Algal Research 57 (2021) 102353

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Review

Two-stage cultivation of microalgae for production of high-value compounds and biofuels: A review

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ARTICLE INFO

Keywords:
Microalgae
Two-stage cultivation
High-value compounds
Biofuels
Wastewater treatment
Techno-economic assessment

ABSTRACT

The biochemical composition of microalgae is significantly altered by growth conditions, thereby necessitating cultivation in precise culture conditions to synthesize biomass as feedstock for production of high-value compounds and biofuels. Nonetheless, culture conditions which promote rapid microalgal growth yields biomass with low concentrations of target metabolites (carotenoids, lipids, carbohydrates, etc.). Conversely, stress conditions introduced to trigger the induction of desired compounds in microalgal cells have an inhibitory effect on growth. Due to the contrasting conditions required for biomass production and accumulation of target compounds, a trade-off is often necessitated to increase the overall product yields.

Two-stage microalgae cultivation, wherein biomass growth and product accumulation are separated into two discrete steps, has been identified as a viable approach to enhance the productivity of target compounds. In the first stage, optimal growth conditions are provided to achieve high biomass productivities, followed by exposure of cells to stress conditions for accumulation of target metabolites in the second stage. Microalgae cultivation systems constitute of open or closed reactors operated in batch, fed-batch, continuous or semi-continuous modes; under photoautotrophic, heterotrophic or mixotrophic metabolisms. In two-stage cultivation, two such configurations are integrated sequentially to exploit the inherent advantages of distinct cultivation systems. Nonetheless, the design of two-stage systems should be application specific as optimal culture conditions of each stage are reliant on the microalgal strain and the desired output.

The present review provides an in-depth analysis on engineering approaches used for two-stage microalgae cultivation from an application-specific perspective, inclusive of discussion on techno-economic assessment and life cycle analysis of systems used for the biosynthesis of valuable compounds, generation of biofuel feedstock and wastewater bioremediation.

1. Introduction

Microalgal biomass is rich in valuable metabolites such as lipids, proteins and carbohydrates, as well as numerous high-value compounds including carotenoids, phycobiliproteins and polyunsaturated fatty acids (PUFA) [1,2]. Moreover, in comparison to conventional crops, microalgae possess favorable traits such as superior capability of fixing carbon, higher photosynthetic efficiencies and biomass productivities, as well as the non-requirement of arable land or freshwater for growth [3,4]. Therefore, microalgae have been recognized as a potential feedstock for sustainable production of food, feed, chemicals, fuel and energy in the context of a bio-based economy [1]. Microalgae can also be utilized for bioremediation applications such as phycoremediation of wastewater streams and sequestration of carbon dioxide (CO_2) emissions from flue gas [5–7].

Despite the multitude of plausible applications, the commercialization of a diverse spectrum of microalgal bioproducts is hindered by low product yields and high costs associated with biomass production and downstream processes [4,8]. Thus, microalgae-centric industries are generally limited to niche markets in food, feed, nutraceutical and

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https://doi.org/10.1016/j.algal.2021.102353
Received 16 December 2020; Received in revised form 24 April 2021; Accepted 28 April 2021
Available online 27 May 2021
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pharmaceutical sectors [1]. In order to exploit microalgae to their full potential, further research is required in terms of enhancing product yields and reducing overall costs. To this end, extensive research has been conducted on the development of novel cultivation systems and strategies.

Microalgal cultivation systems constitute of open or closed reactors operated in batch, fed-batch, continuous or semi-continuous modes; under phototrophic, heterotrophic or mixotrophic metabolisms [9]. These cultivation systems are employed to provide the desired physiochemical conditions (nutrients, salinity, light intensity, temperature, pH, etc.) for biomass production and synthesis of target metabolites [10]. The selection of a suitable cultivation system and physiochemical conditions for microalgae-based production is mainly influenced by the microalgal strain, source of nutrients, desired product and investment costs [11].

Fig. 1 illustrates a summary of physiochemical conditions employed for microalgal biomass production and biosynthesis of a several target metabolites. In general, growth conditions required to enhance the productivity of biomass is detrimental for the accumulation of desired metabolites, and vice versa [12,13]. Biomass productivity is improved by provision of conditions which increase the rate of cell proliferation, whereas the synthesis of desired metabolites such as lipids, carotenoids, carbohydrates, etc. is often enhanced under stress conditions such as nutrient deprivation, extreme irradiance, high temperature, etc. which cause inhibition or cessation of microalgal growth [14]. Hence, it is evident that contradictory conditions are required for the production of biomass and synthesis of target metabolites.

The cultivation of microalgae in two distinct stages has been identified as a solution for this issue [12]. In the two-stage approach, optimal conditions are provided for biomass production in the first stage, whereas stress conditions are administered for the accumulation of target compounds in the second stage [12,13]. The shift of cultivation from the first stage to the second is carried out by altering one or more of growth mode (photoautotrophy, heterotrophy, mixotrophy), operation mode (batch, semi-batch, fed-batch and continuous), physiochemical conditions (nutrients, light intensity, salinity, temperature and pH) and cultivation system (closed or open systems). Different combinations of the aforementioned systems could be incorporated to develop two-stage cultivation strategies for the production of various target compounds from microalgae, as well as to synthesize valuable biomass concurrent to wastewater treatment (Fig. 2). Furthermore, two-stage strategies would allow the integration of unconventional methodologies to improve product yields via elimination of bottlenecks in conventional cultivation processes.

Therefore, comprehensive analysis of techniques utilized for two-stage cultivation would be beneficial to discern potential approaches for the enhancement of product yields in existing industrial systems, as well as for the commercialization of novel bioproducts. Table 1 summarizes existing literature on reviews focusing on two-stage microalgae cultivation. Majority of these studies have focused on methodologies used for the generation of microalgal biomass as feedstock for biofuel production, whilst lower emphasis has been placed on comprehensively discussing potential strategies for the production of high-value compounds. Furthermore, the possibility of integrating multiple cultivation systems, metabolic modes, operating modes and physiochemical parameters has not been discussed holistically. In contrast, the current

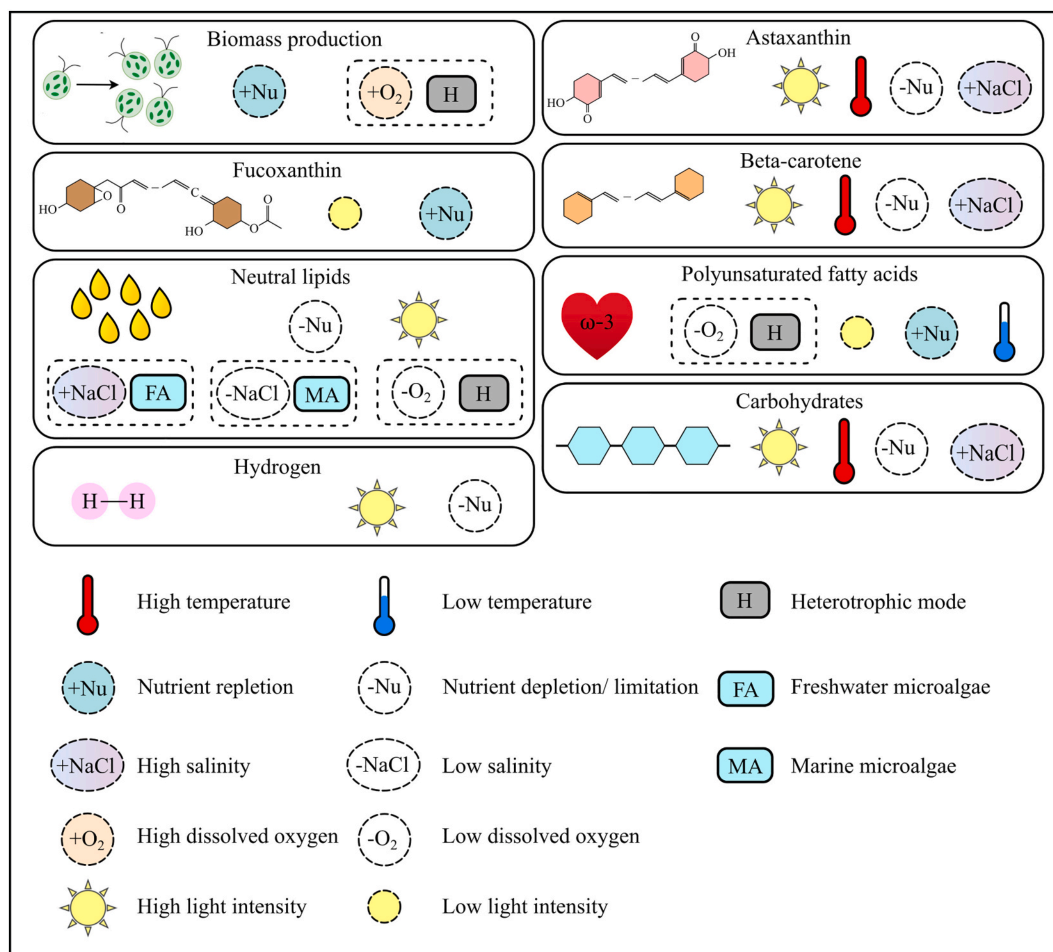

Fig. 1. Physiochemical culture conditions employed for microalgal biomass production and biosynthesis of target metabolites.

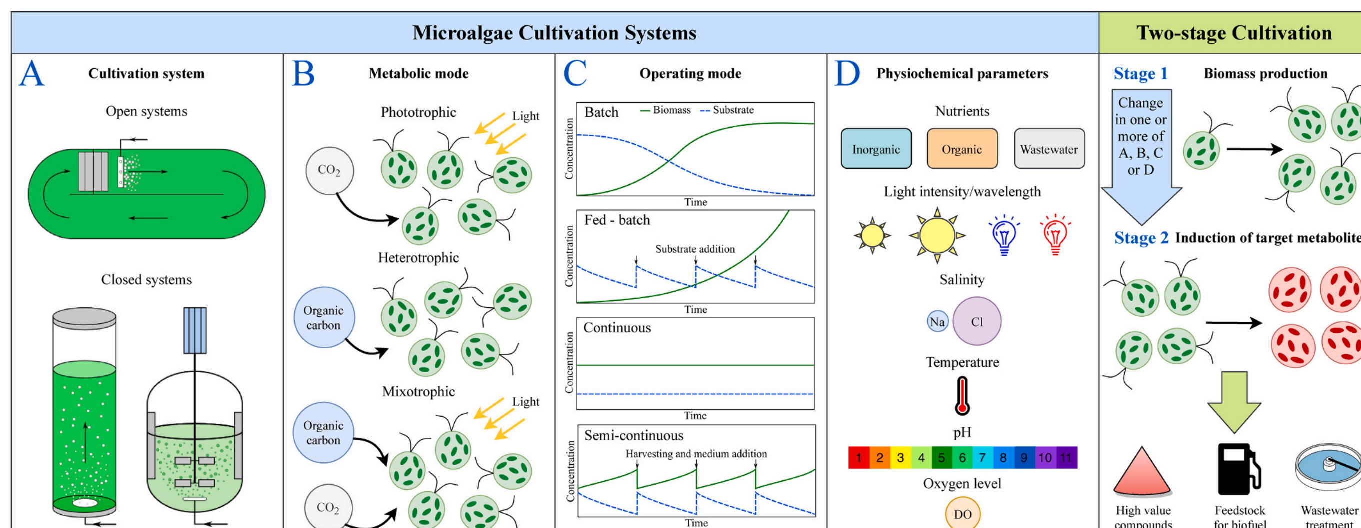

Fig. 2. Two-stage cultivation of microalgae via alteration of cultivation system, growth metabolism, operation mode and physiochemical conditions.

study aims to comprehensively discuss two-stage cultivation strategies using an application specific approach, with thorough analysis of techniques utilized for bioremediation of wastewater as well as synthesis of microalgal bioproducts such as carotenoids (astaxanthin, beta-carotene, lutein, etc.), lipids and carbohydrates. Accordingly, the present review provides an in-depth assessment of recent developments in two-stage microalgae cultivation, focusing on technical aspects, economic feasibility and sustainability.

