Article

Effect of Different TiO$_2$ Morphologies on the Activity of Immobilized Lipase for Biodiesel Production

Kholoud El-Kady, Mai Raslan, and Ayman H. Zaki*
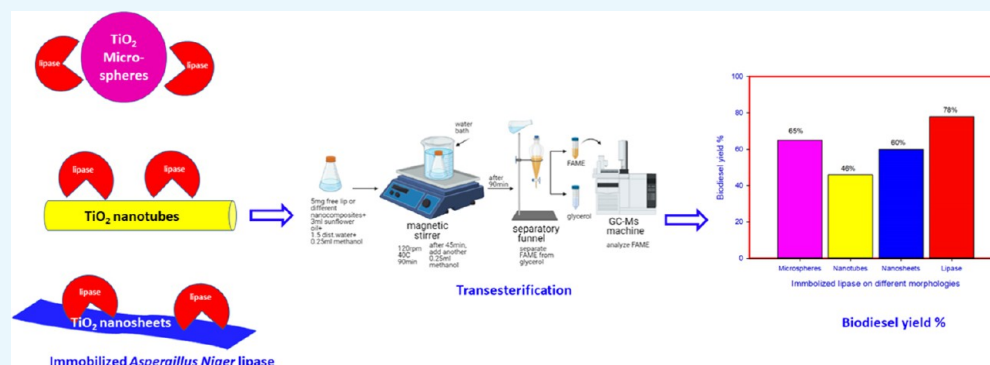
Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Lipase catalytic activity is greatly influenced by immobilization on nanoparticles. In this study, lipase from *Aspergillus niger* was immobilized on TiO$_2$ nanoparticles with different morphologies: microspheres, nanotubes, and nanosheets. All TiO$_2$ samples were prepared by a hydrothermal method. Lipase/TiO$_2$ nanocomposites were prepared by a physical adsorption method through hydrophobic interactions. The prepared composites were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), and high-resolution transmission electron microscopy (HRTEM). The catalytic activity of free and immobilized lipases was tested using sunflower oil in the presence of methanol to produce biodiesel at 40 °C for 90 min. The lipase immobilized on TiO$_2$ microspheres showed the highest activity compared to the lipase immobilized on TiO$_2$ nanotubes and nanosheets. To optimize the lipase-to-microsphere ratio, lipase was immobilized on TiO$_2$ microspheres in different microspheres/lipase, w/w, (S/L) ratios of 1:1, 1:0.75, 1:0.5, and 1:0.25. It was noticed that the hydrolytic activity follows the order 1:0.25 > 1:0.5 > 1:75 > 1:1. The immobilization yield activities were found to be 113, 123, 125, and 130% for the microspheres/lipase (S/L) ratios of 1:1, 1:0.75, 1:0.5, and 1:0.25, respectively.

1. INTRODUCTION

Enzymatic catalysts are employed in different industrial applications, including ester synthesis reactions.[1] Enzymatic catalysts are preferred over chemical catalysts because chemical catalysts have many drawbacks, such as high energy consumption, low product purity, and generation of wastewater.[2] Enzymes play an effective role in industrial biotechnology and microbiology. Lipase is one of the most important enzymatic catalysts because it has applications in several areas, such as in the food industry, wastewater treatment, textiles, leather, cosmetics, biofuels, emulsifiers, flavors, fragrances, pharmaceuticals, and enzymology, as well as in the synthesis of many organic and lipophilic antioxidants.[3,4]

Lipase is part of the hydrolases family, which can be defined as a triacylglycerol acyl hydrolase that acts on carboxylic ester bonds.[5] Lipase can be obtained from plants, animals, and microorganisms such as *Aspergillus niger*. Microbial lipase is more economical, faster, and easily obtained compared to those obtained from plant and animal cells.[6] Microbial lipases are largely applied in biocatalysis due to their versatility, catalytic properties, and high stability in the reaction media.[4] Lipase catalyzes various reactions such as hydrolysis, esterification, and transesterification.[7]

Transesterification is the reaction between triglycerides and alcohols to produce fatty acid alkyl esters and glycerol. Short-chain alcohols like methanol and ethanol produce esters called biodiesel. Biodiesel is defined as a mixture of fatty acid alkyl esters.[8] Biodiesel is considered an alternative fuel to petroleum diesel because of its environmental advantages. It decreases environmental pollution and is renewable and biodegradable;
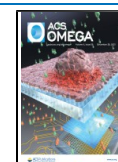
moreover, it is a promising alternative due to the increasing petroleum diesel price, increasing need for energy, and high biodiesel yield.[9] Transesterification is considered as the best technique for biodiesel yield compared with other techniques, especially in the presence of catalysts, because they accelerate the reaction rate and improve the solubility of alcohols in oil as alcohols are sparingly soluble in oil.[10,11] Many catalysts such as acidic and basic catalysts and enzymes are being employed in biodiesel production. The enzymatic catalysis by lipase is used in biodiesel production since it produces biodiesel at low temperatures in the presence of free fatty acids and water; in addition, it increases product purity via an eco-friendly technique.[8] Despite all of these advantages of free lipase in biodiesel production, it could not accomplish the requirements in industrial biocatalysis, such as long-term storage stability, preserved activity, and efficient reusability.[12] Lipase immobilization can overcome these problems and improve stability in reaction media.[13]

Immobilization is the process where the enzyme attaches to the surface of solid supports, leading to the loss of enzyme mobility and retention of enzymatic activity.[7,14−16] Immobilization is an important technique to create a stable biocatalyst with certain features, including high catalytic activity, high enzyme loading, and easy recovery.[14] Moreover, immobilized lipase is preferred over free lipase because immobilized lipase is more stable to environmental changes and can be recycled while the process operates continuously, thereby reducing the production cost. Also, immobilization enhances the efficiency of lipase by increasing its purity, activity, specificity, selectivity, and resistance to inhibitors.[7,15]

Lipase can be immobilized by different methods, such as cross-linking, covalent attachment, encapsulation, or physical adsorption on an inert support.[16] The different types of physical adsorption include ion exchange, hydrophobic adsorption, immobilized metal affinity chromatography (IMAC) adsorption, van der Waals, and hydrogen bonding.[4,17,18] The successful immobilization of an enzyme by adsorption on a solid support can be achieved by the presence of specific functional groups on the surface of both the support and enzyme, which makes the interactions sufficiently strong for the support−enzyme binding (adsorption) to occur.[18]

The choice of immobilization technique and support is an important step. The most common and preferred technique in lipase immobilization is physical adsorption on various supports via interfacial activation (hydrophobic interaction) because of the low cost and the ease and simplicity of the technique without a need to activate the support.[1,3] The support should be chemically, mechanically, and thermally stable; insoluble in the solution involved in the technique; cheap; and compatible with the enzyme to be immobilized.[8]

Recently, great achievements have been noticed at the synergistic action of biotechnology with nanotechnology by applying modern nanoparticles as a support in the immobilization process. Lipase can be immobilized on different supports such as graphene oxide (GO), iron oxide (Fe_3O_4) nanoparticles, and graphene oxide/iron oxide (GO/Fe_3O_4) nanocomposites, silica aerogel, different types of chitosan, hybrid zinc oxide−iron oxide (ZnOFe) magnetic nanoparticles, polydopamine-coated iron oxide ($Fe_3O_4\_PDA\_lipase$), flexible nanoporous materials, $Cu_3(PO_4)_2$-based inorganic hybrid nanoflower, polyurethane nanosupports, silica magnetic nanoparticles, and titanate and TiO_2 nanoparticles.[12,19−26] Nanoparticles are considered as an ideal support in immobilization since they supply better

selectivity along with thermal stability, higher enzymatic activity, easy recovery and purification, very small size, large surface area-to-volume ratio, high adsorption ability, and adaptability toward a wider pH range.[10,27] Using nanoparticles reduces the diffusion hindrance, leading to the availability of high concentrations of the immobilized biocatalysts compared to the enzymes immobilized onto larger materials.

