Review

Recent Advances in Feedstock and Lipase Research and Development towards Commercialization of Enzymatic Biodiesel

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Abstract: Biodiesel is a biodegradable, renewable, and carbon-neutral alternative to petroleum diesel that can contribute to the global effort of minimizing the use of fossil fuels and meeting the ever-growing energy demands and stringent environmental constraints. The aim of this work was to (1) review the recent progress in feedstock development, including first, second, third, and fourth-generation feedstocks for biodiesel production; (2) discuss recent progress in lipase research and development as one of the key factors for establishing a cost-competitive biodiesel process in terms of enzyme sources, properties, immobilization, and transesterification efficiency; and (3) provide an update of the current challenges and opportunities for biodiesel commercialization from techno-economic and social perspectives. Related biodiesel producers, markets, challenges, and opportunities for biodiesel commercialization, including environmental considerations, are critically discussed.

Keywords: biodiesel; feedstock; lipase; transesterification; commercialization

1. Introduction

Biodiesel is gaining acceptance as a substitute for petrodiesel due to its renewability, biodegradability, recyclability, and carbon neutrality. Major economic and environmental factors drive the broader use of biodiesel, such as growing demand, fast drop-off supply and price hikes of crude oil, depleting oil reserves, air pollution, and increased levels of greenhouse gases (GHG) emissions [1,2]. For the reasons above, biodiesel production in the last decade has increased at an annual growth rate of 11.4%, which is higher than for any other biofuel such as bioethanol, biobutanol, bio-2,5-dimethylfuran biomethanol, renewable aviation, and biojet fuels [3,4].

Owing to its lower density (860–900 kg/m³), the energy per gallon biodiesel is about 10% lower (118,000 British Thermal Unit, BTU/gallon) than petrodiesel (130,000 BTU/gallon). However, there are several advantages of biodiesel that make it a competitive, renewable alternative to petrodiesel. Biodiesel can be blended at any level with petrodiesel and can be used in any diesel engine with only minor engine modifications. These modifications that mainly concern the fuel pump design and related injection timing are needed to address some limitations of biodiesel, such as cold starting, engine clogging, and storage [5]. Biodiesel has a higher flashpoint and cetane number than diesel and the emissions from burning biodiesel in a conventional diesel engine contain lower levels of particulate matter, sulfur oxides, hydrocarbons, carbon monoxide, carbon dioxide, and odor [6]. Overall, biodiesel emits 3-fold lower amounts of GHG than petrodiesel and 85% ethanol–gasoline fuel (E85), and 4.5-times
lower than gasoline [7]. Biodiesel has better lubricating properties compared to diesel fuels, which increases the usable life of high-pressure fuel injection equipment that relies on the fuel for its lubrication [8]. Furthermore, biodiesel is safer than petrodiesel because it is less combustible and, therefore, causes less damage if spilled or released to the environment [9].

Biodiesel is typically produced by reacting vegetable oils or animal fats with methyl alcohol in the presence of a chemical (alkali, acid) or enzymatic catalyst. The transesterification process produces biodiesel, fatty acid methyl esters (FAME), and glycerol as a co-product [10,11]. Biodiesel (B100) is marketed as a fuel, whereas glycerol can be used as an ingredient for making soaps, detergents, shampoos, and cosmetics. Vegetable oils such as sesame seed oil [12], palm oil [13], white mustard oil [14], and soybean oil [15] that consist of long chain of fatty acids have been all used for biodiesel production. In addition, waste biomass, microalgae, waste cooking oils, oily sludge, meat processing waste, and animal fat waste from slaughterhouses [16,17] are all gaining importance as inexpensive feedstock. Related information on these feedstocks is also helpful in the design of strategies for low-cost biodiesel production that can at the same time address waste disposal problems related to waste diversion, recycle and re-use [18]. The direct use of vegetable oils has been controversial because of the elevated viscosity [19,20]. For this reason, strategies have been designed to minimize the vegetable oil viscosity, such as dilution, microemulsion, pyrolysis, and transesterification [21].

The different routes for biodiesel production are homogeneous catalysis, heterogeneous catalysis, enzymatic, non-catalytic supercritical, hydro-esterification, microwave, and ultrasound. The most cost-efficient method for the production of biodiesel with higher quality is transesterification of vegetable oils and animal fats using chemical or enzymatic catalysts [22]. The enzymatic catalysis of biodiesel production offers some apparent advantages over the chemical method, which include: room-temperature reaction conditions, elimination of treatment costs associated with the recovery of chemical catalysts, enzyme reuse, high substrate specificity, the ability to convert both free fatty acids and triglycerides to biodiesel in one step, lower alcohol to oil ratio, avoidance of side reactions and minimized impurities, easier product separation and recovery; biodegradability and environmental acceptance [23–27].

In order to extend the viability of the enzymatic biodiesel process, major efforts have been recently made towards process improvement in terms of biodiesel efficiency, the impact of water and fatty acid content, adding value to glycerol co-product and bioreactor design [28]. Research has focused on optimizing process parameters that: (1) directly impact biodiesel production (e.g., oil to alcohol ratio, lipid content, need for treatment and purification, etc.); and (2) have the potential to reduce the number of unit operations further and, consequently, the overall cost of biodiesel production [29]. The success and efficiency of the enzymatically catalyzed biodiesel production are dependent on many factors, such as feedstock, pretreatment, enzyme properties, temperature, time, mixing speed, etc. [17,30] The focus of this review is on the choice of feedstock and enzyme catalyst as two of the major factors affecting the production of enzymatic biodiesel and its properties. Related producers, markets, challenges, and opportunities for biodiesel commercialization, including environmental considerations, are critically discussed.

