Comparative studies of free and immobilized partially purified lipase from *Aspergillus niger* NRRL-599 produced from solid-state fermentation using gelatin-coated titanium nanoparticles and its application in textile industry

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Background and objective
Lipases (triacylglycerol acylhydrolase, EC. 3.1.1.3) belong to a class of hydrolases that are specific for the hydrolysis of fats into fatty acids and glycerol that have much application in different industrial processes. Fungi, yeast, and bacteria have been reported to be sources of lipase. There are many immobilized methods for enzyme, and the commonly used methods are physical adsorption, entrapment, and cross-linkage. This study aimed to evaluate lipase production by *Aspergillus niger* NRRL-599 in solid cultivation using agro-industrial waste as a substrate. Partial purification of the crude enzyme and its characterization and immobilization using nanoparticles were carried out. The potential application of the immobilized and partially purified enzyme was also studied in the field of textile.

Materials and methods
Partially purified *A. niger* NRRL-599 lipase was immobilized by physical adsorption onto modified titanium dioxide nanoparticles using gelatin and palmitic acid binders and characterized by transmission electron microscopy, dynamic light scattering, and Fourier-transform infrared.

Results and conclusion
In our study, lipase produced by *A. niger* NRRL-599 was partially purified by ammonium sulfate at 60% saturation and immobilized on gelatin-coated titanium dioxide. Comparison between the properties of the free and the immobilized *A. niger* NRRL-599 lipase forms was carried out. The optimum pH was 9.0 and 10.0 for the free and immobilized forms, respectively. The half-life of the soluble-free lipase at 50 and 55°C was 17.3 and 23.1 min, respectively, whereas for the immobilized form was 23.1 and 34.6 min, respectively. At 50 and 55°C, the deactivation rate constants (kD) for soluble lipase were 6.6×10^{-3} and 5×10^{-3}, respectively, and 6.6×10^{-3} and 3.3×10^{-3}, respectively, for immobilized lipase. The K_m was 11.11 and 12.5 mM for the immobilized and free forms, respectively. The V_{max} was 416.6 U/mg protein and 296.3 U/mg protein for immobilized and free lipase forms, respectively. This confirms that the apparent affinity toward the substrate increases by immobilization. Partially purified lipase and immobilized enzymes were used in the textiles in the treatment of wool fibers before dying to improve the color strength.

Keywords:
immobilization, lipase, partial purification, textile dyes application, titanium dioxide nanoparticles

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Introduction

Lipids represent a large part of the earth's biomass, and lipolytic enzymes take part in their degradation. Recently, microbial lipases were preferred over plant and animal lipases for industrial applications owing to their reusability [1,2]. Different species of bacteria, yeasts, and fungi can produce lipase with different biological properties [3,4]. Lipases are used in several industries such as fat oleo-chemical industry for initiation of hydrolysis and glycolysis of mixed substrates [5], detergent industry, food processing industry for flavor improvement and to improve quality of food by lipolytic hydrolysis of fats and cream [2], paper making industry to remove hydrophilic compounds from pulp [6], and textile industry to increase fabric absorbability [7]. Lipases are also commercially used in biodegradable polymer production, cosmetics [8], tea processing, and resolution of the racemic mixtures [9].

Enzyme immobilization has many advantages that were already discussed such as improving both the stability of biocatalyst as well as the mass transfer in case of nanoparticles, in addition to their reuse in the reaction of interest [10]. The application of enzyme immobilization in large-scale applications depends on the support, which may be rigid, elastic, permeable, nonpermeable, macroporous particles, or nanoparticles [11].

Materials and methods

Chemicals

All media components were purchased from Adwic Company. Chemicals and reagents were purchased from Aldrich (St. Louis, MO, USA) and were of analytical grade.

Microorganism and culture maintenance

Aspergillus niger NRRL-599 was obtained from the National Research Center Culture collection (Department of Natural and Microbial Products) and was refreshed in potato dextrose agar slants that were kept at 30°C for 7 days. The slants were then placed at 4°C. The culture was maintained in 50% (v/v) glycerol stocks at −80°C.

Solid-state fermentation and extraction of the enzyme

Overall, 3 g of olive oil cake as substrate was taken in 250-ml Erlenmeyer flasks and supplemented with 2.5% tap water to maintaining initial moisture. The flasks were sterilized by autoclave at 121°C for 15 min. After cooling, 4 ml of inoculum of the fungal strain *A. niger* NRRL-599 was added to the solid substrate and incubated at 25°C for 16 days [12]. At the end of the fermentation period, the crude enzyme was extracted by mixing the fermented substrate with 100 ml of tap water and then shaking the mixture in a reciprocal shaker at 200 rpm for 15 min. The obtained extract was filtered, and the supernatant was used as crude lipase enzyme [13].

Solid substrate

Olive oil cakes were supplied by the oils department, National Research Center. The olive oil cakes used in all the experiments were from the same batch of the oil production and were frozen at −80°C before use.

