Review Article

Optimization of microbial cell factories for astaxanthin production: Biosynthesis and regulations, engineering strategies and fermentation optimization strategies

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ARTICLE INFO

ABSTRACT

The global market demand for natural astaxanthin is rapidly increasing owing to its safety, the potential health benefits, and the diverse applications in food and pharmaceutical industries. The major native producers of natural astaxanthin on industrial scale are the alga *Haematococcus pluvialis* and the yeast *Xanthopyllomyces dendrorhous*. However, the natural production via these native producers is facing challenges of limited yield and high cost of cultivation and extraction. Alternatively, astaxanthin production via metabolically engineered non-native microbial cell factories such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* is another promising strategy to overcome these limitations. In this review we summarize the recent scientific and biotechnological progresses on astaxanthin biosynthetic pathways, transcriptional regulations, the interrelation with lipid metabolism, engineering strategies as well as fermentation process control in major native and non-native astaxanthin producers. These progresses illuminate the prospects of producing astaxanthin by microbial cell factories on industrial scale.

1. Introduction

Astaxanthin is a keto-carotenoid with dark reddish color, and is widely distributed in algae, shrimp, crab, shellfish and other organisms [1,2]. Due to its safety and the stable reddish color, astaxanthin became an ideal source for pigmentation in aquaculture and food industry [3]. Additionally, several studies showed potential health benefits associated with astaxanthin supplementation including antioxidant, anti-inflammation, anti-cancer, neuroprotection and immuno-enhancement activities [4]. Owing to these benefits and uses, the demand and market size of astaxanthin have been rapidly increasing. The global astaxanthin market is expected to reach USD 3.5 billion by 2026, from USD 1.37 billion in 2020, at a compound annual growth rate (CAGR) of 16.8% [5]. The animal feed application dominated the market, with a share of 65.5% in 2020, while China dominated the Asia Pacific market for astaxanthin with a share of 40.4% [6].

The chemical structure of astaxanthin (3,3′-dihydroxy-4,4′-dione-β,β′-carotene) resembles that of many other carotenoids. It contains a main chain composed of four isoprene units connected by conjugated double bonds, and one β-ionone ring at each end of the chain. Each ionone ring carries a characteristic 3-hydroxy (OH) and a 4-keto (C═O) groups. These structural arrangements enable the compound to attract free radicals and provide them electrons resulting in termination of the free radical chain reaction, which explains the strong antioxidant activity of astaxanthin [2,7]. Astaxanthin has many optical and geometric isomers [8]. The 3 and 3′ hydroxygroup of each ionone ring can exist in the S or R form. Accordingly, three different optical isomers have been reported ((3S, 3′S), (3R, 3′R), and (3R, 3′S)) for astaxanthin. The anti-oxidant activity is influenced by the form of astaxanthin isomer used, and the (3S, 3′S) form showed stronger antioxidant activity than that of (3R, 3′R) and (3R, 3′S) [9]. Several astaxanthin derivatives have been reported in nature including esterified astaxanthin (mono- and di-ester),

glycosylated astaxanthin (astaxanthin β-D-glucoside, astaxanthin β-D-diglucoside and astaxanthin dirhamnoside) and glycosylated-esterified astaxanthin (astaxanthin diglucoside diester) [10–14]. These modifications take place at the 3 and 3′ hydroxygroup of the ionone rings and are believed to influence the polarity, solubility and the biological activity of astaxanthin [4,10,15].

Astaxanthin can be obtained through extraction from natural sources or chemical synthesis [16]. Astaxanthin biosynthesis has been reported in several organisms including microalgae, bacteria, yeast and plants [4, 17]. Although, astaxanthin has been detected in several aquatic animals, these animals cannot synthesis astaxanthin, but they obtain it through feed [16]. Several microalgae have been extensively studied for their ability to synthesize and accumulate astaxanthin naturally when cells are cultured under stress [18]. Amongst these algae, *Haematococcus pluvialis* is recognized as the best natural source of astaxanthin with the highest astaxanthin content, which can reach up to 4% of the dry cell weight under optimal cultivation conditions [18]. Therefore, *H. pluvialis* astaxanthin covers over 50% of the natural astaxanthin market in the global nutraceutical industry [19,20]. In addition to *H. pluvialis*, *Chlorella zofingiensis* is also considered as a promising source of natural astaxanthin production [21]. However, the large scale production using these algae requires high light intensity, large cultivation areas and complex cultivation process which makes the industrial production challenging [22]. Bacterial astaxanthin biosynthesis have been reported in many strains, such as *Brevundimonas* sp., *Sphingomonas* sp. and *Paracoccus* sp [23–25]. The marine bacterium *Paracoccus carotinifaciens* is considered the best among bacterial astaxanthin producers, with a high level at 480 mg/L by fed-batch fermentation [18,26]. One of the major astaxanthin producing yeasts is *Xanthophyllomyces dendrorhous* [27]. *X. dendrorhous* is the earliest strain used in the industrial production of astaxanthin, and contains about 0.2–0.5 mg/g DCW carotenoids, of which 40–95% is astaxanthin [28,29]. Several strategies have been extensively applied for enhanced production of astaxanthin from *X. dendrorhous*, and the highest yield reported has reached 9.7 mg/g dry cell weight (DCW) [18,30]. In addition to the above mentioned microorganisms, astaxanthin biosynthesis has been detected in some plant species of the genus *Adonis* [17,31].

At present, the human health consumption of astaxanthin is strictly limited to *H. pluvialis* [32]. However, natural astaxanthin cannot meet the market demand due to the low yield and high costs of cultivation and extraction [16]. Therefore, chemically synthesized astaxanthin is the most dominant source for commercial use due to its low cost and higher profitability. Nevertheless, the demand is still in the favor of natural astaxanthin due to safety concerns related to the byproducts formed during the chemical synthesis of astaxanthin [20]. Additionally, natural astaxanthin has higher antioxidant activity, as it mainly exists either in (3S, 3′S) or (3R, 3′R) forms. For instance, more than 95% of astaxanthin from *H. pluvialis* is in (3S, 3′S) form [33], while, the (3R, 3′R) is the dominant form in the red yeast *X. dendrorhous* [34]. However, the chemically synthesized astaxanthin is composed of a mixture of the three isomers at a ratio of 1:2:1 (3R,3′R:3R,3′S:3S,3′S). Thus, to overcome the imperfections of the native producers or chemical synthesis and benefiting from the advances in biotechnology, the engineering of the non-carotenogenic microbes, such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, to produce astaxanthin shows promising results to become better alternative cell factories on industrial scale.

In this review we intend to address the recent advances in microbial astaxanthin production with a main focus on the major native and non-native producers (Table 1) in regards to astaxanthin biosynthesis regulations, engineering strategies and fermentation optimization attempts.

Table 1
Major native and non-native astaxanthin producing microorganisms.

Organism	Highest Yield or Titer	Fermentation scale	Pros	Cons	Ref
Native					
Haematococcus pluvialis	**Modified:** 87.4 mg/L	500 mL volume glass tubular airlift photobioreactors 8 days	High astaxanthin content; safe for human consumption; environment-friendly; stable non-GMO; well accepted by laws and regulations of different countries	Complex cultivation; slow growth rate; large space required; easy to be contaminated in open culture system; high cost of production; difficult to scale up and extract; limited by light and nutrition; astaxanthin in esterified form	[3,19,220, 239,240]
	Unmodified: 77.2 mg/g DCW	Shake-flask 18 days			
Chlorella zofingiensis	73.3 mg/L	Shake-flask 14 days	High growth rate and high cell density when cultivated in heterotrophic conditions; high lipid content; non-GMO	Complex cultivation; easy to be contaminated; difficult to scale up and extract; slow growth rate and large space required when cultured in photoautotrophic conditions, astaxanthin in esterified form	[3,21,36, 241–243]
	6.8 mg/g DCW	NA			
Xanthophyllomyces dendrorhous	**Modified:** 9.7 mg/g	Fermenter 216 h	Simple requirements for growth; easier to scale up and achieve high biomass; high growth rate; can use a variety of carbon sources; can act as a non-GMO	Low yield; the growth rate of yeast cells is inversely proportional to the accumulation of astaxanthin; low market demand for its astaxanthin configuration; harder to obtain pure free astaxanthin; unknown regulatory pathways and thus harder to be rationally engineered	[3,30,80, 198]
	Unmodified: 420 mg/L	1 L bioreactor 240 h			
Non-native					
Escherichia coli	18.7 mg/g DCW	1 L bioreactor 85 h	Easier to scale up and extract; high biomass; fastest growth rate; clear genetic background and mature genetic manipulation; low cost of using glucose as a carbon source, simple equipment requirements	Multiple purification steps and high purification cost are required to remove recombinant DNA and endotoxin	[3,124, 138,244]
	1.18 g/L	5 L bioreactor 60 h			
Saccharomyces cerevisiae	404.78 mg/L	5 L bioreactor 180 h	Easier to scale up and extract; high biomass; fast growth rate; GRAS; environment friendly; clear genetic background; mature genetic manipulation	Purification is required to remove recombinant DNA; low yield; complex intermediate metabolites	[3,172, 225,244]
	13.8 mg/g	5 L bioreactor 68 h			
Yarrowia lipolytica	858 mg/L (16.7 mg/g)	Shake Flask (Fed-Batch) 288 h	Easier to scale up and extract; high biomass; fast growth; GRAS; high lipid content	Purification is required to remove recombinant DNA; low yield; complex intermediate metabolites	[3,117, 149,245]

2. Astaxanthin biosynthesis: pathways, regulations and distribution

2.1. Astaxanthin biosynthetic pathways

As a terpenoid, astaxanthin biosynthesis starts from the 5-carbon isomers isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Fig. 1). IPP and DMAPP can be natively synthesized by mevalonic acid (MVA) pathway in eukaryotic microbes using acetyl-CoA as precursor, or 2-methyl-D-erythritol-4-phosphate (MEP) pathway in prokaryotic microbes and plant plastids using glyceralehyde-3-phosphate and pyruvate as precursors [35,36]. In the MVA pathway, acetyl-CoA acetyltransferase (AACT) condensates two acetyl-CoA molecules to give acetoacetyl-CoA, which is further condensed with additional molecule of acetyl-CoA by hydroxymethylglutaryl-CoA synthase (HMGS) to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) [37]. Then, a reduction step by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) results in mevalonate formation. Afterwards, IPP is formed by a three step reactions mediated by mevalonate kinase (MK), phosphomevalonate kinase (PMK), and at last mevalonate diphosphodecarboxylase (MVD). The formed IPP is then isomerized to DMAPP by isopentenyl diphosphate isomerase (IDI) [37].

