Review

Genetic and non-genetic tailoring of microalgae for the enhanced production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) – A review

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HIGHLIGHTS

- Microalgal biomass is a sustainable source of EPA and DHA.
- The production of EPA and DHA from microalgal biomass is uneconomical.
- Genetic and non-genetic tailoring methods can economically produce EPA and DHA.
- Enhanced biomass productivity positively affects total yield of EPA and DHA.

GRAPHICAL ABSTRACT
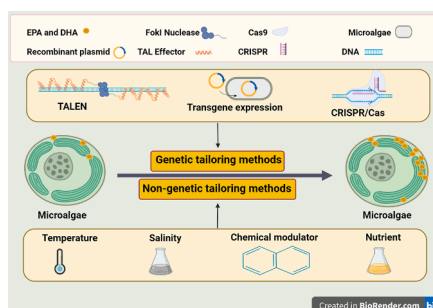
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ABSTRACT

The myriad health benefits associated with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) laid the path for their application in the functional foods and nutraceutical industries. Fish being primarily exploited for extraction of EPA and DHA are unsustainable sources; thus, oleaginous microalgae turn out to be an alternative sustainable source. This review paper aims to provide the recent developments in the context of enhancing EPA and DHA production by utilising non-genetic tailoring and genetic tailoring methods. We have also summarized the legislation, public perception, and possible risks associated with the usage of genetically modified microalgae focusing on EPA and DHA production.

1. Introduction

As people become aware of the benefits (anti-inflammatory, improved cardiovascular health, cognitive development, *etc*.) associated with consuming omega-3 fatty acids, consumer demand for these is increasing (Tiwari et al., 2021). Dietary intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) improves cardiovascular health, as these are incorporated in the phospholipid bilayer of cardiomyocytes, which modulate ion channels, thus preventing lethal arrhythmia (Endo and Arita, 2016). EPA and DHA have also shown additional benefits such as anti-thrombotic, blood pressure-lowering, endothelial relaxation, anti-atherosclerotic and anti-fibrotic effects (Endo and Arita, 2016). DHA is the predominant omega-3 fatty acid in the brain. DHA intake improves cognitive abilities; epidemiologic studies have shown that increased uptake of DHA reduces the risk of dementia up to 50% (Cole et al., 2009). EPA and DHA can also reduce cancer risk; for instance, incorporation of EPA and DHA with doxorubicin has shown changes in membrane lipid, rafts which increases the surface expression, and death receptor clustering (CD95) in mammary cancer cell lines (Ewaschuk et al., 2012).

The market for omega-3 fatty acids will be expanding at the compound annual growth rate of 7.7% by 2027, according to Grand View Research (Oliver et al., 2020). Omega-3 fatty acids include alpha-linolenic acid (ALA)(18:3, n-3), stearidonic adic (STA) (18:4, n-3), eicosapentaenoic acid (EPA)(20:5, n-3), docosapentaenoic acid (DPA) (20:5, n-3), docosahexaenoic acid (DHA) (22:6, n-3). Among all omega-3 fatty acids, EPA and DHA have proven to contribute significantly to health and thus are niche within the nutraceutical industry. Omega-3 fatty acids have traditionally been produced from animal sources such as fish. As a conventional source of EPA and DHA, fish have many associated challenges, indicating the need for alternative sources. The biggest hurdle associated with the use of fish is their overexploitation which has severely damaged marine ecosystems (Sumaila and Tai, 2020). Fish may be contaminated with heavy metals, pesticides, polychlorinated biphenyls (PCBs) etc. and long-term consumption of contaminated fish can cause different types of health disorders (Basu et al., 2021). As EPA and DHA are heat-sensitive, therefore, cooking fish before consumption can lead to a minimal amount of beneficial EPA and DHA left for consumption (Peinado et al., 2016). The benefits associated with EPA and DHA through fish consumption are compromised by these associated shortcomings.

To meet omega-3 demand, alternative sources such as microalgae, genetically modified organisms (GMO) (GM plant, GM fungus) have been explored (Zhao et al., 2016). Table 1 summarizes different genetically modified sources used for EPA and DHA production and the corresponding EPA and DHA yield. Microalgae can naturally produce omega-3 fatty acid and does not compete for fertile land or fresh water (for marine algae). Microalgae naturally assimilate CO_2, making their use in industry environmentally friendly and sustainable. However, the process of nutraceutical production from algae needs work to become economically feasible. New strategies are required to reduce the

capital operational costs of algal cultivation, extraction, and harvesting. According to Davis et al., a significant cost reduction can be achieved by improving biological productivity (Davis et al., 2011). Genetic engineering to enhance lipid productivity is one viable solution, as studies have shown a significant increase (up to 8-fold) in lipid accumulation than that obtained by exploiting the physiological potential or via selective breeding (Chauton et al., 2015). The lack of knowledge in annotated genes involved in algae's metabolic pathway needs to be addressed (Shaikh et al., 2020). Therefore, there is a need to maintain repositories for biological material such as genome sequences with overexpression and knockout libraries, which will help in building bioproduct chassis (Poliner et al., 2018).

The biorefinery approach with cascading extraction efficiently uses all the algal cell components, such as lipids, protein, carbohydrate, antioxidants etc. and aids in making the omega-3 fatty acids production process economical (Derwenskus et al., 2020). The simultaneous utilisation of microalgae pigments and omega-3 fatty acid has been reported (Chua et al., 2020). Similarly, studies have been conducted on the use of protein along with omega-3 fatty acids (Cui et al., 2021). The cyanobacterium *Lyngbya* has been utilised for a sequential biorefinery to produce lipids and the UV protectant, mycosporine-like amino acids (Chandra et al., 2019). *Phaeodactylum tricornutum*, a diatom, the integrated biorefinery process aided in the extraction of fucoxanthin (34% yield), chrysolaminarin (43% yield), and EPA (23% yield) (Zhang et al., 2018b). In another study on *Phaeodactylum tricornutum*, biorefining yielded 92% extractable EPA and 40.9% protein from defatted biomass (Cui et al., 2021). Similarly, the biorefinery approach can be applied in *Nannochloropsis* species for the extraction of EPA, followed by the use of defatted biomass for extraction of protein or utilisation as animal feed (Chua and Schenk, 2017). The biorefinery approach helps in making the process of high-value product production feasible. However, there remains a need to identify suitable extraction methods that efficiently utilize the remaining biomass for other products through the downstream processes (Marella and Tiwari, 2020). Modern investigation of sustainable omega-3 fatty acid sources brought algae in focus as many species have an efficient biosynthetic pathway for EPA and DHA. Conventional methods for improving EPA and DHA accumulation focused on changes in abiotic conditions such as irradiance, temperature, and nutrients. Stress also directs the metabolic pathway towards storage lipids such as triacylglycerols (TAG) instead of carbohydrate synthesis, thus increasing the proportion of EPA and DHA to TAG. The phyla *Bacillariophyta* and *Rhodophyta* predominately produce EPA, whereas *Dinophyta*, *Haptophyta*, *Cryptophyta* produce both EPA and DHA under nutrient replete conditions (Morales et al., 2021). A study utilizing non-genetic method was performed in *Crypthecodinium cohnii* that was cultured with a gradual increment in glucose concentration (9 to 54 g/L) up to 650 days, which led to 15.49% increase in DHA content in total fatty acid (Li et al., 2017b). Similarly, in *Schizochytrium* species, abiotic conditions such as low temperature (4 °C) and high salinity (30 g/L NaCl) lead to 57.52% increment in DHA content (Sun et al., 2018a). Benefits of non-genetic tailoring include low costs and easily available

Table 1
Different genetically modified sources used for EPA and DHA production and the corresponding EPA and DHA yield.

Source		Modification	EPA	DHA	Reference
Genetically modified plant	*Brassica napus*	The incorporation of a transgenic pathway (microalgal/yeast) of seven consecutive enzymatic steps	–	9.7% of total fatty acids (seed)	(Petrie et al., 2020)
	Camelina sativa	The expression of three constructs (p7_DHA5, RRes_B7.2, DHA2015.1) directed the synthesis towards EPA and DHA	20% of total fatty acids (seed)		(Han et al., 2020a)
Genetically modified fungus	*Mortierella alpina* ST1358*	Heterologous expression of Δ17- desaturase gene	26.4 (%, wt/wt) in total lipids	–	(Okuda et al., 2015)
	Mortierella alpina ATCC16266	Three desaturase gene expression (Δ6-desaturase gene; Δ12-desaturase gene; ω3-desaturase gene	6.23% of total fatty acids	–	(Yu et al., 2011)
Genetically modified algae	*P.tricornutum* UTEX646	Expression of Δ5-desaturase	–	10.4% in total fatty acid	(Hamilton et al., 2014)
	Schizochytrium sp. ATCC 20,888	Overexpression of ATP citratelyase (ACL), Acetyl-CoA carboxylase (ACC)	–	37.9% in total lipids	(Han et al., 2020b)

resources (such as LED lamps for light intensity, salt for changing salinity, etc.). However, the drawback of non-genetic tailoring methods is that they are time-consuming. The other shortcoming of non-genetic tailoring is that abiotic stress inevitably reduces biomass yield. Therefore, although abiotic stress triggers metabolic pathways towards EPA and DHA synthesis, it ultimately results in reduced total yield of EPA and DHA (Wang et al., 2019).

