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# Exploring strategies to enhance microalgae nutritional quality for functional poultry-sourced food products

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## ABSTRACT

Microalgae have emerged as a promising feedstock for the poultry feed industry due to their high nutritional potential, containing essential polyunsaturated fatty acids, essential amino acids, vitamins, and other high-value products compared to conventional poultry feed supplements. Microalgae-based feed can enhance broilers' body weight, feed intake, and, more interestingly, improve the nutritional quality of poultry-sourced food products with omega-3 fatty acids and carotenoids. This study aims to review all available metabolic quality-enhancement strategies, including conventional approaches dealing with physicochemical parameters of the cultivation condition and unconventional approaches dealing with advanced molecular biology techniques. These strategies are considered promising for enriching microalgae biomass with valuable metabolites for high-quality feed production, consequently enhancing the nutritional value of poultry meat and specialty eggs. Finally, techno-economic challenges were discussed, and recommendations were delivered.

## 1. Introduction

The global population increases with a significant escalation rate and is expected to reach more than 9700 million in 2050, which has increased the food demand, and is expected to exceed 60 % (Yarnold et al., 2019). For human food consumption, the main animal sources of protein and fat include livestock, poultry, and fish (Mancinelli et al., 2022). Therefore, the reliability of production in the poultry and aquaculture industries is of prime importance. However, poultry feed is primarily derived from plant sources that are high in protein and lipids such as corn and soybean (Madeira et al., 2017). This potentially leads to competition in the allocation of these edible crops between human consumption and animal feed (Kpomasse et al., 2021). Therefore, the utilization of edible plant sources for animal feed is unsustainable with the ever-increasing human population. Furthermore agricultural activities are responsible for 70 % freshwater volume withdrawal thereby being the most water intensive sector (Aivazidou et al., 2016). And the unsustainable use of groundwater extraction for over 40 % of the agricultural needs has created an urgent need for more sustainable agricultural systems (Levintal et al., 2022). According to Ingrao et al., 2023 modified cultivation and agricultural strategies need to implemented for water and food security. Thus, finding a sustainable feed replacement for

poultry plays a greater role in the future of food security globally. In addition to sustainability, the demand for healthier food production has also become a greater quest with such increasing food requirements (Santo et al., 2020).

Although various fatty acids are synthesized by humans, there are certain essential fatty acids (EFAs) that need to be supplemented through food. It is well known that regular intake of n-3 polyunsaturated fatty acids (PUFAs) can improve lipid metabolism and reduce the risk of cardiovascular and metabolic disease (Jump et al., 2012). In addition, PUFAs are also known to have significant potency in enhancing immunity and anti-inflammation properties (Gholamhosseinian et al., 2020). Although fish is considered the conventional source of these PUFAs, depletion of marine sources, lower proportions of PUFA, and the associated risk of heavy metal accumulation in fish have shifted the focus towards other potential sources of PUFAs (Wang et al., 2018b).

Among other potential sources, enriching poultry feed with essential fatty acids is increasingly attractive and practiced. The available potential strategy is to add marine PUFAs or flaxseeds to poultry feed, which results in PUFA enrichment in poultry meat and products (Moghadam and Cherian, 2017). However, microalgal supplements as a source of PUFAs for enrichment in poultry meat and eggs have been grabbing attention for the past few years because of their potential

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advantages, such as high productivity, higher sustainability, and lesser anti-nutritional properties (Neijat et al., 2017; Yonke and Cherian, 2019). According to the National Academy of Medicine the recommended intake of essential omega-3 fatty acids for an adult to maintain a healthy lifestyle is between 1.6 g and 1.1 g for males and females respectively (Office of Dietary Supplements, 2018). Additionally, through enrichment, concentrations of omega-3 fatty acids can reach up to 200 mg per egg (Bruneel et al., 2013). In this context, omega-3 fatty acid-enriched eggs are increasingly appealing, accounting for about 10 % of total egg consumption in the United States. Similarly, worldwide interest in omega-3 eggs is growing, as getting the recommended amount of omega-3 fatty acids from eggs would be an added benefit. Even though hens have an innate ability to synthesize omega-3 PUFAs, significant deposition of such fatty acids on eggs and tissues is primarily determined by the rate of intake of the various omega-3 PUFAs (Ehr et al., 2017).

Microalgae are typically unicellular eukaryotic microorganisms and are globally known as one of the leading producers of PUFAs (Dubey et al., 2023). They are to grow in various different environments, and produce multiple high value products currently applied in various sectors such as food, feed, energy, cosmetics and pharmaceuticals (Patel et al., 2020). Although microalgae produced different classes of lipids such as PUFAs, monounsaturated fatty acids and saturated fatty acids, it is PUFAs which are deemed of great importance (Vadrle et al., 2023). These fatty acids are known to have many health benefits such as lowering inflammation, reducing blood cholesterol, and even as precursors for antihyperglycemic and antiviral activities (Ardiles et al., 2020). Furthermore, these beneficial properties and metabolites found in microalgae also make them a great candidate for feed supplement in poultry industry (Ritu et al., 2022).

Although flaxseed, hempseed, and fish oil were reported to be excellent dietary omega-3 sources for poultry, microalgae possess inherent advantages such as no extensive land requirements, very high productivity, environmental benefits, and biorefinery potential (Chaves et al., 2021). Microalgal species such as *Nannochloropsis oculata*, *Phaeodactylum tricornutum*, *Isochrysis galbana*, and *Chlorella fusca* have previously been used as a dietary supplement in poultry feed to improve the omega-3 fatty acids concentration in eggs (Lemahieu et al., 2013). Although the strategy of supplementing microalgae as a feed ingredient

for poultry has been widely reported, there is a lack of comprehensive accounts about development of the field, and approaches for enhancing the PUFAs in microalgal biomass. Moreover challenges for sustainable omega egg production through microalgal supplementation in poultry feed are not reviewed extensively. Hence, the present review aims to shed light on a holistic way of analyzing the various strategies of supplementing microalgae as a poultry feed ingredient to produce omega-3 eggs while considering the challenges as well.

## 2. Potentiality of microalgae as functional feed supplement in the poultry industry

Microalgae have an exceptional nutrient profile with carbohydrates, lipids, proteins and fiber that are beneficial for nutritive addition to human diets, animal and poultry feeds, and fish feeds. A detailed account of the notional properties of various microalgae can be accessed in the study of Saadaoui et al. (2021). Moreover, significant proportions of phenolic content and flavonoids are also present in microalgal species (Tibbetts et al., 2014). These microcellular factories of nutrient reservoirs as feed ingredients tend to positively impact the physicochemical characteristics of hen such as body weight gain, meat carcass, nutrient quality of the meat, lipid profile, production performance, egg yolk pigmentation, etc. (Khan et al., 2021a). The main benefits of microalgal supplementation to poultry feed is summarized in Fig. 1.

