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Review

Biodiesel production through lipase catalyzed transesterification: An overview

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ABSTRACT

Recently, with the global shortage of fossil fuels, excessive increase in the price of crude oil and increased environmental concerns have resulted in the rapid growth in biodiesel production. The central reaction in the biodiesel production is the transesterification reaction which could be catalyzed either chemically or enzymatically. Enzymatic transesterification has certain advantages over the chemical catalysis of transesterification, as it is less energy intensive, allows easy recovery of glycerol and the transesterification of glycerides with high free fatty acid contents. Limitations of the enzyme catalyzed reactions include high cost of enzyme, low yield, high reaction time and the amount of water and organic solvents in the reaction mixture. Researchers have been trying to overcome these limitations in the enzyme catalyzed transesterification reaction. This paper is meant to review the latest development in the field of lipase catalyzed transesterification of biologically derived oil to produce biodiesel.

Contents

1. Introduction

The idea of biodiesel is not new and it has been demonstrated as early as 1900 in Paris exposition where the French Otto Company operated a small diesel engine on peanut oil [1], but it was not implemented due to the high viscosity and low volatility of vegetable oils. Recently, with the global shortage of fossil fuels, increase in the crude oil prices and environmental concerns to reduce pollution has resuscitated the interest in biodiesel production. The idea is to reduce the viscosity of the oil by replacing glycerol with methyl or ethyl alcohol through the transesterification reaction (Fig. 1). Catalysis of the transesterification reaction can be broadly classified into two categories—chemical and enzymatic. Chemically, transesterification reaction can be acid/base catalyzed. The mixture of oil with excess of ethanol when refluxed at 70 °C for 1 h gave ethyl esters of fatty acids with a yield of 93% [2]. Though the yield is high, the process has many disadvantages such as high energy consumption, difficulty in the transesterification of triglycerides with high free fatty acid content [2]. Moreover, the downstream processes such as the recovery of the glycerol, the removal of the inorganic salts and water from the product, and the treatment of alkaline wastewater are complex and incur extra cost [3,4].

In contrast, biocatalysts allow the synthesis of specific alkyl esters, easy recovery of the glycerol, and the transesterification of triglycerides with high free fatty acid content [2,5,6]. In this approach, lipase catalyzed transesterification is carried out in non-aqueous environments. Reaction mechanism and enzyme kinetics for transesterification have been reviewed by Fjerbaek et al. [7]. Kumari et al. [6] have optimized the conditions for the transesterification of oil from *Madhuca indica* having high free fatty acid content (about 20%). A high yield (99% in 2.5 h) using 50 mg of *Pseudomonas*

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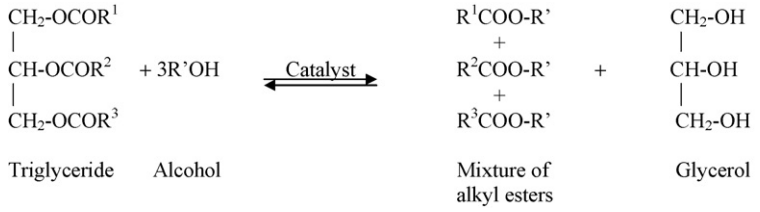

Fig. 1. Transesterification of triglyceride.

cepacia lipase in the form of protein coated microcrystals (PCMCs) has been reported. This is an added advantage when considering the use of low cost feedstock such as tallow [8] and recycled restaurant grease [8,9] having high free fatty acid content to improve the sustainability of biodiesel production.

Lipases are important enzymes in nature responsible for the turnover of lipids. They are also involved in the breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another [10]. They are classified under hydrolases EC 3.1.1.3. They have both hydrolytic as well as synthetic activity [11]. Much work has been done on the lipase catalyzed transesterification of triglycerides. Researchers across the globe have been trying to overcome the limitations of an enzyme catalyzed biodiesel production such as the high cost of enzyme, low yield, high reaction time, need of organic solvents and need of water in the reaction mixture. This paper is meant to review the latest developments in the field of lipase catalyzed transesterification of biologically derived oils to produce biodiesel.

