Journal of Biotechnology 360 (2022) 23–36

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Fourth generation biofuel from genetically modified algal biomass for bioeconomic development

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ARTICLE INFO

Keywords:
Fourth generation biofuel
Microalgae biomass
Techno-economic analysis
Open raceway pond
Bioeconomic assessment
Microalgae cultivation

ABSTRACT

Biofuels from microalgae have promising potential for a sustainable bioeconomy. Algal strains' oil content and biomass yield are the most influential cost drivers in the fourth generation biofuel (FGB) production. Genetic modification is the key to improving oil accumulation and biomass yield, consequently developing the bioeconomy. This paper discusses current practices, new insights, and emerging trends in genetic modification and their bioeconomic impact on FGB production. It was demonstrated that enhancing the oil and biomass yield could significantly improve the probability of economic success and the net present value of the FGB production process. The techno-economic and socioeconomic burden of using genetically modified (GM) strains and the preventive control strategies on the bioeconomy of FGB production is reviewed. It is shown that the fully lined open raceway pond could cost up to 25% more than unlined ponds. The cost of a plastic hoop air-supported greenhouse covering cultivation ponds is estimated to be US 60,000$ /ha. The competitiveness and profitability of large-scale cultivation of GM biomass are significantly locked to techno-economic and socioeconomic drivers. Nonetheless, it necessitates further research and careful long-term follow-up studies to understand the mechanism that affects these parameters the most.

1. Introduction

Biomass-derived fuels, also known as biofuels, are promising solutions for reducing greenhouse gas emissions by decreasing people's dependency on fossil fuels. Biofuels can be classified into first, second, third, and fourth generation biofuel (FGB) based on their biomass feedstock (Mat Aron et al., 2020). First generation of biofuel derived from edible biomass that can compete with food production (Bharathiraja et al., 2022). Second generation biofuel is generally produced from non-edible cellulosic biomass resources (Huzir et al., 2018). Algae were considered a feedstock for second generation biofuel until it was found that much more energy could be produced from algae than other second generation biofuel feedstocks. Thus, biofuel made from algae is now classified as third generation biofuel. FGB is the biofuel produced from genetically modified (GM) algae.

Several researchers used the term fourth generation biofuels for various biofuel types and technologies. Fatih Demirbas (2009) defined the fourth generation of biofuel as biogasoline obtained from the conversion of vegoil and bio-diesel. Barrett (2009) was the first to use the term fourth generation of biofuel to describe the fuel obtained from genetically GM algae. The proposed method used synthetic biology to construct microorganisms with unusually high levels of CO_2 absorbance characteristics. Janda et al. (2012) highlighted three technologies that had the potential to be introduced as the fourth generation of biofuel, pushing the conventional boundaries and facilitating the development of a more efficient and resilient biofuel industry. The technologies included genetically modifying organisms-based biofuel, biofuels decomposed at high temperatures known as solar-to-fuel, and artificial photosynthesis reactions. Though great improvements were achieved by

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https://doi.org/10.1016/j.jbiotec.2022.10.010
Received 11 July 2021; Received in revised form 3 September 2022; Accepted 17 October 2022
Available online 19 October 2022

Nomenclature

Acetyl-CoA carboxylase ACCase
Acyl carrier protein ACP
Adenosine triphosphate ATP
Adenosine diphosphate glucose pyrophosphorylase AGPase
Clustered regularly interspaced short palindromic repeat-associated protein CRISPR-Cas9
Diacylglycerol acyl transferase DGAT
Diacylglycerol acyl transferase type-I DGAT1
Diacylglycerol acyl transferase type-II DGAT2
Diacylglycerol acyl transferase type-III DGAT3
Dihydroxyacetone phosphate DHAP
Discounted cash analysis DCA
Eicosapentaenoic acid EPA
Enoyl-ACP reductase EAR
Fourth generation biofuel FGB.
Genetically modified GM
Glycerol-3-phosphate acyltransferase GPAT
Glycerol-3-phosphate dehydrogenase GPDH
Internal rate of return IRR
Investment payback period PBP
Lysophosphatidic acid acyltransferase LPAT
Malic enzyme ME
Malonyl-CoA ACP transacylase MAT
Minimum selling price MSP
Net present value NPV
Nicotinamide adenine dinucleotide phosphate NADPH
Open raceway pond ORP
Payback period PBP
Pentose phosphate pathway PPP
Phosphatidic acid phosphatase PAP
Photobioreactor PBR
Total capital investment TCI
Transcription, activator-like effector nuclease TALEN
Triacylglyceride TAG
Uridine diphosphate glucose pyrophosphorylase UGPase
Water footprint WF
Wax ester synthase diacylglycerol acyltransferase WS/DGAT
Zinc-finger nuclease ZFN
β- hydroxyacyl-ACP dehydrase HAD
β-ketoacyl-ACP reductase KAR
β-ketoacyl-ACP synthase KAS

then in manipulating and reconstructing microalgal metabolic networks, Lü et al. (2011) were the first to propose biofuels produced by metabolically engineered algae as the fourth generation of biofuel. Some researchers still use FGB for other technologies (Balwan and Kour, 2021); there is a general consensus in the literature that FGB is biofuels from genetically modified biomass (Aamer Mehmood et al., 2021).

However, the cultivation of terrestrial plants to produce first and second generation biofuel is raising concerns regarding their potential adverse impact on bioproductive land and freshwater supplies. Researchers worldwide have worked extensively to mitigate the drawbacks of production (Bórawski et al., 2019). Algal biomass is a promising feedstock due to its potential to be cultivated in non-arable or low-quality agricultural lands without access to potable water. Clarens et al. (2010) determined the impacts of algae production using a stochastic life cycle model and compared this to the impacts associated with switchgrass, canola, and corn farming. Algae cultivation was reported using land roughly 3.3, 4.3, and 5 times more efficiently than corn, canola, and switchgrass, respectively.

Third generation biofuels are made from microalgae, macroalgae, and cyanobacteria as biomass feedstock. Microalgae grow fast and have 20–300 times more oil contents than traditional biomass crops. A shorter harvesting cycle of microalgae than the biomass of first and second generation biofuels leads to greater yields. Comparative studies on the oil yields of microalgae and other biodiesel feedstocks from first and second generation sources indicated lower yields for (L/ ha) corn (172), soybean (446), canola (1190), jatropha (1892), coconut (2689), and palm oil (5950) than for microalgae biomass (58,700) with 30% oil content by weight (Chisti, 2007). CO_2 sequestration and high photosynthesis efficiency are additional advantages of using algae for third generation biofuel production (Bajpai, 2019).

Over 50,000 species of microalga are estimated to exist in aquatic and terrestrial environments, only 30,000 of which are studied and analyzed. The list of the most studied microalgae strains for third generation biofuel production and their lipid, protein, and carbohydrate contents—is shown in Table 1. Microalgae can be classified into four main groups based on their size: microplankton (20–1000 μm), nanoplankton (2–100 μm), ultraplankton (0.5–15 μm), and picoplankton (0.2–2 μm) (Sajjadi et al., 2018).

Microalgae are categorized into four main groups of molecules (lipids, carbohydrates, proteins, and nucleic acids), the proportions of which vary based on the microalgae class. Lipids are more energy-rich (8.99 kcals g^{-1}) than proteins (3.99 kcal g^{-1}) and carbohydrates (3.75 kcal g^{-1}). The main focus is increasing lipid content in microalgae (Hu et al., 2008).

Table 1
Properties of the most widely used microalgae in algal biofuel production (Abdullah et al., 2019).

Class /Microalgae strain	Lipids (%)	Proteins (%)	Carbohydrates (%)
Eustigmatophyceae			
Chlorella vulgaris	41–58	51–58	12–17
Chlorella sorokiniana	22–24	40.5	26.8
Chlorella pyrenoidosa	2	57	26
Chlorella protothecoides	40–60	10–28	11–15
Chlorella minutissima	14–57	47.89	8.06
Chlorophyceae			
Botryococcus braunii	25	–	–
Scenedesmus obliquus	30–50	10–45	20–40
Haematococcus pluvialis	25	–	–
Tetraselmis suecica	15–23	–	–
Scenedesmus dimorphus	16–40	8–18	21–52
Dunaliella salina	6–25	57	32
Dunaliella tertiolecta	11–16	20–29	12.2–14
Scenedesmus quadricauda	1.9	40–47	12
Bacillariophyceae			
Phaeodactylum tricornutum	18–57	30	8.4
Thalassiosira pseudonana	20	–	–
Cyanophyceae			
Spirulina platensis	4–9	46–63	8–14

The technoeconomic analysis of third-generation biofuel production has been reported in many research studies, and it was the focus of several economic assessments (Ianda et al., 2022; Kalavathy et al., 2022; Roles et al., 2021). The cost estimate in third generation biomass production facilities is generally carried out on the basis of capital and operational costs, which can be derived from biomass production and inoculum system, CO_2 delivery, water delivery, dewatering, and storage subsystems (Davis et al., 2016). Though the process design in the third generation and FGB are similar in CO_2 delivery and storage facilities, some differences exist in biomass production, water delivery, and dewatering processes, impacting the production cost. These differences are mainly attributed to the engineered strains' higher lipid and biomass

content and the diffusion risk of plasma and DNA of modified species (Beacham et al., 2017).

