.	*Bruguiera gymnorrhiza*	 Xiamycin A	Anti-HIV	Ding et al. (2010)
Emericella sp.	*Aegiceras corniculatum*	isoindolones	Anti-influenza A virus (H1N1)	Zhang et al. (2011)
Cytotoxic/Anticancer				
Endophytic fungi *Alternaria* sp.	*Aegiceras corniculatum*	Alterporriols K and L	Cytotoxic	Huang et al. (2011a)
Endophytic fungus *Penicillium expansum*	*Excoecaria agallocha*	 (Expansol A) Expansol B	Moderate cytotoxicity against A549 and HL-60 cells	Lu et al. (2010)
Endophytic fungi *Irpex hydnoides Pestalotiopsis microspora* VB5	*Rhizophora mucronata Avicennia officinalis*	Tetradecane	Cytotoxic	Bhimba et al. (2011) Joel and Bhimba (2012)
Fusarium oxysporum	*Rhizophora annamalayana*	Taxol	Cytotoxic	Elavarasi et al. (2012)
Endophytic fungus *Halorosellinia* sp. and *Guignardia* sp.	Kandelia Woody tissue	 Anthracenedione derivatives	Anticancer	Zhang et al. (2010)

(continued)

Table 16.2 (continued)

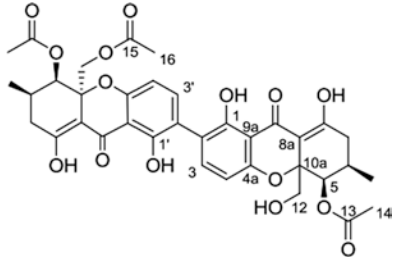
Endophytes	Mangrove species	Isolated bioactive compound/class of compound	Biological activity	References
Endophytic fungus Talaromyces sp.	*Kandelia candel* (L.) Druce	 7-epiaustdiol (R=H), 8-O-methylepiaustdiol (R=CH_3), stemphyperylenol, skyrin, secalonic acid A, emodin and norlichexanthone	*In vitro* cytotoxic activities against human epidermoid carcinoma cell lines KB and KBv200	Liu et al. (2010)
Endophytic fungi *Curvularia*, *Fusarium, Cladosporium, Alternaria*, *Gloeosporium, Diaporthe, Talaromyces.*	*Sonneratia caseolaris*, *Brine fern*, *Pluchea indica*, *Bruguiera gymnorrhiza, Sonneratia apetala, Kandelia candel, Angier's corniculatum Heritiera littoralis*	(+)-3,3′,7,7′,8,8′- hexahydroxy-5,5′-dimethylbianthraquinone, ermistatin and methoxvermistatin, tenellic acid A and alternin, lichexanthone and bikaverin	Cytotoxic	Xiaoling et al. (2010)
Talaromyces flavus	*Sonneratia apetala*	 (Pullularins E) Talaperoxides, and F	Cytotoxic	Li et al. (2011)

Endophytic fungi *Alternaria* sp.	*Myoporum bontioides*	 resveratrodehydes A : R_1 =H, R_2 = CHO resveratrodehydes B: R_1 =CHO, R_2 = CHO	Cytotoxic	Wang et al. (2014)
Endophytic fungi (Unidentified)	*Xylocarpus granatum.*	 Merulin A, B, C	Cytotoxicity against human breast (BT474) and colon (SW620) cancer cell lines	Chokpaiboon et al. (2010)
Antioxidant				
Endophytic fungi *Alternaria* sp.	*Myoporum bontioides*	 resveratrodehydes A : R_1 =H, R_2 = CHO resveratrodehydes C: R_1 =CHO, R_2 = H	DPPH	Wang et al. (2014)
Endophytic fungus *Aspergillus flavus*	*Avicennia offi cianlis, Kandelia candel, Excoecaria agallocha, Rhizophora mucronata*	-	DPPH, hydrogen peroxide, hydroxyl radical scavenging assay, reducing power assay, metal cheating assay and β-carotene-linoleate lipid peroxidation	Ravindran et al. (2012)

(continued)

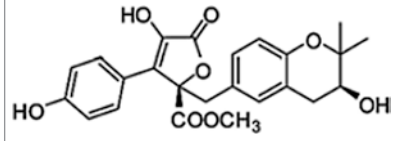
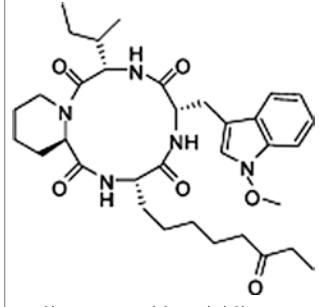
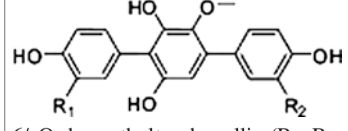
Table 16.2 (continued)

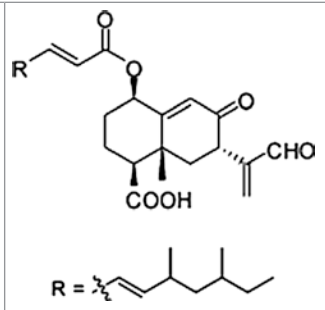
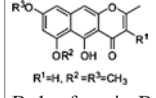
Endophytes	Mangrove species	Isolated bioactive compound/class of compound	Biological activity	References
Endophytic fungus Trichoderma species	*Lumnitzera littorea* (Jack) Voigt, *Scyphiphora hydrophyllacea*, *Xylocarpus mekongensis* Pierre, *Excoecaria agallocha* Linn, *Bruguiera gymnorrhiza* (L.) Lamk., *Rhizophora mucronata* (Lam.), *Aegiceras corniculatum* (L.), *Rhizophora stylosa* Griff, *Sonneratia griffithii* Kurz, *Ceriops tagal* (Perr.), *Avicennia marina* (Forstk.) Vierh, *Bruguiera cylindrica* (L.)	pregnane-3,20β-diol, 14α,18α-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)], diacetate; 4-piperidineacetic acid, 1- acetyl-5-ethyl-2-[3-(2-hydroxyethyl) -1-H-indol-2-yl] -a-methyl, methyl ester; corynan-17-ol, 18,19-didehydro-10-methoxy and oleic acids	Total antioxidant activity, DPPH radical scavenging, activity, NO_2 radical scavenging activity, H_2O_2 radical scavenging activity and total reducing power	Kandasamy and Kandasamy (2014)
Endophytic fungi *Phomopsis amygdali*	*Avicennia marina, Suaeda monica and Rhizophora mucronata*	–	DPPH and ABTS	Bharathidasan and Panneerselva (2012)
Endophytic fungus *Fusarium oxysporum*	*Ipomoea pes-caprae* (Linn.)	galactofuranose-containing polysaccharide	hydroxyl, DPPH, and superoxide radicals	Chen et al. (2015)

Fungal endophyte *Ascomycota* sp.	*Kandelia candel*	2,3-diaryl indone derivatives, ascomindones A-C, methyl 2- (2,6 dihydroxy- 4- methylbenzoyl) -3-hydroxy-5-methoxybenzoate, 2-(2-carboxy-3-hydroxy-5-methylphenoxy)-3-hydroxy-5-methoxy benzoic acid, 2-hydroxy-6-(2-hydroxy-6-(hydroxymethyl)-4-methoxyphenoxy) -4-methylbenzoic acid and emodin	DPPH, hydroxyl radical scavenging activities and the ferric reducing ability power (FRAP	Tan et al. (2016)
Endophytic fungus *Pestalotiopsis* sp.	*Rhizophora mucronata*	pentadecanoic acid, 14-methyl-, methyl ester, octadecanoic acid, methyl ester, nonanedioic acid, dimethyl ester and *n*-hexadecanoic acid	DPPH	Li et al. (2013)
Endophytic fungi *Phomopsis amygdale, Trichoderma sp* and *Alternaria sp*	*Rhizophora mucronata Excoecaria agallocha*	–	DPPH	Salini et al. (2015)
Antimalarial				
Endophytic fungus *Diaporthe* sp.	*Avicennia marina; Kandelia obovata; Lumnitzera racemosa*	 Cytochalasin, tricothecene, polyketide, Dicerandrol D	*In vitro* study against *Plasmodiun falciparum*	Calcul et al. (2013)

(continued)

Table 16.2 (continued)

Endophytes	Mangrove species	Isolated bioactive compound/class of compound	Biological activity	References
Penicillium expansum	*Excoecaria agallocha*	 Butyrolactone V	*In vitro* study against *Plasmodiun falciparum*	Wang et al. (2012)
Endophytic fungus ZSU-H16	Avicennia	 cyclic tetrapeptide apicidin	*Plasmodium berghei* malaria	Singh et al. (1996)
Antidiabetic				
Endophytic fungus *Penicillium chermesinum*	*Kandelia candel*	 6′-O-desmethylterphenyllin (R_1=R_2=H), 3-hydroxy-6′-O-desmethylterphenyllin (R_1=OH, R_2=H) 3,3″-dihydroxy-6′-O-desmethylterphenyllin	α-glucosidase inhibitor	Huang et al. (2011b)

Endophytic fungus *Xylaria* sp. BL321	*Acanthus ilicifolius*	 Uncharacterized compound 07H239	α-glucosidase inhibitor	Song et al. (2012)
Endophytic fungus *Penicillium sp*. HN29-3B1	*Cerbera manghas*	Vermistatin derivative, 6-demethylpenisimplicissin and 2”-epihydroxydihydrovermistatin	α-glucosidase inhibitor	Liu et al. (2014)
Aspergillus tubingensis	*Pongamia pinnata*	 Rubrofusarin B	Mild α-glucosidase inhibitor	Huang et al. (2010)

aureum against bacteria *Bacillus subtilis, Enterococcus* sp., *Klebsiellapneumoniae, Pseudomonas aerugionsa, Salmonella typhi* and *Staphylococcus aureus* and fungi *Candida albicans* and *Trichophyton metagrophytes*. Chaeprasert et al. (2010), reported that the fungal endophytes such as *Cladosporium*, *Colletotrichum*, *Phomopsis* and *Xylaria*isolated from leaf tissues of mangrove plant *Sonneratia caseolaris*exhibited antimicrobial activities against *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus.* Further, the extract from theendophytic fungus *Bionectria ochroleuca*, led to the isolation of two new peptides, pullularins E and F together with three known compoundsverticillin D, pullularin C and pullularin A that may be responsible for their antibacterial activity. Huang et al. (2008), reported the isolation, structural elucidation and biological activities of three new metabolites, phomopsin A, B and C, and two known compounds cytosporone B and C from the mangrove endophytic fungus, *Phomopsis* sp. which was isolated from the stem of the mangrove tree *Excoecaria agallocha* that showed antifungal activities against two fungi *Candida albicans* and *Fusarium oxysporum.* Eighteen endophytic bacteria viz. *Staphylococcus sciuri, Staphylococcus intermedius, Bacillus licheniformis, Bacillus coagulans, Staphylococcus lentus, and Bacillus pumilus* isolated from five mangrove plants such as *Avicennia lanata, Rhizophora mucronata, Rhizophora apiculata, Sonneratia caseolaris*, and *Xylocarpus moluccensis* of east coast of peninsular Malaysia exhibited antibacterial activity against Gram-positive *Bacillus cereus* and *Staphylococcus aureus* and two Gram-negative *Escherichia coli* and *Salmonella typhimurium* (Eldeen 2014). Two endophytic fungi *Guignardia* sp. *and Neusartoya* sp. isolated from mangrove plant *Avicennia* sp. showed antibacterial activities against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and strong antifungal activities against *Candida albicans* and *Aspergillus niger.* Metabolites isolated from these endophytes contained trimeric catechin and helenalin (Ling et al. 2016). Job et al. (2015) isolated endophytic fungi viz. *Acremonium, Cladosporium, Curvularia* and *Saccharomyces* fromleaves and stem of *Avicennia officinalis* that showed antimicrobial activities against different microbes such as *Pseudomonas aeruginosa*, *Escherichia coli, Aeromonas hydrophila*, *Bacillus cereus, Staphylococcus aureus, Edwardsiella tarda*, *Vibrio harveyi, Vibrio fluvialis, Vibrio cholera,Vibrio parahaemolyticus, Vibrio proteolyticus, Vibrio vulnificus, Candida albicans*, *Aspergillus flavus,* and *Aspergillus fumigates.* In another study, endophytic fungus BUEN 880 isolated from leaf samples of different mangrove plants *viz*. *Bruguiera gymnorrhiza* (L.) Savigny, *Bruguiera sexangula* Poir., *Thespesia populnea* (L.) Soland. ex Correa, *Avicennia marina* (Forsk.) Vierh., *Ceriops tagal* (Perr.) C. B. Rob., *Sonneratia alba* J. Smith, *Sonneratia ovata*, *Xylocarpus granatum* Koen., *Xylocarpus rumphii*, *Acrostichum aureum* L., *Rhizophora mucronata* Poir., *Rhizophora apiculata* Bl. and *Excoecaria agallocha* L. exhibited promising antifungal activity against five indicator fungal phytopathogens such as *A. brassicicola* DOAC 0436, *C. capsici* DOAC 1511, *C. Gloeosporioides* DOAC 0782, *F. oxysporum* DOAC 1808 and *Pestalotiopsis* sp. DOAC1098 (Khrueayu and Pilantanapak 2012). Metabolites like xiamycin A, B, indosespene and sespenine were isolated from *Streptomyces* sp. HKI0595, a bacterial endophyte derived from the mangrove tree *Kandelia candel* displayed moderate

to strong antimicrobial activities against several bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* (Ding et al. 2011). Endophytic fungi such as *Fusarium* sp., *Penicillium* sp., *Alternaria* sp., *Nigrospora* sp., *Rhizopus* sp. isolated from leaves of mangrove plant *Rhizophora annamalayana* exhibited antimicrobial activity against different bacteria like *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Vibrio cholerae* and *Staphylococcus aureus* and dermatophytic fungi that includes *Trichophyton rubrum*, *Epidermophyton floccosum* and *Trichophyton mentagrophytes*. The metabolites isolated from these endophytes were 5-Eicosene, (E) – and 1Dodecanol, 2-methyl (S) (Elavarasi et al. 2014). Pestalolide isolated from endophytic fungi *Pestalotiopsis* sp. derived from *Rhizophora mucronata* displayed weak antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (Klaiklay et al. 2012). Six genera of endophytes viz. *Colletotrichum, Alternaria, Phomopsis, Pestalotiopsis, Guignardia, Cladosporium* isolated from mangrove plant *Aegiceras corniculatum* showed antimicrobial activities against different microbes such as bacterial strains *Bacillus cereus*, *Pseudomonas aeruginosa*, *E. Coli*, along with against some multi drug resistant bacteria like *Klebsiella pneumoniae* and *Acinetobacter baumanii* (Bin et al. 2014). Jose and Christy (2013) reported antibacterial and antifungal properties of five endophytic bacterial genus *Serratia, Bacillus, Pseudomonas, Micrococcus and Enterobacter* isolated from mangrove plant *Rhizophora mucronata* (Jose and Christy 2013). Maria et al. (2005) reported antimicrobial and antifungal activities of endophytic fungi such as *Cumulospora marina* and *Pestalotiopsis* sp. and *Aspergillus* sp. isolated from mangrove plants *Acanthus ilicifolius* and *Acrostichum aureum* collected from south east of India. The metabolites viz.7-epiaustdiol, 8-O-methylepiaustdiol, stemphyperylenol, skyrin, secalonic acid A, emodin and norlichexanthone isolated from endophytic fungus *Talaromyces* sp. from stem bark of *Kandelia candel* (L.) Druce reported to exhibit antimicrobial activities (Liu et al. 2010).Other mangrove derived endophytes along with their metabolites responsible for antimicrobial activities are shown in Table 16.2.

16.3.2 Antiviral

The discovery of the potential of endophytes for the production of antiviral compounds is very limited. Only limited numbers of compounds have been reported as antiviral agents from endophytes. The antiviral potential of mangrove derived endophytes are summerized in Table 16.2. Endophytic fungus *Nigrospora* sp. isolated from mangrove plant *Bruguiera sexangula* exhibited prophylactic effects against human rhinoviruses. Metabolites isolated from this endophyte were reported as anthraquinones (Kjer 2009). In another study, altenusin obtained from endophyte *Alternaria* sp., isolated from *Sonneratia alba* showed prophylactic effects against human rhino viruses (Kjer 2009). Xiamycin A obtained from *Streptomyces* sp. strain GT 2002/1503 an endophyte from *Bruguiera gymnorrhiza* exhibited selective anti-HIV activity (Ding et al. 2010). Further, two isoindolones from a fungal endophyte

Emericella sp. (HKZJ), isolated from the inner bark of *Aegiceras corniculatum* exhibited antiviral activity against influenza A virus (H1N1) (Zhang et al. 2011).

16.3.3 Anticancer

Chemotherapy is currently the primary treatment modality in many tumors. However, the development of multidrug resistance (MDR) to chemotherapeutic drugs is a main obstacle for the successful treatment of malignant tumors. The chemical, biological and ecological diversity of the marine ecosystem has contributed immensely for development of potent antitumor compounds (Singh et al. 2008). It is believed that a rich source of anticancer drug candidates could be obtained from marine organisms or their metabolites. Since the discovery of taxol from the endophytic fungus *Taxomyces andreanae* (Stierle et al. 1993) as anticancer compound, the endophytes have been extensively studied for their anticancer potential. In the last decade, there has been a dramatic increase in the number of preclinical anticancer lead compounds extracted from metabolites of marine-derived fungi (Yanagihara et al. 2005; Zhang et al. 2007, 2009). The cytotoxic/anticancer potential of mangrove derived endophytes are summerized in Table 16.2.

Endophytic fungi *Irpex hydnoides* isolated from leaves of *Rhizophora mucronata andAvicenna officinalis* showed cytotoxic activities against Hep2 cell lines. The GC-MS analysis of the bioactive fraction from the endophytic extract revealed the presence of tetradecane as the cytotoxic compound (Bhimba et al. 2011). The mangrove endophytic fungi *Halorosellinia* sp. and *Guignardia* sp. isolated from mangrove plant Kandelia woody tissue inhibited the growth of KB and KBv200 cells, amongst which an anthraquinone derivative displayed strong cytotoxicity against KB and KBv200 cells (Zhang et al. 2010). In another study, Xiaoling et al. (2010) reported different endphytic fungi viz. *Fusarium* sp., *Trichoderma* sp., *Cephalosporium* sp., *Phomopsis* sp., *Penicillium* sp., *Aureobasidium sp., Diaporthe* sp., *Pleosporaceae* sp., *Ascomycota* sp., *Chaetomium* sp., *Cladosporium* sp., *Gloeosporium* sp., *Talaromyces* sp., *Alternaria* sp., *Hypocreales* sp., *Stemphylium* sp., *Colletotrichum* sp. isolated from leaves and branches of eight mangrove plants viz. *Sonneratia caseolaris*, *Brine fern*, *Pluchea indica*, *Bruguiera gymnorrhiza, Sonneratia apetala, Kandelia candel, Angier's corniculatum* and *Heritiera littoralis* for their cytotoxic and antitumor potential against KB and KBv200 cells. The antitumor properties of the isolate endophytic fungi may be attributed topoisomerase I inhibitory property. The antitumor compounds isolate from these endophytic fungal isolates were (+)-3,3′,7,7′,8,8′- hexahydroxy-5,5′-dimethylbianthraquinone, ermistatin and methoxvermistatin, tenellic acid A and alternin, lichexanthone and bikaverin. Endophytic fungus *Alternaria* sp. isolated from *Aegiceras corniculatum* showed cytotoxic activities against two breast cancer cell lines MDA-MB-435 and MCF-7. The isolated antitumor compounds were alterporriols K and L (Huang et al. 2011a). In another study, *Alternaria* sp., an endophytic fungus isolated from the root of a marine semi-mangrove plant *Myoporum bontioides* exhibited broad-spectrum

inhibitory activities against three human cancer cell lines such as human breast MDA-MB-435, human liver HepG2, and human colon HCT-116 as revealed by MTT assay Two new resveratrol derivatives, resveratrodehydes A and B isolated from the metabolites of this endophyte exhibited marked cytotoxic activities against MDA-MB-435 and HCT-116 cell lines (Wang et al. 2014). An unidentified endophytic fungus isolated from *Xylocarpus granatum* displayed cytotoxic activities against human breast (BT474) and colon (SW620) cancer cell lines. The metabolites isolated from this endophyte was reported as nor-chamigrane endoperoxide, merulin A along with two chamigrane endoperoxides, merulins B and C (Chokpaiboon et al. 2010). Endophytic fungus Talaromyces sp. isolated from the stem bark of mangrove plant *Kandelia candel* (L.) Druce, exhibited in vitro cytotoxic activities against human epidermoid carcinoma cell lines KB and KBv200. The metabolites isolated from this endophytic fungus that showed cytotoxic activities are 7-epiaustdiol, 8-O-methylepiaustdiol, stemphyperylenol, skyrin, secalonic acid A, emodin and norlichexanthone (Liu et al. 2010). Endophytic fungus *Penicillium expansum* derived from mangrove plant *Excoecaria agallocha* exhibited moderate cytotoxicity against A549 and HL-60 cells. The compound isolated from this endophyte was Expansol A and Expansol B (Lu et al. 2010). The other mangrove derived endophytes along with their metabolites responsible for cytotoxic activities are shown in Table 16.2.

16.3.4 Antioxidant

Mangrove plants growing in the ecological stressful condition are known to produce some anti-stress metabolites that help them to withstand under the stress condition (Thatoi et al. 2014). So it is obvious that endophytes that inhabit the mangrove plants will be associated with abundant source of novel bioactive compounds with huge potential for exploitation of pharamaceutical drugs. Earlier studies have shown endophytes can activate host stress responses to various abiotic and biotic stresses (Redman et al. 2002; Baltruschat et al. 2008; Swarthout et al. 2009). The antioxidant potential of mangrove derived endophytes are summerized in Table 16.2.

Ravindran et al. (2012) have reported that endophytic fungus *Aspergillus flavus* isolated from leaf and roots of four mangrove plants *Avicennia officinalis, Kandelia candel, Excoecaria agallocha,* and *Rhizophora mucronata* showed potent antioxidant as revealed by different *in vitro* antioxidant assays such as DPPH, hydrogen peroxide, hydroxyl radical scavenging assay, reducing power assay, metal cheating assay and β-carotene-linoleate lipid peroxidation assay. In another study, *Alternaria* sp., an endophytic fungus isolated from the root of a marine semi-mangrove plant *Myoporum bontioides* exhibited antioxidant activity against DPPH radical. Two new resveratrol derivatives, resveratrodehydes A and C isolated from the metabolites of this endophyte exhibited moderate antioxidant capacity against DPPH radical (Wang et al. 2014). Eight different strains of fungal endophytes of Trichoderma species isolated from young mangrove leaves of *Lumnitzera littorea* (Jack)Voigt,

Scyphiphora hydrophyllacea, *Xylocarpus mekongensis* Pierre, *Excoecaria agallocha* Linn, *Bruguiera gymnorrhiza* (L.) Lamk., *Rhizophora mucronata* (Lam.), *Aegiceras corniculatum* (L.), *Rhizophora stylosa* Griff, *Sonneratia griffithii* Kurz, *Ceriops tagal* (Perr.), *Avicennia marina* (Forstk.) Vierh, *Bruguiera cylindrica* (L.) Blum from Andaman and Nicobar Islands, India showed considerable antioxidant potential as revealed by various in vitro antioxidant assays viz. total antioxidant activity, DPPH radical scavenging, activity, NO_2 radical scavenging activity, H_2O_2 radical scavenging activity and total reducing power. The metabolites isolated from these endophytic fungal strains such as pregnane-3,20β-diol, 14α,18α-[4- methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)], diacetate; 4-piperidineacetic acid,1- acetyl-5-ethyl-2-[3-(2- hydroxyethyl)-1-H-indol-2-yl]-a- methyl, methyl ester; corynan-16-ol, 18,19-didehydro-10-methoxy and oleic acids may be attributed to their antioxidant capacity (Kandasamy and Kandasamy 2014). The endophytic fungi *Phomopsis amygdali* isolated from mangrove plants *Avicennia marina, Suaeda monica and Rhizophora mucronata* showed antioxidant activity against stable DPPH and ABTS free radicals (Bharathidasan and Panneerselvam 2012). The endophytic fungus *Fusarium oxysporum* isolated from mangrove plant *Ipomoea pescaprae* (Linn.) produces extracellular polysaccharides that possess antioxidant potential as evident by the in vitro antioxidant assays viz. hydroxyl, DPPH, and superoxide radicals. The study further demonstrated that presence of a novel galactofuranose-containing polysaccharide could be a potential source of antioxidant compounds (Chen et al. 2015).The fungal endophyte *Ascomycota* sp. from mangrove plant *Kandelia candel* exhibited strong antioxidant activities as evident by 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging activities and the ferric reducing ability power (FRAP) antioxidant assays.Presence of novel 2,3-diaryl indone derivatives, ascomindones A-C, methyl 2- (2,6 dihydroxy-4-methylbenzoyl)-3-hydroxy-5-methoxybenzoate, 2-(2-carboxy-3-hydroxy-5-methylphenoxy)-3-hydroxy-5-methoxy benzoic acid, 2-hydroxy-6-(2-hydroxy-6-(hydroxymethyl)-4-methoxyphenoxy)-4-methylbenzoic acid and emodinin these endophytes attributed to their antioxidant potential (Tan et al. 2016). In another study, endophytic fungus *Pestalotiopsis* sp. isolated from Chinese mangrove plant *Rhizophora mucronata* produces several metabolites viz. pentadecanoic acid, 14-methyl-, methyl ester, octadecanoic acid, methyl ester, nonanedioic acid, dimethyl ester and *n*-hexadecanoic acid that showed antioxidant activity against DPPH free radical (Li et al. 2013). Salini et al. (2015) reported endophytic fungi *Phomopsis amygdale, Trichoderma* sp. and *Alternaria* sp. isolated from mangrove plants *Rhizophora mucronata and Excoecaria agallocha* exhibited DPPH scavenging activity.

16.3.5 Antidiabetic

The α-glucosidase inhibitors are considered as one of the most common oral antidiabetic agents used to decrease postprandial hyperglycemia, as they can decrease glucose intake with low hypoglycemic effect. The mangrove endophytic microbe's

ability to produce bioactive compounds in association with its host plants is an opportunity to get sources of natural, inexpensive and eco-friendly antidiabetic drugs. The antidiabetic potential of mangrove derived endophytes are summerized in Table 16.2. Recently, bioactivity studies of secondary metabolites of endophytic actinomycetes from mangrove have shown PTP1B inhibitor and glucosidase inhibitor properties. Huang et al. (2011b) have reported compounds like 6′-O-desmethylterphenyllin, 3-hydroxy-6′-O-desmethylterphenyllin and 3,3″-dihydroxy-6′-O-desmethylterphenyllin from the endophytic fungus *Penicillium chermesinum*, isolated from *Kandelia candel* that exhibited strong α-glucosidase inhibition activity. In another study, an uncharacterized compound 07H239- isolated from the endophytic fungus*Xylaria* sp. BL321 from mangrove plant *Acanthus ilicifolius* showed inhibitory activity on *α*- glucosidase enzyme (Song et al. 2012). Similarly, in another study a vermistatin derivative, 6-demethylpenisimplicissin and 2″-epihydroxydihydrovermistatin isolated from the mangrove endophytic fungus *Penicillium* sp. HN29-3B1 from *Cerbera manghas*, exhibited α- glucosidase inhibitory activity (Liu et al. 2014). Huang et al. (2010) reported that Rubrofusarin B isolated from endophyte *Aspergillus tubingensis* from *Pongamia pinnata* exhibited mild α- glucosidase inhibition activity.

16.3.6 Antimalarial

Mangrove plants and their associated fauna are rich sources of bioactive molecules and are reported for their potential in treatment of different ailments. However, only limited studies have been reported for their antimalarial potential. The antimalarial potentials of mangrove derived endophytes are summerized in Table 16.2. Calcul et al. (2013) reported presence of different metabolites viz. cytochalasin, tricothecene, polyketide, dicerandrol D from endophytic fungus *Diaporthe* sp. from mangrove species *Avicennia marina, Kandelia obovata, Lumnitzera racemosa* showing antimalarial activity against *Plasmodiun falciparum*. Butyrolactone Visolated from endophytic fungi *Penicillium expansum* isolated from mangrove plant *Excoecaria agallocha* have shown antimalarial activity (Wang et al. 2012). Singh et al. (1996) reported that unidentified endophytic fungi ZSU-H16 obtained from *Avicennia* produce metabolites like aapicomplexan histone deacetylase that showed antimalarial activity against *Plasmodium berghei* malaria.

16.4 Conclusion and Future Perspectives

Mangrove plants thriving under extreme abiotic and biotic stress conditions have developed certain special metabolic pathways to produce unique chemicals that enable them to tolerate such stressful environmental conditions. Some of these chemicals are confirmed to be of great potential as a source of novel agents for

various pharmaceutical and other industrial applications. So it is quite obvious that mangrove derived endophytes that remain in a very close association with their host plants are rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity. These endophytes are a good and reliable source of novel natural compounds and also produce several compounds of pharmaceutical significance which can be exploited for discovery of antimicrobial, antiviral, anticancer, antioxidant, antidiabetic, antimalarial drugs which is currently attracting scientific investigations worldwide. Mangrove endophytes are a poorly investigated group of microbes that represent abundant and dependent source of bioactive and structurally diverse compounds which needs to be exploited for pharmaceutical applications. Systematic approaches for isolation and development of bioactive compounds along with the metagenomics, metatranscriptomics, and metaproteomics techniques should be employed to mine these endophytes from mangrove plants for potential natural product discovery.

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Chapter 17
Application of Oncolytic Virus as a Therapy of Cancer

Sushil Kumar Sahu and Mukesh Kumar

17.1 Introduction

A large number of viruses possess specific cellular tropism that determines preferential human cells or tissue to be infected. For instance, Hepatitis-B virus and Hepatitis-C virus infects hepatocytes, Human Immunodeficiency virus infects T-helper cells and macrophages, Human T-lymphotropic virus-1 infects cytotoxic T-cells, Rabies virus infects neurons, Influenza virus infects airway epithelium (Woolhouse et al. 2012). Likewise, viruses which preferentially infect and lyse cancer cells with a very little or no harm to normal cells are called oncolytic viruses. The concept of an oncolytic virus in the treatment of cancer was proposed at the beginning of the twentieth century when a study reported that cervical carcinoma patient showed tumor regression after vaccination with rabies virus (Dock 1904). Subsequently, many groups studied the effect of oncolytic viruses on laboratory cell lines, primary cells, animal models as well as in human trial (Sinkovics and Horvath 1993). In 1956, investigators at National Cancer Institute injected cell culture inocula having wild type adenoviruses in 30 patients with epidermoid cervical carcinomas (Huebner et al. 1956). Most of the patient in this study exhibited tumor regression with a little toxicity whereas control patients treated with inactivated viruses showed no response This seemed to be a remarkable success but during the course of time all these patients again developed tumors. The apparent lack of anticancer efficacy was reflected in other human trials of that time (Cassel and Garrett 1965; Newman and Southam 1954; Southam et al. 1956). At that time chemotherapy and radiotherapy were enjoying success in cancer treatment and sidetrack the

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attention of researchers from viral oncotherapy. Also, the molecular biology techniques required to engineer viruses were not in place and the field of virology was also not sufficiently understood. Recent advances in knowledge in cancer biology, genetic, molecular biology and virology have provided tools and techniques needed for viral engineering and develop an effective cancer treatment. So the neglected area of oncolytic viral research has gained renewed interest.

17.2 Oncolytic Virus Engineering

Oncolytic viruses selectively enter into the cancer cell, destroy them directly by virus-mediated cytotoxicity or indirectly by stimulating host anti-tumor immune response (Ferguson et al. 2012; Lichty et al. 2014). Many wild-type viruses show an intrinsic selectivity for replication within cancer cells while others are genetically engineered to change their cellular, tissue or organ tropism to cancer. Due to their intrinsic characteristics, cancer cells are supposed to be more susceptible targets than normal cells for the viral infection such as resistance to apoptosis, high non-suppressible metabolic activity, poorly developed lymphatics, poor responsiveness to interferons (IFNs) and intrinsic suppression of immune effector response. The altered host microenvironment of cancer cells provides a suitable milieu for oncolytic viruses (Kaufman et al. 2015; Singh et al. 2012). Specific targeting of these viruses to cancer cells could be achieved by either deleting the viral gene required for survival in normal cells but dispensable in tumor cells or using tumor specific promoter for critical viral genes. The following strategies are used for targeting the oncolytic viruses specifically to tumors:

(i) **Targeting specific tumor biomarkers expressed on the cell surface**: Many surface antigens in cancer cells ectopically expressed highly in comparison to normal cells. Oncolytic viruses use them to enter via receptor-mediated endocytosis (Aghi et al. 2008). For example, Intracellular adhesion molecule-1 (ICAM-1) and decay accelerating factor (DAF) are over expressed in many cancer cells which are use by coxsackie viruses for infection (Johansson et al. 2004). CD155 is over-express in colorectal carcinoma (Masson et al. 2001) and in brain tumors (Merrill et al. 2004) which is use by polio virus for infection. However, other defective signaling pathways are use for selective targeting and replication of these viruses in cancer cells if these surface molecules are also expressed on normal cells. Single chain variable fragments (scFv) antibodies and peptide ligands have also been displayed on the viral surface to provide the specificity to the tumor cells.

(ii) **Defective interferon signaling of cancer cells for selective viral replication**: Defective IFN signaling, a common characteristic shared by many cancer cells provides a major privilege to viral replication (Stojdl et al. 2000). Viral infection stimulates type-1 IFN production in normal cells that inhibits protein synthesis in the neighboring cells which protect them from viral infection and

replication. The defective IFN pathway of cancer cells is used for specific targeting of oncolytic viruses. Some viruses synthesize protein to suppress the antiviral type-1 IFN response of normal cells. Such viruses are engineered to mutate the gene of IFN antagonizing protein to maintain the anti-viral immunity of the normal cells to restrict the viral replication only in cancer cells defective in IFN signaling (Elankumaran et al. 2010). Mutated matrix protein (M) of Vesicular stomatitis virus is unable to block the IFN response of normal cells and so cannot infect it while they propagate in cancer cells defective in IFN pathway.

(iii) **Pro-apoptotic molecules targeting host cell for virus survival**: For continuous replication of the virus after infecting the host cells, the proliferation pathway is manipulated by the virus to avoid or delay apoptosis. Mutation or deletion in the viral gene responsible for manipulating the apoptosis of the host cell to prolong the survival of the virus has opted for successful engineering for oncolytic viruses. Adenoviral proteins E1A and E1B inactivate pRb and p53 respectively in normal cells to delay apoptosis and alterations in these genes are utilized for specific targeting of cancer cells. Viral replication, packaging, and production occur more efficiently in cancer cells than normal cells. Tumor-driver mutations augment this process in cancer cells (Coffey et al. 1998). As the cells are destroyed by oncolysis, new viruses are released to destroy the remaining cancer cells (Russell et al. 2012).

(iv) **Use of tumor specific promoters in oncolytic virus engineering**: The DNA viruses can be engineered to specifically replicate in tumor cells by regulating the expression of some of the essential viral genes under the control of tumor specific promoters. Human telomerase reverse transcriptase has been used for a broad range of tumors while prostate specific antigens (PSA) and α-fetoprotein promoter are used for specific type of tumors like prostate and liver cancer respectively. Selective expression of E1 gene in adenovirus has been achieved with such strategies for targeting a particular tumor type (Lanson et al. 2003).

(v) **Viral engineering for tumor microenvironment targeting**: Hypoxic nature, as well as various activated proteins of the tumor microenvironment, can be used for targeting virus to some of the solid tumors. With this concept, an adenovirus has been designed for breast cancer therapy in which E1A promoter has been put in the control of dual specificity promoters of hypoxia and estrogen receptor elements (Hernandez-Alcoceba et al. 2002).

(vi) **Cellular vehicles for oncolytic viruses delivery**: Therapeutic oncolytic viruses develop antibodies in the host that restricts the repeated administration of the oncolytic viruses. To take care of such issues, carriers have been developed based on the tumor type to deliver the oncolytic viruses. Tumor secretes various chemokines and attracts immune cells to promote and metastasize the malignancy. These immune cells can also be manipulated to deliver the oncolytic viruses to tumors. Stem cells, as well as cancer cells, can also be manipulated for specific delivery of oncolytic viruses (Guo et al. 2008).

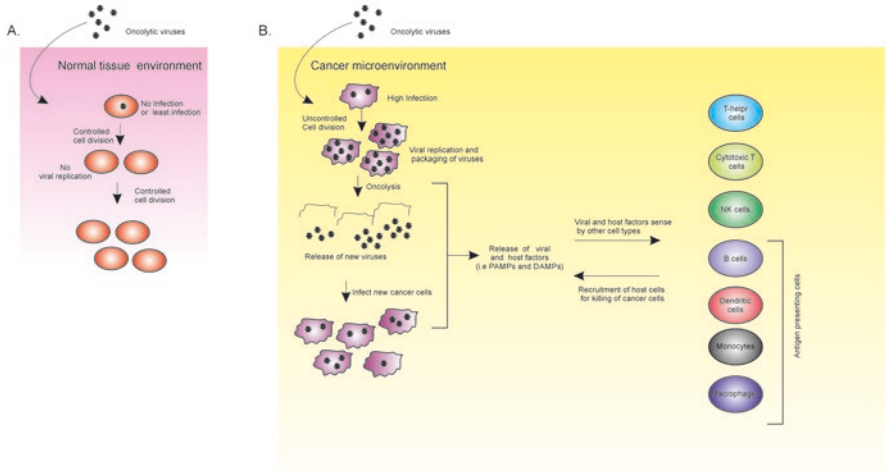

Fig. 17.1 Mechanism of action of oncolytic viruses. (**a**) Oncolytic viruses cannot infect or least infect to normal cells. If some normal cells are infected by viruses they are clear by immune system and does not pass in subsequent cell division. (**b**) Oncolytic viruses infect very highly to cancer cells in comparison to normal cells. These viruses utilizes host cell machinery to replicate and package for production of new viral particles. When the viral load inside the cell increases, oncolysis occurs and new viruses are released into cancer micro-environment. These viruses are now ready to infect new cancer cells. During the process of oncolysis pathogen-associated molecular patterns (PAMPs) from viruses (e.g. viral antigens) released into micro environment. Also, host cell debris called damage-associated molecular patterns (DAMPs) are release. PAMPs and DAMPs recruit immune cells such as NK cells, T helper cells (i.e. CD4 cells) and cytotoxic T cells (i.e. CD8 cells) for killing of cancer cells. Antigen-presenting cells (APCs) which includes B-cells, dendritic cells, monocytes and macrophages present the antigens to T helper cells and cytotoxic T cells during the above process of cell cancer cell killing

In a tumor microenvironment, viral replication leads to innate and adaptive immune activation. This usually is caused by the release of pathogen-associated molecular patterns (PAMPs) from the virus (e.g. viral antigens which may include DNA, RNA, lipid or protein molecules). The activated immune system puts a cheek on virus spread. However, the release of damage-associated molecular patterns (DAMPs) from host cells (e.g. host cell debris) inhibit the above process and favors viral replication. PAMPs and DAMPs recruit natural killer (NK) cells, T helper cells (i.e. CD4 cells) and cytotoxic T-cells (i.e. CD 8 cells) for killing of cancer cells (Escamilla-Tilch et al. 2013; Tang et al. 2012). Antigen-presenting cells (APCs) which include B-cells, dendritic cells, monocytes, and macrophages present the antigens to T helper cells and cytotoxic T-cells during the process that modulate the immunity of the host from pro-tumor to anti-tumor. A schematic for mechanism of action of oncolytic viruses has been shown in figure (Fig. 17.1).

17.3 Oncolytic Viruses and Their Application Against Cancers

A large number of viruses have been discovered with oncolytic activities. A few of them are naturally occurring (e.g. Reo virus and Seneca virus) but most of them are genetically engineered (e.g. Herpes simplex virus, Measles virus, Newcastle disease virus and Vaccinia virus) for clinical trials in cancer treatment (Donnelly et al. 2012; Roberts et al. 2006). ECHO-7, a live strain of enterovirus is the world's first natural oncolytic virus for treatment of skin melanoma was approved by a national agency in Latvia in 2004 (Chumakov et al. 2012). H101, a genetically modified oncolytic adenovirus for the treatment of head and neck cancer was approved in China in the year 2005. Talimogene laherparepvec, a modified herpes simplex oncolytic virus was approved by multiple countries for the treatment of advanced inoperable melanoma. The individual characteristic and research advancement for major DNA and RNA viruses were discussed here in more details. Also, a table has been given for oncolytic viruses and their implication in clinical studies (Table 17.1).

