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Lipase-catalyzed Production of Biodiesel: A Critical Review on Feedstock, Enzyme Carrier, and Process Factors

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Abstract: Biodiesel is a green and renewable alternative energy source which can be produced through lipase-catalyzed transesterification process. Enzymatic biodiesel production has generated much interest due to its environmentally friendly process, low

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wastewater treatment requirements and easy product separation. In this work, we attempt to review some of the recent patents and state-of-the-art technology in lipase-catalyzed transesterification for biodiesel production including feedstock, lipases, novel carriers and process factors. This work will provide useful guidance for researchers to develop cost efficient process for production of biodiesels.

Key word: biodiesel; lipase; transesterification; carrier

1. Introduction

Fossil fuels (coal, natural gas and oil) are world's major energy supply. Due to high consumption and non-renewable nature of fossil fuels, energy crisis is imminent. In addition, fossil fuels cause land degradation, and water and atmospheric pollution. Therefore, there has been increased interest in developing alternative energy sources such as biodiesel [1]. Several countries such as Germany, Malaysia, and America, emphasize and encourage the use of biodiesel through government and regulatory approaches [2]. Global biodiesel production increased from 3.5 million tons in 2005 to 29 million tons in 2016, and was expected to exceed 36.5 million tons by 2025 [3]. Many experts believe that global biodiesel production will reach about 277 million tons per year by 2050 to meet future growing demand for biodiesel [4, 5].

Biodiesel is a mixture of fatty acid methyl esters (FAME) and/or ethyl esters (FAEE) obtained by transesterification of triacylglycerol (TAG) in edible, non-edible or waste oils (Figure 1) [6]. It is a form of sustainable and renewable energy with significantly reduced emissions of particulate matter, greenhouse gasses, unburned hydrocarbons, nitrogen oxides, carbon monoxide, and carbon dioxide [7, 8]. Biodiesel can be directly used in pure form or mixed with diesel in any proportion [9]. It has similar or even better fuel characteristics than petroleum-derived diesel such as better combustion performance (higher combustion efficiency, lower sulfur and aromatic content, higher oxygen content and flash point) [10, 11].

Transesterification is considered to be the best process for producing high quality

commercial biodiesel. The most commonly used catalysts in biodiesel production industry are alkaline catalysts (KOH and NaOH) which have the characteristics of high catalytic activity, short reaction time and high yield of nearly 99 % [12]. However, alkaline process has the disadvantages of high energy consumption, difficult recovery of catalysts and glycerol, and environmental pollution [13].

Despite the cost of lipase, lipase-catalyzed transesterification for biodiesel preparation has attracted significant researchers' interests. Some of the reasons include no side reactions, easy product separation and mild reaction condition (reaction temperature of 40 to 60 °C, reaction time of 12 h to 24 h, pH of 6.0 to 8.0) [14]. Lipase-catalyzed reactions are more environmentally friendly with lower requirements for wastewater treatment and lesser emissions of particulates and greenhouse gases. However, challenges in terms of lipase inactivation by short-chain alcohols, separation and recycling of the catalyst need to be overcome. Thus, there is a need to review the state-of-the-art technology in lipase-catalyzed transesterification for biodiesel production to provide useful guidance for researchers to develop cost-efficient process for production of biodiesel.

2. Trends in Feedstocks for Biodiesel Production

Feedstocks for biodiesel production can be obtained from a variety of sources, and selection of feedstocks is dependent on ease of availability, cost and composition of the feedstock [15]. Generally, feedstocks for biodiesel production can be divided into first, second, third and fourth generations (Figure 2).

The first-generation of feedstock refers to oil from edible sources which account for

about 95 % of biodiesel production. Soybean oil, palm oil, corn oil, sunflower seed oil, coconut oil and other vegetable oils are common first-generation feedstock [16-19]. Although there is a wide range of first-generation feedstock for biodiesel production, the competition between fuel and food industries has increased the final cost of biodiesel. In addition, the use of edible oils for biodiesel production will cause ecological consequences and potential food shortages [20].

Second generation feedstocks are more economical efficient than first generation feedstocks as they are composed of either by-products and wastes of agriculture or food processing industry such as waste cooking oil (WCO), Jatropha seed oil, and etc. Another advantage of using waste oil as biodiesel feedstock is decreased damage to water and aquatic organisms caused by improper disposal of waste oil [21]. The use of WCO as feedstock for biodiesel production has attracted much attention [22, 23]. Biodiesel synthesized from WCO contains large amounts of saturated and unsaturated fatty acids with high cetane number and flash point indicating good fuel properties. Biodiesel may need to be blended with diesel to enhance combustion characteristics due to its possible low energy density [24].

Third-generation feedstocks refers to oil-producing algae, microalgae, bacteria, fungi and yeast [25]. Oil-producing microorganism is a new type of biodiesel feedstock with higher productivity which can avoid the ongoing food vs. fuel conflict [26]. Artificially cultivated algae reproduce quickly, and some high-yield oil algae can reach 70 % oil w/w dry biomass [27]. Therefore, considering factors such as biomass productivity and efficiency, downstream technology and cost-effectiveness, it is feasible to commercially produce biodiesel using

algal oil.

Development of solar-based decomposition of photosynthetic water into energy is expected to become the fourth generation feedstock of biodiesel. This feedstock can be integrated with second or third generation to obtain high photon of fuel conversion efficiency using synthetic biological methods [28]. Fourth generation feedstock is expected to produce biodiesel of high yield, high efficiency and high performance from photobiosolar fuel system in the near future.

3. Lipases for Biodiesel Production

At present, there are three types of catalysts commonly used in the preparation of biodiesel, including alkaline, acidic and lipase-based catalysts. As mentioned above, homogeneous alkaline catalysts (NaOH, KOH) and heterogeneous alkaline catalysts (MgO, CaO) are cheap and have high catalytic activity, but could produce harmful wastewater downstream and is sensitive to free fatty acids. Acidic catalyst (H_2SO_4, HCl) needs longer reaction time and higher reaction temperature and pressure. Therefore, lipase catalyst, which has mild reaction conditions and can be recycled easily, has been studied and developed in recent years.

Lipases, also known as triacylglycerol hydrolases (EC 3.1.1.3), are widely employed to catalyze hydrolysis of free fatty acids from long-chain triacylglycerides, by breaking the ester bond between the glycerol and the fatty acid of the triacylglycerides. It can also catalyze alcoholysis, esterification and transesterification as a widely applied multifunctional enzyme [29, 30]. Lipases catalyze the transesterification of triglycerides in

feedstock oil with methanol or ethanol to form FAME or FAEE, that is, biodiesel (Figure 1). Lipases have diverse origins and can be produced by microbes, animals and vegetables. Microbial lipases from bacteria, yeast and fungi have greater advantages over other sources of lipases for biodiesels production because they have diverse properties such as ease of culture, thermal and pH stability, substrate specificity, etc [31]. Some of the commonly used microbial lipases for biodiesels production include *Rhizopus oryzae* [32], *Aspergillus oryzae* [33], *Aspergillus niger* [34], *Thermomyces lanuginosus* [35], *Geobacillus thermocatenulatus* [36], *Burkholderia cepacia* [37], and etc. Microbial lipases can be easily genetically modified. For example, Zhang et al. synthesised and cloned gene BTL2 of thermophilic lipase from *Geobacillus thermocatenulatus* and then efficiently expressed in *E. coli* BL21 (DE3) cells in order to make use of the advantages of simple culture conditions, short growth cycle and excellent protein expression ability of *E. coli*. The recombinant lipase exhibited high catalytic activity (the yield of FAMEs reached 91.04 % at 45 ℃ within 12 h) after bionic-immobilization process [36].

3.1. Lipase immobilization and its application in biodiesel production

Lipases can be immobilized on carrier material to overcome the disadvantages of poor stability, difficult recovery and poor recycling of free enzyme in biodiesel production. In addition, immobilized lipases can be regenerated and reused in packed bed reactors which greatly reduce lipases cost. Moreover, immobilization can improve lipases tolerance to temperature and organic solvents [38].

There are four main enzyme immobilization techniques namely surface adsorption, in-

situ encapsulation, covalent binding and cross-linking [39]. Surface adsorption of enzymes to different carriers is achieved through van der Waals forces, hydrophobic forces and hydrogen bonds. As one of the most commonly used immobilization methods, surface adsorption has many obvious advantages including simple operation and mild reaction conditions. However, the weak interaction force in surface adsorption do not favor reusability and may cause changes in enzyme conformation [33]. In encapsulation, porous polymer matrix is formed around lipase. Unlike surface adsorption, encapsulated lipases can minimize the inactivation effect. However, encapsulation technique may reduce catalytic activity due to potential increase in the mass transfer limitation between enzyme and macromolecular substrate. Covalent binding is a method to covalently bind lipase to carrier which significantly improves lipases tolerance to heat, organic solvents, pH and other denaturants [40]. In cross-linking method, cross-linking agent form intramolecular and intermolecular crosslinks with specific groups of amino acids on the surface of the lipase to form cross-linked lipase [39].

Table 1 summarizes the recent studies on preparation of biodiesel through transesterification process catalyzed by immobilized lipases. Some of the commonly used commercially available lipase carrier includes silica, carbon nanotubes and electrospun nanofibers. Silica has been widely used as a stable carrier for enzyme immobilization due to its high specific surface area and controllable pore size. Lipase was bionic-immobilized in silica and used as catalyst, and biodiesel yield reached 91.04% using 20% immobilized lipase dosage in 12 h at 45 °C [36]. Carbon nanotubes are also a carrier for lipase

immobilization. Through surface modification and functionalization, the loading capacity of 20 mg protein / g could be achieved, and biodiesel yield reached 92 % [41]. In the past two decades, nanofibers have been used in various fields including immobilization of enzymes, as polymers with ideal physical, chemical and mechanical properties. Electrospun nanofibrous membranes immobilized lipase showed about 2.85 folds higher stability than the free enzyme at 60 °C and the yield of biodiesel prepared from castor oil was 63 % [42]. The morphologies of these carriers are shown in Fig 3.

Nanoscale materials which have large surface area such as magnetic nanoparticles, metal organic frameworks (MOFs), nanoflowers, covalent organic frameworks (COFs) etc. are emerging enzyme carriers for lipase imobilization. However, downstream ultracentrifugation or nanofiltration are required to separate the nanoenzymes from end product which will increase operating costs [43]. In the following section, we will review some of the novel nanomaterials used in lipase immobilization for biodiesel production.