2. Two-stage cultivation of microalgae

Cultivation of microalgae for the commercial production of bioactive compounds and biofuels is hindered by economic infeasibility due to the high cost of production and low product yields, especially under photoautotrophic conditions [21,22]. Hence, commercial cultivation of microalgae is limited to a few species capable of synthesizing lucrative compounds whose market value outweighs the cost of production, or robust strains capable of growth in low-cost cultivation systems with minimum risk of contamination [23]. Thus, extensive research has been performed to develop two-stage cultivation systems to enhance product yields.

2.1. Open/closed hybrid systems

The numerous systems which have been developed for the cultivation of microalgae can be broadly classified as open and closed cultivation systems [24]. Open systems such as raceway ponds (RWPs) are more economical with respect to capital requirements and operating costs as compared to closed systems such as photobioreactors (PBRs). However, open systems yield lower biomass productivities, and the ensuing cultures are more dilute than in closed systems, thereby resulting in higher harvesting costs as larger volumes of culture need to be processed [8]. Conversely, closed systems entail higher capital and operating costs, but offer superior control of culture conditions [13,25]. Therefore, cultivation in closed systems would yield higher and more reproducible volumetric product yields [1,25].

Two-stage cultivation systems which incorporate two sequential cultivation phases in both closed and open systems would enable the exploitation of the inherent advantages of each system whilst mitigating the adverse effects of their drawbacks. For example, a closed PBR could be utilized to yield higher biomass productivities under controlled conditions in the first stage, followed by a brief product accumulation phase in an open RWP at a lower operational cost [26]. In addition to obtaining biomass of the desired composition, two-stage cultivation systems which utilize closed-open hybrid systems would be less susceptible to culture contamination as compared to prolonged cultivation in an exclusively open system [27]. The contamination often occurs in the initial phase of growth, when culture densities are low. As the culture densities increase, the extracellular environments are also manipulated to favor the growth of the desired culture. For example, high pH conditions created by a dense microalgal culture reduce the risk of bacterial contamination. It has been reported that employing extreme conditions such as high pH and high salinity in the second stage enhances target compound productivity whilst mitigating the risk of contamination [18,28].

2.2. Metabolic switch

Two-stage cultivation systems can be utilized to exploit the advantages of different microalgal growth metabolisms. Since microalgae are inherently photosynthetic organisms, the photoautotrophic metabolism is the most commonly adopted mode for their cultivation. Photoautotrophy enables the use of freely-available sunlight for the generation of biomass and biosynthesis of light induced metabolites [29]. However, photoautotrophic cultivation is constrained by light attenuation in cultures as a consequence of self-shading of cells with increasing biomass growth. Thus, the biomass density is usually limited below 10 g/L under photoautotrophy, even in PBRs with minimal light paths [30]. The lower cell densities also increase the energy intensiveness of biomass harvesting, thereby increasing the cost of production [31]. Thus, the feasibility of employing photoautotrophic cultivation to generate microalgal feedstock to produce low-value products such as biofuels is questionable. In contrast, certain microalgal strains are capable of growth under heterotrophy, without the requirement of a source of light. In heterotrophy, organic substrates are used as the source of energy as well as the source of carbon for microalgal growth. Photoautotrophic and heterotrophic metabolisms occur simultaneously in mixotrophic cultivation, where microalgae proliferate by assimilating inorganic and organic carbon in the presence of light [32]. Heterotrophic or mixotrophic modes can be employed to achieve much higher cell densities (>100 g/L) in microalgal cultures, as biomass productivity is not limited by the light penetrability [33]. Since the heterotrophic mode does not require the illumination of reactors, microalgae could be cultivated in conventional bioreactors used for fermentation [34]. Hence, issues related to photoautotrophic cultivation of microalgae, such as the design of PBRs/open systems to achieve efficient illumination and cultivation conditions, can be overcome via heterotrophic growth [34,35]. However, as cultures are not exposed to light, the synthesis of high

Table 1
Reviews published on two-stage cultivation of microalgae.

Review	Methodologies used for two-stage cultivation	Products/applications of two-stage cultivation	Description
This review	HS[1], PC[2], MM[3]	Carotenoids/pigments, PUFA[4], lipids, carbohydrates, H_2[5], WWT[6]	• Two-stage cultivation is the primary focus of the review. • Two-stage cultivation has been discussed using a product/application-specific approach. • Various configurations used for two-stage cultivation of microalgae have been detailed. • Key factors affecting the techno-economic feasibility and environmental sustainability of two-stage cultivation strategies are discussed.
Koller et al., 2012 [15]	MM[3]	Carotenoids/pigments, PUFA[4], lipids, carbohydrates, H_2[5], WWT[6]	• Two-stage cultivation is briefly discussed in a sub-section detailing a case-study on *Nannochloropsis oculata*, cultivated via MM[3]. The applicability of this system for numerous applications is hypothesized. • Does not review primary literature on product/application-specific two-stage cultivation strategies.
Markou and Nerantzis, 2013 [16]	PC[2]	Carotenoids/pigments, PUFA[4]	• Two stage cultivation is briefly mentioned. However, it is not the main focus of the review. • The production of high-value compounds under stress conditions is detailed comprehensively. • Although biofuel production is mentioned in a biorefinery context, primary literature on two-stage cultivation for generation of biofuel feedstock is not reviewed.
Ho et al., 2014 [17]	PC[2]	Lipids, carbohydrates	• Two-stage cultivation is a brief subsection of the review, discussed in the context of manipulating cultivation conditions to synthesize lipid or carbohydrate rich biomass. • Primary focus is on discussion microalgal cultivation strategies for biofuel production. • Co-production of high-value products and simultaneous wastewater treatment is briefly mentioned, despite not being discussed in the context of two-stage cultivation.
Minhas et al., 2016 [18]	PC[2], MM[3]	Carotenoids/pigments, PUFA[4], lipids, carbohydrates	• The main focus of the review is on the manipulation of stress conditions to synthesize microalgal biomass as feedstock for production of high-value metabolites and biofuels. • Two-stage cultivation has been mentioned as a possible methodology to generate feedstock of the required composition, although is not explicitly discussed as a sub-section. • Accordingly, provision of stress conditions via the use of two-stage systems, and the use of heterotrophy-autotrophy regimes have been discussed. However, primary literature on two-stage methodologies (especially HS[1] and MM[3]) have not been discussed comprehensively.
Chen et al., 2017 [19]	PC[2], MM[3]	Carotenoids/pigments, PUFA[4], lipids, carbohydrates	• Main focus is on manipulating environmental stresses and stress tolerance of microalgae for enhanced production of lipids and value-added products. • Two-stage cultivation is a brief subsection of the review, discussed mainly in the context of manipulating stress conditions for production of lipids and carotenoids. • In depth analysis of different configurations of two-stage cultivation (especially HS[1] and MM[3]) is not presented with discussion on primary literature.
Sun et al., 2018 [20]	PC[2], MM[3]	Carotenoids/pigments, lipids	• Main focus of the review is on manipulation of stress conditions and adaptative laboratory evolution (ALE) techniques for lipids and carotenoid production. • Two-stage cultivation is discussed with focus on techniques used: abiotic stress supplementation and autotrophy/heterotrophy regimes. • However, an in-depth review of primary literature on two-stage cultivation strategies is not performed in a product/application-specific context.
Nagappan et al., 2019 [12]	HS[1], PC[2], MM[3]	Lipids, carbohydrates, H_2[5], WWT[6]	• Two-stage cultivation is the main focus of the study and is discussed with focus on biofuel production and wastewater treatment. • Primary literature on two-stage cultivation strategies employed for production of high-value products is not reviewed. • Future perspectives of two-stage cultivation is mainly discussed with consideration of technical aspects, without significant emphasis on techno-economic feasibility and environmental sustainability.
Aziz et al., 2020 [13]	HS[1], PC[2], MM[3]	Lipids, WWT[6]	• Two-stage cultivation is the main focus of the study and is discussed with focus on lipid production. • Primary literature on two-stage cultivation strategies employed for production of high-value products is not reviewed. Although integration of wastewater in microalgae cultivation is mentioned, an in-depth review of two-stage systems for phycoremediation is not presented.

[1] HS: hybrid systems with closed photobioreactors and open ponds.
[2] PC: alteration of physiochemical conditions.
[3] MM: Shift in metabolic modes.
[4] PUFA: polyunsaturated fatty acids.
[5] H_2: hydrogen.
[6] WWT: wastewater treatment.

concentrations of light-induced target metabolites (for example, carotenoids) may be limited under heterotrophy [9]. Two-stage approaches which integrate heterotrophy with other metabolisms have been developed as a workaround for this limitation. For instance, heterotrophic cultivation could be employed in the first stage to rapidly achieve high biomass densities [35], followed by photoautotrophic or mixotrophic cultivation in the second stage for the synthesis of light-induced target metabolites [36,37].

Nevertheless, several important considerations should be made when incorporating heterotrophic metabolism in two-stage microalgae

cultivation systems. Unlike photoautotrophic cultivation of microalgae which fixes CO_2, the heterotrophic metabolism emits CO_2 [38]. Thus, heterotrophic cultivation of microalgae is not viewed as a "green" process, unlike photoautotrophic cultivation. Moreover, in contrast to photoautotrophy, the use of selective conditions to minimize the growth of contaminants in open systems is impossible in heterotrophic cultures [33]. In fact, heterotrophic and mixotrophic cultures are more susceptible to contamination by bacteria due to the use of organic carbon sources in the culture media [34]. Therefore, the maintenance of axenic conditions during heterotrophic and mixotrophic cultivation of microalgae is vital [33]. Furthermore, the high cost of pure organic carbon sources such as glucose increases the production cost of microalgal biomass under heterotrophy and mixotrophy [39]. Thus, research focus has shifted to the use of low-cost alternatives such as crop flours, wastewater, liquid anaerobic digestate, waste from the food and dairy industries and hydrolysates of cellulosic or lignocellulosic materials [9,40,41].

2.3. Wastewater treatment via two-stage cultivation

The cultivation of microalgae in wastewater streams could lower the cost of organic carbon in heterotrophic/mixotrophic cultivation, whilst simultaneously treating the effluent and reducing the freshwater footprint of producing microalgae biomass [42,43]. The use of two distinct stages has numerous advantages during wastewater-based cultivation of microalgae, including the superior control of culture conditions, ability to exploit multiple metabolic modes [44,45], reduced susceptibility of bacterial contamination and enhanced productivity of target metabolites [43]. Furthermore, the efficacy of wastewater treatment, which is characterized by reduction of chemical oxygen demand (COD) and removal of nutrients, could be improved via re-cultivation of microalgae in the waste effluent from the first stage of cultivation [42,46]. Additionally, two-stage cultivation can be employed to exclusively focus on bioremediation of effluent in the first stage and stress induction in the second stage to obtain biomass of the required biochemical composition [47]. Providing stress conditions from the onset of wastewater-based