2. Feedstock for Biodiesel Production

The feedstock and its availability as a raw material is a prerequisite for the successful establishment of a large-scale biodiesel production process [31]. While there has been considerable growth in the biodiesel industry, the feedstock availability has acted as a natural barrier that applied pressure on biodiesel producers and impeded the biodiesel scale up. Feedstock cost and supplies are a function of various factors, including currency strength, domestic and global production capacity for biofuels, energy costs, global demand for food, etc. It has been reported that oilseed crops are only capable of meeting a limited biodiesel demand [32]. This illustrates the need for more viable alternative biodiesel feedstocks such as low-cost waste and non-edible oils. In addition, oleaginous microorganisms such as
algae [33], yeast [34], and bacteria [35] are aggressively studied as promising feedstocks for biodiesel that can help reduce the feedstock shortage [36].

The total cost of producing biodiesel varies and depends on a number of factors, including production method, production scale, quality, and trade cost of raw materials used. Biodiesel production has to look after the investment and operating costs [37]. In terms of availability and cost, the feedstock is a major issue for biodiesel production. Currently, high-quality food-grade vegetable oils, such as soybean, rapeseed, palm, and groundnut oil, are utilized in biodiesel manufacturing. These feedstock expenses account for more than 60% of the overall biodiesel production costs [38]. Thus, to reduce the cost of biodiesel, many low-cost feedstocks and many alternative techniques need to be explored.

Depending upon the source and processing techniques used, the biodiesel feedstocks are grouped into first, second, third, and fourth generations. The first-generation feedstock implies the direct use of food crops, such as corn, soybean, sugarcane, etc., for biodiesel production [39]. The second-generation feedstock includes non-edible raw materials that are no longer suitable for human consumption. Examples include waste vegetable oils and animal fats. Third-generation feedstock comprises high oil yield producing microalgae [40]. Solar energy-based technology of photosynthetic water splitting as a photobiological solar fuel system represents the fourth-generation feedstock of the future. Table 1 presents examples of all four generation feedstocks and their fatty acid composition.

### Table 1. Biodiesel feedstocks and their fatty acid composition.

| Generation | Feedstock                  | Fatty Acid Composition                                                                 | References |
|------------|----------------------------|---------------------------------------------------------------------------------------|------------|
| 1st        | Rapeseed                   | Monounsaturated fatty acids (Oleic acid + Alpha Lipoic Acid + Linoleic acid)           | [41]       |
|            | Olive oil                  | Monounsaturated fatty acids (Oleic acid + Alpha Lipoic Acid + Linoleic acid)           | [41]       |
|            | Tea Seed (Camellia Sinensis) | Palmitic acid + Stearic acid + Oleic acid + Linoleic acid + Gadoleic acid              | [42]       |
|            | Groundnut                  | Monounsaturated fatty acids (Saturated fatty acids + Linoleic acid)                    | [41]       |
|            | Amaranth seeds             | Monounsaturated fatty acids                                                           | [43]       |
|            | Grapeseed                  | Polyunsaturated fatty acid (Linoleic acid)                                           | [41]       |
|            | Sesame                     | Polyunsaturated fatty acid (Linoleic acid + Monounsaturated fatty acids)              | [41]       |
|            | Sunflower oil              | Saturated fatty acids + Monounsaturated fatty acids + Polyunsaturated fatty acid       | [41]       |
|            | Okra (Hibiscus esculentus) seed | Behenic acid + Arachidic acid + Linoleic acid + Oleic acid + Stearic acid + Margaric acid + Palmitoleic acid + Palmitic acid + Myristic acid | [44]       |
|            | Depot margarine            | Rapeseed Palmitic acid + Stearic + Oleic + Linoleic + Linolenic                       | [45]       |
|            |                            | Polyunsaturated fatty acid + saturated fatty acid                                     | [46]       |
Table 1. Cont.

| Generation | Feedstock | Fatty Acid Composition | References |
|------------|-----------|------------------------|------------|
| 2nd Plants | Jatropha tree (*Jatropha curcas*) | Palmitic acid + Oleic acid + Alpha lipoic Acid | [47] |
|            | Karanja (*Pongamia pinnata*) | Oleic acid + Linoleic acid + Palmitic acid + Stearic acid | [48] |
|            | Mahua (*Madhuca indica*) | Oleic acid + Palmitic acid + Stearic acid + Lipoic Acid + Adipic acid | [49] |
|            | Castor bean seed (*Ricinus communis*) | Palmitic acid + Oleic acid + Pentanoic acid + Octanoic acid + Ricinoleic acid | [50] |
|            | Neem (*Azadiractha indica*) | Lipoic + Oleic acid + Oleic acid + Palmitic acid + Arachidic acid + Behenic acid + Lignoceric acid + Palmitoleic acid | [51] |
|            | *Salicornia begelovii* (dwarf saltwort) seed | Linoleic acid + palmitic acid + oleic acid + stearic acid + Linolenic acid | [52] |
|            | Nagchampa tree | Linoleic acid + Oleic acid + Stearic acid + Palmitic acid | [53] |
|            | Rubber seed tree (*Hevea brasiliensis*) | Oleic acid + Linoleic acid + Linolenic acid | [54] |
|            | Tobacco seed (*Nicotiana tabacum*) | Palmitic acid + Oleic acid | [55] |
|            | Meadowfoam (*Limnanthes alba L.*) seed | Eicosenoic acid + Docosadienoic acid + Erucic acid | [56] |
| Waste oil  | (Bakery) Depot margarine | Saturated fatty acid + Monounsaturated fatty acids | [46] |
|            | Sunfoil (triple refined sunflower oil from Restaurant) | Saturated fatty acid + Monounsaturated fatty acids | [46] |
|            | Frying oil | Palmitic acid + Stearic acid + Oleic acid + Linoleic acid + Linolenic acid | [57] |
|            | Waste activated sludge | Palmitic acid + heptadecanoic acid + ginkgoid acid + stearic acid + oleic acid + linoleic acid | [58] |
| Animal oil/fat | Pork Lard | Myristic + palmitic + palmitoleic + stearic + oleic + linoleic + linolenic + Arachidonic + docosapentaenoic | [59] |
|            | Beef Tallow | Myristic + palmitic + palmitoleic + stearic + oleic + linoleic + linolenic | [60] |
|            | Animal fat | Myristic + palmitic + palmitoleic + stearic + oleic + linoleic + linolenic | [61] |
|            | Poultry Fat | Myristic + palmitic + palmitoleic + stearic + oleic + linoleic + linolenic + Arachidonic + docosapentaenoic + docosahexaenoic | [62] |
|            | Tallow | Palmitoleic + oleic + stearic + palmitic + myristic | [63] |
|            | Meat Processing Waste | Myristic + palmitic + palmitoleic + stearic + oleic + linoleic + linolenic + eicosadienoic + saturated fatty acids | [64] |
| 3rd        | Cyanobacteria (*Fremyella diplosiphon*) | Methyl palmitate + hexadecanoic acid + methyl dodecanoate + methyl myristate + hexadecenoate + octadecanoate + octadecenoate + octadecadienoate | [35] |
|            | Algae | Palmitic + stearic + oleic + linoleic | [45] |
| Generation | Feedstock | Fatty Acid Composition | References |
|------------|-----------|------------------------|------------|
| 4th        | Dunaliella tertiolecta | - | [65] |
| Marine alga Nannochloropsis oceanica + oleaginous fungus Mortierella elongata | - | [66] |
| Phaeodactylum tricornutum | Saturated fatty acid + monounsaturated fatty acids + polyunsaturated fatty acid | [67] |
| Phaeodactylum tricornutum Pt4 | Co-expression of a yeast diacylglycerolacyl-transferase (ScDGA1) and a plant oleosin (At-OLEO3) | - | [68] |
| Chlamydomonas reinhardtii with overexpressing a Dof-type transcription factor | - | [69] |