Lipase assay

Lipase activity was determined using 1 ml of culture filtrate mixed with 3-ml emulsion of olive oil in Arabic gum (10% w/v) and 2.5 ml of deionized water in 1 ml of 0.1 M Tris-HCl buffer (pH 7.5). After incubation for 2 h at 37°C and 160 rpm, the reaction was stopped by the addition of 10 ml of 99% acetone or ethanol solution. The mixture was then titrated against 0.05 N NaOH using thymolphthalein as an indicator. Blank assays were conducted by boiling the enzyme. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μmol of free fatty acids per minute under assay conditions [14].

Protein determination

The protein content was determined according to the Lowry method [15] using bovine serum albumin as a standard.

Enzyme partial purification

Crude lipase was concentrated and fractionated using ammonium sulfate. Ammonium sulfate was added to the crude lipase with gentle stirring at 4°C to bring saturation to 20%. The precipitation was collected by centrifugation (10 000 rpm, 15 min, and 4°C) and suggested as fraction 0–20% (F20). Additional ammonium sulfate was added to the supernatant to bring the saturation to 40% and the same treatment was done as 20% saturation to give a fraction of 20–40% (F40). The same work was done for saturation 60% to give a fraction of 40–60% (F60), 80% to give a fraction of 60–80% (F80), and then 100% to give a fraction of 80–100% (F100). All fractions were dissolved in 5 ml of distilled water. The fractions were then dialyzed using a dialysis membrane at 4°C against cold distilled water and then dissolved with 0.1 M Tris-HCl buffer (pH 7.5). The lipase activity and protein content of each fraction were measured, and the specific activities were determined [16].

Immobilization of lipase on gelatin-coated titanium dioxide nanoparticles

Adsorption of partially purified lipase on TiO_2 nanoparticles was achieved by incubating 1 ml of the enzyme (10 mg of lyophilized partial purified enzyme containing 200 U/mg, dissolved in distilled water, pH 6.6) with 50 mg of TiO_2 and 50 mg of palmitic acid with the addition of tween 80 under stirring, followed by the addition of 50 mg of gelatin dissolved in traces of distilled water. The mixture was incubated at 30°C overnight with continual shaking and then centrifuged at 10 000 rpm for 15 min at 10°C. The adsorbed enzyme-containing TiO_2 nanoparticles were removed from the solution and dried. The enzyme activity was measured [17].

Characterization of the TiO_2 nanomaterials

Fourier-transform infrared spectroscopy

Samples were lyophilized, and the powder was mixed with potassium bromide (KBr) to form a thin pellet.

Fourier-transform infrared analyses were performed in a Perkin Elmer 2000 spectrophotometer used to record between 4000 and 400 cm^{-1}.

Transmission electron microscope

Morphological and dimension of samples were characterized by a transmission electron microscopy (TEM) (JEOL Electron Microscopy JEM-100 CX; JEOL). Zeta potential analyzer (Nano-ZS, Malvern Instruments Ltd, Worcestershire, UK) was used to determine the average diameter and size distribution. The samples were diluted (five times) by deionized water just before assessment [18]. All of the analyses were carried out at the National Research Center.

Determination of free and immobilized enzyme properties

Optimum pH and pH stability

Enzyme activities of both free and immobilized partially purified enzymes were measured at 37°C at different pH values using citrate buffer pH from 3.0 to 5.0, 0.2 M phosphate buffer pH from 6.0 to 8.0, and glycine-NaOH buffer pH from 9.0 and 10. The pH stability in the range of 3.0–10 was examined by incubating the enzyme solution at different times (15, 30, and 60 min) at 30°C with the tested buffer, and then, the residual activity was determined.

Optimum temperature and thermal stability

The optimum temperature was determined by measuring the enzyme activity for the free and immobilized partially purified enzyme at temperatures from 20 to 45°C in pH 9.0 and 10 for free and immobilized partially purified enzymes, respectively. For thermal stability, both free and immobilized partially enzymes were incubated at 25°C to 60°C with different incubation periods (15, 30, and 60 min) in the absence of substrate, and the relative activity was determined in glycine-NaOH buffer pH (9.0 and 10) for free and immobilized partially purified enzymes, respectively.

Determination of kinetic parameters and thermal properties of lipase

The activity of free and immobilized partially purified lipase was assayed with olive oil concentration from 0.7–3.6 mM. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were established from the Lineweaver-Burk plot [19]. The activation energy (E_a) was determined by plotting the log of the relative activity of the assayed temperature against $1/T$ (Kelvin) of free and immobilized enzymes [20].

$$E_a = \text{slope} \times 2.303R.$$

Where E_a is the activation energy and R is the gas constant (1.976).

Half-life and the deactivation constant rate were determined [21] by plotting the log of relative activity against time according to the following equation: half-life=0.693/slope and deactivation energy=slope of the straight line.

Pretreatment of wool by enzyme

Wool fibers were treated by the microwave technique. The wool fibers were treated by different enzyme concentrations (1.5–5.5%, v/w) for 5 min [22].

Dyeing wool fibers using the microwave method

In a dye bath containing 2 g/l of saffron natural dye with a liquor ratio of 1 : 50, the wool fibers treated with different concentrations of free and nanoenzymes were dyed by microwave heating at pH 5 for 5 min. The dyed samples were rinsed with warm water and then cold water, washed in a bath containing 5 g/l nonionic detergent at 50°C for 30 min and then rinsed and dried in air at room temperature [22].

