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Enhanced microalgal lipid production for biofuel using different strategies including genetic modification of microalgae: A review

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ABSTRACT

Microalgae have gained considerable attention as an alternative feedstock for the biofuel production, particularly in combination with genetic modification strategies that target enhanced lipid productivity. To tackle climate change issues, phasing out the usage of fossil fuels is seen as a priority, where the utilization of biofuel from microalgae serves as a potential sustainable energy source for various applications. These photosynthetic microalgae utilize solar energy and carbon dioxide to produce energy-rich compounds (i.e., starch and lipids), that can be further converted into biofuels of different types. Among different types of biofuels, biodiesel from the transesterification of triacylglycerols stands out as the most sustainable replacement of transportation fuel over fossil-based petroleum diesel. However, hurdles such as limited productivity, overall production cost and challenges in upscaling the algal technology leaves a huge gap on the road to commercialized microalgae-based biofuel. This review article first presents a comprehensive overview of imperative knowledge regarding microalgae in terms of algal classification, factors affecting the growth of microalgae during cultivation and different steps in upstream processing. This review also discusses recent advances in downstream processing of microalgal biorefinery. Additionally, this review paper focuses on deliberating various recent strategies of genetic modifications and their feasibility for enhanced lipid productivity in microalgae. Finally, the current challenges and future perspectives of microalgae-based biofuels are highlighted in this review discussing several aspects, including sustainability of microalgae-based biofuel production, current status of algae-based industry, risks and legislation considerations of genetic modification of microalgae.

1. Introduction

Energy plays an important role in the lives of every living organism, including humans. From simple processes like heating and cooking to transportation and electricity, a large amount of energy is consumed. Currently, we are heavily dependent on fossil fuels as the main source of energy [1]. Fossil fuels refer to fuels that have been formed from biomass and stored underground millions of years ago. The major types of fossil fuels include coal, oil and natural gas [2]. Fossil fuels like coal play an important role in modern life since the industrial revolution that took place in the 18th century. The reliance then gradually shifted towards oil and gas in the 20th century with the introduction of internal combustion engines [3]. Fossil fuels are widely used as they provide high energy

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Abbreviation

[BMIM][HSO_4]	1-Butyl-3-methylimidazolium hydrogen sulfate
[Emim]DEP	1-Ethyl-3-methylimidazolium diethyl phosphate
[Emim]Oac/[Emim](CF_3SO_2)$_2$N	1-Ethyl-3-methylimidazolium bis (trifluoromethylsulfonyl)imide
2-MeTHF	2-Methyl-tetrahydrofuran
3 PG	3-Phosphoglyceric acid
ACC/ACCase	Acetyl-CoA carboxylase
ACD	Acyl-CoA dehydrogenase
aCh-O	Aqueous choline chloride-oxalic acid
ACOX	Acyl-CoA oxidase
ACP	Acyl carrier protein
ACS	Acyl-CoA synthetases
ADES	Aqueous deep eutectic solvent
ADP	Adenosine diphosphoglucose
AGP/AGPase	ADP-glucose pyrophosphorylase
ATP	Adenosine triphosphate
BE	Branching enzyme
CDTA	Cyclohexanediaminetetraacetic Acid
Ch-Aa	Choline chloride-Acetic acid
CoA	Coenzyme A
CPME	Cyclopentyl methyl ether
CPP	Cell penetrating peptide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAG	Diacylglycerol
DBE	Debranching enzyme
DCW	Dry cell weight
DES	Deep eutectic solvent
DGAT	Diacylglycerol acyltransferase
DGDG	Digalactosyl diacylglycerol
DICER	Helicase with RNase motif
DMHCA	N,N-Dimethyl-3beta-hydroxycholenamide
DNA	Deoxyribonucleic acid
Dof	DNA binding with one finger
DSB	Double strands break
DW	Dry weight
EBA	N-ethyl butylamine
EMS	Ethyl methanesulfonate
ENR	Enoyl-acyl carrier protein reductase
EtLac	Ethyl lactate
EtOAc	Ethyl acetate
F6P	Fructose-6-phosphate
FA	Fatty acid
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FFA	Free fatty acid
G6P	Glucose-6-phosphate
GHG	Greenhouse gas
GM	Genetic modification
GMO	Genetically modified organism
GPAT	Glycerol-3-phosphate acyltransferase
GPDH	Glyceraldehyde 3-phosphate dehydrogenase
gRNA	Guide ribonucleic acid
HAD	3-Hydroxyacyl-CoA dehydrogenase
KAR	3-ketoacyl-ACP reductase
KAS I	Beta-ketoacyl-ACP Synthase I
KAS II	Beta-ketoacyl-ACP Synthase II
KAS III	Beta-ketoacyl-ACP Synthase III
LEC	Lectin-like protein
LHC	Light harvesting complex
LPAT/LPAAT	Lysophosphatidic acid acyltransferase
MAG	Monoacylglycerol
MCAT	Malonyl coenzyme A:acyl carrier protein transacylase
MeOH	Methanol
MGDG	Monogalactosyl diacylglycerol
miRNA	Micro RNA
MUFA	Monounsaturated fatty acid
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-homologous end joining
NO_x	Nitrogen oxides
NP	Nanoparticle
PAP	Phosphatidate phosphatase
PBR	Photobioreactor
PDAT	Phospholipid:diacylglycerol acyltransferase
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
ppt	Part per thousand
pre-miRNA	Pre-microRNA
PUFA	Polyunsaturated fatty acid
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoproteins
rpm	Revolutions per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SAFA	Saturated fatty acid
SBPase	Sedoheptulose-1,7-bisphosphatase
shRNA	Small hairpin RNA
SHS	Switchable hydrophilicity solvent
siRNA	Short interfering RNA
SPS	Switchable polarity solvent
SQDG	Sulfoquinovosyl diacylglycerol
SS	Starch synthase
TAG	Triacylglycerol
TALEN	Transcription activator-like effector nucleases
TCA	Tricarboxylic acid cycle
TEPDA	N,N,N′,N′-tetraethyl-1,3-propanediamine
TF	Transcription factor
TFA	Total fatty acid
TWh	Terawatt-hour
UV	Ultraviolet
VOC	Volatile organic compound
WT	Wild type
Wt%	Percentage by weight

efficiency and are convenient to be obtained with appropriate infrastructure compared to solar or wind energy, which is dependent on weather and geographical location [4]. In 2019, global fossil fuel consumption was found to be 136,761 TWh, which was around 1500 times more compared to two centuries ago [5].

The major issue associated with fossil fuels is the emission of large amounts of greenhouse gases into the atmosphere. The whole supply chain of fossil fuel from drilling wells to burning as fuel results in the emission of greenhouse gases such as methane (CH_4), carbon dioxide (CO_2), sulphur oxides (SO_x) and nitrogen oxides (NO_x). Emissions of these anthropogenic gases contribute to global warming which further leads to climate change, ocean acidification and environmental pollution. Sulphur oxides and the particulate matter or soot released from the burning of fossil fuel cause pulmonary disease in humans. Additionally,

exposure to the chemicals used in hydraulic fracturing for drilling oil and gas may cause water contamination and can cause cancer in humans. Other negative effects of using fossil fuels include degrading soil quality, damaging the ecosystem and environmental pollution due to oil spillage or gas leakage [6]. As a matter of fact, the concentration of atmospheric CO_2 was projected from 280 ppm to 419 ppm from 1900 to 2019 [7], however, in 2021, atmospheric carbon dioxide level was found to be 416.45 ppm. Eventually, in the next 25 years, it is expected that the global surface temperature will rise by 1.5–5.9 °C due to uncontrolled CO_2 emissions [7]. To prevent the worst effects of climate change, governments around the world agreed to reduce emissions of greenhouse gases as part of the 2015 Paris Climate Agreement. One of the targets in the agreement is to phase out and make fossil fuels obsolete while moving towards the goal of net zero emission (an indicator to balance between carbon emission and carbon sink in the atmosphere) by replacing them with alternative and renewable energy sources [2,3]. As a result, tremendous research has been done to explore the opportunities in renewable, green and carbon-neutral biofuels, as this strategical pathway would be the main contributor in reducing greenhouse gas emissions, as well as to provide sustainable solutions towards greener production processes.

Biofuel is seen as a key alternative to the usage of fossil fuel because of its lower emission profile of greenhouse gases, particularly for CO_2 emission, predominantly employed in the transportation sector [8]. The chemical characteristics of biofuels can either be liquid (e.g., bio-methanol, bioethanol, biobutanol, biogasoline and biodiesel), solid fuel (e.g., biocoal) or gaseous (e.g., biohydrogen, biogas, syngas and biomethane) mainly from biomass [9]. Liquid biofuels are commonly used for combustion engines in most transportation sectors, whereas solid and gaseous biofuels are usually used for heat and power production [9,10]. Biofuels can be categorized into first-, second-, third and fourth-generation of biofuels depending on the type of feedstock used [11]. First-generation biofuels are prepared using food crops and dedicated bioenergy crops composed of sugar, starch and vegetable oil. To-date, Brazil is the foremost country that embarked on utilizing sugar cane as raw material for biofuel production, in which the extracted juice consists of high sucrose content and is fermented to produce bioethanol as fuel [12]. Whereas, in the United States, corn and soybean are utilized, while the European countries have used sugar beet as the feedstock for bioethanol production [13]. However, it is a controversial issue to prepare the biofuel using food crops, which has raised questions and serious constraints about whether food crop-producing biofuels are actually beneficial toward subsidized biofuel programs, or if it has had a detrimental impact on food pricing and food security [14]. To overcome these issues, second-generation biofuels focus on utilizing waste and residual feedstocks from non-food-related biomass such as waste vegetable oil and forest/agricultural residue (i.e. lignocellulosic biomass) for the production of biofuels [15]. These types of biomass are derived from lignocellulosic-based material (e.g., leaves, tree barks, sawdust, pulp waste, thinned wood, corn stover, bagasse, straws) which are one of the most abundant biological resource that can be obtained anywhere in the world [16]. While second generation biofuels offer great abundancy and cost effective route over food crop producing biofuels, the challenges of utilizing this feedstock are related to its intrinsic physical structure (e.g. cellulosic composition such as lignin, hemi-celullose and lignin) during the conversion process of lignocellulosic biomass into biofuels [15,17].

Algae (i.e., macro- or microalgae, commonly microalgae are used) are the feedstock for third and fourth-generation biofuel [18]. The main difference between third and fourth generation biofuel is the implementation of genetic modification/engineering of algae for algal-based biofuel production [19]. Microalgae are photosynthetic microorganisms that have a higher growth rate compared to any terrestrial plant. Microalgae are ubiquitous in aquatic ecosystems across the world, even in various types of wastewaters. Via photosynthesis, microalgae convert light and carbon dioxide to produce highly valuable bioactive compounds such as carbohydrates, proteins, lipids, vitamins and pigments which can be converted into various bioproducts for the chemical and pharmaceutical industries [20,21]. It was reported that 1 kg of algal biomass can assimilate about 1.83 kg of carbon dioxide along with some sulphur oxides and nitrogen oxides [22]. Microalgae species accumulate mostly lipids which can be converted into biofuel and may contain up to 70% of lipids on a dry weight basis [23]. Other advantages of algal-based biofuels compared to fossil fuels include low or negligible sulphur emission and high oxygen levels in fuel combustion [22].

Although the process of biofuel production from microalgae is well studied, the economic feasibility of the overall biorefinery process remains a challenge for its commercialization. For instance, the cost attribution on the chemicals involved in its cultivation (upstream processing), strategic ways to recover high value-added components from algal biomass and the optimization requirement for product conversion (downstream processing) remain a hurdle. Various studies have been conducted to resolve these limitations by seeking low-cost alternative nutrients (as the culture medium) found in different types of wastewaters and integrating multiple processing (i.e. pretreatment and recovery process) into a single system to overcome the high cost associated with biofuel production from microalgae [24,25]. Other than economic cost-related issues, the production of high biomass concentration accumulated with a high lipid yield is also essential for efficient biofuel production [26]. Other than applying stress and altering the microalgae cultivation conditions (e.g., cultivation medium pH, low/high light intensity and nutrient starvation), fourth-generation biofuel production involves multidisciplinary research in the field of biotechnology, biology and biochemistry and chemical engineering, with the generic term referred to as "**genetic engineering** or **genetic modification**" to facilitate microalgae modification at the molecular level of cellular activity to overexpress genes or enzymes specifically for lipid synthesis [27]. This strategy aims to develop and improve strains of microalgae with high lipid production and accumulation abilities via genetic and metabolic modification tools [28]. In recent years, there are some studies relevant to gene functions and genome sequencing in microalgae for the production of biofuels. For instance, the enzyme catalyzed by diglyceride acyltransferase (DGAT2) was reported for the formation of triglycerides from diacylglycerol and fatty acyl-CoA [29]. Phosphoenolpyruvate carboxylase (PEPC1) gene in *Chlamydomonas reinhardtii* showed 74.4% lipid content enhancement [30] and overexpression of acetyl-CoA synthetase (ACS2) achieved 2.4-fold higher accumulation of triacylglycerol [31]. Beside, further possibilities including sequencing strategies such as heavy-ion irradiation mutagenesis, sequencing-by-synthesis, single-molecule real-time sequencing, and pyrosequencing may be worth exploring, however, these options are limited as of now [32]. To realize these blue-sky possibilities, the current trend of genetic engineering research should incorporate computational biology (i.e., artificial intelligence (AI) logarithms and machine learning), bioinformatics and multiomic datasets to further discover the precise assembly and annotations of the genome annotation in microalgae research [25,26,33,34].

This review aims to explore the prospects of microalgal lipid as a source of biofuel production, its importance, current trends and possibilities of applying genetic modification on microalgae as metabolic modification tools for enhancement of lipid accumulation. For readers, this review will provide a comprehensive overview covering different aspects of upstream and downstream processing of microalgal technology. In particular, the upstream processing section includes critical discussion about topics related to microalgal cultivation modes (i.e., phototrophic, heterotrophic and mixotrophic) and the classification of microalgae. In downstream processing section, various recent advances in extraction, recovery technologies for value-added products and their respective mechanistic studies have been evaluated. Besides that, the mechanistic pathways of the lipid production and lipid profiles in microalgae are examined to understand the applications and quality of microalgal lipids in biofuel production towards a sustainable alternative over fossil-based fuels. Besides conventional methods, the advanced

methods such as genome editing, and altering metabolic pathways for the genetic modification of microalgae are also discussed, which have been used in achieving higher lipids production and accumulation. Overall, this review will provide a comprehensive resource highlighting that microalgae are an important feedstock for producing biofuels. However, more research is needed to fill the knowledge gaps to improve the lipid production in microalgae for practical applications. Table 1 comprehensively compiles a series of specific topics involved in the present review paper and compares the distinguished features of the present review with previously published reviews on the topic.

2. Microalgae: a general overview

Microalgae research has been conducted extensively across the world. Due to their remarkable cultivation abilities over terrestrial plants, microalgae have gained wide interest in the research community for the production of a wide range of high-value bioproducts (i.e., biodiesel, bioethanol, biomethane, biohydrogen, bioenergy, biofertilizer, algae-feed and supplements) from the algal biomass. Despite the major advantages, there exist many challenges in utilizing microalgae as feedstock especially in the overall upstream and downstream processing.

Microalgae are simple plant-like structure (thallophytes) without roots, stem and leaves, and primarily consist of chlorophyll *a* and *b* as their principle pigment which play an important role in photosynthesis. The chemical structure of chlorophyll *a* is responsible for the absorption/capture of light energy for oxygenic photosynthesis, while chlorophyll *b* is the accessory pigment that helps to collect light energy and transfer it to chlorophyll *a* [45,46]. This process is similar to photosynthetic characteristics of terrestrial plants in capturing sunlight irradiance and assimilating atmospheric carbon dioxide (CO_2) allowing them to grow and reproduce [47]. Both CO_2 and light energy are absorbed by chloroplasts which is reduced into adenosine triphosphate (ATP) and oxygen (O_2) as the "energy currency" to support the growth of microalgae [48,49]. In this way, photosynthesis is a regulating process in the microalgal cell metabolism which enables the growth and biosynthesis of biological macromolecules (i.e., protein, lipid, carbohydrates, carotenoids and micronutrient) stored within the cells of microalgae. Once microalgal cells have reached maturation (stationary phase), the harvested algal biomass is further processed into their respective high-value bioproducts.

2.1. Upstream processes in microalgal cultivation: growth modes and growth conditions

Microalgae are phototrophic organisms that undergo photosynthetic process and assimilate atmospheric CO_2 from aquatic habitats [50]. On the other hand, microalgae also require organic carbon and inorganic nutrients for their growth. The growth of microalgae can be categorized into four different types of growth patterns, namely: i) lag phase, exponential phase, stationary phase and death phase [51]. Lag phase is the initial phase in which microalgae adapt to its surrounding (e.g., culture medium, system pH, temperature, and light intensity). Exponential phase takes place once microalgae are able to adapt to the culture medium and surrounding environment. Since microalgae have regulating mechanism capabilities, eventually they will adapt and sustain themselves to reach stationary phase. Throughout the growth process, biological macromolecules such as protein, lipid, carbohydrates are biosynthesized and developed within the organelles of microalgae. Subsequently in the stationary phase, the nutrients in the culture medium are decreased after consumption by microalgae for cell growth and cell devision, and eventually, some mature algae will undergo cell lysis (i.e. cell death). Lastly, during death phase, there is an equal ratio between new cells and dead cell which may show a decrease in the concentration of microalgal biomass.

In most cases, microalgal cultivation is carried out on a smaller scale

Table 1
Specific topics of microalgae which have been reviewed in literature.

Specific topics included	Biofuel from microalgae	Factors effecting the biomass production and lipids accumulation	Biodiesel from microalgae	Lipids production from microalgae	Methods of extraction of lipids	Genetic modification of algal species for enhanced lipid production	Genetic modification tools; approaches of lipid improvement	Sustainability aspect to produce biodiesel from microalgae	Significant global projects to produce biodiesel	Techno-economic analysis for biofuel production using different cultivation systems	Production of other valuable products from microalgal biomass (food supplement, animal feed, cosmetics, nutraceuticals)
Studies											
[35]	×	×	✓	✓	×	×	×	×	×	✓	×
[36]	✓	×	✓	✓	×	×	×	×	×	×	×
[37]	✓	✓	✓	✓	✓	×	×	×	×	×	✓
[38]	×	×	✓	✓	✓	×	×	✓	×	×	×
[39]	×	×	✓	✓	×	×	✓	×	×	×	×
[40]	×	×	✓	✓	×	✓	✓	×	×	×	×
[41]	✓	×	×	✓	×	×	✓	×	×	×	×
[42]	×	×	✓	✓	✓	×	×	×	×	✓	×
[43]	×	✓	✓	✓	×	✓	×	×	×	×	×
[44]	×	✓	✓	✓	×	×	✓	×	×	×	×
This paper	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×

(in 50 mL–100 mL tissue culture flask) or in a petri dish consisting of agar culture medium. This stage can also be used to store and sustain the microalgae culture before transferring it to a larger cultivation scale. It is usually suggested to inoculate and transfer about 10–20% of the stationary phase microalgal biomass into a new batch culture medium (i.e. preculture stage) for effective growth. The following section will provide a clearer explanation and focus on the growth processes that include autotrophic (i.e., photoheterotrophic and photoautotrophic), heterotrophic and mixotrophic growth modes.

2.1.1. Photoheterotrophic and photoautotrophic growth of microalgae

Photoheterotrophic growth is a method that requires both natural sunlight and organic carbon substrate (e.g., glucose, acetic acid, glycerol, fructose, sucrose, maltose and molasses) for the cultivation of microalgae. It is considered a feasible approach for large-scale production of algal biomass as it involves the use of free natural resource e.g. sunlight in most open pond production systems. Sunlight is sufficient in countries with tropical climate which have two main seasons (wet/rainy and dry season) throughout the year. Most open pond systems may also be installed with submerged aerators or mechanical paddlewheels to increase the atmospheric CO_2 absorption by agitation and help in homogenization of the cultivation system [47]. But, the challenges remain in this method due to the contamination problems that arise due to organic carbon substrates being used as carbon source [52].

Unlike photoheterotrophic growth which utilizes additional organic carbon sources for the cultivation, photoautotrophic growth involves photosynthesis process by utilizing inorganic carbon from atmospheric carbon dioxide sources, natural sunlight and water to generate chemical energy to be used in cellular function (i.e., biosynthesis and respiration) [53]. In most cases, photoautotrophic growth is considered the economically feasible cultivation method as it does not require any additional supplement of organic sources. Many studies have reported that lipid content ranges between 5% and 68% depending on the type of microalgal species [54–56]. Photoautotrophic growth is an efficient cultivation method for carbon sequestration (i.e., capturing and storing of atmospheric carbon dioxide) and contributes to the reduction of global carbon dioxide as microalgae consume CO_2 (present in the atmosphere) as the carbon source for cell growth. Both phototrophic cultivation conditions should be performed in outdoor cultivation systems (e.g. raceway pond or open pond) to ensure it receives sufficient amount of sunlight. However, the challenge of outdoor cultivation systems may involve the risk of contamination, low biomass concentration and high harvesting cost for microalgal cultivation [57]. Fig. 1 shows the illustration of different growth modes for the cultivation of microalgae.

2.1.2. Heterotrophic growth of microalgae

Some microalgae species have also been found in the environment (e.g. polar regions, buried in sediments, or underneath the periphyton) where natural sunlight is unable to reach [58–60]. Due to these light-deprived environments, these microalgae adapt by undergoing heterotrophic metabolism, mainly dependent on glucose for their growth. Heterotrophic growth of microalgae involves the use of external organic carbon sources (i.e. glucose, sucrose, lactose, galactose, glycerol, acetate and fructose) under dark conditions [61]. Glucose has a high energy content and it can enhance physiological growth, especially in microorganisms [62]. The metabolic pathway of carbon sources in heterotrophic conditions involves enzymatic reactions such as transportation, activation (i.e., phosphorylation), intermediary anabolic and catabolic metabolism. The cytoplasmic membrane of microalgae allows the transportation of substrate between the microalgae cells and the environment [63]. This allows them to control the intake and movement of nutrients via translocation and transduction functionality. Several metabolic pathways have been discussed in literature such as the oxidative pentose phosphate (PPP) pathway in dark conditions, Embden–Meyerhof–Parnas (EMP) in light conditions and GS/GOGAT pathway for ammonium assimilation [64,65]. It has also been reported that heterotrophic growth conditions induce high biomass, lipid productivity and provide better growth rates [65,66]. However, some heterotrophic microalgae species may not induce or produce low amount of metabolites such as carotenoid/pigments, and it requires a controlled fermenter to hinder external light penetration during their cultivation process [65,67].

2.1.3. Mixotrophic growth of microalgae

Mixotrophic growth of microalgae is performed using both organic and inorganic carbon sources integrated with dark and light cycles [68–70]. In general, the term “mixotrophic” means the mixture of phototrophic and heterotrophic growth for microalgal cultivation. As a result, mixotrophic growth is characterized as “carbon fixation” which allows microalgae to assimilate inorganic source from CO_2 during the dark condition (heterotrophic) and simultaneously increases its ability to drain high organic carbon concentration in the presence of light (phototrophic) [67,71]. In fact, mixotrophic growth allows microalgae to produce and accumulate photosynthetic carotenoids such as astaxanthin, lutein, fucoxanthin, phycocyanin and β-carotene under controlled illuminated conditions [72]. The biosynthesis of high-value metabolites in the mixotrophic growth of microalgae requires intensive optimization of each parameter, including light intensity, temperature and optimal culture medium ratio (carbon (C), nitrogen (N) and

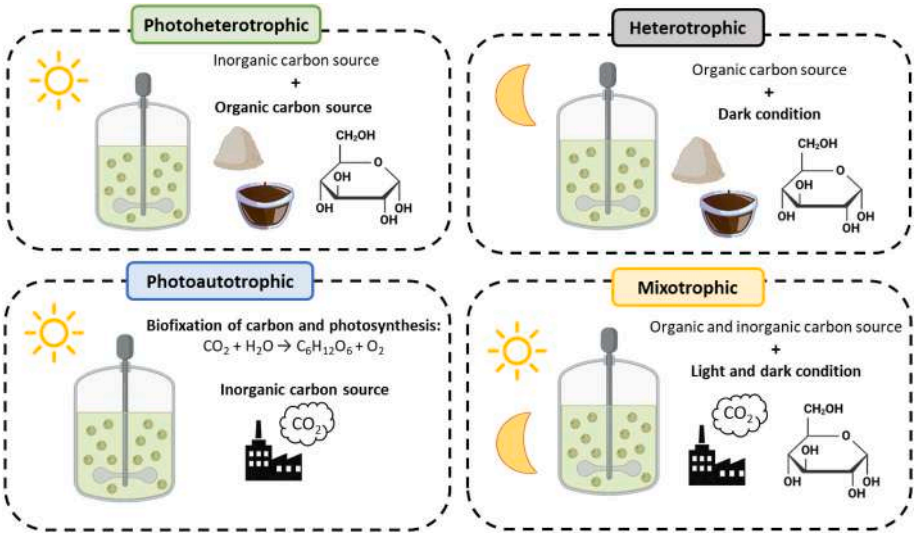


Fig. 1. Different growth modes (photoheterotrophic, photoautotrophic, heterotrophic and mixotrophic) for the cultivation of microalgae.

phosphorus (P)). Recent studies have reported the ability of microalgae for bioremediation of various wastewaters (i.e, piggery, furfural, sewage, municipal and textile) and industrial flue gases under mixotrophic and heterotrophic growth conditions [73–78]. However, microalgae alone cannot bioremediate complex wastewater types, and sometimes algal growth is inhibited [79]. Therefore, co-culturing other microorganisms (e.g., bacteria) with microalgae is important [80–84]. This practice allows the creation of symbiotic interaction (mutual relationship) between bacteria and microalgae to undergo the exchange of oxygen, carbon dioxide and ammonium ions during the bioremediation [85]. Table 2 shows the effect of different organic substrates used and biomass productivity in different growth modes of microalgae.

