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Review

Nanobiocatalysts for Biodiesel Synthesis through Transesterification—A Review

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Abstract: Converting useless feedstock into biodiesel by utilizing the process of transesterification has been regarded as an alternative approach recently used to address the fuel and energy resources shortage issues. Nanobiocatalysts (NBCs), containing the biological component of lipase enzyme immobilized on nanomaterials (NMs), have also been presented as an advanced catalyst to effectively carry out the process of transesterification with appreciable yields. This study highlights the fundamentals associated with NBCs and the transesterification reaction catalyzed by NBCs for summarizing present academic literature reported in this research domain in recent years. Classification of the NBCs with respect to the nature of NMs and immobilization methods of lipase enzyme is also provided for organizing the recently documented case studies. This review is designed to act as a guideline for the researchers aiming to explore this domain of biodiesel production via NBCs as well as for the scholars looking to expand on this field.

Keywords: lipase; enzymatic transesterification; optimization; monitoring; ASTM methods

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1. Introduction

Due to ever-increasing world population and modernization, the total consump-tion of energy has increased tremendously in recent years. For any country, the availa-bility of energy resources is considered a crucial factor influencing the socioeconomic development of that country. The unplanned utilization of these resources particularly petroleum products in transportation and industrial processes is increasing the deple-tion problems in the case of energy resources. Consequently, the prices of these prod-ucts are becoming unaffordable for a common man. The burning of these fossil fuels is also responsible for introducing high levels of carbon dioxide in the atmosphere which directly affects the environment in terms of global warming and is causing sudden climate changes. Several health problems such as cancerous diseases and respiratory ailments are also found to be directly associated with the emissions of these fossil fuels [1–3].

Owing to the aforementioned issues, it is necessary to find alternative energy sources which not only provide similar results in terms of fuel efficacy but must also be eco-friendly

and cost-effective. In this regard, the domain of biofuel has gained much attention from scientific communities during the last few decades as an alternative to these conventional fuels. The remarkable characteristics of biodiesel have made it a promising candidate to fulfill the upcoming world's future power demands. The unique features of biodiesel include biodegradability, environment friendly, less tox-icity, high combustion efficacy, renewability, and reduced emissions of sulfur oxides (SO_x) and nitrogen oxides (NO_x) on burning [4].

To highlight the reasons for increasing demand of this alternate energy source, recent studies are reviewed comprehensively for organizing the literature in terms of the utilized feedstock, the nature of the nanomaterials (NMs) used in nanobiocatalysts (NBCs), and the immobilization method used for fabrication of lipase enzyme in NBCs. This review article will act as a guideline to thoroughly understand the NBCs used as the catalysts for the production of this cutting-edge energy source of biofuel.

2. Resources of Biodiesel Production

A wide variety of feedstock has been tested for the production of biodiesel including the potential sources of oilseeds, algae, and animal fats. The selection of feedstock for biodiesel production is a crucial parameter as some of the feedstock resources are of high cost and utilization of the high-cost raw materials will make production of biodiesel an uneconomical process. Here, we have represented the three major classes of feedstock, along with their characteristics and availability, which are usually utilized for the production of biodiesel.

2.1. First-Generation Feedstock Oils

The use of vegetable oil has drawn much attention in this regard as the manufacturing of biodiesel from this source is relatively easy to prepare and is environmentally safe. If a country is rich in agricultural resources and can cultivate numerous oily feedstock providing crops then the edible oils can be used for biodiesel production. These oils include safflower [4], rapeseed [5,6], cardoon [7], coconut, soybean, sunflower, olive, almond, and canola oil, etc. [8–10]. These oils are suitable for biodiesel production due to their unique physicochemical properties such as low sulfur content and presence of aromatic constituents as well as their abundance but the use of these edible oils as feedstock competes with their utilization as an essential food [11]. Due to the increase in the human population, the demand for these edible oils is increasing day by day and its consumption in biodiesel manufacturing may result in the high cost of these edible oils in markets [1]. Therefore, using edible oil for biodiesel production is not considered a good practice. These issues have motivated the researchers to search for alternative ways for the feedstock which may not create a shortage in the human nourishment resources.

2.2. Second-Generation Feedstock Oils

Despite using edible oil for biodiesel production, the non-edible oils can be used for feedstock as well. These oils are classified as second-generation feedstock oils. Examples of these oils include waste frying oils, jatropha, palm, and linseed oils [12,13]. To overcome the issue of food/fuel debate associated with the first-generation feedstock, this second-generation feedstock is very helpful as it is non-edible and does not compete with the essentiality of food. However, there appeared to be a big issue of consumption of fertile land for the cultivation of these non-edible crops in comparison to the utilization of land and water for food production. Owing to these problems, the non-edible feedstock could not get much fame even when it was observed that these were much more efficient and eco-friendly as compared to the first generation feedstock oils [14].

The essentials such as animal fats including lard, chicken fat [15], fish fat, beef, and duck tallow are usually considered as kitchen waste [16,17]. The problem associated with the use of animal fat as feedstock is the presence of high levels of saturation which make it unsuitable to use in colder regions where the annual temperature is quite

low. Besides this, this kind of feedstock is more prone to oxidation due to the lesser amount of natural anti-oxidants present in these oils [18].

Similarly, frying waste oil as source material is also included in this feedstock class [19,20]. The price of this feedstock is much lower than others because in many restaurants and hotels, this oil is disposed of after successive frying. The disposal of this oil may cause severe problems such as a blockage in drain pipes and also a threat to aquatic life. Owing to the high levels of impurities and free fatty acids, these gutter oils need ultrafiltration and purification for further use in the manufacturing of high purity biodiesel.

2.3. Third-Generation Feedstock Oils

Third-generation feedstock includes algae, microbes, and yeast for oil production. This kind of source materials has successfully cleared the objections of competition with food and land as observed in the first and second-generation feedstock. The ex-traction of oil from these organisms yields a hundred times (up to 300) higher than the above-mentioned classes [21]. Moreover, the growth rate of these organisms is more than conventional oil crops. Some other remarkable properties of these sources are increased carbon dioxide fixation, no need for growth land, and production of biomass is higher [22].

All the above-mentioned generations of feedstock have their advantages and disadvantages along with some challenges, these are summarized in Table 1 [23].

Table 1. Summary of advantages and disadvantages of different feedstock oils used for the synthesis of biodiesel.

Feed Stock	Oil Sources	Benefits	Challenges
First Generation	Edible oil	• Ecofriendly • Easily available • Renewable	• In competition with food crops. • Shortage of fertile land for food crops cultivation
Second Generation	Non-edible oil	• Easily available • Renewable • No competition with food crops • Effective barren land utilization	• In Comparison with food crops for the utilization of water and land • Needs sophisticated methods for purification
Third Generation	Algae Microorganisms yeast	• Renewable • Higher yield of oil • Higher cell lipid contents • No competition with food crops for water and land usage.	• Initial set is not cost-effective • Commercially not viable

The extracted oil from the source material needs to be converted into biodiesel for practical use in engines. The conversion of crude oil to biodiesel modifies its chemical properties along with the reduction in the oil kinematic viscosity which is ten times higher than that of petroleum diesel. The as-obtained biodiesel converted from the oil can be used with or without little modification in the engine.

3. Biodiesel Production Strategies

Conversion of oil into biodiesel can be acquired from different ways such as pyrolysis, microemulsion, and transesterification. Among the conventional methodolo-gies, transesterification is regarded as the most effective way for synthesizing biodiesel as the prepared biodiesel has comparable physicochemical properties with the petro-leum diesel. There is no need for any modification in the vehicle engines for the intro-duction and consumption of the biodiesel obtained from this method. The transesteri-fication process can be enhanced by utilizing different types of catalysts. With respect to the types of catalyst used, the transesterification can be classified into the following typical categories including acid catalyzed transesterification; alkaline catalyzed transesterification; lipase

catalyzed transesterification, transition metal compound catalyzed transesterification, and silicates catalyzed transesterification [24,25].

Transesterification is a three-step reaction that requires a temperature around sixty to seventy degrees centigrade as it relates to the activation/boiling point of the used alcohol. It is a reversible reaction and the reactants need a catalyst to enhance the efficacy of the reaction. Generally, the starting material of this reaction is triglycerides (oils and fats) and alcohol (Figure 1). As mentioned earlier, it is a multistep reaction so the first step proceeds with the reaction of methanol and triglycerides resulting in the formation of diglycerides. Then in the next step, these diglycerides react with the remaining methanol and form monoglycerides. In the final step of this reaction, the monoglycerides react with the methanol and glycerol is produced. The pictorial description of the transesterification process is represented in Figure 1. These reactants are heated in the presence of a catalyst. Mostly, the basic catalyst is used in this process [11,26]. In recent years, the academic literature has shifted to the use of NBCs instead of typical catalysts for carrying out the process of transesterification.

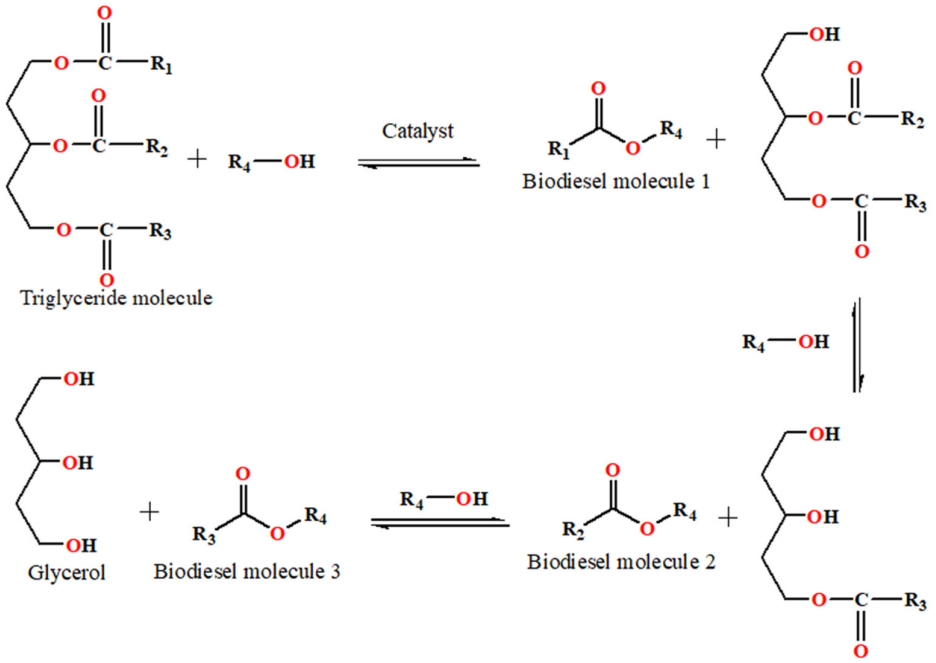

Figure 1. Three-step transesterification process.

In order to reduce the drawbacks of uneconomical raw materials, lengthy mul-ti-steps and low conversion rates, the scientific community is always searching for new ideas to develop a process that could address these issues. In this regard, the enzymatic method has gained much attention due to its ubiquitous properties of recovery/purity of the synthesized products, absence of soap formation in the reaction chamber, and higher conversion rates. Besides this, the use of lipase as an enzyme for transesterifica-tion is of great interest as this enzyme can work with broader ranges of feedstock. While working with the used frying oil and animal fats, one has to overcome the issues of moisture and free fatty acids. However, lipase enzyme can even work efficiently in such troublesome situations as well [2].