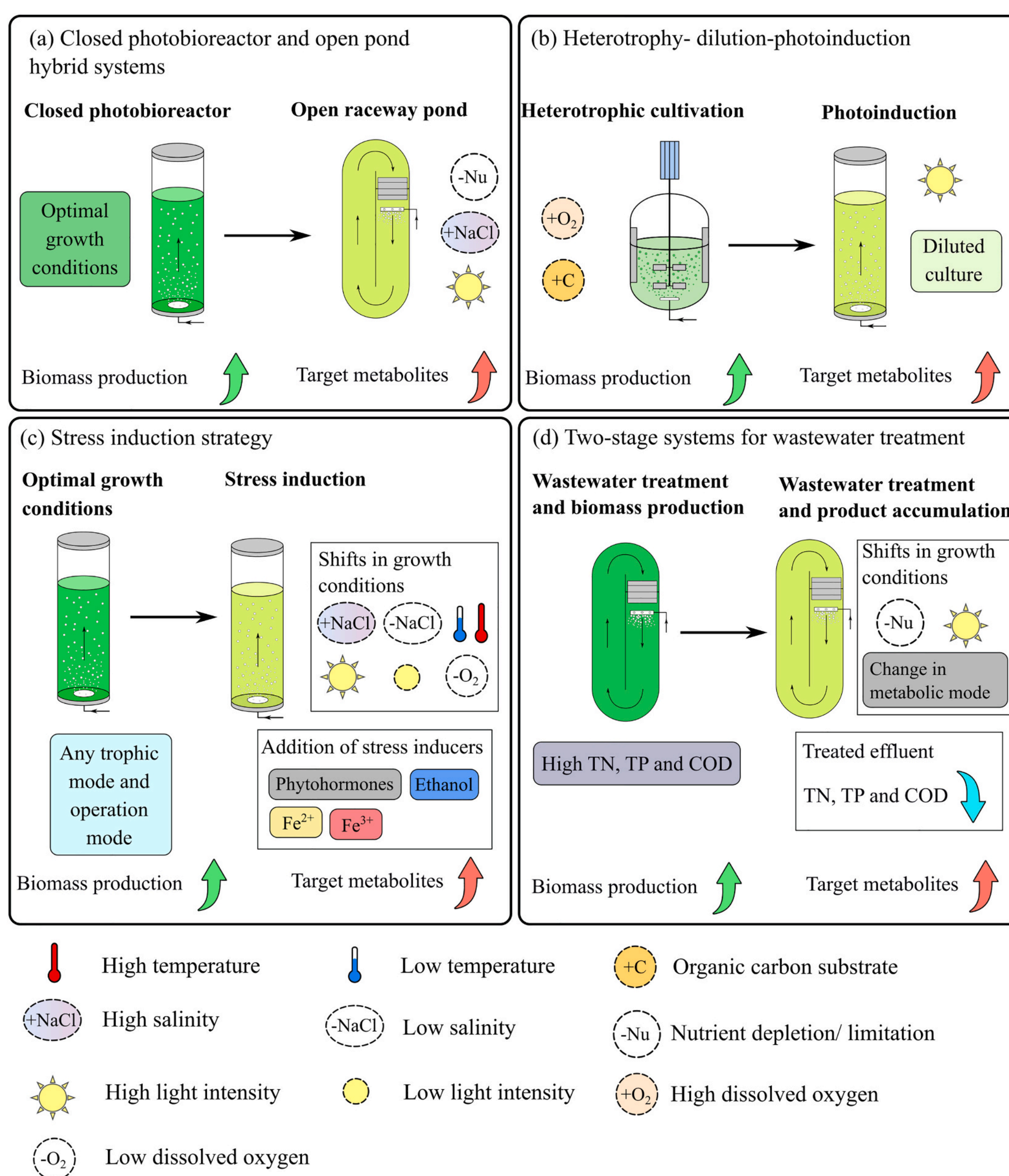

Fig. 3. Common methodologies of two-stage cultivation of microalgae: (a) closed photobioreactor and open pond hybrid systems, (b) heterotrophy-dilution-photoinduction, (c) stress induction and (d) two-stage systems for wastewater treatment.

cultivation may inhibit microalgal growth, and consequently result in low efficiencies in phycoremediation. Hence, two-stage cultivation is an attractive strategy for bioremediation of wastewater and simultaneous generation valuable biomass of the desired biochemical composition.

Some common methodologies employed for two-stage cultivation of microalgae for various applications are illustrated in Fig. 3.

3. Production of high-value compounds

High-value compounds are lucrative metabolites derived from microalgae, with applications in pharmaceutical, nutraceutical and cosmetics industries. Two-stage microalgae cultivation is often employed for production of such compounds including astaxanthin, beta-carotene, fucoxanthin, lutein and important PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Table 2 includes a detailed summary of various two-stage systems used to produce high-value compounds, including the cultivation conditions employed and the productivity of target metabolites.

3.1. Astaxanthin

Astaxanthin is a secondary carotenoid renowned for its excellent antioxidant capacity [76]. Consequently, it has numerous benefits and applications related to human health, including amelioration of inflammation, protection from UV-mediated photooxidation and treatment of age-related macular degeneration and Alzheimer's and Parkinson's diseases [77,78]. The microalga *Haematococcus pluvialis* is the most potent source of astaxanthin, typically accumulating it up to 3–5% of dry cell weight (and even exceeding 7% in specific conditions) [79–81].

Generally, cultivation of *H. pluvialis* for commercial production of astaxanthin is carried out in two-stage systems. The first stage emphasizes on the photoautotrophic production of vegetative cells under near-optimal growth conditions. Thereafter, stress conditions such as high irradiance, high temperature, nutrient depletion or increased salinity are provided in the second stage to induce astaxanthin accumulation [82]. The use of closed PBRs for biomass production followed by a brief carotenogenesis cycle in open ponds (known as “reddening” ponds) is a technique adopted by commercial producers such as Cyanotech Corp., USA [48,83]. Conversely, companies such as Algatechnologies Ltd., Israel employ closed systems of vertical flat panel reactors in greenhouses for biomass production, and outdoor tubular PBRs for biosynthesis of astaxanthin [84]. Producers such as AstaReal Inc., Sweden grow the microalgal inoculum in relatively small-scale glass reactors, before they are transferred to internally illuminated steel fermenters where growth conditions are manipulated to produce astaxanthin-rich biomass [83]. Regardless of the technique used, it is evident that two stages of cultivation which incorporate different cultivation systems and/or stress conditions is often used to produce microalgae-derived astaxanthin [85]. In fact, this is also true for most studies focusing on novel technologies for laboratory or pilot-scale production of astaxanthin by *H. pluvialis* (Table 2).

Studies reported in literature have demonstrated the feasibility of two-stage cultivation systems which employ various operating modes. Fábregas et al. [49] subjected semi-continuously cultivated cells to high light stress under batch conditions to achieve high astaxanthin productivities. Similarly, a study by Park et al. [50], showed that the combination of perfusion culture and stepwise irradiation had synergistic effects which led to improved biomass and astaxanthin concentrations in mixotrophically grown *H. pluvialis*.

It is well-established that subjecting the cultures to nutrient starvation and light stress is effective for carotenogenesis [80,86]. Numerous researchers have demonstrated that these techniques could be supplemented with other stress factors to enhance the biosynthesis of astaxanthin in *H. pluvialis*. Christian et al. [57] exhibited that the use of elevated CO_2 levels (up to 15%) in combination with light stress resulted in astaxanthin yields which were 2–3 times higher than individual stresses. Wen et al. [59] showed that periodic addition of ethanol to the culture media in tandem with nutrient deprivation and light stress resulted in a marked improvement of astaxanthin concentration. This was attributed to the formation of reactive oxygen species upon ethanol addition, which induces astaxanthin accumulation [59]. A similar mechanism was used by Hong et al. [56], who showed that supplementation of cultures with 50 μM Fe^{2+} in the product accumulation phase alleviated the issue of cell clumping during exposure to heat stress in the summer, thus increasing the astaxanthin productivity in outdoor cultures by 147% as compared to the spring. It was hypothesized that the formation of more reactive oxygen species via the iron (II)-catalyzed Haber–Weiss reaction may have consequently facilitated lipid peroxidation and synthesis of astaxanthin in microalgal cells.

Moreover, numerous studies in literature have indicated that compounds such as fulvic acid [52], methyl jasmonate, gibberellin A3 [53], salicylic acid [55] and jasmonic acid [54] could be employed to enhance the biosynthesis of astaxanthin in the second stage. It is plausible that the astaxanthin accumulation is promoted by the addition of such phytohormones due to the upregulation of metabolic pathways which lead to astaxanthin production [20]. Hence, further studies should focus on identifying alternative stress inducing agents and evaluating their cost-effectiveness as compared to existing methodologies. Two-stage cultivation strategies which incorporate the supplementation of cultures with stress inducing agents/additives would be beneficial to reduce capital and operating costs, as carotenogenesis could be induced without the transfer of cultures to a separate cultivation system.

Heterotrophic cultivation could also be adopted to enhance the productivity of astaxanthin from microalgae. Wan et al. [51], developed a novel strategy for the production of astaxanthin using a sequential heterotrophy–dilution–photoinduction process. The higher biomass concentration in the first stage of cultivation (26 g/L) and comparable cellular content of astaxanthin (4.6%) achieved using this method indicated that it could be a promising alternative to increase the productivity of large-scale systems as compared to conventional photoautotrophic cultivation [51]. Nonetheless, the maintenance of axenic conditions would be a significant challenge during heterotrophic cultivation, as the presence of organic carbon increases the risk of culture contamination with heterotrophic bacteria [34].

Since *H. pluvialis* exhibits slow growth rates, its cultivation is associated with the inherent risk of culture contamination [87]. In contrast, the robust microalgal strain *Chromochloris zofingiensis* can proliferate rapidly whilst accumulating astaxanthin, albeit at lower cellular concentrations than *H. pluvialis* [87]. Hence, researchers have employed two-stage cultivation strategies to identify the potential of *C. zofingiensis* for astaxanthin production. For instance, Chen and Wang [61] employed statistically optimized media for the two-stages of biomass production and astaxanthin accumulation in *C. zofingiensis*. Results of the study indicated that the astaxanthin yield from the two-stage cultures were approximately 74% and 15% higher than conventional batch and fed-batch cultures [61]. Furthermore, Zhang et al. [60] employed a novel PBR for production of astaxanthin from *C. zofingiensis* using the heterotrophy-dilution-photoinduction approach. Fed-batch cultivation with periodic glucose addition, and the combination of high light stress and nitrogen deficiency were used to achieve high astaxanthin productivities [60]. These studies showcase the potential of *C. zofingiensis* as a viable substitute to *H. pluvialis* in two-stage astaxanthin production.

3.2. Beta-carotene

Beta-carotene is a precursor of vitamin A and an antioxidant with applications in the food industry as a natural colorant. It has been reported that the dietary intake of beta-carotene may reduce the risk of developing chronic and age related diseases [88]. The halophilic microalga *Dunaliella salina* produces beta-carotene up to 14% by dry cell weight under stress conditions such as extreme salinity, high light intensity, high temperature and nutrient depletion [76,89]. However,

Table 2
Two-stage cultivation strategies employed for synthesis of high-value compounds in microalgae.

