

Immobilization of Thermoalkalophilic Lipase from *Bacillus atrophaeus* FSHM2 on Amine-Modified Graphene Oxide Nanostructures: Statistical Optimization and Its Application for Pentyl Valerate Synthesis

Atefeh Ameri¹ · Mojtaba Shakibaie^{1,2} · Mehdi Khoobi³ · Mohammad Ali Faramarzi⁴ · Alieh Ameri⁵ · Hamid Forootanfar^{2,6}

Received: 17 August 2019 / Accepted: 11 November 2019 / Published online: 10 December 2019
© Springer Science+Business Media, LLC, part of Springer Nature 2019

Abstract

Synthesis of (3-aminopropyl) triethoxysilane (APTES)-functionalized graphene oxide (GO) nanosheets, statistical optimization of conditions for immobilization of *Bacillus atrophaeus* lipase (*BaL*) on as-synthesized support, and application of the immobilized *BaL* for esterification of valeric acid were carried out in this investigation. The optimum specific activity of the immobilized *BaL* ($81.60 \pm 0.28 \text{ U mg}^{-1}$) was achieved at 3 mg mL^{-1} of GO-NH₂, 50 mM of phosphate buffer, pH 7.0, 60 min sonication time, 100 mM glutaraldehyde, 25 U mL⁻¹ of enzyme, and 8 h immobilization time at 4 °C. The immobilized *BaL* retained about 90% of its initial activity after 10 days of storage. Moreover, about 70% of the initial activity of the immobilized *BaL* was retained after 10 cycles of application. The results of esterification studies exhibited that maximum pentyl valerate synthesis using the free *BaL* (34.5%) and the immobilized *BaL* (92.7%) occurred in the organic solvent medium (xylene) after 48 h of incubation at 60 °C.

Keywords *Bacillus atrophaeus* · Lipase immobilization · Graphene oxide · Specific activity · D-optimal design

✉ Mojtaba Shakibaie
shakiba@kmu.ac.ir

✉ Hamid Forootanfar
h_forootanfar@kmu.ac.ir

Introduction

Enzymes are biologically derived catalysts with excellent properties which speed up chemical reactions in industrial processes such as pharmaceutical and medical, biofuel, and food industries [1, 2]. These biological macromolecules represent high activity at mild conditions, high selectivity, and high specificity during the applied processes [3, 4]. The usage of lipases of microbial origins (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) as industrial biocatalysts has recently gained attention due to their potential application [5, 6] in numerous reactions such as ester synthesis, transesterification, interesterification, acidolysis, and aminolysis especially in organic solvents [6, 7]. Hence, lipolytic enzymes of microbial origin have been widely used in pharmaceuticals, detergent, cosmetics, food and flavor, and agrochemical industries for biocatalytic resolution of pharmaceuticals, synthesis of aromatic esters and amino acid derivatives, production of fine chemicals and biodiesel, development of biosensors, and bioremediation of agrochemical's wastes [7, 8].

High cost of enzymatic-assisted processes persuaded investigators to develop procedures by which the applied biocatalyst could separate from the reaction media and recycle again. Moreover, most of the applied enzymes might be unstable under the industrially used conditions such as presence of organic solvents, elevated temperatures, extreme pH, etc. [2, 3]. Immobilization of enzymes on nanocomposite structures not only allows reusing them for several times and as results reducing the final cost but also could increase catalytic activity, stability, and selectivity of the immobilized biocatalysts. In addition, decreasing protein contamination of the final product could also be attained via enzyme immobilization [9, 10]. Generally, there are five approaches to immobilize enzymes including adsorption, entrapment, ionic interface, covalent binding, and cross-linking [3, 11]. In recent years, the effort of enzyme immobilization technology has been modified by using nanostructure materials [3, 12–14] due to their high surface area which allow the improvement of enzyme immobilization efficiency [12, 15]. So, various nanomaterials (even synthetic or natural) have been exploited as carriers or supports to immobilize the enzymes including magnetic nanoparticles [16, 17], carbon nanotubes [18], silica-based nanomaterials, cellulose-based membrane, polymers, and resins [3, 16, 19]. Among the numerous types of nanomaterials, graphene oxide (GO) have mostly been used for enzyme immobilization [2, 20–22]. The general specifications of the GO containing the high surface area, two-dimensional structures, and the capability for surface functionalization permit the improvement of enzyme immobilization efficiency parameters [23, 24]. In addition, both methods of physical adsorption and covalent attachment of enzymes could be carried out for immobilization of enzyme on the GOs' surface [24]. For example, the serine proteases, horseradish peroxidase (HRP), cold-adapted esterase, lipase, and glucose oxidase have successfully been immobilized onto GO through various routes [25–31].

In the present study, the GO nanosheets were firstly prepared and subjected to functionalization using (3-aminopropyl) triethoxysilane. The as-synthesized nanostructure was then applied to immobilize the thermoalkalophilic lipase of *Bacillus atrophaeus* FSHM2, and the most important parameters influencing the immobilization yield was optimized using statistical experimental of design (DOE). The reusability, storage stability, and the influence of pH, temperature, solvent, and metal ions on the immobilized lipase activity were also investigated. Finally, the free and immobilized lipases were employed for synthesis of pentyl valerate (green apple flavor) in various solvents.

Materials and Methods

Chemicals

ρ -Nitrophenol (ρ -NP), ρ -nitrophenyl palmitate (ρ -NPP), glutaraldehyde 25% (v/v), (3-aminopropyl) triethoxysilane (APTES), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial graphite flakes were supplied by Sigma-Aldrich (St. Louis, MO, USA) with a particle size of 325 meshes and purity of 99%. Pure pentyl valerate was provided by Merck Chemicals (Darmstadt, Germany). In the case of other applied materials, the analytical grade was supplied.