On the conflicting side, there appeared some disadvantages regarding the use of lipase enzyme. Use of enzyme makes this process costly and time-taking. Furthermore, disadvantages including the low stability, low recovery rates, and the associated low efficacy of free enzymes at the elevated conditions (high temperatures and pH) are considered hurdles against choosing this process on an industrial scale [27,28].

To overcome the aforementioned problems associated with the use of enzymes as a catalyst, the use of lipase immobilization techniques on solid supports is considered a novel state of the art technique extensively used in the recent years for the production of biodiesel. However, owing to less conformational restrictions and greater Brownian movement, nanomoieties are considered to be better support for enzyme immobilization as compared to the macro-sized conventional supports. In this context, NBCs comprising of various shapes, compositions, and sizes of nanoparticles (Figure 2), have appeared as potent nanocatalysts for biodiesel production. This technique provides a very promising solution in terms of reducing the cost owing to the high reusability rates and for increasing the shelf life of the enzyme. A detailed overview of the conventional methodologies and recent advancements is presented in Figure 2.

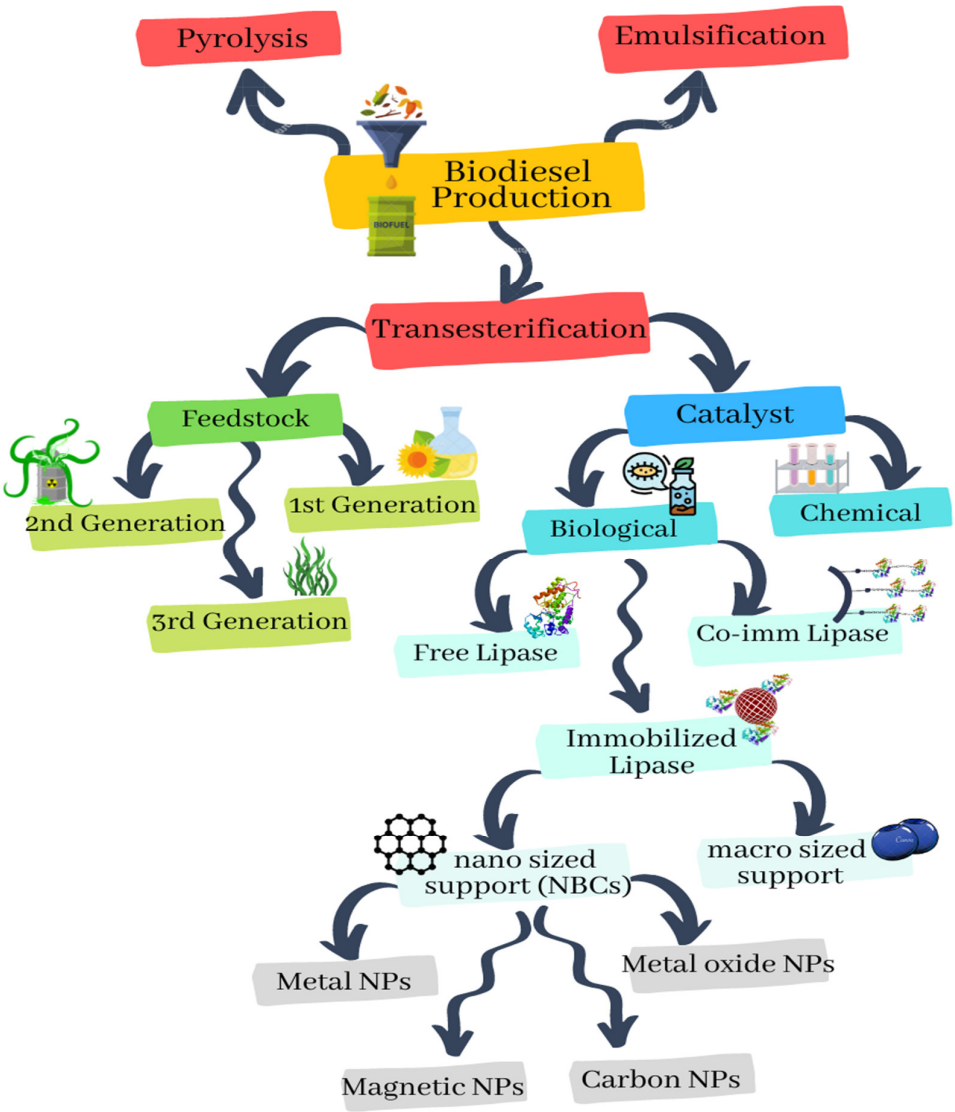

Figure 2. Fundamentals of biodiesel production through enzymatic transesterification.

4. Nanobiocatalysts (NBCs)

The NBCs utilized for the process of transesterification are primarily composed of two components i.e., the nanocatalyst component and the biological component. The peculiar characteristics of both components are essential in determining the efficacy of the NBCs for the process of biodiesel production. Typical steps involved in the transesterification reaction utilizing the NBCs are similar to as employed for simple nanocatalysts. It includes

the addition of specified amount of NBCs (usually sonicated for dispersion formation), alcohol, water and the raw material/feedstock in the conical flask. The acquired reaction mixture is homogenized by placing in an incubating shaker till the completion of the transesterification reaction. Formed glycerol is easily separated from medium by using various physical methodologies including fractionating funnel or simple pouring based separation of the acquired layers. The obtained product is then purified by using the rotatory evaporator functioning under reduced conditions. The NBCs are separated from the residual mixture by the process of centrifugation or magnetic field-based separation depending upon the nature of nanocatalytic component of NBCs [11,16,18]. Upon the basis of the nature of the nanocatalyst systems, the utilized NBCs are broadly classified into NBCs containing magnetic NMs, inorganic NMs, carbon scaffolds based NMs, and ordered mesoporous NMs. In the case of biological component, the NBCs are further classified with respect to the immobilization strategy utilized for the conjugation of the NMs with the biological component. Details of the case studies documenting various NBCs used for carrying out the process of transesterification are presented in the following subheadings.

4.1. Nanocatalytic Component of NBCs

The component of the assembly that lies within the nanometer range constitutes the nanocatalytic portion of the NBCs. Depending upon the nature of the utilized nanocatalytic system, the NBCs can be divided into the following categories.

4.1.1. NBCs Containing Magnetic NMs

Recent academic literature survey indicated that among all the NMs, magnetic NMs have been the most extensively utilized material as a nanocatalytic component of NBCs. Generally, the NMs having mean diameter in nanometer range have been documented as the lipase enzyme immobilization medium for the synthesis of NBCs. Dimensions in the range of nanometers scale for the magnetic NMs ensures the peculiar superparamagnetic properties, enhanced surface area values, larger number of the available active site, cheap preparative methodology and facile separation procedures (removal from the medium by using the influence of external magnetic field) to be present in the NBCs. Commonly, the two types of ferrite colloids including magnetite (Fe_3O_4) [29] and maghemite (γ-Fe_2O_3) [30] are utilized as the magnetic nanocatalytic component of the NBCs. As indicated by the literature survey performed in Table 2, the NBCs containing Fe_3O_4 NMs are most widely used as the main representative element for the magnetically active NBCs. Although the preparation methodology required for Fe_3O_4 NMs (i.e., co-precipitation procedure) is quite facile, additional functionalization steps are required for the immobilization of the lipase enzyme on these NMs. This additional requirements of modification are attributed to the facts that bare/pure Fe_3O_4 NMs are susceptible to air oxidative reactions, unstable under acidic conditions, and unable to develop interactions with the biological molecules owing to their hydrophobic inorganic nature [31]. Keeping in mind the above-stated shortcomings of Fe_3O_4 NMs, numerous surface engineering techniques (most widely used grafting strategy) are utilized for making these NMs appropriate to be utilized for biological/enzymatic applications. Modification of the surface of the NMs by functionalizing it with specific functional groups (such as amines or carboxylic groups) further facilitates the attachment of the lipase enzyme (a biological component of NBCs) with the NMs by developing covalent bonds at the specific sites of the immobilizing enzyme [32].

For the amino functionalization of Fe_3O_4 NMs, the amino silanes (particularly 3-aminopropyl triethoxysilane (AP)) are utilized as the most commonly used functionalizing reagent for NMs. The hydrophobic nature of the silanes, owing to the presence of strong silicon-oxygen (inorganic) backbone and flexible hydrophobic carbon tail, makes the grafting of the silanes over NMs quite easy as the nature of both the grafting medium and grafting agent complements each other. The presence of an amino group is further utilized for converting the hydrophobic surface of the AP coated NMs into the more hydrophobic

surface by using the functionalizing reagent of glutaraldehyde (GA). This functionalization is essential for immobilizing the enzyme on the surface of NMs. The interaction between AP and GA is facile as the natural affinity of the amino group with the carboxylic group to form amide bonds is involved during this bifunctional surface engineering of NMs. Costa et al. [33] engineered Fe_3O_4 NPs by utilizing the co-precipitation method and functionalized it with AP and GA, respectively. The functionalized NMs were used to covalently immobilize the *Candida antarctica* lipase B to synthesize the NBCs. Touqeer et al. [20] synthesized PDA stabilized Fe_3O_4 NPs by utilizing the solvothermal method. The lipase from the *Aspergillus terreus* AH-F2 was covalently immobilized over the synthesized NPs and the prepared NBCs were used for catalyzing the transesterification of the waste cooking oil to produce biodiesel. Case studies reporting these magnetic NBCs are summarized in the Table 2.

4.1.2. NBCs Containing Inorganic Nanoscaffolds

Inorganic nanoscafolds, including graphene oxide (GO), carbon nanotubes (CNTs), silica (SiO_2), and alumina (Al_2O_3), etc. are also one of the most extensively utilized nanocarriers for the synthesis of the NBCs. These inorganic rigid nanoscaffolds provide numerous advantages over other NMs for the immobilization of the lipase enzyme. GO is an exceptional material known for its extremely large surface area, extremely high thermal stability, high mechanical strength, and availability of two-dimensional surfaces for the grafting or functionalization procedures [34]. The presence of various functional moieties including carboxylic groups, hydroxyl groups, and epoxy groups facilitates the successful immobilization of the lipase without using peculiar cross-linking and functionalizing agents. In terms of immobilization of the lipase on GO to form NBCs, the use of immobilization methods including covalent, physical, and entrapment methodology has been documented in the academic literature [34]. Li et al. [35] utilized carboxylic groups of GO for immobilizing the lipase enzyme to synthesize NBCs. The NBCs exhibited 80% hydrolytic efficacy which was found to be far greater than the activity shown by the free lipase enzyme indicating that GO is an exceptional material for the immobilization of the lipase enzyme. Rather than using GO as a nanocatalytic component of the NBCs, the GO is often utilized in the nanocomposite form where certain other NMs are embedded in the single atomic honey-comb carbon sheet to acquire the supporting matrix of the NBCs. The extensive utilization of this approach is attributed to the fact that the two-dimensional sheet-like morphology of GO makes it susceptible to the reaction medium surroundings. Incorporating rigid NMs into the GO sheets ensures that the synthesized NBCs retains the characteristics of its nanocatalytic component even when some of the potentials is lost owing to the modifications in the functional moieties of GO due to medium conditions. Xie et al. [36] incorporated Fe_3O_4 NPs into the GO matrix and utilize it to covalently immobilize the *Candida rugosa* lipase on its surface. The synthesized NBCs carried out the transesterification reaction for soybean oil with a maximum yield of 92.8% under optimum conditions.

