CHAPTER TWO

Seaweed Metabolomics: A New Facet of Functional Genomics

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Abstract

Metabolomics is one of the emerging areas of functional genomics and provides newer insights in systems biology. The integration of metabolome with transcriptome analysis facilitates our understanding of gene functionality and its regulation in various metabolic pathways. Marine organisms have a variety of unique biological processes and adaptations suitable for their successful propagation. Marine macroalgae, known as 'seaweeds', are one of the economically important renewable resources of the oceans and have characteristic morphological and physiological processes quite different from terrestrial plants. Seaweeds being attached forms in intertidal region undergo periodic diverse chronic stresses arising from variations in desiccation, irradiance, ultraviolet radiation, salinity, tidal currents and others from anthropogenic activities. Despite the advancement in transcriptomics for seaweeds in recent times, the genetic regulations controlling various biochemical pathways are still in its inception and largely remain unexplored. The study of metabolomics provides a snapshot of cell's catalytic and regulatory processes and also deciphers metabolic responses involved in plant and environment interactions. While summarizing the recent advancements made in analytical technology platforms, a comprehensive review of metabolomics was prepared and discussed from the context of functional genomics, systems biology and biotechnology to gain newer insights into various regulatory networks functioning in the seaweeds.

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2.1 INTRODUCTION

Marine macroalgae are the large photoautotrophic multicellular benthic plants (occasionally free floating) occurring in marine environments and consist of as many as 25,000 species worldwide with considerable morphological and functional diversity (Holdt & Kraan, 2011). The continuous exploration of seaweeds for various chemicals of immense commercial value has significantly expanded their utility in agrichemicals, cosmetics, biomaterials, bioenergy and their long standing conventional utilisation as food, feed and hydrocolloids (Bixler & Porse, 2011; Holdt & Kraan, 2011). This has resulted spurt in seaweed production from 3.8 million tons in 1990 to whopping 15.8 million tons fresh in 2008 ranking them as one of the major mariculture crops with an annual market value over US$7.4 thousand millions. (FAO, 2010). Seaweeds represent a unique marine environment that is distinct from terrestrial one and experience various chronic stresses arising from fluctuations in various environmental factors such as desiccation, salinity, radiation, temperature and nutrients (Liu & Pang, 2010; Ross & Van Alstyne, 2007). Recently, transcriptome analysis has been employed to gain deeper insight into their evolution and adaptation to hyper-variable environmental conditions (Coelho, Simon, Ahmed, Cock, & Partensky, 2013; Collen, Guisle-Marsollier, Leger, & Boyen, 2007; Dittami et al., 2011; Dittami, Michel, Collen, Boyen, & Tonon, 2010; Dittami et al., 2009; Gravot et al., 2010; Heinrich, Valentin, Frickenhaus, John, & Wiencke, 2012; Pearson et al., 2010). The release of whole genome sequence data of a brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngbye and a red alga *Chondrus crispus* (Irish moss) revealed genome features that have been evolved in this group of organisms for their successful propagation and proliferation in coastal environment. For example, the *Ectocarpus* genome explored the presence of a complex photosynthetic system facilitating its propagation even in highly variable light conditions, flavonoid pathway genes homologous to plants synthesizing high phenolic contents protecting the alga from ultra-violet radiations and also an uncommon halide metabolism has been deciphered based on the presence of 21 putative dehalogenases and 2 haloalkane dehalogenases. Moreover, the genes and gene families associated with the development of multicellularity and evolution of the brown algal lineage have been identified (Cock et al., 2010). Similarly, the genome sequence of red alga has also elucidated metabolic adaptations pertaining to halogen metabolism, synthesis of oxylipins, microRNA and transcription factors for the development of multicellularity (Collen et al., 2013). This study has also revealed unique metabolic features that are otherwise part of bacterial and fungal metabolism (cellulose synthesis and

cell-wall remodelling) and are absent in genome of brown alga. A most recent study by Konotchick et al. (2013) conceptualises a depth-dependent physiology of seaweed by transcriptome analysis.

Though whole genome sequence studies explored some uncommon genomic features related to primary and secondary metabolism in seaweeds, the large part of the genomic information remained unannotated. Integration of data sets from functional- and comparative genomics with proteomics and metabolomics can generate a more accurate and holistic view of genes of unknown function (Xu, Ismail, & Ronald, 2014). A few studies have recently been carried out to understand the metabolic processes in response to salinity and oxidative stress in a brown alga linking with transcriptome data (Dittami et al., 2011; Gravot et al., 2010; Konotchick et al., 2013). These studies have revealed a few unknown pathways functioning under specific stress condition, and warranted for correlation of metabolites-genomic regulatory networks to disclose the specialised mechanisms, which are quite distinct from terrestrial counterparts.