2.2. Classification of prokaryotic and eukaryotic microalgae

Microalgae are unicellular or multi-cellular microorganisms that are commonly found in freshwater, seawater, saline water and even deserts and the arctic. Microalgae perform photosynthesis by assimilating atmospheric CO_2 and release oxygen (O_2) to the atmosphere [103]. There are around 400,000 different species of microalgae identified and the species can be categorized into eukaryotic or prokaryotic [22,104]. Prokaryotic microalgae are single-celled or unicellular organisms that do not contain nuclei or membrane-bounded organelles. On the other hand, eukaryotic microalgae can be classified into organisms which contain single-membrane-bound organelles (e.g., endoplasmic reticulum, Golgi apparatus, lysosome, peroxisomes and vacuoles) and double-membrane-bound organelles (including nuclei, chloroplast and mitochondrion), however, this depends on their respective species [22,105]. The common classification of macroalgae (also known as seaweed) or microalgae can be grouped into: i) Rhodophyta (red algae), ii) Phaeophyta (brown algae), iii) Chlorophyta (green algae), iv) Bacillariophyta (diatoms) and v) Chloroxybacteria (cyanobacteria). To be specific, prokaryotic microalgae consists of Chloroxybacteria (cyanobacteria), while eukaryotic microalgae include Rhodophyta (red algae), Phaeophyta (brown algae), Chlorophyta (Bacillariophyta) and Bacillariophyta (diatoms) [22,47].

Eukaryotic microalgae have a structure similar to plant cells, with organelles such as nuclei, chloroplasts and mitochondria surrounded by a membrane. Prokaryotic microalgae possess a simple cellular structure similar to bacteria without a nuclear membrane [104,106]. In terms of size, eukaryotic microalgae are relatively larger than prokaryotic microalgae. Both types of microalgae undergo the process of photosynthesis, however, chloroplasts only exist in eukaryotic microalgae. Prokaryotic microalgae have their chlorophyll stored in thylakoids in the cytoplasm [107]. Eukaryotic microalgae include green algae and diatoms [108], while prokaryotic microalgae are commonly known as blue-green algae or cyanobacteria [105]. The eukaryotic microalgae are more favorable in the field of molecular biotechnology as they have advantages over the prokaryotic microalgae. Many eukaryotic microalgae carry genetic information consisting of a mixture of bacterial type, animal type and plant type genes and they are referred to as multifunctional 'planimals'. Many of them can switch between phototrophic growth and heterotrophic growth under both aerobic and anaerobic conditions. Furthermore, harvesting eukaryotic microalgae is relatively easier due to their larger size. Eukaryotic microalgae are also able to perform synthesis directly in the chloroplasts or store products temporarily in the vacuoles inside the cell compartment. This increases flexibility and efficiency in the aspect of design-driven biology. Lastly, green microalgae which belong to eukaryotic domain are widely used for genetic modification for biofuel production [108].

2.2.1. Use of microalgae for the production of biofuels

Algae consist mainly of carbon, nitrogen and phosphorus and these elements form protein, carbohydrates, lipids and nucleic acid [42]. The carbohydrates and lipids are the key biomolecules to be converted into organic compounds such as ethylene, propylene, adipic acid and furanics. These organic compounds are the building blocks for biofuels [108]. Microalgae perform photosynthesis by capturing sunlight and carbon dioxide, just like higher plants. Starch and lipids are the key products produced and they are the products that could be harvested for biofuel production. Based on that, microalgae are also said to be an efficient cellular system for the harvesting of solar energy. In terms of dry biomass, up to 60–70% lipids can be present in microalgae. Thus, lipids in microalgae are considered as a potential feedstock for biodiesel [23,108].

There are two ways to convert microalgal biomass into energy; these are thermochemical and biochemical conversions. Thermochemical conversion is subdivided into gasification, pyrolysis, hydrogenation and liquefaction [109,110]. Gasification converts microalgal biomass into combustible gas such as hydrogen, methane, carbon dioxide and ammonia. Modified membranes and metal–organic frameworks can be used to separate hydrogen gas from the gas stream during the process [111,112]. Hydrogenation involves the addition of hydrogen to organic compounds in microalgae to produce liquid hydrocarbons [109,113]. Pyrolysis converts microalgae into bio-oil, pyro gas and char in the absence of air [114]. If flash pyrolysis is used, the conversion of biomass to bio-oil has an efficiency of up to 80%. Liquefaction involves the conversion of wet microalgal biomass in aqueous alkali into heavy oil [115]. Bio- and chemical conversion methods include fermentation and transesterification. In the fermentation method, an enzyme is used to convert starch to sugar, and sugar is then converted to ethanol by yeast [116]. Transesterification converts fatty acids into biodiesel in esters form and glycerol as a by-product [117]. In the process, methanol is often used as the alcohol reagent with sodium hydroxide (NaOH) as the catalyst [118]. Fig. 2 shows a summary of different pathways of conversion of microalgae to biofuels and their respective products.

2.2.2. Types of fuels produced and their applications

Most of the microalgal-based biofuels exist in liquid and gaseous forms. Organic compounds such as lipids and starch can be converted into gaseous biofuels (e.g. biogas and biohydrogen) and liquid biofuels (e.g. biodiesel and bioethanol) [119].

2.2.2.1. Gaseous biofuels from microalgae. Gaseous biofuels mainly consist of methane, carbon dioxide and biohydrogen [113]. These gases can be used directly for combustion in gas engines or gas turbines. Biohydrogen consists of majorly hydrogen gas with a mixture of other gases. Hydrogen gas tops the energy content per unit weight of any known fuel with an energy density of around 142 kJ/g [120]. Hydrogen fuel cells have three times higher efficiency than gasoline fuel, but high purity of hydrogen gas is required as feedstock. Thus, hydrogen gas separation and purification needs to be done in the case of biohydrogen production [120,121]. Microalgae are capable of producing biohydrogen by adjusting their metabolism during their cultivation. There are several ways to produce biohydrogen from microalgae, including dark fermentation, photo-fermentation, direct and indirect photolysis [122,123]. Fig. 3 shows an illustration of these processes. By conducting an in-depth study, the production of biohydrogen from microalgae, using the aforementioned processes, can be further improved to make them commercially viable.

2.2.2.2. Liquid biofuels from microalgae. Microalgae contain significant concentrations of several carbohydrates, including starch, glycogen, agar, and cellulose, which are easily converted to fermentable sugars for the production of bioethanol. These algae-based liquid biofuels are majorly used in transportation industry as their high-octane number prevents knocking and lowers the emission of greenhouse gases. Therefore, most of this algae-based bioethanol can either be directly used in cars or blended with petroleum-based gasoline [124]. Some years ago, the global use of bioethanol increased dramatically, from 1 to 39 billion liters. Brazil and the USA are the dominant industrial players

Table 2
Evaluation of biomass growth under different growth modes, organic substrate and cultivation conditions.

Algae species	Growth mode	Organic substrate	Biomass growth	Cultivation conditions	References
Asterarcys sp. SCS-1881	Phototrophic	No addition of glucose	0.75 g/L	BG-11 medium, 100 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[86]
Asterarcys sp. SCS-1881	Mixotrophic	10 g/L glucose	3.71 g/L	BG-11 medium, without the addition of $NaHCO_3$, 100 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[86]
Asterarcys sp. SCS-1881	Heterotrophic	10 g/L glucose	–	BG-11 medium, 0:24 h (light/dark period)	[86]
Asteracys sp.	Mixotrophic	500 mg/L glucose	4.35 g/L (900 μmol m^{-2} s^{-1}) 0.08 g/L (100 μmol m^{-2} s^{-1})	Modified BG-11 medium, 12:12 h (light/dark period)	[87]
Auxenochlorella protothecoides (SAG 211-7a)	Heterotrophic	20 g/L of glucose	5.65 g/L	Bold's basal medium, 43 μmol photons m^{-2} s^{-1}, 18:6 h (light/dark period)	[70]
Chlamydomonas sp.	Mixotrophic	5% of vinasse	0.010 g/L	Modified BG-11 culture medium, 130 μmol photons m^{-2} s^{-1}, 12:12 h (light/dark period)	[88]
Chlorella sp.	Mixotrophic	0.10 mol/L of acetate 0.10 mol/L of glucose 0.10 mol/L of glycerol	5.1 g/L 6.25 g/L 3.52 g/L	BG-11 culture medium, 120 μmol photons m^{-2} s^{-1}, 12:12 h (light/dark period)	[89]
Chlorella sp.	Phototrophic	0.50 g/L of glycerol	0.22 g/L	Modified f/2 medium, 60–70 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[90]
Chlorella sp.	Mixotrophic	0.5 g/L of glycerol	0.37 g/L	Modified f/2 medium, 60–70 μmol photons m^{-2} s^{-1}, 14:10 h (light/dark period)	[90]
Chlorella sp.	Heterotrophic	0.5 g/L of glycerol	0.18 g/L	Modified f/2 medium, 0 h: 24 h (light/dark period)	[90]
Desmodesmus sp. WC08	Mixotrophic	67 mmol/L of glucose 67 mmol/L of sodium acetate 67 mmol/L of glycerol	0.79 g/L 1.01 g/L 0.45 g/L	Basal BG-11 medium, 8000~10000 lx light intensity, 12:12 h (light/dark period)	[91]
Desmodesmus sp. WC08	Phototrophic	67 mmol/L of glucose 67 mmol/L of sodium acetate 67 mmol/L of glycerol	1.83 g/L 1.83 g/L 1.83 g/L	Basal BG-11 medium, 8000~10000 lx light intensity, 24:0 h (light/dark period)	[91]
Desmodesmus sp. WC08	Heterotrophic	67 mmol/L of glucose 67 mmol/L of sodium acetate 67 mmol/L of glycerol	0.58 g/L 0.38 g/L 0.13 g/L	Basal BG-11 medium, 0:24 h (light/dark period)	[91]
Haematococcus pluvialis (SCCAP K-0084)	Mixotrophic	1.33 g/L of ribose 1.33 g/L of gluconate 1.33 g/L of sodium acetate	1.03 g/L 1.12 g/L 0.77 g/L	BG-11 medium, 45 ± 3 μmol m^{-2} s^{-1}, 12:12 h (light/dark period)	[92]
Haematococcus pluvialis (SCCAP K-0084)	Heterotrophic	1.33 g/L of ribose 1.33 g/L of gluconate 1.33 g/L of sodium acetate	– – –	BG-11 medium, 0:24 h (light/dark period)	[92]
Haematococcus pluvialis (SCCAP K-0084)	Phototrophic	1.33 g/L of ribose 1.33 g/L of gluconate 1.33 g/L of sodium acetate	0.70 g/L 0.70 g/L 0.70 g/L	BG-11 medium, 45 ± 3 μmol m^{-2} s^{-1}, 24:0 h (light/dark period)	[92]
Monodus subterraneus (ATCC30593)	Mixotrophic	5.00 g/L of pure glycerol (with aeration) 5.00 g/L of pure glycerol (no aeration)	45.77 mg/L/day 16.10 mg/L/day	BG-11 medium, 55 μmol photons m^{-2} s^{-1}, 14:10 h (light/dark period)	[93]
Monodus subterraneus (ATCC30593)	Heterotrophic	5.00 g/L of pure glycerol	1.52 mg/L/day	BG-11 medium, 0:24 h (light/dark period)	[93]
Monodus subterraneus (ATCC30593)	Phototrophic	5.00 g/L of pure glycerol (with aeration) 5.00 g/L of pure glycerol (no aeration)	42.21 mg/L/day 9.40 mg/L/day	BG-11 medium, 55 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[93]
Nannochloropsis salina	Phototrophic	0.5 g/L of glycerol	0.34 g/L	Modified f/2 medium, 60–70 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[90]
Nannochloropsis salina	Mixotrophic	0.5 g/L of glycerol	0.42 g/L	Modified f/2 medium, 60–70 μmol photons m^{-2} s^{-1}, 14:10 (light/dark period)	[90]
Nannochloropsis salina	Heterotrophic	0.5 g/L of glycerol	0.22 g/L	Modified f/2 medium, 0 h: 24:0 h (light/dark period)	[90]
Nannochloropsis sp. BR2	Mixotrophic	5 g/L of sugarcane bagasse 10 g/L of sugarcane bagasse	0.063 g/L/d 0.058 g/L/d	f/2 medium, 120 μmol m^{-2} s^{-1}, 16:8 h (light/dark period)	[94]
Nannochloropsis sp. BR2	Phototrophic	5 g/L of sugarcane bagasse	0.051 g/L/d	f/2 medium, 120 μmol m^{-2} s^{-1}, 24:0 h (light/dark period)	[94]
Nannochloropsis gaditana B-3	Mixotrophic	1 g/L of glycerol 10 g/L of acetate	1.07 g/L 0.64 g/L	Modified culture, 190 μmol photons m^{-2} s^{-1}, N/A (light/dark period)	[95]
Nannochloropsis salina SAG 40.85	Mixotrophic	3 g/L of glycerol 3 g/L of glycerol (with pure CO_2)	4.30 g/L 0.24 g/L	f/2 medium, 260 μmol m^{-2} s^{-1}, 12:12 h (light/dark period)	[96]
Oscillatoria sp.	Mixotrophic	Cheese whey water	0.032 g/L	BG-11 medium, 1600 lx, N/A (light/dark period)	[97]
	Mixotrophic	0.5% of glucose	15.36 g/L		[98]

(*continued on next page*)

Table 2 (*continued*)

Algae species	Growth mode	Organic substrate	Biomass growth	Cultivation conditions	References
Porphyridium purpureum (CoE1)		0.1% of sodium acetate 0.5% of glycerol	12.71 g/L 15.72 g/L	Artificial seawater medium, 165 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	
Scenedesmus dimorphus NT8c	Mixotrophic	5 g/L of sugarcane baggase 10 g/L sugarcane baggase	119.5 mg/L/d 105.92 mg/L/d	Bold's Basal Medium, 120 μmol photons m^{-2} s^{-1}, 16:8 h (light/dark period)	[99]
Scenedesmus dimorphus NT8c	Phototrophic	5 g/L of sugarcane baggase	96.44 mg/L/d	Bold's Basal Medium, 120 μmol photons m^{-2} s^{-1}, 16:8 h (light/dark period)	[99]
Scenedesmus obliquus KGE-17	Heterotrophic	500 mg/L of sodium acetate	0.68/d	Modified BG-11 medium, 0:24 h (light/dark period)	[100]
Scenedesmus obliquus KGE-17	Mixotrophic	500 mg/L of sodium acetate	0.96/d	Modified BG-11 medium, 50 μmol m^{-2} s^{-1}, 12:12 h (light/dark period)	[100]
Scenedesmus obliquus KGE-17	Phototrophic	500 mg/L of sodium acetate	0.79/d	Modified BG-11 medium, 50 μmol m^{-2} s^{-1}, 24:0 h (light/dark period)	[100]
Spirulina sp. LEB 18	Mixotrophic	2.5 g/L of glycerol	0.43 g/L/day	Zarrouk medium, 60 μmol m^{-2} s^{-1}, 12:12 h (light/dark period)	[101]
Spirulina sp. LEB 18	Phototrophic	2.5 g/L of glycerol	0.1 g/L/day	Zarrouk medium, 60 μmol m^{-2} s^{-1}, 24:0 h (light/dark period)	[101]
Tetradesmus obliquus (No. 276-1)	Mixotrophic	10 g/L of beet vinasses (molasses)	0.39 g/L/day	Bold's Basal Medium, 80 μmol m^{-2} s^{-1}, 12:12 h (light/dark period)	[101]
Tetradesmus obliquus (No. 276-1)	Phototrophic	10 g/L of beet vinasses (molasses)	0.18 g/L/day	Bold's Basal Medium, 80 μmol m^{-2} s^{-1}, 24:0 h (light/dark period)	[101]
Tetraselmis gracilis	Mixotrophic	5% of vinasse	0.0128 g/L/day	Modified BG-11 culture, 130 μmol photons m^{-2} s^{-1}, N/A (light/dark period)	[88]
Tetraselmis gracilis	Phototrophic	5% of vinasse	0.0087 g/L/day	Modified BG-11 culture, 130 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[88]
Tribonema minus (SAG 880-3)	Heterotrophic	20 g/L of glucose	14.3 g/L	BG-11 medium, 0:24 h (light/dark period)	[102]
Tribonema minus (SAG 880-3)	Phototrophic	20 g/L of glucose	5.36 g/L	BG-11 medium, 100 μmol photons m^{-2} s^{-1}, 24:0 h (light/dark period)	[102]

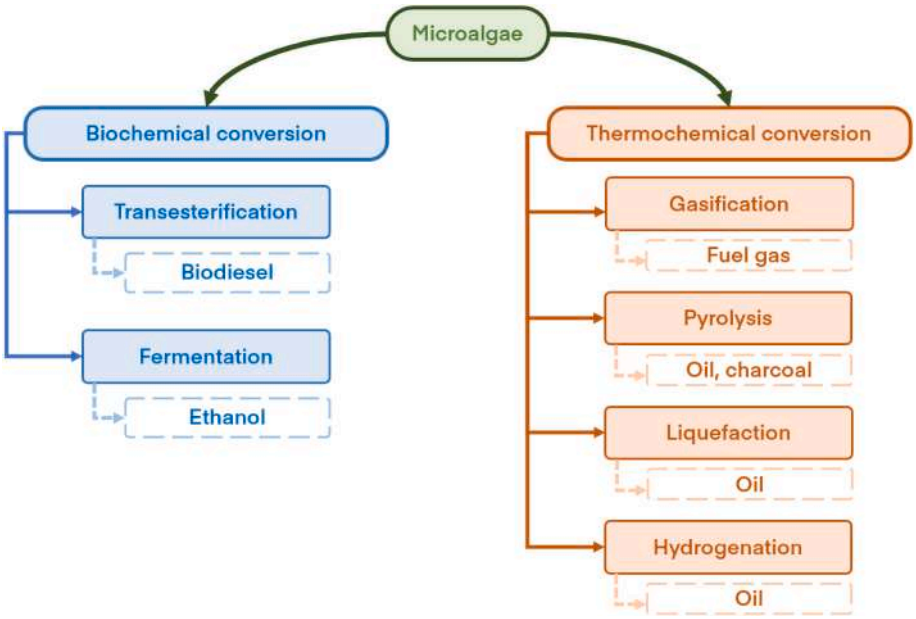


Fig. 2. Different pathways of microalgal biomass conversion to biofuels and their respective products. Modified from Amin [109] with permission from Elsevier.

accounting for over 80% of total production [125]. *Chlamydomonas reinhardtii* and *Chlorella vulgaris* species of microalgae are carbohydrate-rich and can be used in the techno-economic assessment (TEA) for the production of bioethanol. TEA of microalgae-based bio-ethanol production calculates the plant's appropriateness in terms of total investment, total cost, and total net profit [126]. Companies such as Seambiotic, Algenol and Sapphire Energy produce bioethanol on commercial scale with an annual output of 1 billion gallons at a cost of 85 cents/L [127].

Biodiesel is a mixture of fatty acid methyl esters (FAME) which can be synthesized from hydrocarbons or bio-oil from pyrolysis, liquefaction, hydrogenation and transesterification [128]. Bio-oil has high water content which reduces the viscosity and enhances the fluidity which is good for atomization and combustion. However, the high-water content can also reduce the heating value and flame temperature. Therefore, further purification is needed to improve the heating value in biodiesel. Biodiesel has similar engine performance compared to petroleum diesel with provided advantageous such as lower sulphur and particulate matter emissions [118,129]. Microalgae can produce 58,700 L of algal oil per hectare from which 121,104 L of biodiesel per hectare can be produced [130]. Certain microalgal species are advantageous for the production of biodiesel because they are likely to contain high levels

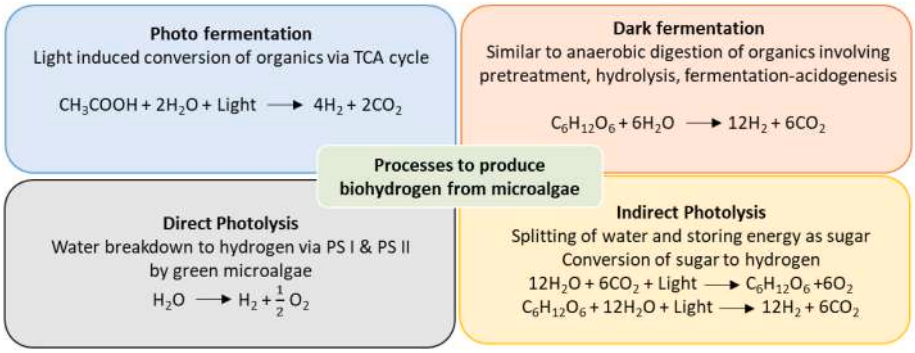


Fig. 3. Different routes to produce biohydrogen from microalgae.

of lipids (50–80%), such as *Botryococcus braunii*, which has up to 80% oil in its biomass [55]. It has been reported that microalgae-derived biodiesel exhibits several characteristics similar to petroleum-derived diesel, including flash point, density, viscosity, solidifying point, cold filter plugging point, and heating value. The majority of these factors are within the biodiesel quality standards set by the American Society for Testing and Materials (ASTM) [131]. Therefore, algae-based biodiesel can be used directly as a fuel with 100% purity or a blended mixture of gasoline fuel. For instance, flex-fuel engines in Brazil can perform duty with all ethanol, all gasoline or any mixture of both as fuel [132]. While in the USA, flex-fuel engines can run on fuel consisting of up to 85% ethanol.

2.2.3. Significance of microalgae in producing biofuel compared to other sources

Chisti [131] predicted that microalgae-based feedstock has the potential and capacity to replace fossil fuel sources and meet the global oil requirement. From the environmental perspective, producing biofuel from microalgae has several advantages such as a high microalgal growth rate, high energy yield and small land area usage [133]. As compared to other biomass sources, microalgae have a high yield per unit of area and light required for their cultivation [134,135]. They do not need fresh water and have the capability to utilize nutrients from wastewater [136]. Although the first-generation of biofuel feedstocks have been commercialized with matured technologies, the drawbacks associated with the sustainability of the food resources remain a critical issue. First-generation biofuel feedstocks are mostly food crops acting as biofuel feedstocks which increase the price of agricultural commodities. Increasing demand for these food crops also causes intensive usage of fertilizers and agrochemicals which degrade water and soil quality [137]. On the other hand, microalgae-based biofuel production can be cultivated in closed photobioreactors or open raceway ponds and does not require arable and productive land [118]. From a socio-economic perspective, the production cycle of microalgal biofuel does not affect the human food chain supply system directly and food against fuel conflict can be avoided [138]. In addition, microalgal biofuel can be produced domestically which will reduce the dependency of a country on foreign oil, creating job opportunities and uplifting the local economy at the same time [139]. As shown in Table 3, the cultivation of microalgae does not require a large area of land compared to the cultivation of terrestrial plant sources.

Microalgae can double their biomass concentration every 2–5 days which is a significant advantage against other feedstocks which take a longer time to grow [24]. Besides, microalgal biomass can be harvested batch-wise or continuously almost all year round compared to the first- and second-generation feedstocks which can only be harvested once or twice a year [33,138]. Thereby, the continuous production of microalgal biomass would largely compensate for the harvesting and transportation costs of microalgal biofuel production over conventional feedstocks [140,141]. Looking at its energy yield, microalgal oil yield is five times higher than that achieved from palm oil which is the best plant for oil yield [142]. The calorific value of bio-oil from microalgae is about 1.4 times that of wood due to lower oxygen contents [143]. Microalgae do not contain lignin and have low hemicellulose levels, this results in increased hydrolysis and fermentation efficiencies and yields [144]. Apart from biofuel, various co-products that are non-toxic, biodegradable and valuable such as soap and glycerine can also be produced from microalgae [118]. For detailed information concerning the comprehensive compilation of lipid content and biomass production from various marine and freshwater microalgae species, readers are suggested to refer to a published work by Mata et al. [55].

Table 3
Significance of microalgae over other feedstocks in producing biodiesel. (The data was adapted with permission from Elsevier [55] for comparison purpose).