CNTs are also another widely used inorganic nanoscaffold that is utilized for the synthesis of NBCs. The peculiar characteristics of the CNTs include low diffusion capabilities, high surface area, high mechanical stability, and high enzyme loading capabilities, etc. Enzymes have been documented to be immobilized on the multi-walled CNTs (MWCNTs) by utilizing the covalent and physical adsorption as an immobilization methodology. Fan et al. [37] documented the formation of NBCs by covalently immobilizing the lipase acquired from *Rhizomucor miehei* on the Fe_3O_4-MWCNTs. The synthesized NBCs exhibited the transesterification reaction for wasted vegetable oil with a yield of 94% under optimal conditions. Mohamad and co-workers [38] utilized a simple adsorption procedure for immobilizing the lipase enzyme onto the surface of acid-functionalized MWCNTs for engineering NBCs. The functionalization is achieved by mixing the MWCNTs in the acid dispersion of sulfuric acid (H_2SO_4) and nitric acid (HNO_3). The presence of charged carboxylic group actively interacted with the other polar moieties (NH_2 and OH) of the enzyme to develop the NBCs. The comparison of free lipase with the immobilized enzymes

in terms of hydrolytic activity and thermal/mechanical stability revealed that the activity was found to be two-fold in the case of immobilized lipase.

Other inorganic nanoscaffolds include Al_2O_3 and SiO_2 NMs that are also utilized for the synthesis of NBCs. Gardy et al. [39] engineered SO_4/Fe_3O_4-Al_2O_3-TiO_2 NMs as a nanocatalytic assembly and utilized the synthesized assembly for the transesterification of the waste cooking oil. Literature survey indicates that the case studies reporting the use of Al_2O_3 for the nanocatalytic assembly are available in the academic literature [40]. However, the utilization of this inorganic nanoscaffold for the formation of nanobiocatalyst for biodiesel production is quite scarce and further research is required in this domain. In comparison, SiO_2 is explored quite extensively in the academic literature for the synthesis of biodiesel by using NBCs. Fathi et al. [41] synthesized NBCs by utilizing the AP functionalized SiO_2 NPs. The AP functionalized silica NPs were further modified by modifying with the zwitter ions. A non-covalent attachment was achieved by immobilizing lipase (acquired from *Yarrowia lipolytica*) onto the NPs without the addition of any cross-linker or precipitating agent. Ashjari et al. [42] synthesized NBCs by utilizing a novel methodology involving the simultaneous immobilization of the lipase enzyme acquired from two different sources (i.e., *Rhizomucor miehei* and *Thermomyces lanuginose*) on the functionalized Fe_3O_4@SiO_2 NMs via chemical grafting method. The synthesized NBCs were used for the transesterification of the waste cooking oil. Case studies documenting the inorganic nanoscaffolds as the nanocatalytic component of NBCs are summarized in the Table 2.

4.1.3. NBCs Containing Metal Oxide NMs

Although literature documenting the formation of NBCs containing metal oxides as a nanocatalytic component are quite abundant in academic literature [43–45] but these NBCs are not generally utilized for the process of transesterification. More work is needed to be done for the expansion of work in this domain. Fatima et al. [46] synthesized PDA stabilized CeO_2 NMs and covalently immobilized the *Aspergillus terreus* lipase on the NMs by using the chemical grafting technique. The NBCs were utilized as the catalyst for the transesterification reaction of *Eruca sativa* oil. The reaction was optimized by using the techniques of response surface methodology (RSM) and gas chromatography-mass spectroscopy (GC-MS).

4.1.4. NBCs Containing Metal-Organic Frameworks (MOFs)

MOFs are one of the advanced classes that have recently emerged in the academic literature as the nanoscaffold utilized for the immobilization of the enzyme to synthesize NBCs [47]. These highly porous and crystalline materials (containing high surface area, opened pores, versatile composition, suitable pore volume, and unique topographical characters) have been documented to host diverse species particularly enzymes. Three different immobilization strategies have been used for the immobilization of the enzymes into the MOFs. These strategies include the surface immobilization method (grafting active species on the outer surface of the MOFs for developing the interaction between enzymes and MOFs), a ship-in-a-bottle methodology (Enzymes are diffused into the pores of pre-formed MOFs), and a bottle-around-a-ship strategy (the MOFs are assembled around the enzyme also termed as in-situ encapsulation) [48]. Among these strategies, the in-situ encapsulation is regarded as the most appropriate immobilization method for immobilization of enzymes in terms of resistance to degradation, minimization of the enzyme leaching problems, and reduction of the biodiesel cost owing to multiple recycling and reuse potential of these MOFs [48].

Table 2. Case studies representing the summary of the Nanobiocatalysts utilized.

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
NBCs containing magnetic NMs							
GA-Fe_3O_4 NPs	Lipase from *Candida rugosa*	NPs by co-precipitation method Chemical grafting for lipase immobilization	NaOH was used as precipitating agent for the mixture of iron salts 100 mM phosphate buffer, pH = 7, 25% GA, nanocatalyst and lipase addition at T = 35 °C with continuous stirring for 2 h	AFM XRD FTIR VSM	80–100 nm	Covalent attachment	[51]
Fe_3O_4 NPs AP-Fe_3O_4 NPs GA-AP-Fe_3O_4 NPs	Lipase from *Rhizopus oryzae*	Fe_3O_4 NPs by co-precipitation method Functionalization by chemical grafting Chemical grafting for lipase immobilization	For the functionalization with AP and GA, the NPs were sonicated in the solution of functionalizing moiety 5 mg of catalyst, 1 cm^3 100 mM phosphate buffer, pH = 7.5, 0.300 cm^3 enzyme solution at 4 °C and 120 rpm shaking speed for 17 h in incubator shaker.	FESEM EDX XRD FTIR VSM	20–30 nm	Physical attachment in case of Fe_3O_4 NPs. Covalent attachment and electrostatic attachment in case of AP-Fe_3O_4 NPs and GA-AP-Fe_3O_4 NPs	[52]

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
AP-Fe_3O_4 NPs	CLEAs from *Candida Antarctica*	Fe_3O_4 NPs by co-precipitation method Functionalization with AP by chemical grafting	NH_4OH added in the $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ for acquiring precipitates followed by functionalization with AP in presence of CH_3OH and glycerol.	SEMFTIR	–	Covalent attachment of CLEAs with the AP-Fe_3O_4 NPs	[53]
		Formation of CLEAs	Purified lipase solution was mixed with GA solution at T = 30 °C for 3 h to get CLEAs				
		Formation of CLEAs-AP-Fe_3O_4 NPs by chemical grafting	5 mg NPs were mixed with the CLEAs solution in the presence of the GA (precipitating agent) for acquiring complex system				
AP-GO-Fe_3O4 NPs	CLEAs from *Enterobacter cloacae*	GO by Hummers method Fe_3O_4 NPs by co-precipitation method Functionalization with AP by chemical grafting	–	SEM EDX FTIR	–	Covalent attachment of CLEAs with the AP-GO-Fe_3O_4 NPs	[34]
		Formation of CLEAs	Purified lipase solution was mixed with GA solution at T = 30 °C for 4 h to get CLEAs				
		Formation of CLEAs-AP-GO-Fe_3O_4 NPs by chemical grafting	3 mg/mL CLEAs were mixed with the NPs in the presence of the GA (precipitating agent) for acquiring complex system				

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
PDA-Fe_3O4 NPs	Lipase from *Aspergillusterreus* AH-F2	Fe_3O_4 NPs by solvothermal method PDA coating by grafting method	Mixture of 1 g of $FeCl_3.6H_2O$, 20 mL ethylene glycol, 3 g $C_2H_3NaO_2$ and 10 mL ethylene diamine followed by the autoclave at 180 °C for 8 h. Functionalization with 0.1 g of dopamine hydrochloride.	XRD FTIR TEM	24–27 nm	Covalent attachment of Lipase with NPs	[20]
		Chemical grafting for lipase immobilization	0.4 g Lipase, 40 mL phosphate buffer, drop wise addition of NPs at 4 °C and continuous stirring for 3 h				
GA-AP-Fe_3O_4 NPs	Lipase from *Candida antarctic*	Fe_3O_4 by co-precipitation Functionalization with AP and GA by grafting	Ionic liquid of ([BMIN]BF4) was used as template during co-precipitation.	XRD FTIR TEM EDX SQUID	150–220 nm	Covalent attachment of lipase with NMs	[54]
		Chemical grafting for lipase immobilization	800 mg Lipase, 25 mL of phosphate buffer, pH = 7.0 and 2 g GA-activated NMs was incubated for 2 h.				
P(GMA-co-MAA)-Fe_3O_4 NPs	Lipase from *Candida rugosa*	Fe_3O_4 NPs by solvothermal method P(GMA-co-MAA)-Fe_3O_4 NPs by blending	0.1 g of Fe_3O_4, 60 mL of acetonitrile, 0.4 g of GMA, 0.4 g of MAA, 0.2 g DVB and 0.02 g of AIBN was reflexed for 20 min.	FTIR XRD SEM TEM XPS VSM N_2 adsorption-desorption studies	200–400 nm	Covalent attachment of lipase with NMs	[55]
		Chemical grafting for lipase immobilization	0.1 g NBCs, lipase enzyme, 100 mL phosphate buffer, pH = 7, T = 30 °C and incubation reaction time = 5 h				

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
Fe_3O_4@PDA NPs	Lipase from *Pseudomonas cepacia*	Fe_3O_4 NPs by co-precipitation Coating with the PDA to get core@shell morphology	200 mL NaOH solution, 0.2 mol/L $FeCl_3$ and 0.1 mol/L $FeCl_2$ solutions were magnetically stirred at 1100 rpm.	DLS AFM FTIR TEM	11 nm Fe_3O_4 NPs	Covalent attachment of lipase NPs	[56]
		Chemical grafting for lipase immobilization	10 mL phosphate buffer, pH = 7, T = 4 °C, 200 mg lipase solution, 4 mL NBCs dispersion and reaction time = 1 h				
GA-Fe_3O_4 NMs	Lipase B from *Candida antarctica*	Fe_3O_4 NMs by co-precipitation method	GA was utilized as the precipitating and activating agent	SEM DLS	293 ± 87 nm	Covalent attachment of lipase	[57]
		Formation of CLEAs by cross-linking	Purified lipase solution was cross-linked in the presence of GA				
		Immobilization of CLEAs by	Both dispersions were mixed in the presence of ammonium sulphate and GA as the cross-linkers/precipitating agent. 150 mM phosphate buffer, pH = 4.4 and stored at 4 °C				
NBCs containing metal oxide NMs							
PDA-CeO_2 NRs	Lipase from *Aspergillus terreus*	Hydrothermal process for PDA-CeO_2 NPs	Hydrothermal process: autoclave for 24 h at 120 °C, calcination in furnace for 2 h at 500 °C followed by coating of NRs with PDA by grafting method/covalent attachment	SEM XRD FTIR EDX	Diameter 50–60 nm and length 150–200 nm	Chemical attachment of Lipase with NRs	[46]
		Chemical grafting for lipase immobilization	0.4 g Lipase, 40 mL phosphate buffer, drop wise addition of NRs at 4 °C and continuous stirring for 3 h				