Several studies analyzed the influence of microalgae as a feed ingredient on broiler meat qualities. For example, doses of up to 1.5 % *Arthrospira platensis* increased the body weight gain by 8 % (Park et al., 2015), whereas another study by Perdana et al. (2021) showed that weight was reduced (18 %) at higher doses of up to 15 %. This can be attributed to the fact that lower dosages have relatively higher digestibility and lower digesta viscosity than higher doses (Venkataraman et al., 1994). Similar observations were made using *Chlorella vulgaris* as a feed ingredient, where lower dosages were found to be effective in increasing the body weight gain and amino acid profile of the meat (El-Bahr et al., 2020). In addition to the poultry weight, the color of the meat was found to be a major influencing factor under the supplementation of microalgae as a feed ingredient. The presence of iron and pigments ( $\beta$ -carotene) in microalgal biomass increased the redness and yellowness of meat respectively (Toyomizu et al., 2001). Moreover, microalgal

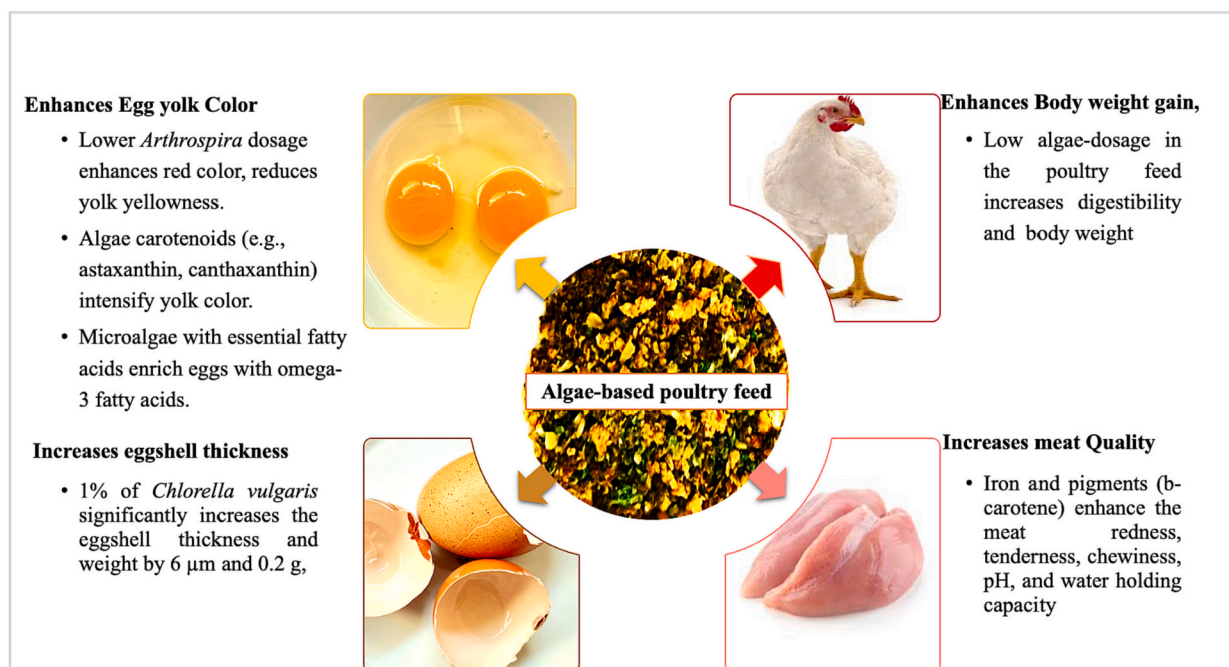


Fig. 1. Prospective benefits of using microalgae for poultry feed supplementation.

supplementation as a feed ingredient also influences the other features of meat such as tenderness, chewiness, pH, and water holding capacity. These changes are due to microalgae's influence on enzymes such as enolases ( $\alpha$  and  $\beta$ ), kinases (pyruvate and creatinine), and calpain (Huang et al., 2020). Lipid peroxidation of the meat was also decreased by the supplementation of *Hematococcus pluvialis*, which was mainly attributed to the presence of higher levels of antioxidants ( $\beta$ -carotene, astaxanthin and vitamin E) (Sun et al., 2018). Moreover, in recent time, steroids have also become a trending supplement for broilers. In a study investigating dexamethasone which is a growth promoter, it was found that, not only was the feed intake and efficiency decreased, the total weight gain decreased as well (Islam et al., 2022). On the other hand, supplementation of 20 ppm astaxanthin, produced in algae was supplemented to broilers and an increase of 5.7 % was noted for body weight gain (Ritu et al., 2022).

Apart from the meat quality, microalgal supplementation as a feed ingredient significantly influences the egg quality of laying hens. For instance, 0.5 % of *Chlorella vulgaris* increases the eggshell thickness and weight by 4  $\mu\text{m}$  and 0.5 g, respectively (Kim et al., 2023). Similarly, the color of egg yolk was significantly influenced by the supplementation of a lower dosage of *Spirulina* which resulted in increased red color and reduced yellowness in the yolk (Omri et al., 2019). It was stated that the presence of pigments, including xanthophylls and carotenoids, would be the major reason for increasing the color of the yolk (Khan et al., 2021b). Moreover, it has been found that using microalgae as feed also profoundly enhances the production rate of eggs. For example, supplementation of doses of up to 1 %, 2.5 %, and 7.5 % of different microalgal species such as *Schizochytrium* sp., *Arthrospira platensis*, and *Chlorella vulgaris* significantly increased egg production by 6 % (Kalia and Lei, 2022). Although in some other studies, varying results were found where supplementation of around 12 % (*Nanochloropsis oceanica*) and 15 % (*Staurorsira* sp.) did not have any effects on the egg production (Ekmay et al., 2015; Manor et al., 2019). Such results display the need for insights about dosages, biochemical composition, and species-specific influences in laying hens. Similarly, the nutrient profile of eggs is magnificently influenced by the incorporation of microalgae as a feed ingredient, as proven by multiple reports. For example,

*Schizochytrium* sp. was fed at low dose (0.2–1 %) to laying hens, which resulted in considerable enrichment of EPA and DHA in eggs (Park et al., 2015). In another study, *Aurantiochytrium limacinum* up to 2.5 % of the dosage increased the DHA in eggs from 0.6 to 4  $\text{mg g}^{-1}$  (Moran et al., 2020). These omega-3 fatty acid-enriched eggs are considered omega-3 eggs, and they are also more flavorful and appealing to people than those enriched with fish oil (Feng et al., 2020). This deems microalgal nutritive profile highly beneficial as the ratio (1:1) of omega 6 and omega 3 is preferred for inclusion in a healthy diet for humans (Michalak et al., 2020). While in supplementation of 0.008 % astaxanthin from *Haematococcus pluvialis* enriched the yolks with astaxanthin (not detected–36.2  $\text{mg/kg}$ ), carotenoids (13.8–86.1  $\text{mg/kg}$ ) and DHA (not detected– 6.35  $\text{mg/egg}$ ) (Magnuson et al., 2018). Hence, from the reports so far, doses vary based on species and chemical composition. Conversion of microalgal biomass to poultry feed is also summarized in Fig. 2.

