Environmental and Climate Technologies
2025, vol. 29, no. 1, pp. 742–755
https://doi.org/10.2478/rtuect-2025-0049
https://content.sciendo.com

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Lipid Extraction and Transesterification Process for Microalgal Biodiesel

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Received 23.04.2025; accepted 04.10.2025

***Abstract* – The growing global demand for sustainable energy solutions indicates the need for biofuels to reduce dependence on fossil fuels. Microalgae have emerged as a promising biodiesel feedstock due to their minimal resource requirements, rapid growth and higher carbon fixation efficiency compared with terrestrial crops. However, challenges such as energy-intensive production at high cost and biosafety concerns currently limit large-scale implementation. Ongoing research is focused on low-cost cultivation systems, innovative reactor design, co-product utilisation and risk mitigation practices to achieve scalable and sustainable microalgae-based biodiesel. In this contribution, the main current trends in the microalgae-derived biodiesel process are presented, with emphasis on lipid extraction and transesterification. Meaningful improvements in upstream processes – cultivation methods, harvesting, and dewatering – and downstream processes, with lipid extraction, conversion, and purification are discussed. The function of alkaline and acidic homogenous and heterogeneous, chemical and enzymatic catalysts in improving transesterification effectiveness is examined. Additionally, emerging non-catalytic approaches, as with supercritical fluid technology, are highlighted for the ability to achieve 85 % yields in a single-step conversion of algal lipids to biodiesel. Finally, the genetic and biochemical engineering capacity to increase lipid productivity and metabolic efficiency for advanced fourth-generation biofuels is examined.**

***Keywords* – Biofuel; catalyst efficiency; fatty acid ethyl ester; genetic engineering; lipid extraction; microalgae.**

Nomenclature

ABBs	Algae-based biofuels
FAAE	fatty acid alkyl esters
FAEEs	fatty acid ethyl esters
FAME	fatty acid methyl esters
FFA	Free fatty acid
FGBs	fourth-generation biofuels
GM	genetically modified
SCFs	supercritical fluids
TAGs	triglycerides

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1. INTRODUCTION

Alternative fuels are needed because of growing global fuel demands, declining petroleum resources, and rising greenhouse gases emissions. Energy resources are mainly fossil-based non-renewable fuels – which currently dominate the world's energy production – renewable fuels, and nuclear-based fuels. To meet the Paris Agreement target of 2 °C, energy related CO_2 emissions must decrease to 9.7 Gt/yr by 2050, with renewable energy and energy efficiency accounting for 94 % of the reductions [1], [2]. Next-generation green fuels offer a real sustainable alternative, capturing CO_2 through photosynthesis and emitting no toxic pollutants. In this context, biomass from organic materials, represents a valuable resource. Biomass has the potential to generate globally 3000 TWh electricity by 2050 and reduce CO_2 emissions by 1.3 Bt/yr [1]–[4]. Biofuels derived from biomass are a viable renewable energy option as they are biodegradable and have carbon-neutral combustion. Two main methods are used to manufacture plant-based biofuels: (a) microbial fermentation and (b) transesterification, both of which have been employed in the past and remain viable [4], [5]. Biofuels exist in solid, liquid, and gaseous forms, with liquid biofuels like biodiesel and bioethanol being more viable and eco-friendly alternatives to traditional fuels in the transport sector. Based on their feedstock, liquid biofuels can further be divided into four categories: (a) first-generation biofuels (from food crop plants); (b) second-generation biofuels (from non-edible crops, plant biomass residues, waste oils, and animal fats); (c) third-generation biofuels (from microalgae); (d) fourth-generation biofuels (FGBs) (from genetically modified algae). The main drawback of biofuels is the higher cost compared to traditional fuels, mostly due to feedstock expenses, which account for 75 % of total production costs [3], [6]. Nevertheless, biodiesel offers a series of advantages over petro-diesel. As shown in Table 1, microalgal biodiesel has a higher cetane number (48–65) compared to petro-diesel (40–55), which results in more effective combustion.

TABLE 1. COMPARATIVE PROPERTIES OF MICROALGAL BIODIESEL AND PETRO-DIESEL

	Microalgal Biodiesel	**Petro-Diesel**	**ASTM* Standards**	**References**
Cetane Number	48–65	40–55	D613	[7]
Total Sulphur	<5 ppm	<10 ppm	D5453	[9]
Flash Point, °C	>160	75	D93	[8]
Cloud Point, °C	–5.2–3.9	–35–5	D2500	[8]
Oxygen Content, wt %	10–12	<0.05	–	[13]
Viscosity at 40 °C, mm²/s	4.519–4.624	1.9–4.1	D445	[7], [8]
Lubricity	Excellent	Baseline	–	[9]
Emission Reduction	CO up to 50 % CO_2 up to 78 %	Not applicable	–	[8]
Blending	Up to 10 % of gasoline or conventional diesel	Not applicable	–	[8]
Environmental Impact	Renewable, biodegradable, non-toxic	Fossil fuel, non-renewable	–	[5]
Economic Challenge (2020)	2.93 USD/L	0.79 USD/L	–	[10], [11]

*ASTM: American Society for Testing and Materials

The low sulphur concentration of biodiesel prevents sulphur dioxide emissions and acid rain. Additionally, the high flash point of microalgal biodiesel improves handling and storage [7], [8]. Furthermore, biodiesel contains more oxygen content (10 % to 12 % by weight), which improves lubricity and reduces CO_2 emissions by up to 78 %, making biodiesel cleaner than standard diesel. Biodiesel can be blended with up to 10 % gasoline or standard diesel and utilised in existing engines without modification. These features make biodiesel a renewable, biodegradable, and non-toxic fuel. However, the economic viability is currently undermined by the costly character of microalgal oil production. Reducing the price of biodiesel from approximately 2.93 USD/L to 0.79 USD/L is crucial to achieving cost competitiveness [8]–[12].