Microalgae strain	Freshwater/ marine species	Product	Two-stage cultivation strategy		Target compound concentration/ productivity	References
			First stage	Second stage		
Haematococcus pluvialis	Freshwater	Astaxanthin	Photoautotrophic cultivation in PBRs[a] under near-optimal growth conditions	Carotenogenesis in RWPs[b] under stress conditions	>1.5% w/w	[48]
Haematococcus pluvialis CCAP 37/4	Freshwater	Astaxanthin	Photoautotrophic semi-continuous cultivation in closed PBRs[a] under low light intensity (40 μmol/m^2/s)	Photoautotrophic batch cultivation in closed PBRs[a] under increased light intensity (240 μmol/m^2/s)	9.6 mg/L/d	[49]
Haematococcus pluvialis NIES-144	Freshwater	Astaxanthin	Mixotrophic perfusion culture (carbon source: acetate) under standard nitrogen concentration and stepwise irradiation (120-150 μmol/m^2/s)	Mixotrophic batch cultivation (carbon source: acetate) under nitrogen depletion and stepwise irradiation (150–450 μmol/m^2/s)	602 mg/L and 15.8 mg/L/d	[50]
Haematococcus pluvialis ZY-18	Freshwater	Astaxanthin	Heterotrophic batch cultivation (carbon source: acetate) in a fermenter under high nitrogen and low temperature (25 °C)	Dilution of heterotrophic culture (carbon source: acetate) and cultivation in PBR[a] under 100 μmol/m^2/s at 28 °C until nitrogen depletion; followed by cultivation under nitrogen depletion and increased light intensity (250 μmol/m^2/s)	4.6% and 6.4 mg/L/d	[51]
Haematococcus pluvialis	Freshwater	Astaxanthin	Optimum growth conditions for biomass production	Use of stress inducing agents (fulvic acid, methyl jasmonate, gibberellin A3, jasmonic acid and salicylic acid) for astaxanthin accumulation	Increased astaxanthin accumulation compared to control cultures	[52–55]
Haematococcus pluvialis NIES-144	Freshwater	Astaxanthin	Photoautotrophic outdoor cultivation in nitrogen-replete media and low light intensity (25–45 μmol/m^2/s)	Photoautotrophic outdoor cultivation in nitrogen-deplete media and high light intensity (315-380 μmol/m^2/s), with Fe^{2+} supplementation (50 μM)	40.5 mg/g and 5.5 mg/L/d	[56]
Haematococcus pluvialis UTEX 2505	Freshwater	Astaxanthin	Photoautotrophic batch cultivation under low light intensity (80 μmol/ m^2/s)	Photoautotrophic batch cultivation under increased light intensity (300 μmol/m^2/s) and increased CO_2 (15 %)	36 mg/g astaxanthin	[57]
Haematococcus pluvialis UTEX 2505	Freshwater	Astaxanthin	Photoautotrophic cultivation for vegetative biomass growth until exponential phase	Mixotrophic cultivation with the addition of 100 mM potassium acetate as the carbon source	10.2 mg/L/d	[58]
Haematococcus pluvialis NIES-144	Freshwater	Astaxanthin	Photoautotrophic batch cultivation under low light intensity (25 μmol/ m^2/s) and nitrogen repletion	Fed-batch cultivation under increased light intensity (150 μmol/m^2/s) in nitrogen-free media with periodic ethanol supplementation	138.7 mg/L	[59]
Chlorella zofingiensis ATCC 30412	Freshwater	Astaxanthin	Heterotrophic batch cultivation in indoor conditions with glucose as the carbon source	Culture dilution, followed by mixotrophic fed-batch cultivation with periodic glucose addition (carbon source) and nitrogen depletion in outdoor "rotating floating" PBR[a] under high light intensity (200-1500 μmol/ m^2/s)	5.3 mg/L/d	[60]
Chlorella zofingiensis ATCC 30412	Freshwater	Astaxanthin	Heterotrophic cultivation in growth media optimized for biomass production (with 46.7 g/L glucose as a carbon source, and 1.13, and 0.125 g/ L of $NaNO_3$ and MgSO4·7H_2O)	Heterotrophic cultivation in growth media optimized for astaxanthin accumulation (with 35.2 g/L glucose as a carbon source, and 0.281, and 0.023 g/L of $NaNO_3$ and $MgSO_4$·7H_2O)	0.9 mg/g and 15.1 mg/L	[61]
Dunaliella bardawil ATCC 30861	Marine	Beta-carotene	Photoautotrophic outdoor cultivation in small RWPs[b] for biomass production under nitrogen-replete conditions	Culture dilution, followed by photoautotrophic outdoor cultivation in large RWPs[b] for carotenoid production under nitrogen-deplete conditions	Stage 1–450 mg/m^2/ d; Stage 2–300 mg/m^2/ d	[62]
Dunaliella salina	Marine	Beta-carotene	Photoautotrophic cultivation in outdoor RWP[b] under nitrogen repletion (5 mM KNO_3) at a salt concentration of 2 M NaCl	Photoautotrophic cultivation in outdoor RWP[b] under nitrogen limitation (0.1 mM KNO_3) and increased salinity (2.5 M NaCl)	7.1 mg/L	[63]
Dunaliella salina CCAP 19/18	Marine	Beta-carotene	Photoautotrophic cultivation in flat panel PBR[a] under low light intensity (200 μmol/m^2/s) with turbidostatic control	Photoautotrophic cultivation in flat panel PBR[a] under increased light intensity (1400 μmol/m^2/s) with turbidostatic control	37 mg/L/d	[64]
Scenedesmus incrassatulus CLHE-Si01	Freshwater	Lutein	Heterotrophic batch cultivation (carbon source: glucose) in a stirred tank bioreactor	Photoautotrophic batch cultivation in airlift PBR[a] under a light intensity of 230 μmol/m^2/s	1.5 mg/g and 3.1 mg/L/d	[36]
Chlorella sorokiniana MB-1-M12	Freshwater	Lutein	Heterotrophic fed-batch cultivation under periodic addition of glucose as a carbon source, maintained at 2.0–7.5 g/L	Heterotrophic semi-batch cultivation (carbon source: glucose) where 50% of culture volume was replenished with fresh growth media	5.9 mg/g and 16.2 mg/L/d	[65]
Chlorella sorokiniana MB-1	Freshwater	Lutein	Mixotrophic semi-batch cultivation under 2.5% CO_2 and acetate under 100 μmol/m^2/s light intensity	Photoautotrophic batch cultivation under 2% CO_2 and increased light intensity (150 μmol/m^2/s)	7.6 mg/L/d and 3.9 mg/g	[66]
Scenedesmus obliquus FSP-3	Freshwater	Lutein	Photoautotrophic cultivation under high light intensity (300 μmol/m^2/s) until 95% nitrogen consumption	Photoautotrophic cultivation in closed under decreased light intensity (75 μmol/m^2/s) until nitrogen starvation	4.2 mg/L/d and 4.7 mg/g	[67]

(*continued on next page*)

Table 2 (*continued*)

Microalgae strain	Freshwater/ marine species	Product	Two-stage cultivation strategy		Target compound concentration/ productivity	References
			First stage	Second stage		
Nitzschia laevis UTEX 2047	Marine	Fucoxanthin	Heterotrophic fed-batch cultivation (carbon source: glucose) in fermenter	Mixotrophic batch cultivation (carbon source: glucose) in bubble column PBR illuminated by white and blue light in the ratio of 1:1	16.5 mg/L/d	[37]
Spirulina platensis AG20590	Freshwater	Phycocyanin	Photoautotrophic batch cultivation under red (660 nm) and blue light (450 nm) at a gradually increasing light intensity (from 75 to 100 μmol/ m^2/s)	Photoautotrophic batch cultivation under blue light (450 nm) at increased light intensity (150 μmol/m^2/s)	1.3 mg/mL	[68]
Arthrospira maxima LJGR1	Freshwater	Phycocyanin	Photoautotrophic cultivation in RWP[b] under outdoor conditions (year-round cultivation)	Controlled cultivation in mini-RWP[b] under blue light (450 nm) at a light intensity of 450 μmol/m^2/s	130 mg/g during end-summer	[69]
Arthrospira platensis SAG 21.99	Freshwater	Phycocyanin	Batch cultivation under 50% POME[c] and high nutrients (urea)	Semi-continuous cultivation with 30% culture media replacement using 100% POME[c] supplemented 800 mg/L urea	4.4 mg/L/d	[70]
Schizochytrium limacinum SR 21	Marine	DHA	Heterotrophic cultivation (carbon source: glycerol) in fermenter with controlled DO[d] levels (50%)	Heterotrophic cultivation cultivation (carbon source: glycerol) in flasks in low-DO[d] environment	6.6 g/L	[71]
Schizochytrium sp. HX-308	Marine	DHA	Heterotrophic fed-batch cultivation (carbon source: glucose) in fermenter under a volumetric oxygen mass transfer coefficient K_La[e] of 150 L/h	Fed-batch cultivation in fermenter under a decreased K_La[e] of 150 L/h	17.7 g/L, 111 mg/L/ h	[72]
Crypthecodinium cohnii ATCC 30772	Marine	DHA	Heterotrophic fed-batch cultivation (carbon source: dark fermentation effluent consisting of volatile fatty acids) in a fermenter with nitrogen-replete feed	Heterotrophic fed-batch cultivation (carbon source: dark fermentation effluent consisting of volatile fatty acids) in a fermenter with nitrogen deficient feed	1.8 g/L	[73]
Nannochloropsis sp. CCNM 1081	Marine	EPA	Photoautotrophic cultivation in stirred tank PBR[a] under 100 μmol/m^2/s light intensity at 25 °C	Photoautotrophic cultivation in flask under low light intensity (30 μmol/m^2/ s) and temperature (10 °C)	~11 mg/L/d	[74]
Phaeodactylum tricornutum 2038	Marine	EPA	Photoautotrophic cultivation at 25 °C	Photoautotrophic cultivation under lowered temperature (10 °C)	6.6 mg/L	[75]

[a] PBR: photobioreactor.
[b] RWP: raceway pond.
[c] POME: palm oil mill effluent.
[d] DO: dissolved oxygen.
[e] K_{La}: volumetric oxygen mass transfer coefficient.

unlike the production of astaxanthin from *H. pluvialis*, the cultivation of *D. salina* for commercial-scale beta-carotene production generally uses a single-step process. Typically, these cultivation systems are hypersaline open ponds exposed to high temperatures and light intensities, whose extreme conditions limit the risk of contamination. It has been reported that salinities employed for cultivation are selected to achieve a compromise between biomass production and carotenoid synthesis. Although the microalgal growth rate and cellular carotenoid content are lower than their maximum values, this strategy ensures the maximum productivity of beta-carotene [83].

Nonetheless, despite of the commercially adopted single-step strategy, numerous studies focusing on two-stage cultivation of *Dunaliella* sp. for production of beta-carotene have been reported (Table 2). In one such study, Ben-Amotz [62] cultivated *Dunaliella bardawil* in nitrate-rich media in a small nursery pond to attain optimal cell growth. Thereafter, cells were transferred to large ponds and diluted with nitrate deficient media to one third of their volume. Beta-carotene productivities were increased by 125% and 50% in first stage and second stage respectively, as compared to single stage cultivation [62]. Lamers et al. [89], also observed that the beta-carotene productivity increased up to 18.5 mg/L/d upon feeding of nitrogen depleted media to cultures. The same researchers have demonstrated that the sudden increase in light intensity was a more potent strategy to achieve higher yields of beta-carotene (37 mg/L/d) [64]. However, it is noteworthy that the nitrogen deprivation strategy was more energy efficient as compared to provision of light stress, which demonstrated that evaluation of the cost of production is necessary to determine the most effective two-stage cultivation strategies. Lamers et al. [89] also remarked that the productivity of beta-carotene under both strategies were significantly higher than existing commercial-scale cultivation systems (i.e. approximately 1.5 mg/L/d), indicating the potential for improvement in the industry. Moreover, in a two-stage method employed by Tafreshi and Shariati [63] for cultivation of *D. salina* in outdoor open ponds, a maximum beta-carotene concentration was obtained via the combined effect of nutrient starvation and upshift in salinity. Nonetheless, Coesel et al. [90] showed that the upshift in salinity was not as significant as high light stress or nutrient deprivation to enhance the biosynthesis of beta-carotene in *D. salina*. However, it is noteworthy that *D. salina* demonstrated the ability to retain its biomass production upon moderate shifts in salinity, which would enable its cultivation under hypersaline conditions to mitigate culture contamination in open systems [90].

3.3. Lutein

Lutein is a carotenoid pigment used in the food and feed industry due to its bright yellow color. It is an effective functional ingredient beneficial for ameliorating cardiovascular diseases, cancers and age-related macular degeneration [91]. Lutein is synthesized by microalgae such as *Scenedesmus* sp., *Mureillopsis* sp. and *Chlorella* sp. under high temperature, nutrient limitation and lower light intensities [18,20]. However, the cellular accumulation of lutein is comparatively lower in these species (0.2–0.7% of dry weight) as compared to astaxanthin in *H. pluvialis* and beta-carotene in *D. salina*, thus necessitating high biomass productivities for feasible lutein production [67]. Table 2 includes a summary of two-stage cultivation strategies employed for production of lutein from microalgae.

Ho et al. [67], studied lutein accumulation using a two-stage strategy in which a lower irradiance level and nitrogen depletion were utilized in

the second stage. A considerable increase in cellular lutein content of *Scenedesmus obliquus* was observed when the downshift in light intensity (from 300 μmol/m^2/s to 75 μmol/m^2/s) was administered at 70% nitrogen consumption. In microalgae, the cellular accumulation of light-harvesting carotenoids such as lutein is increased as a response to enhance the photosynthetic efficiency under the lower availability of light [92,93]. Nonetheless, the lower light availability for photosynthesis may have caused the inhibition of biomass production in the second stage, thereby resulting in an inferior lutein productivity as compared to single-stage cultivation [67]. Thus, the study reinforces the requirement of achieving a compromise between biomass productivity and target compound accumulation to maximize product yields.

Flórez-Miranda et al. [36], employed the heterotrophy-photoinduction strategy to improve biomass and lutein production in *Scenedesmus incrassatulus*. After 24 h of photoinduction, the lutein content increased to sevenfold that of the first stage, whilst the overall productivity of the two-stage heterotrophy-photoinduction system was 1.6-fold higher than the photoautotrophic control [36]. Chen et al. [65], observed that production of biomass and lutein in *Chlorella sorokiniana* under heterotrophic conditions was improved when using a novel two-stage system integrating fed-batch and semi-batch modes. The lutein content and productivity showed substantial improvement over single-stage batch cultures due to the increased biomass production under the two-stage strategy. Chen et al. [65], remarked that the two-stage strategy not only enhanced lutein yields, but was also more cost-effective. Furthermore, in a study by Chen and Liu [66], *C. sorokiniana* was cultivated in a two-stage mixotrophic system integrating two sequential stages of semi-batch and batch cultivation. The lutein productivity of the two-stage strategy showed a 85.9% enhancement over conventional single-stage batch cultivation [66]. Therefore, it is evident that two-stage cultivation strategies could be employed to take advantage of heterotrophic and mixotrophic metabolisms as well as various modes of operation to consequently achieve higher lutein yields as compared to conventional photoautotrophic cultivation.

