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A Concise Review on Immobilized Enzyme-Catalyzed Biodiesel Production

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Abstract:

The increasing demand for sustainable and eco-friendly energy sources has accelerated research on biodiesel as a promising alternative to conventional fossil fuels. Enzyme-catalyzed biodiesel production, particularly using immobilized lipases, has emerged as an efficient and environmentally benign approach due to its high selectivity, mild operating conditions, and reduced by-product formation. Immobilization techniques, including adsorption, covalent bonding, entrapment, and encapsulation, significantly improve enzyme stability, reusability, and catalytic efficiency, thereby reducing production costs and enhancing process feasibility. This review provides a concise overview of recent advancements in immobilized enzyme systems for biodiesel synthesis, highlighting various support materials, immobilization strategies, and reactor configurations. Additionally, the effects of key operational parameters such as temperature, pH, alcohol-to-oil ratio, and substrate type on biodiesel yield are critically discussed. Challenges related to enzyme deactivation, mass transfer limitations, and economic scalability are also addressed. Finally, future perspectives focusing on nanostructured supports, hybrid biocatalysts, and process optimization are presented to advance the commercialization of enzyme-based biodiesel production. This review emphasizes the potential of immobilized enzyme technology as a sustainable pathway for efficient biodiesel synthesis

Keywords: Enzyme, Nanozyme, Biodisel

1. Introduction:

About 80% of the energy worldwide is derived from fossil fuels, and the global energy demand in the next two decades is expected to increase by 48% to satisfy growing population needs. As the amount of fossil fuels is not indefinite and at the current energy consumption rate, there is a rapid depletion of fossil fuels. Moreover, the adverse environmental impact of fossil fuels due to the emission of greenhouse gases (GHGs) has motivated the development of alternative fuels from renewable resources such as solar, hydro, wind, geothermal, and bioenergy.[1-3]Biofuels, in this regard, have attracted ample attention due to the available feedstock in the form of biomass. Bioethanol, bio hydrogen, bio methane, and biodiesel are biofuels with great potential. Biodiesel as a biofuel is a very attractive alternative to petro-diesel because it is renewable, biodegradable, and non-toxic. The energy content, viscosity, and certain number of biodiesel are close to petro-diesel, but the quantity of carbon monoxide, particulate matter, and GHGs emitted from the biodiesel-powered engines is significantly low compared to petro-diesel-powered engines.[4-7]Based on the biomass sources, the various feed stocks utilized for biodiesel production fall into four primary categories i.e. edible oils (first-generation), non-edible oils (second-generation), algal biomass (third-generation), and genetically engineered micro-organisms (fourth-generation) (Figure 1).[8-9]Among the existing edible oils, rapeseed oil (84%) and sunflower oil (13%) contribute significantly to biodiesel production compared to other edible oils.[10] However, producing biodiesel from edible biomass is not viable as

it may impact food supplies. On the other hand, third and fourth-generation feed stocks obtained from phototrophic organisms are emerging as potential candidates due to high lipid yield compared to plant oils, and their CO_2 sequestration property help combat environmental concerns. The drawback associated with the industrial-scale utilization of algal biomass for biodiesel production is the cost-effectiveness of the entire process, including algal cultivation, harvesting, and lipid extraction. Therefore, an abundantly available, inexpensive feedstock is crucial for sustainable biodiesel production.

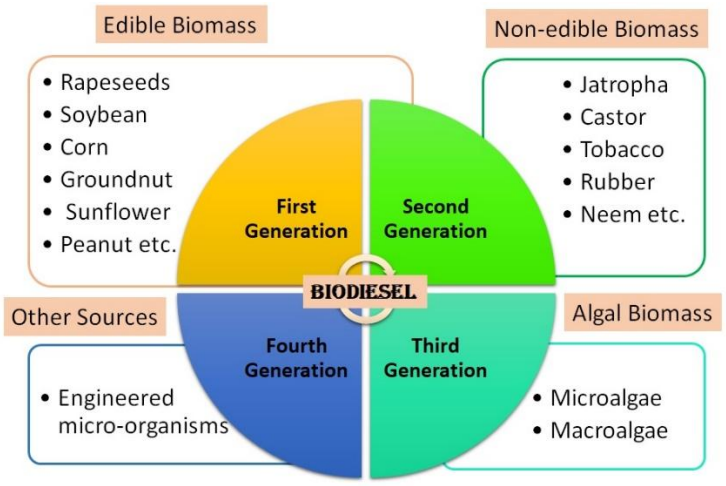

Figure 1. Schematic illustration of different feed stocks utilized for biodiesel production.