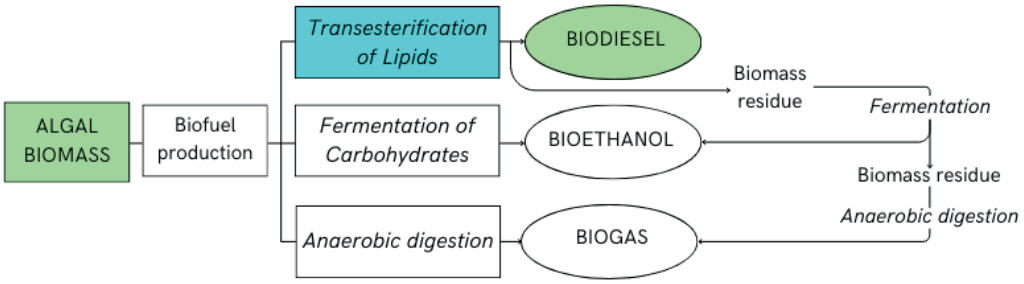

Fig. 1. Integration of direct transesterification for biodiesel production with bioethanol and biogas production pathways.

Mass production of microalgae biofuels is currently limited by high production costs, energy-intensive processing, and technological limitations. Biodiesel yield can be increased by improvements in transesterification methods, such as homogeneous, heterogeneous, enzymatic or non-catalytic approaches. Integrating direct transesterification with biomass residue utilization for bioethanol and biogas production (as shown in Fig. 1) can further optimize resource use. Addressing these challenges requires innovative approaches in genetic engineering, process optimisation, and biorefinery integration for better process control and catalytic efficiency [8], [14], [15]. Some key aspects are discussed in the current paper.

2. MICROALGAE: DEVELOPING POTENTIALS

Generally, algae comprise a diverse group of photosynthetic organisms, from prokaryotic to eukaryotes species. Composed of 70–90 % water, algae efficiently fix atmospheric CO_2 into valuable products like lipids for biodiesel, carbohydrates for ethanol, and proteins for consumption [16]. Based on size and morphology, algae are classified as macroalgae or microalgae, both of which possess excellent adaptability, endure harsh temperatures and desiccation and thrive in diverse saline environments [12], [15]. Microalgae are a diverse collection of unicellular, photosynthetic eukaryotes, including diatoms, which are classified as third-generation biomass feedstock and typically smaller than 0.4 mm in diameter [5], [14].

Microalgae excel in biofuel production due to high productivity per unit area and adaptability to diverse aquatic environments. Microalgae exhibit carbon fixation rates up to 10 times higher than traditional crops, with marine phytoplankton contributing ca. 20 % of global carbon fixation (ca. 12 Gt CO_2/year). Productivity varies with cultivation mode: phototrophic (0.01–0.7 g/L/day biomass, 0.2–505 mg/L/day lipids), heterotrophic (1.2–2 g/L/day biomass, 586.8–3 700 mg/L/day lipids), and mixotrophic (0.25–0.879 g/L/day biomass, 54–250 mg/L/day lipids) [6], [12].

Microalgae cultivated in nutrient-rich conditions contain 30–50 % protein, 20–40 % carbohydrates, and 8–15 % lipids, along with valuable compounds (pigments, antioxidants, and vitamins, e.g., docosahexaenoic acid, astaxanthin, and β-carotene). Exponential growth rates range from 3.5 to 24 h doubling time, with lipid accumulation occurring under stress conditions (e.g., nutrient deprivation, osmotic stress, carbon supplementation) [6], [14], [15].

Microalgae bioproducts are classifiable into two main families: (a) high-value, low-volume products, being used in nutraceuticals (e.g., pigments, food supplements), and (b) low-value, high-volume biofuels for bioenergy. Both may be cost-effectively produced simultaneously [2].

Oil and fatty acid methyl esters (FAMEs) extraction from microalgal biomass yields residues rich in carbohydrate, ideal for bioethanol production. Residual biomass from biodiesel and bioethanol production can be further processed for biogas production through anaerobic digestion. Therefore, algal biorefinery integrating biodiesel, bioethanol, biogas, and other bioproducts has been explored for the economic advantages [2], [6], [17].

Water availability, cultivation medium, and environmental factors like solar radiation and temperatures influence algae growth. *Nannochloropsis oculata* exhibits a 14.92 % lipid increase from 20–25 °C, whereas lipid content reduces from 15 % to 8 % between 15–20 °C before rebounding to 14 % at 25 °C. The growth rate of this microalga rises from 15–20 °C but declines at 25 °C [5], [8]. Generally, alkaline pH enhances lipid accumulation but inhibits growth. For *Nannochloropsis oleoabundans,* triglycerides (TAGs) rise from 13 % to 35 % in the 8–10 pH range, though growth rates halve. Elevated salinity (NaCl up to 2 %) increases lipid and carbohydrate content but inhibits growth, as seen for *Chlorococcum* sp. (lipids rising from 10.3 % to 29.8 %), and also for *Dunaliella* sp. and *Botryococcus braunii* [5], [8], [14]. Lipid production for algae-based biofuels (ABBs) depends mainly on light intensity, nutrients, temperature, retention time, and strain. Nitrogen deprivation (≤0.1 mM or ~0.014 g/L) induces lipid accumulation, with *Nannochloropsis oculata* reaching 48 % lipid content at nitrogen concentrations lower than 0.02 g/L [8], [14]. Finally, ABBs face environmental challenges, like land competition with food crops, resulting in increasing ecological pressures. Indeed, non-edible vegetable oils help mitigate food security concerns. With ca. 44 000 identified species and estimates up to one million, ongoing discoveries of high-growth and high-lipid strains highlight ABBs' potential as a scalable, environmentally friendly energy solution [2], [6], [12], [14].

Feedstock selection is dependent on the bioproduct. Microalgae are ideal for biodiesel production due to high oil yields (58 700–136 900 L/ha) and biodiesel productivity (up to 121 104 kg/yr), i.e., higher than conventional feedstocks. However, efficiency relies on biomass density, lipid accumulation, and species type. Some species offer high lipid content with slow growth rates, increasing production costs. Key species in biodiesel production include *Dunaliella, Nitzschia, Nannochloropsis, Nannochloris, Neochloris, Isochrysis, Phaeodactylum*, and *Porphyridium*, with *Chlorella* having high growth rates and biodiesel yields of up to 256 g/kg [5], [6].