Also, it's noteworthy that supplementing microalgae as a potential feed ingredient shows an array of positive influences on quantitative features of meat, physical features, muscle quality, egg production pattern, color of yolk, flavor, and nutritive value of eggs especially enrichment of omega fatty acids (Saadaoui et al., 2021). However, the exact mechanism of these beneficial changes, in meat and eggs, by microalgal supplementation has not yet been investigated to its full extent. Although some justifications have been discussed such as enhancement of essential fatty acids being mainly due to the upregulation of mRNA levels of genes encoding FADS desaturases, malic enzymes, elongases which are all involved in synthesis of these essential fatty acids. Moreover, microalgal supplementation was also identified to decrease the mRNA expression of genes encoding cytochrome P450 whereas mRNA expression of fatty acid synthases was increased significantly (Tao et al., 2018). Moreover, plasma amino acid proportions in hen were altered under the supplementation of microalgae. Hence, these are the predicted mechanisms of microalgal supplementation as a feed ingredient in hens, and undoubtedly, it induces vital gene expression that is beneficial for producing eggs with higher nutritive value. Overall, the species-specific response, dosage variation, and biochemical composition of microalgae show potential for further exploration of



Fig. 2. Process chart of producing functional poultry feed enhanced with microalgal biomass.



tailoring microalgal biomass to be enriched with specific components that need to be supplemented in poultry. In addition, microalgal technology is yet to be fully cost-effective, hence the targeted high value products needs to be produced efficiently. This leads to an analysis of the existing technologies and strategies for enhancing the essential fatty acids in microalgae in the following sections. The list of methods enhancing the fatty acid profile in previous studies is also summarized in Table 1.

### 3. Enhancing essential fatty acid production in microalgae: Conventional approaches

#### 3.1. Chemical-based approaches

As a natural adaptation and for survival, microalgae produce a variety of high value metabolites, including fatty acids at different concentrations based on the adaptive requirements. The major role of fatty acids in microalgae is related to growth function and energy reservation for stress management. Usually, microalgae accumulate lipid content between 10 % to 70 % under different environmental conditions (Moraes et al., 2021). Among fatty acids, omega fatty acids are accumulated in microalgal cells for their high energy content and flow properties, which are required for their cellular functions. Ample proportions of omega fatty acids were noted in several microalgal species such as 29 % and 25 % EPA of total fatty acid content in *Nannochloropsis* and *Phaeodactylum tricornutum* respectively (Alves et al., 2018), whereas, up to 40 % of DHA was achieved in *Schizochytrium limacinum* under heterotrophic conditions (Şahin et al., 2018). Thus, selecting strains with a higher proportion of omega-3 fatty acids or enriching fast-growing microalgae with engineering (culture/molecular) strategies could be a preferred method for supplementing laying hens in order to produce omega-3 eggs.

The most practiced and proven strategy of improving microalgal biomass and lipid production which is enormously exploited is the basic growth-lipid trade off principle which states that microalgae accumulate lipids by diverting metabolism. The most observed lipid induction strategy includes variation, limitation, and starvation of nutrients such as nitrogen and phosphorus, variation of iron, carbon, calcium, magnesium, etc. Even though these lipid induction strategies are necessarily not reflected in omega fatty acid enhancement as triacylglycerol (TAG) is the post profound effect, certain reports show the profound enhancement of omega fatty acids (Zhang et al., 2022).

#### 3.1.1. Nitrogen

Nitrogen starvation leads to diversion of most of the metabolic flux for lipid biosynthesis, which is one of the earlier strategized techniques for lipid induction. Usually, the assimilation of nitrogen happens through glutamine synthetase, and glutamate synthase pathways, followed by the redistribution to other amino acid molecules through aminotransferases (Sanz-Luque et al., 2015). On the other hand, nitrogen starvation significantly influences the arginine-succinate pathway, which results in enhancement of lipids, and in turn shows vitality of the arginine pathway for lipid enhancement. Hence, the metabolic flux generated by nutrient deprivation are usually cascading, however, the nitrogen assimilation towards nucleic acid metabolism does not interfere with lipid enhancement. Such a mode of lipid enhancement is also reflected in omega-3 fatty acid enrichment. Previous reports have shown that although nitrogen deprivation increased total PUFA, particularly  $\alpha$ -linolenic acid, the EPA fatty acid was significantly reduced (Chen et al., 2011).

Similarly, Cointet et al. (2019) combined high light stress with nitrogen limitation (10 % nitrogen), which resulted in enhanced neutral lipid productivity, but was not reflected in the PUFA content. On the other hand, there are reports supporting that the type of nitrogen sources used can influence the omega fatty acids profile. The omega-3 fatty acids were significantly doubled by using urea, whereas other nitrogen sources (nitrate/nitrite) decreased the PUFAs along with the enhancement of saturated fatty acids. By inducing nitrogen deprivation (starting concentration of 100 mg/L NaNO<sub>3</sub>) and varying inorganic carbon availability, Guihéneuf and Stengel (2013) demonstrated the changes in n-3 long-chain PUFAs. About 70 % of TAG was accumulated with 55 % and 67 % of EPA and DHA, respectively. This was accomplished by the ability of microalgae to use inorganic carbon through the Calvin-Benson cycle using carboxylases and carboxykinase enzymes, where bicarbonate is fixed into glyceraldehyde-3-phosphate which are then converted to lipids and carbohydrates (Guihéneuf et al., 2011). In addition, bicarbonate to carbon dioxide conversion could also be possible through carbonic anhydrase, which is accomplished by a few of the microalgal species (Reinfelder, 2011). While in another study, *Nannochloropsis oceanica* showed a decrease in DHA and EPA under nitrogen depletion with increased TAG accumulation, whereas EPA and DHA were enriched under increased nitrogen supply (concentration of medium kept at 12.4 mg/L NaNO<sub>3</sub>) especially in structural lipids (phospholipids and glycolipids) (Meng et al., 2015). However, an intriguing carbon labeling study in *Nannochloropsis graditana* by Janssen

**Table 1**

Summary of the various methods adopted in studies for Omega-3 fatty acid enhancement in microalgae.

