

# Biosynthesis of Lipase-Immobilized Zinc Oxide Nanoparticle and its Role as a Nanocatalyst in the Biodiesel Production of the Lipid obtained from *Anabaena* Cyanobacterial Species Isolated from Saline Water

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**Abstract:** In the present research work, lipase-immobilized zinc oxide nanoparticles were synthesized and used for the production of biodiesel. The fungus *Aspergillus niger* (KP001169) was exposed to gamma radiation to produce the lipase enzyme, which was later immobilized into ZnO nanoparticles. The synthesized zinc oxide nanoparticle was characterized using FTIR, XRD, and SEM-EDAX. Varying temperature and pH levels were analysed to determine the lipase enzyme's free and immobilized activity. According to the experiment, ZnO NPs lipase (5%) can be reused in the production of biodiesel without being treated for up to five cycles, and its activity was decreased by 15% after the 5<sup>th</sup> cycle. The optimized reaction conditions for the production of biodiesel from the lipid obtained from *Anabaena* species were found to be a methanol: oil ratio (1:4), Reaction temperature (45°C), Reaction time (6 hours), and Reaction speed (400 rpm).

**Keywords:** *Anabaena* species; lipids; biodiesel; ZnO; lipase enzyme; transesterification.

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## 1. Introduction

In recent years, fossil fuels have been crucial to the global economy because they satisfy the energy requirements for heating, lighting, and transportation purposes. The need for fossil fuels in communities has increased as the population has grown. Additionally, the growing use of fossil fuels has raised the atmospheric CO<sub>2</sub> concentration, a major contributor to global warming [1]. Scientists and researchers are moving towards an alternative energy source based on these factors. Biofuels are produced from various feedstocks existing in the natural resources. Algal-based biofuels are currently receiving a lot of interest because of their high lipid content, which is used for the production of biodiesel [2, 3], bioethanol production from sugars [4], biomethane production from biomass [5, 6] and biohydrogen from algal biomass [7]. Various types of catalysts were used in biodiesel production. Among the various categories, lipase-immobilized nanocatalysts enhance the conversion rate of lipids into biodiesel. In the field of nanotechnology, there are numerous industrial uses for various kinds of nanoparticles [8,9]. Generally, enzymes, acids, and alkalis catalysts were used in the production of biodiesel [10-12]. The merits of heterogeneous catalysts include corrosiveness,

easy separation, and the ability to be reused than homogeneous catalysts [13-16]. Currently, a variety of heterogeneous catalysts are used in biodiesel production, such as metal-oxides mixed catalysts, carbon-based catalysts, ion exchange resins, etc [17-22]. The demerits of conventional solid catalysts are low activity, porosity, high cost, and significant leaching. The merits of heterogeneous catalysts are effective and stable in the production of biodiesel to produce high-quality biofuel [23]. Previous research studies simplified the synthesis of nanomaterials. As a result, continuous research has been conducted in the field of nanomaterials, which are used as an effective catalyst in the production of biodiesel [24-27]. Generally, metal oxide-based nanocatalysts enhance biodiesel production. To increase the yield of biodiesel, zinc oxide was modified using various carrier loading techniques, acid-base alteration, and other techniques [28, 29]. Numerous investigation reports are available on the usage of metal oxide-based catalysts in biodiesel production, which have been carried out over the last two decades with significant outcomes [30-32]. Nowadays, metal oxide-based nanoparticles play a major role in the vital applications of medicine, food, agriculture, electronics, and industry [33-40]. In the present research work, lipase-immobilized zinc oxide nanoparticles were used as a nanocatalyst for biodiesel production from the lipid obtained from the *Anabaena* species.

## 2. Materials and Methods

### 2.1. Chemicals and reagents.

SD Fine Chemicals like ammonium acetate, methanol, hexane, N-hydroxysuccinimide, chloroform, EDTA, 3-aminopropyltriethoxysilane, sodium taurocholate, CTAB, zinc nitrate, sodium chloride, EDC and sodium hydroxide were used. Hi-media, ASN III was used to isolate cyanobacteria from saline water.