17.3.1 DNA Viruses as Oncolytic Viruses

17.3.1.1 Adenovirus

Adenoviruses belong to family Adenoviridae. They are nearly 70–90 nm in diameter, naked (outer lipid bilayer is absent) with an icosahedral capsid. They possess a linear double stranded DNA of about 24–26 kb. Human is the natural host for them. They infect both dividing and nondividing cells and are maintained as an episome inside the cells. Lytic replication of engineered adenovirus causes cell lysis. Characteristic of this virus is well understood and can be manipulated genetically to generate more effective virus for oncolysis. ONYX-015 is an adenoviral vector that lacks E1B-55K coding gene, whose encoded protein is needed for adenoviral replication in normal cells. This was the first adenoviral vector to be examined in the clinical trials (Heise et al. 1997). In general, E1B proteins inhibit the host cell apoptotic response which helps the virus to survive in the cell. It also facilitates export of adenoviral late mRNA from nucleus to the cytoplasm, a function shared by many cancer cells, allow them to replicate in cancer cells but not so well in normal cells (O'Shea et al. 2004). E1A is an immediate early gene which helps the virus to infect nondividing cells. The conserved region-2 (CR2) of E1A protein binds with members of Retinoblastoma family (pRB) of tumor suppressors releasing E2F transcription factor from pRB-E2F transcription repressor complex and inactivates pRB. The free E2F initiates transcription of genes in the S-phage of the cell cycle and trigger the cell cycle of the quiescent host cells that efficiently replicates the viral DNA. Mutation or deletion in the CR2 region of E1A, which is conserved among human adenovirus serotypes, has been exploited for specific targeting of highly

Table 17.1 Oncolytic viruses and their impliction in clinical studies

Oncolytic virus	Design of virus	Representative implication in clinical trial
Adeno virus		
CG0070	Deletion of E3 and insertion of human GM-CSF	Phase-I trial for bladder cancer
DNX-2401	Δ24 arginine -glycine -aspartate peptide insertion	Phase-I trial for recurrent malignant gynecologic disease; intraperitoneal delivery is safe
ICOVIR5	Opimitization of modified DNX-2401-E2F promoter	Exploratory trial for glioma patient; mesenchymal stem cell loaded virus and intra-arterial injection is safe.
Ad5PTD (CgAE1AmiR122)	Adenovirus serotype-5; deletion of E1B; protein transduction domain motif in capsid, conditional neuroendocrine E1A expression with miR122 in UTR	Not known
Ad5-yCD/ mutTKSR39rephIL12	Adenovirus serotype-5, insertion of IL12, yeast cytosine deaminase and thymidine kinase mutant TKSR39	Not known
Enadenotucirev (ColoAd1)	Chimeric strain of Adenovirus-11/3 group B	Not known
VCN-01	Insertion of PH20 hyaluronidase; targeting of arginine -glycine -aspartate peptide	Not known
Herpes virus		
G207	Deletion of ICP34.5 and disruption of UL39	Phase-I trial for glioma
HF10	Deletion of UL56; single copy of UL52	Phase-I trial for pancreatic cancer
HSV-1716	Deletion of ICP34.5	Exploratory trial for melanoma; phase-I trial for glioma
Talimogene laherparepvec	Deletion of ICP34.5 and US11; insertion of human GM-CSF	Phase-III trial for melanoma
Parvo virus		
ParvOryx	Wild strain Parovirus H1	Phase-I and Phase-II trial for recurrent glioblastoma
Vaccinia virus		
PexaVec (JX 594)	Deletion of thymidine kinase and insertion of human GM-CSF	Phase-Ib trial for colorectal cancer; Phase-II trial for hepatocellular carcinoma

(continued)

Table 17.1 (continued)

Oncolytic virus	Design of virus	Representative implication in clinical trial
Psostvac	Artificially engineered to express prostate specific antigen and 3 costimulatory genes; administer with a subsequent fowl-pox	Phase-I and Phase-II trial for prostate cancer
Coxsackievirus		
Cavatak	Wild strain of coxsackievirus A21	Phase-II trial for melanoma
Measles virus		
MV-NIS	Edmonston vaccine strain of measles; insertion of sodium-iodide symporter	Phase-I trial for ovarian cancer
Polio virus		
PVS-RIPO	Attenuated strain of Sabin type 1 polio virus; IRES replaced with human rhinovirus type-2 IRES to prevent neurotoxic side effects	Phase-I trial for glioma
Reo virus		
Reolysin	Wild strain Reovirus serotype-3	Phase-I trial solid tumor; Phase-II trial for melanoma
Retro virus		
Toca 511	Murine leukemia virus: Insertion of yeasr cytosine deaminase	Not known
Vesicular stomatitis virus		
VSV-hIFNB	Insertion of interferon-β	Phase-I trial for multiple myeloma, T-cell lymphoma, acute myeloid leukemia

Note: *GM-CSF* granulocyte macrophage colony stimulating factor, *UTR* Untranslated region, *IRES* Internal ribosome entry site

dividing cancer cells by the replication competent oncolytic adenovirus. However, deletion of the E1A gene results in replication deficient adenovirus vector affecting the critical function of the gene, which is used for heterologous transgene expression. Replacement of E1A promoter/enhancer with tissue specific promoter/enhancer such as PSA promoter/enhancer has been used for designing of CV706 oncolytic virus that can preferentially replicate in PSA positive cancer cells (DeWeese et al. 2001). In another attempt, the promoter of E1A has been replaced by hTERT promoter in Ad3 backbone (Ad3-hTERT-E1A) which is mostly activated in cancer cells giving specificity to the viral replication in cancer cells (Hemminki et al. 2012). Similarly, other tissue can be targeted by using this approach. The binding receptors of the adenovirus differ among serotypes and so efficient targeting of

these viruses to different cancers sometimes requires further manipulation. Such viruses are called infectivity enhanced adenoviruses. Adenovirus-5 serotype generally infects the host through the natural receptor called coxsackie adenovirus receptor (CAR), but many cancers tissue (e.g. ovary and bladder cancer) express a low level of these receptors. In such cases, fiber gene is engineered and fiber knob is modified to better interact with CAR receptor. Even the fiber-knob of adenovirus-5 serotype has been replaced by adenovirus-3 serotype to enhance the tumor specific infectivity. The adenovirus-3 knob binds to CD46 (Trinh et al. 2012) or desmoglein-2 (Wang et al. 2011), which is found on the surface of cancer cells (Tuve et al. 2006). In some cases, an arginine-glycine-aspartate (RGD) peptide is incorporated into H1 loop of the adenovirus-5 fiber knob, allowing the vector to infect cells through the αvβ- integrins that are express highly on the cell membrane of many multiple types of cancer (Krasnykh et al. 2000). The strategies of specific deletions in viral genes or substitution of viral promoter with tumor specific promoters have been applied to provide the tumor specificity to the adenoviruses whereas other methods were implemented to increase the infectivity along with specificity. But some modifications have also been applied to these engineered adenoviruses to increase the efficiency of the modality. Infectivity enhanced adenoviruses with a deletion in CR2 regions (Δ24) has also been modified to express human granulocyte macrophage colony stimulating factor (GM-CSF) from the deleted E3 region of the vector genome. The E3 gene is not crucial for adenovirus replication but it protect infected cells from host immune system. These infected cancer cells secrete cytokine which stimulates growth and differentiation of dendritic cells. The dendritic cells, in turn, engulf tumor antigens and migrate to lymph nodes where it activate anti-tumor responses. Oncolytic adenovirus vectors engineered with human GM-CSF have shown anticancer activity in clinical reports (Cerullo et al. 2010, 2011; Chang et al. 2009; Koski et al. 2010). Ideally, a single round of treatment should be efficient enough to eradicate the disease but intra-tumor barriers and size of the tumor lower the efficacy of the modality in case of advanced tumor masses (Bilbao et al. 2000; Sauthoff et al. 2003). However intra-tumoral injection would not get affected by auto-antibodies but metastatic disease would require either prolonged systemic dissemination resulting from intra-tumoral injection or systemic administration of the virus. Hence, re-administration of the virus is likely to be required for increased efficacy but the neutralizing antibody response induced by the virus is the limiting key factor in systemic re-administration of the virus (Chen et al. 2000; Sarkioja et al. 2008). To circumvent the issue, different serotypes of the adenoviruses (e.g. adenoviruses-3 and -5) are engineered to avoid the neutralization by the other serotype. Even, chimeric virus, colo adenoviruses-1, has also been developed by pooling an array of serotypes which were then subjected to recombination and further selection to generate specific chimeric virus for the purpose (Kuhn et al. 2008). Presence of neutralizing antibodies in host cell against this virus, toxicity in liver and variable expression of CAR in cancer cells put some limitation on these viruses to explicitly use for cancer therapy.

17.3.1.2 Herpesvirus

Herpesviruses belong to family Herpesviridae. They are nearly 200 nm in diameter, enveloped with a lipid bilayer and an icosahedral capsid. They possess a double stranded DNA of about 152 kb. Human is the natural host for them. Lytic replication of engineered viruses causes lysis of cancer cells. These viruses first bind to heparan sulfate on target cell followed by entry of viral nucleocapsid into the cytoplasm and then transport into the nucleus for the release of DNA followed by replication. The large genome size of herpesvirus creates an option for gene manipulation to make it more efficient for oncolytic action. Thymidine kinase gene allows treatment with ganciclovir. Oncolytic viruses generated by engineering herpes simplex viruses (HSVs) is a hop for cancer treatment. For instance, Talimogene laherparepvec is an immunostimulatory engineered oncolytic HSV. It is derived from JS1-strain of HSV in which viral ICP34.5 and ICP47 gene are removed and human GM-CSF gene is cloned. The function of ICP34.5 and ICP47 genes were to block antigen presentation by HSV1 major histocompatibility class-I on the surface of the infected cell causing immune evasion (Goldsmith et al. 1998; Liu et al. 2003). Intratumoral injection of this one is effective in melanoma patient especially with stage IIIB, IIIC, IVM1a, or treatment-naive disease. Regression of tumor was also seen in distant noninjected lesions, suggesting a systemic antitumor immunity effect (Andtbacka et al. 2015; Kaufman et al. 2010). The reasonable effect of Talimogene laherparepvec (also called OncoVex or T-VEC) may be due to the inherent property of clinical HSV1 strain, immunostimulation by insertion of human GM-CSF and the selection of melanoma patient treatment which is an immunogenic tumor. However, this virus is toxic and function of many genes are not well understood. Systemic delivery is limited by preexisting immunity and hepatic absorption.

17.3.1.3 Parvovirus

Parvoviruses belong to family Paroviridae. They are nearly 25 nm in diameter, naked (outer lipid bilayer is absent), with an icosahedral capsid. They possess a single-stranded DNA of about 5 kb. They normally infect the rodents and are non-pathogenic to humans. Parvoviruses are intrinsically tumor selective because of the permissive nature of the cancer cells for virus survival. These viruses are promising anti-cancer medicine as they infect the cancer cells and lyse them. The cognate receptor for the virus entry is yet to be identified but sialic acid and transferrin receptors have been reported to be involved in membrane recognition and entry of many of these viruses. Recently, activated pyruvate dehydrogenase kinase-1 has been reported to be essential for virus survival and replication in the cancer cells (Bar et al. 2015). Out of two non-structural proteins NS1 and NS2 of -parvovirus-H1 (H-1PV), the former (NS1) plays an important role in DNA replication, protein expression and virus induced cytotoxicity (Geletneky et al. 2015). ParvOryx (Oryx translational medicine, Germany) is an oncolytic wild type H-1PV which can infect

and lyse various tumor cells types spreading from glioblastoma, breast cancer, lung cancer, pancreatic cancer to cancer stem cells. The clinical trial for glioblastoma has been completed and for the metastatic pancreatic cancer is in progress. Preclinical models have also shown that H-1PV can also kill the blood cancer cells (B –cell lymphoma and T-cell lymphoma/leukemia) via productive infection and are even efficient for some of the chemoresistant hematological malignancies (Angelova et al. 2009).

17.3.1.4 Vaccinia Virus

Vaccinia viruses belong to family Poxviridae. They are nearly 70–100 nm in diameter, enveloped with brick-shaped geometries and complex capsid. They possesses a positive sense double stranded DNA of about 190 kb. Human is the natural host for them. Lytic replication of engineered viral causes lysis of cancer cells with EGFR and E2F activity. This virus bind to the glycosaminoglycans and lamin on target cell. Then it forms a transient macropinocytosis and enters into the cell by endocytosis. Vaccinia virus penetration factors regulate the entry of the virus. Vaccinia virus has high efficiency of transduction. Large genome size create an option for manipulation of many genes to make it efficient for oncolytic action and spread into nearby cells and tissues. It has a broad range of tumor tissue infectivity. However, the unknown function of many viral genes, the rapid emergence of neutralizing in host cell against the virus and inefficient systemic delivery put a check on these viruses to be use in cancer therapy (Albelda and Thorne 2014; Guse et al. 2011; Laliberte et al. 2011).

17.3.2 RNA Viruses as Oncolytic Viruses

17.3.2.1 Coxsackie Virus

Coxsackie viruses belong to family Picornaviridae. They are nearly 28 nm in diameter, naked (outer lipid bilayer is absent) with an icosahedral capsid. They possess a double single stranded RNA of about 7.3 kb. Human is the natural host for them. This virus attack cancer cells over expressing intercellular adhesion molecule-1 (ICAM-1) and decay accelerating factor (DAF). Virus enters into host cells from tight junctions through interaction with DAF. It also requires occludin, Rab 34 and Rab 35 for the entry. This virus can be targeted to multiple tumor types. However, it is reported to cause limb paralysis in mice as well as in humans. Host also produce rapid and strong neutralizing antibody response (Au et al. 2011; Bergelson and Coyne 2013; Kemball et al. 2010). Coxsackie virus A21 (CV-A21) is the one of the most studied and promising oncolytic virus. Commercial manufacturing of CV-A21 is done by Viralytics Limited (Sydney, Australia) as Cavatak. Many studies are ongoing at preclinical or clinical trials at various stages of cancer. Because

of the strong neutralizing antibody effect in the host, the efficacy of the virus is compromised and so combinatorial strategies with immune modulatory molecules are also under consideration to extend the therapeutic window of the virus. Recently, a combination of CV-A21 with anti-CTLA-4 drug ipilimumab (Yervoy; Bristol-Myers Squibb) or PD-1 inhibitor pembrolizumab (Keytruda; Merck) has shown promising results in melanoma.

17.3.2.2 Measles Virus

Measles virus belong to family Paramyxoviridae. They are nearly 100–200 nm in diameter, enveloped with a lipid bilayer is present and an icosahedral capsid. They possess a single-stranded, negative-sense RNA of about 14 kb. Human is the natural hosts of this virus where it causes measles, an infection of the respiratory system. Vaccine strain is used to target cancer cells which cause intracellular fusion and cell death. Characteristic of this virus is well understood and very safe to use in human. Measles virus posses hemagglutinin (H) and membrane fusion protein (F) on its surface which are needed for binding to host cell followed by an invasion. Three known receptors for the H protein on the human cell are cell adhesion molecule Nectin-4, signaling lymphocyte activation molecule (SLAM) and CD46 (Yanagi et al. 2009). It causes fusogenic syncytia formation and cell death (Msaouel et al. 2013). It was engineered to express the human sodium/iodide symporter SLC5A5 (MV-NIS) to make an effective oncolytic virus (Msaouel et al. 2009). MV-NIS allows imaging of infected cells, monitoring treatment response and radio-virotherapy with I131–labeled sodium iodide. Clinical trials suggested tumor regression and safe use of this virus in human (Galanis et al. 2015). However, it is susceptible to pre-existing antiviral immune response.

17.3.2.3 Newcastle Disease Virus

Newcastle disease viruses (NDV) belong to family Paramyxoviridae. They are nearly 100–500 nm in diameter, enveloped with a lipid bilayer is present and a helical capsid. They possesses a single-stranded, negative-sense RNA of about 15.2 kb. Chicken is the natural host for this virus. Entry of virus into host cells occurs by fusion of viral envelope with the plasma membrane of host cell called endocytosis. The virus is not pathogenic to in humans. It is safe to use in human because it does not integrate into the human genome. Infection of NDV to cancer cells leads to loss of mitochondrial membrane potential and activation of caspase-9 leading to apoptosis. The process is independent of IFN signaling and so the tumor cells defective in IFNα production or response can also be treated with the virus (Elankumaran et al. 2006). The viral F-protein helps in the fusion of the virus with the cell membrane to spread from cell to cell through syncytia formation which is responsible for cell cytotoxicity (Zamarin and Palese 2012). Rac1 protein has been implicated in tumorigenesis and sensitivity of the cancer cells to the oncolytic NDV (Puhlmann

et al. 2010). The attenuated lentogenic virus is engineered for increased fusogenic efficiency by inserting multiple polybasic sites in F protein to be cleaved and activated by a broad range of proteases. Further mutations can also be inserted in F protein to increase the efficiency of syncytia formation leading to oncolysis (Altomonte et al. 2010; Vigil et al. 2007). Demonstrated IFN1 antagonistic property of the V protein is also used in association with V gene mutation for decreased pathogenicity of NDV to make it a suitable modality for cancer therapy (Huang et al. 2003; Park et al. 2003). Over-expression of apoptin or influenza virus NS1 protein which is the antagonist of mammalian IFNs has also been reported to increase the efficacy of the virus. The IFN sensitive strain (Hitchner B1) of recombinant NDV expressing NS1protein targets cancer cells with both defective and intact immune responses efficiently without compromising the therapeutic safety (Zamarin et al. 2009). NDV-PV701 and NDV-HUJ have been used for clinical trials in cancer therapy and are found to be promising modalities to be further evaluated for a better outcome (Freeman et al. 2006; Pecora et al. 2002). Type-I IFN sensitive recombinant NDV results in selective oncolysis in a mice experiment (Elankumaran et al. 2010). Early reports suggested its promising effect and safety but this virus has poor systemic delivery and is rapidly clear by host immune response (Cripe et al. 2015; Zamarin and Palese 2012).

17.3.2.4 Polio Virus

Polioviruses belong to family Picornaviridae. They are nearly 30 nm in diameter, naked (outer lipid bilayer is present), with an icosahedral capsid. They possess a positive-sense RNA of about 7 kb. It infect neuronal cells in human. Lytic replication of engineered Polio virus has reduced neurotoxicity. Characteristic of this is well understood and it possesses potent antitumor response that can be manipulated genetically to generate more effective virus for oncolysis. However, the virus is not easy to manipulate and in many cases genetically associated with serious disease. It has demonstrated oncolytic activities in preclinical studies and has shown substantial results in brain tumors (Brown et al. 2014). In cells of neuronal origin, internal ribosome entry site (IRES) present in the 5′ untranslated region allows viral gene translation without a 5′ cap (Pelletier and Sonenberg 1988). The IRES on the oral polio virus Sabin type-1 was replaced with the IRES from human rhino virus type-2 (HRV2) which was named as PVS-RIPO. This was done to remove the neurovirulence of the native virus. Once administered, these modified viruses begins duplicating within cells that express CD155/Necl5 and led to selective destruction malignant glioma cells without harming normal neuronal cells. CD155/Necl5 trap the virus in a metastable state. Then the receptor binding followed by internalization release the virus metastable state. Then the membrane fusion occurs. It seems to be breakthrough in recurrent glioblastoma therapy (Goetz and Gromeier 2010; Gromeier et al. 2000).

17.3.2.5 Reovirus

Reoviruses are belong to family Reoviridae. They are nearly 75 nm in diameter, naked (outer lipid bilayer is absent) with an icosahedral capsid. They possess a double stranded RNA of about 23 kb. This virus has a low pathogenicity after infection. Selective replication and toxicity occurs in cancer cells. They generally infect gastrointestinal tract and respiratory tract in humans but mostly their infection is asymptomatic (Sabin 1959). Viruses binds to cell surface carbohydrate and JAM-A on a target cell. Then it enters into cell by clathrin dependent endocytosis, which is promoted by β-1 integrin. They have oncolytic properties and encourage the development of reovirus based cancer therapy (Lal et al. 2009). Oncolytic properties of reovirus depend upon activated Ras signaling (Norman et al. 2004). Reolysin (Serotype-3 Dearing, T3D strain) was studied for the treatment of many cancers and is the only wild type reovirus in clinical development to pass Phase, II and III trial (Gong et al. 2016; Lal et al. 2009). Replication of virus in the host cell is sensitive to the status of a tyrosine kinase receptor called epidermal growth factor receptor (EGFR). If EGFR is stimulated it activate Ras, which then activates multiple proteins in the pathway to induce genes associated with cell survival and proliferation (McCubrey et al. 2007). Over activation of Ras pathway by a mutation in Ras and/or other genes in the pathway leads to cancer and reovirus oncolysis takes advantage of this over activation of Ras pathway resulting in efficient reoviral replication. Interestingly, the involvement of EGFR in reovirus replication was found to be dependent on signaling pathways initiated through the tyrosine kinase receptor rather than receptor binding specificity. Ras signaling cascades which are downstream molecules to EGFR play a major role in selective replication of the reovirus in cancer cells. Ras activation disrupts anti-viral defense in normal cells (Coffey et al. 1998). Both, the activated Ras and Sos are important for permissiveness of the virus replication in the cell. Double stranded RNA dependent protein kinase (PKR) phosphorylates α-subunit of eukaryotic initiation factor-2 and prohibit translational initiation in normal cells (Brostrom and Brostrom 1998; de Haro et al. 1996; Wek 1994). Replication of reovirus produces dsRNA which binds to the N-terminal domain of PKR resulting its dimerization followed by autophosphorylation and activation (Feng et al. 1992; Vorburger et al. 2004). The activated PKR inhibit translation of viral mRNA in normal cells. Elemets of activated Ras pathway inhibits phosphorylation of PKR which is generally responsible for viral protein synthesis in tumor cells. In viral-infected cancer cells, PKR activation is inhibited by elements of Ras pathway and allow translation of viral mRNA (Strong et al. 1998). The reoviral dsRNA binding protein σ-3 sequester the viral genome and help to inhibit PKR activation (Imani and Jacobs 1988). In absence of PKR, virus undergoes lytic cycle. Activated Ras signaling affect reovirus replication in many different ways. Ras activation enhances viral uncoating and disassembly, increases the generation of viral progeny with enhanced infectivity, and accelerates the release of progeny through enhanced apoptosis (Marcato et al. 2007). The specificity of reovirus to Ras activated cancers has been well established but the mechanism of cell death is yet disputed. Among the theories of necrotic cell death, apoptotic cell death and immune

mediated cell death, the concept of virus induced apoptosis is dominant which is initiated by viral capsid proteins during viral endocytosis (Connolly and Dermody 2002). However, the virus is susceptible to anti-viral immune response.

17.3.2.6 Retrovirus

Retroviruses belong to family Retroviridae. They are nearly 100 nm in diameter, enveloped with a lipid bilayer is and a complex capsid. They possess two identical single-stranded RNA of about 7–10 kb. They can infect multiple species and viruses are not lytic in host cell. It mostly replicates in dividing mitotic cells. It is good for gene delivery and can be engineered for the toxic transgene. The virus enters into host cells by endosomal trafficking. After entering into host cell cytoplasm, the virus produces DNA from its RNA using reverse transcriptase enzyme. The newly synthesized DNA is then incorporated into the host cell genome using integrase enzyme and stay as provirus for an indefinite period. When these viruses infect mitotic cells they rapidly spread without cell lysis (Tai and Kasahara 2008). Toca511 is an engineered retrovirus. It is derived from murine leukemia virus and manipulated to express yeast cytosine deaminase enzyme, which converts 5-fluorocytosine to the toxic metabolite 5-fluorouracil. In case of implanted gliomas in mice, Toca511 therapy resulted in long-term survival and systemic antitumor immunity caused by memory T cells (Huang et al. 2015; Perez et al. 2012). Toca511 is currently in a phase-II clinical trial for malignant glioma and has shown promising interim results. However, the viruses are potential for insertional mutagenesis.

17.3.2.7 Rigavirus

Rigavirusis belong to family Picornaviridae. They are nearly 28–30 nm in diameter, naked (outer lipid bilayer is absent) with an icosahedral capsid. They possess a positive-sense single stranded RNA of about 7.5 kb. The enteric cytopathic human orphan (ECHO) type 7 (i.e. ECHO-7 also called RIGVIR) contains a natural strain of rigavirus. RIGVIR was registered for cutaneous melanoma treatment in Latvia on 2004. A study has shown that RIGVIR possess immunomodulating and cytolytic effects in cancer cells and therapy among IB-IIC melanoma patients decreases the mortality rate to near about fivefold (Donina et al. 2015). Also, it has shown promising treatment for lung cancer and histiocytic sarcoma (Alberts et al. 2016).

17.3.2.8 Semliki Forest Virus

Semliki forest viruses (SFV) belong to family Togaviridae. They are nearly 70 nm in diameter, enveloped with a lipid bilayer is present and an icosahedral capsid. They possesses a positive sense single stranded RNA of about 13 kb. SFV was isolated from mosquitoes. These viruses causes encephalitis by naturally infecting cells of

the central nervous system in rodents and very mild symptoms in humans (Atkins et al. 1985; Mathiot et al. 1990). However, one lethal human infection was reported. In that case, the patient was immunodeficient and was exposed to a large number of viruses (Willems et al. 1979). This virus encodes nine different proteins. The 5′ side of the genome code four nonstructural proteins needed for RNA synthesis and 3′ side code for structural proteins (Atkins et al. 1999). Replication of this virus occurs via a negative strand intermediate which gives rise to a full-length viral RNA. SFV has been pre-clinically tested as an oncolytic virus against glioblastoma. A microRNA target sequence is used to modify the virus so that it will survive and replicate in brain tumor cells but not in normal brain cells. This modified virus causes shrinkage of tumor size and lengthened the life span of mice with brain tumors. It was also tested to kill human glioblastoma cell lines (Ramachandran et al. 2017).

17.3.3 Senecavirus

Senecaviruses belong to family Picornaviridae. They are nearly 30 nm in diameter, naked (outer lipid bilayer is absent), with an icosahedral capsid. They possesses a positive sense single-stranded RNA of about 7.5 kb. SVV-001 strain replicates intracellularly and cause cancer cell lysis and reduce their proliferation. The selective tropism of virus replication involves receptor-mediated endocytosis (Hales et al. 2008; Venkataraman et al. 2008a, b). In 2001, it was discovered as a tissue culture contaminate. The SVV-001 strain (names as NTX-010 by Neotropix, Inc.) was developed as an anti-cancer therapeutic against small cell lung cancer (SCLC) and many pediatric solid tumors (Reddy et al. 2007). In a Phase-I trial, SVV-001 was used to treat 30 patients including six with SCLC cases. SVV-001 was well tolerated among patients. Viral clearance was seen in most cases with development of antiviral antibodies (Rudin et al. 2011).

17.3.3.1 Vesicular Stomatitis Virus

Vesicular Stomatitis viruses (VSV) belong to family Rhabdoviridae. They are nearly 80 nm in diameter, enveloped with a lipid bilayer and a helical capsid. They possess a negative sense single stranded RNA of about 11.2 kb. It encodes five major proteins: G-protein, large (L)-protein, phosphoprotein, matrix (M)- protein and nucleoprotein. VSV naturally infect insects, cattle, horses, and pigs. These viruses stay in an environment with deficient IFN signaling (Hastie and Grdzelishvili 2012; Lichty et al. 2004). So hardly survive in healthy human cells because of the IFN response. However, cancer cells possess very low IFN response, which allows VSV to grow and lyse them. Virus binds to cell surface glycoprotein G to LDLR, membrane fusion, and endocytosis. VSV is neurotoxic when injected intracranially or intranasally. IFN-β over-expressing VSV was engineered by inserting the human IFN-β gene between the viral glycoprotein (G) and polymerase (P) protein (Obuchi et al.

2003). The VSV-human IFN-β is in the clinical trial for liver cancer, relapsed multiple myeloma, acute myeloid leukemia, and T-cell lymphoma. It is hypothesized that IFN-β will act synergistically with VSV to protect normal cells from infection, cause the destruction of tumor cell and support antitumor immune responses. VSV was attenuated by deletion of Met-51 from the M- protein which successfully increases its oncolytic properties (Stojdl et al. 2000). Heterologous envelope proteins are also being used to minimize the neurotoxicity of the VSV considering the neurovirulent nature of the G-protein without compromising the efficacy of the virus (Muik et al. 2014). VSV has shown to shrink tumor size and spread in melanoma, breast cancer, prostate cancer, colon cancer, lung cancer, and certain brain tumors (Ahmed et al. 2004; Bridle et al. 2010; Ebert et al. 2005; Ozduman et al. 2008).

17.4 Conclusion

Treatment of cancer by using oncolytic viruses as an immunotherapeutic modality independently and/or in combination with chemo or radiotherapy has shown promising results. It is important to broaden the horizon of tumor targeting components for viruses capable of replicating specifically in the tumor cells. The efficiency of the virus could be further augmented keeping in mind the heterogeneity of the tumor as well as their associated architecture such as stroma and vasculature. These viruses could be engineered to target new or multiple molecules associated with different aspects of cancer survival simultaneously such as metabolism, cellular proliferation, pro-tumor immunity etc. The immunogenic nature of the viruses can also be further manipulated to minimize the effect of auto-antibodies that restricts the administration of these viruses below the required level to achieve the effective cytotoxicity. At the end, better understanding of the complex network of a heterogeneous population of a tumor would provide the key to design oncolytic viruses with better replication efficacy specifically in cancer cells that would be more cytotoxic either alone or in combination with other modality to effectively control or eradicate the malignancy.

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Chapter 18
Microbes in the Treatment of Diabetes and Its Complications

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18.1 Introduction

Diabetes mellitus affects 415 million people globally and incidence of diabetes may increase to 642 million by 2040 (Ogurtsova et al. 2017). It is characterised by hyperglycemia, resulting from decreased insulin secretion from pancreas or insulin resistance to insulin sensitive tissues (Forbes. and Cooper 2013). Diabetes mellitus can be primarily categorized as type 1 or 2 diabetes (ADA 1997). There are other forms such as gestational diabetes, seen during pregnancy and diabetes due to genetic disorders, chemical toxicity, endocrinopathies etc. (ADA 1997). Type 1 diabetes, an autoimmune disorder is characterized by the loss of pancreatic β-cells. The loss of function of β-cells leads to decreased or no release of insulin, resulting in high blood glucose level (Atkinson et al. 2014; Keenan et al. 2010). Eighty-five percent of the total diabetic population suffer from type 2 diabetes. The pathogenesis starts with resistance of muscle, adipose and liver to insulin signalling resulting in a reduced glucose uptake. To compensate this, pancreas produces more insulin leading to eventual deterioration of pancreatic beta cells; decline in insulin production and secretion. Therefore, in type 2 diabetic subjects, hyperglycemia is a result of both reduced insulin secretion and action (Kahn et al. 1993; Kahn et al. 2014) (Fig. 18.1).

Based on the duration and intensity of hyperglycemia in diabetes, microvascular and macrovascular disorders develop. Microvascular complications such as neuropathy-diabetic foot ulcers, retinopathy and nephropathy are due to chronic

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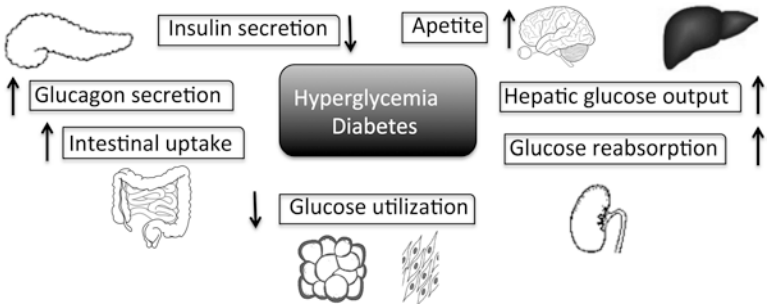

Fig. 18.1 Organ or tissue functions altered in type 2 diabetes

exposure of glucose. Proteins are glycated i.e. glucose forms covalent adducts with plasma proteins, called Advanced Glycation End products (AGEs) (Negre-Salvayre et al. 2009). Macrovascular complications such as cardiovascular disease (CVD) incidence increases in diabetic patients (Mozaffarian et al. 2016). Coronary artery disease (CAD) and myocardial infarction (heart failure) are common CVD disorders. Oxidative stress and inflammation leads to plaque instability, changes in blood coagulation and blood clots in type 1 diabetes patients (Schnell et al. 2013), while patients with type-2 diabetes develop CVD due to hypertension, abnormal cholesterol, triglycerides, obesity and unhealthy lifestyle.

Diabetes or its complications can be alleviated with good glycemic control. This can be accomplished physiologically by increasing insulin level and insulin action, reducing glucagon effect, increasing energy expenditure or glucose utilization, decreasing intestinal energy uptake, lowering food intake and reducing hepatic glucose production. The main organs systems targeted to achieve these are pancreas, liver, muscle, adipose tissue, brain, intestine and kidney. For enhancing insulin level, insulin is administered to both type1 and 2 diabetes subjects. The main class of drugs currently in use for treating diabetes are sulfonylureas, biguanides, thiazolidinedione (TZD), meglitinide, dipeptidyl peptidase 4 (DPP-4) inhibitors, α-glucosidase and amylase inhibitors, sodium-glucose cotransporter (SGLT2) inhibitors, GLP-1 receptor agonists and dopamine receptor agonist (Kahn et al. 2014). Many different medications are given for diabetic complications depending on the nature of complication. For cardiovascular diseases, cholesterol-lowering agent, statins are in use (Grundy 2016). Angiotensin-converting enzyme (ACE) inhibitors are used as therapeutics for diabetic nephropathy (Harris 2005). Some of these therapeutics are obtained from microbes or genetically engineered microbes. This chapter discusses some of the known applications of microbes or its products in diabetes management as well as potential lead molecules identified from microbes and new alternative strategies developed using microbes for diabetic treatment.

18.2 Microbial Intervention

18.2.1 Manipulation of Gut Microbes to Treat Diabetes and Its Complications

Gut microbes are the community of microorganisms residing in intestine of humans and animals. In humans, gut microbes are transferred from mother at birth and composition is established at about 2 years of age (Quigley 2013). Composition of gut flora is uniform in adulthood and is influenced by diet in elderly (Guaraldi and Salvatori 2012). Change in gut microbiota is associated with several metabolic disorders like obesity and diabetes (Greiner and Bäckhed 2016). High plasma level of trimethylamine-N-oxide (TMAO) is a potential risk factor for CVD. Microbes in intestine can produce TMAO from choline and carnitine rich foods (red meat, eggs, fish and high fat dairy), indicating that flora in our intestine can be a possible risk factor for developing CVD. Vegetarians and vegans have lower plasma levels of TMAO. Presence of TMAO activates platelets and increases formation of blood clots, eventually leading to coronary heart disease and stroke. The major phyla of bacteria in gut are firmicutes and bacteroides, higher ratio of firmicutes to bacteroides seem to affect plasma levels of TMAO in human body (Tang et al. 2013; Tang and Hazen 2014; Wang et al. 2011). Firmicutes convert choline and carnitine from eggs and red meat to TMAO. Intestinal bacteria of people suffering from type 2 diabetes differ from healthy individuals. Intestinal microbiota is impaired in obesity and glucose intolerance, with increase in firmicutes and reduction in bacteroides (Kassaian et al. 2017). Lower abundance of bacteroides is associated with inflammation and developing insulin-resistance (Cani et al. 2007). Reduced abundance of butyrate-producing bacteria is seen in type 2 diabetic patients; showing that gut microbiota play an important role (Arora and Bäckhed 2016). Altering gut microbial composition is beneficial for managing diabetes and its complications.

Probiotics are live microorganisms, which can have a positive effect on intestinal microbiota, whereas, prebiotics are plant fibers which are not digested by our body, both makes changes to the composition of intestinal bacteria. When both probiotics and prebiotics are used together, they are called synbiotics. High plasma levels of TMAO, a potential risk for developing CVD can be decreased with the use of probiotics (for example: *lactobacillus*, *bifidobacterium* and yeast) or prebiotics (Tang et al. 2013; Tang and Hazen 2014). Resveratrol is an antioxidant found in grapes, berries and other fruits. Diet rich in resveratrol reduces TMAO by remodelling gut bacteria (Chen et al. 2016). Resveratrol decreases insulin resistance, protects beta cells of pancreas by reducing oxidative damage and improves insulin secretion (Szkudelski and Szkudelska 2015). Indolepropionic acid, produced by gut microbiota protects against type 2 diabetes by preserving beta cell function. A diet rich in fiber and whole grains helps to increase levels of indolepropionic acid and amount of insulin produced from pancreas (de Mello et al. 2017).

Altering ratio of firmicutes to bacteroides has enhanced insulin sensitivity in mice. Cold exposure in mice improved ratio of firmicutes to bacteroides. Transplanting microbiota from cold exposed mice to germ-free mice improved insulin sensitivity, browning of white adipose tissue and energy expenditure. This approach might be useful to manage obesity, dyslipidemia and onset of diabetes (Chevalier et al. 2015; Suarez-Zamorano et al. 2015). *Lactobacillus acidophilus and Lactobacillus casei* delayed onset of type 2 diabetes by reducing total cholesterol, glucose intolerance, insulin resistance, plasma glucose, and oxidative stress in rats (Yadav et al. 2007). *Lactobacillus casei* and *Lactobacillus rhamnosus* administration improved insulin sensitivity and reduced endoplasmic reticulum (ER) stress in skeletal muscle and macrophage activation in white adipose tissues in mice, thereby, improving glucose tolerance (Park et al. 2015). Faecal transplantation with butyrate-producing bacteria improves glucose and lipid metabolism (Udayappan et al. 2014). Supplementing gut microbiome with *Lactobacillus reuteri* through drinking water accelerates ischemic wound healing in mice by upregulation of oxytocin. *Lactobacillus reuteri* supplementation could be a potential treatment for diabetic wound healing (Porporato et al. 2012; Poutahidis et al. 2013).

18.3 Microbial Products

18.3.1 Enzyme Inhibitors

Inhibiting or delaying intestinal glucose absorption is one way of treating type 2 diabetes. It can be achieved by inhibiting enzymes involved in breaking down insoluble starch and glycogen to its soluble monomers (Sales et al. 2012). α-amylase and α-glucosidase are the two main enzymes targeted to lower hyperglycemia. Alpha amylase is mainly found in pancreatic secretions and saliva (Horii et al. 1987; Lorentz 1982), and acts as an endo-enzyme that catalyses breakage of α-(1, 4)-d-glycosidic linkage of insoluble starch and glycogen. *α*-glucosidases on the other hand reside in mucosal brush border of small intestine and catalyse final degradation step of starch and disaccharides to glucose (Matsui et al. 2006; Krishnan and Chandra 1983). Inhibitors of these enzymes reduce or delay digestion and absorption of carbohydrates (Goke and Herrmann-Rinke 1998) and thereby, lower blood glucose. Some of the amylase inhibitors also show promising results for weight loss (Tucci et al. 2010).

Amylase inhibitors are classified into three groups, oligostatins, amino sugars and proteinaceous inhibitors. They are obtained from plant and microbial sources. Examples of microbial based amino sugar- amylase inhibitors are SF638-1 from *Streptomyces* strain PW638, Acarviostatins (I03, II03, III03, IV03) from *S.*

coelicoflavus, Trestatin A, B, C from *S. dimorphogenes* (Meng et al. 2011; Geng et al. 2008; Yokose et al. 1983). Oligostatin C,D,E are obtained from *S. myxogenes,*. Amylostatin Y, Amylostatin S –AI and Adiposin are obtained from *S- amylostaticus*, *S. distaticus* and *S. calvus* respectively (Deshpande et al. 1988; Namiki et al. 1982). Proteinaceous amylase inhibitors obtained from microbial sources are Tendamistat, Haim II, Paim I, Paim II, Parvulustat, AI-409, T-76, AI-3688 from *S. tendae, S. griseosporeus, S. corchorushii, S. corchorushii, S. parvulus, S. chartreusis, S. nitrosporeus and S. aureofaciens* repectively (Vertesy et al. 1984; Hirayama et al. 1987; Rehm et al. 2009; Katsuyama et al. 1992; Sumitani. et al. 2000; Vertesy and Tripier 1985).

The alpha glucosidase inhibitors (AGIs) isolated from microbial source include acarbose made from *Actinoplanes* sp. *SE50*. Acarbose inhibits intestinal -amylase and sucrase with its aminocyclitol moiety valienamine (Truscheit et al. 1981). Miglitol, a semisynthetic derivative of 1-deoxynojirimycin is another glucosidase inhibitor in use. They are obtained from *Bacillu*s and *Streptomyces* strains. Miglitol is also reported to have anti-obesity effect mediated mainly through brown adipose tissue thermogenesis (Hamada et al. 2013; Sels et al. 1999). Voglibose is another α-glucosidases obtained from *Streptomyces hygroscopicies var. limonons* (Dabhi et al. 2013). It is a derivative of validamycin A produced by this species. Voglibose has also been shown to enhance glucagon -like peptide 1 (GLP-1) thereby enhancing insulin sensitivity in humans (Goke et al. 1995).

18.3.2 Hypocholesterolemic Drugs from Microbial Origin

Statins are lipid-lowering medications currently in use. They inhibit enzyme involved in cholestrol biosynthesis, HMG-CoA reductase (hydroxymethylglutaryl coenzyme A) (Ginsberg 2006; Tobert 2003). Mevastatin (a.k.a compactin) was the first statin to be discovered and was obtained from *Pencillium brevicompactum* and *Pencillium citrinum,* However, Lovastatin was the first statin to be approved for treatment of Hypercholestrenemia in United States, FDA (Tobert 1987). Lovastatin, also known as mevinolin was originally obtained from *Aspergillus terreus* (Alberts et al. 1980). Later it was also isolated from different strains of genus *Monascus* (Endo 1979). For commercial production *Penicillium* species, *Monascus ruber* and *Aspergillus terreus* are utilized. Many semi synthetic forms of Lovastatin are currently in use for treating atherosclerosis and cardiovascular disorders. Compactin has been biotransformed using *Streptomyces carbophilus and Actinomadura* sp. to obtain another statin named as Pravastatin. Many synthetic statins modelled from natural statins are also in use (Endo 2010).

18.3.3 Microbes as a Resource for Anti-obesity Molecules

Obesity is a confounding factor for diabetes and associated comorbidities (Van Gaal et al. 2006). Obesity occurs when energy intake is higher than expenditure (Hill et al. 2012). Recently many anti-obese molecules have been identified from microbial origin. Salinomycin, a polyketide produced by *Streptomyces albus*, widely used in poultry industry has exhibited anti adipogenic properties Szkudlarek-Mikho et al. 2012). Monascin and Ankaflavin, two secondary polyketide metabolites isolated from *Monascus purpuresus NTU 568* fermented products have inhibited differentiation of adipocytes as well as lowered lipids (Jou et al. 2010; Lee et al. 2013).

18.3.4 Hypoglycemic Agents from Microbial Source

Type 2 diabetes is often characterised by reduced glucose uptake to insulin sensitive tissues, therefore one of the strategies to combat hyperglycemic state in diabetes is to enhance glucose uptake to insulin sensitive tissues such as adipose and muscle, thereby, maintaining blood glucose levels. There are a few potential lead molecules from microbial sources identified to have hypoglycemic effect. A compound named NFAT 133, enhances glucose uptake in L6 myotubes and reduces plasma glucose in diabetic mice model. This compound is isolated from *Streptomyces strain PM0324667 (*Kulkarni-Almeida et al. 2011). Geodin, a metabolite from fungus *Pencillium glabrum AJ1175* has shown to increase glucose uptake in rat adipocytes (Sato et al. 2005).Glyceollins, a phytoalexin produced from soya bean under fungal stress has shown to stimulate glucose uptake in prediabetic rats (Boue et al. 2012). Adenovirus Ad36 enhances glucose disposal via its E4orf1 protein. E4orf1 exerts its effect through pAKT and Glucose transporter 4 (Hedge et al. 2016).

18.4 Engineered Microbial-Based Therapy

18.4.1 Humulin

Insulin therapy is the most important therapy used for type 1 and 2 diabetes patients to control glucose levels. Insulin in human body is produced from pancreas (beta cells of the Islets of Langerhans) and is mainly involved in maintaining glucose homeostasis. In diabetes, pancreas fails to secrete appropriate amount of insulin required hence resulting in high blood glucose levels (Cernea and Dobreanu 2013). Therefore, patients are administered external insulin. Insulin is produced using different biological systems (animal or genetically engineered microbes) (Ladisch and Kohlmann 1992; Baeshen et al. 2014). Insulin (human) also known as humulin is

produced in non-disease-producing strains of *Escherichia coli and Saccharomyces cerevisiae.* It has been commercially produced several years now (Baeshen et al. 2014).

18.4.2 Engineered GLP1 Secreting Commensal Bacteria for Diabetic Therapy

Reprogramming non-insulin producing cells to insulin secreting cells will be one of the potential therapies to treat diabetes. Recent research has shed light on this aspect. Suzuki et al. discovered that rat epithelial cells secrete insulin upon induction with full length GLP1 in a glucose responsive manner (Suzuki et al. 2003). This paved way for more research on engineered GLP1 secreting cells. Engineered human commensal bacteria (*Lactobacilli*) that secrete GLP-1 were orally administered to diabetic rat and these rats developed insulin producing intestinal cells (Duan et al. 2015). These observations give hope for efficient treatment strategy for enhanced insulin production in the body by orally administered engineered microbes.

18.5 Current and Future Prospectives: The Concluding Remarks

Targeting gut microbial flora either by supplementation, transplantation or altering with prebiotics or probiotics is a promising therapy for diabetic patients. Genetically engineered microbes have been used for producing recombinant insulin for therapeutic use. Recent research is focussing on developing more genetically engineered microbes that can induce insulin production in intestinal cells thereby, compensating the function of beta cells of pancreas. More researchers are also focussing on identifying more potent biomolecules from the microbial mine.

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Chapter 19
Microbe-Based Metallic Nanoparticles Synthesis and Biomedical Applications: An Update

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19.1 Introduction

Nanotechnology has emerged as a new and promising discipline of modern science that encompasses interdisciplinary topics such as material science, physics, chemistry and biology. Thus, nanotechnology has become more fascinating field of study by several group of scientific community including physicists, chemists, material scientists, electronics and mechanical engineers, biomedical researchers, and biologists. The advancement of nanoparticle synthesis and characterization has witnessed novel applications of nanoparticles (NPs) in various fields and it is evolving very rapidly in recent times (Rudramurthy et al. 2016). Typically, NPs are structural materials that possess a specific dimension within the nanoscale range (1–100 nm) (Li et al. 2011; Kato 2011). NPs are composed of three layers namely, the surface layer, shell layer and the core. Mostly, the surface layer is fabricated with different macromolecules, surfactants, metal ions or polymers to impart novel and unique properties (Khan et al. 2017).

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These nanosized particles have greatly attracted because of their uncommon, but attractive physicochemical, electronic, dielectric, electrical, magnetic, mechanical, and biological properties (Narayanan and Sakthivel 2010). In general, NPs exhibit relatively a greater surface to volume ratio, higher Raman scattering and Rayleigh scattering effect in metallic nanomaterials that imparts diverse properties to NPs making them more advantageous as compared to their bulk material counterparts (Narayanan and Sakthivel 2010; Swamy et al. 2015a, b; Akthar et al. 2015). Overall, unique and enhanced properties of nanomaterial are mostly due to their altered shape, size, distribution pattern and other morphological features compared to their bulk particles from which they are synthesized (Swamy et al. 2015a; Raza et al. 2016; Khan et al. 2017). Therefore, a precise control over these factors will govern the characteristic features of NPs and their applications. The controlled synthesis of metallic nanostructures with a definite dimension and organized monodispersity is one of the greatest challenge in nanotechnology research (Maliszewska 2011). In addition, some of the other issues such as easy recovery and purification of the synthesized NPs for their effective uses are equally important. The synthesized nanomaterials with a definite shape, size, and composition are widely explored in numerous fields such as biomedicine, food, cosmetics, energy science, electronics, optoelectronics, agriculture, chemical industries, textiles, optical devices, electrochemical applications, and environment (Iravani et al. 2014; Rudramurthy et al. 2016).

NPs with a size ranging between 0.1 and 1000 nm are frequently synthesized via bottom-up and top-down approaches (Narayanan and Sakthivel 2010; Rudramurthy et al. 2016). In bottom-up approach of nanofabrication, the substrates are added with a building blocks to form nanostructure. Otherwise, molecular nanostructures are formed by the assembly of atoms or molecules that give rise to crystal planes which further stack onto each other to form nanostructures. Usually, bottom-up approach is employed for biological or chemical synthesis of nanostructures. On the other hand, top-down approach employs a gradual degradation or breakdown of larger bulk materials into nano-sized structures. In this method, the nanomaterials are produced by incising out crystals planes that are previously coexistent on the bulk substrate.