The main challenge for ABB's economic feasibility lies in the capital-intensive production. Annual costs for microalgal biomass range from € 3 600 000 to € 3 640 000 per year (€ 2.00–€ 2.02 per kg) with biodiesel cost estimates exceeding conventional fossil fuel prices (€ 1.50–€ 2.00 per litre). ABBs' feasibility is dependent on revenue from by-products like residual biomass and glycerol, which could reduce biodiesel costs [18].

3. METHODOLOGY FOR MICROALGAL-DERIVED BIODIESEL PRODUCTION

Biodiesel production from microalgal biomass consists of two main phases: upstream and downstream processes, as shown in Fig. 2. Upstream processes cover cultivation, dewatering, and harvesting, preparing microalgae for biofuel production. Downstream processes convert harvested biomass into biodiesel through oil extraction, transesterification, and subsequent refining and purification [6].

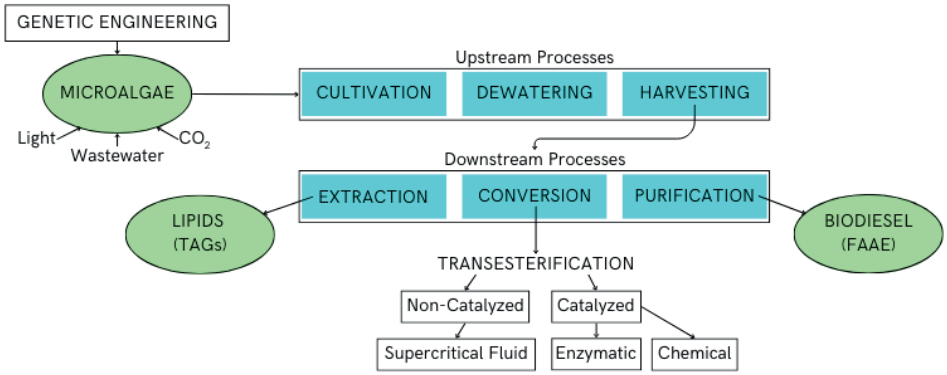

Fig. 2. Key steps in the mechanism of microalgae biodiesel production.

3.1. Upstream Processes

Cultivation of microalgae involves growing and maintaining these microorganisms under controlled conditions to produce biomass, with techniques mainly differentiated by the type of reactor. Microalgae can be grown in outdoor open ponds or indoor in photobioreactors, with each having advantages and limitations, especially for large-scale cultures.

Open ponds are a traditional method of mass production, which relies on phototrophic cultivation but limited by high construction and operational costs. The open nature makes the cultivation susceptible to microbial contamination, while temperature control is problematic, requiring temperature-resistant strains. Light availability also requires an important consideration: while microalgae have the potential to convert up to 12 % of light supplied for biomass production under optimal conditions, open ponds typically achieve 7 % efficiency. On the other hand, closed system photobioreactors offer higher biomass and lipid productivity while minimizing contamination risks. They integrate maximum light capture using transparent materials and constant aeration to enhance CO_2 assimilation, preventing clumping, and improving efficiency [4], [12].

Cultivation parameters, including medium composition, are important in supplying essential nutrients. An acceptable medium should be cost-effective and productive. Rathinasamy *et al*. showed that vegetable waste extracts significantly improve biomass and lipid yield in native microalgae like *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC [19].

Strain selection is crucial at commercial-scale production, where high biomass and oil yield is achieved. The selected strain must grow rapidly and efficiently, based on sustainability, environmental conditions, energy-harvesting ability, and carbon source availability [4], [20].

Dewatering (see Fig. 2) increases downstream extraction efficiency and bio-oil yield. Techniques include centrifugation, freeze drying, heated-drum drying, spiral-plate drying, spray drying, pressure drying, sun drying, vacuum drying, and membrane filtration.

Finally, in the upstream processes, the microalgae harvesting step involves the separation of algal cells from culture medium or water, a time- and energy-demanding process with no single method fully optimized for industrial use. Centrifugation, filtration, flocculation, electrolysis, and magnetophoretic separation are some of the generally used techniques. Among these, flocculation is the most widely used due to low costs, ease of application, and eco-friendliness. Current advancements, such as magnetic nanoparticle-mediated flocculation, enhance reusability and efficacy while reducing the reliance on chemical flocculants such as aluminium sulphate and cationic polymers, which are prone to contaminating biomass [4].

3.2. Downstream Processes

Oil extraction refers to the process of isolating lipids from harvested microalgae biomass and is a crucial process that accounts for 30–40 % of production costs.

The efficiency of solvent extraction is influenced by factors such as hydrogen bonding interactions between polar lipids and organic solvents, which vary depending on microalgae species. Developments like nanoparticles coated with cationic surfactants, show potential for enhancing processes for both harvesting and extraction [4], [17].

Microalgae have cell walls with thickness in the micrometre range, consisting of a polysaccharide matrix (including hemicellulose and cellulose) and membranes. Post-harvesting treatments are designed to disrupt such cell walls, enhancing solvent penetration. Mechanical methods alone are often insufficient due to the small size of microalgal cells, ranging between 3 to 10 µm. For instance, *Chlorella* species have cell walls approximately 0.3–0.5 µm thick, while diatoms range from 0.2 to 2 µm in thickness. To address such challenges, solvent systems such as ionic liquids, supercritical CO_2, and enzymatic treatments are preferred, as they reduce energy and solvent usage.