Enzyme

The applied lipase of the present study was a thermoalkalophilic lipolytic enzyme from *Bacillus atrophaeus* FSHM2 (designated as *BaL*) which has been purified and characterized in a previous survey [32]. In brief, the purified *BaL* was obtained using three sequential steps of ethanol precipitation and dialysis, Q-Sepharose XL anion-exchange chromatography, and SP Sepharose cation-exchange chromatography by a purification factor of 31.8. The purified *BaL* (Mw, 85 kDa) exhibited its maximum activity at 70 °C and pH 9.00 in the existence of 4 M NaCl. It was also observed that the activity of *BaL* was considerably increased when incubated in the presence of organic solvents, especially xylene (25%).

Synthesis of GO

Synthesis of GO was performed according to the modified Hummer's method [33]. Briefly, graphite flakes (1 g) were firstly treated by $K_2S_2O_8$ (0.5 g) and P_2O_5 (0.5 g) in concentrated H_2SO_4 (23 mL) for 6 h. Thereafter, the oxidation of graphite oxide was carried out by adding $KMnO_4$ (3 g) to the reaction mixture and the reaction was kept at a temperature below 20 °C. Then, the mixture was incessantly stirred for 120 min at 35 °C, followed by addition of deionized water (46 mL) into the reaction mixture. Afterwards, the obtained mixture was redispersed in HCl solution (10%) to remove ions and frequently centrifuged and redispersed again in deionized water to reach the neutral pH. The obtained graphite oxide was exfoliated into GO by dispersing in water and sonicating for 90 min under ultrasound (300 W) followed by drying it in a vacuum oven (60 °C, 48 h).

Silanization of the GO Nanosheets

Synthesis of amine-modified GO (GO-NH₂) was performed based on the technique described by Akbari et al. [34]. To functionalize the GO surface via APTES, 250 mg of as-synthesized GO was dispersed in toluene while sonicating for about 15 min and subsequently, 2 mL of APTES (99%) was added to the reaction mixture and allowed to stir under reflux conditions at 110 °C for 24 h. The produced sediment was finally washed three times with toluene and dried using a vacuum oven (60 °C for 24 h).

Characterization

In order to achieve the related Fourier transform infrared (FTIR) spectrum of GO and GO-NH₂, they were firstly dried in an oven at 60 °C followed by preparation of their KBr tablet and analysis using a Bruker Alpha instrument (Bruker Optics, MA, USA) at a resolution of 4 cm⁻¹. The related pattern of thermogravimetric analysis (TGA) of GO and GO-NH₂ were drawn using the STA 503 TG analyzer (YEONJIN Co., Seoul, South Korea, resolution; $\Delta G = 0.5 \mu\text{g}$, $T = 0.05 \text{ }^{\circ}\text{C}$, $\Delta T = 0.01 \text{ }^{\circ}\text{C}$) assisting a heating gradient from 25 to 800 °C (10 °C min⁻¹) under argon flow. The surface morphology of the samples was scanned by field emission scanning electron microscope (FESEM, ZEISS-SIGMA VP, Germany) equipped with energy-dispersive spectroscopy (EDS, X-max, Oxford, England) apparatus.

Immobilization of *BaL* and Statistical Experimental Design

An experimental design, fractional factorial design (FFD), was employed for optimization of factors influencing *BaL* immobilization using a covalent binding method. Statistical software, Design-Expert version 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA), was utilized for planning a 2⁸⁻² FFD with 3 center points. The selected factors include GO-NH₂ concentration (X_1), buffer concentration (X_2), pH (X_3), sonication time (X_4), enzyme concentration (X_5), glutaraldehyde concentration (X_6), time (X_7), and temperature (X_8) (Table 1). Sixty-seven runs were designed by the mentioned software (Table 2, run 1–67) for optimization of the immobilization parameters of *BaL* on amine-functionalized GO. The designed runs of FFD were carried out and analyzed. A significant curvature was found in the analyzed results; so the previously mentioned 2⁸⁻² FFD was augmented to a D-optimal design of response surface methodology (RSM) by adding five new experiments (Table 2, run 68–72) to the previous design. In order to increase the leverage, the applied design was improved by addition of two further experiments (Table 2, run 73 and 74). The actual and predicted data in terms of specific activity (U mg⁻¹) are shown in Table 2. The second-order polynomial equation which contains all interactions was exploited to estimate the predicted response Eq. 1:

$$Y = b_0 + \sum_{i=1}^n b_i x_i + \sum_{i=1}^n b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n b_{ij} x_i x_j \quad (1)$$

In this equation, Y is the response (specific activity, U mg⁻¹), x_i ($i = 1-8$) are the variables of the experiment, b_0 is the model constant, b_i ($i = 1-8$) are the linear effects, b_{ij} ($i \neq j$) are the second-order coefficients, b_{ii} ($i = 1-7$) are the second-order interaction coefficients, e is the

Table 1 Applied levels of independent variables in the fractional factorial design for optimization of *BaL* immobilization conditions on GO-NH₂ support

Variable	Name	Unit	Low level (− 1)	High level (+ 1)
X_1	GO-NH ₂ concentration	mg mL ⁻¹	1	5
X_2	Buffer concentration	mM	25	50
X_3	pH		6	8
X_4	Sonication time	min	15	60
X_5	Enzyme concentration	U mL ⁻¹	25	60
X_6	Glutaraldehyde concentration	mM	10	100
X_7	Time	h	4	8
X_8	Temperature	°C	4	25

Table 2 The fractional factorial design (FFD) augmented to D-optimal design for optimization of factors (with coded values) affected on specific activity of *BaL*