Various physical and chemical procedures are broadly utilized for the synthesis of unique monodispersed NPs. The major concern is that both employs toxic chemicals such as such as surfactants, strong reducing agents, polymer capping agents, organic solvents for the synthesis of nanostructures. Also, the nanomaterials synthesized from these methods are unstable for long time and the cost of the chemicals are very expensive. Hence, the adsorption of toxic chemicals on NPs surface and organic solvents used during the chemical synthesis greatly limit their uses in biomedical fields (Narayanan and Sakthivel 2010; Maliszewska 2011; Rudramurthy et al. 2016). Consequently, developing an ecofriendly, cost effective, clean, nontoxic and biologically compatible NPs synthesis procedures are more appreciated.

Accordingly, recent times have witnessed the development of biological approaches for nanoparticle synthesis. Biological or green synthesis approaches comprise mainly polysaccharides, mixed-valence polyoxometalates, biosynthetic and irradiation methods. A careful selection of an appropriate green route synthesis methods must be considered with two important facts such as the right selection of solvent system and toxic free reducing or stabilizing agents (Iravani et al. 2014).

Biosynthesis methods employs an eco-friendly unicellular and multicellular biological entities including plants, fungi, yeast, bacteria, actinomycetes and viruses (Kowshik et al. 2003; Kuber and Souza 2006; Merzlyak and Lee 2006; Swamy et al. 2015a, b; Chengzheng et al. 2018; Akhtar et al. 2015; Rudramurthy et al. 2016; Shah et al. 2015). Among these, plant based NPs synthesis approach is relatively more advantageous and can be converted into a resourceful bio factories (Thakkar et al. 2010; Iravani et al. 2014; Shah et al. 2015). Nevertheless, microbe based approaches have few disadvantages such as microbes isolation, culturing and maintenance that requires more time, difficulty in obtaining better control over NPs size, shape, dispersion, crystallinity, and slow production rate while, phytosynthesis of NPs is a straight forward process which do not require any complex or multi-step processes and is rapid. Phytosynthesis approaches are simpler, economical and can be easily scaled up for producing NPs in bulk quantities (Swamy et al. 2015a, b; Iravani et al. 2014; Shah et al. 2015). Nevertheless, the understandings on microbial strain selection, manipulation of culture conditions such as pH, temperature, time, and metal ions concentrations has given some hope on the possible implementation of microbe based approaches for large scale production of NPs. A possible use of genetically manipulated microbes to overproduce definite reducing agents can further simplify the process in obtaining NPs with a definite sizes and shapes (Narayanan and Sakthivel 2010; Musarrat et al. 2011a, b). In addition, the NPs synthesized through biogenic route possess specific surface area with a greater catalytic activity and an enhanced interaction between the enzymes and metal ions (Bhattacharya and Mukherjee 2008; Li et al. 2011; Shah et al. 2015).

It is well established fact that microorganisms adsorb and accumulate metals from their environment and thus, they are widely used in various biological applications such as biomineralization, bioremediation, and bioleaching. Also, microbes are effective in reducing environmental pollution (Darnall et al. 1986; Huang et al. 1990; Kapoor et al. 1999; Lloyd and Lovley 2000; Klaus-Joerger et al. 2001). Microbes has the potential to reduce metals to form metallic NPs and therefore, microbes including bacteria, yeast and fungi are considered as best suitable source for synthesizing environmental friendly, cost-effective nanomaterials in recent times (Ahmad et al. 2002; Musarrat et al. 2011a, b). Therefore, this chapter highlights the recent research outcomes on NPs synthesized through microbial routes and their possible applications in various biomedical fields.

19.2 Biosynthesis of Metallic NPs Using Bacteria

Among natural sources, bacteria are expansively utilized for the synthesis of various metallic NPs. This is due to the fact that bacterial cells can be easily manipulated and control over their culture conditions. At present, research focus is towards understanding microbes interacting with inorganic molecules. It is reported that microorganisms can secrete inorganic constituents having nanoscale dimensions either within or outside their cells. Microbes resist heavy metal compounds mainly due to several mechanisms. These include, permeability barrier exclusion, chemical detoxification using enzymes, intra- and extra-cellular sequestration, reduction reactions, and electrochemical potential change caused by metal ion efflux between the cell membrane mediated by membrane proteins or proton anti-transporter proteins (Bruins et al. 2000; Narayanan and Sakthivel 2010). Thus, bacteria can effectively detoxify heavy metal ions through reduction reactions or by precipitating soluble inorganic ions into insoluble and non-toxic metallic nanostructures. The bacterial detoxification process could be both extracellular or intracellular way and thus, biosynthesis of several metallic NPs could be achieved through both the ways i.e., intracellularly and extracellularly. In this section, biosynthesis of different metallic NPs using actinomycetes, cyanobacteria and bacteria are discussed.

19.2.1 Silver Nanoparticles (AgNPs)

The most common bacterial species that are utilized for the biosynthesis of metal NPs include *Bacillus cereus, Escherichia coli, Klebsiella pneumonia*, *Actinobacter* sp., *Corynebacterium* sp., *Lactobacillus* sp., and *Pseudomonas* sp. (Tollamadugu et al. 2011; Iravani et al. 2014; Shah et al. 2015). Different bacterial strains utilized for synthesizing AgNPs are listed in the Table 19.1. The biosynthesis of AgNPs using a bacterial strain, *Pseudomonas stutzeri* AG259 isolated from a silver mine was achieved (Klaus et al. 1999). These AgNPs were synthesized in the periplasmic region of the bacterial cell. The particles had a pyramidal and hexagonal shapes and their size ranged up to 200 nm. The occurrence of the Ag-binding proteins act as a source of amino acid moieties for initiating the nucleation reaction during the biosynthesis of AgNPs. It is reported that small silver-binding proteins are found in the periplasmic layer of the bacterial cell bind to silver ions and through ion efflux, prevents the further entry of metals and thus, protect the cell from metal toxicity (Li et al. 1997; Gupta and Silver 1998). The silver ions in the aqueous solution of silver nitrate were shown to get precipitated by the specific silver precipitating peptides namely, AG3 and AG4 to produce silver crystals with a face centered cubic structure (Naik et al. 2002). According to them, these peptides contains specific amino acids mainly, methionine, arginine, lysine, and cysteine which recognize and reduce the silver ions. The correct mechanism of AgNPs biosynthesis from bacteria is still not clear. However, a study by Parikh et al. (2008) has highlighted the possible

Table 19.1 Silver nanoparticles (AgNPs) biosynthesized from bacteria

Microorganisms	Size (nm)	Shape	References
Aeromonas sp. SH10	–	–	Mouxing et al. 2006)
Arthrobacter kerguelensis	4.2–26.1	Spherical	Shivaji et al. (2011)
Arthrobacter gangotriensis	3.6–22.8	Spherical	Shivaji et al. (2011)
Bacillus amyloliquefaciens	15.9–80	Spherical	Fouad et al. (2016)
Bacillus indicus	2.5–13.3	Spherical	Shivaji et al. (2011)
Bacillus cecembensis	2.8–18.2	Spherical	Shivaji et al. (2011)
Bacillus cereus PGN1	4–5	Spherical	Babu and Gunasekaran (2009)
Bacillus flexus	12–61	Spherical/triangular	Priyadarshini et al. (2013)
Bacillus licheniformis	3–170	Spherical	Sarangadharan and Nallusamy (2015)
Bacillus licheniformis	~50	–	Kalimuthu et al. (2008)
Bacillus licheniformis	~40	Spherical	Kalishwaralal et al. (2008)
Bacillus megaterium	80–98.56	Spherical, truncated irregular	Saravanan et al. (2011)
Bacillus megaterium	46.10–80.62	–	Banu and Balasubramanian (2015)
Bacillus subtilis	20–60	Spherical	Paulkumar et al. (2013)
Bacillus subtilis	5–60	Spherical and triangular	Saifuddin et al. 2009)
Bacillus subtilis	15.9–80	Spherical	Fouad et al. (2016)
Bacillus sp.	42–92	Spherical	Das et al. (2014)
Bacillus sp. AZ1	~7–31	Spherical	Deljou and Goudarzi (2016)
Bacillus stratosphericus	2–20	Spherical/triangular/cubic/hexagonal	Hosseini-Abari et al. (2014)
Bacillus thuringiensis	43.52–142.97	–	Banu et al. (2014)
Corynebacterium sp. SH09	10–15	–	Zhang et al. (2005)
Corynebacterium glutamicum	5–50	Irregular	Sneha et al. (2010)
Escherichia coli	40–60	–	Kannan et al. (2010)
Escherichia coli	5–25	Spherical	Divya et al. (2016)
Escherichia coli	42.2–89.6	Spherical	Gurunathan et al. (2009)
K. pneumonia	28.2–122	–	Shahverdi et al. (2007a)
Lactobacillus sp.	100–300	Hexagonal/triangular/other shapes	Nair and Pradeep (2002)
Morganella sp.	~20	Spherical	Parikh et al. (2008)
Pseudomonas mandelii SR1	1.9–14.1	Spherical	Mageswari et al. (2015)
Pseudomonas stutzeri AG259	3–200	Pyramidal/hexagonal	Klaus et al. (1999)
Pseudomonas antarctica	3.4–33.6	Spherical	Shivaji et al. (2011)
Pseudomonas proteolytica	2.8–23.1	Spherical	Shivaji et al. (2011)
Pseudomonas meridiana	2.2–21.5	Spherical	Shivaji et al. (2011)

(continued)

Table 19.1 (continued)

Microorganisms	Size (nm)	Shape	References
Staphylococcus aureus	160–180	–	Nanda and Saravan (2009)
Streptomyces sp.	20–40	Spherical	El-Naggar et al. (2016)
Streptomyces sp. VITSTK7	20–60	Spherical	Thenmozhi et al. (2013)
Streptomyces sp. VITPK1	20–45	Spherical	Sanjenbam et al. (2014)
Streptomyces ghanaensis VITHM1	30–50	Spherical	Abirami and Kannabiran (2016)
Ureibacillus thermosphaericus	10–100	Spherical	Juibari et al. (2011)

molecular mechanism involved in the extracellular biosynthesis of AgNPs by *Morganella* sp. RP-42, isolated from the insect midgut. They observed that *Morganella* sp. produced crystals of spherical AgNPs with an approximate size of 20 nm. The molecular identification revealed the existence of three silver resistant genes namely, silE, silP and silS in *Morganella* sp. The nucleotide sequence of the gene silE obtained from *Morganella* sp. Showed 99% similarity match with known gene, silE known to express periplasmic silver-binding proteins. The study clearly indicated that *Morganella* sp. secretes silver-binding proteins to their extracellular environment during their growth and these proteins probably involve in the reduction of silver metal ions into AgNPs formation and provides stability to the formed nonocrystals (Parikh et al. 2008). Using *Corynebacterium* sp. SH09 cells isolated from a silver mine, Zhang et al. (2005) showed the biosorption and bioreduction of diamine silver complex. The *Corynebacterium* sp. SH09 showed a stronger biosorption capability for [Ag(NH3)2]+. According to them, ionized carboxyl of amino acid moieties involves in trapping [Ag(NH3)2]+ onto the cell walls. The presence of certain reducing agents for example, aldehydes and ketones involve in bioreduction of [Ag(NH3)2]+ to elemental Ag(0). The biosynthesized AgNPs on the cell walls of this bacterium were having the size ranged between 10 and 15 nm. Likewise, Sneha et al. (2010) synthesized irregular shaped AgNPs from *Corynebacterium glutamicum*. The bioreduction of Ag^{+} to Ag^{o} was achieved through an airborne *Bacillus* sp. and importantly, the synthesized AgNPs were localized in the periplasmic region of the cells. Further characterization using Transmission Electron Microscopy (TEM) and Eenergy Dispersive X-ray (EDX) analysis established the synthesized AgNPs with a size of 5–15 nm. A monodispersed uniform sized AgNPs were synthesized from the dried cells of *Aeromonas* sp. SH10. The bioreduction of [Ag(NH3)2]+ to elemental Ag^{0} took place rapidly in the solution and on the cells (Mouxing et al. 2006). A new isolate, *Bacillus cereus* PGN1 resistant to metal was used to form intracellular AgNPs (Babu and Gunasekaran 2009). The characterization of these particles revealed that AgNPs had a spherical shape with 4–5 nm of size. Likewise, a spherical shaped AgNPs with a size range of 3–170 nm were produced from *B. licheniformis* (an isolate from Al Thwara hot spring, Oman) (Sarangadharan and Nallusamy 2015). Similarly, *B. licheniformis* culture supernatant was used to synthesize AgNPs with an

approximate size of 40–50 nm (Kalishwaralal et al. 2008; Kalimuthu et al. 2008). The biosynthesis of silver chloride NPs having the size range of 20–60 nm and spherical shape was prepared from *Bacillus subtilis* (Paulkumar et al. 2013). In another study, a rapid synthesis of AgNPs using *B. subtilis* culture supernatant with microwave irradiation was achieved (Saifuddin et al. 2009). The NPs were having spherical and occasionally triangular shapes. Using *B. stratosphericus*, Hosseini-Abari et al. (2014) synthesized AgNPs having various shapes (spherical, triangular, cubic, and hexagonal) and a size of 2–20 nm. Likewise, Das et al. (2014) synthesized BACILLUS sp. mediated AgNPs with size ranging 42–92 nm and the particles were having spherical shape. Likewise, a thermophile, *Bacillus* sp. AZ1, *Bacillus megaterium* and *Bacillus flexus* were also capable of synthesizing AgNPs extracellularly (Saravanan et al. 2011; Priyadarshini et al. 2013; Deljou and Goudarzi 2016). *Bacillus thuringiensis* and *Bacillus megaterium* mediated AgNPs were also synthesized (Banu and Balasubramanian 2015; Banu et al. 2014). More recently, used *Bacillus amyloliquefaciens* and *Bacillus subtilis* to produce spherical shaped AgNPs (Fouad et al. 2016). A rapid biosynthesis of AgNPs from the culture supernatant of *Klebsiella pneumonia* was achieved. AgNPs sizes ranged between 28.2 and 122 nm. When piperitone was added, reduction process was inhibited and thus, suggesting the role of an enzyme, nitroreductase involvement in the process (Shahverdi et al. 2007a, b). *Escherichia coli* was used to biosynthesize well dispersed AgNPs with a size ranging between 40 and 90 nm (Gurunathan et al. 2009; Kannan et al. 2010). Likewise, green synthesis of AgNPs using *E. coli* has been achieved by Divya et al. (2016). A thermophilic bacteria, *Ureibacillus thermosphaericus* was capable to biosynthesize polydispersed spherical 10–100 nm sized AgNPs through extracellular mechanism (Juibari et al. 2011). Extracellular synthesis of AgNPs was reported by Shivaji et al. (2011) by using culture supernatants of *Arthrobacter kerguelensis, Bacillus cecembensis, Bacillus indicus, Pseudomonas meridiana, Pseudomonas antarctica, Pseudomonas proteolytica, Arthrobacter gangotriensis*. Bioreduction of silver ions to AgNPs using *Staphylococcus aureus* was shown by Nanda and Saravanan (2009). *Pseudomonas mandelii* SR1 assisted in synthesizing AgNPs having an average diameter of 1.9–10 nm. These AgNPs were highly stable even after 19 months of storage period (Mageswari et al. 2015). Several *Streptomyces* sp. isolates were used to biofabricate spherical shaped AgNPs with the size in the range of 20–60 nm (Thenmozhi et al. 2013; Sanjenbam et al. 2014; El-Naggar et al. 2016). Likewise, *Streptomyces ghanaensis* VITHM1 strain was used to produce highly stable spherical shaped AgNPs with an average size between 30 and 50 nm range (Abirami and Kannabiran 2016).

19.2.2 Gold Nanoparticles (AuNPs)

A variety of AuNPs have been successfully synthesized by using various bacterial strains (Table 19.2). These AuNPs with different shapes have a unique properties to be considered for biomedical applications. The application of AuNPs for curing

Table 19.2 Gold nanoparticles (AuNPs) biosynthesized from bacteria

Bacterial strain	Size (nm)	Shape	References
Bacillus clausii	20–30	Spherical	Zhang et al. (2016)
Bacillus licheniformis	38	Spherical	Singh et al. (2014)
Bacillus licheniformis	10–100	–	Kalishwaralal et al. (2009)
Bacillus marisflavi	~14	Spherical	Nadaf and Kanase (2016)
Bacillus niabensis 45	10–20	Spherical	Li et al. (2016a, b)
Deinococcus radiodurans	43.75	Spherical/triangular/ irregular	Li et al. (2016a, b)
Escherichia coli DH5α	25 ± 8	Spherical	Du et al. (2007)
Klebsiella pneumoniae	4–10	Spherical	Srinath and Rai (2015)
Klebsiella pneumoniae	35–65	Spherical	(Malarkodi et al. 2013)
Lactobacillus sp.	100–300	Hexagonal/triangular/other	Nair and Pradeep 2002
Marinobacter pelagius	2–10	Spherical/triangular	Sharma et al. (2012)
Pseudomonas aeroginosa	15–30	–	Husseiny et al. 2007)
Pseudomonas aeruginosa	10–20	Spherical	Singh and Kundu (2014)
Shewanella oneidensis	12 ± 5	Spherical	Suresh et al. (2011)
Rhodopseudomonas capsulata	10–12	Spherical	He et al. (2007)
Rhodopseudomonas capsulata	50–400	Spherical	Singh and Kundu (2014)
Shewanella algae	10–20	–	Konishi et al. (2004)
Stenotrophomonas maltophilia	~40	Oval	Nangia et al. (2009)

numerous diseases has been practiced ever since from 2500 BC to till date. The importance of AuNPs was recognized over 150 year back when Michael Faraday observed different properties of colloidal gold solution differing from their bulk material, gold (Li et al. 2011). In recent times, biosynthesis and applications of AuNPs has been highly acknowledged. Microbe assisted synthesis of AuNPs is an eco-friendly approach thus, gained more attention compared to other methods as synthesis occurs immediately in the surrounding temperature and pressure (Das et al. 2014; Srinath and Rai 2015). *Lactobacillus* strains, commonly occurring in buttermilk were used to synthesize AuNPs crystalline structures with submicron dimensions (Nair and Pradeep 2002). *Shewanella algae* and *Shewanella oneidensis* were also successfully employed to biosynthesize monodispersal AuNPs in nano regime (Konishi et al. 2004; Suresh et al. 2011). AuNPs formed extra-cellularly from *Pseudomonas aeruginosa* possessed size in the range of 15–30 nm (Husseiny et al. 2007). The bacteria, *Rhodopseudomonas capsulata* was successfully used to produce AuNPs of varied size and shape (He et al. 2007). An average size of AuNPs formed on the bacterial cells (*E. coli* DH5α) surface was found to be 25 ± 8 nm (Du et al. 2007). For the first time, Sharma et al. (2012) employed the marine bacteria,

Marinobacter pelagius to biosynthesize AuNPs Du et al. (2007). They obtained spherical AuNPs in the range of 10–20 nm. Likewise, AuNPs from *Stenotrophomonas maltophilia* were having an approximate size of ~40 with oval shape (Nangia et al. 2009). A successful biosynthesis of AuNPs from *Rhodopseudomonas capsulata* and *Pseudomonas aeruginosa* was achieved by Singh and Kundu (2014). *Rhodobacter capsulatus*, one of the photosynthetic bacteria was shown to have a strong biosorption capability for the $AuCl_4^-$ The presence of carotenoids and certain enzymes on the cell membrane and/or found in their surroundings are believed to involve in the bioreduction of Au^{3+} to Au^0. Further, they speculated that due to this phenomenon the bacterium can exhibit metal tolerance capability (Feng et al. 2007). Likewise, *B. licheniformis, B. niabensis* 45 and *B. clausii* were also used for extracellular biosynthesis of AuNPs (Kalishwaralal et al. 2009; Singh et al. 2014; Zhang et al. 2016; Li et al. 2016a). Further, Li et al. (2016a) predicted the role of a cyclic peptide (P2) with a molecular weight of about 1122 Da involvement in the stabilization of nanocrystals. AuNPs had a spherical shape with an average diameter of 38 nm. Similarly, *Klebsiella pneumoniae* was employed to synthesize spherical shaped AuNPs having monodispersal nature with a spherical shape and their size ranged between 4 and 65 nm (Malarkodi et al. 2013; Srinath and Rai 2015). *Deinococcus radiodurans*, known for its extreme radiations and oxidant stress resistance was used to reduce Au (III) and AuNPs synthesis (Li et al. 2016b). The particles showed polydisparity with spherical, triangular and irregular shapes and had an average size of 43.75 nm. Nadaf and Kanase (2016) used *Bacillus marisflavi* to produce spherical shaped AuNPs with an average size of ∼14 nm.

19.2.3 Magnetic Nanoparticles

Biomineralization is a naturally occurring process mainly mediated by certain microbes commonly called as magnetotactic bacteria. These bacteria use biomineralization proteins to form a well-organized magnetite nanocrystal structures (Bazylinski and Frankel 2004; Arakaki et al. 2008). Most of these magnetotactic bacteria have a diverse morphological features and exist mainly in the sediments of freshwater as well as marine region (Narayanan and Sakthivel 2010). Many kinds of magnetic NPs are currently produced due to their potential nanobiotechnological applications including cancer cure through magnetic hyperthermia. The bacterial nanoparticles are synthesized under stringent biological conditions with bacterial cell membrane lipid layer and definite membrane proteins (Chen et al. 2016). More recently, nanoscience research is focused on magnetic NPs due to the fact that they possess a unique nanoconfiguration with superior properties including high coercive force and super paramagnetic, thus broadly applied in various biomedical fields (Li et al. 2014; Rudramurthy et al. 2016; Chen et al. 2016). The isolated magnetotactic bacteria from marine sulfide-rich water and sediments produced intracellular nanocrystals of ferromagnetic iron sulfide and greigite (Fe_3S_4). They were aligned in chains in association with iron pyrite (FeS_2) and each chain

contained about ten NPs with irregular shape and 75 nm in size. Most of the particles have irregular shape, whereas some exhibit octahedral and cubo-octahedral symmetry with strong diffraction contrast (Mann et al. 1990; Narayanan and Sakthivel 2010). *Magnetospirillum magneticum* has the capability to form 50–100 nm sized ferromagnetic particles (Fe_3O_4 or Fe_3S_4). These NPs were surrounded by magnetosomes, an intracellular phospholipid membrane (Schuler and Frankel 1999). Magnetosomes from *Desulfovibrio magneticus* RS-1 were irregular/bullet shaped with ~35 nm sized were produced by (Posfai et al. 2006). Each magnetosome contained with 1–18 NPs per chain. Likewise, *Magnetobacterium bavaricum*, another magnetic bacterium had a more than 100 NPs per chain of one magnetosome measuring 110–150 nm. The magnetosome had a irregular shape (Hanzlik et al. 1996). Magnetite crystals with a uniform size were produced by magnetotactic bacteria. *M. magnetotacticum* produced ~50 nm sized Fe_3O_4 NPs (Lang and Schuler 2006). Likewise, bacterial magnetic NPs (Fe_3O_4) with an average diameter of 70 nm were synthesized from *M. gryphiswaldense* MSR-1 cells (Guo et al. 2008). Magnetic iron sulfide (FeS) NPs measuring 2 nm in size were produced on the cell surface of *Desulfovibrio vulgaris*, a sulfate-reducing bacteria (Watson et al. 1999). A study by Bose et al. (2009) reports the biosynthesis of Fe_2O_3 by *Shewanella oneidensis* MR-1 with an average size of 30–43 nm. An iron reducing bacterium, *Shewanella oneidensis* was used by Perez-Gonzalez et al. (2010) to produce Fe_3O_4 NPs with 40–50 nm size. Bharde et al. (2005) synthesized iron oxide NPs by using aqueous iron complexes with *Actinobacter* spp. bacterium. Ferromagnetic particles in the size range of 10–50 nn were produced from *Geobacter metallireducens* GS-15 and *Magnetospirillum* strain AMB-1 and (Vali et al. 2004; Elblbesy et al. (2014). Different bacterial strains utilized for synthesizing magnetic NPs are listed in the Table 19.3.

19.2.4 Sulfide Nanoparticles

Sulfide NPs, being nano-sized possess a unique novel optical and electronic properties and thus, widely applied as used as quantum dots fluorescent biomarker, for cancer diagnosis, radio/chemosensitizing, drug delivery and labeling of cells (Juzenas et al. 2008; Smith et al. 2008; Li et al. 2011). *Clostridium thermoaceticum* precipitated CdS from 1 mM $CdCl_2$ on its cell surface and to the surrounding medium when cultured in the growth media containing cysteine hydrochloride. It was proclaimed that cysteine acted as the sulfide source (Cunningham and Lundie 1993). *Klebsiella aerogenes* and *Klebsiella pneumoniae* formed CdS on their cell surface measuring 5–200 nm when exposed to cadmium ions in the culture medium (Holmes et al. 1995; Smith et al. 1998). These NPs demonstrated optical as well as photoactive features comparable to inorganically synthesized CdS systems (Smith et al. 1998). Likewise, zinc sulfide (ZnS) NPs synthesis has been reported by a sulfate-reducing bacteria (*Desulfobacteriaceae*). In another study by Gong et al. (2007), PbS NPs of 13 nm were synthesized from *Desulfotomaculum* sp. under

Table 19.3 Magnetic nanoparticles biosynthesized from bacteria

Microorganisms	Metal NPs	Size (nm)	Shape	References
Desulfovibrio vulgaris	FeS	2	–	Watson et al. (1999)
Desulfovibrio magneticus RS – 1	Magnetosome	~35	Irregular/bullet	Posfai et al. (2006)
M. gryphiswaldense MSR-1	Fe_3O_4	60	Irregular	Guo et al. (2008)
Magnetospirillum magneticum	Fe_3O_4 or Fe_3S_4	50–100	–	Schuler and Frankel (1999)
Magnetospirillum gryphiswaldense	Fe_3O_4	~50	Octahedral prism/ hexagonal prism	Lang and Schuler (2006)
Magnetospirillum strain AMB-1	Fe_3O_4	48	–	Elblbesy et al. (2014)
M agnetobacterium bavaricum	Magnetosome	110–150	Irregular	Hanzlik et al. (1996)
Geobacter metallireducens GS-15	Fe_3O_4	10–50	–	Vali et al. (2004)
Shewanella oneidensis	Fe_3O_4	40–50	Rectangular/rhombic/ hexagonal	Perez-Gonzalez et al. (2010)
Shewanella oneidensis MR-1	Fe_2O_3	30–43	Pseudohexagonal/ irregular/rhombohedral	Bose et al. (2009)

mild conditions. In this biosynthetic process, *Desulfotomaculum* sp. used sulfate as electron acceptor to form sulfide which acts as sulfur source for forming PbS nanocrystals. The particles were found to have a spherical shape with 2–5 nm diameter (Labrenz et al. 2000). Similarly, Bai et al. (2006) reported a novel synthesis of ZnS NPs of 8 nm by an immobilized *Rhodobacter sphaeroides*. In another study, *E. coli* was shown to synthesize intracellular CdS crystals in nanoregime when cultured along with sodium sulfide and cadmium chloride. These nanocrystals showed the wurtzite crystal structure and their size was about 2–5 nm. Further, biosynthesis of CdS nanocrystals was found to increase up to 20-fold when cells were in the stationary phase as compared to their log phase (Sweeney et al. 2004). *Lactobacillus* sp. mediated biosynthesis of CdS NPs has been reported by Prasad and Jha (2010). According to them *Lactobacilli* sp. with a negative electro-kinetic potential readily attracted cations during the process of biosynthesis. CdS NPs had an average size of 4.93 ± 0.23 nm. Li et al. (2009) reported the CdS nanofibres formation by using the bacterial cellulose obtained from *Gluconoacetobacter xylinus* strains. Similarly, *Rhodopseudomonas palustris* assisted extracellular CdS nanocrystals formation was reported by Bai et al. (2009). According to them, the lyase activity of an enzyme (cysteine desulfhydrase) was responsible for the synthesis of these spherical shaped NPs with an average diameter of 8.01 ± 0.25 nm. Similarly, Bai and Zhang (2009) demonstrated a reliable method of biosynthesizing extracellular lead

Table 19.4 Sulphide nanoparticles biosynthesized from bacteria

Microorganisms	Metal NPs	Size (nm)	Shape	References
B. anthracis PS2010	PbS	–	–	El-Shanshoury et al. (2012)
Desulfobacteraceae	ZnS	2–5	Spherical	Labrenz et al. (2000)
Desulfotomaculum sp.	PbS	13	–	Gong et al. (2007)
Escherichia coli	CdS	2–5	Wurtzite crystal	Sweeney et al. (2004)
Recombinant *Escherichia coli*	CdS	6	–	Kang et al. (2008), Chen et al. (2009) and Mi et al. (2011)
Gluconoacetobacter xylinus	CdS	30	–	Li et al. (2009).
Klebsiella aerogenes	CdS	20–200	Spherical	Holmes et al. (1995)
Klebsiella pneumoniae	CdS	5–200	Spherical/ elliptical	Smith et al. (1998)
Lactobacillus sp.	CdS	4.93 ± 0.23	Spherical	Prasad and Jha (2010)
Klebsiella planticola	CdS		–	Sharma et al. (2000)
Pseudomonas spp.	CdS	10–40	Spherical	Gallardo et al. (2014)
Rhodopseudomonas palustris	CdS	8.01 ± 0.25	Cubic	Bai et al. (2009)
Rhodobacter sphaeroides	ZnS	8	Hexagonal lattice	Bai et al. (2006)
Rhodobacter sphaeroides	PbS	10.5 ± 0.15	Spherical	Bai and Zhang (2009)

sulfide (PbS) NPs of 10.5 ± 0.15 nm size by using immobilized *R. sphaeroides*. *Likewise*, *B. anthracis* PS2010 was demonstrated to biosynthesize PbS NPs (El-Shanshoury et al. 2012). Interestingly, CdS quantum dots in genetically engineered *E. coli* by overexpressing CdS binding peptide (Kang et al. 2008; Chen et al. 2009; Mi et al. 2011). Using Antarctic psychrotolerant bacteria (*Pseudomonas* spp.) quantum dots were biosynthesized at low temperature (Gallardo et al. 2014). The hypothesized mechanism of PbS nanocrystals biosynthesis could be because of Pb precipitation. *Lately*, Plaza et al. (2016) *explored the biosynthesis of* CdS and CdTe quantum dots by using Antarctic bacteria (*Pseudomonas* strains) resistant to cadmium and tellurite. Different bacterial strains utilized for synthesizing sulphide NPs are listed in the Table 19.4.

19.2.5 Selenium Nanoparticles

Selenium NPs possess optical, photochemical, and semiconducting properties. Thus, they are widely used in electronic circuit devices and photocopiers (Narayanan and Sakthivel 2010). Different bacterial strains utilized for synthesizing selenium NPs are listed in the Table 19.5. A rhizobacterial strain (*Stenotrophomonas maltophilia* SELTE02) from the soil of selenium successfully transformed selenite to

Table 19.5 Selenium nanoparticles biosynthesized from bacteria

Bacterial strain	Size (nm)	Shape	References
Agrobacterium sp.	185–190	Spherical	Bajaj et al. (2012)
Azoarcus sp. CIB	123 ± 35	Spherical	Fernández–Llamosas et al. (2016)
Bacillus cereus	150–200	Spherical	Dhanjal and Cameotra (2010)
P. aeruginosa SNT1	–	–	Yadav et al. (2008)
Stenotrophomonas maltophilia SELTE02	–	–	Gregorio et al. (2005)
Halococcus salifodinae BK18.	–	–	Srivastava et al. (2014)
Pseudomonas putida KT2440	100–500	Spherical	Avendaño et al. (2016)
Duganella sp.	140–200	Spherical	Bajaj et al. (2012)
Thauera selenatis	20–300	–	Debieux et al. (2011)
Enterobacter cloacae SLD1a–1	200	Spherical	Yee et al. (2007)
Bacillus mycoides SeITE01	50–400	Spherical	Lampis et al. (2014)
Pantoea agglomerans	30–300	Spherical	Torres et al. (2012)
Pseudomonas aeruginosa	47–165	Spherical	Kora and Rastogi (2016)

elemental selenium (Se^0). They found that selenium granules accumulated in the cell cytoplasm and extracellular space (Gregorio et al. 2005). In a study by Bajaj et al. (2012) produced spherical shaped selenium NPs from *Duganella* sp. and *Agrobacterium* sp. with an approximate diameter of 140–200 and 185–190 nm respectively. Also, *P. aeruginosa* SNT1 from seleniferous soil was found to biosynthesize spherical amorphous selenium nanostructure both intracellularly and extracellularly (Yadav et al. 2008). Selenium NPs were also synthesized from *Enterobacter cloacae* SLD1a-1 (Yee et al. 2007), *Bacillus cereus* (Dhanjal and Cameotra 2010), *Thauera selenatis* (Debieux et al. 2011), *Pantoea agglomerans* (Torres et al. 2012) and *Bacillus mycoides* SeITE01 (Lampis et al. 2014). The size of these NPs ranged from 20 to 400 nm. Srivastava et al. (2014) synthesized selenium NPs from *Halococcus salifodinae* BK18. More recently, *Pseudomonas putida* KT2440 was employed to biosynthesize selenium NPs. Measurements by the transmission electron microscope showed that selenium NPs had a size between 100 and 500 nm. These particles were found either attached to the cell membrane or in the surrounding medium (Avendaño et al. 2016). Similarly, Kora and Rastogi (2016) reported the biosynthesis of selenium NPs with a size range of 47–165 nm from a Gram-negative bacterial strain, *Pseudomonas aeruginosa.* Lately, *Azoarcus* sp. CIB, a facultative anaerobic β-Proteobacterium mediated synthesis of selenium NPs was reported by Fernández-Llamosas et al. (2016). The NPs had a spherical shape and possessed an average size of 123 ± 35 nm.

Table 19.6 Other nanoparticles biosynthesized from bacteria

Microorganisms	NPs type	Size (nm)	Shape	References
Aeromonas hydrophila	TiO_2	40.5	Spherical	Jayaseelan et al. (2013)
Actinobacter sp.	Si	10	Quasi-spherical	Singh et al. (2008)
Brevibacterium casei	Co_3O_4	5–7	Cubic	Kumar et al. (2008)
Enterobacter sp.	PbO	–	–	El-Shanshoury et al. (2012)
Escherichia coli	CuO	10–40	Quasi-spherical	Singh et al. (2010)
Lactobacillus sp.	$BaTiO_3$	20–80	Tetragonal	Jha and Prasad (2010)
Lactobacillus sp.	TiO_2	50–60	Spherical	Prasad et al. (2007)
Lactobacillus sp.	TiO_2	8–35	Spherical	Jha et al. (2009a)
Lactobacillus sp.	Sb_2O_3	3–12	Cubic	Jha et al. (2009b)
Lactobacillus sporoge	ZnO	5–15	Hexagonal	Prasad and Jha (2009)
Propionibacterium jensenii	TiO_2	<80	Dumb-bell	Babitha and Korrapati (2013)

19.2.6 Other Nanoparticles (Palladium, Uranium, Lead, Cobalt etc.)

Various other types of NPs including Pd, Ti, Ba, Co, Pb, Zn etc. NPs synthesized from different bacteria are shown in Table 19.6. Soluble palladium (Pd) salts like Na2PdCl4 was reduced to produce palladium Pd (0) metallic NPs on the cell walls and within the periplasmic space of the bacterium, *Shewanella oneidensis* MR-1 cells (Windt et al. 2005). Pd NPs were also synthesized form *Citrobacter braakii* (Hennebel et al. 2011). The presence of H_2, lactate, formate, ethanol and pyruvate were reported to function in the process of biosorption and succeeding bioreduction of Pd (II). Likewise, bioreduction of uranium (VI) by *S. oneidensis* MR-1 and *Desulfovibrio desulfuricans* was also reported (Burgos et al. 2008; Bargar et al. 2008). The average size of uranite NPs was 3 nm as determined by high-resolution transmission electron microscopy and X-ray absorption spectroscopy. Also, scanning electron microscopy revealed that uraninite (UO2) NPs were observed to be formed and accumulated on exopolymeric substances of the cell surface (Marshall et al. 2006; Wall and Krumholz 2006; Bargar et al. 2008; Burgos et al. 2008). Likewise, the bioreduced UO2 NPs from *S. putrefaciens* were found deposited extracellularly or in the periplasmic space (Baranska and Sadowski 2013). Similarly, *S. oneidensis* MR-1 synthesized a long, unique uranium (VI) nanowires that differed from other biogenic uraninite NPs (Jiang et al. 2011). Kumar et al. (2008) synthesized cubic shaped Co_3O_4 NPs attached with proteins by using *Brevibacterium casei*, a metal-tolerant bacterium and aqueous cobalt acetate. These extracellularly synthesized particles had a size in the range of 5–7 nm. Both *Enterobacter* sp. and *Bacillus anthracis when treated with* dried $Pb(NO_3)_2$- produced lead oxide (PbO) NPs (El-Shanshoury et al. 2012). *Enterobacter* sp. showed intracellular synthesis

within the periplasmic space and later exported exterior to the cell wall. While, *B. anthracis* showed extracellular route of biosynthesis. *Lactobacillus* sp. assisted titanium dioxide (TiO_2) NPs were produced at ambient room temperature by Prasad et al. (2007). The NPs were in spherical form and their size ranged from 40 to 60 nm. Similarly, the biogenic synthesis of TiO_2 NPs from *Aeromonas hydrophila* and *Propionibacterium jensenii was* described by Jayaseelan et al. (2013) and Babitha and Korrapati (2013), respectively. Likewise, a cost-effective and reproducible green synthesis of $BaTiO_3$ NPs was reported by using *Lactobacillus* sp. (Jha and Prasad 2010). NPs were having crystalline structure of tetragonal shape with the size of 20–80 nm. According to them, reactive oxygen species and hydrogen (rH2) gas of the culture solution possibly play a role in the process of biosynthesizing nano-$BaTiO_3$. Similar mechanistic view was proposed by Jha et al. (2009a) for the biosynthesis of TiO_2 NPs by using *Lactobacillus* sp. TiO_2 NPs had a spherical shape and the size ranged from 8 to 35 nm. Further, cubic shaped antimony trioxide (Sb_2O_3) NPs of 3–12 nm size were also synthesized from *Lactobacillus* sp. (Jha et al. 2009b). Zinc oxide (ZnO) NPs of 5–15 nm size were biosynthesized from *Lactobacillus sporoge* (Prasad and Jha 2009). Biosynthesis of silicon/silica nanocomposites by *Actinobacter* sp. was demonstrated by Singh et al. (2008). These extracellularly produced quasi-spherical particles were having a size of about 10 nm. Using *E. coli,* extracellular biosynthesis of copper oxide (CuO) NPs was reported by Singh et al. (2010). The spectral characterization revealed the occurrence of a quasi-spherical shaped and 10–40 nm sized CuO NPs.

19.3 Different Nanoparticles from Cyanobacteria

A filamentous cyanobacteria (*Plectonema boryanum* UTEX 485) when interacted with $Au(S_2O_3)_2^{3-}$ solution promoted the biosynthesis of cubic AuNPs of size <10–25 nm (Lengke et al. 2006a). A research work on the mechanistic approach revealed that *P. boryanum* UTEX 485 interacts with aqueous $AuCl_3$ and initially promote gold(I)−sulfide precipitation on the cell walls, followed by depositing octahedral metallic gold of nanosize range between ∼10 nm and 6 μm near the cell surface and in the solution (Lengke et al. 2006b). Further, the same strain was used for the extracellular biosynthesis of platinum (Pt) NPs of size ranging between 30 and 300 nm (Lengke et al. 2006c). Using some cyanobacterial strains (*Limnothrix* sp. 37-2-1, *Anabaena* sp. 66-2 and *Synechocystis* sp. 48-3), irregular and elongated shaped AgNPs measuring between 14 and 31 nm were synthesized by Patel et al. (2015). Likewise, the extracts of 30 Cyanobacterial strains were used to synthesize AgNPs (Husain et al. 2015). Among them, *Cylindrospermum stagnale* NCCU-104 was observed to produce smallest sized (38–40 nm) extracellular AgNPs with different shapes (Table 19.7). More recently, *Nostoc* sp. strain HKAR-2 mediated AgNPs synthesis has been reported with particle size of 51–100 nm (Sonker et al. 2017). Various NPs synthesized from cyanobacteria are shown in Table 19.7.

Table 19.7 Nanoparticles biosynthesized from cyanobacteria

Microorganisms	Metal NPs type	Size (nm)	Shape	References
Arthrospira indica PCC7940	Ag	48	Spherical	Husain et al. (2015)
Arthrospira indica SAE-85	Ag	67	Spherical	Husain et al. (2015)
Arthrospira indica SOSA-4	Ag	48	Spherical	Husain et al. (2015)
Arthrospira maxima SAE-49-88	Ag	61	Triangular	Husain et al. (2015)
Arthrospira platensis NEERI	Ag	46	Triangular	Husain et al. (2015)
Chroococcus NCCU-207	Ag	48	Spherical	Husain et al. (2015)
Gloeocapsa gelatinosa NCCU-430	Ag	88	Spherical	Husain et al. (2015)
Lyngbya NCCU-102	Ag	54	Spherical	Husain et al. (2015)
Oscillatoria sp. *NCCU-369*	Ag	80	Spherical	Husain et al. (2015)
Phormidium sp. *NCCU-104*	Ag	48	Cubic	Husain et al. (2015)
Plectonema sp. *NCCU-204*	Ag	61	Spherical	Husain et al. (2015)
Spirulina CFTRI	Ag	47	Hexagonal	Husain et al. (2015)
Spirulina NCCU-477	Ag	49	Cubic	Husain et al. (2015)
Spirulina NCCU-479	Ag	52	Spherical	Husain et al. (2015)
Spirulina-481	Ag	64	Spherical	Husain et al. (2015)
Spirulina NCCU-482	Ag	42	Spherical	Husain et al. (2015)
Spirulina NCCU-483	Ag	51	Pentagonal	Husain et al. (2015)
Spirulina platensis NCCU-S5	Ag	46	Spherical	Husain et al. (2015)
Synechocystis NCCU-370	Ag	80	Spherical	Husain et al. (2015)
Anabaena ambigua NCCU-1	Ag	48	Spherical	Husain et al. (2015)
Anabaena variabilis NCCU-441	Ag	50	Spherical	Husain et al. (2015)
Aulosira fertilissma NCCU-443	Ag	58	Spherical	Husain et al. (2015)
Calothrix brevissema NCCU-65	Ag	42	Cubic	Husain et al. (2015)
Cylindrospermum stagnale NCCU	Ag	38	Pentagonal	Husain et al. (2015)
Hapalosiphon fontinalis NCCU-339	Ag	50	Triangular	Husain et al. (2015)
Microchaete sp. *NCCU-342*	Ag	40	Spherical	Husain et al. (2015)
Nostoc muscorum NCCU-442	Ag	42	Spherical	Husain et al. (2015)
Scytonema sp. *NCCU-126*	Ag	70	Spherical	Husain et al. (2015)
Tolypothrix tenuis NCCU-122	Ag	44	Spherical	Husain et al. (2015)
Westiellopsis prolifica NCCU-331	Ag	52	Spherical	Husain et al. (2015)
Anabaena sp. 66-2	Ag	24.13 ± 2	Irregular	Patel et al. (2015)
Synechocystis sp. 48-3	Ag	14.64 ± 2	Irregular	Patel et al. (2015)
Limnothrix sp. 37-2-1	Ag	31.86 ± 1	Elongated	Patel et al. (2015)
Plectonema boryanum UTEX 485	Au	<10–25	Cubic	Lengke et al. (2006a)
Plectonema boryanum UTEX 485	Au	10–6000	Octahedral	Lengke et al. (2006b)
Plectonema boryanum UTEX 485	Pt	30–300	Spherical	Lengke et al. (2006c)
Nostoc sp. strain HKAR-2	Ag	51–100	Spherical	Sonker et al. (2017)

19.4 Different Nanoparticles from Actinomycetes

The secondary metabolites produced from Actinomycetes serve as a source of medicaments including antibiotics. Besides, easy genetic manipulation of actinomycetes allow to better control over size (Bhosale et al. 2015; Abd-Elnaby et al. 2016). Exploration of Actinomycetes have proved to be suitable for potential application of NPs synthesis. Actinomycetes assisted NPs exhibit relatively good stability and polydispersity (Bhosale et al. 2015; Abd-Elnaby et al. 2016). Using a *Rhodococcus* sp. (alkalotolerant actinomycete), AuNPs were synthesized intracellularly with the dimension of 5–15 nm (Ahmad et al. 2003a). Electron microscopic data revealed that AuNPs had a monodispersity and were produced on both cell walls and cytospasmic membranes. They also, predicted the possible role of the cell wall and cytoplasmic membrane specific enzymes in carrying out reduction of metal ions. Likewise, by using an extremophile, *Thermomonospora* sp. Ahmad et al. (2003b) have proved the biosynthesis of monodispersed, and spherical AuNPs with an average size of 8 nm. Further, characterization data of Fourier transform infrared spectroscopy confirmed the possible role of amide (I) and (II) bands of protein acting as capping and stabilizing agents on NPs surface. Likewise, *Actinomycetes sps.* mediated synthesis of AgNPs was reported by many researchers (Chauhan et al. 2013; Abdeen et al. 2014; Bhosale et al. 2015; Mohamedin et al. 2015; Składanowski et al. 2016). Most of these particles were in the nanoregime (<100 nm) and possessed spherical or oval shapes. The presence of reductase enzymes are believed to possibly involve in reducing metal ions. An industrially reliable green approach of synthesizing extracellular AgNPs using *Streptomyces hygroscopicus* has been proposed by Sadhasivam et al. (2010). AgNPs (20–30 nm) were observed to possess a spherical shape. Lately, AgNPs and AuNPs biosynthesized from *Streptomyces* sp. possessed spherical and oval shapes. The size of AgNPs remained in the nanoregime of about 8–44 nm while, AuNPs had 10 nm size (Składanowski et al. 2016). Using *Streptomyces hygroscopicus* (Waghmare et al. 2014), synthesized spherical AuNPs spherical in the range of 10–20 nm. Lately, Abd-Elnaby et al. (2016) biosynthesized AgNPs of 22–85 nm from *S. rochei* MHM13. Extracellular synthesis of CuO NPs with 100–150 nm size was recorded using *Streptomyces* sp., an isolate of Pichavaram mangrove, India (Usha et al. 2010). Various NPs synthesized from cyanobacteria are shown in Table 19.8.