Currently, a combination of mechanical press and solvent extraction is used industrially. For large-scale biodiesel production, extraction methods must be efficient, time-efficient, secure, and selective to minimise contamination with proteins and carbohydrates [14], [15], [21]. Several widely recognised techniques for lipid extraction are available, including the Soxhlet, Folch, and Bligh and Dyer methods. The Soxhlet method efficiently dissolves non-polar lipids and volatile components from solids. It employs hexane as solvent and involves a continuous recirculation process. However, the process is labour-intensive and energy-consuming. The Folch method uses 1:2 (v/v) mixture of methanol and chloroform and enables rapid and effective lipid extraction from large quantities of material. Nevertheless, it involves hazardous chemicals (chloroform, methanol, and sometimes dichloromethane), increasing cost of operation. The Bligh and Dyer method utilises a monophasic solution of methanol, chloroform, and water with a 1:1 ratio of chloroform-methanol. It produces two separate phases: lipids in the lower chloroform phase and non-lipid content in the upper phase which consists of water and methanol. Unlike the Soxhlet method, the chloroform-methanol method isolates both neutral and polar lipids. Any residual biomass at the end of the extraction is removed by microalgae biomass filtration. The Soxhlet method is reported to achieve nearly complete recovery of microalgal lipids and is therefore among the most effective methods [14], [21].

Among innovative approaches, supercritical fluid extraction stands out for high efficiency, reduced reliance on toxic reagents, lower energy consumption and less pollution. Carbon dioxide is the most widely used supercritical fluid, due to its low toxicity and low costs. Nevertheless, capital and operation costs are higher than conventional extraction techniques. Therefore, economic feasibility is more favourable when the extracted lipids are targeted for high-value and low-volume products rather than biodiesel production (low-value

and high-volume product) [22]. Performance differences for the main extraction methods are summarised in Table 2.

TABLE 2. COMPARISON BETWEEN EXTRACTION METHODS [14], [23]–[25]

	Soxhlet Method	**Folch Method**	**Bligh & Dyer Method**	**Supercritical CO_2 extraction**
Solvent	Hexane	Methanol and chloroform (1:2 v/v)	Water, methanol and chloroform (2:2:1.8 v/v/v)	Compounds in a critical state
Lipids type	Non-polar	Polar and neutral	Polar and neutral	Neutral
Characteristics	Low cost, simple operation Efficient but time- and energy-consuming	Fast and simple, but harmful reagents are used	Simultaneous lipid extraction and separation	Cleaner and non-toxic, tends to have lower yields
Performance (wt. % crude lipid yield of *Pavlova* sp.)	18.5	–	18.5–21.0	10.4
Efficiency	80–90 % of total recoverable lipids	~80–95 % of total recoverable lipids	For lipid content <2 %: similar yields to Folch For lipid content >2 %: underestimation up to 50 % compared to Folch method	~95 % of total recoverable lipids with optimized conditions
References	[14], [23], [24]	[14], [25]	[14], [23], [25]	[14], [23], [24]

After lipid extraction, various methods are applicable for the conversion to biodiesel, including thermochemical and catalysed chemical conversions. The thermochemical conversions include pyrolysis and hydrothermal liquefaction. During the pyrolysis, dried biomass is decomposed into gases, liquids, and solids at temperatures ranging from 200 °C to 750 °C in the absence of oxygen. On the other hand, hydrothermal liquefaction yields bio-crude oil from wet biomass at temperatures 250–450 °C and pressures of 100–350 bar. Both approaches are effective but have limitations, such as variable biocrude quality, requiring additional purification and high energy demands.

The chemical conversion occurs through transesterification and extracted oils react with methanol in the presence of either an acidic or alkaline catalyst. This process faces challenges due to the use of hazardous chemicals – such as methanol, sodium hydroxide, potassium hydroxide, and sulfuric acid – and difficulties in recovering the final product. Nevertheless, it remains the most commonly used method [6], [9], [15], [17], [20].

4. CONVERSION PATHWAY: TRANSESTERIFICATION

During biodiesel production, the properties of biomass-derived oils must be compatible with those of hydrocarbon-derived fuels. One major challenge with the use of microalgal oils arises from the high viscosity, ranging from 4.519 to 4.624 mm²/s at 40 °C. By comparison, petrodiesel has a viscosity of 1.9–4.1 mm²/s, as per ASTM Standard D445 (see Table 1). High-viscosity oils can lead to oil sludge formation as well as potential engine damage. To overcome these challenges, transesterification is used to convert raw microalgal lipids into biodiesel, specifically long-chain fatty acid alkyl esters (FAAEs). FAAEs, which includes

FAMEs and fatty acid ethyl esters (FAEEs), possess lower molecular weights compared to the original microalgal lipids.

Transesterification may be achieved using either direct (reactive) or indirect (extractive) methods. The reactive method utilises an in-situ process which directly converts microalgal biomass into FAME. This approach integrates lipid extraction and transesterification into a single-step process, reducing production costs by eliminating consecutive steps. In contrast, the extractive method involves a two-step process: extracting lipids from algae, and trans esterifying the extracted lipids into FAME. Experimental results with *Chlorella sorokiniana* (UTEX 1602) reveal that a two-step process – initial pre-transesterification with the Amberlyst-15 catalyst followed by base-catalysed transesterification – achieved a FAME percentage recovery of 94.9±0.9 %, outperforming the one-step in-situ process, which yielded only 60.89 % FAME after 70 minutes at 90 °C [6], [17]. The microalgae lipids converted into biodiesel are primarily triglycerides (TAGs). TAGs are glycerol esters consisting of three fatty acid chains, typically with carbon chains ranging from C14 to C20, attached to a glycerol backbone. In transesterification, TAGs react with an alcohol, which acts as an acyl acceptor, in the presence of a catalyst (acidic, alkaline, or enzymatic) to produce biodiesel and glycerol. Common alcohols employed in transesterification are methanol, ethanol, and isopropanol. Methanol is preferred due to its low cost, high reactivity, excellent solubility in water and oil, low boiling point (64.7 °C), and effectiveness with acidic oils [4], [8], [14], [17], [21].