Run order	Block	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Specific activity (U mg ⁻¹)	
										Experimental	Predicted
1	Block 1	-1	-1	-1	-1	-1	-1	1	1	57.36	56.24
2	Block 1	1	-1	-1	-1	-1	-1	-1	-1	65.56	64.4
3	Block 1	-1	1	-1	-1	-1	-1	-1	-1	62.36	61.28
4	Block 1	1	1	-1	-1	-1	-1	1	1	62.37	61.28
5	Block 1	-1	-1	1	-1	-1	-1	-1	1	62.59	61.6
6	Block 1	1	-1	1	-1	-1	-1	1	-1	65.17	64.08
7	Block 1	-1	1	1	-1	-1	-1	1	-1	63.09	62
8	Block 1	1	1	1	-1	-1	-1	-1	1	63.16	62.32
9	Block 1	-1	-1	-1	1	-1	-1	-1	1	63.16	62
10	Block 1	1	-1	-1	1	-1	-1	1	-1	63.56	62.64
11	Block 1	-1	1	-1	1	-1	-1	1	-1	65.32	64.32
12	Block 1	1	1	-1	1	-1	-1	-1	1	63.87	62.8
13	Block 1	-1	-1	1	1	-1	-1	1	1	65.17	64.08
14	Block 1	1	-1	1	1	-1	-1	-1	-1	66.79	65.68
15	Block 1	-1	1	1	1	-1	-1	-1	-1	65.17	64.16
16	Block 1	1	1	1	1	-1	-1	1	1	65.94	64.88
17	Block 1	-1	-1	-1	-1	1	-1	1	-1	65.17	64.24
18	Block 1	1	-1	-1	-1	1	-1	-1	1	64.79	63.76
19	Block 1	-1	1	-1	-1	1	-1	-1	1	64.12	63.04
20	Block 1	1	1	-1	-1	1	-1	1	-1	65.98	65.12
21	Block 1	-1	-1	1	-1	1	-1	-1	-1	66.38	65.28
22	Block 1	1	-1	1	-1	1	-1	1	1	67.18	66
23	Block 1	-1	1	1	-1	1	-1	1	1	65.96	64.88
24	Block 1	1	1	1	-1	1	-1	-1	-1	67.18	66.08
25	Block 1	-1	-1	-1	1	1	-1	-1	-1	67.59	66.56
26	Block 1	1	-1	-1	1	1	-1	1	1	67.18	66.08
27	Block 1	-1	1	-1	1	1	-1	1	1	57.91	56.88
28	Block 1	1	1	-1	1	1	-1	-1	-1	68.39	67.28
29	Block 1	-1	-1	1	1	1	-1	1	-1	63.66	62.48
30	Block 1	1	-1	1	1	1	-1	-1	1	68.13	67.2
31	Block 1	-1	1	1	1	1	-1	-1	1	62.36	61.36
32	Block 1	1	1	1	1	1	-1	1	-1	67.61	66.48
33	Block 1	-1	-1	-1	-1	-1	1	1	-1	67.86	66.96
34	Block 1	1	-1	-1	-1	-1	1	-1	1	68.16	67.04
35	Block 1	-1	1	-1	-1	-1	1	-1	1	63.56	62.48
36	Block 1	1	1	-1	-1	-1	1	1	-1	63.97	62.96
37	Block 1	-1	-1	1	-1	-1	1	-1	-1	65.58	64.48
38	Block 1	1	-1	1	-1	-1	1	1	1	63.97	62.96
39	Block 1	-1	1	1	-1	-1	1	1	1	64.81	63.68
40	Block 1	1	1	1	-1	-1	1	-1	-1	63.97	62.96
41	Block 1	-1	-1	-1	1	-1	1	-1	-1	56.82	55.68
42	Block 1	1	-1	-1	1	-1	1	1	1	68.83	67.84
43	Block 1	-1	1	-1	1	-1	1	1	1	65.36	64.24
44	Block 1	1	1	-1	1	-1	1	-1	-1	62.36	61.28
45	Block 1	-1	-1	1	1	-1	1	1	-1	64.77	63.76
46	Block 1	1	-1	1	1	-1	1	-1	1	65.17	64.24
47	Block 1	-1	1	1	1	-1	1	-1	1	64.49	63.6
48	Block 1	1	1	1	1	-1	1	1	-1	67.18	66.08
49	Block 1	-1	-1	-1	-1	1	1	1	1	65.17	64.08
50	Block 1	1	-1	-1	-1	1	1	-1	-1	67.39	66.32
51	Block 1	-1	1	-1	-1	1	1	-1	-1	65.56	64.4
52	Block 1	1	1	-1	-1	1	1	1	1	64.77	63.76
53	Block 1	-1	-1	1	-1	1	1	-1	1	65.17	64.24

Table 2 (continued)

Run order	Block	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Specific activity (U mg ⁻¹)	
										Experimental	Predicted
54	Block 1	1	-1	1	-1	1	1	1	-1	65.17	64
55	Block 1	-1	1	1	-1	1	1	1	-1	66.82	65.68
56	Block 1	1	1	1	-1	1	1	-1	1	66.78	65.76
57	Block 1	-1	-1	-1	1	1	1	-1	1	66.77	65.76
58	Block 1	1	-1	-1	1	1	1	1	-1	66.55	65.6
59	Block 1	-1	1	-1	1	1	1	1	-1	65.66	64.8
60	Block 1	1	1	-1	1	1	1	-1	1	66.78	65.68
61	Block 1	-1	-1	1	1	1	1	1	1	67.18	66.08
62	Block 1	1	-1	1	1	1	1	-1	-1	71.61	70.56
63	Block 1	-1	1	1	1	1	1	-1	-1	68.39	67.2
64	Block 1	1	1	1	1	1	1	1	1	68.31	67.2
65	Block 1	0	0	0	0	0	0	0	0	73.73	72.56
66	Block 1	0	0	0	0	0	0	0	0	73.42	72.56
67	Block 1	0	0	0	0	0	0	0	0	73.75	72.56
68	Block 2	-1	1	-1	-1	1	-1	-1	1	60.44	63.04
69	Block 2	-1	1	-1	-1	-1	1	-1	-1	65.17	67.68
70	Block 2	-1	-1	-1	1	1	1	1	-1	65.58	68.16
71	Block 2	1	1	-1	1	-1	1	1	1	66.78	69.36
72	Block 2	-1	-1	-1	1	-1	-1	1	1	64.77	67.36
73	Block 3	1	-1	1	-1	-1	-1	1	-1	65.58	64.08
74	Block 3	1	-1	1	-1	1	1	1	1	66.81	65.28

error of the experiment, and x are the independent variables. The specific activity was also calculated according to Eq. 2:

$$\text{Specific activity} = \text{enzyme activity (U)} / \text{total amount of enzyme (mg)} \quad (2)$$