19.5 Different Nanoparticles from Yeast

AgNPs with a size range of 2–5 nm were biosynthesized extracellularly by Kowshik et al. (2002a) using a silver tolerant yeast strain, MKY3. Likewise, Korbekandi et al. (2016) biologically synthesized spherical AgNPs with 2–20 nm in diameter by using dried yeast cells. Lately, Abdehgah et al. (2017) used *Candida albicans to biologically synthesize crystalline AgNPS ranging* 20–80 nm in size with spherical

Table 19.8 Nanoparticles synthesized from cyanobacteria and yeast

Microorganism	Metal NPs	Size (nm)	Shape	References
Actinomyces				
Rhodococcus sp.	Au	5–15	Spherical	Ahmad et al. (2003a)
Streptomyces sp.	CuO	100–150	–	Usha et al. (2010)
Streptomyces hygroscopicus	Ag	20–30	Spherical	Sadhasivam et al. (2010)
Actinomycetes sp.	Ag	10–20	Spherical	Abdeen et al. (2014)
Actinomycetes sp.	Ag	68.13	–	Chauhan et al. (2013)
Actinomycetes sp.	Ag	8–44	Spherical/oval	Składanowski et al. (2016)
Actinomycetes sp.	Au	10	Spherical/rod	Składanowski et al. (2016)
Streptomyces hygroscopicus	Au	10–20	Spherical	Waghmare et al. (2014)
Streptomyces rochei MHM13	Ag	22–85	Spherical	Abd-Elnaby et al. (2016)
Streptomyces viridodiastaticus SSHH-1	Ag	15–45	Spherical	Mohamedin et al. (2015)
Yeast				
Candida glabrata	CdS	2	Hexagonal	Dameron et al. (1989)
Candida albicans	Ag	20–80	Spherical/oval	Abdehgah et al. (2017)
Yarrowia lipolytica	Au	15	Hexagonal/ triangular	Agnihotri et al. (2009)
Sachharomyces cerevisiae	Au	13.0 ± 0.9	Spherical	Attia et al. (2016)
Sachharomyces cerevisiae	CdS	3.57 ± 0.21	Spherical	Prasad and Jha (2010)
Schizosaccharomyces pombe	CdS	1–1.5	Hexagonal	Kowshik et al. (2002b)
S. cerevisiae	Sb_2O_3	2–10	Spherical	Jha et al. (2009b)
S. cerevisiae	CdTe	2–3.6	Cubic	Bao et al. (2010)
Sachharomyces cerevisae	TiO_2	8–35	Spherical	Jha et al. (2009a)
Sachharomyces cerevisae	Ag	2–5	Spherical	Kowshik et al. (2002a)
Sachharomyces cerevisae	Ag	2–20	Spherical	Korbekandi et al. (2016)
Sachharomyces cerevisae	Fe_3O_4	80–130	Spherical	Fakhrullin et al. (2010)
Rhodotorula mucilaginosa	Cu_2O	10.5	Spherical	Salvadori et al. (2014)

and oval morphologies. Agnihotri et al. (2009) reported the biosynthesis of hexagonal and triangular shaped AuNPs with a mean size of 15 nm from the non-conventional yeast *Yarrowia lipolytica*. Dameron et al. (1989) reported the biosynthesis of quantum crystallites from *C. glabrata* and *Schizosaccharomyces pombe* when grown in the presence of cadmium salts. CdS NPs synthesis from *Sachharomyces cerevisiae* has been reported by Prasad and Jha (2010). These CdS NPs had an average size of 3.57 ± 0.21 nm. Likewise, intracellular precipitation of CdS NPs was observed when a strain, *S. pombe* was challenged with cadmium (1 mM) solution (Kowshik et al. 2002b). Jha et al. (2009b) biosynthesized TiO_2 NPs using *Lactobacillus* sp. and the nanocrystals were having a spherical shape with 8–35 nm diameter. Sb_2O_3 NPs were synthesized from *S. cerevisiae.* These nanostructures were spherical in shape with 2–10 nm size and possessed a face centered cubic unit cell structure (Jha et al. 2009b). Intracellular biosynthesis of copper NPs using *Rhodotorula mucilaginosa* showed the occurrence of spherically shaped particles with an average size of 10.5 nm (Salvadori et al. 2014). An efficient biosynthesis and easy harvesting method was proposed by Bao et al. (2010) to obtain biocompatible cadmium telluride (CdTe) quantum dots by using yeast cells. Fakhrullin et al. (2010) reported the possible preparation of viable yeast cells deposited with Fe_3O_4 NPs on the exterior of the cell's wall. Similarly, yeast cells modified with magnetic NPs have been achieved lately (Gorobets et al. 2011, 2013; Safarik et al. 2015). AuNPs of size 13.0 ± 0.9 nm were biologically synthesized using an *S. cerevisiae* cells extract. The presence of yeast cell metabolites including glucose, trimethylsilyl derivatives of butan-2,3-diol, undecanoic acid and indole-3-acetic acid acted as a capping/reducing agents during AuNPs formation (Attia et al. 2016). Various NPs synthesized from cyanobacteria are shown in Table 19.8.

19.6 Fungal Based Nanoparticles

Fungi commonly called as decomposing organisms are considered as eukaryotic organisms which reside in almost every corner of the earth. Fungi are capable of performing several tasks which include digestion of extracellular food, conversion of complicated molecules into simpler one by enzymatic hydrolysis, and utilize the energy (Blackwell 2011). Fungi are also causative agents of many medical complications in humans as well as animals. However, apart from being pathogenic, fungi play significant role in medicine field for instance, production of antibiotics, and their role in the synthesis of NPs which are playing a key role in biomedical field and many more. NPs have been synthesized through many different techniques; one among these techniques is the biological synthesis, which includes plants, yeast, bacteria and fungi. The application of fungi in biosynthesis NPs was first reported in the production of CdSe NPs by *Candida albicans* (Dameron et al. 1989; Xue et al. 2016). Fungi are regarded as more advantageous compared to any other microorganisms in the biosynthesis of NPs due to several characteristic features, which include, fast growth and easy to handle (many species), fungal mycelia can better

withstand bioreactor conditions such as, high pressure and agitation, secretion of more extracellular enzymes, large biomass capacity, economic livability, high metal accumulation capacity, and many more (Castro-Longoria et al. 2011; Musarrat et al. 2011a; Moghaddam et al. 2015). The tolerance and metal bioaccumulation capability of fungi has also attracted and put them to research in the biological production of metallic NPs (Sastry et al. 2003). Moreover, the scale-up of fungi is easy and is an additional advantage in utilizing them in biosynthesis of NPs, the effective secretion of enzymes (extracellular enzymes), also helps in achieving the vast production of enzymes a feasibility (Castro-Longoria et al. 2012). Most of the fungi have intracellular metal uptake and high wall-binding capacities (Volesky and Holan 1995; Moghaddam et al. 2015), which are significant in the nanoparticle synthesis. The metabolic diversity of fungi attracted them as potential candidates in biosynthesis of NPs. Fungi synthesize NPs either by intracellular or extracellular process and reduction is the key process involved in the synthesis of metallic NPs (Sadowski et al. 2008). Intracellular process involves attachment of metal ions on to the fungal cell surface, through electrostatic interaction. The opposite charges on the surface of metal ion and fungal cell surface generate electrostatic force of attraction. After the absorption of metal ions, enzymes of fungal cell wall which contain positively charged groups reduce metal ions leading to aggregation of nano structures and finally metal NPs. However, In case of extracellular synthesis the enzymes secreted by fungi (Eg. nitrate reductase) reduce metal ions, which finally leads to the formation of stable NPs (Khandel and Shahi 2016). Several different NPs such as gold, silver, zinc oxide and many more have been synthesized using different species of fungi including *Fusarium oxyporum, Aspergillus niger, Aspergillus fumigatus* etc. Various fungi employed in the synthesis of different NPs is shown in Table 19.9.

19.6.1 Silver Nanoparticles (AgNPs)

Silver and its compounds are known to have potential applications in biomedicine field. Several different techniques are available for the synthesis and characterization of AgNPs. Fungi play significant role in synthesis of AgNPs; several different species of fungi have been explored in the biosynthesis. *Fusarium oxysporum* has been widely employed in NP synthesis and the secretion of proteins in to the aqueous solution helps in the development of sulfate diminishing enzyme-based protocol for NPs production. AgNPs with varying morphology with a size ranging from 5 to 50 nm (Ahmad et al. 2003c) and spherical structures with size range 20–50 nm were synthesized from *F. oxysporum* (Durán et al. 2005). The variation in morphology and size could be due to variations in temperature, and different structures can be obtained based on the metallic ion solution and incubation parameters (Riddin et al. 2006; Moghaddam et al. 2015). Earlier studies have reported that *F. oxysporum* synthesizes various sizes and forms of NPs by extracellular synthesis mechanism (Moghaddam et al. 2015). Mukherjee et al. (2001) reported that *F. oxysporum*

Table 19.9 Different nanoparticles biosynthesized from fungi

Microorganisms	NPs Type	Size (nm)	Shape	References
Fusarium oxysporum	Ag	5–50	Varying	Ahmad et al. (2003)
Fusarium oxysporum	Ag	20–50	Spherical	Durán et al. (2005)
Rhizopus stolonifer	Ag	10–25	Quasi-spherical	Binupriya et al. (2010)
Arthroderma fulvum	Ag	15.5 ± 2.5	Spherical	Xue et al. (2016)
Fusarium acuminatum	Ag	5–40	Spherical	Ingle et al. (2008)
Aspergillus fumigatus	Ag	13.88 ± 4.1	Spherical	Magdi et al. (2014)
Aspergillus ochraceus	Ag	13.88 ± 4.1	Spherical	Magdi et al. (2014)
Fusarium oxysporum	Ag	13.88 ± 4.1	Spherical	Magdi et al. (2014)
Penicillium italicum	Ag	13.88 ± 4.1	Spherical	Magdi et al. (2014)
Syncephalastrum racemosum	Ag	13.88 ± 4.1	Spherical	Magdi et al. (2014)
Aspergillus fumigatus	Ag	15–45	Spherical	Alani et al. (2012)
Trichoderma viride	Ag	2–4	Spherical	Fayaz et al. (2010)
Aspergillus fumigatus	Au	22 ± 2	Variable	Gupta and Bector (2013)
Aspergillus flavus	Au	22 ± 2	Variable	Gupta and Bector (2013)
Aspergillus niger	Au	12.79 ± 5.61	Spherical	Sawle et al. (2008)
Aureobasidium pullulans	Au	29 ± 6	Spherical	Bhambure et al. (2009)
Fusarium semitectum	Au	25	Spherical	Philip (2009)
Neurospora crassa	Au	32	Spherical	Zhang et al. (2011)
Volvariella volvacea	Au	20–150	Spherical	Castro-Longoria et al. (2011)
Colletotrichum sp.	Au	20–40	Decahedral/icosahedral	Shankar et al. (2003)
Rhizopus stolonifer	Au	1–5	Irregular	Sarkar et al. (2012)
Nigrospora oryzae	Au	6–18	Spherical	Kar et al. (2014)
Aspergillus terreus	Au	10–20	Spherical	Balakumarana et al. (2016)
Aspergillus terreus	Au	10–50	Anisotropic	Balakumarana et al. (2016)
Fusarium oxyporum	Fe_3O_4	20–50	Irregular	Bharde et al. (2006)
Verticillium sp.	Fe_3O_4	20–50	Cubo-octahedral and quasi-spherical	Bharde et al. (2006)
Aspergillus japonicus	Iron oxide	60–70	Cubical	Bhargava et al. (2013)
Aspergillus oryzae	$FeCl_3$	10–24.6	Spherical	Tarafdar and Raliya (2013)
Coriolus versicolor	CdS	100–200	Spherical	Sanghi and Verma (2009)

(continued)

Table 19.9 (continued)

Microorganisms	NPs Type	Size (nm)	Shape	References
Phanerochaete chrysosporium	CdS	2.56	Face-centered cubic	Chen et al. (2014)
Fusarium sp.	ZnS	100–2000	Irregular	Velmurugan et al. (2010)
Fusarium oxyporum	CdS	9–15	Spherical	Kumar et al. (2007)
Fusarium oxysporum	CdS	5–20	Hexagonal	Ahmad et al. (2002)
Aspergillus terreus	Se	–	–	Sarkar et al. (2011)
Aspergillus terreus	Se	–	–	Vetchinkina et al. (2013)
Lentinula edodes	Se	–	–	Zare et al. (2012)
Fusarium sp.	Se	–	–	Gharieb et al. (1995)
Trichoderma reesei	Se	–	–	Gharieb et al. (1995)
Neurospora crassa	PtNPs	4–35	Spherical	Castro-Longoria et al. (2012)
Aspergillus flavus	TiO_2	62–74	Spherical	Rajakumar et al. (2012)
Aspergillus versicolor	Hg NPs	20.5 ± 1.82	–	Das et al. (2008)

decreases the metal ions through NADH-based reductases and also by shuttle Quinone extracellular procedure (Mukherjee et al. 2001).

Several groups have synthesized and characterized AgNPs from different species of fungi. AgNPs with size range of 10–25 nm and Quasi-spherical were successfully produced using *Rhizopus stolonifer* by extracellular process exploring purified α-NADPH-dependent nitrate reductase which acts as an electron carrier (Binupriya et al. 2010). Furthermore, AgNPs synthesized using *Arthroderma fulvum* range in size from 15.5 ± 2.5 nm, and are spherical in shape (Xue et al. 2016). Earlier study by Gade et al. reported that AgNPs with a definite size range of 5–25 nm were synthesized within 72 h by *Aspergillus niger* when incubated with silver nitrate solution. The synthesized NPs were observed as aggregates on the surface of cell wall, which were then reduced to stabilize using reducing enzymes and proteins produced by the fungus *A. niger* (Khandel and Shahi 2016). The NPs synthesized by exposing aqueous silver nitrate solution to *Fusarium acuminatum* cell extract were found to be spherical in shape with a size range of 5–40 nm with an average diameter of 13 nm. The nitrate-dependent reductase could be responsible for reduction of silver ions which is found in the extra-cellular medium (Ingle et al. 2008). Further, a study by Magdi et al. (2014), reports the reduction of silver salt into AgNPs by *Aspergillus fumigatus*, *Candida albicans*, *Penicillium italicum*, *Syncephalastrum racemosum*, *Fusarium oxysporum* and *Aspergillus ochraceus*. These AgNPs were spherical in shape, with a mean size of 13.88 ± 4.11 nm (Magdi et al. 2014). Furthermore, AgNPs synthesized using *Aspergillus fumigatus* by extracellular process were mostly spherical with a size range of 15–45 nm (Alani et al. 2012). However, AgNPs obtained from *Trichoderma viride* were intracellular and mostly spherical with size range of 2–4 nm (Fayaz et al. 2010).

19.6.2 Gold Nanoparticles (AuNPs)

The characteristic features of AuNPs such as biocompatibility, stability and resistant to oxidation, bio-available surface, allowed them gain significant importance in medicine and biology. The chemical methods of AuNPs synthesis which raise the environmental concerns due to release of toxic residues may be replaced by low-cost and environment friendly process such as, biological synthesis. Several viable microorganisms and their cell-free extracts have been employed by different groups in the synthesis of AuNPs. The biosynthetic process of AuNPs involves two main precursors such as, gold chloride ($HAuCl_4$) and AuCl. $HAuCl_4$ dissociates to Au^{3+} ions (Khan et al. 2013) and AuCl dissociates to Au^{+} (Zeng et al. 2010). Like AgNPs, AuNPs can also be synthesized by either intracellular or extracellular process. In extracellular process Au^{3+} ions are reduced through proteins by trapping them in the cell wall. However, in the case of intracellular process, cytosolic redox mediators reduces $Au3^{+}$ ions upon diffusion through the cell membrane (Das et al. 2012; Kitching et al. 2015). Nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) oxidoreductases reduce Au ions either in the cytoplasm or in the cell surface. However, the mechanism of enzymatic reduction of $Au3^{+}$ remains the same in both intra and extracellular process (Gupta and Bector 2013).

AuNPs synthesized by fungi vary in their shape and size, a study by Gupta and Bector (2013) reports the intracellular production of AuNPs by *Aspergillus fumigatus* and *Aspergillus flavus*, with an average diameter of 22 ± 2 nm. Spherical shaped AuNPs with varying size were synthesized using different fungi including *Aspergillus niger* (12.79 ± 5.61 nm), *Aureobasidium pullulans* (29 ± 6 nm), *Fusarium semitectum* (25 nm), *Neurospora crassa* (32 nm) and *Volvariella volvacea* (20–150 nm) (Sawle et al. 2008; Bhambure et al. 2009; Philip 2009; Zhang et al. 2011; Castro-Longoria et al. 2011). Furthermore, AuNPs produced from *Colletotrichum* sp. were decahedral and icosahedral in shape with a size range of 20–40 nm, while AuNPs from *Rhizopus stolonifer* were reported as irregular (uniform) with size ranging from 1 to 5 nm (Shankar et al. 2003; Sarkar et al. 2012). Gold NPs with a diameter range of 6–18 nm and spherical shape were synthesized by treating gold chloride solution with culture filtrate of the *Nigrospora oryzae* (Kar et al. 2014). On the other hand, Balakumarana et al. (2016) reported the synthesis of AuNPs from mycelial free filtrate of *Aspergillus terreus* which were spherical shape and 10–20 nm in size with; however some particles were observed in anisotropic morphology with a size of 10–50 nm. Several other fungi species have been explored in the successful synthesis of AuNPs which include *Volvariella volvacea*, *Phanerochaete chrysosporium, Fusarium oxyporum, Rhizopus stolonifer*, *Cylindrocladium floridanu* and many more through extracellular process. However, the species including *Fusarium oxysporum, Verticillium luteoalbum,* produces AuNPs through intracellular process. Some species are capable producing AuNPs

by both extracellular and intracellular processes which include *Coriolis versicolor, Rhizopus oryzae, Aspergillus niger, Candida albicans*. Organisms such as *Verticillium* sp. and *Rhizopus oryzae* produce NPs on cell wall and cell surface respectively (Moghaddam et al. 2015).

19.6.3 Magnetic Nanoparticles

Magnetic NPs have broad and potential applications in biological and biomedicine fields due to their super-paramagnetic, high coercive force and unique micro-configuration properties. Magnetic NPs exhibit remarkable characteristic features such as super-paramagnetism, high saturation field, high field irreversibility, extra anisotropy contributions/shifted loops after field cooling. For biomedical applications, iron oxide NPs such as magnetite (Fe_3O_4) or its oxidized form maghemite (γ-Fe_2O_3) are most commonly employed. Even though, cobalt and nickel exhibit higher magnetic property, they are of less importance in biomedical science, due to their toxicity and susceptibility to oxidation (Akbarzadeh et al. 2012). The well-known biocompatible magnetic NPs such as Fe_3O_4 (magnetite) and Fe_2O_3 (maghemite) have been employed various biological and medicine field. Magnetite (Fe_3O_4) NPs have been synthesized extracellularly with the aqueous mixture of ferricyanide/ferrocyanide using fungi *F. oxysporum* and *Verticillium sp*. The proteins secreted by these fungi hydrolyze iron precursors to form magnetite (Fe_3O_4) at room temperature. The particles obtained from *Fusarium oxysporum* were found to be irregular in shape with an overall quasi-spherical morphology and size range from 20 to 50 nm; however, particles from *Verticillium* sp. were cubo-octahedral and quasi-spherical in shape (Bharde et al. 2006). However, iron oxide NPs synthesized by extracellular process using *Aspergillus japonicus* varied in size from 60 to 70 nm having cubical shape with crystal structure which corresponds to magnetite (Bhargava et al. 2013). Further, $FeCl_3$ NPs synthesized extracellularly from *Aspergillus oryzae* were 10–24.6 nm in size with spherical shape (Tarafdar and Raliya 2013).

19.6.4 Fluorescent/Luminescent NPs

The unique size-dependent properties of fluorescent/luminescent colloidal NPs or quantum dots (QDs) which are in the size range of 1–20 nm and composed group II–IV, III–V or IV–VI elements such as, Cd, Te, Se, Zn, In, As), have attracted the researchers in both basic and applied field (Kovalenko et al. 2015; Plaza et al. 2016). Several different groups have synthesized these fluorescent/luminescent NPs and characterized for their applications in biology and medicine. Cadmium sulfide (CdS) QDs were synthesized extracellularly from the white rot fungus *Phanerochaete chrysosporium* by incubating in cadmium nitrate tetrahydrate solution. X-ray analysis of the CdS NPs showed face-centered cubic crystal structure with an average

size of approximately 2.56 nm. Furthermore, the formation and stabilization of CdS QDs may be attributed to the secretion of cysteine and proteins from *Phanerochaete chrysosporium* (Chen et al. 2014). Zinc sulphide (ZnS) and CdS NPs have also been synthesized using *Fusarium sp.* by exposing $CdSO_4$ and $ZnSO_4$ solutions having sizes from 20 to 150 nm. Cadmium NPs *Fusarium oxyporum* produced extracellular process were found to be spherical with the size range of 9–15 nm (Kumar et al. 2007). Moreover, ZnNPs produced by an intracellular process using *Fusarium spp.*were irregular in shape and 100–200 nm in size range (Velmurugan et al. 2010). In another study CdS NPs have been produced extracellularly from $CdSO_4$ solution using *Fusarium oxysporum* and it has been reported that CdS particles were in the size range 5–20 nm with hexagonal shape. The possibility of synthesizing PbS, ZnS, and MoS_2 NPs by enzymatic reduction of sulfate ions by *Fusarium oxysporum* was also tested and preliminary investigations indicated the possible realization of such chemical biotransformations using fungal extracts (Ahmad et al. 2002). Several reports have described synthesis of selenium NPs (SeNPs) using fungi (Gharieb et al. 1995; Sarkar et al. 2011; Vetchinkina et al. 2013; Zare et al. 2012). Monodispersed spherical SeNPs have been extracellularly produced using *Aspergillus terreus* and *Alternaria alternate* and intracellularly by *Lentinula edodes* (Sarkar et al. 2011; Vetchinkina et al. 2013; Zare et al. 2012). *Fusarium* sp. and *Trichoderma reesei* have also been reported in the production of SeNPs (Gharieb et al. 1995).

19.6.5 Other Nanoparticles

Apart from the above discussed NPs several other NPs having potential biomedical applications have been mycosynthesized and characterized by several groups. Platinum NPs (PtNPs), unlike the widely researched AgNPs and AuNPs, have been synthesized from the fungus *Neurospora crassa*. It has been reported that intracellular single PtNPs are in the size range of 4–35 nm in diameter, while nanoagglomerates which are spherical in shape are of 20–110 nm in diameter (Castro-Longoria et al. 2012). A study by Riddin et al. (2006) also reports the production of PtNPs by another fungus *F. oxysporum*, which were formed both intracellularly and extracellularly (Riddin et al. 2006). A novel eco-friendly approach of producing CdS NPs was achieved under ambient conditions by immobilizing the fungus, *Coriolus versicolor* in a continuous column (Sanghi and Verma 2009). The CdS nanocrystals had a spherical shape with size ranging 100–200 nm. *Aspergillus flavus* has been employed in the synthesis of TiO_2NPs which range in size from 62 to 74 nm having spherical shape (Rajakumar et al. 2012). Furthermore, Hg NPs have been synthesized using *Aspergillus versicolor* mycelia, the synthesized NPs accumulated on the surface of mycelia which are 20.5 ± 1.82 nm in size (Das et al. 2008).

19.7 Applications of Microbial Nanoparticles

Different types of NPs synthesized using fungi have found potential applications in various fields of medicine and biology including cancer treatment, treatment of bacterial infections (antibacterial activity), acts as biosensors and bioimaging agents. Antibiotics such as amoxicillin, erythromycin, clindamycin, penicillin G, and vancomycin when used along with AgNPs enhanced their antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli* (Shahverdi et al. 2007b). The AgNPs synthesized from bacterial strains have shown superior antimicrobial activity against several pathogens including methicillin-resistant *S. aureus*, vancomycin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis* and *Streptococcus pyogenes*, *E. coli, B. subtilis, B. cereus S. pyogenes* and *P. aeruginosa*, *Salmonella typhi*, *S. typhimurium, Haemophilus influenzae* and *Klebsiella pneumonia* (Nanda and Saravan 2009; Priyadarshini et al. 2013; Abirami and Kannabiran 2016). BioNPs synthesized from *B. megaterium* were effective against multi-drug resistant pathogens *Streptococcus pneumoniae* and *Salmonella typhi* (Saravanan et al. 2011). The biosynthesized AgNPs from *Streptomyces* sp. exhibited antifungal activity against *Aspergillus niger*, *A. flavus* and *A. fumigatus* (Thenmozhi et al. 2013). *Likewise*, anticandidal properties of AgNPs from *Streptomyces* sp. VITPK1 was shown against *Candida albicans, C. tropicalis and C. krusei* (Sanjenbam et al. 2014). Biosynthesized AgNPs from *S. hygroscopicus* significantly inhibited several microbial pathogens such as *B. subtilis, E. faecalis*, *E. coli, S. typhimurium* and and *C. albicans* (Sadhasivam et al. 2010; Chauhan et al. 2013; Abdeen et al. 2014; Mohamedin et al. 2015; Składanowski et al. 2016). CuO NPs from a actinomycete showed greater activity against *E. coli, S. aureus,* and *A. niger* (Usha et al. 2010). Likewise, the microbes, *E. coli*, and *S. typhi* were greatly inhibited by AgNPs synthesized from *Bacillus* sp. AZ1 (Deljou and Goudarzi 2016). The AgNPs fabricated to cotton cloth significantly showed antimicrobial activity against *E. coli, S. aureus,* and *C. albicans* (El-Naggar et al. 2016). **E. coli assisted AgNPs exhibited inhibitory activity against pathogens namely, Salmonella typhi, Bacillus subtilis, Vibrio cholerae, and Klebsiella pneumoniae** (Divya et al. 2016). AgNPs synthesized from yeast cells effectively inhibited a haploid pathogenic *yeast, Candida glabrata* (Abdehgah et al. 2017). Further, microbially synthesized AgNPs function as an anti-biofouling agents (Abd-Elnaby et al. 2016). Likewise, cyanobacterial assisted AgNPs against *B. megaterium, E. coli, B. subtilis, M. luteus and S. aureus* have shown a significant antibacterial effect (Patel et al. 2015). Likewise, *Aspergillus niger, Trichoderma harzianum*, *Ralstonia solanacearum* and *Xanthomonas campestris* were inhibited by *Nostoc* sp. strain HKAR-2 mediated AgNPs (Sonker et al. 2017). Several groups have reported the potential applications of mycosynthesized NPs. Silver NPs produced from *Arthroderma fulvum* had shown considerable antifungal activity against *Candida* spp., *Aspergillus* spp., and *Fusarium* spp. (Xue et al. 2016). The effective antibacterial activity of AgNPs synthesized from *F. acuminatum* was observed against several pathogenic bacteria, including multidrug resistant *Staphylococcus aureus, Staphylococcus epidermidis,*

Salmonella typhi, and *Escherichia coli* (Ingle et al. 2008). Gold NPs derived from *Trichoderma viride* exhibited potential antimicrobial activity (Mishra et al. (2014). Mycosynthesized AuNPs have potential applications in various fields such as in agriculture (pesticidal agents), in optro-electronics, in cancer treatment, and as antibacterial agents (Das et al. 2009; Sawle et al. 2008; Mishra et al. 2011; Moghaddam et al. 2015). *B. megaterium* mediated biosynthesized AgNPs exhibited a potent larvicidal activity against *Culex quinquefasciatus* and *Aedes aegypti* (Banu and Balasubramanian 2015; Banu et al. 2014). *Anopheles subpictus* and *Culex tritaeniorhynchus* larvae were inhibited by AgNPs synthesized from psychrotolerant *Pseudomonas mandelii* (Mageswari et al. 2015). Likewise, AgNPs synthesized using *B. amyloliquefaciens* and *B. subtilis* were effective in controlling the growth of mosquito colonies of *Culex pipiens pallens* (Fouad et al. 2016). The biosynthesized AuNPs from *E. coli* DH5a are demonstrated to be beneficial for understand the electrochemistry of hemoglobin (Du et al. 2007). The immobilized doxorubicin on bacterial magnetic NPs easily dissolved in an aqueous solution and possessed very high magnetic and ferromagnetic properties. Also, these particles exhibited strong cytotoxicity against cancer cells namely, HePG2 and MCF-7 (Guo et al. 2008). Selenium NPs by *Halococcus salifodinae* BK18 exhibited potent anti-proliferative activity against HeLa cell lines (Srivastava et al. 2014). AuNPs from yeast cells shows potent anticancer activity against Ehrlich ascites carcinoma cells. The cell toxicity is mainly due to the photothermal properties of AuNPs (Attia et al. 2016). More recently, *Nostoc* sp. strain HKAR-2 mediated AgNPs showed potent anticancerous activities against MCF-7 cells (Sonker et al. 2017). The AgNPs synthesized using fungi had shown anticancer activity against several human cancers such as, colon carcinoma, breast cancer, and hepato-cellular carcinoma cells (Magdi et al. 2014). The AgNPs synthesized from *Aspergillus fumigatus* exhibited antiviral against HIV-1(Alani et al. 2012); while AgNPs obtained from *Trichoderma viride* finds potential applications in biosensor and bio-imaging (Fayaz et al. 2010). Further, AuNPs obtained from *Penicillium brevicompactum* showed in vitro cytotoxic activity against mouse mayo blast cancer cells (C2C12) (Mishra et al. 2011). AuNPs produced using mycelia-free culture filtrate of *Nigrospora oryzae* exhibited detrimental effect on the ultra-structure of parasite belonging to *Raillietina* sp.; (Kar et al. 2014). Antibody-conjugated AuNPs/gold nanoparticle-based probe can serve as a simple diagnostic tool in the diagnosis of cancer that is they can differentiate between cancer and normal cells (Chuhan et al. 2011). Yeast cells with magnetic NPs can be used for biotechnological applications such as bioreactors, biosensors, and bioseparations (Fakhrullin et al. 2009). NPs that possess super paramagnetic behavior at room temperature and magnetic particles which are stable in water at pH 7 have significant and potential biomedical applications such as in therapy, biology and medical diagnosis (Cabuil 2004; Morcos 2007; Ersoy and Rybicki 2007; Akbarzadeh et al. 2012). Magnetic NPs such as Fe_3O_4 (magnetite) and Fe_2O_3 (maghemite) have been employed in magnetic resonance imaging (MRI), categorization and manipulation of stem cell, magnetic hyperthermia (cancer therapy), gene therapy, examination of DNA and trained drug delivery (Xiang et al. 2007). Magnetic iron oxide NPs coated on viral nanofibers can be explored as biomarkers in the

detection of human serum antibody (Wang et al. 2015). QDs are more photostable, brighter, and display narrower fluorescence emission spectra which upon excitation by a single wavelength allow multiplexing applications (Liu et al. 2005). SeNPs have exhibit several activities including anticancer activity against breast, lung, kidney, and osteosarcoma, antibacterial, and antioxidant activity. Moreover, they have potential environmental applications also (Sweety et al. 2016).

19.8 Conclusion and Future Directions

Overall, there is an increased awareness towards the better utilization of cost-effective, economically feasible, biological approaches for biosynthesizing eco-friendly biosynthesis of toxic-free NPs. Over the past few decades, various metal based functional nanomaterials from microorganisms including bacteria, fungi, yeast and cyanobacteria have been successfully synthesized. These nanostructures have been shown to have monodisparity and occur in different sizes, shapes and morphology. Importantly, the microbe assisted nanoparticle biosynthesis do not involves any toxic chemicals or cumbersome processes. Further, NPs from microbe origin have shown to exhibit an excellent physic-chemical or material characteristics with an improved biological properties. Therefore, microbes have been widely used as a new source for synthesizing several metallic NPs with different crystalline structures and properties in the current era of nanobiotechnology. Microbes have high diversity and adopt metal tolerance capabilities and thus, can innately bioreduce metal ions to form metallic NPs which is also sometimes due to a detoxification process in the harsh environment. However, there may occurs many more possible mechanisms during the biological process of nanoparticle biosynthesis which yet to be completely elucidated in detail. Some of the studies have highlighted that metal ions are trapped on the cell surface due to ionic interaction between hydroxyl, carboxyl and other chemical groups occurring on the cells. In some studies, it is reported that metal ions are bioreduced due to enzymatic reactions involving NADPH as a reducing agents. However, Further, high diversity, easy growth, maintenance and genetic manipulation of microbial cells are quite easier, they could be considered as probable biofactories to synthesize NPs in large scale. However, controlled synthesis of a nanostructures with a definite shape, size depends on various factors including the concentration of metal ions, reaction time, temperature, pH and the type of organism used in the bioreduction process. In specific, the microbially produced NPs should possess high stability which is proposed to occur due to proteins expressed by the microbes. Hence, future research should focus on developing a simple, reliable microbe based biological protocol for synthesizing different metallic NPs with uniform shape, size, monodisperity and high stability. In addition, manipulation of genes at the genetic level to overexpress specific enzymes or proteins that involve in reduction reaction or acts as capping agents during the NPs synthesis is another trust area of future discovery. Likewise, the understanding of NPs synthesis by microbes will certainly benefit in mediating the controlled

synthesis of functional nanostructures. Thus, microbes based biosynthesis of NPs can be more advantageous for various biomedical applications due to their incredible physic-chemical, optoelectrical, electronic and biological properties.

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Chapter 20
Role of Silver Nanoparticles in Treatment of Plant Diseases

Jyotsna Sharma, Vivek K. Singh, Anil Kumar, Raju Shankarayan, and Sharada Mallubhotla

20.1 Introduction

Nanotechnology is the production and application of materials and various devices through the management of the properties and structure of matter at nanometric scale. It is the ability to measure and create substances between 1 and 100 nm. Nanoparticles have a wide range of usage in areas such as healthcare, medicines (Prabhu and Poulose 2012), cosmetics, chemical substances, food industry, environmental protection, etc. (Dong et al. 2012). It is a rapidly growing scientific FIELD of producing and constructing devices. An important area of research in nanobiotechnology is the creation of nanoparticles with contrasting chemical compositions, sizes and morphologies, and controlled variations (Chen and Schluesener 2008). Moreover, nanobiotechnology also serves as an imperative technique in the development of simple, hygenic, less detrimental, and eco-friendly procedures for the synthesis and congregation of metal nanoparticles having the intrinsic ability to reduce metals (Logeswari et al. 2013). Nowadays, there is a burgeoning demand to foster eco-friendly processes, which do not use lethal chemicals in the protocols for synthesis of nanoparticles. Although conventional methods involving chemical agents are in use to develop nanoparticles but there are also associated discrepancies with it as these are hazardous for the environment. Thus, green synthesis procedures constitute mixed-valence polyoxometalates, polysaccharides, tollens, biological, and irradiation methods having advantages over the conventional procedures (Rao and Kamalakar 2014). The most important issues which must be considered in preparation of

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nanoparticles by biological means are careful selection of the solvent medium and also choosing suitable eco-friendly nontoxic reducing and stabilizing agents (Oke et al. 2015).

Nanoparticles portray properties that are completely unique and upgraded as compared to bulk material and are believed to have more applicability in different areas like plant disease management (Jain and Kothari 2014), cancer diagnosis and treatment (Popescu et al. 2010), etc. Materials which are nanosized have now appeared as peculiar antimicrobial agents attributed to their large surface area to volume ratio (Khalil et al. 2013) and their typical chemical as well as physical properties which raises their association with microorganisms and also their ability to permeate cells (Sondi and Sondi 2004; Danilcauk et al. 2006; Kim et al. 2007). Plant disease management is an essential practice, as these dreadful diseases are one of the dominant aspect in limiting agricultural crop productivity. A variety of methods are being practiced for the protection of plants from diseases, amongst which the use of pesticides is most popular. Use of conventional approaches in agriculture like integrated pest management, and application of chemical pesticides like DDT have adverse effects on health of animals and human beings apart from the deterioration caused to soil fertility. The hazards caused by excessive use of pesticides on environment and human health have been an issue of great concern; therefore, agricultural scientists are searching for alternative measures against pesticides. Use of silver nanoparticles as antimicrobial agents have been found to have detrimental effects on fungal hyphae and conidial germination (Woo et al. 2009). Silver exhibits numerous modes of blockage action against microorganisms; therefore, it may be used with utmost safety for management of various plant pathogens in comparison to synthetic fungicides (Park et al. 2006; Min et al. 2009).

Silver nanoparticles have been the topic of great importance and interest for researchers because of their exclusive properties (size and shape depending on optical, antimicrobial, and electrical properties). A range of manufacturing procedures have been reported for the production of silver nanoparticles that include laser ablation, gamma irradiation, electron irradiation, chemical reduction, photochemical methods, microwave processing and biological synthetic methods (Mehrdad and Farhadi 2010; Iravani et al. 2014). Top-down approach of synthesizing nanoparticles, refers to the creation of nanoscale formations from smallest structures by machining, templating and lithographic techniques, by the application of photonics in nanoelectronics and nanoengineering; while bottom-up approach refers to the phenomenon of preparing organic and inorganic materials into particular structures, atom by atom or molecule by molecule, often by self-assembly or self-organization, which are applicable in several biological processes. Biological scientists are seriously committed towards the development of different nanomaterials (inorganic, organic, hybrid) having distinguished optical, physical and biological properties. Due to these enormous unique properties, nanoparticles have vital applications in fields like medicine, pharmaceuticals, engineering, agriculture, etc. Nanotechnology could be used as an efficient tool to reduce the destruction of agricultural production due to various plant diseases and could provide the much needed trigger for a second green revolution.

20.2 Preparation of Silver Nanoparticles

20.2.1 Chemical and Physical Synthesis of Silver Nanoparticles

The preparation of nanoparticles predominantly includes two main procedures: physical and chemical. The top-down approach comprises the grinding of bulk metals by mechanical means and also stabilization of the resulting nanosized materials by the addition of colloidal protecting agents (Gaffet et al. 1996; Amulyavichus et al. 1998) whereas the bottom-up methods comprise of electrochemical methods, reduction of metals and sono-decomposition. Nanoparticles that fall in the size range of 3–40 nm are authenticated by characterizing them through transmission electron microscopy (TEM) and UV-visible (UV-Vis) absorption spectroscopy and X-ray diffraction (Zhu et al. 2001; Arasu et al. 2010). The electrochemical method of synthesis involves the reduction of $AgNO_3$ in aqueous solution in the presence of polyethylene glycol by electric means.

Another approach for production of silver nanoparticles is sono-decomposition in which ultrasonic waves are used to generate cavitations. Such processes of synthesis of silver nanoparticles requires sonochemical reduction of an aqueous $AgNO_3$ solution in an atmosphere of argon-hydrogen because of the induction of hydrogen radicals during the process of sonication. The silver nanoparticles thus produced are also characterized by different techniques viz. TEM, X-ray diffraction, absorption spectroscopy, differential scanning calorimetry, and EPR spectroscopy. Nanoparticles are also prepared within aqueous foams as a template by electrostatically combining silver ions with an anionic surfactant aerosol in highly stable liquid foam. The foam is then drained off and reduced by adding $NaBH_4$. These silver nanoparticles are 5–40 nm in diameter and stable in solution, suggesting that the aerosol assists in their stabilization (Mandal et al. 2001).

Synthesis of silver nanoparticles is also performed using microwaves that involves the reduction of silver nanoparticles using varied frequencies of microwave radiation. This procedure gives a comparatively faster reaction and also a higher concentration of silver nanoparticles with the same range of temperature and duration of exposure. It can also be inferred from the studies that an increase in the concentration of silver nitrate, increases the reaction time and also a subsequent elevation of temperature, size of the particle, whereas an increase in the concentration of poly(N-vinylpyrrolidone) leads to a decrease in the particle size (between 15 and 25 nm). There are numerous other approaches available for synthesizing silver nanoparticles, such as thermal decomposition, inorganic solvents, chemical substances, photoreduction in reverse micelles, spark discharge, and cryochemical methods which have been reported to result in production of nanoparticles between the ranges of 5–80 nm in diameter (Jiang et al. 2006).

20.2.2 Biological Synthesis of Silver Nanoparticles

Biological synthesis of nanoparticles is becoming more popular because of its easiness of rapid synthesis, controlled toxicity, control on size characteristics, reasonable, and eco-friendly approach. Innumerable natural sources are available for synthesis of nanoparticles using organisms such as plants, fungi, yeast, bacteria, etc. (Table 20.1). Additionally, unicellular and multicultural organisms are able to synthesise intracellular and extracellular inorganic nanoparticles.

20.2.2.1 Nanoparticle Synthesis by Plant Extracts

Using plants for the synthesis of nanoparticles has gathered immense interest amongst scientists because it provides a single step biosynthetic process. This method of synthesis is a superior option, as protocols involving plant sources are free from toxicants, since, natural capping agents are readily supplied by plants. Exposure of silver in the form of an aqueous solution to plant extract prepared from different organs results in the reduction of silver, which consequently generates bimetallic silver nanoparticles. Such reports are available for the synthesis of silver nanoparticles, using *Plumeria rubra* (plant latex), (Patil et al. 2012), *Szyygium aromaticum* (bud extract) and *Murraya koenigii* (leaf extract). This synthesis is possible owing to the natural reducing agent eugenol or carbazoles present in the extracts respectively (Singh et al. 2010; Christensen et al. 2011).

20.2.2.2 Nanoparticle Synthesis by Bacteria

Synthesis of nanoparticles using bacteria as a reducing agent has gained momentum in recent years due to its immense application. It has been reported that *Bacillus species* has the ability to prepare metallic nanoparticles and the potentiality of the

Table 20.1 Examples of some organisms used for biological synthesis of silver nanoparticles

Bacteria	Plants	Fungi	Algae
Aeromonas sp.	*Aloe vera*	*Fusarium oxysporium*	*Spirulina platensis*
Klebseilla pneumonia	*Azadirachta indica*	*Phanaerochaete chrysosporium*	*Oscillatoria willei*
Lactobacillus strains	*Cinnamomum camphora*	*Verticillium*	*Gelidiella acerosa*
Pseudomonas stutzeri	*Emblica officinalis*	*Aspergillus flavus*	
Corneybacterium	*Pelargonium graveolens*	*Aspergillus fumigatus*	
Enterobacter cloacae	*Pinus eldarica*	*Fusarium oxysporium*	

Source: Iravani et al. (2014)

bacteria to convert bulk silver to its nanoform and fabrication of extracellular nanoparticles has also been investigated, and were found to range in size from 10 to 20 nm (Sunkar and Nachiyar 2012).