Plant source	Oil composition by wt. in biomass (%)	Oil productivity (L oil/ha/year)	Land use (m^2 year/kg biodiesel)	Biodiesel productivity (kg biodiesel/ha/year)
Corn/Maize (*Zea mays*)	44	172	66	152
Soybean (*Glycine max*)	18	636	18	562
Jatropha	28	741	15	656
Canola/Rapeseed	41	974	12	862
Sunflower (*Helianthus annuus*)	40	1070	11	946
Castor (*Ricinus communis*)	48	1307	9	1156
Palm oil (*Elaeis guineensis*)	36	5366	2	4747
Microalgae (low oil content)	30	58,700	0.2	51,927
Microalgae (medium oil content)	50	97,800	0.1	86,515
Microalgae (high oil content)	70	136,900	0.1	121,104

2.2.4. Genetic engineering of microalgae for enhanced lipid production

To produce biofuels from microalgae, the aim is to produce hydrocarbons that have similar physical and chemical properties as diesel and kerosene from crude oil. As lipid has the highest energy content with 37.6 kJ/g in microalgae, the lipid is seen as a promising source for biodiesel production [145–147]. However, there is speculation about whether lipid from microalgae is sufficient to provide renewable

biofuels for replacing fossil fuels. Biofuel production from microalgae can be relatively expensive at the moment. Therefore, improving microalgal lipid production and quality could further improve the economics of biofuel production. Traditional ways to improve lipid productivity include culture conditions, chemical additives and applying stress. Currently, engineering approaches, which involve the modification of genetic and metabolic properties of microalgae, are gaining more attention due to genetic and biochemical diversity of microalga as demonstrated in literature [147,148].

The algae industry has currently reached a few milestones in the engineering of microalgae cells. These include nuclear transformation, gene isolation, transformation, and whole-genome sequencing [27]. Although genetic engineering has yet to be proven to cause overproduction of lipids in microalgae, the approach has built up a solid concept about the biosynthetic pathway of the types of fatty acids favoured in biofuel production [149]. The concept is generally applicable to most of the species, but there are variations in the structure of key enzymes and the location of reactions. The results are building up a strong foundation for further development of the genetic engineering approach in microalgae to enhance lipid production [146].

3. Lipid production: type of lipids produced in microalgae

In microalgal cells, lipids can be found in the form of droplets, stored in cellular membranes and organelles. There are two classes of lipids in microalgae, polar and non-polar lipids [150]. The distribution of these lipids varies as it depends on the growth phases of microalgae. Polar lipids are also known as structural lipids and they are key components found in organelle membranes (e.g. thylakoid membranes) of the chloroplasts [151]. Glycolipids and phospholipids are common polar lipids found in the cell membrane which aid in protecting and maintaining the shape of the cell [152]. Each phospholipid molecule has a polar hydrophilic head and two hydrophobic acid tails, which could be pure saturated tails or a mixture of saturated and unsaturated tails. Some polar lipids are also responsible for signaling and reacting to changes in the surroundings [145].

Non-polar lipids are also known as neutral lipids or storage lipids. Common neutral lipids are triacylglycerols (TAGs), diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids (FFAs), hydrocarbons and other pigments [153]. The TAGs are key components in energy production and storage as they undergo catabolism to supply metabolic energy. TAGs are synthesized in the presence of light and accumulated in cytosolic lipid bodies [154]. TAGs can also be used for the synthesis of polar lipids in dark conditions. Most microalgae do not accumulate many TAGs during exponential growth, but rather they are accumulated quickly during the stationary phase [155].

Different fatty acid compounds can be found in different species of algae. Generally, polar lipids are made up of polyunsaturated fatty acids (PUFAs), while non-polar lipids are made up of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SAFAs) [145,146]. Microalgae are also the primary producers of important fatty acids such as omega-3 fatty acids and omega-6 fatty acids. Among all fatty acids, TAGs are usually found in in different species of microalgae [147] and are ready to be converted into biodiesel by the transesterification process [156].

Microalgae generate lipids during photosynthesis and most of their composition include unsaturated fatty acids, mainly palmitoleic (16:1), oleic (18:1) and linoleic acid (18:2) [157]. Saturated fatty acids can be found in the form of stearic acid (18:0). *Chrypthecodinium cohnii* is found to generate a large quantity of polyunsaturated fatty acids such as docosahexaenoic acid (DHA), a ω-3 fatty acid (22:6ω3) which is composed of 28.0% (w/w dry cell weight) of total lipid content (separation achieved up to 91.6% DHA, 88.2% of saturated and monounsaturated fatty acid ethyl ester, respectively) [158]. Table 4 summarises the type of lipids found in microalgae and their respective key components and functions.

Lipid feedstocks of biodiesel consist of 90–98% (by weight) of TAGs and other components including small amounts of mono and diglycerides, free fatty acids and residues of phospholipids, sulphur compounds, phosphatides, carotenes and water [159,160]. The fuel properties of biodiesel from microalgal lipid depend greatly on the composition of fatty acids. The biodiesel produced from microalgal oil, which is low in saturated fatty acid content, has better fuel properties at colder temperatures as saturated fatty acids can drastically increase the pour and cloud point of biodiesel. However, biodiesel with high unsaturated compounds gets oxidized easier than normal diesel. This results in insoluble sediments and deposits, that will interfere with the working of the engine [145].

3.1. Lipid production mechanism in microalgae

In “light” photosynthesis, microalgae use light irradiation to split water into oxygen, protons and electrons, while in “dark” photosynthesis, protons and electrons are utilized to reduce carbon dioxide into starch by the Calvin cycle. Starch can be catabolized to form lipids in the form of fatty acids and TAGs [145,156,170]. Energy is stored as starch which provides energy for crucial metabolic processes without extra energy sources. Neutral lipids such as TAGs function as a secondary source of energy and accumulate at every stage of the growth of microalgae, but with the highest accumulation rate during the stationary phase. The production and accumulation of starch and lipids are similar to buffer mechanisms, which allow cells to cope with different growth conditions and varying light intensity. As energy from starch is the primary source for metabolism in microalgae cells, lipids that act as a secondary source have less energy output and accumulate more energy. By yield from complete oxidation, lipids provide 35 kJ/g, which is more than that of starch which provides 17 kJ/g [171]. Therefore, blocking metabolic pathways that produce starch and other energetic compounds is a strategy to increase TAGs content [172].

According to Vitova et al. [171], starch in green algae is exclusively stored in chloroplasts and neutral lipids, and is mostly found in structures called lipid bodies in the cytoplasm, as well as in the chloroplasts. Lipid bodies are organelles that mainly consist of neutral lipids surrounded by a single layer of phospholipids and some proteins. Besides storing lipids, these proteins also regulate the synthesis and transport lipids to the membranes of other organelles [173]. The distributions of starch and lipids differ depending on strain, growth conditions and external stress. Some studies have reported that under optimal growth conditions, algae biomass can increase significantly, but the lipid contents are relatively low, which range from 5 to 20% of the dry cell weight. However, when given favorable environmental or stress conditions, the formation and accumulation of lipids, particularly TAGs, in microalgae are found to improve as a result of the altered lipid biosynthetic pathways, to cope with the unusual conditions. Increasing TAGs can lead to alterations in the composition of fatty acids and other lipids in microalgae [174].

Lipids can be biosynthesized in three locations via a *de novo* pathway. Fatty acids are synthesized in chloroplasts, while TAG in the endoplasmic reticulum and thylakoids [175]. CoA denotes coenzyme A, and ACP denotes acyl carrier protein. The process starts with the conversion of acetyl-CoA into malonyl-CoA by the reaction between acetyl-CoA-carboxylase and bicarbonate ion. Next, malonyl-CoA is converted into malonyl-ACP by transacylase. Then, malonyl-ACP is converted into 3-ketoacyl-ACP by a synthase enzyme in the next stage and restarts the fatty acid cycle. Finally, thioesterase catalyzes the hydrolysis of the acyl-ACP thioester bond to produce free fatty acids and acyl groups [176]. The free fatty acids generated are either used to synthesize cellular lipids or glycerols which are the building blocks of TAGs. Enzymes involved in the process could be different based on gene expression levels. Alternate sources of acyl groups other than acyl-CoA and acyl-ACP could be phospholipids [172,177]. Fig. 4 shows a schematic diagram illustrating the process of lipid production in microalgal cells.

Table 4
Various types of lipids, their key components and respective functions.

Type of lipids	Name	Key components	Function	References
Polar lipids	Glycolipids	Monogalactosyl diacylglycerol (MGDG), Digalactosyl diacylglycerol (DGDG) and Sulfoquinovosyl diacylglycerol (SQDG)	Maintain cell stability by preventing inflammation, microbes, and microfouling	[161]
	Phospholipids	Phosphate, glycerol, and alcohol form a hydrophilic head while fatty acids which form a hydrophobic tail	Form the bilayer that controls the enter and exit of molecules between the cell membrane and intercellular organelles, gives a response when stimuli are received, and signals the production of some macromolecules	[162,163]
Neutral lipids	Triacylglycerols	Glycerol and three fatty acids are linked by an ester bond in each molecule	A form of storage lipids turned into biodiesel in the form of FAME through transesterification	[156,164, 165]
	Diacylglycerols	Glycerol and two fatty acids are linked by an ester bond in each molecule	Act as a precursor for TAG, phospholipids and phosphatidic acid	[22,166, 167]
Free fatty acids	Monounsaturated free fatty acids	Oleic acid, palmitoleic acid	Fundamental components in neutral lipids	[168,169]
	Polyunsaturated free fatty acids	Linoleic acid, arachidonic acid	Fundamental components in polar lipids	
	Saturated free fatty acids	Lauric acid, stearic acid	Fundamental components in neutral lipids	

3.2. Factors affecting lipid production in microalgae

This section discusses various factors and parameters such as light intensity, temperature, required nutrients, supply of carbon dioxide, system pH and salinity that usually influence the microalgal cultivation. In most cases, some strains may require stress treatment (e.g. nutrient limitation or depletion) to overproduce lipids and some strains may be affected by external factors such as temperature or other environmental factors for enhanced lipids production.

3.2.1. Light intensity

Since microalgae are capable of performing photosynthesis, light is a crucial parameter affecting lipid production. Biodiesel is composed of non-polar lipids, particularly TAGs [178]. The production of non-polar lipids is promoted by low intensity of light, as polar lipids content is reduced under low light intensity to protect the photosystem [40]. It has been found that increasing light intensity will promote the synthesis of oxidizing agents (i.e., reactive oxygen species (ROS) and oxidative stress) which damage polyunsaturated fatty acids [179], thus reducing the polar lipids content and as a result, the content of non-polar lipids will increase [180]. In other words, the accumulation of lipids requires optimum conditions in which high light intensities (above saturation level) may trigger photoinhibition and lead to cessation of starch synthesis and low lipid productivity [181,182]. However, very low light intensity (or heterotrophic condition) might cut-off both starch and lipid synthesis [171,183]. Therefore, the optimum light intensity for the growth of most microalgae species is within the range of 200–400 μmol photons $m^{-2}s^{-1}$ [184]. For *Scenedesmus obliquus*, Nzayisenga et al. [185] reported that fatty acids content doubled from 5.8% to 11.6% under a light intensity of 300 μmol photons m^{-2} s^{-1}. Another study showed that both *Chlorella* sp. and *Monoraphidium* sp. had increased neutral lipids content when cultivated below 400 μmol photons m^{-2} s^{-1} light intensity [186].

The cultivation of microalgae under mixotrophic condition involves the cycles of light and dark reactions. Under this condition, the light reaction cycle undergoes the conversion of light energy into chemical energy which primarily occurs in thylakoids (membrane-bound compartments). During the dark reaction cycle, the chemical energy is further transformed into stable chemical energy [187,188]. As this light/dark cycle period persists simultaneously, it may influence the microalgal photosynthetic process unit turnover time, therefore a maximum photosynthetic efficiency can be attained under this growth

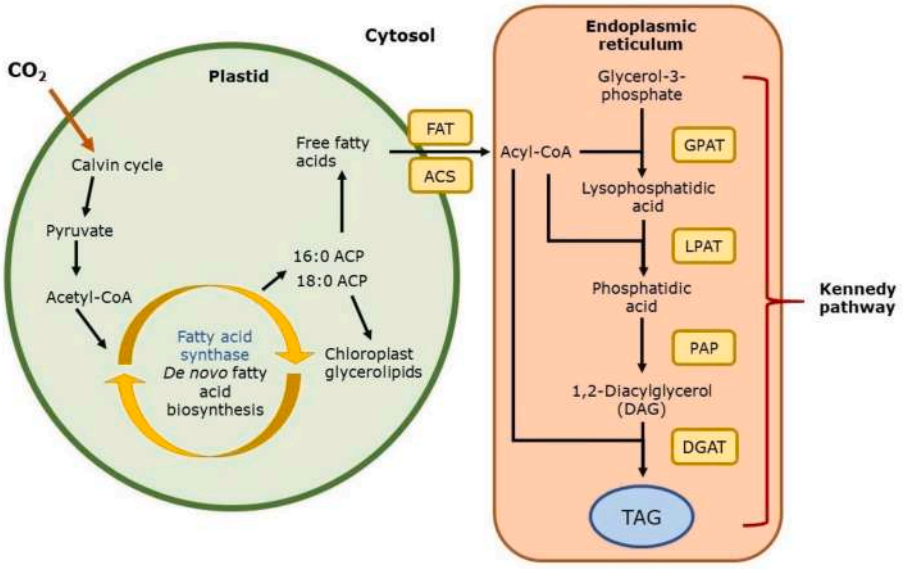


Fig. 4. Schematic illustration of the lipid production process in a microalgal cell. Modified from Chen and Smith [176] with permission from Elsevier.

condition [189]. Additionally, variable light intensities and light–dark cycles have been shown to change the lipid profile in microalgae via changing the metabolism of lipids. Triacylglycerides are produced in the presence of light, and various species require varying light intensities. In addition, the composition of triacylglycerides varies depending on the species and the amount of light exposure. To summarize, light often promotes the production of fatty acids, growth, and the development of membranes, especially chloroplast membranes [190].

In order to fully utilize microalgae as a feedstock for the production of biodiesel, several aspects such as biomass growth and lipid productivity must be taken into consideration. The capacity of microalgae to biosynthesize lipids is influenced by a variety of factors, and the relationship between light intensity and production is important. The studies with continuous illumination (i.e. autotrophic growth mode) demonstrated that lipid formation is increased by strong continuous light [191]. Algal lipid synthesis has been hypothesized to be influenced by light intensity, with intense illumination leading to their build-up. It was revealed that switching from low light to high light conditions boosted lipid accumulation in *Haematococcus pluvialis* species as well [192]. A study by Wahidin et al. [193] examined the lipid production at different light/dark cycles at a uniform light intensity for *Nannochloropsis* sp. The highest percentage of total lipids (31.3%) was attained when the culture was exposed to 18 h of light and 6 h of darkness, on day eight of cultivation. While, at 24:0 light, the total lipid content was substantially lower (27.95%) as compared to 18:06 light/dark cycles at an identical light intensity of 100 μmol $m^{-2}s^{-1}$. The lipid content further decreased to 25.59% because of the photoperiod cycle of 12 h light, 12 h dark. The findings showed that a high lipid content typically corresponds to faster biomass growth rates.

Light penetration and angle of incidence also has a strong effect on microalgal cells. Generally, only 7–10 cm of water at the top level of the water column experiences enough light penetration for efficient photosynthesis. Therefore, the microalgal cells in the deeper parts do not receive enough natural light [40]. This can be resolved by using a photobioreactor during cultivation [180]. Under ideal conditions, a photobioreactor should be able to capture all the available light from the surroundings of the system and supply it to the culture [194]. It is suggested to provide sufficient mixing using a magnetic stirrer or an airstone aerator to enhance light distribution in the culture medium. The chlorophyll of microalgae absorbs light spectrum bands in regions of blue and red. Therefore, providing the light radiation that lies in those wavelength regions could improve the rate of photosynthesis and lipid productivity, as well as reduce energy consumption if not using sunlight as the source. A study reported that blue light promotes the synthesis of enzymes such as carbonic anhydrase and ribulose biphosphate carboxylase, which helps in lipid production [178]. Besides, the composition of lipids by weight in dry biomass of *Chlorella* sp. was also found to be doubled when cultivated under red light [40].

3.2.2. Temperature

Temperature affects metabolic processes such as photosynthetic rate, growth and lipid accumulation in microalgae [195]. Most microalgae are capable of producing lipids in the temperature range between 15 and 40 °C. The most favourable temperature range for microalgal growth is reported to fall between 20 and 30 °C. Thermophile species such as *Anacystis nidulans* and *Chaetoceros* sp. can survive and grow well at a temperature of ca. 40 °C [178,196]. The ideal temperature for growing algae varies depending on the species and desired algal response. However, it is difficult and costly to regulate the temperature in outdoor conditions. The evaporation of medium, overheating and cooling in outdoor conditions, and lipid composition are some of the significant obstacles for algal growth, in addition to the severe temperatures. According to Chaisutyakorn et al. [195], higher temperatures cause the accumulation of saturated lipids whereas lower temperatures cause the accumulation of unsaturated lipids. On the other hand, numerous researchers reported that temperature has little impact on lipid accumulation. Therefore, there are opposing viewpoints on this subject. Wu et al. [197] reported that *Monoraphidium* sp. grew well under 25–35 °C, with optimum lipid productivity of 29 mg $L^{-1}d^{-1}$ at 30 °C. The cells then decayed at 40 °C, with no lipid productivity and negligible biomass productivity, compared to those under 25–35 °C. Another study on *Chaetoceros* sp. showed an optimum growth at 25 °C with a lipid content of about 20.42% [195]. Converti et al. [198] have also reported that lipid accumulation in *Chlorella vulgaris* and *Nannochloropsis oculata* increased from 7.90 to 15.31% and 5.90–16.41% at 25–30 °C, respectively.

3.2.3. Nutrients: nitrogen and phosphorus macronutrients

Besides carbon and oxygen, microalgal cells also consist of nitrogen and phosphorus compounds. Nitrogen and phosphorus are macronutrients, required for microalgal growth. Nitrogen can be obtained from urea and nitrates while phosphorus can be obtained from phosphates [199]. Nitrogen-deficient conditions have been shown to promote lipid biosynthesis by affecting other biochemical pathways in microalgae [200]. According to Hess et al. [147], nitrogen starvation alters the cellular carbon metabolism from protein to lipid synthesis and also the fatty acid composition by promoting the production of saturated and monounsaturated fatty acids. Shi et al. [180] suggested that the degradation of nitrogenous compounds such as chlorophylls and proteins results in more carbon and energy available for lipid accumulation.

On the other hand, phosphorus also affects carbon flow between the synthetic pathways of starch and lipids by inhibiting the activity of ADP-glucose pyrophosphorylase, an enzyme committed in the first step of starch synthesis [201]. This reduces the activity of starch synthesis, thus shifting the metabolic flux toward lipid accumulation [180]. Therefore, nitrogen and phosphorus starvations are commonly used to promote lipid production in microalgae. A study using *Neochloris oleoabundans* microalgae, cultivated under nitrogen-deprived conditions reported TAG productivity of 46.19 mg L^{-1} d^{-1}, which was almost 2-fold compared to initial productivity (23.47 mg L^{-1} d^{-1}) [202]. Yang et al. [203] tested *Chlamydomonas reinhardtii* under nitrogen deprivation conditions and different concentrations of phosphorus. Both nitrogen and phosphorus deprivation conditions yielded the highest total fatty acid (TFA) content of 105 μg/mg, which was 104.7% higher than the control. Other micronutrients such as potassium, cobalt, manganese, iron and zinc are needed in small amounts only, but can significantly affect the growth by triggering enzymatic activities [204].

3.2.4. Carbon dioxide

Carbon is the main element in microalgal cells, which can represent 50% of the microalgal biomass. The content of elemental carbon determines growth conditions as well as the carbon sequestration rate. In most cases, the CO_2 requirement in the cultivation of microalgae is about 2% (v/v) of CO_2 with an aeration gas mixture, yet this depends on case-to-case basis. Carbon dioxide and bicarbonate ions are preferred sources of inorganic carbon for microalgae. Variation in carbon concentration also affects the fatty acid composition and saturation [145]. With a 15% increment in CO_2 concentration, some strains generate more long-chain fatty acids such as C18:0, C18:1 and C20:0 [178]. Uniform distribution of carbon dioxide can be achieved by providing constant mixing. Increasing CO_2 aeration has been proven to enhance lipid content in microalgae [205]. When CO_2 concentration increased to 15% in a photobioreactor with blue light, *Chorella vulgaris* and *Gloeothece membranacea* yielded lipid content of 25.6% and 36%, respectively [205]. Another study on *Scenedesmus dimorphus* was conducted under different CO_2 concentrations and nitrogen: phosphorus (N:P) ratios. The lipid productivity peaked at 6.6 mg L^{-1} d^{-1} and maximum lipid content of 31.6% dry weight (DW) of biomass was achieved, at 6% CO_2 in supplied air and N:P ratio of 1:1 [206]. On the other hand, considering the supply of CO_2 in large-scale cultivation may incur an increase in the cost of biomass. Therefore, it is suggested to build an algal plant and valorize free sources of CO_2 from nearby local industry (e.g., biogas plant, lime

works and power station).

3.2.5. pH and salinity

The pH plays an important role in carbon supply, algal growth as well as in the species diversity in mixed cultures [207]. The pH can fluctuate during day and night in closed cultures, as bicarbonate and carbon dioxide consumption for photosynthesis during the day shifts the pH of the medium to alkaline, while the production of carbon dioxide during respiration at night shifts the pH to acidic [147,208]. The most favorable pH range for microalgal growth lies between pH values 6 to 8.76. Most species are sensitive to pH change, but species such as *Chorella vulgaris* can survive a broad range of pH. Variation in pH can affect the cell growth and lipid accumulation [209]. Species such as *Chlorella* sp., *Nannochloropsis salina* and *Pavlova lutheri* were found to have optimum growth at pH 8, with lipid content of 23%, 24.75% and 35%, respectively [210,211]. According to BenMoussa-Dahmen et al. [212], *Amphora subtropica* and *Dunaliella* sp. had optimum growth at pH 9 and 10, respectively. Under optimized parameters, lipid content was found to increase from 150 to 190 g lipid kg^{-1} biomass in *Amphora subtropica* while lipid content increased from 190 to 280 g lipid kg^{-1} biomass in *Dunaliella* sp. microalgae.

Salinity stress is also a strategy to improve lipid production in microalgae. Under conditions with high salinity stress (the lipid content enhanced from 60 to 70% by increasing the sodium chloride (NaCl) concentration from 0.5 to 1.0 M, for *Chlorella vulgaris*), carbon distribution is likely to shift from starch to lipids synthesis. Under high salinity stress conditions, lipids content increased while the starch and protein content decreased. As reported, the lipid content increased from 60 to 70% when the concentration of NaCl increased from 0.5 to 1.0 M [156,213]. It is because of the upregulation of ACCase (the main enzyme responsible for lipid synthesis) and the downregulation of AGPase (the main enzyme for the synthesis of starch) [180]. High pH also promotes the production of more saturated fatty acids. However, the optimal range of pH and salinity varies between the microalgae strains and their specific needs to determine their respective lipid productivity.

Ho et al. [214] reported that carbon distribution is shifted from starch to lipids synthesis under salinity stress. Results have shown a 13.5-fold increase and a 10.5-fold increase in mRNA levels of enzymes responsible for starch degradation and lipid synthesis, respectively. It is also reported that up to a 56.9% increase in lipid content, along with a decrease in starch content is obtained. Rai et al. [209] studied the effect of salinity on *Chlorella* sp. by growing the microalgae in different concentrations of NaCl (0, 0.2, 0.5, 0.8, and 1.1 M). The lipid production increased with NaCl concentration from 0.0 M to 0.5 M, and peaked at 0.5 M NaCl with lipid production of 0.1842 g L^{-1}, then decreased at 0.8 M and 1.1 M. The lipid content at 0.5 M was 7.93% higher than 0.0 M (control). Shah et al. [211] reported that the lipid content of *Pavlova lutheri* increased with the concentration of NaCl in the range of 15–35 ppt, which peaked at 35 ppt with lipid content of 36%, then decreased to 34% at NaCl of 40 ppt. Various factors affecting lipid production in microalgae are shown in Table 5.

Table 5
Factors affecting lipid production in microalgae.