Method	Experiment	Microalgae	Efficiency	Reference
Nutrient variation	Nitrogen	<i>Phaeodactylum tricornutum</i>	increase in DHA (61 %) and EPA (75 %) when Nitrogen concentration increased (1.24 to 49.40 mg/L)	(Qiao et al., 2016)
	Carbon	<i>Aurantiochytrium</i> sp.	DHA concentration was higher in glycerol (9 g/L) compared to glucose (8 g/L) as carbon source.	(Chang et al., 2013)
Physical	pH	<i>Pinguicoccus pyrenoidosus</i>	EPA concentration tested in pH 5–9, showed optimum production (23.13 % of total fatty acids) at pH 6	(Sang et al., 2012)
	Light	<i>Phaeodactylum tricornutum</i>	relatively constant concentration of DHA and EPA with increase in light irradiance (50 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	(Qiao et al., 2016)
	Temperature	<i>Phaeodactylum tricornutum</i>	decrease in DHA (37 %) and EPA (15 %) when temperature increased (15 to 25 °C)	(Qiao et al., 2016)
	Salinity	<i>Phaeodactylum tricornutum</i>	highest concentration of DHA (1.08 mg/L) and EPA (32.18 mg/L) was achieved at 15 and 28 ppt respectively	(Qiao et al., 2016)
	overexpression of malonyl-CoA: ACP transacylase	<i>Schizochytrium</i>	Increase in DHA (81.5 %) and EPA (172.5 %)	(Li et al., 2018)
Molecular methods	overexpression of long chain fatty acid elongase genes	<i>Thalassiosira pseudonana</i>	Increase in DHA by 4.5 fold and EPA by 1.4 fold	(Cook and Hildebrand, 2015)
	TAG pathways- overexpression of AGPAT1	<i>Phaeodactylum tricornutum</i>	increase in DHA (1.5 fold) and EPA (1.55 fold)	(Balamurugan et al., 2017)
	targeted knockdown of lipid catabolic pathway genes	<i>Thalassiosira pseudonana</i>	increase in DHA and EPA	(Trentacoste et al., 2013)
	Overexpression of transcriptional factors	<i>Chlorella ellipsoidea</i>	increase in overall lipid content of cells	(Liu et al., 2021)

et al. (Janssen et al., 2019) found that under nitrogen starvation, EPA was translocated from phospholipid to neutral lipid, implying that carbon reshuffling within the cell occurred and fatty acid translocating enzymes had a higher specificity towards EPA. Regardless of the contradictory results, these studies have demonstrated that the metabolic flux dynamics induced by nitrogen limitation have a significant impact on EPA production and its allocation between polar and neutral lipids.

### 3.1.2. Carbon

Carbon assimilation in microalgae is widely utilized to adjust the lipid and metabolite accumulation behaviors of microalgae. Glucose and glycerol are commonly preferred as external carbon sources and are assimilated by microalgae through glycolysis initially and then converted to acetyl CoA through the citric acid cycle which in turn is finally utilized for lipids (Winwood, 2013). Under heterotrophic cultivation, microalgae typically incorporate carbon directly for cell energy expenditure, and chlorophylls are less synthesized and downregulated with the relative energy expended for lipid biosynthesis. *Chlorella* in a heterotrophic condition, with carbon source as 10 g/L glucose and 0.1 g/L glycine, produces lipids and carbohydrates about 2.8 and 0.45 times higher than the autotrophic culture, respectively (Miao and Wu, 2006). A pretreated lignocellulosic biomass (78 % polydextrose, 19 % polyxylose) was used as medium for heterotrophic cultivation of *Chlorella pyrenoidosa*, where the shortest generation time as compared to glucose medium (10 g/L glucose, 2.5 g/L xylose) was observed along with a tremendous increase in PUFAs of about 66 % of total fatty acids (Zhang et al., 2019). Although heterotrophic mode produces more biomass and

PUFAs, it has drawbacks such as contamination risks, limited metabolite production, not all species respond well to heterotrophic cultivation, and cost of the carbon source (Lee, 2001). Contamination risks on a large scale are usually due to lower carbon supply, a continuous feeding strategy and competition from other microorganisms, such as bacteria which don't need light to grow (Perez-Garcia et al., 2011).

Though there are reports that support heterotrophic mode for enhancing PUFAs and omega-3 fatty acid production, predominantly, autotrophic mode has better production of omega-3 fatty acids. For instance, ALA was relatively higher under autotrophic mode than under the supplementation of glucose (Cho et al., 2011). Similarly, EPA was relatively improved in *Nannochloropsis* in the autotrophic culture as compared to mixotrophic culture (Hoffmann et al., 2010).

### 3.2. Physical factors

#### 3.2.1. Temperature

Among physical factors, temperature is one of the key factors involved in influencing the fatty acid profile of microalgae. Usually, lower temperatures promote the desaturation of fatty acids. For instance, omega-3 fatty acids such as DHA and EPA were higher at 15 °C and lower at 26 °C (Aussant et al., 2018). Similarly, at 17 °C, EPA and total fatty acid content were significantly increased. This is because microalgae produce higher levels of polyunsaturated fatty acids under higher or lower temperature stress conditions for the purpose of altering membrane fluidity (Hoffmann et al., 2010). During temperature stress, this membrane fluidity protects not only the cellular membrane but also