Metabolomics is another addition to 'omic' techniques and presents the information of biological relevance as it reflects the immediate biochemical consequences of genomic and transcriptomic activity. In recent times metabolomics is gaining prominence in the area of integrated systems biology, and is emerging as an essential analytical tool in the post-genomic era (Blow, 2008; Hall 2006; Saito & Matsuda, 2010). Since the term 'metabolome' was coined in 1998, metabolomics has now become an ancillary high-resolution biochemical phenotyping tool to advance our understanding of primary and secondary metabolism. It has made unprecedented success in assessing responses to environmental stress, biomarker analysis, chemotaxonomy, comparing mutants and different growth stages, drug discovery, studying global effects of genetic manipulation, and natural product discovery (Higashi & Saito, 2013; Kim, Choi, & Verpoorte, 2011; Muranaka & Saito, 2013; Putri et al., 2013). The extensive metabolites information generated from various analytical platforms at different laboratories necessitated to frame international standards for metabolomics studies, most importantly on sample collection, precision in data interpretation and analysis. Sumner et al. (2007) proposed minimum reporting standards for chemical analysis. Subsequently, Nicholson and Lindon (2008) in Nature's Q&A news reported the facts and factual about metabolomics. Roessner and Bacic (2009) published a technical feature in Australian Biochemist on 'Dos and Don'ts of Plant Metabolomics'.

The discovery of genes by networking metabolite information with functional genomics information has led to the development of softwares

enabling to analyse the huge array of data generated from various analytical platforms, attributing their roles in metabolic pathway networks and fluxome (Table 2.1). This has also led to constitute various metabolite libraries (Table 2.2). The most encouraging example of biological component

Table 2.1 Detailed Description of Software Packages Used for Data Processing, Metabolite Signature Matching in Instrument Specific Manner as well as for Metabolic Pathway Mapping

Program	Web Site	Data Type
COLMAR	http://spinportal.magnet.fsu.edu/	NMR data
FiD	http://www.cs.helsinki.fi/group/sysfys/software/fragid/	Tandem mass spectrometry
MeltDB	https://meltdb.cebitec.uni-bielefeld.de/cgi-bin/login.cgi	GC-MS
MetaboloAnalyst	http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp	NMR/MS
MetaboMiner	http://wishart.biology.ualberta.ca/metabominer/	NMR
MolFind	http://metabolomics.pharm.uconn.edu/Software.html	HPLC/MS
Fiehn Lab	http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Structure_Elucidation/	NMR/MS
OpenMS	http://open-ms.sourceforge.net/	LC-MS
Peak alignment	http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Peak_Alignment/	NMR/MS
SetupX	http://fiehnlab.ucdavis.edu/projects/binbase_setupx/	GC-MS
Seven Golden Rules	http://fiehnlab.ucdavis.edu/projects/Seven_Golden_Rules	MS
XCMS	http://metlin.scripps.edu/xcms/	LC-MS
PRIMe	http://prime.psc.riken.jp/	NMR/MS
Metabolic Pathway Databases		
KEGG	http://www.genome.jp/kegg/	
MetaCyc	http://metacyc.org/	
BioCyc	http://biocyc.org/	
Reactome	http://www.reactome.org/	
BRENDA	http://www.brenda-enzymes.org/	
BioPath	http://www.molecular-networks.com/databases/biopath	
Biochemical Pathways	http://web.expasy.org/pathways/	
PathCase	http://nashua.cwru.edu/pathwaysweb/About.aspx#sb	
BioSystems	http://www.ncbi.nlm.nih.gov/Structure/biosystems/docs/biosystems_about.html	
Plant Metabolic Network	http://www.plantcyc.org/	

networking or co-expression analysis studies is the model plant *Arabidopsis* wherein transcriptome and metabolome analysis have successfully discovered the function of genes involved in various metabolic pathways (Quanbeck et al., 2012; Tohge et al., 2005). Such systems biology approaches have recently been gaining importance in medicinal plant research. A specialised metabolic pathway in plants is involved with synchronised

Table 2.2 Description of Databases Often Employed for Metabolites Identification for Metabolomics Studies