In MEP pathway, the first step starts by condensation of glyceralehyde-3-phosphate and pyruvate by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) to form 1-deoxy-D-xylulose-5-phosphate (DXP), followed by a reduction step by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) to form 2C-methyl-D-erythritol-4-phosphate (MEP) [38]. The formed MEP is then converted into 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME) which is catalyzed by 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT). Then 2C-methyl-D-erythritol-2,4-cyclodiphosphate (MECDP) is generated through phosphorylation and cyclization by the activity of CDP-ME kinase (CMK) and MECDP synthase (MDS), respectively. The MECDP is then converted into 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP) under the action of 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (HDS). Then IPP and DMAPP are derived from HMBPP by the activity of 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) [38]. In addition to these differences between MVA and MEP pathways, the two pathways differ in carbon use efficiency, energy and cofactor requirements, and IPP yields [39,40]. Starting from glucose, the carbon loss in the form of CO_2 Via MVA pathway is higher than that of MEP. In the course of the precursors formation, one molecule of CO_2 is generated to form a single acetyl-CoA molecule as the MVA pathway precursor, while, the formation of the MEP pathway precursors, pyruvate and G3P, does not involve any carbon loss [39]. For each IPP molecule formation in the MVA pathway, a total of 1.5 molecules of
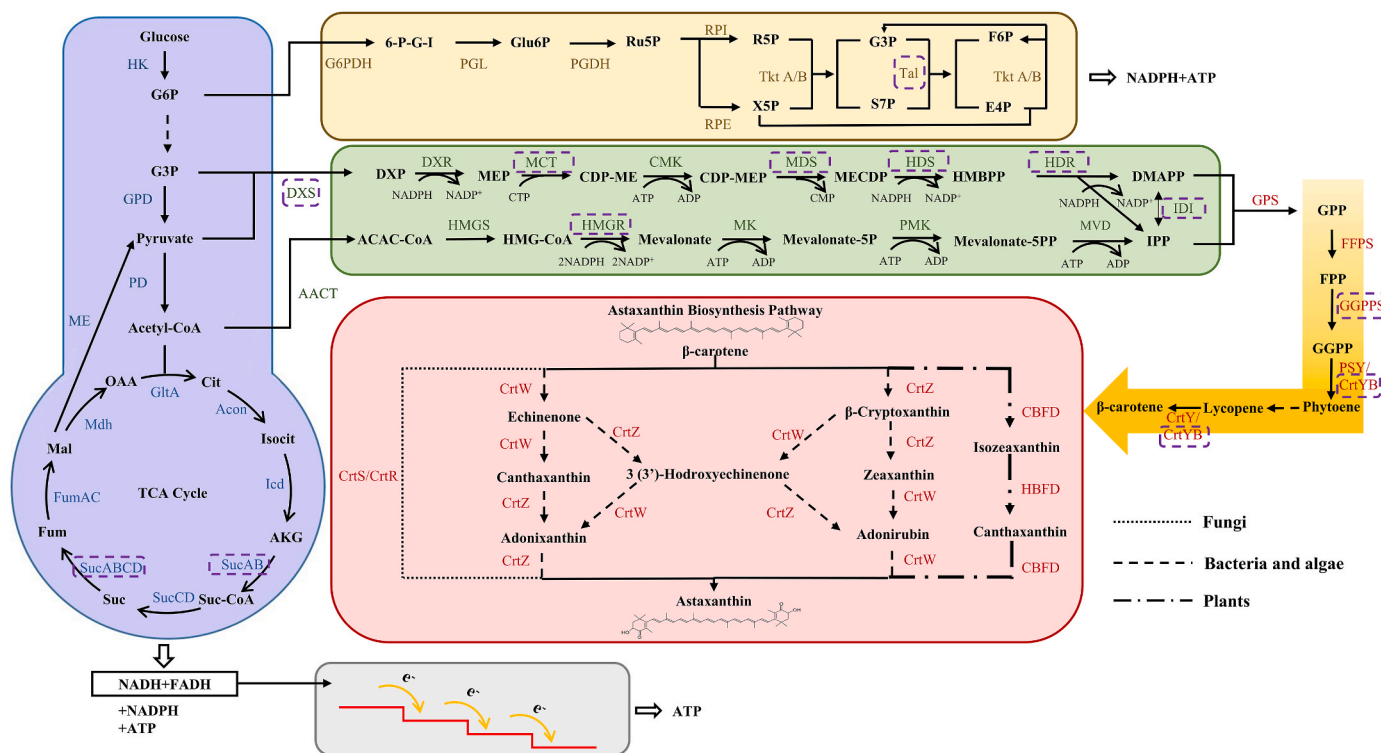

Fig. 1. Scheme for astaxanthin biosynthesis and strategies for enhancing precursors and cofactors supply. Enzymes are as follows: (1) EMP-TCA pathway (pruple): HK, Hexokinase; GPD, glyceraldehyde 3-phosphate dehydrogenase; PD, pyruvate dehydrogenase; GltA, citrate synthase; Acon, aconitate hydratase; Icd, aconitate hydratase; SucAB, α-ketoglutarate dehydrogenase; SucCD, succinyl-CoA synthetase; SdhABCD, succinate dehydrogenase; FumAC, fumarate hydratase; Mdh, malate dehydrogenase; ME, malic enzyme. (2) Pentose phosphate pathway (gold): G6PDH, glucose 6-phosphate dehydrogenase; PGL, 6-phosphogluconolactonase; PGDH, 6-phosphogluconate dehydrogenase; Rpe, ribulose 5-phosphate 3-epimerase; Rpi, ribose-5-phosphate isomerase; Tkt A/B, transketolase subunit A/B; Tal, transaldolase. (3) DMAPP/IPP moldule (green): MVA Pathway: AACT, acetyl-CoA acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MVD, diphosphodecarboxylase; IDI, isopentenyl pyrophosphate isomerase; MEP Pathway: DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, CDP-ME kinase; MDS, MECDP synthase; HDS, 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, IDI, isopentenyl pyrophosphate isomerase. (4) β-carotene biosynthesis (orange): GPS, geranyl pyrophosphate synthase; FFPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl pyrophosphate synthase; CrtY(CrtYB), lycopene cyclase. (5) Astaxanthin biosynthesis (red): CrtZ, β-carotene hydroxylase; CrtW, β-carotene ketolase; CBFD, carotenoid beta-ring 4-dehydrogenase; HBFD, 4-hydroxy-beta-ring 4-dehydrogenase; CrtS, cytochrome P450 mono-oxygenase; CrtR, cytochrome P450 reductase. (6) Electron transport chain (grey). The Solid arrows indicate a single step reaction. The dashed arrows indicate multistep reactions. The dashed purple boxes indicate the engineered steps via overexpression of the corresponding gene in the engineered host. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

glucose, three ATP molecules and two molecules of NADPH are needed [40]. However, the MEP pathway requires 1.25 molecules of glucose, three molecules of NADPH and three ATP molecules (two for the regeneration of CTP, one for CDP-ME conversion to CDP-MEP) (Fig. 1). Thus, the theoretical IPP yield on glucose from MEP pathway is expected to be higher than that of MVA pathway, while more cofactors are needed.

The IPP and DMAPP produced through MEP or MVA pathways are then condensed to give geranyl pyrophosphate (GPP; C10) by the activity of geranyl pyrophosphate synthase (GPS), which is subsequently converted to farnesyl pyrophosphate (FPP; C15) by farnesyl diphosphate synthase (FFPS), and then to geranylgeranyl pyrophosphate (GGPP; C20) by the action of GGPP synthase (GGPPS) [41]. GGPP is then catalyzed by phytoene synthase (PSY or CrtB) to produce the first C40 carotenoid phytoene, which is desaturated and converted into lycopene by the consecutive action of phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS). β-carotene is then formed from lycopene by the activity of lycopene cyclase (LCY, CrtY, CrtYB, CarRRP or LCYB). Afterwards, β-carotene is converted into astaxanthin in enzymatic reactions dependent on the producing organisms [42].

The biosynthesis of astaxanthin in bacteria and algae from β-carotene is mediated by two enzymes, the β-carotene hydroxylase encoded by the *crtZ* (*crtR-B* or *CHYB* in algae) gene, and the β-carotene ketolase encoded by the *crtW* (*bkt* in algae) gene [43–45]. In response to different environmental stress conditions, three different BKTs have been reported to be expressed in *H. pluvialis* [46–48]. The expression level of these proteins varies among each other on a manner dependent on the stress conditions [47]. Luo et al. identified six copies of *bkt* genes (*bkt1a*, *bkt1b*, *bkt1c*, *bkt2a*, *bkt2b* and *bkt3*) in *H. pluvialis* genome, and they are believed to be a result of gene duplications during genome evolution [49]. Similarly, two different β-carotene hydroxylase genes (*crtR-B1* and *crtR-B2*) were also found in *H. pluvialis* genome [50]. In addition to the multi-copies of these genes in *H. pluvialis*, the products of these genes are believed to be localized to different cellular compartments [50–52]. These findings might explain the significant high level of astaxanthin in *H. pluvialis* over other producers. Given that the conversion from β-carotene to astaxanthin involves four reactions that takes place by the addition of two carbonyl groups and two hydroxyl groups at 4, 4′-C and 3, 3′-C positions of the β-ionone ring, respectively (Fig. 1), the hydroxylation and ketolation reactions may occur alternatively dependent on the substrate preferences of the different enzymes which also leads to the accumulation of several intermediates [53].