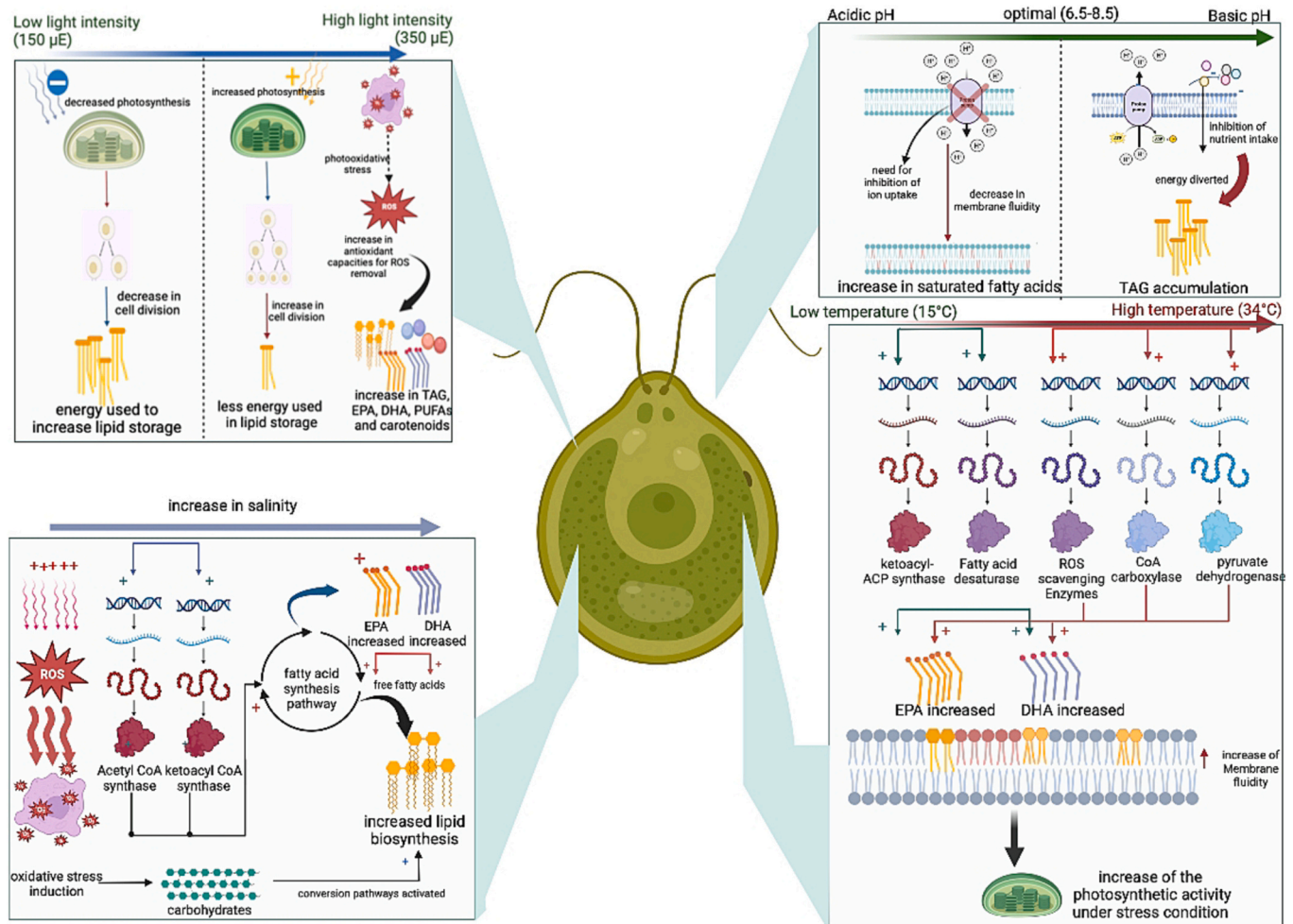


Fig. 3. Physical stress induction on microalgae and the possible Omega-3 enrichment mechanism. Figure created with BioRender.com.

the thylakoid membranes, thereby protecting photosynthesis from damage caused by extreme temperatures (Refsgaard et al., 2000). Microalgae release enzymes such as catalase, glutathione reductase, and superoxide dismutase in response to high levels of reactive oxygen species (ROS) which in turn cause damage to photosynthetic membranes (Mallick and Mohn, 2000). Hence, this temperature stress induces the expression of the pyruvate dehydrogenase complex, which results in the enhancement of acetyl CoA production. Summary of physical factors and the pathways in which they influence the fatty acid production is shown in Fig. 3.

Integrated analyses of the transcriptome, proteome, and fatty acid profiling of the oleaginous microalga *Auxenochlorella protothecoides* UTEX 2341 under low temperatures demonstrated that Fatty Acyl Synthase (FAS) and Fatty Acyl Desaturase (FAD) are upregulated to enhance the synthesis of omega-3 fatty acids in the chloroplast (Xing et al., 2018). Therefore, FAS performs Acyl-CoA synthesis, which is ultimately converted into C18:2 prior being desaturated into omega-3 fatty acids (C18:3) via FAD (Xing et al., 2018).

An adaptive laboratory technique was studied in *Schizochytrium* species with increasing temperature, and the microalgal species adapted up to 34 °C with increased (4 %) DHA production and reduced ROS (Hu et al., 2021). Similarly, eight microalgal species were investigated under temperature variation stress, where *Nannochloropsis oculata* *Isochrysis galbana* showed the highest EPA and DHA production under 14–20 °C temperature (Aussant et al., 2018).

### 3.2.2. pH

The pH of microalgal growth medium could significantly affect cellular growth as well as microalgal metabolism and relevant biochemical proportions. The pH of medium mainly influences the nutrient uptake through altering membrane permeability, influencing H<sup>+</sup> and electron transports (Lavoie et al., 2012). As a result, changing the pH can have a significant impact on cellular intake of nutrients and molecules, which can then be used to manipulate lipid accumulation (Fig. 3). The usual pH range of marine and freshwater microalgae is between 5 and 11 (Moheimani, 2012; Qiu et al., 2017). Generally, freshwater microalgae acclimatize under a wider range of pH, whereas marine microalgae are confined to shorter ranges due to their exposure levels (Perdana et al., 2021). For instance, a maximum EPA content of about 23 % and a maximum PUFA content of about 38 % was observed under a pH of 7 in *Pinguicoccus pyrenoidosus* (Sang et al., 2012). While in *Nannochloropsis salina*, maximum growth and lipid contents were observed at pH 8, whereas no considerable changes were noted in the PUFA or overall fatty acid profile (Bartley et al., 2013). It is noted even in other studies, pH is rarely found to be the direct influence on PUFA production, except for the few reports as mentioned above.

### 3.2.3. Salinity

Salinity induces osmotic stress and triggers changes such as the ionic ratio within the cell through selective ion permeability in the membrane (Fal et al., 2022). However, the salinity induced stress conditions are not uniform for all the microalgal species, as freshwater species are less exposed to salt conditions, whereas marine species are halotolerant. A few examples of halotolerant species include *Dunaliella salina* in 150 ppt of salinity (Oren, 2014) although it is widely known that microalgal growth is not optimum above 35 ppt. Salinity stress causes a series of changes within microalgal physiology, such as carbohydrate content reduction and lipid enhancement, which influence lipid homeostasis by controlling phosphatidic acid. Glycolysis process was increased, and lipid accumulation was triggered with less carbohydrate production. In addition, salinity stress could potentially induce ROS mediated signaling and thereby facilitate the conversion of carbohydrates to lipids (Wang et al., 2018a). Induction of salinity stress also increases the levels of proteins like acetyl CoA carboxylase and ketoacyl-CoA synthase, which are involved in crucial steps of fatty acid synthesis (Chen et al., 2017; Singh et al., 2019). Since these initial steps of fatty acid synthesis are

significantly induced by salinity stress, the chances of unsaturated fatty acids are reduced. However, because of increased membrane permeability and to maintain fluidity, unsaturated fatty acid levels were increased under salinity stress induction. This can be exemplified by a few reports and described in Fig. 3. EPA was magnificently improved in *Chlorella minutissima* under high salinity conditions (Seto et al., 1984). Similarly, DHA and ALA were potentially increased in *Dunaliella salina* and *Chlorella vulgaris* under high supplementation of NaCl (Rismani and Shariati, 2017). Adaptive laboratory evolution was performed in *Phaeodactylum tricornutum* in which 70 % of salinity improved the PUFA production followed by light, temperature, and fulvic acid treatments that resulted in the enrichment of EPA (Wang et al., 2019). It was identified that hypo salinity along with fulvic acid significantly improved the lipogenic genes expression and this was attributed to essential fatty acid enrichment. Apart from these reports, salinity did not appear to have a significant impact on the fatty acid profile, implying that the response is species-specific.