#### 2.1.1. Production and purification of lipase from *Aspergillus niger* (KP001169).

To obtain extracellular lipase using the ideal medium conditions, *Aspergillus niger* (KP001169) was exposed to gamma radiation. It contains 5% yeast extract and 5% olive oil, which is used to produce lipase. 250 mL of broth was seeded with 1.0 mL ( $4.60 \times 10^7$  CFU/ml) of spore inoculums, incubated at 25°C, pH-7, and 200 rpm, and stirred for 72 hours. 0.14 kGy of gamma radiation was induced in the *Aspergillus niger* to increase its ability to break down the lipase [41]. The crude lipase was isolated from the culture broth by stirring at 10,000 rpm for 30 minutes. Ammonium sulphate precipitation was used to purify the lipase. The photometric test was used for the determination of the extracellular lipase activity [42].

### 2.2. Green synthesis of zinc oxide nanoparticle using *Chlorella vulgaris* extract.

Initially, *Chlorella vulgaris* extract (50 mL) was mixed with zinc acetate(4.0 g), and deionized water (200 mL) was mixed in a 1000 mL beaker, then adjusted the pH to 8 with 1 mol/L sodium hydroxide at 80°C stirring for 2 h. The ZnO nanoparticles were obtained, washed repeatedly with suction under a vacuum pump, and dried at 60°C. The obtained ZnO nanoparticle was dried under a muffle furnace at 500°C for 2 hours and used for further experiments.

### 2.3. Immobilization of lipase on ZnO nanoparticles.

The ZnO nanoparticle (0.5 g) was treated with 0.30 mL of 3-Aminopropyltriethoxysilane, and 30 mL of ethanol was added, and the mixture was ultrasonically processed at 35 kHz. ZnO-APTES product was obtained and separated. The reactant solution was treated with 10% isopropanol and dried at 40°C agitated at 200 rpm for 3 hours at RT. A lipase solution of 5 mL and 1 mL of a 5 mg/mL EDC solution were added to the reactant solution, and 5 mg of N-Hydroxysuccinimide was added and incubated for about 2 hours. It was incubated for two hours after adding 0.5 g of ZnO-APTES nanoparticles to the mixture. Lipase-immobilized zinc oxide nanoparticle was separated, washed with distilled water, dried, and maintained at 4°C for further studies [45-48].

### 2.4. Assay for lipase activity.

The reaction mixture was mixed with various enzyme dosages (100–500 mg) and treated with 5 mL phosphate buffer to maintain pH 7 before adding 10 mL of olive oil. After an hour at 40°C of incubation, 20 mL of ethanol-acetone (1:1) was added to the enzyme and substrate mixture in an orbital shaker. The obtained solution was titrated with 0.05 N sodium hydroxide, and phenolphthalein was used as an indicator [41, 45].

### 2.5. pH and thermal stability of enzymes.

The enzymes were pre-incubated for 2 hours at 20 to 80°C at pH 7 to investigate the effect of temperature on immobilized and free enzyme esterase activity. To assess the effect of pH on the lipase immobilized enzyme and free enzyme, pH was changed from 2 to 10 at room temperature by using phosphate buffer.

### 2.6. Reusability and storage stability of immobilized lipase enzyme.

The stability of the immobilized enzyme was analyzed. Immobilized nanoparticles were analyzed by a magnet after each run of an enzyme, rinsed with hexane and deionized water to remove the residual substrate, and their hydrolysis rate in olive oil. To control their storage stabilities, the free and immobilized lipase was kept at pH 7 at 4°C for 100 days. The titration method was used to estimate and analyze the enzymatic activity every 10 days.