Lipase solutions with different activities (X_5 , U mL⁻¹; -1, 25; +1, 60) were prepared in phosphate-buffered solution (X_2 , mM; -1, 25; +1, 50) with various pH values (X_3 ; -1, 6; +1, 8). Next, the support (X_1 , mg mL⁻¹; -1, 1; +1, 5) containing glutaraldehyde (X_6 , mM; -1, 10; +1, 100) was sonicated (X_4 , min; -1, 15; +1, 60) and suspended into enzyme solutions and the prepared mixture was stirred for various immobilization times (X_7 , h; -1, 4; +1, 8) at different temperatures (X_8 , °C; -1, 4; +1, 25) to complete the covalent attachment. After that, the suspension was centrifuged and separated; the immobilized *BaL* was washed three times with phosphate buffer to remove the unbounded enzyme and dried. The centrifuge and washing solutions were collected for protein determination and measurement of lipase activity.

Measurement of Lipase Activity

The colorimetric assay protocol based on the ability of lipase to hydrolyze the esteric bond of ρ -NPP as substrate was applied [35] in order to evaluate the lipase activity (U mL⁻¹). The enzymatic reaction mixture contained 0.8 mL of 0.1 M carbonate buffer (pH 9.00) accompanied by 0.4% of Triton X-100, 0.1 mL of the freshly prepared ρ -NPP solution (0.01 M), and 10 mg of sample was prepared and incubated at 60 °C and 120 rpm for 30 min. Afterward, 0.25 mL of 0.1 M Na₂CO₃ was added to the reaction mixture and centrifuged at 10,000×*g* for 15 min. The amount of released ρ -NP which represented a yellow color was determined by recording the absorbance of the samples at 410 nm using a Shimadzu double beam

spectrophotometer (UV-1800, Shimadzu Corporation, USA). One unit (U) of the lipase activity was defined as the amount of enzyme caused the liberation of 1 μmol of p -NP per min from p -NPP substrate under the standard assay conditions.

Protein Assay

The total amounts of protein were determined using the Bradford's colorimetric method [36]. Bovine serum albumin (BSA) was employed as a standard to draw the calibration curve.

Immobilization Yield and Efficiency

The yield and efficiency of immobilization were demonstrated by the following equations:

$$\text{Immobilization yield (\%)} = [(Y_0 - Y_1) / Y_0] \times 100 \quad (3)$$

where Y_0 and Y_1 show the amounts of total protein and bounded protein to the support during the immobilization process, respectively.

$$\text{Immobilization efficiency (\%)} = [(E_0 - E_1) / E_0] \times 100 \quad (4)$$

where E_0 and E_1 were the total activity and the immobilized enzyme activity, respectively.

pH and Temperature Stability of the Immobilized *BaL*

The pH stability of the free and immobilized *BaL* was assessed by pre-incubating the enzyme for 120 min in 100 mM buffer solutions of citrate buffer (pH 4–5), phosphate buffer (pH 6–8), and carbonate buffer (pH 9–11), and the percentage of residual enzyme activities was determined using substrate (p -NPP) under the previously mentioned assay conditions (Sect. [Measurement of Lipase Activity](#)). To evaluate the influence of temperature on the stability of the free and immobilized *BaL*, the enzyme were pre-incubated at temperatures of 50, 60, 70, 80, and 90 °C for 120 min and the residual lipase activity was then measured.

Stability of the Immobilized *BaL* in the Presence of Metal Ions, Chelating Agent, and Surfactants

The effects of different metal ions (Mg^{2+} , K^+ , Ca^{2+} , Cu^{2+} , Na^+ , Mn^{2+} , Zn^{2+} , and Fe^{3+} all at final concentration of 5 mM), enzyme inhibitor (EDTA, 2.5 mM), as well as surfactants (SDS, Triton X-100, Tween-40, and Tween-80, 1 mM) on the activity of the free and immobilized *BaL* were determined by pre-incubating the enzyme with each of them for 120 min followed by determination of the residual lipase activity under standard assay conditions.

Stability of Immobilized *BaL* in Organic Solvents

The free and immobilized *BaL* was incubated in different organic solvents including ethanol, 2-propanol, chloroform, iso amyl alcohol, *n*-hexane, acetonitrile, methanol, acetone, 1-butanol, xylene, and toluene at a final concentration of 25% (w/v) at 25 °C for 120 min. The residual

activity was evaluated based on the similar procedure as described above (Sect. [Measurement of Lipase Activity](#)).

Reusability and Storage Stability of the Immobilized *BaL*

In order to survey the reusability of the immobilized *BaL*, the used biocatalyst of the first run was separated by centrifugation ($12,000\times g$ for 5 min at 4 °C) and washed three times with phosphate buffer (100 mM, pH 7.2) and the enzyme activity was determined by the above-mentioned assay (Sect. [Measurement of Lipase Activity](#)). Considering the lipase activity of the first run as 100%, the relative activity of the subsequent runs (ten runs) were measured based on the first run. The reusability of the immobilized *BaL* was measured in triplicates. The storage stability was assessed by incubating the immobilized enzyme for 60 days at 25 °C followed by calculation of the residual activity of the samples.