Measurements of color strength (*K/S* value)

An UltraScan PRO spectrophotometer was used to measure the reflectance of the samples, and hence, the *K/S* was measured spectrophotometrically at wavelengths (λ_{max}) of 370 nm. The *K/S* of untreated and pretreated wool fibers with free and immobilized enzymes and dyed with saffron natural dye was evaluated.

Scanning electron microscopy

The surface morphology of untreated and treated wool fibers was investigated using scanning electron microscopy (SEM), with a JSMT-20, JEOL, Japan. Before the examination, the wool fiber surface was prepared on an appropriate disk and randomly coated with a spray of gold.

Results and discussion

The maximum lipase activity was recorded in the fraction precipitated by 40–60% ammonium sulfate saturation (F60), where the enzyme showed an increase in SEA from 976.7 to 3200±1 U/mg protein (purification fold 3.2).

The profile of ammonium sulfate precipitation indicates that the specific enzyme activity increased

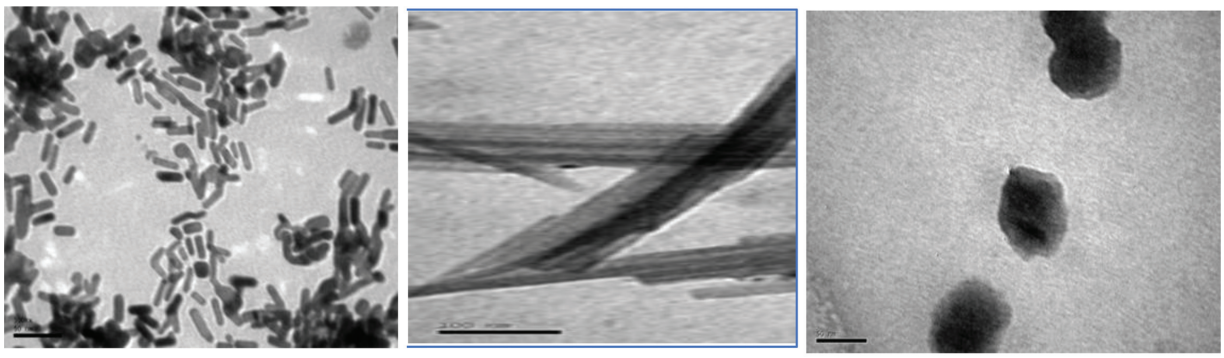
from 0.1-fold with fraction 1 precipitated at 0–20% (F20) to 3.2-fold with fraction three precipitated at 40–60% (F60). The partial purification profile shows a marked decrease in protein content at fractions F20 and F60, which may be attributed to the possible removal of interference. Similar results were also observed for the purification of lipase from *Pseudomonas aeruginosa* srt 9 [23]. The high recovery of lipase activity in the successive fractions may be owing to its high stability [24]. The low recovery of lipase activity in fraction 4 (F80) may be owing to the increase in addition of $(NH_4)_2SO_4$, which may result in a drop in pH and consequently a loss in enzyme activity even in a well-known buffered system [25]. Thus, fraction 3 (F60) contained most of the enzymatic activity, and it was considered to be a partially purified enzyme and was used for further studies.

Characterization studies of the partially purified lipase bound to TiO_2 nanoparticles

The morphology of the naked and lipase-bound titanium dioxide was studied using a TEM (Fig. 1). The carrier appeared to be rod-shaped in Fig. 1A, the free lipase has different morphologies in Fig. 1B, and the enzyme appears to be adsorbed on the surface of the modified TiO_2 nanoparticles in Fig. 1C was spherical like shape. As a result, the enzyme adsorption and incorporation in gelatin–TiO_2 nanocomposite was discovered in matrices. Immobilization could be considered as a powerful tool that helps in the stabilization of the free enzyme. The support material used for enzyme immobilization is an important key parameter owing to the effect of this material on the properties of the catalytic system [26]. These observations were confirmed by the particle size distribution analysis. The particle size and the distribution were measured using the dynamic light scattering method, which is the most popular and common technique for determining the size distribution particles of nano-micelle in suspension [27,28].