Inherent limitations associated with abiotic stress make it essential to modify the genes of microalgae. Genetic tailoring can help to achieve enhanced production of value-added products, biomass accumulation or biorefinery capabilities in microalgae (Fayyaz et al., 2020). Therefore, genetic tailoring paves a strategic practice for modification of the microalgal strains for the high yield of EPA and DHA without impeding the growth kinetics of microalgae. Advances in molecular biological tools helped in facilitating genetic engineering in microalgae. Gene editing tools such as transcription activator-like (TAL) effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) aids in modification of specific genes to enhance lipid accumulation in microalgae. Lipid production has been reportedly increased by knocking down genes (phosphoenolpyruvate carboxylase) involved in shifting the metabolic flux towards carbon (Yao et al., 2017). Nonetheless, there is no comprehensive data on the effect of gene knock-out or knock-down on increased EPA and DHA accumulation in microalgae. Therefore, genetic engineering in microalgae is targeted to shift the flux towards EPA and DHA production by overexpression of native fatty acid elongase and desaturase gene, or by heterologous expression of the interspecies gene construct. The limitation of genetic engineering is that most research has been focused on model microalgae whose genomes have been sequenced (Bhattacharjya et al., 2021). Microalgal species with available genome information need different genetic tools due to interspecific variability in genetic construct. Another limitation associated with microalgal production of nutraceutical PUFA is the requirement of food-grade flocculants for harvesting, and non-toxic solvents for extraction of PUFA while preventing it from getting oxidized. The process also needs to be made economical by reducing the harvesting cost, which can comprise up to 40% of the total production cost (Chua and Schenk, 2017). Genetic tailoring has been executed in microalgal species such as *Phaeodactylum, Nannochloropsis, Chlamydomonas reinhardtii,* etc. In a study, *Nanochloropsis oceanica,* the expression of NoDGAT2K cDNA resulted in 34.7-fold increase in EPA proportion (3.12% dry basis) in TAG (Xin et al., 2019). In comparison, the expression of Δ5-desaturase gene (PtD5b) involved in EPA biosynthetic routes contributed to a 58% increase in EPA from a 64% increase in polyunsaturated fatty acid in *Phaeodactylum tricornutum* (Peng et al., 2014). Similarly, the expression of Δ6-desaturase gene in *Phaeodactylum tricornutum* results in EPA content 18.64% higher than wild type (Zhu et al., 2017). However, genetic engineering in microalgae for the production of value-added products such as EPA and DHA is at its primitive stage and warrants more research. Genetic tailoring methods have the benefit of overcoming the inherent limitations associated with metabolite production capacities of microalgae, which will aid in economising the production process. Optimization of metabolite production via genetic engineering is beneficial in microalgae as it takes less time than manipulation of animal cell lines for the same purpose. Other challenges associated with genetic tailoring methods include risks associated with the use of GM microalgae. Prolonged exposure of microalgae with selectable markers such as herbicide and antibiotic-resistant markers to the environment can lead to superbugs. Studies have also shown the toxic effect on animals due to the use of herbicides (Peillex and Pelletier, 2020).

Implementing modern techniques such as riboswitches, coupling nuclear promoters for gene regulation and RNAi technology for post-transcriptional downregulation of specific genes has helped advance genetic engineering (Patel et al., 2018). Genetic engineering can modify microalgae strains and helps to study their biology, which would aid in achieving high-value products such as protein, pigments, EPA, and DHA.

In microalgal chloroplast the transgene can be expressed for over-accumulation of protein with the desired post-translation modifications like disulphide bond formation, avoiding undesired glycosylation (Kwon et al., 2018). Along with, recent advancements in genetic engineering technologies of microalgae such as high throughput next-generation sequencing, genomics, proteomics, transcriptomics, epigenomic, metabolomics and lipidomics putatively help in predicting DNA sequence, gene structure and its expression after successful integration into the host genome (Salama et al., 2019).

The enormous gap between global EPA and DHA production and recommended dietary intake indicates the need for increased and sustainable production of EPA and DHA. This gap can be bridged by methods such as non-genetic and genetic tailoring methods in sustainable bioresources such as microalgae. This review paper comprehensively describes the non-genetic and genetic tailoring methods that can enhance the EPA and DHA yield in microalgae. This review also comprises the recent development in genetic and non-genetic modifications of algae for the enhanced production of EPA and DHA, while most review papers in the past focused on lipid accumulation via non-genetic tailoring. Table 2 compares the distinguished features of present review with previously published reviews (2017-2021) on EPA and DHA production. This review paper culminates the recent developments for the enhanced EPA and DHA production, exploring both methods.

2. Microalgae as a rich source of EPA and DHA

2.1. Limitations of the current source of EPA and DHA

The animal-derived source of omega-3 fatty acid includes fish, seafood, meat, dairy products etc., of which fish is the primary source of eicosapentaenoic acid (EPA) (20:5, n-3), docosahexaenoic acid (DHA) (22:6, n-3). The aquaculture sector has the capability to provide sufficient amounts of cost-effective EPA and DHA to billions of people. However, the supply of fish has been declining due to unsustainable fishing. Moreover, EPA, and DHA derived from fish have the disadvantage of being non-organoleptic and contaminated with lipophilic pollutants organochlorine pesticides (OCs) (up to 168 ng/g lipid weight)), heavy metals (mercury (up to 0.624 mg/kg wet weight) and cadmium (up to 0.918 mg/kg wet weight) (Davodi et al., 2011; Djedjibegovic et al., 2020; Vassilopoulou et al., 2017). The shortcomings associated with the consumption of non-vegetarian sources (such as *Epinephelus* species, 48% EPA and 108% DHA mg/100 g wet wt) of EPA and DHA is that these sources are cooked before consumption, leading to thermal deterioration of EPA and DHA (Mohanty et al., 2016). Thus, there is a need for alternative sources of n-3 long-chain polyunsaturated fatty acid (LC-PUFA). Vegetarian sources of omega-3 fatty acid such as plant seeds (e.g walnut, chia) are rich in alpha-linolenic acid (ALA) (18:3, n-3), which is converted into EPA and DHA, but with low conversion efficiency. High consumption of these sources is therefore required to obtain adequate EPA and DHA from microalgae and GM plants (such as *Camelina sativa,* 20% of EPA and DHA out of total fatty acids (seed)) (Han et al., 2020a). Plants are disadvantageous in requiring arable land and long generation time (Sharma et al., 2020). Unlike plants, microalgae (such as *Rhodomonas baltica,* 6.31% EPA and 3.83 % DHA in total fatty acid (TFA)) do not compete for arable land and have much faster growth rates, and additionally contain high levels of EPA and DHA (Wang et al., 2019). Table 3 shows EPA and DHA production using different microalgal species. Some microalgae species, along with EPA and DHA, contain value-added nutrients, such as carotenoids which greatly reduce the risk of diseases, and phytosterols that reduce serum cholesterol and increase shelf-life (by preventing oxidation of fatty acids) (Mao et al., 2020).

2.1.1. Benefits associated with the consumption of EPA and DHA

The synthesis of EPA has a crucial step of conversion of ALA to EPA and subsequent EPA conversion to DHA. However, the conversion

Table 2
Comparison between the present review paper with previously published reviews (2017–2021) on the production of EPA and DHA from microalgae.

Genetic engineering	Abiotic stress	Adaptive lab evolution	Photosynthetic efficiency	Risk assessment of GM microalgae	Omics resources	Biorefinery	References
✗	✓	✗	✗	✗	✗	✓	(Paliwal et al., 2017)
✓	✓	✗	✗	✗	✓	✓	(Chen et al., 2017)
✗	✓	✓	✗	✗	✓	✗	(Sun et al., 2018b)
✓	✓	✗	✗	✗	✗	✗	(Ramesh Kumar et al., 2019)
✓	✓	✗	✗	✗	✗	✗	(Aratboni et al., 2019)
✓	✓	✗	✗	✓	✗	✗	(Liang et al., 2020)
✗	✓	✗	✗	✗	✗	✓	(Katiyar and Arora, 2020)
✗	✓	✗	✗	✗	✗	✗	(Remize et al., 2021)
✗	✓	✗	✗	✗	✗	✗	(Ramos-Romero et al., 2021)
✓	✓	✓	✓	✓	✗	✓	This review

Table 3
EPA and DHA production using different microalgal species.