Synthesis of Pentyl Valerate by Free and Immobilized *BaL*

The immobilized *BaL* on the GO-NH₂ prepared at the optimum conditions of immobilization and free *BaL* were used as biocatalysts for the esterification reaction in the both solvent-free and organic solvent media based on the protocol described by Khoobi et al. [37]. In the case of aqueous media, the reaction mixture was prepared by addition of equimolar amount (50 mM) of valeric acid and pentanol into 25-mL glass-stoppered flasks followed by addition of free (10 U mL⁻¹, in carbonate buffer 100 mM, pH 9) or immobilized *BaL* (5 mg) to it and incubation at 60 °C under mild shaking. For organic solvent media, the esterification procedure was performed in *n*-hexane or xylene by addition of a similar amount of valeric acid and pentanol (50 mM) to the reaction and insertion of free or immobilized *BaL* and incubation at the similar conditions as said above. In both kinds of esterification procedure, molecular sieve was added after 2 h of reaction initiation to absorb excess amount of water. Interval samples (1, 2, 4, 6, 12, 24, 36, and 48 h) from each experiment was then taken and analyzed for amount of ester formation by a gas chromatography apparatus (Agilent, 7890A) connected to a flame ionization detector (FID, Agilent). The applied capillary column was an HP Innowax with length of 30 m, inner diameter of 0.32 μm, and thickness of 0.5 μm. The nitrogen gas was used as carrier at flow rate of 90 mL min⁻¹ while the temperature of detector and injector were adjusted to 280 and 250 °C, respectively. The temperature of the oven was programmed as follows: (i) 100–150 °C with the rate of 10 °C min⁻¹, (ii) 150–180 °C at the rate of 1 °C min⁻¹, and (iii) 180–280 °C with the rate of 3 °C min⁻¹. The peak area and retention time of the sample were identified and calculated via comparison by pure pentyl valerate as standard.

Data Analysis

Analysis of the results was performed using the statistical software package, Design Expert version 6.0.4 (Stat-Ease Inc., Minneapolis, MN). The qualities of the reduced cubic models were examined by the *R*-squared value. The lipase activity was evaluated by analysis of

variance (ANOVA) and F test ($p \leq 0.05$) to establish the optimum conditions for a specific activity (U mg^{-1}).

Results and Discussion

Characterization of GO and GO-NH₂ Nanosheets

The characterization of GO and GO-NH₂ nanosheets were assessed using FTIR, AFM, TGA, and SEM. Figure 1a shows the FTIR spectra for GO and GO-NH₂. The peak shown at 3117 cm^{-1} in the FTIR spectrum of GO could be attributed to the hydroxyl groups. The absorption peak of GO at 1733 cm^{-1} is associated to the carboxylic groups. Moreover, the absorption peak at around 1049 cm^{-1} corresponds to the epoxide group. GO nanosheets were silanized and reacted with the amine group of the APTES, and the peak that newly emerged at 2926 cm^{-1} can be allotted to the symmetric vibration of the $-\text{CH}_2-$ groups of the alkyl chains of the APTES while the peak at 2860 cm^{-1} were contributions from the asymmetric vibrational stretching (Fig. 1a). The absorption band at around $1500\text{--}1650 \text{ cm}^{-1}$ could be associated to the C–N stretching and N–H bending of the amide functional group. The presence of the new absorption peaks at around 1109 and 1037 cm^{-1} can be assigned to the Si–O–C groups, which offer suggestion of effective chemical functionalization (Fig. 1a). The disappearing peak at around 1049 cm^{-1} , confirming to the epoxide group in the GO, indicated silanization reaction between the epoxide groups of the GO and the amine group of the APTES (Fig. 1a). The spectra of the GO and GO-NH₂ were similar to results of previous works [24, 25, 38]. The C=O stretching vibration of carbonyl and carboxyl groups located at the edges of the GO nanosheets, C=C, and C–OH vibrations, as well as C–O stretching vibration of epoxide group were observed in the FTIR spectrum of GO [25]. Tian et al. [24] reported that when the amine group of the APTES reacted with GO, the peaks at 2933 and 2860 cm^{-1} were created and assigned to the symmetric and asymmetric vibrations of the $-\text{CH}_2-$ groups of the alkyl chains of the APTES, respectively.

The thermal properties of the GO and GO-NH₂ were studied using a TGA instrument. The TGA curves of GO nanoplates and GO-NH₂ nanosheets under nitrogen atmosphere are shown in Fig. 1b. The GO is unstable at high temperatures. Heating below 150°C , the GO samples lose weight due to the release of water. The major weight loss followed below 300°C , and the main reason was the slow destruction of oxygen-containing functional groups such as hydroxyl and epoxy. Though, the weight loss of GO-NH₂ nanosheets below 300°C was greatly lower (less than 20%) caused by the reaction of the oxygen-containing groups with APTES. The main weight loss of the GO-NH₂ nanosheets observed in the $300\text{--}600^\circ\text{C}$ temperature range is possibly ascribed to the decomposition of the silane chains attached to the GO nanosheets. Similar results were achieved by Tian et al. [24] showing the silanization of the graphene oxide nanoplates using 3-aminopropyltriethoxysilane to obtain functional graphene nanoplates. They found that the functional graphene nanoplates showed better thermal stability than the raw GO nanosheets (based on the TGA curves).

