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Strategies for producing high value small molecules in microalgae

Michael H. Cagney [a,b], Ellis C. O'Neill [a,b,*]

[a] School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, UK
[b] Biodiscovery Institute, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

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ABSTRACT

Eukaryotic microalgae are a diverse group of organisms that can be used for the sustainable production of a wide range of high value compounds, including lipids, flavours and dyes, bioplastics, and cosmetics. Optimising total biomass production often does not lead to optimal product yield and more sophisticated biphasic growth strategies are needed, introducing specific stresses to induce product synthesis. Genetic tools have been used to increase yields of natural products or to introduce new pathways to algae, and wider deployment of these tools offers promising routes for commercial production of high value compounds utilising minimal inputs.

1. Introduction

Eukaryotic microalgae are a diverse group of organisms which exhibit great metabolic diversity and are able to synthesise a wide range of high value natural compounds. These products, including lipids, carbohydrates, terpenes, and bioplastic polymers, are increasing in global demand with applications in numerous industries. The photoautotrophic nature of microalgae makes them inherently more sustainable than cultivation of other organisms, requiring minimal resource inputs for growth whilst fixing and converting significant quantities of environmental CO_2 into their products (Rodolfi et al., 2009). They can be grown in closed photobioreactor systems, controlling physical and biological conditions, or more cost-effective open raceway ponds (Greenwell et al., 2010). Many microalgae are capable of heterotrophic growth comparable to microbial fermentative systems, which can generally achieve faster growth rates and biomass accumulation compared to photoautotrophic growth alone. Alternatively, some cultivation systems are mixotrophic, combining phototrophic and heterotrophic growth schemes.

Unlike plant-based products, microalgae require much less land, can use unpurified water unsuitable for agriculture and do not compete with food crops (Rodolfi et al., 2009). To achieve optimal productivity, biotechnological cultivation often employs a biphasic growth system. In the first stage, high biomass is achieved using ideal growth conditions, followed by a second stage in which desired compounds are produced by upregulating associated metabolic pathways, often by introducing abiotic stresses (Bhola et al., 2021).

1.1. Microalgal genetic engineering

Microalgal genetic engineering is in its infancy compared to other well-established organisms. Significant advances have been made in the development of genetic engineering toolkits over the past 30 years primarily in the model chlorophyte *Chlamydomonas reinhardtii*, in turn allowing for expression of transgenes, strain development and improved productivity in other microalgal species (Doron et al., 2016).

Endogenous metabolic pathways can be engineered by expression of appropriate recombinant enzymes, increasing expression of native enzymes, heterologous expression of alternative highly active enzymes, or disruption of competing pathways (Diao et al., 2018). Non-native compounds can also be produced in microalgae by expressing the biosynthetic pathways from other organisms, using their highly efficient metabolism and low inputs to make high value products. Eukaryotic microalgae are also valuable expression systems for recombinant proteins, particularly complex proteins requiring post-translational modifications (Rasala and Mayfield, 2015). Here, the introduced transgene encodes for a recombinant protein that is itself a high value product.

Several transformation techniques have been developed for microalgae including glass bead agitation, electroporation, *Agrobacterium tumefaciens*-mediated transformation, biolistic bombardment and

* Corresponding author. School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, UK.
E-mail address: ellis.oneill@notttingham.ac.uk (E.C. O'Neill).

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CRISPR-Cas, and *Escherichia coli* conjugation (Doron et al., 2016; Slattery et al., 2018). These techniques all depend upon expression cassette integrating into the nuclear or plastid genome. Transformation efficiencies are often low, (Slattery et al., 2018; Fajardo et al., 2020) particularly in the nuclear genome, due to a combination of physical barriers of the cell and random integration of expression cassettes by non-homologous end joining, causing 'position effects', which results in variable expression between transformants and requires extensive screening (Fajardo et al., 2020; D Adamo et al., 2019).

Transgene expression levels can be more consistent on self-replicating vectors (episomes) as they do not integrate into the genome and are unaffected by position effects (US Pat, 2016; Karas et al., 2015). Episomes are dependent on self-replicating autonomous sequences (ARSs) for maintenance in subsequent generations but few ARSs have been identified for microalgal episomes. Dehoff et al. created a method for identifying endogenous ARS sequences in microalgal ribosomal DNA (US Pat, 2016). Additionally, Karas et al. developed stably maintaining episomes in the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* using sequences derived from the yeast *Saccharomyces cerevisiae* (Karas et al., 2015). These episomes were repurposed by Slattery et al. in *P. tricornutum* as a CRISPR-Cas vector, and modified to host eight biosynthetic genes required for vanillin biosynthesis. The eight enzymes were stably expressed after 4 months; however, the pathway was non-functional due to point mutations introduced in the first month (Slattery et al., 2018). Despite being unsuccessful, this presents a significant achievement in expressing an entire biosynthetic pathway in a microalga using one expression construct.

Much effort has been centred on optimising expression cassette design. The great diversity displayed across microalgae has meant using expression cassettes with heterologous 5′ and 3' untranslated regions (UTRs), i.e. promoters and terminators, such as the CaMV35S promoter, has often resulted in poor or even undetectable expression (Fajardo et al., 2020; Schroda et al., 2000). Instead, expression cassettes are tailored to the host species using endogenous UTRs from genes encoding highly abundant housekeeping proteins. Among the most widespread UTRs is the chimeric HSP70A-RBCS2 promoter, created by fusing the individually strong heat shock protein 70 A (HSP70A) and Rubisco small subunit promoters (RBCS2) (Doron et al., 2016; Schroda et al., 2000).

Gene silencing mechanisms in microalgae can specifically target gene transcripts for degradation, often supressing expression of heterologous genes (Fajardo et al., 2020). A major improvement in expression cassette design employs the use of self-cleavable 2A peptides from the Picornaviridae viral family. When using 2A peptides, the gene of interest and selectable marker are expressed polycistronically. During translation, the 2A peptide self-cleaves to release the mature protein of interest, circumventing gene silencing and increasing expression levels (Rasala et al., 2012). This strategy is now routinely used in transgenic expression in various microalgal species.

Introns are known to have significant impact on microalgal gene expression and are present in both 5′ UTRs and throughout gene coding regions of microalgal genomes (Doron et al., 2016). Their effects are exerted through several regulatory mechanisms, and can upregulate gene expression through the phenomenon termed 'intron mediated enhancement', as demonstrated by Baier et al. who systematically spread the first intron of RBCS2 throughout a transgene coding region expressed in *C. reinhardtii* (Baier et al., 2020). Including introns in expression cassettes has much potential for increasing transgene expression levels, although the practice is not currently widespread.

This review will discuss the use of industrially significant eukaryotic microalgae to manufacture a wide range of valuable compounds, including lipids, terpenes, bioplastics, and cosmetics. Yields can be increased using a variety of physical, biological, and genetic engineering interventions with different products being targeted with different techniques.

2. Lipids

2.1. Biofuels

Diminishing fossil fuel reserves and global warming has prompted development of low-cost renewable fuels with reduced emission outputs. Lipid-based biofuels are inherently much more sustainable compared to existing petroleum fuels and have lower emission outputs. Fatty acid methyl esters (FAMEs) are used in biodiesel with physical properties (cold-flow, low viscosity, and storage suitability) determined by degree of saturation and alkyl chain length (Razeghifard, 2013; Li et al., 2008). Traditionally, these lipids have been sourced from higher plants, but this is impractical for meeting increasing demands (Chen and Wang, 2021). Microalgae instead offer a more sustainable platform for biofuel production due to greater lipid contents, carbon fixation, and greater general lipid productivity per area than plants (Rodolfi et al., 2009). As of 2023, the global microalgal biofuel market is valued at $9.14 bn and is projected to reach $17.94 bn by 2030. (Grand View research)

2.1.1. Biofuel production in algae

Lipid content of several oleaginous species has been investigated with lipids often reaching at least 50% total dry cell weight (DCW) (Mallick et al., 2016). Interestingly, general cell productivity and lipid content are inversely related due to metabolic burdens imposed by lipid synthesis. In other words, microalgal species with greatest lipid content often have poor biomass accumulation which complicates cultivation of otherwise promising species (Rodolfi et al., 2009). Furthermore, not all oleaginous species are appropriate for biofuel production. *Lobospira incisa* lipids are mostly polyunsaturated fatty acids and are therefore unsuitable for use as biodiesel due to susceptibility to oxidation requiring further hydrogenation (Razeghifard, 2013; Li et al., 2008). *Botrycoccus braunii* produces a range of large hydrocarbon lipids which can be easily fractionated but cannot be transesterified into biodiesel (Banerjee et al., 2002). The viability of *B. braunii* is also limited by its poor generation time of 3–4 days in optimal conditions.