Large-scale commercial lipid production from microalgae faces challenges due to high cost and low productivity (Sun et al., 2019). It is now known that the congenital metabolic pathway in the wild-type microalgae species is not suitable for industrial fatty acid production. The ideal biodiesel should contain a well-balanced composition of saturated (mono-saturated and poly saturated) and unsaturated fatty acids for fuel efficiency as an alternative to fossil fuels (Coniglio et al., 2013). The hydrocarbon chain compositions and the saturation status or length of the biodiesel produced from fatty acids in microalgae are not ideal quality specifications (Muñoz et al., 2021).

Besides the incurred advantages of the manipulated strain, the discharge of the GM microalgal strains into surrounding water bodies may pose a potential risk that could lead to horizontal gene transfer and subsequent health and environmental concerns (Shokravi et al., 2021). Therefore, each process involved in the cultivation, harvesting, and processing of GM biomass must consider precautionary measures to ensure environmental sustainability and the conservation of natural resources. The challenges posed by each cultivation system are not equal. The enclosed cultivation system offers better control and minimized contamination risk but higher capital expense (Abdullah et al., 2019). Open raceway pond (ORP) has lower capital and operational costs than enclosed systems but is prone to leakage and dispersion by wind current or animal interference. ORP has stringent quality requirements that must be met before scale production (Hannon et al., 2010). Hence the effect of these considerations should be addressed in the process modeling and cost analysis of the FGB production. Specific remediation processes must be undertaken before wastewater discharge into the environment from the harvesting and dewatering systems. Nevertheless, the financial burdens of such control measures in FGB production should be calculated and justified. The present review is intended to fill this gap in the literature by addressing the absence of reviews on FGB commercialization. A summary of the most relevant literature on the commercialization of biofuels is presented in Table 2.

Several studies have been published on FGB; however, the focus in most of those works are on metabolic engineering and genetic modification of algal strains than other aspects such as bioeconomy. Studies like those of Aamer Mehmood et al. (2021) have mainly emphasized developing metabolically engineered microbial platforms, and the environmental and bioeconomy of the genetic modification was rather cursory, lacking in depth or specificity. Meanwhile, Dutta et al. (2014) provided a general discussion on the economy of first to fourth generations of biofuels for comparison purposes, while Meadows et al. (2018) investigated the role of the final product in commercializing metabolically engineered biofuels.

Many studies have reported different features of FGB. The main focus of these papers is the metabolic manipulation of algal strain to enhance the oil content and biomass yield. However, very few studies have considered the bioeconomy of the FGB and the perceived savings achieved due to promoted higher lipid and biomass yield. The incurred cost of applying preventive controls in producing FGB is also a topic that must be thoroughly investigated and accounted for before commercialization. Hence this study focused on the bioeconomic performance of FGB by discussing the techno-economic and socioeconomic factors. The effectiveness of genetic modification is mainly related to the manipulation target in the host microorganism. It could result in an enhancement in oil content and biomass yield or the increase in one may adversely affect the other. Hence it is of utmost importance to elucidate the mechanism which regulates the lipid accumulation and biomass yield and ensures the highest possible quantity and qualities. This paper addresses the gap in the literature by exploring the factors influencing bioeconomic assessments of FGB.

2. Essential factors in bioeconomy of FGB

The studies on the economic analysis of microalgae-based biofuels emerged that the oil and algal biomass yield are the main cost factors in commercial microalgal biofuel production, and their increase could positively impact the economic feasibility of the FGB (Aziz et al., 2020; Shokravi et al., 2020b). Higher lipid content and yield in microalgae lead to lower greenhouse gas emissions and higher energy gain in life cycle assessment (Delrue et al., 2012; Shokravi et al., 2022). Ponnusamy et al. (2014) indicated that a 10% increase in lipid contents of *Nannochloropsis salina* 1776 microalgae strain could bring the net energy ratio (energy produced/energy consumed) from less than 1 to greater than 1 while keeping other parameters constant, leads to greater energy outputs. Fischer et al. (2011) conducted a techno-economic analysis to obtain the viability of commercial microalgal biodiesel using net present value (NPV). They found that a 6.22% increase in lipid content could promote the probability of economic success from 50% to 90%. It was indicated that increasing biomass production from 0.122 g/L/day to 0.133 g/L/day could increase the economic success of the investment by 40%. Therefore the parameters in enhancing the lipid accumulation and biomass yeld of fourth generation biomass are discussed as following.

2.1. Enhancing lipid accumulation

Implementing metabolic engineering strategies to develop high-performance strains with enhanced lipid accumulation is invaluable

Table 2
Summary of the literature on the commercialization of biofuels.

Title	Challenges	Metabolic Engineering	Health and Environment	Bioeconomy	Authors/ References
"A critical perspective on the scope of interdisciplinary approaches used in fourth-generation biofuel production"	✓	✓	×	×	Godbole et al. (2021)
"Fourth generation biofuel from genetically modified algal biomass: Challenges and future directions"	✓	✓	✓	×	Shokravi et al. (2021)
"Developing fourth-generation biofuels secreting microbial cell factories for enhanced productivity and efficient product recovery; a review"	✓	✓	×	×	Malik et al. (2021)
"Advances in developing metabolically engineered microbial platforms to produce fourth-generation biofuels and high-value biochemicals"	✓	✓	✓	✓	Aamer Mehmood et al. (2021)
"Recent advances and future directions in plant and yeast engineering to improve lignocellulosic biofuel production"	✓	✓	×	×	Ko et al. (2020)
"Chapter 20 - The fourth generation of biofuel"	✓	✓	×	×	Moravvej et al. (2019)
"Fourth generation biofuel: A review on risks and mitigation strategies"	✓	×	✓	×	Abdullah et al. (2019)
"Metabolic engineering for advanced biofuels production and recent advances toward commercialization"	✓	✓	×	✓	Meadows et al. (2018)
"Evolution retrospective for alternative fuels: First to fourth generation"	✓	×	✓	✓	Dutta et al. (2014)
"Metabolic engineering of algae for fourth generation biofuels production"	✓	×	×	×	Lü et al. (2011)

for improving microalgae-based biodiesel's efficiency and industrial relevance (Sun et al., 2019). The lipid biosynthesis pathway is critical to producing oil in microalgae, and genetic modification of the lipid biosynthesis pathway could enhance biodiesel production's economic viability and ensure successful commercialization (Ranjbar and Malcata, 2022).

Lipids are microalgae's highly concentrated metabolic energy reserve, and their oxidation yield is around 38 kJ g^{-1} double that of carbohydrates (17 kJ g^{-1}). The lipid productivity in microalgae is higher than in traditional oil-bearing crops, such as corn, soybean, and palm tree, and can synthesize 58,700 L of oil per hectare (Chisti, 2007). The microalgal lipid yield varies based on the species, cultivation environment, and conditions (Chen et al., 2018). *Botryococcus braunii*, *Chlorella protothecoides*, *Nannochloropsis* sp., *Neochloris oleoabundans*, and *Schizochytrium* sp. are among the microalgae species with lipid above 50% of dry cell weight (Luangpipat and Chisti, 2017).

Microalgal lipids can be classified into polar and nonpolar lipids. Generally, 41–92% of total lipids in the microalgae are comprised of polar lipids, while it is 5–51% for nonpolar lipids (Courchesne et al., 2009). Glycolipid and phospholipid are polar lipids commonly found in the microalgae biomass membrane's cellular wall, which maintains the cell structure. Moreover, polar lipids with long-chain fatty acids can produce polyunsaturated fatty acids undergoing a series of metabolic reactions (Tang et al., 2020). Triacylglycerides and triacylglycerols (TAGs) are among the group of nonpolar lipids which do not have an electric charge in their molecular structure. Triacylglycerides comprise organelles and cellular membrane components, while TAGs are the physiological energy, carbon reservoir, and biodiesel precursor (Xue et al., 2021). The composition, occurrence and abundance of TAG in specific microalgae are regulated by its genetic makeup (Hu et al., 2008). Fatty acids are the main constituents of lipids in aquatic organisms, which may be composed of branched or straight carboxylic acids with long aliphatic chains. These aliphatic chains can be unsaturated or saturated, for instance, saturated, monounsaturated, and polyunsaturated fatty acids. Saturated fatty acids are essential in determining fuel properties (Tang et al., 2020).