Database	Web Site	Data Type
The Human Metabolome Database	http://www.hmdb.ca/	Various spectral information
SetupX and BinBase	http://fiehnlab.ucdavis.edu/projects/binbase_setupx	GC-MS data
MetaboLights Database	http://www.ebi.ac.uk/metabolights/index	Various experimental studies
The BioMagResBank	http://www.bmrb.wisc.edu/metabolomics/	NMR spectra
The Madison Metabolomics Consortium Database	http://mmcd.nmrfam.wisc.edu/	MS and NMR data
MassBank	http://www.massbank.jp/	MS search
Golm Metabolome Database	http://gmd.mpimp-golm.mpg.de/	GC-MS search
METLIN	http://metlin.scripps.edu/index.php	MS/MS search
Fiehn Lib	http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/	GC-MS search
Birmingham Metabolite Library Nuclear Magnetic Resonance database	http://www.bml-nmr.org/	NMR search
mzCloud	https://mzcloud.org/	MS search
PRIMe Standard Spectrum Search	http://prime.psc.riken.jp/?action=standard_index	NMR and MS search
PRIMe MS2T	http://prime.psc.riken.jp/lcms/ms2tview/ms2tview.html	LC-ESI-Q-TOF/MS
HIFI	http://spectra.psc.riken.jp/menta.cgi/netcdf/index	LC-FTICR-MS
SpinAssign	http://prime.psc.riken.jp/?action=nmr_search	NMR search
AtMetExpress	http://prime.psc.riken.jp/lcms/AtMetExpress/	LC-MS

action of various genes. Decoding the information of those genes, gene regulatory networks and the association of gene-metabolite are of paramount importance to design metabolic engineering and synthetic biology approaches to have an organism producing high value chemicals in environmentally safe manner and with low input.

Metabolite profiling in seaweeds has been carried out for decades but restricted to targeted identification of one group of metabolites particularly lipids and their derivatives (Bouarab et al., 2004; Goulitquer, Potin, & Tonon, 2012; Kumari et al. 2013a, b). Mycosporine-like amino acids and halogenated compounds are yet another targeted metabolite families often used to study the stress responses in seaweeds (Kundel et al., 2012; La Barre, Potin, Leblanc, & Delage, 2010; Yuan, Westcott, Hu, & Kitts, 2009). The metabolomic information generated for seaweeds is at its infancy in comparison to the reference libraries of metabolites constructed for terrestrial plants and can be visualised from the publications released in last one decade in comparison to the same in terrestrial plants (Figure 2.1). Recently, an attempt has been made to constitute a reference library for seaweed metabolites (Davis & Vasanthi, 2011).

This review discusses the significance of co-expression studies integrating metabolomics information with genomics to derive a comprehensive view of biological system and specialised regulatory networks. An overview of the advances made on analytical platforms for metabolite analysis is also summarised. Further, the metabolomics is presented as a new facet to functional genomics approaches in seaweeds in order to draw a holistic view of their physiological regulations and responses arising due to highly dynamic marine environment.

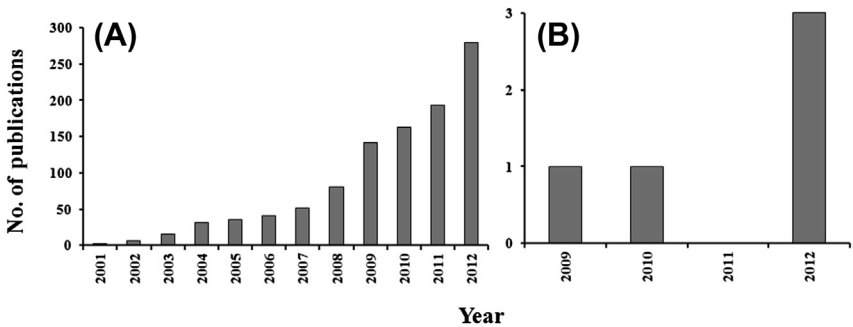

Figure 2.1 The number of publications on metabolomics studies on (A) plants and (B) seaweeds as obtained by searching web of knowledge with the key words 'metabolomics SAME plant' and 'metabolomics SAME seaweed' for former and later, respectively.

2.2 SAMPLE COLLECTION

The vast array of metabolites in the biological system coupled with their dynamic variations resulting from subtle environmental fluxes pose an incredible challenge to analyst while identifying metabolites. Metabolite profile varies with the diurnal fluctuation, availability of nutrients, variation in photosynthesis and respiration depending on the light (Davis, Fiehn, & Durnford, 2013; Dunn & Ellis, 2005; Ho, Tang, Hoefel, & Vigneswaran, 2012; Lee, Perdian, Song, Yeung, & Nikolau, 2012). Therefore, it is very crucial to fix the time and condition for plant collection before metabolite extraction. Utmost care has to be taken in such a way that all sampling should be done at the same conditions. Metabolites have dynamic exchange rates from one form to another thereby warranting for fixing the state of plant immediately after harvest and flash freezing is the best way. Freeze-drying of the harvested sample is another crucial step before metabolite extraction, as difference in water content will lead to biased quantification of metabolites. Later, sample extraction, which is to be optimised with respect to the sample type such as whole plant, specific cell type as well as according to the analytical platform to be used for analysis, is followed.