Bio-based feedstocks, including oils (vegetable, algal, microbial) and animal fats, are considered a potent and promising renewable source for biodiesel production. Depending on the feedstock composition, biodiesel with different degrees of purity and properties can be produced [27]. The feedstock selection, therefore, has a direct impact on the choice of the bioprocessing method used, the biodiesel yield, and the cost.

The biodiesel feedstocks are categorized into edible, non-edible, and waste-based [19]. The feedstock availability and selection for biodiesel production are country and region-specific [70]. For instance, biodiesel production in Canada is based on canola oil, whereas soybean meal is the feedstock of choice in the USA and Brazil. European countries (Italy, Finland, Germany, and UK) predominantly use rapeseed oil as biodiesel feedstock. The abundance of coconut and palm oil dictates the biodiesel production from these feedstocks in Indonesia and Malaysia, respectively [15,71,72]. In India, karanja and jatropha oils are the main biodiesel feedstock [73,74]. The growth of non-edible feedstocks for biodiesel production is gaining momentum. China, for instance, has recently set aside a region just to grow jatropha and other non-food oil plants, whereas India has around 60 million hectares of non-cultivated land, which may be used for jatropha production [75].

2.1. First Generation Feedstock

Among the edible vegetable oils, sunflower, rapeseed, soybean, and mustard oil are all first-generation feedstocks. However, due to food security issues, biodiesel commercialization from edible oils is a challenging one [76]. Small-scale biodiesel production at a farm level utilizes first-generation feedstocks, such as coconut [77], palm tree [78], soybean [79], and sunflower [80]. Some added advantages of first-generation feedstocks are their abundance, availability, biodegradability, easy production in available infrastructure, and technology [81]. However, the major constraints in the production of first-generation biodiesel are the feedstock price and availability, limited production capacity, and competition with food production.

2.2. Second Generation Feedstock

The application of second-generation feedstocks from non-edible oil sources (waste cooking oil, tallow oil, animal fats, fish oil, etc.) avoids the ongoing food vs. fuel conflict, with no direct effect on the food supply chain and added benefits of biodegradability, low sulfur and aromatic content [82,83]. Although the second-generation feedstocks are mainly low-value non-edible oils, their processing to biodiesel may add extra cost and time. Non-edible feedstocks, also called energy crops, include: jatropha [84–87], jojoba [88], tobacco seed [89], salmon oil [90], and sea mango [91]. Biodiesel generated through energy
crops is a clean alternative fuel to petrodiesel; however, the supply of this feedstock in large quantities is unfortunately not sustainable [92]. Additionally, the cooking waste oils, restaurant grease, and animal fats [93], such as beef tallow and pork lard [94], are also considered second-generation feedstocks. Non-edible oils crops could be grown on wasteland for maximum utilization of the country’s resources. Because of limited availability, however, the second-generation feedstocks cannot fulfill the transportation fuel demand alone. Moreover, biodiesel generated from non-edible vegetable oils and animal fats showed poor engine performance in cold environments due to their high concentration of saturated FAME that is known to have inferior cold flow properties, namely cloud and pour point [95]. For example, the undesirable high melting point and high viscosity of beef tallow (a second-generation feedstock) are due to the presence of nearly 50% saturated fatty acids from the total amount of fatty acid in beef tallow [94]. The cold flow of properties of biodiesel containing high levels of saturated FAME can be improved through winterization, adding fuel additives or branched branched-chain esters, blending with petrodiesel or vegetable oils of lower crystallization temperature, etc. [96]. These additional processing steps, however, render the production economics of biodiesel less attractive, with a low to moderate commercialization potential. Therefore, due to efficiency and sustainability issues, the large-scale biodiesel production from first- and second-generation feedstocks has faced a major hurdle of high biodiesel cost [97,98]. A recent development of a second-generation feedstock is fat, oil, and grease (FOG) that is recovered from wastewater discharges from restaurants, kitchens, food processing plants, and slaughterhouses [99]. FOG is a potential biodiesel feedstock due to its lower price, better oxidative stability, flash point, cetane number, and total emissions compared to other feedstocks, and reduced carbon footprint that is realized through wastewater management [100].