3.1.1. Magnetic nanopaticles-immobilized lipases

The use of magnetic nanoparticles as lipases' carriers has garnered much interest as they can be easily separated from reaction medium under external magnetic field (Figure 4). Two of the most commonly used magnetic carriers for lipase immobilization are magnetite (Fe_3O_4) and magnetic hematite (γ-Fe_2O_3) [44]. Surface functional modification of magnetic nanoparticles is usually carried out to enhance the interaction with biological molecules and improve enzyme carrying capacity [45, 46]. Aminosilanes, such as aminopropyltriethoxysilane (APTES), bind to the surface of iron oxide through covalent

bonds, which are the most widely used amino functional reagents in the modification of magnetic nanoparticles. Lipase can be covalently coupled with the amino modified on the surface of the carrier through glutaraldehyde (GA) to form a magnetic biocatalyst. Through this strategy, Bento et al. prepared bare iron oxide magnetic particles by co-precipitation method and functionalized with γ-APTS as lipase carrier [47].The result showed that immobilized enzyme maintained about 53 % of the enzyme activity and obtained better enzymatic kinetic parameters (Km increased by three times, from 410 to 1262 mmol L^{-1} and Vmax decreased by seven times, from 12,390 to 1786 $U \cdot g^{-1}$) compared with free enzyme. Immobilization was also conducive to the thermal stability of the enzyme, and the half-life of the enzyme increased by about 10 times at 50 °C. Similarly, the reaction system in the fixed bed reactor was more stable, and the yield of ethyl ester could be maintained at a high level for 15 consecutive days (about 96 %). Similarly, co-precipitation method was also used to synthesize magnetic nanoparticles and functionalized with organic-inorganic hybrid tetraethyl orthosilicate (TEOS) and N-[3-(trimethoxysilyl)propyl] ethylenediamine (TSD), then was used as a carrier for immobilization [48]. The thermal stability of immobilized lipase was also observed to be significantly improved. Under the conditions of 40 ° C, methanol / oil molar ratio of 4 : 1 and catalyst dosage of 1 g, the maximum yield of biodiesel was 96 % in 30 h.

3.1.2. Metal-organic framework-immobilized lipases

Metal-organic framework (MOF) is a three-dimensional network material widely studied in recent years, which is self-assembled by metal nodes and organic ligands [49]. The

characteristics of high specific surface area and flexible pore size adjustment make MOFs as excellent carriers for immobilized enzymes. Another remarkable advantage of MOF-enzyme biocomposites is that they can exhibit excellent biocatalytic properties with improved stability and reusability [49]. Lipase Eversa Transform 2.0 was successfully encapsulated into ZIF-8, which was formed by the coordination of Zn^{2+} ions with 2-methylimidazole (HmIm) and considered as a common enzyme immobilization material due to extremely mild preparation conditions, and the encapsulated lipase exhibited good stability and reusability. Under the condition of methanol ratio of 12 : 1, the residual activity of biodiesel production remained 83 % after 5 cycles [50].

Besides ZIF-8, other MOF materials such as ZIF-L, ZIF-67, IRMOF, MIL, UiO, HKUST and etc. can also be used for lipase immobilization [51]. MOFs can be hydrophobically modified to enhance its functionality. Hydrophobic ZIF-L coated with polydimethylsiloxane (PDMS) was prepared by chemical vapor deposition and used for immobilization of lipase from *Aspergillus oryzae* (AOL) to produce biodiesel (Figure 5). PDMS coating enhanced the stability of ZIF-L in PBS. AOL@PDMS-ZIF-L can be used to produce biodiesel with a yield up to 94 %. Catalytic activity of the AOL@PDMS-ZIF-L was about 85 % of its initial catalytic activity after five consecutive cycles [33].

3.1.3. Hybrid nanoflower-immobilized lipases

Biomimetic mineralization is a strategy for transforming self-assembly processes in natural biological systems into encapsulation of bioactive molecules within protective exteriors. Hybrid nanoflower-immobilized lipase formed by $Cu_3(PO4)_2$ and enzyme is a

promising enzyme immobilization with high protein loading and low diffusion limit [52]. Owing to the interaction between metal ions and enzyme, it exhibits high activity and stability [53]. Li et al. reported that when Ca^{2+} was used, not only the self-assembly of lipase hybrid nanoflowers was induced, but also the enzymatic activity was activated by inducing conformational changes in the lipase from *Aspergillus oryzae* [54]. In addition, hybrid nanoflowers have the advantage of simple synthesis under mild conditions [55]. However, the nano-structure of hybrid nanoflowers is fragile, resulting in low mechanical properties and reusability, which limits its application [56]. Jiang et al. applied lipase-inorganic hybrid nanoflowers to prepare biodiesel from sunflower seed oil. The conversion of biodiesel reached 96.5 %, and remained 72.5 % after five cycles (Figure 6) [57].

In order to enhance the reusability of hybrid nanoflower-immobilized lipases, Zhong et al. integrated Fe_3O_4 magnetic nanoparticles (MNPs) into surfactant-activated lipase hybrid nanoflowers by co-precipitation and covalent cross-linking. The magnetic lipase-inorganic hybrid nanoflowers retained 84 % of its initial activity, while the lipase-inorganic hybrid nanoflowers retained only 26 %, indicating good reusability after being reused 10 times [56].

3.1.4. Covalent Organic Frameworks-immobilized lipases

Covalent organic frameworks (COFs) is crystalline material connected by covalently organic building units and its backbone is composed of light elements (B, C, N, O, Si) [58]. Unlike MOFs, COFs do not contain toxic metal ions which may damage the conformation of biological molecules. It also has long-term water and/or chemical stability [59-61]. In addition, the composition of COFs does not change significantly once covalently connected,

so it is easy to predict and prepare COFs with desired composition and pore size for penetration of biological molecules and hinder aggregation of enzymes [62].

Enzymes can be immobilized onto COFs through different strategies such as physical adsorption, covalent bonding and etc [63]. At present, there are very few studies that focused on the immobilization of enzymes in COFs [64]. Zhou et al. was the earliest that attempted to produce biodiesel using COFs-based immobilized enzyme [65]. The synthesized core-shell magnetic COF complex (Fe_3O_4@COF-OMe) had the advantages of large specific surface area, good chemical stability and can be easily recovered and reused. The COFs immobilized lipases maintained its initial catalytic activity and can be used to produce biodiesel from Jatropha curcas oil with a yield of about 70 % under the optimized conditions (Figure 7).

In conclusion, some newly developed carriers such as magnetic nanoparticle, MOF, hybrid nanoflower and COF have been developed for lipases immobilization. Lipases immobilized on these carriers can be easily recovered, have good stability and potential to replace homogeneous or heterogeneous acid and base catalysts.

4. Process factors affecting lipase-catalyzed biodiesel production

There are many factors affecting lipase-catalyzed esterification and/or transesterification process for production of biodiesels such as lipase concentration, type of alcohols, molar ratio of alcohol-to-oil, presence of water in the reaction systems, solvents, ultrasonic assistance and other parameters [66]. The aforementioned factors must be carefully considered in order to obtain high yield of biodiesels.

4.1. Effects of lipase concentration on lipase-catalyzed biodiesel production

Table 2 shows the effects of lipase concentration on biodiesel yield. Enzyme concentration has significant effects on lipase-catalyzed biodiesel production. Higher lipase concentration increases the amount of active centers available for catalysis of transesterification process for biodiesel production. However, as lipase concentration continue to increase, biodiesel yield does not increase but rather remains constant or starts to decrease. Excessive lipase concentration causes lipases to accumulate and hinder the contact between substrate and enzyme active center resulting in decrease of enzymatic activity and biodiesel production [67, 68].

Zhang and colleagues [36] employed immobilized *Geobacillus thermocatenulatus* lipase 2 (GTL2) for biodiesel production. They found that the yield of biodiesel improved rapidly as the amount of enzyme increased from 1 to 10 %. No further increase in biodiesel yield was observed as the dosage of immobilized lipase exceeded 10 % (wt / wt based on the oil). Similar findings were observed in other study [33]. In another study, Binhayeeding et al. reported a decrease in biodiesel yield as the concentration of immobilized *C. rugosa* and *R. Miehei* lipase exceeded 3 % [22].

In conclusion, enzyme concentration can accelerate reaction rate but excessive enzyme concentration often lead to decrease in biodiesel conversion rate. High enzyme concentration also lead to increase in production cost. Generally, 0.5-1 wt % enzyme concentration is sufficient to produce high yield of biodiesel at an acceptable cost efficiency.

4.2. Effects of alcohols on lipase-catalyzed biodiesel production

The effects of alcohol on the yield of biodiesel are summarized in Table 3. Methanol, ethanol, isopropanol, n-butanol and different types of alcohols have been applied for biodiesel production [69-71]. Among them, methanol and ethanol are most widely used to prepare FAME and FAEE, respectively [69].

Methanol is easily available and relatively cheap alcohol. Lipase-catalyzed production of biodiesel using methanol can be conducted under mild reaction condition with high reaction rate [72]. Nevertheless, it also has some obvious drawbacks. Methanol causes lipases denaturation and inactivation. Besides that, methanol is mainly produced from natural gas which is a non-renewable fossil energy source and thus not attractive in biodiesel preparation [73].

Ethanol is another cheap source of alcohol which is abundantly available in lignocellulosic materials like crop residues, grasses, sawdust, wood chips, etc [74]. Biodiesel composed of FAEE contains better characteristics including higher cetane number, higher heat combustion and better storage properties [72]. In addition, it has lower nitrogen oxide and carbon monoxide emissions as compared to FAME [75, 76]. However, FAEE is difficult to separate from reaction system due to the stable emulsion formed from glycerin and FAEE during ethanolysis [77]. Some study also found longer reaction time is required to produce biodiesel when ethanol was used as acyl acceptor [78].

The molar ratio of alcohol-to-oil is another factor affecting biodiesel yield. Thereotically, a molar ratio of alcohol-to-oil of 3:1 is needed to produce 1 mol of FAME (Figure 1). As this is a reversible reaction, excess reactants can push reaction forward. Therefore, high

amout of alcohol is often used to maximize the yield of biodiesel. However, excessive alcohol (alcohol-to-oil molar ratio above 3:1) which is not soluble in reaction system may reduce lipases activity resulting in the decrease of biodiesel yield [14]. Solubility of excessive alcohol can be increased by addition of organic solvent which protects lipases from inactivation and improves the reaction rate [79].