Microalgae	EPA (% TFA*)	DHA (% TFA*)	Reference
Isochrysis aff. *galbana* clone T-Iso CCAP83 927/14	_	13.1	(Wang et al., 2019)
Rhodomonas baltica NIVA-5/91	6.31	3.83	(Wang et al., 2019)
Nannochloropsis gaditana	_	37.83	(Mitra et al., 2015)
Nannochloropsis oceanica CCMP1779	5.43	_	(Wang et al., 2019)
Pavlova lutheri	12–13 (in TAG)	5–6 (in TAG)	(Guihéneuf and Stengel, 2017)
Aurantiochytrium sp	_	39–48	(Chang et al., 2013)
Aurantiochytrium sp. T66 (ATCC PRA-276)	_	35.76	(Patel et al., 2019a)
Phaeodactylum tricornutum CCMP 2561	17.7	2.38	(Wang et al., 2019)

(*Total fatty acid (TFA))

efficiency of ALA into EPA is low (Zhang et al., 2018a). Furthermore, omega-6 fatty acid is easily available (unlike omega-3 fatty acid), so it is consumed more often (Chirmade et al., 2016). As such, an imbalance is created in omega-6/omega-3 fatty acid ratios. This noted imbalance leads to inflammatory diseases, as omega-6 fatty acid interacts with inflammation-causing agents (e.g. prostaglandins and leukotrienes). However, omega-3 fatty acid, specifically EPA and DHA, leads to the formation of a lipid-derived compound named resolvin which can mitigate inflammatory response (Balta et al., 2017). DHA helps in producing lipid-derived protectins that have an anti-inflammatory and neuroprotective functions (Bosviel et al., 2017). DHA also decreases the production of reactive oxygen species and inflammatory cytokines (interleukin-1 and tumor necrosis factor) (Saenz de Viteri et al., 2020). The inclusion of EPA and DHA in the membrane of cardiomyocytes alters the lipid microenvironment of the membrane, thus affecting the ion channels, reducing the risk of arrhythmia and improving cardiovascular health. EPA and DHA can directly reduce cardiac fibrosis by inhibiting TGF-b1-induced smad2/3 nuclear translocation, which is involved in cardiac remodelling (Chen et al., 2011). EPA and DHA can also increase nitric oxide production and GMP/PKG pathway activation, suggesting an anti-inflammatory role. DHA is the predominant omega-3 fatty acid in brain tissues. Studies have shown that with increased dietary uptake of DHA, the risk of dementia can be reduced up to 50%, thus improving cognitive health (Cole et al., 2009). EPA and DHA have shown promise in reducing cancer risk, especially when administered concomitantly with chemotherapeutic drugs. One such study has shown the increased chemotherapeutic cytotoxicity with DHA and anthracyclines administration (Colas et al., 2006). Another study on mammary cancer cell lines showed the increased clustering and surface expression of death receptors (CD95) on treating with EPA, DHA, and doxorubicin (Ewaschuk

et al., 2012). The International Maritime Organisation (IMO) has established intake recommendations only for alpha-linolenic acid (ALA) and not for n-3 LC-PUFA; recommended ALA intake is 1.1 g/day for females and 1.6 g/day for males (Wong et al., 2015). However, the American Dietary Guidelines (2015–2020) recommend consumption of 226.80 g per week of a variety of seafood for approximately 250 mg per day of EPA and DHA for the general population and 226.80–340.20 g per week of seafood for pregnant and lactating women (Zhang et al., 2018c). Nevertheless, to restore a balance in omega-6 fatty acid and omega-3 fatty acid, a ratio between 5:1 and 3:1 could be considered as an optimum for humans, thus making it crucial to incorporate omega-3 fatty acid in a diet (Boumil et al., 2017).

2.1.2. Challenges in bridging the gap between demand and supply of EPA and DHA

The estimated global annual gap for EPA and DHA supply is 1.1 million tons, which can be seen as an opportunity to apply innovative methodologies that would help circumvent this wide gap (Schade et al., 2020). The major PUFA producing microalgae such as *Crypthecodinium cohnii*, *Schizochytrium* species, *Ulkenia* species have high DHA content, but EPA content is not comparable. Thus, it is crucial to focus on microalgal strains, producing high levels of both EPA and DHA, to elicit benefits associated with both fatty acids. The accumulations of EPA and DHA in microalgae are highly contingent on abiotic stresses (nitrogen limitation, low temperature, low light, high salinity). Specifically, stresses during the cultivation stage could expedite cellular accumulation of EPA and DHA (Wang et al., 2019). Stress also causes lipid peroxidation, which reduces the quality of PUFA, making it unfit for consumption and unfeasible for the commercial market (Ismail et al., 2016). The crucial issue from the consumers' perspective is the high cost of EPA and DHA in the market, likely due to the expensive production processes. Genetic and non-genetic tailoring of microalgae can increase EPA and DHA yield without compromising biomass growth. These altered strains can be modified to incorporate antioxidant activity to protect PUFA from deterioration due to oxidation. Whereas in the aquaculture industries, the deficiency of EPA and DHA content in fish can be fulfilled by incorporating fishmeal and fish oil, but these sources are limited. However, the incorporation of marine microalgae feed, naturally rich in EPA and DHA, has proven to be a valuable alternative (Betancor et al., 2017).

3. Biotechnological tools for the improvement of EPA, DHA production

3.1. Genetic engineering approach for the enhanced EPA and DHA production

Conventional production of EPA and DHA from microalgae relies on selection of appropriate abiotic conditions. Abiotic conditions trigger

the metabolic pathway for increased EPA/DHA production, but also decrease cell division rates. It has been reported that abiotic stress such as low light intensity (250 μmol m^{-2} s^{-1}) and low temperature (5C) lead to enhancement in EPA (96%), and DHA (77%) production in microalgae, but these conditions prove to be hostile for cell division (Sirisuk et al., 2018). Genetic engineering is an indispensable tool which provides economic benefits associated with enhanced metabolite yield and productivity. These techniques also provide the ease of cultivation associated with the growth under selective conditions and can be extended to provide feasibility of harvesting (Alam et al., 2016). Therefore, the focus needs to be on strain improvement at the genome level. Microalgal strains can be improved at the genomic level via random mutagenesis (by utilising physical, chemical, and insertional mutagens), transgene expression, or genome editing.

3.1.1. Gene editing tools

Genetic engineering involves several steps. For instance, transformation (expression of foreign DNA into the target cell) includes DNA penetration into the cell, integration of foreign DNA into the target cell's genome, then stable and desired expression of the integrated DNA (Park et al., 2019). The successful genetically modified microalgae are further selected based on selection markers (Park et al., 2019). Therefore, genetic engineering sometimes may result in undesired modification. For example, using the CRISPR/Cas9 system in *Nannochloropsis* sp. has shown to result in cell death, but the use of alternatives such as FnCas12a and AsCas12a have prevented cytotoxicity (Naduthodi et al., 2019). However, there are other strategies such as optimization of codon, intron and promoters, and modification of the host genome to increase the compatibility for transgene expression (Douchi et al., 2021). The primary mechanism of genome engineering is using tools to intentionally alter specific genes in the target cell. Gene editing involves the nuclease enzyme, which cuts specific DNA segments, followed by the replacement with desired DNA segments. The desired DNA segment inserted into the host carries selectable markers and promoters. Selectable markers help in distinguishing the transformants from non-transformants, while the promoters help to efficiently express the gene of interest. Selectable markers can be grouped based on the specific function or characteristic conferred by them. The selectable marker may be antibiotic-resistant, herbicide-resistant, metabolic, or photosynthetic selection markers. Genetic tools significantly improve metabolite production, as observed in some microalgae. For example, in a study on *Nannochloropsis gaditana*, the use of RNA-seq analysis helped identify 20 transcription factors that were negative regulators of lipid production. Further, CRISPR/Cas9 was used in a reverse genetic approach to knock out genes, leading to a mutant strain with the capability to accumulate twice the amount of lipid (5.0 g m^{-2} d^{-1}) than the wild strain (Ajjawi et al., 2017). Genetic engineering has also paved a path to obtain markerless mutations in microalgae. For instance, *Aurantiochytrium limacinum* was transformed with a transgene containing enhanced green fluorescent protein gene (*egfp*), antibiotic resistance marker, chloramphenicol resistance gene (*Cmr*) with two *lox*P loci. Further, the *egfp* gene was successfully integrated into the genome of *A. limacinum* without antibiotic resistance marker genes with the application of Cre/*lox*P system (Sun et al., 2015).

The strategy involved in increasing the content of PUFA (EPA and DHA) comprises of overexpression of gene involved in PUFA biosynthesis or shunting of genes involved in diverting the metabolic flux away from PUFA biosynthesis. Studies have been conducted showing the use of TALEN for genome editing targeted towards lipid accumulation in microalgae. A study conducted by Hao et al. showed that the use of TALEN for knocking out Acyl-CoA hydrolysis (ptTES1) gene targeted towards lipid accumulation in microalgae. The use of TALEN in *Phaeodactylum tricornutum* for disruption of the hotdog-fold thioesterase gene involved in acyl-CoA hydrolysis (ptTES1) had been conducted. The disruption of the hotdog-fold thioesterase gene has shown a 1.7-fold increase in TAG. However, the level of EPA decreased in the mutants

(Hao et al., 2018) (Fig. 1). The nuclease, FokI (cleavage domain) fused to TALEN protein (DNA-binding domain), forms a TALEN monomer, and two Fok I domains can cause cleavage only on dimerization (Matsumoto et al., 2020). Two TALEN monomers lead to an effective cleavage. Thus, TALEN can be created easily with better cleavage activity and possesses the advantage of increased specificity associated with the cleavage at a specific spacer length. However, protein engineering for the synthesis of these specific TALEN protein tends to be expensive and time-consuming, along with the requirement of two proteins (two TALEN monomers) for a single target (Sanagala et al., 2017).