2.3. Third Generation Feedstock

The third-generation feedstock is of microbial origin and includes oleaginous microalgae, bacteria, fungi, and yeast. The cultivation and production of these oil-generating microorganisms require no land or special growth supplements [101]. The third-generation feedstocks have higher biomass productivity than the conventional crops, which is due to their high photosynthesis efficiency, especially in the photosynthetic microbes [102]. In addition, the oleaginous microbes present a new biodiesel feedstock that overcomes the challenges of availability, adaptability to climatic conditions, and controversy of the food supply chain that are typically faced by the first and second-generation feedstocks [103].

Lipids from microalgal origin appear as the most promising third-generation biodiesel feedstock. Microalgae have very high growth rates and accumulate high levels of lipids (oil) through photosynthesis [104,105]. Algal cultivation is easier than plant cultivation. Cultivated algae show fast multiplication [65]. High oil yielding algae can reach up to 70% oil w/w dry biomass with an annual oil production of 136,900 L/ha and biodiesel productivity of 121,104 kg biodiesel/ha [106]. Algal oil-based biodiesel production could become feasible at a large scale provided a continuous supply of feedstock is in place. Hence, algae cultivation or algal farming need to be further explored in both rural and urban areas to ensure feedstock sustainability. This approach could have an added benefit of creating employment opportunities, thereby strengthening the agricultural sector. More research is also needed to address the inherent drawbacks of algal biodiesel associated with its instability and degradability at higher temperatures [107].

2.4. Fourth Generation Feedstocks

Considering the feedstock cost for biodiesel development, it is desirable to research and exploit opportunities to develop a very inexpensive, high-energy density feedstock that is easily accessible and of unlimited availability. Solar energy-based technology of photosynthetic water splitting into energy constituents holds promise to become a future biodiesel feedstock [108]. This feedstock could be integrated with a second and or third-generation
feedstock for a high photon to fuel conversion efficiency (PFCE) using a synthetic biological approach. Thus, the future photobiological solar fuel system will be utilized for the production of high-quality, high-yield, high-efficiency, and high-performance biodiesel. In general, microorganisms can be cultivated in a photobioreactor for a solar-aided fuel conversion in a two-step process that involves microbial biomass production followed by photo-bio-solar fuel production by engineered and immobilized algae or cyanobacteria (Table 1). Current research has focused on achieving a 10% PFCE that requires prior organism and bioreactor design and construction. Further enhancement of the PFCE may be possible through developing microbial electrosynthesis technologies that utilize electro-biofuel/synthetic-cell hybrid systems as the fourth-generation feedstock [109].

3. Lipases for Biodiesel Production

Applications of conventional techniques such as micro-emulsification, pyrolysis, and transesterification with alkaline/acid catalysts are well developed for the processing of biodiesel [21]. While these procedures are widespread and well investigated, there are some pitfalls and technological difficulties that need to be addressed and overcome, such as catalyst recovery, purity of the glycerol co-product, high energy consumption, significant volumes of wastewater that require treatment, etc. The development and implementation of the lipase enzymatic path have greatly improved the efficiency of biodiesel processing, thus decreasing the size of the process equipment to the microlevel [110]. Lipases are hydrolytic enzymes that catalyze the production of free fatty acids, diacylglycerols, monoaerylglycerols, and glycerol by cleaving off the ester bonds of triacylglycerols (TAG) from fats and oils [111]. As lipases possess an unusual trait of hydrolysis over oil-water interfaces, they can also trans-esterify triacylglycerols to fatty acid alkyl esters in the presence of short-chain alcohols, such as methanol and ethanol [112]. Biodiesel production is one of the most significant and spectacular applications of lipases [113]. However, as lipases are ubiquitous in nature, they could find applications in various industries [114]. Their ubiquity is evident from Table 2, which shows that lipases are produced in plants and microorganisms, including bacteria, fungi, and yeasts.

| Source | Species | Habitat | Reference |
|--------|---------|---------|-----------|
| Plants | Triticum aestivum L. | - | [115] |
| | Pachira aquatica | Seed of Tree, UNESP, Brazil | [116] |
| | Coconut (Cocos nucifera linn) seed | NIFOR substation Abak, Akwa Ibom State of Nigeria | [117] |
| | Castor Beans | Chiltemn Seeds (Ulverston, Cumbria, UK) | [118] |
| | Bay Laurel (Laurus nobilis L.) Seeds | Hatay, Turkey | [119] |
| | French bean (Phaseolus vulgaris L.) | Landraces of Himachal Pradesh, India | [120] |
| | Nigella sativa L. Seed | Denizli region of Turkey | [121] |
| | Brassica napus L. | Bangladesh Agriculture Research Institute, Irshardi, Pabna and Rajshahi Local Shaheb Bazar Market. | [122] |
| | Rice Bran | Bangalore, India | [123] |
| | Jatropha curcas L. | Isiuwa quarters of the Nigerian Institute for Oil Palm Research, Benin City, Nigeria | [124] |
| | Lupine seeds | Poland | [125] |
### Table 2. Cont.