Nitrogen deprivation has been reported to be highly effective for increasing lipid content in several algal species due to carbon flux relative to growth favouring lipid synthesis (Mallick et al., 2016). The oleaginous genus *Nannochloropsis* has gained much interest as the lipid content of several species in standard growth conditions reaches ~30% DCW (Rodolfi et al., 2009). Up to 80% of *Neochloris oleoabundans* lipids are saturated long chain fatty acids, making this species particularly promising for biodiesel production (Li et al., 2008).

Salt stress has also been effective for increasing lipid accumulation in *Dunaliella* sp. with NaCl increasing lipid content up to 210 mg/g, or 70% DCW, with TAGs making up 56% of total lipids (Takagi et al., 2006). *Nannochloropsis* and *Dunaliella* are attractive due to being relatively easily to manipulate, however, their cultivation is complicated by their reliance upon saltwater, in turn limiting potential for biofuel production (Li et al., 2008). Wax esters produced by the Euglenid *Euglena gracilis* can be used directly as biofuel feedstock without requiring additional processing. The relative proportion of wax esters can be readily increased by anaerobic growth conditions (Gissibl et al., 2019). The total lipid yield of *E. gracilis* could be improved by 40%, up from 13% to 18%, under hypoxic conditions by using Fe-ion mutagenesis followed by selecting of the highest producing cells using fluorescence-activated cell sorting (Yamada et al., 2016).

2.1.2. Engineering biofuel production

Among metabolic engineering approaches to increase neutral lipid productivity, overexpression of endogenous or heterologous enzymes central to TAG biosynthesis (Fig. 1) has generally been an effective strategy. Chungjatupornchai et al. overexpressed endogenous plastidial LPAT in *N. oleaoabundans* under the control of either HSP70A-RBCS2 or β-tubulin 2 (βTUB2) promoters (Chungjatupornchai and Fa-aroonsawat,

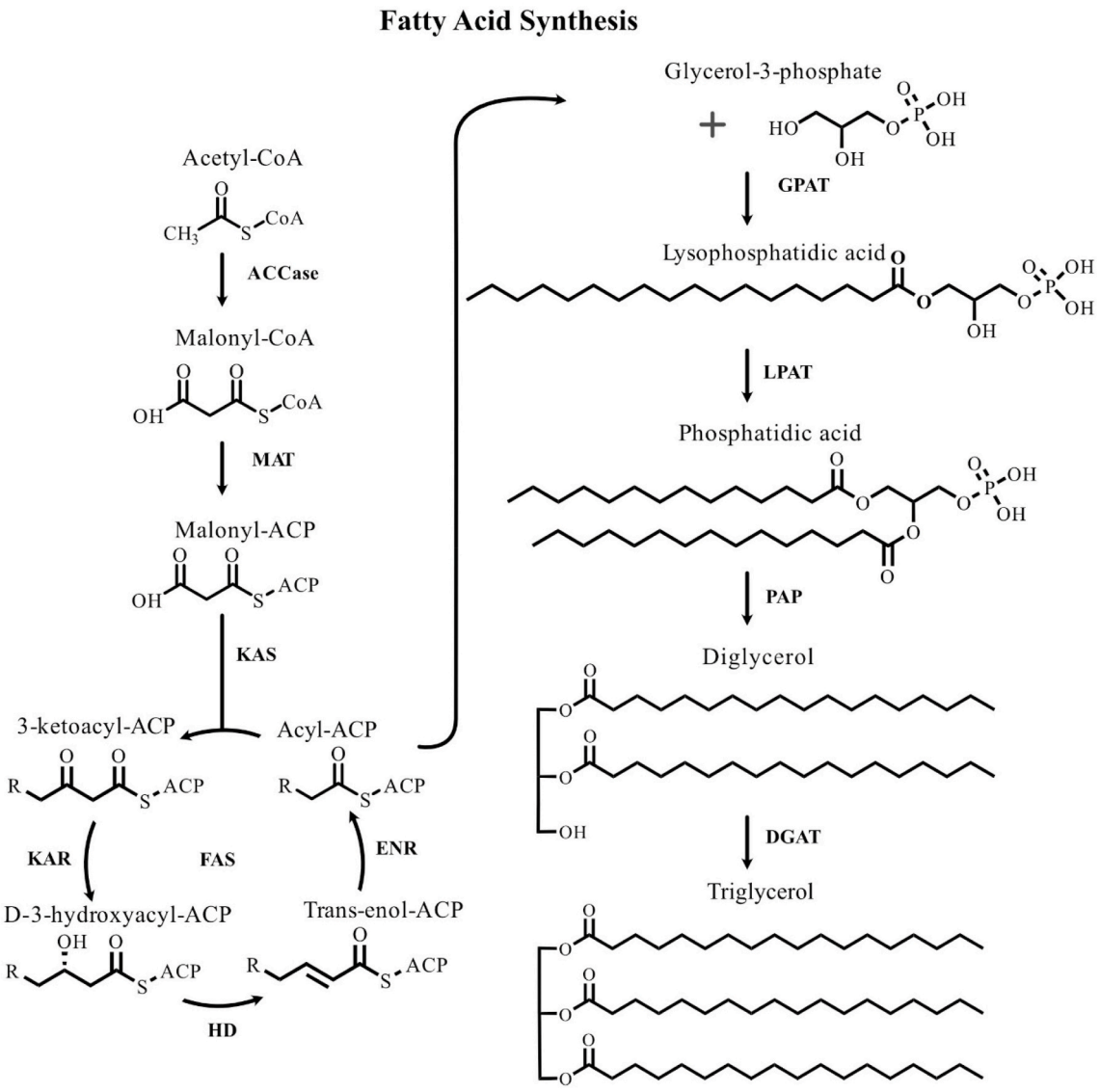

Fig. 1. Fatty acid synthesis and triglyceride biosynthetic pathways. Photosynthesis- and glycolysis-derived pyruvate is converted into acetyl-CoA. *De novo* fatty acid synthesis in chlorophytes occurs in the chloroplast and endoplasmic reticulum, where acetyl-CoA is converted into malonyl-CoA. Enzymes of the fatty acid synthase complex (FAS) catalyse acyl chain formation followed by sequential condensation, reduction, dehydration, and reduction steps generating saturated fatty acid chains C14:0, C16:0 and C18:0 in length before addition to glycerol to form the acylglycerols. Key enzyme steps in biosynthesis are highlighted in bold. ACCase: acetyl-CoA carboxylase, MAT: malonyl-CoA ACP transacylase, KAS: 3-ketoacyl-ACP synthase, KAR: 3-ketoacyl-ACP reductase, HD: 3-hydroxylacyl-ACP dehydrase (HD), ENR: enoyl-ACP reductase, GPAT: glycerol-3-phosphate acyltransferase, LPAT: acyl-CoA-dependent acyl-CoA: LPA acyltransferase, PAP: phosphatidic acid phosphatase, DGAT: diglyceride acyltransferase.

2021). This resulted in a 1.6-fold increase in TAG content to 73% DCW, 20% higher than previously reported overexpression of DGAT, a 2.1-fold increase in TAG content to 50% DCW, a 1.9-fold increase in total lipid productivity to 16.84 mg/L/day, and a 2.1-fold increase in TAG productivity to 11.68 mg/L/day. Chien et al. found that co-expression of endogenous GPAT, LPAT, PAP and DGAT in a single expression cassette in Chlorella sp did not increase biomass accumulation above 0.22 g/L/day, but TAG-content increased 2.3-fold to 46% total lipid content. This demonstrates expressing multiple genes is likely the most effective strategy for increasing TAG production (Chien et al., 2015).