CRISPR is composed of recognition sequence called guide RNA, which helps in recognizing the DNA sequence to be cleaved and ribonucleoprotein which facilitates cleavage. CRISPR has been used as a biotechnological tool in microalgae since 2014, but it has been limited due to toxicity associated with its nuclease. Nevertheless, CRISPR is still used and targeted towards homologous DNA repair to conduct site-specific genome editing. CRISPR, as a biotechnological tool, is highly efficient and easy to use. CRISPR has been used to edit the genomes of bacteria, plants, and animals, as well as microalgae (Patel et al., 2019b). Although CRISPR/Cas9 is simpler, more accurate, and more efficient than previous genome editing techniques, it has been difficult to apply this technique in microalgae. As such, a great deal of effort has been devoted to preventing off-target mutation and cytotoxicity associated with Cas9 ribonucleoproteins (RNPs). However, the main problem in CRISPR is expression and delivery of guide RNA into the genome (Shin et al., 2016). The first report on the use of CRISPR in *Chlorella* species for
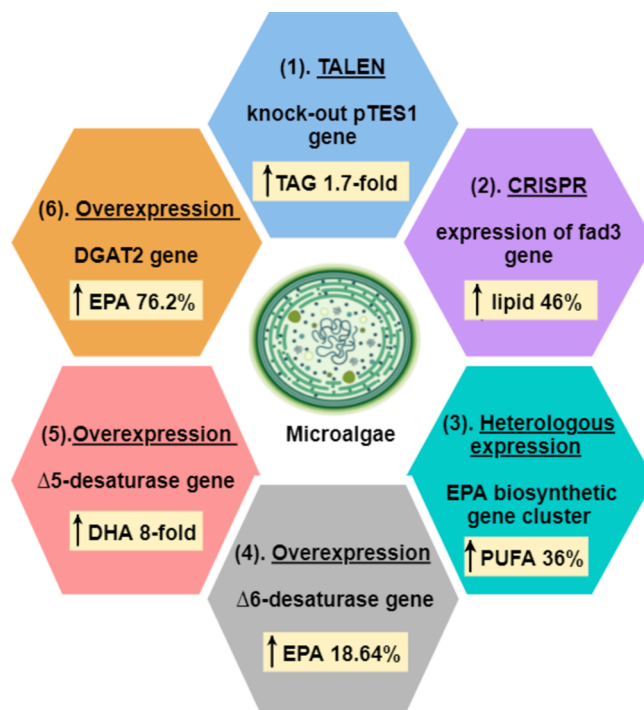

Fig. 1. The application of genetic engineering to enhance the lipid production in microalgae. (1) TALEN has been used in *Phaeodactylum tricornutum* for the knock-out of Acyl-CoA hydrolysis (ptTES1) gene which resulted in 1.7-fold increase in TAG (Hao et al., 2018); (2) CRISPR technology used to target fad3 gene in *Chlorella vulgaris* to improve lipid up to 46% (w/w) (Lin and Ng, 2020); (3) The heterologous expression of EPA biosynthetic gene cluster (from *Shewanella japonica*) into *Aurantiochytrium* species enhances PUFA up to 36% (Wang et al., 2020); (4) The overexpression of Δ6-desaturase gene in *Phaeodactylum* sp. enhances EPA up to 18.64% strain (Zhu et al., 2017); (5) The overexpression of Δ5-desaturase gene in *P. tricornutum* UTEX646 resulted in 8-fold increment in DHA (Hamilton et al., 2014); (6) The overexpression of DGAT2 in resulted in enhanced EPA production up to 76.2% (Li-Beisson et al., 2019).

gene manipulation was reported in 2020. This work used CRISPR/Cas9 system integrated into a plasmid delivered in the host cell via electroporation. The CRISPR/Cas9 system targeted omega-3 fatty acid desaturase (fad3) gene, targeting lipid accumulation up to 46% (w/w) (Lin and Ng, 2020) (Fig. 1). The Cas9 nuclease has proven to be successful in systems such as bacterial and mammalian cell lines, but, in microalgae, it has caused mutations that lead to cytotoxicity. In one study aiming to address this, toxicity was not only reduced by direct delivery of Cas ribonucleoprotein via electroporation, but also by the use of alternative Cas variants. The use of FnCas12a, AsCas12a in *Nannochloropsis oceanica* IMET1 resulted in highly efficient transformants obtained up to 93 % by using FnCas12a (Naduthodi et al., 2019).

Studies on the application of genetic engineering in microalgae triggering the metabolic pathway towards the enhanced production of EPA and DHA have been reported. In a study using microalgae *Phaeodactylum tricornutum*, the pHY18 vector was ligated with gene of interest DGAT2 and then transformed by electroporation protocol (Niu et al., 2012). This enzyme is involved in the last step of the biosynthesis of triacylgylceride. Overexpression of DGAT2 results in increment in neutral lipid content up to 35%. Consequently, this also leads to increment in EPA content in fatty acid up to 76.2%, without compromising the biomass growth in the genetically modified microalgae (Li-Beisson et al., 2019) (Fig. 1). Similarly, in microalgae *Nannochloropsis oceanica*, DGAT2 gene was cloned using the vector pNa03 and the DGAT2 gene was expressed under the Hsp20 promoter. The diacylglycerol acyltransferase (DGAT2), which is a rate limiting enzyme, has also shown its role in increasing the neutral lipid content by 69% in the genetically modified one (in reference to the wild type). However, the overexpression of DGAT2 decreased PUFA content by 74.6% without hampering biomass accumulation (Li et al., 2016). In another study, *Nannochloropsis oceanica* showed that the role of DGAT2s in exhibiting substrate preference to incorporate a particular PUFA CoA in triacylglycerols (TAGs). Therefore, NoDGAT2J showed its preference for incorporation of linoleic acid and NoDGAT2K for EPA. In *N. oceanica* IMET1, the NoDGAT2J and 2 K cDNA were contained in pXJ427 and pXJ428 plasmid DNA. Expression of NoDGAT2J and NoDGAT2K in *N. oceanica* resulted in proportion of linolenic acid (18.7-fold increase) and EPA (34.7-fold increase) in TAG up to 3.92 and 3.12% (dry basis), respectively (Xin et al., 2019). Increase in EPA can also be achieved by altering enzymes involved in the EPA biosynthetic routes such as Δ5-desaturase, Δ6-desaturase. In a study conducted with *Phaeodactylum tricornutum*, the cDNA of Δ5-desaturase gene (PtD5b) was cloned into

pHY18 plasmid and transformed by electroporation. Overexpression of Δ5-desaturase enzyme in the engineered microalgae lead to 64% increase in polyunsaturated fatty acid, out of which 58% increase was observed in EPA (Peng et al., 2014). In another study, overexpression of Δ6-desaturase gene in *Phaeodactylum tricornutum* lead to increased EPA (38.101 mg/g dry cell weight) content up to 18.64% higher than the wild strain (Zhu et al., 2017) (Fig. 1). Similarly, the enhancement in DHA production in microalgae is associated with Δ6-desaturase, Δ5-elongase enzymes. Acyl-CoA-dependent Δ6-desaturase is rate limiting for the PUFA biosynthetic pathway. Δ5-elongase catalyses the conversion of EPA into docosapentaenoic acid which further lead to DHA biosynthesis via Δ4-desaturase. In *P. tricornutum* UTEX646, the expression of Δ5-desaturase resulted in the increment in DHA production up to 8-fold in reference to the native strain. Expression of Δ6-desaturase in the same strain also resulted in increased DHA production, although this increase was not significant (Hamilton et al., 2014) (Fig. 1). The *Aurantiochytrium* species can produce high DHA levels (more than 25% of dry cell weight), but substantially lower levels of EPA. As such, heterologous expression of EPA biosynthetic gene cluster from bacterium, *Shewanella japonica* into *Aurantiochytrium* species lead to the accumulation of lipid and PUFA up to 26.9 and 36.0%, respectively (Wang et al., 2020) (Fig. 1). Table 4 represents genetically modified microalgae with their respective EPA and DHA yield under different experimental conditions.