Factor	Parameters	Strains	Results	References
Light	Light intensity of 300 μmol m^{-2} s^{-1}	*Scenedesmus obliquus*	Fatty acid contents doubled from 5.8% to 11.6%	[185]
	Light intensity of 400 μmol m^{-2} s^{-1}	*Chlorella* sp.	Neutral lipid content comprised 71.66% of total lipid content	[186]
		Monoraphidium dybowskii	Neutral lipid content comprised 60.65% of total lipid content	
	Light intensity of 8000 lux	Marine *Chlorella* sp.	Lipid content peak at 397.8 mg/L	[57]
	Light intensity of 5000 lux	*Nannochloropsis* sp.	Lipid content peak at 572.8 mg/L	
	LEDs of red light 220 lux	*Chlorella* sp.	Lipid content increase to 60% (w/w) compare to white light (30% (w/w))	[215]
Temperature	30 °C	*Monoraphidium* sp.	Optimum lipid productivity of 29 mg/L d	[197]
	25 °C	*Chaetoceros* sp.	Optimum lipid content of 20.42%	[195]
Nutrients	3 days under nitrogen starvation	*Neochloris oleoabundans*	TAG productivity of 46.19 mg/L d and TAG content of 26.38% DCW were achieved.	[216]
	Nitrogen and phosphorus starvation, sodium acetate added	*Chlamydomonas reinhardtii*	TFAs content of 105 μg/mg, which is 104.7% higher than the control group.	[203]
Carbon dioxide	20% CO_2 aeration	*Botryococcus braunii*	Total lipid content of 12.71%	[217]
	15% CO_2 aeration with 200 μmol m^{-2} s^{-1} blue light	*Chlorella vulgaris*	Total lipid content of 25.6%	[205]
		Guzmania membranace	Total lipid content of 36%	
	6% (v/v) CO_2 aeration with NP ratio 1:1	*Scenedesmus dimorphus*	Maximum lipid productivity of 6.6 mg/L d and maximum lipid content of 31.6% DW of biomass	[206]
pH and salinity	pH 8	*Nocardiopsis salina*	Lipid accumulation of 24.75% by mass	[210]
	pH 8	*Pavlova lutheri*	Lipid content of 35%	[211]

(continued on next page)

3.3. Methods for lipids extraction from microalgae

The lipid extraction process from microalgae refers to the separation of the desired neutral lipids and fatty acids from the cells and culture medium [218]. Microalgae have complex and strong cell walls made of polysaccharides and proteins. Prior to the lipid extraction, a suitable cell disruption process or pretreatment is necessary depending on the cell type [23]. After cell disruption, the lipid extraction process is carried out using solvents as the conventional method for lipid extraction from microalgae. This section will evaluate various conventional and recent advanced strategies available for lipid extraction from microalgae. An overview of the upstream and downstream processing of microalgae is illustrated in Fig. 5.

Table 5 (*continued*)

Factor	Parameters	Strains	Results	References
	35 ppt NaCl		Lipid content of 36%	
	pH 8	*Chlorella* sp.	Maximum lipid production of 0.1995 g/L and lipid accumulation of 23%	[209]
	0.5 M NaCl		Lipid production of 0.1842 g/L and lipid content of 21.40%	
	pH 9, light period 21 h and 31 °C	*Amphora subtropica*	Lipid content increased from 150 to 190 g/kg	[212]
	pH 10, light period 24 h and 34 °C	*Dunaliella* sp.	Lipid content increased from 190 to 280 g/kg	

3.3.1. Conventional mechanical methods

Conventional mechanical methods involve the use of a solid physical surface to disrupt or pretreat the cell wall of the microalgae. The common conventional mechanical methods are expeller press, grinding and beat beating. While, the recent mechanical methods involve microwave, ultrasound, osmotic pressure and pulsed electricity methods. This section will discuss in detail each of these methods and respective mechanisms.

3.3.1.1. Expeller press and grinding. This is a traditional method similar to extracting oil from seeds and crops where high mechanical pressure is applied to microalgae [219]. This can disrupt the cell wall and also squeeze out the lipids directly. Extraction yield increases with pressure, but too much pressure can lead to the downgrading of extracted oil due to the heat generated. If there is high heat generated during the process, cooling is needed, and this will increase operational costs. Moreover, it is reported that colour pigments are extracted along with the extracted oil, thus removing them incurs additional costs [220,221]. Zheng et al. [222] reported a lipid extraction efficiency of 29% from *Chlorella vulgaris* after grinding the cells with liquid nitrogen. Kim et al. [223] obtained a lipid extraction efficiency of 60.1% from *Scenedesmus* sp. before solvent extraction. With expeller press, Topare et al. [220] obtained a yield of 1250 mL of algae oil from 70 kg of algae without further solvent treatment.

3.3.1.2. Bead beating. Bead beating disrupts the cell by treating biomass slurry together with fine beads spinning at high speed to grind the cells. There are two types of bead mills: shaking vessels and agitated beads. In the shaking vessel, the entire culture vessel is shaken to damage the cells. Normally, more than one vessel will be used to enhance disruption. In the agitated type, the cell culture is agitated with beads. The second type has more effective disruption on the cells as it combines the effects of agitation, collision and grinding of the beads. The beads can be made from zirconia-silica, titanium carbide or zirconium oxide and the ideal bead diameter is 0.5 mm [219]. Zheng et al. [222] obtained a lipid yield of around 10% dry weight (DW) from *Chorella vulgaris* by beating with beads of diameter ranging from 0.40 to 0.60 mm with a rate of 1500 rpm for 20 min. Alavijeh et al. [224] combined bead beating and enzyme hydrolysis to extract lipid from *Chorella vulgaris*. The lipid yields were found to be 0.05 and 0.06 g lipid/g biomass for biomass treated with protease and lipase, respectively, after bead beating.

3.3.2. Recent advanced mechanical methods

3.3.2.1. Microwave-assisted pretreatment. Microwave electromagnetic radiation lies in the range of frequency of 0.3–300 GHz. It can resonate the polar components such as water molecules and this damages the algal cell membrane as well as generating heat. This method can be used to treat both wet and dry microalgal biomass, but with better performance on wet biomass. This is a method that only causes cell disruption, and solvent treatment is needed afterward [42,221]. Nogueira et al. [225] obtained a lipid recovery of around 24.2% in *Chaetoceros calcitrans* with microwave pretreatment. Rokicka et al. [226] used the microwave as a pre-treatment for lipid extraction from *Botryococcus braunii* and *Chorella vulgaris*. The lipid yields were obtained as 56.42% and 41.31% for *Botryococcus braunii* and *Chorella vulgaris*, respectively.

3.3.2.2. Ultrasound-assisted pretreatment. This method applies sound waves of frequency above 20 kHz to the culture medium, generating a cycle of alternating compression and rarefaction, which are parts with high pressure and low pressure, respectively. This method aims to disrupt the cytoplasm of cells and release lipid molecules. One of the advantages of using this method is the process of cavitating cytoplasm with ultrasonic waves [42,221]. Before extraction with the solvent mixture, Ido et al. [227] treated *Scenedesmus obliquus* by ultrasonication and the overall recovery yield of the extraction was found to be 26.63% by weight. Rokicka et al. [226] used ultrasonication on *Botryococcus*

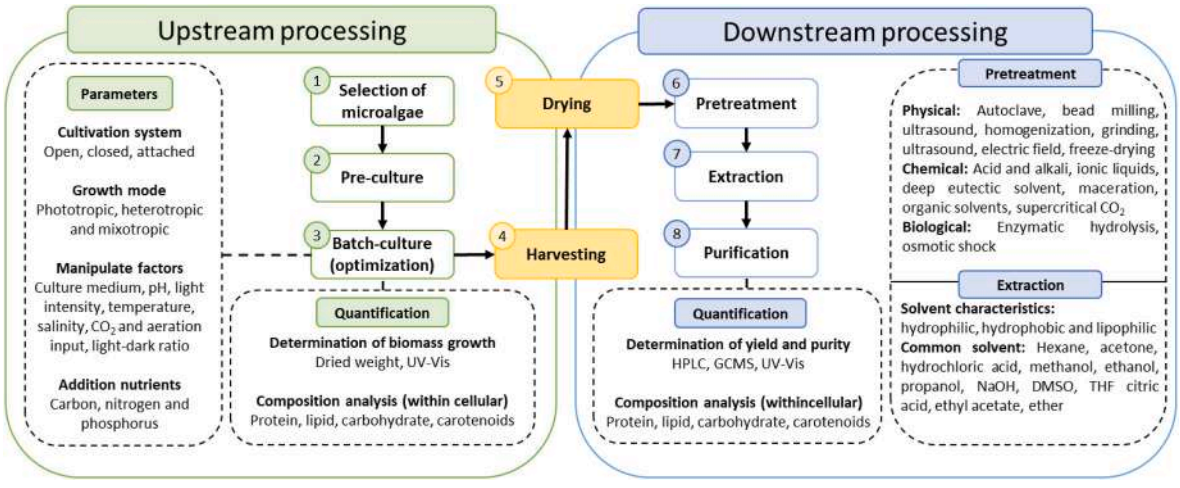


Fig. 5. Detailed steps involved in the upstream and downstream processing of microalgae.

braunii and *Chorella vulgaris* and obtained lipid yields of 39.61% and 35.28%, respectively. In another study conducted by González-Balderas et al. [228], ca. 73% lipid recovery was achieved from *Desmodesmus* sp. biomass when ultrasound was used as pre-treatment before extraction. Fig. 6 shows the pretreatment before and after ultrasound-assisted pretreatment and the mechanism involved during the cavitation process produced by the ultrasound.

3.3.2.3. Osmotic pressure method. This method alters the osmotic pressure by altering the salt concentration in the culture media to levels much higher or much lower than the normal level. The imbalance of osmotic pressure between regions inside and outside of the microalgal cells disrupts the cell walls. This method can be achieved by hyper-osmotic and hypo-osmotic conditions. Hyper-osmotic refers to the condition where the exterior of the cell has a higher salt concentration. The fluids diffuse out from the cell and cause the cell to shrink, damaging cell envelopes. While hypo-osmotic refers to the condition where the exterior of the cell has a lower salt concentration, causing the fluid to diffuse into the cell and resulting in the cell to burst if the stress is too high [219]. Yoo et al. [232] used osmotic shock to treat wet *Chlamydomonas reinhardtii* biomass for lipid extraction. After treating with sorbitol and NaCl as osmotic agents for 7 days, the final lipid yields were found to be 5.96 and 6.67 mg/L, respectively, which represented a lipid recovery of 9.06 and 34.5%, respectively. González-González et al. [228] tested osmotic shock as pre-treatment on *Dunaliella salina* and *Chaetoceros muelleri*, and the results showed that this treatment was more effective on *Chaetoceros muelleri* than *Dunaliella salina*. After treatment with osmotic shock, lipid recoveries for *Chaetoceros muelleri* and *Dunaliella salina* were noted as 72% and 21%, respectively. Fig. 7 shows the diffusion of fluid concept for isomostic, hypoosmotic and hyperosmotic conditions of the microalgae cell.

3.3.2.4. Pulsed electric field-assisted pretreatment or electro-permeabilization. This cell disruption method is used to create micro-pores at the cell membrane by applying short electric pulses with a large electric field force. It is also known as electroporation or electro-immobilization. This method has been successfully used to extract lipid molecules from *Chlorella vulgaris* [233]. The study also showed that higher exposure time leads to higher lipid yield. However, this method requires a large amount of energy and costs, thus it is not feasible for large-scale applications [42]. Silve et al. [234] applied PEF on *Auxenochlorella protothecoides* cultivated mixotrophically and autotrophically before solvent extraction. Lipid recovery was found to be 97% and 90%, respectively, after 20 h of extraction. Papachristou et al. [235] observed up to 83% of lipid recovery from *Scendesmus almeriensis* with PEF applied before solvent extraction. Fig. 8 shows the pretreatment before and after pulsed electric field-assisted pretreatment and the mechanisms involved during the permeabilization process produced by the electric field. Table 6 summarizes the recent advances in mechanical methods used in microalgal lipid extraction.

3.3.3. Advanced chemical methods for lipid extraction

Advanced chemical methods for lipid extraction involve techniques such as supercritical carbon dioxide (SC–CO_2), enzymatic biocatalysis, use of engineered nanoparticles, bio-based solvents, ionic liquids (ILs), switchable solvents and deep eutectic solvents. Each of these respective extraction methods requires an optimization procedure in order to obtain high lipid productivity. Most of these chemical methods will implement green and environmental-friendly solvents for the recovery of lipids from microalgae biomass.

3.3.3.1. Supercritical carbon dioxide. Carbon dioxide achieves a supercritical state at 72 bar and 32 °C [42]. In this state, it possesses solvent properties of liquid and mass transfer properties of gas, which means a greater diffusion coefficient [243]. Its selectivity towards non-polar lipids can be further enhanced by using it with polar co-solvents such as toluene, methanol and ethanol. The polar co-solvents aid in delinking the bond between non-polar lipids and polar lipids, so more non-polar lipids can be extracted by supercritical carbon dioxide [218]. Lorenzen et al. [243] performed extraction with CO_2 at 120 bar and 20 °C on *Scenedesmus obliquus* and obtained 92% of the total lipids. The yield obtained by Obeid et al. [244] from *Chlorella vulgaris* was up to 97% when treated with supercritical CO_2 at 450 bar and 50 °C, with the addition of co-solvent ethanol (10% v/v). Supercritical carbon dioxide is gaining more attention as a greener solvent compared to conventional organic solvents based on VOCs. It has higher selectivity towards the desired non-polar lipids and poses greener characteristics such as lower toxicity, negligible environmental effects and inert chemical properties,

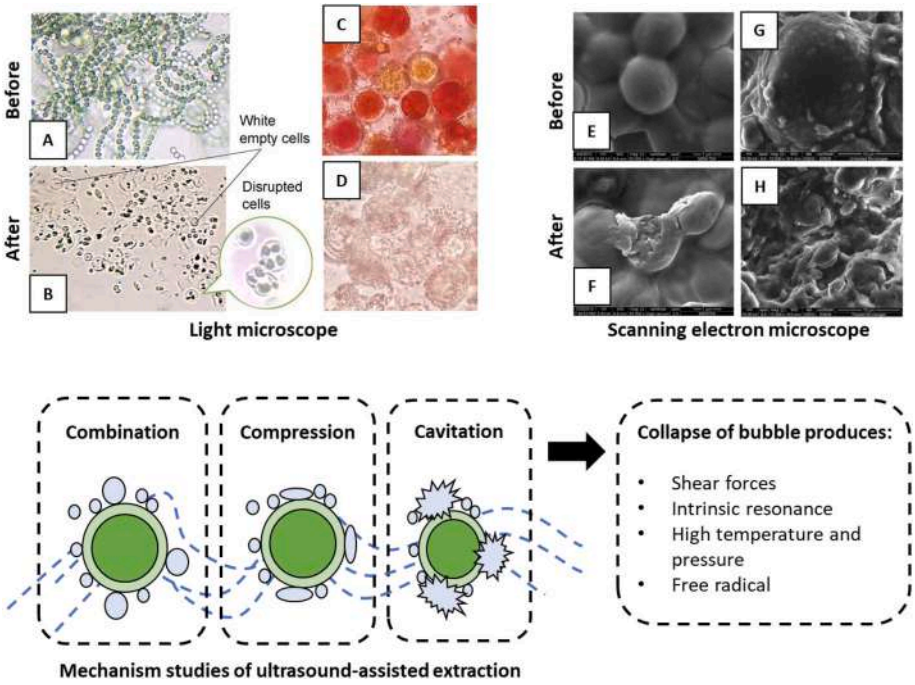


Fig. 6. Effect of ultrasound-assisted pretreatment before and after shown in the light microscope and scanning electron microscope. **(A)** *Trichormus variabilis* microalgae before treatment (light microscopic), **(B)** *Trichormus variabilis* microalgae after treatment of 50% amplitude, 20 kHz frequency for 15 min (light microscopic) adapted from Haque et al. [229] with permission from Elsevier, **(C)** *Haematococcus pluvialis* microalgae before treatment (light microscopic), **(D)** *Haematococcus pluvialis* microalgae after treatment of 40% amplitude for 25 min (light microscopic) adapted from Khoo et al. [230] with permission from Elsevier, **(E)** *Chlorococcales* (*Scenedesmus* sp., and *Chloroccum* sp.) microalgae before treatment (scanning electron microscope), **(F)** *Chlorococcales* (*Scenedesmus* sp., and *Chloroccum* sp.) microalgae after treatment of 30 kHz, 0.4 kW/L for 1 h (scanning electron microscope) adapted from Keris-Sen et al. [231] with permission from Elsevier, **(G)** *Haematococcus pluvialis* microalgae before treatment (scanning electron microscope), **(H)** *Haematococcus pluvialis* microalgae after treatment (scanning electron microscope) adapted from Khoo et al. [230] with permission from Elsevier.

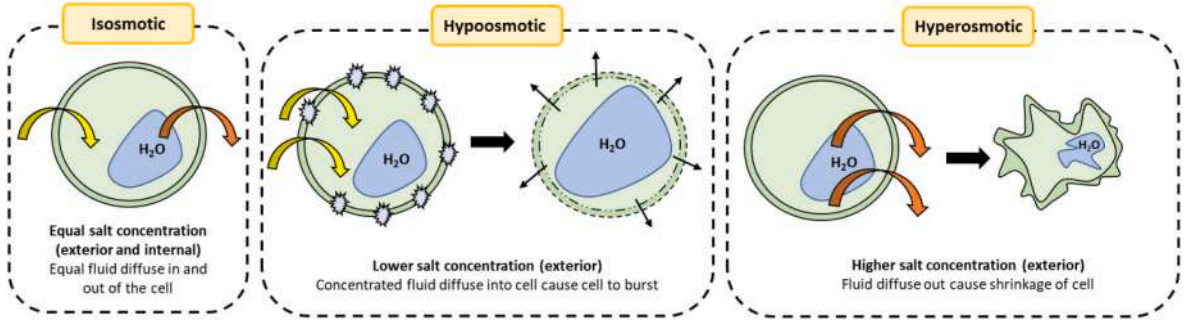
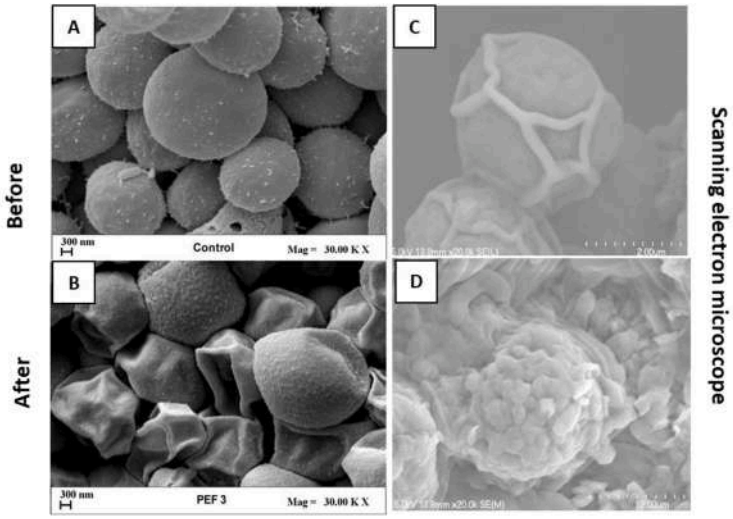


Fig. 7. Conceptual figure of osmotic pressure for isosmotic, hypoosmotic and hyperosmotic conditions of the microalgae cell.



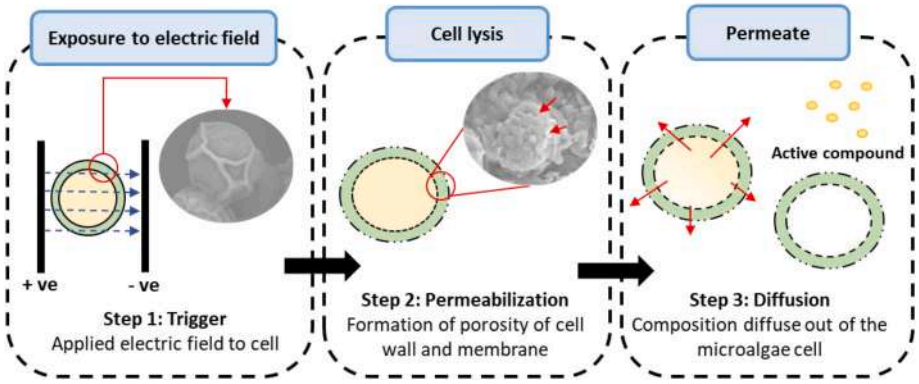


Fig. 8. Mechanism studies and effect of pulsed electric field-assisted pretreatment before and after shown in the scanning electron microscope. **(A)** *Chlorella vulgaris* (CCAP 211) before treatment (control), **(B)** *Chlorella vulgaris* (CCAP 211) after treatment (100 kJ/kg, 30 kV/cm) adapted from Carullo et al. [236] with permission from Elsevier, **(C)** *Chlorella pyrenoidosa* before treatment (control) **(D)** *Chlorella pyrenoidosa* after treatment (20 kV/cm, 130 Hz, 6 μs) adapted from Han et al. [237] with permission from Elsevier.

all of which contribute to its increasing popularity as part of the biofuel production chain. This method has been commercially available due to its wide applications in the pharmaceutical and food industries.

3.3.3.2. Enzymes. Enzymes are useful biocatalysts as they can bind to specific molecules in the cell wall and hydrolyze the bonds causing membrane rupture in the process of lipid extraction [245]. Recent research has focused on the application of different enzymes such as chitinases, pectinase, amylase, cellulose and lysozyme on *Chlorella vulgaris* for lipid extraction [246]. Chitinases, pectinase and lysozyme successfully damage the cell wall of the cells. Moreover, using a combination of enzymes also results in higher lipid extraction yield [42]. Zhang et al. [247] demonstrated enzymatic hydrolysis with a mixture of cellulose, xylanase and pectinase on *Scendesmus* sp. and recovered 86.4% of lipid. He et al. [248] used a mixture of cellulose, hemicellulose, papain and pectinase as enzymes for three-phase partitioning and it yielded 92.59% of FAMEs in *Nannochloropsis oculata*.

3.3.3.3. Engineered nanoparticles. Nanoparticles (NPs) are less hazardous, more stable and more reusable than conventional solvents in extraction steps. High lipid extraction of 74.29% was obtained in *Chlorococcum* sp. by using titanium (Ti) nanoparticles at a dosage of 15 mg L^{-1} [42]. Besides, zirconium dioxide (ZrO_2) nanoparticles are reported to improve lipid release in *Chlorococcum* sp. by two times in the presence of chloroform:methanol solvent [42]. Huang and Kim [249] reported a yield of 98.75% lipid from *Chlorella vulgaris* with NiO-NPs followed by Folch's method. Khanra et al. [250] used ZrO_2 NPs followed by Bligh and Dyer technique to extract lipids in *Chlorococcum* sp., and the yield was found to be 78.52%. It can be observed that the use of engineered nanomaterial enhances the capabilities of microalgae to

Table 6
Conventional and recent mechanical methods used in microalgal lipid extraction.

Method	Parameters	Strains	Solvent (v/v)	Lipid recovery	References
Expeller press	Pressure that creates 140–210 °F	Algae from the open pond system	–	1250 mL algae oil yielded from 70 kg of algae	[220]
Grinding	–	*Scenedesmus* sp.	$CHCl_3$:MeOH (2:1 v/v)	Extraction efficiency of 60.1%	[221]
Bead beating	Glass beads diameter of (0.40–0.60 mm), 1500 rpm, 20 min	*Chorella vulgaris*	–	Lipid yield of around 10% DW	[222]
	Bead beating: 0.4 mm Y_2O_3 stabilized zirconium dioxide, ZrO_2 beads, 2039 rpm, 25 °C Hydrolysis: lipase (2% v/w), 37 °C, 24 h, pH 7.4	*Chorella vulgaris*	Tris–HCl, $CHCl_3$:MeOH (1.78:2.22 v/v)	0.06 g lipid/g biomass yielded	[224]
	Bead beating: 0.4 mm Y_2O_3 stabilized zirconium dioxide, ZrO_2 beads, 2039 rpm, 25 °C Hydrolysis: protease (2% v/w), 37 °C, 24 h, pH 7.4	*Chorella vulgaris*	Tris–HCl, $CHCl_3$:MeOH (1.78:2.22 v/v)	0.05 g lipid/g biomass yielded	[224]
Microwave	2.45 MHz, 1.4 kW, 40 s	*Chaetoceros calcitrans*	Water: propan-2-ol: cyclohexane (11:8:1, v/v/v)	~24.2%	[225,238]
	2.45 GHz, 400 W, 60 s	*Botryococcus braunii* *Chlorella vulgaris*	$CHCl_3$:MeOH (2:1, v/v)	56.42% 41.31%	[226]
	400 W, 70 °C, 1 bar, 15 min	*Arthrospira platensis*	Methanol:ethyl acetate: light petroleum (1:1:1, v/v/v)	1.59% of fatty acid	[239]
	1000 W, 100 °C, 10 min	*Scenedesmus obliquus*	Chloroform:methanol (1:1, v/v)	19.25%	[234]
	400 W, 70 °C, 1 bar, 15 min	*Arthrospira platensis*	Methanol:ethyl acetate: light petroleum (1:1:1, v/v/v)	1.59% of fatty acid	[240]
	2.46 GHz, 2800 W, 200 μs, 9 Hz pulse	*Auxenochlorella protothecoide*	16.1 mL ethanol and 6.6 mL hexane	37.29%	[239]
Ultrasound	24 kHz, 400 W	*Scenedesmus obliquus*	n-hexane: isopropanol (1:10, w/v)	26.63% by weight	[227]
	24 kHz, 400 W, 60 s	*Botryococcus braunii* *Chlorella vulgaris*	$CHCl_3$:MeOH (2:1, v/v)	39.61% 35.28%	[226]
	Step 1: 42 kHz, 100 W Step 2: 40 kHz, 300W	*Desmodesmus* sp.	$CHCl_3$:MeOH (2:1, v/v)	~73%	[241]
	50/60 Hz, 620 W, 60 °C for 3 h	*Chlorella vulgaris* *Scenedesmus obliquus* *Nannochloropsis oculata*	n-hexane:isopropanol (2:1, v/v)	21.0 g per g biomass 0.31 g per g biomass 0.32 g per g biomass	[242]
Osmotic pressure	60 g L^{-1} sorbitol, n-hexane:methanol (7:3)	*Chlamydomonas reinhardtii*	$CHCl_3$:MeOH (2:1, v/v)	5.96 mg/L lipid yielded after 7 days	[232]
	60 g L^{-1} NaCl, n-hexane:methanol (7:3)			6.67 mg/L lipid yielded after 7 days	
	Biomass:water (1:15)	*Dunaliella salina*	–	21%	[228]
	Biomass:water (1:5)	*Chaetoceros muelleri*	–	72%	
Pulsed electric field	1.5 MJ kg_{DW}^{-1}, 1 μs, 40 kV cm^{-1}	*Scenedasmus almeriensis*	Ethanol:hexane (1:0.41, v,v)	~70% total lipids without incubation, 83% after 24 h incubation before solvent treatment	[235]
	1.5 MJ kg_{DW}^{-1}, 1 μs, 40 MV m^{-1}	*Auxenochlorella protothecoides*	Water:Ethanol: Hexane (1:18:7.3, v/v/v/v)	After 20 h of extraction, 97% and 90% recovery for microalgae from mixotrophic and autotrophic cultivation, respectively	[234]

biosynthesize more lipids, whereas several studies have also reported its ability to promote effective cell harvesting of microalgae [251]. Such nanoparticle-engineering also aids micro-environmental stress inducement, micronutrient supplementation, backscattering of light, magnetic separation and nanoparticle-based flocculation which would greatly influence the economic viability of microalgal biorefinery industry [251,252].