Several studies have attempted to overexpress ACCase to increase abundance of malonyl-CoA as a precursor for lipid synthesis, but this has not been successful in microalgae (Mallick et al., 2016). Malic enzyme activity may be significant in oleaginous species, where extra NADPH for fatty acid synthesis is generated by conversion of excess mitochondrial citrate to acetyl-CoA (Razeghifard, 2013). Jeon et al. overexpressed endogenous malic enzyme in *Nannochloropsis salina* under control of a β-TUB promoter-terminator pair resulting in a 53% increase in fatty acid content to 1.5 g/L suitable for conversion into FAMEs (Jeon et al., 2021).

Overall, despite being a promising renewable biofuel platform, microalgae are impractical due to numerous issues regarding their cultivation and downstream processing. Microalgal biodiesel is uncompetitive with petroleum fuels due to costing approximately $2.2/kg to produce, with cell harvesting and drying alone accounting for 50% of total production costs (US Pat, 2016; Mallick et al., 2016). Interest for many commercial producers has since transitioned to comparably more economic polyunsaturated fatty acids for use as nutritional supplements.

2.2. PUFAs

Polyunsaturated fatty acids (PUFAs) are very long chain fatty acids (C20-22 long) with two or more double bonds. PUFAs are further categorised into groups depending on the distance of the first double bond from the methyl end of the hydrocarbon chain including omega-3,

omega-6, and omega-9 fatty acids (Adarme-Vega et al., 2012). Essential fatty acids to human health include the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) acid. In the human body, EPA (C20:5 n-3) is converted into eicosanoids which function as signalling molecules in the innate immune response, whereas DHA (C22:5 n-3) is a structural lipid of the brain, retina, and heart (Ward and Singh, 2005).

As of 2023, the global omega 3 oil market is valued at $9.3 bn and is projected to reach $22 bn by 2033 (Future Market Insights). EPA and DHA are typically sourced from aquaculture, however, issues with over-fishing and climate change threaten long-term sustainability of this option. Microalgae have gained increasing interest due to being able to produce significant amounts of PUFAs, notably EPA and DHA, which are mostly present as membrane or storage lipids (Liu et al., 2022). The economic viability of microalgae as a source of PUFAs is emphasised by the presence of commercial EPA- and DHA-rich microalgal oils with DHA making up most of the market (Ward and Singh, 2005).

PUFA accumulation is linked to growth stages functioning as an energy reserve during unfavourable growth conditions. Omega-3s accumulate due to their high energy content and necessity for cellular functions, such as membrane repair (Adarme-Vega et al., 2012). Culture nutrients impact on PUFA productivities with nitrogen deprivation often being used to induce high PUFA productivities under light provision to maintain continuous photosynthesis (Adarme-Vega et al., 2012). Several species have been explored for omega-3 production with few having been successfully used in industry.

2.2.1. DHA production in algae

Members of the *Crypthecodiniaceae* and *Thraustochytriaceae* families are among the most used microalgae for the production of DHA, with the latter being preferable due to their cell walls being easier to disrupt during downstream processing (Peltomaa et al., 2018). Oil content of these microalgae is rich in DHA with negligible EPA (Ward and Singh, 2005). The dinoflagellate *Crypthecodinium cohnii* is widely used in industry and can accumulate up to 109 g/L biomass with 19 g/L being DHA, and less than 1% of other PUFAs in optimal conditions, favourable for downstream processing (Diao et al., 2018). Thraustocrytids, including the genus *Schizochytrium*, are among the best microalgal sources of DHA due to their high growth rates, reaching 100 g/L biomass within as few as four days. Lipid levels of highly productive strains can reach up to 70% DCW with DHA making up to 70% total fatty acids (TFA) (Aasen et al., 2016).

2.2.2. EPA production in algae

EPA levels of *P. tricornutum* are among the highest reported in wild-type organisms reaching 52% TFA, (Gu et al., 2022) and is therefore highly promising for industrial EPA production. Diatom biomass productivity is comparably poor even in heterotrophic cultures. Gu et al. demonstrated potential for scalability using an optimised modelling and repeated-batch culture strategy increasing biomass by 50% to 160 mg/L/day, and EPA levels by 20% to 55 mg/g DCW, respectively (Gu et al., 2022).

Nannochloropsis sp. are promising for industrial EPA production due to high lipid content reaching up to 60% DCW, and established genetic engineering toolkit permitting feasible strain optimisation (Peltomaa et al., 2018; Ma et al., 2016; Xu, 2022). EPA levels often range between 5 and 35% TFA in several species (Adarme-Vega et al., 2012; Ma et al., 2016) with the highest reported EPA content at 42% TFA in *Nannochloropsis oceanica* (Xu, 2022). However, EPA is only up to 4% DCW (Liu et al., 2022). *Nannochloropsis* is limited to autotrophic growth schemes with few reports of high productivity using heterotrophic growth in terms of both biomass and lipid content. Biomass achieved through autotrophic growth in open ponds often does not exceed 1 g/L, although 3 g/L per day has been reported using more costly closed bioreactor systems (Ma et al., 2016). PUFA content of cryptophytes can rival that of *Nannochloropsis* with *Storeatula major* being a rich source of all PUFAs, up to 12.5 μg/mg DCW, and *Chroomonas mesostigmatica* EPA content rivalled that of *Nannochloropsis*, up to 6.1 μg/mg DCW. Ultimately, these cryptophyte species are not competitive with *Nannochloropsis*, as *Nannochloropsis* volumetric yields were up to 100-fold greater (Peltomaa et al., 2018).

2.2.3. Engineering PUFA production

Attempts to improve PUFA content has involved upregulation of enzymes involved in overall lipid synthesis (Fig. 1) and PUFA pathway-specific enzymes such as fatty acid desaturases (FADs) (Fig. 2). Previous PUFA metabolic engineering attempts in *Schizochytrium* proved challenging, often compromising biomass and lipid yields (Li et al., 2018). Li et al. overexpressed endogenous MAT in Schizochytrium sp under control of translation elongation factor promoter and cytochrome 1 terminator (Li et al., 2018). This proved an effective strategy as total lipid yield increased by 39.6% to 110.5 g/L, and biomass also increased by 3.2%. Overall PUFA content increased by 24.5% to 8.57 g/L with DHA increasing by 81.5% to 47.39 g/L, and EPA increasing by 172.5% to 1.65 g/L, respectively.

The highly abundant protein Rubisco is involved in the first step of carbon fixation in photoautotrophic growth and is therefore unnecessary in heterotrophic growth. Diao et al. performed a knockout of '*Rubisco*' in *C. cohnii* to favour carbon and energy flux towards lipid accumulation under heterotrophy. The authors observed a 10.6% increase in overall lipid accumulation, although they did not comment on specific DHA fold-change (Diao et al., 2018).

Niu et al. successfully improved lipid productivity of *P. tricornutum*, increasing PUFA content by 41%, by overexpressing endogenous GPAT using an expression cassette under the control of fucoxanthin chlorophyll *a*/c binding protein (fcp) C promoter and fcpA terminator (Niu et al., 2016). By overexpressing endogenous Δ6 FAD under control of fcpA promoter, Zhu et al. increased *P. tricornutum* EPA content by 48% to 38.101 mg/g DCW, although the authors observed up to a 4% decreased growth rate (Zhu et al., 2017).

Poliner et al. explored the effects of singular and co-expression of Δ5, Δ9 and Δ12 FADs using optimal expression cassettes in *N. oceanica* employing a constitutive elongation factor promoter. Individual expression of Δ5 and Δ12 FADs increased EPA content by 25% due to greater carbon flux towards the omega-6 pathway, whereas co-expression saw no further increase above this (Poliner et al., 2018). The authors also noted increased growth rates and decreased TFA content in all cell lines except for Δ9 FAD expression.

Overexpression of endogenous enzymes is not necessarily effective for increasing EPA levels. When Liu et al. overexpressed endogenous DGAT in *N. oceanica* under a ubiquitin extension protein promoter they found negligible impact on EPA levels. Instead, heterologous expression of *C. reinhardtii*-derived DGAT under the same promoter significantly increased EPA levels by 5.9-fold. Co-expressing endogenous Δ0 elongase-1 under a lipid droplet surface protein promoter further increased EPA levels by 12.3-fold to 12.6 mg DCW (Liu et al., 2022).

3. Terpenes

Terpenes, with the general chemical formula $(C_5H_8)n$ are a structurally diverse class of organic compounds constituting the largest group of natural products synthesised by microalgae (Papaefthimiou et al., 2019). They are made by conjoining isoprene precursor 5C units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), synthesised by the plastid-localised methyl-D-erythritol pathway (MEP) and cytoplasmic-localised mevalonate (MVA) pathway, followed by various cyclisation and elaboration reactions (Fig. 3). These include sterols, carotenoids, flavours, and scent compounds which function in both primary and secondary metabolism.