Metabolic pathways in microalgae are categorized into several organelles (Kang et al., 2021). The CO_2 assimilated by the Calvin cycle in chloroplast, photosynthesis, glycolysis, and central carbon metabolism are used for synthesizing fatty acids. The synthesized fatty acids are generally stored as TAGs. The Kennedy pathway is the elementary metabolic process required for the TAGs accumulation in microalgae and plants, where TAG is produced through a sequential transfer of acyl groups from acyl-CoA to various positions (Lenka et al., 2016). Overexpression or inhibition of important enzymes that regulate lipid accumulation is generally used to increase TAG accumulation in algae. Diacylglycerol acyl transferase (DGAT) is the most studied enzyme to increase TAG synthesis. Deng et al. (2012) showed that overexpression of native DGAT2s in *C. reinhardtii* could increase neutral lipids up to 44%. Niu et al. (2013) characterized diacylglycerol acyl transferase type 2 (DGAT2) isoform in marine diatom *Phaeodactylum tricornutum* and found that DGAT2 overexpression augments the neutral lipid content up to 35%. It was observed that this overexpression could increase the polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA), up to 76.2%.

The lipid biosynthesis pathway is engineered by two main techniques: manipulating lipid biosynthesis and bypassing the regulation of lipid biosynthesis pathways (Godbole et al., 2021). Fig. 1 shows essential genetic modification approaches to improving lipid yield in microalgae.

2.1.1. Manipulating microalgal lipid biosynthesis

The algal chloroplast is the core metabolic pathway for carbohydrates, fatty acids, tetrapyrroles, and terpenoids biosynthesis; therefore, the metabolic engineering and modification of the chloroplast genomes is a routine method (Füssy et al., 2019). For example, microalgal chloroplast genomes in *Nannochloroposis* sp. possess approximately 120

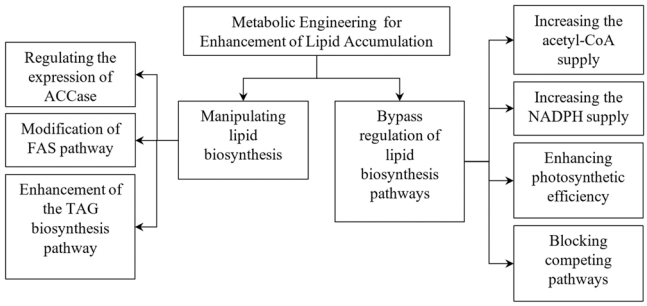

Fig. 1. Genetic modification approaches to improving lipid yield in microalgae.

genes involved in the photosynthesis of solar energy to convert atmospheric carbon to blocks of lipids (Lu et al., 2021). Lipid biogenesis in microalgae is an interconnected network of multiple metabolic pathways that begins with the carboxylation of acetyl-CoA to malonyl-CoA by ACCase (Ranjbar and Malcata, 2022).

2.1.1.1. Regulating ACCase expression. Acetyl-CoA carboxylase (ACCase) is a key regulatory enzyme for *de novo* TAG biosynthesis in chloroplast and cytosol, which was first isolated from the microalga *Cyclotella cryptic* (Zhang et al., 2018). Most fermentation culture improvements by induction stress conditions are attributed to upregulating ACCase (Sun et al., 2019). Rismani-Yazdi et al. (2012) and Li et al. (2014) found reduced ACCase genes' expression in *Nannochloropsis oceanica* and *Neochloris oleoabundans* under nitrogen deprivation conditions leads to a significantly increased lipid accumulation. Babu et al. (2017) showed that phytohormones supplementation under nitrogen limitation in *Chlorella sorokiniana* could substantially upregulate the intracellular levels of ACCase and consequently increase lipid productivity. Liu et al. (2011) found that a glucose carbon source could upregulate the ACCase gene in *Chlorella zofingiensis,* which may enhance the accumulation of fatty acids.

Gomma et al. (2015) showed that the fatty acid content of *Scenedesmus quadricauda* could increase up to 1.6-fold by overexpression of ACCase. ACCase overexpression in *Schizochytrium sp.* and *Dunaleilla salina* resulted in a 11.3% and 140% increase in fatty acid and lipid contents, respectively (Talebi et al., 2014). The kinetic characterization of the ACCases in algal strains and plants are similar. However, it was found that successful metabolic engineering strategies in plants and prokaryotes may not necessarily be amenable to microalgae (Blatti et al., 2013).

The overexpression of ACCase doesn't always guarantee increased lipid production, and several attempts to produce increased TAG accumulation through overexpressing ACCase protein have failed to achieve a satisfactory outcome (Bengoechea-Alonso and Ericsson, 2007). For example, Dunahay et al. (1996) reported that increasing the ACCase enzyme activity did not increase the accumulation of fatty acid in *Cyclotella cryptica* and *Navicula saprophila.* Zhang et al. (2014) indicated overexpression of heterologous *GmDof4* from soybean could upregulate the enzyme activity and expression of ACCase in transgenic *Chlorella ellipsoidea* cells. Efforts to increase fatty acid by upregulating ACCase were only modestly successful due to the complex regulation and interaction among controlling factors such as light, thioredoxin, phosphorylation, and PII protein.

2.1.1.2. Modifying fatty acid synthesis pathway. As fatty acids act as the building blocks of lipids, enhancing the fatty acid metabolic precursors such as acetyl-CoA and malonyl-CoA is considered the initial step in lipid biosynthesis (Marella et al., 2018). In fatty acid synthesis, the carbon sources are converted into pyruvate via glycolysis and further transformed into acetyl-CoA. Carboxylation of acetyl-CoA by ACCase produces malonyl-CoA is the primary carbon donor for the extension of the acyl-chain (Tian et al., 2013). In the next step of the fatty acid synthesis,

malonyl-CoA is converted to an acyl carrier protein (ACP), forming malonyl-ACP, which is catalyzed by the enzyme malonyl-CoA ACP transacylase (MAT) (Ranjbar and Malcata, 2022). A significant correlation was reported between transcript abundance of MAT and stress-induced fatty acid accumulation, so it was of great interest as a target for manipulating the synthesis of the fatty acid pathway (Sun et al., 2019). Lei et al. (2012) indicated that applying high temperature, high salinity, and nitrogen depletion could influence MAT gene expression in *Haematococcus pluvialis*. They showed that the high temperature (42 °C) and the combined salinity (Actinium and iron salts) could increase ACP expression by 8.7 and 9-fold rise, respectively, resulting in about a quarter increase in fatty acid accumulation. Chen et al. (2017) found that MAT's overexpression in *Nannochloropsis oceanica* increases dry weight lipid content by 36% more than the wild strain. Li et al. (2018) achieved 39.6% in total lipids yield by MAT overexpression in *Schizochytrium* sp. A schematic fatty acid synthetase pathway diagram is shown in Fig. 2.

The complex of fatty acid synthase enzyme in the chloroplast synthesizes the saturated fatty acids chains via a highly energy-intensive cyclical and incremental addition of malonyl-CoA (Hill and Alper, 2016). ACCase catalyzes the formation of MAT activated by ATP depletion, while in the enzymatic complex of fatty acid, the synthase acyl chain is formed by two carbon fragments derived from MAT. The saturated fatty acids chain formation process encompasses repetitive rounds of condensation, reduction, dehydration, and repeated reduction, which is conducted by β-ketoacyl-ACP synthase (KAS), β-ketoacyl-ACP reductase (KAR), β-hydroxyacyl-ACP, dehydrase (HAD), and enoyl-ACP reductase (EAR), respectively. KAS is the first enzyme in the fatty acid synthesis complex targeted to achieve higher lipid production (Naghshbandi et al., 2019). Records of upregulating genes encoding expression of MAT, KAS, HAD, and EAR in *Neochloris oleoabundans* under nitrogen-limited conditions are presented by Rismani-Yazdi et al. (2012). It was indicated that overexpression of the KAS or other subunits of the fatty acid synthesis complex does not always increase lipid production (Naghshbandi et al., 2019). For example, the KAS overexpression in *Phaeodactylum tricornutum* by Fan et al. (2018) did not induce any change in lipid contents.