2.3 ANALYTICAL PLATFORMS FOR METABOLITE SEPARATION AND IDENTIFICATION

Because of the vast metabolite diversity, there have been 100,000 known and 200,000 unknown metabolites profiled through employing a wide range of analytical tools and techniques (Saito & Matsuda, 2010). The analytical platforms for metabolite analysis fractionate them based on their (1) different masses, (2) mass/charge ratio, (3) ionisation. Therefore, a range of analytical platforms are used for metabolite profiling and mainly includes gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), capillary electrophoresis–mass spectrometry (CE-MS), nuclear magnetic resonance (NMR) spectroscopy, Fourier Transform infrared and Raman spectroscopy. Among these, MS-based and NMR spectroscopic approaches are most popular. Most recently, matrix-assisted laser desorption/ionisation (MALDI) has also been introduced as another analytical platform for metabolite profiling (Ye et al., 2013). MS-based approaches are the most preferred techniques

for metabolite profiling because of their moderate to high sensitivity, high reproducibility and superior separation potential based on electron ionisation and charge to mass ratio.

GC-MS method has been the most popular and preferred analytical platform for metabolite profiling because it is rapid, reproducible and less affected with matrix effect. The only condition for GC-MS-based analysis is to vaporise the samples by derivatization mainly by silylation reaction. This step is considered to be the most crucial step and required to be optimised in terms of the volume ratios of pyridine × methoxyamine to N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), preferred is 1: 9 but the ratio of 1:2 or 1:1 have also been reported (Fiehn et al., 2008; Hill & Roessner, 2013; Lisec, Schauer, Kopka, Willmitzer, & Fernie, 2006). The utmost care has to be taken to avoid the moisture after derivatization of samples, which otherwise lead to abundance of ions m/z 221 and m/z 281 for in GC-MS spectra that is attributed to polysiloxanes arising from hydrolysis (Fiehn et al., 2008). After derivatization, most polar metabolites or metabolites with highly polar functional groups such as sugars, amino acids, amines and fatty acids can easily be identified by GC-MS. One limitation with trimethylsilyl derivatization is the low-ion abundance, therefore, required to be optimised for derivatization duration and in some cases the absence of the molecular ion ($M^{+\cdot}$), and in many cases an M-15$^{+\cdot}$ fragment ion. Precision in the mass of the molecular ion is important for the determination of molecular formula aiding to metabolite identification (Dunn et al. 2013). Another cautionary note applies to data analysis after obtaining the GC-MS spectrum. The GC-MS spectrum is highly complex and contains numerous overlapping peaks because of the co-elution of low abundant metabolites peaks. This necessitate for deconvolution of the spectrum. While deconvolution of the spectrum, defining thresholds for peak finding is most difficult task as it leads to either false negative or false positive signals for metabolites, if set to very high and very low values respectively (Fiehn et al., 2008). In addition to software available from instrument manufacturers, softwares freely available for deconvolution are Automated Mass Spectral Deconvolution and Identification System (http://chemdata.nist.gov/mass-spc/amdis/), and Metabolite Detector (http://md.tu-bs.de/). The metabolite libraries available for metabolite identification are NIST08, as generalised chemical library (http://chemdata.nist.gov/mass-spc/ms-search/), specifically for metabolites are the Golm Metabolome Database (Kopka et al., 2005) and FiehnLib (Kind et al., 2009).

LC-MS is another emerging platform for metabolomics studies preferably for semipolar metabolites and includes soft ionisation techniques

such as electrospray ionisation, atmospheric pressure chemical ionisation, atmospheric pressure photoionisation, and fast atom bombardment. The major advantages offered by this technique are no derivatization required and metabolites can be effectively extracted with aqueous alcohol solution and can directly be analysed mainly on reverse phase column. The hydrophilic interaction chromatography (HILIC) is the recent advancement in metabolomics studies, which gives more sensitivity and reproducibility in comparison to reverse phase (Gika et al. 2013). The LC-MS holds promising potentials of reproducibility in m/z values but significant variations may be observed in retention time and peak intensities. It is cautionary to run standard quality control (QC) sample every time with the sample. As retention times are prone to drift significantly, peak identification for LC-MS is based on centroiding over the m/z range generally performed on extracted ion chromatograms (EIC) (Tautenhahn, Bottcher, & Neumann, 2008). The instrument manufacturer software packages are enabled with direct acquisition of centroided data. EIC is 2D distribution intensity signals vs retention time over a small m/z interval. The other algorithms making their way for improving the peak picking include Mexican Hat wavelet analysis (Tautenhahn et al., 2008), a bi-Gaussian mixture model (Yu & Peng, 2010). Ion annotation is another critical factor for data analysis in LC-MS-based metabolomics studies because one metabolite shows many peaks in LC-MS with different m/z values. The reference mass spectral library and software packages for peak assignments for LC-MS data is under progress. A few software tools available publically for preprocessing and analysis of LC-MS data include Markerlynx, Mzmine, XCMS and MetAlign. The preprocessed results can be exported for further statistical analysis. LC therefore allows separation of compounds of wide range of polarity with little efforts comparative to GC based analysis. Advancement in LC techniques is ultra performance LC, which speeds up the run time and provides better chromatographic resolution (Flamini et al. 2013).