3.3.3.4. Bio-based solvents. Wan Mahmood et al. [253] conducted a study in which they compared the effectiveness of bio-based solvents and conventional solvents in extracting lipids from microalgal biomass. Four bio-based solvents were compared with a conventional solvent (hexane) for lipid extraction from *Chlorella vulgaris* and *Nannochloropsis* sp. The four bio-based solvents tested were ethyl acetate (EtOAc), ethyl lactate (EtLac), cyclopentyl methyl ether (CPME), and 2-methyl-tetrahydrofuran (2-MeTHF). In case of microalgal crude lipid yields, all the bio-based solvents exhibited better results compared to hexane for both strains of microalgae. In case of extraction of trans-esterifiable lipids, bio-based solvents showed similar yields as hexane for *Chlorella vulgaris*, but higher yields than hexane for *Nannochloropsis* sp. Moreover, all the bio-based solvents performed better than hexane in the yields of FAME. EtLac provided the highest FAME yield of 33.02% for *Chlorella vulgaris* while 2-MeTHF provided the highest FAME yield of 41.29% for *Nannochloropsis* sp. Huang et al. [254] used biodiesel in the form of FAME as one of the extractants in the lipid extraction from wet microalgae with 70% water content. They reported the highest extraction efficiency of 68.19% with a mixture of biodiesel and methanol in a ratio of 6:4. The yield was 108% higher than the system using a mixture of chloroform and methanol.

3.3.3.5. Ionic liquids. Ionic liquids are organic salts that exist in a liquid state at a wide range of temperatures, typically from 0 to 140 °C. They can disrupt the cell structure of wet microalgal biomass, which then improves the access of co-solvents towards lipids or promotes auto-partitioning of the lipids [255]. This method is suitable for wet biomass as it can eliminate the drying and dewatering steps, therefore reducing the usage of energy by 59% and further reducing additional operating costs [42]. The advantages of using ionic liquids for lipid extraction from microalgae include characteristics such as non-flammability, recyclability, non-volatility and thermal stability [256]. Choi et al. [257] obtained a yield of 250 mg lipid/g cell by treatment with ([Emim]DEP). They also used a mixture of two ionic liquids for lipids extraction. Among the mixtures, the combination of [Emim]OAc/[Emim]$(CF_3SO_2)_2N$ had the highest yield which was 255.6 mg lipid/g cell. Pan et al. [258] used [BMIM][HSO_4] as an ionic solvent on microwave pre-treated *Chlorella sorokiniana, Nannochloropsis salina* and *Galdieria sulphuraria*, and lipid extraction yield was found to be 0.23 g, 0.10 g and 0.19 g per g of dry algae, respectively.

3.3.3.6. Switchable solvents. There are two types of switchable solvents (i.e., smart solvents) namely switchable polarity solvents (SPSs) and switchable hydrophilicity solvents (SHSs) [259]. SPSs switch their polarities depending on the concentration of carbon dioxide, where polarity is increased with the concentration of carbon dioxide [260]. Non-polar or low-polarity lipids are extracted by the low-polarity form of SPSs. SHSs change their properties between hydrophobic and hydrophilic. The two states could also be altered by the addition or removal of carbon dioxide. Detailed mechanistic understanding of switchable ability of switchable solvents from low to high polarity is reported in several studies [261,262]. Lipids were dissolved in hydrophobic form, then carbonated water was added to change SHSs into hydrophilic form. This forms a two-layer mixture that consists of a lipid phase and an aqueous SHSs-containing phase. Lastly, the lipids were removed and SHSs were separated from the aqueous phase and reused [68,75]. A study conducted by Du et al. [261] used N-ethyl butylamine (EBA) as a switchable solvent on *Neochloris oleoabundans* and obtained crude lipid yield of 13 wt% after 18 h. While, Al-Ameri and Al-Zuhair [263] obtained a biodiesel yield of 47.5% when *Chlorella* sp. was treated with N, N-dimethylcyclohexylamine (DMCHA). Another study by Cheng et al. [264] compared the extraction efficiency of N,N,N′,N′-tetraethyl-1, 3-propanediamine (TEPDA) and chloroform-methanol on *Nannochloropsis oceanica*. The results showed a 5% higher extraction yield by TEPDA than chloroform-methanol. Based on these findings, it can be perceived that these switchable solvents exhibit promising results for the extraction of lipids and the reactants such as CO_2 are considered environmentally friendly. However, there are limited studies reporting their feasibility on a larger scale. Thus, it is worth exploring switchable solvents in future studies.

3.3.3.7. Deep eutectic solvents. Deep eutectic solvents (DESs) are an emerging class of green solvents (i.e., transformation of ionic liquid solvents) that are formed from a eutectic mixture of Lewis or Brønsted acids and bases which can contain a variety of anionic and/or cationic species [265,266]. The mechanism of DES solvents is derived via their hydrogen bonding extraction abilities from the high polarity characteristics of water. In most cases, the organic salts (e.g. chloride and choline chloride) act as the hydrogen bond acceptor and the organic solvent (e.g. amides, amines, alcohols, and carboxylic acids) acts as a hydrogen bond donor [267]. Since the cell wall of microalgae is composed of α-cellulose and hemicellulose, the combination of hydrogen bond acceptor/donor in DESs creates an H-bonding interaction that disrupts the cellulose structure composed of hydroxyl (-OH) group [268]. Aqueous deep eutectic solvents (ADES) are found to enhance lipid extraction from microalgae [269]. Several methods such as centrifugation and washing with water are used to separate the biomass from ADES. In a study using *Chlorella,* three different DES were used, and all three ADES-tested strains showed lipid recovery yield of 80.90%, 66.92% and 75.36% which was up to 50% higher than untreated biomass [268]. In another study, *Chlorella* sp. and *Chlorococcum* sp. were treated with choline chloride-acetic acid, which yielded 21% and 15% of FAME content, respectively [270]. Table 7 comprehensively evaluates various recent advanced chemical methods in microalgal lipid extraction.

3.3.4. Conventional chemical methods for lipid extraction

Conventional chemical methods use chemicals (solvents) to extract the lipid from microalgae. The conventional solvents used in lipid extraction include n-hexane, chloroform, methanol, ethanol, petroleum ether, dichloromethane and any mixture of these solvents based on the method and desired lipids [274]. Methanol and chloroform are examples of polar solvents and hexane is a non-polar solvent [42]. The polar and non-polar solvents are often combined to improve the efficiency of extraction. This is because lipid yields depend on the type of solvents and the ratios of non-polar solvents to polar solvents. The theory behind this states that the polar solvent can break the bonds between the polar lipid-non-polar lipid complexes and the non-polar solvent will solubilize the non-polar lipids which are desired for the production of biodiesel [218]. The drawback of polar solvent is that it extracts chlorophyll along with lipids [42].

Hexane, benzene and ether are often used as solvents after the expeller press [275]. The remaining pulp from the expeller is treated with cyclohexane to further extract the remaining oil in the pulp. By using the distillation process, the oil and cyclohexane are easily separated. This method can yield more than 95% of the total oil produced by the algae [118]. The solvents break intermolecular forces such as van der Waals forces, hydrogen bonding and covalent bonding between the lipids and other organelles [276]. The solvent molecules form linkages with lipids depending on polarity, non-polar solvent to non-polar lipids and vice versa. The solvent–lipids complex diffuses across the cell membrane and static organic solvent film, into the bulk solvent, which is the driving force moving algal lipids through the concentration gradient. The process continues until an equilibrium state is reached [218].

Bligh and Dyer method and the Folch method are two of the common methods for lipid extraction [277]. The Folch method uses a mixture of chloroform and methanol with a ratio of 2:1 by volume. The biomass is first mixed and diluted with saline solution, then the resulting mixture develops two separate layers. The lipid in the upper phase is extracted. Bligh and Dyer's method is similar to the Folch method, but it needs the addition of water as a co-solvent to improve the separation [221]. Chloroform and methanol are hazardous, so research is underway to find alternatives [278,279]. The selection of solvents has to be carefully made based on different parameters such as toxicity, efficiency, selectivity towards the desired lipids, ability to recycle, and ability of the solvent to protect lipids from degradation. Table 8 compiles the conventional chemical methods used for microalgal lipid extraction.

3.4. Algae-based biodiesel: Hope for future energy

Microalgae are a well-known renewable resource that contains multiple useful constituents such as lipids, proteins and carbohydrates which can be used to produce many value-added products [21]. Besides, it is considered a promising substitution for fossil fuels in producing biofuel for energy generation [23]. Genetic modification of microalgae is now being widely investigated to improve its resistance to pathogens or chemical controls such as herbicides and insecticides. There are some regulations governing genetically modified (GM) algae depending on the procedures and applications. In the USA, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) applies to the algae-based pesticides [283]. The Food and Drug Administration (FDA) regulates the algae which are made for food, medicines or nutrition supplements. The Federal Food, Drug, and Cosmetics Act applies depending on the

Table 7
Recent advanced chemical methods in microalgal lipid extraction.

Method	Species	Parameters	Solvents	Results	References
Supercritical CO_2	*Scenedesmus obliquus*	120 bar, 20 °C, 14 h	–	92% of total lipids extracted	[243]
	Chlorella vulgaris	450 bar, 50 °C, 3.5 h	Ethanol (10% v/v)	97% of neutral lipids extracted	[244]
	Chlorella vulgaris	5.5 MPa, 85 °C, 30 min	1.0 mL/min of methanol	84.8 wt% of total lipids extracted	[271]
	Nannochloropsis oculata	450 bar, 50 °C, 3.5 h	Ethanol (10% v/v)	83% of neutral lipids extracted	[244]
	Dunaliella tertiolecta	200–370 bar, 40–80 °C, 60 min	Hexane:ethanol (1:1)	54.83% of total lipid extracted	[272]
Enzymes	*Scenedesmus* sp.	Cellulose, xynalase, pectinase, 45 °C, pH 4.4	$CHCl_3$:MeOH (1:1 v/v)	86.4% lipid recovered after 190 min	[247]
	Nannochloropsis oculata	Cellulase, hemicellulase, papain, pectinase, 80 °C, 1 h incubation, 2 extraction cycles	Dipotassium phosphate (K_2HPO_4) (6% g/100 mL), ethanol (65% v/v)	92.59% FAMEs recovery	[248]
Nanoparticles	*Chlorella vulgaris*	Nickel(II) oxide, pH 7	$CHCl_3$:MeOH (2:1 v/v)	98.75% lipid recovered after 1 min	[249]
	Chlorococcum sp.	Zirconium dioxide, ZrO_2	$CHCl_3$:MeOH (2:1)	78.52% lipid recovered	[42]
Bio-based solvents	*Chlorella vulgaris*	–	EtLac	33.02% FAME yielded	[253]
	Nannochloropsis sp.	–	2-MeTHF	41.29% FAME yielded	
	Wet microalgae with 70% moisture content	–	FAME: Methanol (6:4 v/v)	68.19% lipid recovered	[249]
Ionic liquids	*Chlorella sorokiniana*	Heated to 120 °C, on hold for 60 min at 800 W microwave irradiation	[BMIM][HSO_4]	0.23 g/g dry algae	[258]
	Nannochloropsis salina		[BMIM][HSO_4]	0.10 g/g dry algae	
	Galdieria sulphuraria		[BMIM][HSO_4]	0.19 g/g dry algae	
	Chlorella vulgaris	120 °C, 2 h	[Emim]DEP	250.0 mg/g cell	[257]
	Chlorella vulgaris	120 °C, 2 h	[Emim]OAc/[Emim]$(CF_3SO_2)_2N$ (1:1 w/w)	255.7 mg/g cell	
Switchable solvents	*Chlorella* sp.	35 °C, 1 h cell disruption, 1 h extraction, 1 h separation	DMHCA Methanol: oil (6:1 mol/mol), 30% enzyme	47.5% biodiesel yield achieved	[263]
	Nannochloropsis oceanica	–	TEPDA (TEPDA)	5% higher recovery yield than chloroform-methanol	[264]
	Neochloris oleoabundans	–	EBA	13.0 wt% crude lipid yielded at 18h	[273]
Deep eutectic solvents	*Chlorella* sp.	28 °C, 24 h	Aqueous choline chloride-oxalic acid (aCh-O)	80.90% lipid recovered	[268]
	Chlorella sp.	130 °C, 60 min	Choline chloride-Acetic acid (Ch-Aa)	21% FAME content obtained	[270]
	Chlorococcum sp.	110 °C, 60 min		15% FAME content obtained	

Table 8
Conventional chemical methods for the extraction of lipids from microalgae.

Method	*Strains*	Solvent	Volume ratio	Results	References
Folch method	*Chlorella pyrenoidosa*	CPME: Isoamylase	2:1	75.88 mg lipids/g biomass	[280]
		2-MeTHF:Isoamylase	2:1	107.24 mg lipids/g biomass	
		$CHCl_3$:MeOH	2:1	113.47 mg lipids/g biomass	
	Chlorella vulgaris	$CHCl_3$:MeOH	2:1	Lipid yield of 16.1% w/w	[274]
	Chaetoceros muelleri	$CHCl_3$:MeOH	2:1	Lipid recovery of 27.5%	[281]
	Chlorella sp.	$CHCl_3$:MeOH	2:1	Lipid recovery of 10.0%	
	Nannochloropsis oculata	$CHCl_3$:MeOH	2:1	Lipid recovery of 79.5%	
Bligh and Dyer method	*Chlorella pyrenoidosa*	$CHCl_3$:MeOH	1:2	115.05 mg lipids/g biomass	[280]
		2-MeTHF:MeOH	1:1.7	61.42 mg lipids/g biomass	
		CPME:MeOH	1:1.7	89.35 mg lipids/g biomass	
		2-MeTHF: Isoamylasee	2:1	95.73 mg lipids/g biomass	
		CPME:Isoamylase	2:1	74.6 mg lipids/g biomass	
	Chlorella vulgaris	$CHCl_3$:MeOH	1:2	Lipid yield of 52.5%	[274]
	Schizochytrium sp.	$CHCl_3$:MeOH	2:1	Lipid yield of 22%	[282]
	Thraustochytrium sp.	$CHCl_3$:MeOH	2:1	Lipid yield of 10.7%	

product application. Additionally, in Malaysia, the Biosafety Act 2007 aims to regulate the release, importation, exportation and contained use of living modified organisms, and the release of products of such organisms, for protecting human, plant and animal health, the environment and biological diversity [284]. Research on the use of genetic modification of algae is increasing to obtain better lipid productivity [27,284]. The upscaling from lab scale to commercial plant imposes societal, cultural and economic risks with the release of GM algae into the environment. Some potential threats include changes in natural food web structure, the displacement of native species, local extinctions or formation of algal blooms. Furthermore, with higher triacylglycerides (TAGs) content, more reduced and shorter chain fatty acids (C_{10}–C_{12} FA)

are desirable for fuel production. The GM-specific implication for ecological impacts caused by TAG/FA content should be concerned as well.

3.4.1. Transesterification of extracted lipids from microalgae to biodiesel application

Transesterification is the process in which fat or oil chemically reacts with alcohol to produce methyl esters and glycerol in the presence of a catalyst. Refining and separation of microalgal biomass are required before proceeding to transesterification [285]. The details of different catalysts used to enhance the production of biodiesel are shown in Fig. 9.

The refining includes drying, extraction of oil, and cell disruption which constrains the production of biodiesel because of the following: (i) cell wall strength and thickness of some microalgae species lead to higher energy requirement for oil extraction and cell disruption; (ii) wet biomass hinders the extraction of oil because of the water present in it [286]. Different mechanisms of transesterification are dependent on the type of catalysts used. Various studies for the production of biodiesel from algae utilizing different types of catalysts under optimized conditions are presented in Table 9.

3.4.2. Viability of algae-based biodiesel and their energy efficiency ratio

The viability of biodiesel obtained from different feedstocks has been examined for its specific properties with the standards given by the American Society for Testing and Materials (ASTM) [293]. Some of the significant properties of microalgal biodiesel as compared to biodiesel obtained from other feedstocks are shown in Table 10 [294–296]. The biodiesel productivity of different terrestrial plants as compared to microalgal biodiesel is shown in Fig. 10. It is clearly seen that there is a considerable difference in the oil yield and biodiesel productivity of different feedstocks and microalgae. The yield and productivity of microalgae also depend on the content of lipids in the biomass.

The current algae-based biodiesel production technologies are still being developed to be considered for sustainable energy. Energy efficiency factor, defined as the ratio of energy produced to the energy consumed, is used as an indication of a product's energy sustainability [297]. Energy efficiency factor greater than one implies the production of net positive energy, and vice versa [298]. In the production of biodiesel from jatropha and palm oil, the energy efficiency factor values are more than one, compared to the microalgae, as shown in Fig. 11 [299]. Understandably, the overall upstream processing cost that involves cultivation, stressing conditions and harvesting may indirectly contribute to the mass production of algal biomass.

3.4.3. Techno-economic analysis and leading countries using biofuels

The cost of per kg of algal biomass ranges from 16 USD to 33 USD from open raceway pond (ORP) and closed photobioreactor (PBR) systems. One study estimates that for every kg of algal biomass production in 1 acre of area, it costs about 11 USD, but it can be reduced to 4.4 USD for 100-acre production by using advanced technologies and optimized conditions [300]. Although cost can be minimized, it is still high as compared to biodiesel obtained from other terrestrial plants and conventional biodiesel from petroleum. In one study, *Tetraselmis suecica* was cultivated in 1 Ha and 100 Ha and the cost of per kg of biomass obtained was 15.8 USD and 5.5 USD, respectively [301]. However, the cost was still high as compared to diesel obtained from petroleum. The cost of algal biodiesel obtained from closed PBR is about 60–70% more than the open ponds. The selling price of biodiesel produced from soybean, cooking oil, and Jatropha are 1.38 USD/L, 0.73 USD/L, and 1.4 USD/L, respectively, which is higher than the diesel obtained from petroleum, but still less than the algal biodiesel [4]. The cost of producing algal biodiesel can be reduced by curtailing the capital cost and increasing productivity [302].

Based on the economic point-of-view of biodiesel production, the recovery and conversion process of algae-based oil to biodiesel is not affected by its cultivation process whether in an open pond or closed photobioreactor. However, the major economic hurdle in producing microalgal biomass is the cost of synthetic culture media. In the case study, the estimated cost for producing a kilogram of microalgae biomass in a closed photobioreactor and open pond (raceway) was estimated to be USD 2.95 and 3.80, respectively (assumption for zero-cost availability for carbon dioxide supply) [303]. Table 11 provides an estimated cost of biodiesel produced by different sources.

Many countries have started to use biofuels to power their futures, and their native plants have been chosen as significant feedstocks. The USA, Brazil, and Indonesia are the top three countries out of the seven countries listed in Fig. 12. Solazyme, Blue Marble Production, Algenol, Solix Biofuel, Reliance Life Science, Culture Biosystem Organization, Oil Inc., and Proviron industries are some of the established firms working to produce bulk biodiesel from microalgae. Algenol, a huge company based in Florida with a stock market valuation of USD 3.1 million, produces 8000 gallons of biodiesel per acre of algal harvest each year. Reliance Biodiesel, based in India, delivers 100 barrels of biodiesel each day. Biofuels are being developed in developing nations, for example, India, and Williamson Magor Bio Fuel Limited (North East India) and Oils of the United Kingdom have formed a joint venture [36].

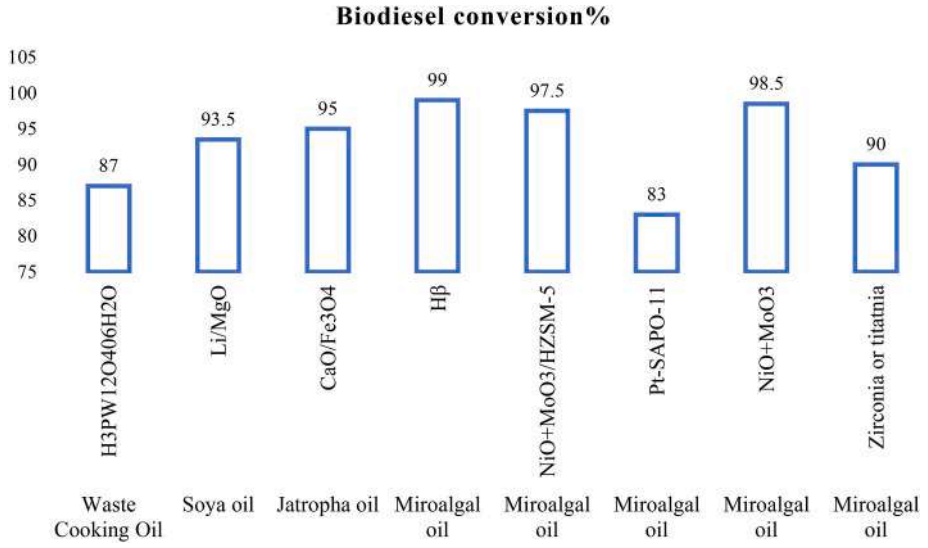


Fig. 9. Conversion of biodiesel using different catalysts.

Table 9
Conversion of microalgae to biodiesel via transesterification.

Microalgae Species	Moisture content% (microalgal biomass)	Transesterification condition	Process conditions	Yield of Biodiesel	References
Aurantiochytrium sp.	0 to 2 (v/v)	Enzyme catalysed, In situ esterification in dimethyl carbonate	50 °C for 12 h, the DMC to biomass ratio is 5:1 (v/w) and the enzyme to biomass ratio is 30% (w/w).	89.5%	[287]
Botryococcus braunii	7.8	Continuous methanol reflux with an acid catalyst and a co-solvent	Methanol: mol lipid = 151:1, 5 h, 47% v/v hexane, 75% catalyst/total lipid	95%	[285]
Chlamydomonas sp.	68.7	Alkaline catalyst	Sample = 12 ml/8 ml/2 g, 15 min, 45 °C, 600 rpm 0.5 wt% NaOH in MeOH, hexane/MeOH/	100%	[288]
Chlorella vulgaris	80	Supercritical method	325 °C, 2 h	100%	[289]
Chlorella pyrenoidosa	90	Acid catalyst and Co-solvent	4 ml of methanol, 0.5 M H_2SO_4 and 8 ml n-hexane at 120 °C for 3 h	92.5%	[290]
Nannochloropsis sp.	90	Supercritical method	wet algae to methanol = 1:9 (wt./vol), 255 °C, 25 min	85.8%	[291]
Nannochloropsis gaditana	65	Acid catalyst with co-solvent	0.3 mL H_2SO_4, 2 mL chloroform, 1 ml ethanol, ethanol to biomass ratio = 3.3:1, 125 °C, 2 h	96.4%	[292]

Table 10
Comparison of significant properties of microalgae-based biodiesel, other feedstocks biodiesel and ASTM standards.