2.1.1.3. Modifying TAG synthesis pathways. The Kennedy pathway in the chloroplast is the TAG synthesis pathway, in which acyl-CoA are converted into glycerolipids. In the Kennedy pathway, TAG is produced through sequential acylation of a glycerol-3-phosphate backbone by the contribution of the diacylglycerol acyltransferase (DGAT),

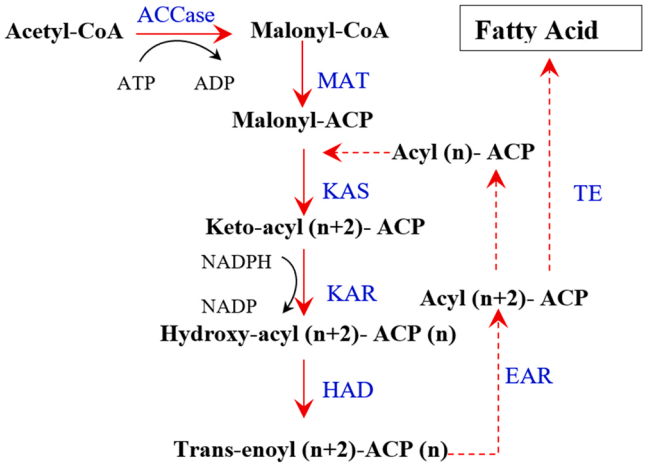

Fig. 2. Schematic diagram of the fatty acid synthetase pathway. ACCase: acetyl-CoA carboxylase; MAT: malonyl-CoA ACP transacylase; HAD: β-hydroxyacyl-ACP dehydrase; KAS: β-ketoacyl-ACP synthase; KAR: β-ketoacyl-ACP reductase; EAR: enoyl-ACP reductase.

dihydroxyacetone phosphate (DHAP), glycerol-3-phosphate acyltransferase (GPAT), glycerol-3-phosphate dehydrogenase (GPDH), lysophosphatidic acid acyltransferase (LPAT), and phosphatidic acid phosphatase (PAP) enzymes (Kang et al., 2021). Kennedy pathway is a target to increase the production of TAG and lipids. Yao et al. (2014) found that the overexpression of endogenous GPDH in oleaginous marine diatom *Phaeodactylum tricornutum* lead to 60% increase in lipid content. The overexpression of GPATs in *P. tricornutum* promoted two-fold higher TAG or neutral lipid contents (Niu et al., 2016). The overexpression of the LPAT led to a 2.4-and 2.8- fold improvement in the lipid and TAG productivities in *Neochloris oleoabundans*.

DGAT is the most studied enzyme involved in lipid synthesis, participating in the final acylation process in TAG production, committing a rate-limiting step. Diacylglycerol acyl transferase type-I (DGAT1), diacylglycerol acyl transferase type-II (DGAT2), diacylglycerol acyl transferase type-III (DGAT3), and wax ester synthase/acyl-coenzyme A (acyl-CoA): diacylglycerol acyltransferase (WS/DGAT) are the four types of DGAT in microalgae (Kang et al., 2021). Wei et al. (2017b); Wei et al. (2017a) overexpressed endogenous DGAT1 leading to a 47% and 2.4-fold increase in the yield and TAG content in *Nannochloropsis oceanica* (Li et al., 2016). The overexpression of the endogenous DGAT2 under nitrogen repletion and depletion conditions, respectively, increased TAG contents by 69% and 129% in *N. oceanica* (Li et al., 2016). Several studies showed that the DGAT2 overexpression in *P. tricornutum, N. oleoabundans* and *C. reinhardtii* enhance TAG and total lipid contents (Ahmad et al., 2015; Klaitong et al., 2017; Niu et al., 2013). It was found that WS/DGAT can utilize short and long-chain fatty alcohols as substrates.

2.1.2. Bypassing the regulation of lipid biosynthesis pathways

Blocking competitive pathways that catabolize lipids is another avenue that has been explored. Bypassing the lipid synthesis pathway regulatory could improve acetyl-CoA, nicotinamide adenine dinucleotide phosphate (NADPH), and photosynthetic efficiency or block competing pathways, which are discussed as follows.

2.1.2.1. Increasing of Acetyl-CoA. Acetyl-CoA is the central metabolite in fatty acid biosynthesis, malonyl-CoA-derived metabolism, isoprenoid biosynthesis, mitochondrial respiration, and various reactions of acetylation (Avidan et al., 2015). Several efforts to manipulate competing lipid biosynthesis pathways have been reported to improve lipid productivity in microalgae (Ranjbar and Malcata, 2022). It was also shown that the acetyl-CoA content in oleaginous microalgae is significantly higher than in moderate oil-producing strains (Avidan et al., 2015). Acetyl-CoA synthetase, pyruvate dehydrogenase complex, and adenosine triphosphate (ATP) citrate lyase are the primary sources of acetyl-CoA supply in microalgae. Upregulating and overexpressing these acetyl-CoA suppliers enhanced lipid accumulation and synthesis in microalgae (Yan et al., 2013). In microalgae, the pyruvate dehydrogenase kinase enzyme inhibits the activity of the pyruvate dehydrogenase complex, resulting in a decrease in lipid content. Ma et al. (2014) indicated that antisense knockdown of pyruvate dehydrogenase kinase enzyme in *Phaeodactylum tricornutum* increased lipid content up to 82% of cell dry while keeping the biomass yield nearly constant.

2.1.2.2. Increasing the NADPH. NADPH is a crucial reducing equivalent needed for lipid production. Lipids are highly reduced metabolites, and their biosynthesis requires a high amount of reducing power sourced by a constant supply of NADPH to reduce acetyl groups into the growing fatty acid acyl chain (Xue et al., 2017). The pentose phosphate pathway (PPP) generates NADPH, and the transhydrogenase cycle, plays a critical role in maintaining the NADPH biosynthetic capability (Kang et al., 2021). Fatty acid biosynthesis demands 14 NADPH molecules as a reducing co-factor to produce one molecule of palmitoyl-CoA from acetyl-CoA (Marella et al., 2018). enzymatic activity of the malic

enzyme (ME) is linked with NADPH, and it was shown that Overexpression of cytosolic ME elevated NADPH content and lipid content in engineered *P. tricornutum* (Xue et al., 2015). A 3.2-fold increase in lipid content in engineered *Chlorella pyrenoidosa* was achieved by heterologous expression of ME (Xue et al., 2016).

The couping of the PKS pathway with the PPP pathway was shown to supply NADPH for the polyunsaturated fatty acids biosynthesis. The transhydrogenase system could be combined with the fatty acid synthesis pathway to supply NADPH for saturated fatty acid biosynthesis (Sun et al., 2019). The overexpression of the G6PDH gene in *Aurantiochytrium* sp. increased NADPH via the PPP pathway, enhancing the polyunsaturated fatty acid contents by 10.6% (Cui et al., 2016). Increasing the supply of NADPH and acetyl-CoA is a common metabolic engineering approach that has shown higher potential in improving lipid accumulation in microalgae among the methods based on bypassing the regulation of lipid biosynthesis pathways.

2.1.2.3. Enhancing photosynthetic efficiency. The photosynthetic efficiency in wild microalgae strains is a function of the light intensity and duration, and the growth rate rises in proportion to light intensity until saturation. Enhancing photosynthetic efficiency could increase microalgal biomass yield and lipid accumulation. Photosynthesis supplies reducing power force triggering the synthesis pathways and provides both assimilated carbon sources for lipid biosynthesis to be used as an indirect approach (Park et al., 2019). The complexes of light-harvesting antenna are the primary component in capturing and transferring light energy to the reaction center (Nagao et al., 2013). It is speculated that reducing the light-harvesting antenna could increase photosynthetic efficiency; hence, it is used as a platform, combined with other promising targets, to improve lipid contents in microalgae (De Mooij et al., 2015).

Up to a point, an increase in illumination stimulates growth by providing the required energy for exciting electrons in the light-harvesting complex in the chloroplast. Once the absorption of light by chlorophyll surpasses the photosynthetic capacity, growth is progressively inhibited. Therefore, photoinhibition causes the cells in external layers to have low photosynthetic efficiency due to excessive light exposure, while most other cells receive lower illumination needed for their growth (Carvalho et al., 2011). Reducing the photosystem antenna could enhance light distribution and biomass productivity (Ranjbar and Malcata, 2022). A 65% reduction of chlorophyll antenna size in *C. reinhardtii* resulted in improved photosynthetic efficiency (Kirst et al., 2012) and greater solar conversion efficiencies under mass culture conditions (Polle et al., 2003) compared to wild-type. RuBisCO is the crucial enzyme responsible for carbon dioxide fixation (Zhou et al., 2020). Wei et al. (2017a); Wei et al. (2017b) showed that RuBisCO activase overexpression could increase biomass and lipid content in *Nannochloropsis oceanica*. Increasing RuBisCO activity could increase carbon assimilation through the Calvin cycle.