20.2.2.3 Nanoparticle Synthesis by Fungi

Production of nanoparticles by biological means using fungi has been attempted in recent times, because of their reception towards toxicity, higher bioaccumulation, comparatively economic, effortless synthesis method, simple downstream processing and biomass handling. There are several reports of extracellular biosynthesis of silver nanoparticles using various fungi *viz. Aspergillus niger* (Gade et al. 2008), *Fusarium solani* (Ingle et al. 2009), *Aspergillus oryzae* (Binupriya et al. 2010) and *Pleurotus sajor caju* (Nithya and Ragunathan 2009). The genus *Penicillium* is reported to be a good choice for extracellular synthesis of silver nanoparticles.

Synthesis of spherical nanoparticles using *Trichoderma viride* has also been described (Thakkar et al. 2010). Prologue of silver ions to *Fusarium oxysporum* leads to the synthesis of stable Ag hydrosols (Ahmad et al. 2003). *Phoma glomerata* has been traced to produce silver nanoparticles whose antimicrobial activity against *E. coli*, *S. aureus* and *P. aeruginosa* has been assessed (Birla et al. 2009).

20.2.2.4 Nanoparticle Synthesis by Yeast

Kowshik et al. (2002) reported the extracellular synthesis of nanoparticles in bulk by using the procedure of straight forward downstream processing. A silver tolerant yeast strain MKY3 has also been reported by this research group which could produce silver nanoparticles of 2–5 nm size by simply inoculating the culture with aqueous silver nitrate. Biosynthesis of silver and gold nanoparticles using an extremophile strain of yeast that was isolated from acid mine drainage has also been investigated by Mourato et al. (2011). Another member of aquatic yeasts, *Rhodosporidium diobovatum*, has been probed for intracellular synthesis of stable lead sulphide nanoparticles (Seshadri et al. 2011).

20.3 Characterization of Nanoparticles

Nanoparticle characterization is an important step for appreciation and introduction of controlled synthesis of nanoparticles and their applications. Characterization of nanoparticles is performed by making use of diverse techniques like scanning and transmission electron microscopy (SEM, TEM), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), Atomic force microscopy (AFM), Dynamic light scattering (DLS), powder X-ray diffractometry (XRD), and UV-Vis spectroscopy. These analytical tools aid in resolving diverse criteria

such as, particle size, shape, crystallinity, fractal dimensions, pore size and surface area. These techniques could also be used to determine additional specifications like orientation, intercalation and dispersion of nanoparticles and nanotubes in nanocomposite materials, besides, analysis of size and morphology using TEM, SEM and AFM. 3D images are analysed by AFM technique in order to intend the particle height and volume, while, particles size distribution can be performed using dynamic light scattering. Furthermore, X-ray diffraction is exercised for analysing the crystallinity, while confirmation of formation of sample is analysed by means of UV-Vis spectroscopy by displaying surface Plasmon resonance (Sun et al. 2000; Khomutov and Gubin 2002; Lee et al. 2003; Chimentão et al. 2004; He et al. 2004; Hutter and Fendler 2004; Zhang et al. 2004; Vilchis-Nestor et al. 2008).

20.4 Applications of Nanoparticles with Reference to Plant Disease Management

There has been great interest in applying nanotechnology in the area of agriculture in solving many loopholes: increasing yield, increase efficiency of resource utilization, minimizing the waste production, nanofertilizers, nanopesticides, nanobased treatment of agricultural waste, nanosensors, etc.

20.4.1 Nanoparticles in Restraining Plant Diseases

For managing and controlling plant associated diseases various salts (both organic and in organic) have been used. Apart from this, other modified organic materials are also used for the purpose (El-Sherif et al. 2007; Abd El-Hai et al. 2010; Kundu and Saha 2014). The latest approach in management of plant diseases is the use of nanoparticles which may prove very fruitful. The most basic way to protect plants from attack by pathogens is a direct application of nanoparticles in the soil or treatment of seeds prior to sowing/storage or foliar spray that result in suppression of the pathogens in the same manner as that of chemical pesticides. When nanoparticles are to be applied directly in soil, their effects on non-target organisms especially the mineral fixing/solubilising microorganisms will be of great significance. Various nanomaterials like carbon tubes, cups, etc., can also be used for SAR inducing chemicals, carrying chemicals (such as pheromones), polyamine synthesis inhibitors or even concentration of active ingredients of pesticides for their controlled release especially under flooded conditions. In order to fully utilize the advantage of nanotechnology in plant disease protection and management, it becomes essential to analyse the effect of nanosized particles on microbes and also their application in synthesizing pesticides. Nanoparticles may also prove to be very effective agents in the diagnosis of plant pathogens/diseases and pesticide residue analysis owing to their minute size and high reactivity.

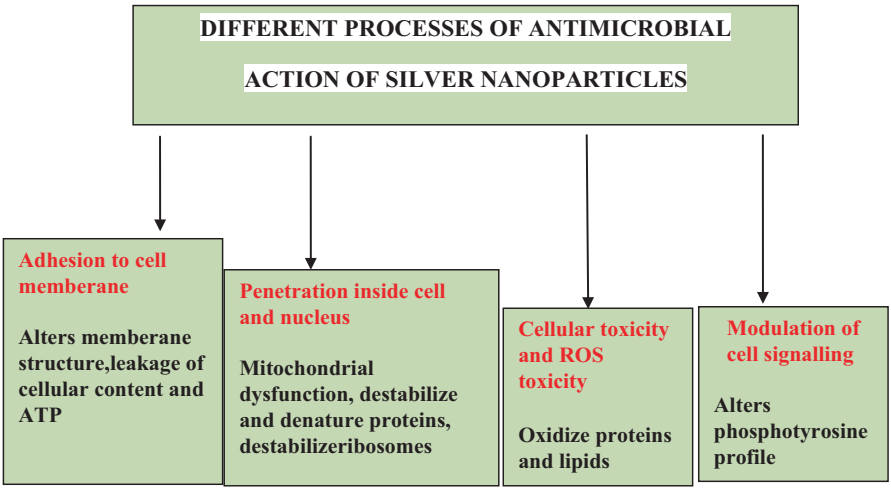

Fig. 20.1 Different methods of antimicrobial action of silver nanoparticles (Source: Dakal et al. 2016)

20.4.2 Effect of Nanoparticles on Pathogens/Microorganisms

Since, physio-chemical characteristics of nanosized particles vary immensely from their macroform, it becomes important to examine the effect of nanoparticles on microorganisms to harness the benefit of this technology in plant protection especially against phytopathogens. Nanoparticles because of their ultra-small size, even smaller than a virus particle and high reactivity, may affect the pathogenic activity of the microorganisms.

20.4.3 Effect of Nanoparticles on Bacteria

Bryaskova et al. (2011) studied the effect of three different bacteria (*Staphylococcus aureus* – gram +ve, *E. coli* – gram −ve bacteria and *P. aeruginosa* non-ferment gram −ve bacteria) in order to study the antibacterial potential of synthesized silver nanoparticles. Guzman et al. (2009) described excellent antimicrobial activity of silver nanoparticles against methicillin resistant gram +ve bacteria such as *E. coli*, *P. aureginosa* and *S. aureus*. Concentration, physiology, metabolism, intracellular selective permeability of membranes and the type of microbial cell are the different factors for the basis of antimicrobial activity of the nanoparticles. There are different methods by which silver nanoparticles suppress the activity of the microbes as illustrated in Fig. 20.1.

20.4.4 Effect of Nanoparticles on Plant Pathogenic Fungi

Major limiting factors for the production of food material are diseases caused by various plant pathogens viz., bacteria, fungi, viruses and nematodes. Large number of procedures are used for the control of plant pathogens but none exhibit perfect protection as they are in one or the other way very harmful for the environment and human beings. Hence, a great scope exists for the exploitation of nanotechnology for the management of plant pathogens. The nanoparticles have also been found to be suppressive for fungal growth. The silver nanoparticles were tested for fungicidal activity against different yeasts and moulds – *Candida albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata* and *Aspergillus brasiliensis*. The hybrid materials confirmed the strong antifungal effects against these microbes. The antifungal effect of zinc oxide nanoparticles (ZnO) against two fungal pathogens – *Botrytis cinerea* and *Penicillium expansum* (present in post-harvest stages) has been established. The treatment with nanoparticles resulted in deformation in the hyphae of *B. cinerea* and also suppressed the development of conidiophores and conidia in *P. expansum* which further led to the degradation of fungal hyphae.

20.4.5 Nanopesticides

At the starting phase of crop growth, fungal bioinoculants are added an active ingredient in the root area that assist in protecting the plant from invading pathogens (Khan and Anwer 2011). Gradual release of this ingredient is favoured because all the spores do not invade the host at once and it also decreases the quantity of the pesticide needed for suppression of disease which automatically protects the man as well as the environment from ill effects caused due to the release of chemical pesticides in the environment (Khan and Jairajpuri 2012).

Controlled release of the active ingredients of bioinoculants can be further improved through a nanotechnological approach by using nanomaterials as carrier for these chemicals. Such formulations may greatly decrease the amount of pesticide input and associated environmental hazards. Nanopesticides shall reduce the rate of application because the quantity of the chemical actually being effective is at least 10–15 times lesser than that applied with classical formulations. Hence a much lower than normal amount could be required to achieve satisfactory control of the disease. One of the reliable pesticide delivery systems is nanoemulsion owing to properties of better kinetic stability, small size, lower viscosity and improved optical transparency. Micro or nanoemulsions, as carriersfor pesticide delivery, can improve the solubility and bioavailability of the active ingredients of these chemicals.

Nanopesticides contain very small particles of the active ingredients or other small engineered structures with useful pesticidal properties (Bergeson 2010). Nanopesticides can enhance the rate of dispersion and wettability of agricultural

formulations (thus reducing organic solvent runoff) and unwanted pesticide movement. Characteristics of nanopesticides such as, permeability, crystallinity, stiffness, thermal stability, solubility and biodegradability are well revealed by nanomaterials. Nanopesticide formulations also offer large specific surface area and therefore help in providing an increased affinity to the target. Nanoemulsions, nanoencapsulates, nanocontainers and nanocages are some of the available nanopesticide delivery techniques that may prove effective in plant protection programmes. Corradini et al. (2010) explored the possibility of utilizing chitosan nanoparticles, a highly degradable antibacterial material for slow release of NPK fertilizer. Liu et al. (2006) developed kaolin clay-based nanolayers that could be used as cementing and coating material for the controlled release of fertilizers.

20.4.6 Nanoparticles in the Detection of Phytopathogens and Pesticide Residue

20.4.6.1 Detection of Phytopathogens

In order to protect the crops from the dreadful diseases, it is needed to have beforehand apprehension of the pathogens so that pesticides would be timely used for their treatment. Various phytobacteria, viruses, fungi can be detected by using nanoparticles as the biomarkers. Nanoparticles based sensors might offer improved detection limits in detecting viral pathogens in plants. Nanoparticles can either be directly modified for use in pathogen detection or for a diagnostic tool to detect compounds indicative to a diseased condition. Nano-chips are types of microarrays that contain fluorescent oligo capture probes through which the hybridization can be detected and are also used for detecting single nucleotide change in bacteria and viruses due to their high specificity as well as sensitivity (Lopez et al. 2009). For seed certification and plant quarantines, SPR sensor may prove highly efficient in the detection of infection in plants. Research on pathogen detecting nanosensors for their field application would be highly valuable for rapid diagnosis and disease management.

20.4.6.2 Pesticide Residue Detection

Around 1050 chemicals are reported as pesticide residues by Food and Drug Administration (FDA 2005). There are traditional techniques like gas or liquid chromatography-mass spectroscopy for detection but these are more time consuming as compared to the latest nanosensors which have high sensitivity, superselectivity and responds faster. Now a day's Nanosensors are recommended for pesticide residue detection because of many advantages like it has high sensitivity, even low concentrations can be detected by nanosensors, super selectivity and the response is much quicker.

20.5 Review on Use of Silver Nanoparticles in Management of Different Plant Diseases

The pathogens responsible for most plant diseases can only be diagnosed accurately in the laboratory, which is essential before recommendations can be made on integrated disease management strategies. Plant pathologists are attempting to develop a fruitful solution for safeguarding food and agricultural crops from bacteria, fungal and viral agents. Ultra-modern technologies such as nanoscale platforms, biological sensors, miniature detection devices and nanosensors could have a significant part in pathogen detection and disease management. Nanotechnology integrated methods need to be integrated into present practices in order to protect crops and lessen crop loss due to pests and the associated diseases.

A number of nanotechnology approaches have advanced the existing crop protection procedures in a better way (Pérez-de-Luque and Rubiales 2009). Advent of nanotechnology can facilitate in administering momentous opportunities to deal with agricultural hazards associated with fungicides and herbicidal fertilizers by effectively regulating their release (Li et al. 2007). For example, an eco-friendly fungicide is under development that uses nanomaterials to liberate its pathogen killing properties, only when it is inside the targeted pathogen (Choudhary et al. 2010). Control of pathogens in treatment of food crop diseases is essential. Recently, efforts have been made to develop harmless management methods that pose fewer hazards to humans and animals, and have focused on overcoming lack of synthetic fungicides.

Silver in nano forms exhibits good has a high antimicrobial potential and thus have extensive applications in various areas like sterilization purposes including materials of medical devices and water sanitization but have comparatively less reports in management of plant associated diseases. Therefore, Jo et al. (2009) performed an experiment silver ions and nanoparticles in their different forms were tested in order to investigate the fungicidall activity on *Bipolaris* sorokiniana and *Magnaporthe grisea* – plant-pathogenic fungi. Results obtained indicate profound effect of silver ions and nanoparticles on colony formation in these two pathogens. Effective concentrations of silver compounds blocking colony formation by 50% (EC_{50}) were higher for *B. sorokiniana* than for *M. grisea*. The inhibitory effect on colony formation significantly diminished after silver cations were neutralized with chloride ions. Another assay was also performed in under growth chamber conditions by conducting inoculation assays in order to confirm that both ionic and nanoparticle silver significantly reduced these two fungal diseases on perennial ryegrass (*Lolium perenne*). Particularly, silver ions and nanoparticles effectively reduced severity of disease. The *in vitro* and *in planta* evaluations of silver indicated that both silver ions and nanoparticles influence colony formation of spores and disease progress of plant-pathogenic fungi. The efficacy of silver ions and nanoparticles in *in planta* is much greater and that too with various preventative applications.

Another study conducted by Ocsoy et al. (2013) proved that Bacterial Spot, is a severe disease found in tomatoes caused by *Xanthomonas perforans*. They forged DNA-directed silver (Ag) nanoparticles (NPs) grown on graphene oxide (GO) which efficiently decreased the cell viability of *X. perforans* cell. At the very low concentrations (16 ppm of Ag@dsDNA@GO) composites showed sterling antibacterial potential in culture with significant advantages in improved stability, enhanced antibacterial activity, and stronger adsorption properties. Application of Ag@dsDNA@GO at 100 ppm on tomato transplants in a greenhouse experiment effectively lessen the harshness of bacterial spot disease compared to untreated plants, giving results similar to those of the current grower standard treatment, with no phytotoxicity.

Lamsal et al. (2011a) conducted experiments on disease pepper anthracnose caused by *Colletotrichum* species which is vital hindering factors for pepper production in Korea, its management being strongly dependent on chemicals. Anthracynose rot is a very cumbersome disease to handle if it gets established in the field. Thus, the aim of this group was to evaluate the possibilities of using silver nanoparticles instead of commercial fungicides to control the disease. In this study, they evaluated the effect of silver nanoparticles against pepper anthracnose under different culture conditions. Silver nanoparticles (WA-PR-WB13R) were applied at various concentrations to determine antifungal activities in vitro and in the field. The application at 100 ppm concentration of silver nanoparticles produced highest inhibition of the growth of fungal hyphae as well as conidial germination in comparison to the control in vitro. In field trials, the inhibition of fungi was significantly high when silver nanoparticles were applied before disease outbreak on the plants. Scanning electron microscopy results indicated that the silver nanoparticles caused a detrimental effect on mycelial growth of *Colletotrichum* species.

Disease causing fungi get their energy from the plants on which they reside. They are liable for a great deal of devastation and are characterized by various morphological symptoms such as, wilting, scabs, mouldy coatings, rusts, blotches and rotten tissue. Effect of silver nanoparticles on the phytopathogenic fungal growth was also investigated by Min et al. (2009). Fungal phytopathogens, especially for sclerotium-forming species *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotinia minor,* were selected due to their important roles in survival and disease cycle. Tests for the fungal hyphal growth revealed that silver nanoparticles effectively inhibited hyphal growth in a dose-dependent manner. Differential antimicrobial efficiency of silver nanoparticle was detected among the fungi on the basis of their hyphal growth in the following order, *R. solani* > *S. selerotiorum* > *Sclerotinia minor*. Tests for the sclerotial germination growth revealed that the nanoparticles showed significant inhibition potential. In particular, the sclerotial germination growth of *S. sclerotiorum* was most efficaciously inhibited at low concentrations of silver nanoparticles. A microscopic observation affirms that hyphae exposed to silver nanoparticles were critically impaired, resulting in the separation of layers of hyphal wall and collapse of hyphae. This experimental study proposed the possibility to use silver nanoparticles as a substitute to pesticides for scleotium-forming phytopathogenic fungal controls.

Japanese oak wilt *(Raffaelea quercivora)* is a vector-borne disease transmitted by the flying ambrosia beetle, *Platypus quercivorus*, and causes heavy destruction in different species of Oak. Kim et al. (2009) investigated the antifungal activity of three different forms of silver nanoparticles against unidentified ambrosia fungus R. QUERCIVORA, which has been responsible for the mortality of a large number of oak trees in Korea. In the presence of silver nanoparticles, growth of fungi was significantly reduced in a dose dependent manner. They also determined the effectiveness of combining the different forms of nanoparticles. Microscopic observation revealed that silver nanoparticles caused detrimental effects not only on fungal hyphae but also on conidial germination.

In a study by Krishnaraj et al. (2012) they developed silver nanoparticles by adopting green chemistry approach using *Acalypha indica* leaf extract as reducing agents. The reaction medium used in the synthesis process was optimized to get improved yield, controlled size and stability. Further, the biosynthesized silver nanoparticles were confirmed through UV-Vis spectrum, XRD and HR-TEM analysis. Different concentration of silver nanoparticles were evaluated to determine the inhibitory and suppressive effect of fungal plant pathogens *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Botrytis cinerea* and *Curvularia lunata*. Interestingly, 15 mg concentration of silver nanoparticles showed excellent inhibitory activity against all the tested pathogens. The results clearly recommends that plant extract mediated silver nanoparticles may haveparamount applications in controlling various plant diseases created by fungi.

Fungal disease **powdery mildew** affects a wide range of plants and fetches out plant's nutrients. This results in less blooming and weakening of the plants leading to decline in crop yield as the disease severity increased. Lamsal et al. (2011b) investigated and reported the effect of silver nanoparticles against powdery mildew under different cultivation conditions in both in vitro and in vivo conditions. Silver nanoparticles obtained from Bio-pulse Ltd., at various concentrations were applied before and after disease outbreak in plants to determine their antifungal activities. In the field tests, the application of 100 ppm silver nanoparticles showed the highest inhibition rate for both before and after the outbreak of disease on cucumbers and pumpkins. Also, the application of 100 ppm silver nanoparticles showed maximum inhibition for the growth of fungal hyphae and conidial germination in in vivo tests. Scanning electron microscope results indicated that the silver nanoparticles caused detrimental effects on both mycelial growth and conidial germination.

Lee et al. (2013) reported a rapid, simple, and in-expensive method for synthesizing AgNPs using an easily available bioresource material – milk. The synthesized AgNPs were mostly circular in shape with an average size of 30–90 nm, and elemental composition confirmed the significant presence of Ag without other contaminants. The strong antagonistic activity (87.1%, 86.5%, and 83.5%) against phytopathogens *Colletotrichum coccodes*, *Monilinia* sp., and *Pyricularia* sp. suggests that these AgNPs could be used as a powerful fungicidal agent in agricultural applications.

In an independent investigation Paulkumar et al. (2014) synthesized silver nanoparticles using the leaf and stem extracts of *Piper nigrum*. Antibacterial activ-

ity of the green synthesized silver nanoparticles against agricultural plant pathogens *Citrobacter freundii* and *Erwinia cacticida* isolated from infected agricultural crops *Abelmoschus esculentus* and *Citrullus lanatus* respectively was analyzed. The antibacterial property of silver nanoparticles is a constructive application in the field of agricultural nanotechnology. In order to authenticate the nature of the synthesized nanoparticles, their characterisation was done using UV-vis spectroscopy, X-ray diffraction (XRD), scanning electron microscope (SEM), transmission electron microscope (TEM), energy dispersive X-ray analysis (EDAX), and Fourier Transform Infrared Spectroscopy (FTIR). The measurement of peaks at 460 nm in the UV-vis spectra for leaf and stem synthesized silver nanoparticles affirms the reduction of silver metal ions into silver nanoparticles. Further, XRD analysis proved the crystalline nature of the synthesized silver nanoparticles. The TEM images displayed that the synthesized silver nanoparticles were within the size of about 7–50 nm and 9–30 nm, respectively. The FTIR analysis assisted in identification of the possible functional groups and the plant peptides that are involved in the preparation of silver nanoparticles.

Kasprowicz et al. (2010) noted a momentous decrease in growth of mycelia when spores incubated with silver nanoparticles of *Fusarium culmarum* a plant pathogen responsible for many plant diseases – Seedling Blight, foot rot, stalk rot, etc. The incubation of 24 h of *Fusarium culmarum* spores with a 2.5 ppm solution of silver Nanoparticles greatly inhibited the number of germinating fragments and sprout length in comparison to the control. Another study reported by Kamran et al. (2011) evaluated the potential of nanosilver (NS) and nanodioxide titanium (TiO_2) for removal of bacterial contaminants in Potato plant tissue culture procedures.

People around the world depend on rice as a staple food and a crop failure, for any reason, poses a real threat of starvation. Rice blast, a disease, induced by a fungus, causes lesions on leaves, stems, peduncles, panicles, seeds, and roots. So, this disease poses a potential threat for crop failure that it has been ranked among the most important plant diseases of all. Elamawi and EL-Shafey (2013) reported that silver nanoparticles can be applied efficiently for restraining of rice blast disease and obstruction of detrimental infections, even though there were no phytotoxicity reports on the rice grains produced. Evaluation of silver nanoparticles was also tested against rice leaf blast fungus was evaluated under different cultivation conditions both in vitro and in vivo. Under lab conditions, the application of four concentrations of silver nanoparticles to the culture of *Magnaporthe grisea* showed significant inhibition of both hyphal growth and number of colonies formed in a dose-dependent manner. Number of spores/ml decreased in all treatments. Under greenhouse conditions, silver nanoparticles were sprayed in concentrations 0, 25, 50, 100 and 200 ppm on rice seedling leaves thrice (3 h before inoculation, 1 and 5 days after artificial inoculation with spore suspension). Damaged Leaf Area Percentage (DLA %) indicated that the application of 100 ppm silver nanoparticles was highly efficient before and after inoculation (26.7, 15.3 and 20%), respectively compared to the untreated plants of 80%. The chemical fungicides isoprothiolane (Fuji one) and azoxystrobin (Amistar) at a concentration of 100 ppm each showed the lowest DLA (19.6% and 14.7%, respectively). Scanning electron microscope

results revealed that the silver nanoparticles have a detrimental effect on mycelial growth.

The land snail *Eobania vermiculata* is an important crop pest causing serious devastation in agriculture. Ali et al. (2015) investigated the possibilities of application of silver nanoparticles (AgNPs) to control the pest. The AgNPs were synthesized biologically using white radish (*Raphanus sativus* var. *aegyptiacus*). The biosynthesis was regularly checked by UV-Vis spectroscopy. X-ray diffraction spectra revealed peaks of crystalline nature of AgNPs and the transmission electron micrographs further confirmed the size of the synthesized nanoparticles ranging from 6 to 38 nm. When exposing snails and soil matrix to AgNPs in a laboratory experiment resulted in reduction of the activity and the viability of the land snail (20% of AgNPs treated snails died) as well as the prevalenceof fungal population in the surrounding soil. Moreover histology and ultrastructure alterations have been found in both kidney and the digestive gland of AgNPs treated land snails. The collaborative effect of synthesized AgNPs as antifungal agents was evaluated and clearly revealed that AgNPs can be effectively used against various plant pathogenic fungi. Utilization of silver in managing of plant diseases has also been evaluated and reported by Jo et al. (2009) by using two fungal pathogens of cereals viz. *Bipolaris sorokiniana* (spot blotch of wheat) and *Magnaporthe grisea* (rice blast). Results of the *in vitro* assays indicated that silver both in ionic and nanoparticle forms suppressed colony growth of both the pathogens but *M. grisea* was comparatively more sensitive to silver application. When tested *in vivo* with perennial ryegrass (*Lolium perenne*) silver ions and nanoparticles brought significant reduction in disease severity when applied 3 h prior to pathogen inoculation.

Resistant plant pathogens have emerged against the conventional antibiotics, whose alternative could possibly be the application of eco-friendly nanoparticles as an important strategy to manage plant disease. Antimicrobial activity of silver nanoparticles (AgNPs) i.e. Ag, AgP, AgIB, AgAE and AgBE and antibiotics (Nystatin and Streptomycin) were evaluated against plant pathogens *Erwinia carotovora* pv. *carotovora* and *Alternaria solani* in an experiment carried out in completely randomized Block Design with three replicates. The antibiotic Nystatin was used as a standard antibiotic reference in case of antifungal activity while streptomycin in case of antibacterial activity. For the antifungal and antibacterial activity different concentrations were prepared of 150 ppm, 200 ppm and 250 ppm and zone of inhibition (mm) for all silver nanoparticles (AgNPs) and antibiotics were prepared and inhibition zone was measured in millimetres (mm). Results revealed that the silver nanoparticles (AgNPs) i.e. AgAE and AgIB showed largest inhibition zone with the tested *E. carotovora* pv. *carotovora* where the activity was 14.33 mm and 13.13 mm respectively followed by AgBE (10.40 mm), AgP (10.33) and Ag (7 mm) while the reference antibiotic streptomycin produced lowest inhibition zone (5.66 mm). In case of *A. solani* maximum inhibition zones were achieved from silver nanoparticles (AgNPs), AgAE and AgIB where the antifungal activity was 27 mm and 24 mm followed by AgBE (22.33 mm), AgP (21.66 mm) and Ag (18.66 mm) while the reference antibiotic nystatin produced a minimum inhibition zone (4 mm). Further it was noticed that increasing the concentration of silver

nanoparticles (AgNPs) significantly increased the inhibition zones of the test plant pathogen and higher concentration (250 ppm) possessing strong antimicrobial activity concluding that silver nanoparticles (AgNPs), had maximum inhibitory effect against *Erwinina carotovora* pv. *carotovora* and *Alternaria solani* when compared with antibiotics (Abbas et al. 2015).

Mishra et al. (2014) conducted a study that was concentrated on the synthesis of silver nanoparticles extracellularly (AgNPs) by using supernatant of a culture of an agriculturally essential bacterium, *Serratia* sp. BHU-S4 and indicated its important role for the management of spot blotch disease in wheat. The biosynthesis of AgNPs by *Serratia sp.* BHU-S4 (denoted as bsAgNPs) was checked by UV-visible spectrum that exhibit the surface plasmon resonance (SPR) peak at 410 nm which isan important characteristic feature of AgNPs. Moreover, characterization of bsAgNPs was performed by using the X-ray diffraction (XRD), electron and atomic microscopy's, energy dispersive X-ray (EDAX) spectrometer, FTIR spectroscopy and thermogravimetric analyzer (TGAfor its structural, morphological, elemental, functional and thermal analysis. The XRD and EDAX analysis confirmed successful biosynthesis and crystalline nature of AgNPs which were spherical in shape with size range of 10–20 nm. The bsAgNPs showed efficient antifungal potential against *Bipolaris sorokiniana*, the spot blotch pathogen of wheat. Two, 4 and 10 mg/ml concentrations of bsAgNPs alleged for complete blockage of conidial germination, whereas in the absence of bsAgNPs, conidial germination was 100%. A disease named detached leaf reported obtrusive conidial germination on leaves of wheat spoiled with *B. sorokiniana* conidial suspension alone, it is reported that on treating leaves with bsAgNPs, germination of conidia was totally suppressed. The results were further validated under greenhouse conditions, where utilization of bsAgNPs notably weakens *B. sorokiniana* infection in wheat plants. Histochemical staining revealed a significant role of bsAgNPs treatment in inducing lignin deposition in vascular bundles. These discoveries serve as an important utilization of bsAgNPs in managing different plant diseases, demonstrating the exciting potentialities of nanofungicide employing agriculturally important bacteria.

El-Batal et al. (2016) reported that silver and selenium nanoparticles reduced disease severity in potato and also improved the vegetative and chemical parameters of the plant. Here silver nanoparticles were prepared by adopting a biological approach using *Trichoderma viride* cell free supernatant and additional chemical synthesis was also done using polyvinyl pyrrolidone (PVP) through gamma irradiation. The antimicrobial activity of the nanoparticles was evaluated in the field against *Alternaria solani* which causes early blight disease of potato. Disease severity, vegetative and biochemical parameters of plant were determined. Application of the nanoparticles 150 µg/ml + K_2SO_4 (2%) as plant foliar resulted as a highly effective inhibitor of disease severity (8.39%) as compared to the untreated plants (86.17%). The treatment also resulted in a significant increase in the growth and physiological parameters. A combined treatment of Selenium nanoparticles (SeNPs) 0.5 µg/ml, ascorbic acid 300 µg/ml and K_2SO_4 (2%) was found to reduce disease severity significantly (9.81%). SeNPs 0.5 µg/ml, AgNPs 150 µg/ml and K_2SO_4 (2%) when used simultaneously showed a disease severity of 12.63% but all plant physi-

ological parameters were found to be enhanced including yield. They concluded and recommended the application of AgNPs and SeNPs as plant foliar for controlling plant pathogen and improving plant yield.

Abdelmalek and Salaheldin (2016) conducted a study on pathogenic fungal infections and their tremendous harmful effects on human health and economy. Citrus fruit crop has immense economic, social and health impact on humans all over the world. Citrus fruits are invaded by a numerous pathogens in its various stages from blossoming to reaping and harvesting stages that impinge fruit production and considerably deteriorate the fruit quality. In the present study, a survey was done to investigate incidence of citrus leaf spots and fruit decay in some orchards in Menofiea and Beheira during 2014 and 2015. Three different fungi were isolated from samples of citrus. *Alternaria alternata* was the most abundant fungal pathogen isolated from citrus leaf and fruit spots followed by *Alternaria citri* and *Penicillium digitatum*. Silver nanoparticles were tested as a potent fungicide that may replace the dangerous cytotoxic fungicides. Silver nanoparticles were synthesized with 10 ± 5 nm average diameter size. An in vitro experimental assay was carried out on Potato Dextrose Agar (PDA) media treated with 50, 100 and 150 ppm of silver nanoparticles in addition to two control traditional fungicides products, namely Iprodione and Difenoconazole at 150 ppm concentrations. Results revealed that silver nanoparticles (150 ppm) showed potent antifungal activity against the isolated fungi that will open the gate for a new generation of less harmful fungicides replacing the cytotoxic dangerous currently used fungicides.

Gupta and Chauhan (2015) highlighted the fungicidal properties of silver nanoparticles against *Alternaria brassicicola* that causes Black Spot of cauliflower, radish, cabbage, kale which results in severe agricultural loss. They tested the synthesised silver nanoparticles (AgNPs) of 10, 25, 50, 100 and 110 ppm concentrations against *A. brassicicola* cultured on PDA in petridishes. They calculated the inhibitory rate (%) with a view to investigate the antifungal capacity of silver nanoparticles against varied pathogens. Treatment with 100 ppm AgNPs resulted in maximum inhibition of *A. brassicicola* i.e. 92.2%. 110 ppm of AgNPs also shows the same result, therefore 100 ppm AgNPs was treated as optimize concentration. AgNPs effectively inhibited the growth of *A. brassicicola*, which suggests that AgNPs could be used as fungicide in plant disease management. Further research and development are necessary to translate this technology into plant disease management strategies.

20.6 Conclusion and Future Prospects

The main purpose of the usage of nanomaterials in agriculture is to particularly reduce plant disease which otherwise results in deterioration of crops and may ultimately result in hunger and starvation, especially in the countries which are less developed. Despite having number of plausible advantages, the approach of nanoresearch has still not been utilised in the agricultural sector to its fullest. Various tools

and devices of nanotechnology, such as, nanocapsules, nanoparticles and even viral capsids, are few examples that can be used for the detection and treatment of plant diseases. The use of target specific nanoparticles can reduce the damage to other plant tissues that are not part of the target and also the extent of release of unwanted chemicals into the environment. Nanotechnology holds a great potential in agriculture, but a few technical issues are yet to be addressed, such as increasing the scale of production processes, lowering costs, as well as risk assessment issues. In this aspect, nanoparticles derived from biopolymers such as proteins, carbohydrates and plant extracts are particularly striking with low impact on human health and the environment. Nanomaterials and nanostructures with unique chemical, physical, and mechanical properties; e.g. electrochemically active carbon nanotubes, nanofibers and fullerenes have been recently developed and applied for preparation of highly sensitive biochemical sensors. These nanosensors also have relevant implications for applications in agriculture, particularly for soil analysis, easy bio-chemical sensing and control, water management and delivery, pesticide and nutrient delivery.

There is no doubt that nanotechnology will play an integral part in the upliftment of the agricultural sector, as it is proficient of being used in varied agricultural products that safeguard plants and oversee plant growth and spot diseases. Scientific researchers have been heading towards delving advanced applications and functions of nanotechnology in agriculture and the food industry. In the coming years, if these discoveries are applied sensibly, our environment and agricultural sector both will indeed see tremendous positive changes for the betterment of one and all.

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Chapter 21
Endophytic Fungi and Bioactive Metabolites Production: An Update

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21.1 Introduction

Alexander Fleming invented world's first antibiotic penicillin on 28th September 1928, which revolutionized the class of anti-bacterial drugs. Since then more number of antibiotics were discovered towards the treatment of infectious diseases. But, there is no end for new discoveries due to the drug resistance in bacteria (Xing et al. 2011). Provision of drugs has always been a challenge in medicinal field, seeking therapeutic drugs from natural products. Research in natural products for drug discovery methods are competitive with other synthetic drugs, due to lesser toxicity and broad spectrum activities in less quantity of compound administration. Research on plant based products requires continuous improvements in the screening process, extraction, isolation and structure interpretation. Moreover, various issues related to large-scale supply of novel compounds should be addressed in order to evaluate

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their bioactivities so that novel drug molecules can be discover based on the available traditional knowledge (Butler 2004; Kumara Swamy et al. 2011; Swamy and Sinniah 2015; Arumugam et al. 2016). Many of the plant based products are having several biological properties and hence, they are widely used in therapeutics to treat many diseases. Interestingly, most of the modern drugs are either plant based compounds or their synthetic derivatives. Many of these bioactive compounds are derived from medicinal plants based on the available traditional practices and knowledge (Mohanty et al. 2014; Swamy and Sinniah 2015, 2017).

In addition, medicinal plants have become the source of natural compounds for the discovery of novel drug molecules with improved biological activities and less toxicity. Plant extracts and their purified compounds provide immense possibilities in the discovery of new lead molecules with higher effectiveness. Plants produce enormous secondary metabolites with high chemical diversity (Sasidharan et al. 2011; Kumara et al. 2012; Swamy and Sinniah 2015; Swamy et al. 2015; Arumugam et al. 2016). The utilization of medicinal plant materials may result in species deterioration and cause loss of biodiversity. Moreover, obtaining natural products from plants is pretty expensive. Therefore, microorganisms residing inside the medicinal plants are suitable alternatives in drug discovery process as they minimize such problems and can act as important sources of natural products with vast potential for the discovery of new molecules for pharmaceutical use (Nisa et al. 2015). These microorganisms, so called as endophytes, can be isolated and grown in the laboratory and tested for bioactivity. Endophytes includes mainly bacteria and fungi and among them, fungi are most frequently isolated. However, occurrence and significance of endophytes associated with many number of plant species are yet to be screened and studied in detail. Hence, there is a large scope for the discovery of novel bioactive metabolites from these microbes associated with therapeutically valued medicinal plants (Nisa et al. 2015). Conversely, endophytic fungi remain under explored because of their inability to produce higher quantities of bioactive compounds required for drug discovery. Endophytic fungi may be at least one million species distributed in plants worldwide suggesting a rich source of novel and undescribed diverse species (Chandra 2012). Previous reports showed that endophytic fungi live inside the plant tissues causing no harm or any disease. In addition, endophytic fungi promote plant growth and produce secondary metabolites which are required for plant defense mechanisms (Tan and Zou 2001). These metabolites synthesized or derived from the endophytic fungi are biologically active compounds. Endophytic fungal species are reported to produce vast number of antimicrobial, antioxidant and anticancer compounds (Chandra 2012; Nisa et al. 2015; Demain 2014). In this chapter, we highlight the importance of endophytic fungi and their symbiosis with the host plants. In addition, several biological implications of endophytic fungi with special reference to the production of various classes of bioactive compounds are discussed in detail.

21.2 About Endophytic Fungi

Endophytic fungi are generally noticed in most of the plant species on earth. They reside in the host plant tissues established either through symbiotic or pathogenic relationships. These microbes are reported to produce a plethora of biomolecules involved in promoting plant growth or providing plant protection (Strobel et al. 2004). Endophytes are defined by many researchers in many ways which is mainly based on the viewpoints of endophytes isolated, examined and identified from host plants. Bacon and White (2000) confer a comprehensive and ordinarily accepted explanation of endophytes as "Microbes that colonize and live in the internal tissues of plants without causing any immediate, adverse effects". While, Tan and Zou (2001) explains endophytes as "an endophyte is a bacterial (including Actinomycete) or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intra-cellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease". Schulz and Boyle (2005) explained "Endophytic fungi, which colonize plants internally without apparent adverse effects, occur mutualistically, ubiquitously in plants and they produce number of compounds which can inhibit pathogens". Although the asymptomatic nature of endophytes living within plant tissues has incited focus on mutualistic or symbiotic relationships among host plants and endophyes, the biological diversity of endophytic microbes suggest that they can also be aggressive opportunistic or saprophytic pathogens (Strobel and Daisy 2003).

21.3 Origin and Evolution of Fungal Endophytes

The occurrence of endophytic fungi associated with plants has been revealed in the fossilized plant stems and leaf tissues (Taylor and Taylor 2000). Many harmless endophytic fungi are inactive pathogens, and can result in plant diseases when the plant is under stress or aged. Gene mutation could have occurred leading to an endophytic mutant during their evolution in association with its host plant. In vitro conditions, both the host and the endophytes are able to produce secondary metabolites which are toxic to each other (Peters et al. 1998). It has been suggested that endophytic fungi achieve asymptomatic colonization by a balanced antagonism between fungal virulence and response of plant defense mechanisms (Rodriguez et al. 2009). The multilocus phylogenetic framework of ancestral-state reconstructions suggests that endophytes have arisen from ancestors of insect-parasites and later got differentiated through a successions of inter-kingdom host jumps (Torres et al. 2007). Endophytic fungi such as *Neotyphodium* and *Balansia* are reported to be evolved from insect parasites before progressing to become epibiotic plants. The microbes initially obtained nutrients by infecting insects and whiteflies. Later, they entered grass hosts directly and aroused as different forms of endophytes (Rodriguez et al. 2008, 2009; Nisa et al. 2015).

The interactions between host-endophyte and host-pathogen involve perpetual mutual antagonisms that are mediated by the secondary metabolites secreted by the symbionts. Any imbalance in these host-pathogen interactions results in diseases whereas, host-endophyte interactions will always have a balanced antagonism. Thus, it can be speculated that these long-held associations have resulted in the possible transfer of genetic information between themselves and the host plants and vice versa. Interaction of endophytes is specific to host which confers the evolution of endophytes when they particularly infect the plant. Among numerous endophytes, at least one host specific species can be observed. The endophytic fungi, *Acremonium* species inhabiting *Lolium perenne* L. and a lot of grasses are anticipated to be host specific to grasses. Interestingly, *Epichloe* species are known to colonize with the intercellular space of host tissues systemically and can remain asymptomatic during their lifecycle and when the infected plants produce seeds, they get transmitted vertically. Likewise, some of the other *Epichloe* species can cause choke disease, where inflorescence maturation stops to produce no seeds (Clay and Schardl 2002). A naturally occurring non-pathogenic endophyte, *Colletotrichum magna* was developed from the mutation of wild-type strain causing anthracnose diseases in cucurbit plants resulting in dark, sunken lesions on stems, leaves, flowers as well as fruits. Also, this mutant has the ability to propagate systemically within the host plant showing no symptoms of diseases. However, retains the characteristics such as host specific infection, wild-type levels of in vitro sporulation, appressoria formation and spore adhesion (Nisa et al. 2015).

21.4 Symbiosis Between Plant and Endophytic Fungi

Endophytes are a representative symbiotic association of the fungi and their host plants. The description of symbiosis refers to the living together of dissimilar organisms where both hosts and symbionts are benefited (Akhtar et al. 2015; Swamy et al. 2016a, b). These symbiotic fungal associations relatively influence on rhizospheric soil nutrition and plant fitness (Brundrett 2006; Akhtar et al. 2015). Research efforts have witnessed that most of the plants in nature have a symbiotic relationship with endophytic fungi. Endophytic fungi live inside the plant, analogous to an epiphytes living on the plant surface. For example, some plant pathogens like smuts can exist internally and asymptomatically within host plant for many years before they finally become evident. Nevertheless, a large variety of heterotrophic organisms exist within the plant tissues during their life cycle and produce no disease symptoms. Asymptomatic endophytic fungi may be ever-present in the plant kingdom, rivaling insects in their species diversity (Arnold et al. 2000). Most attention has been emphasized on endophytic fungi existing in plant leaves, roots and stems (Clay and Schardl 2002; Park and Eom 2007). Many researchers have showed that there exists a intra and inter specific competition and mutualistic symbiosis of mycorrhizae and endophytes. These interactions are further reported to affect the variety of the plant community and evolution of the species. This symbiosis between

host plants and fungi is distributed widely effecting the diversity and structure of ecosystem (Park and Eom 2007).

The mode of penetration and colonization of endophytic *Fusarium* species and pathogen *Drechslera* sp. in bean and barley plants proved that the endophytic fungi are different from pathogenic fungi (Barbara et al. 2002). The endophytic fungi penetrated through the stomata along the anticlinal epidermal cells and colonization was limited, localized and intercellular in the shoots of bean and barley; in contrast, the pathogen was penetrated directly through the cell wall and colonized extracellularly. These differences of endophytes with respect to pathogenic fungi may not be able to cause disease to the host plant. Endophytic fungi are reported to promote the host plant growth and development as well as plant physiology (Muhammad et al. 2010; Swamy et al. 2016a). Endophytic fungi receive nutrition, protection and multiplication opportunities from their associated host plants. Likewise, host plants are also benefited from this symbiosis (Clay and Schardl 2002). The endophytes produce cytokinins, indole acetic acid and many other plant growth promoting elements which help to control or regulate germination, growth, metabolism, or other physiological activities (Tan and Zou 2001). Partial involvement of these fungi can enhance the uptake of soil nutrients such as nitrogen and phosphorus by host plants (Reis et al. 2000). Inoculation of an endophyte, *Colletotrichum gloeosporioides* to in vitro cultures of *Artemisia annua* L. have shown to promote their growth effectively (Tan and Zou 2001). The presence of these endophytes enhances the fitness of host plants. These endophytic fungi release a wide range of secondary metabolites for instance alkaloids and these act as metabolic inhibitors of insects, anti-feedants, promotes drought tolerance of host plant, and reduces microbial infections. The herbs infected with endophytic fungi shows better resistance towards higher temperature (Rodriguez et al. 2009). Few endophytic fungi, like *Acremonium lolli* of perennial Ryegrass produce poisonous secondary metabolic products (Ergopeptine alkaloids) that are involved in protecting the plants from pathogens (Philippe 2016). Some of the endophytic *Neotyphodium* species can only spread by infecting seeds of the host plants (Schardl and Phillips 1997). Plant-endophytic fungal association also provides defensive mutualism by producing several classes of bioactive metabolites such as ergopeptine, lolitrems, loline (alkaloids) and pyrrolopyrazine alkaloids and (Bush et al. 1997; 76. Saikkonen et al. 2010; Philippe 2016). Ergovaline produced from endophytic fungi is expected to be the key agent of fescue toxicosis observed in livestock (Philippe 2016).

