Nano-Immobilized Biocatalysts for Biodiesel Production from Renewable and Sustainable Resources

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Abstract: The cost of biodiesel production relies on feedstock cost. Edible oil is unfavorable as a biodiesel feedstock because of its expensive price. Thus, non-edible crop oil, waste oil, and microalgae oil have been considered as alternative resources. Non-edible crop oil and waste cooking oil are more suitable for enzymatic transesterification because they include a large amount of free fatty acids. Recently, enzymes have been integrated with nanomaterials as immobilization carriers. Nanomaterials can increase biocatalytic efficiency. The development of a nano-immobilized enzyme is one of the key factors for cost-effective biodiesel production. This paper presents the technology development of nanomaterials, including nanoparticles (magnetic and non-magnetic), carbon nanotubes, and nanofibers, and their application to the nano-immobilization of biocatalysts. The current status of biodiesel production using a variety of nano-immobilized lipase is also discussed.

Keywords: nanomaterials; nano-immobilized enzyme; immobilization; biodiesel; lipase

1. Introduction

Demand for energy has risen continuously [1], and the world’s oil reserves can be depleted by 2050 with the current consumption rate [2]. This causes the price volatility of petroleum-based fuels, and has stimulated research on the renewable and sustainable production of biofuels. Biomass can be a promising feedstock to meet current and future demand for renewable and sustainable fuel. Biofuels generated from biomass include biodiesel, bioethanol, biohydrogen, and biomethane. Bioethanol is produced based on a three-step process: pre-treatment, saccharification, and fermentation. Bioethanol has been produced using sugarcane, corn, wheat, and potatoes, which has resulted in an increase in world grain prices. In order to avoid the moral issue of using food for fuel production, lignocellulosic biomass is being considered as the feedstock for the production of bioethanol. Biodiesel is produced by transesterification to convert long-chain triglycerides into fatty acid methyl esters (FAMEs). Biodiesel is an attractive alternative fuel because it is nontoxic, renewable, and biodegradable. Its combustion emission profile is favorable because of the low emissions of CO, NOx, sulfur content, and particulate matter [3]. The benefits of biofuels over traditional fuels are greater energy security and reduced socioeconomic issues of environmental pollution [4]. Biodiesel is expected to minimize the greenhouse effect [5]. Furthermore, compared to petro-diesel, biodiesel has shown a higher combustion efficiency, flash point, and cetane number, and better lubricant efficiency.

The renewable feedstock for biodiesel production is divided into three types: edible oil, non-edible oil, and waste edible oil. Edible oil, such as vegetable oil, as the raw material is unfavorable for biodiesel production because of its high cost and the issue of using food for fuel production [6]. Recently, alternative biofuel resources, such as non-edible oil, waste oil, and microalgae oil have been explored to address the issues of the edible oil-derived biodiesel production. Jatropha, karanja, mahua,
polanga, rubber, and castor oil are non-edible oils for biodiesel production and are cheaper than edible oils [7]. Waste oil, such as waste cooking oil, grease, and soap stock, is an abundant feedstock with low cost. The total amount of waste cooking oil is 16.6 million tons per year [8]. Microalgae is a non-edible crop that can produce 25 times more oil than plants. Some microalgae can accumulate oil up to 50% by weight of dry biomass [9]. Microalgae is an environmentally friendly biomass due to its high capacity for carbon dioxide fixation, and it has a higher growth rate than terrestrial plants [9,10].

Transesterification reactions of oils require short-chain alcohols as an acyl acceptor in the presence of catalysts, such as acid/base chemocatalysts or enzymes. Non-edible oil and waste cooking oil are problematic in the conventional biodiesel production process using alkaline catalysts because they have a high content of free fatty acid (FFA) [11]. A feedstock with high FFA content can be used in the lipase-catalyzed process [12]. The lipase-catalyzed process has environmental and economic advantages, such as a lower energy consumption requirement, a low post-treatment cost, and a broader feedstock specificity than the chemical catalytic process [13,14].

One drawback of the lipase process is the high cost of the enzyme. Thus, the use of immobilized lipase is important to reduce production cost [14]. In addition, the immobilized lipase is easier to handle than free lipase, and in some cases, it shows improved performance in terms of pH tolerance, substrate selectivity, thermal stability, and functional stability [15,16].