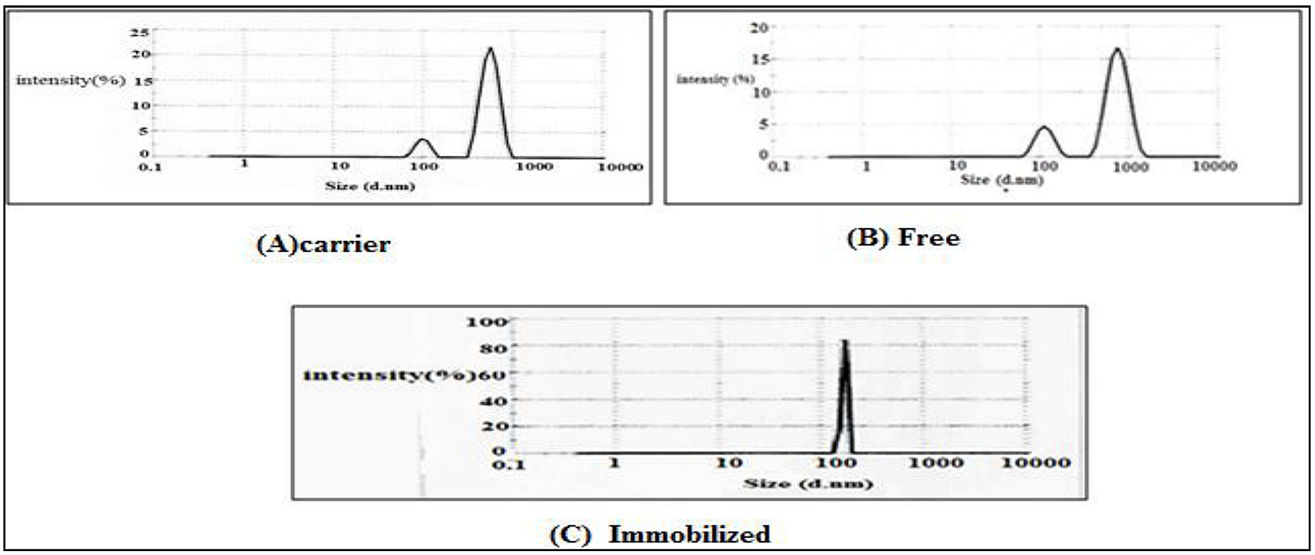
The results in Fig. 2 and Table 1 show that the carrier had two peaks of particle size around 729.4 and 108.2 dnm, with PDI of about 0.702. The free enzyme had particle sizes around 534.7 and 95.19 dnm, with PDI of 0.482. On the contrary, immobilized lipase had one peak, with a small particle size around 160.5 dnm and PDI of 1.000. Thus, it is important to note that the strategy used to synthesize nanoparticles was efficient because of the presence of agglomeration of more than one particle in the solution. This agreed with what was stated by Yang *et al.* [28]. To verify the incorporation of partially purified enzyme on the nanocomposite, Fourier-transform infrared spectroscopy absorption spectra were measured for the samples (Fig. 3): gelatin titanium dioxide nanocomposite (a), free partially purified lipase (b), and immobilized lipase (c). It was observed that in case of the carrier, the characteristic bands were assigned at 3610, 3420, 2870, 2840, 1735, 1650, 1450, and 1250 cm^{-1} corresponding to OH (str) vibration of water adsorbed on the TiO_2 surface [27], the OH stretching and N-H stretching of primary and secondary amines, C-H_2 stretching, C-H stretching of carboxylic acid, and C<td:glyph name=″dbnd″/>O of ester groups. N-H bending, C-H bending, and C-N stretching groups proved the formation of gelatin coated to TiO_2 nanoparticles. After lipase immobilization, all of the bands mentioned before were also observed but with low intensities. This may be owing to the physical

Figure 1



TEM images of the immobilized lipase (c) on gelatin-coated TiO_2 nanoparticles in comparison with the carrier (a) and free enzyme (b) at 50 and 100 nm. TEM, transmission electron microscopy.

Figure 2



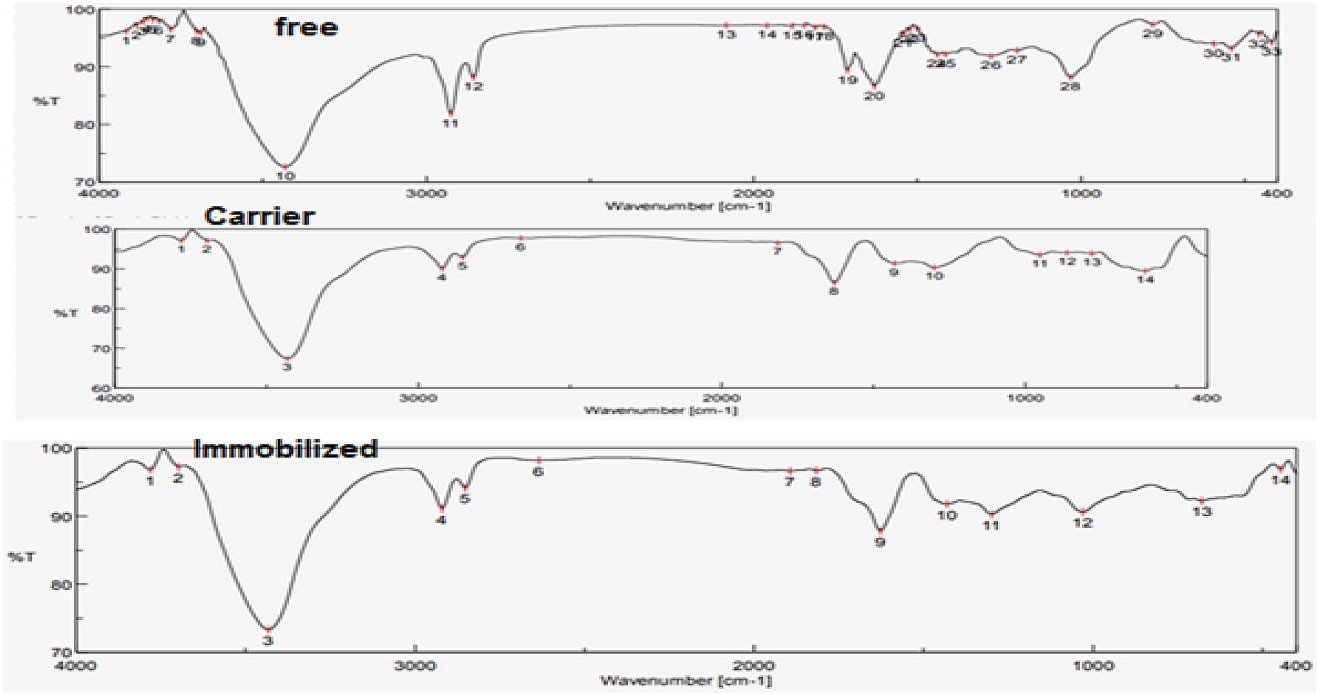
Particle size distribution analysis of the immobilized lipase in comparison with the carrier and free enzyme.

