

## REVIEW

# Mixotrophic cultivation of microalgae for carotenoid production

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## Abstract

The intrinsic ability of microalgae to accumulate high amounts of carotenoids has made them the preferred aquatic organisms of biotechnological exploration for carotenoid production. To continuously innovate and modify microalgal bioprocesses, aquaculture scientists have been working hard for the past decades in a forward-looking way, and mixotrophic cultivation of microalgae is deemed as a promising strategy to decrease production cost. This review is intended to summarise the recent research advancement of carotenoids production from mixotrophically cultivated microalgae, starting from the structure, biosynthesis, physiological roles and applications of carotenoids and followed by the production processes both currently established and under development. Most importantly, the microalgal physiology of mixotrophic cultivation is reviewed in depth both in general and specifically for the most studied species, and the prospects of commercially viable mixotrophic microalgal processes for carotenoid production along with the insight of future research are of course discussed. Finally, we conclude that mixotrophy might be a promising strategy for large-scale cultivation of microalgae to produce carotenoids although some technical obstacles need to be overcome.

## KEYWORDS

aquaculture, carotenoid, microalga, mixotrophy

## 1 | INTRODUCTION

Carotenoids are a group of tetraterpenoids, which contribute the purple, yellow, orange or red colour of some plants and microorganisms in nature.<sup>1,2</sup> Except being precursors of phytohormones with roles in plant development, carotenoids are essential light absorption pigments assembling light harvesting apparatus for photosynthesis systems and are deemed to play key functions in photoprotection in plants and microalgae.<sup>3,4</sup> As colourants and antioxidants, carotenoids are extensively used as ingredients or additives for functional foods,

cosmetics and animal feed.<sup>5</sup> It is also reported that carotenoids might be able to prevent the onset of some chronic diseases, such as certain cardiovascular diseases, cancers and eye diseases.<sup>6</sup>

Carotenoids might be obtained from colourful plants such as tomato, carrot, annatto, grapes and paprika. Various categories of microorganisms like bacteria, yeast, filamentous fungi and microalgae are preferred natural sources for biotechnological exploration of production. In fact, microorganisms are more diversified than plants in nature and known to be able to produce higher amounts of carotenoids, and in addition they can be cultivated more conveniently without seasonal or geographical variations.<sup>7</sup> Among the microorganisms, microalgae are the promising industrial organisms for current

biological carotenoid production.<sup>8</sup> Microalgae have intrinsic capability to utilise both sunlight and organic carbon as energy sources to grow and metabolise complex biomolecules. Similar to plants, microalgae can grow autotrophically with simple inorganic nutritional elements, and unlike microorganisms, microalgae can multiply rapidly and have the ability to produce numerous secondary metabolites. This well-balanced and useful combination has made microalgae unique category of microorganisms for biomanufacturing of phytochemicals. Moreover, microalgae constitute a distinctive reservoir of biodiversity due to their enormous number of species existing in nature, and thus could produce a wide range of bioactive compounds, such as pigments, vitamins, fatty acids.<sup>9</sup>

Some microalgal species can be cultivated either phototrophically, heterotrophically or mixotrophically, depending on the light and carbon source availability. Mixotrophic mode of microalgal cultivation can offer the benefits of both autotrophy and heterotrophy. It involves the utilisation of organic and inorganic nutrients under light by aerobic respiration and photosynthesis. The merits of mixotrophic cultivation of microalgae include amelioration of photo-inhibition, higher growth rates than both autotrophy and heterotrophy, reduction in nighttime loss of biomass, and less photo-oxidative damage during the cultivation.<sup>10,11</sup> The compounds synthesised under mixotrophic conditions have characteristics of both photosynthetic and heterotrophic metabolisms but with higher production rates. Furthermore, the energy cost is lower in mixotrophic cultivation, compared with photoautotrophic cultivation, due to reduced light demands. At the same time, the CO<sub>2</sub> produced by aerobic respiration can be used by photosynthesis under mixotrophic conditions, and thus the demand of CO<sub>2</sub> supply is reduced, compared with photoautotrophic cultivation of microalgae.<sup>12</sup> In the presence of inorganic carbon, the net oxygen exchange between the cells and the culture medium can be minimised, and a balance between respiration and photosynthesis (internal recycling of O<sub>2</sub> and CO<sub>2</sub>) can be attained.<sup>13</sup> Because of these advantages, the mixotrophic cultivation is well-thought-out as preferred methods for microalgal carotenoids production with increased growth rates and boosted pigment production.<sup>10,11</sup>

Though simultaneous assimilation of inorganic and organic carbon in mixotrophic mode may provide a prospect to ameliorate microalgal cultivation with improved energy economics in comparison with autotrophic cultivation, some demerits of mixotrophic cultivation do exist, such as the need for light, carbon dioxide, organic carbon and oxygen at the same time, lower energy conversion efficacy and net consumption of CO<sub>2</sub> regardless of its release in respiration.<sup>14</sup> Additionally, the cost of the organic carbon source could be approximately 80% of the total cost of culture medium; therefore, economical organic substrates might need be screened out in order to overcome the higher cost.<sup>15</sup> Grama et al. were among the first to show that a low-cost waste product of glycerol from biodiesel industry could be used as an algal bioprocess co-substrate not only to enhance the biomass production but also to reduce or eliminate the aeration energy demand.<sup>13</sup> High risk of bacterial contamination is also a major concern in mixotrophic cultivation,<sup>16</sup> and a great challenge for large scale mixotrophic

cultivation of microalgae is to develop inexpensive photobioreactor systems since the cost of current systems is extremely high, compared to that of bioreactors of heterotrophic cultivation.<sup>17</sup>

This review intends to summarise the recent research advances of mixotrophic microalgal cultivation for carotenoid production, to shed light on future researches in microalgal physiology under the conditions of mixotrophic cultivation, and to prospect commercially viable biotechnological processes for carotenoid production from mixotrophic microalgae. With our efforts to fulfil the purpose of this review, we first introduce the structures and biosynthesis, physiological roles and applications of carotenoids, then describe current biotechnological production processes of commercially important microalgal carotenoids, and lastly discuss the physiology of mixotrophic cultivation both in general and specifically for some promising microalgal species for carotenoid production.

## 2 | CAROTENOIDS

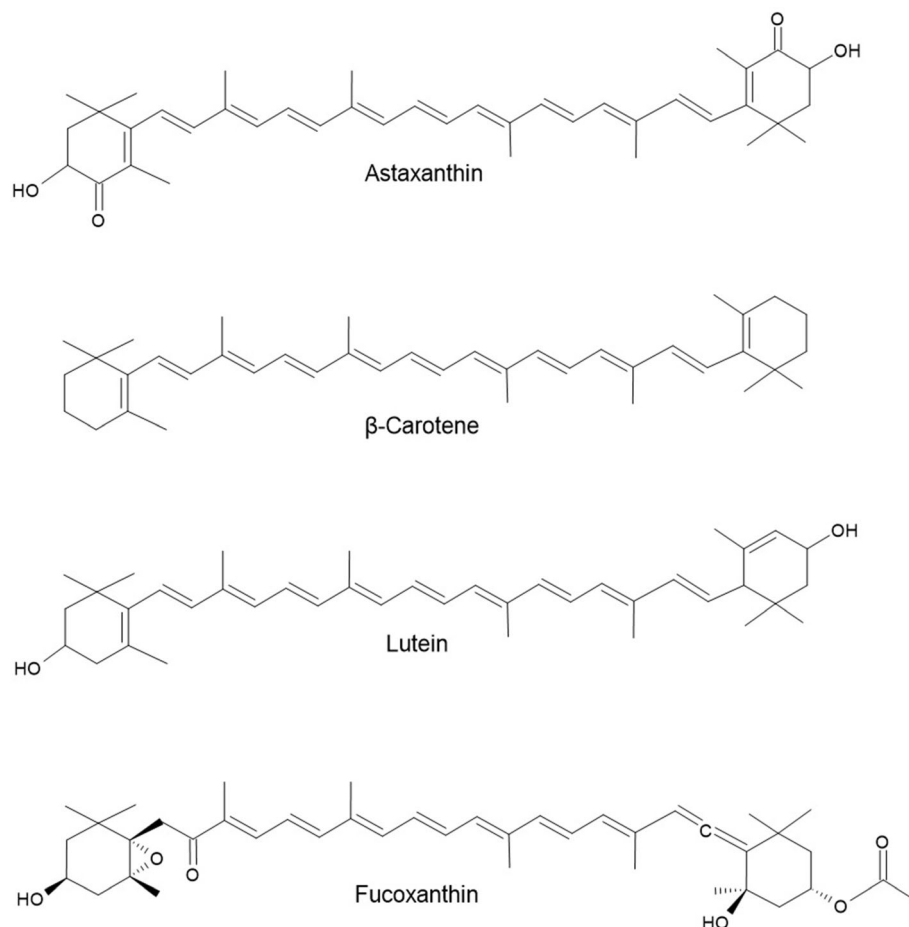
Natural pigments generally termed as 'Carotenoids' include more than 1100 kinds of molecules obtained from various plants, algae, fungi, bacteria and archaea.<sup>1,18</sup> Most animals and humans are incapable of synthesising carotenoids in vivo although some animals can accumulate substantial amounts through their diet.<sup>1,5</sup> To date, aphids are the only animals known to have genes essential for carotenoid biosynthesis.<sup>19</sup> In order to familiarise our audience with our topic, we describe up to date information about the classification, structure, biosynthesis, physiological roles and applications of carotenoids in this section.

### 2.1 | Chemical structures and categories of carotenoids

Carotenoids are a class of natural fat soluble non-chlorophyll accessory pigments consisting of a 40 carbon polyene structure (tetraterpenes with eight isoprenoids units). There are two main categories of carotenoids: (1) carotenes that contain only hydrocarbons without oxygen groups, for instance,  $\beta$ -carotene and lycopene and (2) xanthophylls that are the oxygenated derivatives of carotenes, such as, lutein, astaxanthin, zeaxanthin and neoxanthin.<sup>5,18,20</sup> Chemical structures of some important carotenoids are presented in Figure 1.

Carotenoids can absorb light between 400 and 550 nm wavelengths and range from colourless to deep red, depending upon the number of conjugated double bonds.<sup>5</sup> The central C<sub>40</sub> conjugated double bond system of carotenoids in the polyene chain functions as a 'chromophore' to absorb the blue light for photosynthesis and is responsible for their characteristic yellow, orange or red colour. Oxygenated functional groups present at terminal  $\beta$ -ionone rings of xanthophylls play a role in protecting the cell from oxidative damage by scavenging singlet oxygen species.<sup>21</sup>

Microalgae can synthesise carotenoids and all types of xanthophylls found in plants. Additionally, they can produce a huge array of pigments that are specific to algae, for example, fucoxanthin is found



**FIGURE 1** Chemical structures of carotenoids

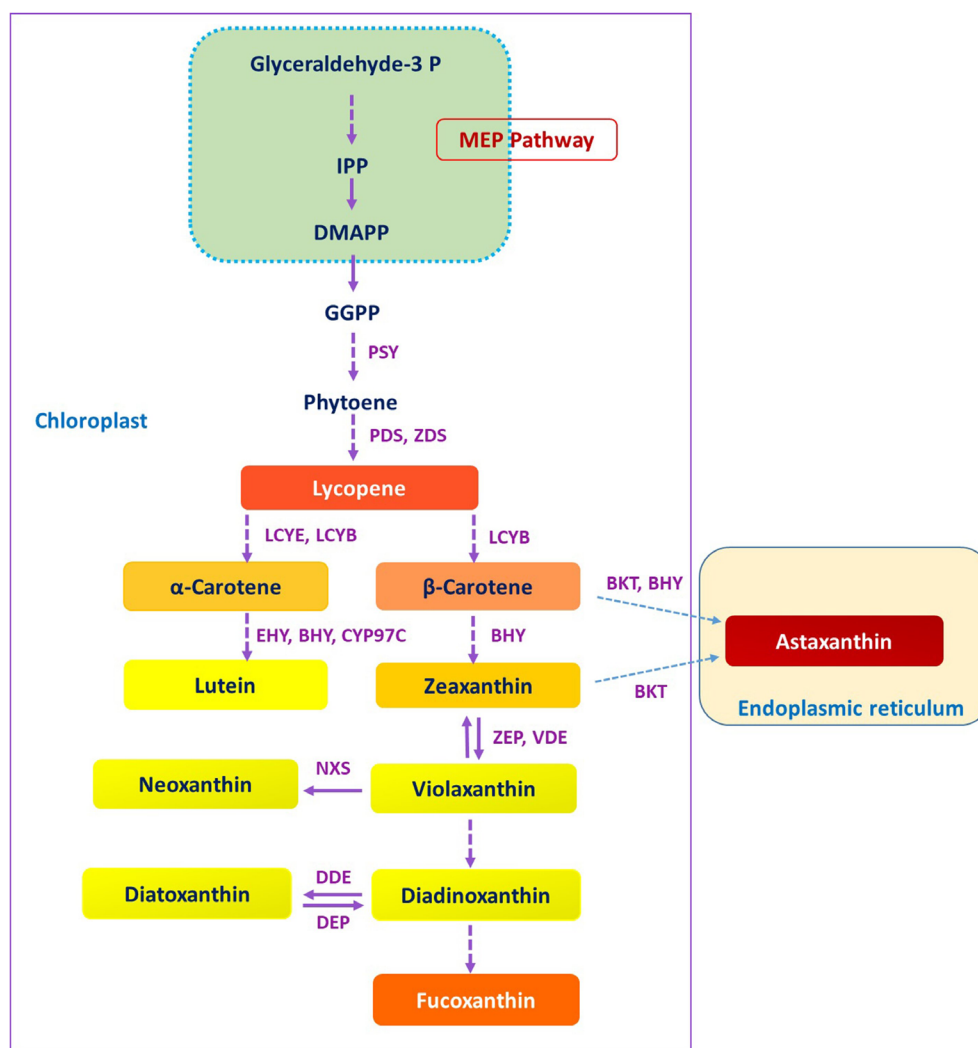
only in brown algae and diatoms, peridinin only in dinoflagellates, and alloxanthin only in Cryptophytes.<sup>5,21</sup> Microalgal carotenoids can be categorised as primary carotenoids (some xanthophylls and  $\beta$ -carotene) and secondary carotenoids (astaxanthin, lutein, violaxanthin, antheraxanthin and zeaxanthin). Primary carotenoids are structural as well as functional constituents of the photosynthetic apparatus and participate directly in photosynthesis, whilst secondary carotenoids are extra-plastidic pigments synthesised in bulky amounts only under specific environmental stimuli through carotenogenesis.<sup>5,22</sup> They protect chlorophyll from light damage by creating a protective layer in the microalgal cells upon exposure to extreme light intensities. Therefore, secondary carotenoids are accumulated in high quantity under stressed conditions for protection purposes.<sup>23</sup>