Biodiesel belongs to the family of fatty acid methyl esters (FAMEs), and the four routes to produce biodiesel from vegetable and animal fats include direct use a blender, thermal cracking known as pyrolysis, micro emulsions, and transesterification.[11]The transesterification of triglycerides in the vegetable and animal fats with small alcohols such as methanol is the most viable biodiesel production pathway in terms of cost and quality. The transesterification reaction rate significantly depends upon the molar ratios (triglycerides: alcohol), catalyst, temperature, reaction time, and moisture content in the oil. The transesterification reaction can be carried out in the presence or absence of the catalyst. The uncatalyzed transesterification requires elevated temperatures for better yields, and temperatures greater than 400 ^{o}C often lead to the degradation of the ester. The reaction yield of transesterification can be enhanced by using methanol in the supercritical state. Still, the operational conditions of the reaction are critical, and the overall process is cost-ineffective.[12]The catalytic transesterification is classified as homogeneous and heterogeneous, including acid-base catalysts and biocatalysts. In base-catalyzed transesterification of fats, sodium hydroxide (NaOH) or potassium hydroxide (KOH) is used as the catalyst, whereas in acid-catalyzed reactions, the common catalysts are either sulfuric acid or sulfonic.[13-14] The acid-base catalyzed transesterification is accompanied by low yield and purity due to side reactions like saponification and hydrolysis. Furthermore, separating products followed by purification and neutralization is another setback for acid-base catalyzed transesterification.[15]Conversely, biocatalytic transesterification offers the advantage of forming highly pure products under mild, environmentally benign conditions with minimal downstream processing. Both heterogeneous and homogeneous enzymatic transesterification have been explored for biodiesel production.[16-18]The current review explicitly describes the opportunities and challenges associated with enzymatic biodiesel production, including lipase engineering, liquid formulations, and immobilization techniques.

2. Enzymatic Biodiesel Production

Lipases are biocatalysts in the category of hydrolases (acyl glycerol acyl hydrolases, EC 3.1.1.3), which hydrolyze the triglycerides to glycerol and fatty acids. They are also termed carboxyl esterases as they catalyze the hydrolysis of ester and long-chain acyl glycerol synthesis.[19-21]Based on their origin, these enzymes are classified as plant, animal, and microbial lipases. Microbial lipases have gained popularity among the three due to their stability, bulk availability, and low cost. The microbial sources for lipase include bacteria, fungi, and yeast. The microbial lipases are extracellular with an average molecular weight of 30-40 kDa. The optimum pH of lipases is 7.5-9, and the temperature range for mesophilic

lipases is below 70 °C, whereas the thermophilic lipases retain activity up to 100 oC. The lipases derived from the extreme thermophilic bacteria *Thermoanaerobacter thermohydrosulfuricus* and *Caldanaerobacter subterraneus,* in addition to retaining activity up to 90 °C also exhibited stability to organic solvents making them suitable candidates for biodiesel production in water-free environment.[22-25]

The active site of lipase consists of histidine, serine, and aspartate/glutamate as three amino acid residues. In enzyme-catalyzed transesterification, the first step is the formation of an enzyme acyl complex followed by a nucleophilic attack on the carbonyl carbon. In the final step, the complex is resolved to release a fatty acid, and the enzyme is regenerated (Scheme 2).[26]