3.4. Other pigments

Fucoxanthin is a carotenoid pigment with potential applications in the pharmaceutical and nutraceutical industries, owing to its antioxidant, anti-inflammatory, anti-cancer and anti-obesity effects [94]. Marine microalgal strains such as *Tisochrysis lutea*, *Odontella aurita* and *Phaeodactylum tricornutum* can accumulate fucoxanthin up to 0.5–2.5% of dry cell weight [95]. Lu et al. [37] employed high-density fermentation of *Nitzschia laevis* to achieve excellent levels of fucoxanthin productivity by means of a two-stage cultivation strategy. In the first stage, biomass production was enhanced using fed-batch cultivation under heterotrophic culture conditions. In the second stage, a mixture of blue and white light was supplied to cultures in batch mode to induce fucoxanthin under mixotrophy [37].

Furthermore, phycocyanin, which has anti-inflammatory, antioxidant, antitumoral and antibacterial effects [96], has also been produced using two-stage microalgae cultivation systems. Lee et al. [68], developed a two-stage system for cultivation of *Spirulina platensis*, yielding 1.28 mg/mL phycocyanin within two weeks of cultivation. Initially, cultures were illuminated with a combination of blue and red light to accelerate biomass growth. Subsequently, blue light was used to induce phycocyanin accumulation in the second stage [68]. The phycobilisomes of cyanobacteria (blue-green algae), which contain phycocyanin and aids in light-harvesting, do not absorb blue light. Consequently, more phycobilisomes (and by extension, phycocyanin) are synthesized upon cultivation under blue light [97]. Blue light was also employed for the production of phycocyanin from *Arthrospira maxima* in a two-stage bioprocess developed by García-López et al. [69]. It was noteworthy that the product yields were significantly affected by climatic conditions, since the first stage of cultivation was performed under outdoor conditions in RWPs. Thus, substantial phycocyanin induction was only observed in cultivation cycles subjected to favorable growth conditions during outdoor cultivation [69]. Moreover, a sequential batch and semi continuous system which incorporated the use of palm oil mill effluent (POME) was developed by Nur et al. [70], to produce phycocyanin from *Arthrospira platensis*. The two sequential stages of cultivation yielded a phycocyanin productivity which was comparable with control cultures cultivated under batch mode in standard media [70].

Considering the numerous studies reported in literature, it is evident that two-stage cultivation is a viable strategy, and often a necessity, for the production of high-value pigments. Nonetheless, most studies have been performed in relatively small scale, thus necessitating scaled-up cultivation and comprehensive techno-economic analyses to assess the feasibility of large-scale implementation.

3.5. Polyunsaturated fatty acids (PUFA)

Microalgae are rich sources of PUFA, including the omega-3 fatty acids EPA and DHA, which have significant health benefits including the improvement of cardiovascular health, lowering high contents of blood fat, reducing the risk of strokes, and positively aiding in the development of infants [98]. Microalgae-based PUFA have been viewed as a possibly sustainable alternative to fish oil [99]. Considering their market values, production of PUFA from microalgae would be a more attractive prospect for valorization of microalgal lipids, in comparison to biofuel production [100]. Therefore, there has been increasing efforts to develop technologies for microalgae-based production of PUFA [101], including the use of two-stage cultivation systems (Table 2).

Chi et al. [71] suggested that DHA production by *Schizochytrium limacinum* could be enhanced via two stages of heterotrophic cultivation. Higher DO levels were maintained to improve the cell proliferation in the first stage whilst DO levels were reduced to significantly enhance DHA accumulation in the second stage [71]. Similarly, Qu et al. [72], also demonstrated the applicability of the two-stage O_2 supply strategy to produce DHA from *Schizochytrium* sp. The DHA concentration was 63.88% higher than product yields obtained via single-stage cultivation [72]. Furthermore, Chalima et al. [73], studied the accumulation of DHA during heterotrophic cultivation of *Crypthecodinium cohnii*. The two-stage cultivation mode which used fed-batch cultivation with nitrogen feeding in the early exponential phase, and nitrogen starvation in the second stage resulted in slightly higher DHA concentrations as compared to the control.

Two-stage cultivation strategies under photoautotrophy have also been employed for the production of PUFA. Mitra et al. [74], studied the use of *Nannochloropsis* sp. for the production of EPA via the reduction of temperature and incident light intensity of cultures in the second stage. The two-stage system successfully increased the EPA yield by approximately 3.4 fold in the second stage [74]. Similarly, the temperature reduction strategy was also employed in a study by Jiang and Gao [75], who reported an 120% increment of EPA production in *P. tricornutum* as compared to the control. Nonetheless, further studies are required to assess the economic feasibility of this strategy as reducing the temperature of large volumes of culture by approximately 15 °C would be associated with high energy costs (especially under higher ambient temperatures). Moreover, it is evident that product yields of PUFA obtained via heterotrophy far exceeds the corresponding yields from photoautotrophic cultivation, and therefore reflects the more feasible avenue for production of microalgae-based PUFA [102].

4. Production of microalgal biomass as feedstock for biofuels

Microalgae produce high contents of lipids and carbohydrates which makes them a promising feedstock for the production of numerous biofuels such as biodiesel, bioethanol, biogas and pyrolysis products [17,103–105]. In the context of biofuel production, increasing the lipid content of microalgal biomass from 20–40% to 60% would ensue in a substantial reduction of capital costs associated with cultivation, as the

size of production facilities would be halved [13]. Therefore, in order to improve the feasibility of producing microalgae-based biofuels, various two-stage cultivation strategies which separate the biomass growth phase and the product accumulation phase have been employed, as summarized in Table 3.

4.1. Lipids

4.1.1. Two-stage cultivation strategies for lipid production

The use of hybrid cultivation systems which integrate both open RWPs and closed PBRs has been identified as a viable strategy for lipid production. Such systems aim to exploit the high biomass productivity of PBRs and low capital costs of RWPs whilst mitigating the inherent disadvantages associated with each distinct system. The two-stage hybrid cultivation system developed by Narala et al. [26] for lipid production using *Tetraselmis* sp. showcased significantly higher growth rates as compared to the single-stage systems of closed PBRs or open RWPs [26]. The hybrid system could effectively mitigate contamination issues associated with open systems, as the cultures were not held in the RWPs for a prolonged period of time. Nonetheless, comprehensive techno-economic analyses are required to assess the viability of employing such hybrid systems for the production of low value products such as microalgal lipids as biodiesel feedstock.

Numerous studies in literature have also focused on the synthesis of lipids via the use of abiotic stress conditions in the second stage. These include stimuli such as high irradiance levels, elevated temperature, shifts in salinity, nutrient deprivation, addition of lipid inducing agents, etc. Nitrogen starvation is a commonly used mechanism to trigger the accumulation of lipids in the second stage [106,108]. Lucas-Salas et al. [107] successfully enhanced the lipid productivity in *S. obliquus* by administering nitrogen starvation through the use of effluent from the first stage of cultivation as the input stream of the second stage PBR. Chu et al. [118] used a modified strategy wherein phosphorous was added from the onset of nitrogen depletion in the culture media to achieve a 2.2-fold increment of lipid productivity in *Chlorella* PY-ZU1. The converse approach of phosphorous deprivation is also used to enhance lipid accumulation in the second stage, as evident by the study conducted by Álvarez-Díaz et al. [133] using *Ankistrodesmus falcatus*. Aléman-Nava et al. [123] also showed that a 4-fold increase in lipid content of *Nannochloropsis oculata* was observed via the second stage of nitrogen and phosphorous starvation. The study also demonstrated that alkaline flocculation could be employed as a pre-harvesting strategy to transfer microalgae from nutrient replete media in the first stage to the nutrient deplete media in second stage [123].

The lipid accumulation potential could be further enhanced by integration of high light stress. For instance, Su et al. [111], employed the combined effect of nitrogen starvation and light stress in the second stage of *N. oculata* cultivation to achieve lipids yields which were 2.82 times higher than the control. Furthermore, Sun et al. [121] demonstrated that cultivation of *Isochrysis* sp. in two PBRs with different light paths was effective in enhancing lipid yields and mitigating the effect of photoinhibition in outdoor systems. A low-density inoculum was first grown in a flat plate PBR with a longer light path (7 cm), before being transferred to PBRs with a shorter light path (1.8 cm). The self-shading of cells due to higher biomass densities in the second stage alleviated the photoinhibitory effect of intense solar irradiation in the PBR with the shorter light path.

Additionally, the wavelength of the light source used for illumination of microalgal cultures has a significant effect on lipid accumulation. Jung et al. [119] demonstrated that green light (520 nm) was the most effective stress condition to induce lipids in microalgae, surpassing the performance of salt stress and nitrate depletion. The stress caused by green light is attributed to the lower light absorption ability of microalgae at the aforementioned wavelength, and the consequent decrease in photosynthetic efficiency [119]. This effect was also evident in a study by Ra et al. [134], where green light appeared to be a more capable light source for induction of lipids as compared to blue or red lights. Nonetheless, the cost effectiveness of employing different light sources should be evaluated.

Furthermore, numerous studies have demonstrated that salinity stress is effective in the synthesis of lipids in microalgal biomass [135,136]. For instance, Xia et al. [109] showed that the lipid production in *Scenedesmus obtusus* could be increased by approximately 1.2-fold by addition of 20 g/L NaCl in the second stage. The potential of this strategy in scaled-up cultivation was demonstrated in an 140 L outdoor PBR, although the biomass productivity was reportedly lower due to fluctuation of weather conditions [109]. The duration of salt stress is a vital consideration to be made in the production of microalgal lipids. Prolonged salt stress (400 mM NaCl) up to 9 days resulted in the highest cellular lipid content of 31% in *Scenedesmus* sp., compared to 18.23% of the control cultures [124]. However, biomass production was significantly inhibited. In comparison, the overall lipid yields could be enhanced as compared to the control by limiting the salt stress to either 3 or 6 days, thus achieving a compromise between lipid induction and biomass production [124]. In contrast to salt addition, a study conducted by Ra et al. [112] demonstrated that the lipid content of numerous marine microalgal strains could be enhanced via the reduction of salinity [112].

Two-stage cultivation strategies which utilize various lipid inducing agents have also been reported in literature. For instance, the lipid contents of *Monoraphidium* sp. and *Dunaliella tertiolecta* were increased by the addition of fulvic acid, triethylamine and sodium azide in the second stage of cultivation [110,113,117]. In a study by Sun et al. [106], Fe^{3+} supplementation was used in tandem with nitrogen starvation and high light intensity to enhance the lipid accumulation of *Neochloris oleoabundans* in the second stage.

Different metabolic modes have also been exploited in two-stage cultivation to improve biomass and lipid productivity. Yen and Chang [126] showed that a two-stage cultivation under photoautotrophy followed by mixotrophy could increase biomass growth and reduce the risk of contamination. The heterotrophy–dilution–photoinduction strategy, previously discussed in Sections 3.1 and 3.3, has been applied to significantly enhance the lipid production in various microalgal strains of the genus *Chlorella* [114,137]. Xiong et al. [115] used the converse approach of cultivating *Chlorella protothecoides* under the photoautotrophy, followed by heterotrophy or mixotrophy in the second stage to increase the lipid yield on glucose by up to 69% as compared to cultivation under pure heterotrophy [115]. The use of mixotrophy or heterotrophy in the second stage is advantageous as it alleviates the limitation of biomass density of cultures by mitigating the effect of self-shading.