## 2.2 | Carotenoid biosynthesis in microalgae

Building blocks of carotenoids are isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), the common C5 precursors of terpenoids. Isopentenyl pyrophosphate and DMAPP are synthesised by the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. These are the two distinct biosynthesis pathways found in nature, both of which were discussed

elsewhere in details.<sup>24–26</sup> In plants, the MVA pathway is located in the cytoplasm and the MEP pathway in plastids.<sup>27</sup> Bacteria, cyanobacteria and unicellular green microalgae are thought to possess MEP pathway in plastids.<sup>25,28</sup> This pathway is relatively recently discovered in 1990s firstly in bacteria,<sup>29</sup> but it is not found in Archae, fungi and animals.<sup>30</sup> The MVA pathway has long been found to be present in the cytosol of Archaea, fungi and animals.<sup>25</sup> Isopentenyl pyrophosphate and DMAPP produced through both pathways can be elongated into geranyl diphosphate (GPP), farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) by GPP synthase (GPPS), FPP synthase (FPPS) and GGPP synthase (GGPPS) respectively, which are the direct precursors of mono-, di-, tri-terpenes and carotenoids. Two 20-carbon GGPP molecules can be condensed to form one 40-carbon phytoene, a colourless acyclic carotene containing three conjugated double bonds and serving as precursor of all the 40-carbon carotenoids.<sup>24,27</sup>

Phytoene synthase (PSY) is considered as a key enzyme, which executes the rate-limiting entry step into the biosynthesis pathways of carotenoids in photosynthetic organisms. Phytoene synthase catalyses the condensation of two GGPP molecules, which in turn yields phytoene. The next step in the pathway is a desaturation reaction leading to the formation of lycopene, a red coloured molecule containing 13 double bonds and a chromophore of 11 conjugated double bonds.<sup>25</sup> Two enzymes involved in creating conjugated double bonds



**FIGURE 2** Microalgal carotenogenesis. BHY,  $\beta$ -carotene hydroxylase; BKT,  $\beta$ -carotene ketolase; CYP97C, carotene  $\epsilon$ -hydroxylase; DDE, diadinoxanthin deepoxidase; DEP, diatoxanthin epoxidase; DMAPP, dimethylallyl pyrophosphate; EHY,  $\epsilon$ -carotene hydroxylase; GGPP, geranyl geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; LCYB, lycopene  $\beta$ -cyclase; LCYE, lycopene  $\epsilon$ -cyclase; MEP, 2-C-methyl-D-erythritol 4-phosphate; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin deepoxidase; ZDS,  $\zeta$ -carotene desaturase; ZEP, zeaxanthin epoxidase. Modified from Cezare-Gomes et al.<sup>31</sup> and Goff et al.<sup>36</sup>

to yield lycopene are phytoene desaturase (PDS) and  $\zeta$ -carotene desaturase (ZDS).<sup>31</sup> Lycopene serves as a substrate for the formation of both  $\alpha$ - and  $\beta$ -carotene along with their oxygenated xanthophyll derivatives.<sup>32,33</sup> At this step, the pathway splits into branches, one leads to lycopene cyclisation by lycopene  $\beta$ -cyclase (LCYB) yielding  $\beta$ -carotene, and another is a concerted action of LCYB and lycopene  $\epsilon$ -cyclase (LCYE) to form  $\alpha$ -carotene. The  $\alpha$ - and  $\beta$ -carotene undergo hydroxylation via  $\beta$ -carotene hydroxylase (BHY) to synthesise lutein and zeaxanthin, respectively. Further the epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) yields antheraxanthin and violaxanthin.<sup>34</sup> Under stress conditions, such as high light intensity, violaxanthin can be de-epoxidated back to zeaxanthin by violaxanthin de-epoxidase (VDE). The reversible interconversion of zeaxanthin and violaxanthin is denoted as the violaxanthin cycle, which is crucial for photoprotection.<sup>35</sup> The rearrangement of ionone ring of violaxanthin forms neoxanthin, a precursor for the acetylenic xanthophylls peridinin,

vaucheriaxanthin, fucoxanthin and its 19'-acyloxy derivatives (heteroxanthin, diadinoxanthin, diatoxanthin and alloxanthin).<sup>21</sup> Possible pathways of microalgal carotenogenesis are depicted in Figure 2.

After biosynthesis, carotenoids can be deposited inside or outside of the chloroplast. The primary carotenoids have direct roles in photosynthetic mechanism and are located in the chloroplast, whereas, secondary carotenoids are stored outside the chloroplast. It is considered that secondary carotenoids are transferred from the chloroplast to the lipid vesicles, playing significant roles in communication between the two biosynthesis processes that occur in the cytosol and in the chloroplast.<sup>31</sup>

### 2.3 | Physiological roles of carotenoids

In microalgae, the carotenoids generally function as accessory light harvesting pigments for photosynthesis and also protect the

photosynthetic apparatus from the damage of excessive light by quenching reactive oxygen species (ROS) such as single oxygen and free radicals. For example, carotenoids can prevent oxidative damage of intracellular lipids in microalgae by scavenging free radicals using their core functional groups. The carotenoids are also known to play roles in phototaxis and phototropism.<sup>19,23</sup> Photo-protective carotenoids protect the cell against oxidative degradation through numerous mechanisms including dissipation of excessive energy into heat through the xanthophyll cycle, scavenging of single oxygen, scavenging of peroxy radicals and inhibiting the formation of singlet oxygen by deactivating photosensitisers, for instance, triplet-state chlorophyll.<sup>52</sup>

Due to their structures, carotenoids can be dispersed in different cell compartments in different ways. Particularly, xanthophylls can be present within the interior portion of the lipid bilayer of cell membranes, with their hydrophilic or polar ends protruding into an aqueous environment. Fucoxanthin, lutein, neoxanthin and xanthophyll cycle pigments are the examples of these more hydrophilic carotenoids. The presence of these hydrophilic carotenoids may possibly affect the thickness, flexibility or permeability of membrane and in turn influences the stability of the cell membrane. Roles of carotenoids in cell cycle regulation, growth factors regulation, cell differentiation, immunostimulating, intracellular signalling and modulation of various types of receptors have also been proposed.<sup>38</sup>

## 2.4 | Applications of carotenoids

Carotenoids have numerous applications in different sectors including feed, food, cosmetic, health, pharmaceuticals, etc. As feed ingredients for poultry, carotenoids not only can enhance yellow colour of egg yolks and skin of broilers but also promote the wellbeing of these oviparous animals.<sup>39</sup> As feed ingredients for aquaculture, carotenoids have roles in promoting growth and survival, increasing disease resistance and stimulating immune functions of aquatic animals. For some aquatic animals, some carotenoids work as essential nutrients since they are not being able to be synthesised by these animals.<sup>40,41</sup> Maybe more important, carotenoids in aquaculture feeds are essential to give the characteristic colours of some farmed aquatic animals, which is an attractive feature in the market and affects consumer's choices of consumption.<sup>40</sup> Besides growth improvement, pigmentation is the best-known function of carotenoids in aquaculture.<sup>42</sup> Carotenoids showed better effects on skin pigmentation compared to xanthophylls.<sup>43</sup> Carotenoids can also be used in aquaculture to enhance the quality of brood stock and to improve the quality of live feeds.<sup>44,45</sup> Carotenoids can be of help increasing the resistance of aquatic animals to environmental stresses, attributed to their antioxidant activity.<sup>46</sup> Carotenoids can also promote embryonic development of aquatic animals, stimulate growth and maturation of gonads and increase significantly the performance and reproductive animal precocity.<sup>40,47</sup> Aquatic animals conspicuously accumulate carotenoids in their gonads, which is assumed to be essential for their reproduction and the successful development of their eggs and early larval stages.

Astaxanthin supplementation in cultured salmon and red sea bream increased ovary development, fertilisation, hatching and larval growth.<sup>48</sup> Furthermore, carotenoids may contribute to photo-protection by capturing excess radiation, dissipate it in the form of heat and thus prevent peroxidative damage to DNA, sugars, proteins, lipids.<sup>40</sup> Nakano and Wiegertjes<sup>49</sup> reported antioxidant activity, anti-stress, anti-inflammatory, egg quality enhancement, growth improvement, immune stimulation, lipid metabolism and liver function improvement, photoprotection, provitamin A activity and enhanced reproduction as general biological functions of carotenoids in fish. Astaxanthin is best known as an essential aqua feed additive for imparting the pinkish-red coloration to the flesh of aquatic animals resulting in a better quality and acceptance in the consumer market. Astaxanthin plays a fundamental role not only in the cultivation but also in the breeding of diverse kinds of aquaculture species. Physiological benefits of astaxanthin include improvements in survival, growth performance, reproductive capacity, stress tolerance, disease resistance and immune-related gene expression.<sup>50</sup> In a recent study, as an alternative of astaxanthin, supplementation of dietary  $\beta$ -carotene improved growth performance, coloration, fatty acids profile, antioxidant status and nonspecific immune response of *L. vannamei*. Moreover, using a combination of both  $\beta$ -carotene and astaxanthin proposed a synergistic effect between them.<sup>51</sup>

In food and beverages sectors, carotenoids are used not only as the colourant, making them more appealing to the consumers, but also as essential health nutrients.<sup>19</sup> Roles of carotenoids in skin health are well-known,<sup>52</sup> and carotenoids can also provide protection against sunlight and UV.<sup>53–55</sup> Plenty of studies have shown that carotenoids have beauty-enhancing effects, and thus carotenoids are widely used in cosmetic products.<sup>56</sup>

Owing to the pro-vitamin A and strong antioxidant activity of carotenoids, their demand is very high in health and pharmaceuticals sectors.<sup>31</sup> Among the carotenoids,  $\beta$ -carotene is accountable for producing retinol and retinoic acid and triggering various transcription factors, for example, retinoid X receptor, peroxisome proliferator-activated receptors and hormone receptors.<sup>36</sup> Carotenoids also have immunostimulatory activity,<sup>57,58</sup> which contributes to the health benefits and disease prevention mechanisms. There are numerous experimental evidences indicating the roles of carotenoids in inhibiting the onset of many serious diseases such as age-related macular degeneration, diabetes, skin diseases, depression, cardiovascular diseases and various types of cancer.<sup>59–62</sup> Carotenoids can also play roles in eye protection because they absorb damaging blue light that enters the eyes.<sup>63</sup> Vertebrates accumulate carotenoids to shield photoreceptors from short-wavelength light induced damage.<sup>64</sup> Due to their antioxidant and anti-inflammatory activities, carotenoids have significant preventive and therapeutic benefits against age-related ocular abnormalities including cataract, glaucoma, diabetic retinopathy and macular degeneration.<sup>65</sup> The positive association of ocular health with consumption of carotenoids has been proven by clinical trials and epidemiological studies.<sup>66</sup> Research has also suggested carotenoids can be therapeutic solutions against degenerative diseases specially brain injuries.<sup>67</sup> For instance, Alzheimer's disease (AD) is a serious

neurodegenerative disorder and the most common cause of dementia characterised by weakened memory and cognition. Carotenoids can provide neuronal protection in AD by decreasing amyloid- $\beta$  ( $A\beta$ ) secretion in human neurons via  $\beta$ -secretase inhibition since  $A\beta$  is one of the major pathological hallmarks in AD patient brains.<sup>68</sup> Additionally, higher total plasma concentrations of carotenoids are associated with lower possibility of depression in older persons and vice versa.<sup>69</sup> Memory Health in Birmingham and Howard Foundation UK have been issued a U.S. Patent for a product designed to prevent and treat neurodegenerative disease, or dementia. Patent (10,835,509) shows xanthophyll carotenoids, and omega-3 fatty acids have a significant positive impact on memory, mood and patient quality of life.<sup>70</sup> In a recent study, with reference to diabetes, serum  $\beta$ -carotene levels have been testified to be inversely associated with levels of HbA1c that are linked to impaired insulin sensitivity.<sup>36</sup> Plenty of evidences are found in literature indicating the positive effects of carotenoids against cardiovascular diseases.<sup>71-74</sup> Likewise, studies have demonstrated the efficacy of carotenoids as anti-osteoporotic,<sup>75</sup> neuroprotective,<sup>76</sup> anti-ageing,<sup>77</sup> anti-obesity<sup>78,79</sup> and antibacterial agents.<sup>80</sup> In addition to above mentioned applications, carotenoids might be used in the classification of algae as chemotaxonomic markers because some carotenoids are specific to certain algal classes.<sup>21</sup>

### 3 | PRODUCTION OF CAROTENOIDS FROM MICROALGAE

Natural carotenoids are considerably preferred over those prepared chemically since synthetic pigments might be detrimental to health and environment. Natural  $\beta$ -carotene contains a mixture of *cis* and *trans* isomers, which contribute to its superior biological properties. Such a mixture is rarely obtained from synthetic methods. Additionally, natural  $\beta$ -carotenes can be absorbed more easily by the body compared to synthetic ones.<sup>19,81</sup> Due to these benefits, production of natural carotenoids is gaining more and more importance in recent years, and microalgae might be the most favoured industrial organisms for biotechnological exploration of this purpose. Production of some important carotenoids from different microalgae is briefly discussed in this section.