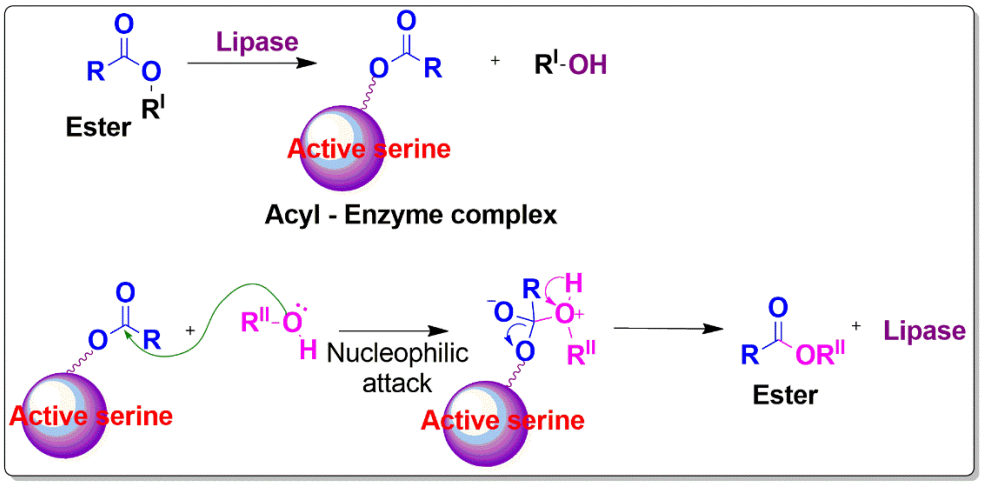

Figure 2. Transesterification reaction mechanism using lipase as a biocatalyst

2.1 Enzyme Immobilization Strategies

2.1a Adsorption Immobilization: The most popular method for immobilizing lipase is adsorption immobilization due to its ease of usage and viability. Research on lipase immobilization by adsorption in the recent years (2013-2018) has been shown to have a large proportionate benefit of up to 42%.[29]Adsorption immobilization of lipase is a process used to attach lipase enzymes onto a solid support or surface through adsorption interactions. This immobilization technique has several benefits, including improved stability, reusability, and ease of separation from the reaction mixture. Lipase leaching from support does however, have the drawback of reversibility due to the absence of long-term links between them.[27] The rate of oil conversion to biodiesel is boosted by nanomaterials high surface-to-volume ratio, higher lipase loading capacity, and resistance to lipase leaching.[28] Silk fabric was created using an amino functionalized polydimethylsiloxane, and the treated silk tissue was immediately adsorbed with Candida sp. 99-125 lipase. After being reused more than 80 times, the immobilized lipase on the silk fabric retained esterification activity without noticeably declining and displayed 1.6 times increased hydrolytic activity and better resistance to polar solvents (methanol and ethanol). Furthermore, the magnetic nano-particle's superior performance, low toxicity, chemical stability, and ease of separation from the reaction system it was widely utilised to immobilise lipase. However, lipase immobilisation is not only possible by adsorption independently but also by using nanomaterial as carriers.[30]

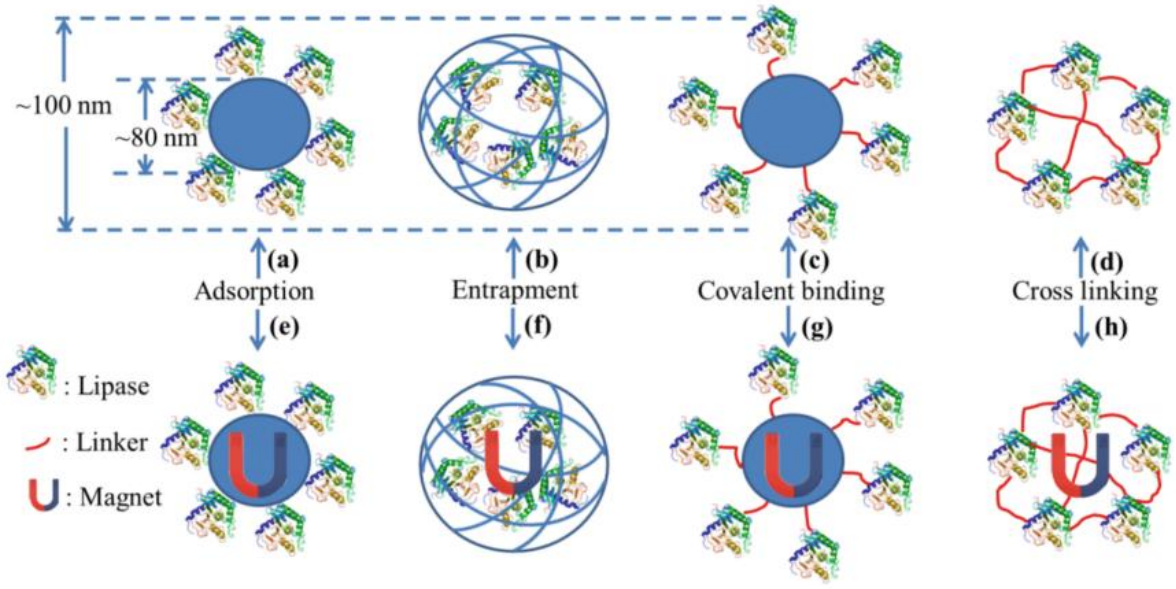

Figure 3. Schematic illustrations of lipase immobilizations[31].