### 2.7. Cyanobacterial mass cultivation for lipid extraction.

By using the serial dilution technique, the cyanobacterial strain was isolated from saline water. The green-blue-green cyanobacterial species were identified using compound microscopic. The lipid bodies present in the isolated cyanobacterial strains were identified using the Nile red strain method. Out of five pure colonies, one strain was identified as an *Anabaena* using 18s rRNA molecular sequencing method. Mixotrophic culturing techniques were used to cultivate the *Anabaena* species under three media: BG-11, BBM, ASN III, and modified ASN III medium [46]. *Anabaena* was found to have high lipid content and was used for the production of biodiesel.

### 2.8. Soxhlet extraction – lipid extraction from wet cyanobacterial biomass.

The wet cyanobacterial biomass was collected and dried at 60°C until a stable weight was achieved. 350 mL of n-hexane was added to a round bottom flask connected with a soxhlet

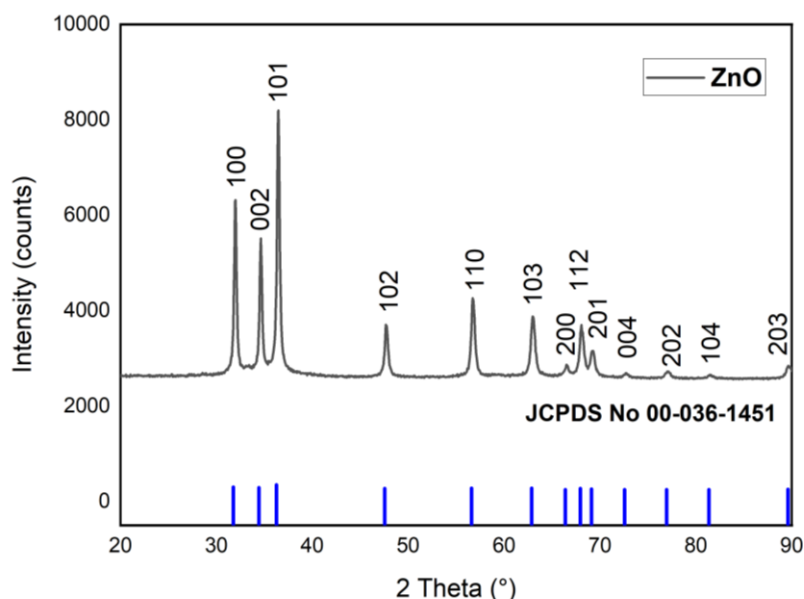
tube containing 250 g of cyanobacterial biomass, and the reaction solution was refluxed for 48 hours at 80°C. Finally, the distillation technique was used to remove the excess solvent, and the obtained crude lipid was used to produce biodiesel using a lipase-immobilized zinc oxide nanocatalyst.

### 2.9. Transesterification of biodiesel from the lipid of cyanobacterial biomass.

The lipid obtained from *Anabaena* was treated with methanol: oil (4:1), followed by the addition of 300mg of nanobiocatalyst, and the reaction mixture was stirred for 4 hours in a magnetic stirrer at 45°C. The final product obtained, biodiesel, was treated with 25 mL of chloroform. The obtained organic layer containing biodiesel was distilled in a rotary evaporator to remove the excess solvent and other impurities [41, 45].

## 3. Results and Discussion

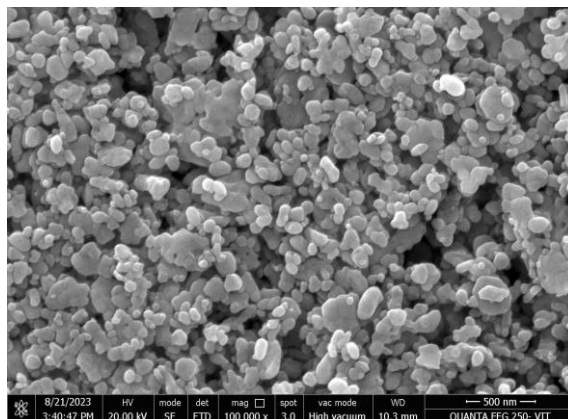
As shown in Figure 1, the synthesized ZnO nanoparticles were analyzed using an X-ray diffractometer. The phases of ZnO nanoparticles were analyzed, and the results showed, a high conformity to the peaks found in the synthesized ZnO. The peaks at (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), (202), (104), and (203) was due to the formation of ZnO nanoparticles which have similar lattice parameter values of the reported ZnO nanoparticles with JCPDS file 00-036-1451.