Characteristics	*Jatropha curcus*	Soybean biodiesel	*Ceba pentandra*	Biodiesel from chicken waste	Algae-based biodiesel	Diesel fuel	ASTM Methods	ASTM Limits
Acid number (mg/g)	0.46	0.07	0.51	0.4	**0.01**	0.5 max	D664	0.50
Carbon residue	–	0.019	–	–	**0.018**	0.15–0.35	D4530	0.050
Cetane number	–	50	–	–	**71.67**	40–45	D613	47
Calorific value or heating value (MJ/kg)	39.46	–	39.46	39.71	**37–41**	40–45	–	–
Density (g/cm^3)	0.846	0.838	0.876	0.926	**0.864**	0.830	–	–
Kinematic viscosity(kg/m.s)	4.5	4.5	4.7	4.9	**11–35.4**	1.9–4.1	D445	1.9–6.0
Flash point(°C)	125.5	122	120.5	177	**149**	38–52	D93	93
H/C Ratio	–	1.81	–	–	**1.81**	1.81	–	–

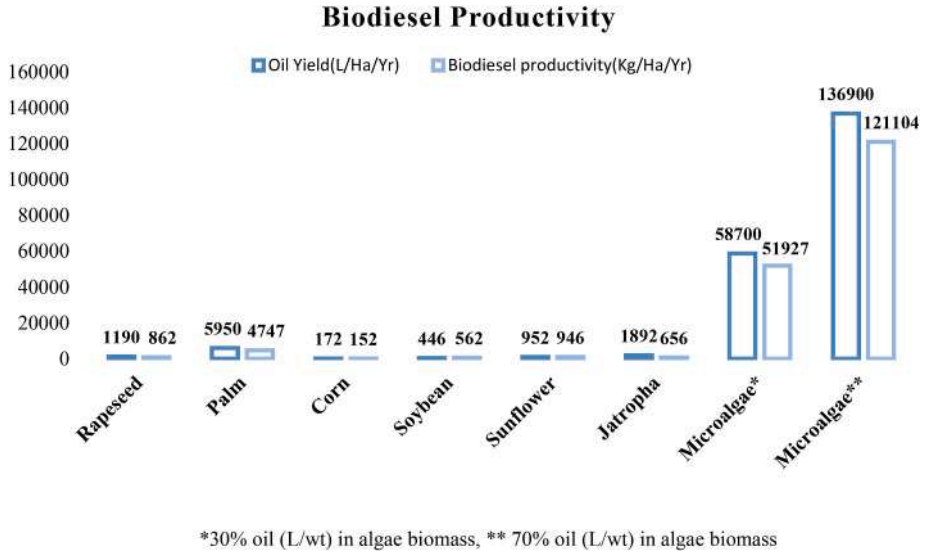


Fig. 10. Productivity of microalgal biodiesel vs other feedstocks.
*30% oil (L/wt) in algae biomass, ** 70% oil (L/wt) in algae biomass.

3.5. Global projects for the production of biodiesel from microalgae

Israel is working on a project under i-CORE, an acronym for the Israeli Center of Research Excellence. This includes four pilot-scale projects for obtaining renewable energy using cutting-edge technologies. One of these four pilot studies is going to fund about 17 million USD for renewable energy sources. As the industry still implements conventional methods using corn as a feedstock and converting its sugar to ethanol, most of the research is focused on finding out the possibilities and viable alternatives by introducing biowaste from wood chips and even algae to obtain biofuels. Researchers at the Technion-Israel Institute of Research are working on examining the photocatalysis of CO_2 and H_2O into fuels; biomass gasification; and producing liquid biofuels from biomass, while researchers at Ben Gurion University are focused on cutting-edge technologies to obtain green alternative fuels and algal biotechnology. Weizmann Institute of Science is working for cross-university linkage for the successful accomplishment of i-CORE [305].

3.5.1. European projects for microalgal energy

3.5.1.1. Future european league for microalgal energy (FUEL4ME). Microalgae have a lot of potential, but the technology for generating

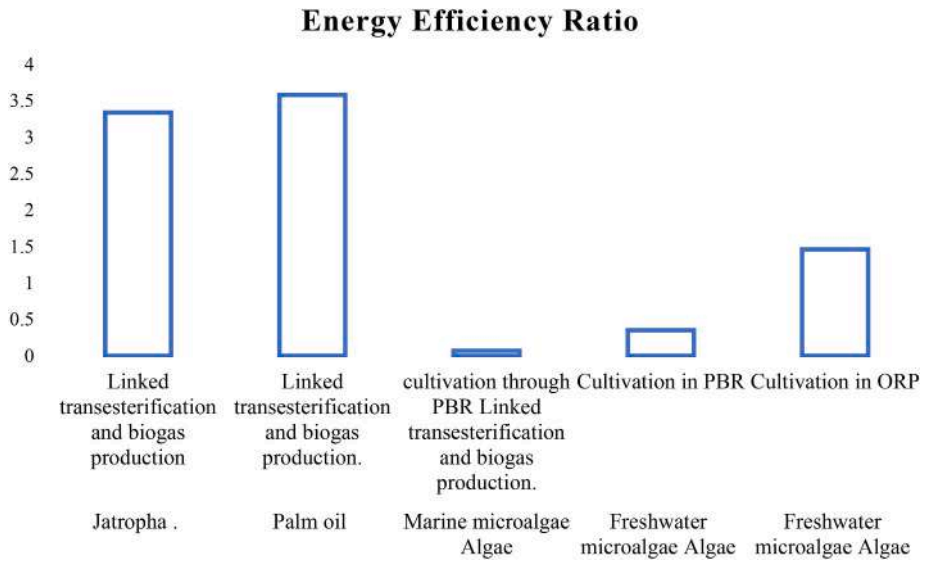


Fig. 11. Energy efficiency ratio for the production of biodiesel using various feedstocks.

Table 11
The estimated cost of biodiesel produced by different sources.

Source	Cost (USD/L)	References
Open ponds	2.65	[304]
Open ponds	2.75	[301]
Closed photobioreactor	5.5	[304]
Closed photobioreactor	4.5	[301]
Petroleum diesel	0.7	[35]

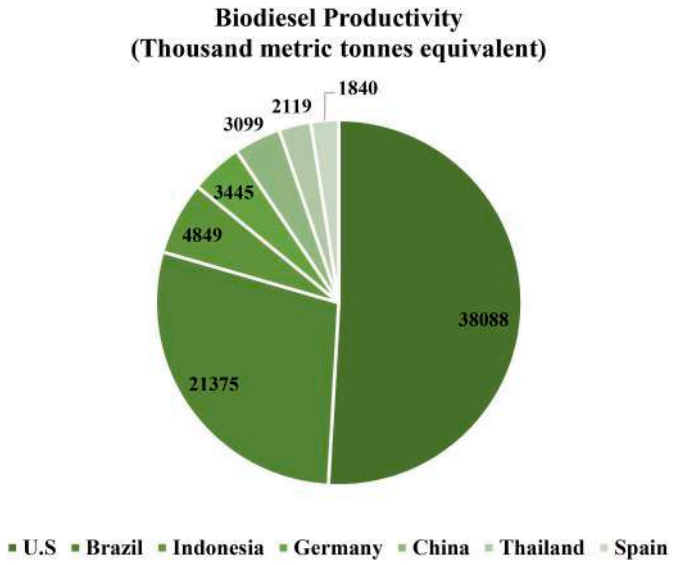


Fig. 12. Leading countries in the production of biodiesel.

biofuels from them is still in its infancy. It is critical to lower operational expenses and develop a positive energy balance in order to make microalgae a source of biofuels, competitive with fossil fuels (fossil energy input is higher than energy output). Many collaborative research projects in Europe are investigating/have investigated the potential of microalgae for biofuel production. For example, 4-year FUEL4ME project aimed to create a sustainable chain for continuous biofuel production using microalgae as a production platform, allowing 2nd generation of biofuels to compete with fossil fuels. The project started on 1st January 2013 and was completed on 31st December 2016. The overall budget of the project was € 5,369,514,10 out of which € 4,014,981,50 was contributed by European Union [306]. The main aims of this project were to:

(i) convert the two-step algal biomass production process into a continuous one-step process with high lipid content (production process);
(ii) develop a continuous downstream process using all components of the algal biomass (conversion process); and
(iii) combine the production and conversion processes.

Following the setup and proof of concept under controlled indoor settings, the continuous process was tested outdoors in four distinct locales under real-world production conditions (NL, IL, IT ES). A continuous downstream process was developed along with research on biomass production. Finally, the entire process (both biomass production and biofuel conversion) was integrated and put through an economic and life cycle analysis [306].

Partners in this project included DLO-Plant Research International, FeyeCon Carbon Dioxide Technologies BV, Ben Gurion University of the Negev, Fotosintetica & Microbiologica S.r.l. BioTopic, Wageningen University, Norsker Investigaciones, DLO-Food & Biobased Research, Proviron, Evodos B.V. PDX, Cellulac, Neste Oil, JOANNEUM RESEARCH Forschungsgesellschaft mbH, and IDConsortium S.L [306].

3.5.1.2. Genetic improvement of algae for value-added products (GIAVAP). The GIAVAP project started on 1st January 2011 and ended on 31st December 2013. The budget allocated for the project was € 7,184,970,60. Researchers applied genetic engineering approaches to a variety of economically important algal strains, with a focus on carotenoid and PUFA synthesis, as well as the overexpression of commercially valuable peptides. Existing model algal strains were used to investigate different cultivation technologies, as well as harvesting and extraction procedures for lipids, carotenoids, and proteins. In addition, products were planned to be examined for energy, pharmaceutical, nutritional, or medical purposes to assess the economic viability of manufacturing techniques and their commercialization [307].

3.5.1.3. Biofuel from algae technologies (BIOFAT). The BIOFAT project aimed for the microalgal conversion to biofuels comprising all the processes starting from growth optimization, accumulation of oil and starch, continuing to downstream processing with the production of

biofuels. BIOFAT was implemented in two phases:

(i) the optimization of the process was carried out at two pilot-scale facilities (each sized 0.5 ha) located in Portugal and Italy.
(ii) Economic modeling and scaling up to a demo facility of size 10 ha.

Both pilot plants named BIOFAT Pataias Pilot Plant (BPPP) and BIOFAT Camporosso Pilot Plant (BCPP) were designed and equipped with state of art technologies and consisted of stabilized systems for microalgal cultivation and biomass production, such as tubular photobioreactors, green wall panels, raceways and cascade raceways. The microalgae species utilized were *Nannochloropsis oceanica* and *Tetraselmis suecica* [308]. The specific aim of Biofat was to get the maximum benefit from algae by causing minimum impacts on the environment. BIOFAT was planned for four years (from 1st May 2011 to 30th April 2015) to get 900 tons/yr of yield [308].

EU FP7 Energy program promoted three projects for the evaluation of technical viability of Algal Biofuels. This Algal cluster targeted the production of biofuels from the selection of strain, cultivation, scaling up, biomass production, extraction of oil, production of biofuels and its testing in various transportation modes. Three projects bear an estimated cost of 42 million USD, out of which 27 million USD was contributed by the EU [308].

Besides these, **All-gas** project evaluated the large-scale biofuel production via the cultivation of low-cost microalgal strains with municipal wastewater. **InteSusAl** focused on the innovative and cutting-edge technologies to produce biofuels by optimizing algal production through phototrophic and heterotrophic routes.

4. Genetic modification of green microalgae to improve lipid production and yield

4.1. Overview of genetic engineering in microalgae

Microalgal species that belong to the genera *Phaeodactylum*, *Nannochloropsis*, *Chlorella,* and *Dunaliella* have been widely investigated in the last decade for various applications. When these species are subjected to nutrient starvation, neutral lipids are accumulated in them [309]. These neutral lipids as TAGs are later converted to biofuels by transesterification. At the same time, microalgal-based lipids are gaining interest, especially in the food industry because they can become a substitute for fish oil and an alternative source for PUFAs such as omega 3 and omega 6 fatty acids providing dietary and health benefits to humans [310]. Regardless of the benefits hidden in microalgae, large-scale production of the commodities/chemicals derived from microalgae is an uphill task due to the upstream and downstream processing costs. Continuous progress and advancement in research such as genetic engineering and synthetic biology can contribute to achieving an economically viable process/system. Strain improvement by indirect/direct genetic modification is a recommended methodology for the enhancement of growth and lipid productivity in the microalgal strain of interest [311].

When the wild microalgae strains are nutrients-starved, they accumulate high lipid content, but the biomass yield is decreased. Therefore, genetic modification is a potential strategy for producing the strains that can accumulate lipids without affecting biomass productivity and impairing growth. In the last decade, substantial research has been carried out to develop genetic tools and produce genetically modified and improved strains. Genetic modification is carried out by employing direct genetic engineering, adaptive laboratory evolution (ALE), and random mutagenesis. Physical mutagens (i.e. x-rays, gamma-rays, and UV light), ALE, and chemical mutagens [ethyl methanesulfonate (EMS) and N′-nitro-N-nitrosoguanidine (NTG)] are being investigated to introduce random mutations in microalgae [312]. However, effective strategies based on genetic engineering can generate particular deletions or insertions in the parent (host) genome to achieve the required improvements and avoid the associated risks. Advancements in the development of efficient, accurate, and speedy delivery systems, highly improvised genome editing tools, and the versatile technology of sequencing are considered to be vital to produce genetically modified and improved microalgal strains [313]. The nuclear transformation of *Chlamydomonas reinhardtii* was the first milestone achieved in the field of microalgal biotechnology in 1990. Agitation of cells was performed in the presence of DNA-coated glass beads, allowing the formation of micro-sized pores in the cell membrane by the effect of friction and facilitating entry of DNA molecules into the cell [314].

Nowadays, many methods are taken into practice in delivering DNA (exogenous) into various microalgal strains. Some of the significant and accomplished techniques are *Agrobacterium tumefaciens*-mediated transformation, electroporation, agitation with glass beads, and particle bombardment [315]. The bottlenecks of these techniques are that they are (i) applicable to limited species, (ii) lead to cell death because of harsh treatments, and (iii) require strenuous optimization to be applied to other species [316]. The omics technology for microalgae has provided a methodology for identifying regulatory components required to develop the strategies of genetic engineering. As such, the identification of selection markers, splicing signals, reporter genes, terminators and promoters has contributed to the expression of heterologous genes in microalgae. This led to the increase in momentum in the progress of molecular tools included in genome editing system which are highly equipped such as Transcription Activator-Like Effector Nuclease (TALEN), RNA interface (RNAi), Clustered Regularly Inter-Spaced Palindromic Repeats and associated proteins (CRISPR-Cas), and Zinc Finger Nulease (ZFN). These genetic tools were used extensively in studies during the last decade [317]. Table 12 summarizes the strategies and the tools developed for the enhancement of lipid production in microalgae such as the Kennedy pathway, transcription factors, fatty acid synthesis (FAS), and PUFA/TAG metabolism.

Green algae are categorized in the phylum of Chlorophyta, and they are mostly unicellular and commonly found in freshwater. Green algae differ from red algae and brown algae by the types of chlorophyll they possess. The types of chlorophyll found in algae influence the colour of the algae. All of the algae contain chlorophyll *a*, green algae contain chlorophyll *b* while red algae and brown algae contain chlorophyll *d* and chlorophyll *c*, respectively [337]. Chlorophyll *b* aids in absorbing a wider range of light energy for photosynthesis, but it is not found in every photosynthetic organism [338].

Green algae are favored in biofuel particularly biodiesel production because of their high population densities and high growth rates [339]. The number of chlorophylls is found to be relatively higher than the number of other accessory pigments in green algae, while other algae have several accessory pigments higher than the number of chlorophylls. This shows that green algae have better photosynthetic efficiency than other types of algae. In some green algae, more than 50% of biomass dry weight is comprised of lipid. TAGs and DAGs make up most of the lipids found in green algae, with other components such as carotenoids and oil constituents [340]. TAGs and DAGs are the neutral lipids favored in biodiesel production. The genetic modification of microalgae improves the yield and qualities of the products, in this case, lipids to compensate for the costs. This strategy targets mainly modifying metabolic pathways to overexpress or silence certain genes to obtain higher biomass and desired product yield.

DNA encodes genes, which provide congenital characteristics to produce other molecules such as proteins to form a microalgae cell. Genes are encoded in DNA which is transcribed into RNA. RNA is converted into proteins that form other complexes and catalysts for the whole organism. The network between genomes is studied to modify the gene toward the accumulation of desired products [341,342]. The genetic modification process starts with picking and collecting a gene with an interesting trait from the DNA of the donor organism that naturally possesses the specific gene. Next, plasmid DNA which serves as a vector

Table 12
Strategies and tools employed for the enhancement of lipid production in microalgae.

Strain	Target Genes	Strategy	Effect on lipids	Comments	Reference
Genetic engineering (FAS metabolism)					
Scenedesmus quadricauda	GUT1	HE	GUT1 increased lipid content 1.9-fold	No significant effect on growth	[318]
Nannochloropsis oceanica	*NoMCAT*	OE	36% and 31% increase in TFA and neutral lipid content respectively. 8% increase in C20:5	High photosynthetic efficiency and growth rate compared to wild-type strains	[319]
Chlamydomonas reinhardtii	*DtTE*	HE	69% increase in neutral lipids content and 56% improvement in TFA	No effect on growth	[320]
Genetic engineering (Kennedy pathway)					
Phaeodactylum tricornutum	DGAT2	OE	35% and 76.2% increase in neutral lipids and EPA content	The growth rate of transgenic strains was the same as WT	[321]
Nannochloropsis oceanica	DGAT2	OE	129% and 69% increased TAG content during deplete and replete conditions. 53% and 74% decreased MUFA and PUFA content, respectively	overexpression of DGAT2 did not cause any negative impact on the growth	[322]
Phaeodactylum tricornutum	DGAT2D	OE	Total lipid content was doubled	>50-fold DGAT2D mRNA levels and >30 -fold increase in enzyme abundance. The growth rate decreased by 15%	[323]
Nannochloropsis oceanica	DGAT1A	OE	TAG content increased by 39% under nitrogen depletion. 2.4-fold increased TAG under nitrogen-replete. TAG yield was 47% higher than the wild strain	NoDGAT1A overexpression does not cause any negative effect on growth. knockdown of NoDGAT1A caused a 25% reduction in TAG content with nitrogen depletion	[324]
Tetraselmis chui	EpDGAT1 ScDGAT2	HE	40–115% increase in TAG content	No significant impact on growth	[325]
Chlamydomonas reinhardtii	LiGPAT	HE	TAG content increased by 50%. Decrease in C18:3 n-3 and C16:4 n-3, Increase in C18:1 n-9 and C16:0	Overexpression did not affect the growth	[326]
Genetic engineering (PUFA metabolism)					
Phaeodactylum tricornutum	*PtD5b*	OE	64% and 75% increment in PUFA and MUFA content 65% and 58% increase in neutral lipids and EPA, respectively	No significant effect on growth	[327]
Nannochloropsis oceanica	*NoD12*	OE	50–75% increase in AA under N-starvation. 32.6% increase in LA in PC	Overexpression under the control of stress-inducible endogenous lipid droplet surface protein (LDSP) promoter, (Higher expression under N-starvation conditions)	[328]
Dunaliella salina	*TpFADS6* *DsFADS6*	OE, HE	EPA enhancement up to 21.3 mg/L, from 1.6 mg/L (WT). Up to, 554, 193, and 91 mg/L EPA in TFA when supplemented with PeSM, CO_2, and myoinositol, respectively	The use of PeSM, CO_2, and myoinositol assists in the growth	[329]
Chlorella vulgaris	*ω-3 FAD*	OE	Under N-deficient conditions, there was a 7% increase in TFA. 2.8% increased ALA in TFA	No significant effect on growth	[330]
Genetic engineering (transcription factors)					
Nannochloropsis salina	*NsbHLH2*	OE	43% increased lipid productivity	The growth rate increased by 55%	[331]
Nannochloropsis oceanica	*NobZIP1*	OE	65–100% increased lipids in OE strain. Further, 40% of lipids per dcw are present in the medium. 40% decreased lipid content in KD strain	The main mode of action is likely the regulation of *UGDH*. No effect on the growth rate but the cell wall thickness reduced	[332]
Chlorella ellipsoidea	*GmDOF4*	HE	53% increase in lipids content during the stationary phase	No effect on growth. Decrease in protein and carbohydrate content in mutant	[333]
Genetic engineering (NADPH generation)					
Chlamydomonas reinhardtii	PETC FDX5	OE	lipid content increased and varied between 50 and 250% during the depletion for both proteins	110–170% increased starch concomitantly	[334]
Phaeodactylum tricornutum	G6PD	OE	170% increase in the total lipids content during the replete phase, 100% during depletion		[335]
Chlorella pyrenoidosa	AtNADK3	HE	45–110% increased total lipids during growth		[336]

OE, overexpression; HE, heterologous expression.

is isolated from another organism, mostly bacteria. The plasmid DNA and donor DNA are then mixed with the enzyme that cuts the DNA into pieces. The pieces of DNA combines according to chemistry and form a new complete plasmid. This plasmid DNA now contains the gene from the donor organism. After that, the new plasmids with the new gene are either inserted or accepted by the bacterial cells by transformation. The bacteria with the new gene are later grown in large amounts and desired results can be observed [343]. In this case, green algae have the same role as bacteria.

4.1.1. Gene modification tools

In genetic modification, genome editing is a crucial part that involves knockout, knockdown, mutations, insertion and deletion of genes [344]. Gene knockout aims to delete a gene permanently, while gene knockdown aims to down-regulate the activities of the gene [343]. RNA interference (RNAi) is a common gene knockdown method [344,345]. In this method, microRNA (miRNA) or short interfering RNA (siRNA) are cleaved by the enzyme DICER from pre-microRNA (pre-miRNA) or small hairpin RNA (shRNA), respectively. Then, miRNA or siRNA binds to a molecule containing an enzyme called RNA-induced silencing complex (RISC). Next, the complex consists of RISC and miRNA or siRNA bind to complementary or targeted messenger RNA (mRNA) sequences. This causes cleavage and then malfunction of the targeted mRNA, which further inhibits the formation of a certain protein [346]. According to Wei et al. [345], RNAi is established for only a few species such as *Chlamydomonas reinhardtii, Dunaliella salina* and *Penium margaritaceum*. The method was tested on *Nannochloropsis oceanica* strain and achieved up to 79% transcript level suppression in the strain. Some of the drawbacks of this method include silencing the desired phenotypes, incomplete repression of transformants and target genes, and off-target changes [344]. Fig. 13 illustrates the mechanisms of RNAi as a genomic editing tool.

CRISPR and TALEN are examples of gene knockout methods [348, 349]. Transcription Activator-Like Effector Nuclease (TALENs) is a man-made enzyme that cut the targeted part of DNA and results in a double-stranded break (DSB) at that part. Then the cleavage can either combine or a new gene with the desired trait is added to the part, thus the genomic functions of the DNA are mutated [39]. Kurita et al. [350] applied the Platinum TALENs in *Nannochloropsis oceanica* and obtained the result of efficient mutations in two genes: nitrate reductase and acyltransferase in the strain. The study revealed over-accumulation of lipids by employing TALEN-guided UDP-glucose pyrophosphorylase gene disruption. Fig. 14 depicts the mechanism of TALENS as a type of genomic editing tool.

Clustered Regularly Interspaces Short Palindromic Repeat (CRISPR) creates DSBs by recombinant nucleases. The nuclease Cas9 protein forms a complex with a guide RNA (gRNA). The gRNA can identify the target sequence which needs to be edited and leads the Cas9/gRNA complex to search for it [352]. Saey [353] found the target sequence, the Cas9 protein cleaves it and a DSB is formed at the site. DSB is then repaired by a mechanism called non-homologous end joining (NHEJ) and causes mutations to happen at the cleavage site. Moreover, the complex of Cas9 and gRNA can be delivered as ribonucleoproteins (Cas9 RNPs) into the cells to reduce probabilities of off-targeting and cytotoxicity [354]. Chang et al. [355] have successfully applied this technique to green marine microalgae, *Tetraselmis* sp., in which they modified the ADP-glucose pyrophosphorylase (AGP) protein in the strain which triggered an increase in lipid productivity. The lipid content considerably increased by 2.7 and 3.1-fold (i.e. 21.1 and 24.1% dry cell weight). The illustration of the mechanism of CRISPR is shown in Fig. 15.

4.1.2. Delivery of modified gene

The basic steps involved in algal genetic modification include host selection followed by identifying and collecting the gene of interest, then transformation, and lastly selection and screening. The stages of transformation and selection play crucial roles in the whole process [344]. The gene transformation of microalgae is challenging as the DNA is needed to be transferred through the cell, plasma membrane and nuclear membrane. Pre-treatment is often needed, so that the cells can survive chemical and mechanical treatments [357]. Various techniques are therefore studied for effective gene transformation in microalgae.

Traditional methods of gene delivery include agitation of cells in agitators, electroporation and microparticle bombardment. In the agitation method, the cells needed to be treated with enzymes to degrade the cell wall before being agitated with glass beads coated with foreign DNA, and non-ionic surfactants such as polyethylene glycol (PEG). Electroporation applies electric pulses to form micropores on the phospholipid bilayer for molecules to pass through. Microparticle bombardment uses non-reactive metals such as gold or tungsten as microprojectiles that carry DNA and shoot them at the targeted cells at a speed of around 500 m/s. All of these methods have been proven to be effective on *Chlamydomonas reinhardtii* [357,358].