Solvents like ethyl acetate, methyl acetate, and dimethyl carbonate help separate glycerol from biodiesel and prevent microemulsion formation while enhancing the solubility of hydrophobic TAGs in alcohols. The efficiency of transesterification methods and the biodiesel yield are influenced by factors such as oil-to-alcohol molar ratio, temperature, moisture, stirring rate, reaction time, alcohol concentration, glyceride content, and catalyst type [26], [27]. Fatty acid composition affects viscosity, iodine value, cetane number, cold flow properties, and polyunsaturated fatty acid content. Optimal biodiesel performance is achieved with a high FAME yield, short reaction time, low temperatures, low molar ratio of reactants, and minimal catalyst concentration [6], [8], [17], [21].

4.1. Homogeneous Catalysis

Homogeneous catalysed transesterification is the most widely used method in the production of biodiesel due to its effectiveness in promoting mass transfer between lipids and catalysts under mild conditions. It requires highly active catalysts and is conducted at low pressures (1–2 atm), moderate temperatures and short reaction times (30 minutes to 2 hours). However, saponification in the reaction can lead to complications in the purification of the final product, as well as the separation, recovery, and recycling of the catalyst from the reaction mixture. Homogeneous-catalysed transesterification can be carried out using either an alkaline or an acidic catalyst [8], [19].

Homogeneous alkaline catalysed transesterification can achieve high FAME yields at moderate temperatures (30–65 °C) and atmospheric pressure. A maximum biodiesel yield of 97.66 % was reported for *Chlorella protothecoides* oil using 0.5 % NaOH as the catalyst (w/w), a methanol-to-oil molar ratio of 7:1, a reaction temperature of 60 °C, and a reaction time of 60 minutes [17], [21].

The most widely used alkaline catalysts include sodium and potassium salts with anion groups like aluminate, carbonate, hydroxide, methoxide, and ethoxide. Sodium hydroxide and potassium hydroxide are the most used base catalysts in commercial applications. A flowchart of the production steps using NaOH as alkaline catalyst is presented in Fig. 3.

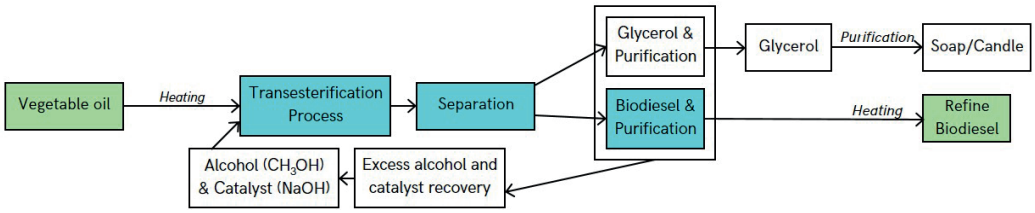

Fig. 3. Flowchart of biodiesel production process from vegetable oil via alkaline-catalysed transesterification.

The disadvantages of homogeneous alkaline catalysts are the need to neutralize the alkali water generated during downstream processing and the applicability only to algal lipids with low free fatty acids (FFAs) content (<2–3 wt %) [4], [8], [17], [21].

Due to the high FFAs content of microalgal biomass, alkaline catalysts are generally unsuitable for in-situ transesterification of microalgae. For vegetable oils containing greater than 5 wt % FFAs content, acidic catalysts are preferred. Common examples of acidic catalysts include sulfuric acid, sulfonic acid, hydrochloric acid, phosphoric acid, ferric sulphate and boron trifluoride. Acidic catalysts enable a high FAME conversion rate, require low-cost feedstocks, and prevent FFAs from forming soap. Inconveniently, acidic catalysts require higher reaction temperatures (55–80 °C), and longer reaction times (12–50 hours), which can lead to corrosive conditions. The reaction rate is up to 4000 times slower in comparison with alkaline catalysts, primarily due to the requirement of high alcohol-to-oil molar ratio. Acidic catalysts can also be deactivated by water content through competition for available protons. Studies on the transesterification of *Chlorella* oil with high FFAs content established the effectiveness of sulfuric acid as a catalyst, working at optimised conditions, including a reaction temperature of 65 °C, a reaction time of 24 hours, a methanol-to-oil molar ratio of 30:1, and a catalyst concentration of 0.5 mol/L. Despite the high FFAs content, a biodiesel yield of approximately 99 % was achieved [4], [8], [17], [21].

4.2. Heterogeneous Catalysis

Heterogeneously catalysed transesterification offers eco-friendliness, non-corrosiveness, recyclability, and extended operational lifespan [8], [21]. Common heterogeneous base catalysts possess high activity and reaction rates and include alkali earth metal oxides (e.g., CaO, MgO, SrO, ZnO, CuO, BaO, Li-CaO), basic polymers (e.g., polyethyleneimine, polyvinylamine, poly(2-vinylpyridine)), and functionalised polystyrene and polyurethane. Carbonates like $CaCO_3$, $MgCO_3$, $SrCO_3$, and $BaCO_3$ are also used. Heterogeneous acid catalysts are advantageous for their stability in the presence of water and FFAs. Experiments have shown that solid acid catalysts, such as sulphated/tungstate zirconia, sulphated tin oxide, and sulfonated polystyrene/saccharides, possess multiple active sites with varying acidity strengths, making them preferable over liquid acids. Reaction conditions of 350–400 °C and 17 MPa for microalgal biodiesel production have yielded up to 90.2 % conversion. However, there are challenges related to undesired side reactions and relatively low reaction rates [8], [17], [21]. One significant limitation of heterogeneous catalysts is their suboptimal porous structure, which may be underdeveloped or unevenly distributed. This inefficiency in the structure may inhibit effective mass transfer between the algal lipids and the active catalytic sites, thereby reducing catalytic performance. To compensate for this, stringent reaction conditions must be used, making the process highly energy-intensive to achieve acceptable catalytic efficiency. Alternatively, non-conventional heterogeneous catalysts present several

significant advantages, including ease of separation from the reaction mixture, thus simplifying downstream processing and reducing the cost of production. Examples of these catalysts include titanium-zirconia, potassium-doped zirconia, and amorphous zirconia [4], [8], [19].