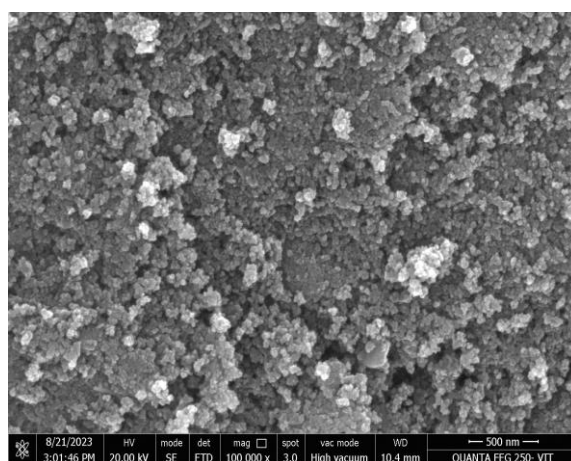
**Figure 1.** XRD pattern for the biosynthesized ZnO nanoparticles.

The morphology of the ZnO nanoparticle was confirmed by scanning electron microscopy studies. As shown in Figure 2, the surfaces of the ZnO nanoparticles were investigated using an SEM with an electron beam energy of 5 kV. The structures and surfaces of the ZnO nanoparticles were clear, smooth, and nearly, and the nanoparticle size was found to be 100-500 nm. The lipase enzyme becomes inactive by enzyme aggregates that are present on the surface of the nanoparticle. The existence of nano-sized holes in the morphology suggests that enzymes have bound to the ZnO nanoparticles. Figure 3 depicts the coarse surface of the ZnO nanoparticles through nanoscale pores that represented the protein layer when the lipase enzyme was immobilized on them. In this study, the rate of enzyme immobilization on ZnO nanoparticle surfaces was quantified and found to be 86%. The covalent bonds that form

between the lipase enzyme's  $-NH_2$  and  $-CHO$  groups of ZnO nanoparticles cause the enzyme to immobilize nanoparticles that bind to surfaces. Meanwhile, they discovered that the coupling of the enzyme to the carrier was affected by altering the key covalent linkages and magnetic support before enzyme immobilization.



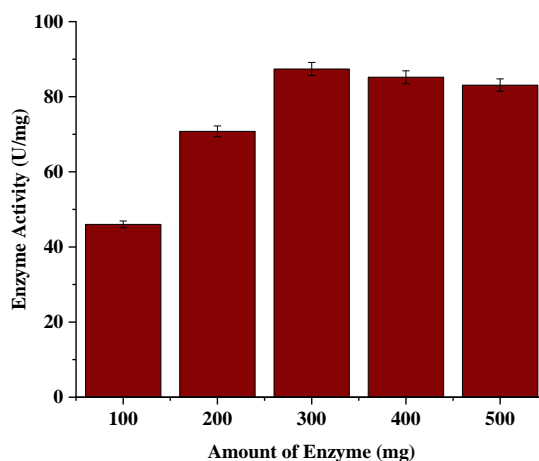
**Figure 2.** SEM morphology of the synthesized ZnO nanoparticles.



**Figure 3.** SEM morphology of the lipase immobilized ZnO nanoparticle.

### 3.1. Esterase activity of different enzyme concentrations.

Esterase activity began to increase quickly and peaked at 300 mg (87.4 U/mg), as shown in Figure. 4. When enzyme dosages were increased, a little reduction in esterase activity was observed. Because of raised protein-protein interaction leading to substrate conversion inhibition, esterase activity decreased as the concentration of the enzyme increased.