Adebami and colleague investigated the effects of methanol-oil molar ratio on biodiesel yield and catalytic activity of *Aspergillus oryzae* GP11 immobilized on glyoxyl-agarose. As the molar ratio of alcohol-to-oil increased from 1 : 1 to 5 : 1, the yield of biodiesel also increased from 68.2 ± 3.41 % to 97.7 ± 4.89 %. A decrease in yield was observed at a molar ratio of alcohol-to-oil of 6 : 1 indicating deactivation of the immobilized lipase [80]. Similar results were observed in a study by Parandi et al. who employed *Candida Antarctica* Lipase B immobilized on the Fe_3O_4 magnetic hybrid sol-gel nanocomposite for transesterification of WCO. Highest biodiesel yield (96 %) was obtained at a methanol-oil molar ratio of 4 : 1 in a 24 h transesterification conducted at 40 ℃ [48].

Stepwise addition of alcohol, especially in the case of high alcohol-oil molar ratio, can be used to prevent enzyme denaturation and inactivation and to improve biodiesel yield [81, 82]. Wang and colleagues found short chain alcohol resulted in obvious inactivation of lipases with a FAME yield of only 65.4 %. Stepwise addition of alcohol (three times) improved FAME yield significantly to 90.4 % [83]. In another study, stepwise addition of alcohol to the reaction sytem (five times of equal alcohol volume every 60 min) resulted in a 17-fold improvement of FAME yield (from 2.84 % to 48.06 %) and a improvement of

17 % of FAEE yield [84].

In conclusion, most of the current work in enzymatic production of biodiesel has chosen methanol as an acyl acceptor due to the shorter reaction time and simpler separation steps. Stepwise addition of methanol can help to increase biodiesel yield with less denaturing effects on lipase activity.

4.3. Effects of water on lipases-catalyzed biodiesel production

Water has profound influence on catalytic activity and stability of lipases. In some lipases, the catalytic site is hidden within the structure of the enzymes. Most lipases has a lid that covers the enzymes catalytic site which will open at the presence of an water-oil interface resulting in exposure of the enzymes active site. This phenomenon is known as interfacial activation and is critical to ensure substrate gets access to lipases active site [85].

Table 4 summarized the effects of water content on biodiesel yield. Babaki and colleagues found 10 % of water (w / w of oil) in reaction system can achieve a high conversion (nearly 100 %) of canola oil into biodiesel in 96 h using *Rhizomucor miehei* lipase immobilized on epoxy-functionalized silica [86]. Meanwhile, Li and colleagues found an optimal amount of 20 % water (w / w, based on the oil weight) was needed in transesterification using *Rhizopus oryzae* lipase immobilized on macroporous resin or anion exchange resin to achieve 81.8 % and 91.8 % of biodiesel yield, respectively [32]. Due to hydrolysis, a gradual decrease in the yield was observed when the water content exceeded 20 %.

The presence of water also has significant impact on production, refining, storage and

quality of biodiesel fuel [87]. Moisture and free fatty acids in feedstocks reduces formation of fatty acid alkyl esters [88, 89]. Water and FFAs forms soap which reduce catalytic efficiency of enzymes, conversion and yield of biodiesel.

In another study, Chesterfield et al. evaluated the effects of initial water addition on reaction rate and ethyl ester yield, using Novozym 435 (lipase from *Candida antarctica* supported on macroporous acrylic resin). Increase in total initial water content resulted in decrease of more than 90 % of initial reaction rate and yield [90]. Similar findings were observed by Deng et al. [91]. It might be possible that the inherent water content of Novozym 435 was already at or above the moisture level needed by optimal lipase activity [91, 92], indicating that any free water added to the reaction mixture was not conducive to the reaction speed and yield. Therefore, the optimum water content required to reach the max enzymatic activity is dependent on the physicochemical property of feedstock, structure of lipase, immobilized support used and the organic solvent.

In conclusion, 10 % water content (based on oil) is beneficial to achieve the ideal biodiesel yield.

4.4. Effect of solvents on lipase-catalyzed biodiesel production

Solvents (n-hexane, alcohols, etc.) are sometimes added into transesterification reaction system to create a homogeneous mixture to eliminate problems of reactants in two phases system and also to decrease viscosity of the reaction mixture. Solvent can also increase enzyme diffusion rate and reduce substrate mass transfer rate [93]. The effects of solvents on lipase-catalyzed biodiesel production is summarized in Table 5.

Cao et al. found n-hexane and methanol (3 : 1 volume ratio) increased the biodiesel yield up to 93.33 % through one-step *Burkholderia pyrrolica* lipase-catalyzed transesterification process (35 °C in 4 h) [94]. However, methanol and glycerol have poor solubity in hexane resulting in poor stability of lipases. Tertiary alcohols (t-amyl alcohol and t-butyl alcohol), which are not a substrate for lipase, can dissolve both methanol and glycerol [95]. Thus, they can be good solvents for the conversion of oil to biodiesel mediated by immobilized lipase [96, 97]. Babaki and colleagues found 30-40 % of t-butanol resulted in complete methanolysis of rapeseed oil within 96 h by using CALB immobilized on epoxy-functionalized silica [86].

Pollardo et al. found that low concentrations of polar organic solvents (acetone, acetonitrile and tetrahydrofuran) increased FAME yields by 50-100 % as compared to high concentrations of polar organic solvents [98]. Polar organic solvent interact with the enzymes active sites resulted in changes of secondary structures [99]. *Burkholderia cepacia* lipase (BCL) demonstrated alterations in the secondary structure in various solvent systems. In tert-butyl alcohol and tert-amyl alcohol systems, the α-helix content in BCL tended to decrease forming "open" conformation which increased the contact between active site and substrate easier to enter. Thus, BCL had a higher biodiesel yield in tert-butyl alcohol and tert-amyl alcohol systems as compared to n-hexane, petroleum ether and isooctane systems [100].

4.5.Effect of ultrasonic assistance on lipase-catalyzed biodiesel production

The chemical and physical effects of ultrasound are generated by cavitation collapse, and it will produce extreme local conditions, such as high temperature, high pressure,

turbulence, and high shear force. These effects could help to generate conducive emulsions between unmixing fluids, thereby enhancing mass transfer and accelerating the rate of transesterification [101].

Substantial works have been carried out with introduction of ultrasound either in acid and base catalyzed biodiesel synthesis or enzymatic transesterification reaction [102-104]. Most part of these studies used ultrasonic bath in order to provide sonication [104, 105], which has a lower ultrasonic power density, reported by Bhangu et al [106]. In his work, an ultrasonic probe (20 kHz, 400 W) was inserted in the reaction mixture to provide sonication and lipase from *Candida rugosa* was used to catalyze the conversion of canola oil into biodiesel. The result showed that the reaction was carried out completely with the help of ultrasonication in about 1.5 h as compared to 22-24 h without ultrasonic treatment. The advantage of ultrasound assistance to remarkably shorten reaction time was also reported. The reaction time reduced from 28 to 3 h with ultrasonic frequency of 16 kHz and conventional mechanical stirring, lipase concentration of 30 kUnit, methanol-to-oil molar ratio of 4 : 1, and reaction temperature of 40 °C, reported by Patchimpet et al. [23]. Ultrasonic emulsification was also applied in packed-bed reactor to avoid the problem of methanol stratification, and a shorter reaction time and increase of yield were observed [107].

Therefore, ultrasonic assistance has been proved to become a promising method to reduce reaction time of enzymatic catalysis in biodiesel production.

4.6. Other influencing factors

Reaction time is considered to be one of the key factors in biodiesel preparation

catalyzed by lipases. Generally, the yield of FAME increases with the extension of reaction time until reaches equilibrium [108]. Though, overlength reaction time may cause unnecessary cost increases in preparation. Biodiesel synthesis by transesterification of WCO with Nile tilapia visceral lipase as low-cost raw material and catalyst was invesgated [109]. With the reaction time ranged from 4 to 36 h, the yield of FAME increased to 93.24 %, as an equilibrium conversion. However, no more increase of yield was obtained with prolongation of the reaction time beyond 28 h due to the lack of substrate for the enzyme. Fan et al. reached the reaction equilibrium point in a relatively short reaction time (2 h) with a hydroxyl-functionalized ionic liquid as solvent in lipase-catalyzed transesterification [110].

Lipase mainly comes from mesophilic and thermophilic microorganisms, with the optimum activity at 35 - 50 °C and 60 - 80 °C, respectively [111]. Most mesophilic lipases are unstable at temperatures above 70 °C, while thermostable lipases can remain active at up to 100 °C in the presence of organic solvents and detergents [112]. For example, thermostable lipases from the extreme thermophilic bacteria *Caldanaerobacter subterraneus* and *Thermoanaerobacter thermohydrosulfuricus* still exhibit catalytic activity at 90 °C [113]. Lower temperature inhibits catalytic activity, while higher temperature destroys the molecular structure of lipase, resulting in inactivation. Based on this, Wang et al. chose 34.9 °C as the optimum temperature using central composite design of response surface methodology when lipase was immobilized on multiwall carbon nanotube, and reached a highest yield of 95.2 % [114].

Stirring rate is another indispensable factor in lipase catalyzed biodiesel production. Proper agitation can promote the mass transfer at the liquid-liquid interface to fully disperse the methanol molecules in the oil phase since methanol and oil are immiscible. A notable increase in the initial reaction rate and final FAME content, from 78.9 to 94.0 wt %, was observed with stirring speed from 250 to 750 rpm using a low-cost liquid lipase Eversa® Transform 2.0 as the biocatalyst [17], owing to the probability of formation of enzyme-substrate complexes raised by the improved mass transfer [115]. Similar increasing trend was also observed for the conversion of WCO into biodiesel using immobilized lipase [22]. However, excessive stirring speed will not improve the conversion of FAME. Some studies speculated that high stirring speed may promote the evaporation of methanol, resulting in poor interfacial contact between enzyme and substrates, or produce shear force and bring mechanical damage to the enzyme [22, 116].

5. Recent Patents on Biodiesel

Table 6 summarizes some of the recent patents on biodiesel production in the past ten years. Few patents mentioned the use of lipase as catalyst for preparation of biodiesel mainly focusing on novel carriers for lipase immobilization. Most of the patents were related to production of high quality biodiesel such as improvement of stability, decreased in acid value and removal of impurities.