In general, enzymes are immobilized on macro/micro materials for various applications, including use in biosensors, biofuel production, and drug delivery [15]. The immobilized enzymes on macro/micro materials have some technical issues, such as distortion of protein configuration, steric hindrance, and a low diffusion rate [17]. To address these issues, many researchers have used nanomaterials as an enzyme support. Nanomaterials as enzyme immobilization agents have many advantages. The large surface-area-to-volume ratios of nanomaterials is one of the main advantages, which allows high enzyme loading and increases mass transfer [18]. In aqueous suspensions, enzyme-bound nanomaterials exhibit Brownian motion, which is different from free enzymes and endow higher enzyme activity than free enzymes [16,19]. In this paper, we review and discuss the advances in enzyme immobilization techniques (Section 2), lipase immobilization using nanostructured materials (Section 3), and biodiesel production using nano-immobilized lipase (Section 4).

2. Enzyme Immobilization Techniques

2.1. Cross-Linking Immobilization

The cross-linking immobilization process attaches the enzymes to one another using a multifunctional reagent [16]. This method does not require a support matrix and the resulting enzyme maintains 100% activity [20]. However, a loss of enzyme activity via conformational change can occur during immobilization. The control of the cross-linking reaction is difficult; thus, it is not easy to obtain an enzyme with high activity retention [16]. Sheldon et al. [20] developed a cross-linked enzyme aggregates (CLEAs) method for an effective enzyme immobilization. The CLEAs method is very simple and involves enzyme precipitation from aqueous solutions by the addition of non-ionic polymers, salts, or organic solvents. The lipases from Thermomyces lanuginosus and Rhizomucor miehei were precipitated with ammonium sulfate in the presence of SDS as surfactant followed by enzyme crosslinking with glutaraldehyde [21]. The hydrolytic activities of the CLEAs and the lipases were enhanced threefold and twofold, respectively, over those of free enzymes.

2.2. Adsorption Immobilization

The adsorption of enzyme on the surface of a support is an old technology and a simple method. It is based on a physical binding mechanism, such as a dipole-dipole, hydrophobic, or van der Waals interaction or hydrogen bonding [18,22]. Physical binding was performed in relatively ambient conditions and showed a high enzyme loading [16,22]. Adsorption immobilization does not provide a high stability and might cause a loss of enzyme molecules during operation and washing.
because of weak binding between the enzyme and the supports [23]. Tang et al. [23] immobilized glucose oxidase (GOD) on a platinum nanoparticle-modified carbon nanotube (CNT) electrode via adsorption. To avoid a loss of GOD, the surface of the GOD/Pt/CNT electrode was coated by Nafion. The Nafion/GOD/Pt/CNT electrodes showed good characteristics: a short response time (~5 s), a large current density (1.176 mA/cm²), a large determination range (0.1–13.5 mM), and high sensitivity (91 mA/M·cm²). After 22 days, the stability of immobilized GOD on the Nafion-coated Pt/CNT electrodes still maintained a 73.5% value based on an initial response. To overcome the enzyme leaching, the enzyme was immobilized into the pores of polyanilin nanofibers (PANFs) through a three-step process, which included enzyme adsorption, precipitation, and cross-linking (EAPC) [24,25]. Kim et al. [24] have compared GOD activity and the stability of enzyme adsorption (EA), enzyme adsorption and cross-linking (EAC), and EAPC. The relative activities of EA, EAC, and EAPC were 11%, 24%, and 100%, respectively, and EAPC showed the highest thermal stability at 50 °C. The high stability of EAPC can be explained by the increase in enzyme loading and the prevention of enzyme leaching and denaturation.