Unfortunately, there are some drawbacks of using nanoparticles in enzyme immobilization, including the high cost of the immobilization process, the limitations of applicability on a large scale, and the need for separation of reaction media.[17,28] TiO_2 nanoparticles and also titanate with various morphologies, especially microspheres, are used in many applications since they are inexpensive, easy to prepare, nontoxic, and commercially available.[29]

The aim of this work is to study the effect of TiO_2 with different morphologies on the catalytic activity of lipase toward biodiesel production, where no papers are found in the literature concerning this issue.

2. MATERIALS AND METHODS

2.1. Materials. Sunflower oil and olive oil were purchased from the local market. Lipases from the fungus *A. niger* and TiO_2 powder were purchased from Loba Chemie, India. Sodium hydroxide, methanol, ethanol, phenolphthalein, hydrochloric acid, and phosphoric acid were purchased from EL Nasr Company, Egypt.

2.2. Synthesis and Characterization of TiO_2 Nanostructures. *2.2.1. Preparation of Nanotubes.* The desired TiO_2 and/or titanate nanotubes or nanosheets were prepared by the conventional eco-friendly hydrothermal method. In the typical synthesis, 5 g of the as-purchased TiO_2 powder was added to 500 mL of 10 M sodium hydroxide. The mixture was subjected to vigorous stirring for about 0.5 h. The milky suspension formed was then transferred to a 1 L Teflon-lined autoclave and heated in an oven at 160 °C for 4 and 16 h to form nanosheets and nanotubes, respectively. The obtained powder was collected and washed several times with distilled water to obtain pure sodium titanate nanotubes and nanosheets (Na-TNTs and Na-TNSs, respectively). The obtained sodium titanates were washed with 0.1 M HCl to form the corresponding H-titanate nanotubes and nanosheets (H-TNTs and H-TNSs, respectively). Finally, the powder was annealed at 500 °C for 4 h.[30]

2.2.2. Synthesis of Mesoporous TiO_2 Microspheres. H-titanate nanotubes were used as a starting material to prepare TiO_2 microspheres. In brief, 4 g of H-TNTs was added to 650 mL of distilled water; this mixture was mixed for about 0.5 h using a magnetic stirrer. Then, 10 mL of HF was added to this suspension, followed by the addition of 19.5 g of Urea. After 1 h of stirring, this mixture was transferred to a Teflon-lined stainless steel autoclave of 0.5 L capacity and subsequently placed in an oven for 12 h at 180 °C. The formed powder was washed with distilled water and then dried at 80 °C for 2 h.[31]

2.3. Immobilization of Lipase. First, different nanocomposites were prepared using a 1:1 ratio (w/w) of TiO_2 nanostructures (nanotubes, nanosheets, and microspheres) to immobilize lipase. Nanotubes with lipase (T-LIP), nanosheets with lipase (Sh-LIP), and microspheres with lipase (S-LIP) were prepared by physical adsorption at a 1:1 ratio (w/w). Second, nanocomposites of different microspheres/lipase with different ratios of 1:1, 1:0.75, 1:0.5, and 1:0.25 (w/w) were prepared. In a typical synthesis, 0.5 mL of pure lipase suspension (0.005 g of
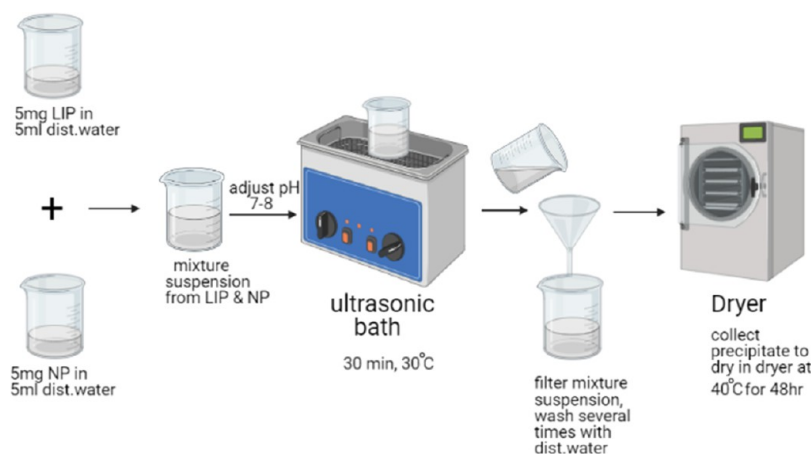
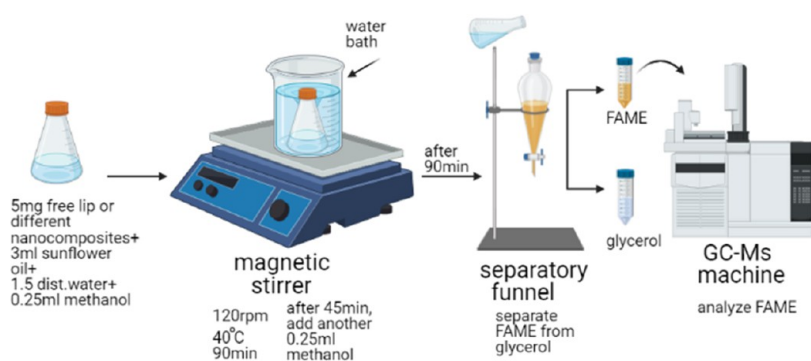**Figure 1.** Preparation steps of immobilized lipase.

Figure 2. Transesterification steps of sunflower to biodiesel.

lipase in 5 mL of water) was added to a 0.5 mL (T, Sh, and S) suspension (0.005 g of NPs in 5 mL of water). The pH of the mixture was adjusted to 7−8 using phosphate buffer.[32−35] The suspension was subjected to sonication for 30 min at 30 °C. The mixture was then filtered using filter paper, washed several times with distilled water, and finally left to dry at 40 °C for 48 h. All steps are presented in Figure 1.

2.4. Hydrolytic Activity Assay and Immobilization Yield (IY %). Various lipase suspension samples (free lipase and different S-LIPs) (1 mL) were incubated separately with a reaction mixture formed from 1 mL of 0.1 M Tris-HCl buffer (pH 8.0), 2.5 mL of deionized water, and 3 mL of olive oil at 30 °C. After 30 min, 3 mL of 95% ethanol was added to stop the reaction, and each sample solution was transferred to a 50 mL Erlenmeyer flask. The liberated fatty acids were titrated against 0.1 M NaOH using phenolphthalein as an indicator, which turns pink at the endpoint. Both test and blank were employed. The same technique was applied separately for each concentration of immobilized lipases (S-LIPs). Enzyme activity was expressed as units per mL enzyme.[20]

The immobilization yield activity was calculated using the following equation[36,37]

immobilization yield activity (IY , %)

= (activity of immobilized lipase/activity of free lipase)

× 100

2.5. Transesterification Reaction. Transesterification reactions were carried out in 100 mL flasks on a shaking plate at 120 rpm at 40 °C. Five milligrams of each sample (free lipase,

T, Sh, S, and different nanocomposites) was added separately to the reaction mixture including 3 mL of sunflower oil, 1.5 mL of distilled water, and 250 μL of methanol. After half-time of the reaction, another 250 μL of methanol was added; alcohol was added in this way to avoid loss of lipase activity by excess alcohol. By the end of the second addition, the molar ratio of methanol/oil reached. The formed biodiesel samples were collected after 90 min and were analyzed by GC/MS.[38] All reaction steps are illustrated in Figure 2.