On the other hand, cell-penetrating peptides and bacterial conjugation are two of the emerging gene delivery methods that work on some strains of microalgae. CPPs are peptides with less than 30 amino acids and act as media to carry the gene into cells. It has been applied to green algae such as *Chorella vulgaris*, *Chlamydomonas reinhardtii*, and *Dunaliella salina,* respectively [358]. Bacterial conjugation involves the sharing of genetic information from bacterial to cells by transferring plasmids through pili [358]. Muñoz and his team [357] succeeded in transferring plasmids to *Acutodesmus obliquus* and *Neochloris oleoabundans* from *Echerichia coli* via conjugation.

4.2. Lipid metabolism

Lipid metabolism refers to the synthesis and degradation of lipids for energy generation or cell structure formation [359]. The synthesis of lipids is known as lipid biosynthesis and the degradation of lipids is known as lipid catabolism.

4.2.1. Lipid biosynthesis

Most of the genetic modification efforts for lipid metabolism focus on promoting lipid biosynthesis in microalgae [360]. Lipid synthesis pathways can be summarised into three main steps: malonyl CoA synthesis, elongation of acyl chains and TAG formations [176,344]. The mechanism and the targeted enzymes for genetic modifications are shown in Fig. 16.

Malonyl-CoA is used for fatty acid synthesis. It is formed from acetyl-CoA through carboxylation facilitated by acetyl-CoA carboxylase (ACC) [177]. Gomma et al. [318] carried out a study, in which they tested the effect of overexpression of ACC in *Scenedesmus quadricauda*. The gene ACC1 was first amplified from brewer's yeast *Saccharomyces cerevisiae* and then transformed into *Scenedesmus quadricauda* by electroporation. *Scenedesmus* cells (1×10^5) were treated with buffer and distilled water, and 80 μl of the suspension was mixed with 40 ng of the gene expression cassette before electroporation. After electroporation, overexpression of ACC1 in *Scenedesmus quadricauda* resulted in 1.6 folds increment in TFA content compared to wild strain.

Another enzyme to be used in genetic modification for lipid production improvement is diacylglycerol acyltransferase (DGAT) as its overexpression has been reported to result in improved accumulation of TAG [344]. DGAT acts as a catalyst in the esterification of fatty acids into DGAT molecules and shifts DAG to TAG synthesis instead of phospholipids synthesis [358]. DGATs are often referred to separately as DGAT1 and DGAT2. They have different structures, but both are primary enzymes in TAG synthesis in the *de novo* pathway [361]. However, La Russa et al. [362] reported that overexpression in DGAT genes in *Chlamydomonas reinhardtii* did not enhance TAG accumulation or result

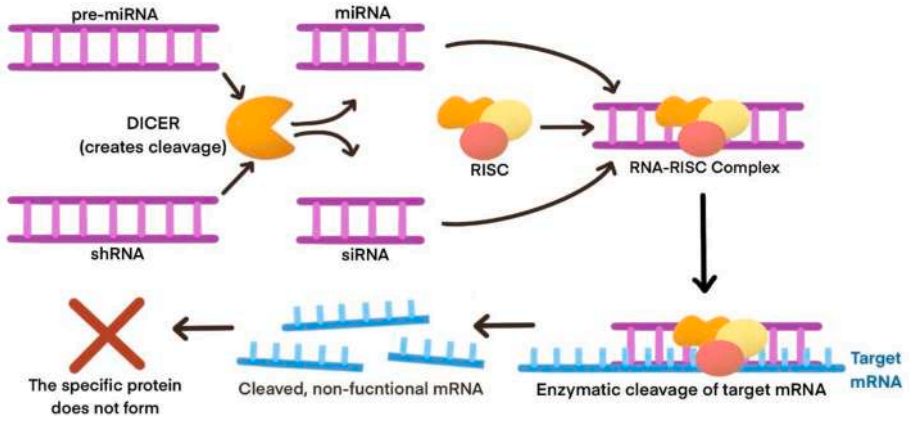


Fig. 13. The mechanism of RNAi as a genomic editing tool. Modified from Majumdar et al. [347] with permission from Frontiers.

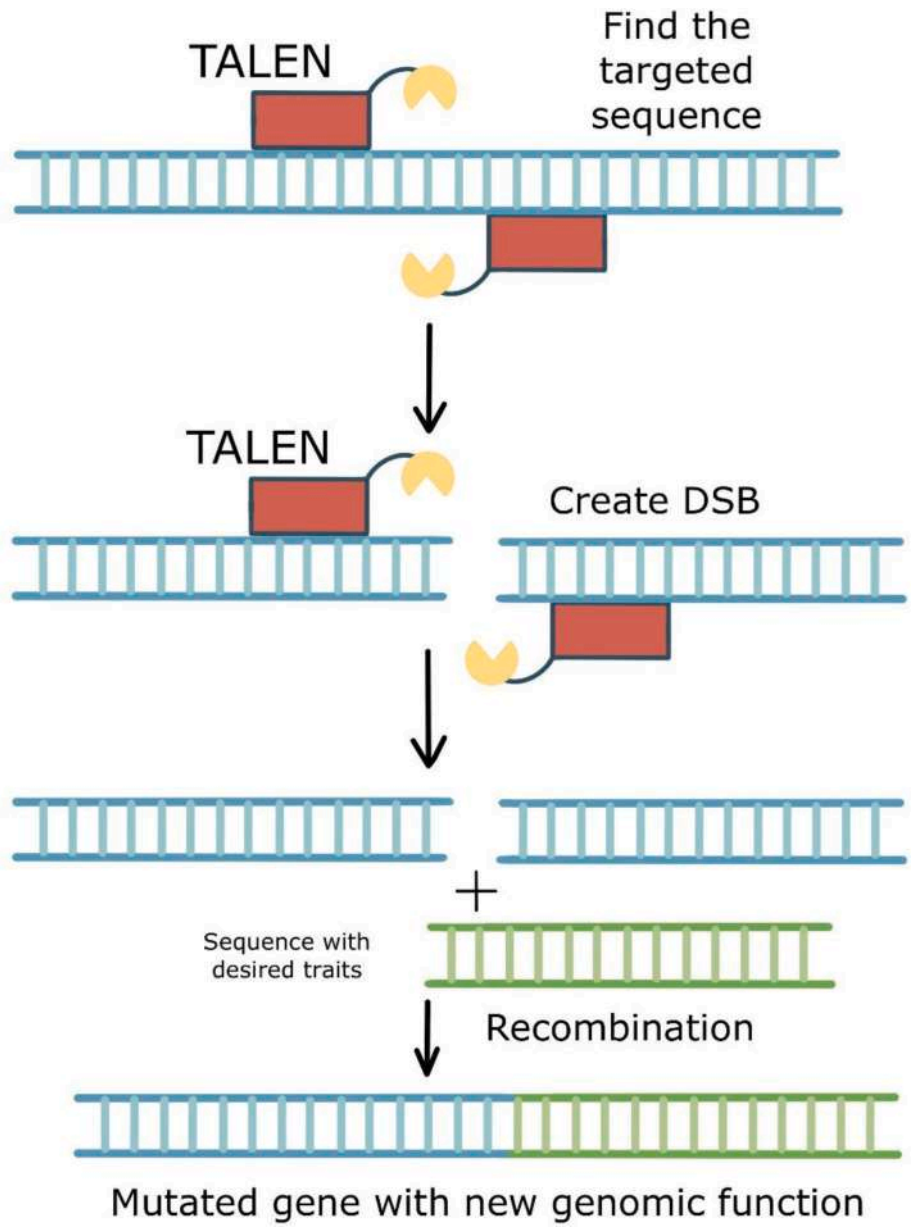


Fig. 14. The mechanism of TALENs. Modified from Li et al. [351] with permission from Springer Nature.

in any changes in fatty acid profile. This method has been tested on other species, however. Úbeda-Mínguez, García-Maroto and Alonso [325] performed experiments using this strategy on the green alga *Tetraselmis chui*. They obtained DGAT1 from *Echium pitardii* and DGAT2 from *Saccharomyces cerevisiae* and used them to transform the genome of *Tetraselmis chui* by electroporation. The results showed that there is up to a 111% increase in TAG content in a set of strains compared to the wild type (WT). Another study was conducted by Klaitong et al. [363] where they tested endogenous DGAT on *Neochloris oleoabundans* which also showed an increase in TAG production and accumulation. One of the strains showed a 1.9-fold increment in neutral lipids content compared to WT and overall TAG content in biomass of *Neochloris oleoabundans* was found to be ca. 46.1% after genetic transformations.

Besides DGAT, lysophosphatidic acid acyltransferase (LPAAT/LPAT) is also involved in TAG synthesis by generating an intermediate, phosphatidic acid [364]. Phosphatidic acid is then converted to DAG by lipid phosphate phosphatases [365]. DAG is then converted into TAG [366]. Wang et al. [367] resynthesized the LPAAT gene obtained from *Brassica napus* and transformed it into *Chlamydomonas reinhardtii* for overexpression. The TFA contents of the transgenic strain increased by 17.4%. Chungjatupornchai et al. [364] applied endogenous LPAAT1 on *Neochloris oleoabundans*, cultivated under both N-depletion and repletion conditions. The transgenic strain showed an increase in TAG content and productivity under the N-depletion condition, by 4.8 and 2.2-fold, respectively.

4.2.2. Lipid catabolism

Altering lipid catabolism is a strategy of blocking competing pathways. One of the pathways targeted for blocking the beta-oxidation of fatty acids. In beta-oxidation, fatty acids are degraded in the mitochondria and peroxisomes, and this is promoted by enzymes such as acyl-CoA dehydrogenase (ACD) and acyl-CoA oxidase (ACOX). Therefore, it is suggested that ACD and ACOX could be the target enzymes to be silenced by microRNA [368]. Degraded fatty acids are used for polar lipid synthesis, particularly membrane reorganization [360]. Kong et al. [369] experimented with blocking beta-oxidation by genetically modifying *Chlamydomonas reinhardtii* with a mutant deficient in the ACD and ACOX family. Along with N-depletion, an increase of 20% in TAG

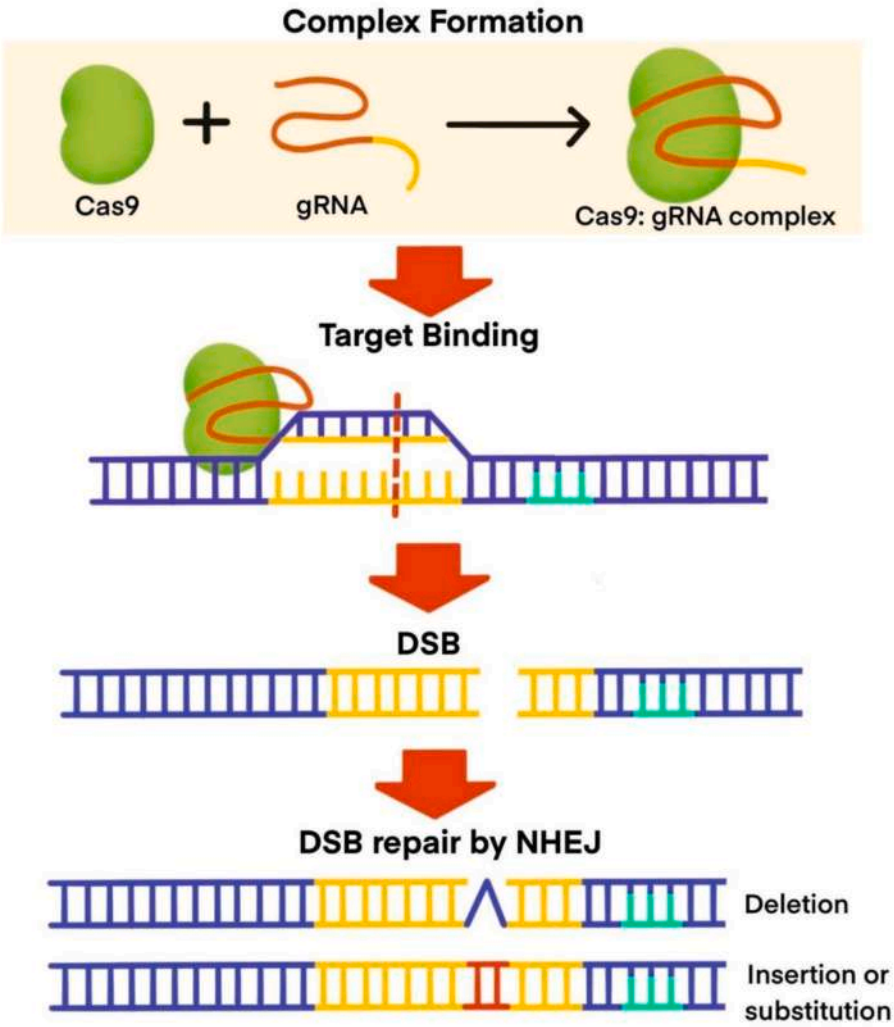


Fig. 15. The mechanisms of CRISPR. Modified from El-Mounadi et al. [356] with permission from Frontiers.

content was observed.

Since TAG is the favored lipid for biofuel production, lipid degradation and reconstruction of membrane lipids can be considered as a strategy to enhance TAG production. Phospholipase is responsible for destruction of membrane lipids for TAG biosynthesis or lipid droplet deposition [370]. Shin et al. [370] used CRISPR-Cas9 to prepare a mutant of *Chlamydomonas reinhardtii* with the phospholipase gene removed. During the growth phase, one of the mutant strains had shown up to a 190.42% increase in TAG content. Under N-depletion conditions, the mutants also demonstrated an increase in both TAG and DAG content. One of the mutant strains reached a 68.21% increase in TAG content and 117.97% in DAG content. The increase in the accumulation of DAG corresponds to the increment in the TAG content.

Targeting the acyl-CoA independent pathway is another way to accumulate TAG in microalgae by degrading membrane lipids. In this strategy, phospholipid: diacylglycerol acyltransferase (PDAT) is utilized to degrade membrane lipids for TAG synthesis [371]. PDAT catalyzes the process of phospholipid contributing an acyl group and turning it into DAG for TAG synthesis [372]. The mechanism of degrading membrane lipids for the synthesis of TAG and the enzyme involved is depicted in Fig. 17. Phosphatidylcholine is a phospholipid with choline attached to it and lysophospholipid is the product of hydrolyzation of the phospholipid [373,374]. Zhu et al. [372] transformed an exogenous PDAT from *Saccharomyces cerevisiae* into *Chlamydomonas reinhardtii*. The TFAs and TAG content in the mutant strain increased by 22% and 32%, respectively.

4.3. Starch metabolism

Starch is the major carbon and energy storage compound in microalgal cells [376]. Synthesis of starch and lipids involves competition of carbon skeletons, hence blocking the synthesis of starch is proposed as a strategy to enhance TAG production [377]. de Jaeger et al. [378] developed and selected five mutants of *Scenedesmus obliquus* with decreased or completely null starch content by UV mutagenesis and these mutants were exposed to N depletion condition. All mutants demonstrated an increase in their TFA and neutral lipid production without adversely affecting the growth rates. One of them achieved a TAG content comprising 49.4% dry weight of the cell after 4 days under N depletion condition. Another study on starch-deficient *Dunaliella tertiolecta* mutants reported an increase in dry weight TAG content by 42-92% after UV mutagenesis [377].

In starch synthesis, an enzyme called ADP-glucose pyrophosphorylase (AGPase) converts glucose-1 phosphate and ATP to ADP-glucose and inorganic phosphate. ADP-glucose is then elongated into starch granule chains by starch synthase (SS) and branching enzymes (BE) [376]. Some studies which involved mutation in the subunit of AGPase, known as sta6 mutant, observed an increase in lipid accumulation along with N-deprivation in *Chlamydomonas reinhardtii* [379]. Mi-Ae et al. [380] inhibited AGPase in the cytosol with a chemical CDTA which resulted in a 27% increase in FA production along with N-deficiency. Li et al. [381] applied the strategy of silencing AGPase in *Chlamydomonas reinhardtii* and observed the TAG content increased by 10-fold. However,

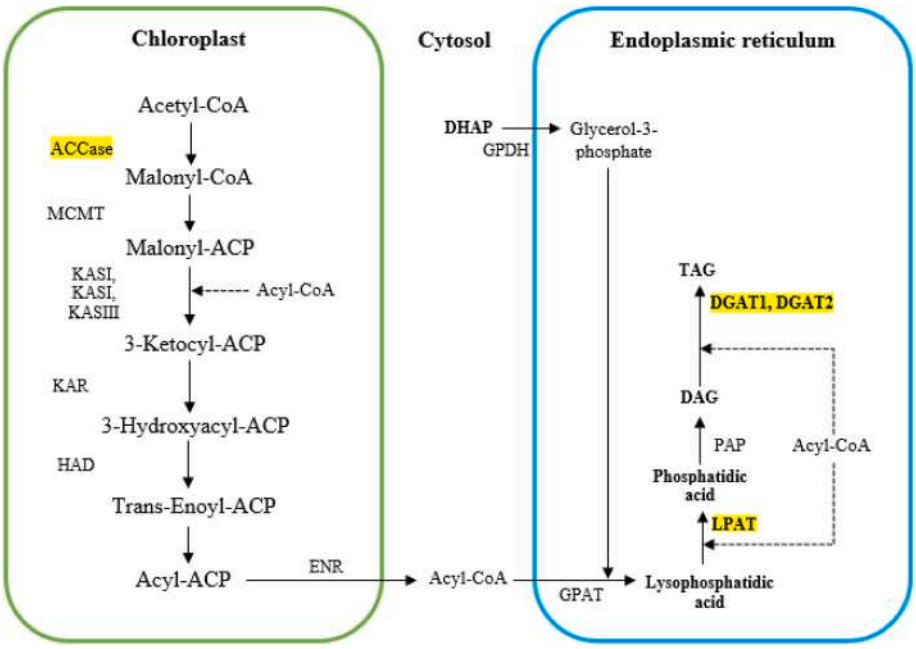


Fig. 16. The targeted enzymes for genetic modifications are highlighted in the mechanism of TAG biosynthesis. Modified from Fayyaz et al. [344] with permission from Elsevier.

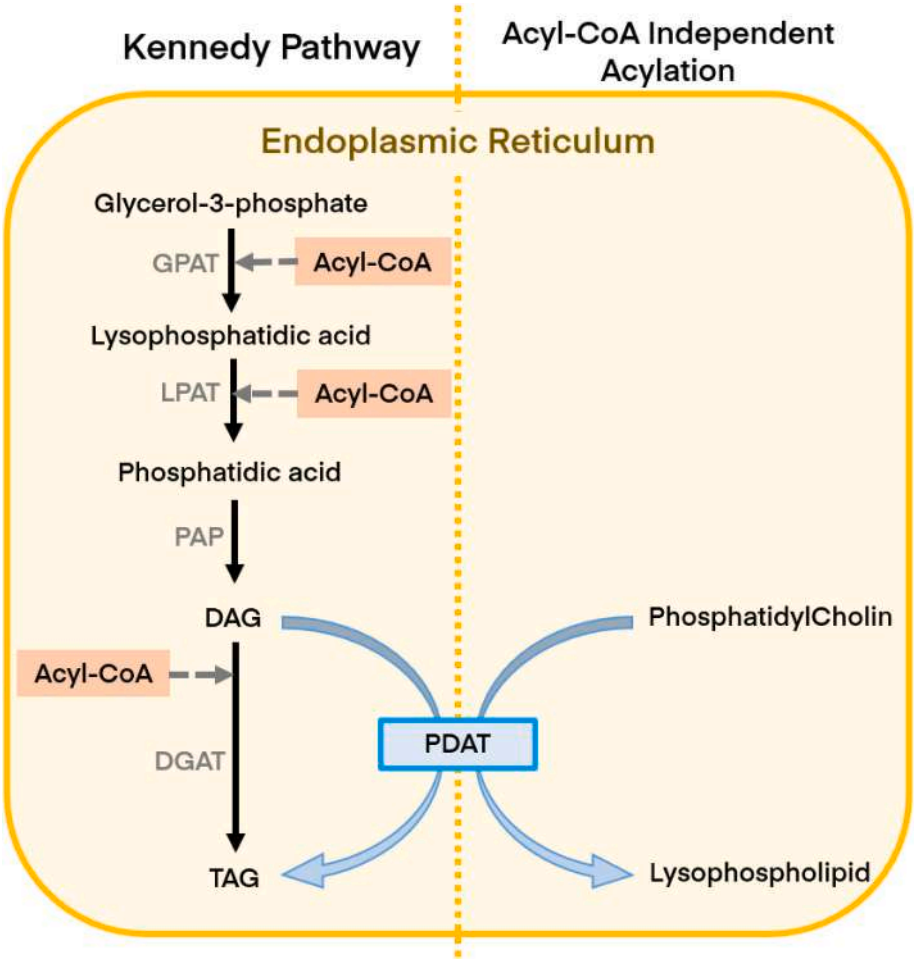


Fig. 17. The mechanism of degrading membrane lipids for the synthesis of TAG and the enzyme involved is highlighted. Modified from Torabi et al. [375] with permission from Springer Nature.

the study also showed inhibited growth of the mutated strain.

Kato et al. [382] genetically modified *Chlamydomonas reinhardtii* by mutating a gene encoding a starch debranching enzyme (DBE) in the isoamylase family. This aimed to promote carbohydrate degradation and redirect carbon resources into lipids and carotenoids. The microalgae were cultivated under outdoor conditions and cultivation was examined under light and dark conditions by changing cycles which is commonly unfavorable for lipid accumulation [382]. However, the mutant successfully achieved a 1.46-fold increase in lipid productivity under the condition. The mechanism illustrating the repartitioning of carbon flux to lipids in DBE-deficient microalgae is shown in Fig. 18.

4.4. Photosynthetic efficiency

Light is considered as one of the vital factors for microalgal growth and composition of biomass. The adequate intensity of light favors the production of lipids. This is because adequate light intensity aids in storing surplus photoassimilates, which get converted to chemical energy in the later stage. In a study, *Nannochloropsis* sp. exhibited the accumulation of the highest lipid content of 47% of dry weight at a high light intensity of 700 μmol photons $m^{-2}s^{-1}$. One the other hand, the microalgal species *Scenedesmus abundans* and *Botryococcus* sp. produced a lipid content of 32.77% and 35.9%, respectively at a light intensity of 6000 lux [39]. Although starch was the primary product, the content of lipids as secondary product increased along with rate of photosynthesis in microalgae. An increase in biomass yield indicated that more carbon was available for distribution among starch and lipid. Thus, improving the photosynthetic efficiency of microalgae was a good strategy to improve lipid production. However, limited studies are currently available on photosynthetic efficiency enhancement for green algae.

In microalgae, light-harvesting complexes (LHCs) are responsible for the capture of light energy along with reactions in Photosystem I (PSI) and Photosystem II (PSII) [383]. They are also known as light-harvesting chlorophyll antennae or antenna complex. They are normally large which reduces light use efficiencies. When given a condition of high light intensity, the light energy releases a number of electrons that exceeds the ability of the electron transport system to handle. The imbalance between the photon absorption rate and photon utilization rate causes the light energy to dissipate as heat. Therefore, it is suggested that reducing the size of the antenna complex can improve the photosynthetic efficiency [384]. Shin et al. [384] used a chemical mutagen, ethyl methanesulfonate (EMS) to generate random mutations to cause antenna truncation in *Chlorella vulgaris*. The biomass productivity had reached 0.224 g L^{-1} day^{-1}, which was 44.5% higher than the WT.

Carbon dioxide fixation takes place during photosynthesis and the Calvin cycle is the initial stage of the process [385]. A rate-limiting enzyme for this cycle is called sedoheptulose-1,7-bisphosphatase (SBPase) which regenerates the precursor for ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco). Rubisco catalyzes the carboxylation of the CO_2 acceptor molecule, which then improves CO_2 fixation [385]. Rubisco and its role in carbon dioxide fixation is depicted in Fig. 19. Fang et al. [386] transformed the SBPase gene obtained from *Chlamydomonas reinhardtii* into *Dunaliella bardawil* and grew the mutant strains in culture media with different concentrations of NaCl. All strains exhibited ca. 5% higher glycerol production than the WT. Other photosynthetic efficiency-related strategies include inserting additional chlorophyll pigments and widening the photosynthetic active radiation upto 750 nm. Achieving these strategies by genetic modifications is theoretically feasible, but limited studies were conducted on microalgae, particularly green algae [383,387].