2.3. Covalent Immobilization

The covalent immobilization of the biocatalyst is the attachment of enzyme to the nanomatrix by covalent bonding between the enzyme and the supports [16]. The strong binding of enzyme to the support matrix via the covalent bond prevents enzyme leaching from the surface and improves the thermal stability in some cases [18,22]. This technique, however, often provokes the deactivation of enzyme because of the conformational restriction of the enzyme by covalent binding [22,26]. Hong et al. [26] immobilized α-chymotrypsin on an amine-functionalized superparamagnetic nanogel by covalent binding and compared the stability of the immobilized enzyme and free enzyme. The optimal pH value of the free enzyme and the immobilized enzyme was at pH 7.8, and the pH profile was similar. With respect to thermal stability, the free enzyme had no activity above 75 °C, but the residual activity of the immobilized enzyme retained 88.7% until 85 °C. The storage stability of the free enzyme dramatically decreased down to almost zero after 22 days at 25 °C. In contrast, the activity of the immobilized enzyme decreased only 10% of its initial activity during 35 days at 25 °C. Kinetic parameters (K_m and V_max) for native and immobilized enzymes were also determined. The K_m value of the immobilized enzyme was 1.57 times higher than that of the native enzyme, whereas the V_max value of the immobilized enzyme was smaller than that of the native enzyme. This indicates that the substrate affinity of the immobilized enzyme decreased in comparison with the native enzyme, which might be related to a steric effect and diffusion limitation due to immobilization onto the supports. Kim et al. [27] developed an enzyme aggregate coating method on nanofibers. In the first step, α-chymotrypsin (CT) molecules were attached onto the surface of nanofibers via covalent binding. Then, additional enzyme molecules were cross-linked to the attached enzyme molecules via glutaraldehyde treatment, which forms the enzyme aggregate coating. The initial activity of the enzyme aggregate increased nine times compared to the case of only a covalent immobilization enzyme, because the enzyme coating consisted of multiple layers of enzymes on the nanofibers. With respect to enzyme stability, a CT-aggregate-nanofiber was still stable without loss of its activity during a month. This technology can be applied to various nanomaterials and has potential applications in bioconversion, bioremediation, and biosensors [27–29].

2.4. Entrapment Immobilization

The entrapment technology entraps the enzyme in a porous gel or fibers [16,18]. From a TEM analysis, the diameter of entrapped magnetite crystallites was approximately 20 nm. The entrapment process can protect enzyme activity because of the indirect contact with the confined environment, which minimizes the effects of gas bubbles, mechanical sheer, and hydrophobic solvents [18]. Entrapment immobilizations using nanoparticles are generally based on the reverse-micelle or sol-gel technique [30–34]. Reetz et al. [32] reported the simultaneous entrapment of a lipase Amano PS (from
Pseudomonas cepacia) and nanostructured magnetite (Fe₃O₄) containing hydrophobic sol-gel material. The colloidal magnetite-containing lipase was characterized by enzyme activity and was 2–3 times higher than that of free enzyme. Yang et al. [33] reported the simultaneous entrapment of horseradish peroxidase (HPR) and spherical silica-coated nanomagnetite. This technique consisted of two procedures performed in two steps: reverse-micelle and sol-gel processes. Entrapment immobilization by reverse-micelle microemulsion can generate uniform-size nanoparticles, which leads to a strong monodispersion of nanoparticles. Compared to free HPR, the nanoentrainment-immobilized HPR exhibited high stability toward temperature and pH changes. However, this method required a rigorous optimization process because it was difficult to control the reverse-micelle size [30]. Additionally, the sol-gel process involved harsh reaction conditions for the entrapment immobilization [35]. The new entrapment technology for enzymes and nanoparticles using biomagnetic silica was performed under mild conditions with an improvement in enzyme stability, immobilization efficiency, and loading density [35,36].

The single-enzyme nanoparticles (SENs) method was developed by Kim and Grate in 2003 [37]. Each enzyme molecule was surrounded by a porous composite organic/inorganic network with a thickness of a few nanometers. The SENs method involves a two-step procedure: first, vinyl polymers were grafted onto the enzyme surface by radical polymerization; then, the polymer network around the enzyme surface was formed by silanol condensation. During the vinyl polymerization, the thickness of the polymer network could be controlled. The catalytic efficiency of SENs-CT based on a $K_{cat}/K_{m}$ value of SENs-CT (3.44 × 10⁵ M⁻¹ s⁻¹) value decreased to half that of free enzyme. However, the $K_{m}$ value of SENs-CT was similar to that of free enzyme. This indicates that the SENs had no mass-transfer limitation for the substrate. Yan et al. [38] reported a simple development of nanogels containing a single enzyme. The SENs of HPR showed similar Michaelis–Menten parameters ($K_{m}$ and $K_{cat}$), but the thermal stability of the SENs was enhanced up to 65 °C. Moreover, the enzyme activity was maintained in the presence of polar organic solvents, such as methanol, tetrahydrofuram, and dioxane. The enzyme immobilization techniques are summarized in Figure 1 [18,22].