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
$PPy/MA\text{-}TiO_2$ NPs	Lipase from *Rhizopus oryzae*	$PPy/MA\text{-}TiO_2$ NPs are synthesized by in-situ chemical polymerization	In TiO_2 dispersion, pyrrole and methyl anthranilate was added into the suspension to get the matrix containing embedded NPs	FTIR XRD TGA DTA TEM SEM EDX	38–50 nm functionalized NMs	Chemical attachment when immobilization is performed in the presence of GA.	[58]
		Lipase grafting by chemical method	5 mL lipase solution, 5 mL NBCs dispersion, 0.1 M TrisHCl-buffer with pH = 8 and incubation time = 24 h			Physical attachment in the absence of GA	
NBCs containing inorganic nanoscaffolds							
$Fe_3O_4@SiO_2$ NPs PEI-$Fe_3O_4@SiO_2$ NPs PAA-$Fe_3O_4@SiO_2$ NPs	Lipase from *Pseudomonas cepacia*	Co-precipitation for Fe_3O_4 NPs and Stober Method for $Fe_3O_4@SiO_2$ NPs	TEOS, ethanol and NH_3 for preparation followed by drying at 50 °C for NPs	-	-	Physical attachment for $Fe_3O_4@SiO_2$ NPs	[59]
		Chemical grafting for lipase immobilization	2 mg/L lipase, 2.5 mL phosphate buffer, addition of dispersed NPs at 4 °C with continuous stirring for 2 h.			Chemical attachment for PAA and PEI modified NPs	

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
Fe_3O_4 NPs GO/Fe_3O_4 NPs AP-GO/Fe_3O_4 NPs GA-GO/Fe_3O_4 NPs	Lipase from *Rhizopus oryzae*	GO by Hummer's Method Fe_3O_4 NPs by co-precipitation method GO/Fe_3O_4 NPs by in-situ deposition of NPs on GO surface	T = 80 °C, N_2 flow, 25% NH_3 solution was utilized for in-situ deposition For the functionalization with AP and GA, the NPs were sonicated for 2 h in the solution of functionalizing moiety	AFM FTIR XRD EDX FESEM VSM ZPM BET	20–30 nm for Fe3O4 NPs	Physical attachment for Fe_3O_4 NPs and GO/Fe_3O_4 NPs	[60]
		Chemical grafting for lipase immobilization	1 mL phosphate buffer solution, 5 mg of each NPs, pH = 7.5 followed by the addition into lipase solution. Mixture was shaken at 4 °C for 17 h in shaking incubator at a stirring speed of 120 rpm.			Electrostatic attractions and covalent attachment for AP-GO/Fe_3O_4 NPs and GA-GO/Fe_3O_4 NPs	
GA-PMAM-Fe_3O_4/MWCNTs	Lipase from *Burkholderiacepacia*	MWCNTs-NH_2 was prepared by chemical grafting. Fe_3O_4 NMs was incorporated in the MWCNTs-NH_2 by deposition	MWCNTs were purchased from Nanotech Port Corporation Limited. Functionalization was carried out in the order: MWCNTs-COOH, MWCNTs-NH_2, Fe_3O_4-MWCNTs, PMAM-Fe_3O_4-MWCNTs and GA-PMAM-Fe_3O_4-MWCNTs	TEM SQUID XRD CLSM XPS	40–60 nm for MWCNTs	Chemical attachment of lipase with the NBCs	[61]
		Chemical grafting for lipase immobilization	0.1 g of nanocomposite, 25% GA, 5 mL phosphate buffer, pH = 7, shaker speed 200 rpm at 30 °C and reaction time of 10 h.				

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
Fe_3O_4@SiO_2 NPs AP-Fe_3O_4@SiO_2 NPsMP-Fe_3O_4@SiO_2 NPs	Lipase (NS81006) from the genetically modified *Aspergillus niger*	Fe_3O_4 NPs by co-precipitation Functionalization with AP and MP by Stober Method	Fe_3O_4 NPs synthesis: stirring = 600 rpm, T = 30 °C, reaction time = 30 min, 25% ammonia solution followed by heating at 85 °C for 30 min. Ultrasonication for 30 min for functionalization.	XRD SEM TEM FTIR VSM	20.05 nm	Chemical attachment of Lipase with the NBCs	[62]
		Chemical grafting for lipase immobilization	0.5 g NBCs, 10 mL ethanol, 10% GA, T = 25 °C, reaction time = 2 h, phosphate buffer solution and pH = 7.				
$NHCS_2H$-Fe_3O_4/GO NMs	Lipase from Porcine pancreas	GO by Hummer's methodFe_3O_4 prepared via co-precipitation Functionalization of GO and Fe_3O_4/GO NMs	GO dispersion, 1.29 g $FeCl_2$, 3.51 g $FeCl_3$, 3%wt. acetic acid, T = 80 °C, 20 mL NH3 solution and oven drying of the Fe_3O_4/GO NMs at 60 °C for 12 h. Functionalization with dithiocarbamate by doing reflex in the presence of AP and CS_2	SEM TEM EDX UV-VIS	10–25 nm	Chemical attachment of Lipase with GO as well as Fe_3O_4/GO NMs	[63]
		Chemical grafting for lipase immobilization	3 mg/mL Lipase solution, pH = 7.5, phosphate buffer solution, incubation at T = 38 °C for reaction time 10 h				
PDA@Co-MWCNTs	Lipase from *Candida rugosa*	Co-MWCNTs by in-situ oxidative polymerization Functionalization of MWCNTs with PDA	Co doped MWCNTs were generated by using the precursor salt of $CoCl_2$. Polymerization of the aniline was used to stabilize MWCNTs while PDA coating was carried out to stabilize the Co-MWCNTs.	FTIR TEM TGA DTA CLSM CD	70–90 nm	Chemical attachment of lipase with the PDA@Co/MWCNTs	[64]
		Chemical grafting for lipase immobilization	1 mg/mL lipase solution, 2.5% GA solution, pH = 7.4, phosphate buffer solution 50 mM and incubation time of 6h				

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
Fe_3O_4/GO NMs	Lipase from *Candida rugosa*	GO by Hummer's method Fe_3O_4 prepared via co-precipitation Functionalization of GO and Fe_3O_4/GO NMs Chemical grafting for lipase immobilization	500 mg GO suspension, 3 g $FeCl_3.6H_2O$, 2.1 g $FeSO_4.7H_2O$ and NH_3 solution was stirred at 80 °C followed by ultrasonication for 45 min. Vacuum drying at 60 °C Powdered lipase, phosphate buffer 100 mL, pH = 7 and NMs suspension was incubated at 30 °C for 5 h.	TEM XRD XPS FTIR VSM N_2 absorption-desorption studies	10–20 nm	Covalent attachment of lipase with Fe_3O_4/GO NMs	[36]
PANI/Ag/GO NMs	Lipase from *Aspergillus niger*	Formation of PANI/Ag/GO by cross-linking method Chemical grafting for lipase immobilization	In-situ polymerization by combining GO (obtained by Hummer's method) and PANI/Ag by crosslinking via ammonium sulphate in hot air incubator. Activation of nanocatalyst via GA. 10 mg of lipase solution, 100 mL phosphate buffer, pH = 7 and NMs suspension was incubated at 1800 rpm at 4 °C	FTIR TEM SEM XRD DLS AFM TGA	50 nm	Covalent attachment of lipase with PANI-Ag/GO NMs	[65]
NBCs with advanced nanocatalytic systems							
ZIF-8 (Zn-MOFs)	Lipase from *Rhizomucor miehei*	one-step encapsulation method for synthesizing X-shaped L@ZIF-8	Coordination between zinc (Zn^{2+}) ions and 2-methylimidazole generated ZIF-8	FESEM EDX TEM FTIR Powder XRD BET	-	Encapsulation	[66]

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
(MOFs) Cu-NFs	Lipase from Porcine pancreas	L@Cu-NFs by biomimetic mineralization strategy	1 mL $CuSO_4$, 20 mL phosphate buffer and 20 mg lipase enzyme mixture was incubated for 3 days at 4 °C. Centrifugation was used for the collection of NBCs.	FTIR TGA SEM XRD EDX BET CLSM N_2 absorption-desorption studies	-	Encapsulation	[67]
ZIF-67 (Co-MOFs)	Lipase from *Candida rugose*	in situ encapsulation of lipase with cobalt 2-methylimidazolate framework ZIF-67	4 mL lipase solution, 2 mL cobalt nitrate and 5 mL 2-methylimidazole were stirred at T = 25 °C. Centrifugation at 6000 rpm for 20 min and was washed with phosphate buffer solution.	Powder XRD TGA FESEM NMR EDX FTIR BET UV-VIS	5.03 nm MOFs after the lipase encapsulation	Encapsulation	[48]
UiO-66 (Zr-MOFs)	Lipase from *Aspergillus niger*	UiO-66 was synthesized by the hydrothermal methodology Modification with PDMS was done by CVD	$ZrCl_4$ was ultra-sonicated in the presence of DMF and BDC and was autoclaved to get the UiO-66. Modification of the UiO-66 was done by placing the UiO-66 powder on thin glass placed under fresh PDMS stamp	Powder XRD BET N_2 adsorption-desorption studies Contact angle studies	-	Physical adsorption	[49]
		Immobilization by physical adsorption	30 mg UiO-66, 22.5 mg/mL lipase, 0.05 mol/L phosphate buffer solution, pH = 5.6, incubation at 45 °C with the shaker speed of 200 rpm carried out for 4 h.				

Table 2. *Cont.*

Nanomaterials	Biomaterials/ Stabilizing Agents	Formation Process	Formation Conditions	Characterization Techniques	Size of the Nanomaterials	Nature of Lipase Attachment with NMs	Reference
Zn-MOFs	thermophilic lipase from *Alcaligenes* sp.	L@Zn-MOFs by biomimetic mineralization strategy	Centrifugation at 6000 rpm at 4 °C. 50 mg lipase and zinc acetate 200 mg incubation at room temperature for 48 h, the immobilizedL@Bi-MOF was collected by the centrifugation 8000 rpm for 5 min at 4 °C.	SEM TEM PXRD TGA FTIR CLSM CD BET	800 nm	Encapsulation	[50]

Scanning Electron Microscopy (SEM), Energy dispersive X-ray analysis (EDX), Brunauer-Emmett-Teller analysis (BET), Fourier Transform Infrared Spectroscopy (FTIR), Ultraviolet Visible Spectroscopy (UV-Vis), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Transmission Electron Spectroscopy (TEM), Nanorods (NRs), Nanoparticles (NPs), Polydopamine (PDA), Polyacrylic acid (PAA), Polyethyleneamine (PEI), Tetraethyl orthosilicate (TEOS), Field Emission Scanning Electron Microscope (FESEM), Vibrating Sample Magnetometer (VSM), Zeta potential Measurements (ZPM), 3-aminopropyl triethoxysilane (AP), Glutaraldehyde (GA), Atomic Force Microscopy (AFM), Cross-linked Lipase Enzyme Aggregates (CLEAs), 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIN]BF4), Superconducting Quantum Interference Device (SQUID), multi-walled carbon nanotubes (MWCNTs), polyamidoamine (PMAM), confocal laser scanning microscopy (CLSM), 3-mercaptopropyltrimethoxysilane (MP), Tetraethyl orthosilicate (TEOS), poly(glycidyl methacrylate-co-methacrylic acid) (P(GMA-co-MAA),glycidylmethahacrylate (GMA), Divinylbenezene (DVB), methacrylic acid (MAA), 2,2′-azodiisobutyronitrile (AIBN), Thermogravimetric analysis (TGA), Differential thermal analysis (DTA), Circular dichroism spectroscopy (CD), Attenuated total reflection (ATR), Dynamic Light Scattering (DLS), Atomic force microscopy (AFM), Metal organic frameworks (MOFs), zeoliticimidazolate framework (ZIF), nanoflowers (NFs), polyaniline (PANI), polypyrrole (PPY), methyl anthranilate (MA), Universiteteti Oslo (UiO-66), polydimethylsiloxane (PDMS), Zirconium chloride ($ZrCl_4$), 1,4-benzenedicarboxylic acid (BDC), chemical vapour deposition (CVD), N,N′-dimethylformamide (DMF).