| Source     | Species                          | Habitat                                      | Reference  |
|------------|----------------------------------|----------------------------------------------|------------|
|            | **Bacteria**                     |                                              |            |
|            | *Streptomyces* sp. Al-Dhabi-49   | Soil, Saudi Arabia                           | [126]      |
|            | *Chryseobacterium polytrichastri*| ERMR1:04 Glacier, Sikkim Himalaya            | [127,128]  |
|            | Seeds of African oil bean        | NIFOR, Benin City                            | [129]      |
|            | *(Pentaclethra macrophylla Benth)*|                                              |            |
|            | *Chryseobacterium* sp. strain IHBB10212 | Glacier top-surface soil, Himalaya, India | [130]      |
|            | *Bacillus cereus* HSS            | Mediterranean Sea, Eastern Harbor, Al Shatby, and Abu-Qir | [131]      |
|            | *Thalassospira permensis* M35-15 | Sea water and sediments samples             | [132]      |
|            | *Bacillus subtilis* strain Kakrayal_1 | Katra region of Jammu and Kashmir, India | [133]      |
|            | *Geobacillus thermoleovorans* DA2 | Desert, Southern Sinai                       | [134]      |
|            | *Pelosinus fermentans*           | Groundwater, Germany                         | [135]      |
|            | *Micrococcus luteus*             | Agriculture field and garden                 | [136]      |
|            | *Bacillus aerius*                | Soil and water of hot spring, Shimla         | [137]      |
|            | *Ralstonia species*              | Soil sample, Germany                         | [138]      |
|            | *Trichoderma harzianum*          | Soil sample, Turkey                          | [139]      |
|            | *Acinetobacter baylyi*           | Marine sludge, Thailand                      | [140]      |
|            | *Serratia marcescens*            | Raw milk                                     | [141]      |
|            | *Aspergillus fumigatus*          | Oil contaminated soil, HRTC workshop, Himachal Pradesh | [142]      |
|            | *Trichoderma reesei* strain RF10625 | Fungal Biodiversity Institute, The Netherlands | [143]      |
|            | *Aspergillus niger* (strain LFS) | DSM Food Specialties B.V.                   | [143]      |
|            | *Geotrichum* sp.                 | UNICAMP, Brazil                              | [144]      |
|            | *Cunninghamellamella verticillata*| Oil-mill waste                               | [145]      |
|            | *Aspergillus niger*              | Dept of Biochemistry and Microbiology, University of Plovdiv, Bulgaria | [146]      |
|            | *Rhizopus chinensis*             | (CCTCC) China Center for Type Culture Collection | [147]      |
|            | *Penicillium simplicissimum*     | Waste from the babassu oil industry          | [148]      |
|            | *Limtongella siamensis*          | Grease traps, Kasetsart University, Thailand | [149]      |
|            | *Yarrowia lipolytica*            | Marine oil-contaminated sludge               | [150]      |
|            | *Candida rugosa*                 | Sigma-Aldrich Co. (Germany)                  | [151]      |

3.1. Lipase Activity and Specificity

As lipases are obtained from a variety of biological sources (Table 3), they possess diverse substrate and catalytic specificity [152]. The selection of lipase for a specific application must be carried out on the basis of enzyme specificity and stability in various solvents [153]. According to their substrate specificity, lipases are divided into three groups: sn-1,3-specific lipases (hydrolyze ester bonds in the sn-1 and sn-3 position in TAG); sn-2-specific lipases (lipases preferentially cleave acyl chains in the sn-1 and sn-3 position in TAG); and nonspecific lipases [154,155]. Lipases with a sn-1,3-regioselectivity are most common, while the sn-2 fatty acids in TAG are less accessible to lipases due to steric hindrances [154].
Table 3. Microbial production of lipase on different substrates.

| Lipase Producer                | Lipase Activity | Substrate                | Reference |
|-------------------------------|-----------------|--------------------------|-----------|
|  *Pseudomonas* sp. LSK25      | 50.5 U/mL       | Rice bran oil            | [156]     |
|                               |                 | Coconut oil              |           |
| *Antarctic Pseudomonas* sp.   | 130.7 U/mL      | Olive oil                | [157]     |
|  *Candida viswanathii*        | 101.1 U/mL      | Olive oil                | [158]     |
| *Pseudomonas* sp. LSK25       | 0.35 to 0.4 U/mL| Olive oil                | [156]     |
| *Aspergillus terreus* NCFT 4269.10 | 475 U/mL       | Sunflower oil            | [159]     |
| *Bacillus amyloliquefaciens* PS35 | 361 mU/ml      | Palm oil                 | [160]     |
| *Pseudomonas* fluorescens Strain AMS8 | 226.69 U/mL  | Olive oil                | [161]     |
| *Pseudomonas aeuriginosa*     | 528.54 U/L      | Olive oil                | [162]     |
|                               | 422.0 U/mL      | Jojoba oil               |           |
|                               | 92.8 U/mL       | Corn oil                 |           |
| *Penicillium camembertii* Thom PG-3 | 128.0 U/mL  | Soybean oil              | [163]     |
|                               | 146.5 U/mL      | Rape seed oil            |           |
|                               | 180.0 U/mL      | Linseed oil              |           |
| *Colletotrichum* gloesporioides 41 | 18.8 U/mL     | Olive oil emulsion       | [164]     |

3.2. Lipase Thermostability and Half-Life

The enzyme thermostability is an important characteristic that determines the enzyme potential and robustness for industrial applications. Hence, biotechnological research has been focused on the development of thermostable enzymes by 1) genetic engineering, protein engineering, and strain mutation to improve enzyme stability; and 2) biosurveying and discovery of new thermophilic organisms capable of producing unique thermostable enzymes [165]. For example, a recent study reported the isolation of a novel thermo-halophilic bacteria from a hot spring area in Indonesia. This strain produced a thermostable lipase with a temperature optimum of 70 °C, which was able to catalyze both hydrolysis and transesterification reactions [166].

The half-life of any chemical reaction is defined as the time elapsed to reach half (50%) of the initial reactant concentration [167]. The half-life of select lipases and their temperature optima are displayed in Table 4. Strain mutation has been used as a strategy to alter both the half-life and thermostability of produced lipases [58,165,168]. Protein engineering of the enzyme structure through introducing a salt bridge in the enzyme macromolecule significantly increased the thermostability of a *Stenotrophomonas maltophilia* lipase from 40 °C to 90 °C, with an additional improvement in half-life [165]. Recombinant DNA technology has been applied to enhance lipase production and reduce the overall cost [169].