![Figure 1](image-url)

**Figure 1.** Comparison of different enzyme immobilization techniques (modified from the references of [18,22]).

3. Development of Nano-Immobilized Lipase Biocatalyst

Lipases have been obtained from fungi, bacteria, animals, and plants [39]. They catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at the oil–water interface. In addition to hydrolytic catalysis, lipases catalyze synthetic reactions, such as esterification and transesterification [40]. Lipases
have been used as catalysts in the cosmetics, chemical synthesis, detergent formulation, food, and pharmaceuticals industries and in biodiesel production \[40,41\]. In lipase-catalyzed biodiesel production, the major issue is the high enzyme cost. Thus, the use of immobilized lipases is important for operational cost reduction by the increasing of enzyme reusability. Additionally, in some cases, immobilized lipases have shown higher enzyme activity than free lipases \[41\]. Recently, many researchers are using nanomaterials as a carrier for enzymes. Nanomaterials have many advantages as an enzyme carrier. The large surface-area-to-volume ratio of nanomaterials allows for high enzyme loading and enhanced mass transfer \[18\]. In an aqueous suspension, enzyme-bound nanomaterials exhibit Brownian motion, which exhibits higher enzymatic activity than the free enzyme. The illustration of enzyme immobilization to nanomaterials is presented in Figure 2.

**Figure 2.** Illustration of enzyme immobilization techniques to nanoparticles (A) (modified from the references of \[25,42\]), nanotubes (B) (modified from the references of \[43\]), and nanofibers (C) (modified from the references of \[44\]). APTS: 3-aminopropyltriethoxysilane, TEOS: tetra-ethoxy silane, PEOS: poly-ethoxy silane, PAN: polyacrylonitrile, EDC: N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride.

3.1. **Nanoparticles-Based Lipase Immobilization**

3.1.1. Non-Magnetic Nanoparticles

Nanoparticles with a 1–100 nm nanoscale diameter have been used for biological applications \[45\]. Nanoparticle-based carriers have increased the catalytic efficiencies of various lipases, including enhanced activity, stability, and reusability due to their unique size and physical properties \[12,46\].
Among nanoparticles, the non-magnetic nanoparticle-attached enzyme is well dispersed in the reaction solution. Thus, regeneration for reuse is often a difficult task and requires high-speed centrifugation for a long time. Non-magnetic nanoparticle carriers include zirconia, silica, polystyrene, chitosan, and polylactic acid (Table 1) [39,47–51]. When immobilized on a hydrophilic inorganic surface, lipases have poor binding ability because a polypeptide chain “lid” of lipases secludes the catalytic site [52]. Chen et al. [47] immobilized Pseudomonas cepacia lipase (PCL) on zirconia nanoparticles grafted with various carboxylic acids (valeric acid, capry acid, stearic acid, oleic acid, linoleic acid, and 1,10-decanedicarboxylic acid). The stearic acid as carboxylic acid surfactant exhibited better activity and enantioselectivity. The initial activity of PCL-stearic-ZrO$_2$ was 10.5 and 16.6 times higher than the unmodified ZrO$_2$-PCL and crude lipase powder, respectively. The long hydrophobic chain induced an interfacial activation effect because of the interaction between the polypeptide chain of the lipases and stearic acid. Kim et al. [48] studied the immobilization of Mucor janaicus lipase (MJL) using covalent attachment to ethylene diamine (EDA)-activated silica nanoparticles. The silica nanoparticles were activated using a coupling agent (glutaraldehyde (GA) or 1,4-phenylene diisothiocyanate (NCS)). Using GA or NCS as a coupling agent can avoid side binding between large enzyme molecules and the carrier. Thus, the EDA-GA- and EDA-NCS-activated nanoparticles could access surface areas for enzyme immobilization. The immobilized MJL-attached EDA-GA and EDA-NCS silica nanoparticles enhanced the enzyme loading and activity. The immobilized MJL had a wide range of pH tolerance and high thermal stability [48]. The relative activity of the lipase immobilized on the EDA-GA and EDA-NCS silica carriers was 115% and 107% compared to free enzyme, respectively.