2. Source of enzyme

Lipases from different sources have been investigated for their transesterification activity on different oils. These have been listed in Table 1. The most desired characteristics of the lipase are its ability to utilize all mono, di, and triglycerides as well as the free fatty acids in transesterification, low product inhibition, high activity and yield in non-aqueous media, low reaction time, reusability of immobilized enzyme, temperature and alcohol resistance.

Clearly, lipases from different sources have different properties suitable for the process. Thus, there has been a search for an ideal enzyme. Luo et al. [12] screened a psychrophilic lipase producing bacterium *Pseudomonas fluorescens* (strain B68) from soil samples of China. This psychrophilic lipase (lipB68) was immobilized on cellulose fabric tested for the transesterification activity in the production of biodiesel. A yield of 92% in 12 h with an optimum temperature of 20 °C has been reported [12]. A lower optimum temperature greatly reduces the energy requirement for the process for many geographical locations. Another study has explored the activity of marine lipase from *Bacillus pumilus* B106 which has high pH, salinity and temperature tolerance [13]. Lipases from other sources like *Penicillium* spp., *Rhizopus* spp. and *A. niger* have been shown to be economically produced by solid state fermentation [14]. Comparison of the economics of large-scale lipase production in submerged and solid state fermentation from *Penicillium restrictum* with babassu oil cake as substrate has been done. The capital investment for submerged fermentation was found to be 78% higher than the solid state fermentation process. Also the unitary product cost for solid state fermentation process was 47% lower than the product selling price in contrast to submerged fermentation process, where it is 68% higher than the product selling price [15]. Recently, Li et al. [16] have used immobilized *Penicillium expansum* lipase to catalyze the production of methyl esters from waste oil with high free fatty acid content. They reported a yield of 92.8% in 7 h using silica gel to control excess water activity. Efforts in our laboratory are on for the screening of efficient lipases in different microorganisms isolated from the natural environment.

3. Optimization of the process

Different strategies have been employed to improve the yield of the enzyme and to make the reaction time as short as possible. The optimum reaction conditions such as temperature, time duration and agitation speed vary greatly and have to be optimized with different combinations of the source of the lipase, the immobilization media, and alcohol used (Table 1). Recently, Rodrigues et al. [17] have studied the differential activity of three different commercial lipases (Novozyme 435, Lipozyme TL-IM and Lipozyme RM-IM) on three different sources of oil (soya bean, sunflower and rice bran) with four different short chain alcohols (methanol, ethanol, propanol and butanol) under different conditions of temperature and molar ratio of alcohol in the reaction. Shah et al. [2] optimized the conditions for the activity of immobilized lipase from *Chromobacterium viscosum* for the transesterification of jatropha oil with ethanol and obtained the yield up to 92%. Shimada et al. [18] used an alternative technique of stepwise addition of methanol. It has been shown that lipase from *Candida antarctica* was inactivated at more than 1.5 molar equivalents of methanol against oil. According to stoichiometry of the reaction, three molar equivalents of methanol are needed for the complete methanolysis of oil. Thus, one molar equivalent of methanol was added three times after 10, 14 and 24 h, respectively to obtain a final conversion of 98.4%. Such a high conversion rate demonstrates the potential of the technique but the reaction time in all the reactions is considerably high when compared to 1 h for chemical transesterification reaction. Recently, Lee et al. [19] have reported 98.92% conversion yield in 6 h by using supercritical fluid (carbon dioxide) and stepwise addition of methanol. The use of supercritical carbon dioxide increases the mass and thermal transfer which increases the reaction rate. However, due to high mass transfer, the methanol concentration has to be maintained at a lower level to prevent inhibition of the lipase. Although this process has many advantages, the optimized conditions reported by Lee et al. [19] include the use of very high enzyme concentration (20%) and water content (10%) in the reaction.

The amount of water in the reaction mixture is a critical parameter. Thermodynamic water activity (a_w) determines the mass action effects of water on hydrolytic equilibria. It also describes the distribution of water between various phases that can compete in binding water [20]. Thus water activity is a better parameter than water content to study the effect of water on enzymatic reactions in non-aqueous media as it eliminates the ambiguity due to the competition for binding water by immobilization support or other substances/impurities in the reaction mixture [21]. It has been reported that a minimum amount of water (or water activity), which may vary from system to system, is required for the activity of the enzyme [22–24]. It has been found that an enzyme requires less than a monolayer of water for biological activity. As the level of water increases, it increases the enzyme flexibility and the

Table 1
Comparison of transesterification activities of different lipases on different substrates.