2.1. b. Entrapment and encapsulation immobilization: Lipase is restricted by entrapment (inclusion) or encapsulation immobilization (Figure 3b, f) in a polymeric matrix made of agar, alginate, and collagen as well as in a porous support like zeolite that is produced by co-precipitation. The lipase from Bacillus licheniformis NCU CS-5 was immobilized on magnetic nanocomposites made of chitosan and Fe_3O_4 by encapsulation and amino propyl-functionalization and cyclodextrin grafting. After being utilized for 15 cycles of esterification and 28 days of storage, the cyclodextrin encapsulated lipase demonstrated noticeably better thermos stability and pH stability over the free lipase and maintained more than 80% of its relative activity.[32] The thermophilic lipase from Alcaligenes sp. was embedded in bio-based metal-organic frameworks (Bio-MOFs) using a biomimetic mineralization technique, with high recyclability during transesterification and no detectable changes in crystal and morphological structure after three repeated cycles. The leaking issue can be effectively solved using this technique. The manufacture of biodiesel encounters considerable mass transfer resistance due to the packed lipases and compact outer matrix. Due to this, the use of entrapment or encapsulation for lipase immobilization in the biodiesel industry has received relatively few reports.

2.1. c. Covalent immobilization: The covalent link between the surfaces of the enzyme and the carrier is necessary for the covalent immobilization of lipase (Figure 3c, g). Aldehydes are typically used to activate the carriers so that the enzyme protein can be immobilized there by creating an amine-aldehyde Schiff connection between the amino and aldehyde groups.[33] Due to its two aldehyde groups, glutaraldehyde serves as a bifunctional linking agent that can interact with amino groups on both the main modified carrier and enzyme. Eq. 1 is a schematic expression of the reaction of covalent immobilization of lipase on a carrier with glutaraldehyde.

$$\text{Carrier-NH}_2 + \text{OHC (CH}_2)_3\text{CHO} + \text{H}_2\text{N-Lipase} \rightarrow \text{Carrier-N = CH (CH}_2)_3\text{CH}$$

$$= \text{N-Lipase} + 2\text{H}_2\text{O} \quad (1)$$

For the purpose of covalently immobilizing the enzyme for the manufacture of biodiesel, lipase from Rhizopus oryzae (ROL) was physically adsorbable and covalently attached to mesoporous silicate magnetic nanoparticles (MS-MNPs). The performance of the immobilized lipase is occasionally enhanced in addition to helping to separate the used lipase from the biodiesel products. Thermomixers lanuginosa (TLL) and Rhizomucor miehei (RML) lipases were immobilized on silica core shell coated magnetic nano-particles (Fe_3O_4@SiO_2) with aldehyde group functionalized for covalent immobilization. By maintaining 97% and 54% of their initial activities at 65 °C, respectively, the thermos stabilities of the immobilized TLL and RML were significantly improved.[34]Candida rugosa lipase was covalently coupled with glutaraldehyde and immobilized onto a Fe_3O_4@SiO_2 nanoparticle dip-coated nanocomposite membrane with 3-aminopropyletriethoxysilane (APTS) modification. The decreased Km and Vmax of the immobilized enzyme represent

an increase in substrate affinity and a decrease in catalytic activity respectively. In order to prevent activity loss and improve operational stability of the immobilized Burkholderia cepacia lipase on magnetic nanoparticles as core shells, a novel hetero functional carrier was developed by strengthening anion exchange and weakening covalent binding to overcome the drawbacks of adsorption and covalent immobilizations.[35]Research on covalent immobilization of lipase for biodiesel generation has been widely reported and will remain a research hotspot in the future due to the mechanical strength, leakage prevention, and simple recycling.