21.5 Endophytic Fungi Versus Mycorrizae

Mycorrizae and endophytic fungi in plants represent symbiotic association. There is no significant difference between their association and mechanism with the host plants, but few differences are present in their appearance and structure (Jumpponen 2001). Mycorrhizal fungi associates with plants by colonizing roots and nurture in the surrounding rhizospheric soil, while endophytes live exclusively inside the plant

tissues and emerge to sporulate when the host plant tissue or whole plant is senesced (Rodriguez et al. 2009). Both mycorrizae and endophytic fungi are beneficial to the fungi and the host plants (Smith and Read 1997). However, endophytic fungi appear ubiquitously in roots and shoot of monocotyledonous and dicotyledonous of the plant and have been found in all the species of plants. Whereas, Mycorrizae of most groups appear in higher plants of the root parts (Hamayun et al. 2009). Mycorrhizal association can be internal, where the fungus colonizes the host plant's roots (Endomycorrhizae) or as observed in Vesicular Arbuscular Mycorrhizal fungi (VAM), or extracellular (Ectomycorrhizae) (Akhtar et al. 2015; Swamy et al. 2016a). VAM association closely attaches plants through their hyphal networks which are usually found in excess of 100 m of hyphae per cubic centimeter of soil (Parniske 2008). VAM significantly enhances the nutrient and water uptake ability of plants through roots from soil. Moreover, VAM also increases the plant ability to tolerate various harsh environmental situations including mineral deficiencies, water stress, soil toxicities, and soil erosion (Parniske 2008; Akhtar et al. 2015). Endophytes are a diverse cluster of fungi having superficial implications on plants through plant ecology, fitness, and provides biotic and abiotic stress tolerance, decreases water consumption and increases plant biomass (Brundrett 2006). Therefore, the mycorrhizae of plants are grouped under root endophytes (Schulz et al. 2002; Lima et al. 2012).

21.6 Biological Implications of Endophytic Fungi

Endophytic fungi can stimulate plant growth, increase resistance towards disease causing pathogens, suppress the weed, increase tolerance to abiotic and biotic stresses (Sturz et al. 2000). In addition, they also have potential to produce vast bioactive secondary metabolites with pharmaceutical importance (Tan and Zou 2001; Demain 2014). Many endophytic fungi provide protection to the host by inducing defense mechanisms in plants against a broad range of pathogens. The endophytes are known to produce an antibiotic substance which inhibits the pathogen growth, or may compete with pathogen for space and nutrition. For example, Barley plants associated with endophyte *Piriformospora indica* have exhibited antagonistic to vascular pathogen, *Fusarium culmorum* and leaf pathogen, *Blumeria graminis* (Johnson et al. 2014). Endophytic fungi, namely *Cordana* sp. and *Nodulisporium* sp. showed effective activity against Anthracnose disease cause fungi *Colletotrichum musae* in wild Banana (Nuangmek et al. 2008). Endophytic fungi also produce herbicidal, nematicidal and pesticidal compounds and have the ability to trigger plant defense mechanism. Endophytic fungi like *Acremonium*, *Paecilomyces*, *Trichoderma*, *Fusarium*, *Chaetomium,* and *Phyllosticta* species have exhibited nematicidal activity against *Radopholus similis* and *Meloidogyne incognita* (Vu et al. 2006; Kalele et al. 2007; Goswamia et al. 2008; Yan et al. 2011). The endophytic fungus *Hypoxylon pulicicidum* sp., produce novel indole diterpenes, Nodulisporic acids (NAs) which exhibited insecticidal activity against wide range

of insects such as mosquitoes, fruit flies and dog flea (Bills et al. 2012). *Neotyphodium coenophialum* and *Neotyphodium lolii* are the grass (*Lolium arundinaceum*) endophytes which produced ergot alkaloids, toxic to herbivores (Simons et al. 2008). The metabolites of endophytic fungi can also be successfully used in weed management. For example, Brefeldin A produced by *Cladosporium* sp. suppress the pollen tube maturity of weed *Picea meyeri* (Wang et al. 2007) and Ascotoxin isolated from *Paraconiothyrium* sp. affect the seed germination of weed *Lactuca sativa* and *Echinochloa crus-galli* (Khan et al. 2012).

Endophytic fungi provide defense mechanism and also significantly influence the development of plant community. The interaction between the endophytic fungi with host plants increases plant productivity as well as the species diversity (Park and Eom 2007). Endophytes can direct or indirectly promote the plant growth through different mechanisms. Endophytes produce some kind of growth hormones or induces the host plants to secrete hormones and thus stimulates the host growth by improving nutrient metabolism (Schulz and Boyle 2005). For example, an endophytic fungus *Sebacina vermifera* significantly promoted the growth of *Nicotiana attenuata* by inhibited the ethylene signaling leads decrease in ethylene production (Barazani et al. 2007) and seed-borne fungal endophyte, *Stagonospora* species isolated from *Phragmites australis* enhanced reed biomass formation in axenic microcosms by providing a competitive advantage to germlings (Ernst et al. 2003).

Endophytic fungi enhancing drought tolerance in host plant is a great benefit under dry environmental conditions; the mechanisms vary from plant to plant. For example, tall fescue grass growth increased with an endophyte *Neotyphodium* association and its growth more than uninfected tall fescue due to water stressed (West et al. 1993). Drought stress resulted in altered stomatal behavior and osmotic regulation in plants (West 1994). *Piriformospora indica* provides tolerance to drought stress in Chinese cabbage leaves through stimulating the expression of drought linked genes and plastid-localized Ca^{2+}sensing receptor protein. Endophytic fungi significantly alter the antioxidant activity of its host which enhances tolerance from abiotic stress. The accumulation of loline alkaloids produced by endophytes affected osmotic potential and increased tolerance to other environmental stresses like heat, low light and low soil fertility (Siegel and Bush 1997)

Endophytic fungi are involved in biodegradation of ecological wastes. The fungal endophytes have the ability to degrade several kinds of organic compounds including carbon and nitrogen sources by producing enzymes. Endophytic fungi extensively metabolize benzoxazinones to less toxic metabolites by the oxidoreductases (Zikmundova et al. 2002). In addition, endophytes have been reported to improve the soil micro-environment by decomposing environmental pollutants. For example, 89.51% of phenanthrene was removed by *Ceratobasidum stevensii* isolated from the *Euphorbiaceae* (Dai et al. 2010). Endophytic fungus *Pestalotiopsis microspora* degraded the synthetic polymer polyester polyurethane, polycyclic aromatic hydrocarbons and phenanthrene with bioremediation applications (Russel et al. 2004; Wang and Dai 2011).

The secondary metabolites from endophytic fungi play a wide range of biological activities. In the recent years, abundance of secondary metabolites extracted

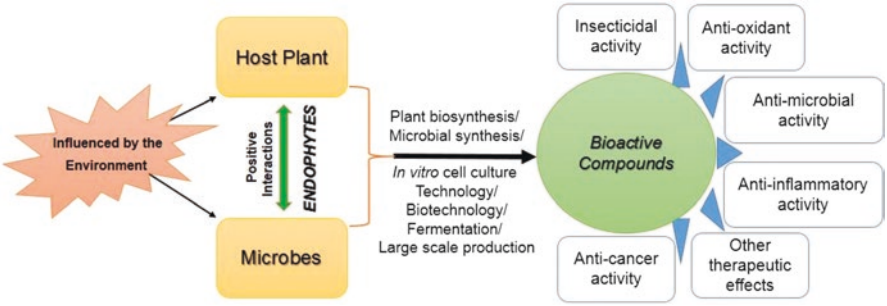

Fig. 21.1 Various bioactive compounds synthesized from the endophytic microbes associated with plants under the influence of their surrounding environment

from endophytic fungi are found to have applications in medicinal drug as antimicrobial agents, anticancer and antitumor agents, antidiabetic agents, cholesterol inhibitors and immunosuppressive agents (Strobel 2003). In the search of new bioactive molecules, endophytic microorganisms have been demonstrated to produce many metabolites that are reported to possess antimicrobial, antioxidant, anti-inflammatory, insecticidal, anticancer and other biological properties (Fig. 21.1) (Sun et al. 2004; Luo and Fang 2008; Demain 2014; Gubiani et al. 2014; de Felício et al. 2015). Apart from biological activity, they have been successfully screened for application in diverse areas, in agriculture as pesticides and herbicides (Tétard-Jones and Edwards 2016).

21.7 Endophytic Fungi and Their Secondary Metabolites

The secondary metabolites of fungal origin display a wide array of structural types, and their study has certainly improved the field of organic chemistry during the past several decades. Endophytic fungi are reported to produce a huge number of low molecular weight compounds, also grouped as secondary metabolites. "The metabolites which are produced after the active growth phase and are not directly involved in growth and development of the organism are collectively known as secondary metabolites". In comparison to the primary metabolites which are essentially same for all living systems, the secondary metabolites are usually species specific and are derived from some of the intermediates of primary metabolism. Secondary metabolites from endophytic fungi comprises a wide variety of structures including terpenoids, alkaloids, quinones, xanthones, peptides, steroids, flavonoids, phenols and phenolic compounds. The secondary metabolites obtained from grass endophytic fungi includes alkaloids like peramine, ergovaline, ergotamine, lolitrem etc. (Wang et al. 2004; Kunkel et al. 2004; Nisa et al. 2015; Demain 2014). As stated by Strbel, (2002), fungal endophytes occurring within the plants have the capability to secrete metabolites which are either similar to, or with higher activity than that of their respective hosts. The secondary metabolites of fungal origin exhibit a very wide

range or structural types, and their study has significantly improved the modern discovery. However, recovery of the fungal metabolites still remains as the major challenge in drug discovery (Demain 2014). Apart from anti-microbial activity, several secondary metabolites have been successfully screened for application in diverse areas. These include their application in agriculture as pesticides and herbicides, in pharmaceuticals as cholesterol inhibitors, immunosuppressive agents, anticancer and antitumor agents (Tables 21.1 and 21.2). In the last few years, many secondary metabolites obtained from endophytic fungi are reported to have many applications in the field of biomedicine and agrochemistry (Strobel 2003; Huang et al. 2007; Nisa et al. 2015). Plants are potential reservoir of many endophytic fungi and may be the treasure for many useful bioactive metabolites. There is a need and increasing demand to hunt for the bioactive metabolites turned in recent years towards hidden metabolites in the endophytic fungi which are used as antibacterial, antifungal and anticancer drugs. Endophytic fungi from medicinal plants have played an important role as an alternative source of novel bioactive metabolites (Nisa et al. 2015; Demain 2014). Some of these bioactive secondary metabolites produced from endophytic fungi are mentioned in the Tables 21.1 and 21.2 and discussed below.

21.7.1 Antibacterial Compounds

There are a number of antibacterial metabolites reported from endophytic fungi and few of them have been reviewed in this section. Krohn et al. (1999) reported antibacterial compound Ketodivinyllactonic steroid, herbarulide from *Pleospora herbarum,* an endophytic fungus. Lu et al. (2000) determined bactericidal property of ten steroid compounds isolated from *Colletotrichum* sp. isolated from the medicinal plant, *Artemisia annua*. Among the tested compounds 3-oxo-ergosta-4,6,8(14),22-tetraene, 3b-hydroxy-ergosta-5-ene, 3b-hydroxy-5a,8a-epidioxy-ergosta-6, 3b,5a-dihydroxy-6b-acetoxy-ergosta-7,22-diene, 22-diene 6-isoprenylindole-3-carboxylic acid and 3b,5a-dihydroxy-6b-phenylacetyloxy-ergosta-7,22-diene showed effective inhibition against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas* sp. and *Sarcina lutea.* Wagenaar and Clardy (2001) isolated antibacterial compound, dicerandrols A-C from the endophytic fungus, *Phomopsis longicolla* of *Dicerandra frutescens*. Strobel et al. (2001) reported antibacterial activity of 5 classes of volatile compounds including esters, alcohols, ketones, lipids and acids from a novel endophytic fungus, *Muscodor albus* associated with *Cinnamomum zeylanicum.* The compounds showed effective inhibition for *E. coli*, *S. aureus*, *Micrococcus luteus*, and *B. subtilis*. Out of them, esters of 1-butanol 3-methyl- acetate were found to the most effective class of inhibitory constituents. A steroid, ergosterol compound isolated from *Penicillium janthinellum* associated with *Melia azedarach*, exhibited antibacterial activity against *Leishmania* sp. (Marinho et al. 2005).

Table 21.1 Antimicrobial activity of compounds/extracts of fungi associated with medicinal plants

Endophytic fungi	Medicinal plant (host)	Extract/compound	Effective against	References
Colletotrichum sp., *Pleospora herbarum*	*Artemisia annua*	3-oxo-ergosta-4,6,8(14), 22-tetraene, 3b-hydroxy-ergosta-5-ene, 3b-hydroxy-5a,8a-epidioxy-ergosta-6, 3b,5a-dihydroxy-6b-acetoxy-ergosta-7,22-diene, 22-diene 6-isoprenylindole-3-carboxylic acid and 3b,5a-dihydroxy-6b-phenylacetyloxy- ergosta-7,22-diene, Herbarulide	*Bacillus subtilis, Staphylococcus aureus, Sarcina lutea, Pseudomonas* sp., *Candida albicans* and *Aspergillus niger*	Lu et al., (2000)
Phomopsis Longicolla	*Dicerandra frutescens*	Dicerandrols A-C	*S. aureus, S. pyogenes, B. subtilis,*	Wagenaar and Clardy (2001)
Muscodor albus	*Cinnamomum zeylanicum*	Esters, alcohols, ketones, lipids and acids	*Pythium ultimum, Phytophthora cinnamomi, Rhizoctonia solani, Ustilago hordei, Stagnospora nodorum, Aspergillus fumigatus, Fusarium solani, Verticillum dahlia, Cercospora beticola, Tapesia yallundae, Xylaria* sp., *C. albicans, E. coli, S. aureus, Micrococcus luteus, B. subtilis*	Strobel et al. (2001)
Fusarium sp. YG-45	*Maackia chinensis*	Fusapyridons A	*S. aureus* and *P. aeruginosa*	Tsuchinari et al. (2007)
Nerium oleander	*Chaetomium* sp.	Phenolic acids, flavonoids	*E. coli*, *B. cereus*, *S. annatum*, *S. aureus*, *L. monocytogenes* and *C. krusei*	Huang et al. (2007)
Phomopsis sp.	*Vitex negundo*	Mycelial extract	*E. coli, S. typhimurium, B. cereus, B. subtilis, K. pneumoniae and S. aureus*	Desale and Bodhankar (2013)
Phomopsis sp., *Botryosphaeria* sp.	*Garcinia atroviridis, G. dulcis, G. mangostana, G. nigrolineata, G. scortechinii*	Ethyl acetate extracts	*S. aureus, Candida albicans and Cryptococcus neoformans, Microsporum gypseum*	Phongpaichit et al. (2007)

Acremonium curvulum, Aspergillus ochraceus, Gibberella fujikuroi, Myrothecium verrucaria and Trichoderma piluliferum	*Bauhinia forficate*	Mycelial extract	*Salmonella typhi, S. aureus, S. pyogenes, B. subtilis, E. faecalis*	Bezerra et al. (2015)
Nigrospora sphaerica, Pestalotiopsis maculans, Colletotrichum gloeosporioides, M. sterilia	*Indigofera suffruticosa*	methanolic extract, ethyl acetate extract	*S. aureus, B. subtilis, E. coli, K. pneumonia, P. aeruginosa*	dos Santos et al. (2015)
Cylindrocarpon sp, *Phoma* sp., *Fusarium* sp, *Phaeosphaeria avenaria*	*Saussurea involucrate*	Mycelia extract	*A. fumigates, S. aureus, B. subtilis, E. coli, C. neoformans*	Lv et al. (2010)
Fusarium, Gibberella sp.,	*Ophiopogon japonicus*	Extract of mycelia	*S. aureus, C. neoformans*	Liang et al. (2012)
Phoma sp.	*Cinnamomum mollissimum*	5-hydroxyramulosin	*A. niger*	Santiago et al. (2012)
Diaporthe sp.	*Pandanus amaryllifolius*	Diaportheone A, Diaportheone B,	*M. tuberculosis*	Bungihan et al. (2011)
Chloridium sp.	*Azadirachta indica*	Naphthaquinone Javanicin	*Pseudomonas* sp.	Kharwar et al. (2008)
Fusarium sp.	*Paris polyphylla*	5alpha, 8alpha-epidioxyergosta-6, 22-dien-3beta-ol , 22-dien-3beta, 5alpha, 6beta, 7alpha-tetraol, and butanedioic acid	*B. subtilis, S. haemolyticus Agrobacterium tumefaciens, E. coli, P. lachrymans, X.* vesicatoria and *Magnaporthe oryzae*	Huang et al. (2009)

(continued)

Table 21.1 (continued)

Endophytic fungi	Medicinal plant (host)	Extract/compound	Effective against	References
Aspergillus spp.	*Gloriosa superb*	(5-(hydroxymethyl furan-2-carbaldehyde, 4-hydroxyphthalic acid dimethylester, and ergosterol, 6-Methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene-11-one-5,6,7,8-tetralene-7-acetamide	*S. aureus, B. subtilis, E. coli, P. aeruginosa, S. typhimurium, S. cerevisiae, C. albicans, C. gastricus*	Budhiraja et al. (2012)
Fusarium sp. YG-45	*Maackia chinensis*	fusapyridons A	*S. aureus, P. aeruginosa*	Tsuchinari et al. (2007)
Chaetomium sp.	*Nerium oleander*	Fermentation broth	*E. coli*, *B. cereus*, *S. annatum*, *S. aureus*, *L. monocytogenes*	Huang et al. (2007)
Penicillium sp.	*Cerbera manghas*	4-(3-hydroxybutan-2-yl)-3,6-dimethylbenzene-1,2-diol, and 3,4,5-trimethyl-1,2-benzenediol	Methicillin Resistant *S. aureus*	Zhuang et al. (2008)
Alternaria sp.		xanalteric acids I and II	*E. faecium*, *E. cloacae*, *S. pneumonia, P. aeruginosa*	Kjer et al. (2009)
Alternaria alternata	*Coffea arabica*	Extract	*S. aureus, E. coli*	Fernandes et al. (2009)
Aspergillus fumigatus		Deoxypodophyllotoxin	*S. aureus*, *K. pneumonia*, *P. aeruginosa*	Kusari et al. (2009)
Phomopsis sp.	*Plumeria acutifolia*	terpenoid	*E. coli*, *Pseudomonas* sp., *Klebsilla* sp., *B. subtilis*, *S. aureus, S. typhi, C. albicans*	Nithya and Muthumary (2010)
Xylaria sp.	*Piper aduncum*	Extract	*Cladosporium cladosporioides* and *C. sphaerospermum.*	Oliveira et al. (2010)
Fusarium solani	*Taxus baccata*	1-tetradecene, 8-pentadecanone, 8-octadecanone, 10-nonadecanone and octylcyclohexane	*S. epidermidis*, *S. aureus*, *S. flexneri, B. subtilis*, *E. coli*, *C. albicans, C. tropicalis*	Tayung et al. (2011)
Xylaria sp. and *Diaporthe* sp.		Extract	*S. paratyphi* and *E. faecalis*	Nath et al. (2012)

Pestalotiopsis sp.	*Biota orientalis*	Extract	*S. faecalis*, *S. typhi*	Subbulakshmi et al. (2012)
Botrytis sp.	*Ficus benghalensis*	Extract	*E. coli, Klebsiella* sp.	Senthilmurugan et al. (2013)
Aspergillus sp.	*Bauhinia guianensis*	Fumigaclavine C, Pseurotin A	*E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*	Pinheiroa et al. (2013)
Colletotrichum gloeosporioides, *Penicillium* sp. and *Aspergillus awamori*	*Rauwolfia serpentina*	Extract	*S. pyogenes, E. coli, E. faecalis, S. enterica* ser *paratyphi*, *C. albicans, E. nidulans*	Nath et al. (2015)
Nigrospora sphaerica, Pestalotiopsis maculans	*Indigofera suffruticosa*	Methanolic extract	*S. aureus*	dos Santos et al. (2015)
Curvularia australiensis, *Alternaria. alternate, Alternaria citrimacularis*, *Aspergillus. niger* and *Cladosporium cladosporioides*	*Aegle marmelos*	Extracts	*S. epidermidis*, *S. aureus*, *Shigella* sp., *P. aeruginosa*, *E. faecalis*, *E. coli*, *K. pnuemoniae*, *P. mirabilis* and *S. typhi*	Mani et al. (2015)
Chaetomium globosum	*Nymphaea nouchali*	Chaetoglobosin A and C	*S. aureus*, *B. cereus*, *P. aeruginosa*, *E. coli*	Dissanayake et al. (2016)
Cunninghamella sp., *Rhizoctonia* sp., *Colletotrichum* sp., *Phomopsis* sp, *Penicillium* sp.	*Cinnamomum mercadoi*	Ethyl acetate extract of mycelia	*S. aureus, B. cereus E. coli, E. aerogenes*	Marcellano et al. (2017)

Table 21.2 Anticancer activities of compounds/extracts of fungi associated with medicinal plants

Endophytic fungi	Medicinal plant (host)	Compound/s	Effective against	References
Phoma sp.	*Cinnamomum mollissimum*	5-hydroxyramulosin	Murine leukemia cells	Santiago et al. (2012)
Paecilomyces sp.	*Taxus mairei, Cephalataxus fortune, Torreya grandis*	Fermentation broth	Human cancer cell lines, HL-60 and KB cells	Huang et al. (2001)
Alternaria sp.	*Polygonum senegalense*	Exctract	L5178Y leukemic cells	Aly et al. (2008)
Aspergillus sp.	*Gloriosa superb*	(5-(hydroxymethyl furan-2-carbaldehyde, 4-hydroxyphthalic acid dimethylester, and ergosterol, 6-Methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene-11-one-5,6,7,8-tetralene-7-acetamide	Leukemic cancer cell line (THP-1), Breast cancer cell line (MCF-7)	Budhiraja et al. (2012)
Penicillium sp.	*Centella asiatica*	Ethyl acetate extract	HeLa, A431, MCF7 human cancer cells	Devi and Prabakaran (2014)
Chaetomium sp.	*Salvia officinalis*	Isocochliodinol	L5178Y mouse lymphoma cells	Debbab et al. (2009)
Ascomycetes sp.	*Mimosops elengi*	Ergoflavin	ACHN, H460, Panc1, HCT116, and Calu1 cancer cell lines	Deshmukh et al. (2009)
Cephalotheca faveolata	*Eugenia jambolana*	Sclerotiorin	Colon cancer (HCT-116) cells	Giridharan et al. (2012)
Hypoxylon truncatum	*Artemisia annua*	Daldinone C (1) and daldinone D (2), altechromone A and (4S)-5,8-dihydroxy-4-methoxy-alpha-tetralone	Colon cancer cell line, SW1116	Gu et al. (2007)
Phomopsis archeri	*Vanilla albidia*	Phomoarcherins, kampanol A (4), R-mevalonolactone, ergosterol, and ergosterol peroxide	cytotoxicity against cholangiocarcinoma cell lines	Hemtasin et al. (2011)
Penicillium sp. SXH-65	Plant collected from saline–alkaline soil	Meroterpenoids	Hela, HL-60 and K562 cell lines	Sun et al. (2013)
Phlebiopsis sp.	*Morinda citrifolia*	Ethanol extract	LU-1, MCF-7, PC-3 cacner cells	Wu et al. (2015)
Phomopsis sp.	*Annona muricata*	Ethyl acetate extract	MCF-7	Artika et al. (2017)

Tsuchinari et al. (2007) found the antibacterial activity of 3,4,5-trisubstituted N-methyl-2-pyridone alkaloid, fusapyridons A characterized from the extract of *Fusarium* sp. YG-45 isolated from *Maackia chinensis*. The compound showed activity against *S. aureus* and *P. aeruginosa* with the minimum inhibitory concentration (MIC) at 50 and 6.25 μg/mL respectively. Huang et al. (2007) evaluated the efficiency of metabolites extracted from endophytic fungi of *Nerium oleander* against the growth of *E. coli*, *B. cereus*, *S. annatum*, *S. aureus*, *L. monocytogenes* and *C. krusei*. They found that the extract of seven predominant fungal isolates exhibited antimicrobial activity than the host plant metabolites. Zhuang et al. (2008) characterized anti-bacterial compounds from *Penicillium* sp. isolated from mangrove plant *Cerbera manghas*. They identified compounds 4-(3-hydroxybutan-2-yl)-3,6-dimethylbenzene-1,2-diol, and 3,4,5-trimethyl-1,2-benzenediol which possessed the inhibitory effects on Methicillin Resistant *Staphylococcus aureus* (MRSA) while 4-(3-hydroxybutan-2-yl)-3-acetyl-6-methylbenzene-1,2-diol had no effect on MRSA. Kjer et al. (2009) conducted antibiotic activity of the compounds from *Alternaria* sp., against multidrug-resistant bacteria. Two new compounds xanalteric acids I and II exhibited less bactericidal activity against *S. aureus* while, another compound altenusin exhibited broad spectrum activity against several bacteria like *Enterococcus faecium*, *Enterococcus cloacae*, *Streptococcus pneumonia* and *Pseudomonas aeruginosa* with an MIC between 31.5 and 125 μg/mL.

Fernandes et al. (2009) determined the MIC and minimum bactericidal concentration (MBC) for crude extract of *Alternaria alternata* endophyte obtained from *Coffea arabica* L. The ranges of MIC and MBC values were found to be 50–100 μg/mL for *S. aureus* and 400–800 μg/mL for *E. coli.* Kusari et al. (2009) conducted the antibacterial activity for Deoxypodophyllotoxin, isolated from endophytic fungus *Aspergillus fumigatus,* which revealed effective inhibition towards the pathogenic bacteria *S. aureus*, *K. pneumonia* and *P. aeruginosa***.** Lima et al. (2011) documented the 15 culture filtrates of endophytic fungi isolated from *Piper aduncum* L. inhibited the growth of *M. tuberculosis* by more than 90% as determined by qualitative and quantitative bioassays. Lv et al. (2010) studied the antimicrobial potential of endophytes isolated from *Saussurea involucrate*. Extracts isolated from the fermentation broth of 49 endophytic fungi were tested for their antimicrobial properties against pathogenic microorganisms *S. aureus*, *E. coli*, *B. subtilis, A. fumigatus, C. albicans* and *C. neoformans*. The study showed that at least one test microbe was inhibited by 12 fungi isolated. Among them, five strains were reported to possess broad spectrum antimicrobial activity. However, only four strains were effective against the tested fungal pathogens.

Nithya and Muthumary (2010) reported antibacterial compound from endophytic fungus *Phomopsis* sp. associated with *Plumeria acutifolia*. The compound was extracted with ethylacetate and identified as terpenoid by thin layer chromatography (TLC), UV, FT-IR spectroscopic analysis. The compound was found to be active against the growth of the bacterial pathogens *E. coli*, *Pseudomonas* sp., *Klebsilla* sp., *B. subtilis*, *S. aureus* and *S. typhi* whereas there was no significant effect on *C. albicans*. Similarly, antibacterial terpene compound was found in *Phomopsis* sp. isolated from the medicinal plant, *Allamanda cathartica.* Twenty

microlitres of ethyl acetate fraction of terpene compound revealed effective inhibitory activity against *E. coli*, *Pseudomonas* sp., *Klebsilla* sp., *S. aureus, S. typhi* and *B. subtilis* with the zone of inhibition ranging from 15 ± 0.30 to 25 ± 0.50 mm (Nithya and Muthumary, 2011). Tayung et al. (2011) isolated *Fusarium solani* from the bark of *Taxus baccata* which produced antibacterial compounds 1-tetradecene, 8-pentadecanone, 8-octadecanone, 10-nonadecanone and octylcyclohexane displaying considerable bactericidal activity against *S. epidermidis*, *S. aureus*, *S. flexneri, B. subtilis*, *E. coli* and *K. pneumonia*. Ho et al. (2012) examined the endophyes (*Lasmenia* sp., *Ophioceras tenuisporum*, *Xylaria cubensis* and *Cyanodermella* sp.) obtained from *Citrus* and *Zanthoxylum* of *Rutaceae* and *Cinnamomum* of *Lauraceae* against three phytopathogenic bacteria like *Erwinia carotovora, Xanthomonas campestris* and *Ralstonia solanacearum.* Among tested isolates, *Cyanodermella* sp. showed better inhibition of all the pathogens. In another study, Nath et al. (2012) showed that the crude extracts of *Xylaria* sp. and *Diaporthe* sp. with superior antimicrobial activity against clinical pathogens namely, *Salmonella paratyphi* and *Enteroccocus faecalis* (Nath et al. 2012). Subbulakshmi et al. (2012) tested antibacterial activity of endophytic fungi *Alternaria* spp., *Colletotrichum gloeosporioides*, *Pestalotiopsis* sp., *Fusarium* sp., *Pestalotiopsis* sp. isolated from the leaf samples of *Biota orientalis*, *Pinus excels* and *Thuja occidentalis.* Methanol extract of *Pestalotiopsis* sp. isolated from *Biota orientalis* had significant inhibition of growth of the bacteria *Streptococcus faecalis* and *Salmonella typhi*.

Senthilmurugan et al. (2013) reported the antibacterial activity of crude extract of endophytic fungus *Botrytis* sp. isolated from *Ficus benghalensis* which inhibited the growth of *Escherichia coli* and *Klebsiella* sp. Pinheiroa et al. (2013) conducted the antibacterial activity for two alkaloids, fumigaclavine C and pseurotin A produced by endophytic fungus *Aspergillus* sp. EJC08 associated with medicinal plant *Bauhinia guianensis*. The compound fumigaclavine C inhibited *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* with MIC at 62.50, 31.25, 15.62 and 7.81 µg/mL respectively. Whereas, pseurotin A inhibited *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* with MIC at 31.25, 31.25, 15.62 and 15.62 µg/mL respectively. Subban et al. (2013) reported antibacterial activity of the compound 4-(2,4,7-trioxabicyclo[4.1.0]-heptan-3-yl) extracted from *Pestalotiopsis mangiferae*, an endophyte of *Mangifera indica* Linn. The MIC of this compound for *B. subtilis* and *K. pneumoniae* was at 0.039 mg/mL, whereas for *E. coli* and *M. luteus* it was 1.25 mg/mL followed by *P. aeruginosa* at 5 mg/mL. de Felício et al. (2015) isolated 45 endophytic fungi from *Bostrychia tenella* (Ceramiales). The crude extracts of these fungi were evaluated for antifungal, antibacterial and cytotoxic activities and the results revealed that endophytes *Penicillium decaturense* and *P. waksmanii* had the highest bioactivities and for the first time they isolated cytochalasin D, a well-known antibiotic and antitumor compound. More recently, Mani et al. (2015) have isolated 169 endophytes from the medicinal plant, *Aegle marmelos.* The strains belonged to *Curvularia australiensis*, *Alternaria. alternate, Alternaria citrimacularis*, *Aspergillus. niger and Cladosporium cladosporioides*. The extracts of these fungi were effective against the clinical pathogens such as clinical pathogens *S. epidermidis*, *S. aureus*, *Shigella* sp., *P. aeruginosa*, *E. faecalis*, *E. coli*, *K. pnuemoniae*, *P. mirabilis* and *S. typhi.*

21.7.2 Antifungal Compounds

Endophytic fungi exhibit antagonism towards pathogenic fungal organisms. Krohn et al. (1999) reported herbarulide, a novel ketodivinyllactonic steroid from *Pleospora herbarum,* an endophyte. Lu et al. (2000) conducted antifungal activity for secondary metabolites isolated from the endophytic fungus *Colletotrichum* sp. of *Artemisia annua.* The metabolites 3b, 5a-dihydroxy-6b-phenylacetyloxy-ergosta-7,22-diene, 3-oxo-ergosta- 4,6,8(14),22-tetraene, 3b,5a-dihydroxy-6b-acetoxy-ergosta-7,22-diene and 3b-hydroxy-ergosta-5-ene effectively inhibited *Aspergillus niger* and *Candida albicans* with the MICs between 50 and 100 μg/mL. Another compound, 6-isoprenylindole-3-corboxylic acid showed inhibitory activity against fungal pathogens such as *Phytophthora capsici*, *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia cerealis*. Likewise, *Cryptosporiopsis* cf. *quercina* obtained from the stem explants of *Tripterygium wilfordii* produced cryptocin and this compound was found to effectively inhibit the growth of *Pyricularia oryzae* and other plant pathogens (Li et al. 2000).

Strobel et al. (2001) reported a novel endophyte *Muscodor albus* associated with *Cinnamomum zeylanicum* and extracted 5 classes of volatile compounds including alcohols, ketones, esters, acids and lipids that exhibited antifungal activity against the pathogens namely, *Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora cinnamomi*, *Stagnospora nodorum*, *Ustilago hordei*, *Sclerotinia sclerotiorum*, *Fusarium solani*, *Aspergillus fumigates*, *Verticillum dahlia*, *Tapesia yallundae*, *Cercospora beticola* and *Candida albicans.* Harper et al. (2003) isolated pestacin from endophytic fungus *Pestalotiopsis microspora* exihibited moderate antifungal activity for root-invading pathogen *Pythium ultimum*. Liu et al. (2004) isolated 12 compounds from endophytic fungus *Aspergillus fumigatus* CY018 of *Cynodon dactylon*. These compounds showed antifungal activity against human pathogens *C. albicans*, *A. niger* and *T. rubrum*. The compounds, asperfumoid, fumitremorgin C, fumigaclavine C, helvolic acid and physcion showed inhibitory activity against *C. albicans* with MICs of 75.0, 62.5, 31.5, 31.5 and 125 μg/mL respectively and no significant activity was found against *A. niger* and *T. rubrum*. However, other compounds were observed with no antimicrobial effect.

Silva et al. (2006) evaluated antifungal activity of five compounds derived from *Phomopsis cassia*e associated with *Cassia spectabilis*. Among them, the metabolite 3,12-dihydroxycadalene exhibited activity against phytopathogens *Cladosporium cladosporioids* and *C. sphaerospermum*. Kjer et al. (2009) isolated altenusin metabolite from endophytic fungus *Alternaria* sp., which showed broad spectrum activity against multidrug-resistant fungi *Aspergillus faecalis* and *Candida albicans*, with an MIC 125 and 62.5 μg/mL respectively. Oliveira et al. (2010) reported dihydroisocoumarin (3R,4R)-3,4-dihydro-4,6-dihydroxy-3-methyl-1-oxo-1H-isochromene-5-car- boxylic acid from the endophyte, *Xylaria* sp. associated with *Piper aduncum.* The compound showed moderate antifungal activity against *Cladosporium cladosporioides and C. sphaerospermum.* In addition, they also isolated two known compounds, (3R,4R)-4,7-dihydroxymellein and (R)-7-hydroxymellein from *Penicillium*

sp. associated with *Alibertia macrophylla*. These compounds effectively inhibited *C. cladosporioides and C. sphaerospermum.* From the same plant, Gubiani et al. (2014) isolated two novel eremophilane-type sesquiterpenes (xylarenones F and G) which showed potent anti-inflammatory properties.

Tayung et al. (2011) isolated and identified antifungal compounds from *Fusarium solani* associated with the bark of *Taxus baccata*; 1-tetradecene, 8-octadecanone, 8-pentadecanone, octylcyclohexane and 10-nonadecanone, which displayed activity against *C. albicans* and *C. tropicalis*. Sugijanto et al. (2011) determined the fungicidal activity of lecythomycin extracted and purified from the endophytic fungus *Lecythophora* sp.. The results showed activity against the fungal strains such as *Aspergillus fumigatus* and *Candida kruzei* with MIC of 62.5-125 mg/mL. Nath et al. (2012) study revealed the crude extracts of *Xylaria* sp. and *Phomopsis* sp. inhibited the growth of *C. albicans.* Subbulakshmi et al. (2012) tested fungicidal activity of endophytic fungi *Alternaria* spp., *Colletotrichum gloeosporioides*, *Pestalotiopsis* sp., *Fusarium* sp., *Pestalotiopsis* sp. obtained from the leaf samples of *Biota orientalis*, *Pinus excels* and *Thuja occidentalis.* Methanol extract of *Pestalotiopsis* sp. isolated from *Biota orientalis* had higher antifungal property against *C. albicans and Beauveria bassiana.*

Santiago et al. (2012) isolated 5-hydroxyramulosin, a polyketide compound from the culture filtrate of *Phoma* sp., an endophyte of the medicinal plant, *Cinnamomum mollissimum*. The extracted compound was reported to exhibit higher activity against *Aspergillus niger*. Ho et al. (2012) isolated endophytic fungi *Lasmenia* sp., *Ophioceras tenuisporum*, *Xylaria cubensis* and *Cyanodermella* sp. from *Citrus* and *Zanthoxylum* of *Rutaceae* and *Cinnamomum* of *Lauraceae* which showed different level of antagonistic effects against phytopathogens like *Alternaria solani*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Colletotrichum higginsianum*, *Cylindrocladiella lageniformis*, *Fusarium oxysporum*, *Monilinia fructicola*, *Penicillium digitatum*, *Pestalotiopsis psidii*, *Pythium aphanidermatum*. Among these endophytic fungi *Cyanodermella* sp. was extremely antagonistic to the fungal pathogens *Cynlidocladiella lageniformis*, *Fusarium oxysporum* and *Monilinia fructicola.* Gherbawy and Gashgari (2013) tested antifungal activity of 33 endophytes isolated from the leaves of *Calotropis procera*. All the isolates were tested against four plant pathogens namely, *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum* and *Pythium ultimum*. Among the isolates, *Chaetomium globosum* and *Myrothecium verrucaria* exhibited superior activity against the pathogenic fungal strains. Wu et al. (2013) evaluated the fungicidal property of the isolated steroid compounds of *Phomopsis* sp. associated with the plant, *Aconitum carmichaeli.* The identified compounds 6-ethoxy-5,15-dihydroxyergosta-7,22-dien-3-one, 9,14-dihy droxyergosta-4,7,22-triene-3,6-dione, ganodermaside D and calvasterols possessed moderate or weak antifungal activities. Subban et al. (2013) reported antifungal activity of 4-(2,4,7-trioxa-bicyclo[4.1.0]-heptan-3-yl), a phenolic compound isolated from an endophytic fungus *Pestalotiopsis mangiferae* associated with *Mangifera indica* Linn. The MIC of the compound for *C. albicans* was found at 0.039 mg/mL.

21.7.3 Anticancer Compounds

Cancer is one of the major causes of health hazard all over the world. The identification of secondary metabolites with cytotoxicity improved the development of new approach for anticancer therapy for several decades. Endophytes are able to produce novel metabolites that exhibit anticancer activities (Strobel and Daisy 2003). For the first time, Stierle et al. (1993) obtained Paclitaxel, an anticancer agent from *Taxomyces andreanae* (endophytic fungus) isolated from the Yew tree (*Taxus brevifolia*). Paclitaxel, a highly functionalized diterpenoid which is found in many species of *Taxus* (Strobel et al. 1993; Suffness 1995). Zhang et al. (2000) reported anticancer compound vincristine from endophytic fungus *Fusarium oxysparum* isolated from the phloem (inner bark) of *C. roseus* L. Metabolic products of cultured fungus were analysed by TLC and HPLC. Similarly, Yang et al. (2004) selected the endophytic fungi which produced vincristine by isolating fungi from the leaves of *Catharanthus roseus* (L.) G. Don. An endophytic fungus, *Mycelia sterilia* 97CY was isolated from the leaves of *Cantharanthus roseus* and the zymotic extracts were analyzed by TLC and HPLC found an anticancer agent vincristine. Puri et al. (2005) reported an anticancer compound camptothecin from an endophytic fungus *Entrophospora infrequens* obtained from *Nothapodytes foetida*. Silva et al. (2006) determined the antiproliferative activity of five compounds identified from *Phomopsis cassia*e isolated from *Cassia spectabilis* against the HeLa cervical cancer cells, the metabolite 3,12-dihydroxycadalene inhibited at an IC_{50} of 20 μM/L and 3,12-dihydroxycalamenene and 3,11,12-trihydroxycadalene showed weakly inhibition. Similarly, Teles et al. (2006) extracted secondary metabolites from *Periconia atropurpurea*, an endophyte isolated from *Xylopia aromatica*, using ethyl acetate. Before identifying, these metabolites were tested for their biological activity and proved the cytotoxic activity of the compounds.

Phongpaichit et al. (2007) screened the antiproliferation and cytotoxicity of 65 crude extracts of 51 chosen endophytes of *Garcinia* species (5 species from *Garcinia atroviridis*, 23 from *G. dulcis*, 6 from *G. nigrolineata*, 16 from *G. mangostana*, and 1 from *G. scortechinii*). Among them 11.1% of extracts showed the activity against the proliferation of NCI-H187 cells and 12.7% against KB cells. Forty percent of extract showed cytotoxicity against normal Vero cell lines. Gangadevi and Muthumary (2008) isolated endophytes from different plants and screened their anticancer properties. Taxol is one of the widely studied fungal metabolite successfully used in the treatment of cancer. The fungus *Colletotrichum gloeosporioides* (strain JGC-9) isolated from *Justicia gendarussa,* produced 163.4 μg/L of taxol which showed higher cytotoxicity towards the human cancer cell lines BT 220, Int 407, H116, HLK 210 and HL 251. They also observed the dose dependent cytotoxicity of the extracted taxol suggesting the potential of this fungus in producing taxol in vitro. Ge et al. (2009) reported cytotoxic alkaloids from *Aspergillus fumigatus* associated with the stems of *Cynodon dactylon*. Compounds 9-deacetylfumigaclavine C and 9-deacetoxyfumigaclavine C revealed cytotoxicity against K562 cells (leukemia cancer cell line) with IC_{50} values of 41.0 ± 4.6 and 3.1 ± 0.9 μM respectively. The compound 9-deacetoxyfumigaclavine C exhibited similar activity as that of

doxorubicin hydrochloride (1.2 ± 0.2 μM), an approved drug to treat leukemia. The metabolite 14-norpseurotin considerably induced neurite outgrowth of rat pheochromocytoma cells (PC12) at a concentration of 10 μM and observed to show higher activity compared to the other natural drug, pseurotin A. Secalonic acid D, a mycotoxin (ergochrome class) extracted from the mangrove endophyte was reported to have high cell toxicity against K562 and HL60 leukemia cells. It was shown to induce toxicity through apoptosis (Zhang et al. 2009). Nithya and Muthumary (2009) characterized an anticancer compound using UV, thin layer chromatography, and Fourier transform infrared spectroscopy (FTIR) analysis revealed the presence of taxol from culture filtrate of endophytic fungus *Colletotrichum gloeosporioides* associated with *Plumeria acutifolia.* Similarly, Srinivasan and Muthumary (2009) reported that the endophytic fungus *Pestalotiopsis* sp. associated with *Catharanthus roseus* was able to produce taxol.

Fernandes et al. (2009) isolated *Alternaria alternate*, an endophyte from *Coffea arabica* L. and its crude extracts was determined with antitumor activity. The cytotoxic activity against HeLa cells showed IC_{50} at 400 μg/mL. Likewise, Kjer et al. (2009) reported that xanalteric acids I and II of endophytic fungus *Alternaria* sp., exhibiting higher growth inhibition against L5178Y cells at a concentration 10 μg/mL. In another report by Deshmukh et al. (2009), a novel anticancer agent (ergoflavin) was isolated from *Claviceps purpurea* (PM0651480) associated with *Mimusops elengi* (*Sapotaceae*). Zhou et al. (2009) reported anticancer compound taxol from *Mucor* sp. an endophytic fungus associated with *Taxus chinensis*. Shweta et al. (2010) found camptothecin derivatives from endophytic fungal strains of *Fusarium solani* MTCC 9667 and MTCC 9668. The camptothecin derivatives identified as 9-methoxycamptothecin and 10-hydroxycamptothecin. Pandi et al. (2011) also reported Taxol from endophytic fungus *Lasiodiplodia theobromae* isolated from the medicinal plant *Morinda citrifolia* and revealed the cytotoxic effect of fungal taxol on MCF-7 cells with an IC_{50} at 300 μg/mL.

An active metabolite, Sclerotiorin isolated from *Cephalotheca faveolata* was shown to induce apoptosis in cancer cells (Giridharan et al. 2012). Sclerotiorin was found to be a potent anti-proliferative agent against different cancer cells. It induced apoptosis in colon cancer (HCT-116) cells via BAX and inhibition of BCL-2 proteins those further activated cleaved caspase-3 enzyme causing apoptosis of cancer cells. Santiago et al. (2012) isolated 5-hydroxyramulosin (a polyketide compound) from the culture filtrate of *Phoma* sp. obtained from the plant, *Cinnamomum mollissimum*. The compound showed cytotoxicity against P388 murine leukemic cells with IC_{50} value 2.10 μg/mL. Likewise, Lu et al. (2012) reported that the extracts of endophytic fungi from *Actinidia macrosperma* with cytotoxic and antitumor activities against brine shrimp and five types of cancer cell lines. Cytotoxic activity was found in most of the isolates and also comparatively higher toxicity against brine shrimp. From the MTT assay, it was found that about 82.4% of fungal isolates revealed growth inhibitory activity against cancer cells (50% inhibitory concentration IC50 <100 μg/mL). Some of the fungal isolates showed strong antitumor activity against all cancer cell lines tested suggesting the role of endophytic fungi as a novel metabolite against cancer. Sun et al. (2013) evaluated cytotoxicity of seven

terpenoid compounds arisugacins B, F, G, I, J, territrem B and territrem C isolated from an endophytic fungus *Penicillium* sp. SXH-65 against Hela, HL-60 and K562 cell lines. Compound arisugacin B and F revealed the cytotoxicity at IC_{50} values ranging from 24 to 60 μM.