Patent EP2687588A1 provided a method for preparation of biodiesel by using a mixture of free and immobilized lipase [117]. Free lipase was used in the early stage of the catalytic process to produce biodiesel with a conversion rate of more than 90 %. The yield of

biodiesel was improved by using immobilized lipase at a later stage to produce biodiesel with a conversion rate of more than 98 %. This method also described the use of membrane or molecular sieve for dehydration to reduce the acid value of biodiesel to less than 0.5 mg KOH / g. This is beneficial for raw material with water content greater than 0.5 % (based on oil weight) and prevent the tedious alkali neutralization process. Therefore, this invention has good economic and environmental benefits.

Patent 2022227122A1 descibed a method for biodiesel preparation using dual MOFs immobilized lipases [118]. ZIF-8 and macroporous ZIF-8 were used to immobilize *Aspergillus niger* lipase. After 8 hours of reaction, the conversion rate of ANL@ZIF-8 and ANL@M-ZIF-8 were 80 % and 92 %, respectively. The invention also described for the first time a larger scale preparation of biodiesel (200 – 500 g) using MOFs which provided evidence on the possibility for industrial preparation of biodiesel by MOF immobilized lipase.

Besides MOF, some recent patents have discussed the use of other novel carriers for lipase immobilization. Patent CN104404023A described the used of gluconic acid modified magnetic particles as carriers to immobilize *Candida antarctica* lipase [119]. The reusability and storage stability of immobilized lipase had greatly improved as compared to that of free form. Biodiesel conversion rate of more than 90 % can be achieved following 7 times of repeated usage.

Patent CN105274157A revealed the usage of static emulsion silica gel microspheres to immobilize *Candida Antartica B* and *Burkholderia Cepactia* [120]. The static emulsion silica

gel is composed of a large number of small droplets containing lipase which enhances the interfacial enzymatic activity with a biodiesel yield of 95.22 %. The emulsion structure can be retained for at least 20 times of repeated use (480 hours of continuous cycle operation) with a biodiesel yield of more than 75 %.

Patent EP20170164255 employed phosphate and/or sulfate of lithium and aluminum as heterogeneous acid catalysts for both transesterification and esterification in batch and continuous flow reaction systems to prepare biodiesel with yield exceeding 80 %. More importantly, the catalyst invented by this patent could be used alone or in combination with other common alkaline catalytic materials, such as magnesium oxide, alumina and sodium oxide, to simultaneously promote esterification and transesterification reactions in a biodiesel yield of 87.9 % [121]. Patent EP20150700342 provided method for production of oxidation-stabilized biodiesel using a combination of antioxidants. By combining antioxidants of different potency, i.e. at least one C1 - C4 alkylated monophenol and at least one C1 - C4 alkylated bisphenol, the oxidative stability of biodiesel can be greatly improved (storage more than 90 days) at low antioxidant content (e.g. 100-200 ppm), and by adding dihydroxyphenol, such as tert-butylhydroquinone (TBHQ), this effect can be further improved. This production method also inhibitted corrosion of the production equipment (tanks, pipelines, etc.) [122].

Biodiesel produced from transesterification needs to be purified in order to remove catalyst, water, glycerol and excess alcohol. In addition, biodiesel produced from waste cooking oils also contains polar compounds such as dimers, mono and diacylglycerols and

free fatty acids that need to be removed [123]. Wet washing using filter aids (such as diatomite) is one of the main technologies used for industrial purification of biodiesel. However, treatment of the filter aid increases production costs and brings operational difficulties. In order to improve the quality of biodiesel produced from plant oil (which has solid particles derived from the plant metabolism) and reduce the cost of purification, patent US10266781B2 proposed a filter-free purification technology for the removal of solid particles (mixture consisting of monotriglycerides and sterol glycosides) [124]. After contact with the dilute acid stream, the ester rich stream went through washing step and sufficiently intense stirring (greater than 800 rpm) to remove impurities, alternating with the decanting step to remove the dense phase from the stream. The resulting flow was exposed to drying and cooling process, which is then filtered without the use of filter aids. In the purification process of biodiesel extracted from food grade soybean oil, this method could reduce the impurity concentration to an acceptable value (10.5 mg / kg, lower than the maximum limit of 24 mg / kg required by Brazilian regulatory authority).

Patent US10982159B2 proposed a method to reduce monoglycerides, especially saturated monoglycerides, in crude biodiesel [125]. Alkaline earth metal hydroxide solution (sodium hydroxide) was added to crude biodiesel to hydrolyze glycerol esters in biodiesel which can be removed by centrifugal separation (< 0.3 % of MAG in biodiesel).

Reactor technologies and process control are also important part of lipase-catalyzed biodiesel production. Patent US11034984B2 introduced on-line dehydration system in lipase-catalyed preparation of biodiesel from short-chain alcohol to form a gas recycle unit.

The volatile gas in the enzyme reactor enter low-temperature absorption tank prior to return to enzyme reactor. Water in the enzyme reactor could be continuously taken out, thereby improving the conversion rate of the reaction [126]. Patent US2014259886A1 implemented a model predictive control algorithm using an advanced process controller to control many aspects of biodiesel production system including material flow rate system, glycerol and methanol treatment system, separation system, etc [127]. The invention can be applied in a wide range of environments, in a variety of vegetable oil sources, and in any required industrial, commercial, private or other systems.

6. Future perspectives

The major hurdle in application of immobilized lipases for large scale biodiesel preparation is the cost of the immobilized lipases. Development of low-cost and stable immobilized lipase carrier will be a mainstream research direction in future. This is essential for the overall production costs and benefits of industrial-scale biodiesel production. In addition, new immobilization strategies such as multi-enzyme co-immobilization system should be explored to improve the stability and reusability of immobilized enzyme and also to improve the yield of biodiesel in complex substrates environment.

7. Conclusions

Lipase catalyzed transesterification is a green and efficient way for biodiesel preparation. Most current work in biodiesel preparation focuses on the second- and third-generation feedstocks which are non-edible oil and waste cooking oil. Meanwhile, fourth generation

feedstocks is still in its infancy. Although most large scale biodiesel production is achieved using free enzymes, immobilization technology including development of new carriers (cost effective and stable carriers) and immobilization strategies (multi-enzyme co-immobilization) should be explored for continuous production of high quality biodiesel. Some novel carriers like MOF, COF and magnetic nanoparticle have been applied in lipase immobilization and show good catalytic ability. They have potential to replace homogeneous or heterogeneous acidic and alkaline catalysts in large-scale biodiesel production. Many studies have been conducted to optimize lipase catalyzed transesterification for biodiesel preparation. In terms to reaction conditions, high molar ratio of alcohol to oil, appropriate amount of lipase content, low water content in feedstock and ultrasonic processing have been proven to have a positive impact on the yield of biodiesel.

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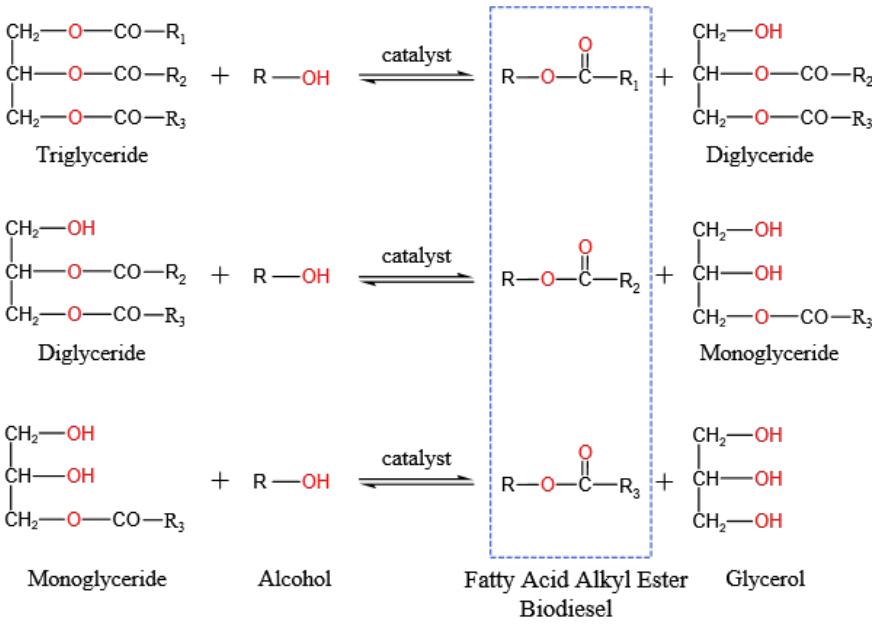