2.1.2.4. Blocking competing pathways. Another effective strategy in metabolic engineering is blocking competing metabolic pathways. Absolute inhibition of competing pathways is unsuitable for cell growth, and the target of blocking competing pathways is to reduce the activity of the targeted site. Lipid catabolism and carbohydrate biogenesis share the same carbon precursors with lipid biosynthesis (Park et al., 2019). Many microalgae strains use carbon sources for carbohydrate synthesis as the primary storage metabolite. Thus, the carbohydrate synthesis pathway's blocking can drive the metabolic carbon flux towards lipid accumulation. Uridine diphosphate -glucose pyrophosphorylase (AGPase) is the predominant enzyme for catalyzing carbohydrate synthesis (Sun et al., 2019). Inactivation of AGPase in *C. reinhardtii* increased lipid accumulation during nitrogen starvation (Work et al., 2010). Li et al. (2010a) reported a 10-fold increase of TAG contents in *Chlamydomonas* mutant with inactivated AGPase.

UGPase is a rate-limiting enzyme for the accumulation of chrysolaminarin that contributes to carbon allocation in an algal cell (Hong et al., 2016). Suppression of UGPase in a diatom could decrease the chrysolaminarin content while promoting lipid overproduction. Zhu et al. (2016) showed that a 69% decrease in UGPase activity in *P. tricornutum* led to 4.89 fold reduction in chrysolaminarin biosynthesis. Daboussi et al. (2014) found that disruption of the UGPase gene increased the TAG contents in the *Phaeodactylum tricornutum* strain by 45 fold. Reducing the lipid catabolism rate is another option for promoting higher lipid biosynthesis; however, disrupting lipid catabolism also may decrease biomass production and growth (Chu, 2017). Another competing pathway comprises the reaction that converts phosphoenolpyruvate into pyruvate or oxaloacetate. By engineering the metabolite, the phosphoenolpyruvate is converted into oxaloacetate only through the tricarboxylic acid cycle (Ng et al., 2017). Consequently, many reports have shown that the knockdown of the phosphoenolpyruvate carboxylase gene could enhance lipid content (Deng et al., 2014; Tian et al., 2014; Yang et al., 2016).

2.1.3. Challenges of metabolic engineering

Although data from metabolic engineering studies in microalgae are limited compared to unicellular organisms such as bacteria and yeast, recent development in genetic engineering tools and available data for omics accumulation has facilitated advanced metabolic engineering in FGB production (Brar et al., 2021). Numerous techniques have been used to overexpress certain microalgaes' metabolic or regulatory genes by transforming the nucleus, chloroplast and mitochondria to improve biomass and biofuel production in industrial microalga (Godbole et al., 2021). However, finding efficient methods to knock down or knock out unwanted genes remains a challenge in microalgae's genetic engineering (Shin et al., 2016).

Unlike unicellular organisms such as bacteria and yeast, the cells in microalgae are diploid, and conventional genetic engineering tools such as homologous recombination and episomal plasmid expression may lead to the significant genetic variability of progeny. Although the report of specific disruption of genes in knockout based on zinc-finger nuclease (ZFN) and transcription, activator-like effector nuclease (TALEN) are few, these techniques are very hard to achieve in most microalgae (Shin et al., 2016). Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) have been successfully applied to heterologous genome editing (Chang et al., 2020; Nguyen et al., 2020; Shin et al., 2019). Deployment of CRISPR/Cas9 has enabled a more systematic metabolic engineering, producing more stable transformants with improved lipids. However, using this system for a knockdown in microalgae has proven difficult. Jiang et al. (2014) tested Cas9 for targeted gene disruption in *Chlamydomonas reinhardtii* and observed extremely low targeting efficiency.

Recently sophisticated synthetic biology tools, which include coordinated expression of several transgenes to identify the molecular regulatory circuits, metabolic fluxes, sensing and transferring redox signals, have been widely used in metabolic engineering (Larrea-Alvarez, 2018). Sophisticated metabolic engineering could impart many benefits, such as fine control of metabolic fluxes, robust control of regulatory circuits, generating more efficient enzyme cascade reactions, creating metabolic reactions that do not exist in nature, and many others (Lee, 2012). However, examples of sophisticated metabolic engineering to microalgae are limited, mainly due to having several organelles with many gene homologs in microalgal metabolic pathways (Kang et al., 2021). A summary of studies conducted dealing with manipulating lipid biosynthesis is presented in Table 3.

2.2. Enhancing biomass yield

Enhancing biomass yields for industrial applications is desirable due to minimizing culture space and reducing extraction and downstream processing expenses (Wan et al., 2011). Increasing biomass yield plays

Table 3
A summary of studies conducted dealing with manipulating lipid biosynthesis.

Approach	Species	Improvement	Reference
Regulating the expression of ACCase	*Scenedesmus quadricauda*	1.6-fold increase in fatty acid content	Gomma et al. (2015)
Modification of the fatty acid synthase pathway	*Haematococcus pluvialis*	24% increase in total fatty acids	Lei et al. (2012)
	Nannochloropsis oceanica	36% higher lipid contents	Chen et al. (2017)
	Schizochytrium	39.6% higher lipids yield	Li et al. (2018)
Enhancement of the TAG biosynthesis pathway	*Chlamydomonas reinhardtii*	50% increase in TAG content	Iskandarov et al. (2016)
	Phaeodactylum triornuum	57.5% increased lipid content in dry weight	Zou et al. (2018)
	Chlamydomonas reinhardtii	44.5% higher lipid content	Wang et al. (2018)
Increasing the acetyl-CoA supply	*Schizochytrium sp.*	11.3% higher fatty acid	Yan et al. (2013)
	Yarrowia lipolytica	60-fold increment in lipid accumulation	Blazeck et al. (2014)
Increasing the NADPH supply	*Mucor circinelloides*	2.5-fold increase in lipid content	Zhang et al. (2007)
	Phaeodactylum tricornutum	57.8% increase in lipid content of dry weight	Xue et al. (2015)
	Chlorella pyrenoidosa	3.2-fold enhanced neutral lipids up to	Xue et al. (2016)
	Crypthecodinium cohnii	20% improved DHA productivity	Liu et al. (2015)
	Aurantiochytrium sp.	10.6% enhancement in the percentage of PUFAs in total lipids	Cui et al. (2016)
	Phaeodactylum tricornutum	2.7-fold increase in lipid content	Xue et al. (2017)
Enhancing photosynthetic efficiency	*Synechococcus* sp. PCC 7002	3-fold increase in free fatty acid production	Ruffing (2014)
	Nannochloropsis oceanica	41% increase in lipid productivity,	Wei et al. (2017b); Wei et al. (2017a)
Blocking competing pathways	*Chlamydomonas*	A 10-fold increase in TAG	Li et al. (2010a)
	Chlamydomonas	3.5-fold higher lipid	Li et al. (2010b)
	P. tricornutum	24.58% increment in lipid accumulation	Zhu et al. (2016)
	Thalassiosira pseudonana	3-fold increment in TAG yield	Hildebrand et al. (2017)
	Chlamydomonas reinhardtii,	74.4%enhance lipid content	Kao and Ng (2017)

an important role in enhancing the energy density and total energy contents of FGB. Several factors govern biomass productivity, including CO_2 fixation, abiotic stress, and light utilization (Muthukrishnan, 2022). Enhancing biomass yield without negatively affecting lipid accumulation is an important strategy in the genetic modification of algal strains, which the following techniques can achieve.

2.2.1. Enhancing CO_2 fixation

Manipulation of CO_2 assimilation is critical to improving microalgae's photosynthesis rate. Photosynthetic carbon fixation in photosynthetic organisms takes place in the Calvin pathway (Sharma et al., 2018). Calvin cycle could be divided into three main steps of carbon fixation, reduction, and regeneration, that supply precursors for the biosynthesis of carbohydrates by consuming NADPH and ATP during photosynthesis (Andrade et al., 2021). The strategies for improving the photosynthetic efficiency required a breakthrough to regulate the Calvin pathway. Rubisco is the key target enzyme of the Calvin cycle in chloroplasts, significantly influencing the carbon assimilation rate. However, direct manipulation of Rubisco has had limited success due to the complexity of the Rubisco enzyme kinetics (Tcherkez et al., 2006).