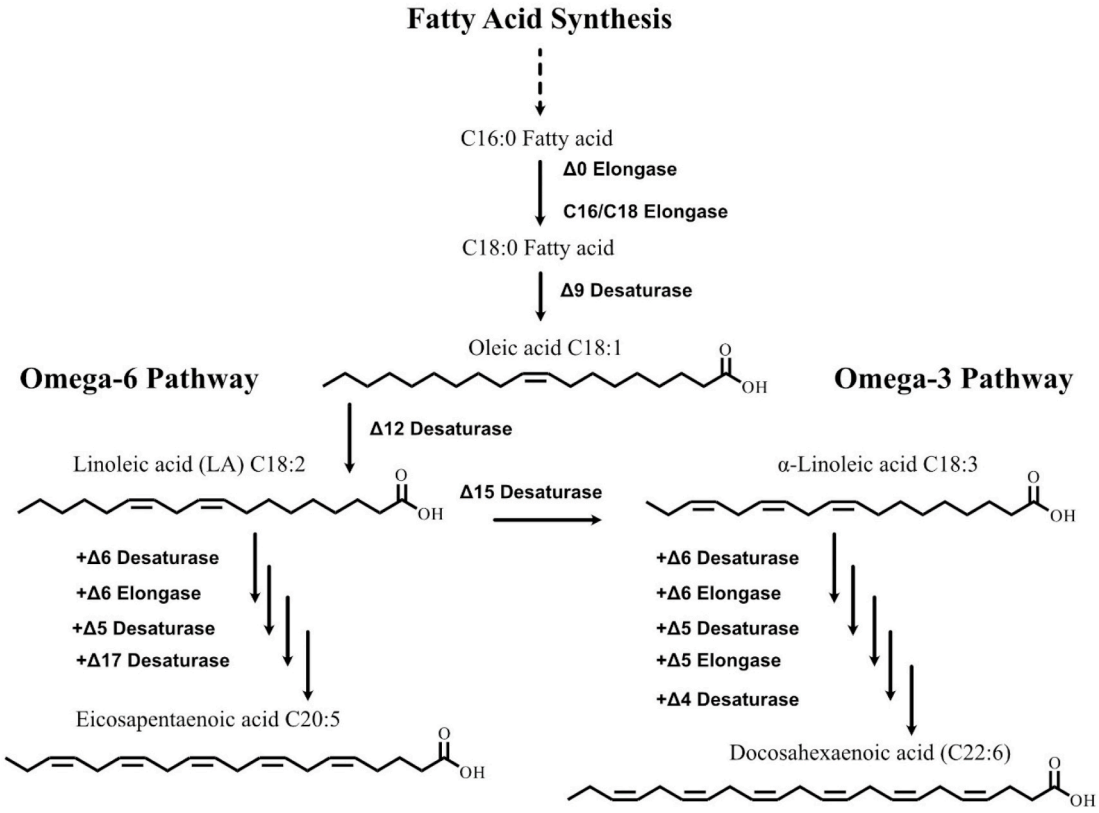

Fig. 2. PUFA biosynthetic pathways. Simplified main omega-6 and omega-3 pathways of microalgal PUFA biosynthesis. PUFAs are synthesised in the chloroplast from the precursor oleic acid and are then generated through the fatty acid synthesis pathway involving sequential desaturation and elongation of intermediate fatty acid chains. Fatty acid desaturases (Δ desaturase) introduce *cis* double bonds at specific locations whereas elongases (Δ elongase) catalyse chain elongation. Intermediate fatty acids and alternate biosynthesis pathways are not shown.

3.1. Engineering heterologous terpenes

Terpenes have been produced in a variety of microorganisms by overexpression of heterologous terpene synthases, often derived from higher plants (Fig. 3). Commercial heterologous production of terpenes is well established in bacteria and yeast fermentative systems. Due to their high production of carotenoids. Microalgae already produce terpene precursors at high levels and may be better suited for heterologous terpene production than more conventional microbes (Lauersen, 2019). Heterologous expression of terpene synthases has been successful in microalgae, demonstrating their utility as a production platform.

The monoterpene geraniol, a component of rose essential oils and a platform chemical for pharmaceutically valuable monoterpene indole alkaloids, is synthesised from geraniol pyrophosphate. Heterologous expression of the Madagascar periwinkle (*Cantharanthus roseus*) geraniol synthase in *P. tricornutum*, under an alkaline phosphatase promoter on episomal DNA produced 0.309 mg/L geraniol under photoautotrophic conditions, comparable to 0.56 mg/L reported in genetically engineered yeast (Fabris et al., 2020).

The sesquiterpene alcohol patchoulol, sourced from patchouli (*Pogostenum cablin*), is used as a fragrance ingredient in perfumes. Lauersen et al. heterologously expressed *P. cablin*-derived patchouli synthase in *C. reinhradtii* chloroplast under the control of HSP70A-RBCS2 promoter, yielding 922 μg/g DCW or 1.03 mg/L (Lauersen et al., 2016)

Other sesquiterpenes are feedstocks for biodiesel, such as (E)-α-bisabolene, which is synthesised by cyclisation of farnesyl pyrophosphate by bisabolene synthase. Expressing grand fir-derived (*Abies grandis*) bisabolene synthase in the nucleus of *C. reinhradtii,* under the control of HSP70A-RBCS2 promoter, yielded 10.3 mg/g DCW, or up to 3.9 mg/L under phototrophic conditions and 11 mg/L under mixotrophic conditions (Wichmann et al., 2018). Relatively low yields of terpenes are partly due to unoptimised genetic cassettes. Einhaus et al. compared the effectiveness of common high performing promoters controlling bisabolene synthase expression including photosystem I reaction centre subnit II (PSAD), RBCS2i1 (RBCS2 containing first intron), and β-TUB2 to a synthetically optimised β-TUB2 promoter, AβSAP(i), combining elements of HSP70A, β-TUB2 and RBCS2 intron 1 paired with ferredoxin 1 terminator. The synthetic AβSAP(i) outperformed all other promoters producing (E)-α-bisabolene at 3.2 mg/g DCW, or 2.5 mg/L, 18-fold greater than native β-TUB 2 promoter, and 4-fold greater than PSAD and RBCS2i1, respectively (Einhaus et al., 2021).

Coleus forskohlii synthesises the diterpene 13R(+) manoyl oxide, a precursor of the medicinal compound forskolin, using the synthases CfTPS2 and CfTPS3. Expressing both enzymes under the control of the HSP70A-RBCS2 promoter and RBCS2 3' UTR, and targeted to the chloroplast using PSAD transit peptide, produced 80 mg/g DCW 13R(+) manoyl oxide in photoautotrophic conditions (Lauersen et al., 2018).

The labdane diterpene sclareol, produced by clary sage (*Salvia sclarea*), is a fixative agent in the fragrance industry and is a highly valued sustainable alternative to ambergris sourced from sperm whales. Sclareol is synthesised by geranylgeranyl pyrophosphate cyclisation into the intermediate molecule copal-8-ol diphosphate by the type II diterpene synthase copal-8-ol diphosphate synthase. Papaefthimiou et al. heterologously expressed *Cistus creticus*-derived copal-8-ol diphosphate synthase in the chloroplast of *C. reinhardtii* under the control of photosystem II D2 (PSBD) promoter and photosystem II D1 (PSBE) terminator, yielding 0.038 mg/g DCW along with three other diterpene products *ent*-manoyl-oxide, *ent*-13-*epi*-manoyl-oxide and labda-13-ene-8α,15-diol totalling 1.172 mg/g DCW (Papaefthimiou et al., 2019).