**Figure 4.** Effect of enzyme concentrations on Esterase activity.

### 3.2. Effect of free and immobilized enzyme on pH and temperature.

The free and immobilized lipase activity was affected by temperature. Free and immobilized lipase both remain active at temperatures below 50°C. Free lipase lost some of its enzyme activity at 50°C; however, lipase immobilized on ZnO NPs maintained 80% of its activity at 70°C. The immobilized enzyme had a maximum activity of 89.1 U/mg at 45°C compared to the free enzyme's highest activity of 83.4 U/mg at 35°C. Yang et al. [49] reported that the immobilized form of the enzyme increases its heat stability, resulting in less protein denaturation. When the temperature was raised, the reaction confirmed that the activity of the free lipase enzyme rapidly decreased. Figure 5 demonstrates that the activity of the lipase immobilized on NPs continued to decrease in comparison to the free enzyme. The highest enzymatic activity (89.1 U/mg) was recorded at 45°C, whereas the esterase activity of the enzyme immobilized on magnetic nanoparticles was shown to stay stable until the temperature reached 65°C (81.1 U/mg). The effect of pH on the relative activity of free and immobilized lipase is shown in Figure 6. This study showed that the activity of lipase-immobilized NPs was sustained throughout a wider pH range than that of free enzymes. Lipase was immobilized, and free lipase enzyme activity was severely damaged at pH 6–8. Similar results were revealed, showing that the pH changed from 6.0 to 7.0 when the enzyme was immobilized. At pH 9.0, natural lipase was more active at pH 10, but immobilized lipase was more active [46]. According to Xie et al., the optimal pH for an immobilized enzyme is higher because of its attachment to the support surface and increased tolerance to pH change, which investigated whether pH affected the activity of free and enzyme-immobilized nanoparticles [42].

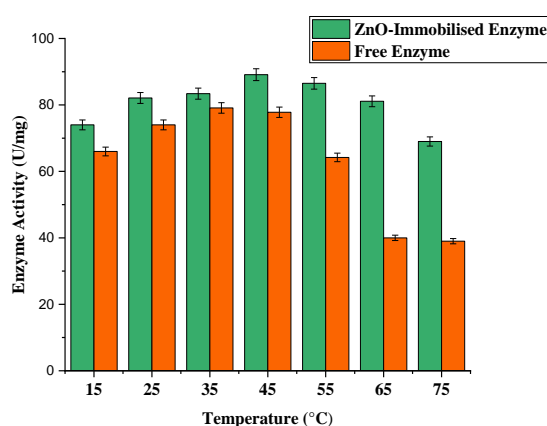


Figure 5. Effect of temperature on the free and immobilized enzyme.

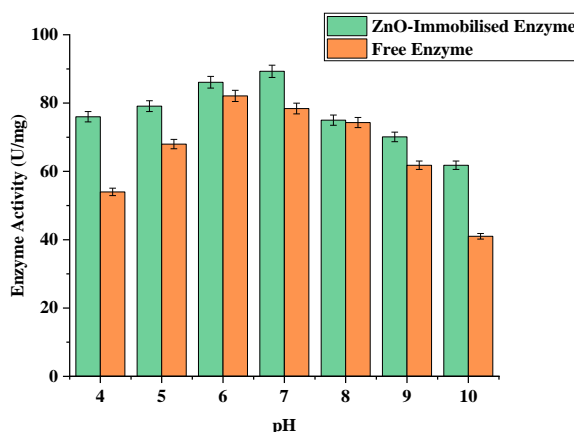


Figure 6. Effect of pH on the free and immobilized enzyme.

### 3.3. Storage stability of the free and immobilized enzyme.

This study analyzed that after 5 cycles, the enzyme's activity did not significantly reduce. In cycle six, there was a sharp reduction in enzyme activity, as illustrated in Figure 7, in a sequence from 86.1 to 79.8 U/mg for the first five times. The main cause of reduced enzyme activity was washing time, which caused damage to the enzyme to escape from the carrier [50]. The immobilized enzyme's activity significantly declined after 60 days because of superior storage stability than the naturally occurring free enzyme. A similar result was observed by Yigitoglu et al. in the storage stability [51].