Source of oil	Source of enzyme	Alcohol	Optimum conditions	Immobilization medium	Maximum yield	Other details	Reference
Jatropha	*Chromobacterium viscosum*	Ethanol	40 °C, 10 h, 200 rpm	Celite-545	92%	Water content 0.5%	Shah et al. [2]
	Burkholderia cepacia	Ethanol	40 °C, 24 h, 200 rpm	–	79%	Ultrasonic pretreatment, 110 W, 2–3 h	Shah and Gupta [31]
	Enterobacter aerogenes	Methanol	30 °C, 60 h, 200 rpm	Silica	94%	Used t-butanol as solvent	Kumari et al. [64]
Tallow	*Mucor miehei*	Methanol	45 °C, 5 h, 200 rpm	–	98.4%	Hexane solvent	Nelson et al. [5]
		Ethanol			98.3%		
		Propanol			98.6%	Used hexane as solvent, 6 mol% water based on triglyceride was added	
		Butanol			99.6%	Used hexane as solvent	
		Isobutanol			99.4%	Used hexane as solvent, 6 mol% water based on triglyceride was added	
Soybean oil	*Thermomyces lanuginosa*	Methanol (stepwise addition)	40 °C, 50 h, 150 rpm	Silica gel	>90%	Molar ratio of methanol to oil 1:1, lipase 10%	Du et al. [4]
	Mucor miehei	Ethanol	45 °C, 5 h, 200 rpm	–	97.4%	Hexane solvent	Nelson et al. [5]
	Pseudomonas fluorescens (psychrophilic strain B68)	Methanol (stepwise addition)	20 °C, 12 h, 180 rpm	Cellulose fabric	92%	n-Heptane solvent	Luo et al. [12]
	Pseudomonas fluorescens	Ethanol (initial molar ratio to oil 3:1)	70 °C, 24 h, 180 rpm	–	71%	Iso-octane solvent, solubilized lipase AK	Zhao et al. [30]
		Methanol (molar ratio to oil 3:1)	40 °C, 72 h, 200 rpm	–	83.8%	5 mmol/l Ca^{2+}, 5% water	Yang et al. [35]
	Rhizomucor miehei (*Lipozyme IM-77*)	Methanol	36.5 °C, 6.3 h, 200 rpm	Macroporous weak anionic resin beads	92.2%	*n*-Hexane solvent, water content 5.8%, molar ratio of methanol to oil 3.4:1	Shieh et al. [41]
Mixture of soybean and rapeseed oils	*Candida antarctica*	Methanol (stepwise addition)	30 °C, 48 h, 130 rpm	SM-10	98.4%	Water content <400 ppm	Shimada et al. [18]
Sunflower oil	*Candida antarctica* (Novozyme 435)	Methanol (continuous addition)	50 °C, 15 h, 130 rpm	Macroporous resin support, diameter 0.3–0.9 mm	97%	Water content 400 ppm	Belafi-Bako et al. [40]
Waste cooking oil	*B. subtilis* 1.198	Methanol (stepwise addition)	40 °C, 72 h, 220 rpm, pH 6.5	Hydrophobic carrier with magnetic particles (Fe_3O_4)	90%	–	Ying and Chen [43]