CE-MS has been shown as another technique with potentials for untargeted metabolite profiling for metabolomics studies. This is a complementary technique to LC, relatively inexpensive as it does not require expensive LC columns and also requires very small amount of samples and solvents. The separation of metabolites is based on the same principal of charge to mass ratio as in LC. Because of the small sample size required this technique suffers from poor sensitivity. The improvement in hardware configuration i.e. replacement of stainless steel needle for sample spraying with platinum needle resulted in better results for anionic compounds

in negative ionisation mode with improved sensitivity to about 63-fold and limit of detection (LODs) between 0.03 and 0.87 μM (Soga et al., 2009). The ionisation efficiency was further enhanced by the development of background electrolyte (BGE) and sheath liquid composition. Replacing ammonium acetate with triethylamine in BGE and sheath liquid was found to be effective for enhancing the metabolite ionisation as well as improved the coverage (Kok, de Jong, & Somsen, 2011). Moreover, Ramautar et al. (2012) developed a sheath less CE-MS method for the detection of polar and charged metabolites in human urine even at a scale of subnanomolar. Even then the application of CE-MS in metabolomic studies is limited compared to other MS-based approaches mainly because of lower sensitivity, resolution and coverage, and also less experience of researchers. The potential of this technique has been described in various reviews (Barbas, Moraes, & Villasenor, 2011; Monton & Soga, 2007; Ramautar, Demirci, & de Jong, 2006; Ramautar, Somsen, & de Jong, 2013).

MALDI-MS is another powerful tool to investigate the de novo imaging and dynamics of metabolites by direct analysis of the tissue (Kaspar, Peukert, Svatos, Matros, & Mock, 2011; Lee et al., 2012; Stoeckli, Chaurand, Hallahan, & Caprioli, 2001). Though matrix generates small interfering ionised peaks on desorption/ionisation process, matrix-free laser desorption/ionisation was developed and shown to be applicable to image UV-absorbing metabolites from plant tissues (Holscher et al., 2009). The metabolite imaging application was further improved with the development of various matrix-free surface-modified/functionalised techniques including metal and carbon materials (Cha et al., 2008; Jun et al., 2010; Northen et al., 2007; Woo, Northen, Yanes, & Siuzdak, 2008). More recently, Ye et al. (2013) showed the efficiency of MALDI matrix [1,8–bis (dimethyl-amino) naphthalene, DMAN] over conventional matrix 2,5–dihydroxybenzoic acid (DHB) revealing the better resolution and coverage with the identification of large array of organic acids, amino acids, sugars, lipids, flavonoids and their conjugates.

NMR Spectroscopy is another approach employed extensively for various metabolomics studies. Spectra of nuclei of atoms having odd atomic number (^{1}H) or odd mass number (^{13}C) is acquired by application of radio frequency excitation under strong magnetic field. An NMR spectrum consists of peaks characterised by their position, multiplicity, and width and all these parameters are governed by local electronic (or chemical) environment. Recently, NMR spectroscopy has shown extensive utilisation in plant metabolomics mainly for discrimination of species, ecotypes and mutants

(Broyart et al., 2010; Kim, Choi, Erkelens, Lefeber, & Verpoorte, 2005; Ward, Harris, Lewis, & Beale, 2003), transgenic plants from their wild type siblings (Barros et al., 2010; Charlton et al., 2004; Choi, Choi, et al., 2004), infected plants from healthy ones (Choi, Tapias, et al., 2004) and also led to understand the metabolic transitions during disease (Ward et al., 2010). It also allows detection and quantification of metabolites in a complex mixture without prior knowledge of sample composition even under *in vivo* state (Oikawa & Saito, 2012). According to Fernie, Obata, Allen, Araujo, and Bowler (2012) "NMR represents the gold standard in structure identification." The usefulness of NMR spectroscopy to both species management and monitoring of agricultural product quality (Ryan & Robards, 2006) led to compile a reference library of metabolites for terrestrial plants. Extracts prepared for NMR based metabolomics have delivered comparable spectra for multiple laboratories when standardised with respect to solvent type, pH and runtime temperature (Ward et al., 2010). The application of magic angle spinning is getting popularity to evaluate the physiological state of intact cells or tissues (Dunn & Ellis, 2005). Although NMR is less sensitive than MS, NMR offers the advantage of simpler and reliable extract preparation and measurements in nondestructive and nontargeted manner. The natural abundance of ^{1}H, ^{31}P, ^{19}F, ^{13}C and ^{15}N atoms is the limiting factor to the sensitivity of NMR spectroscopy. The recent developments in hardware configurations along with the availability of coil cooling with liquid helium or liquid nitrogen (to reduce the magnitude of noise generated by thermal motion of electrons) delivers sensitivity enhancement and considerably shorten the experimental time.