Table 4. Optimum temperature and half-life of microbial lipases.

| Source            | Strain   | Optimum Temperature | Half-Life (t½, min) | References |
|-------------------|----------|---------------------|---------------------|------------|
| *Geobacillus zalihae* | D43E     | 70 °C               | 135                 | [168]      |
|                   | T118N    |                     | 75                  |            |
|                   | E226D    |                     | 165                 |            |
|                   | N304E    |                     | 120                 |            |
| *Rhizopus chinensis* | r27RCL   | 60 °C               | 0.85                | [170]      |
|                   | m28      |                     | 6.5                 |            |
|                   | m26      |                     | 4.5                 |            |
|                   | m28      |                     | 6.5                 |            |
|                   | m29      |                     | 12.3                |            |
3.3. Lipase Reusability

The harsh conditions of industrial processes require the use of robust enzymes that retain activity over the entire duration of the enzymatic catalysis. Enzyme immobilization on a solid support has been shown to improve the enzyme resistance to denaturation by alcohol and promote enzyme reusability [112,179]. Immobilized enzymes have several advantages over free enzymes, which include prolonged enzyme-substrate contact, enzyme recycling, improved process control, and more efficient product recovery [23].

Enzyme immobilization has revolutionized biocatalysis as it enabled the development and establishment of more cost-efficient biotechnologies that offer higher quality bioproducts. Immobilization methods include physical adsorption, covalent binding, encapsulation, and bio-selective adsorption [179,180]. In one study, a lipase enzyme isolated from Arachis hypogaea seeds was immobilized with Ca-alginate and agarose gel, which significantly improved enzyme stability [112]. Another study reported on the immobilization by physical adsorption and entrapment of commercial lipases (Pseudomonas fluoresces AKL, Pseudomonas cepacia PSL, Hog pancreas PHL, Porcine pancreas PPL, and Mucor javanicus MJL) on polyhydroxybutyrate (PHB), sodium alginate and chitosan [179]. Lipase from the marine yeast Yarrowia lipolytica was immobilized on microporous resin, which improved lipase activity over the free enzyme and allowed enzyme re-use [181]. The use of nanotechnology (carbon nanotubes, metal-based nanoparticles, etc.) has become another groundbreaking technology for lipase immobilization [176,182].

3.4. Lipase-Catalyzed Biodiesel Production

As can be seen from Table 5, the yield of biodiesel produced by means of lipases varies depending on the lipase source, substrate source, lipase activity, and reaction parameters, such as temperature, time, and alcohol to substrate ratio. Clearly, in each case, optimization
of the reaction conditions is necessary. Overall, fungi and yeasts have shown strong abilities to produce lipase (Table 5). Bacterial lipases used for biodiesel production have been sourced from *Chromobacterium viscosum*, *Burkholderia cepacia*, *Enterobacter aerogenes*, *Thermomyces lanuginosa*, *Pseudomonas fluorescens*, etc. [183].

**Table 5.** Lipase reaction parameters and biodiesel yield.

| Lipase Feedstock | Lipase Source | Lipase Name | Lipase Conc. (%) | Temp. (°C) | Time (h) | Molar Ratio (Alcohol/Oil) | Biodiesel Yield (%) | Reference |
|------------------|---------------|-------------|------------------|------------|----------|--------------------------|---------------------|-----------|
| Marine microalga Nanochloropsis | Candida antarctica | Candida antarctica lipase A (CALA) | 10 | 35 | 72 | 8:1 | 40.8 | [184] |
| Microalga Chlorella vulgaris | Candida antarctica | Lipase B (Novozyme 435) | 40 | 40 | 72 | 13:1 | 66.7 | [185] |
| Waste sardine oil | Aspergillus niger | Lipase | 10 | 30 | 72 | 9:1 | 94.5 | [186] |
| Kernel oil | Thermomyces lanuginosus | Lipozyme TL IM | 0.25 | 45 | 4.03 | 1.50 | 83.9 | [187] |
| Chinese Tallow Kernel oil | Burkholderia cepacia | PS lipase | 20 | 40 | 24 | 4:1 | 55.2 | [188] |
| Soapstock from rice bran oil | Candida antarctica | Novozyme 435 | 10 | 40 | 24 | 5:1 | 93.0 | [189] |
| Soapstock from rice bran oil | Thermomyces lanuginosus | Lipozyme TL IM | 10 | 30 | 24 | 5:1 | 88.0 | [189] |
| Palm oil fatty acid distillate (PFAD) | Candida antarctica | Novozyme 435 | 1 | 60 | 2.5 | 3:1 | 93.0 | [190] |
| Tung oil | Rhizopus oryzae | Chimeric lipase | 13 | 40 | 48 | 3.88 | 91.9 | [191] |
| Jatropha oil | Enterobacter aerogenes | Lipase | - * | 55 | 48 | 4:1 | 94.0 | [192] |
| Waste tallow | Candida antarctica | Lipase B (CALB) Candida antarctica lipase B | 1.25 | 50 | 24 | 30:1 | 99.0 | [193] |
| Nanochloropsis oculata microalga | Bacillus sp. S23 | Lipase | 1.5 | 35 | 60 | 12:1 | 95.7 | [194] |
| Beef tallow | Burkholderia cepacia | Immobilized lipase | 20 | 50 | 48 | 12:1 | 89.7 | [195] |
| Animal fat | Candida antarctica | Immobilized lipase | 10 | 40 | 6 | 50:6 | 79.0 | [196] |
| Lard | Candida sp. | Lipase | 20 | 40 | 30 | 3:1 | 87.4 | [197] |
| Lard | Candida antarctica | Lipase | 10 | 30 | 72 | 1:1 | 74.0 | [198] |
| Lard | Candida antarctica | Lipase | 2–6 | 50 | 20 | 5:1 | 97.2 | [199] |
| Used cottonseed oil | Pseudomonas sp. | Lipase | 30 | 37 | 48 | 6:1 | 70.0 | [200] |
| Palm oil | Rhodotorula mucilagenosa P 11B9 | Lipase | 0.5 | 30 | 72 | 3:1 | 51.3 | [201] |
| Palm oil | Aspergillus niger | Lipase | 2–3 | 25 | 72 | 3:1 | 87.0 | [202] |
| Palm oil | Aspergillus niger | Lipase | 2–3 | 40 | 72 | 3:1 | 69.0 | [202] |
| Used cooking oil | Rhizopus oryzae PTCC 5174 | Lipase | 15.5 | 35 | 72 | 3:1 | 98.0 | [203] |
| Soybean oil | Rhizopus oryzae | Lipase | 5 | 35 | 72 | 3:1 | 89–92 | [204] |