| Nanomaterials | Strain | Carrier | Type of Binding | Special Feature | Refs. |
|---------------|--------|---------|-----------------|-----------------|------|
| Nanoparticles | *Pseudomonas cepacia* | Zirconia | Covalent | Increased activity and enantioselectivity | [47] |
| *Mucor japonicus* | Silica | Covalent | Enhanced enzyme loading and enzyme stability | [48] |
| *Candida antarctica* | Polystyrene | Adsorption | High hydrolytic activity | [49] |
| *Candida rugosa* | Chitosan | Covalent | High enzyme loading and activity retention | [50] |
| *Candida rugosa* | Polylactic acid | Adsorption | Enhanced activity and stability | [51] |
| *Candida rugosa* | γ-Fe$_2$O$_3$ | Covalent | Enhanced stability | [53] |
| *Porcine pancreas* | Magnetic | Adsorption | Good reusability | [54] |
| *Pseudomonas cepacia* | SWNT | Adsorption, covalent | Increased retention of enzyme activity | [55] |
| *Rhizopus arrhizus* | MWNT | Covalent | Enhanced resolution efficiency | [43] |
| *Candida rugosa, C. antarctica B, Thermomyces lanuginosus* | MWNT | Adsorption | Enhanced stability | [56] |
| *Candida rugosa* | MWNT | Adsorption | High enzyme activity | [57] |
| *Candida rugosa* | MWNT | Adsorption | Enhanced activity and thermal stability | [58] |
| *Candida antarctica* | MWNT | Adsorption | Enhanced activity and stability | [59] |
| *Candida antarctica* | Polycrylonitrile | Covalent | High enzyme stability | [44] |
| *Candida rugosa* | Poly-(acrylonitrile-co-maleic acid) | Covalent | High activity and enzyme loading | [60] |
| *Candida rugosa* | Cellulose acetate | Covalent | Enhanced thermal stability | [61] |
| *Burkholderia cepacia* | Polycaprolactone | Covalent | Enhanced catalytic activity and reusability | [62] |
| *Candida rugosa* | Polyvinyl alcohol (PVA) | Covalent | Equivalent esterification activity to that of Novozyme 435 | [63] |

Table 1. List of various immobilized lipases on different nanomaterials. SWNT: single-walled carbon nanotube; MWNT: multi-walled carbon nanotube.
Candida antarctica lipase B (CAL-B) was immobilized on polystyrene nanoparticles via adsorption [49]. Polystyrene nanoparticles were synthesized using a nanoprecipitation technique. Since the enzymes were adsorbed by hydrophobic interactions, the immobilization efficiency was not affected by the change in pH. Nevertheless, the activity of immobilized CAL-B depended on the change in pH. The ionization state of the lipase’s active site can be affected by pH changes. Therefore, the adsorption immobilization of lipases was performed at pH 6.0 to avoid the possibility of enzyme conformation change. The hydrolytic activity of the immobilized lipase on polystyrene nanoparticles was compared with Novozyme 435 and crude enzyme powder, and its activity was 1.16-fold higher than Novozyme 435 and 1.81-fold higher than free enzyme.