#### 3.1 | Current production processes of astaxanthin

In 2018, global market size of astaxanthin was estimated at US\$ 600 million and predicted to reach US\$ 880 million in 2026. More than 95% of this market refers to synthetically derived astaxanthin, and the production cost of synthetic astaxanthin is about US\$ 1000 kg<sup>-1</sup> for a market price above US\$ 2000 kg<sup>-1</sup>. Astaxanthin extracted from *Haematococcus pluvialis* accounts for less than 1% of the total world production, and its market price is estimated at US\$ 7000 kg<sup>-1</sup> due to its higher production cost, estimated at best at about US\$ 1800 kg<sup>-1</sup> in an adequate location.<sup>82</sup> For instance, the production cost in the EU is approximately 1500 euro kg<sup>-1</sup> in Greece and 6400 euro kg<sup>-1</sup> in the

Netherlands, whilst for synthetic astaxanthin is around 880 euro kg<sup>-1</sup>. However, synthetic astaxanthin is not allowed to be used as food ingredients in the EU and in the United States, and therefore, natural astaxanthin is the only source of astaxanthin for food applications.<sup>83</sup> Due to increasingly recognised health benefits and growingly required market demand for natural astaxanthin, a significant amount of recent research has focused on better understanding the production processes of microalgae-based astaxanthin. To date, *Chlorella zofingiensis*,<sup>84,85</sup> *Haematococcus pluvialis*,<sup>86-88</sup> *Chlorococcum* sp.,<sup>89,90</sup> *Tetraselmis* sp.,<sup>91,92</sup> *Chlorella sorokiniana*,<sup>91,93</sup> *Chlorella protothecoides*<sup>94</sup> and *Scenedesmus obliquus*<sup>95</sup> have been delineated as species of microalgae that might be able to produce natural astaxanthin. Among these species, *H. pluvialis* has been best developed and eventually employed to produce astaxanthin at commercial scales.

Following around 40 years of industrial-scale development, tens of *Haematococcus* astaxanthin manufacturing facilities have been established in different countries. These existing production facilities differ in terms of scale and methods of cultivation. As such, they have different costs of production.<sup>88</sup> *H. pluvialis* contains the highest concentration of astaxanthin, ranging from 4% to 7.7%.<sup>96,97</sup> As much as 95% of the total carotenoids in *H. pluvialis* can be astaxanthin, which is significantly higher than those found in other types of microalgae, where only up to 85% of total carotenoids can be astaxanthin.<sup>98,99</sup>

Astaxanthin is typically produced from *H. pluvialis* via either a one-stage or two-stage approach. During the one-stage process, when nutrients start to deplete, algal cells convert into cysts whilst astaxanthin synthesis pathways are triggered.<sup>100</sup> The two-stage cultivation method involves a growth stage followed by an induction stage. During the growth stage, the microalgae cells are provided with sufficient nutrients to support the accumulation of biomass. During the induction stage, the conditions are adjusted to prompt the algal cells to develop into resting cells.<sup>101,102</sup> Previous research has found that the two-stage process produces a significantly higher yield of astaxanthin than the one-stage process. For instance, the titre of astaxanthin in dry biomass has been found to reach 4.0% of dry weight when the two-stage cultivation method is used, whilst only 0.8% was obtained using the one-stage cultivation approach.<sup>96</sup> A research comparison of the approaches reveals that the two-stage approach offers a lot of advantages over the one-stage method.<sup>103,104</sup> As such, it might be reasonable to conclude that the two-stage process represents a more effective strategy which enhances the economic viability of astaxanthin production from *H. pluvialis*.

*H. pluvialis* is typically cultivated in two types of bioreactors for commercial astaxanthin production: closed photobioreactors (PBRs) and open raceway ponds (ORPs). In both systems, the cells develop photoautotrophically and use non-organic carbon source.<sup>105,106</sup> Whilst both bioreactors have been used in industrial settings, more than 90% of the biomass is produced in ORPs.<sup>107</sup> ORPs are in fact the most cost-effective and straightforward platform for large scale microalgal cultivation. However, as they are open systems, they exhibit elevated water evaporation losses, suffer high risk of contamination and produce cell culture of low concentration, which results in an energy-intensive downstream processes. Furthermore, ORPs are

only suitable in areas in which the climate conditions are suitable. Closed systems do not have many of the problems associated with ORPs, but they are more costly to develop.<sup>108,109</sup> In combination with the relatively high costs, a further issue associated with PBRs is the lack of scalability. To address the above issues, multiple modifications have been tried in closed PBR systems to boost usability of PBRs, including intensifying the mixing, using artificial sources of light, and making the external surface larger. The PBRs that are most commonly used for industry are the Green Wall Panel (GWP) PBR, the Flat Panel Airlift (FPA) and the tubular PBR. Each of these schemes exhibits different characteristics, including the types of materials utilised and the principles of design employed. However, despite the fact that PBRs have been investigated in depth, there is still a need to improve the underlying configuration to optimise environmental and technological aspects to ensure that the systems are environmentally and economically viable.<sup>110</sup> To provide the required production scales and optimised cultivation conditions, there is a need to develop bioreactors that allow process automation, enable parameters to be controlled, reduce the risk of chemical and microbial contaminations and foster culture autonomy under varied climate conditions. A modular airlift photobioreactor system has been designed to meet these requirements. The semi-industrial system has a total volume of 1000 dm<sup>3</sup> and can be operated using a computer control system which monitors and adjusts LED lighting, air bubbling and carbon dioxide supply. The efficacy and efficiency of the system were verified by cultivating *H. pluvialis* via a two-stage process with the prospect of modular scaling up.<sup>111</sup>

Large-scale cultivation of *H. Pluvialis* to produce astaxanthin is inherently challenging as the cultures rely on stringently controlled environments during the green stage of development.<sup>112</sup> Microbio Resources Inc. performed the first large-scale evaluation of astaxanthin fabrication from *Haematococcus* in an industrial setting (500,000 L bioreactor, 4500 m<sup>2</sup>) in California back in 1987. The biomass powder that was produced was sold under the brand Algaxan Red and was used for the aquaculture sector. Since then, more than ten commercial plants have been developed for the production of *Haematococcus*-based astaxanthin, for instance, Mera Pharmaceuticals Inc. and Cyanotech Inc. in the United States; Fuji Chemical Industries Co. Ltd. in Japan; Alga Technologies Inc. in Israel and Parry Pharmaceuticals Inc. in India.<sup>113,114</sup> The majority of these plants use phototrophic cultivation although some companies are also using mixotrophic cultivation, one of which is Fuji Chemicals. Most, if not all, companies have adopted a two-stage cultivation strategy for production according to the most recent reports.<sup>88,109,115</sup> During the first stage of green cell cultivation, the cells are grown in reactors under nutrient replete conditions either phototrophically, mixotrophically or heterotrophically. During the second stage of red cell cultivation, green cell culture is transferred to OPRs or outdoor PBRs and induced to produce astaxanthin under stressed conditions mostly phototrophically.<sup>88</sup>

### 3.2 | Current production processes of $\beta$ -carotene

Natural  $\beta$ -carotene is preferred over synthetic  $\beta$ -carotene due to its higher fat-solubility and superior antioxidant properties.<sup>116</sup> Synthetic

$\beta$ -carotene is nearly in 100% *trans* form, whilst  $\beta$ -carotene from fruits and vegetables (carrots, pumpkins, sweet potatoes etc.) contains 10% of *cis* isomers, and the algal  $\beta$ -carotene contains all-*trans* form and 9-*cis* isomer in almost equal proportions.<sup>117</sup>

Among microalgae, genus *Dunaliella* is well-known for accumulation of large amounts of  $\beta$ -carotene. Carotenogenesis in *Dunaliella salina* is promoted by high salinity and highlight stresses. Furthermore, limitation of nitrogen and phosphorous as well as addition of organic solvent can also be used to boost production.<sup>118</sup> Annual production of *Dunaliella* biomass for  $\beta$ -carotene has been estimated to be about 1200 tons. Currently, *D. salina* has become one of the best producers of natural  $\beta$ -carotene, and it is estimated to supply more than 95% of the total natural  $\beta$ -carotene marketed. Among the *Dunaliella* species, *D. salina* and *D. bandowii* could accumulate a substantial quantity of  $\beta$ -carotene (10–14% of dry weight). *Dunaliella parva* could also produce substantial amount of  $\beta$ -carotene (up to 4% dry weight). *Dunaliella salina* is particularly important and preferred over other species due to its higher growth rates.<sup>116</sup>

For commercial scale cultivation of *Dunaliella*, phototrophic cultivation in open culture systems is the most established method at present, and large outdoor ponds of quite a few hectares can be used. In 1986, two Australian companies for first time started the large-scale production of  $\beta$ -carotene from *D. salina*. They used about 20 cm deep ponds and extensive pumping systems to maintain salinity and provide nutrients. Major challenges in the open culture systems include assuring the homogeneity of nutrient supply and uniformity of temperature and other conditions throughout the whole pond. To overcome the above challenges, closed culture systems or photobioreactors were considered as the alternative cultivation method. Although the production cost is high, photobioreactors offer various advantages, for instance, flexibility in the design, better control of cultivation parameters, higher biomass production, lower risk of contamination and less CO<sub>2</sub> losses.<sup>116</sup> Some additional advantages of closed systems are possibility of cultivation of sensitive strains that cannot deal with predators in open pond, high productivity and high purity.<sup>119</sup> Therefore, the recent studies are focusing on developing the  $\beta$ -carotene production process of *D. salina* using close cultivation systems.

Two different opinions on the optimal method of utilising *D. salina* cultivation to produce  $\beta$ -carotene have been presented in the literature.<sup>120</sup> According to the first view, growing dense *Dunaliella* culture in a single stage delivers greater carotenoid production. Growing *Dunaliella* within a single-stage typically produces biomass with lower  $\beta$ -carotene content but yields a higher density of cells and more biomass. Moreover, it is simpler to manage and needs less space. Whilst the second opinion asserts that cultivating microalgae in two-stages produces biomass with a higher  $\beta$ -carotene content (up to 10–15% of the dry weight) and supports high  $\beta$ -carotene productivity in the right conditions. In the first stage, the microalgae are cultivated in small size nursery ponds in nitrate-rich medium to achieve optimal biomass. In the second stage, the cells are transferred to large ponds and diluted to about one-third concentration in nitrate deficient medium to induce  $\beta$ -carotene synthesis.<sup>117</sup> Commercial  $\beta$ -carotene production from *D. salina* by two stage strategy is complicated by the fact that

the conditions required for optimal cell growth are different from those required for maximum accumulation of  $\beta$ -carotene. Such as, 18%–21% NaCl is required for the optimal growth of *D. salina*; however, maximum  $\beta$ -carotene accumulation is attained at 27% NaCl. The major disadvantages of this strategy are requirement of large area, higher cost, labour intensive process, predation by protozoa at low salinity and outgrow of non-carotenogenic algal species at low salinity. Therefore, a semi-continuous method, working at an intermediate salinity, was considered as alternative approach. In addition, the pond designs were reconsidered and the suitability of climate at the production facility should also be carefully considered.<sup>121</sup>

*Dunaliella* have been commercially employed to produce natural  $\beta$ -carotene currently in countries such as China, Israel, Australia, the United States and Mexico.<sup>122,123</sup> The leading companies that produce  $\beta$ -carotene from *Dunaliella* are located in Australia and Israel in semi-desert areas (Whyalla, South Australia; Hutt Lagoon, Western Australia; Eilat, Israel), where rain is less frequent and undesired dilution of ponds and decrease of salinity is minimised. These regions also have plentiful sunlight for most of the year, supporting fast growth and carotenoid production. In Australia, *D. salina* is grown in huge (total area > 750 ha), shallow (~30–50 cm deep), and unstirred open ponds, whereas, in Israel raceway ponds are used. In Australia, land is cheap and flat with the availability of unlimited water supply, making the open pond cultivation a success. On the other hand, in Israel, land is expensive, and it is difficult to acquire flat lands, and thus the use of more expensive raceway ponds is obligatory. Although high salinity and high sunlight at Hutt Lagoon and Whyalla results in a high rate of  $\beta$ -carotene production, the continuous mode of cultivation in unstirred ponds in which the solubility of CO<sub>2</sub> is low leads to low biomass comparing with that in the well-mixed raceway ponds used in Israel. In Israel, harvesting is done by centrifugation, but this would be economically impossible in Australia, given the fact that much larger volumes of culture must be processed. Less expensive methods of cell harvesting, such as flocculation and flotation, are employed in Australian plants, which are uniquely developed for this purpose.<sup>124</sup> After harvesting, the biomass may either be dried by spray drying to produce algal powder, or be further extracted to produce  $\beta$ -carotene oil. The major products produced and marketed by the companies include spray dried and stabilised whole-cell *Dunaliella* powder containing about 2%–3%  $\beta$ -carotene for applications in animal feeds and aquaculture, 1.6%–4% solution of  $\beta$ -carotene in vegetable oil to be used as a dietary supplement and 3% or higher titre of  $\beta$ -carotene in water-soluble beadlets for application in the food industry as colourants.<sup>121,124</sup>

Although the productivity data of *D. salina* in open cultivation systems are frequently reported, it is still challenging to draw comparisons between different facilities because they grow the microalgae in different areas that feature different climatic conditions, cultivation media and strains. Moreover, pertinent data on the market performance of the main microalgae manufacturers are frequently treated as confidential business information. Recently reported data of *D. salina* productivity at the Sevastopol region (Crimean Peninsula) suggest that *D. salina* culture may accrue in excess of 18.3 mg L<sup>-1</sup> of carotenoids.<sup>125</sup> This amount parallels the 15–20 mg L<sup>-1</sup>  $\beta$ -carotene