* 50 U of immobilized lipase/g.
4. Lipase and Biodiesel Markets

4.1. Lipase Market

According to the Fior Market survey, the global lipase market is projected to register a CAGR of 8.8% during the forecast period 2020–2025 and reach USD 961.85 million by 2028 [205]. Due to the diverse origin of lipases, their properties, and their abilities to catalyze different biotechnical reactions, the market for lipases is expanding beyond the food and biofuels industry to include animal feed, pharmaceutical, detergent, cosmetic, pulp and paper, leather, and textile industries [206]. The use of lipase in biology and electronics, biosensors, and nanotechnology is also on the rise. The global lipase market is segmented into animal lipases and microbial lipases. In 2020, the microbial and animal feed lipases segments held the largest market share of 61.64% and 26.6%, respectively. The key players in the global lipase market are Amano Enzymes Inc. (Elgin, IL, USA), Advanced Enzymes (Thane, Maharashtra, India), Clerici-Sacco Group (Cadorago, Como, Italy), Chr. Hansen Holdings A/S (Boege Alle, Hoersholm, Denmark), Enzyme Development Corporation (New York, NY, USA), E. I. Du Pont De Nemours (Wilmington, DE, USA), Genencor (Rochester, NY, USA), Novozymes A/S (Franklinton, NC, USA), Koninklijke DSM N.V. (Heerlen, Limburg, The Netherlands), and Renco New Zealand (Eltham, Taranaki, New Zealand) [207]. The major limitations for the wider, large-scale use of lipases have been their high cost and the lack of transparency in the laws and regulations related to lipase patents around the world.

4.2. Biodiesel Market

In 2016, the size of the biodiesel market was USD 34.1 billion and should reach USD 41.2 billion by 2021 at a 3.8% CAGR. However, over the period 2021–2028, the global biodiesel market is forecasted to grow at a higher CAGR of 5.25%. The biodiesel demand is expected to exceed 41.4 billion liters by 2025 [208]. The biodiesel market is driven by the increasing demand for environmentally safe fuels that reduce GHG emissions. Other major growth-inducing factors are the thriving automotive industry, the surging prices of fossil fuels, the recent advancements and emergence of the third-generation biodiesel from algae, and the implementation of favorable government policies to promote biodiesel usage [209]. Chemical biodiesel is manufactured globally by several major producers, such as Archer Daniels Midland Company (Chicago, IL, USA), Wilmar International Limited (Chinatown, Singapore), Bunge Limited (Chesterfield, MO, USA), Neste Corporation (Espoo, Finland), Renewable Energy Group Inc. (Ames, IA, USA), Louis Dreyfus Company (Rotterdam, The Netherlands), Cargill Inc. (Wayzata, MN, USA), BIOX Corporation (Hamilton, ON, Canada), Munzer Bioindustrie (Vienna, Austria), and Emami Group (Kolkata, West Bengal, India).

Although the enzymatic routes for biodiesel production have been intensively investigated, the large-scale production of enzymatic biodiesel is currently limited (Table 6). The U.S. has several commercial-scale production facilities; however, the largest single plant (Aemetis Biorefinery, Inc., Cupertino, CA, USA) for enzymatic biodiesel production of 50 million gallons per year has been built in India. International collaborative efforts and strategic partnerships between biodiesel manufacturers and lipase-producing companies may accelerate the progress towards the development of commercially viable technologies for enzymatic biodiesel.
Table 6. Commercial production of enzymatic biodiesel: lipase source, feedstock, and annual production of biodiesel.

| Country   | Company Name                          | Lipase Source                                      | Main Feedstock                                | Annual Production          | Reference |
|-----------|---------------------------------------|----------------------------------------------------|-----------------------------------------------|---------------------------|-----------|
| USA       | Viesel Fuel LLC                        | Eversa Transform\(^\text{®}\) from A. oryzae       | Waste cooking oil, brown grease               | 11 million gallons        | [210]     |
| USA       | SRS International Co.                  | Immobilized lipase                                 | Used restaurant oil                          | 5 million gallons         | [211]     |
| USA       | Buster Biofuels                        | Callera\(^\text{®}\) Trans L lipase from Thermomyces lanuginosus | Brown grease, fish oil                       | 5 million gallons         | [212]     |
| USA       | Blue Sun Energy                        | Callera\(^\text{®}\) Trans L lipase from Thermomyces lanuginosus | Used cooking oil, palm fatty acid distillate, corn oil | 30 million gallons        | [213]     |
| Israel    | TransBiodiesel Ltd.                    | TransZyme A                                        | Used cooking oil, animal fat, acid oil, brown grease | 50,000 tons               | [214]     |
| Israel    | EnzymeCore                             | TransZyme A                                        | Low-cost oils and fats with high free fatty acid and polar lipid content | 1500 tons                 | [215]     |
| South Korea | M-Energy                          | TransZyme A                                        | Brown grease extracted from grease trap, fat, oil, grease | 30,000 tons               | [216]     |
| India     | Aemetis Biorefinery, Inc.              | -                                                  | Brown grease, low grade used cooking oils, palm fatty acid distillate and other plant oil waste feedstocks | 50 million gallons        | [217]     |
| China     | Lyming and Environmental Protection Technology Co. Ltd. | Candida sp. 99–125 lipase                          | Waste cooking oil                            | 10,000 tons               | [218]     |
| China     | Hunan Rivers Bioengineering Co. Ltd.  | Immobilized Novozym 435\(^\text{®}\) (lipase B from Candida antarctica) | Beef tallow, soybean oil                     | 20,000 tons               | [218]     |
| Brazil    | Olfar                                  | Immobilized Callera\(^\text{®}\) Trans L lipase from Thermomyces lanuginosus | Recovered vegetable oil, animal fat, soybean oil | 378 million liters        | [219]     |