Another feature of NMR spectroscopy is the ease of comparative quantification of metabolites as the spectral peak intensity obtained is directly proportional to the quantity of metabolite present. The position of NMR peaks are determined by the chemical environment around those sites and thus the technique is better suited for mixtures containing different variety of compounds i.e. a mixture of amino acids, sugars, aromatics, fatty acid etc. With this, NMR spectroscopy can be an effective tool for chemical fingerprinting and fluxome studies. The assignment of peaks to respective compounds is a daunting task in NMR spectroscopy owing to often observed peak congestion. The assignment is facilitated by dispersing the 1D spectra to 2D experiments where correlations between chemically bonded pairs are acquired. The most commonly used 2D experiments are TOCSY (total correlation spectroscopy; ^{1}H-^{1}H correlation), HSQC (hetero-nuclear single quantum correlation; ^{1}H-^{13}C correlation) and HMBC (hetero-nuclear multiple bonds

correlation; ^{1}H-^{13}C correlation). TOCSY involves application of two pulses (preferably of 90° flip angle) wherein the duration between them is incremented to generate second dimension along with efficient mixing using a 17-pulse train designed by Levitt (2001). HSQC experiments involve excitation of proton magnetisation followed by Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) transfer to ^{13}C followed by evolution period to generate indirect dimension. Signal is often detected on ^{1}H channel after INEPT transfer from ^{13}C channel. J-Resolved spectroscopy is additional useful 2D experiment that delivers spectra free from J-coupling in the indirect dimension and is better suited for quantitative studies.

Among various solvents employed for extraction of metabolites for NMR-based studies, perchloric acid was shown to be the most suitable for extraction of polar metabolites but found to be unsuitable for acid-labile compounds like fructose-2,6-bio-phosphate, Nicotinamide adenine dinucleotide phosphate (NADPH) and Deoxyguanosine triphosphate (dGTP) (Kruger, Troncoso-Ponce, & Ratcliffe, 2008). A common extraction method employed for GC-MS analysis i.e. aqueous $CHCl_3$/methanol suffers from the inferior extraction efficiency for number of metabolites, e.g. amino acids and nucleotides in comparison to acid-based extraction. Two-phase extraction system including the aqueous methanol in combination with chloroform is also employed to yield polar and nonpolar metabolites (Choi, Choi, et al., 2004; Choi, Tapias, et al., 2004). The additional separation step while using two-phase extraction system is a limiting factor towards high-throughput analysis. Recently, Kim, Choi, and Verpoorte (2010) showed an extraction system with aqueous methanol as most suitable for metabolite profiling. The cautionary remark while extraction of metabolite is to stabilise pH of the extraction system whose fluctuation leads to broadening of peaks in ^{1}H spectrum. Phosphate buffers of pH 6 (Kim et al., 2010) and oxalate buffer of pH 4 (Pereira et al., 2005; Son et al., 2009) are some of the most commonly used buffering systems.

Authors have made an attempt to study metabolomics of seaweeds using NMR spectroscopy. This was initiated with the simplification of sample preparation together with NMR spectral acquisition. The aqueous extract obtained from the physical crushing of the alga representing the native state was analysed. While recording the spectra from the aqueous medium, pre-saturation pulse, a weak radio frequency pulse of long duration, was applied on H_2O peak to saturate the transition leading to its suppression for both 1D as well as 2D experiments (Figure 2.2). During this experiment a drop of D_2O containing Trimethylsilyl propanoic acid (TSP) was added for controlling the drift of magnetic field as well as an internal reference respectively. Application