4.5. Other transcription factors

It is not necessary to directly apply genetic modification to the gene, controlling the synthesis or degrading of lipids. The idea of utilizing other transcription factors (TFs) that indirectly affect the synthesis or degradation of lipids can be considered as well [389]. TF is a gene that has a DNA-binding protein encoded and is responsible for gene expression regulation [390]. According to Liu et al. [391], LEC1 is a gene that controls seed development, and applying overexpression of this gene will affect other genes such as ABI3 and FUS3 which further affects genes that control fatty acid synthesis. The researchers of this study overexpressed AtLEC1 in *Chlorella ellipsoidea* and cultivated the mutant strains under mixotrophic and autotrophic conditions. The

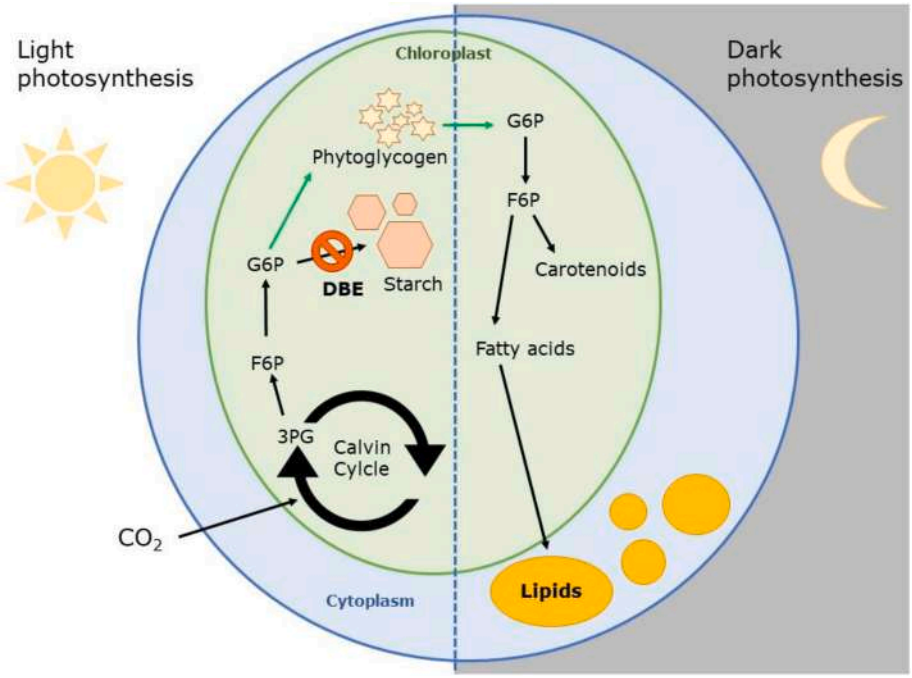


Fig. 18. The mechanism showing the repartitioning of carbon flux to lipids in DBE-deficient microalgae. Modified from Kato et al. [382] with permission from Springer Nature.

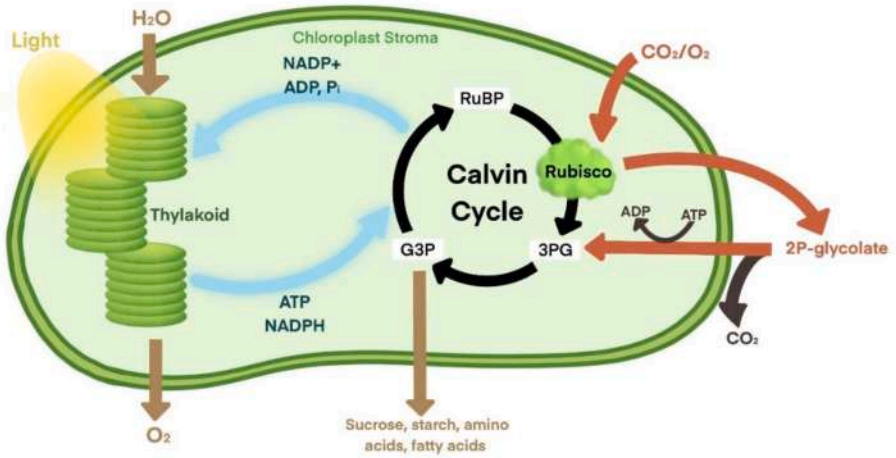


Fig. 19. An illustration of the involvement of Rubisco in carbon dioxide fixation. Modified from Bhat et al. [388] with permission from Frontiers.

overexpression of the gene did not affect the growth of mutant strains. The content of fatty acids and lipids was increased by a maximum of up to 32.65% and 29.91%, respectively.

A protein called DNA binding with one finger (Dof) is a potential TF to be genetically modified in microalgae for lipid productivity improvement. Dof recognizes either AAAG or CCCT sequences in the promoter region of the binding genes. In the promoter region, a gene, BCC1 can be found and it promotes the formation of ACCase [389]. Ibáñez-Salazar et al. [392] obtained a Dof type TF from soybean and applied it to *Chlamydomonas reinhardtii* via bacteria conjugation by co-cultivation with *Agrobacterium*. Besides the increase in total lipids content up to 2.3-fold, authors also reported that a subunit of the fatty acid synthase (FAS), ENR1 was overexpressed. Zhang et al. [333] reported the transformation of GmDof4 from soybean into *Chlorella ellipsoidea* and observed an increase in lipid content from 46.4 to 52.9%. They also observed upregulation in the ACCase gene and FAS gene. Jia et al. [389] overexpressed the Dof TF endogenously in *Chlamydomonas reinhardtii* and the transgenic strain had resulted in 23.24% higher in TFAs content compared to the WT.

On the other hand, phosphoenolpyruvate carboxylase (PEPC) is an enzyme which can improve lipid production when knocked down. Phosphoenolpyruvate (PEP) is a product of glycolysis and can act as a substrate for both protein and lipid synthesis. PEPC is the catalyst in one of the stages of the PEP to protein pathway. Thus, it is suggested that the knockdown of PEPC can redirect the carbon flow to the lipid synthesis pathway [392]. The suggested mechanism is shown in Fig. 20. Deng et al. [393] knocked down PEPC1 in *Chlamydomonas* sp. by RNAi which resulted in a 20% increment in TAG level in transgenic cells. Kao and Ng [30] used CRISPR-Cas9 technology to knockdown the PEPC genes in *Chlamydomonas* sp. and successfully reduced the activity of the genes by 94%. The transgenic cells achieved the highest lipid productivity of 34.9 mg/L d, which was 94.2% higher than the WT. Table 13 summarizes the genetic modifications of green algae based on different aspects and corresponding genes or enzymes.

5. Current challenges and future perspectives: How far are we in utilizing microalgae-based biofuels?

As good as it sounds, the challenges of genetic manipulation persist due to the limited availability of bioinformatic and genomic databases. This section will discuss the concerns related to commercialization, net-zero emission, environmental sustainability, risks and legislation considerations of genetic modification in microalgae.

5.1. Current status of the algae-based industry

Biofuel is important in this era while humans are tackling pollution and climate change issues caused by the consumption of fossil fuels during the past decades. The human population is growing fast and an increasing population means growing energy demand. According to data provided by Sönnichsen [394] on biofuel production in leading countries in 2019, the USA and Brazil are leading the other countries, with biofuel production of 1551.1 and 992.2 PJ, respectively. European countries such as Germany, France, Netherlands and Spain have a total

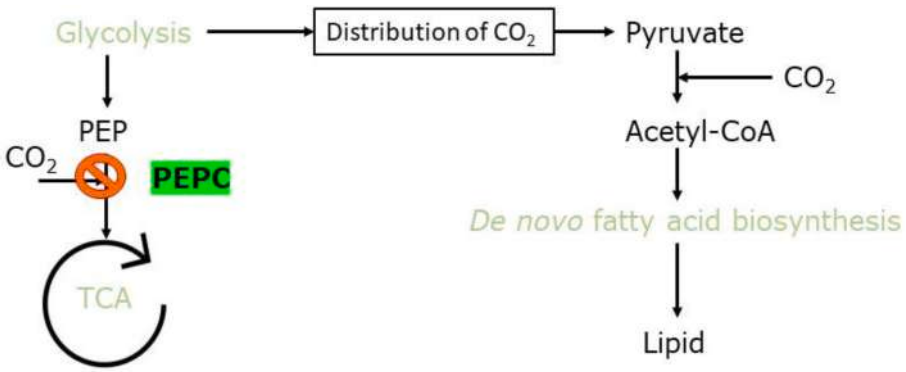


Fig. 20. The strategy to knockdown PEPC for redirecting the distribution of CO_2 from the tricarboxylic acid cycle (TCA) to the lipid synthesis pathway. Modified from Kao and Ng [30] with permission from Elsevier.

Table 13
Results of genetic modifications on green algae based on different aspects and corresponding genes or enzymes.

Process/Aspect	Types of genetic modification	Gene/Enzyme	Strain	Results	References
Lipid biosynthesis	Overexpression	ACC1 from *Saccharomyces cerevisiae*	*Scenedesmus quadricauda*	1.6 fold increment in TFA content compared to WT	[318]
		DGAT	*Chlamydomonas reinhardtii*	No changes in TAG accumulation and fatty acid profiles	[362]
		DGAT1 from *Echium pitardii* and DGAT2 from *Saccharomyces cerevisiae*	*Tetraselmis chuii*	111% increase in TAG content compared to WT	[325]
		Endogenous DGAT	*Neochloris oleoabundans*	1.9-fold increment in neutral lipids content compared to WT, TAG content of 46.1%	[363]
		LPAAT from *Brassica napus*	*Chlamydomonas reinhardtii*	17.4% increase in TFA content	[367]
		LPAAT1	*Neochloris oleoabundans*	4.8-fold and 2.2-fold increment in TAG content and productivity respectively, under N-depletion condition	[364]
Lipid catabolism	Knockout	Phospholipase	*Chlamydomonas reinhardtii*	190.42% increase in TAG content during the growth phase, 68.21% and 117.97% increase in TAG and DAG content respectively under N-depletion condition	[370]
		ACD, ACOX	*Chlamydomonas reinhardtii*	20% increase in TAG content under N-depletion condition	[369]
	Overexpression	PDAT from *Saccharomyces cerevisiae*	*Chlamydomonas reinhardtii*	22% and 32% increase in TFA and TAG content	[372]
Starch metabolism	Strains deficient in starch content	–	*Scenedesmus obliquus*	One strain achieve 49.4% DW of TAG content after 4 days under N depletion, TFA and TAG content of all strains increased	[378]
		–	*Dunaliella tertiolecta*	42–92% per DW increase in TAG content	[377]
	Inhibition by chemical CDTA	AGPase	*Tetraselmis* sp.	27% increase in FA production under N-depletion condition	[380]
	Knockdown	AGPase	*Chlamydomonas reinhardtii*	10-fold increment in TAG content	[381]
	Overexpression	DBE in isoamylase	*Chlamydomona reinhardtii*	1.46-fold increment in lipid productivity	[382]
Photosynthetic efficiency	Reduce in antenna size	–	*Chlorella vulgaris*	44.5% higher in biomass productivity compared to WT	[384]
	Overexpression	SBPase from *Chlamydomonas reinhardtii*	*Dunaliella bardawil*	All strains cultivated under different concentrations of NaCl achieved around 5% higher glycerol production than in WT	[386]
Other transcription factors	Overexpression	AtLEC1	*Chlorella ellipsoidea*	32.65% and 29.91% increase in FAs and lipids, respectively	[382]
		Dof from soybean	*Chlamydomonas reinhardtii*	2.3-fold increment in total lipids content	[392]
		GmDof4 from soybean	*Chlorella ellipsoidea*	46.4–52.9% increase in lipid content	[333]
		Endogeneous Dof	*Chlamydomonas reinhardtii*	23.24% higher in TFA compared to WT	[389]
	Knockdown	PEPC1	*Chlamydomonas* sp.	20% increment in TAG level	[393]
		PEPC	*Chlamydomonas* sp.	Highest lipid productivity of 34.9 mg/L/d (94.2% higher than WT)	[30]

biofuel production of 402.3 PJ. These countries use a mixture of biodiesel and petrodiesel in vehicles [127,130,395]. Governments around the world have started to develop policies to include biofuel in the transport fuel sector and it is estimated that biofuels will comprise 20% of liquid fuels by 2050. These actions have grown the demand for biofuels and increased the burden on biofuel producers and, as such, issues including land availability and cultivation resources are emerging [127]. Biofuel from food crops (i.e. first-generation biofuels) is not feasible due to the competition with land for the food supply chains of humans [396]. Production of biofuel with microalgae will be able to avoid competition with food supplies and provide economic potential to rural communities at the same time [395]. Other advantages of microalgal biofuel include CO_2 mitigation and reduction in GHGs emissions.

5.2. Sustainability of microalgae-based production

In terms of sustainability, microalgal biofuel is a cleaner option compared to fossil fuels as it has lower emissions of GHGs, hydrocarbons and particulate matter, except for NO_x [395]. Despite the advantages, microalgal biofuel is neither economically nor commercially feasible due to limited production which leads to high production prices when compared to fossil fuels. Currently, industrial microalgal biomass production is around 15,000 tons per year globally, which is very low compared to the demand [397]. The cultivation, harvesting and processing stages in microalgal biofuel are costly [398]. Light is one of the important parameters in microalgae cultivation and enough light penetration can increase microalgal biomass accumulation. Cultivation in closed PBRs can provide better light penetration and distribution to microalgae during cultivation when compared to open pond cultivation, but PBR cultivation can cost 2 to 2.5 times higher than open ponds [399].

Furthermore, the energy requirement of microalgal biofuel production is reported to be higher than the energy content in the biofuel itself [400]. The biofuel from microalgae is commonly argued to give lower power output and torque compared to fossil fuel [395]. Therefore, genetic modification strategies are explored to improve the feasibility of microalgal biofuel by improving biomass production rates and qualities. Genetic modification can reduce the production cost of microalgal biofuel by 15–20% and generate profit from other by-products. Omics studies are explored in the context of GM and they can provide virtual

predictions and insight into the experimental genome [344].

However, only a few strains such as *Chlamydomonas reinhardtii, Dunaliella salina* and *Ostreococcus tauri* have been investigated in some studies [401]. The cost of genetic modification itself is high and other aspects such as stability, toxicity, regulatory affairs and safety are still under investigation. Moreover, to protect intellectual property, most companies prefer to prevent the sharing of technology. This can limit and even prevent the genetic modification of microalgae for large scale application and can delay the commercial application of microalgal biofuel in the market [402].

5.3. Feasibility of algae-based biodiesel for environmental sustainability

The biodiesel obtained from microalgae should comply with the regulations provided by European Union Renewable Energy Directives [403]. The CO_2 footprint of several algae farming systems was studied by Azari et al. [404] and the results demonstrated that an open-pond system, that uses wastewater, produces the least amount of greenhouse gases, 67 g CO_2 equivalent/MJ of energy produced. This is because in an open pond, solar energy is used for lighting and drying. Heterotrophic culture (PBR) generates 89 g CO_2 equivalent/MJ. The need for a continuous supply of CO_2 is negated during heterotrophic farming, resulting in lower GHG emissions. The ORP system using recycled water emits 95 g CO_2 eq./MJ, while the ORP system using fresh water emits 149 g CO_2 eq./MJ. There are considerable differences in estimates of CO_2 eq/MJ emissions of biofuel produced in life cycle analyses (LCAs) of biodiesel production, with many values exceeding fossil diesel emissions. The differences in the study are mostly attributable to the use of several models, which are accompanied by significant differences in microalgae treatment, harvesting methods, and fuel extraction [300].

The findings of these studies suggest the need for more effective and efficient cultivation and harvesting technologies in the process of LCA of algae biodiesel production to reduce GHG emissions. To produce 1 kg of dry algal biomass, at least 1.83 kg of CO_2 is required, assuming the algae has a carbon content of 50%. The CO_2 concentration should be several times higher than the value given. The potential efficiency of CO_2 absorption varies from 20% to 90% depending on operational conditions, while the actual efficiency in open ponds is around 35%. CO_2 absorption efficiency is higher in PBRs, at around 75% [405]. The availability of CO_2 supply (as well as fresh water) determines the location of algae cultivation sites. If flue gas CO_2 is used, the algae farm should be adjacent to the power plant or another substantial CO_2 source. For optimal algae cultivation, flue gases with a temperature of 20–35 °C and CO_2 content of 15–20% by volume can be employed. Flue gases from traditional power plants must be cooled before usage since their temperature is close to 95 °C, which can harm the algae [406].

5.4. Risks and legislation considerations of genetic modification in microalgae

Currently, most of the GMs on microalgae are performed on a laboratory scale. When applied on an industrial scale, there are possibilities for leakage or release of these genetically modified strains into natural ecosystems, which may cause unwanted impacts on the ecosystem. The safety of exposing GM strains to the natural ecosystem remains uncertain as they might be unfit to survive, or become invasive, or produce toxins that harm other organisms in the system. Thus, the risks of genetically modified microalgae are debatable and have led to the halting of the application of GM on microalgae [401,407]. As genetically modified organisms (GMOs) are still controversial, algomics need to be well studied and developed to have a better understanding of the whole process of GM on microalgae [344].

The impacts on humans, animals and the environment of turning microalgal biofuel into mass production are also debated. Some microalgae species such as *Chaetoceros* and *Prototheca* are classified as pathogens, which means they could produce toxins that are harmful to animals and humans. Besides, a microalgal biofuel processing plant could cause contamination in surface water and groundwater, which could affect the humans' water supply chain [401]. Usher et al. [127] suggested to study the impacts on the environment of a large microalgal biofuel plant. Water qualities for the cultivation of microalgae vary with microalgae strains. Industrial and domestic wastewater is often suggested as an alternative water source for microalgae cultivation, as these wastewaters might contain high content of nitrogen and phosphorus compounds, which are essential nutrients for microalgal growth. However, excess nitrogen and phosphorus compounds can lead to eutrophication which further causes low oxygen concentrations due to the respiration of microalgae at night. Furthermore, wastewater might contain viruses and pathogens. Viruses can result in the death of the whole microalgae population and affect the supply chain due to the loss of the product. Pathogens can coexist with microalgae and are present in the harvested product. It is recommended to give post-treatment to the product to avoid any negative health impact.

On terrestrial impact, Usher et al. [127] stated that an open pond design will increase the likeliness of contamination. This is because failure in design or poor construction might release the pond contents into the ground by leaching. Although the pond contents might not be toxic, they could lead to the contamination of groundwater. They suggested the use of PBR as a cultivation container to prevent leakage. In addition, Environmental Impact Assessment surveys must be done to assess the possible threats of large-scale ponds on the local ecosystem. According to Usher et al. [127], microalgae also produce some gaseous products that cause atmospheric pollution such as methane, organohalogens and volatile organic compounds (VOCs). Methane is emitted under anaerobic conditions by methanogenic bacteria. This can be prevented by constant aeration of water. If the methane emission is not controlled, it will cause emissions of dihalo- and trihalomethanes, as well as other halogenated compounds, which are destructive to the ozone layer in the stratosphere. Production of VOCs such as isoprene from microalgae is observed. VOCs react with sunlight and NO_x and lead to ozone formation in the troposphere. Tropospheric ozone molecules are air pollutants that can harm the respiratory system in humans and animals [408]. Before microalgal biofuel production begins, environmental policy should be reviewed to prevent destructive impacts on the environment. Other than that, certification should be given as a guide to producers and consumers to have a better understanding of the production [127].

6. Concluding remarks

The present review elucidated different approaches including genetic modification to enhance the accumulation of lipids in microalgae for biofuel production. Still, there remains a critical question: Will microalgae provide a future alternative biofuel and simultaneously address the challenges to net zero emission by 2050? The answer depends on how stakeholders allign with the green transition of microalgae-based biofuels and their economical viability. Based on the techno-economics analysis, the major cost barrier lies in the cultivation of microalgae compared to other energy crops even though wastewater and flue gas are used to cultivate microalgae. Thus, tremendous efforts are being made in developing algae-based biofuels for sustainable energy production to replace fossil-based fuels. The ability of microalgae to harness solar energy to produce lipids has resulted in their classification as third-generation biofuel feedstock. Lipids are chosen as feedstock for biofuel rather than starch due to the simplicity in the conversion of lipids like TAG and DAG into biodiesel via transesterification. Despite advantages, such as easy cultivation and help in CO_2 mitigation, the utilization of microalgae to produce biofuel is not yet feasible from commercial and economic perspectives due to limited productivity and technology.

To achieve the goal of making microalgal biofuel feasible and

accessible worldwide, genetic modification is studied to improve lipid productivity in microalgae. This strategy involves the upregulating or downregulating of the metabolisms that affect lipid synthesis and degradation, as well as blocking competitive pathways in lipid synthesis. However, research based on this strategy needs to be accelerated as only limited studies are available. At the same time, it is advised that risks and considerations, as well as policies in the development of GM of microalgae, need to be well studied to prevent further issues. The main conclusions of this review are summarized below:

- The overall cost of biomass production, harvesting, lipid extraction and conversion to biofuels has restrained the commercialization of microalgae-based lipids and biofuels production.
- To overcome this bottleneck, genetic modification of microalgae is suggested to improve strains in terms of biomass and lipid productivity.
- In the last decade, substantial research has been carried out to develop genetic tools and produce genetically modified and improved strains.
- Genetic modification is carried out by employing direct genetic engineering, adaptive laboratory evolution, and random mutagenesis.
- Advancements in the development of efficient, accurate, and speedy delivery systems, highly improvised genome editing tools, and the versatile sequencing technology are considered to be vital to produce genetically modified and improved microalgal strains for enhanced lipid and biofuel production.

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.

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Associate Professor Dr. Koji Iwamota, affiliated to Malaysia–Japan International Institute of Technology, Universiti Teknologi Malaysia, **Koji Iwamoto** is an Associate Professor at the Malaysia–Japan International Institute of Technology, Universiti Teknologi Malaysia, in the department of Environmental Engineering and Green Technology (EGT). He is in charge of the Algae and Biomass Research Laboratory and is working on projects under the auspices of different grants. He obtained his PhD degree from the University of Tsukuba, Japan, and then worked there from 2003 to 2014 as a researcher and associate professor. His areas of interest include, bioengineering, biochemistry, applied microbiology, environmental microbiology, phycology, and plant physiology. He has been working on various microalgal species in recent years, including some novel species having oleaginous and phytoremediation potential. He is also working on acido-thermophilic species having the potential to absorb rare metals. He has published papers in peer reviewed and reputed journals. He has also presented papers at international conferences. He has been actively working on the joint degree programme (JDP), which is a collaboration between UTM and UT. Koji Iwamoto was the recipient of the Marine Biotechnology Research Paper Award and the Best Presentation award at the 5th World Fisheries Congress. He also chaired the International Conference on Environmental Science and Green Technology (ICEGT-2022).

Professor Dr. Pau Loke Show, affiliated to University of Nottingham (Malaysia), Malaysia, Prof. Ir. Ts. Dr. Pau-Loke SHOW is the President of International Bioprocess Association in Malaysia. He is also the director of Sustainable Food Processing Research Centre and Co-director of Future Food Malaysia, Beacon of Excellence, in the University of Nottingham Malaysia. He is a Full Professor at the Department of Chemical and Environmental Engineering, University of Nottingham Malaysia. Prof Show obtained the Post Graduate Certificate of Higher Education in 2014, and is now a Fellow of the Higher Education Academy UK. He has graduated more than 20 PhD and MSc students, and is leading a team of 20 members consisting of postdoctoral, PhD and MSc students. So far, he has published more than 650 journal papers. His publications have been cited over 10,000 times over the past 5 years. His current h-index is 68, placing him among the top leaders (Rank #1) of his chosen field (Microalgae Technology). He is also the Primary Project leader for more than 35 projects with total amount more than MYR5.0 million. He is now serving as an Editor-in-chief in Current Nutrition & Food Science, Editor in Scientific Report, Biocatalysis and Agricultural Biotechnology, Associate Editor at Bioengineered; Current Biochemical Engineering, and Editorial board member in Bioresource Technology and, Biochemical Engineering Journal.

Professor Amit Bhatnagar (Listed as *Highly Cited Researcher by Clarivate, Web of Science, 2018–2022*) is a Full Professor at LUT University, Finland, since August 2020. He is also the Head of Master's Programme in Water Technology at LUT University. Earlier, he had worked as Full Professor, Associate Professor and Assistant Professor at the University of Eastern Finland, Finland. He obtained his Ph.D. degree from the Indian Institute of Technology (IIT), Roorkee, India in 2003. Later, he worked in various international universities/institutes such as Linnaeus University, Sweden; University of Porto, Portugal; Hamburg University of Technology (TUHH), Germany; University of Kuopio, Finland; Yonsei University, South Korea; Swiss Federal Institute of Environmental Science and Technology (EAWAG), Switzerland; Central Building Research Institute (CBRI)/CSIR, India. Prof. Bhatnagar is the recipient of many reputed international fellowships including Center of Excellence in Analytical Chemistry (CEAC) World Laboratory Wilhelm Simon Fellowship; Brain Korea-21 (BK-21); Marie-Curie More Experienced Researcher; Erasmus Mundus visiting fellowship; and postdoc fellowship from Portuguese Foundation for Science and Technology. He has published >260 research/review articles in SCI/SCIE journals. His current H-index is 71 with >20,000 citations (Scopus). Prof. Bhatnagar currently serves as Associate Editor of *Critical Reviews in Environmental Science and Technology*, and he is Editor/Editorial Board Member of *Environmental Pollution* (Elsevier), *Environmental Technology & Innovation (Elsevier)*, *Biochar*, *Scientific Reports*, and *PLOS One etc*.