3.2. Mutagenesis

Mutagenesis caused by mutagens (physical, chemical, or insertional) leads to mutations in the host genome when the host cell is exposed. Mutagens can include ultraviolet radiation, gamma rays, intercalating agents (e.g. acridine orange, ethidium bromide), and alkylating agents (e.g. ethyl methane sulphonate (EMS) and N-methyl-N-nitro-N-nitrosoguanidine (NTG)). Physical mutagens exert their mutagenic effect directly or by creating free radicals (Sobieh et al., 2018). These radicals can lead to chemical bond formation between nitrogenous bases of DNA, such as the exposure of UV radiation results in the construction of thymine dimer (covalent bond) in DNA strands (Antusch et al., 2017). Chemical mutagens are chemicals that cause mutation in the target cell via abnormal base pairing or structural changes (Ackerman and Horton, 2018). Insertional mutagen causes insertional mutagenesis in which a foreign DNA sequence present in a plasmid with a selectable marker is inserted into the host cell and integrated into the microalgal genome.

Table 4
Summary of genetically modified microalgae used for the enhanced production of EPA and DHA under different experimental conditions.

Microalgae	Experimental conditions	Genetic modification executed	EPA yield	DHA yield	Reference
Phaeodactylum tricornutum	f/2 medium, 21 ± 1 °C, light intensity 200 μmol photons $m^{-2}s^{-1}$, 15 h light and 9 h dark period	Overexpression of DGAT2	7.42% of dry cell weight	–	(Niu et al., 2013)
N. oceanica IMET1	Modified f/2 medium, 22 °C, light intensity 55 μmol photons $m^{-2}s^{-1}$, 24 h light period	Expression of NoDGAT2K	3.12% of dry cell weight	–	(Xin et al., 2019)
Phaeodactylum tricornutum	f/2 medium by omitting Si, 21 ± 0.5 °C, 15 h light and 9 h dark period	Overexpression of Δ5-desaturase enzyme	38.9 mg/g of dry cell weight	0.44 mg/g dry cell weight	(Peng et al., 2014)
Phaeodactylum tricornutum	f/2 medium, 22 ± 1 °C, 100 μmol photons $m^{-2}s^{-1}$, 12 h light and 12 h dark period	Overexpression of Δ6-desaturase gene	38.101 mg/g dry cell weight	–	(Zhu et al., 2017)
P. tricornutum UTEX646	Enriched Seawater, Artificial Water (ESAW) medium, 20 °C, light intensity 60 μmol photons $m^{-2}s^{-1}$, 24 h light period	Expression of Δ5-desaturase	–	10.4% in total fatty acid	(Hamilton et al., 2014)
Aurantiochytrium species	PYG medium (60 g/L glucose, 10 g/L yeast extract, and 5 g/L artificial seawater, 25 °C	Expression of EPA biosynthetic gene cluster	2.7 g/L of dry cell weight	–	(Wang et al., 2020)
Pavlova lutheri	Artificial sea water medium, 22 °C, 8.6 W m^{-2}, 24 light period	UV irradiation (254 nm wavelength)	23.1 mg/g dry cell weight	10.6 mg/g dry cell weight	(Meireles et al., 2003)
Aurantiochytrium species	M4 liquid medium, 23 °C	UV irradiation (50 W, 30 s)	–	624.93 mg/L in dry cell weight	(Liu et al., 2020)
Nannochloropsis oculata	Natural sea water with Guillard's f/2 enrichment solution, 25 °C, light intensity of 90 μmol photon $m^{-2}s^{-1}$, 12 h light period	Ethyl methane sulphonate (EMS)	30.8 mg/g dry cell weight	–	(Chaturvedi and Fujita, 2006)
Schizochytrium species	40 g/L glucose and 0.4 g/L yeast extract in artificial sea water, 25 °C	N-methyl-N-nitro-N-nitrosoguanidine along with UV irradiation	–	56.22% of total fatty acid	(Xie and Wang, 2015)

The foreign gene can result in gene deletion resulting in a metabolic shift towards lipid synthesis pathways (Ryu et al., 2020). Mutagenesis is followed by utilising high throughput screening methods such as fluorescence-activated cell sorting (FACS), which helps identify the mutated strain by staining with lipophilic fluorescent dyes. The advancement in sequencing techniques for mutated genes has also led to identifying critical genes involved in metabolite production, which can be heterologously expressed to obtain enhanced lipid production. A study on *Scenedesmus* sp. led to identifying critical genes involved in lipid production in the mutant strain (Ma et al., 2014). In another study, two key genes (acetyl-CoA carboxylase (ACCase) and phosphoenolpyruvate carboxylase (PEPC) genes) were expressed in the mutant strain, leading to increment in lipid synthesis (Ma et al., 2019). The mutations in the host genome can have beneficial, deleterious, or no effect on the host. The application of physical/chemical mutations is more prevalent than the use of insertional mutagens due to the ease of use and the broad extent of current knowledge. Mutagenesis in particular has been utilised to generate microalgal strains which can produce high lipid content without compromising the biomass growth which otherwise leads to uneconomical downstream processing (Li et al., 2017a).

3.2.1. Physical mutagens

Physical mutagens include radiation sources such as ion beams, ultraviolet, X-rays, gamma radiation, or atmospheric and room temperature plasma (ARTP). Insertional mutagenesis requires prior knowledge of molecular biological techniques such as identification, construction, and insertion of a specific plasmid in the host cell. Chemical mutagens are prepared to a specific concentration, and after the exposure, the cells need to be washed with a chemical agent (such as sodium thiosulphate) for some time to stop the effect (Dinesh Kumar et al., 2018). In contrast, physical mutagens such as UV irradiation can be exposed to the sample by placing it under a UV lamp. The UV irradiation intensity can be maintained by adjusting the distance between the lamp and the sample (closer to the lamp, the higher the intensity) (Meireles et al., 2003). Therefore, the ease of use associated with UV has made it the method of choice compared to other methods. In a study using microalgae *Pavlova lutheri*, the use of UV irradiation of 254 nm resulted an increase in 32.9% DHA and 32.8% EPA in dry biomass as compared to the wild strain (Meireles et al., 2003). Studies performed on some microalgae with UV irradiation resulted in enhanced lipid and DHA accumulation with absence of EPA accumulation. This has been observed in *Aurantiochytrium* sp., treated with UV irradiation (50 W, 30 s), which resulted in an increased accumulation of lipid (1417.37 mg/L) and DHA (624.93 mg/L), up to 1.79 and 1.9 times higher than the native strain, respectively (Liu et al., 2020). Similarly, *Thraustochytriidae* sp. has been reported to increase the lipid and DHA production up to 78.88 and 23.77%, respectively (Hu et al., 2020). In both the studies, EPA was absent and instead, docosapentaenoic acid (DPA) was observed because of the up regulation of m-RNA levels of CoA-transferase (CoAT), acyltransferase (AT), enoyl reductase (ER), dehydratase (DH), and methyltransferase (MT). ARTP has been shown to produce a mutant microalgal strain with enhanced metabolite production ability. ARTP in *Crypthecodinium cohnii* has been shown to produce an exopolysaccharides (EPS) volumetric yield of 1.02 g/L that was 33.85% higher than the wild type strain (Liu et al., 2015a). ARTP increased the lipid content up to 61% in a mutant strain of *Desmodesmus species* (Sun et al., 2020). The application of ARTP in *Chlorella pyrenoidosa* resulted in a mutant with enhanced dry weight and lipid, up to 22.07% and 16.85%, respectively (Cao et al., 2017). However, the use of ARTP for a mutant microalgal strain with enhancement EPA and DHA has not been observed. The treatment with gamma-ray on *Scenedesmus* sp. led to increased lipid content up to 71.3% compared to the wild strain (Liu et al., 2015b). Gamma ray treatment on *Nannochloropsis* sp., however, showed the loss of EPA and 4-fold decrease in phosphatidylglycerol levels in the mutant strain (Al-Hoqani et al., 2017), indicating the need for more targeted research.

3.2.2. Chemical mutagens

Chemical mutagens interact with DNA leading to abnormal base paring and structural changes which alter replication and transcription of DNA. Chemical mutagens cause mutation by agents causing base transition (such as 5-bromodeoxyuridine, and 2-aminopurine), non-alkylating agents (nitrous acid, formaldehyde, hydrazine), intercalating agent (acriflavine, acridine orange, ethidium bromide, and proflavine) and alkylating agents (diethyl sulphate (DES), ethyl methane sulphonate (EMS), methyl methane sulfonate (MMS), and N-methyl-N-nitro-N-nitrosoguanidine (NTG).