2.6. Characterization. *2.6.1. FAME Analysis.* The obtained biodiesel was analyzed by gas chromatography using Agilent GC (7890A), equipped with a mass spectrometer 5975C. The initial oven temperature at the start was 50 °C and was maintained at this temperature for 0 min. The second temperature was 210 °C, where the temperature was increased from 50 to 210 °C at a rate of 10 °C/min. The temperature was maintained at 210 °C for 13 min and then increased to 230 °C at 5 °C/min and held for 15 min. GC−MS was set to electron ionization mode and was adjusted to operate with 70 eV.[39,40]

2.6.2. Characterization of Materials. HRTEM micrographs were obtained from a JEOL-JEM 2100 (Japan) at an acceleration voltage of 200 kV. XRD patterns were recorded on a PANalytical (Empyrean) X-ray diffractometer at a scan range of 5−80° and scan step of 0.02°. Fourier transform infrared spectroscopy (FTIR, Bruker Vertex 70) was used to examine the chemical bond vibrations of samples. Field-emission scanning electron microscopy (FESEM), elemental mapping, and energy-dispersive X-ray spectroscopy (EDXS) were performed (Carl Zeiss, Germany).

3. RESULTS AND DISCUSSION

3.1. Characterization of Free and Immobilized Lipase.
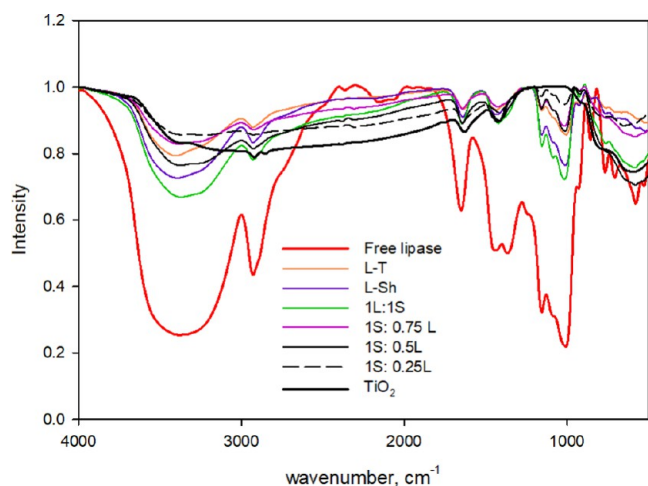Figure 3 displays the FTIR spectra of the prepared titania, free

Figure 3. FTIR of lipase, TiO$_2$, and their nanocomposites (L-T, L-Sh, L-S).

lipase, and prepared nanocomposites. The spectra of the prepared nanoparticles exhibit peaks at ~3400 and 1620 cm^{-1} indicating the presence of a OH group. This may be due to the presence of large amount of water and hydroxyl groups in samples. The peaks located at 1620 cm^{-1} indicate the presence of physically adsorbed water molecules H−O−H, while the broad peaks positioned at 3400 cm^{-1} indicate O−H stretching vibrations.[41] Free lipase displayed certain distinguishing peaks at 1660 and 1541 cm^{-1}, representing the amide bands I and II, respectively.[42] Additionally, lipase displayed main peaks at 3420−3150 cm^{-1} (−OH stretching vibrations and −NH stretching vibrations), 2924 cm^{-1} (C−H stretching vibrations), 1652 cm^{-1} (N−H bending vibrations), and 1080 cm^{-1} (C−O bond stretching vibrations). The occurrence of the band at 1056 cm^{-1} may be due to C−N and/or C−O stretching. Almost all peaks of lipase were found in the corresponding composites; this confirms the successful loading of lipase over different morphologies of titania.

Figure 4a−f shows the HRTEM images of lipase composites with TiO$_2$ nanotubes, nanosheets, and microspheres. The obtained images confirm the successful preparation of the desired composites. As can be seen in Figure 4a,b, the nanotubes are randomly distributed over the lipase layers, while Figure 4c,d shows that the TiO$_2$ nanosheets are stacked to the lipase surface. On the other hand, the images of lipase-microspheres are illustrated in Figures 4e,f and 5. The results revealed that the microspheres are composed of small TiO$_2$ nanoparticles, which aggregate to form the desired microspheres. After mixing with lipase of different ratios, the small nanoparticles tend to aggregate on the lipase layers. Figure 6 shows the FESEM micrographs of lipase/microsphere composite, in addition to the images of elemental mapping of this composite. The images confirm the successful preparation of the desired composite, where the lipase layers can be seen on the surfaces of microspheres brighter than the microsphere surface. The inset in Figure 6 is grouped images with changed brightness degree to differentiate between the microspheres and lipase layers, where the dark is titania and the light parts are lipase layers. This was also confirmed by the elemental mapping results, where the C

atoms (represents lipase) are uniformly distributed over TiO$_2$ microspheres.

3.2. Immobilization Mechanism and Interfacial Activation.
Lipase was immobilized over TiO$_2$ by an adsorption method. The adsorption method is subclassified into different types: ion exchange, hydrophobic adsorption, immobilized metal affinity chromatography (IMAC) adsorption, van der Waals, and hydrogen bonding.[4,17] Since TiO$_2$ is hydrophobic in nature, the expected mechanism of immobilization is hydrophobic interaction. It is worth mentioning that adsorption methods have many advantages compared to other methods: there is no significant change in the lipase structural configuration, in addition to its simplicity and low cost.[4] Lipases are complex and special enzymes and have two different conformations: a closed form, in which the active center is hidden from the medium by a polypeptide chain called a lid, and an open form, in which the lid moves and exposes the active center of lipase to the medium.[17,43,44] This open form shows a very large hydrophobic pocket exposed to the medium, which is the active form; it is formed from the hydrophobic groups in the lid internal face and the hydrophobic residues in the active center of lipase; hence, it was found that exposure of this hydrophobic pocket to the hydrophobic medium is highly favorable.[45,46] Consequently, lipase shows a peculiar mechanism of action called interfacial activation when attached to a hydrophobic support where the active center of lipase is exposed outside the lid to link to the substrate (oil drops), which enhances the catalytic activity of lipase.[43,44,47] As discussed in the activity assay part, the immobilization of lipase in different ratios over TiO$_2$ microspheres greatly enhanced the catalytic activity relative to the free enzyme activity, where the samples showed activities of 130, 125, 123, and 113% for ratios of 1:0.25, 1:0.5, 1:0.75, and 1:1, respectively. The results revealed that as the enzyme concentration increases, the catalytic activity decreases; this may be attributed to the enzyme molecule−enzyme molecule interaction at higher concentrations,[36,48−50] since high lipase concentration results in lipase−lipase dimer formation through certain interactions between the open forms of the two lipase molecules, and these aggregates differ in activity and stability compared to the monomeric enzymes, as shown in Figure 7.

3.3. Hydrolytic Activity Assay and Immobilization Yield (IY, %).
In this study, the hydrolytic activity of S-LIP at a ratio of 1:0.25 (w/w) is higher than those at ratios of 1:0.5, 1:0.75, and 1:1 (w/w) because high enzyme concentrations lead to enzyme−enzyme interactions between the open forms of enzyme molecules, which affects the enzyme activity. Therefore, as the enzyme concentration increases, the enzyme activity decreases. Immobilization yield activity of S-LIP were 113, 123, 125, and 130% for ratios of 1:1, 1:0.75, 1:0.5, and 1:0.25, respectively. In certain conditions, IY% can be higher than, which explains the enzyme hyperactivation phenomenon as it occurs in lipase, especially when lipase comes in contact with hydrophobic supports via interfacial activation.[37]