Table 1 Particle size analysis of the carrier, free enzyme, and immobilized enzyme using dynamic light scattering

Sample code	Particle size (dnm)	PDI
Carrier	729±190.6, 81.2%	0.702
	108±23.19, 18.8%	
Free enzyme	534±115.4, 88.3%	0.482
	95±15.46, 11.7%	
Immobilized enzyme	160±8.293	1.000

attachment of immobilized lipase. On the contrary, in the case of the free enzyme, the pattern exhibited a characteristic band at 1713 cm^{-1}, which disappeared after immobilization of the enzyme. The bands at 1631 and 1628 cm^{-1} for free enzyme and at 1628 and 1628 cm^{-1} for carrier were transferred to 1628 and 1627 cm^{-1}, respectively, after immobilization [29,30].

Figure 3



FTIR spectra of immobilized lipase compared with the free enzyme and carrier. FITR, Fourier-transform infrared.

Characterization of free and immobilized partially purified enzyme

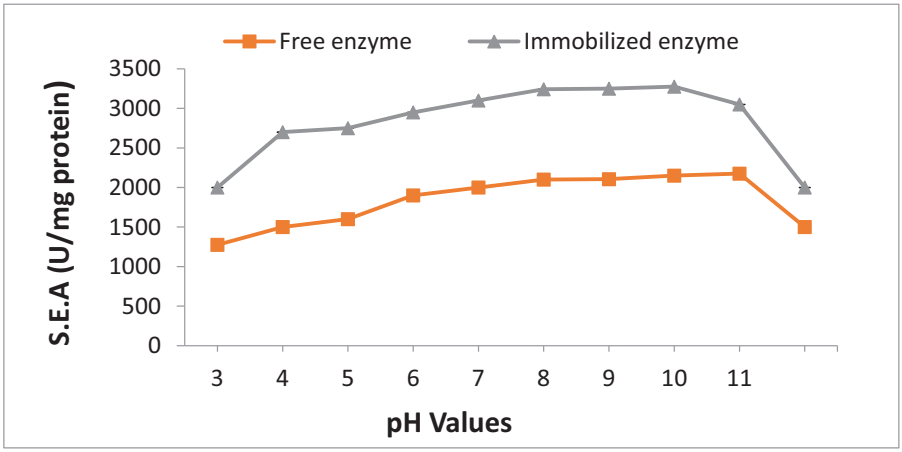
Effect of pH value on the activity of free and immobilized enzymes

Specific enzyme activities for both free and immobilized lipase enzymes were detected at different pH values (Fig. 4). pH values 10.0 and 9.0 were found to be the optimum for immobilized and free enzymes, respectively. The immobilized lipase had a higher specific enzyme activity (3275±0.4 U/mg protein) than the free one (2175±0.42 U/mg protein). The pH stability of both the free and immobilized enzymes was studied at broad pH range values (6–10) (Fig. 5). The immobilized lipase was stable in pH ranges 7.0–8.0 for 60 min, whereas the free one was stable in pH ranges 7–10 for 15 min only. This indicates that the immobilization appreciably improved the lipase stability in the neutral and alkaline regions for a longer time, indicating that it has a great potential for applications in textiles and detergent industries [31]. Similar results were obtained using *Rhizopus oryzae* [32] and *Fusarium globulosum* [33].

Effect of temperature on activity and thermal stability of free and immobilized partially purified lipase enzymes

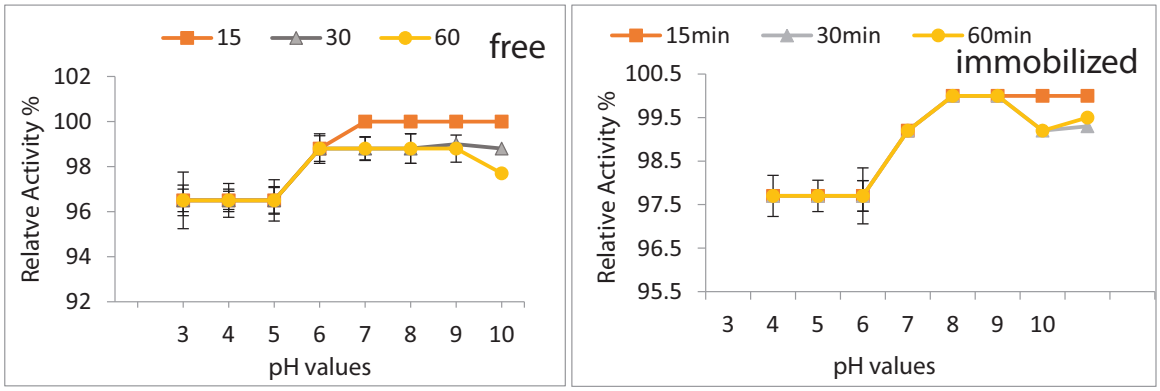
The temperature dependence of both free and immobilized lipase enzymes is illustrated in Fig. 6. Maximum specific enzyme activity of the free and immobilized occurred at 40°C (2225±0.5 and 3300 ±0.9 U/mg protein, respectively). Although a gradual decrease of the specific enzyme activities was detected on increasing the temperature, the activity of the immobilized lipase was still higher than the free enzyme. Similar results were observed by Bruno *et al.* [34] who found that the optimum temperature was (40°C) for both immobilized and free lipase of *Mucor miehei*.