Earlier studies utilizing chemical mutagens such as acriflavine, 2-aminopurine in *Plectonema boryanum* have shown to result in phage-resistant mutation. However, their effectiveness was less than that of alkylating agent (Singh and Kashyap, 1977). The initial studies utilized different chemical mutagens for the induction of mutation in microalgae. However, recent research has focused on using alkylating agents, as they appear to be the most promising chemical mutagen. Alkylating agents such as MMS, EMS and NTG have also been widely used as chemical mutagen triggering lipid accumulation in oleaginous microalgae (Ravindran et al., 2017). MMS cause SOS-dependent mutation and can induce base substitution of all classes, mainly GC-to-AT transversions. In comparison, EMS can cause mutation in the absence of SOS activity with predominant GC-to-AT transitions (Du et al., 2017). However, NTG can induce a wide range of mutations by alkylating purines and pyrimidines. Studies have shown that chemical mutation is preferred over physical mutation because of the mechanistic differences. The use of the chemical mutagen ethyl methane sulphonate (EMS) in *Nannochloropsis oculata* has shown to increase the production of EPA in the mutant strain up to 29% as compared to the wild-type strain. The accumulation of EPA up to 30.8 mg/g in *Nannochloropsis oculata* strain is postulated to be the result of the extrachloroplastic lipid desaturase (Chaturvedi and Fujita, 2006). In *Schizochytrium* sp., the use of NTG along with UV radiation resulted in 34.84% increment in lipid and 56.22% in DHA accumulation than the native strain. This could be plausible due to the increase in expression of malic enzyme, ATP–citrate lyase, and glucose-6-phosphate dehydrogenase enzymes, responsible for the accumulation of DHA (Xie and Wang, 2015).

3.2.3. Insertional fragments

Random mutagenesis caused by physical/chemical mutagens can result in mutant microalgae with enhanced lipid production, but it remains difficult to identify which genes underly target phenotypes. Therefore, the utilisation of insertional mutagenesis has benefited from being high-throughput and allowing the identification of perturbed genes through molecular techniques (Shin et al., 2017). Insertional mutagenesis has been used in *Chlamydomonas* sp. to identify a set of conserved genes named GreenCut2 that can be directly involved in photosynthesis and provide a collection of phenotyped insertion mutants of algae (Dent et al., 2015). In *Nannochloropsis salina*, the application of insertional mutagenesis by inserting pNs*Sh*ble plasmid resulted in a mutation at trehalose-6-phosphate synthase (TPS) domain which leads to reduced trehalose synthesis. This mutant strain exhibited enhanced growth and lipid accumulation up to 53 and 34%, respectively, than the native strain (Ryu et al., 2020). Similarly, in *Nannochloropsis oceanica,* an insertional mutagenesis library was developed with the promising mutant (HLM23) strain accumulation up to 40% of neutral lipid (Südfeld et al., 2021).

3.3. Non-genetic tailoring

3.3.1. Epigenetic changes associated with abiotic culturing conditions

An epigenetic change refers to the reversible or heritable changes in gene expression pertaining to mi-RNA expression, DNA methylation, histone modification etc. The changes in abiotic conditions lead to epigenetic changes which do not alter the DNA sequences, but rather the pattern of chromatin binding to DNA which alters gene expression.

Microalgae with sufficient nutrient supply will thrive and accumulate biomass, but not store compounds such as starch or lipids. Therefore, the lipid production process can be economically unfeasible due to the trade-off between lipid and biomass accumulation. The mechanism by which nutrient starvation induces lipid accumulation is not entirely understood. Although, in the case of nitrogen starvation, studies have shown that lipid induction occurs due to the downregulation in mitochondrial gene expression. Moreover, research suggests that acetyl-CoA and NAD(P)H, which is completely utilised in mitochondrial respiration, would be partially available for fatty acid synthesis, thus leading to enhanced lipid production (Carpinelli et al., 2014). A study on phosphate limitation, however, showed an increase in total lipid production mainly due to the increase in TAG in *Monodus subterraneus*. Whereas in digalactosyldiacylglycerol (DGDG), the proportion of EPA increased up to 21.5%. However due to phosphate starvation that leads to lower biomass yield, the total EPA content in total fatty acid decreased up to 15.5% (Khozin-Goldberg and Cohen, 2006). Epigenetic modifications can cause adaptive changes in the phenotype of microalgae and enhance their stress tolerance (Kronholm et al., 2017). The identification of different regulatory mechanisms can be analysed via transcriptome and genome sequencing analysis, which will help to elucidate the relationship between specific abiotic stresses and epigenetic changes in microalgae. However, there is very limited knowledge regarding epigenetic memory in microalgae, despite extensive work on model plants such as *Arabidopsis thaliana* (Thiebaut et al., 2019).

A recent study exposed *Chlamydomonas reinhardtii* to salt stress and revealed that the regulation of stress responsive genes were downregulated by prolonged exposure to high salinity. This downregulation was attributed to epigenetic changes resulting in strain adaptability to high salinity (Perrineau et al., 2014). Some studies have shown the importance of proteins involved in epigenetic modifications, which can result in gene silencing. These proteins include KMT1, which causes methylation and leads to epigenetic changes, in turn contributing to heterochromatin development and gene silencing (Blanc et al., 2012). In a study on *Chlorella* sp., the epigenetic modifications were postulated to be responsible for the increment in TAG. Although, in this study, these changes were not sustained (Rumin et al., 2015). Additionally, a relationship between TAG accumulation and culture stage has also been established based on epigenetic changes in some microalgae (*Nannochloropsis* sp., *Chlorella* sp.) (Schüler et al., 2017). A study on *Picochlorum soloecismus* demonstrated that a 66% increase in lipids corresponded to nitrogen starvation. This stress condition caused the hypomethylation of CpG related to the epigenetic modification, thus providing an insight in the utilisation of the epigenetic modification targeting towards desirable metabolite production (Steadman et al., 2020). Another study in *Chlamydomonas reinhardtii* based on whole-genome sequencing has shown that effects due to transgenerational epigenetics resulted in adaptive evolution (Kronholm et al., 2017). However, the changes in evolutionary outcome due to differences in methylation pattern are complex for quantitative traits such as cell division (Kronholm et al., 2017).

3.3.2. Adaptive lab evolution

Adaptive lab evolution (ALE) utilises the capability of microorganism to thrive in diverse environmental conditions. ALE involves the prolonged growth of microorganisms in a stressful environment by varying selective pressures. Selective pressures range from abiotic conditions such as thermal, nutrient, and light stress to stress induced by chemical agents such as antibiotics, sesamol, sethoxydim, butanol etc. ALE causes insertions, deletions, and single nucleotide polymorphisms in microorganisms. ALE enhances growth rates and survivability. It can also trigger the metabolic pathway towards metabolites of interest, such as lipids and carotenoids. (Reyes et al., 2014). The general method of ALE involves batch cultivation of microalgae, e.g. in Erlenmeyer flasks. The methodology consists of transferring an aliquot of culture into a new flask with fresh medium at regular intervals, cultivated in the same controlled culture conditi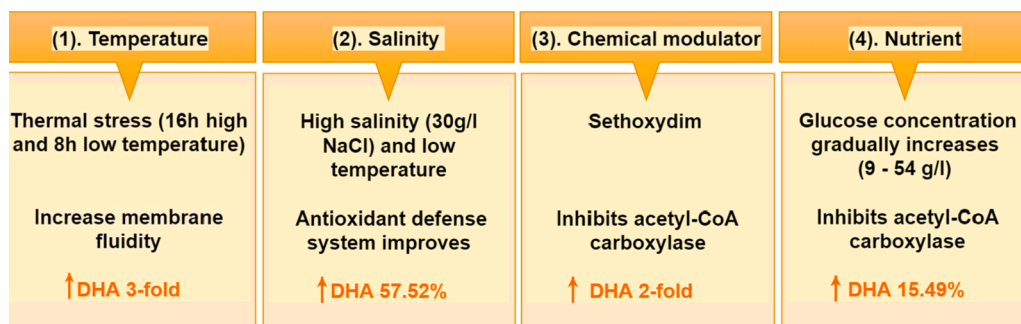ons for an additional round of growth. The repetitive culture cycle, ranging from weeks to years, results in strains with improved phenotypes associated with the growth environment (Stella et al., 2019). However, batch cultivation for ALE can have disadvantages such as fluctuating nutrient supply, growth rate, pH, CO_2. This shortcoming can prevent the usage of complex culturing conditions for microalgal selection. Therefore, bioreactors with continuous culture (chemostat) can provide controlled conditions and alternative to batch culture (Stella et al., 2019).