3.4. Biodiesel Yield and Transesterification Kinetics.
The effects of TiO$_2$ morphology and immobilization w/w ratio on the biodiesel yield were evaluated using sunflower oil as a substrate at a reaction temperature of 40 °C for 90 min using a methanol-to-oil molar ratio of 6:1 because it was noticed that a high methanol-to-oil molar ratio improves the reaction between methanol and triglyceride, which shifts the reaction forward to completion and avoids a reversible reaction; hence, it produces a
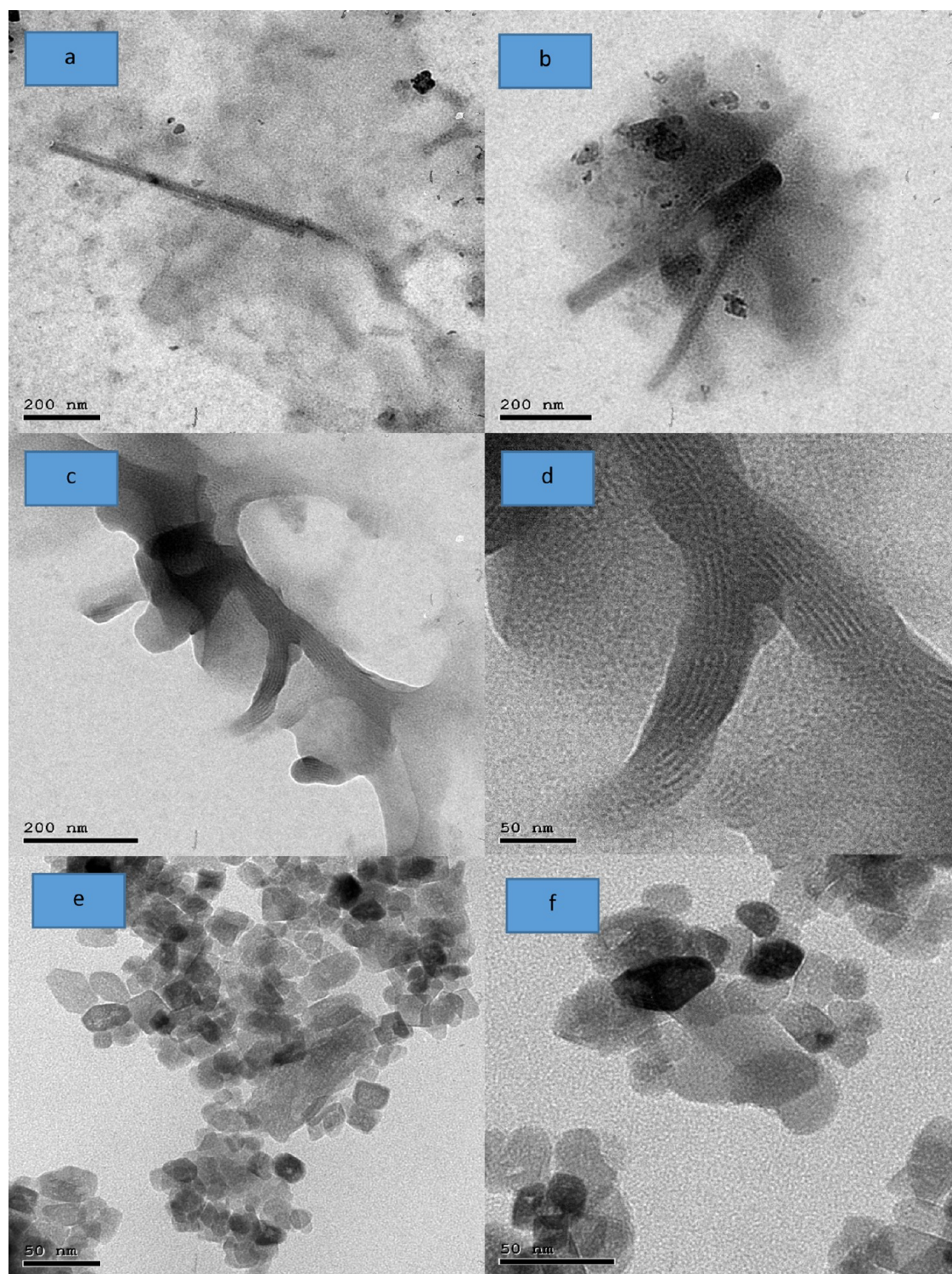
Figure 4. HRTEM images of (a, b) T-LIP, (c, d) Sh-LIP, and (e, f) S-LIP nanocomposites.

higher biodiesel yield in a shorter time.[51] The yield was calculated using the following equation[52−54]

$$\text{biodiesel yield (\%)} = (\text{mass of biodiesel obtained}/\text{mass of oil used}) \times 100$$

The results revealed that when comparing the biodiesel yield of lipase immobilized over different morphologies of TiO_2, the highest yield was achieved using lipase immobilized over microspheres. Therefore, the sample S-LIP achieved a biodiesel yield of 65%, and the samples T-LIP and Sh-LIP achieved 46 and 60% yields, respectively (Figure 8). Additionally, S-LIP of different ratios of 1:0.75, 1:0.5, and 1:0.25 exhibited biodiesel yields of 79, 76, and 80%, respectively (Figure 9). The results revealed that the sample with a microsphere-to-lipase ratio of 1:0.25 achieved the highest biodiesel yield among all materials; this means that only 25% of lipase can be used to achieve a higher percentage than pure lipase. Using these microspheres as
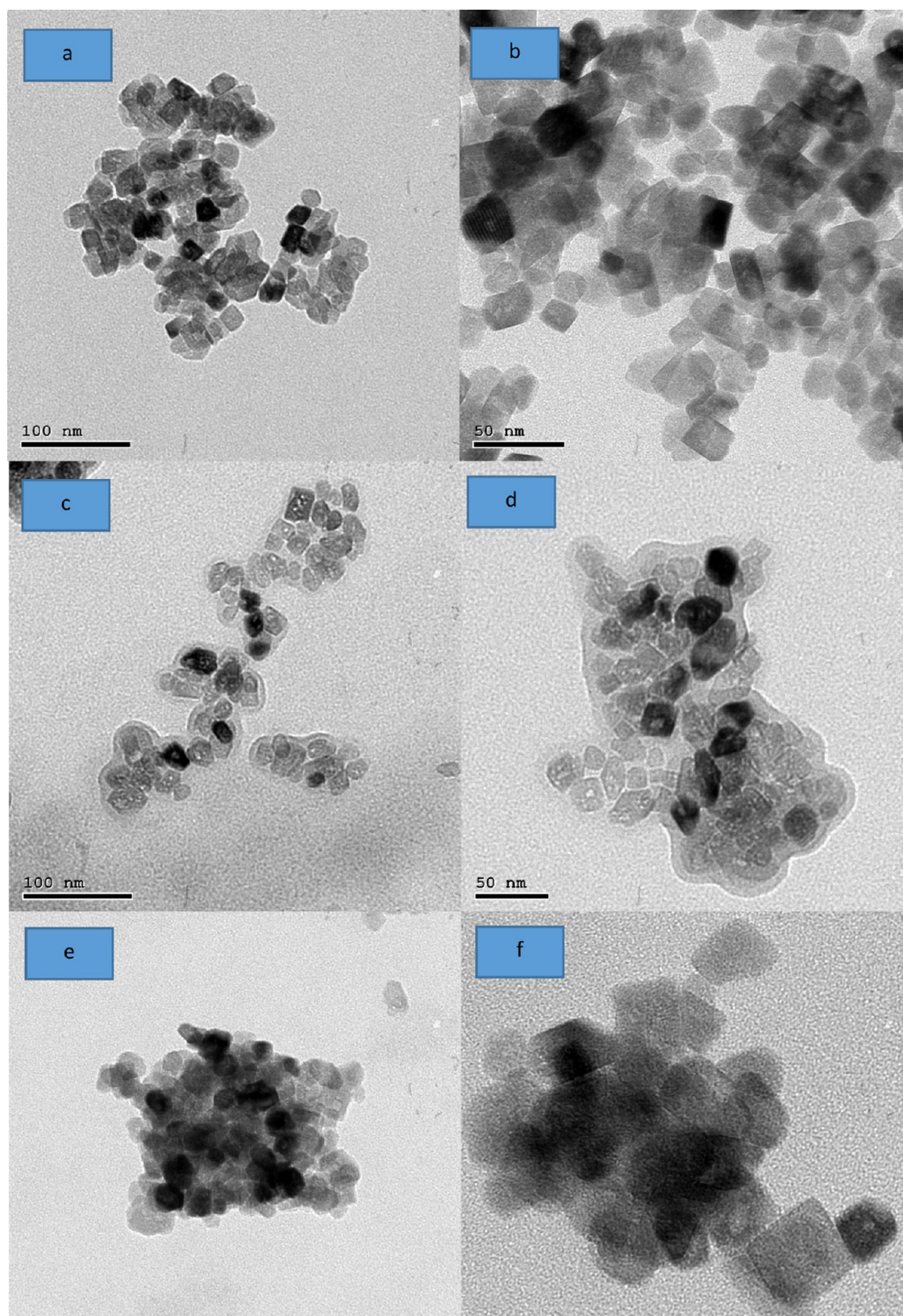
Figure 5. HRTEM images of (a, b) 1S:0.75lip, (c, d) 1S:0.5lip, and (e, f) 1S:0.25lip nanocomposites.