Hu et al. [49] synthesized typical Zirconium (Zr) based MOFs and utilize it to immobilize *Aspergillus niger* lipase enzyme via facile physical adsorption. Before immobilization, MOFs were coated with the polydimethylsiloxane (PDMS) by using chemical vapour deposition (CVD) which modifies the MOFs surface and impart it with the hydrophobic nature along with the retention of the intrinsic properties of the NBCs. For immobilization of the enzyme, hydrophobic interactions were used for carrying out the physical absorption. The synthesized NBCs were then utilized for the transesterification reaction of soybean oil to form biodiesel. Li et al. [50] engineered L@Zr-MOFs by utilizing the biomimetic mineralization strategy. The synthesized L@MOFs was used alongside the methanol for carrying out the transesterification of sunflower oil. No significant change in morphology of the NBCs was observed after three cycles of reuse. Case studies summarizing the NBCs containing MOFs are summarized in Table 2.

4.2. Biological Component of NBCs

NBCs containing enzymes as biological components have been frequently documented in recent years for various applications [68,69]. One of the main applications of these enzymes-based NBCs is to catalyze various industrially important reactions with high specificity, stereoselectivity, and efficacy. Owing to the presence of the biological component in the NBCs, the reactions must be carried out under mild reaction conditions. Otherwise, the biological component may suffer degradation or denaturing due to the extreme reaction conditions [69]. NBCs utilized for the production of biodiesel usually carries out the process of enzymatic transesterification with the help of the lipase enzyme (which is naturally capable of performing hydrolysis of the triglycerides into fatty acids and glycerol present at the interface of oil/water) [70]. Furthermore, the lipase enzyme catalyzes numerous esterification and transesterification processes [71]. Chemically, lipases are regarded as triacylglycerol acyl hydrolases that are naturally present in plants, animals, and microorganisms as indicated by Table 2. Extremely high conversion rates up to 90% in terms of synthesis of fatty acid alkyl esters (FAAE) have been documented in the case of using lipase as a component of NBCs [71]. Moreover, other disadvantages such as the occurrence of side reactions, low conversion rates, extreme reaction parameters, high working/maintenance cost, and requirement of difficult separation techniques can be avoided by utilizing the lipase enzyme-based NBCs. In addition, the utilization of renewable feedstock as raw materials is another advantage that is achieved by using these peculiar NBCs [72]. Numerous immobilization procedures, used for the fabrication of lipase enzyme on/inside the nanocatalytic component of the NBCs, are presented in Figure 3. The selection of the suitable immobilization technique is regarded as the most crucial step for acquiring the high efficacy of the NBCs for catalyzing any chemical reaction. Apart from the efficacy of the lipase based NBCs, the properties such as overall activity, stability, regeneration, inactivation potential, toxicity, and effectiveness of NBCs are influenced by the procedure opted for the immobilization of lipase enzyme. Generally, the immobilization methods for lipase can be divided into following categories.

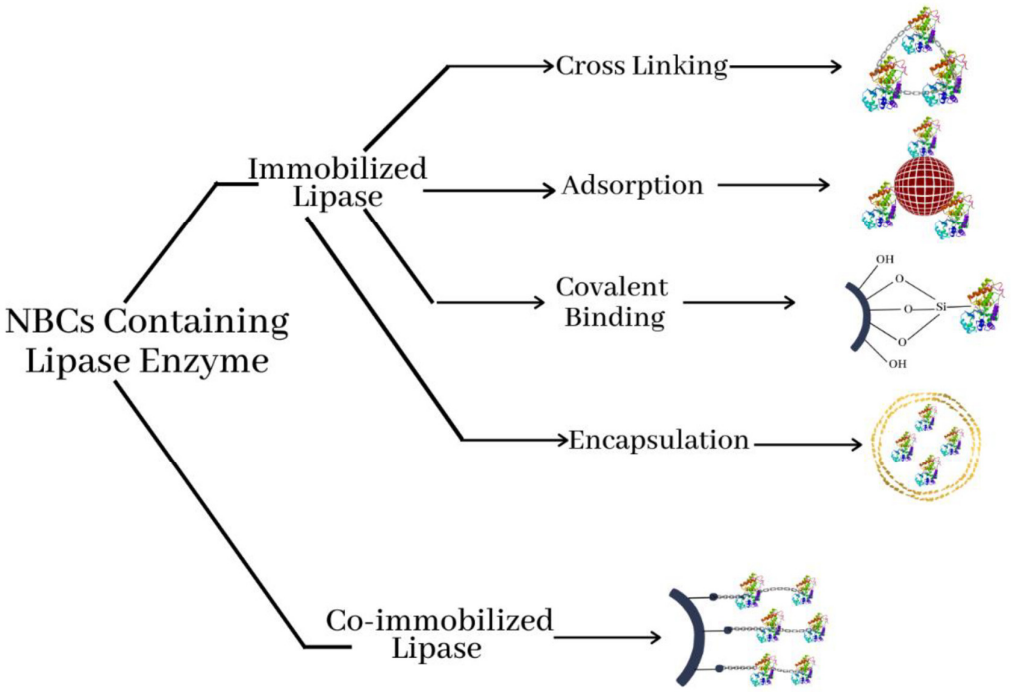

Figure 3. Enzyme immobilization methods used for the production of NBCs.

4.2.1. Physical Adsorption

Immobilization via physical adsorption is carried out by developing weak monovalent interactions (i.e., hydrophobic interactions, van der Waals interactions, ionic bonding, and hydrogen bonding) between lipase and the NMs of the NBCs. It is a reversible immobilization strategy and is regarded as the more advantageous mode of immobilization in terms of activity as no significant configurational changes in the enzyme is observed during the physical adsorption of the enzyme on the NMs. The presence of the hydrophobic surface (NMs) is essential for physically adsorbing the enzyme on the NMs [73]. However, the certain disadvantage of leaching of the enzyme has been observed in case of using this immobilization method. The weak nature of these physical bonds makes the immobilized lipase susceptible in extreme conditions (temperature, pH val-ues, and ionic strength, etc.) and numerous researchers have documented that the re-tention capabilities of the lipase enzymes physically adsorbed on the NMs in NBCs is challenged during varying reaction conditions resulting in the process of leaching and thus, reduced lipase activities was observed during reruns experiments [60].

4.2.2. Entrapment

Immobilization via entrapment also does not involve the formation of covalent bonds. Instead, the lipase enzymes are entrapped within the constraints of the support medium via inclusion procedures. This inclusion is an irreversible process and the synthesized NBCs permits for the reacting molecules and products to interact with the enzyme but the entrapped enzyme itself does not leach out of the assembly. Entrapment utilizes the proper balancing of mechanical forces of the entrapping medium and enzymes to reduce the problems of enzyme inactivation and enzyme leaching by not allowing the enzyme to develop any chemical interaction between the lipase and medium. Entrapment is the most recent and advanced immobilization technique which is frequently utilized in the case of MOFs based NBCs. Some researchers assume that entrapping the lipase with the framework of the supporting matrix should cause problems during the catalysis as entrapping the enzyme would create the diffusional barrier between reactant and enzyme. The reactant molecule would not be able to easily reach the enzyme owing to

the surrounding framework [73]. However, this barrier was not significantly observed in the case of the enzymatic transesterification reaction used for the production of biodiesel. In fact, some of the highest conversion rates for biodiesel production were observed in case of these NBCs at optimum conditions [48,66,67].

4.2.3. Covalent Bonding

Immobilization via covalent bonding is the most commonly utilized irreversible immobilizing procedure for lipase enzyme. The structural and configurational characteristics of lipase are responsible for this type of immobilization. Particular functional groups present in the amino acids (lysine: α-amino group, cysteine: thiol group), aspartic acid/glutamic acids: carboxylic groups and other phenolic and imidazole groups) associated with the lipase enzymes which are not essential for the catalytic activity of the enzyme take part in this type of immobilization [37]. For covalent immobilization, the complementary bond-forming moieties must also be present on the surface of the NMs. The GA and AP are usually used as the surface functionalization agent in the case of the NMs. However, the surface functionalization is not necessary for developing the covalent bond in the case of the inorganic scaffolds that already contain such functional groups as the respective functional moieties [74]. In any of the cases, the nucleophilic sites of the lipase molecules interact with the electrophilic functional moieties of the NMs by forming covalent bonds. The advantages of this type of immobilization included high reusability potential of the NBCs owing to the extremely high stability because of the formation of covalent bonds. Thermal stability as well as the half-life period of the lipase was also found to be highly effective in case of this type of immobilization [75].

4.2.4. Cross-Linking

Immobilization via cross-linking is also one of the recent immobilization methods which are documented for carrying out irreversible immobilization of the lipase enzyme. This procedure for immobilization can be utilized with and without the usage of the NMs in NBCs. If this crosslinking is performed without the use of any stabilization medium/NMs, it is called carrier-free immobilization. If the lipase is cross-linked separately and then introduced into the NMs by covalent attachment it is regarded as co-immobilization. In the case of carrier-free immobilization, the lipase molecules are firstly purified and are then cross-linked by the development of the intermolecular cross-linkages between lipase molecules by using the cross-linking agents. GA was utilized as the enzyme linker for the preparation of the cross-linked *Burkholderia cepacia* lipase [76]. GA works by the reaction of free amino groups (lysine) and GA oligomers by utilizing inter and intra-molecular aldol condensation. In the case of co-immobilization, the cross-linked aggregates of lipase are firstly prepared by using the cross-linking agents. These aggregates, in the second step, are then immobilized on NMs to synthesize co-immobilized NBCs [57].

5. Fundamentals Associated with the Biodiesel Production via NBCs

5.1. Characterization of NBCs

Numerous analytical techniques can be utilized for the characterization of the NBCs utilized for the process of the transesterification process (Table 3).

Table 3. Various analytical techniques utilized for characterization of NBCs.