### 3.2.4. Light

Light was identified as one of the important factors that influences the fatty acid formation in microalgae. Like plants, microalgae require a considerable level of photons for photosynthesizing, and the required wavelength ranges between 400 nm to 700 nm (Katam et al., 2022). Because of the vitality of photosynthesis, light could potentially control the growth and anabolic behavior of microalgae. So far, the different wavelengths of light are known to specifically induce different changes in the biochemical composition of microalgae. Blue light is known to induce a higher level of omega-3 fatty acid accumulation in green algae (Helamieh et al., 2021) (Fig. 3). Furthermore, DHA fatty acid is increased by varying the wavelength of the light between 440 and 660 nm at an intensity of 150 mol m<sup>-2</sup> s<sup>-1</sup> (Maltsev et al., 2021). The light intensity also plays a key role in altering the fatty acid profile. A light intensity of 150 mol photons m<sup>-2</sup> s<sup>-1</sup> irradiance, for example, resulted in a 4-fold increase in chlorophyll and carotenoids production, as well as a 16.5 % increase in PUFA and a 5.7 % increase in EPA, whereas a light intensity of 750 mol photons m<sup>-2</sup> s<sup>-1</sup> significantly reduced the PUFA content of the microalgae. Blue light, on the other hand, is known to influence a higher level of lipid accumulation at the 12:12 h light/dark cycle (Da Conceição et al., 2020). The main mechanism underlying blue light-induced accumulation of omega-3 fatty acids and lipids is the generation of ROS, and cell division is slowed under blue light while it is accelerated under other lights, including red light. Blue light was also found to negatively influence carbohydrate formation and increase lipid accumulation, along with influencing the expression of various genes involved in the metabolic pathways of microalgae (Patelou et al., 2020).

### 3.2.5. Other factors

Aside from the common factors, other effective exogenous factors are used concurrently to induce omega-3 fatty acids. For instance, application of UV radiation to microalgal culture can enhance growth as well as produce mutants through random mutagenesis. EPA was profoundly increased under UV irradiance in *Phaeodactylum tricornutum* (Liang et al., 2006). Similarly, exposure to UV-A radiation increased the PUFA content in *Nannochloropsis oculata* (Maltsev et al., 2021) while the PUFA content of *Pavlova lutheri* was significantly reduced despite significant improvements in storage lipids. Another widely used strategy is supplementing the microalgal growth medium with substances that could elicit PUFA or fatty acid accumulation. Among supplements, phytohormones are incredible elicitors as they are the stress responders in plants which could profoundly impact the growth and metabolic behavior of microalgae. Phytohormones such as auxin has proven to enhance PUFA content of microalgae when exogenously supplied to the microalgal medium. On the other hand, Cytokinin is not recommended for increasing PUFA content because studies show that it decreases PUFA content (Sivaramakrishnan and Incharoensakdi, 2020). Esakkimuthu et al. (2020) identified p-coumaric acid as a potential enhancer of



microalgal biomass and fatty acid accumulation on par with jasmonic acid and salicylic acid. Coumaric acid and jasmonic acid showed considerable increments of PUFA from 12.5 to 15.5 % and 21.5 %, respectively along with total lipid and biomass enhancement. Similarly, polysorbates were also found to influence the biomass and fatty acid production in microalgae. Supplementing the microalgal growth medium with Polysorbate 60 enhanced the PUFA from 12.5 % to 27 % in high lipid production conditions (Esakkimuthu et al., 2019). The major mechanism causing such increase in lipids was due to the increase in membrane permeability and fast uptake of nutrients, which might also eventually lead to an increase in PUFA content.

Vitamins are another crucial compound that are widely supplemented to microalgae for stimulating the growth and biomass. Vitamins such as B1, B7 and B11 are essential vitamins required by microalgae. Vitamins like thiamine is needed for synthesizing a variety of enzymes involved in carbon metabolism, whereas vitamin B7 acts as a cofactor for acetyl CoA carboxylase, which is found to be involved in the first-rate limiting step of lipid biosynthesis (Tandon et al., 2017). Although only a few reports exist, these vitamins have a critical functional role in carbon metabolism and lipid biosynthesis and can potentially be used for omega-3 fatty acid enhancement in microalgae.

#### 4. Essential fatty acid enhancement in microalgae through advanced molecular approaches

Next to conventional approaches, genetic and metabolic manipulation of microalgae for the benefits of accumulating value-added products in microalgae is becoming increasingly attractive. With concurrent advancements and development of techniques in relevant fields, engineering the targeted genes as well as manipulating the metabolic pathways has become feasible in recent years. Overexpression knockdown and heterologous expression of genes that regulate fatty acid synthesis are the most well-known and successful techniques (Santin et al., 2021). There have been potential attempts to carry out multigene transgenic expression and blocking of competing pathways for the enrichment of PUFA (Gonçalves et al., 2016). Selecting approaches or engineering techniques have been discovered to be critical in transforming and mutating genes in the microalgal system. Like other forms of microbial

genetic engineering, techniques such as electroporation, particle bombardment, agrobacterium mediated transformation, and episome introduction through bacterial conjugation (Karas et al., 2015), are attractively employed to achieve nuclear transformation in microalgae. Mutagenesis is achieved with the help of chemical and physical mutagens where the genetic transformation is less feasible. Owing to inconsistent results, random mutagenesis is less preferred nowadays to enrich microalgal PUFAs. On the other hand, the genetic understanding of microalgae is growing rapidly, encompassing information about various genomic sequences and relative advancements in nuclear transformation techniques that grab the attention of random mutagenesis (Arora et al., 2020). Overexpression is carried out through constructs containing respective regulatory sequences, whereas RNAi is commonly used to downregulate the genes. Recently, genome editing techniques have been trending to knock down genes using nuclease proteins which are achieved by cutting the genome at a specific region. TALEN and CRISPR/cas9 systems are the most reliable techniques followed to cut down specific genes. CRISPRi, which works at the transcriptional level, is also commonly used to manipulate genetic expression (Fajardo et al., 2019). The genetic engineering approaches dealing with genes involved in fatty acid synthesis genes and, triglycerides synthesis, blocking competing pathways and RNAi for silencing transcription factors are summarized in the Fig. 4.

##### 4.1. Manipulating lipid biosynthetic genes

Genes involved in the lipid biosynthetic pathway are targeted and overexpressed. For instance, about a 80 % increase in DHA, a 170 % increase in EPA, and around a 70 % increase in DPA were observed by overexpressing Malonyl CoA-ACP transacylase in *Schizochytrium* sp. (Li et al., 2018). Similarly, when genes like Malonyl CoA-ACP transacylase, Ketoacyl-Acyl-Carrier-Protein Synthase, acyl-ACP thioesterase, and fatty acid synthase were overexpressed in *Haematococcus pluvialis*, DHA and EPA levels increased 4-fold and 2-fold, respectively. It is worth noting that acyl-ACP thioesterase oversees the initial condensing reactions in fatty acid synthesis. The results were not always consistent; for example, overexpression of thioesterase resulted in a slight increase in EPA but not in other PUFAs (Lei et al., 2012). In general, the genes encoding the