A bi-functional cytochrome P450 astaxanthin synthase, CrtS, is believed to mediate the conversion of β-carotene to astaxanthin in *X. dendrorhous* (Fig. 1), however the mechanism is not well understood [54]. It should be noted that, the redox partner cytochrome P450 reductase, CrtR, is essential for astaxanthin biosynthesis in this yeast [55]. In this context, the sole expression of *crtS* in β-carotene producing *S. cerevisiae* did not result in accumulation of astaxanthin until *crtR* was co-expressed [54,56].

The biosynthetic pathway of astaxanthin from β-carotene in *Adonis* is different than the above mentioned organisms (Fig. 1). In *Adonis aestivalis*, a carotenoid-β-ring-4-dehydrogenase (CBFD) hydroxylates the fourth carbon of β-ionone ring, then the hydroxylated products are further dehydrogenated into 4-keto group by carotenoid-4-hydroxy-β-ring-4-dehydrogenase (HBFD) [17,57]. The hydroxyl group on the third carbon of β-ionone ring is also introduced by the CBFD enzyme [17].

2.2. Astaxanthin biosynthesis regulation

2.2.1. Transcriptional regulations of astaxanthin biosynthesis

Given their protective rule against stress, carotenoids including astaxanthin are believed to be synthesized in response to different stressors [58,59]. Several studies have been conducted on the natural producers *H. pluvialis, C. zofingiensis* and *X. dendrorhous* to reveal the transcriptional regulations of astaxanthin biosynthesis.

Multiple stressors have been reported to induce astaxanthin synthesis in *H. pluvialis* including high light, nitrogen limitation, and chemical inducers. High Light can cause elevated levels of reactive oxygen species (ROS) which in turn promotes the accumulation of astaxanthin [60]. However, astaxanthin biosynthesis seems to be influenced not only by the light intensity, but the wavelength of light as well. For instance, red light was found to enhance the biomass, while blue light promotes astaxanthin production in a poorly understood mechanism [61,62]. Blue light signaling is known to induce ROS in algae [60]. Thus upon the exposure to the blue light, significant upregulation in the expression level of catalase has been detected in *H. pluvialis* suggesting the exposure of the cells to oxidative stress which in turn led to up-regulation of the genes encoding β-carotene ketolase and hydroxylase [62]. Additionally, changes in the expression levels of the blue light receptors encoded by *CPH1* and *PHOT* genes have been detected and influenced the astaxanthin accumulation, suggesting their rule in mediating the signal for astaxanthin biosynthesis [61,62]. For example, upon the upregulation in expression level of *PHOT*, up-regulation of the carotenoid biosynthesis genes *PSY* and *PDS* was detected [61]. Exogenous supplementation with some chemicals can play a synergetic effect in enhancing astaxanthin production as well. Addition of Fe^{2+} under high light can increase astaxanthin levels indirectly through promoting oxidative stress and influencing photosynthesis by up-regulation of photosynthesis-antenna genes including *Lhca1*, *Lhca3*, *Lhca4*, *Lhcb2*, *Lhcb3*, *Lhcb5*, *Lhcb6* and *Lhcb7* [63]. Under high light conditions, γ-aminobutyric acid supplementation resulted in significant improvement of the biomass and subsequently the astaxanthin yields [64]. The γ-Aminobutyric acid addition up-regulated several stress resistance related genes (*PP2C, SnRK2, CPK, HSP90, WRKY1, PR-1*) and led to increased stress resistance of the algal cells. Additionally, upregulation of *Lhca2* and *PTOX* genes was detected upon the supplementation which resulted in enhancement of light protection and led to improved photosynthetic activity of the cells. The induction of the endogenous methyl jasmonate pathway by ethanol, upregulated DXS, the key enzyme in MEP pathway, and carotenogenic genes such as *PSY*, *bkt* and *crtR-B*, which resulted in enhanced astaxanthin accumulation [65].

Although, many transcriptomic analyses revealed the change in the expression level of key genes in astaxanthin biosynthesis upon the exposure to stress in *H. pluvialis*, the regulatory mechanism of these changes is not clear. Transcription factors (TFs) are important regulatory proteins that play important rule in the activation or repression of the expression of their target genes [66]. The bHLH family TFs are reported to be carotenogenesis suppressors in plants [67]. In consistent with these findings, nitrogen limitation in the presence of high light can induce high level of astaxanthin accumulation in *H. pluvialis*, which was accompanied with downregulation of the TFs from bHLH family [68]. On the other hand, C3H, MYB, Nin-like, MTB_related and ERF TFs were highly expressed TF families under the effects of salicylic acid in the presence of high light [68,69]. In addition to TFs, some miRNAs also have been identified as responding miRNAs to sodium acetate and high light stress [70]. A total of 83 and 46 miRNAs were considered as light and sodium acetate stress responsive miRNAs, respectively, and 14 miRNAs responded to both stresses. The identified miRNAs were targeting several genes involved in signal transduction, heavy metal stress response, and secondary metabolism. Moreover, four miRNAs (miR482d, PC-3p-859521_20, miR167, and PC-5p-411755_80) involved in regulating astaxanthin synthesis by directly targeting the astaxanthin biosynthetic genes, *lcy-B*, *GGPS*, *PDS* [70].

Many studies have been focused on the genetic control of carotenogenesis in the red yeast *X. dendrorhous*, however the regulations of these processes are not well understood yet [71]. The MVA and sterols biosynthetic pathways are important for astaxanthin biosynthesis as precursors supply and storage medium, respectively. The transcription factor Sre1 has been identified as a regulator that regulates these pathways by directly regulating the *ERG10*, *HMGS*, and *HMGR* genes

whose upregulations is beneficial for astaxanthin synthesis [72,73]. Similar to *H. pluvialis*, the medium components can influence astaxanthin production in this yeast. Catabolic repression by glucose has been reported in *X. dendrorhous* and has been proved to affect astaxanthin production [74,75]. Such repression is mediated by a metabolic suppressor encoded by *MIG1* gene along with the co-repressor complex Cyc8-Tup1. The deletion of *MIG1* upregulated *crtI*, *crtYB* and *crtS* transcripts in presence of glucose [74]. Likewise, deletion of *CYC8* and *TUP1*, upregulated the genes involved in the synthesis of carotenoids precursors including *HMGR*, *IDI* and *FPS* genes [75]. Exogenous supplementation with the phytohormone 6-benzylaminopurine (6-BAP) can induce the astaxanthin synthesis by upregulating the transcription levels of related genes *HMGR*, *IDI* and *crtYB* [76].

2.2.2. Crosstalk between lipid and astaxanthin biosynthesis

The astaxanthin biosynthetic pathway is interfering with that of lipids including fatty acids, TAG and sterols (Fig. 2). On the other hand, due to their hydrophobic nature, carotenoids including astaxanthin are incorporated in the lipid rich cellular compartments including plasma membranes and lipid bodies (LBs), which will be discussed in details in section 2.3.2 [77,78]. Thus, the relation between lipid and astaxanthin biosynthesis is complex and seems to be dependent on the organism and the astaxanthin biosynthetic pathway itself. For instance, in the red yeast *X. dendrorhous*, astaxanthin biosynthesis is competing on the substrate acetyl-CoA which is also the precursor for fatty acids and ergosterol biosynthesis [79]. Miao et al. reported an astaxanthin overproducing mutant where fatty acids production was reduced compared to the wild type, suggesting the inverse correlation between the two pathways in this yeast [80]. Furthermore, the inhibition of the fatty acids biosynthetic pathway in the wild-type strain using triclosan resulted in 2 fold and 1000 fold increase in astaxanthin and ergosterol

content, respectively, which might be attributed to increased acetyl-CoA availability and re-directing the flux toward the mevalonate pathway [81].