Figure 1 The chemical equation of transesterification in biodiesel preparation

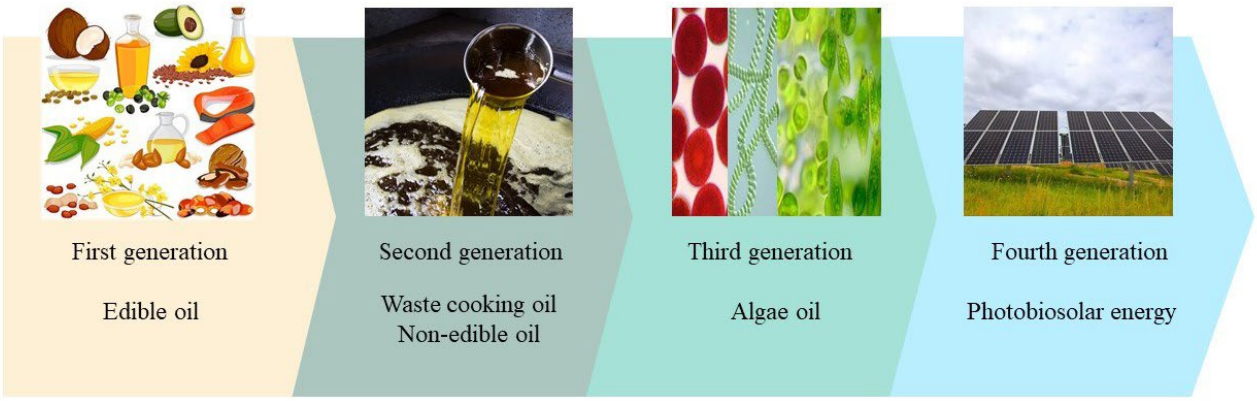


Figure 2 Trend in biodiesel feedstock

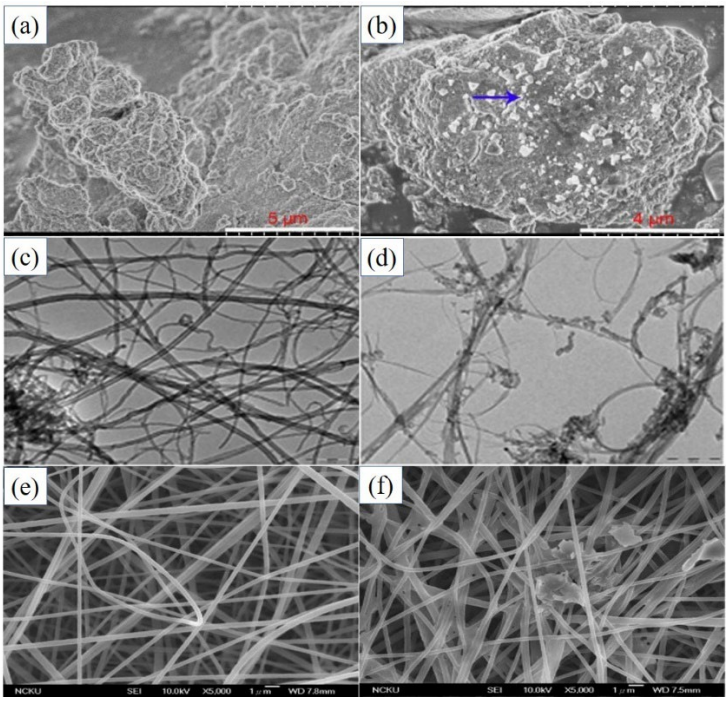


Figure 3 Morphology of commonly used carriers and immobilized lipase. Surface profile of SiO_2 (a) and lipase@SiO_2 (b) by scanning electron microscope [36]. Transmission electron microscope images of 1,3-propane-diamine functionalized carboxylated single-walled carbon nanotubes and CaL-B immobilized carbon nanotubes (c and d) [128]. Field emission scanning electron micrographs polyacrylonitrile nanofibrous membrane and nanofibrous membrane with immobilized *P. cepacia* lipase (e and f) [129].

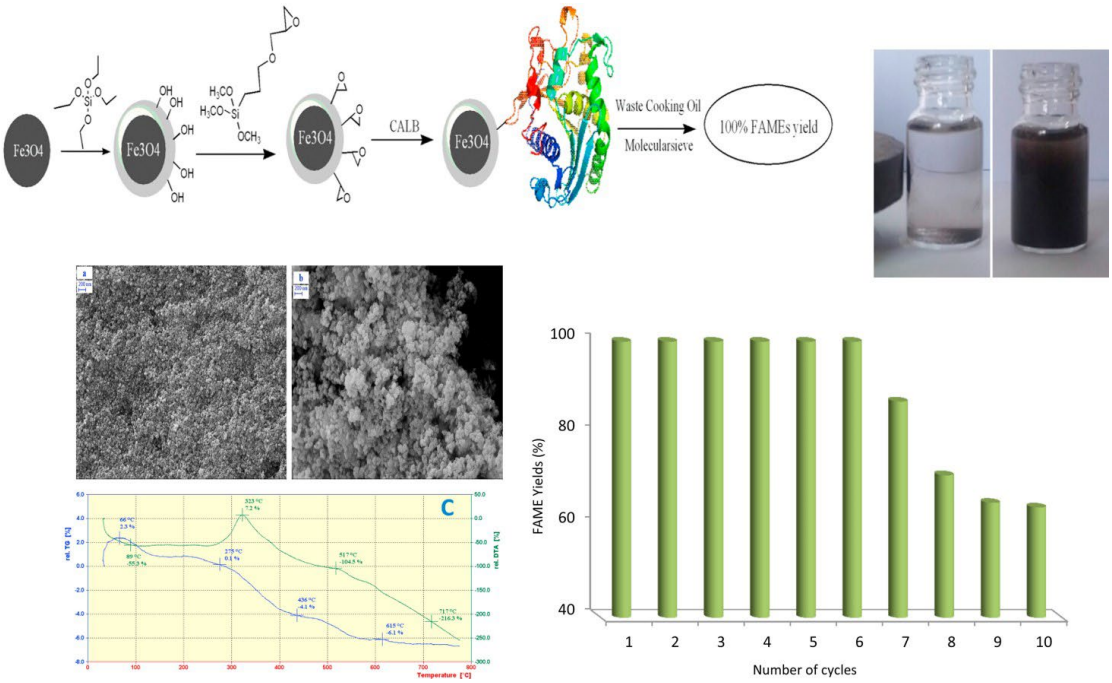


Figure 4 Lipase immobilized on functionalized magnetic nanoparticles (MNPs) to catalyze biodiesel synthesis [130].

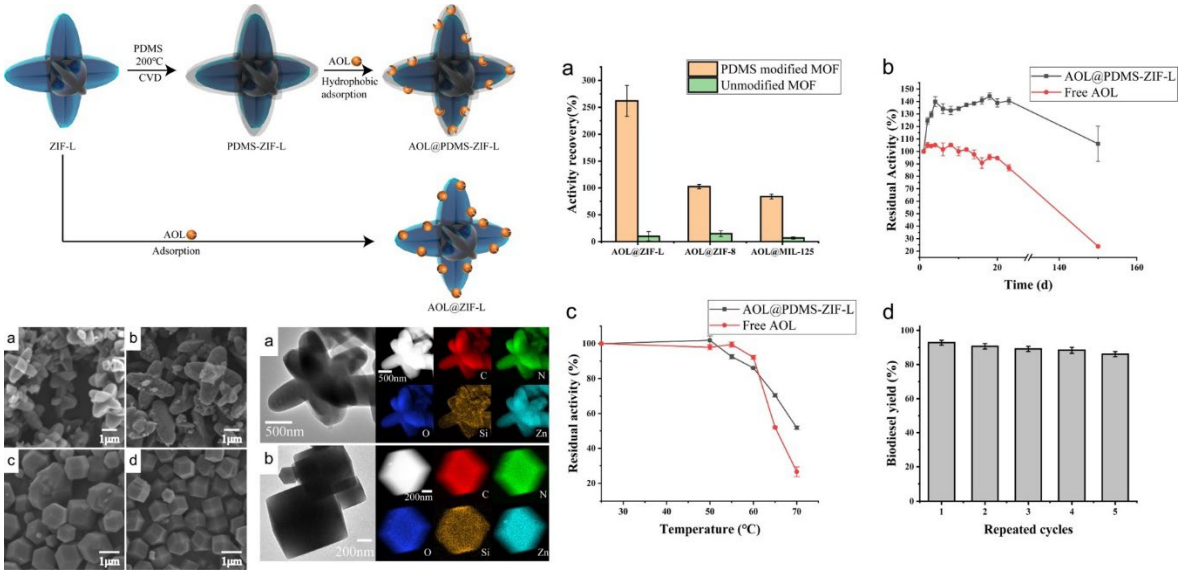


Figure 5 ZIF-L was coated with polydimethylsiloxane (PDMS) and used for immobilization of *Aspergillus oryzae* (AOL) lipase for biodiesel production [131]

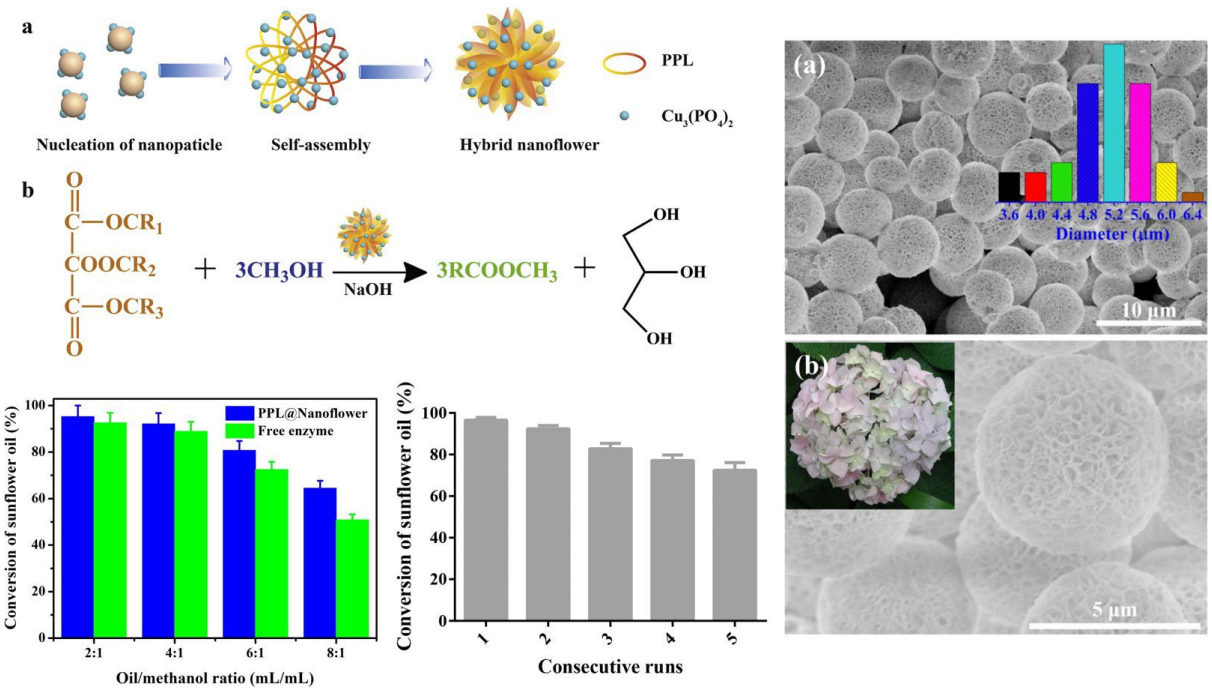


Figure 6 Lipase-inorganic hybrid nanoflowers have the potential to be an economically viable biocatalyst for biodiesel production [57]