4.3. Enzymatic Catalysis

Enzymatic catalysis is a viable alternative to both homogeneous and heterogeneous acid/base catalysis for the synthesis of esters from TAGs and FFAs. Enzymes such as lipases and hydrolases can function under mild conditions, typically at moderate temperatures (35–45 °C), and exhibit high tolerance to FFAs and water. This approach avoids saponification and the need for additional purification steps. However, enzymatic catalysis experiences activity reduction due to excess alcohol, low lipid concentrations, water content, and temperature fluctuations. Additionally, by-products like glycerol can obstruct both free and immobilized enzymes, further restricting the catalytic process [4], [8], [17], [21].

The methodologies chosen for enzyme immobilisation influence the activity and reusability of enzymatic catalysts. Common techniques employed are covalent bonding, entrapment, and adsorption. Recent advancements, such as magnetic nanoparticles, have further enhanced enzymatic catalysis. Enzyme-nanozeolite complexes have achieved over 93 % FAEE yields with increased enzymatic activity. Additionally, biocatalysis using magnetic nanoparticles functionalised with aminopropyl triethoxysilane and glutaraldehyde linked with *Rhizopus oryzae* lipase has been found to have improved stability and a biodiesel yield of 57.2 % from *Chlorella vulgaris* [8], [17].

Organic solvents find extensive use in enzymatic reactions but pose safety risks due to toxicity and flammability. Greener alternatives, such as dimethyl carbonate, are increasingly explored. In transesterification of *Chlorella* sp.-derived TAGs with Novozymes 435 lipase, dimethyl carbonate enhances biodiesel yields and enzyme stability, maintaining immobilized enzyme activity for over ten reaction cycles [8], [21].

4.4. Non-Catalysis

Non-catalytic transesterification offers a viable alternative by enabling a single-step conversion of algal lipids to esters. Supercritical conditions are applied, under which substances are subjected to temperatures and pressures exceeding their critical points, thereby transitioning into a supercritical fluid (SCF) state. SCFs exhibit both gas- and liquid-like properties, including high density and solvent power. Methanol, at 255 °C and ca. 80 bar, functions as a homogeneous SCF, enhancing lipid interactions and enabling efficient reactions even in high-water-content environments, which typically hinder non-supercritical processes.

Supercritical methanol has achieved an 85.75 % FAME yield in the transesterification of wet *Nannochloropsis* sp. with approximately 90 % water content. The tuneable properties of SCFs, including adjustable density and enhanced solvation capabilities, enable their effectiveness in extraction, reaction, and fractionation processes. However, SCF technology presents challenges such as high energy consumption, safety issues in the operation process, the need for advanced wastewater treatment systems and the high cost of expensive equipment. SCF technology requires durable reactors, efficient control devices and high-pressure pumps; additionally, operation and maintenance costs are higher than conventional processes. Finally, it is to note that both catalytic and non-catalytic transesterification in microalgae can be optimised using co-solvents, microwaves, or ultrasound, which reduce reaction times and improve FAME yields [8], [17].

5. POTENTIAL AND RISKS OF GENETIC ENGINEERING IN FGB DEVELOPMENT

Despite the advantages of third-generation biofuels, their use currently remains limited to small-scale systems, mainly due to low yields and economic constraints. Recent research focuses on genetic and biochemical engineering to enhance yields, reduce costs, and improve quality, leading to the development of the so called fourth generation biofuels (FGBs). FGBs aim to improve microalgal biomass production and optimise metabolic pathways for industrial applications. The main differences between third and fourth-generation biofuels are summarised in Table 3.

TABLE 3. COMPARISON BETWEEN THIRD AND FOURTH GENERATION (FGB) BIOFUELS

	Third Generation	**Fourth Generation**
Sources	Algae	Extension of third generation
Feedstock	Microalgae	Genetically modified algae
Production Methods	Biochemical/Thermochemical	Genetic engineering to alter cellular metabolism
Advantages	Cultivable land not required No food-energy conflict CO_2 fixation Wastewater treatment Easy conversion to biofuels	No food-energy conflict Medium CO_2 fixation Medium wastewater treatment Higher production rate Increased photosynthetic efficiency
Disadvantages	Require high carbon and nitrogen inputs Extensive downstream processing Higher costs Risk of marine eutrophication Biomass production too low for commercialisation	Require high carbon and nitrogen inputs Economically viable only in the long run Higher regulations due to safety issues Biomass production too low for commercialisation

The unintentional release of genetically modified (GM) algae – through wind dispersal, waterfowl, or insects – could lead to invasions of natural habitats, threatening biodiversity and potentially causing toxicity or allergic reactions. Algal blooms may also increase as a result.

Russo *et al*. [34] carried out a competitive growth experiment to study the risk in open-pond cultivation of mutant strains of *Chlamydomonas reinhardtii*. The mutant (CC-4333) had twice the exponential growth rate of the wild type (CC-124) in monoculture and contained 200 % more TAGs per cell. However, in co-culture, the wild strain dominated due to the mutant's slow transition phase, reducing environmental risks [28]–[30]. Another concern is the disposal of GM residues because lateral gene transfer via plasmid or chromosomal DNA could persist even after the microalgae's death. Effective waste management strategies are essential to mitigate these risks. Various containment measures have been proposed, including the use of self-cloning and mutagenesis (which avoid foreign DNA sequences) and biocontainment methods [24], [35]. Nevertheless, the lack of large-scale studies on GM algal cultivation in aquatic environments and the absence of internationally coordinated regulations on environmental and health risks have hindered the adoption of FGB technologies. Although most countries follow the Cartagena Protocol on Biodiversity, further research and regulatory advancements are needed to support the commercialization of GM microalgae biofuels [28], [33].

Strain improvement is achieved at the genome level through gene overexpression or repression. Examples include overexpression of genes involved in the TAG or fatty acid

biosynthesis pathways or blocking genes competing against lipid accumulation (e.g., starch biogenesis and lipid catabolism) [28], [29]. Genetic modification methods include (a) random mutagenesis, (b) nuclear transformation for transgene expression, and (c) genome-editing technologies like the CRISPR-Cas9. Molecular approaches to lipid enhancement target competitive pathways, lipid body formation, transcriptional regulation, and photosynthetic efficiency. Notably, overexpressing diacylglycerol acyltransferase accelerated TAG biosynthesis by 69 %. Additional enzymes in the Kennedy pathway, essential for lipid synthesis in the endoplasmic reticulum, have also been targeted [29], [30]. Advances in synthetic biology, bioinformatics and multi-omics (genomic and transcriptomics) continue to improve microalgae biofuel production [18], [21], [31], [33], [34], [36]. However, scaling up FGBs production faces biosafety, environmental and health concerns, particularly in open-pond cultivation systems.