Nevertheless, the most popular astaxanthin producing algae, *H. pluvialis* and *C. zofingiensis*, showed different patterns in astaxanthin accumulation in response to fatty acids inhibition [36,51]. These two algae have been reported to accumulate significant amount of TAG and astaxanthin simultaneously in response to stress conditions [82,83]. Additionally, astaxanthin is predominantly accumulated in esterified form in these two algae and stored in TAG filled LBs which acts as a solvent for the synthesized astaxanthin. Because astaxanthin biosynthesis in algae is derived from MEP pathway, and it competes with fatty acid and TAG biosynthesis on pyruvate, there might be a potential regulatory mechanism between both pathways [84,85]. A previous report suggested the presence of a minimal TAG that might be needed to induce the biosynthesis of esterified astaxanthin and accumulation in LBs in *H. pluvialis* [86]. The chemical inhibition of fatty acids biosynthesis using norflurazon and cerulenin in this alga resulted in significant reduction in TAG content, the major fatty acids in astaxanthin esters, such as oleic acid (C18:1) and linolic acid (C18:2), and total astaxanthin [51,87]. Additionally, the cerulenin treated cells showed higher free astaxanthin content in spite of the decrease in the total astaxanthin level suggesting a possible feedback inhibition of the free astaxanthin on its biosynthesis, which is believed to be relieved through esterification. In consistent with this hypothesis, the total astaxanthin level has been restored upon fatty acids supplementation to the cerulenin treated algal cells. On the other hand, cerulenin treated *C. zofingiensis* showed enhanced total astaxanthin level upon the inhibition of fatty acids biosynthesis which is opposite to *H. pluvialis* [36,84,85,88]. Some Studies tried to find explanations for this behavior. For instance, Liu et al. showed that the increase in astaxanthin might be due to enhanced
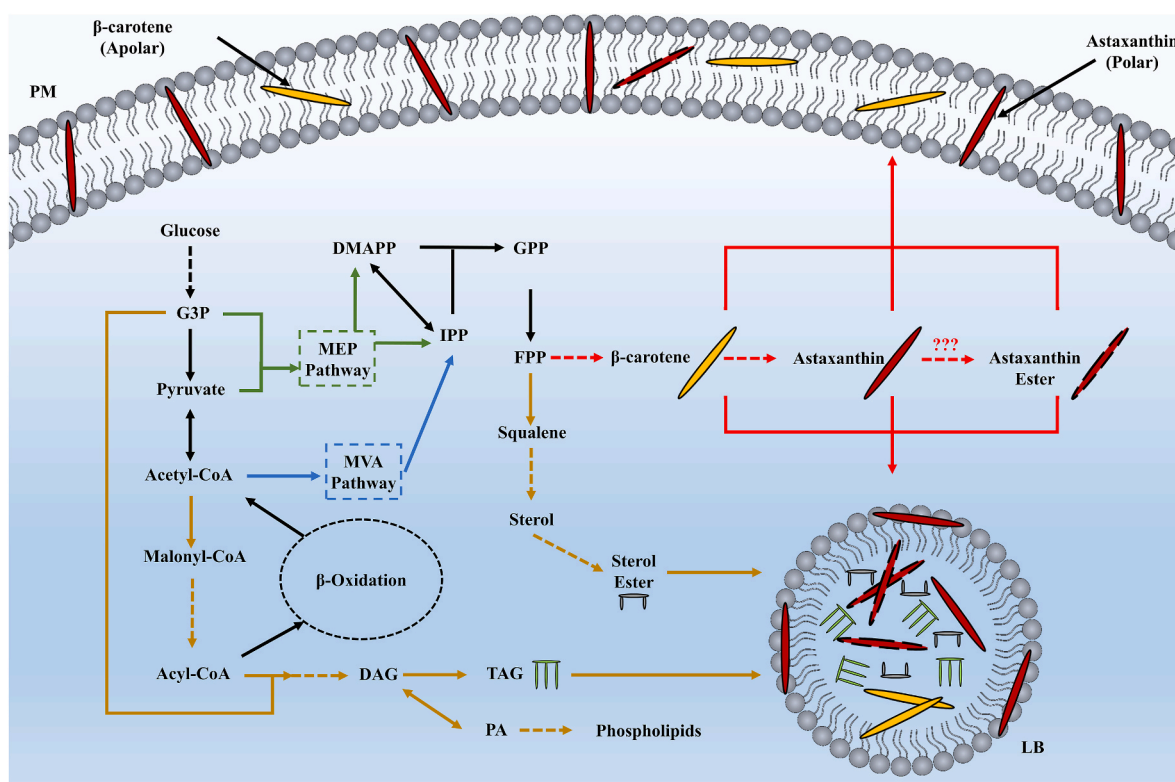

Fig. 2. The crosstalk between lipid and astaxanthin biosynthesis and the hypothetical distribution of astaxanthin in microorganisms. The Solid arrows indicate a single step reaction. The dashed arrows indicate multistep reactions. The gold arrows represent the lipid biosynthesis pathways. The blue arrows represent the MVA pathway. The green arrows represent the MEP pathway. The red arrows represent the astaxanthin biosynthesis pathway. G3P, glyceraldehyde-3-phosphate; DAG, diacylglycerol; TAG, triacylglycerol; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; FPP, farnesyl pyrophosphate; PA, phosphatidic acid; LB, lipid body; PM, plasma membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conversion of other carotenoids into astaxanthin inside the cell, supported by decrease of β-carotene and canthaxanthin in alignment with the increase of astaxanthin and cerulenin concentration [85]. Additionally, cerulenin might induce ROS which might serve as inducer for astaxanthin biosynthesis [36,88]. Another explanation is that, the content of astaxanthin in *C. zofingiensis* is much lower than *H. pluvialis,* however they have almost the same content of total fatty acids. Therefore, in contrast to *H. pluvialis*, the level of TAG might not be a limiting factor for astaxanthin accumulation in *C. zofingiensis* and the fatty acid inhibition might result in enhanced precursor and energy supply for carotenogenesis [88].

Sterols represent a class of lipid that have been found in eukaryotic cells with a great importance in maintaining the membrane integrity and fluidity [89]. Ergosterol is a major lipid component of the fungal cell membranes and helps to maintain the membrane integrity in a manner similar to cholesterol in mammalian cells [90]. In the astaxanthin overproducing *X. dendrorhous* mutant mentioned above, besides the reduced fatty acid content, Miao et al. also reported reduction in the ergosterol content [80]. Similarly, chemical inhibition of ergosterol biosynthesis using fluconazol resulted in 5 fold increase in astaxanthin [81]. Astaxanthin and ergosterol biosynthetic pathways are sharing the substrate FPP, so the flux reduction of the competitive pathway in the mutant is conducive to astaxanthin synthesis, which provides a possible route for metabolic engineering in *X. dendrorhous* [79]. Additionally, the key enzyme HMGR in the MVA pathway have been reported to be negatively regulated by sterols in some fungal species and mammalian cells [89]. The deletion of *CYP61* (*ERG5*), encoding C22-sterol desaturase, which is involved in the late steps of ergosterol biosynthesis, resulted in enhanced HMGR transcription level and 2 fold increase in astaxanthin and carotenoids production in *X. dendrorhous* [91]. Further investigations on this mutant revealed that ergosterol depletion activates the sterol regulatory element-binding protein (SREBP) pathway by the activation of Sre1 protein which is involved in the activation of several genes involved in MVA pathway, sterol biosynthesis and carotenogenesis [72]. However, the deletion of the genes *ERG3* and *ERG4* which are involved in the preceding and the following step to the Erg5 mediated reaction, respectively, had no effect on carotenogenesis, despite the inability to accumulate ergosterol, suggesting the inactivation of SREBP in these mutants [92]. Thus, the activation of SREBP in the *Δerg5* mutant might be dependent on the sterol composition, ergosta-5, 7,24(28)-trienol in particular, rather than ergosterol itself. It worth noting that, along with TAG and astaxanthin, an increase in sterols occurred in response to high light stress in *H. pluvialis* [93]. Additionally, sterols biosynthesis is derived from the IPP synthesized in the chloroplast of *H. pluvialis* through MEP pathway which competes with carotenoids biosynthesis [93]. However, the effect of sterol biosynthesis on astaxanthin or carotenoids have not been studied yet in this algae.

2.3. Astaxanthin biosynthesis and storage distribution

The study of the subcellular location of the biosynthesis and storage of astaxanthin is of great importance for engineering astaxanthin overproducers, however only limited studies have been conducted to reveal this mystery in the native astaxanthin producers.

2.3.1. Astaxanthin biosynthesis localization

Given that the biosynthesis of astaxanthin is a multistep reaction with several enzymes, it is possible that the biosynthesis localization involves several subcellular locations. For instance, astaxanthin biosynthesis in *H. pluvialis* is believed to be divided between the chloroplast and the endoplasmic reticulum (ER), where β-carotene is produced in the chloroplast and then transported to the ER by unknown mechanism to be converted to astaxanthin followed by esterification step [1,51,94]. This is consistent with the fact that, carotenogensis in algae is derived from MEP pathway which is originated in the chloroplast. Additionally, several enzymes involved in β-carotene biosynthesis

were reported to be in the chloroplast [95,96]. At last, *in vitro* assay using several fractions of *H. pluvialis* cell lysates showed that β-carotene conversion to astaxanthin is associated with ER containing fractions [51]. It should be noted that, β-carotene hydroxylase has been detected in LBs and chloroplast membranes [52]. However, ketolase activity was only associated with LBs [52]. LBs are known to be generated from ER in eukaryotes, which might be a possible reason for the detection of astaxanthin biosynthetic activity in the ER containing fractions [51,97].

In contrary to the extensively studied *H. pluvialis*, limited information about the biosynthesis and storage localization are available for the yeast *X. dendrorhous.* Verdoes et al. hypothesized a membrane bound cartoenogenic complex for astaxanthin production in this yeast [98]. Additionally, astaxanthin biosynthesis from β-carotene is mediated by a bi-functional p450 monooxygenase (CrtS) which is believed to be localized to the ER [54,99]. Thus, it is possible that carotenogenesis is localized to the ER, which needs further experiments to be confirmed.

2.3.2. Astaxanthin storage localization

As mentioned previously, carotenoids are hydrophobic and they tend to be stored in membranes and LBs due to their high lipid content, however astaxanthin distribution in the cells appears to be influenced by the type of the astaxanthin produced whether it is esterified or in a free form (Fig. 2). In *H. pluvialis,* 95% of astaxanthin is in esterified form and it is believed to be accumulated in the LBs [51]. However, analysis of extracts from thylakoid membranes of *H. pluvialis* showed accumulation of free and esterified astaxanthin in the membranes [100]. Furthermore, at the late stages of the growth period, the ratio of the thylakoid membranes/whole cell free astaxanthin content were much higher than that of the astaxanthin ester and was around 50% of the whole free astaxanthin content, suggesting higher affinity of the free form to the membranes [100]. However, the possibility of contamination of these membranes with LBs during the cell lysates preparation cannot be ruled out [101].