The prepared SEM of nanostructures (Fig. 2a, b) exhibited the typical sheet-like structure of GO and GO-NH₂. The GO nanosheets were smooth with a single layer sheet (Fig. 2a). GO-NH₂ nanosheets were wrinkled structures that provide high

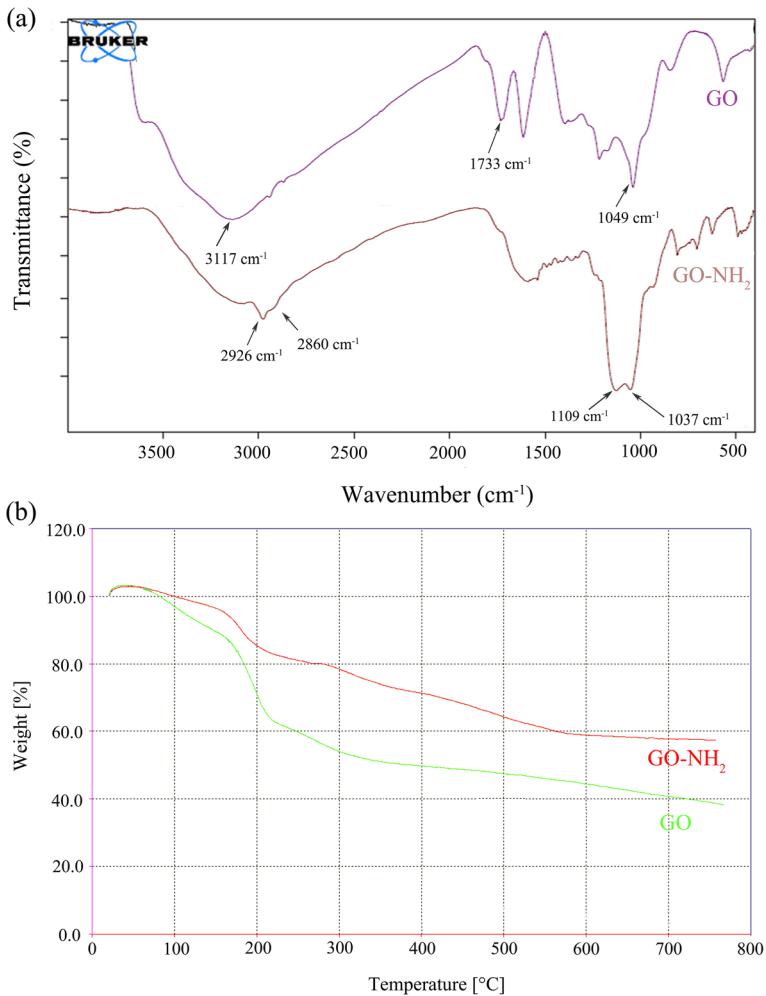


Fig. 1 **a** The FTIR spectra of graphene oxide (GO) and amine-functionalized graphene oxide (GO-NH₂) nanosheets. **b** TGA profiles of graphene oxide (GO) and amine-functionalized graphene oxide (GO-NH₂) under nitrogen atmosphere

specific surface for efficient enzyme immobilization (Fig. 2b). AFM measurements were used to measure the morphology and thickness of GO and GO-NH₂ nanosheets. The GO nanosheets displayed an even more flat morphology and were only a few nanometers thick (Fig. 2c, d).

D-Optimal Model for Immobilization of *BaL* on GO-NH₂

The specific activities of immobilized *BaL* on GO-NH₂ obtained from experimental runs are shown in Table 2, from which the actual and predicted responses of models were represented. Experimental data results were subjected to ANOVA to determine their significant parameters (Table 3). The ANOVA checks the adequacy of the model, and the *F* value analysis was used to assess it. The ANOVA showed that immobilization of lipase was highly significant and

sufficient. The Model F value of 221.79 implied that the model was significant. The reduced cubic model showed a statistical significance at 95% confidence interval. There was only a 0.01% chance that a “model F value” this large could occur due to the noise. A p value < 0.0001 (< 0.05) indicated that the model was statistically significant and confirmed that there was no lack of fitness. The coefficient of determination (R^2) of the model for the response (specific activity) was 0.9992. The obtained high R^2 values confirmed the fitness of the proposed models. Moreover, the high values of the determination coefficients indicated that the models explained 99.92% of the variability data, and the regression was statistically significant (p value < 0.0001) at 95% confidence level. Adjusted and predicted R -squares should be within approximately 0.20 of each other. The “predicted R -squares” and “adjusted R -squares” were 0.9576 and 0.9947, respectively. These amounts are indicated with a reasonable agreement. The signal-to-noise ratio is assessed by adequate precision where a ratio greater than 4 is desirable for models. Our ratio was 83.39, indicating an adequate signal. This model can be used to navigate the design space for response. From the Design Expert, the reduced cubic Eq. (2) is given as below:

$$\begin{aligned} \text{Specific activity (U mg}^{-1}\text{)} = & +72.58 + 0.82X_1 - 0.25X_2 + 0.54X_3 + 0.33X_4 + 0.94X_5 \\ & + 0.54X_6 - 0.080X_7 - 0.35X_8 - 0.27X_1X_2 - 0.14X_1X_3 + 0.38X_1X_4 \\ & + 0.11X_1X_5 - 0.29X_1X_6 - 0.12X_1X_7 + 0.25X_1X_8 \\ & + 0.17X_2X_3 - 0.14X_2X_5 + 0.16X_2X_7 - 0.23X_2X_8 \\ & + 0.27X_3X_4 - 0.11X_3X_6 - 0.091X_4X_6 + 4.11X_4X_7 \\ & + 0.19X_4X_8 - 0.85X_5X_6 - 0.45X_5X_7 + 1.89X_5X_8 \\ & + 0.33X_6X_7 - 0.55X_6X_8 - 8.40X_1^2 - 3.95X_1X_2X_3 \\ & + 1.07X_1X_2X_5 - 2.19X_1X_2X_6 + 0.88X_1X_2X_8 \\ & + 0.21X_1X_3X_5 - 0.079X_1X_3X_6 - 0.25X_1X_3X_8 \\ & + 0.25X_1X_4X_5 + 0.17X_1X_4X_6 - 0.089X_1X_4X_8 \\ & + 0.13X_1X_5X_7 - 0.34X_1X_6X_7 + 0.28X_1X_7X_8 + 0.15X_2X_3X_5 \\ & + 0.19X_2X_3X_6 + 0.14X_2X_3X_8 - 0.44X_2X_4X_5 \\ & + 0.28X_2X_4X_6 - 0.56X_2X_4X_8 - 0.094X_2X_6X_7 - 0.090X_2X_7X_8 - 0.18X_3X_4X_5 \\ & + 0.43X_3X_4X_6 - 0.34X_3X_4X_8 + 0.22X_3X_5X_6 + 0.23X_3X_5X_7 \\ & + 0.32X_3X_5X_8 - 0.48X_3X_6X_7 - 0.62X_3X_6X_8 + 0.67X_3X_7X_8 \end{aligned} \quad (5)$$