Analytical Technique	Explanation of Analytical Technique in Case of NBCs	Reference
FTIR	Detection of functional groups on the surface of NMs. Immobilization methods particularly covalent bond based immobilization is studied by using this technique.	[77]
DLS	The size and disperse nature of the NBCs are investigated by using the DLS technique. The increment in the hydrodynamic radius, as well as the polydispersity values of the NMs after the conjugation with the enzyme, is used as a tool for the validation of the formation of NBCs.	[65]
N_2 adsorption-desorption studies	The information regarding physical absorption based immobilization of the enzymes of the NMs in NBCs is investigated by this technique. The adsorption mechanism and model followed for the adsorption is validated by this technique. Selective separation of the required crystalline MOFs from the sample carried out for the synthesis of the NBCs is also performed by using this technique.	[49]
EDX	The elemental composition and purity of NMs are estimated by this technique. Moreover, the surface modifications and immobilization of lipase on NMs are also affirmed by EDX.	[43]
XRD/Powder XRD	The insights regarding size, composition, and crystallinity of NMs (before and after immobilization of lipase) are obtained by this technique. Generally, lipase immobilization or surface modifications does not change the structure of core NMs.	[78]
Contact angle study	The hydrophobic nature of the NBCs and its interaction with the hydrophobic lipase enzyme is investigated by carrying out contact angle studies. This property can provide useful insights regarding the stability by investigating the interaction between lipase and the NMs present in the NBCs.	[49]
SEM	Morphology of NMs and NBCs is confirmed by using the SEM micrographs. An increase in the size and variation in surface morphology of the NMs after the lipase attachment is considered an indication of successful immobilization.	[79]
TEM	The morphology and more importantly surface of NMs is characterized by TEM. The NMs modified with polymers exhibits typical core-shell morphology, while after immobilization of enzyme relatively aggregative structures appear.	[78]
VSM	To detect the magnetic potential of the synthesized NBCs by developing the hysteresis loop. High saturation magnetization values of the NBCs also provide information regarding the separation potential of the NBCs under the influence of the external fields. Comparison of the saturation magnetization values of pure NMs and functionalized NMs (in NBCs) provide validation regarding the immobilization and modification procedures associated with the NMs.	[80]
CLSM	Superposed field images provided the visual confirmation of the immobilization of the enzyme on the NMs. Two and three-dimensional fluorescence intensity further confirm the immobilization of the enzyme.	[37,80]
XPS	Elemental composition, surface chemistry, and electronic state of nano-material are determined by XPS. The difference in XPS spectra before and after immobilization of lipase on NMs validates the conjugation of lipase with NM. Generally, carbon/nitrogen ratio verifies the attachment of lipase.	[79]
AFM	This technique when utilized with the tapping mode is used to analyze the surface roughness of the NMs. The distance between peak to peak valley values in the micrographs is indicative of the roughness of the NMs. The immobilization of the enzyme also influences the roughness of NMs and the change in the roughness values of NMs is utilized as evidence for the formation of the NBCs.	[65]
DTA	The thermal stability imparted to the enzyme via immobilization on the NMs to generate NBCs is investigated by using this technique.	[58]
CD	To investigate the secondary structure of the lipase enzyme in its immobilized form, this technique is utilized.	[50]

5.2. Monitoring/Optimization of the Biodiesel Process

The monitoring of biodiesel production is carried out by utilizing a number of techniques. GC (Gas chromatography) is the most widely adopted analytical technique utilized for this purpose. Gao et al. [81] utilized the GC technique for the monitoring and optimization of the transesterification reaction carried out in the case of *Jatropha curcas* L. oil in the presence of NBCs. The amount of the acquired biodiesel produced in the reaction mixture was determined by investigating and comparing the retention times and peak areas of standardized ester peaks. For optimizing the transesterification reactions, several experiments were run and the yields were calculated. The parameters were optimized one by one for the transesterification reaction. The parameter that was under observation was varied in the particular elected range while all other parameters were kept constant during the optimization of that particular parameter. The effect of the variation of the under-study parameter was observed on the biodiesel yield and the peculiar value where maximum yield was acquired was termed as the optimized value for that particular parameter. In this particular study, the optimized values of 500 mg, 20% wt., 1:4, 40 °C, and 8 h were observed for the reaction parameters of content of oil, dosage of NBCs, oil/alcohol ratio, temperature, and reaction time respectively. Adnan et al. [66] also utilized the technique of GC for studying and optimizing the transesterification reaction of soybean oil catalyzed by the NBCs of Lipase immobilized on MOFs. Some other techniques are also utilized in conjugation with the GC for studying the synthesized biodiesel. Sarno et al. [82] utilized TGA as a quantitative technique, in combination with the Gas chromatography-Mass spectrometry (GC-MS) analysis, for investigating the amount of biodiesel produced by carrying out transesterification reaction of tomato seed oil catalyzed by NBCs. The comparison of the thermogravimetric profiles, including DTA profiles, of the synthesized biodiesel with the standard biodiesel was used for the validation of the formation of biodiesel. The tomato seed oil exhibited decomposition around 270 °C while the synthesized biodiesel loses 96% of its mass within the range of 50–210 °C. The appearance of these weight loss peaks within this range (well within the standard range of properties affiliated with the changes in the temperature values, such as cloud point, flash point, etc. of the biodiesel standards) validates the formation of the biodiesel in the medium.

Similarly, the technique of response surface methodology (RSM) is also utilized almost as frequently as GC for optimizing/monitoring of transesterification of oil carried out in the presence of NBCs. RSM is one of the main concepts which are utilized for the designing of the experiments. RSM is essentially useful for monitoring and optimizing the transesterification reaction, as during this reaction, more than one or more reaction variables (independent variable taken as a response) are influenced by many other variables during this reaction. This method is advantageous in comparison to other methodologies as the development of the mathematical relationships between the dependent and independent variables associated with biodiesel production significantly reduces the number of trials required for the optimization of the reaction. The effect of qualitative variables upon biodiesel production is also investigated by this method [83]. Touqeer et al. [20] optimized the parameters including dosagsilicae of the NBCs, oil to methanol ratio, reaction temperature, reaction time, and water content for transesterification reaction of the waste cooking oil to obtain the maximum yield of 92%. Rahimi et al. [84] also documented 99% of the transesterification of the *Camelina sativa* seed oil catalyzed by NBCs optimized by using the central composite design in the RSM.

The validation of the successful completion of the biodiesel can also be confirmed via Fourier transform infrared spectroscopy (FTIR), Proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and Carbon-13 nuclear magnetic spectroscopy (^{13}C NMR). Rafiei et al. [48] utilized the technique of ^{1}H NMR for investigating the yield of the crude biodiesel. Bencze et al. [85] carry out the monitoring of the transesterification reaction of sunflower via NBCs by using ^{1}H NMR. Factors including effects of organic solvents, water content, substrate-solvent ratio, ethanol concentration, cross-linking type, cross-linking length, surfactant dosage and temperature were optimized by using ^{1}H NMR in this study.

Khan et al. [77] utilized the techniques of FTIR, ^{1}H NMR and ^{13}C NMR for the characterization of synthesized biodiesel produced by using NBCs for the transesterification of waste cooking oil. The appearance of particular FTIR peaks associated with the methyl esters, including stretching frequency of C=O groups (methoxy ester), asymmetric bending of CH_3 (methyl esters), stretching vibration of OCH_3 (methoxy esters), and stretching vibrations of CH (terminal methyl groups in methyl esters), synthesized as a result of the transesterification process was used for characterization. Similarly, the presence of characteristics carbon peaks associated with the produced biodiesel including carbonyl carbon, methoxy carbon (ester), unsaturated carbon (methyl esters), and methylene carbon (FAME) was used for characterizing the synthesized biodiesel.

Several other uncommon methodologies are also documented for the monitoring of the transesterification reactions carried out by using the NBCs. In the case of CLEAs based NBCs, effects of different reaction conditions and substances (particularly concentration of alcohols/methanols) over the size and aggregate morphology of NBCs can further be evaluated by Dynamic light scattering (DLS) methodology. Pico et al. [57] also utilized the technology of thin-layer chromatography (TLC) and High-performance liquid chromatography (HPLC) to semi-quantitatively assess the bioconversion rates observed in the case of CLEAs-GA-Fe_3O_4 NBCs owing to the highly viscous nature of extracted crude oils. Kalantari et al. [86] also utilized the HPLC technique for studying the conversion rates of transesterification reaction of corn oil into biodiesel. However, these methodologies are rarely documented in the academic literature to be used as a monitoring tool in the case of biodiesel production. Mostly, the focus is kept on the use of the GC technique for carrying out the monitoring of production of biodiesel. Case studies documenting various reaction parameters/optimized conditions associated with the transesterification reactions are summarized in Table 4.

Table 4. Case studies representing the summary of the transesterification process for the production of biodiesel.

Nanobiocatalyst	Transesterification Process	Raw Material	FAME Monitoring Technique/ Optimization Technique	Transesterification Conditions/ Optimized Parameter	FAME Yield	Remarks	Reference
L-PDA-CeO_2 NRs	Enzymatic transesterification	*Eruca sativa* oil	GC-MS	–	C16:0 (1.448%) C18:0 (0.186%) C18:1 (28.181%) C22:0 (65.111%) C20:1 (4.712%)	The utilization of the RSM for the optimization of the transesterification processes allowed numerous advantages to this study. According to RSM, the transesterification process followed the quadratic model with R^2 value and adjusted R2 value of 0.9802 and 0.9903 respectively. Reusability studies revealed that the NCBs were efficient for performing the transesterification up to five cycles without any significant loss in the activity of the NBCs	[46]
			RSM	10% NBCs, 6:1 methanol to oil ratio, T = 35 °C, 0.6% water content and reaction time = 30 h.	Maximum biodiesel yield 89.3%		
L-Fe_3O_4 NPs L-Fe_3O_4/GO NPs L-AP-Fe_3O_4/GO NPs L-GA-Fe_3O_4/GO NPs	Whole cell transesterification	*Chlorella vulgaris* oil	GC-MS	8.6 mg of catalyst, T = 45 °C, reaction time = 24 h, 0.5 mL of extracted lipid, 5 mg cm-3 n-Hexane and 0.15 mL methanol	L-Fe_3O_4 NPs (54.14%), L-GO-Fe_3O_4 NPs (57.05%), L-AP-GO-Fe_3O4 NPs (~62%), L-GA-GO-Fe_3O_4 NPs (~70%)	The functionalization of GO-Fe_3O_4 NPs by GA and AP improved efficacy of the transesterification process by effectively immobilizing lipase enzyme. The amine group in AP and aldehyde group in GA facilitated lipase attachment via electrostatic interaction and covalent bond formation (C=N) respectively. Therefore, functionalized NPs were more stable and exhibited better results in comparison.	[60]

Table 4. *Cont.*

Nanobiocatalyst	Transesterification Process	Raw Material	FAME Monitoring Technique/ Optimization Technique	Transesterification Conditions/ Optimized Parameter	FAME Yield	Remarks	Reference
L-GA-Fe_3O_4 NPs	Enzymatic transesterification	Waste cooking oil	GC-MS	36% w/w NPs, 200 rpm stirring rate, reaction time = 90 min, T = 40 °C and 1:5 oil to methanol ratio	93.58%	Extremely high yield of biodiesel was observed in case of the synthesized NBCs. However, the physicochemical characters of biodiesel were not studied.	[51]
L-Fe_3O_4 NPs L-AP-Fe_3O_4 NPs L-GA-AP-Fe_3O_4 NPs	Whole cell transesterification	*Chlorella vulgaris* oil	GC-MS	8.6 mg of catalyst, T = 45 °C, reaction time = 24 h, 0.5 cm^3 of extracted lipid, 5 mg cm-3 n-Hexane and 0.15 cm^3 methanol	L-Fe_3O_4 NPs (54.14%), L-GA-Fe_3O_4 NPs (~58%) L-GA-AP- Fe_3O_4 NPs (69.8%)	Among the NBCs, the system containing both the moieties responsible for the covalent and electrostatic interactions based attachment of lipase enzyme with the NMs yielded the maximum results in terms of transesterification process.	[52]
CLEAs-AP-Fe_3O_4 NPs	Enzymatic transesterification	Waste cooking oil	GC-MS	2.2 g of waste cooking oil, 1:3 oil to methanol ratio, 0.3% NBCs, 170 rpm stirring rate, T = 35 °C. for 72 h.	49%	The CLEAs although exhibited better results in terms of stability and reusability in comparison to the lipase immobilized without the process of cross linking or precipitation. However, yield was found to be quite low in comparison to the recent literature which may be attributed to the reduction in the surface area of the NBCs owing to the process of crystallization.	[53]
CLEAs-AP-GO-Fe_3O_4 NPs	Enzymatic transesterification	*Ricinus communis* oil	GC-MS	0.4 g oil, 1:3 oil to methanol ratio, 0.2% w/w NBCs, T = 25 °C and reaction time 24 h	78%	Comparison of the NBCs with the free lipase indicated that the yield of biodiesel production increased 3 folds owing to the immobilization and formation of CLEAs.	[34]