Contrarily, *X. dendrorhous* produces astaxanthin in its free form. Electron paramagnetic resonance spectroscopy experiment on *X. dendrorhous* membranes proved the incorporation of astaxanthin in plasma membrane [102]. In another study, laser confocal fluoresce microscopy analysis (LCFM) for the yeast *X. dendrorhous*, suggested the accumulation of the carotenoids in LBs [103,104]. LBs are composed of a core filled with TAG and sterol esters enclosed in a monolayer of phospholipids [97]. A previous study showed that polar carotenoids (zeaxanthin) are mainly localized to the phospholipid layer of artificial LBs, while apolar carotenoids (β-carotene) were in the core of the LBs [105]. Given the polarity of astaxanthin, due to the two hydroxyl and two keto groups, it is possible that free astaxanthin might be incorporated in the LBs membranes which needs further confirmation. However, the incorporation of carotenoids in membranes is believed to be toxic to the cells, and resulted in several responses included cell membrane destruction, oxidative stress, induction of drug stress responses, in addition to decreasing in the plasma membrane fluidity [106–109]. Carotenoids are embedded in membranes in different orientations dependent on their polarity (Fig. 2), where the apolar carotenoids are perpendicular to the membrane lipid chains and the polar carotenoids are in a parallel orientation [102]. Therefore, the effect of the polar carotenoid on the membranes fluidity is stronger.

3. Strategies for microbial cell factories optimization

There is a growing demand for the sustainable production of astaxanthin in microbial cell factories via metabolic engineering. Several efforts have been taken to enhance the production of astaxanthin in the native producers, however the lack of genetic manipulation tools for these organisms limited their development. Thus, the development of non-native producers for astaxanthin using other microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* has attracted a great attention (Table 1) [18]. In the past decade, the

biosynthesis of astaxanthin in these non-native microbes has achieved high yields [3]. Compared to the native organisms, these microbes are rapidly growing on low cost substrates and can easily reach high cell density within few days, in addition to the availability of mature genetic manipulation tools for their metabolic engineering. However, the use of *E. coli* in food grade products biosynthesis has several health concerns due to its ability to produce endotoxins. Alternatively, *S. cerevisiae* is a GRAS organism and was proven to be suitable for the production of carotenoids and terpenoid [27]. Additionally, the non-conventional yeast *Y. lipolytica* has attracted a great attention for the production of several hydrophobic compounds, due to its ability to accumulate lipids up to 60% of its dry weight in its LBs [110]. This oleaginous yeast has been successfully engineered for production of lycopene and β-carotene with significant high yields up to 21 mg/g DCW and 90 mg/g DCW, respectively [111–114]. Although, carotenoids biosynthesis in non-native microbes have achieved high yield in several hosts but the transformation from β-carotene to astaxanthin is still low [18,56, 115–117]. The possible limitations might include enzymes expression levels and their substrates preferences, the accessibility of enzyme to substrates, precursors and cofactors supply, products and intermediates feedback inhibition, the interference with other biosynthetic pathways, and physiological stress due to the accumulation of astaxanthin or its intermediates. In this section we will review the recent strategies that have been applied to overcome these limitations in the above mentioned microbial cell factories to achieve high yields of astaxanthin.

3.1. Directing the flux toward astaxanthin production

Several strategies have been adopted for directing the flux toward astaxanthin through metabolic engineering by enhancing the precursors supply and optimization of astaxanthin biosynthetic enzymes.

3.1.1. Enhancing precursors and cofactors supply

β-Carotene is the common building block for the biosynthesis of astaxanthin in all the reported pathways. The pathway to produce β-carotene starting from MEP/MVA precursors contains several rate limiting steps that influence its accumulation. Accordingly, and in order to achieve high levels of astaxanthin, an optimization to the β-Carotene biosynthesis is needed.

IPP and DMAPP are essential for isoprenoid production including carotenoids. Therefore, adequate supply of these substrates plays a critical role in enhanced isoprenoid production. MVA or MEP pathways are the main source for the native supply of these key precursors. Thus, upregulation of these two pathways is believed to enhance the IPP and DMAPP supply and isoprenoid production, which can be achieved by optimizing the rate limiting steps or the heterologous expression/over-expression of the whole pathway. In the MVA pathway, HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase) is a major rate-limiting enzyme which is regulated at translational and post-translational levels by a feedback inhibition system [118]. HMGR is regulated at the translation level through feedback inhibition that is believed to be mediated by mevalonate [119]. Additionally, the enzyme degradation is modulated through a feedback inhibition mediated by sterols, which can be relieved by the deletion of its N-terminus transmembrane domain [37]. The overexpression of this enzyme or its truncated form showed enhancement in the production of isoprenoids including astaxanthin *in S. cerevisiae* and *Y. lipolytica* [116,120–122]. Similar to HMGR, the rate limiting enzyme 1-deoxy-ᴅ-xylulose-5-phosphate synthase (DXS) in the MEP pathway is feedback inhibited by IPP and DMAPP [123]. The overexpression of DXS resulted in significant enhancement in β-carotene which was optimized for astaxanthin production in *E. coli* [124,125]. The overexpression of the MEP pathway enzymes IspD (MCT) and IspF (MDS) led to 71% increase in astaxanthin [126]. Although, the overexpression of genes involved in MEP or/and MVA pathways is beneficial for isoprenoid production, the toxicity of the intermediates limits the enhancement of the IPP supply and the subsequent increase in

isoprenoid [40]. For instance, the introduction of MVA pathway in addition to the native MEP pathway in *E. coli* has been found to be toxic due to the accumulation of HMG-CoA [127]. Thus, balancing the expression level of the genes involved in the MVA pathway was needed for enhanced isoprenoid production including astaxanthin [127–129]. Additionally, the overexpression of IspG (HDS) enzyme from the MEP pathway results in accumulation of HMBPP and reduced growth, which requires the co-oexpression of IspH (HDR) enzyme to alleviate its toxicity and enhance astaxanthin production [124,130]. Some toxic effects have been detected upon the accumulation of IPP in the cells, thus the overexpression of *idi* to convert IPP to DMAPP is always adopted to reduce its toxicity [124,125,131].

Another important intermediate in β-carotene biosynthetic pathway is FPP. As mentioned previously, carotenoids biosynthesis is competing with sterol biosynthesis on FPP [79]. However, the complete in-activation of sterol biosynthesis is not possible due to its deleterious effects on the cells, fungal hosts in particular. Thus the downregulation of the sterol biosynthetic pathway might be more feasible. In this context, the downregulation of squalene synthase encoded by *SQS1* from *Y. lipolytica* by promoter truncation resulted in enhanced β-carotene which further promoted astaxanthin production [116,117]. Alternatively, similar effect can be achieved by directing the flux toward GGPP formation by overexpressing highly active GGPPS like CrtE03 M mutant [122], GGPPs7 from *Synechococcus* sp. [117], or GGPP synthase from *Archaeoglobus fulgidus* [131]. Direct synthesis of GGPP from IPP was achieved through *in vivo* enzyme assembly of GGPP synthase (CrtE) and Idi by fusing a pair of short peptide RIDD and RIAD to their C-termini, respectively, which guided the construction of multienzyme complex to prevent intermediate diffusion and resulted in 2.7 fold increase in astaxanthin level in *E. coli* [128].

The optimization of the two rate limiting steps of phytoene synthesis and lycopene cyclization catalyzed by phytoene synthase (PSY or CrtB) and lycopene cyclase (CrtY), respectively, is critical for enhanced carotenoids production [132]. Thus for effective β-carotene and astaxanthin production, the bifunctional phytoene synthase and lycopene cyclase CrtYB enzyme from *X. dendrorhous* has been overexpressed in several host for pathway optimization [116,117,122].

Aside from substrates, the cofactors NADPH and ATP are essential for carotenoids biosynthesis. Hence, in order to boost astaxanthin production, it is necessary to balance the supply of the cofactors. The central carbon metabolism plays a vital role in supplying these cofactors, NADPH is mainly generated through the pentose phosphate pathway (PPP) and malic enzyme reaction, while ATP is mainly generated through electron transport chain starting from NADH formed in the TCA cycle [133]. Based on this and in order to optimize the β-carotene biosynthesis in astaxanthin producing *E. coli*, combined upregulation of TCA cycle and PPP was adopted by overexpressing *sucAB*, *sdh* and *talB* [37,38,41,124].

3.1.2. Optimization of astaxanthin biosynthetic enzymes

3.1.2.1. Balancing the levels and activities of *β-carotene hydroxylases and ketolases.* One of the bottlenecks of astaxanthin biosynthesis in engineered microorganisms, is the accumulation of several intermediates during the conversion of β-carotene into astaxanthin (Fig. 1). The astaxanthin biosynthetic enzymes from different sources have different substrate preferences. For example, the CrtW from *Paracoccus* sp. N81106 shows a strong substrate preference for carotenoids with non hydroxylated β-ionone rings [53]. On the other hand, zeaxanthin (hydroxylated) is used as main substrate by CrtW from *Brevundimonas* sp. strain SD212 [134]. The CrtZ from *Erwinia uredovora* showed substrate preference for β-carotene, while the CrtZ from *Alcaligenes* sp. PC-1 showed higher activity toward canthaxanthin [135]. Accordingly, to efficiently convert β-carotene into astaxanthin, selecting the appropriate combination of enzymes with desired substrate preference is necessary.

A screening of nine CrtZ and eight CrtW from different organisms was conducted to efficiently produce astaxanthin in *S. cerevisiae* [136]. As a result of the screening, a CrtW from *B. vesicularis* DC263 and CrtZ from *Alcaligenes* sp. strain PC-1 were verified to be the best combination for highest astaxanthin yield (3.1 mg/g DCW) with less intermediates accumulation.