Moreover, heterotrophic cultivation has also been utilized in two-stage approaches for lipid production without a shift in metabolism. For instance, Wang et al. [116], studied two-stage heterotrophic fed-batch cultivation of *C. protothecoides*, where the combined stress of low O_2 supply and nitrogen starvation was used to improved lipid productivity up to 175.2 mg/L/h. Moreover, it was demonstrated that the two-stage process thus developed could be integrated with photoautotrophic cultivation by recycling of CO_2 generated during the heterotrophic metabolism. A similar two-stage heterotrophic cultivation strategy which employed nitrogen depletion and hyperosmotic stress was developed for lipid production in *C. protothecoides* [120]. The lipid productivity and lipid content exhibited 1.60 and 1.92-fold improvements from single-stage fed-batch cultures without stress induction [120]. Moreover, Cui et al. [122] developed a two-stage mixotrophic cultivation strategy for cultivation of *Chlorella vulgaris*, wherein the addition of 8 g/L of sodium erythorbate in the first stage and aeration with 10% CO_2 in the second stage significantly enhanced biomass and lipid productivity up to 1.85 and 1.64 times that of photoautotrophic cultivation [122].

Table 3
Two-stage cultivation strategies utilized to generate microalgal feedstock for biofuel production.

Microalgae strain	Freshwater/marine species	Product	Two-stage cultivation strategy		Target compound concentration/ productivity	References
			First stage	Second stage		
Tetraselmis sp. M8	Marine	Lipids	Photoautotrophic semi-continuous outdoor cultivation in PBRs[a] in standard growth media	Photoautotrophic semi-continuous outdoor cultivation in RWP[b] under nutrient depletion	n.r[c]	[26]
Neochloris oleoabundans HK-129	Freshwater	Triacylglycerides and carbohydrates	Photoautotrophic batch cultivation under high nitrogen and low light intensity (100 μmol/m^2/s)	Photoautotrophic batch cultivation under nitrogen depletion, increased light intensity (200 μmol/m^2/s) and addition of Fe^{3+} (0.037 mM)	Triacylglycerides - 51.6 mg/L/d; Carbohydrates - 90.7 mg/L/d	[106]
Scenedesmus obliquus CCAP 276/3A	Freshwater	Lipids	Photoautotrophic continuous cultivation under high nitrogen concentrations (85 mg/L $NaNO_3$)	Photoautotrophic continuous cultivation in effluent from first stage PBR (NO3–N under 7 mg/L)	1.6 g/m^2/d	[107]
Chlorella sp. HS2	Freshwater	Lipids	Photoautotrophic batch cultivation in nitrogen-rich media	Photoautotrophic batch cultivation in nitrogen-depleted media	36.7 % w/w and 216.9 mg/L/d	[108]
Scenedesmus obtusus XJ-15	Freshwater	Lipids	Photoautotrophic batch cultivation in standard growth media	Photoautotrophic batch cultivation under increased salinity (20 g/L NaCl)	47.7% and 60.7 mg/ L/d (indoor cultures), 42.1% and 23.2 mg/L/ d (outdoor cultures)	[109]
Dunaliella tertiolecta FACHB-821	Freshwater	Lipids	Mixotrophic cultivation with 1.0 g/L glycerol	Cultivation with the addition of 100 ppm triethylamine	20.2 mg/L/d	[110]
Nannochloropsis oculata	Marine	Lipids	Batch cultivation in rectangular PBR[a] (light path - 16 cm) under nitrogen repletion (1.7 μM urea), low CO_2 (atmospheric air) at a light intensity of 300 μmol/m^2/s	Batch cultivation in flat panel PBR[a] in nitrogen deficiency and increased CO_2 (2%) at a light intensity of 500 μmol/ m^2/s and salinity of 35 g/L	0.3 g/L/d	[111]
Isochrysis galbana	Marine	Lipids	Photoautotrophic, batch cultivation under standard salinity (30 psu)	Photoautotrophic, batch cultivation under decreased salinity (10 psu)	47% w/w	[112]
Monoraphidium sp. FXY-10	Freshwater	Lipids	Heterotrophic batch cultivation (carbon source: glucose) with the addition of fluvic acid (80 mg/L)	Photoautotrophic, batch cultivation under decreased fluvic acid concentration (25 mg/L)	54.7% w/w	[113]
Chlorella pyrenoidosa, Chlorella vulgaris, Chlorella ellipsoidea	Freshwater	Lipids	Heterotrophic batch cultivation until depletion of glucose (carbon source)	Photoautotrophic batch cultivation following dilution with growth media, with continuous illumination at 300 μmol/m^2/s	*C. pyrenoidosa* - 89.9 mg/L/d; *C. vulgaris* - 85.4 mg/L/d; *C. ellipsoidea* - 67.9 mg/ L/d	[114]
Chlorella protothecoides strain 0710	Freshwater	Lipids	Photoautotrophic batch cultivation until the end of the log phase	Heterotrophic batch cultivation (carbon source: glucose) of cells produced under photoautotrophy	58.4% w/w and 11.8 g/L/d	[115]
Chlorella protothecoides IOCAS038F	Freshwater	Lipids	Heterotrophic fed-batch cultivation cultivation (carbon source: glucose) in stirred-tank bioreactor under supplementation with glucose and nitrogen	Heterotrophic fed-batch cultivation cultivation (carbon source: glucose) under nitrogen deprivation and reduced oxygen supply and agitation rate	36.8% w/w and 175.2 mg/L/h	[116]
Dunaliella tertiolecta	Marine	Lipids	Photoautotrophic batch cultivation in standard media with 1.5M NaCl concentration	Cultivation under salinity stress (2.5M NaCl) and supplementation with 50 μM sodium azide supplement	Lipid productivity–10% higher than control; Cellular lipid content–70.5% higher than control	[117]
Chlorella PY-ZU1	Freshwater	Lipids	Photoautotrophic batch cultivation in standard media until nitrogen depletion	Cultivation under nitrogen depletion, with phosphorous supplementation upon nitrogen depletion	42.1% w/w and 191.3 mg/L/d	[118]
Phaeodactylum tricornutum	Marine	Lipids			60.6% w/w	[119]

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Table 3 (*continued*)

Microalgae strain	Freshwater/marine species	Product	Two-stage cultivation strategy		Target compound concentration/ productivity	References
			First stage	Second stage		
			Photoautotrophic batch cultivation under blue LED light (465 nm) in nitrogen replete media	Photoautotrophic batch cultivation under green LED light (520 nm) upon reaching the stationary phase		
Chlorella protothecoides IOCAS038F	Freshwater	Lipids	Heterotrophic fed-batch cultivation (carbon source: glucose) with nitrogen concentration maintained between 150–350 mg/L	Heterotrophic fed-batch cultivation (carbon source: glucose) with no nitrogen supplementation and hyperosmotic stress of 1200 mOsm/kg provided by addition of sorbitol	39.2% w/w and 177.3 mg/L/h	[120]
Isochrysis sp. CS177	Marine	Lipids	Photoautotrophic batch cultivation in panel PBR[a] (light path - 7 cm) under outdoor conditions	Photoautotrophic semi-continuous cultivation in panel PBR[a] (shorter light path - 1.8 cm) under outdoor conditions	0.4 g/L/d	[121]
Chlorella vulgaris FACHB-960	Freshwater	Lipids	Mixotrophic cultivation under low CO_2 (atmospheric air) aeration, addition of 8 g/L sodium erythorbate and supplementation with 6 mL of 250 g/L $NaNO_3$	Mixotrophic cultivation under enriched CO_2 (10% v/v) aeration and supplementation with 30 mL of 250 g/L $NaNO_3$	43.7 mg/L/d	[122]
Nannochloropsis oculata SAG 38.85	Marine	Lipids	Photoautotrophic batch cultivation under sufficient nutrients (nitrogen 10 mM, phosphorus 1 mM)	Photoautotrophic batch cultivation under nutrient depletion (nitrogen 0 mM, phosphorus 0 mM)	40% w/w	[123]
Scenedesmus sp. CCNM 1077	Freshwater	Lipids and carbohydrates	Photoautotrophic batch cultivation in standard growth media	Photoautotrophic batch cultivation under salinity stress (400 mM NaCl) for 9 days	Lipids - 31% w/w; Carbohydrates - 33.3% w/w	[124]
Spirulina platensis	Freshwater	Polysaccharides	Photoautotrophic batch cultivation under low light intensity (96 μmol/m^2/s) and low temperature (28 °C)	Photoautotrophic batch cultivation under increased light intensity (192 μmol/m^2/s) and increased temperature (38 °C)	27.3% w/w and 207.2 mg/L	[125]
Chlorella vulgaris	Freshwater	Biomass	Photoautotrophic batch cultivation until stationary phase	Mixotrophic fed-batch cultivation with 3 g/L of glucose added daily as a carbon source	7.4 g/L	[126]
Chlorella sp. AE10	Freshwater	Starch and carbohydrates	Photoautotrophic batch cultivation under low CO_2 (1% v/v), low light intensity (100 μmol/m^2/s) and high nitrogen (1.5 g/L)	Photoautotrophic batch cultivation under increased CO_2 (10% v/v), increased light intensity (1000 μmol/m^2/s) and decreased nitrogen (0.375 g/L)	Starch - 0.3 g/L/d; Carbohydrates - 0.4 g/L/d	[127]
Chlorella salina	Marine	Starch and carbohydrates	Photoautotrophic batch cultivation until early stationary phase	Photoautotrophic batch cultivation under nitrogen and sulfur limitation	Starch - 30.5 mg/L; Carbohydrates - 146.0 mg/L	[128]
Chlamydomonas reinhardtii C137 (mt^+)	Freshwater	Hydrogen	Photoheterotrophic batch cultivation (carbon source: acetate) in standard media until late logarithmic phase	Photoheterotrophic batch cultivation (carbon source: acetate) in sulfur deprived media	~2 mL/h	[129]
Chlamydomonas reinhardtii Dang 137c	Freshwater	Hydrogen	Mixotrophic batch cultivation in standard growth media (carbon source: acetate) under low light intensity (20–25 μmol/m^2/s)	Mixotrophic batch cultivation in sulfur deprived media (carbon source: acetate) and increased light intensity (110 μmol/m^2/s)	4.5 mmol/L	[130]
Chlamydomonas reinhardtii stm6	Freshwater	Hydrogen	Photoautotrophic batch cultivation in sulfur-limited media	Photoautotrophic batch under sulfur deprivation	90% H_2 in total gas	[131]
Anabaena variabilis ATCC 29413	Freshwater	Hydrogen	Photoautotrophic batch cultivation in standard growth media under low light intensity (40–50 μmol/m^2/s) and supplementation of 5% CO_2	Photoautotrophic batch cultivation in nitrate deprived media under high light intensity (120–140 μmol/m^2/s) and anaerobic conditions	4.1 mL/g/h	[132]

[a] PBR: photobioreactor.
[b] RWP: raceway pond.
[c] n.r: not reported.

4.1.2. Wastewater-integrated lipid production

Research on microalgae-based biofuels has increasingly focused on the integration of wastewater as the growth media, since it can substantially reduce the cost of microalgae cultivation [7,43]. Furthermore, wastewater-based cultivation facilitates simultaneous phycoremediation and generation of valuable microalgal biomass as feedstock for biofuel and bioenergy production [138–140]. As detailed in Table 4, two-stage microalgae cultivation systems could be adopted to manipulate operating conditions required for efficient nutrient removal from wastewater streams and synthesis of lipids in microalgal biomass [42,43].

Ge et al. [45] studied the applicability of different metabolic modes in two-stage cultivation of *C. vulgaris* for simultaneous treatment of centrate wastewater and lipid production. Results of the study indicated that sequential cultivation under photoautotrophy and mixotrophy could be employed to obtain enhanced lipid yields (approximately sixfold that of photoautotrophy) and achieve complete nutrient removal from wastewater [45]. Similarly, Farooq et al. [43] cultivated two strains of the genus *Chlorella* in brewery wastewater, where different combinations of metabolic modes were used under two distinct stages. In the first stage, cell growth was performed under photoautotrophic mode in anaerobically digested brewery wastewater, whilst the second stage focused on lipid accumulation via cultivation in media supplemented with glucose or undigested brewery wastewater under photoheterotrophy or mixotrophy. Photoheterotrophic conditions with glucose supplementation was the effective in the removal of nutrients and boosting lipid productivity to more than threefold that of conventional single stage cultures. Moreover, the use of photoautotrophy to achieve considerable cell densities of microalgae in the first stage was effective mitigating the risk of bacterial contamination as compared to the scenario of direct cultivation in wastewater with high organic carbon load [43].