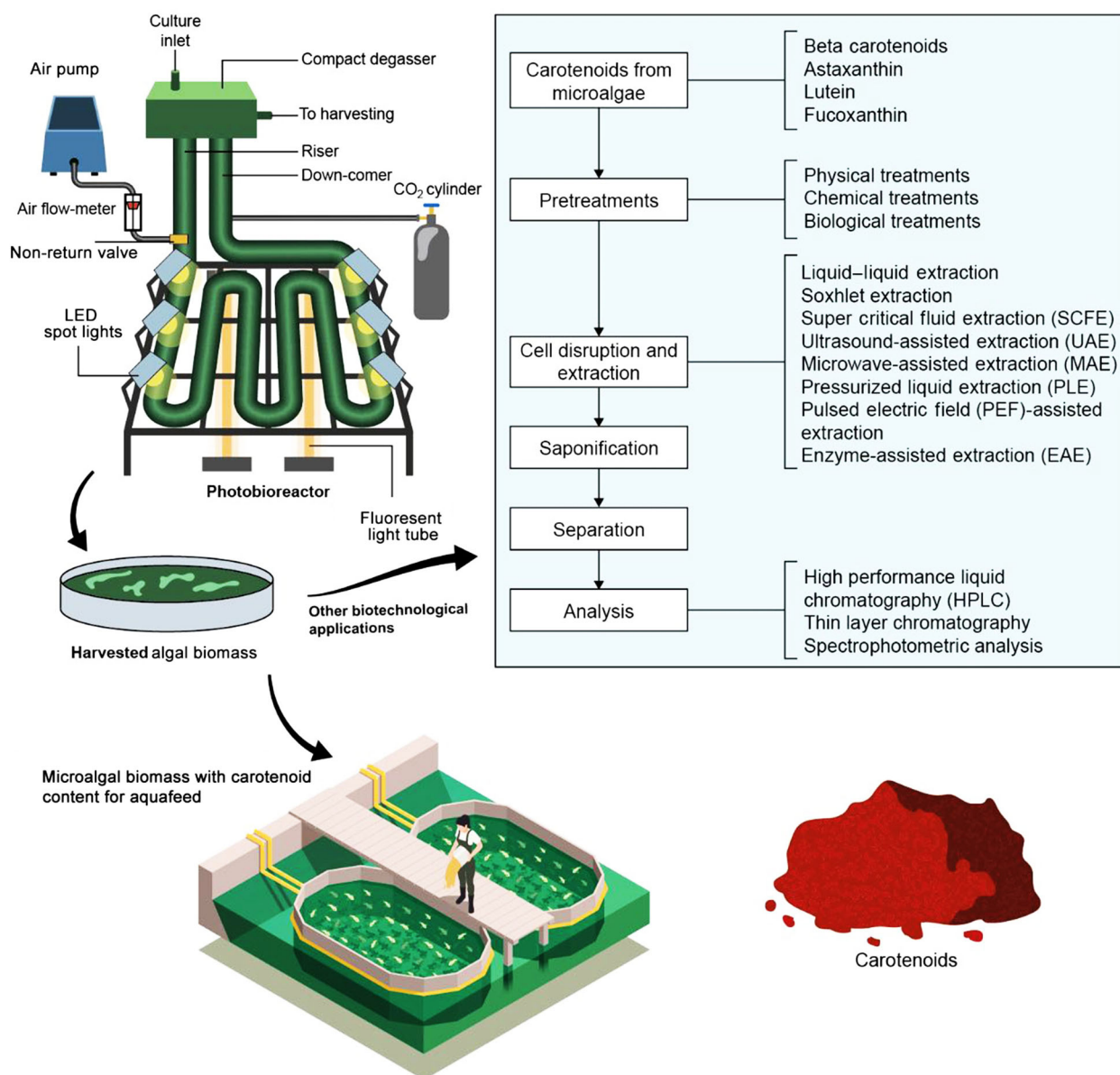
concentration level reported by China, Spain, Israel and Thailand. It is worth noting that  $\beta$ -carotene concentration was acquired under normal illumination in the absence of any further increase in salinity or addition of carbon dioxide. Further research is definitely needed to optimise the cultivation conditions to produce  $\beta$ -carotene.<sup>126–128</sup> A general strategy of carotenoids production from microalgae is given in Figure 3.

### 3.3 | Cultivation of microalgae to produce lutein

Lutein has high demand as a valuable nutraceutical functional food ingredient, and current global lutein market value is about EUR 255 million, which is expected to reach at EUR 409 million by 2027.<sup>129</sup> Chemical synthesis of lutein is not commercially feasible because of low overall yield although recently researchers have discovered a chemical synthesis method with more than 20% lutein yield from its precursor molecules.<sup>129</sup> Currently petals of marigold flower are the major source of commercial lutein, but microalgae are gaining more attention due to their capability to produce higher amounts of lutein than marigold.

Microalgae require less land and labour for cultivation than marigold and thus are regarded as key candidates for bioproduction of lutein.<sup>130</sup> They possess superior levels of biomass productivity, contain high amounts of lutein, can be cultivated in either freshwater, in brackish water or seawater, or even in waste water, and do not depend on arable land or environmental weather conditions.<sup>131</sup> Microalgae can be cultured all through the year, and the environmentally friendly extraction methods of lutein can be tailored for microalgal biomass. Lutein from marigold is esterified, but in microalgae lutein exists in free forms, which can be easily absorbed compared to the esterified forms.<sup>129</sup> In future, in case of socioeconomic changes in the marigold-producing countries that land availability possibly becomes difficult and labour becomes expensive, the microalgal lutein may be the best possible alternative.<sup>132</sup> Nevertheless, the primary difficulty in producing carotenoids from microalgae generally is that the biomass grows at low rates because of the stress conditions required for carotenogenesis. Due to this fact, cultivating microalgae for lutein production on an industrial scale generally requires two stages, first to cultivate the microalgae under optimal conditions for swift biomass growth using a complete growth medium, and then introduce stress conditions for improving the levels of the desired carotenoid.<sup>131</sup> Numerous techniques can be used to increase the level of lutein content in microalgae, including varying the intensity of light, using different light colours, modifying growing conditions and limiting nutrients.

Furthermore, the growth rate of microalgae is 5–10 times higher than plants, and they do not compete for resources with conventional agriculture. Even if the lutein content in microalgae is lower than marigold under normal conditions, microalgal production of lutein can be combined with other valuable algal products including vitamins, polysaccharides and pigments. Therefore, an aquaculture process could be developed for lutein production, coupling with other products and environmental benefits, such as wastewater treatment and CO<sub>2</sub>



**FIGURE 3** General strategy of microalgal-based carotenoids production. The general strategy of mixotrophic cultivation is similar to that of phototrophy except the use of organic carbon sources. The extraction part of the general strategy is only relevant when microalgal carotenoids are extracted for functional foods or any other biotechnological applications. Because microalgal biomass with appropriate carotenoid content can be directly used as aquafeed, extraction steps are generally not required.

fixation. According to estimations, annual microalgae biomass production of 70–150 ton per hectare could be achieved, using microalgae *Scenedesmus almeriensis*, which contain  $8.54 \text{ mg g}^{-1}$  of lutein in biomass. To draw a comparison, annual average production of marigold is 30–60 tons per hectare with lutein content of about  $12.31 \text{ mg g}^{-1}$ .<sup>133</sup>

Research has been undertaken into producing lutein using microalgae, covering a variety of species, such as *Chlamydomonas reinhardtii*,<sup>134</sup> *Chlorella protothecoides*,<sup>135,136</sup> *Tetraselmis suecica*,<sup>137,138</sup> *Dunaliella salina*,<sup>139</sup> *Dunaliella bardawil*,<sup>140</sup> *Scenedesmus almeriensis*,<sup>141</sup> *Chlorella zofingiensis*,<sup>142</sup> *Chlorella sorokiniana*,<sup>143</sup> *Galdieria sulphuraria*,<sup>144</sup> *Muriellopsis* sp.,<sup>145</sup> and *Scenedesmus incrassatulus*.<sup>146</sup> Recent research<sup>147</sup> has examined the influence of cultivation

conditions to produce lutein from *Chlorella vulgaris*, with a pilot plant being developed. It was shown that even at low-intensity light and comparatively high nitrate level conditions, high specific lutein concentration could be achieved, and it can be predicted that more intense light would yield higher cell density although adding 100 mM of NaCl was proved to have no notable impact on lutein levels. This process was scaled up to 50 L bubble column photo-bioreactors, and it was demonstrated that the same level of lutein productivity was achievable as at the lower (5 L) scale. It was also demonstrated that the greatest productivity ( $1.6 \text{ mg L}^{-1} \text{ day}^{-1}$ ) was achievable at the pilot scales. It was shown that this process could run in a semi-continuous manner for 32 days, with high concentrations of lutein being

achieved ( $15\text{--}20\text{ mg L}^{-1}$ ). These findings are clearly encouraging to develop large scale processes for lutein production. In the last few years, researchers have been examining ways to optimise light supply for lutein production from microalgae. A productivity level of  $33.8\text{ mg L}^{-1}\text{ day}^{-1}$ <sup>148</sup> was achieved by cultivating strain *Scenedesmus obliquus* M2-1, which was superior to those achieved by other researchers using *C. protothecoides* ( $10.0\text{ mg L}^{-1}\text{ day}^{-1}$ ),<sup>149</sup> *D. salina* ( $3.68\text{ mg L}^{-1}\text{ day}^{-1}$ ),<sup>139</sup> *S. incrassatus* ( $3.0\text{ mg L}^{-1}\text{ day}^{-1}$ )<sup>146</sup> and *S. obliquus* ( $4.15\text{ mg L}^{-1}\text{ day}^{-1}$ ).<sup>131</sup>

Mixotrophic cultivation has recently been considered as more suitable approaches for microalgal lutein production although more studies are needed to develop scalable and commercially feasible methodologies.<sup>143</sup> Research by Florez-Miranda et al. employed a two-phase cultivation technique, which firstly cultivated *S. incrassatus* heterotrophically and then followed by a photoinduction phase to improve biomass and lutein production.<sup>146</sup> To optimise the first phase cultivation, a variety of nitrogen sources and temperature levels were tested, and the greatest amount of lutein content was found at  $30^{\circ}\text{C}$  and with urea as the nitrogen source. Whilst in the second phase, lutein content rose seven times following 24 h of photoinduction. Comparing between autotrophic and heterotrophic growth, the researchers found that heterotrophic methods increased lutein production by 1.6 times. Thus it was proposed that heterotrophic and/or mixotrophic methods of producing lutein were superior means of cultivation in comparison to autotrophic cultivation.

Although currently commercial facility of lutein production from microalgae has not yet been established, experiments at pilot scales have been undergoing to cultivate *Muriellopsis* and *Scenedesmus* for production process development. The system employed by was a 4000 L closed tubular system configured with a double loop, which was one of the largest single-unit closed photobioreactors ever built solely for the purpose of cultivating *Scenedesmus almeriensis* strain to produce lutein. This system subsequently underwent a redesign to raise the capacity up to a 28,000 L working volume, comprising ten 2800 L units in a fence-style configuration.<sup>150</sup> Despite the photobioreactors having a larger volume and greater tube diameter (10 cm with the 4000 L unit, 9 cm with the fence type unit), the 4000 L system had greater productivity than the 55 L prototype detailed by Del Campo, García-González and Guerrero<sup>114</sup> in terms of both volume and occupied area, reaching  $290\text{ mg lutein m}^{-2}\text{ d}^{-1}$ .<sup>114,151</sup> It was reported that *Muriellopsis* sp. cultivated in the 55 L tubular photobioreactor had the highest level of lutein production at  $180\text{ mg m}^{-2}\text{ d}^{-1}$ .<sup>114</sup> The ability of *Muriellopsis* sp. to accumulate lutein was further evaluated outdoors in open ponds agitated by a paddlewheel. The system was operated semi-continuously under conditions of boosting both biomass and lutein production for a duration of 9 months. The average productivity was  $20\text{ g dry biomass}$  with about  $100\text{ mg lutein m}^{-2}\text{ day}^{-1}$  yield in summer. This cultivation strategy looks promising for the development of commercial lutein production processes from microalgae.

To conclude, commercially viable production of lutein as shown in experiments with the species *Muriellopsis* and *Scenedesmus* might be quite possible, using tubular photobioreactor systems. This conclusion

can be further corroborated by the fact that the same equipment has been successfully employed to produce other carotenoids from microalgae.<sup>129</sup> The primary obstacle for commercial exploitation of lutein production from microalgae lies in the downstream extraction/separation processes, which represent 50%–80% of overall production costs.<sup>152</sup> This is due to the fact that many algal species have rigid cell walls, which limits yields and rates of pigment extraction.<sup>153,154</sup> The problems of high harvesting cost and high energy requirements for cell disruption and extraction can be resolved with further studies.<sup>133</sup> This means there is a requirement for a method of extraction that is rapid, non-complex, selective and economically viable. Though technical obstacles do exist, we still intend to conclude that microalgae could be promisingly able to compete with marigold as organisms of lutein production with current state of art technologies and with further improvements expected in microalgal technology in the near future. Commercial production of microalgal lutein requires the attention of aquaculture engineers to implement already available microalgal cultivation technologies relying upon recent research outcomes.