3.1.2. Magnetic Nanoparticles

The reuse of immobilized enzymes on non-magnetic nanoparticles requires high-speed centrifugation [52]. To overcome these issues, many researchers have studied enzyme immobilization using magnetic nanoparticles, because magnetic nanoparticles can be readily separated the reaction solutions using magnetic attraction [42,52,53,64–68]. Nanoscale magnetic particles have a unique property of superparamagnetism [69,70]. They do not form agglomerates at room temperature; thus, they are well-suspended in reaction solution [70]. The magnetic iron oxides have been mostly used as magnetic nanoparticles because of their low toxicity and biocompatibility [69]. Dyal et al. [53] evaluated the activity and stability of Candida rugosa lipase (CRL) attached on γ-Fe₂O₃ magnetic nanoparticles by covalent binding. The γ-Fe₂O₃ magnetic nanoparticles were activated with either acetyl or amine groups to connect with the amine groups of lipases. The operational stability of immobilized CRL was significantly improved. Lee et al. [54] developed hydrophobic magnetic nanoparticles to immobilize crude porcine pancreas lipase (PPL). The lipases showed high activation when they were immobilized on the hydrophobic surface of nanoparticles [46,47]. Sodium dodecyl sulfate (SDS) was used as the ligand for hydrophobic Fe₃O₄ magnetic nanoparticles. The PPL immobilized on surface-modified magnetic nanoparticles (8–12 nm in size) enhanced the thermal stability compared to free PPL. Both the free and immobilized PPL showed maximum activity at temperatures between 37 and 40 °C, and the specific activity of immobilized PPL was 1.42-fold higher than that of the free enzyme. The SDS ligand on the nanoparticles’ surface acted as a spacer between the nanoparticles and the enzymes, which resulted in a flexible enzyme structure form. CRL was also immobilized on functionalized superparamagnetic nanoparticles (poly(GMA)-grafted Fe₃O₄/SiOₓ) by Lei et al. [64]. Poly-(glycidyl methacrylate) (GMA) was grafted onto the surface of Fe₃O₄/SiOₓ by radical polymerization. The diameter of the functionalized magnetic nanoparticles was 100 nm and showed higher saturation magnetization (8.3 kA/m). The immobilized CRL showed better pH resistance and thermal stability, and its residual activity remained at 83% of the initial activity after being reused six times. CRL was covalently bound to Fe₃O₄ magnetic nanoparticles (12.7 nm) using carbodiimide activation. The Fe₃O₄ magnetic nanoparticles-immobilized lipase showed 1.41-fold enhanced activity, 31-fold enhanced stability, and better tolerance to changes of solution pH compared to the free enzyme [65]. Thangaraj et al. [66] examined the effect of various SiOₓ ratios for coating on Fe₃O₄ and further functionalization of Fe₃O₄/SiO₂ magnetic nanoparticles using organosilane compounds (3-aminopropyltriethoxysilane (APTES) and 3-mercaptpropyltrimethoxysilane (MPTMS)) for lipase immobilization. When the Fe₃O₄/SiO₂ ratio was 1:0.25, the immobilization efficiency was the highest. The immobilized lipase on functionalized Fe₃O₄/SiO₂ magnetic nanoparticles using APTES showed the best catalytic activity. APTES might help to improve the surface characterization of magnetic nanoparticles.

3.2. Carbon Nanotubes-Based Lipase Immobilization

Carbon nanotubes are promising materials for enzyme immobilization. Carbon nanotubes based on graphitic sheets have a unique structure that rolls into a cylindrical shape [71]. Carbon nanotubes, including single-walled nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs), are used
for enzyme immobilization (Table 1) [43,55–59]. Lee et al. [55] immobilized PCL onto SWNTs using two different solvent systems (buffer and ionic liquid). In the buffer solution, carbon nanotubes are basically insoluble because of the van der Waals forces of SWNTs. The use of an ionic liquid increased the immobilization efficiency via providing a better dispersion of carbon nanotubes than buffer solution. MWNTs can load a large amount of enzyme [71]. Lipases were immobilized in carbon nanotube–silica composites in an anion-aqueous reaction system [56]. MWNTs were used as additives to prevent lipase inactivation during the sol-gel process. The activity of three types of lipases (Candida rugosa, Candida antarctica type B, and Thermomyces lanuginosus) with 2.7% (w/w) MWNT was enhanced 10-fold than the lipase immobilized without MWNT in a esterification reaction. The immobilized lipase with 2.7% MWNT retained 96% of the initial activity after five reuses, whereas the immobilized lipase without MWNT was completely deactivated under the same condition.