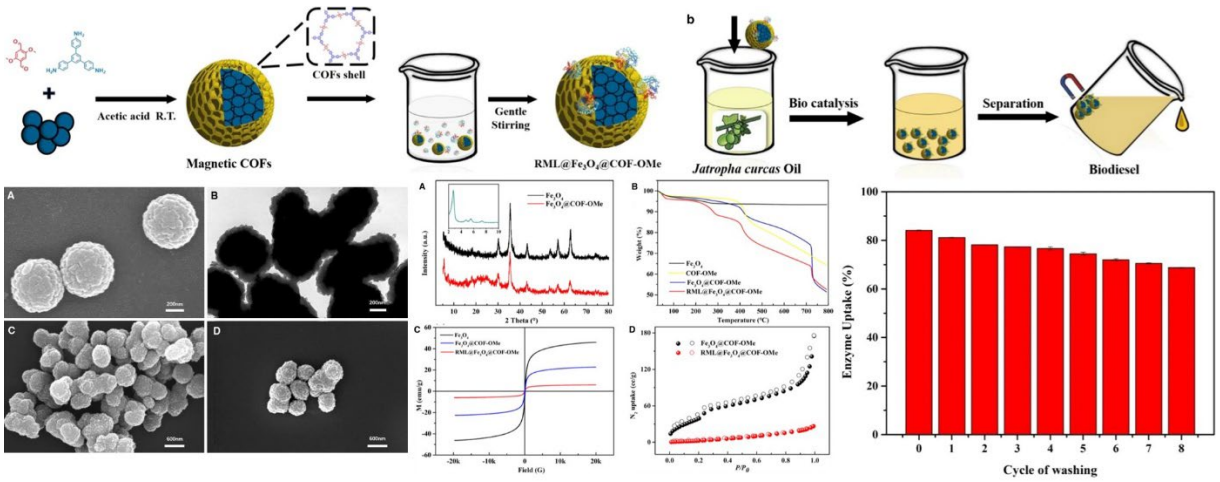


Figure 7 Core-shell magnetic COF composite (Fe3O4@COF-OMe) for immobilization of RML (*Rhizomucor miehei* lipase) for its application in biodiesel production [65]

Table 1 Biodiesel preparation with immobilized lipase

Source of lipase	Immobilization method	Support	Feedstock	Methanol-to-oil molar ratio	Reaction time / h	Temp / °C	Yield / %	Reference
Aspergillus oryzae	cross-linking	Fe_3O_4 magnetic nanoparticles embedded hybrid nanoflowers	soybean oil	4:1	24	40	87.6	56
Aspergillus oryzae	adsorption	hydrophobic modified ZIF-L	soybean oil	4:1	24	40	94.17	33
G. thermocatenulatus	adsorption	silica from bio-silicification	rapeseed oil	5:1	12	45	91.04	132
Aspergillus niger	pore diffusion	macro-ZIF-8-PDMS	soybean oil	4:1	24	45	88	34
T. lanuginosus	covalent binding	Microporous polymeric matrix	sunflower oil	6:1	5	65	63.7	35
Burkholderia cepacia	covalent binding	aminated magnetic nanoparticles	soybean oil	4:1	12	45	96.8	37
Candida Antarctica Lipase B	adsorption	Fe_3O_4 magnetic hybrid sol-gel nanocomposite	WCO	4:1	30	40	96	48
Candida Antarctica Lipase B	adsorption	Carboxylation of multiwalled carbon nanotubes	rapeseed oil	3:1	24	40	92	41
Rhizomucor miehei	absorption	core–shell magnetic COF composite	*Jatropha curcas* oil	3:1	48	50	67.8	65

Enterobacter cloacae	covalent binding	Zr-MOF/PVP electrospun nanofibrous membranes	*Ricinus communis* oil	3:1	12	50	83	[42]
Candida rugosa	absorption	$NiFe_2O_4$ nanoparticles	algal lipids	4:1	24	40	80	[25]

Table 2 Effect of concentration of immobilized lipase on biodiesel yield

Lipase concentration	Biodiesel yield	Other conditions (alcohol-oil molar ratio\reaction time\temperature)	Reference
2 %	94.37 %	4 : 1 \ 24 h \ 45 ℃, water content 9 %	[33]
20 %	91.04 %	5 : 1 \ 12 h \ 45 ℃	[36]
1 %	93.1 ± 0.2 %	3 : 1 \ 12 h \ 35 ℃, water content 5 %	[22]

Table 3 Effects of alcohol addition on biodiesel yield

Alcohol type	Alcohol-oil molar ratio	Times of addition	Other conditions (catalyst amount \ temperature \ time)	Yield	Reference
Methanol	4 : 1	1	0.5 g \ 40 ℃ \ 24 h	97 %	[48]
Methanol	5 : 1	3	37 ℃ \ 60 h	97.7±4.89 %	[80]
Fusel alcohol	3 : 1	3	14 % \ 40 ℃ \ 24 h	90.4 %	[22]
Ethanol	2 : 1	5	30 ℃ \ 200 min	58.16 %	[84]

Table 4 Effect of water on biodiesel yield

Water amount (based on oil)	Other conditions (catalyst amount \ temperature \ time)	Yield	Reference
10 %	0.3 mg \ 50 ℃ \ 96 h	Near 100 %	[86]
20 %	6 IU per gram oil \ 37 ℃ \ 60 h	91.8 %	[32]
From 0.08 to 1.77 %	14 % \ 40 ℃ \ 24 h	Drop by more than 90 %	[90]
2.53 % [a]	10 % \ 40 ℃ \ 24 h	92.2 %	[91]

[a]Water content in this reaction was based on the lipase preparation (wt%)

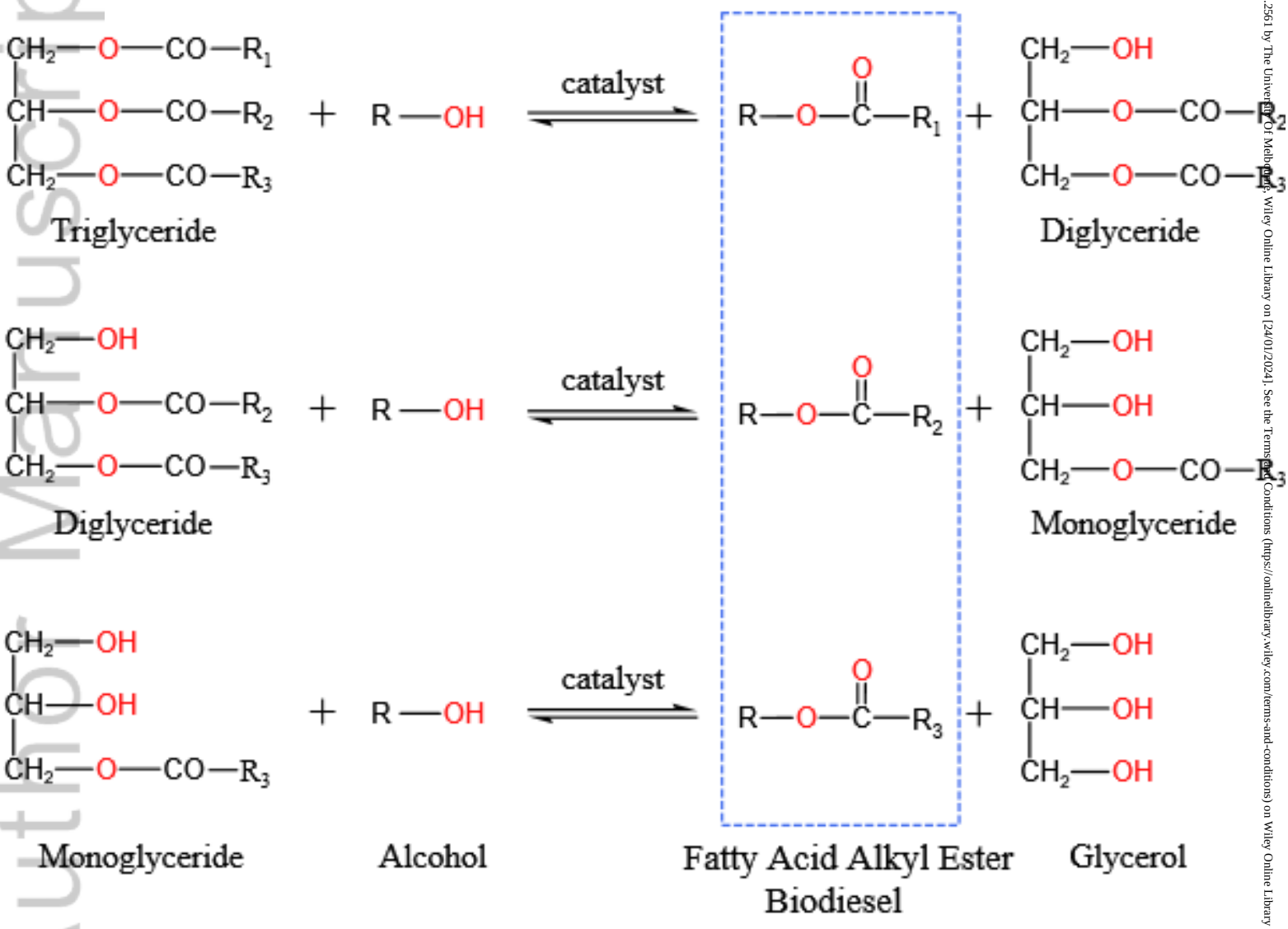
Table 5 Effect of organic solvent on biodiesel yield

Solvent type	Amount	Other conditions (catalyst amount \ temperature \ time)	Yield	Reference
n-hexane	molar ratio of n-hexane to methanol 3: 1	2.5 g \ 35 ℃ \ 4 h	93.33 %	[94]
t-Butanol	50 % (based on oil weight)	300 mg \ 50 ℃ \ 24 h	57.4 %	[86]
Acetonitrile	2 mL/ gram oil	40 ℃	37.9 %	[98]
tert-Amyl alcohol	0.96 mL	87.6 mg \ 40 ℃ \ 12 h	60.1 %	[100]

Table 6 Recent patents on biodiesel production

Patent	Field	Title	Year	Reference
EP2687588A1	Lipase-catalyzed preparation	METHOD FOR PREPARING BIODIESEL	2014	[117]
WO2022227122A1	Lipase-catalyzed preparation	METHOD FOR PREPARING BIODIESEL BY USING DUAL MOFS IMMOBILIZED LIPASE	2022	[118]
CN104404023A	Lipase-catalyzed preparation	Preparation method of magnetic carrier immobilized lipase, and method for preparing biodiesel under catalysis of magnetic carrier immobilized lipase	2015	[119]
CN105274157A	Lipase-catalyzed preparation	Method for producing biodiesel by utilizing immobilized lipase and adopting static emulsion method	2016	[120]
EP20150700342	Preparation	OXIDATION-STABILIZED BIODIESEL	2016	[122]
EP20170164255	Catalyst	USE OF HETEROGENEOUS ACID CATALYSTS BASED ON MIXED METAL SALTS TO PRODUCE BIODIESEL	2017	[121]
US10982159B2	Purification	Method for reducing the content of saturated monoglycerides in a raw biodiesel	2021	[125]