The utilisation of ALE is widely applied in microalgae such as *Crypthecodinium cohnii*, *Schizochytrium* sp., and *Tisochrysis lutea* targeted towards enhanced lipid and PUFA production. The application of ALE in *Crypthecodinium cohnii* helped in obtaining evolved strain showing glucose tolerance by culturing with a gradual increase (9 to 54 g/L) in glucose concentration via 260 cycles (650 d). This evolved strain resulted in an increased DHA content in total fatty acid up to 15.49% when exposed to 45 g/L glucose concentrations (Li et al., 2017b) (Fig. 2). The enhanced accumulation of lipid and PUFA were obtained in *Crypthecodinium cohnii*, with the combined use of ALE with chemical modulators. The application of chemical modulator such as sethoxydim in *Crypthecodinium cohnii*, which inhibits acetyl-CoA carboxylase (ACCase), resulted in an increase in lipid and DHA production followed by the use of sesamol, which increased biomass productivity (up to 30%). This approach resulted in doubled productivity of lipid (0.046 g/L/h) and DHA (0.025 g/L/h) (Diao et al., 2019) (Fig. 2). In *Tisochrysis lutea* microalgae, ALE was applied by using thermal stress as a selection pressure up to six months. This evolved strain (without affecting the growth rate) resulted in three-fold increment in DHA accumulation in polar lipid associated to the change in membrane fluidity in response to temperature change (Gachelin et al., 2021) (Fig. 2). Previous studies have shown that providing low temperature (10 to 25 ℃), PUFA content increases (120%) with simultaneous decrease (30% in palmitic acid and 20% in palmitoleic acid) in lipid, whereas under high salinity (0.4 to 4 M NaCl), the lipid content increases (total saturated fatty acids: 20.4% and total monounsaturated fatty acids: 36.3%) with a concomitant decrease (12.7%) in PUFA production (Jiang and Gao, 2004; Lari et al., 2016). Therefore, to obtain enhanced total lipid and PUFA content in *Schizochytrium* species, both low temperature (4 °C) and high salinity (30 g/L NaCl) in ALE with 30 adaptation cycles was applied. This resulted in DHA and lipid yield of 38.12 and 52.11 g/L, respectively, with increases in DHA and lipid up to 57.52 and 37.32%, respectively (Sun et al., 2018a) (Fig. 2). ALE is time consuming, but it has the advantage of generating multiple mutations simultaneously which provide better adaptability to the microorganisms. The variables associated with ALE are passage size, rate, and frequency of mutation. However, passage size is the only controllable parameter.

4. Improving photosynthetic efficiency for enhanced EPA, DHA yield

4.1. Genetic tailoring for improving photosynthetic efficiency

The improvement in photosynthetic efficiency with increment in EPA and DHA yield can reduce the cost up to 11.9 USD per kg of total EPA and DHA equivalents (Chauton et al., 2015). Therefore, it becomes crucial to focus on improving photosynthetic efficiency along with EPA and DHA production to make the process economical. The EPA and DHA yield depends on biomass density. Thus, the exploitation of genetic engineering techniques to improve total biomass can assist in the commercial production of omega-3 fatty acids from microalgae. The shortcoming of large scale microalgae cultivation is the culture present at surface with the most proximity to light possesses the maximum photosynthetic efficiency, compounded by photo-inhibition if light exposure is prolonged. However, the cells which are shaded by the surface culture are light-limited. Studies have also shown that the reactive oxygen species formed by high light intensity causes lipid peroxidation and accumulation of unstable PUFA with reduced quality

Fig. 2. The application of non-genetic tailoring methods to enhance the lipid production in microalgae (1). The application of ALE using thermal stress in *Tisochrysis lutea* resulted in change in membrane fluidity associated 3-fold DHA enhancement (Gachelin et al., 2021); (2) The application of high salinity (30 g/L NaCl) and low temperature 4 °C) in *Schizochytrium* sp. resulted in 57.52% DHA increment (Sun et al., 2018a); (3) The application of chemical modulator such as sethoxydim in *Crypthecodinium cohnii* inhibited the acetyl-CoA carboxylase (ACCase) and enhanced biomass productivity, respectively (Diao et al., 2019); (4) In *Crypthecodinium cohnii* the application of ALE by providing gradual increase in glucose resulted in 15.49% increment in DHA content at 45 g/l glucose concentration (Li et al., 2017b).

(Sun et al., 2018c). Therefore, strategies should be devised which would protect the microalgae from its adverse effects. One way to achieve this is by modifying light-harvesting antenna. In *Chlamydomonas reinhardtii*, the photosynthetic productivity was increased by targeting the light-harvesting antenna 4 (tla4) which was truncated by insertional mutation. The truncation was caused by disruption of chloroplast signal recognition particle 54 (CpSRP54) gene. In this study, with 65% smaller size of chlorophyll (Chl) antenna than wild type, the Chl per cell was decreased (Jeong et al., 2017). In another study on *Chlamydomonas reinhardtii*, insertional mutagenesis caused truncation of light-harvesting antenna 1 (tla1) which resulted in decrease in antenna size in Photosystem I (50%) and photosystem II (65%) as compared to the wild type of strain (Shin et al., 2018). Similarly in oleaginous microalgae, *Phaeodactylum tricornutum* the light-harvesting antenna was truncated by the CRISPR/Cas9-mediated knockout of ALB3b gene. Thus, the fucoxanthin-chlorophyll *a*/c-binding proteins were decreased which required high light intensity for photosynthetic saturation. However, lipid peroxidation in the mutant strain was not increased by exposure to high light intensity because of the presence of xanthophyll pigments which protect lipids present in thylakoid membrane from peroxidation (Nymark et al., 2019). Therefore, pigments (carotene, astaxanthin, lutein, fucoxanthin, zeaxanthin etc.) quench free radicals and act as antioxidants because of isoprene units, thus protecting lipids from oxidative damage, which would otherwise degrade the quality of EPA and DHA (Ismail et al., 2016).

Photo-inhibition is the phenomenon which occurs due to excessive irradiance and results in excessive photons which are not utilised by carbon fixation or fluorescence or non-photochemical quenching (NPQ) process. Photo-inhibition causes carbon loss, which hampers productivity. Mutation by the use of chemicals such as methylviologen, rose bengal, and hydrogen peroxide in *Chlamydomonas reinhardtii* do not affect productivity, but may lead to resistance to high light and oxidative stress. These evolved strains with very high light resistance (VHLR) are able to produce high zeaxanthin and maintain D1 protein along with PSII repair and detoxification of reactive oxygen species (Vecchi et al., 2020). *Chlorella vulgaris* exposed to high light intensity leads to photo-oxidative damage and photo-inhibition. Therefore, the implementation of phenotypic selection of strain possessing 50% reduction in chlorophyll content per cell and LHCII complement with respect to PSII. Followed by mutant selection, exposure to rose bengal resulted in pale-green strain exhibiting resistance to singlet oxygen. The mutant strain produced 68% increase in biomass compared to wild type strain and enhanced fatty acid content in dry biomass, especially oleic acid (C18:1) (Dall'Osto et al., 2019). Photosynthetic efficiency can be improved by the incorporation of efficient carbon fixation. Therefore, it is crucial to improve the efficiency of the rate limiting enzyme of the Calvin-Benson cycle, RuBisCO. In *Synechococcus elongates*, the overexpression of rbcLS gene (RuBisCO subunits) resulted in improved biomass accumulation along with increased fatty acid production, up to 3 times greater than

the native strain (Ruffing, 2014). The overexpression of RuBisCO activase in *Nannochloropsis oceanica* enhances the photosynthesis up to 28% and biomass accumulation by 46%. The majority of fatty acids included EPA (C20:5), palmitoleic acid (C16:1), palmitic acid (C16:0) and oleic acid (C18:1), but lipid productivity of mutant strain was similar to the native strain and the 40% enhanced lipid yield was due to the increased biomass yield (Wei et al., 2017).

4.2. Non-genetic tailoring for improving photosynthetic efficiency

The economic feasibility in line with the EPA and DHA production process could be realized by improving the photosynthetic efficiency via non-genetic tailoring. Improving photosynthetic efficiency enhances the biomass, leading to increased EPA and DHA yield. Nutrients play a vital role in a proper functioning of photosynthetic and biosynthetic pathways in microalgae. Nutrients such as phosphate are involved in synthesising phospholipids, proteins, and ATP. Nutrients are essential for proper functioning of the photosynthetic apparatus (Saxena et al., 2020). Nitrogen limitation results in lower levels of RuBisCO, which leads to decreased Chl a levels and accumulation of reactive oxygen species. As such, nutrient limitation results in reduced photosynthetic efficiency. Although nitrogen limitation triggers EPA and DHA accumulation, net lipid yield is reduced due to decreased biomass production. A study on three marine diatoms (*Entomoneis paludosa* NCC18.2, *Nitzschia alexandrina* NCC33, and *Staurosira* sp NCC182) has shown that high irradiance along with nitrogen limitation negatively impacts photosynthetic efficiency. To obtain both high PUFA content and biomass, the implementation of low light with no nitrogen limitation would be a profitable approach (Cointet et al., 2019).

Some unconventional methods of non-genetic tailoring of photosynthetic efficiency include the bio-mineralisation of nanoparticles. The improvement in photosynthetic rate by the use of $CaCO_3$ nanoparticles (CN) has been observed in *Neochloris oleoabundans*. The CN-coated cells showed enhanced lipid and biomass accumulation up to 18.4 and 31.5%, respectively than the CN-uncoated cells. The higher biomass and lipid productivity along with decreased photo-inhibition was because of the splitting of high light intensity into moderate light intensity by undergoing refraction by $CaCO_3$ crystal (Hong et al., 2019). The unutilised solar radiation can be better utilised, which otherwise leads to lower biomass productivity. The implementation of organic dyes has shown to improve light utilisation efficiency in the microalga, *Chlorella vulgaris*. The red dye rhodamine101 resulted in cell growth (1.5 g/L) which was the result of red-photons being directly utilised for photosynthesis, as they attained near-optimal energy levels. Blue organic dye, 9,10-diphenylanthracene, resulted in lipid accumulation up to 30%, raising the possibility that colour affects lipid accumulation (Seo et al., 2015).