C. rugosa lipase (CRL) was also attached to MWNTs [43,56–59]. CRL was immobilized on MWNT through physical adsorption, and a high retention of catalytic activity of up to 97% was observed. The immobilized CRL showed 2.2- and 14-fold increases in the initial rate of transesterification in hexane and water-immiscible ionic liquids (Bmim) (PF6), respectively [57]. Mohamad et al. [58] reported a simple adsorption method to immobilize CRL onto acid-functionalized MWNTs (F-MWNTs). The polar groups (COO⁻) were introduced to the MWNTs via stirring with an acid mixture containing H₂SO₄ and HNO₃. The charged carboxyl moieties on the MWNTs’ surfaces could be connected with other polar moieties (NH₂ and OH) on the CRL. The immobilized CRL on acid F-MWNTs had improved structural integrity and mechanical strength. The activity and thermal stability of the immobilized CRL on MWNTs were twofold enhanced compared to those of the free enzyme.

3.3. Electrospun Nanofibers-Based Lipase Immobilization

Some nanoparticles and nanotubes can suffer from mass-transfer limitation and difficulty in recycling because of their good dispersion [72]. Electrospun nanofibers are promising carriers to overcome these issues [44,60–63]. Nanofiber membranes have a large surface area for enzyme loading and high porosity for efficient substrate diffusion. Lipases were attached to the surface of electrospun nanofibers as the carrier or entrapped in the nanofibers [52]. Nanofiber membranes with carboxyl groups were made from poly(acrylonitrile-co-maleic acid) (PANCMA) via an electrospinning process [59]. The immobilized CRL on this nanofiber membrane showed an enhanced enzyme loading (from 2.36 to 21.2 mg/g) and increased activity (from 33.9 to 37.6%) compared to those of the hollow-fiber membrane. The efficiency of the biocatalysis increased because the Kₘ value of the immobilized lipase decreased. Huang et al. [61] reported the development of immobilized CRL on an electrospun cellulose nanofiber membrane via covalent binding. The nanofiber was made by electrospun cellulose acetate. Then, it was oxidized using NaIO₄ to produce aldehyde groups, which can be covalently bonded with enzyme molecules. The activity of immobilized CRL was 29.6 U/g under an optimal condition, and the thermal stability was higher than that of free enzyme. Song et al. analyzed the activity of encapsulated Burkholderia cepacia lipase (BCL) in polycaprolactone (PCL) nanofibers [62]. The activities of a BCL–PCL nanofiber were evaluated using two reaction models: hydrolysis activity in aqueous media and transesterification activity in non-aqueous media. The specific hydrolysis activity was higher than the transesterification activity. The immobilized BCL maintained 50% of its initial activity up to the 10th recycle in non-aqueous media.

4. Biodiesel Production Using Nano-Immobilized Lipase

Recently, enzyme immobilization using nanomaterials as carriers has been developed and used in biodiesel production (Table 2) [67,73–82]. The improvement of enzyme activity, stability, and reusability by employing nano-immobilized enzyme systems can reduce the cost of enzyme use. In the biodiesel production process, the immobilized lipases were disrupted by shear stress from the stirring in the batch reaction [45], indicating that reaction systems need to be developed for biodiesel production. Wang et al. [67] successfully developed a packed-bed reactor system using immobilized lipase on
Fe$_3$O$_4$ nanoparticles. The biodiesel conversion was over 88% for 192 h using a four-packed-bed reactor. The four-packed-bed reactor exhibited higher conversion and stability than a single-packed-bed reactor. As a result, the four-packed-bed reactor has been proved to have a great potential in biodiesel production based on large-scale nanobiocatalytic systems.

Table 2. Summary of investigations on biodiesel production using nano-immobilized lipase. mMWNCT: magnetic multi-walled carbon nanotube.