US11034984B2	Catalyst	Method for improving yield of enzymatic preparation of biodiesel from greases	2021	[126]
US2014259886A1	Control system	ADVANCED PROCESS CONTROL OF A BIODIESEL PLANT	2014	[127]



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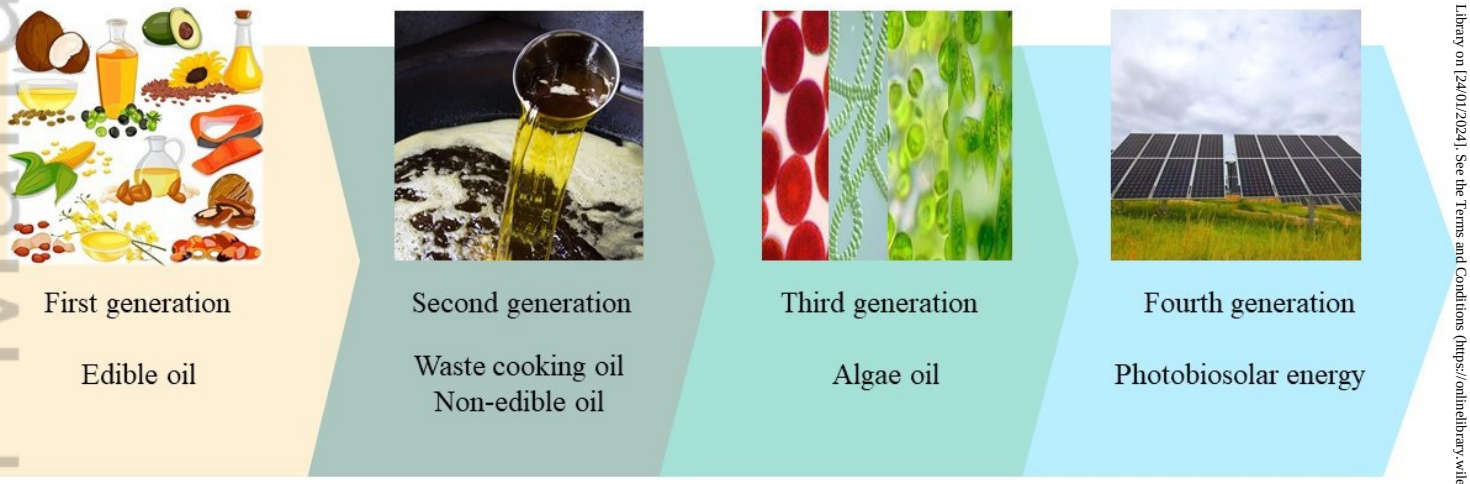

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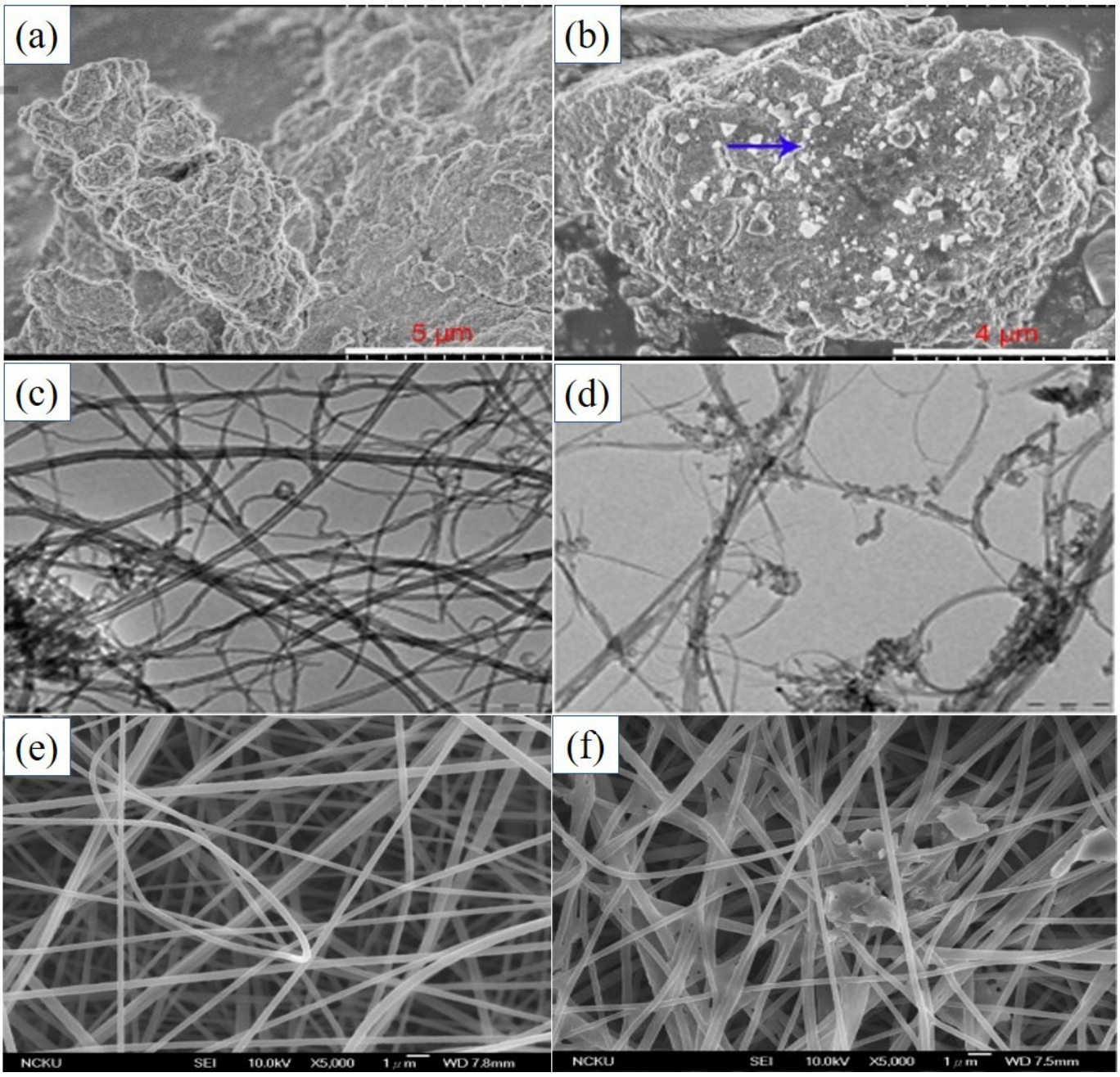

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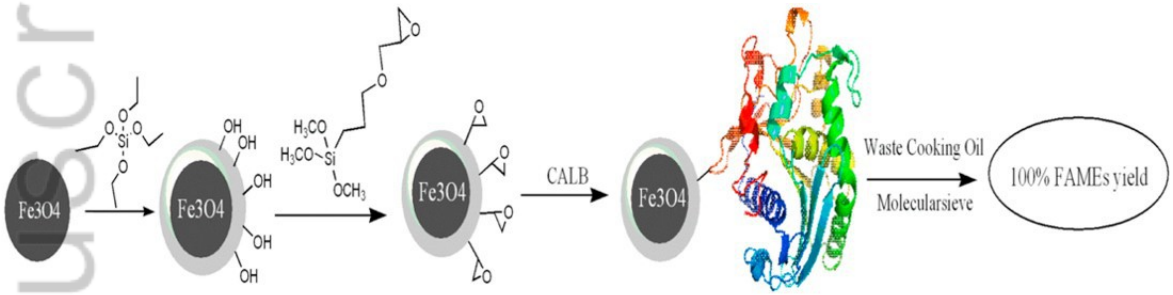