Figure 4



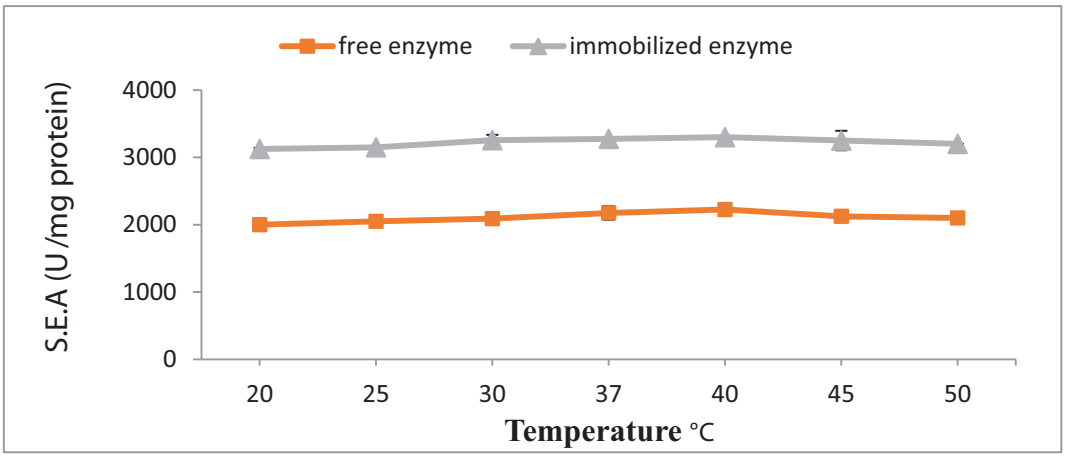
Effect of pH values on lipase activity of *Aspergillus niger* NRRL-599 in case of free and immobilized enzymes.

Figure 5



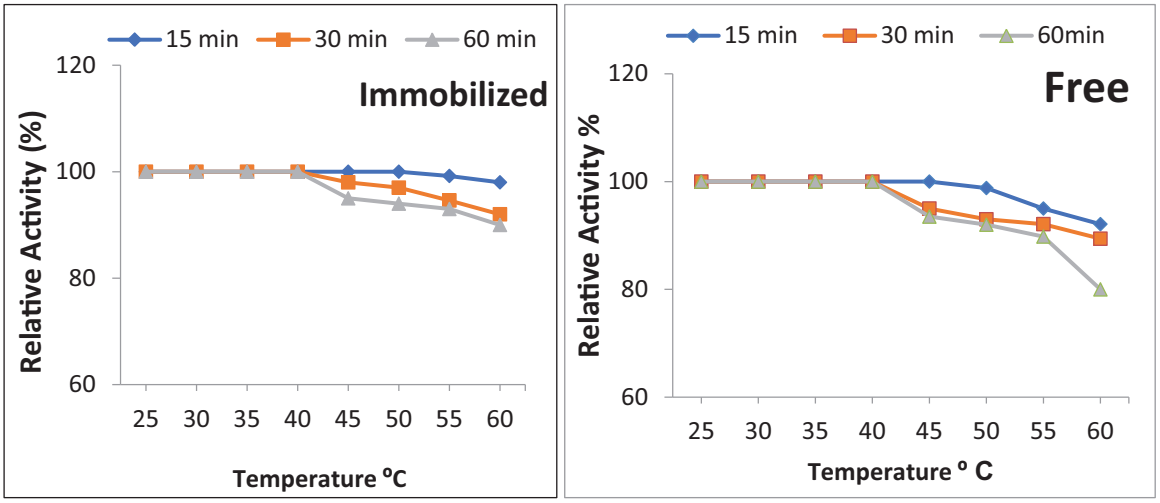
Effect of pH stability on the free and partially purified immobilized lipase forms.

Figure 6



Effect of temperature on the activity of the free and immobilized lipase forms.