Although waste effluents can be integrated as alternative cultivation media for microalgae, the sudden shift of metabolism from photoautotrophy in the standard culture media to heterotrophy in wastewater may be lethal. Hena et al. [44], demonstrated that this issue could be addressed by the introduction of an intermediate photoheterotrophic acclimation stage. The transfer of photoautotrophically grown *C. sorokiniana* to an intermediate stage of photoheterotrophic cultivation before switching the metabolic mode to heterotrophy resulted in significantly higher yields of biomass and lipids as compared to single-stage photoautotrophic cultivation as well as two-stage cultivation under sequential photoautotrophy-heterotrophy. The three-stage process was also highly effective in concurrent removal of COD, nitrogen and phosphorous from dairy farm effluent [44].

Whilst the aforementioned studies aimed to produce biomass under photoautotrophy in the first stage and integration of wastewater-based cultivation in the second stage, research focusing on the converse approach have also been reported in literature. In this strategy, the primary aim of the first stage is to achieve high biomass concentrations under heterotrophic or mixotrophic mode in wastewater, whilst the second stage is employed to accumulate lipids under photoautotrophic cultivation. Álvarez-Díaz et al. [141] employed this method to demonstrate the efficacy of phycoremediation at the end of the first stage, where nitrogen and phosphorous in the medium was below the detection limit. Thereafter, lipid-rich *S. obliquus* biomass was synthesized through the induction of stress conditions via nutrient starvation in combination with other factors such as aeration with CO_2 enriched air, high salinity and presence of light [141].

Zhou et al. [42] proposed an integrated two-stage approach to enhance lipid yields and nutrient removal from municipal wastewater. In this study, hetrotrophically grown *Auxenochlorella protothecoides* from the first stage was harvested via self-sedimentation, and the treated wastewater was recycled as the growth media for a second stage of photoautotrophic growth [42]. Moreover, the study incorporated the use of residual microalgae in the media as an inoculum for the second stage to bypass the requirement of an exogeneous seed culture, thus representing a possible avenue for cost reduction. The approach of media recycling could also be extended for cultivation of multiple microalgal strains with different tolerances to inherent stress factors in wastewater streams [142].

These studies indicate a clear possibility of enhancing lipid yields via manipulation of culture conditions, albeit at incremental costs (addition of nutrients, requirement of multiple reactors, etc.). Thus, the

Table 4
Two-stage microalgae cultivation strategies for simultaneous lipid production and phycoremediation of wastewater.

Microalgae strain	Freshwater/ marine species	Two-stage cultivation strategy		Wastewater treatment efficiency	Lipid concentration/ productivity	References
		First stage	Second stage			
Chlorella vulgaris	Freshwater	Photoautotrophic cultivation in 10% v/v CW[b]	Mixotrophic cultivation with periodic addition of CW[b] and glycerol (carbon sources), maintained at 1% v/v and 2.0 g/L respectively	COD[a] - 89.5–93.4%; Nitrogen - 95.5–99.8%; Phosphorus - 98.5–100%	24.7 mg/L/d	[45]
Chlorella vulgaris UTEX-265	Freshwater	Photoautotrophic batch cultivation in anaerobically digested brewery wastewater	Photoheterotrophic batch cultivation with glucose addition (5 g/L)	More than 80% removal of TN[c] and TP[d] at the end of the first stage	108 mg/L/d	[43]
Chlorella sorokiniana	Freshwater	Photoautotrophic batch cultivation in BG-11 medium	Heterotrophic batch cultivation in dairy farm effluent, with 3-day photoheterotrophic acclimation phase (three-stage cultivation)	COD[a] - 98.8%; Nitrate - 98.1%; Ammonium - ~100%; Phosphate - 98.4 %	4.5 g/L	[44]
Scenedesmus obliquus SAG 276.10	Freshwater	Batch cultivation in wastewater	Batch cultivation in same reactor under nutrient starvation and increased salinity (15 g/L)	Near complete removal of nitrogen and phosphorus at the end of the first stage (below detection limit)	49% w/w	[141]
Auxenochlorella protothecoides UMN280	Freshwater	Heterotrophic batch cultivation in concentrated municipal wastewater. Biomass harvested by self-sedimentation in the first stage.	Photoautotrophic batch cultivation with CO_2 enriched air (5 %) in residual wastewater from first stage. Residual microalgal cells from first stage used as inoculum.	COD[a] -79.1%; Total ammonia - 100%; TN[c] - 90.6%; TP[d] - 98.5%	32.8% w/w	[42]

[a] COD: chemical oxygen demand.
[b] CW: centrate wastewater.
[c] TN: total nitrogen.
[d] TP: total phosphorus.

assessment of the economic feasibility of these cultivation strategies and subsequent downstream processes is of paramount importance to assess the applicability of these methods for production of microalgae-based biodiesel.

4.2. Carbohydrates

Microalgae synthesize carbohydrates under stress conditions, which could be converted into various biofuels including bioethanol, biogas or biocrude oil via processes such as fermentation, anaerobic digestion or hydrothermal liquefaction [105,143]. Several studies focusing on microalgal carbohydrate production using two-stage cultivation systems have been reported in literature, as detailed in Table 3.

For instance, Cheng et al. [127] proposed a two-stage process to improve carbohydrate and starch accumulation in *Chlorella* sp. via nitrogen limitation, high irradiance and increased CO_2 supply in the second stage. Furthermore, a similar strategy which integrated the combined effect of nitrogen starvation, high irradiance and Fe^{3+} supplementation in the second stage was successful in improving the carbohydrate productivity of *N. oleoabundans* [106]. Chong et al. [128], employed nitrogen and sulfur limitation to enhance the accumulation of starch and carbohydrates in *Chlorella salina* after biomass production was maximized under the favorable growth conditions provided in the first stage. Limitation of nitrogen and sulfur in the media caused a shift in photosynthetic carbon partitioning, where synthesis of carbohydrates and starch is favored as compared to synthesis of proteins and chlorophylls [128]. Furthermore, Lee et al. [125] proposed a two-stage cultivation strategy involving the increment of light intensity and temperature to produce polysaccharides in *S. platensis*. The upshift of salinity via the addition of 400 mM NaCl was also successful in enhancing carbohydrate content (33.33%) of *Scenedesmus* sp., although extended administration of salt stress resulted in significant biomass losses [124]. Results of the study showcase the necessity to identify the optimal conditions of stress provision to maximize the productivity of target compounds in microalgae. Upon review of the numerous studies reported in literature, it is evident that majority of two-stage cultivation strategies developed for carbohydrate production emphasized on the manipulation of physiochemical growth conditions. Thus, further strategies involving the use of multiple metabolic modes or cultivation systems could be explored with the goal of carbohydrate production.

4.3. Hydrogen

Certain microalgal strains possess the capability to produce hydrogen (H_2) via photobiological processes [144]. The photoproduction of H_2 using *Chlamydomonas reinhardtii* under sulfur deprivation has been reported in literature [129–131]. Deprivation of sulfur in the culture media causes the inhibition of photosystem II (PSII), thereby leading to the cessation of O_2 evolution. Nonetheless, aerobic respiration is unaffected by sulfur deprivation, which causes the dissolved oxygen in the media to be consumed, subsequently establishing an anaerobic state in the culture [130]. The ensuing anaerobiosis induces hydrogenase mediated photoproduction of H_2 in *C. reinhardtii* [131]. Lehr et al. [131], compared the H_2 productivity of *C. reinhardtii* in a two-stage process where the first stage utilized either batch cultivation under minimal sulfur concentrations or fed-batch cultivation with minimal sulfur addition. When cultivation was performed under minimal nutrient concentrations, it was not necessary to harvest and resuspend the biomass in sulfur deprived media for photoproduction of H_2 [131].

Furthermore, certain species of cyanobacteria possess the capability to produce H_2 via direct and indirect biophotolysis [145]. For instance, species such as *Anabaena variabilis* have been exploited for H_2 production via the activity of the nitrogenase enzyme under anaerobic and nitrogen deficient conditions [145]. Yoon et al. [132] utilized a two-stage strategy for H_2 production using *A. variabilis* wherein biomass was produced under stepwise increments of light intensity, followed by nitrogen depletion and further increment of light intensity in an anaerobic environment.

Nonetheless, the photoproduction of H_2 by microalgae is constrained by the high costs of production and inefficient product storage methods [145]. Thus, further advancements in research are essential for adoption of microalgae-derived hydrogen for bioenergy applications.

5. Feasibility of two-stage cultivation: current status and future perspectives

With the exception of a few highly lucrative or cost-effective products, the widespread exploitation of microalgal biomass is hindered by the energy intensiveness and environmental impact of the production process [146]. Two-stage cultivation systems have been developed for the enhancement of product yields based on the hypothesis that higher product yields would lower the production costs. Nonetheless, most studies focusing on two-stage cultivation have been performed in laboratory scale and discussion on economic feasibility and environmental sustainability has been fairly limited. Therefore, it is vital to perform techno-economic feasibility studies and life cycle assessments (LCA) of two-stage cultivation systems and their downstream processes to identify the potential of large-scale implementation [1,13].

5.1. Techno-economic considerations

5.1.1. Economic feasibility

Although the commercialization of certain high-value compounds is viewed as a viable industrial application of microalgae, there are a multitude of considerations to be made prior to establishment of large-scale production facilities. In a study by Panis and Carreon [147], a modeling approach was used for techno-economic analysis of large-scale astaxanthin production from *H. pluvialis* cultivated in a two-stage system. Results of the study indicated that selection of an ideal location for the production process is of utmost importance, as profitability was significantly affected by the geographical location. The product yields at the two distinct locations used in the study varied due to differences in climatic conditions, which in turn caused variations in revenue and operating costs. Furthermore, the dissimilar costs of land, utility costs, taxation policies, labor costs, etc. at the two geographic locations significantly affected the overall profitability. Therefore, it is evident that the feasibility of two-stage cultivation systems should be studied on a case-by-case basis prior to their large-scale implementation [147].

Moreover, it is important to note that the microalgae-based high-value product market must overcome several hurdles to receive the widespread attention of both consumers and industries. Although numerous species of microalgae synthesize a wide range of carotenoids, only a few of them have been commercialized as their benefits have yet to be studied extensively or have not been conveyed to consumers effectively [148]. Additionally, prior to commercialization, novel food products derived from microalgal sources must conform to the strict regulations established by regulatory bodies and governments [149]. Consequently, microalgae-derived high-value products have only secured niche markets in selected regions of the world. The barriers for new entrants to enter the market are quite high, and could be perceived as a risky venture for investors [146].

Despite of the extensive studies conducted on assessing the potential of microalgae as feedstock for the production of third generation biofuels, the currently available technologies are inadequate for economically feasible biofuel production [150]. This is primarily due to the high costs associated with cultivation (capital costs of equipment and land, cost of nutrients, energy intensive media circulation/aeration, temperature control, etc.) [151] and harvesting (which accounts for 20–30% of total costs) [8]. Two-stage cultivation systems, although effective in increasing the productivity of target metabolites, may incur incremental capital and operating costs depending on the system configuration and operating conditions. For instance, the addition of stress inducing agents

would increase chemical costs, the transfer of cultures between different cultivation systems (i.e. first and second stages) would entail increased pumping costs, and the use of PBR-RWP hybrid systems would increase capital and operating costs compared to exclusively cultivating microalgae in RWPs. Thus, without comprehensive techno-economic analysis, it is difficult to ascertain if the higher product yields in two-stage systems can offset the possibly higher production costs. Nevertheless, from the data available in literature, it is evident that microalgae-based biofuels such as biodiesel cannot be manufactured at costs comparable to petroleum-based diesel or biodiesel derived from conventional feedstock without substantial technological progress [152].

5.1.2. Resource recovery from waste streams

A prospective strategy to reduce the cost of microalgae cultivation is the recovery of carbon and nutrients from wastewater and flue gas, as low-cost alternatives to the conventional process inputs. It has been reported that a 35–86% cost reduction could be achieved by the use waste sources for CO_2 and nutrients [153]. Nonetheless, numerous challenges such as the susceptibility to culture contamination and requirement of robust strains should be addressed during the cultivation of microalgae in wastewater streams. Similarly, the high temperature, elevated concentration of CO_2, and presence of impurities such as nitrogen oxides (NO_x), sulfur oxides (SO_x) and heavy metals in flue gas may inhibit microalgal growth due to culture overheating, acidification and toxicity [6]. Hence, robust microalgae strains should be selected, and efficient mechanisms should be in place for control of pH and temperature if flue gases are employed for microalgae cultivation [25]. Moreover, the bioaccumulation of toxic compounds in microalgae may limit the applicability of biomass generated through the utilization of flue gas and wastewater streams. Thus, it is important to select appropriate waste streams such as flue gas from natural gas combustion and wastewater with low concentrations of heavy metals (possibly from food and beverage industries) in order to secure a diverse spectrum of applications for the generated microalgal biomass [154,155].