### 3.4 | Cultivation of microalgae to produce fucoxanthin

Currently fucoxanthin can be produced from brown macroalgae, but there are two major problems with this feedstock. First, the content of fucoxanthin in macroalgae is relatively low ( $0.01\text{--}2.08\text{ mg g}^{-1}\text{ DW}$ ), and thus the extraction cost is extremely high. Secondly, fucoxanthin from macroalgae would compete with global food production resources since seaweeds are cultivated offshore mostly for food consumption.<sup>155</sup> Alternatively, microalgae are free from competition for food resources and can be a better option for fucoxanthin production because they can accumulate higher content of fucoxanthin ( $2.24\text{--}59.2\text{ mg g}^{-1}\text{ DW}$ ).<sup>156</sup> Rapidly growing diatoms, a category of microalgae, can synthesise fucoxanthin at high levels, which makes them potential sustainable sources of fucoxanthin, but cost-effective production of fucoxanthin from diatoms at commercial scales still requires considerable improvement in productivity. Whether or not diatoms would eventually be commercially utilised for fucoxanthin production depends on the availability of strains of mutagenesis or genetic engineering that can produce higher levels of fucoxanthin, and this would in turn rely on effectiveness of optimising diatom cultivation conditions.<sup>157,158</sup>

Although many species of microalgae are capable of producing fucoxanthin, only a small number of them have been explored for producing the compound commercially.<sup>158,159</sup> Among these are *Chaetoceros calcitrans*,<sup>160</sup> *Chaetoceros gracilis*,<sup>161</sup> *Cylindrotheca closterium*,<sup>162</sup> *Halamphora coffeaeformis*,<sup>163</sup> *Isochrysis galbana*,<sup>164</sup> *Phaeodactylum tricornutum*<sup>165,166</sup> and *Odontella aurita*.<sup>159</sup> A Korean group has found that high levels of fucoxanthin could be accumulated in the microalga *Isochrysis* aff. *galbana* (*Tisochrysis lutea*) and at the same time the molecule could be extracted by a one-step 5-min ethanol procedure with roughly 95% extraction efficiency.<sup>160</sup> Further experiments showed that a two-phase solvent system of n-hexane-

ethanol-water (10:9:1) could separate fucoxanthin and lipids from extracts, making *I. aff. galbana* potentially a useful commercial source for simultaneous production of both lipids and fucoxanthin. Some other investigations have been undertaken to clarify the influence of cultivation conditions on the content of fucoxanthin in various microalgal species cultivated in photobioreactors, and relatively high levels of fucoxanthin have been reached in a few species, such as *Tisochrysis lutea* (18.23 mg g<sup>-1</sup> dried sample) and *Odontella aurita* (18.47 mg g<sup>-1</sup> dried sample). *T. lutea* might be superior to *O. aurita* in terms of commercial production since the former species could be cultivated in continuous/mild conditions, achieving both high levels of biomass and fucoxanthin production.<sup>167</sup> Additionally, Petrushkina, Gusev, Sorokin, Zotko, Mamaeva, Filimonova, Kulikovskiy, Maltsev, Yampolsky and Guglya<sup>168</sup> have found that *heterokont* microalgae (*Mallomonas* sp.) are easy to culture and isolate, which produce high levels of fucoxanthin.<sup>163</sup> To select those strains of microalgae that were the most efficient fucoxanthin producers, seven new strains from across Vietnam and Russia were isolated, and their fucoxanthin content and growth conditions were studied. From this research, a new strain of *Mallomonas* sp. (*Synurophyceae*) was isolated, which had very high levels of fucoxanthin in biomass (26.6 mg g<sup>-1</sup> DW). Analysing extant data related to *heterokont* and *haptophyte* algae revealed very interestingly that a trio of algal groups showed the greatest promise for commercially producing fucoxanthin: diatoms (max 21.67 mg g<sup>-1</sup> DW), *Synurophyceae* (max 26.6 mg g<sup>-1</sup> DW) and *Prymnesiophyceae* (max 18.23 mg g<sup>-1</sup> DW). McClure, Luiz, Gerber, Barton and Kavanagh<sup>169</sup> have found that extremely high levels of fucoxanthin (up to 42.8 mg g<sup>-1</sup> DW) could be accumulated in *Phaeodactylum tricornutum* in moderate light conditions upon process improvement, and surprisingly further addition of nitrate to the medium lead to a substantial increase in both fucoxanthin levels (up to 59.2 mg g<sup>-1</sup> DW) and photobioreactor productivity.<sup>164</sup> In light of the above research, potential is definitely high to develop commercially viable processes for production of fucoxanthin from microalgae in photobioreactors.<sup>114,170</sup> In fact, Algatechnologies Ltd, a microalgal biotechnology company situated in Israel, has already launched the first commercial fucoxanthin product, 3% fucoxanthin oleoresin trademarked as Fucovital®, from microalgae back in 2018 via large scale cultivation of *P. tricornutum* in fully controlled closed tubular outdoor photobioreactors.<sup>171,172</sup> In a recent study, two marine microalgae (*P. tricornutum* and *T. lutea*) have been cultivated in industrial scale (15 m<sup>3</sup>) tubular photobioreactors semi-continuously for fucoxanthin production, and *P. tricornutum* has exhibited better performance in terms of biomass (2.87 g DW L<sup>-1</sup>) and fucoxanthin content (0.2%–0.7%). These results are showing promises for commercial fucoxanthin production from microalgae using semi-continuous operation.<sup>156</sup>

Whilst at present commercial production of fucoxanthin from microalgae is not well established globally, we might still be able to conclude that microalgae can serve as a good feedstock for fucoxanthin production. Microalgae are single-cell microorganisms, and microalgal strains for fucoxanthin production can be conveniently selected and easily engineered. Large-scale microalgal cell culture can be well established and maintained in photobioreactor systems, which have

already been developed for other carotenoid production, such as astaxanthin. To further expand this aquaculture sector, research on strain selection and improvement, process optimisation and physiology of relevant microalgae should be carried out. Specifically, fucoxanthin synthesis and transport pathways and storage mechanisms might need to be further clarified for process improvement and optimisation, and the essential genes for fucoxanthin synthesis and regulation might need to be cloned for strain improvement via metabolic engineering.

## 4 | MIXOTROPHIC CULTIVATION OF MICROALGAE FOR CAROTENOID PRODUCTION

Commercial processes of carotenoid production might be possibly developed only after we sufficiently understand the physiology of microalgae. In this section, we first discuss physiology of mixotrophic cultivation in general and then specifically for several most studied microalgal species.

### 4.1 | Physiology of mixotrophic cultivation

In mixotrophic mode of cultivation, photosynthesis and aerobic respiration take place simultaneously. Less amount of organic carbon is required for energy production due to the use of light energy. In addition, photosynthesis and organic carbon metabolism function independently in a non-competitive manner; therefore, in order to study the physiology and metabolism of mixotrophic cultivation, it is critical to understand assimilation of inorganic and organic carbon in microalgae.

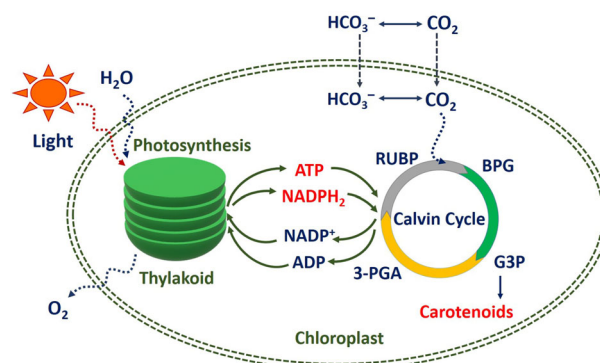
#### 4.1.1 | Assimilation of carbon dioxide in microalgae

Carbon dioxide (CO<sub>2</sub>) is the most important and often limiting substrate for photosynthetic carbon sequestration in plants and other photosynthetic organisms. In photoautotrophic mode, the sunlight is used as energy source, and CO<sub>2</sub> is utilised as carbon source. Cell utilises sunlight to excite the photosystems for the process of photo phosphorylation in order to get energy rich ATP and reductant NADPH<sub>2</sub>. ATP and NADPH<sub>2</sub> are then consumed to convert the inorganic carbon into carbohydrates. Fixation of one molecule of CO<sub>2</sub> requires two molecules of NADPH<sub>2</sub> and three molecules of ATP.<sup>173</sup> Soil-borne and aquatic photosynthetic organisms may encounter intense long-lasting and temporary variabilities over the time and space in the supply of dissolved inorganic carbon (Ci). The rather low amount of CO<sub>2</sub> in the atmosphere frequently limits microalgae growth mainly due to the low CO<sub>2</sub> affinity of ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco), the vital enzyme in net carbon assimilation.<sup>174</sup> In autotrophic organisms, Rubisco is the most abundant enzyme which catalyses the rate-limiting step in light-independent

reaction of C3 cycle. Rubisco catalyses photosynthetic carbon reduction and photorespiratory carbon oxidation by reacting with its substrates ribulose-1,5-bisphosphate (RuBP), and  $\text{CO}_2$  or  $\text{O}_2$ , respectively.<sup>175,176</sup> Carboxylation of RuBP by Rubisco produces two molecules of 3-phosphoglycerate (3-PGA) which then enters the C3 cycle. Efficiency of Rubisco is quite low due to slow turnover rate and requirement of high  $\text{CO}_2$  concentration. Moreover,  $\text{O}_2$  is a competitive inhibitor of the carboxylation reaction, and in the presence of  $\text{O}_2$ , oxygenation reaction occurs to produce phosphoglycolate (2-PG) and 3-PGA. 2-PG is a compound of no use and an uncommon metabolic intermediate. The 2-PG is converted to glycolate by hydrolysis which then enters salvage pathway (photorespiration), and at most 75% carbon is recovered in the form of 3-PGA with 25% carbon lost as  $\text{CO}_2$ . Rubisco oxygenation affects photosynthetic organisms in two different ways: (1) loss of carbon decreases photosynthetic efficiency and (2) photorespiration increases the energetic cost of photosynthesis.<sup>177</sup> Microalgae are able to use distinct pathways for  $\text{C}_i$  assimilation: (1) direct  $\text{CO}_2$  diffusive assimilation through the plasmatic membrane, (2) direct transport of  $\text{HCO}_3^-$  through the plasmatic membrane and (3) conversion of the  $\text{HCO}_3^-$  into  $\text{CO}_2$  by inducing the enzyme carbonic anhydrase (CA).<sup>178</sup> To cope with low  $\text{CO}_2$  concentration problem, microalgae have developed various means of supplying  $\text{CO}_2$  to the Rubisco that are known as carbon-concentrating mechanisms (CCMs). carbon-concentrating mechanisms are an evolutionary characteristic that developed for microalgae to acclimatise to low concentrations of  $\text{CO}_2$  particularly in aquatic environments. carbon-concentrating mechanisms actively uptake  $\text{HCO}_3^-$  via aquaporins to provide high level of  $\text{C}_i$  in microalgal cells and with the aid of CA provide elevated  $\text{CO}_2$  levels for photosynthetic apparatus. Carbonic anhydrase is present on the outer side of plasma membrane, in the cytoplasm and inside the chloroplast. Carbonic anhydrase speeds up the equilibrium of  $\text{CO}_2$  and  $\text{HCO}_3^-$  in order to enrich  $\text{CO}_2$  for photosynthesis. Extracellular CA also catalyses the hydration of  $\text{CO}_2$  that leaks from the cell and thus decreases the efflux of  $\text{CO}_2$  to the surroundings. All the  $\text{C}_i$  that enters into the cell is hydrated into  $\text{HCO}_3^-$  in the cytosol which then diffuses into the carboxysomes where CA converts it to  $\text{CO}_2$ , and as a result, carboxylase activity of Rubisco is favoured over oxygenase activity. On the other hand, at high concentration of  $\text{CO}_2$ , activity of CCM is down regulated, which also leads to the inactivity of CA. In that case, microalgae adopt other carbon fixation mechanism referred to as 3-hydroxypropionate pathway which uses the acetyl-CoA carboxylase and propionyl-CoA carboxylase to fix  $\text{CO}_2$  into glyoxylate and intermediates in carbon metabolism.<sup>179</sup> Inorganic carbon transport and  $\text{CO}_2$  assimilation in microalgal cell is shown in Figure 4.

#### 4.1.2 | Assimilation of organic carbon in microalgae

Some microalgae are capable of growing heterotrophically by consuming organic substrates as sole carbon and energy source, depending on the strain and growth conditions. The utilisation of organic carbon