Figure 7



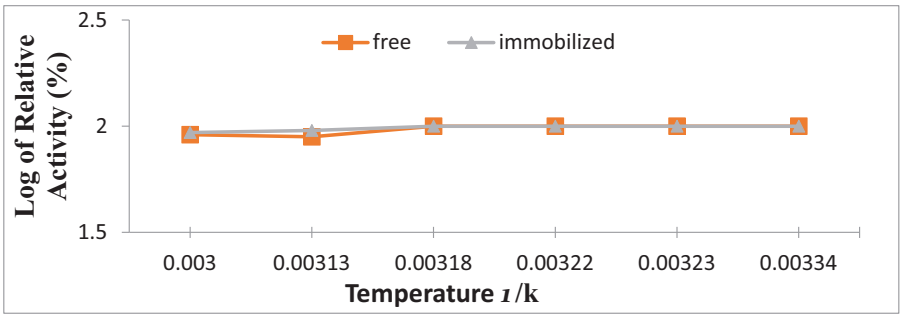
Thermal stability of the free and immobilized partially purified *Aspergillus niger* NRRL-599 lipase activity.

The relative activity of both free and immobilized lipase was determined at different temperatures, and the results are shown in Fig. 7. The temperature profile for the free enzyme showed that 100% residual activity was attained after 15 min incubation at 45°C and 80% residual activity was still retained after 60 min incubation at 60°C. On the contrary, the immobilized enzyme was more tolerant to thermal treatment where it retained 100% residual activity after 15 min incubation at 50°C and only got 90% residual activity after 60 min at 60°C. This phenomenon was similar to previous reports in which the immobilization of lipase is stable in comparison with soluble nature form in terms of a long incubation period at a high temperature [35].

Effect of temperature on kinetic behavior of free and immobilized lipase

The temperature data were plotted in the form of Arrhenius plots (Fig. 8), from which the energy of activation was calculated. E_a for immobilized and free lipase forms was 0.4 and 0.5 kcal/mole, respectively. In the immobilized enzyme form, the energy of activation of lipase is 20% lower. Lower activation energy of immobilized enzyme form compared with that of the free enzyme may be attributed to the diffusion resistance of product and substrate in the case of immobilization [36].The heat activation profile of free and immobilized enzymes provides a relationship between the enzyme substrate structure and function at different temperatures (Table 2). The deactivation rate constant K_d is an important parameter

Figure 8

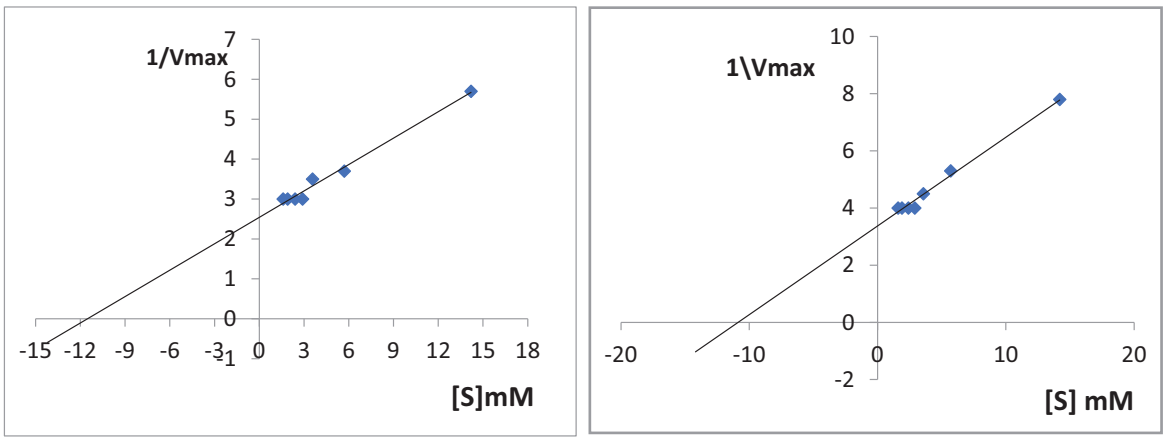


Log of relative activity as a function of temperature for the free and immobilized partially purified *Aspergillus niger* NRRL-599 lipase forms.

Table 2 Thermaldeactivation rate constant (K_d) and times of half-life ($t_{1/2}$) of the free partially purified and immobilized lipase of *Aspergillus niger* NRRL-599 at different temperatures

Kinetic parameter	Partially purified enzyme (free)	Immobilized enzyme
Activation energy (kcal/mol)	0.5	0.4
Half-life (min)		
50°C	17.3	23.1
55°C	23.1	34.6
Deactivation rate constant (h)		
50°C	6.6×10^{-3}	6.6×10^{-3}
55°C	5×10^{-3}	3.3×10^{-3}

Figure 9



Linearweaver-Burk plot for immobilized (a) and free (b) partially purified enzyme of *Aspergillus niger* NRRL-599.

to initiate economic bioprocess at the industrial level. The half-lives of the immobilized enzyme at 50 and 55°C were 23.1 and 34.6 min, respectively, proving to be higher than those of the free enzyme, which were 17.3 and 23.1 min, respectively. The deactivation rate constant K_d at 50 and 55°C was 6.6×10^{-3} and 5×10^{-3} for free lipase, respectively, and 6.6×10^{-3} and 3.3×10^{-3}, respectively, for immobilized form. The deactivation rate constant value at increasing temperatures showed that the immobilized enzyme is highly stable compared with the free one.