a support for lipase suggests a perfect feature for controlling the key factors that regulate the biocatalyst efficacy. Examples of the controlled key factors are surface area, enzyme effectiveness, and mass transfer resistance.[55,56]

Lipases are employed as catalysts in the transesterification reaction to produce biodiesel. Lipase-mediated transesterification of oils in the presence of alcohols results in the formation of long-chain fatty acid methyl esters (FAMEs) called biodiesel. The transesterification of oil to produce FAMEs is a kinetically
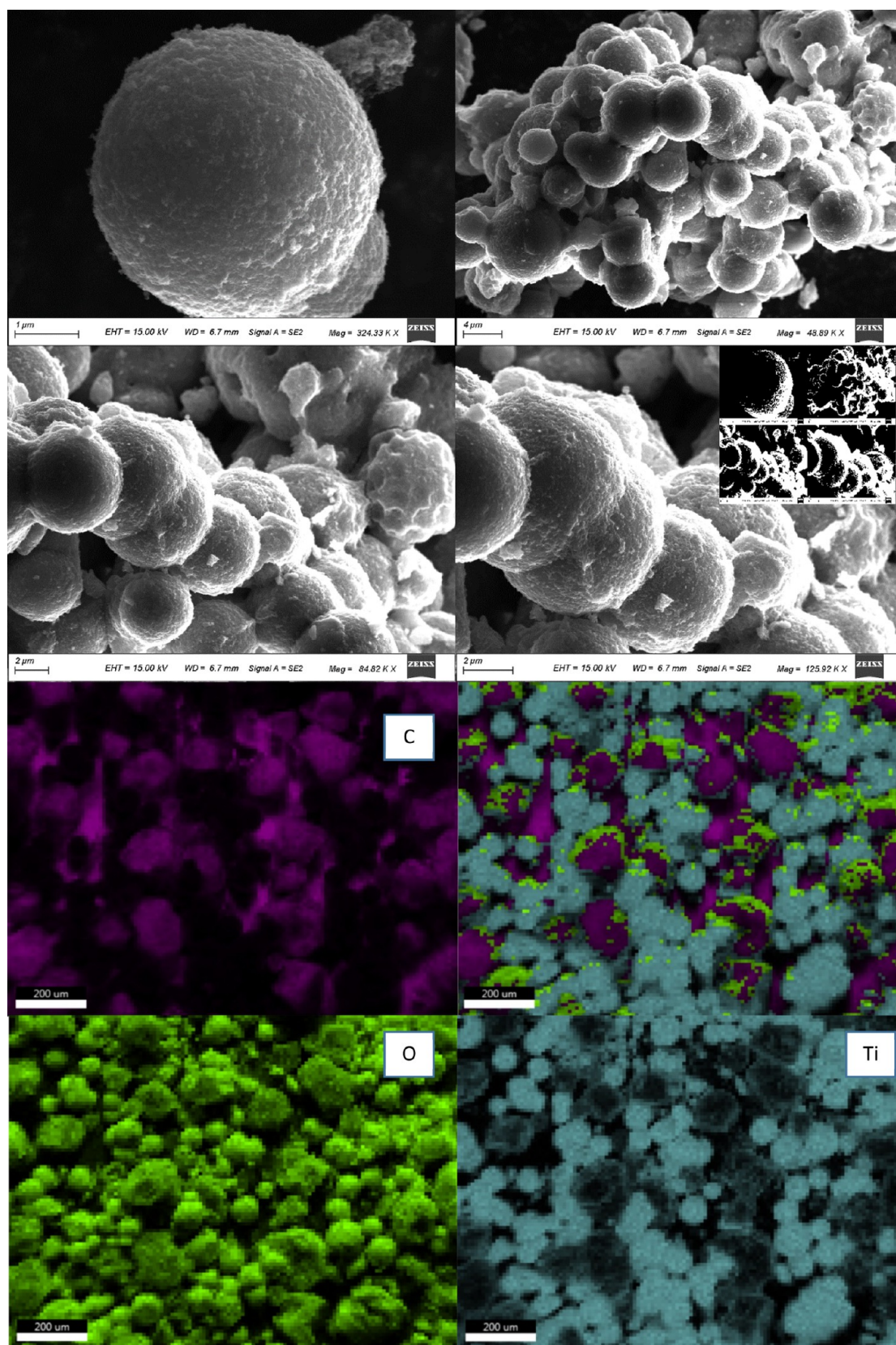
Figure 6. FESEM images and elemental mapping of lipase/microsphere composite.

controlled reaction where the transient yields of FAMEs depend on the catalyst (lipase).[57] Lipase specificity depends on the different types and lengths of fatty acids of triacylglycerol molecules (acyl donor) and the length of alcohol (acyl

acceptor).[58] Lipases should be nonstereospecific to convert all tri-, di-, and monoacylglycerols to the corresponding monoalkyl esters (biodiesel).[59] In kinetically controlled transesterification, the triacylglycerol substrate (acyl donor) reacts with the serine
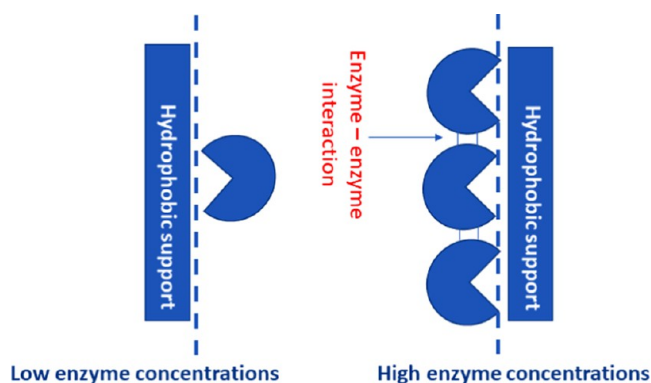
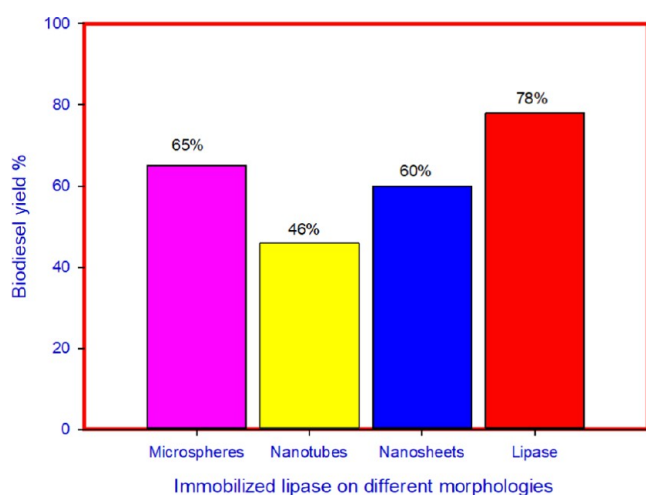**Figure 7.** Interfacial activation and effect of enzyme loading on activity.