In addition to the substrate preferences, the expression level of CrtZ and CrtW is critical for intermediates accumulation control, which can be adjusted through optimization of the copy number of each gene, promoter strength or ribosome binding site engineering [18,27]. For instance, replacement of the CrtZ gene promoter with a stronger one increased the enzyme level ratio of CrtZ to CrtW and yielded 30.4% increase in astaxanthin [136]. In order to finely optimize the ratio the two enzymes, screening of several RBS libraries obtained through introducing six random nucleotides to the RBS region of these genes resulted in optimized cassette with a titer of 3.46 mg/g DCW and 99% astaxanthin [137]. Further increase by 67.6% was obtained by multiple integration of the optimized cassette. In addition, since the expression level of a the heterogeneous gene is generally proportional to its copy number, adjustment of the copy number of CrtZ and CrtW have been successfully applied to enhance astaxanthin biosynthesis in several hosts [116,117,122,138]. For example, introducing additional copy of CrtZ from *P. ananatis* resulted in 68.5% increase in the ratio of astaxanthin to total carotenoid in *E. coli* [138].

Besides the above mentioned strategies to balance the activity of the two enzymes, ensuring the proper folding of the enzymes is critical for obtaining their maximum activity. Thus, to optimize the folding of CrtZ and CrtW, the GroES-GroEL molecular chaperone were overexpressed in *E. coli* which resulted in 1.45 fold increase in astaxanthin with a yield of 1.18 g/L [124].

3.1.2.2. Enzyme engineering of β-carotene hydroxylases and ketolases. As stated previously, astaxanthin biosynthesis from β-carotene is mediated by the activity of CrtW and CrtZ in algae and bacterial cells. The two enzymes are iron (Fe^{2+}) and oxygen dependent oxygenases, which was supported by several *in vitro* assays [53,135,139]. Additionally, the conserved iron binding motifs exist among all sequences of different CrtZ and CrtW strongly supports that the Fe^{2+} is essential for the reaction [135]. The lack of information about the structure of these enzymes up to now is limiting their rational engineering. Thus, the majority of the studies on characterizing and enhancing the properties of these enzymes are mainly focused on alanine-scanning mutagenesis, directed evolution, fusion proteins constructs through artificial linkers, and directed localization of the enzymes through signal peptides.

In order to characterize the amino acid residues which are critical for the enzymes activity, site directed mutagenesis in the conserved amino acid residues among the protein families is of a great help. Similar to other oxygen-dependent and iron-containing integral membrane enzymes, CrtZ and CrtW contain putative iron-binding His motifs and have multiple transmembrane domains with several conserved amino acid residues [18,140]. Therefore, alanine-scanning mutagenesis of these residues at the His motifs and the transmembrane domains was performed to further study the CrtW from *Paracoccus* sp. strain N81106 [140]. Among the conserved amino acids at the three His motifs of CrtW, six His residues (H69, H103, H106, H107, H221 and H222) were essential for its activity, while partial activity was retained upon on the alanine substitution of the three His residues (H65A, H218A, and H219A). Additionally, alanine substitution of the Y134A, F176A, F118A, P116A, W126A, W229A and L232A residues in the transmembrane domains resulted in decreased activity. Site directed mutagenesis to the sumoylation site (K90R) of a β-carotene hydroxylase from *H. pluvialis* resulted in 1.34 fold enhancement in astaxanthin production, which is believed to be due to enhancement on the protein stability [141].

Directed evolution via error-prone PCR has been successfully employed for enhancing the activity of CrtW from different sources. Six mutations (H96L,R203W, A205V, A208V, F213L and A215T) in the CrtW from *Sphingomonas* sp. DC18 showed improved activity toward hydroxylated carotenoids which resulted in enhanced astaxanthin production in *E. coli* [142]. Enhancement of astaxanthin levels was achieved upon three mutations in L175 M, M99V, and M99I residues of the CrtW from *Paracoccus* sp. N81106 [140]. The mutations increased the affinity of CrtW toward adonixanthin without any significant effect on β-carotene conversion rate. A triple mutant (H165R/V264D/F298Y) of β-carotene ketolase from *H. pluvialis* showed enhanced β-carotene conversion rate which resulted in 2.4 fold increase in canthaxanthin level and subsequent increase in astaxanthin level by 93% in *S. cerevisiae* [143]. Additional mutation at the (M1T/N188D/L271R) residues further enhanced increased the canthaxanthin yield by 51% and decreased the β-carotene accumulation by 44% [144]. In the same study, *H. pluvialis* β-carotene hydroxylase mutatnt at L288R residue showed 33% enhanced astaxanthin production compared to the wild type. Furthermore, the co-expression of the these two mutants resulted in 39% astaxanthin increase [144]. Another triple mutant (A6T, T105A and L239 M) of CrtW from *Brevundimonas* sp. SD212 enhanced astaxanthin level by 5.35 fold *in E. coli* [137]. The mutant showed enhanced conversion of the intermediates and astaxanthin reached 92% of the total carotenoids produced by this strain.

Creating artificial scaffolds of the key enzymes of a synthetic pathway has been proved as effective strategy for enhancing the efficiency of the pathway by reducing the undesirable intermediates diffusion [145]. In this context, a fusion construct of CrtZ from plant and CrtW from *Brevundimonas* sp. strain SD212 using flexible linker resulted in 1.4 fold increase in astaxanthin level when expressed in *E.coli* [146]. β-carotene has been reported to accumulate in the plasma membrane of engineered *E.coli*, thus bringing the enzymes to close proximity to their substrate might be beneficial to enhance the productivity [147]. Accordingly, and regardless of their predicted transmembrane domains, the directed localization of a fusion protein of CrtW and CrtZ from *Brevundimonas* sp. strain SD212 and *Pantoea agglomerans*, respectively, by linking them to the membrane protein GlpF led to 215.4% improved astaxanthin levels [148]. Likewise, the N-terminus fusion of a signal peptide of the outer membrane protein ompF to truncated BKT from *Chlamydomonas reinhardtii* led to 31% increase in astaxanthin in *E. coli* [126]. Further increase by 34% was obtained upon enhancing the stability of the protein by C-terminus fusion with *E. coli* thioredoxin (*trxA*) as molecular chaperone. In a recent study on astaxanthin production using *Y. lipolytica*, directed localization of a fusion construct of CrtZ from *H. pluvialis* and CrtW from *Paracoccus* sp to the ER, β-carotene biosynthesis location; LBs and peroxisomes, hypothetical β-carotene storage location; resulted in 4.8 fold (139.4 mg/L) increase in astaxanthin level [149]. Further optimization of the culture using fed-batch shake flask fermentation led to a yield of 858 mg/L which is the highest reported in yeast.

3.2. Membrane stress management strategies

The accumulation of astaxanthin and carotenoids in membranes induces toxic effect to the cells, which subsequently limits their yield. Directing the storage to the LBs has been an effective strategy to increase carotenoids production and relieve their toxicity in *S. cerevisiae* and *Y. lipolytica* [114,150,151]. Therefore, directing the storage of astaxanthin to the LBs is promising for optimizing astaxanthin accumulation in engineered microorganisms. However, free astaxanthin is highly probable to be stored in membranes due to its structure and polarity. Astaxanthin in *H. pluvialis* and *C. zofingiensis* is mainly in esterified form and accumulates in LBs, yet the mechanism and the enzymes involved in esterification are not clearly identified [51,84,152]. Astaxanthin esterification in *H. pluvialis* and *C. zofingiensis* is speculated to be mediated by diacylglycerol acyltransferases (DGATs) and the long-chain-alcohol O-fatty-acyltransferase (AAT), respectively, [51,152]. However, *in*

vitro assay using microsomal fraction of *S. cerevisiae* expressing the *C. zofinginesis* AAT did not show esterification activity toward astaxanthin [153]. Another approach can be adopted by enhancing the water solubility of astaxanthin. Carotenoids glycosylation is known to enhance their solubility [154,155]. For instance, zeaxanthin di-glycosylation mediated by zeaxanthin glycosyltransferase from *Erwinia herbicola* enhanced its water solubility from 12.6 ppm to 800 ppm [154]. Glycosylated astaxanthin has been reported in some microorganisms such as *Paracoccus* sp. N81106 (Agr*obacterium aurantiacum*) and *Sphingomonas astaxanthinifaciens* and showed higher solubility in water based solvents compared to free astaxanthin [11,13]. Few attempts have been made to produce glycosylated astaxanthin in *E.coli* through the heterologous expression of zeaxanthin glycosyltransferase (CrtX) from *E. uredovora* [12,155]. However, several glycosylated intermediates have been detected which might be due to the non-specificity of the enzyme toward astaxanthin [155]. Furthermore, the total carotenoids level has been decreased accompanied with increase in the accumulation of un-converted β-carotene suggesting an interruption of the astaxanthin biosynthetic pathway upon the expression of CrtX. It should be noted that, optimization of the glycosyl donor supply should be considered for efficient production of glycosylated astaxanthin.

Alternatively, enhancing the capacity of the membranes for storage via membrane engineering strategies can be an effective tool for enhanced astaxanthin production. The knock-out of the membrane morphology genes, *lpp*, *bamB*, and *rodZ*, in *E. coli* resulted in increase in the astaxanthin content by 40% [156]. Upon the knock-out, the *E. coli* cells exhibited elongated morphology which in turn increased the membrane surface area for the storage of astaxanthin. Another strategy has been adopted to increase the membrane surface area by introducing inner and outer membrane vesicles in *E. coli* [157]. Inner membrane vesicles were induced by the heterologous expression of the caveolin protein (Cav1) which is involved in the formation of the cell membranes invaginations (caveolae), while the outer membrane vesicles were formed by the repression of *rffD* and *rfaD* genes which are involved in the outer membrane integrity, and an increase in astaxanthin titer by 50% and 62% was achieved, respectively [157].