Effect of substrate concentration on lipase activity

Effect of substrate concentration on lipase activity

The activity of the free and immobilized enzymes was estimated at various olive oil concentrations. The enzyme activity increases with increase in the substrate concentration up to 3.5 mM for both free

and immobilized enzymes (data not shown). The reaction kinetics of two forms of lipase were estimated from the Lineweaver-Burk plot under optimal conditions (Fig. 9A and B). The results indicated that K_m value of immobilized lipase (11.11 mM) was less than that of free (12.5 mM), which in turn pointed to the high affinity of the immobilized enzyme to the substrate [36]. In addition, the V_{max} of immobilized lipase was higher than that of free form by 1.4-fold.

The catalytic efficiency (V_{max}/K_m) for the immobilized form was greater than that of the free form by 1.58-fold, and this indicated that the rate of conversion of substrate product is higher in the immobilized form [37].

Applications of TiO_2 nanoparticles for obtaining smart textiles

Scanning electron microscopy

SEM images (Fig. 10) showed that the scale of the untreated wool fiber is clear and arranged compactly around the fiber. The scales were changed slightly after the treatment with soluble and immobilized enzymes using gelatin-coated titanium dioxide as the foreign material observed in the SEM image.

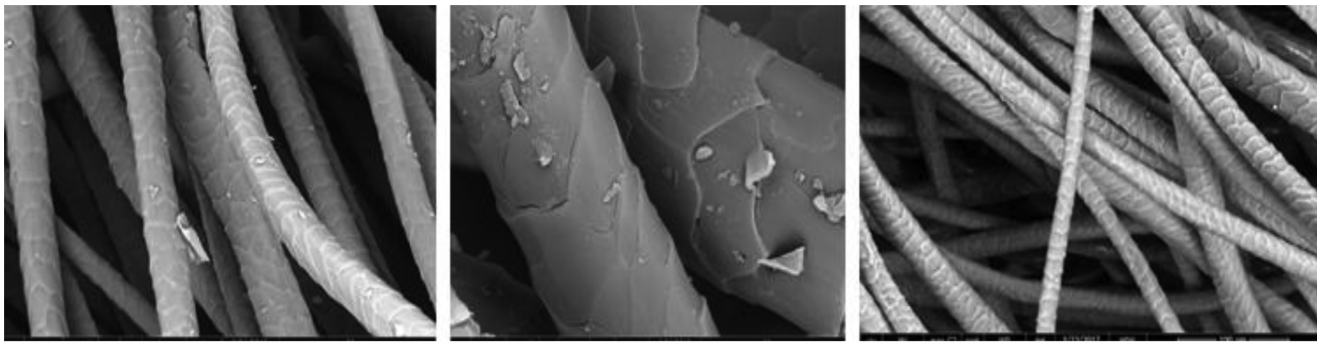
Effect of different concentrations of enzyme on fibers dyed with saffron dye

The obtained results indicated that the high value of color strength was higher than untreated wool fibers. It is also observed that pretreatment with immobilized enzyme exhibited higher values of color strength than pretreatment with enzyme free. The pretreatment using 1.5% (v/g) of immobilized enzyme gave the highest value of color strength (*K/S*) for wool fibers dyed with saffron natural dye using the microwave, as shown in Fig. 11.

Conclusion

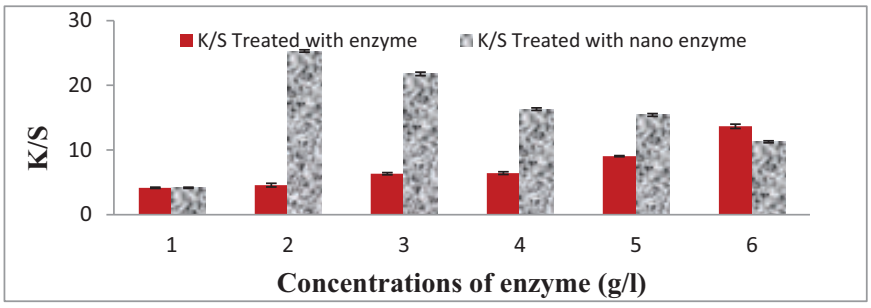
In this study, lipase was produced by *A. niger* NRRL-599 using agro-industrial waste (olive oil cake) as substrate by solid-state fermentation. The exploitation of waste material for the production of valuable products like economic enzymes supports commercialization activities; in addition, it may help in solving pollution problems. Lipase was partially

Figure 10



SEM images of free and immobilized enzyme with nanoparticles in comparison with free wool fibers dyed with saffron natural dye. SEM, scanning electron microscopy.

Figure 11



Effect of different concentrations of free and immobilized enzyme on wool fiber dyed with saffron.

purified by 60% ammonium sulfate and then immobilized on modified titanium dioxide nanoparticles by adsorption. This method was simple and economic. Optimization of the immobilization parameters such as pH and temperature was studied to get the maximum specific enzyme activity (3300 ±0.9 U/mg protein). The research found that there was a significant improvement in pH and heat stability. Kinetic studies showed that the substrate affinity for the immobilized enzyme is great, and it exhibited lower K_m and higher V_{max} compared with the free enzyme. The results represented the potential applicability of immobilized enzymes for industrial applications in the textile industry.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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