Figure 8. Biodiesel yield using lipase supported over nanotubes, nanosheets, and microspheres.
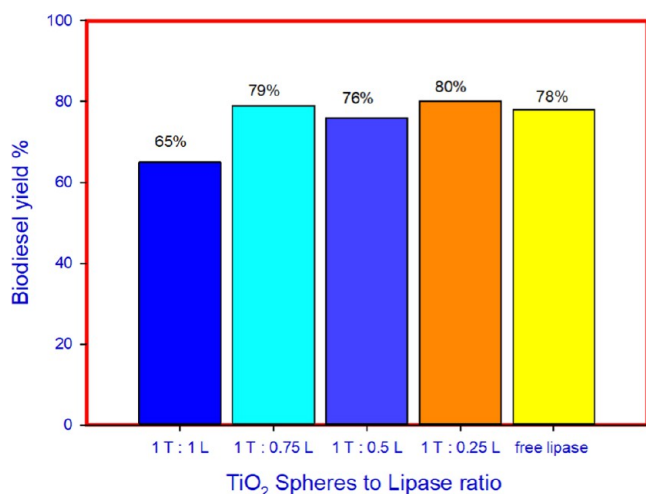

Figure 9. Biodiesel yield using different ratios of microspheres to lipase.

residue of the lipase catalytic triad to form an acyl–enzyme intermediate, which then reacts with the other substrate (acyl acceptor) to form the desired acylated product.[58,60,61] It was noticed that using triacylglycerol as an acyl donor has a positive effect, which accelerates the transesterification rate. Several approaches were attempted to improve the transesterification of vegetable oils,[62] wherever the transesterification reaction took place in two steps. In the first step, triglycerides are hydrolyzed

to free fatty acids, and in the second step, the produced free fatty acids are esterified to fatty acid methyl esters. In this study, the enzymatic kinetic models of oil hydrolysis and FFA esterification are combined together. These results are in agreement with other previously published studies.[63,64] Lipases also catalyze the formation of esters from glycerol and long-chain fatty acids.[65] They include several bioconversion reactions such as interesterification, esterification, hydrolysis, alcoholysis, aminolysis, and acidolysis.[66]

Glycerol is the main byproduct in transesterification reaction, which constrains lipase catalytic effect. It adsorbs onto lipase immobilization supports, which leads to a decrease in lipase activity and process efficiency.[67]

Glycerol forms a hydrophilic layer on the surface of the biocatalyst, which prevents the accessibility of immobilized lipase to hydrophobic substrates (such as residual triglyceride, diglycerides, and monoglycerides). Moreover, unreacted alcohol leaves the reaction mixtures and accumulates on the glycerol layer and further covers the immobilized lipase surface, leading to lipase deactivation because of the local alcohol concentration.[67,68]

Various methods have been applied to solve these problems, such as elimination of glycerol by dialysis or extraction using a polar solvent or adding organic solvents (e.g., *n*-hexane or *tert*-butanol) to decrease the viscosity of the reaction mixture and make it more homogeneous, or alternatively using a highly hydrophobic support that prevents glycerol adsorption.[69,70] It was found that the hydrophobicity of the support avoids clogging of the biocatalyst by glycerol formation. In this study, the support (TiO_2) is hydrophobic, which hinders glycerol adsorption on lipase and enhances the reversible immobilization of lipase by interfacial activation (hydrophobic interactions).[68]

4. CONCLUSIONS

Lipase was immobilized on different morphologies of TiO_2 nanoparticles by a physical adsorption method. The free lipase and their titanate nanocomposites accomplish high biodiesel yield. It was noticed that the lipase/titanate microsphere nanocomposite produces the highest biodiesel yield, and a low concentration of immobilized lipase on titanate microsphere (0.25:1) approximately produced the same biodiesel yield using free lipase. Consequently, a low concentration of immobilized lipase is used instead of free lipase leading to cost-effective results.

■ AUTHOR INFORMATION

Corresponding Author

Ayman H. Zaki − *Materials Science and Nanotechnology Department, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Beni-Suef 62521, Egypt;* orcid.org/0000-0003-0026-1721; Phone: 002-01225047780; Email: ayman.zaki@psas.bsu.edu.eg, Ayman_h_zaki@yahoo.com

Authors

Kholoud El-Kady − *Biotechnology & Life Sciences Department, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Beni-Suef 62521, Egypt*

Mai Raslan − *Biotechnology & Life Sciences Department, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Beni-Suef 62521, Egypt;* orcid.org/0000-0003-3136-6833

Complete contact information is available at:

https://pubs.acs.org/10.1021/acsomega.1c04942

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Brito, M. J. P.; Bauer, L. C.; Santos, M. P. F.; Santos, L. S.; Bonomo, R. C. F.; Fontan, R. D. C. I.; Veloso, C. M. Lipase immobilization on activated and functionalized carbon for the aroma ester synthesis. *Microporous Mesoporous Mater.* 2020, 309, No. 110576.

(2) Kumar, R.; Pal, P. Lipase immobilized graphene oxide biocatalyst assisted enzymatic transesterification of Pongamia pinnata (Karanja) oil and downstream enrichment of biodiesel by solar-driven direct contact membrane distillation followed by ultrafiltration. *Fuel Process. Technol.* 2021, 211, No. 106577.

(3) Okura, N. S.; Sabi, G. J.; Crivellenti, M. C.; Gomes, R. A.; Fernandez-Lafuente, R.; Mendes, A. A. Improved immobilization of lipase from Thermomyces lanuginosus on a new chitosan-based heterofunctional support: Mixed ion exchange plus hydrophobic interactions. *Int. J. Biol. Macromol.* 2020, 163, 550−561.

(4) Ismail, A. R.; Baek, K. H. Lipase immobilization with support materials, preparation techniques, and applications: Present and future aspects. *Int. J. Biol. Macromol.* 2020, 163, 1624−1639.

(5) Chandra, P.; Singh, R.; Arora, P. K.; et al. Microbial lipases and their industrial applications: a comprehensive review. *Microb. Cell Fact.* 2020, 19, No. 169.

(6) Kaur, A.; Rishi, V.; Soni, S. K.; Rishi, P. A novel multi-enzyme preparation produced from Aspergillus niger using biodegradable waste: a possible option to combat heterogeneous biofilms. *AMB Express* 2020, 10, No. 36.

(7) Mortazavi, S.; Aghaei, H. Make proper surfaces for immobilization of enzymes: immobilization of lipase and α-amylase on modified Na-sepiolite. *Int. J. Biol. Macromol.* 2020, 164, 1−12.

(8) Nezhad, M. K.; Aghaei, H. Tosylated cloisite as a new heterofunctional carrier for covalent immobilization of lipase and its utilization for production of biodiesel from waste frying oil. *Renewable Energy* 2021, 164, 876−888.

(9) Hama, S.; Kondo, A. Enzymatic biodiesel production: an overview of potential feedstocks and process development. *Bioresour. Technol.* 2013, 135, 386−395.

(10) Touqeer, T.; Mumtaz, M. W.; Mukhtar, H.; Irfan, A.; Akram, S.; Shabbir, A.; Choong, T. S. Y.; et al. Fe3O4-PDA-Lipase as Surface Functionalized Nano Biocatalyst for the Production of Biodiesel Using Waste Cooking Oil as Feedstock: Characterization and Process Optimization. *Energies* 2020, 13, No. 177.

(11) Karmakar, A.; Karmakar, S.; Mukherjee, S. Properties of various plants and animals feedstocks for biodiesel production. *Bioresour. Technol.* 2010, 101, 7201−7210.

(12) Liu, Y.; Shao, X.; Kong, D.; Li, G.; Li, Q. Immobilization of thermophilic lipase in inorganic hybrid nanoflower through biomimetic mineralization. *Colloids Surf., B* 2020, 197, No. 111450.