2.1.d. Cross-linking: In order to make enzyme aggregation (without matrices), cross-linking immobilization of lipase (Fig. 3d, h) most frequently uses glutaraldehyde as a cross-linker. This does not affect the crystal structure of Bio-MOF. bio-inspired metal-organic frameworks, are a class of materials that combine principles from both biology and chemistry to create novel porous structures. Metal-organic frameworks (MOFs) are typically crystalline materials composed of metal ions or clusters coordinated to organic ligands, forming a porous three-dimensional network. These structures have a wide range of applications, including gas storage, separation, catalysis, and drug delivery. Because two aldehyde residues of one glutaraldehyde molecule act with two reactive amino residues from the lipase encapsulation.

2.2 Micro/Nano-Carriers Used for Lipase Immobilization: Micro and nano-carriers are commonly used in various fields, including biotechnology, for immobilizing enzymes like lipase. Immobilization enhances the stability, reusability, and performance of enzymes, making them suitable for industrial applications.

2.2.a.Carbon Nanotubes: Multi-walled carbon nanotubes (MWCNTs) or single-walled carbon nanotubes (SWCNTs) can serve as carriers for lipase immobilization due to their large surface area and unique properties.[36] However, issues with biocompatibility and dispersion prevent carbon nanotubes (CNTs) from being widely used in the biotechnological field.[37] The functionalization of CNTs, whether covalent or noncovalent, can address the aforementioned shortcomings. Since it allows for the adsorption of polymers or surfactants on the surface of CNTs without impairing their original architecture and functionality, non-covalent CNTs functionalization is chosen among these. To enhance the physicochemical properties of CNTs, various polymers, including polyamides, poly-3-hexylthiophene, poly-L-lysine, and hyper branched polyline, have been used.[38-41]By adopting two distinct techniques, Rhizomucor miehei lipase (RML) was covalently immobilized to both CNTs and silica gels. RML was immobilized via an immobilization/carbodiimide activation procedure on carboxylate MWCNTs (RML@MCNT@COOH) and 3-carboxypropyl silica gel (RML@Si_2@COOH). Additionally, it was immobilized on SWCNTS that had been glutaraldehyde activated and 3-aminopropyl-triethoxysilane (3- APTES) functionalized 3-aminopropyl silica (RML@Si-Glu) as well. Following immobilization, expressed activities for RML@SiGlu, RML@Si@COOH, RML@SCNT-Glu, and RML@MCNT@CO were 90.2, 36.9, 26.1, and 16.9%, respectively. Comparing RML@Si-Glu to its RML analogues, it was notable that it had strong catalytic performance. At 40 °C and pH 7.5, the half lives of RML@Si-Glu, RML@Si@COOH, RML@SCNT-Glu, and RML@MCNT@COOH were, respectively, 7.6, 5.8, 4.6, and 4.2-fold longer than those of the free enzyme. All immobilized nano biocatalysts maintained more than 90% of their original activity after 10 successive cycles of hydrolyzing p-nitrophenyl butyrat, according to the reusability profile.[42]

2.2.b. Magnetic Nanoparticles: Iron oxide nanoparticles coated with polymers can be used to immobilize lipase through physical adsorption or covalent bonding. Magnetic carriers enable easy separation of the enzyme from the reaction mixture using an external magnetic field. Due to their high surface-to-volume ratio, superior physical and chemical stability, low toxicity, variable surface modification, and ease of separation, magnetic nanoparticles (MNPs) have become popular supports for lipase immobilization in recent years.[43]Even after repeated use, immobilized lipase on MNPs has been proven to have great operational stability and good catalytic activity.[44,45]Due to their superior biocompatibility and lack of toxicity, the Fe_3O_4 MNPs, which include γ-Fe_2O_3, $MgFe_2O_4$, $MnFe_2O_4$, $CoFe_2O_4$, and $CoPt_3$ MNPs, have been the most commonly employed for lipase immobilization.[46]However, due to the magnetic dipole-dipole interactions, bare Fe_3O_4 MNPs always have a propensity to aggregate.[47]According to findings in the literature, functionalizing Fe_3O_4 MNPs can significantly increase their chemical stability and dispersity. [48]