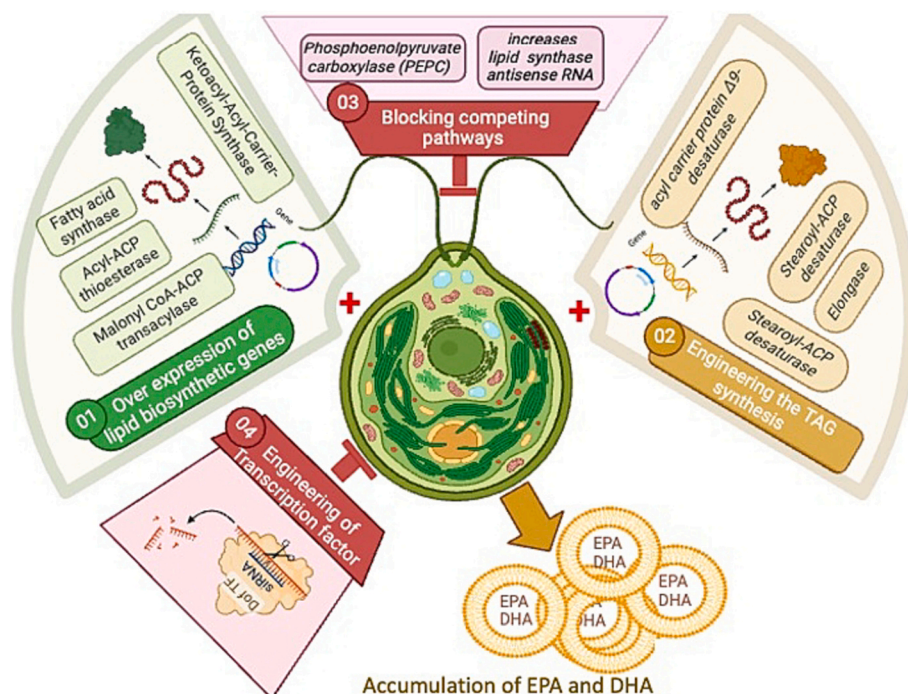


Fig. 4. Genetic engineering approaches to enhance EPA and DHA. Figure created with BioRender.com.



final step of fatty acid biosynthesis are preferred as they can increase the desired product, whereas major positive results for enhancing PUFAs as well as fatty acid biosynthesis were achieved with overexpressing the enzymes that are involved in the initial fatty acid synthesis steps.

#### 4.2. Engineering the elongation and desaturation process

Elongation and desaturation processes are carried out after the fatty acids are synthesized by de novo process, either in the endoplasmic reticulum or on plastids, by elongases and desaturases. These enzymes are ideally suited for modifying the fatty acid profile of microalgae, as unsaturation can be influenced at this stage. For example, Stearoyl-ACP desaturase was overexpressed in *Nannochloropsis oceanica* which resulted in the increased production of linoleic acid by more than 15 % along with triglyceride accumulation (Fofana et al., 2006). Similarly, plastidial acyl carrier protein  $\Delta 9$ -desaturase was overexpressed which resulted in a magnificent alteration of the fatty acid profile by increasing the EPA up to 58 % along with a more than 60 % increase in triglycerides of *P. tricornutum* (Smith et al., 2021). Similarly, to desaturases, overexpression of fatty acid elongase in *Thalassiosira pseudonana* increased DHA and EPA by 4 and 1 fold, respectively (Cook and Hildebrand, 2015). In another report, elongase from *Ostreococcus tauri* was subjected to heterologous expression in *P. tricornutum* and this led to substantially enhanced DHA levels by 8 times as compared to the wild strain (Hamilton et al., 2014). It was also identified that the co-expression of the same elongase along with acyl-CoA-dependent desaturase further improved the DHA yield. For microalgal species like *P. tricornutum*, where the actual content of DHA is low, such heterologous expression becomes an essential tool. Hence, engineering the desaturation and elongation pathways were found to be critical in increasing the PUFA content. Interestingly, these genes controlling the elongation and desaturation of microalgae are heterologous expressed in other organisms and have successfully elevated the levels of DHA and EPA. For example, the desaturase gene of *Micromonas pusilla* was overexpressed, resulting in a higher level of EPA in plant triglycerides (Petrie et al., 2010). Such results of expressing microalgal genes in other organisms and the relevant elevation of PUFAs reinforce its vitality as a PUFA producer.

#### 4.3. Engineering the triacylglycerol synthesis pathways

The triacylglycerol (TAG) synthesis pathways are localized in endoplasmic reticulum and plastids, which are controlled by a cascade of enzyme reactions. Altering these genes is also reflected in the PUFA content of microalgae, as the acyl transferases show substrate specificity, whose alteration could alter the saturation process and length of the fatty acids. Enzymes such as glycerol-sn-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) which catalyze the initial and final steps of TAG synthesis are mainly targeted in this pathway for enriching the unsaturated fatty acids. The enzyme GPAT was overexpressed in *P. tricornutum*, resulting in 40 % more EPA accumulation and a significant reduction in saturated fatty acids (Niu et al., 2016). Similarly, overexpression of the AGPAT1 enzyme in *P. tricornutum* resulted in increased EPA and DHA levels (Balamurugan et al., 2017). On the other hand, DGAT was overexpressed in *P. tricornutum* resulting in more than 70 % of the EPA content (Haslam et al., 2020). The heterologous expression of DGAT2 from *Brassica napus* in *Chlamydomonas reinhardtii* increased the unsaturated fatty acid content significantly (Ahmad et al., 2014). From various reports, it can be emphasized that the alteration of genes encoding LPAT and DGAT will be a highly effective strategy for enhancing fatty acids, especially omega-3 fatty acids.

#### 4.4. Blocking competing pathways

This approach, which primarily involves blocking starch synthesis, has been devised to reduce the carbon footprint and flood the single

pathway with a large amount of energy. To create starchless mutants, physical mutagenesis or molecular tools have been widely used to inactivate the ADP-glucose pyrophosphorylase. A starchless *C. reinhardtii* mutant was created using X-rays as mutagens, which resulted in higher triglyceride accumulation of up to 4-fold due to the consolidation of metabolic flux diverted for lipid biosynthesis (Li et al., 2010). Phosphoenolpyruvate carboxylase (PEPC), which converts phosphoenolpyruvate to oxaloacetate, is one of the essential enzymes for amino acid metabolic pathway that is blocked. Knockdown of PEPC could potentially enhance more pyruvate and eventually lead to higher acetyl CoA pool. Similar knockdown strategy was carried out in *C. reinhardtii* which elevated the lipid content above 70 % (Johnson and Alric, 2013). Recently, suppressing lipid catabolism by knocking down the lipases was identified as effective in two ways such as increasing the lipid content without affecting growth, as other blocking pathway strategies are at the expense of growth. An antisense RNA approach was carried out to knockdown the lipases, which was effective in *T. pseudonana* where the lipid content was elevated along with enrichment of DHA and EPA (Trentacoste et al., 2013). It is noteworthy that blocking competing pathway strategies are effective in inducing the lipid enhancement but do not harbor any control over altering the fatty acids except in a few studies.