(13) Ficanha, A. M. M.; Antunes, A.; Oro, C. E. D.; Dallago, R. M.; Mignoni, M. L. Immobilization of Candida antarctica B (CALB) in Silica Aerogel: Morphological Characteristics and Stability. *Biointerface Res. Appl. Chem.* 2020, 10, 6744−6756.

(14) Hartmann, M.; Kostrov, X. Immobilization of enzymes on porous silicas—benefits and challenges. *Chem. Soc. Rev.* 2013, 42, 6277−6289.

(15) Kochanè, T.; Budrienè, S.; Pielichowski, K.; Pielichowski, J. Application of polyurethane-based materials for immobilization of enzymes and cells: a review. *CHEMIJA* 2006, 17, 74−89.

(16) Mohamad, N. R.; Marzuki, N. H. C.; Buang, N. A.; Huyop, F.; Wahab, R. A. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnol. Biotechnol. Equip.* 2015, 29, 205−220.

(17) Garcia-Galan, C.; Berenguer-Murcia, Á.; Fernandez-Lafuente, R.; Rodrigues, R. C. Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv. Synth. Catal.* 2011, 353, 2885−2904.

(18) Jesionowski, T.; Zdarta, J.; Krajewska, B. Enzyme immobilization by adsorption: a review. *Adsorption* 2014, 20, 801−821.

(19) Coşkun, G.; Çıplak, Z.; Yıldız, N.; Mehmetoğlu, Ü. Immobilization of Candida antarctica Lipase on Nanomaterials and Investigation of the Enzyme Activity and Enantioselectivity. *Appl. Biochem. Biotechnol.* 2021, 193, 430−445.

(20) Ficanha, A. M. M.; Antunes, A.; Oro, C. E. D.; Dallago, R. M.; Mignoni, M. L. Immobilization of candida antarctica b (calb) in silica aerogel: Morphological characteristics and stability. *Biointerface Res. Appl. Chem.* 2020, 10, 6744−6756.

(21) de Sousa Fonseca, T.; de Oliveira, U. M. F.; de Oliveira, M. D. C. F.; de Lemos, T. L. G.; da Silva, M. R.; Rios, N. S.; de Mattos, M. C.; et al. Immobilization of Amano lipase AK from Pseudomonas fluorescens on different types of chitosan-containing supports: use in the kinetic resolution of rac-indanol. *Bioprocess Biosyst. Eng.* 2021, 44, 785−792.

(22) Fotiadou, R.; Chatzikonstantinou, A. V.; Hammami, M. A.; Chalmpes, N.; Moschovas, D.; Spyrou, K.; Stamatis, H.; et al. Green Synthesized Magnetic Nanoparticles as Effective Nanosupport for the Immobilization of Lipase: Application for the Synthesis of Lipophenols. *Nanomaterials* 2021, 11, No. 458.

(23) Ghasemi, S.; Yousefi, M.; Nikseresht, A.; Omidi, H. Covalent binding and in-situ immobilization of lipases on a flexible nanoporous material. *Process Biochem.* 2021, 102, 92−101.

(24) Cipolatti, E. P.; Valério, A.; Henriques, R. O.; Pinto, M. C. C.; Lorente, G. F.; Manoel, E. A.; Pessela, B. C.; et al. Production of new nanobiocatalysts via immobilization of lipase B from C. antarctica on polyurethane nanosupports for application on food and pharmaceutical industries. *Int. J. Biol. Macromol.* 2020, 165, 2957−2963.

(25) Esmi, F.; Nematian, T.; Salehi, Z.; Khodadadi, A. A.; Dalai, A. K. Amine and aldehyde functionalized mesoporous silica on magnetic nanoparticles for enhanced lipase immobilization, biodiesel production, and facile separation. *Fuel* 2021, 291, No. 120126.

(26) Zulfiqar, A.; Mumtaz, M. W.; Mukhtar, H.; Najeeb, J.; Irfan, A.; Akram, S.; Nabi, G.; et al. Lipase-PDA-TiO2 NPs: An emphatic nano-biocatalyst for optimized biodiesel production from Jatropha curcas oil. *Renewable Energy* 2021, 169, 1026−1037.

(27) Khan, M.; Husain, Q.; Asmat, S. Synthesis, characterization, and applications of nanographene-armored enzymes. *Methods Enzymol.* 2018, 609, 83−142.

(28) Klein, M. P.; Nunes, M. R.; Rodrigues, R. C.; Benvenutti, E. V.; Costa, T. M.; Hertz, P. F.; Ninow, J. L. Effect of the support size on the properties of β-galactosidase immobilized on chitosan: advantages and disadvantages of macro and nanoparticles. *Biomacromolecules* 2012, 13, 2456−2464.

(29) Nady, D.; Zaki, A. H.; Raslan, M.; Hozayen, W. Enhancement of microbial lipase activity via immobilization over sodium titanate nanotubes for fatty acid methyl esters production. *Int. J. Biol. Macromol.* 2020, 146, 1169−1179.

(30) Farghali, A. A.; Zaki, A. H.; Khedr, M. H. Control of selectivity in heterogeneous photocatalysis by tuning TiO2 morphology for water treatment applications. *Nanomater. Nanotechnol.* 2016, 6, No. 62296.

(31) Zaki, A. H.; El-Shafey, A.; Moatmed, S. M.; Abdelhay, R. A.; Rashdan, E. F.; Saleh, R. M.; El-dek, S. I.; et al. Morphology transformation from titanate nanotubes to TiO2 microspheres. *Mater. Sci. Semicond. Process.* 2018, 75, 10−17.

(32) Santos, J. C.; Nunes, G. F.; Moreira, A. B.; Perez, V. H.; de Castro, H. F. Characterization of Candida rugosa lipase immobilized on poly (N-methylolacrylamide) and its application in butyl butyrate synthesis. *Chem. Eng. Technol.* 2007, 30, 1255−1261.

(33) Krajewska, B.; Piwowarska, Z. Free vs chitosan-immobilized urease: Microenvironmental effects on enzyme inhibitions. *Biocatal. Biotransform.* 2005, 23, 225−232.

(34) Ratledge, C.; Kristiansen, B. *Basic Biotechnology*, 3rd ed.; Cambridge University Press, 2006; p 605.

(35) Živković, L. T. I.; Živković, L. S.; Babić, B. M.; Kokunešoski, M. J.; Jokić, B. M.; Karadžić, I. M. Immobilization of Candida rugosa lipase by adsorption onto biosafe meso/macroporous silica and zirconia. *Biochem. Eng. J.* **2015**, *93*, 73–83.

(36) Boudrant, J.; Woodley, J. M.; Fernandez-Lafuente, R. Parameters necessary to define an immobilized enzyme preparation. *Process Biochem.* **2020**, *90*, 66–80.

(37) Illanes, A.; Wilson, L. Parameters for the Evaluation of Immobilized Enzymes Under Process Conditions. In *Immobilization of Enzymes and Cells*; Humana Press: New York, 2020; pp 65–81.

(38) Wang, X.; Dou, P.; Zhao, P.; Zhao, C.; Ding, Y.; Xu, P. Immobilization of lipases onto mag-netic Fe3O4 nanoparticles for application in biodiesel production. *ChemSusChem* **2009**, *2*, 947–950.

(39) Mahangani, N.; Vunain, E.; Meijboom, R.; Jalama, K. *Biodiesel Production Over ZnO/TiO₂ Catalyst: Effect of Co-Solvent, Temperature and Reaction Time*, Proceedings of the World Congress on Engineering, 2015.

(40) Ruppel, T.; Huybrighs, T.; Shelton, C. T. Fatty Acid Methyl Esters in B100 Biodiesel by Gas Chromatography (Modified EN 14103), Perkin Elmer's Application Note, 2008.

(41) Mogilevsky, G.; Chen, Q.; Kulkarni, H.; Kleinhammes, A.; Mullins, W. M.; Wu, Y. Solvent and Structural Effects in the N–H Bond Homolytic Dissociation Energy. *J. Phys. Chem. C.* **2008**, *112*, 3239–3246.