The triterpenes lupeol, sourced from seeds of the genus *Lupinus*, and betulinic acid, sourced from bark of several plant species including

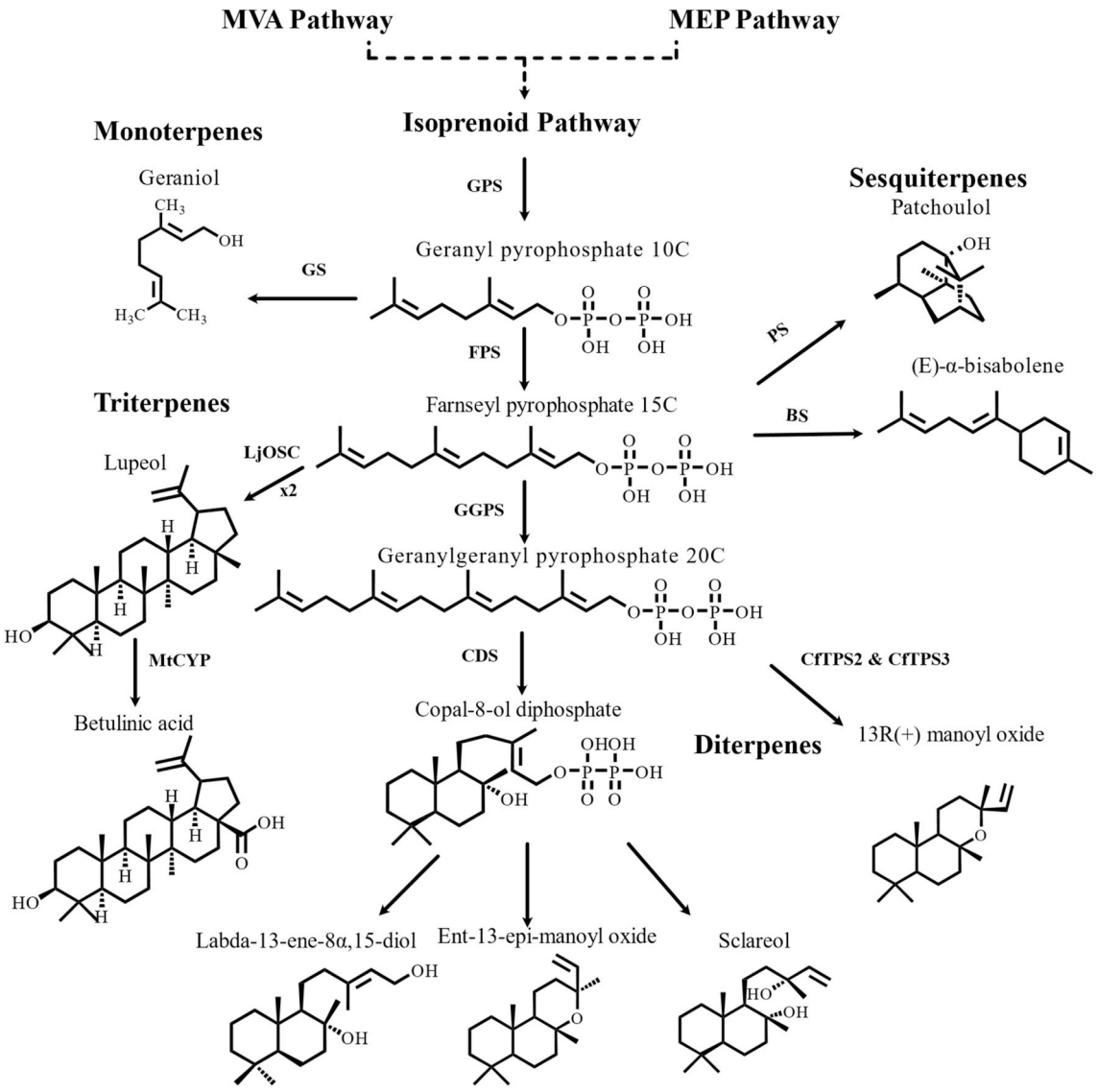

Fig. 3. Heterologous terpene biosynthesis. Isoprenoid biosynthesis occurs in the chloroplast. In the isoprenoid pathway, 5C isoprenoid molecules isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), structures not shown, are synthesised through the mevalonate (MVA) and methyl-D-erythritol phosphate (MEP) pathways and are sequentially connected to create higher-order isoprenoid backbone molecules geranyl pyrophosphate, farnseyl pyrophosphate, geranylgeranyl pyrophosphate. Key enzymes steps in biosynthesis are highlighted in bold. GPS: geranyl pyrophosphate synthase, FPS: farnesyl pyrophosphate synthase, GGPS: geranylgeranyl pyrophosphate synthase. Cyclase enzymes catalyse cyclisation of isoprenoid backbone molecules into monoterpenes, sesquiterpenes, diterpenes, and triterpenes. PS: patchouli synthase, BS: bisabolene synthase, LjOSC: *Lotus japonicus* oxidosqualene cyclase, MtCYP: *Medicago truncatula* cytochrome P450, CfTPS2 and CfTPS3: *Coleus forskohlii* terpene synthases 2 & 3, CDS: copal-8-ol diphosphate synthase.

white birch (*Betula pubescens*), are highly valued pharmaceutical compounds with anti-HIV and anti-cancer properties. Triterpenes are synthesised by conversion of FPP to intermediates such as 2,3-oxidosqualene (naturally present in *C. reinhardtii* and *P. tricornutum*) which is cyclised into basic terpene scaffolds, termed sapogenins, and are further chemically modified into active compounds by a range of enzymes, including cytochrome P450 monooxygenases (D'Adamo et al., 2019). D'Adamo et al. co-expressed *Lotus japonicus*-derived oxidosqualene cyclase, the multifunctional *Medicago truncatula*-derived cytochrome P450 monooxygenase and the native NADPH reductase in *P. tricornutum*, under control of a fcpA promoter. Two-day-old-cultures produced maximum lupeol yield of 5.8 μg/g DCW, or 0.1 mg/L, similar to 0.35 mg/L lupeol yield reported in engineered *C. reinhardtii*, and betulinic acid yield >0.1 mg/g DCW, comparable with concentrations first reported in unoptimised genetically engineered *S. cerevisiae*. Cell growth was not affected, although the authors noted heterologous expression of oxidosqualene cyclase compromised expression of native enzymes in the MEV and sterol biosynthetic pathways, limiting precursor availability (D'Adamo et al., 2019).

Yields of heterologous terpenes in microalgae remain low and currently cannot economically compete with higher yields obtained in established fermentative microbial systems (Lauersen, 2019). Productivity can be increased with further optimisation of cultivation conditions and expression cassettes as genetic toolkits become more available (Einhaus et al., 2021).

3.2. Carotenoids

Carotenoids are lipid-soluble pigments consisting of a tetraterpene (C40) backbone composed of two classes with *cis* or *trans* isomers (Gong and Bassi, 2016). Primary carotenoids, composed of only hydrocarbons (e.g., phytoene, lycopene, α-carotene and β-carotene) are involved in light capture during photosynthesis, energy dissipation, and electron transfer, and are essential to cell survival (Lauersen, 2019). Secondary

carotenoids or xanthophylls (e.g., lutein and zeaxanthin), composed of oxygenated hydrocarbons, are more abundant than primary carotenoids and are upregulated in response to stress (Ambati et al., 2019) (Fig. 4).

Carotenoids possess potent antioxidant properties for quenching reactive oxidative species (ROS) produced during photosynthesis. The antioxidant properties exerted by astaxanthin are greater than other carotenoids, make it a desirable therapeutic agent for the treatment of several degenerative diseases (Patel et al., 2022). Some carotenoids, such as α-carotene and β-carotene, are essential nutrients in the human diet functioning as vitamin precursors (Del Campo et al., 2007). Lutein and its isomer zeaxanthin accumulate in the retina and are effective in long-term prevention of several eye-related diseases (Gong and Bassi, 2016).

Synthetic carotenoids, present as *trans* isomers and accounting for 85–90% of total market share, are mostly used in fish feed, but are unsuitable for human consumption due to lower bioavailability and association with increased risk of several diseases (Patel et al., 2022; Ye et al., 2008). Natural carotenoids are present as complex mixtures of mostly *cis* isomers, which are safer and more bioavailable than *trans* isomers (Gong and Bassi, 2016). Carotenoids are often sourced from higher plants, exemplified by marigold petals being used for commercial lutein production. However, seasonality, low specific carotenoid content, and labour-intensive cultivation compromises their productivity (Ho et al., 2014). Microalgae in contrast have a high turnover of carotenoids,(Lauersen, 2019) high specific carotenoid content, and high biomass accumulation, making them a more viable commercial production platform than plants (Ho et al., 2014).