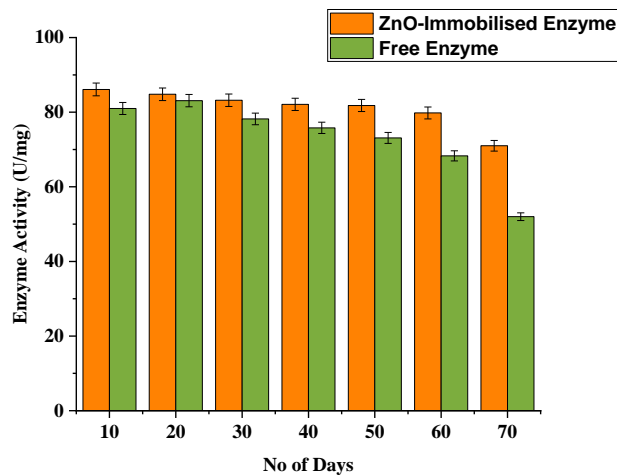


Figure 7. Storage stability of the free and immobilized enzyme.

### 3.4. Biomass and lipid productivity of the isolated cyanobacterial strain.

Four different media were used to cultivate the cyanobacterial species *Anabaena*: modified ASN III, BG-11, BBM, and ASN III are the media which were grown in 500 mL Erlenmeyer flasks (conical flasks). The results established that the isolated *Anabaena* species showed a lag time of two days and an exponential phase long-term six days in all media before ultimately reaching the stationary phase, as shown in Figure 8. As a result, changing the growth media conditions does not cause any change in the *Anabaena* strain biomass dry weight (OD), which remains constant with optical density. The biomass dry-weight concentration of the *Anabaena* strain at the stationary phase was calculated and found to be 33.3 g/L biomass productivity, and the lipid productivity was found to be 19.7 g/L.

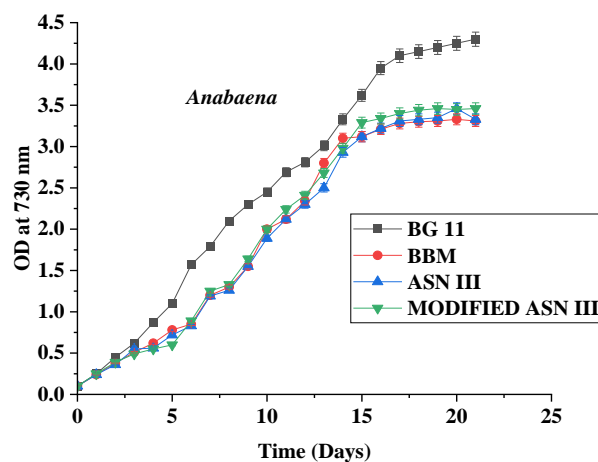


Figure 8. Growth curve of *Anabaena* in four different mediums.