(42) Rabbani, G.; Baig, M. H.; Lee, E. J.; Cho, W. K.; Ma, J. Y.; Choi, I. Biophysical study on the in-teraction between eperisone hydrochloride and human serum albumin using spec-troscopic, calorimetric, and molecular docking analyses. *Mol. Pharmaceutics* **2017**, *14*, 1656–1665.

(43) Mulinari, J.; Oliveira, J. V.; Hotza, D. Lipase immobilization on ceramic supports: An overview on techniques and materials. *Biotechnol. Adv.* **2020**, *42*, No. 107581.

(44) Manoel, E. A.; Dos Santos, J. C.; Freire, D. M.; Rueda, N.; Fernandez-Lafuente, R. Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. *Enzyme Microb. Technol.* **2015**, *71*, 53–57.

(45) Arana-Peña, S.; Rios, N. S.; Carballares, D.; Goncalves, L. R.; Fernandez-Lafuente, R. Immobilization of lipases via interfacial activation on hydrophobic supports: Production of biocatalysts libraries by altering the immobilization conditions. *Catal. Today* **2021**, *362*, 130–140.

(46) Arana-Peña, S.; Rios, N. S.; Carballares, D.; Mendez-Sanchez, C.; Lokha, Y.; Gonçalves, L. R.; Fernandez-Lafuente, R. Effects of enzyme loading and immobilization conditions on the catalytic features of lipase from Pseudomonas fluorescens immobilized on octyl-agarose beads. *Front. Bioeng. Biotechnol.* **2020**, *8*, No. 36.

(47) Mokhtar, N. F.; Muhd Noor, N. D.; Mohd Shariff, F.; Mohamad Ali, M. S. The immobilization of lipases on porous support by adsorption and hydrophobic interaction method. *Catalysts* **2020**, *10*, No. 744.

(48) Palomo, J. M.; Fuentes, M.; Fernández-Lorente, G.; Mateo, C.; Guisan, J. M.; Fernández-Lafuente, R. General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality. *Biomacromolecules* **2003**, *4*, 1–6.

(49) Wilson, L.; Palomo, J. M.; Fernández-Lorente, G.; Illanes, A.; Guisán, J. M.; Fernández-Lafuente, R. Effect of lipase–lipase interactions in the activity, stability and specificity of a lipase from Alcaligenes sp. *Enzyme Microb. Technol.* **2006**, *39*, 259–264.

(50) Palomo, J. M.; Ortiz, C.; Fernández-Lorente, G.; Fuentes, M.; Guisán, J. M.; Fernández-Lafuente, R. Lipase–lipase interactions as a new tool to immobilize and modulate the lipase properties. *Enzyme Microb. Technol.* **2005**, *36*, 447–454.

(51) Musa, I. A. The effects of alcohol to oil molar ratios and the type of alcohol on biodiesel production using transesterification process. *Egypt. J. Pet.* **2016**, *25*, 21–31.

(52) Zaki, A. H.; Naeim, A. A.; El-Dek, S. I. Sodium titanate nanotubes for efficient transesterification of oils into biodiesel. *Environ. Sci. Pollut. Res.* **2019**, *26*, 36388–36400.

(53) Yahya, N. Y.; Ngadi, N.; Jusoh, M.; Halim, N. A. A. Characterization and parametric study of mesoporous calcium titanate catalyst for transesterification of waste cooking oil into biodiesel. *Energy Convers. Manage.* **2016**, *129*, 275–283.

(54) González, E. A. Z.; García-Guaderrama, M.; Villalobos, M. R.; Dellamary, F. L.; Kandhual, S.; Rout, N. P.; Arizaga, G. G. C.; et al. Potassium titanate as heterogeneous catalyst for methyl trans-esterification. *Powder Technol.* **2015**, *280*, 201–206.

(55) Kim, J.; Grate, J. W.; Wang, P. Nanobiocatalysis and its potential applications. *Trends Biotechnol.* **2008**, *26*, 639–646.

(56) Feng, W.; Ji, P. Enzymes immobilized on carbon nanotubes. *Biotechnol. Adv.* **2011**, *29*, 889–895.

(57) Rana, Q. U. A.; Irfan, M.; Ahmed, S.; Hasan, F.; Shah, A. A.; Khan, S.; Badshah, M.; et al. Bio-catalytic transesterification of mustard oil for biodiesel production. *Biofuels* **2019**, *3*, 1–8.

(58) Marsden, S. R.; Mestrom, L.; McMillan, D. G.; Hanefeld, U. Thermodynamically and kinetically controlled reactions in biocatal-ysis—from concepts to perspectives. *ChemCatChem.* **2020**, *12*, 426–437.

(59) Souza, L. T.; Mendes, A. A.; Castro, H. F. D. Selection of lipases for the synthesis of biodiesel from Jatropha oil and the potential of microwave irradiation to enhance the reaction rate. *BioMed Res. Int.* **2016**, *2016*, No. 1404567.

(60) Yang, K.; Wang, Y. J. Lipase-catalyzed transesterification in aqueous medium under thermodynamic and kinetic control using carboxymethyl cellulose acetylation as the model reaction. *Enzyme Microb. Technol.* **2004**, *35*, 223–231.

(61) Kasche, V. Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of β-lactam antibiotics, peptides and other condensation products. *Enzyme Microb. Technol.* **1986**, *8*, 4–16.

(62) Al-Zuhair, S. Biochemical Catalytic Production Of Biodiesel. In *Handbook of Biofuels Production*; Woodhead Publishing, 2011; pp 134–159.

(63) Hari Krishna, S.; Karanth, N. G. Lipase-catalyzed synthesis of isoamyl butyrate: a kinetic study. *Biochim. Biophys. Acta* **2001**, *1547*, 262–267.

(64) Janssen, A. E.; Sjursnes, B. J.; Vakurov, A. V.; Halling, P. J. (1999). Kinetics of lipase-catalyzed esterification in organic media: correct model and solvent effects on parameters. *Enzyme Microb. Technol.* **1999**, *24*, 463–470.

(65) Sharma, R.; Chisti, Y.; Banerjee, U. C. Production, purification, characterization, and applications of lipases. *Biotechnol. Adv.* **2001**, *19*, 627–662.

(66) Rajendran, A.; Palanisamy, A.; Thangavelu, V. Lipase catalyzed ester synthesis for food processing industries. *Braz. Arch. Biol. Technol.* **2009**, *52*, 207–219.

(67) Rodrigues, J.; Canet, A.; Rivera, I.; Osório, N. M.; Sandoval, G.; Valero, F.; Ferreira-Dias, S. Biodiesel production from crude Jatropha oil catalyzed by non-commercial immobilized heterologous Rhizopus oryzae and Carica papaya lipases. *Bioresour. Technol.* **2016**, *213*, 88–95.

(68) Martin, L. S.; Ceron, A.; Oliveira, P. C.; Zanin, G. M.; de Castro, H. F. Different organic components on silica hybrid matrices modulate the lipase inhibition by the glycerol formed in continuous trans-esterification reactions. *J. Ind. Eng. Chem.* **2018**, *62*, 462–470.

(69) Tacias-Pascacio, V. G.; Virgen-Ortíz, J. J.; Jiménez-Pérez, M.; Yates, M.; Torrestiana-Sanchez, B.; Rosales-Quintero, A.; Fernandez-Lafuente, R. Evaluation of different lipase biocatalysts in the production of biodiesel from used cooking oil: Critical role of the immobilization support. *Fuel* **2017**, *200*, 1–10.

(70) Lima, L. N.; Oliveira, G. C.; Rojas, M. J.; Castro, H. F.; Da Rós, P. C.; Mendes, A. A.; Tardioli, P. W.; et al. Immobilization of Pseudomonas fluorescens lipase on hydrophobic supports and application in biodiesel synthesis by transesterification of vegetable oils in solvent-free systems. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 523–535.