expressed activity [25]. In case of the lipases, an increase in water content above an optimum level promotes the hydrolytic activity and the transesterification yield drops [2]. The water content has to be subsequently reduced below 0.05 vol.% water content according to the biodiesel norms in the USA [26]. This would require an additional dehydration step which would incur extra cost. To eliminate the water content from the reaction mixture, the enzymes can be solubilized to achieve homogeneous reactions in non-aqueous media. This can be done by modifying enzymes with polyethylene glycol (PEG) or short alkyl chains. PEG-modified or pegylated enzymes, though biologically active, usually have low solubility (mostly much less than 1 mg/ml) [27,28] which limits their use. However, high solubility (up to 44 mg/ml) can be achieved by modifying them with short alkyl chains which ultimately results in higher activity [29]. Zhao et al. [30] reported a yield of 71% using solubilized lipase AK from *P. fluorescens*. It was also observed that the solubilization with short alkyl chains imparts thermal stability to the enzyme. The optimum temperature for the solubilized lipase AK was found to be 70 °C as compared to 30 °C for the native lipase AK. Thus, solubilization of enzyme with short alkyl chains on one hand reduces the downstream processing cost, but on the other hand makes the process energy intensive. Clearly, further studies are needed to optimize the process. Another strategy to improve the efficiency of lipase catalyzed transesterification in non-aqueous media is through ultrasonic pretreatment. Shah and Gupta [31] have studied the effect of ultrasonic pretreatment of *Burkholderia cepacia* lipase on transesterification activity for biodiesel production from jatropha oil. They have reported 79% conversion rate at 24 h after ultrasonic pretreatment at 110 W for 2–3 h as compared to 34% conversion rate in control. The increase in activity is due to changes in the microenvironment of tyrosine and tryptophan residues, changes in tertiary structure and reduction in particle size which increases the catalytic surface area and reduces mass transfer limitations. In the same context, different enzyme preparations: EPRP (enzyme precipitated and rinsed with n-propanol) [32], PCMC (protein coated microcrystals) [32,33], CLEA (cross-linked enzyme aggregates) [6] have been reported to show enhanced activity and stability in non-aqueous media. Sheldon et al. [34] have reviewed the formulation, properties and applications of CLEAs. Solanki and Gupta [32] have demonstrated the enhanced transesterification activity of EPRP and PCMC of α-chymotrypsin as compared to the lyophilized powder in non-aqueous media. They have attributed the enhanced activity to better retention of essential water layer for catalysis and reduction in particle size in case of EPRP and higher surface area of biocatalyst in PCMC which reduces the mass transfer limitation. CLEA and PCMC prepared using 50 mg of *P. cepacia* lipase gave a conversion yield of 92% and 99%, respectively in 2.5 h as compared to 98% in 6 h by free enzyme with *M. indica* oil [6]. In another study, the stability of PCMC was demonstrated. The PCMC prepared using 50 mg *P. cepacia* lipase was stable at 60 °C giving a 96% conversion in 90 min while free enzyme lost almost all its activity giving 8% conversion [33].

The effect of metal ion and metal chelant on lipase activity has also been studied. Recently, Yang et al. [35] have reported an increase in activity of *P. fluorescens* 26-2 lipase up to 2.8-fold in the presence of 5 mmol/l Ca^{2+} ion. They have reported a biodiesel conversion rate of 83.8% on soya bean oil with methanol (molar ratio 3:1 with oil) at 40 °C, 200 rpm after 72 h but with 5% water content. Nielsen et al. [36] have reviewed the considerations for process design in different bioreactors and have presented an economic analysis of biodiesel production. They have concluded that packed-bed technology using immobilized enzymes holds great promise for the development of a continuous process for biodiesel production. The key patents filed in the area of biodiesel pertaining to innovation in optimization of reaction conditions, plant design and use of glycerol byproduct have been reviewed by Singh et al. [37].

4. Immobilization and reusability of the enzyme

One of the common drawbacks with the use of enzyme-based processes is the high cost of the enzyme. Immobilization of enzymes has generally been used to obtain reusable enzyme derivatives. Immobilized enzymes are also more stable towards temperature, chemical as well as shear denaturation [7]. This enables easy handling, recovery and recycling of the biocatalyst and hence lowers the cost. Immobilized *Thermomyces lanuginosa* lipase has been shown to be thermostable with a half-life of 135 h at 70 °C in a blend of palm stearin, palm kernel oil and sunflower oil (55:25:20, wt%) [38]. In case of the biocatalysts in non-aqueous media, immobilization has been reported to result in better activity [2]. It has been shown that silica gel used as immobilization carrier promotes acyl migration in the transesterification process which increases the yield from 66% to 90% when *T. lanuginose* lipase with 1,3-positional specificity was used [4]. Enzyme activity also depends on the physical and chemical properties of the immobilization carrier. For example, in case of sol–gel immobilized lipases, the free alkyl groups of its precursors (e.g., iso-butyltrimethoxysilane) create a lipophilic microenvironment and interact with the lipase causing its activation in a similar manner as in interfacial interaction [39]. However, immobilization may also cause internal and external mass transfer limitation for large molecules like triacylglycerol and fatty acid alkyl ester [7]. In addition, glycerol has been shown to inhibit immobilized *Candida antarctica* lipase (Novozyme 435) [40]. This may be due to product inhibition [40] or mass transfer limitation due to the effect of glycerol on external film layer formation [7]. Thus continuous removal of glycerol by dialysis using flat sheet membrane module has been suggested [40].