2.2.c. Polymeric Nanofibers: Electrospun polymeric nanofibers can serve as carriers for lipase immobilization. They offer a high surface area-to-volume ratio and can be tailored for specific applications. Due to a number of structural and

functional advantages, including biodegradability, biocompatibility, nontoxicity, hydrophilicity, high porosity, suitable functional moieties, and the presence of various structural compositions, nanofibers have emerged as an appealing material choice with a high potential for enzyme immobilization. All of these distinguishing qualities contribute to the enzyme being loaded more effectively. The pores of the nanofiber contain the enzyme molecules, and the enzyme's three-dimensional structure is also conserved. Immobilization on nanofibers improved the enzyme's stability properties by increasing its resilience to unfavorable conditions (high temperature, alkaline and acidic pH).[49] Phase separation, self-assembly, electrospinning, and template synthesis are some of the various techniques used to make nanofibers. The best method for making nanofibers is electrospinning since it has a number of advantages including flexibility, ease of handling, cost effectiveness, and mechanical strength.[50-52]Recently, a variety of materials, including poly(lactic acid), polyurethane, Fe/SiC, TiO_2/SnO_2, graphene/polyimide, TiO_2/WO_3, $MoS_2/CdSTiO_2$, poly(glycerol sebacate), PVP/SiO_2, graphite/SiC, gelatin/zein, etc. have been used to develop electrospun nanofibers .[52-54]. PVA/Zn^{2+} electrospun nanofibers were used for the first time as support matrices for the adsorption and cross-linking of lipase. Based on operating parameters such as Zn^{2+} concentration (%), PVA concentration (%), voltage (kV), injection speed (ml/h), and needle tip-collector distance (cm), nanofibers were synthesized using the electrospinning technique. Results showed enhanced pH stability, temperature resistance, and recycling efficiency of nanofibers integrated with enzyme-like improvements. Electrospinning has been used to create nanofibers made of polyacrylonitrile (PAN) that range in diameter from 150 to 300 nm. CRL was covalently attached to these nanofibers and immobilized due to the increased surface area for immobilization. After 5 minutes of activation and 1-hour interaction with the enzyme solution, immobilized lipase demonstrated retention of over 80% of activity. When immobilized biocatalysts were kept in the buffer for 20 days at 30 °C, they retained approximately 90% of their initial activity while the enzyme's free state lost 80% of its actual activity. After ten successive reaction cycles, it likewise kept 70% of its specific activity [56]. Under the ideal reaction conditions, Candida rugosa lipase immobilised on cellulose nanofiber membrane displayed high enzyme activity (29.6 U/g of the biocatalyst). It demonstrated significantly greater toughness and thermal stability than the enzyme's comparable free form. In contrast to free enzyme, aggregated lipase was immobilised on modified graphene oxide nanocomposites (maGO-CLEAs-lip), which also increased storage stability by retaining activity at levels of about 75% after 30 days of incubation and resulted in an improved biodiesel yield that was 3.0 times higher than that of free enzyme.[57] Burkholderia cepacia lipase was immobilised on magnetic nanoparticles that demonstrated noticeably improved operational stability, greater reusability, and increased biodiesel output.

2.2.d. Metal-Organic Frameworks (MOFs): MOFs are crystalline materials with a porous structure that can be tailored for enzyme immobilization. They offer a high surface area and tunable properties. Diverse architectures, significant surface area, designable pore diameters, and functionalized pore walls make MOFs superior to other traditional host matrices as a platform for hosting biomolecules and enzymes, which are crucial for industrial and commercial operations.[58] The broad use of MOFs in immobilizing enzymes is being investigated in addition to their defensive functions and this is made possible by a number of remarkable qualities such their high porosity, clearly defined design, and customized functionality. To immobilize Rhizomucor miehei lipase (RML), Adnan et al. created X-shaped zeolitic imidazolate frameworks (ZIF-8) using a one-step encapsulation technique.[59] When compared to the enzyme's free form, the RML@ZIF8 as-developed showed a 26-fold increase in high activity recovery (2632%). Immobilized biocatalyst was used in an isooctane system to convert soybean oil into biodiesel. Under ideal reaction circumstances, the conversion efficiency increased to almost 95%. The immobilized enzyme's conversion yield did not significantly diminish, according to the reusability pattern, and it maintained 84.7% of its initial activity even after 10 more catalytic cycles. The remarkable performance and stability of the immobilized enzyme are attributed to ZIF-8 and RML's harmonious relationship and great biocompatibility. In a different study, lipase encapsulation inside the microporous ZIF-67 was used to create a novel type of heterogeneous bio catalytic system (Figure 4). Soybean oil was converted into biodiesel using the resulting lipase@ZIF-67 bio-composite in a solvent-free environment. The biocatalyst's thermal and storage stability and ability to be recycled were both significantly increased by the immobilization method. Additionally, the stiff ZIF-67 scaffold improved the embedded enzyme's resistance to both temperature and pH, protecting it against deactivation and promoting recyclability. This recently created strong biocatalytic conjugate has outstanding catalytic performance in the multiple subsequent cycles of soybean oil transesterification to biodiesel, making it a sought biocatalyst for commercial use.[60]