As of 2022, the global carotenoid market is valued at $2.0 bn and is projected to increase to $2.7 bn by 2027 (BCC Research). Most carotenoids are commercially significant with applications as colourants and nutritional supplements in the food and pharmaceutical industries (Del Campo et al., 2007). The most valued carotenoids are astaxanthin, β-carotene, lutein, zeaxanthin, and lycopene, with astaxanthin and β-carotene making up almost half of the market due to their nutritional and therapeutic significance (Gong and Bassi, 2016).

3.2.1. *Carotenoid production in algae*

Carotenoid content varies between microalgal species and is

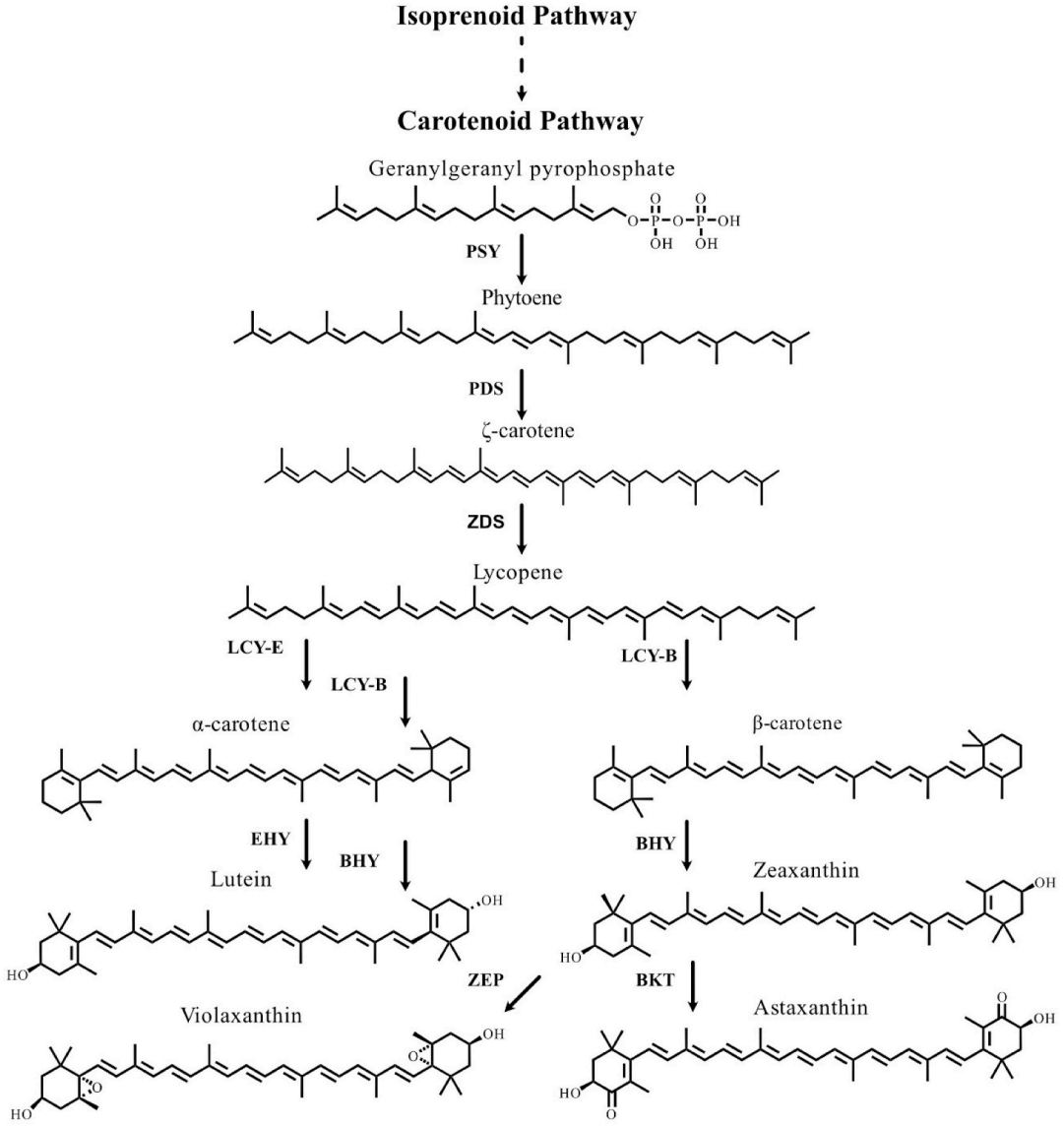

Fig. 4. Carotenoid biosynthetic pathway. The carotenoid biosynthetic pathway occurs in the chloroplast and is an extension of the isoprenoid pathway. Key enzymes steps in biosynthesis are highlighted in bold. PSY: phytoene synthase, PDS: phytoene desaturase, ZDS: ζ-carotene desaturase, LCY-E: lycopene ε-cyclase, LCY-B: lycopene β-cyclase, EHY: ε-carotene hydroxylase, BHY: β-carotene hydroxylase, ZEP: zeaxanthin epoxydase, BKT: β-carotene ketolase. Adapted from,(Gong and Bassi, 2016) and Ye et al. (Ye et al., 2008).

dependent on cultivation conditions including temperature, pH, salinity, and nutrients (Ambati et al., 2019). Microalgal production of carotenoids uses biphasic cultivation, with the first phase accumulating high biomass often under heterotrophic conditions, and then specific stresses are applied in the second phase to stimulate carotenoid production.

Several microalgal species have been investigated for lutein productivity. *C. sorokiniana* is among the most studied for lutein production with yields ranging from 5 to 15 mg/g DCW under autotrophic growth (Zhao et al., 2022). Ho et al. reported *Scenedesmus obliquus* grown under autotrophic conditions in high light intensity increased lutein productivity 3-fold from 1.39 mg/g to 4.52 mg/g DCW (Ho et al., 2014). During abiotic stresses, the phytohormone melatonin induces synthesis of various metabolites in microalgae (Zhu et al., 2022). Zhu et al. reported that melatonin supplemented *Acutodesmus* sp autotrophic cultures increased lutein content 1.53-fold to 17.44 mg/g DCW (Zhu et al., 2022).

Microalgal industrial production of astaxanthin is done almost exclusively by *Haematococcus pluvialis* due to astaxanthin accumulating up to 5% DCW, superior to most other microalgae (Patel et al., 2022). Greatest productivity has been achieved using photoautotrophic conditions where yields range between 20 and 30 mg/g DCW (Del Campo et al., 2007). Pereira and Otero demonstrated using red-blue light increased astaxanthin to 50 mg/g DCW (Pereira and Otero, 2020). The long maturation time compromises cost effectiveness of *H. pluvialis* cultures and has incentivised investigating other species (Patel et al., 2022).

According to Chen et al., *Chromocloris zorofingiensis* cells are easier to disrupt and harvest valuable products from than *H. pluvialis* (Chen et al., 2017a). Astaxanthin yields are generally highest when exposed to blue light, although the authors observed maximum yields of 38.9 mg/g DCW using white light in combination with nitrogen deprivation. Pyruvic acid was later found to be the most effective chemical inducer, increasing biomass and therefore astaxanthin yields up to 11.4 mg/g DCW or 87 mg/L (Chen et al., 2022). Yadavalli et al. observed maximum astaxanthin productivity in iron-supplemented *C. sorokiniana* mixotrophic cultures, yielding 34.4 mg/g DCW or 154 mg/L (Yadavalli et al). Li et al. demonstrated exogenous γ-aminobutyric acid supplementation improved biomass and astaxanthin yields 5.3-fold, up to 354.1 mg/L (Li et al., 2023)

The halotolerant genus *Dunalialla salina* is the most effective production platform for commercial β-carotene, accumulating up to 50 mg/g, or 14%, DCW (Ye et al., 2008). β-carotene is present as lipid globules within the chloroplast and is synthesised in response to ROS generation under a combination of hypersaline, nutrient-deprived, high light intensity, high temperature conditions. Various isomers are synthesised and can be manipulated by light intensity during cell division; the most abundant and desired being the highly bioavailable 9-*cis* isomer (Raja et al., 2007). *D. bardawil* is the best producer of β-carotene, accumulating up to 80 mg/g DCW (Chen et al., 2017a). Srinivasan et al. observed sodium bicarbonate as carbon source supplementation increased β-carotene content two-fold in *D. salina* and *D. bardawil* (Srinivasan et al., 2015).