Many transesterification processes employing lipases have used an immobilized form of the enzyme [2,4,9,18,24,25,40–43]. Lipases from microorganisms like *Mucor miehei*, *Rhizopus oryzae*, *Candida antarctica*, and *P. cepacia* have been found suitable for biodiesel production. Various carriers for immobilization have been used such as celite, cellulose fabric, SM-10, and silica (Table 1). But the activity of the enzyme decreases on recycling immobilized enzyme [42]. This might be due to desorption, substrate inactivation or product inhibition [17,40,42]. However, Shimada et al. [18] have reported more than 95% ester conversion even after 50 cycles (100 days) of the reaction. Further, the efficiency on recycling the enzyme depends on the solvent with which it is washed after each batch and the acyl acceptor used in the reaction. Washing with non-polar solvent *n*-hexane has been shown to cause highest retention of lipase activity [17]. Reusability of immobilized lipases has been reported to decrease when lower linear alcohols such as methanol or ethanol are used as acyl acceptors for biodiesel production. Use of propane-2-ol has been found to maintain the lipase activity up to 12 cycles while no activity was observed when methanol was used [44]. Other acyl acceptors especially methyl acetate has been found to be efficient and gave a yield of 92% with no significant loss of lipase activity even after continuous use for 100 batches [45].

Recently, it has been suggested that the cost of biodiesel production can be minimized by using two immobilized lipases with complementary position specificity instead of one lipase. Combined use of two immobilized enzymes Novozyme 435 and Lipozyme TL-IM using response surface methodology gave a yield of 97.2% of methyl ester from lard oil. These lipases could be recycled 20 times [46].

Another strategy to reduce the cost of the enzyme is to make the whole cell biocatalyst. It involves cells which can be engineered to overproduce enzyme to catalyze the reaction. Utilization of microorganisms such as bacteria, fungi and yeast has appeared as promising whole cell biocatalysts for biodiesel production. Fila-

mentous fungi have been found as most robust whole biocatalyst system [47]. Spontaneous immobilization of the filamentous fungi on biomass support particles allows the separation of the whole cell biocatalyst and facilitates their reuse. In addition, use of packed-bed reactor operated at an appropriate circulation flow rate allows repeated methanolysis reaction by protecting fungal cells from the physical damage. Furthermore, cross-linking of immobilized cells with glutaraldehyde improved the stabilization of whole cell catalyst. However, substrate conversion yield can be limited by the toxicity of the solvent to the host cells and by low mass transfer rate of high molecular weight substrates (oil) from the solvent phase to the whole cell biocatalyst in the aqueous phase. This can be overcome by surface immobilization of enzyme on the bacterial cells [48–50]. Jung et al. [50] have immobilized a thermostable lipase (TliA) from *P. fluorescens* on the cell surface of a solvent resistant bacterium *Pseudomonas putida* GM730. This was achieved fusing the enzyme with anchoring motif of Ice Nucleation Protein (INP) from *Pseudomonas syringae*. Recently, *Pichia pastoris* has been demonstrated to be a more robust expression system for surface display of lipases with better stability and higher dry cell weight per ferment culture [35,51]. In another study, Ying and Chen [43] encapsulated lipase overproducing strain *Bacillus subtilis* 1.198 into divinylbenzene polymicrosphere, a hydrophobic carrier with magnetic particles (Fe_3O_4) to impart superparamagnetic property. This is termed as Magnetic Cell Biocatalyst (MCB). It allows easy magnetic separation and recycling. Further, it can be regenerated under 48-h cultivation to ensure maximum activity of lipase. Thus, it provides a long-term source of uniform biological activity for biodiesel production. There might be a chance of mutational inactivation of overproducing strain during consecutive recycling of the whole cell catalyst but at present it has not been reported. Generation of genetically engineered *E. coli* whole cell biocatalyst expressing lipase (LipK107) from *Proteus* sp. has also been reported. Thus engineered *E. coli* produced yield of nearly 100% biodiesel at optimal condition [52]. Biosynthesis of biodiesel-adequate FAEE (fatty acid ethyl esters) by metabolically engineered *E. coli* in the presence of glucose and oleic acid gave a novel approach that might pave the way for industrial production of biodiesel [53].