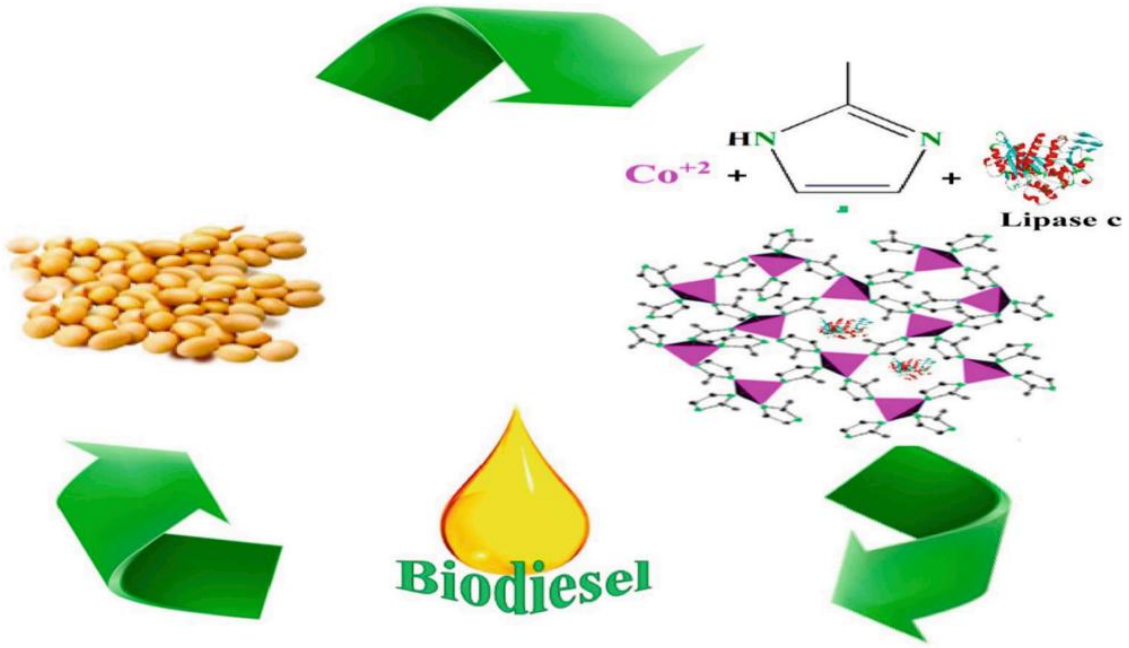

Figure 4. Encapsulation of lipase into the ZIF-67 for biodiesel production. [61]

3. Challenges in Enzymatic Routes for Biodiesel Production and Future Prospects:

The synthesis and use of nano-biocatalysts for the production of biodiesel have drawn a lot of interest due to their improved catalytic performance and stability, exceptional recycling efficiency, and high process ability. In the past 20 years, the number of publications on nano-biocatalyst has grown yearly (Figure 5). The development of nano-biocatalyst research in new domains and for the generation of biodiesel will continue to accelerate in the next years. There are still a lot of obstacles to overcome, though, including the effective manufacture of strong nano-biocatalysts, the avoidance of leaching and loss of enzyme activity from nano-biocatalysts, more affordable and ecologically safe nanomaterials, and simple and effective recycling with high enzyme activity. Physical or chemical approaches were used to immobilize lipases on nano-supports in order to create a powerful nano-biocatalyst. The enzyme that has been physically immobilized in/on a carrier has fewer conformational changes, but is more likely to desorb from the carrier when exposed to temperature and pH variations. The enzyme is strengthened by the covalent bond formed by chemical means between the support and the enzyme, albeit the characteristics of the enzyme may be marginally impacted by the altered enzymatic structure. Increase the robustness and reusability of the nano-biocatalyst by using a more stable approach for immobilizing the nanomaterial and lipase and avoiding the leaching of the enzyme from the carrier. There have been more bench-scale studies on the manufacture of biodiesel to date, and these studies have demonstrated the exceptional performance of the used nano-biocatalysts. There are, however, few examples of the use of nano-biocatalysts in biodiesel production scale-up to achieve significant economic advantages over petro-diesel. The investigation of the production of biodiesel using lipase-nano-particle bio composite in packed-bed reactors [61]and a magnetically stabilized fluidized bed reactor [62] revealed great potential for the design and operation of enzymatic conversion of oil to biodiesel on industrial scales. Because of their small size, nano-biocatalysts can be used in continuous flow reactors without being negatively impacted by the mechanical agitation found in stirred tank reactors (STR), which immobilizes enzymes. The performance of nano biocatalyst in the generation of biodiesel is outstanding because of the drastically improved surface to volume ratio. In contrast to magnetic field separation in usage of magnetic nano-biocatalyst, recycling the nano-particles by conventional centrifugal or natural sedimentation methods presents significant challenges. The Biodiesel Nano additive residues caused some issues in filtering unburned nano-particles from exhaust emissions. Consequently, future investigations are reducing the nanoadditive residues in biodiesel are also being worked on. Capture the unburned nanoparticles coming from diesel