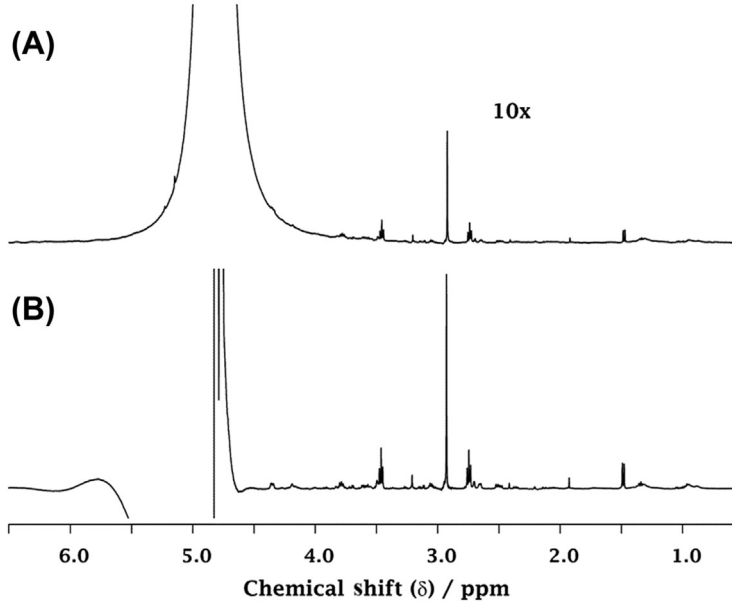

Figure 2.2 ^{1}H NMR spectra acquired from aqueous extract of *Ulva lactuca* after (A) single pulse excitation (B) pre-saturation block applied before single pulse excitation. Sensitivity gain by a factor of 10 was noticed however water peak sometimes distorted.

of pre-saturation block can distort baseline and magnetisation transfer via chemical exchange mediated saturation transfer and can affect quantitation of sugar residues in the seaweed biomass richer in such compounds.

Freeze-drying of samples is recommended for metabolite quantitation studies but in our study spectral information generated from the freeze-dried samples was found different from the spectra recorded from aqueous extract. Some of the spectral peaks were found missing from the former analysis (Figure 2.3).The peak information includes the signature for acetate and coniferaldehyde, which led to know some of the unexplored specialised features in algal species.The existence of coniferaldehyde indicated the existence of lignin precursors in seaweeds, which was then confirmed with additional experiment of derivatization followed by reductive cleavage method (Lu & Ralph, 1997). The chemical signature of acetate provided inferences towards the specialised regulatory mechanisms involved in mitigation of the effect of gaseous flux arising as a result of tidal rhythms (Gupta, Thakur, Reddy, & Jha, 2013). NMR spectra of algal species investigated by the authors seem to be quite distinct from the spectra reported in literature for terrestrial plants, thus indicating a difference in their metabolic complexity related to their distinct habitat.

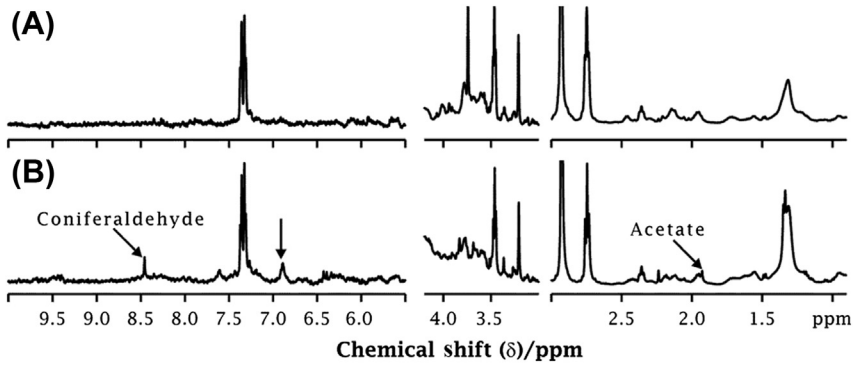

Figure 2.3 ^{1}H NMR spectra of *Ulva lactuca* (A) after freeze-drying and (B) direct aqueous extract. Arrows indicated the peaks found missing after freeze-drying. *(Source: Gupta et al., 2013).*

2.4 INTEGRATION OF METABOLOMICS DATA WITH FUNCTIONAL GENOMICS

Networking of multicomponent information generated from different 'omic' technologies led to a number of gene discoveries towards specialised mechanism in different model and nonmodel plants (Higashi & Saito, 2013). The most encouraging example of gene co-expression networking with metabolomics is the identification of genes responsible for anthocyanin biosynthesis (Tohge et al., 2005). A few more examples of gene-metabolite co-expression analysis are the identification of glycosyltransferase genes (Yonekura-Sakakibara, Fukushima, & Saito, 2013), putative bile acid transporter family protein involved in the transport of glucosinolate-related metabolites (Gigolashvili et al. 2009; Sawada et al. 2009) and genes involved in circadian clock network (Kerwin et al., 2011). The co-expression networking has now been progressed to single cell level and the example of the key findings extracted out from such studies are the role of D-enantiomer of ornithine as a selective regulator to L-arginine biosynthesis and urea cycle in tobacco cells (Gholami et al. 2013). Metabolic features of different root cell types in *Arabidopsis* (Moussaieff et al., 2013) and salt responsive network profile in rice suspension cell culture (Liu et al. 2013) are a few recent examples. Recent reviews by Higashi and Saito (2013), Seaver, Henry, and Hanson (2012), and Schilmiller, Pichersky, and Last (2012) provided exhaustive information on functional genomics of terrestrial plants by gene-metabolomics co-expression studies.