21.8 Conclusion

Endophytic fungi are an excellent source of various bioactive natural compounds and possess considerably effective biological properties. Thus, utilization of these microbes can certainly benefit the current demand for novel lead molecules by medical, pharmaceutical and agriculture industries. However, large scale production of these metabolites in is a major challenge to the scientific world. Also, detailed understanding of the plant-fungal symbiosis may reveal possible pathways involved in their relationship as well the synthesis of secondary metabolites. Hence, application of fungal biotechnology is a need of the future research to reveal the involvement of genetic control mechanisms that exists to control the production of secondary metabolites. Also, research should emphasize on detailed understanding of physiology, defensive roles and biochemical pathways involved in secondary metabolite secretion by endophytic fungi.

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Chapter 22
Fungal Endophytes from Seaweeds: An Overview

Vipin Kumar Singh, Abhishek Kumar Dwivedy, Akanksha Singh, Simran Asawa, Awanindra Dwivedi, and Nawal Kishore Dubey

22.1 Introduction

22.1.1 Seaweeds

Seaweeds are one of the major producers of marine ecosystem, found almost in all part of the coastal regions around the globe. They are defined as evolutionarily diverse assemblages of marine photosynthetic, non-vascular macro-algal forms, inhabiting the littoral zone in sea, which vary in their color, shape and size. The size of marine algal forms may be very small (few mm) or upto several centimeters (Coppejans et al. 2009). Seaweeds fall under three different categories including green (chlorophyceae), red (rhodophyceae), and brown (phaeophyceae). The characteristic color of seaweeds are due to different pigmentation. Major pigments of green forms are chlorophyll *a*, chlorophyll *b*, $\alpha-$, $\beta-$, and $\gamma-$ carotene, and siphonoxanthin; brown seaweeds possess pigments such as chlorophyll *a*, c*1*, c*2*, fucoxanthin and β carotene. Important pigments harbored by red seaweeds are $\alpha-$, $\beta-$ carotene, allophycocyanin, c−phycoerythrin, chlorophyll a, allophycocyanin and r−phycocyanin. Some Asian countries, especially China and Japan utilize seaweed as important part of their dietary materials. However, people of western world are exploiting seaweed extract for application in food and cosmetics industry. Seaweed preparations can be used as raw, dried or cooked form. Although,

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seaweeds have very low calorie value, they are valuable source of important minerals, vitamins, proteins, trace elements, oils, antioxidants and fats (Mouritsen 2013). Earlier, seaweeds were utilized as gelling substance and stabilizers in food and drug industry, but, presently, therapeutic roles of seaweed derived compounds as anticancer, anticoagulant, antihyperlipidemic, immunomodulatory, thyroid stimulating, antiestrogenic, antibacterial, antiviral, antiobesity and antifungal agent have been demonstrated in disease management (Mohamed et al. 2012). So far, several bioactive compounds including sulfated polysaccharides, carotenoids, peptides, phlorotannins and sulfolipids from various seaweeds have been isolated and applied for disease treatment.

22.1.2 Fungal Endophytes

Microorganisms inhabiting within plants are continuously becoming the interest of microbiologists particularly, when the host plants are source of disease curing drug molecules. Very large group of microorganisms acting either as symbiont or pathogen are known to colonize the plants. Among them, endophytes represent a broad group comprised of actinomycetes, bacteria and fungi colonizing several host plant during their life without expressing any visible symptom (Stone et al. 2000; Hyde and Soytong 2008). Wilson (1995) briefly described the term "endophyte" as any organism living within plant (Gr. *Endon*, within; *phyton*, plant) without manifestation of disease symptom. However, not all endophytes are pathogenic in nature but, some bacterial or fungal pathogens causing plant diseases may have endophytic origin. Currently, at many places, the term endophyte is being used as synonym of mutualist (Mani et al. 2015). Across the globe, fungal endophytes are found in all climatic conditions and have been reported from almost every plant family including bryophytes, pteridophytes, gymnosperms and angiosperms (Zhang et al. 2006; Golinska et al. 2015; Santoyo et al. 2016). In nature, fungal endophytes may represent themselves as obligate or facultative organisms (Hardoim et al. 2008). Research is still needed to understand the enigmatic coexisting behavior of plant and endophyte and why plant defense systems do not work out to eliminate the colonizing endophytes. The probable mechanism of coexistence is due to mutual benefits to both associated partners. Plants get advantage in terms of growth promotion (Alvin et al. 2014; Santoyo et al. 2016), prevention from pathogen attack, stress tolerance (Larran et al. 2016) and enhancement in nutrient assimilation while, endophytes derive nutrients from host plant (Wilson 1995). Their entry inside the host tissue may be either through natural opening like stomata or wounds created by secretions of specific degradative enzymes such as cellulases (Zhang et al. 2006).

Plants associated with endophyes have been reported to perform better than that of without endophytes (Zhang et al. 2006) which is probably due to secretion of bioactive compounds by endophytes that facilitate the plant growth and protects from damages arising from pathogen attack (Golinska et al. 2015; Abdalla and

Matasyoh 2014). Metabolic compounds released by endophytes are expected to induce plant defense system and activate plant growth machinery under diseased condition to maintain the cellular loss caused by apoptosis (Alvin et al. 2014). In addition, endophytes are source of several compounds having novel properties. The large scale production of endophyte derived secondary metabolites may gain importance in agriculture and pharmaceutical industries (Golinska et al. 2015) due to their antibacterial, antifungal, anticancerous, immunosuppressive, and antioxidative nature (Zhang et al. 2006). Endophyte association has recently been recognized to minimize the chances of disease development in host plants, thus their application as biocontrol agent can successfully diminish the use of chemical herbicides, and pesticides (Larran et al. 2016) to restrict the phenomenon of food chain bioaccumulation as well as biomagnification.

Various experimental studies and observations suggest that almost all plants under natural environmental conditions are in symbiotic relationship with very diverse group of mycorrhizal or endophytic fungi (Petrini 1986). Fungal endophytes are known to exert strong impact on plant's adaptibility, community structure, evolution (Brundrett 2006) and variety of organisms' associated (Omacini et al. 2001). Palaentological evidences have demonstrated the association of plants with endophytes (Krings et al. 2007) and mycorrhizae (Redecker et al. 2000) from more than 400 million years ago, thus exhibiting the implication of endophytic fungi in origin and evolution of terrestrial life. Although, mycorrhizal fungi are only associated with rhizosphere of higher plants, endophytic fungi colonize within roots, stem and leaves of associated plants.

Endophytic fungi have been categorized by different workers into two major groups i.e. claviciptaceous endophytes (C-endophytes) residing within few grass tissues and non clavicipitaceous endophytes (NC-endophytes) showing symbiotic association with bryophytes, pteridophytes, gymnosperms and angiosperms. C-endophyte denotes small group of evolutionarily associated fungi limited to few grass species. Commonly, they are found within shoot tissues and spread by systemic infection mechanism. Clay and Schardl (2002) classified C-endophytes in three types. Type I includes those species of fungal endophytes which produce symptoms and are pathogenic in nature. However, Type II and III includes fungi displaying the characteristics of mixed and symptomless association, respectively.

22.2 Geographical Distribution of Seaweeds Harbouring Fungal Endophytes

Ample work has been carried out regarding collection of group of macro-algal host as well as single algal host for assessing their fungal endophytic diversity. However, little studies have been made with respect to seaweed screening for potential endophytic fungi. From Tamilnadu coast in India, diversity of fungal endophytes residing within marine macroalgae has been well elucidated by Suryanarayanan et al.

(2010). Later works on isolation and identification of fungal endophytes from the marine habitats of different parts of world including Malaysia (Ariffin et al. 2011), Atlantic coast Canada (Flewelling et al. 2013a, b) and North Atlantic region, United Kingdom (Flewelling et al. 2013a, b) were also reported. Approximately, 100 endophytic fungi from seaweeds have been reported by different workers from different regions of the world, 75% of which are known to occur in Baltic Sea, Canada, China, India, North Sea and United Kingdom. Out of 100 seaweeds collected, 41 host species belong to red algae followed by 32 species of brown algae and 19 species of green algae. In an estimate by Guiry (2012), the total number of algae existing on earth is 72,500, out of which 7000 are red algae, 8000 are green algae and 2000 brown algae, of them only nearly 100 have been reported to act as host for fungal endophyte which necessitates the further investigation of newer host for endophytes which can be explored for novel bioactive compounds. Many fungal endophytes associated with variety of algal hosts are still unidentified. Suryanarayanan et al. (2010) described the fungal endophytes of eleven brown algae, six green algae and eight red algae from Tamilnadu coast, India. Members of phaeophyceae favored the higher diversity of endophytes as compared to chlorophyceae. Ten different seaweeds from Mandapam and Pondicherry coastal region of India, have been tested for production of natural bioactive metabolites from endophytic fungi (Mathan et al. 2013). Total 156 endophytic fungal isolates were reported to inhabit the seaweed. Among collected algae, highest isolation frequency was represented by *Codium species* (80%) followed by *Ulva fasicata* (10%). Nineteen strains of fungal endophytes were screened for their potential activities in terms of antibacterial properties against the human and fish pathogenic bacteria. Only six fungal strains exhibited better effectiveness towards pathogenic bacteria which is expected to be due to presence of active secondary metabolites. Type and quantity of bioactive molecules synthesized by an endophyte depends on the host organisms and environmental factor. Based on detailed literature survey, Sarasan et al. (2017) concluded that dominance of bioactive molecules derived from different endophytic fungi inhabiting within marine algae were found in the order of brown algae (39%) > red algae (28%) > green algae (23%). Poor diversity of fungal endosymbionts and associated bioactive molecules in members of chlorophyceae has been suggested due to short life span of green algae as well as slow growth rate of associated endophyte (Zuccaro and Mitchell 2005).

22.3 Isolation of Endphytic Fungi from Seaweeds

Surface sterilization is the most comon technique for the isolation of endophytic fungi from seaweeds. For this the samples should be rinsed several times with sterile distilled water followed by surface sterilization with 10% sodium hypochlorite or 70% ethyl alcohol for few seconds. Samples should again be washed with sterile distilled water and then small pieces should be transferred onto isolation medium containing choramphenicol for specific period aseptically (Wang et al. 2006; Ariffin

et al. 2014). The treatment time is important for sterilization and may vary from species to species. For microalgae, treatment time of 10–20 s is sufficient while it may be raised to 60–120 s for macroalgae. Reduction in treatment time may result into incomplete surface sterilization whereas treatment more than the optimum time may lead to death of associated endophytic microorganism (Kjer et al. 2010). So every precaution should be practiced while performing the endophyte isolation. There are chances of existence of epiphytic fungi during isolation of endophyte due to incomplete sterilization which can be ascertained by taking imprint of surface sterilized seaweeds on solid agar medium. No growth on imprinted portions of media indicate complete surface sterilization. Fungal cells growing out of seaweed is then aseptically transferred onto separate solid agar medium for obtaining pure culture.

22.4 Seaweed Fungal Endophytes and Bioactive Secondary Metabolite Production

Seaweed fungal endophytes are untapped source of novel bioactive secondary metabolites. Moreover, the search of new sources of bioactive secondary metabolites from endophytes of marine alga would relieve the pressure of overexploitation of terrestrial hosts. In contrast, reports are available indicating a lot of differences in the chemical composition of metabolites synthesized by a particular endophytic fungus isolated from terrestrial and marine sources. The same host from different geographical locations has also been shown to represent different fungal species, indicating the heterogeneity in association. The variation in fungus is reported to produce quite different secondary metabolite under different habitat indicating the impact of environmental conditions.

Zhang et al. (2007) have reported the isolation of endophytic fungus *Aspergillus niger* EN-13 from brown seaweed *Colpomenia sinuosa*. The endophyte was able to produce the antifungal compound naphthoquinoneimine derivative i.e. 5,7-dihydroxy-2-[1-(4-methoxy-6-oxo-6H-pyran-2-yl)-2-phenylethylamino]-[1,4]naphthoquinone as revealed by 2D NMR techniques, EI-MS, and HR-ESI-MS. Hulikere et al. (2016) reported the isolation of endophyte *Cladosporium cladosporoides* from seaweed *Sargassum wightii*. The isolated fungus was identified by its morphological and molecular features. The fungal extract was tested for antioxidant property, reducing power, total phenolic content, flavonoid content, and antiangiogenic activity. Ethyl acetate extract of fungal metabolites when subjected to LC-MS analysis disclosed the mixture of different phenolic compounds responsible for the activities. de Felício et al. (2015) investigated the antibacterial, cytotoxic and antifungal activity of crude and organic solvent extract of some selected endophytic fungi. Fungi identified on the basis of molecular characteristics were *Acremonium implicatum*, *Xylaria* sp., *Trichoderma atroviride*, *Nigrospora oryzae*, *Penicillium decaturense* and *Penicillium waksmanii* which were isolated from seaweed *Bostrychia tenella*.

They firstly demonstrated the antitumorigenic compound cytochalasin D from marine fungi *Xyalaria* sp. showing potential application in pharmaceutical industry. Li et al. (2017) isolated and evaluated the antimicrobial, antioxidant and cytotoxic activity of five different polyhydroxylated hydroanthraquinone derivatives from culture extract of endophytic fungus *Talaromyces islandicus* EN-501 inhabiting within tissues of rhodophyte *Laurencia okamurai*. Structural details of compounds were elucidated by analytical techniques like NMR, ECD and XRD. Endophytic fungi, *Aspergillus wentii* EN-48 was isolated from seaweed *Sargassum* (Sun et al. 2012). Molecular identification of fungus was carried out on the basis of PCR based DNA amplification and sequencing of ITS region. Three different tetranorlabdane diterpenoids along with five analogous compounds were recovered from acetone and ethyl acetate extract of the fungus and culture broth, respectively. Structural details of compounds recovered after chromatographic techniques were revealed by spectroscopic and crystallographic investigations. The external appearances of different recovered compound were colorless, needle shaped, crystal or prism like. Cytotoxicity experiments were performed against a variety of cancer cell lines. Compound 6 (Wentilactone B) (IC_{50} = 17 μM) was noticed as most effective one amongst all. Antimicrobial assays of purified compounds were performed against pathogenic bacterial and fungal strains. Compound 4 (tetranorditerpenoid derivative) was found to exhibited potent antifungal activity against *Candida albicans* having MIC value of 16 μg/ml.

Increasing number of human health problems have promoted the researchers from different part of world to look for innovative metabolites especially from marine sources because of their unique metabolic activities. Feng-Wu (2012) reported the isolation and purification of seven different compounds from ethyl acetate extract of endophytic fungi *Guignardia* sp., inhabiting within the edible marine alga *Undaria pinnatifida*. Structural elucidations of compounds were performed by infra red (IR), nuclear magnetic resonance (NMR) as well as mass spectroscopy (MS). The compounds identified were ergosterol, ergosterol epoxide, and cyclic dipeptides including cyclo-(Tyr-Leu), cyclo-(Phe-Phe), cyclo-(Val-Leu), cyclo-(Phe-Pro) and cyclo-(Leu-Ile). Significant cytotoxic and antifungal activity was exhibited by first and sixth compound. The green seaweed *Ulva pertusa* has been reported as the host of fungal endophyte *Chaetomium globosum* QEN-14 which have been explored for the isolation of cytochalasan derivative, cytoglobosins A-G (Cui et al. 2010a). Among the isolated compounds tested for cytotoxic activity against different cancer cell lines, Cytoglobosins C and D manifested better results in suppressing the activity of cancer cell line A549, having IC_{50} values 2.26 and 2.55 μM, respectively. Rest of the compounds possessed very poor activity with IC_{50} value higher than 10 μM. The endophyte *Aspergillus ochraceus* recovered from brown seaweed *Sargassum kjellmanianum* was found as the novel source of 7-nor-ergosteroid bearing pentalactone B-ring i.e. 7-nor-ergosterolide and two steroidal derivative 3β,11α-dihydroxyergosta-8,24(28)-dien-7-one and 3β-hydroxyergosta-8,24(28)-dien-7-one (Cui et al. 2010b). Structural details of compound were established by Mosher's methodology. Isolated compounds were screened for their potential cytotoxic activity against cancer cell lines NCI-H460, SMMC-7721,

SW1990, DU145, HepG2, Hela, and MCF-7. 7-nor-ergosterolide displayed cytotoxic activity for the cancer lines NCI-H460, SMMC-7721, and SW1990 with IC_{50} values of 12.0, 16.9, and 67.6 μM, respectively while 3β,11α-dihydroxyergosta-8,24(28)-dien-7-one exhibited very weak activity (IC_{50} = 65.4 μM) against SMMC-7721 cancer cell line. Interestingly, none of the isolated compound presented effective antimicrobial efficacy when tested against *Staphylococcus aureus*, *Escherichia coli*, and *Aspergillus niger*.

The red alga *Ceramium* sp. collected from North Sea, Busum, Germany, has been described to host the fungal endophyte *Phaeosphaeria spartinae* as a potential source of Spartinoxide, 4-hydroxy-3-prenyl-benzoic acid and anofinic acid (Elsebai et al. 2010). The structural details were determined with the help of spectroscopic analysis. When efficacy of obtained compound was evaluated against different enzymes including human leukocyte elastase (HLE), acetylcholinesterase trypsin, and cholesterolesterase, Spartinoxide and 4-hydroxy-3-prenyl-benzoic acid established as the potent inhibitor of HLE having IC_{50} values 6.5 and 8.1 μM, respectively. The inhibitors of HLE activity can be used to relieve the patients suffering from pulmonary emphysema, rheumatoid arthritis, and cystic fibrosis. The fungus *Chaetomium* is known for important secondary bioactive metabolites such as chaetoglobosins, chaetoquadrins, chaetosporins, and orsellides. Fungal endosymbiont *Chaetomium species* inhabiting within marine algae as a source of novel polyketide, Chaetocyclinone A, B, and C have been demonstrated by Lösgen et al. (2007). The isolated fungus was cultured in a medium based on malt extract, glucose and yeast extract for the purpose of active compound biosynthesis. Culture filtrate was extracted with ethyl acetate at pH 5.0 and dried to obtain crude extract. Further purifications were performed by the column chromatography technique using Sephadex LH-20 and RP 18 silica gel. Structural details of compound isolated were obtained from IR, NMR and MS studies. With reference to biological activity, Chaetocyclinone A was found to have profound antifungal activity against *Phytophthora infestans* while rest of the compound was observed to devoid of any kind of antibacterial and antifungal activity. Furthermore, isolated compounds had no cytotoxic effect on cancer cell lines of stomach, liver and breast. Pontius et al. (2008) isolated the dimeric xanthone derivative, Monodictyochromes A and B from the endophyte *Monodictys putredinis* residing within the tissue of an unidentified marine green alga. Successful separation of compound was achieved by vacuum liquid chromatography (VLC) and HPLC. Both the compounds were assessed for cancer chemopreventive property with respect to their inhibitory activity against cytochrome P_{450} 1A and determined to have their IC_{50} values 5.3 and 7.5 μM, respectively. Isolated compounds differed in terms of inhibiting the activity of aromatase with their IC_{50} values 24.4 and 16.5 μM, respectively. Enhancement of NAD(P)H:quinone reductase (QR) activity in Hepa 1c1c7 murine liver cancer cell line was observed after treatment with Monodictyochromes A and B. Marine fungi derived bioactive molecules are of immense pharmaceutical importance. A large number of newly reported secondary metabolites accounting for about 33% have been obtained from marine algicolous fungi. Search for new compounds from marine sources led to the isolation of Citrinal A, along with another two compounds citrinin and

2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran from *Penicillium* sp. i-1-1 growing endophytically within *Blindia minima*, a seaweed of family ulvaceae. Analysis of crude extract of the fungus after separation with ethyl acetate using the techniques of column chromatography and reverse phase HPLC resulted into the identification of tricyclic compound Citrinal A. Cytotoxicity experiment revealed the potential inhibitory action of Citrinal A and Citrinin against cell line A-549 and HL-60 with their IC_{50} values 80.7, 43.5 μmol/L and 143.1, 62.5 μmol/L, respectively. The third compound had very weak activity ($IC_{50} > 200$ μmol/L) against the both cell lines tested. Some of the important secondary metabolites along with endophytic fungi and their marine algal host are represented in following Table 22.1.

Table 22.1 Seaweed and associated endophytic fungi secreting important metabolites

Seaweed	Endophytic fungi	Chemical compound	References
Colpomenia sinuosa	*Aspergillus niger* EN-13	5,7-dihydroxy-2-[1-(4-methoxy-6-oxo-6H-pyran-2-yl)-2-phenylethylamino]-[1,4] naphthoquinone	Zhang et al. (2007)
Sargassum wightii	*Cladosporium cladosporoides*	Mixture of phenolics	Hulikere et al. (2016)
Bostrychia tenella	*Acremonium implicatum*, *Xylaria* sp., *Trichoderma atroviride*, *Nigrospora oryzae*, *Penicillium decaturense* and *Penicillium waksmanii, Xylaria* sp.	Cytochalasin D from *Xylaria* sp.	de Felício et al. (2015)
Laurencia okamurai	*Talaromyces islandicus* EN-501	Polyhydroxylated hydroanthraquinone derivatives	Li et al. (2017)
Sargassum species	*Aspergillus wentii* EN-48	Tetranorlabdane diterpenoids and related compounds	Sun et al. (2012)
Undaria pinnatifida	*Guignardia* sp.	Ergosterol and cyclic dipetides	Feng-Wu (2012)
Ulva pertusa	*Chaetomium globosum*	Cytoglobosins (A-G)	Cui et al. (2010a)
Sargassum kjellmanianum	*Aspergillus ochraceus*	7-nor-ergosterolide, 3β,11α-dihydroxyergosta-8,24(28)-dien-7-one, 3β-hydroxyergosta-8,24(28)-dien-7-one	Cui et al. (2010b)
Ceramium species	*Phaeosphaeria spartinae*	Spartinoxide, 4-hydroxy-3-prenyl-benzoic acid and anofinic acid	Elsebai et al. (2010)
Marine algae (Not specified)	*Chaetomium*	Chaetocyclinones	Lösgen et al. (2007)
Marine green algae (Not specified)	*Monodictys putredinis*	Monodictyochromes A and B	Pontius et al. (2008)

(continued)

Table 22.1 (continued)

Seaweed	Endophytic fungi	Chemical compound	References
Blidingia minima	*Penicillium* sp.	Citrinal A	Zhu et al. (2009)
Fucus vesiculosus	*Epicoccum* sp.	Isobenzofuranone derivative	Abdel-Lateff et al. (2003)
Marine red alga	*Paecilomyces variotii* EN-291	Varioxepine A	Zhang et al. (2014)
Sargassum thunbergii	*Eurotium cristatum*	Cristatumins (A-D), Neoechinulin A, Isoechinulin A, Variecolorin G, Preechinulin, Tardioxopiperazine A and Echinulin	Du et al. (2012)
Sargassum thunbergii	*Eurotium cristatum* EN-220	*N*-(4′-hydroxyprenyl)-cyclo(alanyltryptophyl),	Du et al. (2017)
		Isovariecolorin I,	
		30-Hydroxyechinulin,	
		29-Hydroxyechinulin,	
		Rubrumline M	
Laurencia okamurai	*Talaromyces islandicus* EN-501	2,2′,3,5′-Tetrahydroxy-3′methylbenzophenone,	Li et al. (2016)
		2,2′,5′-Trihydroxy-3-methoxy-3′ methylbenzophenone,	
		1,4,7-Trihydroxy-6-methylxanthone,	
		1,4,5-Trihydroxy-2-methylxanthone	
Grateloupia turuturu	*Paecilomyces variotii* EN-291	Butyrolactone IX and Aspulvinone O	Zhang et al. (2015a, b)
Grateloupia turuturu	*Paecilomyces variotii* EN-291	Varioloid A	Zhang et al. (2016a, b)
Brown alga-endophytic strain (cf-27)	*Trichoderma citrinoviride*	Citrinovirin,	Liang et al. (2016)
		Cyclonerodiol,	
		3-(2-Hydroxypropyl)-4-(hexa-2*E*,4*E*-dien-6-yl) furan-2(5*H*)-one,	
		5-Hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone	
Grateloupia turuturu	*Paecilomyces variotii* EN-291	Dihydrocarneamide A, *Iso*-notoamide B	Zhang et al. (2015a, b)
Pterocladiella tenuis	*Penicillium chermesinum* EN-480	Chermesins A−D	Liu et al. (2016)
Pterocladiella capillacea	*Chondrostereum* sp. NTOU4196	Chondroterpenes A–H	Hsiao et al. (2017)

22.5 Diversity of Molecules Produced by Seaweed Endophytic Fungi

Although marine-derived fungi are marine isolates of terrestrial species, they are fertile producers of novel metabolites of unique structures (Oh et al. 2006; Kjer et al. 2010; Gao et al. 2011) which are not produced by terrestrial species of same fungus (Osterhage et al. 2000). There are different types of pure compounds and crude extracts isolated from fungal endophytes of seaweed origin that exhibit diverse biological activities such as antifungal, insecticidal, antibacterial, antialgal, antiplasmodial, anticancer, acetylcholine esterase inhibitor, cytotoxicity, antiviral, antiangiogenic and antioxidant (Bhadury et al. 2006; Newman and Hill 2006; Bhatnagar and Kim 2010; Ohkawa et al. 2010) (Fig. 22.1). Novel secondary

Fig. 22.1 Different biological activities of seaweed associated fungal secondary metabolites

metabolites synthesized by Endophytic fungi associated with marine algae can be chemically categorized as alkaloids (Tsuda et al. 2004), aromatic polyketides (Pontius et al. 2008), terpenes (König et al. 2006; Lösgen et al. 2007), sesquiterpenes (Bugni and Ireland 2004) and steroids (Zhang et al. 2016a, b). Thus, these fungi can be employed for synthesis of pharmacologically active metabolites or compounds for drug biosynthesis (Engel et al. 2002; Blunt et al. 2010).

22.6 Factors Responsible for Enhancement of Secondary Metabolite by Seaweed Fungal Endophytes

Seaweeds comprise second largest, source of diverse assemblage of marine fungi. A number of seaweeds encompassing genera mainly belonging to Phaeophyceae like *Adenocystis*, *Ascophyllum*, *Desmarestia*, *Dictyota*, *Fucus*, *Lobophora*, *Padina*, *Phaeurus*, *Sargassum*, *Stocheospermum*, and *Turbinaria*; members of Rhodophyceae viz. *Gelidiella*, *Gracilaria*, *Grateloupia*, *Halymenia*, *Palmaria*, *Plocamium*, *Portieria*, *Pyropia* as well as Chlorophyceae members such as *Acrosiphonia*, *Caulerpa*, *Halimeda*, *Monostroma*, and *Ulva,* have been studied extensively for their fungal associations (Singh et al. 2015). Red and brown seaweeds showed to have greater fungal species diversity where as green seaweeds harbored lower fungal species diversity (Suryanarayanan et al. 2010). It has been reported that short life cycle of different green seaweeds and slow growth of the endosymbionts may be responsible for their low fungal diversity (Zuccaro and Mitchell 2005).

Seaweeds are well known source for biological activities such as antioxidant, anticancer, anti-fungal, anti-microbial, anti-viral, insecticidal, anti-inflammatory and diuretic activity for a long time. Production of bioactive metabolites by endophytic fungi depends largely on culture conditions. For example, Bode et al. (2002) reported the increase in the synthesis of bioactive metabolites by the endophytic fungi through manipulation of physical factors such as pH, salinity and aeration, and altering growth medium composition or adding certain inhibitors and precursors to the medium (Llorens et al. 2004). Further, Miao et al. (2006) reported that the antibiotic activity of a marine-derived fungus increased with salinity of the growth medium. The synthetic ability of the fungi can also be enhanced by the presence of small molecule elicitors in the growth medium as they specifically alter the transcription of secondary metabolite gene clusters (Pettit 2011).

Mathan et al. (2013) have reported the influence of cultural conditions and environmental parameters like culture media, C and N sources, pH, NaCl concentration and temperature affecting the growth as well as production of the bioactive metabolites of the seaweed endophytic fungi *Aspergillus terreus* KC 582297. Among the various carbon sources (glucose, fructose, maltose, sucrose and starch) and nitrogen sources (ammonium chloride, sodium nitrate, peptone, yeast extract and beef extract), glucose and yeast extract have been reported to be the most suitable carbon and nitrogen source for their optimum growth as well as production of bioactive metabolites, respectively. Maximum bioactive metabolite productions occurred at pH of 5.5 and temperature at 25 °C.

22.7 Future Perspectives

Emergence of antimicrobial resistance against popular drugs has been a global threat as quoted by the then UN secretary general, Ban Ki-moon at United Nations General Assembly meeting in 2016 during a discussion session describing the growing emergence of antimicrobial resistance as a fundamental threat. Development of novel drugs from unexplored sources could be a solution to battle this problem. This practice demands isolation of rare, potential organisms from unique habitats, this signifies seaweed endophytes acting as pool of unique bioactive compounds having diverse bioactivity. However, relatively little information is available about algicolous endophytes and associated novel metabolites.

In an estimate by Strobel and Daisy (2003) and Hawksworth (2004), out of 100,000 explored fungi, only about 0.08% are algicolous (Zuccaro et al. 2003), showing opportunities of exploring new fungal strains and associated unique bioactive compounds.

Despite the prominent biosynthetic potential of endophytes, a regular production scenario during in vitro conditions has so far been not achieved. Thus, following steps can be taken for extending the current research on algicolous endophytic fungi to meet the future demands of bioactive secondary metabolites as well as to explore new species.

(a) The development of fungal culture collection centres (repositories) from marine habitat to facilitate research on their bioactive principle to fulfil the demands of pharmaceutical industry.
(b) Performing research related with the chemical defensive function of the endophytic fungi toward their host algae, an understudied research area, will provide a deeper insight into symbiosis and the associated metabolites of the endophytic fungi and their host algae.
(c) Specific study regarding identification of exact function of potential compounds and the related gene cluster would pave the way to understand the interactions between the endophytes and their host algae.
(d) Studying different physiological and ecological conditions that have led to the activation of secondary metabolism gene clusters might also be useful in optimizing environmental condition for maximum production of metabolites.
(e) Developing special physiological and genetic conditions for identifying silent" gene clusters for exploring more "silent" secondary metabolites under laboratory condition.
(f) Associating modern techniques like metagenomics with state of art isolation techniques and culture condition might be helpful in culturing and studying cryptic algicolous endophytes, which are unexplored as compared to fast growing and culturable endophytes.

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Chapter 23
Probiotic Biosurfactants: A Potential Therapeutic Exercises in Biomedical Sciences

Priyanka Saha, Deepa Nath, Manabendra Dutta Choudhury, and Anupam Das Talukdar

23.1 Introduction

Microbes occupy a strong population in the living world. They possess extra and intracellular vital products such as antibiotics, enzymes, toxins, biopolymers and pigments. Till date, more than 10,000 active broad-spectrum metabolites with medicinal properties have been isolated from these microbes (Kelecom 2002). However, most of the microbial worlds remain unexplored owing to its vastness. Recent studies confirmed that only <0.1% of microbial world has been investigated till date (Augustin et al. 2014). When listing microbial bioactive compounds, biosurfactants (BSs) are such metabolites with many interesting properties due to their multiple diversities in both structures and functions also with their pronounced usage in industries. BSs are basically amphiphilic surface active agents in bacteria, fungi, and classes of actinomycetes. They belong to classes of glycolipid, glycolipoproteins, glycopeptides, or lipoproteins, lipopeptides or derivatives of fatty acids (Banat et al. 2010), and less likely glycoglycerolipids too (Wicke et al. 2000). The major basic properties of BSs comprise of its detergency, wetting, foaming, emulsification, dispersion, penetrating, stimulator of microbial growth, thickening, metal sequestering, antimicrobial agents, and oil recovering. These properties make BSs possible to replace some of the most useful chemical surfactants that are currently in use these days. BSs are however a promising natural surfactants that bear added advantage over chemically synthesized ones, such as mainly in consuming renewable substrates, less toxic, and ecological compatible (Marchant and Banat 2012). Attention in BS research has been on the air past two decades for such beneficial

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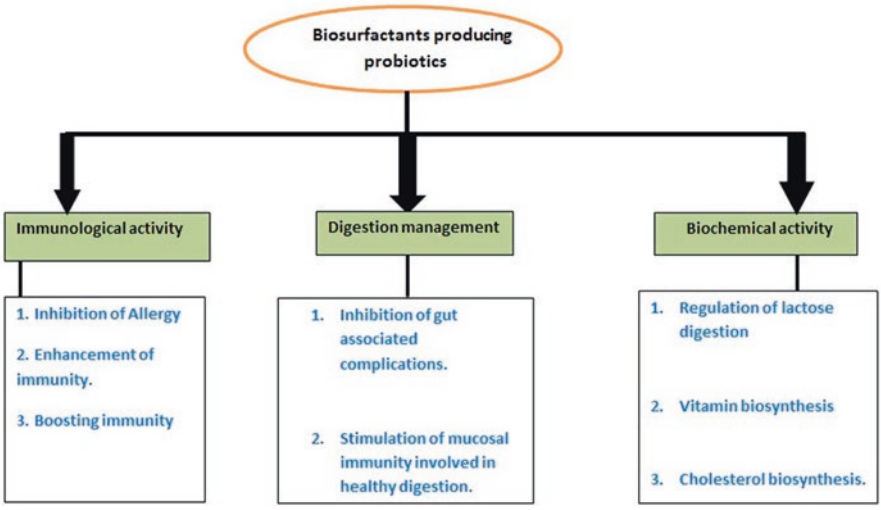

Fig. 23.1 Physiological activity of probiotic biosurfactants

properties; however the prime cause for the production of BSs remains mostly unknown. Several advocated biological roles of BSs have been brought to light. Mainly, it may be due to, expanding of surface area and hydrophobic water-insoluble substrates combination (Carrillo et al. 2003), bacterial infections and quorum sensing (Davey Smith and Ebrahim 2003) (Fig. 23.1), self-defense by producing microbial toxins (Boris and Barbés 2000), and cell multiplying in the bacteria proliferation process (e.g., viscosinamide production) (Cameotra and Makkar 2004). For isolation of BS-producing microbes, several screening methodologies has been applied (Brzozowski et al. 2011; Thavasi et al. 2011) and extensively studied (Nerurkar et al. 2009; Busscher and Van der Mei 1997). Microbial groups like *Acinetobacter, Pseudomonas, Bacillus,* Rhodococcus, *Corynebacterium* and yeast have been stated to produce BSs (Cribby et al. 2009; Desai and Banat 1997; Ceresa et al. 2015) have been establish to have surface action (Falagas et al. 2007).

With environmental compatibility becoming an increasingly important hinder in the selection of industrial chemicals, the use of biosurfactants in environmental is increasing. Moreover, biosurfactants are used in the petroleum industry, such as in microbial enhanced oil recovery and the transportation of crude oil. In various industries, biosurfactants works as emulsifiers (Desai and Banat 1997). However, biosurfactants have hardly been practiced in industry due to costs involved in its recovery. Consideration to the surface-active molecules of biological origin was prioritized because of their application in food molting industries (Donlan and Costerton 2002; Falagas and Makris 2009). Though, nature of the microbial surfactants produced, depend on the producer, yet component like carbon and nitrogen, trace elements, also contribute to their production.

Microbial surfactants are known to be secondary metabolites, play pivotal role for the sustainance of biosurfactant producing microorganisms by aiding in nutrient

transport, acting as biocide agents or in microbe-host interactions (Falagas et al. 2006). It also takes part in biofilm formation (Gudiña et al. 2010a, b) Biosurfactant retain numerous restorative as well as biomedical importance (Gomaa 2013) with potent antifungal antibacterial (Gan et al. 2002) and antiviral properties. It also inhibit fibrin clot formation and anti-adhesive action against several pathogenic microorganisms (Fracchia et al. 2010). Here, in this article role and applications of microbial surfactants were discussed with main focus on the most recent and appealing medicinal and pharmaceutical perspectives.

23.2 Chemical Nature of Biosurfactants

Biosurfactants comprises of microbial kingdom with vivid chemical composition. These surfactants are complex molecules with different chemical configurations starting from peptides, to antibiotics respectively. Microorganisms also yield surfactants that are in combination with multiple chemicals called as polymeric surfactants. Many microbial surfactants have been purified (Deziel et al. 2000; Gandhi and Chikindas 2007). Microbial surfactants sometimes contain both polysaccharides and protein and are termed as high molecular weight surfactants. Living microbial cells show high cell surface hydrophobicity behaves as surfactant. In a few cases, surfactants themselves take part in microbial cell growth. Exocellular surfactants plays role in cell adhesion, emulsification, dispersion, flocculation, cell aggregation and desorption phenomena (Gołek et al. 2009). A broad classification of biosurfactants is given in the subsequent Table 23.1.

Table 23.1 Chemical constituents of a few pronounced biosurfactants

Type of biosurfactants	Microorganism	References
Streptofactin	*Streptomyces tendae*	Portilla-Rivera et al. (2008) and Madhu and Prapulla (2014)
Glycolipids	*Alcanivorax borkumensis*	Pascual et al. (2008)
	Arthrobacter sp.	
	Corynebacterium sp.	
Rhamnolipids	*Pseudomonas aeruginosa*	Reid et al. (2001a, b) and Gudiña et al. (2010a, b)
	Pseudomonas sp.	
	Serratia rubidea	
	Candida apicola	
	Candida bombicola	
Sophorolipids	*Candida lipolytica*	Rodríguez et al. (2013a, b) and Reid et al. (2001a, b)
	Candida bogoriensis	

(continued)

Table 23.1 (continued)

Type of biosurfactants	Microorganism	References
Viscosin	*Pseudomonas fluorescens*	Rodrigues et al. (2004)
Cellobiose lipids	*Ustilago maydis*	Saharan and Nehra et al. (2011)
Polyol lipids	*Rhodotorula glutinus*	Saharan et al. (2011)
	Rhodotorula graminus	
Diglycosyl diglycerides	*Lactobacillus fermentii*	Rodrigues et al. (2006)
Lipopolysaccharides	*Acinetobacter calcoaceticus*	Rodríguez et al. (2010)
	Pseudomonas sp.	
	Candida lipolytica	
Lichenysin A	*Bacillus licheniformis*	Kermanshahi and Peymanfar (2012)
Surfactin	*Bacillus subtilis*	Kermanshahi and Peymanfar (2012)
	Bacillus pumilus	
Fatty acids	*Arthrobactter paraffineus*	Saharan et al. (2011)
	Norcardia erythropolis	
	Talaramyces trachyspermus	
Ornithine	*Thiobacillus thiooxidans*	Rodríguez et al. (2010)
	Streptomyces sioyaensis	
	Gluconobacter cerinus	
	Acinetobacter sp.	
Trehalose lipids	*Arthrobacter paraffineus*	Santos et al. (2013)
	Corynebacterium sp.	
	Mycobacterium sp.	
	Norcardia sp.	
Alasan	*Acinetobacter radioresistens*	Rodríguez-Pazo et al. (2013a, b)
Phospholipids	*T. thiooxidans*	Kermanshahi and Peymanfar (2012)
	Corynebacterium alkanolyticum	
Particulate surfactants (PM)	*Pseudomonas marginalis*	Thavasi et al. (2011)
Sulfonyl lipids	*T. thiooxidans*	Thavasi et al. (2011)

23.2.1 Glycolipids

Glycolipids are the biosurfactants containing carbohydrates associated with long chain aliphatic acids or hydroxyaliphatic acids (Hong et al. 2005) Rhamnolipids, trehalolipids, sophorolipids are best known glycolipids produced by such variety of microorganisms (Heinemann et al. 2000)

23.2.1.1 Rhamnolipids

Glycolipids that unites two molecules of rhamnose linked with one or two molecules of β-hydroxy-decanoic acid are called rhamnolipids. The production of rhamanose with glycolipids was initially studied in *Pseudomonas aeruginosa* (Gupta and Garg 2009). The two important glycolipids produced by *Pseudomonas aeruginosa* are L-rhamnosyl-Lrhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl- β-hydroxydecanoyl-βhydroxydecanoate commonly referred as rhamnolipids I and II individually (Gudiña et al. 2010a, b).

23.2.1.2 Trehalolipids

they are disaccharides trehalose linked at C-6 and C-6′ to mycolic acids which is associated with most species of Mycobacterium, Nocardia and Corynebacterium. Mycolic acids are usually a long-chain, α- branched β- hydroxy fatty acids (Meylheuc et al. 2006). The most common trehalolipids are trehalose dimycolate and anionic trehalose lipid found in *Rhodococcus erythropolis* and Artrobacter sp. downregulates the surface and interfacial tension respectively (Kermanshahi and Peymanfar 2012).

23.2.1.3 Sophorolipids

Sophorolipids are glycolipids of a carbohydrate dimer, sophorose linked to a long chain hydroxyl fatty acid by glycosidic linkage. *Torulopsis bombicola* and *T. apicola* are a few example (Meurman 2005). Frequently, sophorolipids are found as a mixture of macrolactones and free acid form (Inès and Dhouha 2015).

23.2.2 Lipopeptides or Lipoproteins

Lipopeptides and lipoprotein are mostly contains of a lipids attached with polypeptide chain. Peptide like gramicidins (decapeptide antibiotic) and polymyxin (lipopeptide antibiotic) produced by *Bacillus brevis* and *B. polymyxa* posseses amazing biosurfactant activity (Meurman and Stamatova 2007).

23.2.3 Surfactin

Surfactin (lipopeptide from *Bacillus subtilis*), is one of the most dominant biosurfactant. It usually comprises of a seven amino acid welldefined ring attached to a fatty acid chain via lactone linkage (Meurman 2005). Surfactin produced by *B.*

subtilis increases solubility and bioavailability also stimulating indigenous microorganisms for enhanced cleanup of a contaminated soil (Madhu and Prapulla 2014)

23.2.4 Fatty Acids, Phospholipids and Neutral Lipids

Bacteria and some yeast produce fatty acid and phospholipid surfactants during growth on n-alkanes and other hydrocarbons. The HLB (hydrophilic and lipophilic balance) of the surfactants produced in this condition is directly related to structures of the hydrocarbon chain (Murphy et al. 2006). *Corynebacterium alkanolyticum* is a classic example (Vecino et al. 2014) of a phospholipid biosurfactant, with limited production of the biosurfactants. But interestingly use of self-cycling fermentation processes increase the biosurfactant production (Madhu and Prapulla 2014). Production of phosphatidyl ethanolamine by *Rhodococcus erythropolis* grown on n-alkane helps in lowering the interfacial tension between water and hexadecane (Moldes et al. 2013).

23.3 Surfactants Employed as Medical Products Are Primarily Biobased

Surfactants, a specific product either in solid or liquid state tends to lower the surface tension, plays ample part in pharmaceutical world (Salehi et al. 2014). Surfactants are amphiphilic in nature with both hydrophilic and lipophilic domains. Their major role in pharmaceutical industries is to improve the drug solubility for those which are insoluble in water, accumulating new recent and more authentic bioactive agents (e.g.vaccines, vitamins, proteins and oligonucleotides), to aid in in vivo delivery. They also help in encapsulated drugs stabilisation. They are involved in penetration of drugs across membranes, skin, and other various biological interfaces. Surfactants are also vital to improve the fluidity of delivery system and glutinous excipients such as those engaged for suppositories (Sambanthamoorthy et al. 2014). They so sometimes behave as wetting agents to enabling an easier and more reliable mode of drug incorporation to delivery vehicles and serves as promising choice for existing modes like powders, granules, and nanoparticles. The common use of surfactants is for vital systems as drug delivery vehicles. Emulsions are not stable; often agitation is associated with it for its stability. “biobased” surfactants, is on the rise mainly due to the increased feedstock cost, and the for utilization of renewable feedstocks with enhancement of sustainability (Rodríguez et al. 2010; Zobell 1943). Moreover, dependence upon dwindling production of petroleum and constant rapid CO_2 and other greenhouse gases production with continuous presence with its devastating impact on climate change. These factors have amplified

consumer demand for more sustainable products. So, it has been estimated that the market value of biobased surfactants will exceptionally increase in recent years.