Table 4. *Cont.*

Nanobiocatalyst	Transesterification Process	Raw Material	FAME Monitoring Technique/ Optimization Technique	Transesterification Conditions/ Optimized Parameter	FAME Yield	Remarks	Reference
L-PDA-Fe_3O_4 NPs	Enzymatic transesterification	waste cooking oil	RSM	10% catalyst concentration, 1:6 oil to methanol ratio, reaction time 30 h, T = 37 °C and water concentration 0.6%	92%	Optimization of the biodiesel transesterification by RSM revealed that the quadratic model was found to be best fitted in case of this reaction. The synthesized biodiesel was found to be comparable with the ASTM international reference for biodiesel.	[20]
L-PDA-Fe_3O_4 NPs	Enzymatic transesterification	Waste chicken fat oil	GC-MS	–	C16:0 (17.96%) C18:0 (20.85%) C18:1 (42.92%) C18:2 (16.54%)	Comparison between actual and predicted FAME values affirms that the quadratic model was the best fit for the transesterification process. The results of the FTIR analysis were used as the confirmation technique for the immobilization of the enzyme on the NPs. Recovery from the medium was carried out by using the magnetic properties of the NBCs. Reuse studies further confirm that no decrease in the activity of the NBCs was observed for three reuse cycles.	[87]
			RSM	6% catalyst concentration, T = 42 °C, 1:6 oil to methanol ratio and Reaction time 36 h	90.6%		
L-GA-PA-Fe_3O_4 NPs	Enzymatic transesterification	Rapeseed oil	GC-MS	20% NBCs, 1:6 oil to methanol ratio, 2%water content, T = 45 °C, reaction time = 24 h and 250 rpm agitation speed.	89.4%	FAME conversion of greater than 70% was acquired after the 5 rerun cycles of transesterification carried out via NBCs. The external magnetic field was utilized as the recovery tool in case of NBCs. Utilization of ionic liquids as major stabilization medium provided an alternative route for the preparation of NMs.	[54]

Table 4. *Cont.*

Nanobiocatalyst	Transesterification Process	Raw Material	FAME Monitoring Technique/ Optimization Technique	Transesterification Conditions/ Optimized Parameter	FAME Yield	Remarks	Reference
L-AP-Fe_3O_4@SiO_2 NPsL-MP-Fe_3O_4@SiO_2 NPs	Enzymatic transesterification	Soybean oil	GC-MS	oil 10 mL, T = 45 °C, 1:3 oil to methanol, catalyst 0.5 g, stirring = 600 rpm and reaction time = 12 h	L-AP-Fe_3O_4@SiO2 NPs (76%) L-MP-Fe_3O_4@SiO_2 NPs (72%)	The maximum immobilization efficacy for L-AP-Fe_3O_4@SiO_2NPs and L-MP-Fe_3O_4@SiO_2 NPs was found to be 78.7% and 77% respectively. The NBCs could maintain the catalytic efficacy up to five cycles	[62]
L-P(GMA-co-MAA)-Fe_3O_4 NPs	Enzymatic transesterification	Soybean oil	GC-MS	T = 40 °C, 1:4 oil to methanol, NBCs 25% and reaction time 54 h.	92.8%	The leaching test performed in case of NBCs revealed that the lipase immobilization imparted long term stability to the NBCs.	[55]
L-Fe_3O_4@PDA NPs	Enzymatic transesterification	Soybean oil	ATR-FTIR	200 mg NBCs, 1:1 oil to methanol, T = 37 °C and reaction time 12 h	90%	Immobilization was achieved via Michael addition and aldolic condensation at the specific catechol sites eliminated the need for the use of extensive coupling agents. Denaturing owing to methanol medium limited the use of NBCs to three runs.	[56]
L@Cu-NFs	Enzymatic transesterification	sunflower oil	NMR	T = 45 °C, 1:2 oil: methanol, 12 mL oil, 0.1 g NBCs,	96.5%	MOFs possess favorable catalytic activity and stability in the ester hydrolysis. Further, the hybrid nanoflower was used as a catalyst for biodiesel production. It can be reused for 5 cycles. So, the hybrid nanoflower exhibited potential for acting as economically viable biocatalyst for the production of biofuel.	[67]

Table 4. *Cont.*

Nanobiocatalyst	Transesterification Process	Raw Material	FAME Monitoring Technique/ Optimization Technique	Transesterification Conditions/ Optimized Parameter	FAME Yield	Remarks	Reference
L@ZIF-8	Enzymatic transesterification	Soybean oil	GC-MS	2.19 g soybean oil, 1:4 oil to ethanol, 9% wt. water content, reaction time = 24 h, T = 45 °C and 8% wt. lipase dosage.	95.6%	In terms of the recovery studies and retention of efficacy after reruns, the synthesized NBCs exhibited excellent results. 26-fold recovery rate was observed in case of NBCs in comparison to free lipase.	[66]
L@ZIF-67	Enzymatic transesterification	Soybean oil	NMR	0.5 g Soybean oil, 100 mg NBCs, 10% water content, 1:6 oil to methanol, reaction time = 6 h and T = 45 °C.	78%	The rerun experiments indicate that the NBCs can be effectively utilized for 6 runs. The activity loss of 78.5% to 56% was observed in case of utilizing NBCs for 6 runs. Enzyme leaching was not observed during the studies.	[48]
L- Fe_3O_4/GO NBCs	Enzymatic transesterification	Soybean oil	GC	48.5 g oil, 1:4 oil to methanol ratio, 20%wt. NBCs, reaction time = 60 h and T = 40 °C.	92.8%	The NBCs can be effectively separated by using external magnetic field. The NBCs do not exhibit any significant decrease in the catalytic efficacy up to five cycles in terms of biodiesel production.	[36]
L-PMAM-Fe_3O_4/MWCNTs NBCs	Enzymatic transesterification	Waste vegetable oil	GC RSM	1.98 g oil, 1:4 oil to methanol, lipase dosage 6% wt., reaction time = 10 h, T = 50 °C and water content 8%wt.	>93%	Esterification activities of NBCs were found to be 27 fold more in comparison to free lipase enzyme.	[37]
L@UiO-66	Enzymatic transesterification	Soybean oil	GC	10g of oil, 0.2–0.5 mg NBCs and reaction time = 12 h	93%	Physical adsorption was utilized as a mode of immobilization owing to the complementary hydrophobic nature of the lipase and the modified UiO-66.	[49]
L@Zr-MOFs	Enzymatic transesterification	Sunflower oil	1H NMR	100 mg NBCs,15.9 mg lipase dispersion, 12 mL of sunflower oil,1 M NaOH solution for adjusting the pH.		Catalytic activity, stability, recyclability, and reusability potential of synthesized NBCs were increased by using biomimetic mineralization strategy for the preparation of NBCs.	[50]

Gas Chromatography-Mass spectrometry (GC-MS), Fatty Acids Methyl Esters (FAME), Nanoparticles (NPs), Nanobiocatalysts (NBCs), Hexadecanoic acid methyl ester (C16:0), 7,10,13-Hexadecatrienoic acid methyl ester (C16:3), Danish Clean Water (DCW), Nanomaterials (NMs), Temperature (T), Stearic Methyl stearate (C18:0), Cis-9-octadecenoic methyl ester (C18:1) Cis-9,12-octadecenoic methyl ester (C18:2), 9-Octadecnoic acid, 12-hydroxy methyl ester (C18:1(OH)), Eicosanoic acid Methyl ester (C20:0), Response surface methodology (RSM), Erucic acid methyl ester (C22:1), Gondoic acid methyl ester (C20:1), Lipase (L), Nuclear Magnetic resonance (NMR).

In summary, the following key observations can be made by using the literature survey presented in Tables 2 and 4. The utilization of magnetic NBCs is the most commonly exploited approach among all types of NBCs owing to its advantage of facile separation. Although comparing the efficacy of the NBCs with respect to biodiesel yield is a challenging task as the instruments and reaction conditions utilized for optimization/monitoring vary greatly in each study, few generalized trends were observed in the case of NBCs. For magnetic NBCs, the observed biodiesel yield values were found to be quite low in the case of whole cell transesterification (as low as 54.14%) whereas much higher yields were observed in the case of enzymatic transesterification process (Table 4). Covalent immobilization was found to be the most effective immobilization method for acquiring the best results in the case of all types of NBCs. In the case of MOFs, the physical adsorption and entrapment methodologies were found to be quite effective as well but the same can't be said about the other types of NBCs. The preference of entrapment and physical adsorption methodologies in the case of MOFs over the covalent bonding is understandable owing to the commonly utilized biomimetic mineralization methodology opted for the formation of such assemblies. Similarly, the preference of covalent attachment for the NBCs containing magnetic NMs, inorganic nanoscaffolds, and metal oxide NMs also lies in the fact that the NBCs are prepared first (by different methodologies) and then the lipase enzyme is immobilized over it. Physical adsorption is a reversible process while covalent attachment is an irreversible process. Therefore, the covalent attachment is preferred for preparing such assemblies (Table 2). In terms of strength and long term stability, the CLEAs based assemblies exhibited the best results showing almost negligible differences in terms of efficacy with respect to reruns. However, these assemblies showed the least efficacy in terms of the biodiesel yield. This is attributed to the fact that although the cross-linked material imparts stability to the NBCs but this cross-linking reduces the surface area available for the catalytic transesterification resulting in lower yields in the case of biodiesel production (Tables 2 and 4).

5.3. Reuse and Recovery Experiments

Recovery and reusability experiments are also an important experiment that must always be reported whenever novel NBCs are documented in the academic literature as these experiments provides useful insights into the commercial and economical suitability of the synthesized NBCs. In terms of the case studies surveyed for this review, the peculiar NBCs can be broadly divided into the two categories of the magnetic NBCs and non-magnetic NBCs concerning the procedure opted for recovery of the NBCs from the reaction medium. As indicated by the name i.e., magnetic NBCs, the NBCs containing magnetically active component are separated from the reaction medium by using the external magnetic field as the separation technique. Ngo et al. [88] engineered Lipase based aldehyde functionalized magnetic (Fe_3O_4) NBCs and utilize these NBCs for catalyzing the transesterification reaction of converting grease into biodiesel. Authors utilized the external magnetic field as a facile and fast way to completely recover the NBCs from the reaction medium. In case of non-magnetic NBCs, the process of centrifugation is usually utilized as the major recovery method for separating NBCs from the reaction medium. Rafiei et al. [48] synthesized lipase@ZIF-67 by utilizing an in-situ encapsulation method and used it for the transesterification reaction of soybean oil. The opted recovery method for separating NBCs from the reaction mixture used in this study was the process of centrifugation (6000 rpm) carried out for 5 min. After recovery, the recovered NBCs are then washed with the regents such as n-hexane and tertiary butanol. This washing is followed by the drying technique and the acquired NBCs are then again reused for catalyzing the transesterification reaction. The better the recycling potential of the NBCs, the better it is for the economic value of the NBCs. High reuse potentials lower the cost of the overall process. Reuse potential of the catalyst depends on the stability of the synthesized NBCs. The NBCs reported by Jiang et al. [67] exhibited the appreciable catalytic potential up to five cycles of reuse.