6. CONCLUSIONS

Microalgae biofuels have the potential to enhance global energy sustainability by decreasing dependence on fossil fuels and mitigating greenhouse gases emissions. Nevertheless, large-scale deployment remains constrained by technical limitations and high costs. Future research should concentrate on: (i) optimising cultivation parameters—such as medium composition, light management and strain selection—to maximise biomass productivity and resource-use efficiency; (ii) developing advanced reactor design capable to overcome the limited conversion efficiency of current systems; (iii) developing cost-effective lipid extraction, conversion and purification processes.

Integrating microalgal biofuel production with wastewater treatment and carbon capture technologies could enhance cost efficiency while addressing environmental issues [14], [17]. In addition, advancements in synthetic biology and genetic engineering could enable the development of strains with improved lipid yields, faster growth rates and increased stress resistance. Nevertheless, stronger regulatory systems and biocontainment approaches must be designed to enable control of biosafety risks related to fourth-generation biofuels. Ultimately, collaborations between biotechnology, environmental science, and policymaking can enable increased market acceptability of microalgal biofuels.

REFERENCES

[1] Gielen D., Boshell F., Saygin D., Bazilian M. D., Wagner N., Gorini R. The role of renewable energy in the global energy transformation. *Energy Strategy Reviews* 2019:24:38–50. https://doi.org/10.1016/j.esr.2019.01.006.

[2] Yang J., Zhao T., Cui X., Peng M., Wang X., Mao H., Cui M. New insights into the carbon neutrality of microalgae from culture to utilization: A critical review on the algae-based solid biofuels. *Biomass and Bioenergy* 2022:166:106599. https://doi.org/10.1016/j.biombioe.2022.106599.

[3] Antar M., Lyu D., Nazari M., Shah A., Zhou X., Smith D. L. Biomass for a sustainable bioeconomy: An overview of world biomass production and utilization. *Renewable and Sustainable Energy Reviews* 2021:139:110691. https://doi.org/10.1016/j.rser.2020.110691.

[4] Chauhan S., Velramar B., Soni R. K., Mishra M., Sudheer P. D. V. N. Biofuels: Sources, Modern Technology Developments and Views on Bioenergy Management. *Biotechnology for Biofuels: A Sustainable Green Energy Solution* 2020:1:197–219. https://doi.org/10.1007/978-981-15-3761-5_8.

[5] Singhal A., Prashant. Biofuels: Perspective for Sustainable Development and Climate Change Mitigation. *Biotechnology for Biofuels: A Sustainable Green Energy Solution* 2020:1:1–22. https://doi.org/10.1007/978-981-15-3761-5_1

[6] Karpagam R. Algae as a feedstock for biofuel production. *Environmental Sustainability of Biofuels: Prospects and Challenges* 2023:1:201–217. https://doi.org/10.1016/B978-0-323-91159-7.00016-3

[7] Bahadar A., Khan M. B. Progress in energy from microalgae: A review. R*enewable and Sustainable Energy Reviews* 2013:27:128–148. https://doi.org/10.1016/j.rser.2013.06.029

[8] Ponnumsamy V. K., Al-Hazmi H. E., Shobana S., Dharmaraja J., Jadhav D. A., Banu R. J., Piechota G., Igliński B., Kumar V., Bhatnagar A., Chae K. J., Kumar G. A review on homogeneous and heterogeneous catalytic microalgal lipid extraction and transesterification for biofuel production. *Chinese Journal of Catalysis* 2024:59:97–117. https://doi.org/10.1016/S1872-2067(23)64626-1

[9] Amin A. Review of diesel production from renewable resources: Catalysis, process kinetics and technologies. *Ain Shams Engineering Journal* 2019:10(4):821–839. https://doi.org/10.1016/j.asej.2019.08.001

[10] Moriarty P., Honnery D. Microalgal based biofuels: Sources, benefits, and constraints. *Microalgal Biomass for Bioenergy Applications, Woodhead Series in Bioenergy* 2024:23–40. https://doi.org/10.1016/B978-0-443-13927-7.00012-8

[11] Roles J., Yarnold J., Wolf J., Stephens E., Hussey K., Hankamer B. Charting a development path to deliver cost-competitive microalgae-based fuels. *Algal Research* 2020:45:101721. https://doi.org/10.1016/j.algal.2019.101721

[12] Sarwer A., Hamed S. M., Osman A. I., Jamil F., Al-Muhtaseb A. H., Alhajeri N. S., Rooney D. W. Algal biomass valorization for biofuel production and carbon sequestration: a review. *Environmental Chemistry Letters* 2022:20:2797–2851. https://doi.org/10.1007/s10311-022-01458-1

[13] Pham P. X., Nguyen K. T., Pham T. V., Nguyen V. H. Biodiesels manufactured from different feedstock: From fuel properties to fuel atomization and evaporation. *ACS Omega* 2020:5(33):20842–20853. https://doi.org/10.1021/acsomega.0c02083

[14] Tomar S., Agarwal S., Singh H., Kumar R., Qureshi K. A., Jaremko M., Emwas A.-H., Rai P. K. Microalgae: A promising source for biofuel production. *Biocatalysis and Agricultural Biotechnology* 2023:53:102877. https://doi.org/10.1016/j.bcab.2023.102877

[15] Soni V. K., Krishnapriya R., Sharma R. K. Algae: Biomass to Biofuel. *Biofuels and Biodiesel, Methods in Molecular Biology* 2021:2290:31–51. https://doi.org/10.1007/978-1-0716-1323-8_3