Therefore, efforts were made by several researchers to shift the focus from direct manipulation of the enzyme itself to targeting factors that regulate Rubisco activity. Rubisco activase plays a pivotal role in regulating CO_2 by regulating the activity of Rubisco (Hazra et al., 2015). SBPase and Aldolase enzymes also improve carbon fixation due to their role in the regeneration of precursor substrates resulting in enhanced biomass production.

2.2.2. Enhancing stress tolerance

Improving stress tolerance through microalgal engineering strains can ensure the cost-effective production of biomass (Sharma et al., 2018). Kotchoni et al. (2016) designed an RNAi-mediated gene knockdown of adenosine monophosphate deaminase in *C. reinhardtii* to generate algal strains capable of being grown in cold temperate climates. It was found that genetic manipulations displayed ~3-fold enhanced biomass, growth rate, and CO_2 assimilation compared to wild type. Carbohydrates contribute to maintaining a high photosynthetic rate; hence, biomass loss could be observed in mutants with low levels of hydrocarbons. The availability of inorganic carbon in the vicinity of Rubisco is an important abiotic factor that influences the rate of carbon fixation (Wang et al., 2011).

2.3. Enhanced photosynthetic efficiency

The availability of photon energy is an abiotic factor influencing carbon fixation efficiency. The size of photosynthetic antenna systems in photosynthetic microalgae is large to maximize the absorption of the photons; however, large antenna pigments limit light's penetration into the culture's deeper layers, reducing biomass yield (Formighieri et al., 2012). Reducing the number of chlorophyll molecules in the light-harvesting complex is a solution to improve light transmission and absorption capacities in microalgal cells. Mutants with truncated antenna systems increased biomass production and, consequently, reduced production costs downregulating the genes encoding pigment binding proteins (Sharma et al., 2018). However, the shrunk antenna system is susceptible to photodamage due to exposure to intense solar radiation (De Mooij et al., 2015).

Selecting an appropriate oil-rich high-yield indigenous algal strain is essential for large-scale biodiesel production. The genetic engineering methods used to enhance microalgal organisms' growth profile, lipid content, and fatty acid profiles are species-specific. There is no fixed protocol available for all microalgae. The green alga *Chlamydomonas reinhardtii* is a single-celled photosynthetic model that has emerged as the most studied microalgal species for enhancing lipid content through genetic modification. Choosing proper selectable marker genes for screening transgenic algae is crucial to genetic manipulation in microalgae.

3. Socioeconomic analysis

The term socioeconomic has to do with the interactions between economic activity and the social aspects of people's lives. The balance between social and economic indicators is of the utmost importance for developing a sustainable system. The success of a commercial product could be negatively affected when there is an imbalance between two social and economic indicators, and it can cause a significant loss of market value. Hence, socio-economic indicators should be investigated before a product is commercialized. Profitability, social well-being, resource conservation, and social acceptability are the main socio-economic factors related to the production of FGB.

3.1. Profitability

The profitability of a nascent industry is a vital sustainability measure related to operating revenues. Profitability indicators resent the effects of capital costs, inflation, and non-cash items so that it can be determined whether a type of technology should be scaled up and commercialized (Bayai and Ikhide, 2016). Techno-economic analysis and NPV are the most important profitability indicators (Charoensiddhi et al., 2018). Vardanega et al. (2017) reported that algal biofuel projects with 2–5 years of payback time with an NPV > 0 are feasible.

Implementing commercial-scale algal biofuel production is a prerequisite for estimating a reliable cost model. The information on techno-economic analyses can be used to evaluate and compare the costs and benefits of multiple projects, technologies, or facilities (Quinn and Davis, 2015). Information obtained from techno-economic analyses can also be used to evaluate whether production targets are being achieved (Davis et al., 2016), identify major contributors to cost (Slade and Bauen, 2013), and evaluate the economic feasibility of upscaling (Charoensiddhi et al., 2018).

3.2. Social well-being

Potential occupational hazards specific to the cultivation of FGB biomass can be classified into four groups: antibiotic resistance, allergies, carcinogens, and pathogenicity (or toxicity). GM organisms can produce allergenic molecules or act as allergens themselves (Genitsaris et al., 2011; Mandel, 2003; Menetrez, 2012). It was reported in 2004 and 2005 that hundreds of farmers in India exposed to Bt cotton suffered from allergy symptoms (Ho, 2006a). Modified cells are exposed to antibiotics to protect them against foreign DNA during insertion. As the cells continue to express the antibiotic-resistant gene, the antibiotics may transfer to other organisms or into food consumed by humans. Bacterial resistance may increase due to this chain of events (Mandel, 2003).

Carcinogenic substances specific to algae may lead to the development of cancerous tissue in the human body (Menetrez, 2012). Moreover, GM organisms may introduce or increase the presence of pathogens or toxins that may harm humans (Snow and Smith, 2012). Several reports have been published on this topic. For instance, in 2005, scientists found a protein in a transgenic pea that caused inflammation in the lungs of mice and provoked other sensitivities (Ho, 2006b). In another study, affected cells were observed in the pancreases of young mice fed GM soya (Vecchio et al., 2009).

3.3. Risk of catastrophe

The FGB biomass production could cause a catastrophe through its intentional and unintentional release into surrounding water and land bodies. Potential catastrophe risks related to the release of GM algae can be classified into four groups: competition with native species, changes in the natural habitat of protected species, toxicity, and horizontal gene transfer (Hewett et al., 2016). Nonnative species can harm native communities due to their propensity to invade and spread. The situation is worse when genetically enhanced species are involved, as these species have improved assimilation, growth, resistance, and product characteristics. Hence, the potential for GM algae to cause harm in this manner should be thoroughly assessed before such algae are dispersed from their open or contained cultures (Adeniyi et al., 2018). Cultivation of GM algae has stringent quality requirements that must be met before large-scale production is feasible (Hannon et al., 2010). This subsection discusses the microalgal biomass production's environmental and health impact.

3.3.1. Cultivation systems

PBRs and open raceway ponds ORPs are two predominant cultivation systems employed in commercial microalgal biomass production. Although several studies have explored FGB production, limited data are available on the cultivation, harvesting, storage, and transportation of GM algae biomass in pre-commercial or commercial-size facilities. Cultivation systems of various sizes, configurations, and designs are used for microalgae biomass production, and the challenges posed by each system are not equal (Puri et al., 2015). Enclosed cultivation systems offer optimal control and minimize contamination risk, but they have a higher capital expense (Abdullah et al., 2019). GM algae strains could enhance the yield and quality of biofuel, resulting in the commercial sustainability of FGB production.

According to an economic assessment by AquaFUELS, the production price of tubular photobioreactors is $13.8/kg dry biomass. At the same time, the raceway cultivation cost for the same product is about $2.5/kg of dry biomass (Garofalo, 2009). Norsker et al. (2011) calculated the biomass production costs for three commercial-scale microalgal production systems—ORP, horizontal tubular PBRs, and flat-panel PBRs—assuming a 100-hectare facility to be €4.95, €4.16, and €5.96 per kg, respectively. Other cost estimates were reported in the literature, such as Chisti's (2007) estimated costs of ORPs and PBRs biofuels of $3.80/gal and $2.95/gal in a facility with a capacity of 10 tonnes/yr. Davis et al. (2011) calculated MSPs of $8.52/gal and $18.10/gal for algal lipids from ORPs and PBRs to reach an internal rate of return (IRR) of 10% in a facility producing 10 tonnes/yr, while Richardson et al. (2012) indicated that ORPs have a lower production cost ($12.74/gal) than PBRs ($32.57/gal) in a facility producing 10 tonnes/yr. Richardson et al. (2014) estimated total production costs of $109/gal and $77/gal for ORP and PBR, respectively, emphasizing that neither system sufficiently supports economic success based on current technology and prices.

PBR exhibited higher productivity and photosynthesis efficiency and, thus, had lower production costs. However, the investment costs for PBR are higher than for ORP. Huntley et al. (2015) considered large-scale demonstration PBRs and ORP facilities to produce two diatoms *Staurosira* and the chlorophyte *Desmodesmus* marine microalgae strains. The results were used to evaluate the performance of a 100-ha commercial facility, assuming it was built in 2015. The techno-economic analysis and life cycle assessment results indicated that a biomass yield of 100 MT/ha/yr and an algal lipid yield of > 50, 000 L/ha/yr has to be achieved for algae production to become a viable investment. Trostle (2010) conducted a cost estimation to reach 100 MT/ha of NPV/yr. The study showed that a lipid concentration of 35% by weight requires a capital cost of US$112,400/ha and an operating cost of US$39,000/ha (Datta, 2012).