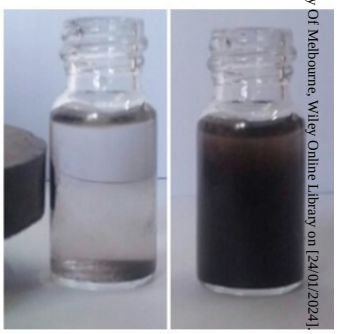

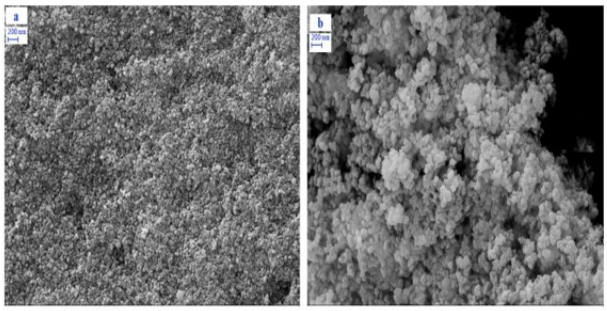

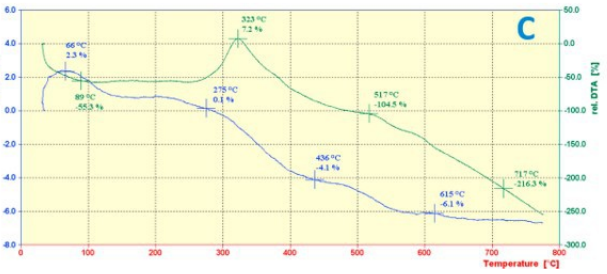

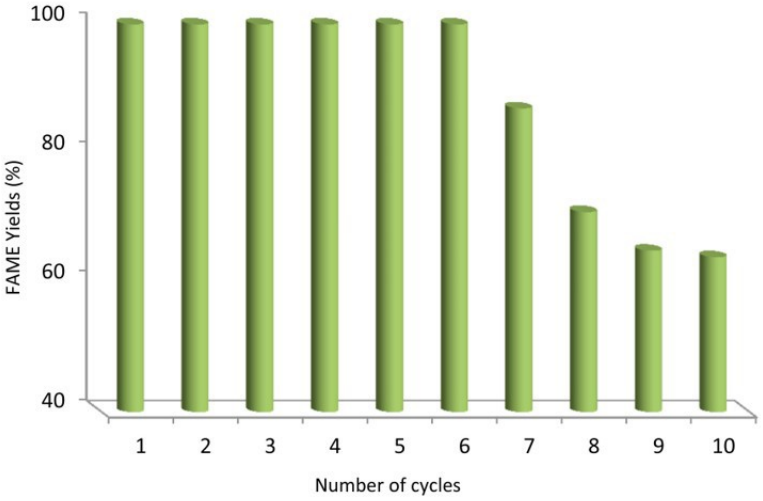

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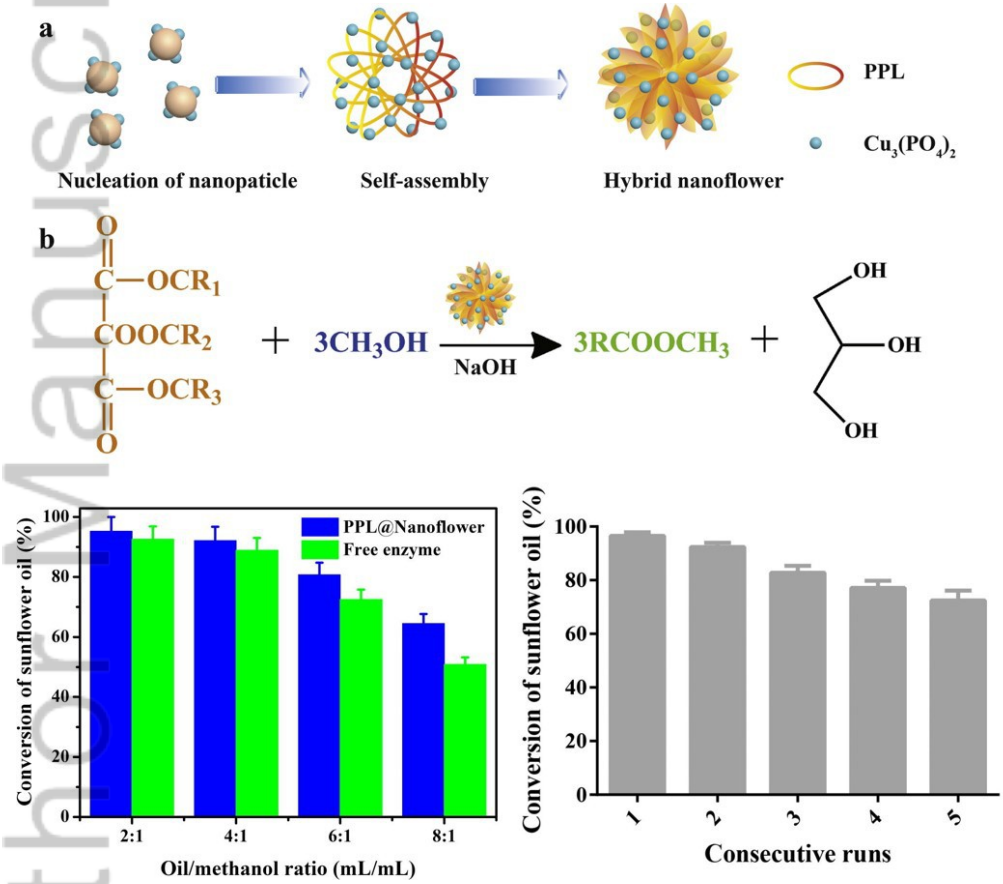

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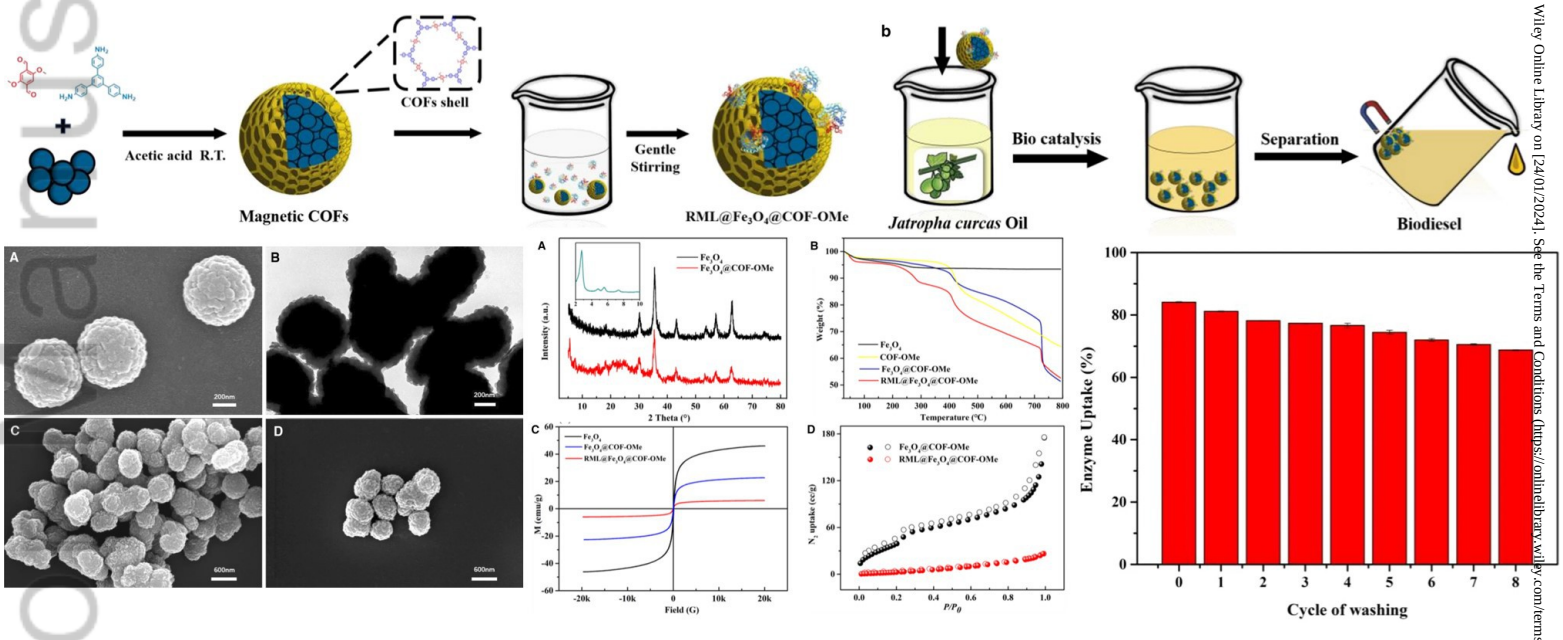

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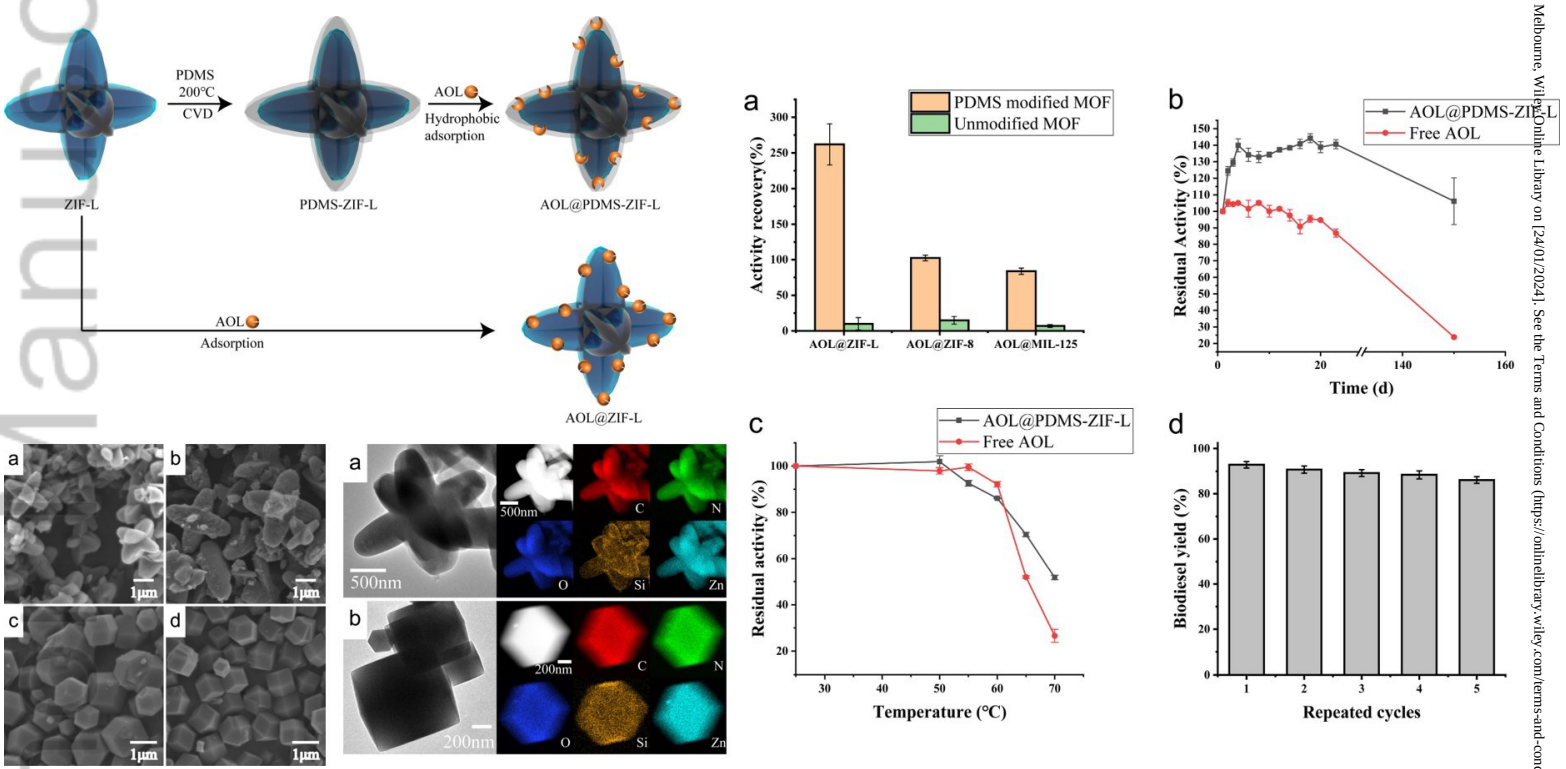

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EP20170164255	Catalyst	USE OF HETEROGENEOUS ACID CATALYSTS BASED ON MIXED METAL SALTS TO	2017	[121]

		PRODUCE BIODIESEL		
US10982159B2	Purification	Method for reducing the content of saturated monoglycerides in a raw biodiesel	2021	125
US11034984B2	Catalyst	Method for improving yield of enzymatic preparation of biodiesel from greases	2021	126
US2014259886A1	Control system	ADVANCED PROCESS CONTROL OF A BIODIESEL PLANT	2014	127