### 3.5. Transesterification – biodiesel production

According to Xie et al. [52], lipase immobilized on magnetic nanoparticles produced the highest biodiesel conversion efficiency at 92.3% from soyabean oil as a feedstock under ideal conditions. Similarly, Yagiz et al., [53] reported that immobilizing lipozyme TL on hydrotalcite used as a biocatalyst converted waste oils into biodiesel with significant biodiesel yield efficiency. The fatty acids present in the lipid obtained from the *Anabaena* were identified using GC-MS Spectral analysis. The five fatty acids were nonadecanoic acid, stearic acid, oleic acid, palmitic acid, and myristic acid, and applicable for good quality biodiesel production. All five fatty acids identified in the lipid of the *Anabaena* cyanobacterial strain were converted into fatty acid methyl ester and analyzed using GC-MS spectral analysis (Table 1). The biodiesel yield achieved up to 96% using 300 mg lipase immobilized zinc oxide nanoparticles, methanol: oil molar ratio (4:1), and 45°C stirred for 6 hours. The qualities of the biodiesel produced are shown in Table 2, where it was found that they became within the standard ranges given by ASTM and EN standards. The cetane number for produced biodiesel was determined to be 54, resulting in smooth engine operation and good cold start performance. If the produced biodiesel contains a low cetane number, partial combustion increases the emission levels. A measure of the relative delay between fuel injection and auto-ignition is called the cetane number [54]. Biodiesel's density at 30°C and viscosity at 40°C were found to be 0.861 g/cm<sup>3</sup> and 1.427 mm<sup>2</sup>/sec, respectively, being in the standard range shown in Table 2. It was suggested in the previous article that viscosity influences penetration, drop size, and atomization quality. High-viscosity fuels have issues with poor combustion, more emissions, and exhaust smoke. Additionally, it causes big fuel droplets to form during injection, which may damage fuel pump components and injectors, increase engine deposits, and result in poor fuel atomization during spraying. Density directly affects both the air-fuel ratio and the volume of biodiesel injected into the combustion chamber [54]. A higher iodine value (IV) indicates a lower fuel oxidation stability. The EN 14214 standard of biodiesel iodine value is around a maximum of 120. With a higher flash point of 136°C, the biodiesel that was produced could be less flammable and safer to handle, store, and transport. A 0.293 mg KOH/g acid value was observed in the biodiesel that was produced from the lipid of cyanobacteria species of *Anabaena* [55]. The cloud point indicates the cold flow properties of biodiesel. Moreover, excessive unsaturation causes oxidation challenges, so adding a stabilizer will help the fuel last longer in storage [56]. Biodiesel produced from *Anabaena* does not produce any ash and water content while running the engine. Since all of the values come up within the biodiesel standard range, it is evident that high-quality biodiesel can be made from *Anabaena* lipid by enzymatic transesterification with the use of an immobilized lipase enzyme on zinc oxide nanoparticles.

**Table 1.** The fatty acid methyl ester is present in the biodiesel produced from the lipids of *Anabaena*.

Retention time	Fame	Mass (m/z)
19.60	Nonadecanoic acid methyl ester	312.53
20.94	Stearic acid methyl ester	298.50
21.46	Oleic acid methyl ester	296.50
24.92	Palmitic acid methyl ester	270.50
25.73	Myristic acid methyl ester	240.42

**Table 2.** Properties of biodiesel.

Properties	Biodiesel	ASTM D0975	ASTM D6751	EN 14214
Density at 30°C g/cm <sup>3</sup>	0.857	0.876	0.875-0.90	0.86 to 0.90
Viscosity at 40°C mm <sup>2</sup> /sec	1.392	1.9 to 4.1	1.6 to 6	3.5 to 5

Properties	Biodiesel	ASTM D0975	ASTM D6751	EN 14214
Flashpoint °C	134	60-80	100 to 170	>120
Pour point °C	-05	-35 to -15	-15 to 16	---
Cloud point °C	04	-15 to 5	-3 to 12	---
Cetane number	52	40 to 55	47 to 65	---
Acid value mg KOH/g	0.3	0.35	<0.8	<0.5
Iodine value I <sub>2</sub> /g	90	---	---	<120

#### 4. Conclusion

Based on the experimental results, the synthesized zinc oxide nanoparticles were immobilized on the lipase enzyme, increasing the amount of biodiesel produced by the lipid isolated from the *Anabaena* cyanobacterial species. Five times without losing any of its activity, the immobilized lipase could be utilized in the biodiesel transesterification process. The ideal reaction conditions were reported to be nanobiocatalyst (300 mg), 400 rpm, 45°C, and a 4:1 molar ratio of methanol to oil. The lipids derived from *Anabaena* were converted into fatty acid methyl esters under these conditions, and the % yield was calculated to be 96.57%.

#### Author Contributions

All authors have read and agreed to the published version of the manuscript.

#### Institutional Review Board Statement

Not applicable.

#### Informed Consent Statement

Not applicable.

#### Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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#### Conflicts of Interest

The authors declare no conflict of interest.

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