[16] Merlo A., Conti F. Bioactive Derivatives from Algae: Properties and Applications in Pharmaceutical. *Environmental and Climate Technologies* 2023:27(1):438–449. https://doi.org/10.2478/rtuect-2023-0032

[17] Karpagam R., Jawaharraj K., Gnanam R. Review on integrated biofuel production from microalgal biomass through the outset of transesterification route: A cascade approach for sustainable bioenergy. *Science of the Total Environment* 2021:766:144236. https://doi.org/10.1016/j.scitotenv.2020.144236

[18] Branco-Vieira M., Mata T. M., Martins A. A., Freitas M. A. V., Caetano N. S. Economic analysis of microalgae biodiesel production in a small-scale facility. *Energy Reports* 2020:6(S8):325–332. https://doi.org/10.1016/j.egyr.2020.11.156

[19] Karpagam R., Abinaya N., Gnanam R. Assortment of native microalgae for improved biomass and lipid production on employing vegetable waste as a frugal cultivation approach for biodiesel application. *Current Microbiology* 2021:78:3770–3781. https://doi.org/10.1007/s00284-021-02643-1

[20] Narayanan M. Biorefinery products from algal biomass by advanced biotechnological and hydrothermal liquefaction approaches. *Discover Applied Sciences* 2024:6:146. https://doi.org/10.1007/s42452-024-05777-6

[21] Ezhumalai G., Arun M., Manavalan A., Rajkumar R., Heese K. A holistic approach to circular bioeconomy through the sustainable utilization of microalgal biomass for biofuel and other value-added products. *Microbial Ecology* 2024:87:61. https://doi.org/10.1007/s00248-024-02376-1

[22] Taher M. A., *et al*. Techno-economic analysis of supercritical carbon dioxide extraction of lipids from microalgae for biodiesel production. *Biochemical Engineering Journal* 2020:159:107612. https://doi.org/10.1016/j.bej.2020.107612

[23] Zhou J., Wang M., Saraiva J.A., Martins A.P., Pinto C.A., Prieto M.A., Simal-Gandara J., Cao H., Xiao J., Barba F.J., Extraction of lipids from microalgae using classical and innovative approaches. *Food Chemistry* 2022: 384:132236. https://www.sciencedirect.com/science/article/pii/S0308814622001972

[24] Luksta I., Spalvins K. Methods for Extraction of Bioactive Compounds from Products: A Review. *Environmental and Climate Technologies* 2023:27(1):422–437. https://doi.org/10.2478/rtuect-2023-0031

[25] Saini R. K., Prasad P., Shang X., Keum Y. S. Advances in lipid extraction methods—A review. *International Journal of Molecular Sciences* 2021:22(24):13643. https://doi.org/10.3390/ijms222413643

[26] Wiedemann L., Conti F., Sonnleitner M., Saidi A., Goldbrunner M., Investigation and optimization of the mixing in a biogas digester with a laboratory experiment and an artificial model substrate. *EUBCE Proceedings* 2017:889–892. https://doi.org/10.5071/25thEUBCE2017-2CV.4.14

[27] Conti F., Wiedemann L., Sonnleitner M., Goldbrunner M., Thermal behavior of viscosity of aqueous cellulose solutions to emulate biomass in anaerobic digesters *New J. Chemistry* 2018:42(2):1099–1104. https://doi.org/10.1039/c7nj03199h

[28] Shokravi H., Shokravi Z., Heidarrezaei M., Ong H. C., Rahimian Koloor S. S., Petrů M., Lau W. J., Ismail A. F. Fourth generation biofuel from genetically modified algal biomass: Challenges and future directions. *Chemosphere* 2021:285:131535. https://doi.org/10.1016/j.chemosphere.2021.131535

[29] Park S., Nguyen T. H. T., Jin E. Improving lipid production by strain development in microalgae: Strategies, challenges and perspectives. *Bioresource Technology* 2019:292:121953. https://doi.org/10.1016/j.biortech.2019.121953

[30] Li D. W., Cen S. Y., Liu Y. H., Balamurugan S., Zheng X. Y., Alimujiang A., Yang W. D., Liu J. S., Li H. Y. A type 2 diacylglycerol acyltransferase accelerates the triacylglycerol biosynthesis in heterokont oleaginous microalga *Nannochloropsis oceanica*. *Journal of Biotechnology* 2016:229:65–71. https://doi.org/10.1016/j.jbiotec.2016.05.005.

[31] Salama E. S., Govindwar S. P., Khandare R. V., Roh H. S., Jeon B. H., Li X. Can Omics Approaches Improve Microalgal Biofuels under Abiotic Stress? Trends in Plant Science 2019:24(7):611–624. https://doi.org/10.1016/j.tplants.2019.04.001

[32] Cavelius P., Engelhart-Straub S., Mehlmer N., Lercher J., Awad D., Brück T. The potential of biofuels from first to fourth generation. *PLOS Biology* 2023:e3002063. https://doi.org/10.1371/journal.pbio.3002063

[33] Abdullah B., Syed Muhammad S. A. F., Shokravi Z., Ismail S., Kassim K. A., Nik Mahmood A., Aziz M. M. A. Fourth generation biofuel: A review on risks and mitigation strategies. *Renewable and Sustainable Energy Reviews* 2019:107:37–50. https://doi.org/10.1016/j.rser.2019.02.018

[34] Russo D. A., Beckerman A. P., Pandhal J. Competitive growth experiments with a high-lipid Chlamydomonas reinhardtii mutant strain and its wild-type to predict industrial and ecological risks. *AMB Express* 2017:7:10. https://doi.org/10.1186/s13568-016-0305-x

[35] Wang F., Zhang W. Synthetic biology: Recent progress, biosafety and biosecurity concerns, and possible solutions. *Journal of Biosafety and Biosecurity* 2019:1(1):22–30. https://doi.org/10.1016/j.jobb.2018.12.003

[36] Bertolini M., Conti F., Algae culture conditions and process parameters for phycoremediation and biomaterials production. *Environmental and Climate Technologies* 2022:26(1):1092–1105. https://doi.org/10.2478/rtuect-2022-0082