The orientation of astaxanthin in the plasma membrane enables its diffusion through the membrane [129]. Therefore, the secretion of astaxanthin to the medium can reduce the membrane stress, the potential astaxanthin mediated feedback inhibition, and the extraction costs. However due to its hydrophobicity, the amount of secreted astaxanthin in the culture medium is very limited. Accordingly, the use of biocompatible organic solvents can facilitate the secretion of astaxanthin in the medium [115].

3.3. Dynamic control systems: a promising strategy for astaxanthin production

The manipulation of cellular metabolism through metabolic engineering strategies including tuning gene expression, gene deletions, as well as protein engineering has enabled improvement in the production levels of many valuable products in microbial cell factories. However directing the flux toward non-native biosynthetic pathways creates burden to the cells due to the competition over the resources and the energy supplies with the native metabolic pathways, in addition to the possible toxicity of the products [158]. Such burden can influence the cell growth and the yield of the desired product. Thus, to overcome these restrictions, decoupling of the cell growth and the biosynthesis of the product of interest is needed, which can be achieved though dynamic control system [158]. Several studies have been conducted to control the production of several products via dynamic control systems, however few examples are available for astaxanthin [159,160]. A temperature dependent dynamic control system has been developed using the temperature-responsive Gal4M9-system in *S. cerevisiae* which resulted in 235 mg/L astaxanthin through fed-batch fermentation [144]. Similarly, decoupling the growth and the production in *E. coli* using the inducible

IPTG promoter resulted in 64% increase in astaxanthin [129]. Although dynamic control systems are promising in optimizing the production of several products, but several limitations still exist including the lack of known sensors for the biosynthetic/toxic intermediates and the limited information about native sensor-response systems in some promising microbial cell factories [161,162].

3.4. Directed evolution of microbial cell factories

The development of ideal microbial cell factories is challenging even with the advances in molecular biology and biotechnology, due to the complexity of intracellular reaction networks in living cells and the limited knowledge about their regulation, which limit the appropriate rational design. Therefore, directed evolution of the whole cell factory is another effective strategy to improve its performance. The majority of the directed evolution research has been made on the native producers *X. dendrorhous* and *H. pluvialis* and little has been done on the non-native producers. In this section we summarize the directed evolution strategies used in astaxanthin producing strains including mutagenesis and adaptive laboratory evolution (ALE) (Tables 2 and 3).

3.4.1. Chemical and physical mutagenesis

Chemical mutagenesis, physical mutagenesis (UV and Gamma irradiation), and atmospheric and room temperature plasma (ARTP) are the most commonly used mutagenesis strategies for microbial cell factories directed evolution [163]. Gamma rays mediated mutagenesis have been employed to increase the production of astaxanthin in *X. dendrorhous* and led to 1.77 fold increase in astaxanthin [164]. ARTP mutagenesis for astaxanthin producing *S. cerevisiae* promoted astaxanthin level by 83% [165]. Treatment of *H. pluvialis* cells with ethyl methane sulfonate (EMS) and UV increased astaxanthin by 2.38 fold and 2.17 fold, respectively [166]. In order to obtain a better mutagenic effect, a combination of physical and chemical mutagenesis is frequently used. A three-stage mutagenesis to *H. pluvialis* has been conducted using UV mutagenesis followed by EMS and screening based on the resistance to the inhibitor diphenylamine (DPA), resulted in selection of a mutant with 1.7 fold enhancement in astaxanthin production compared to the wild type cells [167].

3.4.2. Adaptive laboratory evolution

Over the last few years, adaptive laboratory evolution (ALE) has received a significant attention for the development and optimization of many microbial cell factories [168]. ALE is conducted under laboratory conditions where the cells are subjected to controlled culture conditions until a desired mutant is obtained upon the exposure to several stressors. However, to obtain the desired mutant, a proper selection of the stressors is required. Due to their antioxidant activities, carotenoids producing organisms including astaxanthin producers are believed to be more fit to oxidative stress [32,169]. Accordingly, adaptive evolution of *X. dendrorhous* upon the exposure to six oxidizing agents (ionone, diphenylamine, NaCl, TiO$_2$, H$_2$O$_2$ and NaClO) have been conducted to get an astaxanthin overproducing strain [170]. As a result, an evolved mutant with 48.2% enhancement in astaxanthin was obtained in response to the oxidative stress caused by TiO2. In another study, combined ARTP mutagenesis and ALE using H$_2$O$_2$ led to 4 fold increase in astaxanthin in *S. cerevisiae* [171].

3.4.3. Screening methods

For an ideal directed evolution process at the enzymes or whole cell level, an efficient rapid screening method is needed to select the desired mutant. Owing to its red color, visual color screening depending on the change in color intensity is the most common and easiest method for the selection of astaxanthin overproducers [144,172]. However, the background due to interference of other intermediates (i.e. canthaxanthin) and the color saturation make it challenging [144]. The use of astaxanthin biosynthesis inhibitors diphenylamine (DPA) and nicotine, or the

Table 2
Chemical and physical mutagenesis strategies for astaxanthin production.

Hosts	Mutagenesis methods	Mutagenic conditions	Fermentation scale	Titer (mg/L)	Yield (mg/g DCW)	Fold change	Ref
H. pluvialis	UV + EMS	15min /0.12% (w/w)	–	–	–	3.2	[166]
	UV + EMS + DES	11min/2.0% (V/V) /0.1% (V/V)	shake-flask 15 days	90	47.21	1.7	[167]
X. dendrorhous	γ-irradiation	5.0 kGy	shake-flask 8 days	2.6	15.9	1.77	[164]
	ARTP + UV	50s/40s	shake-flask 96 h	–	–	1.2	[246]
S. cerevisiae	ARTP	30s or 40s	5-L bioreactor 84 h	217.9	13.8	3.26	[165]

Table 3
ALE strategies for astaxanthin production.

Hosts	ALE stress agents	ALE conditions	ALE rounds	Fermentation scale	Titer (mg/L)	Yield (mg/g DCW)	Fold change	Ref
X. dendrorhous	H_2O_2	10 mmol/L	1	shake-flask 120 h	10.4	1.3	1.83	[247]
	TiO_2	300 mg/L	150	shake-flask 48 h	1.22	–	1.48	[170]
	TiO_2	500 mg/L	5	shake-flask 72 h	14.74	–	2	[248]
S. cerevisiae	H_2O_2+ARTP	10 L/min/30%	1	5-L fermenter 210 h	65.9	–	4	[171]

expression of astaxanthin biosynthetic genes under week promoters can help to overcome these limitations and facilitate the screening [144,166, 167]. Other screening methods using flow-cytometry and near-infrared spectroscopy have been employed to screen astaxanthin hyper-producers [173–175]. A high-throughput screening using an Raman-Activated Droplet Sorting (RADS) showed promising results for screening astaxanthin hyper-producers [176]. Using astaxanthin producing *H. pluvialis* variants as a model, a sorting accuracy of 98.3%, enrichment rate of 8 folds, and throughput of ~260 cells/min was achieved.

4. Astaxanthin fermentation optimization

Besides the improvement of the microbial cell factories performance through the above mentioned strategies, the appropriate optimization of the fermentation conditions contributes greatly to boost their productivity and reduce the production cost. Therefore, for optimum and cost efficient industrial scale production of astaxanthin using these organisms, native producers in particular, adequate knowledge of their fermentation process is crucial. As a secondary metabolite, the biosynthesis of astaxanthin is not required for the growth of the cells. Additionally, several reports indicated the dependence of astaxanthin biosynthesis on several environmental stress conditions, which are not the optimum conditions for the cell growth. Thus, the fermentation strategy adopted for astaxanthin production is a two-stage fermentation, where the cell growth is promoted at the first stage, followed by astaxanthin biosynthesis stimulation stage. In this section we will discuss the most significant factors that stimulate astaxanthin biosynthesis including stress conditions, chemical supplementation, pH, temperature and dissolved oxygen (DO).

4.1. Stress and cultivation strategies

High light intensity, nutrients restriction and high salinity are the major stress conditions that trigger astaxanthin biosynthesis in *H. pluvialis* [177]. While, the use of high light intensities requires high amount of energy, nutrients restriction is more convenient in terms of cost and implementation for industrial scale production of astaxanthin. Nitrogen starvation is believed to induce astaxanthin production effectively [178–181]. A recent study showed 25% increase in astaxanthin

using a sequential stress strategy involved extended nitrogen starvation followed by moderate light intensity exposure at the late palmella stage [177]. Moderate sodium chloride concentration promotes astaxanthin production, while high concentration has an inhibitory effect [182,183]. In *C. zofingiensis* separate application of high light (HL) or salinity stress (SS) can moderately enhance astaxanthin accumulation, while the combinatorial HL and SS can significantly enhance the production [83]. The current large-scale cultivation of *H. pluvialis* for astaxanthin production is a complex two-stage culture system, the green stage and red stage. While the green stage is for obtaining high biomass, and the red stage is characterized by the synthesis and accumulation of astaxanthin in response to inducers. At present, studies on the production of astaxanthin from *H. pluvialis* are mainly focused on optimizing the culture and induction conditions [184]. Several fermentation strategies have been developed to save the cost and increase the productivity of astaxanthin using this microalgae including: sequential heterotrophic-phototrophic culture, one-step continuous culture, indoor artificial light culture, biofilm cultivation and sequential mixotrophic culture [183,185–196].