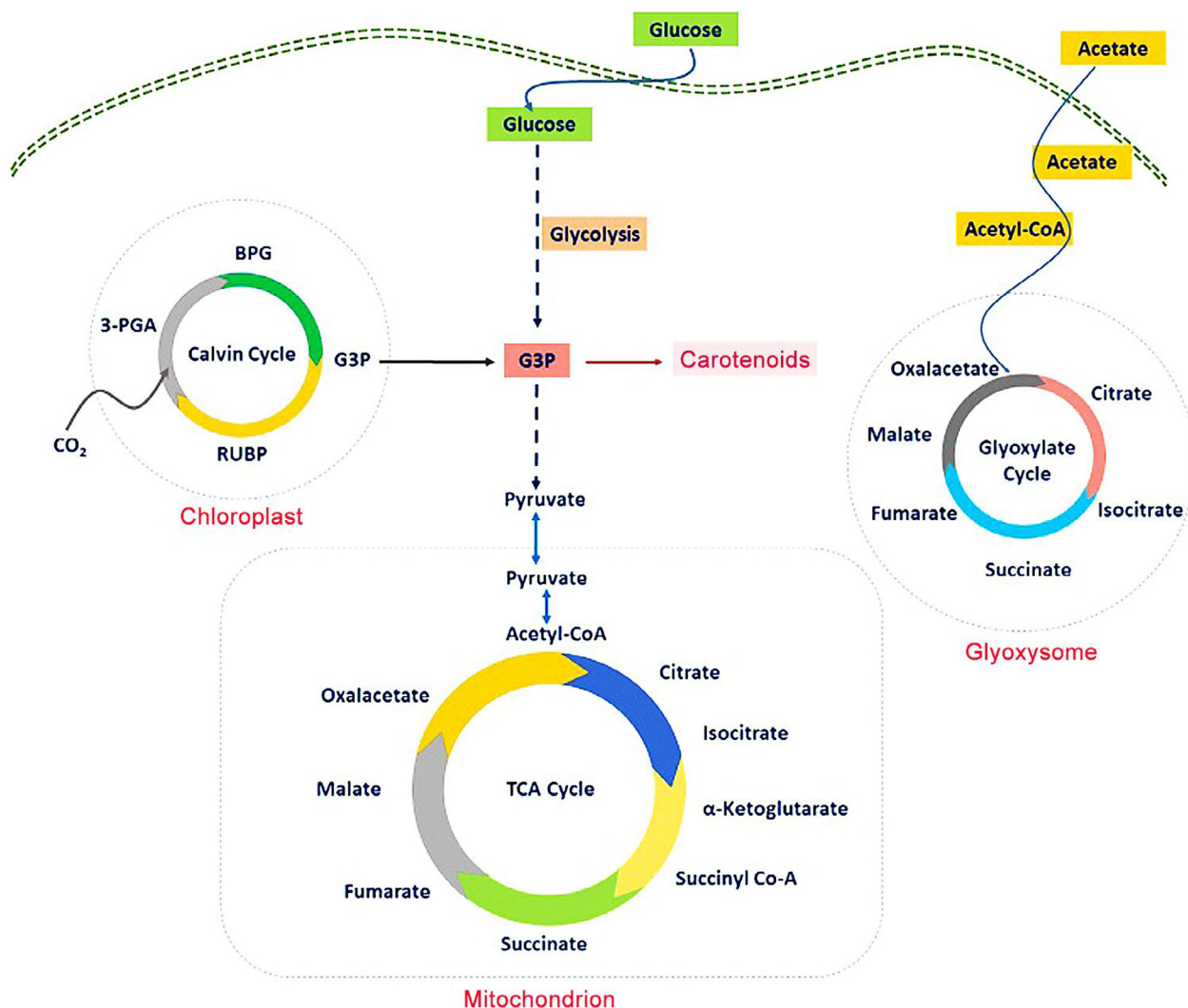


**FIGURE 4** Inorganic carbon transport and  $\text{CO}_2$  assimilation in microalgal cell (autotrophic metabolism). 3-PGA, 3-phosphoglycerate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BPG, bisphosphoglycerate; G3P, glyceraldehyde-3-phosphate;  $\text{HCO}_3^-$ , Bicarbonate;  $\text{NADP}^+$ , nicotinamide adenine dinucleotide phosphate;  $\text{NADPH}_2$ , nicotinamide adenine dinucleotide phosphate (reduced); RUBP, ribulose 1,5-bisphosphate

depends on the transportation of the carbon substrate across the membrane and the enzymatic processes to incorporate organic carbon into the central carbon metabolism. Light is not required for the growth of heterotrophic microalgae, and only a carbon source is sufficient to provide both the energy and carbon skeletons for the cells.<sup>180</sup> In heterotrophic mode of microalgal growth, the organic carbon is catabolised similar to bacteria. The membrane of organelles in microalgae encompasses a range of transporters of different specificities, such as, ion channels, primary and secondary transporters, assisting the transport of metabolites across the membrane and forming the metabolic network consisted by chloroplast, cytosol and mitochondria etc.<sup>173</sup>

Glucose is the most commonly used sugar and utilised by most microorganisms including microalgae. Various studies have used glucose in heterotrophic as well as in mixotrophic cultivation of microalgae.<sup>181–184</sup> Embden-Meyerhof (EM) pathway and Pentose Phosphate (PP) pathway are the two different pathways found in microalgae for the breakdown of glucose.<sup>185</sup> The PP pathway is particularly important as various enzymes found in this pathway also contribute in the Calvin cycle, which indicates that the carbon metabolism and photosynthetic  $\text{CO}_2$  assimilation are regulated by common cellular controls. The PP and EM pathways are both carried out in the microalgal cell cytoplasm, and based on the conditions (glucose and light), the PP pathway presumably has a greater metabolic rate than the other pathway.<sup>173,180</sup> In heterotrophic algae and under aerobic conditions, glucose can be completely oxidised into  $\text{CO}_2$  through EM pathway, PP pathway and tricarboxylic acid cycle (TCA), and ATP is generated by oxidative phosphorylation.<sup>173</sup>

Other than glucose, acetate (or acetic acid) is another common carbon source studied frequently for microalgal heterotrophic and mixotrophic growth.<sup>184,186,187</sup> Acetate uptake in microalgae is ATP dependent, and cells rely on monocarboxylic/proton transporter protein that facilitates the uptake of monocarboxylic molecules across the membrane.<sup>188</sup> The acetate is metabolised via acetylation of



**FIGURE 5** Organic carbon transport and assimilation in microalgal cell (heterotrophic metabolism). 3-PGA, 3-phosphoglycerate; BPG, bisphosphoglycerate; G3P, glyceraldehyde-3-phosphate; RUBP, ribulose 1,5-bisphosphate

coenzyme A by acetyl-CoA synthetase, producing acetyl-CoA, which then enters the TCA cycle or can be metabolised by glyoxylate cycle, an alternative cycle for the transformation of monocarboxylic acids. Glyoxylate cycle bypasses the  $\text{CO}_2$  liberating steps of TCA cycle and is more effective in conversion of carbon into cell biomass. Glyoxylate cycle is also vital in gluconeogenesis, which leads to the starch accumulation in the cells in the absence of light.<sup>173,185,189,190</sup> Glyoxylate cycle takes place in the glyoxysome. Glyoxysomes are specific peroxisomes present in the microalgae in which fatty acids can be metabolised for cell growth when photosynthesis is not active.<sup>191</sup> It is important to note that acetate at high concentration can be toxic and adversely affect the growth of cells.<sup>192</sup> Nevertheless, at low concentrations, numerous microalgae can utilise acetate as sole carbon source. This is indeed favourable since acetate is a convenient and low-cost substrate derived from several industrial processes, large-scale cultivation of microalgae with this molecule would not be limited by the substrate supply.<sup>193</sup> Organic carbon transport and assimilation in microalgal cell (heterotrophic metabolism) is presented in Figure 5.

#### 4.1.3 | Interplay of autotrophy and heterotrophy

Mixotrophic mode of microalgal growth involves the utilisation of organic and inorganic nutrients under light via respiration and photosynthesis, offering the advantages of both photoautotrophic and heterotrophic metabolism. In mixotrophic mode of cultivation, the supplementation of an organic carbon means that cell growth is not strictly dependent on photosynthesis, and therefore light is not an obligatory growth factor.<sup>194</sup> In mixotrophy, both metabolisms significantly contribute to cell growth, and the complete understanding of the contributions of both metabolisms still requires rigorous investigations. In depth research reports on this topic are not extensively available mostly because of the complex features of mixotrophy compared to the autotrophy or heterotrophy alone although understanding the physiology of mixotrophy is imperative to optimise commercial production processes.

Chojnacka and Noworyta<sup>195</sup> have studied the metabolic flux of heterotrophic and autotrophic metabolism under mixotrophy, using

*Spirulina* sp. as a model organism. They proposed that *Spirulina* sp. generates the essential ATP predominantly from light reactions at the initial period of growth. The ATP obtained from photosynthesis possibly speeds up the glucose anabolism in heterotrophic metabolism afterward. Since autotrophic and heterotrophic metabolisms were coupled in mixotrophic mode, the total algal biomass was much greater than in autotrophy or heterotrophy alone. Another study examined the balance of autotrophic and heterotrophic metabolism in mixotrophic culture of *Karlodinium micrum* and observed that it was dominated by heterotrophic metabolism.<sup>196</sup> Previously, Kamiya and Kowalik<sup>197</sup> reported the direct interference of light on glucose uptake under mixotrophic growth conditions, and it was found that autotrophic metabolism was favoured at the early stage of algal growth and glucose uptake could be blocked on Days 0–1. But this inhibition could be mitigated when the cell density was increased, leading to the dominance of heterotrophic metabolism. In a recent report, the contribution of autotrophy and heterotrophy to the central metabolism was studied in mixotrophic culture of *C. sorokiniana*. Results revealed that the ratio of autotrophy and heterotrophy altered dramatically during a mixotrophic growth process. Autotrophic metabolism was dominant at early stage of growth (days 0–1), but with the cell concentration increasing and photosynthesis inhibited by cell self-shading, heterotrophy became to be prevailing from day 1–3. With the consumption of organic carbon undergoing, organic carbon substrate became scarce after day 3 and autotrophy again completely dominated the metabolism. The findings suggested that higher cell density and subsequent self-shading are the key factors that directly influenced the contribution of autotrophy and heterotrophy during a mixotrophic culture.<sup>198</sup>

Previous reports revealed that EM pathway, TCA cycle and mitochondrial oxidative phosphorylation showed high activities in all three growth modes of microalgae. It also revealed that the mode of cultivation had only slight or no effect on these pathways.<sup>199,200</sup> Another investigation reported the detailed comparison of molecular mechanisms under autotrophic and mixotrophic culture of *C. sorokiniana*, using acetate as the substrate in mixotrophic cultivations. The results revealed that Chlorophyll content per cell was decreased in mixotrophy though the transcripts encoding the enzymes involved in the synthesis of chlorophyll precursor (Mg-protoporphyrin-IX) were upregulated.<sup>201</sup> Differential gene expression was detected for the enzymes used in carbon fixation under different modes of microalgal cultivation. Glyceraldehyde 3-phosphate dehydrogenase, an enzyme responsible for producing glyceraldehyde 3-phosphate in Calvin cycle, was downregulated in mixotrophy, endorsing the important role of photosynthates in mixotrophy. Cytoplasmic phosphoenolpyruvate carboxylase (PEPC), the key enzyme for C4 photosynthesis, was upregulated in mixotrophy, whereas all other enzymes of C4-like carbon fixation pathway were equally expressed in both autotrophy and mixotrophy. Upregulation of PEPC in mixotrophy suggested that *C. sorokiniana* shifted to C4-like carbon fixation for acetate metabolism to recover CO<sub>2</sub> released by the oxidation of acetyl-CoA. With increased CO<sub>2</sub> concentration in mixotrophy, CA and Rubisco activase were downregulated, whereas Rubisco accumulation factor (RAF) was

upregulated. In autotrophy, the relatively lower CO<sub>2</sub> concentration compared to mixotrophy maybe is responsible for the upregulation of Rubisco activase; however, in mixotrophy increased expression of RAF adds to the assemblage of Rubisco complex in order to enhance carbon fixation. Mitochondrial pyruvate dehydrogenase kinase (PDK) enzyme, which is accountable for the inactivation of pyruvate dehydrogenase by phosphorylation and preventing acetyl-CoA accumulation from pyruvate, was expressed only in the presence of acetate. In mixotrophy, glycolytic enzymes including pyruvate kinase, fructokinase, phosphoglycerate mutase and glyceraldehyde 3-phosphate dehydrogenase were all downregulated, demonstrating the critical role of those pathways in the absence of acetate, from which ATP and reducing powers were produced in the dark reaction of photosynthesis.<sup>201</sup> In a recent study, *Chlorella vulgaris* showed higher growth rate under mixotrophic cultivation (with acetate and CO<sub>2</sub> supplementation) compared to autotrophic and heterotrophic cultures. The chlorophyll content was highest under mixotrophic condition, and organic carbon metabolism was also enhanced, whereas the activities of CA and Rubisco were downregulated. Under mixotrophic cultivation, the transcript abundances of most enzymes involved in the Calvin and TCA cycle were increased significantly. Moreover, carbohydrate and amino acid metabolisms were more active, which might be one of the reasons for higher biomass production and improved CO<sub>2</sub> sequestration.<sup>202</sup> Through transcriptomic analysis, Lv, Zhao, Feng, Liu, Nan, Liu and Xie<sup>203</sup> described that under mixotrophic mode glucose metabolism primarily depended on the glycolysis and the PP pathway, whereas TCA cycle, oxidative phosphorylation and photosynthesis were considerably suppressed. Results further revealed that optimum concentration of organic carbon not only improved biomass production but also enhanced the organic carbon consumption efficiency. Taking together, all these results discussed above put forward that complex role of organic carbon in mixotrophic mode of cultivation might be an important topic of future investigation.

It is widely accepted that in the presence of organic carbon, photosynthesis and organic carbon metabolism are independent and non-competitive, resulting in the equivalent roles for cell growth of autotrophic and heterotrophic cultivation.<sup>204</sup> However, conflicting results are also found in the literature. For instance, Studies have reported the adverse effects of organic carbons on photosynthesis under mixotrophic cultivations.<sup>13,203,205</sup> On the other hand, upregulation of photosynthesis in the presence of organic carbon has also been observed frequently.<sup>204,206–208</sup> It is important to note here that different algal strains might react differently in balancing the utilisation of light and organic carbon sources, and thus whether selecting mixotrophy as cultivation methodology or not could be strain dependent. In conclusion, the controversial interactions between photosynthesis and organic carbon assimilation under mixotrophic modes are yet to be fully discovered.