engine exhaust [63]. The adverse effects of these repurposed nano-biocatalysts on Environment and health issues need to be addressed [64].

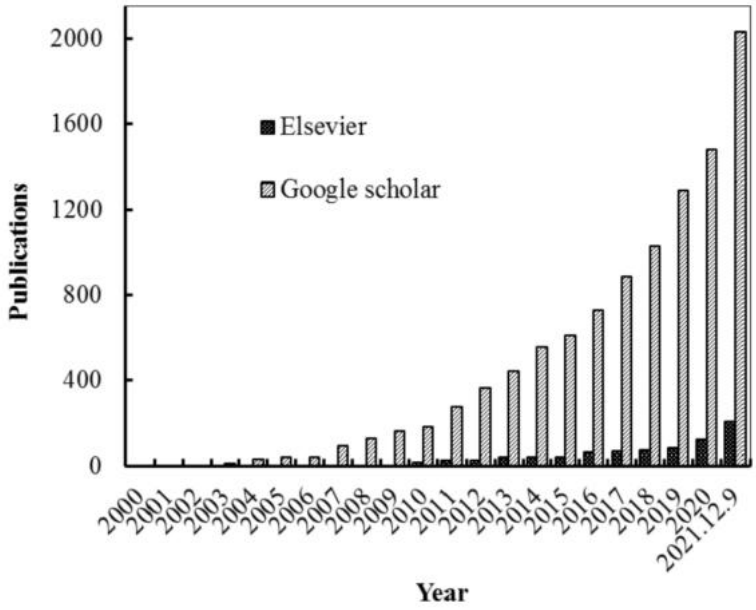

Figure 5. Publications on Elsevier and Google scholar. Searching key words: Nano, biodiesel, and lipase.

4. Conclusions:

In addition to the developing use of nanotechnology in the immobilization of lipase as a nano-biocatalyst for the conversion of fat and oil to FAMEs, biodiesel attracts increasing interest as a superior alternative bio-renewable energy source. Enzyme immobilization on nonmaterial can be accomplished via a variety of techniques, including adsorption, entrapment, encapsulation, covalent immobilization, and cross-linking. Covalent immobilization of lipase on magnetic nano-particles has received significant attention from the general public due to its effect on changes in conformation and activity as well as the reusable nature of the immobilized enzyme.It is common practice to employ the following methodologies to fully characterize a nano-biocatalyst: XRF and EDX for compositional characterization; XRD, FTIR, XPS, Raman spectroscopy, and BET for structural characterization; SEM, TEM, DLS, AFM, and BJH for morphological characterization; and VSM and TGA. Non-magnetic and magnetic nanoparticles, carbon nanotubes, electrospun nanofibers, metal-organic frameworks, and hybrid nanocomposites are among the interesting nano-carriers for lipase-based nano-biocatalyst development. Magnetic nanoparticles and renewable materials are receiving increased attention in recent studies. Nano biocatalysts catalytic efficacy, stability, recycling effectiveness, and process ability are all enhanced by lipase immobilization. Over the past two decades, research into nano-biocatalysts has grown year by year. Studies on nano-biocatalyst will continue to increase significantly in the production of biodiesel and other new fields in the next years.

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