Matsuwaki et al. (2015) studied the diffusion risk of a GM *Pseudochoricystis ellipsoidea* MBIC 11204 strain from an ORP into the surrounding environment. It was reported that *P. ellipsoidea* sequences were detected at a considerable distance from the ORP. Wind currents and leakage were the factors most responsible for spreading algae strains from the cultivation pond. Different control options must be considered when designing ponds for cultivating GM algae to reduce diffusion risks. Using lining to control leakage and using air-supported plastic hoop greenhouses are among the preventive steps that have been taken to reduce this risk (Abdullah et al., 2019). Lundquist et al. (2010) proposed enclosing a cultivation system using a plastic hoop air-supported greenhouse, and it was estimated that covering an ORP in this way would cost $142.

The plastic lining of ORPs is a notable capital cost in any commercial microalgae production system. Nearly all existing algae cultivation ponds are plastic-lined (Davis et al., 2016; Musa et al., 2019). Percolation prevention and enhanced biotic environment control are the main advantages of plastic liners (Benemann et al., 1987). Manufactured liners used to line algae ponds fall into two categories: durable and economical. Durable liners cost nearly $60,000/ha, which translates to roughly $0.5 per kg of algal biomass. However, the extra initial cost is compensated for by reduced repair and maintenance costs and the longer lifetime of the lining system (Benemann et al., 1987). Leidos

estimated that installing lining in a cultivation area costs $30,626/acre (Davis et al., 2016). Davis and Wiatrowski (2020) indicated that the fully lined open raceway pond could cost up to 25% more than unlined ponds.

3.3.2. Water supplies and recycling

Ensuring safe and clean water supplies is an overriding global demand due to freshwater scarcity caused by global warming and pollution. Hoekstra (2003) first introduced the concept of water footprint (WF) to address how much water is consumed and lost in the supply chain (Patzelt et al., 2015). WF is assessed by calculating the type of pollution and the amount of polluted water (Aldaya et al., 2012). Reducing the water footprint in the production process is of the main objectives of a sustainable biofuel system (Quiroz, 2021). Wu et al. (2012) were the first to propose a life cycle water analysis framework using a standardized water footprint methodology to assess blue water, green water, and agricultural grey water discharge in biofuel feedstock production. Green and blue water footprints are the consumed volumes of rainwater and groundwater used in production. Grey water footprint is calculated by quantifying the freshwater required to dilute polluted water into the freshwater quality standard. The feedstock used for biofuel (Holmatov et al., 2019), the energy extraction process (Gerbens-Leenes, 2018), and the final biofuel (Amundson, 2016) are important parameters in the water footprint of the produced biofuel.

The discharged wastewater from dewatering and harvesting facilities is conveyed into a treatment sump through a drainage channel system and disposed of during the following treatment. The filtrate from the treatment process is recycled into the cultivation ponds as blue WF. The culture medium's discharge from the cultivation of GM algal biomass could pose significant health and environmental risks. Therefore, specific remediation must be undertaken before the discharge of wastewater into the surrounding ecosystem. The WF concept in the exploitation of FGB is illustrated in Fig. 3.

Recycling and reusing discharged wastewater effluents from the harvesting step reduce freshwater consumption (González-González et al., 2018). Thus, nutrient-recycling this discharged wastewater could enhance economic efficiency by minimizing material input (Lowrey et al., 2016). Following best practices, an ideal recycling system would produce zero grey WF waste (Aziz et al., 2020). However, limited recycling of the culture medium can be done, and the remnant should be disposed (Crofcheck and Crocker, 2016).

Using wastewater instead of freshwater is a promising method to cultivate microalgae due to its high levels of nutrients. Using wastewater to cultivate algae can make FGB production feasible and sustainable (De Francisci et al., 2018). Makeup water from nearby surface or groundwater resources is sent to the supply system through aqueducts and pipelines. Meanwhile, the medium drained from the harvesting stage is sent to channel networks and routed back to settler ponds by harvest pumps (Davis et al., 2016). Pacific Northwest National Laboratory's biomass assessment tool was developed to calculate the potential biomass and oil yield, along with the required ranges of important upstream resources (Coleman et al., 2014). Applying biomass assessment tools coupled with water scarcity quantifier software, such as the available water remaining system, can provide a useful image of the water used for a wide range of production and biomass yields in techno-economic analyses and life-cycle assessments of FGB (Xu et al., 2019).

Recycling water and nutrients and reusing discharged residue water from the harvesting process could reduce freshwater consumption and enhance the economic efficiency of the process. Ideally, such recycling and reusing will produce zero grey WF. However, discharged water from harvesting can be reused only a limited number of times and must be disposed of safely and environmentally friendly.

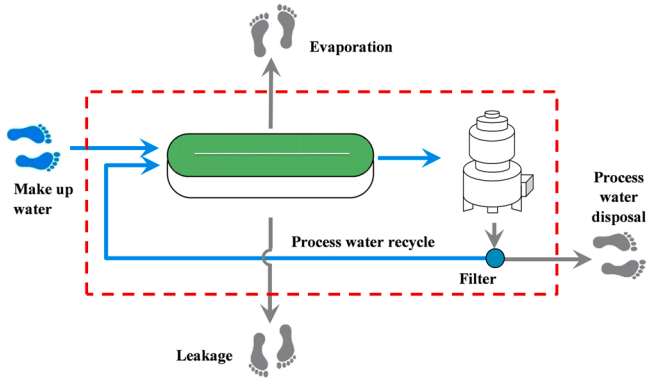

Fig. 3. The concept of water footprint (WF) in the exploitation of fourth generation biofuel (FGB).

3.3.3. Diffusion Risk

Releasing toxic microalgae strains into the environment could have severe societal effects and devastate human and animal health (Assunção et al., 2017). Concerns about toxicity have been raised based on the invasive *Alexandrium minutum* toxic algae, which caused blooms in 1985 (Beacham et al., 2017). Horizontal gene transfer is the mechanism by which materials from one organism to another are transferred in a non-genealogical manner (Goldenfeld and Woese, 2007). Cyanobacteria are attractive candidates for biofuel production, owing to their simple nutrient requirements and fast cell growth. These properties, however, mean that cyanobacteria are likely to cause horizontal gene transfer among cyanobacterial taxa, cyanobacteria, and eukaryotic algae species (Snow and Smith, 2012).

An objection was submitted to the regulatory authorities in Hawaii against the intended outdoor large-scale cultivation of GM *Chlamydomonas reinhardtii* algae. It was found that the released DNA persisted in the environment and was the leading cause of horizontal gene transfer among bacteria (de Vries et al., 2001). Matsuwaki et al. (2015) conducted a study to assess the invasion risk of the uncontained cultivation of GM *Pseudochoricystis ellipsoidea* microalgae. The spread of GM cells into the surrounding area was investigated for over 35 days. The results show that the psbA gene of *P. ellipsoidea* was detected in designated vessels even when they remained 150 m from the cultivation pond. In this case, wind was cited as the primary transport mechanism of the psbA genes into the environment.

The exchange of DNA among organisms of different species through horizontal gene transfer is one of the greatest concerns related to adopting GM biomass for FGB production (Jain et al., 2002). Therefore, the residue obtained from the energy extraction process and the water diffused from the harvesting of GM biomass should be disposed of carefully. Doing so will prevent horizontal gene transfer by exchanging transgenic plasmid or chromosomal DNA (Abdullah et al., 2019; Heidarrezaei et al., 2020; Shokravi et al., 2020a).

3.4. Social acceptability

For an energy strategy to be successful, it must balance the conflicting interests of economic growth and ecosystem health. In recent decades, the genetic modification of plants has become controversial, and conflicts between commercial interests and environmental concerns have escalated (Müller, 2004). The fast-growing market of GM products has provoked considerable opposition from consumers since its introduction in 1994. The advent of GM products in countries like Argentina and the US was followed by a failure to monitor their post-release impact on human health and the environment.

Furthermore, labelling GMO products was not compulsory when such products were new, and, as a result, no records were kept to trace product consumption. Two-fold (up to 10-fold) increases were found in food-related diseases in 1999 compared to the amounts recorded in a survey implemented before the advent of GMO products. This

observation resulted in public regulatory resistance in several countries, including many EU nations (Ho et al., 2007). As a way of dealing with concerns about the social acceptability of FGB, the statistics on the damage to humans and ecosystems caused by GM biomass should be available to the public. It would increase people's awareness of the benefits and risks associated with GM biomass. Table 4 shows a summary of the socio-economic challenges hindering FGB production.

Striking a balance between social and economic factors related to FGB production is crucial for ensuring the successful development of FGB in a competitive business environment. Moreover, businesses will not be protected from risks related to fraud and deception until the appropriate regulations are in place. The presence of gaps overlaps, and conflicts regarding regulatory coverage lower the security of investments in FGB.