5. Ethical challenges for the usage of genetically modified microalgae

5.1. Legislation on the genetically modified organism

According to the rules on living modified organisms (LMO), stated by the United Nations (UN) Cartagena Protocol on Biosafety, a genetically modified organism (GMO) contains a combination of novel genetic material through modern biology. The Cartagena Protocol defines modern biology as applying *in-vitro* nucleic acid technique and fusion of cells that do not belong to the same taxonomic family. Nonetheless, the use of mutagenesis on the organism as GMO has not been treated in a fully transparent manner. As such, microalgae that have been modified by the use of mutagens to enhance the production of EPA and DHA cannot be commercialized without going through the legislative guidelines formulated for GMO (Dederer, 2019). The strict legislative guidelines surrounding the cultivation of GMO are a reason behind no visible conversion of such projects from the research laboratory to commercial scale. However, a few studies are progressing towards the commercial cultivation of genetically modified organisms. An outdoor trial was sanctioned by the U.S. Environmental Protection Agency for the growth of genetically engineered *Acutodesmus dimorphus* for fatty acid synthesis possessing green fluorescent gene as a selectable marker. This 50-day experiment showed that genetically engineered microalgae did not affect native algae strain in outdoor cultivation. Another study used recombinant DNA technology at a large scale (closed cultivation up to 550 L photobioreactor and open cultivation up to 1250 L raceway pond) for enhanced EPA and DHA production has been executed by heterologous expression of Δ5-elongase in *P. tricornutum* (Hamilton et al., 2015).

5.2. Public perception of genetically modified organisms

Globally the perception of people towards GMOs is clouded by misconception, limited understanding, and unfamiliarity. The unfamiliarity can be eliminated by labelling food containing GM ingredients. Thus, recently (from 2020), the United States Department of Agriculture (USDA) has imposed the requirement of labels for indicating GM ingredients (Bovay and Alston, 2018). Misconceptions about GMOs are based on mass media reports, the internet, and protests against GMOs performed by NGOs. However, people with scientific knowledge tend to have a more positive perspective towards GMOs (Wunderlich and Gatto, 2015). GM microalgae with enhanced EPA and DHA production can be presumed to be more favourable for people interested in healthy eating with a positive perception towards GM food. The commercialization of GM microalgae producing value-added products such as EPA, DHA, and carotenoids depends on consumer's positive outlook for their overall acceptance. The commercial production of EPA and DHA from microalgae is executed by heterotrophic microalgae growth to avoid the limitation exposed by photoinhibition and photo limitation. Although such cultivation leads to the high cost of the EPA and DHA, genetic engineering intervention can reduce this cost by introducing high product yield with reduced harvesting cost.

5.3. Risk associated with genetically modified organisms

The risk associated with GM microalgae includes adverse effects on the health of human beings, animals, and the environment. Human beings and animal health can be affected by an allergic reaction in response to a known allergen which can be detected by an IgE binding assay or testing against unknown allergens (Ansotegui et al., 2020). A study has shown that genetically modified soybean in humans causes an allergic response which is absent in response to naturally present soybean (Lee et al., 2017). Genetic modification in microalgae requires selectable markers, which provide it with a selective advantage over non-transformants. However, selectable markers such as antibiotic and herbicide-resistant markers can damage health as their release in the environment can produce resistance in native strain and further result in superbugs. In genetically modified microalgae utilised for EPA and DHA production, such as *Phaeodactylum* sp. and *Nanochloropsis* sp., the widely used zeocin antibiotic is not included in European Food Safety Authority's (EFSA's) antibiotic resistance gene assessment. Nevertheless, zeocin needs to be avoided before commercial production of value-added products, as it is recognized in the list of essential medicines by the World Health Organization (Tagliaferri et al., 2019). The use of herbicide as a selective agent against resistant markers has shown toxicity in animals (Peillex and Pelletier, 2020). The risk associated with the exposure of the GM microalgae to the environment can be due to horizontal gene transfer during intentional release, which is possible on commercial cultivation for a value-added product or unintentional release due to spillage. The commercial cultivation of genetically modified microalgae for value-added products such as omega-3 can be spilt during the dewatering stage of harvesting, but GM *Acutodesmus dimorphus* for fatty acid synthesis showed no significant impact on the native strains (Hamilton et al., 2015).

6. Future perspectives

Currently, most of the commercial production of omega-3 fatty acids utilise the heterotrophic mode of nutrition of microalgae (Fermentalg, XiaoZao Tech), but it is prone to contamination (Oliver et al., 2020). However, non-genetic tailoring via abiotic stress can enhance EPA and DHA production in photosynthetic microalgae without the high risk of contamination, but it is associated with reduced biomass. Thus, future research needs to focus on advancing techniques to obtain enhanced EPA and DHA production without compromising the biomass accumulation, leading to reduced total yield. However, microalgae have inherent limitations that can be overcome by using genetic engineering for metabolite production. The feasibility for large scale production of EPA and DHA from genetically modified microalgae is limited by public perception and strict legislative guidelines on GMOs. Indeed, there is a requirement of scientific intervention to provide information regarding the advantages associated with GM microalgae. Although there have been advances in microalgal fatty acid production, the gap has not been minimized between the theoretical and current maximum lipid yield (Remmers et al., 2018). This indicates the requirement of strategies to achieve a powerful method and efficient strain to attain a surmountable maximum yield. In an effort to obtain efficient strains, genetic modifications need to be conducted. The gene sequencing of only model microalgae was focused initially, which expanded to more than 60 species and counting (Remmers et al., 2018). Therefore, the narrow range of microalgae explored for omega-3 fatty acid production should be expanded to subsume the untapped potential of microalgae for EPA and DHA production. However, only a few successful results have been reported for EPA and DHA production from microalgae via genetic tailoring methods. Therefore, intense research is required to develop genetic engineering tools in microalgae research as it has previously proven to be a promising strategy.

7. Conclusions

The commercial production of omega-3 fatty acids for applications such as infant formula is the most significant sector consuming DHA, but this cannot wholly depend on unsustainable sources (fish, seafood). Therefore, microalgae prove to be a sustainable alternative. However, the current rate of production of EPA and DHA from microalgae is low. The intervention of non-genetic and genetic tailoring methods can enhance EPA and DHA production (Fig. 3), directing the metabolic flux towards EPA and DHA production, thus promises to be a remarkable strategy for renewable, sustainable EPA and DHA generation.
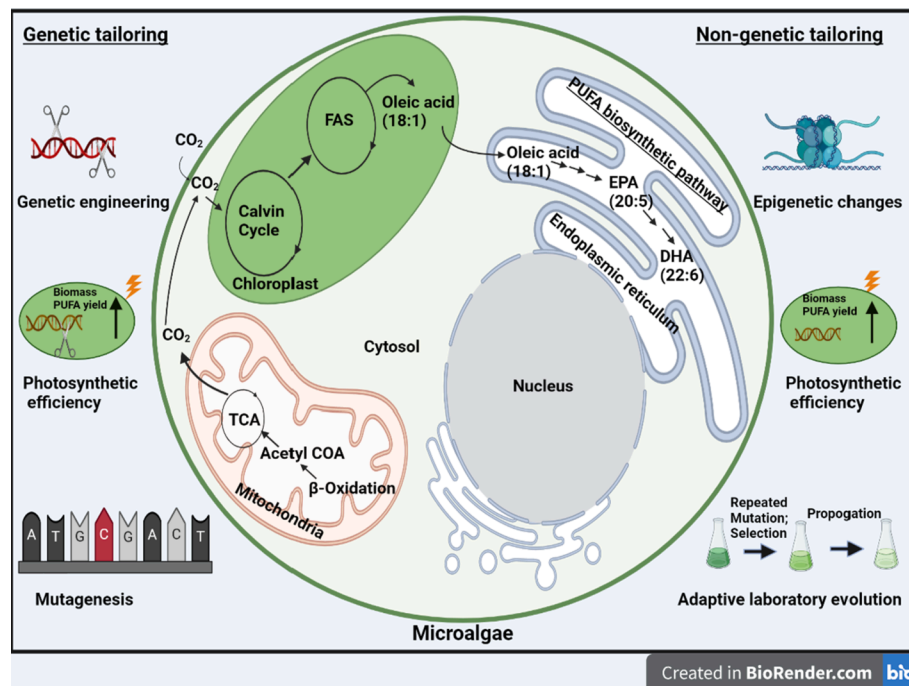
Fig. 3. Genetic and non-genetic tailoring methods for the enhanced production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from microalgae (tricarboxylic acid (TCA) cycle, Fatty acid synthase (FAS)).

CRediT authorship contribution statement

Parul Jakhwal: Conceptualization, Data curation, Validation, Visualization, Writing – original draft. **Jayanta Kumar Biswas:** Writing – review & editing. **Archana Tiwari:** Writing – review & editing. **Eil-hann E. Kwon:** Writing – review & editing. **Amit Bhatnagar:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing, Resources, Project administration.

Declaration of Competing Interest

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

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