5.4. Physicochemical Parameter Study for the Synthesized Biodiesel

Another fundamental that must be taken into account for the transesterification study carried out via NBCs is the physicochemical characterization of the synthesized biodiesel. Comparative analysis of the synthesized biodiesel with the specified standard biodiesel is essential for investigating the practical applicability potential of the synthesized biodiesel. The synthesized biodiesel must exist with the specified range provided by the standards. The American Society for Testing and Material (ASTM) standard provided by ASTM International and European Standard (EN) values provided by the European Standardization Organizations (ESOs) can be utilized for the comparison purposes of the synthesized biodiesel to the standard biodiesel. The details affiliated with the utilized standards are summarized in the Table 5. Most of the academic studies reporting the NBCs do not document this feature of the studies. The documentation of the physicochemical parameters of the synthesized biodiesel is essential as these properties provide information regarding the possible working potential of the biodiesel.

The property of cetane number acts as an indicator of the behavior of the biodiesel during the cold environment. The appreciable value of the cetane number validates that the engine will run smoothly and will exhibit good cold start potential. Low values of the cetane number lead to the increment in the exhaust emission owing to the incomplete combustion of the biodiesel. This property is a relative measure of biodiesel injection and auto-ignition of fuel. The cetane number documented by Jambulingam et al. [89] falls well within the described standard range indicating the high applicability potential of the acquired biodiesel. Similarly, the property of the viscosity influences the atomization quality, penetration, and size of the fuel droplets. Highly viscous fuels lower engine efficacy by hindering the combustion process. Poor atomization of fuel owing to viscosity also leads to the formation of large-sized droplets that require high propelling energy during fuel pumping and place strain on engine pumping elements and injectors. Density influences the air to fuel ratio in the engine and controls the amount of the fuel to be injected into the combustion chamber [58]. Similar to the aforementioned properties, each defined property (for standards) is somehow related to the applicability of the biodiesel in the engine. Therefore, it is extremely important to investigate the physicochemical properties of the synthesized biodiesel and researchers should always document this comparison between the synthesized biodiesel and the standards for providing insights regarding the quality and the commercialization potential of their synthesized NBCs. It should also be mentioned here that most academic studies have reported the FAME yield in case of biodiesel which is the physicochemical property in itself. With respect to the EN 14214 standard, a lower limit of standard biodiesel is 96.5%. Various academic studies have documented comparable results with this standard while synthesizing the biodiesel by using the NBCs [49,55,66,67]. However, attention must be focused on other physicochemical parameter as well for better understanding of this process.

Table 5. Reference table for comparing synthesized biodiesel physicochemical characteristics with the international standards for biodiesel [90–94].

Properties	Units	Standard and Corresponding ASTM Method		Standard and Corresponding ASTM Method		Standard and Corresponding EN Method		Test Requirements and Comments
		ASTM D0975	ASTM Method	ASTM D6751	ASTM Method	EN 14214	EN Method	
Density *	g/cm^3	0.876	-	0.875 to 0.90	-	0.86 to 0.90	EN ISO 3675 or 12185	Densitometer Hydrometer
Kinematic Viscosity **	mm^2/s	1.9 to 4.1	D445	1.9 to 6	D445	3.5 to 5	EN ISO 3104	Methods are equivalent
Specific gravity	–	0.850	D1298 or D4052	0.88		-	NA	–
Flash Point	°C	60 to 80	D93	100 to 170	D93	>120	EN ISO 3679	EN method (Rapid Equilibrium closed cup) and ASTM method (Pensky–Martens closed cup)
Cloud Point	°C	−15 to 5	D2500	−3 to 12	D2500	Location and season dependent	EN ISO 23015	Wax Appearance Temperature
Cetane number	-	40	D613	47	D613	75	EN ISO 5165	Cetane Engine, Methods are equivalent
Acid number	mg KOH/g	-	-	0.5	D664	0.5	EN 14104	EN method (calorimetric titration), ASTM method (Potentiometric titration)
Iodine value	g I_2/100g	-	-	-	-	120	EN 14111 and EN 16300	Titration method
Ash content	%	0.01	D2709	<0.02	D 2709	-	-	Centrifugation
Sulphur	mg/kg	15 mg/kg	D5453 or D2622	15 mg/kg	D5453	10 mg/kg	EN ISO 20846 or 20884 or 13032	UV fluorescence WDXRF
Water content	%	0.02	D2709	0.03	D2709	-	-	Centrifugation
90% recovered Distillation	%	90% 338 °C max	D86	90% 360 °C max	D1160	-	-	Vacuum distillation
Total contamination, max	mg/kg	-	-	-	-	24	EN ISO 12262	-

Table 5. *Cont.*

Properties	Units	Standard and Corresponding ASTM Method		Standard and Corresponding ASTM Method		Standard and Corresponding EN Method		Test Requirements and Comments
		ASTM D0975	ASTM Method	ASTM D6751	ASTM Method	EN 14214	EN Method	
Copper Strip corrosion	-	No. 03	D130	No. 03	D130	Class 1	EN 1SO 2160	Methods are equivalent
Linolenic acid methyl ester, max	% wt.	-	-	-	-	12	EN ISO 14103	GC
Polyunstatured methyl esters, max	% wt.	-	-	-	-	0.2	EN ISO 15779	GC
MG, DG and TG	% wt.	0.4 MG	D6584	-	-	0.70 MG 0.20 DG 0.20 TG	EN ISO 14105	GC
Free Glycerine	% wt.	0.020	D6584	-	-	0.020	EN ISO 14105 And 14106	GC
Total Glycerine	% wt.	0.240	D6584	-	-	0.250	EN ISO 14105	GC
Phosphorus	% wt. and mg/kg	0.001	D4951	-	-	4mg/kg	EN ISO 14107	ICP-MS
Lubricity	µm	520	D6079	-	-	-	-	Lubricity tester
Conductivity	pS/m	25	D2624 D4308	-	-	-	-	Electrical Conductivity meter

* At 30 °C. ** At 40 °C. Not applicable (NA), Wavelength Dispersive X-ray Fluorescence Spectroscopy (WDXRF), Monoglycerides (MG), diglycerides (DG), triglycerides (TG), Gas Chromatography (GC), Inductively coupled plasma-Mass spectrometry (ICP-MS).

6. Conclusions and Future Direction

This review highlights the fundamentals associated with the NBCs recently documented to be utilized for the synthesis of biodiesel by carrying a transesterification reaction. In terms of raw materials, it was observed that the third-generation feedstock has emerged as the most promising alternative of the edible feedstock. New species or biomass (including algae, fungi, yeast, and other microbes, etc.) can be utilized to expand the scope of the available raw materials in case of the transesterification reaction. Lipid content assays, as well as other metabolite profiling assays, can be done beforehand on the feedstock for acquiring the better efficacy for the production of biodiesel that will be done by utilizing these resources. Furthermore, it was observed that the researchers have focused mainly on the Fe_3O_4 NMs during the development of NBCs. Other novel magnetic materials such as ferrofluids can also be explored for acquiring similar desired results. Additionally, a variety of metal oxides utilized for the production of NBCs can also be increased for developing novel NBCs to be utilized for the process of transesterification.

In the case of the biological component, the immobilization method used for the fabrication of the lipase on NMs in NBCs was found to be the most critical aspect influencing the efficacy of the NBCs for the transesterification reaction. The use of the entrapment technique in MOFs requires further exploration as the peculiar mechanisms involved in the immobilization strategies are not completely understood as of yet. The kinetics, thermodynamics, and computational studies (docking studies) can be reconnoitered for exploring the essentials of the immobilization of lipase on the NMs. In the case of characterization/monitoring/optimization techniques, GC-MS and RSM were found to be the most effective techniques for studying the transesterification process. Further, less commonly used techniques such as TLC, FTIR, HPLC, ^{1}H NMR, and ^{13}C NMR, etc. have been documented in the academic literature but these techniques fall short in comparison to the essential information provided by GC-MS and RSM. It was observed that most of the documented case studies reported the reuse and recovery studies in the case of NBCs which is a welcome sight and must be appreciated as this can provide essential insights regarding the industrial applicability potential of the synthesized NBCs. However, a comparison of the synthesized biodiesel with the standard biodiesel was not provided in most case studies. The researchers should also focus on this aspect of their study as this feature is also essential for developing the understanding regarding the working potential of the prepared biodiesel. Overall, it is concluded that the use of NBCs has provided a facile alternative to conventional fuels in terms of synthesizing biodiesel via transesterification reaction carried out with exceptionally high yields, high recovery rates, and high stability.

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Abbreviations

NBCs	Nanobiocatalysts
NMs	Nanomaterials
SO_x	Sulfur oxides
NO_x	Nitrogen oxides
Fe_3O_4	Magnetite
γ-Fe_2O_3	Maghemite
AP	3-aminopropyl triethoxysilane
GA	Glutaraldehyde
GO	Graphene oxide
CNTs	Carbon nanotubes
SiO_2	Silica
Al_2O_3	Alumina
MWCNTs	Multi-walled Carbon nanotubes
H_2SO_4	Sulfuric acid
HNO_3	Nitric acid
SEM	Scanning Electron Microscopy
EDX	Energy dispersive X-ray analysis
BET	Brunauer-Emmett-Teller analysis
FTIR	Fourier Transform Infrared Spectroscopy
UV-Vis	Ultraviolet Visible Spectroscopy
XRD	X-ray diffraction
XPS	X-ray photoelectron spectroscopy
TEM	Transmission Electron Spectroscopy
NRs	Nanorods
NPs	Nanoparticles
PDA	Polydopamine
PAA	Polyacrylic acid
PEI	Polyethyleneamine
TEOS	Tetraethyl orthosilicate
FESEM	Field Emission Scanning Electron Microscope
VSM	Vibrating Sample Magnetometer
ZPM	Zeta potential Measurements
AFM	Atomic Force Microscopy
CLEAs	Cross-linked Lipase Enzyme Aggregates
$[BMIN]BF_4$	1-Butyl-3-methylimidazolium tetrafluoroborate
SQUID	Superconducting Quantum Interference Device
MWCNTs	Multi-walled carbon nanotubes
PMAM	Polyamidoamine
CLSM	Confocal laser scanning microscopy
MP	3-mercaptopropyltrimethoxysilane
P(GMA-co-MAA)	Poly(glycidyl methacrylate-co-methacrylic acid)
GMA	Glycidylmethahacrylate
DVB	Divinylbenezene
MAA	Methacrylic acid
AIBN	2,2′-azodiisobutyronitrile
TGA	Thermogravimetric analysis
DTA	Differential thermal analysis
CD	Circular dichroism spectroscopy
ATR	Attenuated total reflection
DLS	Dynamic Light Scattering
AFM	Atomic force microscopy
MOFs	Metal organic frameworks
ZIF	Zeoliticimidazolate framework
NFs	Nanoflowers
PANI	Polyaniline

PPY	Polypyrrole
MA	Methyl anthranilate
UiO-66	Universitetet i Oslo
PDMS	Polydimethylsiloxane
$ZrCl_4$	Zirconium chloride
BDC	1,4-benzenedicarboxylic acid
CVD	Chemical vapour deposition
DMF	N,N′-dimethylformamide
TiO_2	Titanium dioxide
CeO_2	Cerium dioxide
L	Lipase
FAAE	Fatty acid alkyl esters
GC-MS	Gas chromatography-Mass Spectrometry
RSM	Response Surface Methodology
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
^{13}C NMR	Carbon-13 nuclear magnetic spectroscopy
TLC	Thin-layer chromatography
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance spectroscopy
ASTM	American Society for Testing and Material
ES	European Standard
ESOs	European Standardization Organizations
NA	Not applicable
ICP-MS	Inductively coupled plasma- Mass spectrometry
WDXRF	Wavelength Dispersive X-Ray Fluorescence Spectroscopy
MG	Monoglycerides
DG	Diglycerides
TG	Triglycerides
GC	Gas Chromatography

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