3.2.2. Engineering carotenoid production

Directly expressing biosynthetic enzymes has been used to increase lutein production in microalgae. Phytoene desaturation catalysed by phytoene synthase (PSY) is a rate limiting step in carotenoid biosynthesis (Galarza et al., 2018). Cordero et al. heterologously overexpressed *C. zofingiensis*-PSY in *C. reinhardtii* under the control of HSP70A-RBCS2 promoter and RBCS2 3′ UTR, increasing lutein and violaxanthin content by 2.2-fold and 2-fold, respectively (Cordero et al., 2011). Astaxanthin content in *H. pluvialis* increased by 67% to 34.3 mg/L when endogenous PSY was overexpressed in the chloroplast under the control of native 5′ photosystem subunit II D1 and 3' Rubisco large subunit UTRs (Galarza et al., 2018). Steinbrenner and Sandmann demonstrated overexpressing a mutant PSY under control of native PSY promoter and terminator sequences in *H. pluvialis* resulted in a 26% greater astaxanthin accumulation to 11.4 mg/g DCW (Steinbrenner and Sandmann, 2006).

The enzymes β-carotene ketolase (BKT) and β-carotene hydroxylase (BHY) are required for conversion of carotenoid intermediates into astaxanthin. Zheng et al. were the first group to demonstrate how astaxanthin could be synthesised in transgenic microalgae. The authors simultaneously expressed *H. pluvialis*-derived BKT and BHY in *C. reinhardtii* under control of HSP70A-RBCS2 promoter and RBCS2 3' UTR resulting in a 34% increase in astaxanthin content to 1.6 mg/g DCW (Zheng et al., 2014).

Endogenous *C. reinhardtii* β-carotene ketolase (*Ct*BKT) expression levels are relatively low. To improve this, Cazzaniga et al. re-designed *Ct*BKT *in silico* using codon optimisation, intron inclusion and removal of a redundant C-terminal sequence (Cazzaniga et al., 2022). Several attributes of the engineered *Ct*BKT strain were improved over the parent strain including greater tolerance to high light intensity and high biomass accumulation, outperforming even the fast-growing *Chlorella vulgaris*. Astaxanthin content made up to 74% of total ketocarotenoids, yielding 2.5 mg/g DCW, which accumulated during constitutive growth, meaning adverse physiological effects on cells associated with induced stresses observed in *H. pluvialis* could be avoided (Cazzaniga et al., 2022).

D. salina β-carotene could be converted to xanthophylls by heterologously overexpressing *C. reinhardtii*-derived BHY using endogenous *D. salina* RBCS2 promoter, resulting in a 3-fold and 2-fold increase in violaxanthin and zeaxanthin content, respectively (Simon et al., 2016). Hu et al. increased *D. salina* β-carotene content 2.2-fold to 1.4 μg/mL by using CRISPR-Cas with guide RNAs targeted the endogenous *D. salina* BHY gene (Hu et al., 2021). Chen et al. increased β-carotene content of *Scenedesmus* sp 3-fold to over 30 mg/g DCW by heterologously expressing a synthetic PSY gene with the highest consensus of amino acids derived from a combination of *C. reinhardtii*, *D. salina*, and *Moriella zofingiensis* under control of CaMV35S promoter (Chen et al., 2017b).

Post-transcriptional methylation is a key mechanism involved in fine-tuning mRNA translation. Liu et al. overexpressed an endogenous 18S rRNA methyltransferase, CrBUD23, in *C. reinhardtii* using CaMV35S promoter, increasing cell growth rates with a 65.1% increase in lutein content to 6.54 mg/g DCW (Liu et al., 2023).

4. Polyhydroxyalkanoates

The desirable properties of petrochemical-based plastics have made them among the most used materials in everyday life. These materials are highly unsustainable with significant environmental damage associated with their disposal, and greenhouse gases released during their production contributing to the worsening climate crisis. Thus, there is growing demand for biodegradable bioplastics produced from renewable sources to phase out existing petroleum-based plastics. Bioplastic production currently accounts for 1% of global plastic production (Bhola et al., 2021).

Polyhydroxyalkanoates (PHAs) are a class of inert, non-toxic, biodegradable polymers with a global market value in 2023 of $93 mn and projected to increase to $195 mn by 2028. (Markets and Markets) PHAs are synthesised by a range of organisms in the presence of excess carbon during stationary phase using acetyl-CoA from metabolism (Fig. 5). PHA bioplastics are classified into three major subgroups based on their chain length: short-chain (C3-5), medium-chain (C6-14) and long-chain (C15+) (Bhola et al., 2021). Composition of monomer units determines mechanical properties such as thermoplasticity and elastomasticity: short chain lengths are more brittle whereas long chain lengths are more elastic (Costa et al., 2019). Short chain length PHAs make up most of the market due to having similar properties to conventional plastics, with long chain lengths being used for elastics and rubbers (Costa et al., 2019).

Plant biomass sourced from agricultural crops is a notable source of

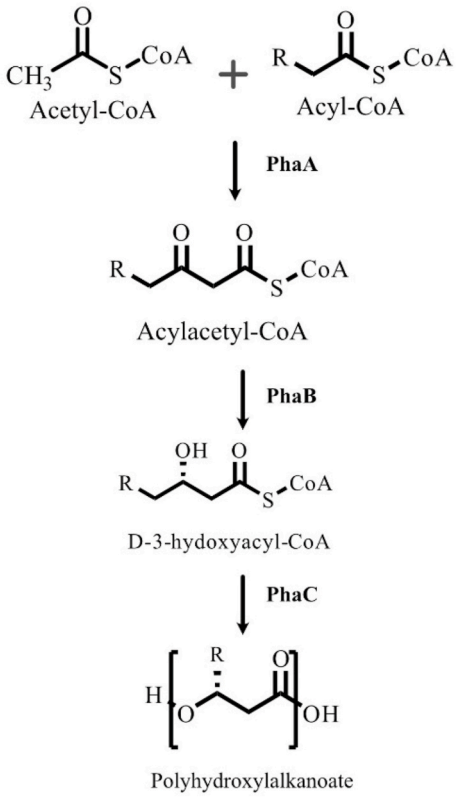

Fig. 5. Polyhydroxylakanoate biosynthesis. Acetyl-CoA and acyl-CoA molecules are condensed together forming acylacetyl-CoA, which is then reduced to D-3-hydroxyacyl-CoA and added to existing PHA monomers through an ester bond, releasing CoA. R═CH_3 for polyhydroxy butyrate (PHB), the simplest PHA. Key enzyme steps in biosynthesis are highlighted in bold. PhaA: β-ketothiolase, PhaB: acetoacetyl CoA reductase, PhaC: PHB synthase.

PHAs, but these plant based PHAs have poor mechanical properties, and compromise food security by competing with agricultural land (Madadi et al., 2021). PHAs can instead be produced by various microorganisms including *Cupriavidus necator* and recombinant *Escherichia coli*, where PHAs accumulate as insoluble cytoplasmic granules serving as carbon and energy reserves under stress conditions (Bhola et al., 2021; Madadi et al., 2021). Carbon supply accounts for up to 50% of bacterial fermentation production costs, in turn being uncompetitive with cheaper petroleum-based plastics (Costa et al., 2019).

4.1. Algal production of PHAs

Generally, microalgae accumulate lower levels of PHA than bacteria, but the photoautotrophic nature presents a significant cost saving measure by permitting synthesis of PHAs without relying on supplemented organic carbon (Madadi et al., 2021). Microalgae are often grown mixotrophically using biphasic cultivation, with the second growth phase under nitrogen- or phosphorous-limiting conditions converting intracellular carbon stored as glycogen into PHA (Bhola et al., 2021). Different PHA monomers and resulting polymers and copolymers can be synthesised depending on carbon source and degree of nitrogen and phosphorous deprivation, which upregulates PHA biosynthetic enzymes (Costa et al., 2019).

A *Chlorella* sp. was found to have higher biomass accumulation of 2.74 g/L DCW than three cyanobacteria strains, with PHAs produced of comparable thermal stability and quality, and further potential to increase yields by optimising growth conditions (Roja et al., 2019). Sodium bicarbonate supplementation increased polyhydroxybutyrate (PHB) synthesis in *C. vulgaris* by maximising biomass up to 2.01 g/L of which 80% was PHB, the highest reported in literature thus far (Selvaraj et al., 2021).