5.1.3. Harvesting strategies

Exploring strategies to reduce the harvesting cost of microalgae is also imperative to enhance the feasibility of biofuel production. This is especially relevant from the perspective of two-stage cultivation, as an intermediate harvesting step is often required to transfer microalgal cells from the first stage to the second stage. To this end, techniques such as alkaline flocculation and chitosan-assisted or fungal-assisted bioflocculation and have been identified as possibly cost-effective solutions [123,156,157]. Moreover, the selection of microalgal strains capable of self-sedimentation would be attractive for cost reduction as simple gravity settling vessels could be employed for harvesting, instead of expensive techniques such as centrifugation and filtration [8]. Nevertheless, it would be preferable to focus on two-stage cultivation strategies which do not require the intermediate harvesting step for synthesis of target metabolites, such as the addition of stress inducing agents or sequential culture dilution and photoinduction.

5.1.4. Strain selection and improvement

In two-stage cultivation, the sudden surge of stress conditions upon transfer of cultures from the first stage to the second may be fatal to microalgal cells. The loss of biomass upon exposure to stress conditions would be highly detrimental, given the costs incurred for biomass production. Therefore, the selection of robust and resilient microalgal strains which can retain biomass production under stress conditions is a major consideration to be made to establish economically feasible microalgae-based industries. A prospective strategy to mitigate this issue of biomass loss is the stepwise administration of the shift in cultivation conditions, which would allow microalgae to gradually acclimate to the stress conditions. For instance, the increment of light intensity could be provided gradually [50], or an intermediate cultivation step under the photoheterotrophic mode could be employed in between two stages of photoautotrophy and heterotrophy [44].

Furthermore, adaptive laboratory evolution (ALE), mutagenesis and genetic engineering approaches could be employed for the development of stress-tolerant microalgal strains [158,159]. ALE involves the cultivation of multiple generations of a selected microalgae strain under extreme conditions for prolonged periods in a laboratory environment [160]. Robust microalgal strains possess the plasticity to acclimate to extreme culture conditions, and subsequent generations would possess the ability to thrive under the physiochemical conditions employed during ALE. Thus, the adapted strains could be utilized to obtain enhanced yields of target compounds under stress conditions. Additionally, numerous physical and chemical mutagens such as ultraviolet radiation, gamma radiation, *N*-methyl-N-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS) have been applied to develop strains with high lipid and carotenoid productivity [161–163]. Genome sequencing and the advancement of omics technologies have had a positive impact on the development of transgenic microalgal strains with enhanced biomass productivity and capability to synthesize high concentrations of valuable metabolites [164]. This is achieved by overexpression and/or down-regulation of transcriptional and translational genes in the required metabolic pathways [165]. The genetic manipulation approach is more precise than random mutagenesis, thus leading to the development of engineered microalgal strains for controlled production of desired metabolites such as lipids, hydrogen and pigments [165]. These techniques can be employed to develop desirable traits such us the retention of biomass production under stress conditions, thus allowing biomass production and concomitant product accumulation in a single stage of cultivation.

5.1.5. Integrated biorefinery concept

The high biofuel production costs could also be alleviated via the co-production of microalgal high-value products in a biorefinery context [166]. The biorefinery approach allows the conversion of residual biomass from recovery of high-value products into biofuels. The lucrative nature of the high-value compounds (i.e. astaxanthin, lutein, beta-carotene, etc.), which are to be the primary products of such biorefineries, would offset the high operating costs of microalgae cultivation and downstream processes [146]. In this context, the costs directly associated with biofuel production would be limited to the costs incurred for the conversion of residual biomass into biofuels.

Nonetheless, a key issue that arises from this approach is the disparity in demand for high-value products and biofuels; i.e. whilst high-value products operate within a niche market, biofuels are required in large quantities. For instance, cultivation of microalgae to supply biofuel required for 10% of the energy demand for transportation in Europe would require 6% of Europe's agriculture land [1]. It would be infeasible for a niche high-value product to secure a market which necessitates microalgae cultivation in the aforementioned scale. Thus, the biorefinery approach to produce microalgal biofuels has limited potential beyond applications such as energy recuperation for existing production processes.

5.2. Life cycle assessment

In addition to the consideration of economic aspects, studies on LCA should be performed to gauge the environmental feasibility of two-stage cultivation systems. LCAs utilize key metrics such as net energy ratio (NER), global warming potential (GWP), acidification potential, eutrophication potential, ozone depletion, human toxicity, ecotoxicity (marine, freshwater, terrestrial), land usage, water footprint, particulate matter formation, mineral resource depletion and fossil resource depletion to evaluate the environmental feasibility of exploiting microalgal biomass [167,168].

NER and GWP are two metrics which have been studied extensively to assess the environmental feasibility of microalgal biofuels. The NER is defined as the energy output of microalgae-based biofuel in relation to

the energy input in its production, expressed as a ratio [169]. GWP is indicative of the greenhouse gas emissions associated with the process, and is quantified through equivalent CO_2 emissions per predefined functional unit [170]. It is noteworthy that the system boundaries of the LCA should include "cradle-to-grave" analysis of all steps in biomass processing (inclusive of cultivation, downstream processing and product transportation) for accurate evaluation of NER and GWP. Due to the differences in scope in LCA studies, contradictory results have been reported with regard to the NER and GWP of microalgae-based production processes. Previous reviews on LCA of microalgae-based biofuel production also indicate that the NER and GWP is significantly affected by the selection of microalgal strains, cultivation systems and downstream processes [169,171]. Due to the wide variation of these parameters, NER and GWP of two-stage systems would undoubtedly change from system to system.

The general consensus of various LCA studies reported in literature is that RWPs showcase more favorable metrics of NER and GWP as compared to PBRs [169,171]. In fact, most studies indicate that the energy input of PBRs far exceed the energy output of microalgal biodiesel, resulting in infeasible NER. Ultimately, due to the energy intensiveness of the manufacturing process, the NER of microalgal biofuel is significantly inferior to conventional petroleum-based diesel (approximately by a factor of 5) [172]. The lower NER of microalgal biofuel necessitates further research for sustainable use of microalgal feedstock for biofuel production. Nevertheless, the GWP of microalgal biodiesel is significantly lower than petroleum-based diesel, and comparable to crop-based biodiesel [167,169,172]. Furthermore, it should be noted that the GWP depends on the type of biofuel manufactured from microalgae. For instance, in a study by Bennion et al. [173], hydrothermal liquefaction of microalgal biomass resulted in a substantially lower GWP as compared to pyrolysis. Therefore, alternative routes for processing of microalgal feedstock should be assessed to identify the most feasible biofuel production processes. Numerous studies reported in literature suggest that the use of renewable energy sources (photovoltaic systems, wind energy, etc.) [167,174], recycling of culture media, use of wastewater for nutrient recovery [168], emphasis on enhancing biochemical composition, improving calorific value of products and cost-effective downstream processing [175] can improve NER and GWP of microalgae-based biofuels.

Despite of the multitude of two-stage microalgae cultivation systems reported in literature, extensive studies focusing on LCA have not been reported. In a study by Khoo et al. [175], a PBR-RWP hybrid system exhibited significantly higher CO_2 emissions and energy intensiveness as compared to conventional RWPs. In fact, the high cost of downstream processing led to poor NER values, with energy inputs exceeding the energy output of microalgal biofuel [175]. Conversely, Adesanya et al. [176] suggested that biodiesel derived from two-stage systems which couple airlift PBRs and RWPs have a significantly lower GWP and fossil energy requirements as compared to fossil-derived diesel. Moreover, the hybrid cultivation system proposed in the aforementioned study incorporated the recycling of spent culture media to reduce freshwater footprint. The recycling of freshwater consequently reduced the GWP and fossil energy requirement as well. Stephenson et al. [177] showed that biodiesel derived from microalgae cultivated in RWPs under a first stage of nutrient-sufficient cultivation and a second stage of nutrient depletion exhibited 78% lower GWP and 85% lower fossil energy requirement as compared to fossil-derived diesel. Contrastingly, utilizing the same two-stage strategy in airlift tubular PBRs resulted in significantly higher GWP (273%) and fossil energy requirement (362%). Due to the contradictory results reported in these studies, it is evident that LCA of two-stage systems should be evaluated on a case-by-case basis to identify their environmental impacts.

Although most LCA studies on microalgal biofuels have evaluated GWP and NER, there is a significant gap in literature on LCA of two-stage microalgae cultivation systems with respect to other impact categories such as particulate formation, eutrophication, ecotoxicity, acidification and land usage. For instance, upon analysis of 266 LCA studies on various biofuels, Carneiro et al. noted that only 48% had assessed land usage despite being a significant impact category, whereas over 89% and 82% of studies had incorporated GWP and NER in LCA [170]. LCA studies which include conventional cultivation scenarios have revealed that the impacts under these numerous categories depend on the culture parameters and cultivation conditions [167,168,178]. Therefore, it is highly likely that LCA of different two-stage cultivation configurations would showcase varying results under different impact categories.

Moreover, LCA could be employed as a tool to identify the most environmentally feasible two-stage cultivation configurations for production of high-value metabolites. In a study conducted to evaluate the environmental impact of astaxanthin production via two-stage cultivation of *H. pluvialis*, it was concluded that more significant reductions in environmental impact could be achieved under artificial illumination as compared to cultivation under sunlight [179]. This was attributed to the lower productivity of biomass under outdoor cultivation. Moreover, flat plate PBRs were identified as the most suitable reactor type for astaxanthin production. Accordingly, the use of flat plate PBRs with artificial lighting resulted in the reduction of impact by 62–79% under various categories. Thomassen et al. [180], showed that the environmental impact of beta-carotene production could be substantially alleviated via recycling of water during two-stage cultivation of *D. salina*. Moreover, it was identified that the environmental impact varied with the geographical location of microalgae cultivation and the power generation mix.

Thus, it is evident that it would be vital to comprehensively assess the environmental feasibility of distinct two-stage systems using LCA. Accordingly, ideal two-stage cultivation systems should be identified on the basis of both techno-economic and environmental feasibility.

6. Conclusions

Cultivation of microalgae in view of manufacturing bioproducts requires the synthesis of biomass of the desired biochemical composition. Nonetheless, due to the opposing culture conditions required for rapid cell proliferation and target compound accumulation, extensive studies on two-stage cultivation strategies have been performed. In this review, the technical aspects of various configurations employed for two-stage microalgae cultivation were discussed in a product-specific context. Whilst two-stage microalgae cultivation systems have been successfully adopted in industrial scale for production of certain high-value compounds such as astaxanthin from *H. pluvialis*, further research is required for feasible production a diverse spectrum of high-value compounds and biofuels. The two-stage strategies with potential to increase the productivity of target compounds provide a base for further improvement in future studies. The use cost-effective cultivation systems, integration nutrient recovery from waste streams and adoption of energy efficient downstream processes would be instrumental for feasible production of microalgal bioproducts via two-stage cultivation. Nonetheless, comprehensive techno-economic analysis and LCA studies are required on a case-by-case basis to assess the feasibility of implementing the various strategies discussed in this review. To this end, further research should focus on scaled-up implementation of potential two-stage cultivation systems and concurrent feasibility studies.

CRediT authorship contribution statement

Vinoj Chamilka Liyanaarachchi: Investigation, Visualization, Writing - Original Draft. **Malith Premaratne**: Investigation, Visualization, Writing - Original Draft. **Thilini U. Ariyadasa**: Conceptualization, Supervision, Resources, Writing - Review & Editing, Project administration, Funding acquisition. **Pemaththu Hewa Viraj Nimarshana**: Visualization, Writing - Review & Editing. **Anushree Malik**: Visualization, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Funding

This work was supported by the State Ministry of Skills Development, Vocational Education, Research & Innovation, Sri Lanka [grant number MSTR/TRD/AGR/3/2/18] and the Department of Science and Technology, Government of India [grant number DST/INT/SL/P-20/2016].

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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