As evident from Table 6, the predominant raw materials for the commercial production of enzymatic biodiesel are waste cooking oil and brown grease, both second-generation feedstock. Waste cooking/frying oils have high FFA content (typically 20–60% w/w) as heat and water are known to accelerate the hydrolysis of TAG to FFA. On the other hand, brown grease is categorized as grease that contains above 15% w/w FFA. The alkali-catalyzed transesterification reaction of waste cooking oil and brown grease results in soap formation, which consumes the alkali catalyst, creates difficulties in the downstream recovery of biodiesel and diminishes the biodiesel yield. Pretreatment with sulfuric acid in the presence of methanol is required to esterify the FFA and reduce their content to
below 1% (w/w) before the alkaline transesterification of TAG to FAME can proceed. The two-step acid-base process adds to the production cost and is considered as one of the major drawbacks of chemical biodiesel [16]. Lipases can trans/esterify both TAG and FFA in one step, hence their use on a large scale. Table 6 also suggests that the commercial process makes use of commercial lipases as the preferred biocatalyst in both free (liquid) and immobilized (solid) states.

5. Concluding Remarks

Biodiesel is a renewable, biodegradable, non-flammable, non-toxic biofuel that has the potential to minimize the use of petrodiesel and strengthen energy security and socio-economic development while reducing the environmental impact. To reach its full potential as the energy source of choice, several techno-economic and social challenges need to be overcome.

The main challenge in enzymatic biodiesel production is its high cost. Lipases are still expensive, and the enzyme-catalyzed process requires more time to complete than the alkali-catalyzed process. If not optimized, lipases may be inhibited by methanol, which results in diminished biodiesel yields. The cost-efficiency of biodiesel can be improved using immobilized enzymes that are recovered and recycled. As the glycerol co-product in biodiesel production remains underutilized, the development of viable technologies for glycerol conversion to value-added products will further strengthen the production economics of biodiesel. The ability of lipase to catalyze biodiesel production from low-cost feedstock with a high free fatty acid content, such as waste cooking oil, grease, and tallow, can also lower the cost of enzymatic biodiesel. Furthermore, the discovery and engineering of new and robust lipases with high activity, thermostability, and resistance to inhibition will accelerate the progress towards the establishment of a cost-effective enzymatic process [23]. Another challenge is the poor cold flow properties of biodiesel in terms of cloud point, pour point, and cold filter plugging point [83,220–223]. The presence of higher amounts of FAME in biodiesel than petrodiesel further aggravates this problem.

In addition, the water content of oily feedstocks may need adjustment to ensure optimal transesterification conditions for lipase catalysis. A further drawback is the low oxidation stability of biodiesel (both enzyme- and alkali-catalyzed biodiesel) that is caused by the presence of polyunsaturated FAME [224]. In addition, the viscosity of biodiesel is 11–17 times higher than petrodiesel, which can make pumping, combustion, and atomization more difficult [225]. Biodiesel combustion may lead to sludge buildup in the injectors and engine heads, and compression ignition engines can wear excessively [226]. The use of fuel additives can improve the oxidation stability, engine, and storage life of biodiesel, and the performance of biodiesel-diesel blends in cold climates [227,228]. However, there is a paucity of studies on the effects of antioxidants on the lubricity of biodiesel while patents regarding methods for improving the cold flow properties of biodiesel remain scarce [229,230]. For this reason, the biodiesel’s physical-chemical properties need to be refined to increase compatibility with compression ignition engines.

A major disadvantage of the first-generation biodiesel is that it is produced from edible oils derived from food crops, hence the concerns about increases in food prices in the global market and the ongoing food vs. fuel debate. While the second-generation biodiesel avoids the use of food crops, it still requires the cultivation of non-edible oil plants, which may lead to competition with food crops for arable land [231]. In addition to the above challenges, the expansion of the biodiesel industry may have direct or indirect negative environmental impacts. For example, to maintain a sufficient feedstock supply, more land may be required to cultivate oil crops, which could lead to deforestation. Rainforests are the largest carbon sinks in the world, and their removal will inevitably release enormous amounts of carbon into the atmosphere. Furthermore, as water is extensively used for cooking, drinking, and irrigation, water-intensive biodiesel production can considerably strain water resources and increase pressures on water supplies [232].
Farmers, researchers, feedstock providers, transporter, investors, and youth are expected to benefit from the emerging biodiesel market. Many policymakers favor biodiesel for its potential benefits, including increased domestic energy stability, lower GHG emissions, economic growth, and job creation, especially in rural areas. Subsidies, grants, campaigns, and biodiesel mandates in national policies can be implemented to facilitate the development of first-generation biodiesel. A study of the socio-economic status in Thailand related to the biodiesel market reported that more workers are needed for the biodiesel than the petrodiesel industry, which will create more employment by the end of 2022 [233]. Likewise, India currently generates around 70 billion liters of wastewater per day, and opportunities exist to upgrade the existing sewage treatment plants for simultaneous production of biogas and biodiesel. This may add an additional 700,000 jobs and allow the production of around 350 million liters of biodiesel. Various national and international organizations currently collaborate with the objective to demonstrate real-world opportunities of innovative biodiesel technology for the production and usage of high-mileage 100% biodiesel (B100) in vehicle applications. A variety of cooperation initiatives engage sustainable diesel supporters around the world to promote biodiesel and its awareness at the regional, national, and international levels. These include National Biodiesel Board (https://www.biodiesel.org, accessed on 20 August 2021), European Biodiesel Board (https://ebb-eu.org, accessed on 20 August 2021), Biodiesel Ambassadors (https://www.nbb.org/join-us/partnership-programs/biodiesel-ambassadors, accessed on 20 August 2021), Biodiesel Alliance (https://www.greennamerica.org/fuels-future/sustainable-biodiesel-alliance, accessed on 20 August 2021), Next Generation Scientists for Biodiesel, (https://biodieselssustainability.org/ngsb/, accessed on 20 August 2021), and others.

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