4.1.1. Engineering PHA production

There are fewer reports of eukaryotic microalgal genetic engineering for PHA production than in cyanobacteria. Genetic engineering strategies centre around heterologous expression of phaA, phaB, and phaC PHA biosynthetic genes (Fig. 5) from the β-proteobacterium *Ralstomia eutropha*, which naturally produces high amounts of PHB (Hempel et al., 2011).

C. reinhardtii possesses PhaA and is capable of synthesising PHB with metabolic engineering. Chaogang et al. demonstrated *C. reinhardtii* accumulated PHB up to 6 mg/g DCW by co-expressing heterologous *R. eutropha*-derived PhaB and PhaC under control of HSP70A-RBCS2 promoter (Chaogang et al., 2010). The authors observed reduced cell growth but were unsure whether due to random nuclear integration of transgenes or if PHB granules were responsible for the metabolic burdens caused.

Similarly, expression of the complete *R. eutropha* PHB biosynthetic pathway into *P. tricornutum* under an inducible nitrate reductase promoter enabled PHB accumulation up to 10.6% DCW in seven-day-old cultures (Hempel et al., 2011). Windhagauer et al. expanded on transgenic P. *tricornutum* PHB synthesis using episomes by investigating different configurations of constitutive and inducible promoters (Windhagauer et al., 2022). None of the twenty-four constitutive promoters produced detectable PHB, likely due to gene silencing or metabolic burdens, whereas using an alkaline phosphatase 1 promoter yielded 2.3% PHB as DCW, or 27.9 mg/L in eleven-day-old cultures (Hempel et al., 2011).

Despite cultivation being considerably more cost-effective than bacterial fermentations, microalgae are not cultivated explicitly for commercial PHA production due to comparably lower growth rates. Companies instead often make PHAs as a side-product from leftover biomass (Bhola et al., 2021).

5. Mycosporine-like amino acids

UV radiation (200–400 nm wavelength light) is harmful to most organisms primarily due to DNA damage. Synthetic UV filters commonly used in sunscreens, such as oxybenzone, are implicated in pollution of water sources and are harmful to aquatic life (Schneider and Lim, 2019). There is growing interest in phasing out of these compounds for environmentally friendly and potent UV-absorbing compounds for use in sunscreens. Aquatic organisms including microalgae can produce mycosporine-like amino acids (MAAs) to mitigate the effects of UV radiation without generating ROS, analogous to melanin produced by animals or phenylpropanoids and flavonoids produced by higher plants (Geraldes and Pinto, 2021; Fathi et al., 2022).

More than 30 MAAs have been characterised with molecular structures consisting of a cyclohexanone or cyclohexenimine ring conjugated to an amino acid or amino alcohol (Fig. 6). (Fathi et al., 2022; Hosseinabadi et al., 2022) High molecular absorpivities of MAAs make them among the strongest UV-A and UV-B absorbing compounds in nature (Geraldes and Pinto, 2021). Coupled with their water solubility and environmental stability, MAAs have great potential as UV absorbing compounds in sunscreens, successfully demonstrated by use of porphyra-334 in the commercial product Helioguard (Geraldes and Pinto, 2021).

5.1. Mycosporine production in algae

Chemical synthesis of MAAs is challenging and difficult to scale, and they are predominantly produced by extraction from natural sources (Fathi et al., 2022). Macroalgae, such as *Porphyra*, generally contain high concentrations of MAAs, but are already required for the food industry and seasonality complicates reliable production. MAAs

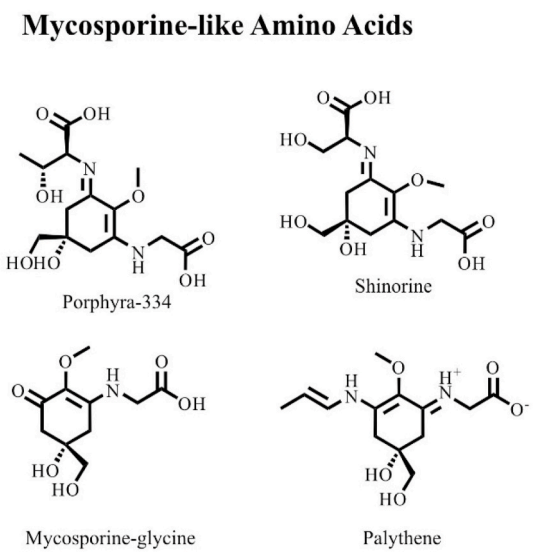

Fig. 6. Chemical structures of mycosporine-like amino acids discovered in microalgae. Biosynthetic enzymes catalysing key steps remain uncharacterised in eukaryotic microalgae.

accumulate in the cytoplasm of several microalgal species and are appropriate for industrial scale production (Fathi et al., 2022).

Accumulation and structure of MAAs is both species-specific and individual MAA-specific (Xiong et al.). Jeffrey et al. screened 152 non-UV exposed marine microalgal species including diatoms, chlorophytes, euglenophytes, eustigmatophytes, and dinoflagellates for the presence of UV absorbing compounds (Jeffrey, 1999). Strains exhibiting most UV absorbing compounds were from bloom-forming dinoflagellates, prymnesiophytes and cryptomonads, whereas strains belonging to chlorophytes, euglenophytes, and eustigmatophytes contained the least UV absorbing compounds. Dinoflagellate genera *Fibrocapsa*, *Woloszynskia* and *Gymnodinium catenatum* absorption maxima corresponded to known MAAs (porphyra-334, shinorine, mycosporine-glycine, palythene); *Gymnodinium catenatum* was the richest source of yet unidentified compounds.

Biosynthesis of MAAs is also induced in response to UV radiation. Xiong et al. observed MAA-specific content of several chlorophyte species resistant or sensitive to UV-B radiation. Following UV-B exposure, they found MAA-specific content to increase for most species investigated, though yields were low, ranging from 0.06 to 0.19 mg/g DCW (Xiong et al). Chekanov et al. observed differing production of UV absorbing compounds in *Haematococcus rubicundus*, *Bracteacoccus aggregatus* and *Deasonia* sp in response to UV-A. All strains accumulated UV-absorbing compounds at 332 nm corresponding to MAAs, with *B. aggregatus* being the most potent UV absorber and most resistant to UV-A (Chekanov et al., 2022).

Nitrate and phosphate were found to increase UV absorption at 332 nm characteristic of shinorine in *C. vulgaris* (Hosseinabadi et al., 2022). Gharib et al. found salt stress in in *Desmodesmus* induced MAA production with absorption observed at 320 nm, whereas non-salt stressed *Desmodesmus* did not produce MAAs (Gharib et al., 2020).

5.2. Application of synthetic Biology approaches to high-yield production of mycosporine-like amino acids

Currently, the only genetic engineering attempts to improve mycosporine yields have been attempted in *E. coli* and cyanobacteria and there are no reports in eukaryotic microalgae (Fathi et al., 2022). This is partly due to lack of understanding of regulatory mechanisms and biosynthetic enzymes involved in key biosynthetic steps. We expect to see more advances in this field as knowledge gaps in eukaryotic microalgal mycosporine biosynthesis are filled.

6. Conclusion

Eukaryotic microalgae produce a wide range of high value compounds from minimal input and represent a sustainable resource for the future. Improvements in yields can be achieved by optimising culture conditions, but often stress responses that increase product synthesis reduce overall biomass and thus reduces unit productivity. Combining high growth rate, followed by a low growth but high product synthesis stage, in biphasic growth regimens, has achieved the best of both worlds. Although requiring minimal input costs for basic algal growth, to produce viable yields requires substantial infrastructure costs, including culturing facilities, harvesting and downstream processing.

Addressing these challenges will require a combination of engineering, biological and genetic contributions. Recent development of genetic engineering has improved yields of their natural products by increasing precursors, decreasing competing reactions and increasing the final product synthesis.

Currently all commercial algal products are natural compounds, but heterologous expression of high value products has undergone a recent renaissance, building on the new genetic engineering tools available in a range of algal species. However, these now need to move from laboratory proof of concept to industrial scale. Combining the improvements and culturing conditions, based upon the knowledge of optimising native products, will allow further developments in microalgae based commercial production of high value compounds.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Michael H. Cagney reports financial support was provided by Biotechnology and Biological Sciences Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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