where $X_1, X_2, X_3, X_4, X_5, X_6, X_7$, and X_8 represent the GO-NH₂ concentration, buffer concentration, pH, sonication time, enzyme concentration, glutaraldehyde concentration, time, and temperature, respectively. The reduced cubic model was highly significant and considerable. The relationship between predicted and experimental response obtained from the final model is illustrated in Fig. 3a. Figure 3b presents the plot of internally studentized residuals vs. the predicted response. Based on Fig. 3, a good agreement was found between the predicted and actual values.

The interaction between the factors and response was investigated by 3D surface plots (Fig. 4). Figure 4a shows the effect of GO-NH₂ concentrations, pH, and their interaction on specific activity (U mg⁻¹) of immobilized *BaL*. GO-NH₂ concentration of 3 mg mL⁻¹ and pH of 8.00 led to a maximum specific activity (73.12 U mg⁻¹). Specific activity of the immobilized *BaL* was low (65.44 U mg⁻¹) in high levels of GO-NH₂ concentration (5 mg mL⁻¹) and pH (8.00). Dutta and Saha [20] showed that the optimum concentration of

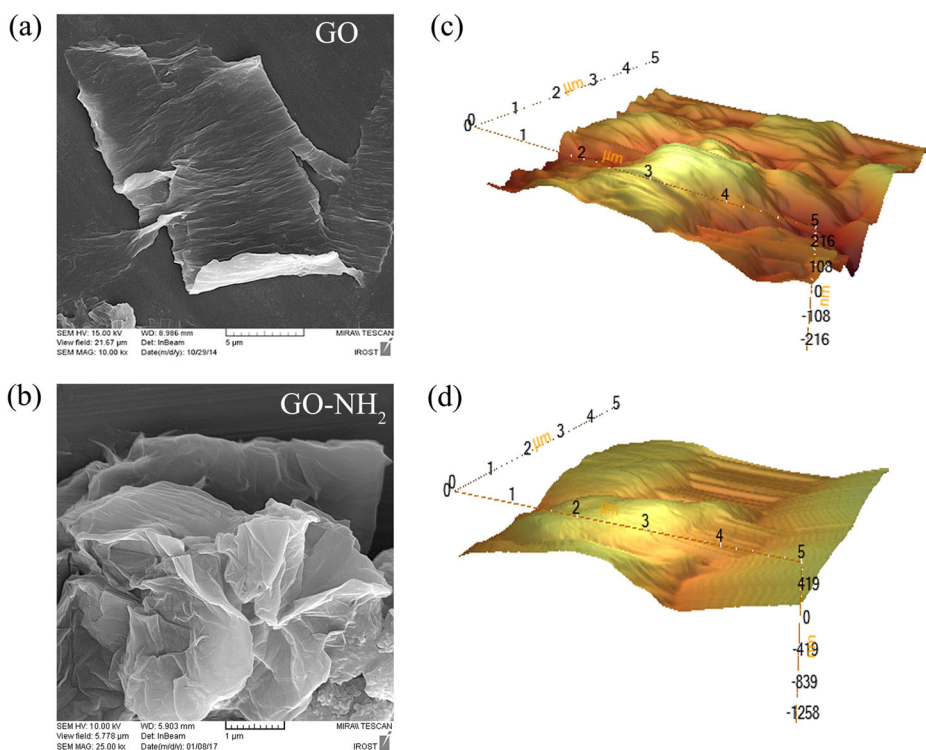


Fig. 2 **a, b** The morphology of graphene oxide (GO) and amine-functionalized graphene oxide (GO-NH₂) obtained by SEM. **c, d** AFM images of graphene oxide and amine-functionalized graphene oxide

graphene oxide for lipase immobilization was 1.2 mg mL⁻¹. They reported that enzyme activity (U mL⁻¹) in this concentration of graphene oxide was 80.9 ± 1.88 U mL⁻¹.

The effect of GO-NH₂ concentration (mg mL⁻¹), varying enzyme concentration (U mL⁻¹), and their interaction on the specific activity of immobilized *BaL* are illustrated in Fig. 4b from which it was obvious that high enzyme concentration (60 U mL⁻¹) increased the specific activity (73.52 U mg⁻¹), while GO-NH₂ concentration was at the center point (3 mg mL⁻¹). Decreasing enzyme concentration (25 U mL⁻¹) in the reaction mixture resulted to decline the specific activity (71.68 U mg⁻¹) of the immobilized *BaL*. The enzyme/support ratio has been reported as one of the most important parameters in the immobilization of enzyme reaction system. For example, Amirkhani et al. [39] who optimized the immobilization parameters of *Candida rugosa* lipase on magnetic silica aerogel, reported that the enzyme-specific activity increased from 14.1 to 15.4 U mg⁻¹ when the enzyme/support ratio was enhanced from 0.3 to 0.5 (w/w), at a constant level of other factors. In another study conducted by Aybaster and Demir [40], the maximum specific activity (7.47 μ mol *p*-NP mg⁻¹ enzyme min) of the immobilized lipase of *Thermomyces lanuginosus* on styrene-divinylbenzene copolymer was observed at an enzyme concentration of 4% (v/v) and pH of 7.5. Asmat et al. [23] applied various ratios of enzyme/nanosupport for immobilization of lipase on facile-synthesized polyaniline-coated silver-functionalized graphene oxide nanocomposites. They reported that optimum residual activity occurred at a ratio of 1:10 of enzyme/nanosupport. Dutta and Saha [20] also showed that the immobilization yield improved by increasing graphene oxide/enzyme (w/w) ratio.