The majority of the surfactants are partially derived from renewable resources (Moldes et al. 2013). A number of derivatives from oleo chemicals which are also the ingredients of the fatty acyl include polysorbates, saccharide esters, and fatty acid ethoxylates. Therefore, the development and opening out of oleo chemical make the biobased surfactants incorporate with biorefineries to spawn chemical intermediates biobased yield and fuels from seed oil plants (Rivera et al. 2007). Coconutt, cuphea oils and feed stocks like palm, palm kernel improved in C_{10}-C_{16} saturated fatty acyl (predominantly palm stearine, a palmitic acyl-rich derived from the fractionation of palm kernel oil) are a classic examples,. Low-cost bases of fatty acyl class comprises of algal oils, ricinoleic acid, soapstick, cooking oils, soapnut oil tallow, jatropha oil used are usually products grown in Brazil, India, and some other countries worldwide. Heterogeneous catalytic reactions is a very effective process catalyzed by petroleum or from fatty acid methyl ester which vintages products like, the lipophilic group of APGs (Alkyl polyglycosides) and sometimes medium-chain fatty alcohols Sharma et al. 2015) Phospholipids are directly obtained from soap stock, gums, and other oleo chemical processing co-products (Saravanakumari and Mani 2010). Bioprocessing affords tremendous added positive benefits compared to chemical processing, chiefly with respect to sustainability improvement, lessening of consumption of energy, reduced amounts of waste and its related end products, absence of metal based toxicity, and safer operating environments. The major disadvantages are the prohibitive costs for enzymes compared to chemical catalysts (although this concern is reduced when enzymes are immobilized to enable reuse) and the lower reaction rates that accompany many enzymatic reactions. For example, fatty acyl-liked products need not contain aldehydes/ketones, phospholipids, peroxides, and other impurities. But, as energy costs increase, the importance of sustainability increases, and as a result, the production and efficacy of the systems increase, enzymatic bioprocessing is estimated to turn out to be further attractive and cost-competitive. When talking of cosmetic production, owing to its water binding capacity, emulsification, wetting properties, spreading nature and foaming effect on viscidness and consistency of the product, biosurfactant are now a product of choice to substitute chemically synthesized surfactants (Table 23.1). These surfactants have the emulsificating features predominantly. Moreover, additive properties like wetting agents, cleansers, antimicrobial agents, solubilizers, intermediaries of enzyme action, foaming substitute, in insect repellents, cosmetics, mascara, lipsticks, toothpaste, dentine cleansers, etc (Sauvageau et al. 2012). Nanoemulsions, generally piece of choice owing to their reduced size are quite easy to be sterilized by microfiltration and are more possible to penetrate interfaces *in vivo* and avoid physiological clearance too. Nanoemulsions are commonly used in parenteral delivery.

Water-oil-surfactant mixtures often form stable microemulsions, characterized by nanometer-sized architectures (Heinemann et al. 2000). Hydrophilic and lipophilic surfactant method yields W/O and O-W--microemulsions, individually, naturally comprising of spherical nanodroplets. Surfactant systems possessing dynamic

intertwined networks of oil and water separated by surfactant monolayers. Microemulsions are generally produced in *in vivo* by providing a waterless combination of the constituents that self-microemulsify (emulsify) on contact with water.

Surfactants finds its way in the world of therapeutic agents. For example, biosurfactants like fatty acid esters, glycolipid biosurfactants and amino acid-based biosurfactants possess antimicrobial activity (Sharma et al. 2014) (Table 23.2). Polyunsaturated fatty acid, glycolipid biosurfactants and monoacyl glycerol (MAGs) have proved to possess anticancer activity (Sotirova et al. 2009). Sophorolipid biosurfactants are activators of immune response (Nerurkar et al. 2009). Surfactants are protuberant machineries of several personal hygiene, dermatological care and cosmetics supplements.

The surfactants possess a highly biocompatible and nonionic features. Cationic surfactants are used for the oligonucleotides delivery. Recently, biobased cationic derivatives of arginine have proved to be operative as biocompatible delivery agents (Sihorkar and Vyas 2001)

Another common surfactant category is. These phospholipids are the major constituents of synthetic lung surfactant, used in the neonatal and acute distress syndrome activity (Sharma et al. 2014) and of liposomes, nothing but spherical objects, can contain of one or multiple number of concentric phospholipid bilayers (known as uni or multi-lamellar), which are common delivery vehicles (Thavasi et al. 2011).

This review discusses the current state of biosurfactant research, with an emphasis on potential therapeutic applications. We aim to provide new insights and understand diverse bio functions for cutting edge applications as improved anticancer drugs or nanoscale microemulsion-based drug delivery vectors.

Table 23.2 Biomedical and therapeutic applications of probiotic bacteria

Probiotic strain	Medical applications	Mechanism
Lactobacillus crispatus and *Lactobacillus jensenii*	Treatment of BV and UTIs	Increase estrogen level
Lactobacillus casei	Control diarrhea	
	Reduce episodes of hypertensions	Produce antihypertensive exopolysaccharides
	Cure for colorectal cancer (CRC)	Promote immune cell activity, immune cell proliferation, apoptosis
Streptococcus thermophillus	Prevent necrotising enterocolitis (NEC)	Interfere with immature intestinal proliferation in preterm infants, intact epithelial integrity
Lactobacillus rhamnosus and *Lactobacillus fermentum*	Treatment of UTI and BV	Cause less yeast, coliforms in vagina by selfcolonization
Bifidobacterium infantis	Important in abdominal pain, bowel movement and bloating	Not specific

(continued)

Table 23.2 (continued)

Probiotic strain	Medical applications	Mechanism
Lactococcus lactis	Cytokine, protein, and DNA delivery	Transient colonization
Escherichia coli Nissle 1917	Infectious diarrhea due to Shigella (highly contagious organism)	Colonization resistance to mucosal pathogens
Lactobacillus casei Shirota (Yakult) and *Lactobacillus acidophilus*	Treatment of malfunctioning in GIT	Colonizing gut epithelial lining
Bifidobacterium and *Streptococcus thermophiles*	Reduce HIV associated immune deficiencies and diarrhea	Increase immune cell count, improvement in stool consistency
Lactobacillus casei	Reduce recurrence of bladder cancer	Immune modulation
Escherichia coli and DNA extract from probiotic organism	Mediate antiinflammatory activity and hypersensitivity conditions	Ameliorate disease toll like receptor signaling
Lactobacillus iners	Reduction of UTIs	Estrogen replacement therapy
Lactobacillus acidophilus	Alleviate lactose intolerance	Ferment lactose to lactic acid
Lactobacillus plantarum	Help in recuperation from surgery	By preventing wound from pathogens etc.
Bifidobacterium lactis and *Lactobacillus rhamnosus* GG	Treatment of allergies that effect intestine (eczema from cow's milk in infants)	
Lactobacillus reuteri SD2112	Treatment of rotavirus diarrhea	Hinder proliferation of retrovirus

Heinemann et al. (2000) and Kermanshahi and Peymanfar (2012)

23.4 Biomedical Applications Cases of Some Probiotic Biosurfactants

23.4.1 Biosurfactants as Antitumor Agents

Biosurfactants can regulate quite a number of mammalian cell functions and therefore their efficacy to act as an antitumor mainly by checking severe abnormal cancer progression courses (Fig. 23.2). Definitely, such molecules act in several intercellular molecular pattern recognition steps more pronouncingly found in signal transduction, cell immune response and cell differentiation (Tahmourespour et al. 2011). For instances, glycolipids have been displayed to be directly associated with cell cycle arrest and death of malignant cells in mouse B16 cells (Velraeds et al. 1998). Exposure to increasing concentrations of such polymeric active molecule, led to the gathering of B16 cells in the sub-G0/G1 phase, which is a signal of approaching towards apoptosis (Walencka et al. 2008). Besides, a categorization of apoptotic actions including the chromatin condensation and DNA fragmentation was confirmed, approving the apoptosis- initiation potential of biosurfactants in these cells (Toribio et al. 2010).

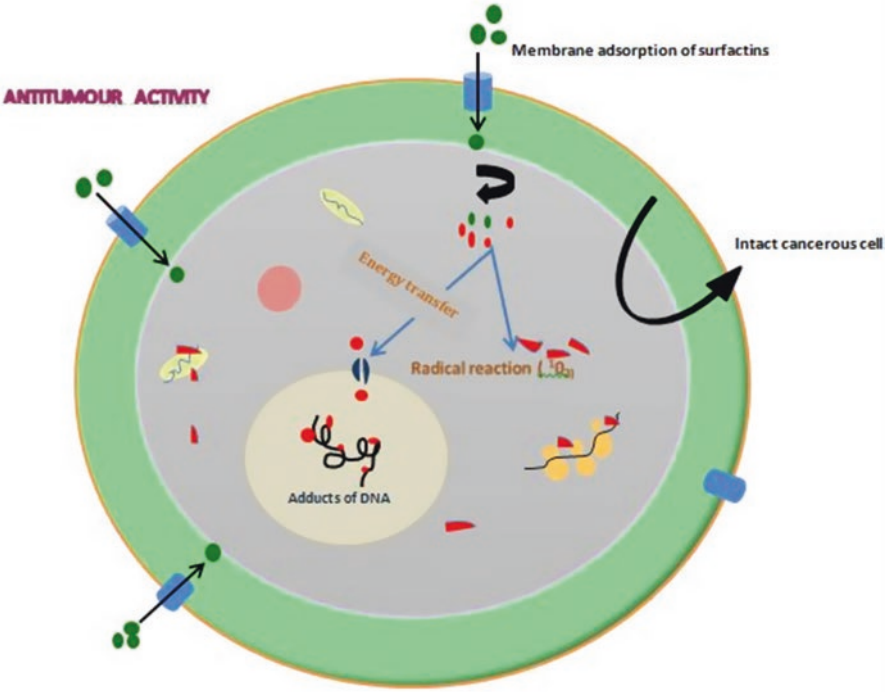

Fig. 23.2 Antitumour activity of biosurfactants (surfactins)

23.4.2 Gene Delivery

Newly marketed potent drugs and their delivery recently creates a vital effect on our ability to treat many complicated sorts of diseases (Toiviainen 2015) Rather, a controlled system mainly hold two important features: (i) ideal drug stacking capability, which results in increased of drug bioavailability and volume to reach the target of interest, and (ii) the following release of the drug in a controlled manner. To carry out essential purposes, many forms of pharmaceutical carriers like polymeric, macromolecular also cellular ones have been currently used (Vecino et al. 2013). They mainly exists in a colloidal nature in forms of microspheres, lipid particles and vesicular systems like sphingosomes and liposomes (Busscher 2004). Microemulsions have occurred as novel form suitable for topical, oral, nasal, ocular and other ways of drug administration (Van Hoogmoed et al. 2006). Significant attention of researchers are focused to simplify its formulation. However, the vital standard that most formulations fail to abide is the judicious use of biocompatible and biodegradable pharmaceutical agents as their ingredients.

23.4.3 Biosurfactants as Anti-adhesives

Biofilm formation and production are the foremost step in the establishment of a bacterial surface, and upsurge the likelihood of nosocomial complications. Biosurfactants have been found to inhibit of pathogenic organisms to infection sites. Surfactin diminishes the formed by *Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis* and *Salmonella typhimurium* in polyvinyl chloride wells, and also extensive use in vinyl urethral catheters (Vujic et al. 2013). Precoating the catheters by treating with the surfactins solution over them prior to inoculation with media is just as effective as adding surfactin directly in the growth medium. Given the position of opportunistic infections with *Salmonella* species, which also includes more severe urinary tract infections witnessed in AIDS patients, these consequences have potentialities for practical uses.

23.4.4 Antimicrobial Activity (Table 23.3)

Table 23.3 Antimicrobial activity of biosurfactants

Microorganism	Biosurfactant type	Activity/application
Pseudomonas aeruginosa	Rhamnolipid	Antimicrobial activity against *Mycobacterium tuberculosis*
		Anti-adhesive action beside many emerging bacterial and yeast strains collected from voice prostheses
		Induced hemolysis and coagulation in a dose dependent manner of plateletless plasma but is not injurious to lung, liver, heart and kidney of chicken. *Bacillus subtilis* Iturin and *Bacillus subtilis* Surfactin
		Mycosis recovery owing to antimicrobial activity and antifungal activity
		Effect on the membrane structure of yeast morphology and cells derived. Upsurge in the electrical conductance of biomolecular lipid crusts
Bacillus subtilis	Surfactin	Antimicrobial and antifungal activities
		Inhibition of fibrin clot formation
		Hemolysis and formation of ion channels in lipid membranes
		Antitumor activity against Ehrlich's ascite carcinoma cells
Bacillus subtilis	Pumilacidin	Antiviral activity against human immunodeficiency virus 1 (HIV-1)
		Induction of apoptosis in human leukemia K562 *Bacillus subtilis* Pumilacidin
Bacillus subtilis	Iturin	Antiviral activity against herpes simplex virus 1 (HSV-1) (surfactin analog)
		Inhibitory action against K+-ATPase, H+, and protection alongside gastric ulcers in vivo

Toribio et al. (2010) and Nerurkar et al. (2009)

23.4.5 Other Biomedical and Therapeutic Applications

Biosurfactants forms an important part of food supplements. pure minerals and vitamins are now a days replaced by food supplements, homeostatic nutrient complexes, which are a mixture of vitamins and minerals liberated and maintained in their natural form by probiotic microorganisms and their byproducts, including enzymes and organic acids are being prescribed these days which in turn increases the effectiveness of the minerals and vitamins, as well as providing many other nutritionally vital compounds. the complex are numerous essential biochemicals that are important for us, including the antioxidant superoxide dismutase, as well as various immune supportive antimicrobial peptides, biosurfactants, biotins, chromium compounds, hydrogen peroxide, lactic acid and lysozymes. One of the vital main groups of organisms studied for their use as an effective probiotic are members of the *Lactobacilli* group, which have the possible preventive role in pathogen colonization and help to restore the normal microbial flora (Vandecandelaere and Coenye 2015; Vaughan et al. 2005).

23.5 Challenges

Interesting features of biosurfactants have led to a range of its applications in the medical sciences. They are beneficial as antimicrobial agents, and they also have the capacity to be used as major immunomodulatory molecules and other adhesive agents in gene therapy. Biosurfactants have found its way to gene transfection, as ligands for binding antibodies, as adjuvants for antigens and fibrin clot inhibitors and fibrin clot lysis inducers. Capable alternatives to yield potent biosurfactants with transformed antimicrobial profiles and diminished toxicity against mammalian cells may be subjugated by genetic variation of biosurfactants. Additionally, biosurfactants have the prospective to be a statement of choice for medical insertional materials, thus reducing hospital contamination and synthetic drugs practice. They may also be assimilated into probiotic preparations to work against severe complications of urogenital tract contaminations. Irrespective of the huge prospective of biosurfactants in this field, their practical utility still leftovers restricted. This status may be possibly due to their over production and also its costlier issue and proper lack of information on their toxicity profile. Additionally, research on human cell line and microbiota are essential to confirm the use of biosurfactants in several biomedical related areas. Yet, there appears to be great prospective for their applications in the medical science ring waiting to be fully exploited.

23.6 Conclusion

Biosurfactants have potent antimicrobial applications that have been extensively used for gene transfection, antigen antibody reactions, as adjuvants for antigens and so forth. Their capability to modify ingredients of bio molecules like cAMP and PLA2 might act as modulators of signal transduction. Genetic alteration could rise upto potent biosurfactants with altered antimicrobial profiles and reduced toxicity against human cell lines. Apart from the use as potential alternative antimicrobial agents, is their use as antiadhesives. Biosurfactants can deferral the pathogenic biofilm growth initiation on medical insertional materials like cathetar, thus creating a chck point on hospital infections without involvement of synthetic drugs and chemicals. They can also be cast-off in pulmonary immunotherapy and incorporated into preparation and production of probiotic recipe to combat urogenital tract infections. *Lactobacilli* species, important probiotic can be a part of the normal healthy human flora. Since they are biological and safe, biosurfactants are a substitute of choice for marketed medicines and antimicrobial agents, and could be utilized as safe and operational probiotics therapeutic agent.

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Chapter 24
Recent Antibiotics Used in Dental Disease Management

Jerusha Santa Packyanathan, C. Elizabeth Rani Juneius, and M. Vinoth

24.1 Introduction

A greater understanding of various dental infections and, in particular, microbes like bacteria which are the causative agents of several dental diseases, has led to further interest in the indications for these drugs as adjunctive measures. Treatment and prophylaxis with antibiotics are inevitable for oral care. The most significant use of antibiotics is preventing dentally induced bacteraemia and metastatic infection. Antibiotics have revolutionized the control of infectious diseases and have a significant role in dental practice. Whilst new indications for the use of antibiotics become more widespread, all members of the healthcare professions need to be aware that these drugs have significant adverse effects and their misuse can lead to life-threatening infection (Seymour 2017).

24.1.1 Oral Microbiome

The oral cavity is a complex biological ecosystem with very large number of organisms living in a biofilm (Filoche et al. 2010). Several microorganisms are present in the oral cavity and they are collectively known as oral microbiota, or microflora or commonly as the microbiome. Joshua Lederberg framed the term microbiome meaning "to signify the ecological community of commensal, symbiotic, and

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pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Lederberg and Mccray 2001).

The presence of Microorganisms in the oral habitat, makes us susceptible to several infections and diseases like periodontitis, caries, root canal infections, infections of the pulp, alveolar osteitis and even tonsillitis. Several studies have established the relationship between bacteria and the occurrence of such systemic diseases (Seymour et al. 2007), including cardiovascular disease (Beck and Offenbacher 2005), stroke (Joshipura et al. 2003), preterm birth (Offenbacher et al. 1998), diabetes (Genco et al. 2005), and pneumonia (Awano et al. 2008).

The interaction of the organisms is complex and the switchover from health to disease is associated with a shift in the balance of the ecosystem usually from resident facultative anaerobes to obligate anaerobes for most pulpal and periodontal diseases (Ruby and Barbeau 2002). Even though only a few of the microorganisms cause odontogenic infections, in disease state, many other non-pathogenic bacterial species contribute by maintaining an ecosystem favourable for survival and growth of the pathogenic species. The onset of disease is due to a shift in microbial flora. Micro-organisms in a biofilm are consistently more resistant to usual dosage of antibiotics by 1000 to 1500-fold (Costerton 1999).

24.1.2 Oral Bacterial Pathogens

The most prevalent oral microbes are *Granulicatella paradiacens, Streptococcus mutans, Streptococcus sp., Streptococcus gordonii, Streptococcus sanguinis, Veillonella sp., Abiotrophia defective, Veillonella parvula, Streptococcus cristatus, Streptococcus mitis and Actinomyces* (Ribeiro et al. 2017). The microflora associated with odontogenic infections are typically mixed and of indigenous origin. *Streptococcus, Peptostreptococcus, Peptococcus, Fusobacterium, bacteroides,* and *Actinomyces* species are the principle microflora isolated from these infections (Karlowsky et al. 1993). In particular, *Tannerella forsythia* (*Tannerella forsythensis*), *Porphyromonas gingivalis* and *Treponema denticola,* known as "red complex" pathogens*,* have been indicated for playing important roles in various forms of periodontal diseases (Haffajee et al. 2000). *Campylobacter sp.*, *Prevotella intermedia/ Prevotella nigrescens*, *Fusobacterium sp.,* members of the "orange complex", are also related to periodontal breakdown as the secondary group of periodontal pathogens and the "green complex," represented by the combination of *Eikenella corrodens*, *Capnocytophaga sputigena, C. ochracea, C. gingivalis, C. concisus* was considered primary colonizers and compatible with periodontal health (Socransky et al. 1998).

24.2 Bactereamia

Manipulation of infected oral tissues, such as measurement of periodontal pockets, calculus removal, over-instrumentation during root canal treatment and especially tooth extraction are known to cause bacteraemia (Parahitiyawa et al. 2009). Infection in a tooth or its surrounding tissues may also lead to spontaneous bacteraemia, if the infection spreads to adjacent blood vessels. Severe underlying diseases including immunosuppressive illnesses and treatments predispose the patient to systemic odontogenic infection complications (Seppanen et al. 2008, Lee et al. 2009).

24.3 Infective Endocarditis

When bacterial infections in the oral tissues come in contact with the cardiac tissue, the patient develops infective (bacterial) endocarditis which may sometimes produce fatal results. Infective endocarditis is popularly identified as infection present in endothelial lining of the cardiac tissue (heart) (Marsh et al. 2009). The most common etiologic agent is *Streptococcus sanguinis* (Newman and van Winkelhoff 2001). Patients (both children and adults) with infective endocarditiss are advised to take high dose amoxicillin prior to any dental intervention. Two grams of oral amoxicillin is administered just a short while before the treatment begins (Dajani et al. 1979). This dose is sufficient to provide considerable hours of antibiotic cover in the patient. Clindamycin is used in those allergic to penicillin (Durack 2005).

24.4 Gingivitis and Periodontitis

Plaque that extends subgingivally can trigger the immune system imbalance, inducing an inflammatory response (Belkaid and Hand 2014). Gingivitis and periodontitis are the most common plaque-induced inflammatory conditions. The most prevalent anaerobic gram-negative bacteria in subgingival region inc *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Tannerella forsythensis*. All are critical in the onset and subsequent development of periodontitis. If untreated, these bacteria can lead to the periodontal pocket, connective tissue destruction, and alveolar bone resorption (Sanz et al. 2000).

24.5 Dental Abscesses

24.5.1 Cellulitis

The preliminary phase of bacterial infection is termed as cellulitis and the causative agents are facultative anaerobic bacterial species like *Streptococci pyogenes*. The secondary phase of the infection is commonly known as Abscess. It is a throbbing pool of pus caused by bacterial activity. This occurs when the infective organisms penetrate into the underlying tissue from the oral cavity through a dental source (Newman and van Winkelhoff 2001). Antibiotics used for treatment include penicillin which aids to prevent progression to the second phase of cellulitis – the abscess.

24.5.2 Lateral Periodontal Abscesses

The formation of abscesses can lead to a blockage in the periodontal pocket and have association with the vital pulp in relation to the tooth (Marsh et al. 2009).

The ideal choice of treatment includes the drainage and irrigation of the periodontal abscess with 0.2% Chlorhexidine like antiseptic mouthwash. However, antibiotic treatment is rarely mandatory.

24.5.3 Acute Dentoalveolar Abscesses

Inflamed or necrotic pulp of vital teeth are the cause of these abscesses. It may be an infection of pulpless root canals in some cases. The invasion of bacteria leads to the death of the pulp due to advanced caries. The choice of treatment in this case is to remove the source of irritation or infectivity by locally invasive measures (Cope et al. 2014). Amoxicillin is used to treat an alveolar dental abscess (Newman and van Winkelhoff 2001). An average of about 3% of the population is sensitive to penicillin, hence erythromycin is recommended for such hypersensitivity.

24.6 Post Dental Implant Infections

It is believed that bacterial contamination leads to dysfunction and eventually failure of a dental implant insertion (Esposito et al. 1998). It is a well-established fact that contaminations involving biomaterials are challenging to handle and require removal sooner or later (Esposito et al. 1998). The likelihood of occurrence of an infection is predisposed by skill of the operator (traumatic and delayed surgery increases susceptibility of infection) and to the degree of disinfection followed.

Earlier antibiotics were prescribed both preoperatively and postoperatively. This protocol is succeeded by the administration of 2 g of phenoxymethylpenicillin per os, for about an hour preoperatively and monitored by 2 g twice daily for the next 10 days (Adell et al. 1985). However the modern protocols (Flemmig and Newman 1990) recommended short-term term prophylaxis which includes 2 g of Amoxicillin or Augmentin given 1 h ahead of the surgery and penicillin-V 500 mg was given four times per day for only 1 day.

24.7 Dental Sinusitis

Sinusitis must be treated immediately with a view to preventing complications. This includes contiguous spread of infection into the infratemporal space or orbital cavity, osteomyelitis of the surrounding maxilla and pansinusitis. The clinical signs of infection were: headache, locoregional pain, cacosmia, inflammation of the oral buccal mucosa, genian and infraorbitary tumefaction and rhinorrhea or unilateral nasal discharge. Amoxicillin or doxycycline is administered. Doxycycline can cause nausea, vomiting, diarrhoea, dysphagia, oesophageal irritation and photosensitivity. It may also cause staining and occasional dental hypoplasia. The anticoagulant effect of warfarin might be enhanced by doxycycline. Table 24.1 depicts various antibiotics used to treat dental diseases.

Table 24.1 Main antibiotics used in dental disease management

Name of the dental pathogen	Disease	Drug of choice	Mode of action
Streptococcus mutans	Dental plaque	Chlorhexidine	It possesses bactericidal effect due to attachment of the cation to the oppositely charged bacterial cell walls. When concentrations of chlorhexidine is reduced, it has bacteriostatic effect instead; and at elevated strengths, membrane disruption leading to cell death occurs
		Various other natural remedies have been suggested or studied to a degree, including deglycyrrhizinated licorice root extract (Ahn et al. 2012; Hu et al. 2011), Tea tree oil (Carson et al. 2006), Macelignan (found in nutmeg Yaanti et al. 2008), curcuminoids (the main components of turmeric, Pandit et al. 2011) and eugenol (found in bay leaves, cinnamon leaves and cloves)	

(continued)

Table 24.1 (continued)

Name of the dental pathogen	Disease	Drug of choice	Mode of action
Streptococcus sanguinis	Dental plaque	Penicillin and aminoglycoside antibiotics. moxifloxacin	Cell wall inhibition
			Protein synthesis inhibition
Aggregatibacter actinomycetemcomitans	Localized aggressive periodontitis, a severe infection of the periodontium	Penicillin or amoxicillin	Cell wall inhibition
Streptococcuss salivarius	Dental carries	Penicillin or amoxicillin	Cell wall inhibition
Streptococcus mitis	Dental carries	Penicillin or amoxicillin	Cell wall inhibition
Streptococcus sanguis	Dental carries	Penicillin or amoxicillin	Cell wall inhibition
Campylobacter (*Wolinella*) *rectus*	Gingivitis	*Amoxicillin* (amoxicilline)-clavulanat	
Tannerella (*Bacteroides*) *forsythia*	Periodontitis in adults and gingivitis	*Amoxicillin*/ metronidazole	
Selenomonas noxia	Putative periodontal pathogens	Azithromycin,	Interfering with their protein synthesis. It binds to the 50S subunit of the bacterial ribosome, thus inhibiting translation of mRNA
Filifactor alocis	Putative periodontal pathogens	Clindamycin	It prevents the production of bacterial protein by hindering ribosomal translocation, in a similar way to macrolides. This is accomplished by fastening to the subunits of 50S rRNA of the huge bacterial ribosome
Lactobacillus casei	Dental carries	Ticarcillin	Prevent cross-linking of peptidoglycan during cell wall synthesis, when the bacteria try to divide, causing cell death

(continued)

Table 24.1 (continued)

Name of the dental pathogen	Disease	Drug of choice	Mode of action
Bifidobacterium	Dental carries	Pristinamycin,	The substance fixes itself to the 50 S ribosomal subunit of the bacteria and hinders the elongation procedure in protein synthesis. Hence, only reasonable bacteriostatic activity
		Vancomycin	Inhibiting the second stage of cell wall synthesis of susceptible bacteria
Prevotella buccae	Dental carries	Pristinamycin,	Each compound binds to the bacterial 50 S ribosomal subunit and inhibits the elongation process of the protein synthesis, thereby exhibiting only a moderate bacteriostatic activity
		Vancomycin	Inhibiting the second stage of cell wall synthesis of susceptible bacteria
Scardovia wiggsiae	Dental carries	Pristinamycin,	The substance fixes itself to the 50 S ribosomal subunit of the bacteria and hinders the elongation procedure in protein synthesis. Hence, only reasonable bacteriostatic activity
		Vancomycin	Inhibiting the second stage of cell wall synthesis of susceptible bacteria

24.8 Prophylaxis in Dentistry

24.8.1 Indications

Antibiotic prophylaxis may be indicated prior to certain dental procedures. Antibiotic prophylaxis is unwarranted unless the person is predisposed, to infection ((ADA) American dental association (2017)). This prophylaxis doesn't not necessarily have to kill bacteria but can constrain bacterial adherence (Gauser et al. 1983).

24.8.2 Prophylaxis for Prosthetic Joint Implants

For patients who have undergone joint replacement surgery and are subjected to dental treatments like gingival manipulation or mucosal incision, only after the discussion with the orthopaedic surgeon antibiotic prophylaxis must be considered (ADA Chairside Guide).

24.8.3 Prophylaxis for Infective Endocarditis

Antibiotic prophylaxis is recommended for patients at high-risk of developing infective endocarditis who need to undergo invasive dental operative procedures. This may include gingival tissue manipulation or damage of the oral mucosa or the manipulation of the periapical region (NICE guidelines 2016) (Table 24.1).

24.8.4 Prophylaxis for Compromised Immunity

Individuals who have compromised immune systems pose a higher threat in acquiring an infection after a dental procedure. Some of the conditions are human immunodeficiency virus (HIV), severe combined immunodeficiency (SCIDS), neutropenia, cancer chemotherapy, hematopoietic stem cell or solid organ transplantation, head and neck radiotherapy, autoimmune disease, sickle cell anemia (Tate et al. 2006), asplenism or status post splenectomy, diabetes and bisphosphenate therapy. (Montefusco et al. 2008; Rogers et al. 2009) mentioned about the ways by which such kind of patients are treated.

24.8.5 Shunts, Indwelling Catheters or Medical Devices

The AHA recommends that antibiotic prophylaxis for nonvalvular devices, including indwelling vascular catheters and implantable cardiovascular devices be specified when the device is placed to prevent infection at the surgical site (Lockhart et al. 2007; Baddour et al. 2003) general antibiotics used for this purpose must be included.

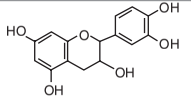
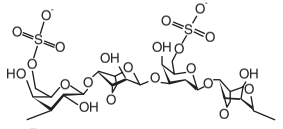
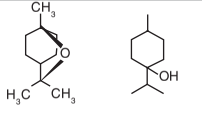
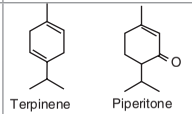
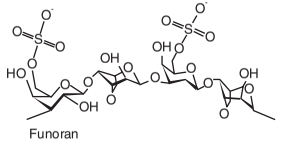
24.8.6 Hurdles of Prophylaxis

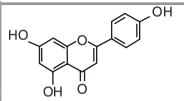
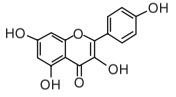
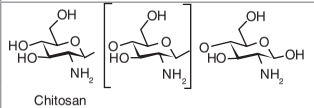
It is accepted that careful use of antibiotics prophylaxis is specified to diminish the danger of developing opposition to presently followed antibiotic routines (Dajani et al. 1997). Considering the growing figures of organisms that are gaining endurance to existing antibiotic treatments, it is best to be prudent in our use of antibiotics. There exists several adverse anaphylactic reaction following the administration of a drug (CDC 2014). Antibiotic prophylaxis for patients at risk does not provide total protection against infection. The occurrence of acute infection like weakness, fever, malaise, lethargy may signify antibiotic failure and pose a further requirement of medical evaluation.

24.9 Using Natural Products Used as Alternative or Adjunctive Anticaries Chemotherapy

Though there are several antibiotics available to treat dental diseases, the occurrence of resistance among the various species of bacteria and also harmful side effects caused by the organisms still strives the scientist to discover more new drugs from various sources. There are several reports for the use of traditional medicinal plants and herbs that may be effective against oral pathogens. For example, the use of chloroform extract of the leaves of (Droseraceae) *Drosera peltata* has been used for several years to manage caries. This extract displays a broad antibiotic spectrum, acting against numerous pathogens in the mouth, with maximum toxicity against *S. sobrinus* and *S. mutans*. Tichy and Novak (1998) determined a collection of 27 medicinal and plants extracts has inhibitory effects of oral streptococci. The most active extracts included those from *Albizia julibrissin* (Fabaceae), *Abies canadensis* (Pinaceae), *Albizia julibrissin* (Fabaceae), *Chelidonium majus* (Papaveraceae), *Pinus virginiana* (Pinaceae), *Juniperus virginiana* (Cupressaceae), *Sassafras albidum* (Lauraceae), *Rosmarinus officinalis* (Lamiaceae), *Tanacetum vulgare* (Asteraceae) and *Thuja plicata* (Cupressaceae). Phytochemicals are having significant effects on these pathogens and following are some examples of phytochemicals with similar characteristics to control dental pathogens (Table 24.2).

Table 24.2 Natural compounds with antibacterial effects

Putative active constituents	Examples of chemical structure of main putative active constituents	Source of natural products	Biological activity	References
Catechin-based polyphenols		Black tea (*Camellia sinensis*)	Glucosyltransferase activity is prevented	Hattori et al. (1990), Touyz and Amsel (2001), and Linke and LeGeros (2003)
Funoran	 Funoran	*Gloiopeltis furcata*	Inhibition of mutans streptococci adherence and desorption effects	Sasaki et al. (2004) and Sato et al. (1998)
			Reduction in dental plaque and caries development in rats infected with *S. sobrinus*	
			Reduction in human dental plaque formation	
Cineole, terpinen-4-ol	 Cineole Terpinen-4-ol	Essential oil from *Melaleuca alternifolia*	Antimicrobial activity against planktonic cells of mutans streptococci	Groppo et al. (2002), Hammer et al. (2003), and Takarada et al. (2004)
			Fall in population of salivary mutans streptococci amounts	
Terpinene, piperitenone oxide piperitone, pinene	 Terpinene Piperitone	Essential oils from *Mentha piperita* and *Rosmarinus officinalis*	Antimicrobial activity against planktonic cells of mutans streptococci	Takarada et al. (2004) and Rasooli et al. (2008)
			Inhibitory effects on human plaque formation	
Funoran	 Funoran	*Gloiopeltis furcata*	Inhibition of mutans streptococci adherence and desorption effects	Sasaki et al. (2004) and Sato et al. (1998)
			Decrease in plaque and caries formation in mice infected with *S. sobrinus*	
			Dental plaque formation is seen to fall	

Apigenin, kaempferol, *tt*-farnesol	 Apigenin 	Propolis	Planktonic and biofilm cells of *S. mutans* are affected by the its antimicrobial activity	Ikeno et al. (1991) and Koo et al. (2000, 2002a, b, 2003, 2005)
			Disruption of acidogenic/aciduric properties of planktonic and biofilm cells of *S. mutans*	
			Inhibitory effects on Gtf activity and *gtfBCD* gene expression	
			Reduction of formation of *S. mutans* biofllms and EPS content	
			Reduction in caries development in rats infected with *S. mutans* or S. *sobrinus*	
			Decrease in dental plaque build-up and its EPS matter	
Low-molecular-weight chitosans, chitooligosaccharide, water-soluble chitosans	 Chitosan	Shells of crustaceans	Planktonic and biofilm cells of *S. mutans* are affected by the its antimicrobial activity	Tarsi et al. (1997), Choi et al. (2001), Fujiwara et al. (2004), Bae et al. (2006), Hayashi et al. (2007), and Busscher et al. (2008)
			Hinders the adherence of *S. mutans*	
			The viability of bacteria is decreased and therefore dental plaque and salivary mutans streptococci counts drops	

(continued)

Table 24.2 (continued)

Putative active constituents	Examples of chemical structure of main putative active constituents	Source of natural products	Biological activity	References
Lenthionine, disulfide derivative, oligosaccharides	 Lenthionine	Shiitake *(Lentinus edodes)*	Planktonic and biofilm cells of *S. mutans* are affected by the its antimicrobial activity	Hirasawa et al. (1999) and Shouji et al. (2000)
			Decrease in formation of biofilm and synthesis of water-insoluble glucan by mutans streptococci	
			Reduction in caries development in rats infected with *S. mutans*	
Polyphenols	Unknown	Oat hulls	Hinders the multiplication of *Lactobacillus acidophilus*	Vogel et al. (1962) and Stookey and McDonald (1974)
			Reduction in caries development in rats	
Polyphenols	Unknown	Hop bracts	Inhibitory effects on water-insoluble glucan synthesis by mutans streptococci	Tagashira et al. (1997) and Shinada et al. (2007)
			Reduction in human dental plaque formation	

24.10 Experimental Study

24.10.1 Insilico Approach on Molecular Docking

24.10.1.1 Pubchem

PubChem, is a component of the NIH's Molecular Libraries Roadmap Initiative, and is designed to provide information on biological activities of small molecules. Since the active components of most commercially available medicines are classified as small molecules (generally those with molecular weight less than 500 Da), these molecules are particularly important for functional studies. PubChem's integration with NCBI's Entrez search engine provides useful information with regard to structures including substructure, superstructure, similarity structure and bioactivity data. We have downloaded the ligand structure from pubchem database.

24.10.2 Chemsketch

Chemsketch is a molecular sculpting program used to generate and change descriptions of chemical organisations. This allows user to work with innovative features that permit the rotation of molecules and add colour to increase visualization.

24.10.3 Open Babel

Open Babel functions as an essential chemical aid which assists in designing to and converting chemical data. It's an open, cooperative development permitting individuals to explore, change, examine, or collect data from molecular demonstration, chemistry, solid-state materials, or other associated areas. As the structure of ligand is not in PDB format we use open babel to convert it into PDB.

24.10.4 PDB (Protein Databank)

Protein data bank is repository of protein molecule along with their crystallographic structure with various information regarding structural functions, family domains etc. We retrieved our receptor structure from here.

24.10.5 Swiss PDB Viewer

It is an application that allows us to examine many proteins simultaneously and has a user friendly interface. In order to comprehend structural alignments and associate their active sites or other relevant aspects, this program lets us superimpose proteins. Amino acid mutations, H-bond angles and distance between atoms are easy to obtain intuitive graphic and menu interface.

24.10.6 Patchdock

Patch dock performs structure prediction of protein-protein and protein-small molecule complexes. Two different molecules of any type are given as: proteins, DNA, peptides, drugs. The results obtained are potential options which are complexes categorised by shape complementarity standards. Molecular docking was done using the patch dock software. It is done to predict the binding of the receptor and the ligand.

24.11 Research Findings

The compounds chosen were used to find out its binding efficiency with an adhesion protein of *Streptococcus mutans* because of its significant role in the pathogenesis of the bacteria (Figs. 24.1, 24.2, and 24.3 and Table 24.3).

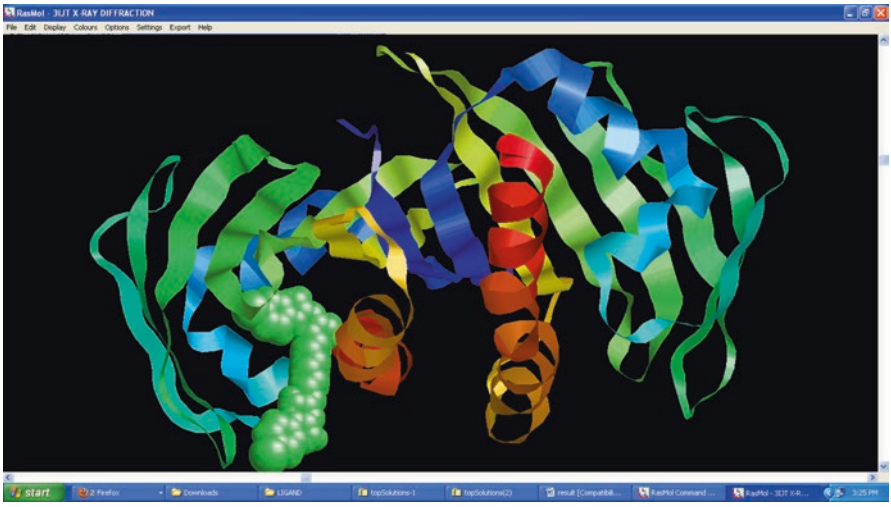


Fig. 24.1 Docking of adhesion protein of *Streptococcus mutans* with ligand chlorhexidine

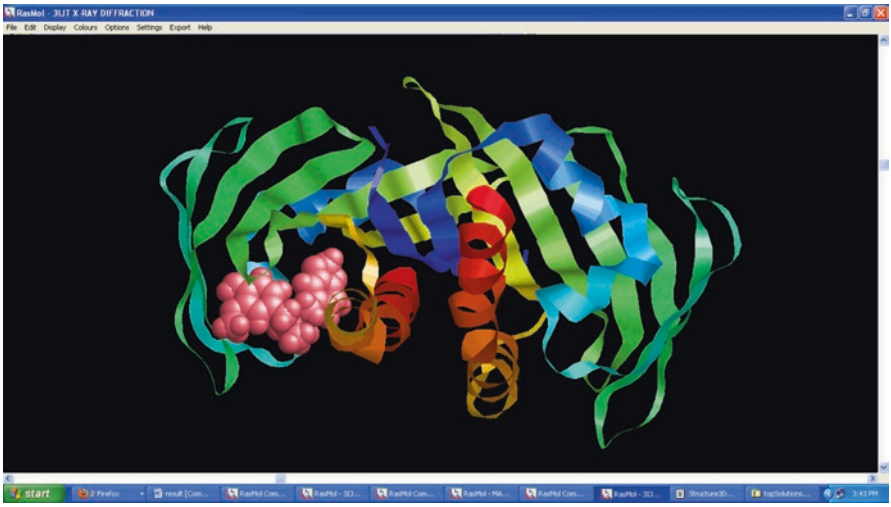


Fig. 24.2 Docking of adhesion protein of *Streptococcus mutans* with ligand macelignan

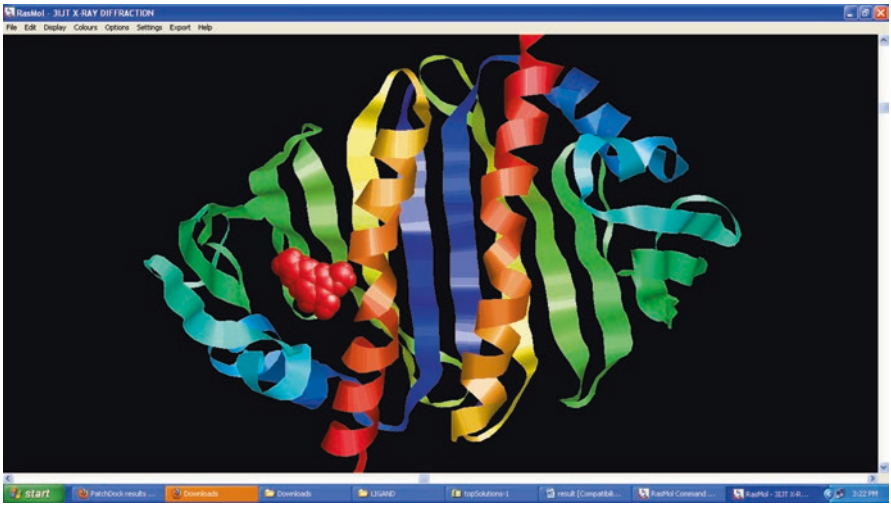


Fig. 24.3 Docking of adhesion protein of *Streptococcus mutans* with ligand eugenol

As per the docking result we could able to find out that the natural compound macelignan is as good as the chemical compound chlorhexidine. This compound has several side effects including staining of tongue and teeth, oral and throat irritation, increased tartar, dry mouth, dysgeusia, decreased taste sensation, tongue swelling and gingivitis. Hence the natural compound macelignan, present in cloves can be used to treat dental carries caused by *Streptococcus mutans.*

Table 24.3 Docking results: binding score for adhesion protein of *Streptococcus Mutans* with ligand chlorhexidine, macelignan and eugenol

Ligand	Binding score	Area	ACE transformation
Chlorhexidine	4958	568.60	1.58 −0.65 −1.64 16.16 92.58 47.26
Macelignan	4942	582.30	−156.16 −1.74 0.25 −1.75 16.86 84.14 39.00
Eugenol	2490	299.00	−70.32 2.33 −0.74 0.17 11.92 78.64 41.17

Solution No: Number of the solution, **Score:** Geometric shape complementarity score the solutions are sorted according to this score, **Area:** Approximate interface area of the complex, **ACE:** Atomic contact energy, **Transformation:** 3D transformation: 3 rotational angles and 3 translational parameters. The transformation should be applied on the ligand molecule

24.12 Conclusion

Dentists should be fully appraised of the benefits of these drugs and when they should be prescribed. However, antibiotics cannot and must not replace interventional procedures Indications for the use of these drugs as prophylactic measures are now reducing. Whilst new indications for the use of antibiotics become more widespread, all members of the healthcare professions need to be aware that these drugs have significant adverse effects and their misuse can lead to life-threatening conditions. It therefore is essential for dentists to be aware of the judicious practice of antibiotics and the risks and price one has to pay for its overuse and misuse. Since there are many natural compounds which has similar effects on dental pathogens but with minimal side effects can be prescribed by the dentist to cure these dental diseases.

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