Light can induce carotenogenesis *X. dendrorhous*, however high light intensities are lethal to the cells [197]. Irradiation with white and ultraviolet light resulted in increase of astaxanthin yield in *X. dendrorhous* by 85% and 97%, respectively in a shake flask [198]. Furthermore, large scale fermentation using 10 L and 800 L fermenters under white light irradiation led to astaxanthin yield of 420 mg/L (4.7 mg/g) and 350 mg/L (4.1 mg/g), respectively [198]. High C/N is believed to promote astaxanthin production in *X. dendrorhous*, however high glucose could inhibit the cell growth in. Accordingly, Yamane et al. developed a two-stage fed-batch culture: where the first stage was controlled at low C/N to promote the cell growth, followed by a production stage at high C/N which significantly enhanced astaxanthin production [199]. Similarly, a two stage fed-batch fermentation was conducted with a controlled glucose concentration of 25 g/L at the lag and the early log phases, and 5 g/L at the late log and the stationary phases and 109% increase in astaxanthin was obtained [200]. Sonication has a positive effect on enzyme activity and microbial processes [201]. In this context, exposure of *X. dendrorhous* culture to ultrasonic stimulation led to 26% enhancement of astaxanthin [201].

4.2. Chemical supplements

In addition to light and nutrient deficiency, addition of chemical supplements to the culture media have a significant influence on astaxanthin production. Exogenous addition of acetate to the culture medium of *H. pluvialis* led to enhancement in astaxanthin by 2 fold compared to the control group [202]. Further increase can be obtained through combined oxidative stress with acetate supplementation by addition of ferrous ions or active oxygen species (singlet oxygen, superoxide anion radical, hydrogen peroxide and peroxy radical) [203]. Addition of the cationic polymer polyethyleneimine led to enhanced oxidative stress which was accompanied with 5 fold increase in astaxanthin [204]. Phytohomrones such as methyl jasmonate, gibberellin, salicylic acid have synergetic effect on astaxanthin accumulation in *H. pluvialis* [48,205]. Other studies indicated that the addition of the antioxidants butylated hydroxyanisole and butylated hydroxytoluene could significantly induce astaxanthin synthesis and led to 2.03 and 1.66 fold increase, respectively, however the mechanism of induction is not clear [206,207]. Astaxanthin content in *X. dendrorhous* was enhanced by 40.7% upon glutamate feeding [208]. Ethanol and acetic acid feeding after glucose consumption increased astaxanthin by 31% and 26%, respectively, in *X. dendrorhous* culture [209]. Other studies showed that plant and fungal extracts are beneficial for boosting astaxanthin production using *X. dendrorhous* [210–212].

4.3. pH

The pH of the fermentation broth has a significant influence on the metabolic activities of the microorganisms. *H. pluvialis* can grow on a wide range of pH with an optimal growth pH of 7.0–7.5, while a pH higher than 8.25 promotes astaxanthin biosynthesis [182,213,214]. The parasitic fungus *Paraphysoderma sedebokerensis* is one of the most serious contaminants of *H. pluvialis* cultures with a significant influence on the growth and subsequently the astaxanthin level [215]. An acidic cultivation strategy was developed to prevent infection of *H. pluvialis* with the fungus in culture for astaxanthin production [216]. The strategy involved cultivation at a pH 4 for the growth and astaxanthin biosynthesis, while to relief the pH dependent reduction in astaxanthin, gradual light irradiation in addition to nitrogen deficiency were employed at the induction stage which resulted in 141 fold increase in astaxanthin levels compared to the control.

The optimal pH for astaxanthin biosynthesis in *X. dendrorhous* appears to be dependent on the strain. For instance, the earliest reports showed that the optimal pH for *X. dendrorhous* growth and astaxanthin production was 4.5 [217]. A mutant strain showed optimal astaxanthin production pH of 5.0 while the optimum growth was achieved at pH 6.0 [218]. However, the change in the pH had a great influence on growth, but little effect on astaxanthin production. Contrarily, culture of the *X. dendrorhous* DSMZ5626 under uncontrolled pH condition showed significant inhibition of astaxanthin below 5.5, while the cell growth was significantly inhibited when pH dropped to 4.2 [219]. These findings suggest that the genetic background of the strain influences their response to the pH.

4.4. Temperature

Temperature is another critical factor that affects the growth of the microbial cells. The change in the temperature significantly influence the enzymatic activities of the metabolic pathways enzymes and subsequently the cell growth and astaxanthin production. The optimal temperature for astaxanthin accumulation in *H. pluvialis* is 27–28 °C; and the growth and production rate would decrease significantly at temperatures lower than 15 °C or higher than 30 °C [220–222]. While, the optimum temperature for *X. dendrorhous* is 20–22 °C with a significant growth and production inhibition at temperatures higher than 22 °C [217]. In engineered non-native carotenoid producers, lower temperatures were favored for carotenoids biosynthesis. For instance, In *S. cerevisiae*, low temperatures were thought to benefit the accumulation of crotein, zeaxanthin, and β-carotene [223,224]. Similarly, a 78.96% increase in astaxanthin and 2 fold increase in the total carotenoids was achieved upon the cultivation of a high-yield astaxanthin producing *S. cerevisiae* strain AX15 at 20 °C [225]. Park et al. studied the effect of different temperature (26, 28 and 30 °C) on astaxanthin accumulation in *E. coli*, and 30 °C was the selected as the optimum temperature for astaxanthin production [226].

4.5. Dissolved oxygen (DO)

DO is a key parameter in astaxanthin fermentation process, due to its direct effect on the energy metabolism of the cells, and the astaxanthin biosynthetic enzymes activity. Yamane et al. found that astaxanthin accumulation in *X. dendrorhous* is directly proportional to the oxygen supply [199]. Further kinetic analysis showed that respiration rate was positively correlated with astaxanthin production and negatively correlated with ethanol production [199]. Wang et al. found that *X. dendrorhous* growth and astaxanthin production would be significantly inhibited when the dissolved oxygen tension was adjusted at 20% saturation [227]. Another study investigated the effect of different agitation speeds (250, 400, and 600 rpm) on *X. dendrorhous* DSMZ5626 in a 2 L bioreactor, and found that DO, biomass and astaxanthin production reached the highest when the speed was the highest, and *vice versa* [219]. Similarly, enhancement of the oxygen level in shake flasks using the biocompatible organic oxygen carrier *n*-hexadecane resulted in 58% increase in the carotenoid yield of *X. dendrorhous* [228]. DO is not critical for astaxanthin biosynthesis by *H. pluvialis*. On the other hand, adequate supply of carbon dioxide is required for the photosynthetic activities, and the oxygen generated through photosynthesis is partially consumed by the astaxanthin biosynthetic pathway [229]. Moderate increase in carbon dioxide supply can lead to increase in astaxanthin production [230]. Furthermore, the introduced CO_2 can alter C/N ration which creates nutrients deficiency condition that act as astaxanthin inducer.

5. Conclusion and outlook

Up to date, the highest yield of astaxanthin was achieved in recombinant *E. coli* at 1.18 g/L by employment of a combination of different engineering strategies, which is much higher than the most popular native producer *H. pluvialis* and *X. dendrorhous* [124]. In contrast to this non-native producer, the optimization of astaxanthin in the native producers *H. pluvialis* and *X. dendrorhous* is mainly through directed evolution and fermentation optimization through chemicals supplementation for the induction of astaxanthin biosynthesis. However, these methods have been successfully implied to increase astaxanthin levels but they are laborious and not cost efficient. This illuminate the power of synthetic biology in optimization of microbial cell factories. However, compared to some high terpenoid producing strains, for example, farnesene high-producing *S. cerevisiae*, there still exist huge room for astaxanthin producing strains for production optimization and enhancement [231]. In addition to the metabolic engineering strategies mentioned above, introduction of non-native pathways to enhance the precursors supply might be of great influence on increasing astaxanthin production. For example, IPP enhancement has been achieved in several organisms via introducing exogenous isoprenol utilizing pathway (IUP), which led to enhanced isoprenoids production [232–234]. Additionally, other promising membrane stress management strategies have to be adopted to relief the burden of astaxanthin accumulation on the cells. Enhancing the membrane fluidity can be achieved through increasing the unsaturated fatty acids content of the membranes [235]. The *OLE1* gene encodes a Δ9-fatty acid desaturase which is catalyzing the dehydrogenation of the 9-position in saturated fatty acids forming the corresponding unsaturated fatty acid [236]. The overexpression of this gene

has been successfully employed to increase triterpenoids and β-carotene production in yeast by reducing the membrane stress [237,238]. Furthermore, deep knowledge about gene expression and regulation is needed to improve these microbial cell factories, such as epigenetic modifications, non-coding RNAs and post-translational regulations etc., which are still mysterious. Additionally, highly efficient genetic manipulation tools and techniques are also in urgent demand for native microbial producers and non-conventional but promising yeast such as *Y. lipolytica*. It worth noting, the design of microbial cell factory needs to be closely connected to the industrial process and market needs and applications. For example, to increase the stability and bioavailability of astaxanthin, different forms of astaxanthin obtained through esterification, covalent binding to proteins, or PEGylation need to be investigated. And considering the huge differences between flask cultivation and bioreactor fermentation, the evaluation or screening of engineered strains is preferred to be based on industrially used culture mediums and parallel-bioreactors.

At the end, the market application of natural astaxanthin transformed by synthetic biotechnology have to face supervisory regulations on market access by different countries. The removal of regulatory obstacles is also the key to unlocking attractive market prospects of astaxanthin.

Author statement

Mostafa Basiony: Writing - original draft; Writing - review & editing. Visualization; **Liming Ouyang:** Project administration; Resources; Supervision; Validation; Writing - original draft; Writing - review & editing. **Danni Wang:** partial writing; **Jiaming Yu:** partial writing; **Liming Zhou:** partial writing, Visualization; **Mohan Zhu:** partial writing; **Xuyuan Wang:** partial writing; **Jie Feng:** partial writing; **Jing Dai:** partial writing; **Yijie Shen:** partial writing; **Chengguo Zhang:** partial writing; **Qiang Hua:** Investigation, Supervision; **Xiuliang Yang:** Funding acquisition, Investigation, Resources; **Lixin Zhang:** Funding acquisition, Investigation, Resources.

Declaration of competing interest

Nothing declared.

Acknowledgements

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