4. Techno-economic analysis

When studying the economic performances of biofuel production facilities, certain operating and capital expenses are incurred and must be determined by defining relevant cost factors (Demirbas, 2009). The techno-economic analysis is the primary tool used by researchers to assess the economic performance of a project throughout its lifetime (Comodi et al., 2017). It provides critical information with which researchers can identify process bottlenecks, guide systems' operations and design specifications, and compare the costs of using different technologies (Sheets and Shah, 2018).

Recently, Rothamsted Research and Plymouth Marine Laboratory undertook projects to study the large-scale cultivation of genetically engineered P. *tricornutum* strains (D'Adamo et al., 2019). Also, a joint venture between Sapphire Energy and UC San Diego is currently being conducted to study the commercialization potential of GM biomass (Beacham et al., 2017). The economy of scale could be achieved by employing an efficient model and designing appropriate facilities. Patel et al. (2021) calculated the capital investment and operational cost needed for a large-scale system producing biodiesel using *Dunaliella tertiolecta* isolates as biomass feedstock. The operating cost for producing biofuel from *D. tertiolecta* strain with a 1.244 g/L dry biomass yield and 37%w/w lipid content was 3.19 $/L. The operating cost was reduced to 0.77 $/L when using natural seawater as the growth medium in large-scale facilities. The NPV was $ 750.91 for an ORP built in an area of one hectare with a 5.18% IRR considering a 10-year investment payback period (PBP). PBP, NPV, and IRR are typical benchmarks used to validate the economic viability of an investment in the long run. Biomass productivity can be increased by 50% by upscaling production five times and increasing the NPV to $ 30,355. Other important parameters considered when calculating NPV include total capital investment, discounted cash analysis (DCA), and minimum selling price (MSP) (Aziz et al., 2020). Fig. 4 illustrates the procedure used to calculate NPV and perform the sensitivity analysis.

Biofuel products and by-products vary from gas to solid, including bioethanol, biodiesel, biohydrogen, liquid biocrude, and biochar. The techno-economic performances of biofuels differ significantly due to differences in final products, models, and economic assumptions (Li, 2018). The market value of biofuel and its by-products is essential to determining the production system's economic feasibility. Lee (2016) evaluated the economic feasibility of some emerging biofuel productions based on market values. Bioethanol is the most widely used type of biofuel. Ahmed et al. (2021) reviewed various techniques to enhance microalgae-based biohydrogen production and the associated costs. They found that the cost of producing biohydrogen ranges from $1.42 kg^{-1} to 7.61 kg^{-1}, which is higher than the cost of other energy sources. Beattie et al. (2021) conducted a techno-economic analysis and life cycle assessment on genetically modified *Synechocystis* sp. PCC 6803 in biorefinery facility to produce biofuels and the oleochemical co-product. The cultivation and separation of the oleochemical, harvesting, and fuel processing stages were carried out at the facility. The cultivation pond was assumed to be covered to prevent the release of GM cyanobacteria biomass. The MSP of the fuel generated by the integrated biorefinery facility was calculated at $2.47 $(dm^3)^{-1}$, while the actual value was $2.01 $(dm^3)^{-1}$.

Table 4
Socioeconomic challenges hindering FGB production.

Challenges	PI	SWI	RCI	SAI	Indicator	Ref.
Strain selection	✓	–	–	–	NPV	Beacham et al. (2017)
Cultivation of GM algae	–	✓	–	✓	Workdays lost due to injury, risk of catastrophe, and public opinion	Beacham et al. (2017)
Potential pond crashes and GM algae diffusion	✓	✓	–	✓	NPV, risk of catastrophe, workdays lost due to injury, and public opinion	Snow and Smith (2012)
Coproducts of GM algae cultivation	–	✓	–	✓	Risk of catastrophe, workdays lost due to injury, and public opinion	Abdullah et al. (2019)
Water footprint	–	✓	–	✓	Risk of catastrophe, workdays lost due to injury, and public opinion	Abdullah et al. (2019)
Potential occupational hazards	–	✓	–	–	Workdays lost due to injury	Ho et al. (2007)
Susceptibility to natural disasters	–	✓	–	✓	Risk of catastrophe, workdays lost due to injury, and public opinion	Charoensiddhi et al. (2018)

* PI: profitability indicator; SWI: social wellbeing indicator; RCI: resource conservation indicator, and SAI: social acceptability indicator.

5. Future prospects

The main potential benefits of using GM algae biomass are the increased yield, growth rate, and tolerance of microalgae species. The market opportunities and future development of FGB greatly depend on implementing sustainable strategies for the large-scale production of GM feedstocks at low costs. ORP systems are the most cost-effective large-scale bioreactors used for microalgal biomass production and, thus, are the most preferred option for cultivating algal biomass. However, implementing an ORP system to cultivate GM microalgal species requires a comprehensive risk assessment. In open bioreactors, preventive controls must be considered throughout the design and production processes to reduce the diffusion risk via horizontal gene transfer caused by releasing chromosomal DNA or plasmid into ecosystem. However, the financial burdens of such control measures in FGB production are not frequently justified or even calculated.

Limited reports on large-scale GM microalgal biomass cultivation have simultaneously considered environmental sustainability and business performance. Meanwhile, the large and economically feasible scale of FGB production is a prerequisite for a robust investigation of its techno-economic viability. Therefore, future research should address the environmental impacts of the large-scale production of GM microalgal species in open bioreactors and the effects of these parameters in the design of control and safety systems. The techno-economic analyses of these control tools can be made through several design runs in

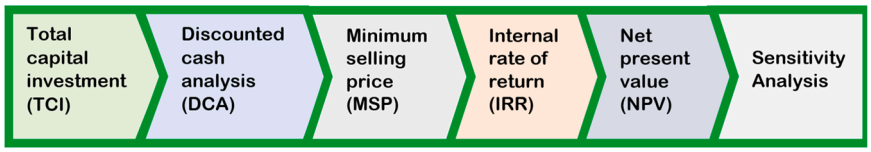

Fig. 4. Procedure for performing the sensitivity analysis and calculating net present value (NPV).

standardized processes. Finally, future research should investigate developing and applying viable and safe genetic engineering techniques that do not involve foreign DNA, reducing biosafety concerns.

6. Conclusions

The oil and biomass content are important cost drivers in the bioeconomic evaluation of algal biofuels. It was indicated that a 6.22% increase in an algal strain's lipid content could promote biofuel production's bioeconomic success probability by up to 90%. Moreover, a 0.011 g/L/day increase in biomass yield could enhance the bioeconomic success of the investment by 40%. Therefore, genetic modification methods for enhancing lipid accumulation and biomass yield could significantly improve the bioeconomics of the FGB. The genetic manipulation of the microalgal strains could lead to a simultaneous increase in oil and biomass yield, or an increase in one may adversely affect the other. Blocking competing pathways is one the most efficient methods in genetic modification and could increase the TAG contents by 45-fold in specific strains.

An OPR is necessary for economically viable large-scale algal biomass production due to the lower production costs. However, using an open pond could increase the diffusion risk of modified species into surrounding ecosystems by wind or animals. The release of GM strains into the environment could cause the exchange of DNA among organisms of different species through horizontal gene transfer. Using plastic hoop air-supported greenhouse covering is suggested as a control measure to prevent the release of the GM strains. Covering cultivation ponds is estimated to add an extra US 60,000$ /ha to the capital cost. On the other hand, using leakage control can lead to extra production and operational costs for FGB production, which should also be considered in bioeconomic analyses. The fully lined open raceway pond could cost up to 25% higher than unlined ponds. The economic performance of FGB production could be improved by recycling water and nutrients and reusing discharged residue. Besides the incurred costs, socioeconomic drivers exist among the most significant determinants in market valuation and commercialization of GM algal biomass.

CRediT authorship contribution statement

H. Shokravi: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Project administration. **M. Heidarrezaei:** Investigation, Writing – original draft, Software. **Z. Shokravi:** Conceptualization, Investigation, Writing – original draft. **H. C. Ong:** Writing – review & editing, Supervision, Funding. **W. J. Lau:** Writing – review & editing, Supervision, Validation. **M. F. M. Din:** Software, Resources, Visualization, Funding. **A. F. Ismail:** Writing – review & editing, Visualization.

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.

Data Availability

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

Acknowledgement

The authors would like to acknowledge the generous support of The Fengtay Cultural and Educational Foundation. Besides, the authors also would like to thank University Teknologi Malaysia for its facilities and support.

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