Integration of metabolite information with other functional genomics is based upon guilt-by-association principle (Saito, Hirai, & Yonekura-Sakakibara, 2008). This principle states that in a certain biological process at a given point of time a set of genes or metabolites are co-ordinately regulated reflecting a common regulatory system. One can designate the function of unknown gene or group of genes co-expressing in a biological function or during specific environmental stress. For example the stress induced by sulphur deficiency in *Arabidopsis* allowed predicting the genes coding for sulphotransferases (Hirai et al., 2005), two MYB transcription factors (Hirai et al., 2007), chain elongation enzymes (Sawada et al., 2009), and leucine biosynthesis and phenylpropanoid pathway as elicitors to adapt to the new environment (Caldana et al., 2011). Identification of cold stress-regulated raffinose by a transcription factor DREB, and dehydration-induced ABA-dependent responses (Urano et al., 2009), specialised metabolism particularly of flavonoids against exposure to UV radiations (Kusano et al., 2011), genes responsible for modifications of flavonoids (Tohge & Fernie, 2010; Yonekura-Sakakibara et al., 2008, 2007), elicitors for aliphatic glucosinolate biosynthesis (Hirai et al., 2007), isoprenoid pathway (Wille et al., 2004), brassinosteroid-regulated genes (Lisso, Steinhauser, Altmann, Kopka, & Mussig, 2005) and many more are the examples of integrative systems biology approach. Similar to model plants, co-expression analysis studies revealed secondary metabolite biosynthesis pathways and their regulatory genes and gene clusters in non-model plants such as cyanogenic glycosides in cassava, sorghum and *Lotus japonicas* (Takos et al., 2011; Takos & Rook, 2012); root of cannabinoid biosynthetic genes in *Cannabis sativa* (van Bakel et al., 2011); identification of diphosphate synthase from the tomato trichome (Schilmiller et al., 2009); tissue specificity of triterpene saponins including pharmacological glycyrrhizin in *Glycyrrhiza* (Seki et al., 2008, 2011).

A few attempts of co-expression analysis have also been made in seaweeds to understand various physiological adaptations inherited by this group of organisms but restricted to only one genus *Ectocarpus*. The study with *Ectocarpus* aimed at understanding the responses to salinity shown the active function of γ-aminobutyric acid synthesised through a salt stress-induced putrescine-degradation pathway (Dittami et al., 2011). Another study by the same research group on same alga showed impregnation of genomic alterations at metabolite level to stabilise the transition of evolutionary colonisation of alga from fresh water to marine habitat (Dittami et al., 2012). Even then much more need to be explored for seaweeds because of the

existence of uncommon physiological state and behaviour of seaweeds in comparison to their terrestrial counterparts. For example, the cell wall biosynthesis in seaweeds is a complex phenomenon as it composed of heteropolysaccharide agar and carrageenan in seaweeds belonging to red, alginates in brown and Ulvan in seaweed species of Ulvophyceae. A close understanding of biosynthesis mechanism of these hydrocolloids may help to initiate genetic manipulation studies to improve the seaweed for these traits. Likewise, the oxylipin biosynthetic machinery in brown seaweeds showed gene cluster homologous to both plant and animal. The sulphinic acid compounds and their derivatives reported by Gupta et al. (2013) in seaweeds are of great interest and their biosynthetic pathways need to be explored for exploring their role in free radical detoxification. A systems biology approach combining both gene and metabolite expression holds the potential to uncover adaptive mechanisms not revealed by whole genome sequencing.

In conventional approach, co-expression study involves simultaneous analysis of metabolomics and transcriptomics of an organism at a given point of time under specific physiological or environmental condition. A co-expression match between mRNA (gene) and targeted metabolite determines their homologous expression. Reverse genetic approach knocking out the gene then leads to identify responsible metabolites and its function. This leads to a discovery of relationship between gene regulatory networks with specialised metabolic pathway opening new avenues for metabolic engineering for the production of targeted specialised metabolites in seaweeds. Alternatively, modern synthetic biology approaches can reconstruct the specialised metabolic pathways *in vitro* for production of large amounts of desired metabolites.

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