Interestingly, the higher biomass yield in mixotrophy is also associated with photosynthetic reutilisation of CO<sub>2</sub> from organic carbon metabolism. Mixotrophic cultures can overcome the drawback of inadequate CO<sub>2</sub> by providing internal CO<sub>2</sub> sources from the respiration of organic carbon. Studies have proved that the reutilisation of

CO<sub>2</sub> could lead to higher biomass growth under mixotrophic cultivations.<sup>209–211</sup> In these situations, both autotrophic and heterotrophic metabolic pathways might have worked synergistically instead of competitively to promote cell growth because assimilation of organic carbon provided an endogenic source of CO<sub>2</sub> to derive photosynthesis and photosynthesis improved availability of O<sub>2</sub> for respiration. Consequently, gaseous transfer requirements became less demanding, and oxygen stripping costs might be reduced since lower amount of aeration was needed, comparing with autotrophic cultivation.<sup>212</sup>

## 4.2 | Mixotrophic cultivation of *Haematococcus pluvialis* for astaxanthin production

*Haematococcus pluvialis* is a unicellular freshwater microalga widely distributed in various water-logged environments and commonly known for its promising potential to produce natural astaxanthin. Astaxanthin is a secondary carotenoid with bright red colour, which can be produced by applying cellular stresses onto *H. pluvialis*.<sup>115</sup> Astaxanthin is mostly used as high value functional foods because of its extraordinary antioxidant activity, which is greater than  $\beta$ -carotene and lutein.<sup>213</sup>

*Haematococcus* can be cultivated phototrophically, heterotrophically and mixotrophically and go through evident morphological changes throughout its life cycle. The green vegetative cells and the red cyst cells are the most common morphological forms, and astaxanthin is accumulated in the red cysts. Photoautotrophic cultivation is extensively used methodology for astaxanthin production, and heterotrophic cultivation is seldom used in commercial process of production since biosynthesis of astaxanthin is light-dependent. Mixotrophic cultivation is considered as the most suitable alternative to improve *Haematococcus* growth and astaxanthin accumulation.<sup>214</sup> Various studies have reported the superior performance of mixotrophic cultivation of *H. pluvialis* for astaxanthin production.<sup>215–220</sup> Nevertheless, the production of astaxanthin from *H. pluvialis* is still very challenging due to high production cost, low growth rate, high risk of contamination and extended cultivation period.<sup>16</sup> Therefore, at present, major research focus is to overcome these problems by testing various cultivation strategies. The investigations of mixotrophic cultivation of *H. pluvialis* for astaxanthin production are summarised in Table 1.

Among some promising developments, a multilevel heuristic LED regime was used to significantly induce the growth and valuable products accumulation in *H. pluvialis* under mixotrophic conditions. The astaxanthin productivity was considerably increased, and the highest astaxanthin content was attained with gluconate as carbon source under the white-blue light regime. This novel multilevel LED strategy could be developed synergistically with many parameter controls according to the characteristics of algal cells for desired bio-product.<sup>222</sup> Varied substrates, such as monosaccharides, oligosaccharides, sodium acetate and polyalcohols, were tested for mixotrophic cultivation of *H. pluvialis*, and glycerol was singled out as the best choice due to its good performance both as carbon source and astaxanthin

inducer. Based on the above findings, a sequential mixotrophy dilution photoinduction strategy (SMDP) was proposed for economical astaxanthin production using glycerol as the carbon source.<sup>221</sup> This idea has been recently further developed by Khazi et al. who proved that mixotrophically cultivated seed culture of *Haematococcus* had at least equal or even better performance than phototrophically cultivated one in ORPs for astaxanthin production.<sup>223</sup>

Growth media and culture conditions used for mixotrophic cultivation of *H. pluvialis* influences the biochemical composition of the microalgae.<sup>224</sup> Marinho, Malafaia, de Araújo, da Silva, dos Santos, de Moraes and Gálvez,<sup>224</sup> evaluated the effect of various culture media on the growth, life cycle, biochemical composition and astaxanthin accumulation in *H. pluvialis* under photoautotrophic and mixotrophic conditions. Higher concentrations of chlorophylls *a* and *b* and protein were found under photoautotrophic cultivation, whereas higher biomass, fatty acid and astaxanthin accumulation were observed under mixotrophic cultivation. Modified Provasoli medium was proved to be the most efficient one to enhance astaxanthin productivity. Wen, Wang, Ding, Geng and Li<sup>214</sup> tested a nitrogen-depleted mixotrophic cultivation methodology in outdoor raceway ponds, using acetic acid as carbon source, and substantial increase in astaxanthin productivity was observed. This method might be promising for mass cultivation of *H. pluvialis* under mixotrophic growth mode. In another experiment, exogenous glycerol was used to promote astaxanthin and lipid accumulation in *H. pluvialis* under low light conditions. Exogenous glycerol could speed up the astaxanthin production by increasing the formation of pyruvate and by facilitating the esterification of astaxanthin.<sup>213</sup> This method could overcome the long cultivation period problem associated with astaxanthin production from *H. pluvialis*.

## 4.3 | Mixotrophic cultivation of *Chlorella zofingiensis* for astaxanthin production

*Chlorella zofingiensis* was previously assigned to the genus *Chlorella*, however, some reports classify this alga to the genus *Muriella*, *Mychonastes* or *Chromochloris*, indicating an evolutionary distance from the true *Chlorella*.<sup>225</sup> Now *C. zofingiensis* is also named *Chromochloris zofingiensis*.<sup>226,227</sup>

The unicellular *C. zofingiensis* has the ability to grow phototrophically, heterotrophically and mixotrophically. It can utilise a variety of organic carbons and can be easily cultured at fast growth rates. Furthermore, it is an excellent source of lipids and carotenoids.<sup>228</sup> It can produce both primary and secondary carotenoids and is considered as a possible alternative source of natural astaxanthin since its yield can reach up to 56 mg L<sup>-1</sup>, which is comparable to that of *H. pluvialis*.<sup>85</sup> In addition, the growth rate of *C. zofingiensis* is higher than *Haematococcus*, and it can be cultivated under various conditions with minimal negative environmental effects. Interestingly, it grows fast in all three modes of cultivation (autotrophy, heterotrophy and mixotrophy) and is easy to be scaled up both indoors and outdoors.<sup>229</sup> Easier cell disruption and greater recovery of pigments are the additional benefits of astaxanthin production from *C. zofingiensis*.<sup>230</sup>

**TABLE 1** Mixotrophic cultivation of *Haematococcus pluvialis* for astaxanthin production

| Strain                | Organic carbon                                                                                                                                   | Cultivation strategy                                                                                | Operational parameters                                                                                                           |                                       |                                           |                 | Outcome                                                                                                                                             | Reference |
|-----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|-------------------------------------------|-----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
|                       |                                                                                                                                                  |                                                                                                     | Light intensity<br>( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )                                                                      | Temperature<br>( $^{\circ}\text{C}$ ) | Aeration<br>rate                          | Agitation (rpm) |                                                                                                                                                     |           |
| UTEX No. 16           | Sodium acetate                                                                                                                                   | Batch and fed-batch                                                                                 | 45 (first 4 days) to 90 (Days 5–7), 120 (Days 8–11), 150 (Days 12–15) and 180 (after Day 15)                                     | 30                                    | 100 L h <sup>−1</sup>                     | 350             | Total astaxanthin production in the fed-batch culture was about 20.5% higher than in the batch culture                                              | 215       |
| 34/7                  | Sodium acetate and sodium malonate                                                                                                               | Batch                                                                                               | 68.25 dark: light cycle of 12:12 h                                                                                               | 18 ± 1                                | –                                         | –               | Sodium acetate and malonate enhanced the accumulation of astaxanthin than in autotrophy                                                             | 216       |
| Steptoe (Nevada, USA) | Sodium acetate                                                                                                                                   | Batch                                                                                               | 35 and 85                                                                                                                        | 23 ± 2                                | –                                         | –               | Highest astaxanthin content per dry weight was obtained at 85 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with 0.8% NaCl                                   | 217       |
| NIES-144              | Acetate                                                                                                                                          | Fed-batch (two-stage perfusion)                                                                     | 30, 60, 90, 120, 150 and 180                                                                                                     | 23                                    | 0.1 vvm                                   | 150             | High concentration of biomass and astaxanthin.                                                                                                      | 218       |
| CCALA 1081            | Sodium acetate                                                                                                                                   | Batch                                                                                               | 90 and 350                                                                                                                       | 25–26                                 | 500–700 cm <sup>3</sup> min <sup>−1</sup> | –               | High light stress led to a significant increase in the amount of astaxanthin both in autotrophic (22-fold) and in mixotrophic cultivation (16-fold) | 219       |
| NIES-144              | Sorbitol, mannitol, inositol, glycerol, arabinose, xylose, glucose, mannose, galactose, fructose, sucrose, maltose, raffinose and sodium acetate | Sequential mixotrophy dilution photoinduction (SMDP) and two-stage strategy in photoinduction stage | 75 (8 h light/16 h dark) in growth stage, 200–400 in dilution stage and 750 in photoinduction stage                              | 24 ± 0.5 and 26 ± 0.5                 | 0.1 vvm                                   | –               | Glycerol and SMDP induced a better growth performance and a higher astaxanthin concentration                                                        | 221       |
| NIES-144              | Sodium acetate                                                                                                                                   | Fed-batch (carbon to nitrogen and pH-stat cultivation)                                              | 4 ± 1                                                                                                                            | 24 ± 1                                | 0.5 vvm                                   | 30              | Higher level of astaxanthin under high carbon to nitrogen ratio                                                                                     | 220       |
| SCCAP K-0084          | Ribose, acetate or gluconate                                                                                                                     | Multilevel LED regimes                                                                              | White light (73 ± 2) at adaptation phase, red/blue light (80 ± 2) at green stage and red/blue light (135 ± 5) at induction stage | 25                                    | –                                         | 150             | Astaxanthin biosynthesis has been significantly enhanced and the highest content of 3.3% was achieved with gluconate at the white-blue regime       | 222       |

TABLE 1 (Continued)

| Strain | Organic carbon                 | Cultivation strategy | Operational parameters                                   |                                    |                          |                                 | Agitation (rpm) | Outcome                                                        | Reference |
|--------|--------------------------------|----------------------|----------------------------------------------------------|------------------------------------|--------------------------|---------------------------------|-----------------|----------------------------------------------------------------|-----------|
|        |                                |                      | Light intensity ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) | Temperature ( $^{\circ}\text{C}$ ) | Aeration rate            |                                 |                 |                                                                |           |
| wz-3   | Sodium acetate and acetic acid | Fed-batch            | 300 and 150                                              | 25                                 | 250 ml $\text{min}^{-1}$ | –                               | –               | Enhanced astaxanthin productivity                              | 214       |
| H6     | Exogenous glycerol             | Batch                | 200 and 150                                              | 25 $\pm$ 1                         | –                        | Manually shaken 5–6 times daily | –               | Accelerated astaxanthin production and increased lipid content | 213       |

Because of these advantages, various studies are now undergoing to explore the potential of this alga for carotenoids production.<sup>231–233</sup>

The mixotrophic physiology of *C. zofingiensis* is an important topic of investigation since mixotrophic cultivation methods are considered as a commercially promising option. A recent study explored the mixotrophic metabolism of *C. zofingiensis* and found that under mixotrophic cultivation glucose metabolism and photosynthesis operated in a dynamic balance in *C. zofingiensis*. The intermediates of glycolysis could enter straightly to chloroplasts, replacing Rubisco fixed  $\text{CO}_2$  to provide carbon for photosynthesis during mixotrophy. This would reduce the wastage of energy which might be the reason of higher biomass productivity.<sup>228</sup> Recently, a *C. zofingiensis* mutant (CZ-bkt1), which can simultaneously accumulate high amounts of zeaxanthin, lutein and  $\beta$ -carotene under various culture conditions, was engineered. CZ-bkt1 was found to have a dysfunctional carotenoid ketolase, leading to the accumulation of zeaxanthin instead of its downstream ketocarotenoid astaxanthin. Currently, CZ-bkt1 is the only species that can accumulate high amounts of these three important carotenoids simultaneously.<sup>142</sup>

Various interesting mixotrophic cultivation approaches have been proposed for *C. zofingiensis*, focusing on cost reduction in biomass and astaxanthin production. For instance, a two-step cultivation method was proposed to cultivate the microalgae in a patented floating photobioreactor which was constantly shaken by the natural flowing water for cost-effective production of astaxanthin.<sup>84</sup> Another study investigated the performance of a vertical tubular outdoor photobioreactor system to cultivate *C. zofingiensis*. The water spray-based cooling system combined with shade tarpaulin successfully reduced the cost of temperature control and improved the biomass productivity in outdoor photobioreactors.<sup>234</sup> Recently, a two-stage strategy was developed to detect the role of phytohormones as positive stimulators enhancing astaxanthin and lipid coproduction in microplate-based mixotrophic cultures of *C. zofingiensis*. The outcomes showed that among various phytohormones auxins were the most efficient stimulators of astaxanthin production.<sup>235</sup> In another study, Chen, Jiang and Wei<sup>16</sup> investigated the effects of urea on cell growth and pigment profile of *C. zofingiensis* under mixotrophic cultivation conditions and suggested that urea could be used as an economical nitrogen source for the production of natural astaxanthin with no significant variations of pH in the culture medium during the cultivation process. Although the research at pilot scales are not available, these recent findings should be able to serve as a starting point towards the development of innovative, commercially viable and cost effective methodologies for mixotrophic production of astaxanthin from *C. zofingiensis*. Research reports about mixotrophic cultivation of *C. zofingiensis* for carotenoids production is summarised in Table 2.

#### 4.4 | Mixotrophic cultivation of other species for carotenoid production

Among other microalgae, *C. sorokiniana*,<sup>236,237</sup> *Desmodesmus* sp.<sup>238,239</sup> and *Dunaliella salina*<sup>240,241</sup> were frequently studied for carotenoid

production under mixotrophic conditions. *Scenedesmus* sp.,<sup>242</sup> *Coccomyxa acidophila*<sup>243</sup> and *Nitzschia laevis*<sup>244</sup> were also cultivated mixotrophically for carotenoid production.