| Strain               | Carrier            | Substrate         | Biodiesel Conversion (%) | Reusability (Days Or Cycles) | Refs.  |
|----------------------|--------------------|-------------------|--------------------------|------------------------------|--------|
| *Pseudomonas cepacia*| Fe$_3$O$_4$        | Soybean oil       | 88                       | 10 days                      | [67]   |
|                      | PAN-nanofiber      | Rapeseed oil      | 94                       | 20 days                      | [73]   |
|                      |                    | Soybean oil       | 90                       | 10 cycles                    | [74]   |
| *Thermomyces lanuginosa* | Amino-Fe$_3$O$_4$ | Soybean oil       | 90                       | 4 cycles                     | [75]   |
|                      | Epoxy-silica      | Palm oil          | 97                       | 5 cycles                     | [76]   |
|                      |                    | Canola oil        | 99                       | 20 cycles                    | [82]   |
| *Burkholderia sp.*   | Amino-Fe$_3$O$_4$-SiO$_2$ | Waste cooking oil | 91                       | 3 cycles                     | [77]   |
|                      | Alkyl-Fe$_3$O$_4$-SiO$_2$ | Olive oil       | 90                       | 10 cycles                    | [80]   |
|                      |                    | Chlorella vulgaris| 90                       | 2 cycles                     | [81]   |
| *Rhizomucor miehei*  | PAMAM-mMWCNT      | Waste cooking oil | 94                       | 10 cycles                    | [78]   |
|                      | Epoxy-silica      | Canola oil        | 95                       | 7 cycles                     | [82]   |
| *Candida antarctica* | Epoxy-Fe$_3$O$_4$-SiO$_2$ | Waste cooking oil | 100                      | 6 cycles                     | [79]   |
|                      | Epoxy-silica      | Canola oil        | 59                       | 15 cycles                    | [82]   |

Raita et al. [76] studied biodiesel production from palm oil using immobilized *Thermomyces lanuginosus* lipase (TLL) on magnetic nanoparticles. The biodiesel production yield was 97.2% under optimal conditions (23.2 wt % enzyme loading, 4.7:1 molar ratio of methanol to oil, and 3.4% water content at 50 °C for 24 h). The attached lipases on the magnetic nanoparticles retained over 80% activity after five recycles. Waste cooking oil was also converted to biodiesel via transesterification using nano-immobilized lipase [77–79]. Karimi et al. [77] immobilized BCL on superparamagnetic iron-oxide nanoparticles (SIONs) for biodiesel production. The conversion of waste cooking oil to biodiesel was 91% within 35 h. Fan et al. [78] obtained a high biodiesel yield of 94% from waste cooking oil using immobilized *Rhizomucor miehei* lipase (RML). The RML was immobilized onto polyamidoamine (PAMAM) grafted with magnetic multi-walled carbon nanotubes (MWCNTs) (m-MWCN-PAMAM). The immobilized RML showed 27-fold higher esterification activity than free RML, and there was no decrease in activity during 10 recycles. These results showed a great potential for biodiesel production.

Tran et al. [80] developed alkyl-functionalized Fe$_3$O$_4$-SiO$_2$ nanocomposites for lipase immobilization. The immobilization efficiency on alkyl-grafted Fe$_3$O$_4$-SiO$_2$ was 1.3-fold higher than non-functionalized Fe$_3$O$_4$-SiO$_2$. The immobilized lipase was used for biodiesel production. The biodiesel conversion was over 90% for 30 h in batch operation. Tran et al. [81] also developed a one-step extraction and transesterification process of biodiesel production from wet microalgae biomass using alkyl-grafted Fe$_3$O$_4$-SiO$_2$-immobilized lipase. The biodiesel conversion was over 90% under optimal conditions. The transesterification of soybean oil was catalyzed by a lipase immobilized on a polyacrylonitrile (PAN) nanofiber [74]. The biodiesel conversion was 90%. The immobilized lipase on the nanofibers maintained 91% of its initial activity for 10 cycles. All of these results represent that nanomaterials can be used as a good carrier for lipase immobilization and that nano-immobilized lipase has potential for commercial application in biodiesel production.

5. Conclusions

Recently, various nanomaterials have attracted much attention as carriers for enzyme immobilization. Nanomaterials as immobilization carriers have many advantages, such as their large surface areas compared to other materials for enzyme immobilization. Many researchers have effectively immobilized lipases on functionalized nanomaterials, and their applications appear promising.
In particular, the application of nano-immobilized lipase in packed-bed reactors resulted in high enzyme loading, multiple reuses, and effective protection from enzyme denaturation in biodiesel production, showing the potential of nano-immobilization technology in the biofuel industry. Further investigation, especially for the scale-up of the biodiesel production process, using nano-immobilized lipase is necessary to implement these technologies on an industrial level. The integrated development of a high enzyme and nano-immobilization technique will play a key role in cost-effective biodiesel production.

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