However, according to a recent analysis [7], the cost of the enzyme per kg ester produced still remains higher as compared to the cost of alkali catalyst (per kg ester produced). It has been suggested that the cost of lipase can be reduced by the use of recombinant DNA technology. Further, protein engineering and directed evolution methods can be used to improve lipase stability, substrate specificity and catalytic efficiency which will facilitate lowering the cost of the overall process [54,55]. Lipase Engineering Database (available at http://www.led.uni-stuttgart.de/) provides information regarding structure–sequence–function relationships of lipase and related proteins sharing the same α/β-hydrolase fold to facilitate protein engineering [56].

5. Utilization of byproducts

Another important consideration to improve the economic feasibility of biodiesel production is the utilization of the primary byproduct, glycerol. One option is to sell it as boiler fuel, as animal food supplement or to a glycerol refinery. But, it is not very attractive economically. For use in manufacture of food and beverages, pharmaceuticals or cosmetics, purified glycerol is required [57]. Purification to remove impurities such as alcohol, salts and water would incur extra cost. Conversion of glycerol to other value added products can be achieved through chemical or biological catalysis. The potential products of chemical conversion include propylene glycol, propionic acid, acrylic acid, propanol, isopropanol, allyl alcohol and acrolein. Biological utilization of glycerol has been observed in bacteria of the family *Enterobacteriaceae* and several species of *Clostridia*. The potential product of anaerobic fermentation is 1,3-propanediol along with other co-products like acetic acid, butyric acid, acetone, butanol, ethanol, 2,3-butanediol, lactic acid, succinic acid, formate and hydrogen [58–60]. These options have been extensively reviewed by Johnson and Taconi [57]. Recently, Xu et al. [61] have reviewed the technology available for the conversion of glycerol to 1,3-propanediol and the prospects of its integrated production with biodiesel. Overall, both chemical and biological catalysis can be used to convert glycerol into value added products. However, additional research and development is required to make these processes lucrative enough to be incorporated into biodiesel production.

Balan et al. [62] have also demonstrated the conversion of extracted oil cake fibres from several sources to bioethanol by a three-step process, including Ammonia Fibre Expansion (AFEX) pretreatment followed by enzymatic hydrolysis and fermentation by *Pichia stipitis*. They have suggested the use of undigested solids as protein-rich animal feed supplement. The integration of these technologies for conversion biogenic raw materials like carbohydrates, lignin, oils and proteins to intermediates and final products through a combination of biotechnological and chemical processes in a single unit is known as a 'Biorefinery' [63]. Further exploration and integration of these technologies into biorefineries would promote commercial biodiesel production.

6. Summary

Lipase catalyzed biodiesel production has been an active area of research and shows great potential to generate an environmental friendly and economic fuel in future. Researchers have tried different parameters to standardize the catalysis of transesterification reaction, a pivotal step in biodiesel production. Various lipases from different sources immobilized on different carriers have shown a range of yields with different oil substrates and different acyl acceptors. Clearly, optimization of reaction conditions is required in each case. Nevertheless, a vast data has been generated and the effort to develop an ideal process continues. It has been shown that short chain linear alcohols, especially methanol, inhibit the activity of lipases and decrease the reusability of immobilized lipases. High retention of immobilized lipase activity is reported when washed with non-polar solvent like *n*-hexane. High conversion rates close to 100%, low reaction times, and high activity in non-aqueous media have been achieved in independent studies. Different strategies applied have their own advantages as well as limitations. A recent study by Fjerbaek et al. [7] indicates that the cost of the enzyme per kg ester produced still remains higher as compared to the cost of alkali catalyst (per kg ester produced). It has been suggested that the cost of lipase can be reduced by the use of recombinant DNA technology. In addition, further research and development is needed for efficient recovery and utilization of the glycerol to make the process economic and eco-friendly. An integration of these approaches to minimize the limitations along with further research on bioreactor design and scale up of the process is required for the commercial production of biodiesel through lipase catalyzed reaction in future.

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