*Chlorella sorokiniana* had been chosen for lutein production for long, and it was discovered not very long ago that lutein production in fact could be enhanced under mixotrophic conditions. Cells of *C. sorokiniana* were first mixotrophically cultivated under fed-batch mode , supplemented with sodium acetate every 12 h for 1.5 days, and then roughly 92.5% of the culture was transferred to another photobioreactor to induce lutein production. Lutein content (9.57 mg g<sup>-1</sup>) and productivity (11.57 mg L<sup>-1</sup> day<sup>-1</sup>) were significantly improved by this methodology.<sup>237</sup> Recently, a very interesting strategy named ‘oxygen balanced’ mixotrophy was proposed for *C. sorokiniana* SAG 211/8 K. Under this strategy, photosynthetic O<sub>2</sub> production was counterbalanced by respiratory oxygen consumption, and CO<sub>2</sub> required for photosynthesis could be supplemented by aerobic conversion of acetic acid. The setup, carried out as 14 h:10 h day/night cycles, did not require substantial amount of gas–liquid exchange during daytime, and 96% of the substrate was found to be converted into biomass. Moreover, under mixotrophic cultivation, 61 times less aeration was applied, and the biomass productivity was doubled, compared to the photoautotrophic culture.<sup>245</sup> The basic idea resides in the balance between respiration and photosynthesis was previously reported.<sup>13</sup> A mutant, *C. sorokiniana* MB-1-M12 showed higher lutein productivity under semi-continuous cultivation with a medium replacement ratio of 75%, simulating outdoor cultivation conditions

(at 35°C/25°C for a 12 h/12 h light/dark cycle).<sup>246</sup> In a recent study, the coproduction of astaxanthin and lipids was estimated for *C. sorokiniana* under mixotrophic cultivation using an external loop airlift photobioreactor, and the results suggested that this method could be economically viable, environmentally sustainable and of greater commercial potential.<sup>93</sup> Previously, internal loop airlift photobioreactor was also successfully used to enhance lutein productivity and decrease the cultivation cost, using crude glycerol for mixotrophic cultivation of *Scenedesmus* sp. In fed-batch mode, step-wise addition of crude glycerol was found to be able to enhance cell biomass and lutein productivity.<sup>242</sup>

*Dunaliella salina* is another unicellular microalga extensively studied for carotenoids production, and the industrial sector of β-carotene production from *D. salina* has already been well established. However, the production cost is still high, and biomass production of *D. salina* is the major challenge. A recent study reported high biomass, protein and carotenoid content could be achieved, using crude glycerol for mixotrophic cultivation of *D. salina*. The microalgae were cultivated in a helical photobioreactor, and continuous cultivation was performed at the dilution rate of 0.52 day<sup>-1</sup>. The biomass productivity was 75% higher than the previously reported values for autotrophic cultivations.<sup>241</sup> In a previous study, the β-carotene and biomass contents of mixotrophically grown *D. salina* were increased 1.95- and 1.75-folds, respectively, through media optimisation using response surface methodology. The optimal conditions were 15 g L<sup>-1</sup> glucose, 1.8 g L<sup>-1</sup> nitrate and 0.013 g L<sup>-1</sup> phosphate.<sup>240</sup>

**TABLE 2** Mixotrophic cultivation of *Chlorella zofingiensis*/*Chromochloris zofingiensis* for astaxanthin production

| Strain                                         | Organic carbon | Cultivation strategy             | Operational parameters                                  |                  |               |                 | Outcome                                                                                                     | Reference |
|------------------------------------------------|----------------|----------------------------------|---------------------------------------------------------|------------------|---------------|-----------------|-------------------------------------------------------------------------------------------------------------|-----------|
|                                                |                |                                  | Light intensity (μmol m <sup>-2</sup> s <sup>-1</sup> ) | Temperature (°C) | Aeration rate | Agitation (rpm) |                                                                                                             |           |
| <i>Chlorella zofingiensis</i> (ATCC 30412)     | Glucose        | Batch                            | 90                                                      | 30               | —             | 130             | High glucose and low nitrate favoured the production of astaxanthin                                         | 231       |
| <i>Chromochloris zofingiensis</i> (ATCC 30412) | Glucose        | Fed-batch                        | 150                                                     | 26 ± 1           | —             | 150             | The highest contents of astaxanthin and fatty acids were simultaneously obtained at 0.2 mM Fe <sup>2+</sup> | 232       |
| <i>Chromochloris zofingiensis</i> (ATCC 30412) | Glucose        | Batch (microplate-based culture) | 80 ± 30, 150 ± 30 and 300 ± 30                          | 26               | —             | 350             | Higher astaxanthin production in nitrogen-deprived culture                                                  | 233       |
| <i>Chromochloris zofingiensis</i> (ATCC 30412) | Glucose        | Batch (microplate-based culture) | 300 ± 30                                                | 26               | —             | 350             | Highest levels of astaxanthin (89.9 mg L <sup>-1</sup> ) in this microalga reported to date                 | 235       |
| <i>Chromochloris zofingiensis</i> (ATCC 30412) | Glucose        | Batch                            | 80                                                      | 28               | —             | 140             | Higher biomass and higher yield of total carotenoids                                                        | 16        |

TABLE 3 Mixotrophic cultivation of various other microalgae for carotenoids production

| Microalgae                                         | Organic carbon             | Cultivation strategy                                   | Operational parameters                                     |                                    |               |                 | Outcome                                                                          | Reference |
|----------------------------------------------------|----------------------------|--------------------------------------------------------|------------------------------------------------------------|------------------------------------|---------------|-----------------|----------------------------------------------------------------------------------|-----------|
|                                                    |                            |                                                        | Light intensity ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )   | Temperature ( $^{\circ}\text{C}$ ) | Aeration rate | Agitation (rpm) |                                                                                  |           |
| <i>Chlorococcum</i> sp.                            | Glucose                    | Batch                                                  | 22                                                         | 25                                 | –             | 150             | Enhanced induction of astaxanthin formation by reactive oxygen species           | 248       |
| <i>Coccomyxa acidophila</i> acidophile microalgae) | Urea (both C and N source) | Batch                                                  | 150                                                        | 25                                 | –             | –               | Lutein accumulation was up to $3.55 \text{ mg g}^{-1}$                           | 243       |
| <i>Desmodesmus</i> sp. F51                         | Sodium acetate             | Batch, fed-batch and repeated fed-batch                | 600 in batch, 200–1050 in fed batch and repeated fed-batch | 26–28                              | 0.2 vvm       | –               | Lutein production was enhanced with fed-batch cultivation.                       | 238       |
| <i>Dundaliella salina</i>                          | Glucose                    | Batch                                                  | 60                                                         | 25                                 | –             | 130             | The $\beta$ -carotene content was increased 1.95-fold                            | 240       |
| <i>Chlorella sorokiniana</i> MB-1 and the mutants  | Sodium acetate             | Batch                                                  | 150                                                        | 25                                 | 0.1 vvm       | 300             | Mutant strain MB-1-M12 had better lutein production                              | 236       |
| <i>Chlorella sorokiniana</i> MB-1                  | Acetate                    | Semi-batch and semi-batch-integrated two-stage process | 100                                                        | 26–28                              | 0.1 vvm       | –               | Enhanced biomass and lutein productivity                                         | 249       |
| <i>Nitzschia laevis</i> (UTEX 2047)                | Glucose                    | Batch                                                  | 15                                                         | 22                                 | –             | 150             | Enhanced fucoxanthin and eicosapentaenoic acid yields                            | 244       |
| <i>Chlorella sorokiniana</i> FZU60                 | Acetate                    | Multistage integrated fed batch                        | 150                                                        | 33                                 | 0.15 vvm      | 300             | Enhanced lutein product was obtained with multi-operation integrated method      | 237       |
| <i>Dundaliella salina</i>                          | Crude glycerol             | Batch                                                  | 75                                                         | 30                                 | –             | 150             | Carotenoid content was more than 60% higher than that of autotrophic cultivation | 241       |
| <i>Chlorella sorokiniana</i> MB-1-M12              | Acetate                    | semi-continuous                                        | 150                                                        | 35 and 25                          | 0.1 vvm       | –               | Higher lutein productivity compared to batch and fed-batch                       | 246       |
| <i>Scenedesmus obliquus</i> CWL-1                  | Glucose                    | Batch and fed-batch                                    | 150                                                        | 30                                 | –             | 150             | Highest lutein production in fed-batch                                           | 250       |

(Continues)

TABLE 3 (Continued)

| Microalgae                               | Organic carbon                                     | Cultivation strategy                                      | Operational parameters                                      |                                       |                             |                    | Outcome                                                                               | Reference |
|------------------------------------------|----------------------------------------------------|-----------------------------------------------------------|-------------------------------------------------------------|---------------------------------------|-----------------------------|--------------------|---------------------------------------------------------------------------------------|-----------|
|                                          |                                                    |                                                           | Light intensity<br>( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) | Temperature<br>( $^{\circ}\text{C}$ ) | Aeration<br>rate            | Agitation<br>(rpm) |                                                                                       |           |
| <i>Chlorella sorokiniana</i> SAG 211/8 K | Acetic acid                                        | 'Oxygen balanced' mixotrophy under 14:10 day/night cycles | 514 $\pm$ 17                                                | 37                                    | 0.1 vvm (during night only) | 500                | Mixotrophic biomass contained 50% w/w of protein and 7.3 mg g <sup>-1</sup> of lutein | 245       |
| <i>Desmodesmus subspicatus</i> LC172266  | Glucose or glycerol in batch, glucose in fed-batch | Batch and fed-batch                                       | 50                                                          | 30                                    | –                           | 100                | Enhanced carotenoid and lipid productivities                                          | 239       |
| <i>Scenedesmus</i> sp. (CAS-173)         | Crude glycerol                                     | Fed-batch                                                 | 3000 lux, 16:8 light-dark cycles                            | 23 $\pm$ 10                           | –                           | –                  | Stepwise addition of crude glycerol enhanced biomass and lutein productivity          | 242       |

Microalga *Nannochloropsis gaditana* has been long studied for lipid production under photoautotrophic cultivations conditions and is also capable of producing carotenoids such as violaxanthin and vaucheraxanthin. No detailed information regarding its mixotrophic cultivation appeared to be available until recently.<sup>247</sup> The microalga was cultivated under both mixotrophic and phototrophic conditions, and the biomass, fatty acid and carotenoid levels in various situations were thoroughly compared. It was proved that *N. gaditana* might be able to grow mixotrophically, using glucose or glycerol, but not acetate, as organic carbon sources. Mixotrophic cultivation with glucose increased the total lipids content in the biomass by 34% relative to phototrophic cultivation, suggesting mixotrophy as a promising strategy for cultivation of *N. gaditana* to produce carotenoids and lipids.<sup>247</sup>

Various recent findings on mixotrophic cultivation of different microalgal species for carotenoids production are presented in Table 3. Frequent research publications in the field indicate that mixotrophy is now under extensive investigation for carotenoid production with encouraging results.

## 5 | CONCLUSIONS

Carotenoids are a group of colourful tetraterpenoids with essential physiological functions in plants and microalgae and have numerous applications in many different industrial sectors including, feed, food, cosmetics, human health, pharmaceuticals etc. particularly, in aquaculture, carotenoids have roles in promoting pigmentation, growth, reproduction and egg quality as well as in enhancing stress tolerance and disease resistance, and stimulating immune functions of aquatic animals. Although some commercially important carotenoids can be produced by chemical synthesis, natural carotenoids are preferred over synthetic ones due to their superior bioactivities. Thus, carotenoid production from microalgae has been explored for decades, and tens of production facilities have been established globally. Among important carotenoids, astaxanthin and  $\beta$ -carotene from microalgae have well-established production facilities. Though lutein and fucoxanthin from microalgae are not yet fully industrialised, intensive research and developments have been under way, and relevant reports strongly indicate commercially viable production processes are promising.

Mixotrophic cultivation of microalgae can offer the advantages of both photoautotrophy and heterotrophy for carotenoid production. Both photoautotrophic and heterotrophic metabolisms contribute to mixotrophic cultivation although their contribution ratio might keep on changing during the cultivation period. According to one viewpoint, in the presence of organic carbon, photosynthesis and organic carbon metabolism are independent and non-competitive, whereas according to a second opinion, both autotrophic and heterotrophic metabolic pathways are activated and can work synergistically due to the availability of an endogenic CO<sub>2</sub> source to drive photosynthesis. Most of the recent studies are reporting the superior performance of mixotrophic cultivation for carotenoids production, using various commercially important microalgal species. However, overall speaking, our understanding of mixotrophic physiology of microalgae is not sufficient

enough to support vigorous bioprocess development for production of carotenoids from mixotrophic microalgae because of the complexity of mixotrophic mechanisms and lack of research. The fact that conflicting results are reported on the active pathways during mixotrophic cultivation suggests that in depth investigations are necessary to advance our understanding of mixotrophic physiology of microalgae.

Challenges and obstacles do exist for us to develop robust and scalable mixotrophic cultivation processes for carotenoid production. First, simultaneous requirements of light supply, carbon dioxide and organic carbon might complicate the process control and thus increase the process costs. Secondly, the much higher risk of contamination associated with organic carbon supply might need to be more carefully managed, compared to phototrophic cultivation. Thirdly, inexpensive bioreactor systems with light supply must be developed, compared to the cases of heterotrophic cultivation. Future research might need to focus on overcoming the above challenges and obstacles, and at the same time, in depth investigations of mixotrophic physiology are needed for us to continuously innovate carotenoid production processes from microalgae. Industrial R&D might concentrate on process innovation, process optimisation and development of photobioreactor systems for mixotrophic cultivation. With innovative research and intensive industrial development over the next decade, mixotrophic cultivation of microalgae might become a promising biotechnological trend for natural carotenoid production.

## AUTHOR CONTRIBUTIONS

**Fakhra Liaquat:** Writing – original draft. **Mahammed Ilyas Khazi:** Writing – original draft. **Ali Bahadar:** Writing – original draft; writing – review and editing. **Lu He:** Writing – original draft. **Ayesha Aslam:** Writing – original draft. **Rabia Liaquat:** Writing – original draft. **Spiros N. Agathos:** Writing – review and editing. **Jian Li:** Conceptualization; funding acquisition; supervision; writing – review and editing.

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## CONFLICT OF INTEREST

The authors have no affiliation with any organisation with a direct or indirect financial interest in the subject matter discussed in this paper.

## DATA AVAILABILITY STATEMENT

Data availability is not applicable to this article as no new data were created or analysed in this study.

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