#### 4.5. Engineering the transcriptional factor

Transcriptional factors (TF) control and regulate gene expression by binding to the cis-acting element of the promoter which can also be engineered to regulate the expression of vital enzymes involved in lipid biosynthesis. *Chlorella ellipsoidea* was subjected to heterologous expression of the DNA-binding one finger TF (Dof TF), which resulted in elevated lipid accumulation along with a high growth rate, and it was observed that several genes were upregulated (Lei et al., 2012). A simultaneous induction of growth and lipid production under nitrogen and salinity stresses was observed in *N. salina* when it was subjected to overexpression of transcription factor with the leucine zipper domain, which was identified as a stress regulator in plants (Kwon et al., 2017). Similarly, heterologous expression of transcription factor NF-Y in plants from *Chlorella ellipsoidea* significantly improved the production of biomass in the *Arabidopsis thaliana*. Thus, this transcriptional engineering was found to be the most reliable and efficient technique, as it could simultaneously influence several genes along the same pathways (Liu et al., 2021). Although several reports dealt with improving the biomass and lipid accumulation of microalgae through TF engineering, reports on altering the fatty acid profile, especially through unsaturated fatty acid enrichments, are hard to find. However, the quality of regulating multiple genes along the same pathways could serve as a better tool to increase unsaturated fatty acids too. With extensive results, the underlying mechanism of regulating the metabolic pathways through transcription engineering needs to be thoroughly studied.

### 5. Economic feasibility, challenges for sustainable microalgal biomass as a feed ingredient

One of the challenges that comes with using microalgae as feedstock/ingredient is the expense, owed mostly to the harvesting process. Approximately 30 % of the microalgal biomass production cost comes from harvesting the algae. Microalgae and cyanobacteria typically have a cell size ranging from 1 to 30  $\mu\text{m}$  in diameter, a negative surface charge, and an insignificant level of density, hence making the harvesting process challenging and energy consuming. An added disadvantage for marine strains is the ionic strength possessed by seawater, which also negates the harvesting of marine microalgae (Liu et al., 2017).

Many harvesting techniques have been investigated such as centrifugation, filtration, and bioflocculation and each method displays its pros and cons. For example, centrifugation may be too disruptive to

microalgal cells and can degrade the quality of the biomass produced. This technique also requires a high energy input, which decreases its economic feasibility. Filtration of cells using membranes and/or filters that separate biomass from the medium is also a widely used method, although it works best for larger and filamentous species with a length of approximately 200  $\mu\text{m}$  (Maity and Mallick, 2022).

A recently suggested method for harvesting technology is bio-flocculation. A variety of bioflocculation techniques are available, such as the use of plant-based and microbial-based biopolymers and auto-flocculation. Plant based biopolymers such as seed powder from the clearing nut *Strychnos potatorum* have shown promising results of 99.7 % settling of the microalgal species *Chlorella vulgaris* in a study conducted by (Razack et al., 2015). Biopolymers from a microbial source still need to be developed as their toxicity and safety for consumption can act as obstacles (Ummalyma et al., 2017). Although a recent study, investigated an unconventional method of bioflocculating low settling microalgal species *Picochlorum* sp. by mixing it with *Tetraselmis* sp., which has a greater cell size and settling capacity. This allowed easier harvestation of the smallest microalgal cells (Saadaoui et al., 2023). Techniques like these can be implemented to reduce cost and energy required to flocculate and harvest mix cultures of microalgae.

Other cost related challenges include the high energy requirements which come with cultivating microalgae. Large scale cultivation and application of microalgae need high biomass in addition to low energy and capital requirements (Saadaoui et al., 2021). Moreover many forms of cultivation have been investigated and adopted such as open raceway ponds, which are widely known for being cost effective in terms of energy and medium compared to closed systems such as photobioreactors. Although the main disadvantage of open systems is contamination which can eventually affect the quality of biomass produced (Hosseini et al., 2023). When considering between the pros and cons of both systems, closed systems are still recommended due to higher quality of biomass. Moreover, a recent study found that opting for continuous cultivation strategies compared to batch in photobioreactors may be more energy efficient and sustainable. Furthermore it was also suggested that continuous cultivation strategies can also become more economically feasible in the future (Peter et al., 2022).

The biggest challenge with using microalgae as feedstock is ensuring its safety for human consumption. A rigorous investigation for the selection of suitable strains is primarily needed. Microalgae strains should be nonpathogenic, non-toxic, fast growing, and produce high levels of omega-3 fatty acids (Perdana et al., 2021). The toxicity of a strain in broilers may differ from that in humans, and this requires further investigation. Additionally, long term studies on use of algae for food production need to be conducted, to exclude any unknown side effects there may be.

Advances in microalgal research have led to the provision of a variety of suitable strains, the prediction of metabolic pathways, and ways to augment metabolite production by exploiting metabolic pathways. One challenge is enhancing the yield of lipids using external factors without altering or compromising the growth of the strain. To fully understand lipid production and metabolic pathways, future research must focus on molecular changes in microalgal strains. As suggested by Brar et al. (2021), conventional approaches in addition to next generation sequencing (NGS) techniques should be applied to recognize genetic regions that are responsible for lipid expression and how these can be altered for hyperproduction.

Moreover, food security is one of the most critical global challenges of the twenty first century (Greene et al., 2016), in addition to health issue concerns which are also on the rise. Omega-3 fatty acids are globally known for their health benefits including reduction in inflammation, cardiovascular diseases, cognitive health to name a few (Kumari et al., 2023). The availability of such essential omega-3 fatty acids, amino acids, proteins, n-3 PUFAs and other lipids in microalgae has allowed for developments in algal based food alternatives (Koyande et al., 2019). It is estimated that replacement of even 5–20 % of the

soybean and corn meal, in poultry diets, with microalgal based feed would save more than 2000 tons of soybean and corn meal for human consumption per year (Bruinsma, 2017). However, research and advancements on optimizing high value metabolites from algae for poultry will be of no use unless the digestibility of such products is verified. Improvement of digestibility of biomass and biomass containing feeds by broilers can be conducted by the addition of hydrolytic enzymes, essential amino acids, and trace minerals which complete a balanced diet (Kalia et al., 2021). Although adding these may contribute to a commercial disadvantage, by increasing the feed production costs.

## 6. Conclusion

Microalgae have emerged as a promising feedstock for poultry feed industry due to their high nutritional potential, containing essential biomolecules such as polyunsaturated fatty acids. These are beneficial as supplements for enhancing the quality of poultry meat and eggs. Despite the multiple benefits of using microalgae as an alternative feed supplement, the implementation is still lacking. Enhancing the production of the metabolites produced in microalgae will make the biomass even more attractive for use regardless of cost and energy constraints. Multiple strategies can be implemented, with the research still ongoing based on conventional and unconventional approaches. Each of which have its own pros and cons, considerations on the best method need to consider. Furthermore considering stress applications and molecular manipulation simultaneously needs to be investigated, as this may have a higher potential than applying a single strategy alone. As the demand for microalgae-based technologies will increase in the future, considerations for the most optimized cultivation and harvesting new generation technologies also need to be considered.

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## CRediT authorship contribution statement

**Sivakumar Esakkimuthu:** Conceptualization, Writing – original draft. **Simil Amir Siddiqui:** Writing – original draft, Writing – review & editing. **Maroua Cherif:** Writing – review & editing. **Imen Saadaoui:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data availability

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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