Table 3 Analysis of variance for the fitted quadratic polynomial model for optimization of immobilization parameters

Source	Sum of squares	df	Mean square	F value	p value (prob > F)
Block	6.11	2	3.06		
Model	641.71	60	10.7	221.79	< 0.0001*
X_1	43.25	1	43.25	896.85	< 0.0001
X_2	4.04	1	4.04	83.74	< 0.0001
X_3	18.76	1	18.76	389.07	< 0.0001
X_4	6.84	1	6.84	141.77	< 0.0001
X_5	56.87	1	56.87	1179.4	< 0.0001
X_6	19.89	1	19.89	412.49	< 0.0001
X_7	0.41	1	0.41	8.52	0.014
X_8	7.66	1	7.66	158.76	< 0.0001
X_1X_2	4.59	1	4.59	95.18	< 0.0001
X_1X_3	1.28	1	1.28	26.62	0.0003
X_1X_4	9.41	1	9.41	195.12	< 0.0001
X_1X_5	0.84	1	0.84	17.46	0.0015
X_1X_6	5.37	1	5.37	111.34	< 0.0001
X_1X_7	0.89	1	0.89	18.44	0.0013
X_1X_8	4.25	1	4.25	88.2	< 0.0001
X_2X_3	1.93	1	1.93	39.94	< 0.0001
X_2X_5	1.35	1	1.35	27.98	0.0003
X_2X_7	1.65	1	1.65	34.26	0.0001
X_2X_8	3.44	1	3.44	71.38	< 0.0001
X_3X_4	4.51	1	4.51	93.54	< 0.0001
X_3X_6	0.79	1	0.79	16.38	0.0019
X_4X_6	0.53	1	0.53	11.09	0.0067
X_4X_7	18.05	1	18.05	374.2	< 0.0001
X_4X_8	2.42	1	2.42	50.23	< 0.0001
X_5X_6	3.63	1	3.63	75.28	< 0.0001
X_5X_7	13.46	1	13.46	279.11	< 0.0001
X_5X_8	10.52	1	10.52	218.1	< 0.0001
X_6X_7	6.87	1	6.87	142.45	< 0.0001
X_6X_8	1.51	1	1.51	31.3	0.0002
X_1^2	202.06	1	202.06	4190.21	< 0.0001
$X_1X_2X_3$	17	1	17	352.59	< 0.0001
$X_1X_2X_5$	6.02	1	6.02	124.76	< 0.0001
$X_1X_2X_6$	14.75	1	14.75	305.91	< 0.0001
$X_1X_2X_7$	4.1	1	4.1	85	< 0.0001
$X_1X_3X_5$	2.85	1	2.85	59.09	< 0.0001
$X_1X_3X_6$	0.41	1	0.41	8.56	0.0138
$X_1X_3X_8$	4	1	4	82.88	< 0.0001
$X_1X_4X_5$	4.04	1	4.04	83.85	< 0.0001
$X_1X_4X_6$	1.87	1	1.87	38.75	< 0.0001
$X_1X_6X_8$	0.51	1	0.51	10.51	0.0078
$X_1X_5X_7$	1.14	1	1.14	23.66	0.0005
$X_1X_6X_7$	7.54	1	7.54	156.35	< 0.0001
$X_1X_7X_8$	4.93	1	4.93	102.15	< 0.0001
$X_2X_3X_5$	1.48	1	1.48	30.77	0.0002
$X_2X_3X_6$	2.33	1	2.33	48.21	< 0.0001
$X_2X_3X_8$	1.19	1	1.19	24.65	0.0004
$X_2X_4X_5$	12.57	1	12.57	260.7	< 0.0001
$X_2X_4X_6$	4.91	1	4.91	101.88	< 0.0001
$X_2X_4X_8$	20.9	1	20.9	433.37	< 0.0001
$X_2X_6X_7$	0.58	1	0.58	11.97	0.0053
$X_2X_7X_8$	0.53	1	0.53	10.95	0.007
$X_3X_4X_5$	2.19	1	2.19	45.42	< 0.0001
$X_3X_4X_6$	11.76	1	11.76	243.85	< 0.0001

Table 3 (continued)

Source	Sum of squares	df	Mean square	F value	p value (prob > F)
$X_3X_4X_8$	7.49	1	7.49	155.4	< 0.0001
$X_3X_5X_6$	3.07	1	3.07	63.76	< 0.0001
$X_3X_5X_7$	3.28	1	3.28	67.98	< 0.0001
$X_3X_5X_8$	6.46	1	6.46	134.03	< 0.0001
$X_3X_6X_7$	14.72	1	14.72	305.31	< 0.0001
$X_3X_6X_8$	24.46	1	24.46	507.27	< 0.0001
$X_3X_7X_8$	28.5	1	28.5	590.93	< 0.0001
Residual	0.53	11	0.048		
Lack of fit	0.46	9	0.051	1.51	0.4602**
Pure error	0.068	2	0.034		
Cor total	648.35	73			

*Significant; **not significant

Figure 4c represents the effects of varying pH and glutaraldehyde concentrations on specific activity of the immobilized *BaL*. An increment in specific activity (73.52 U mg^{-1})

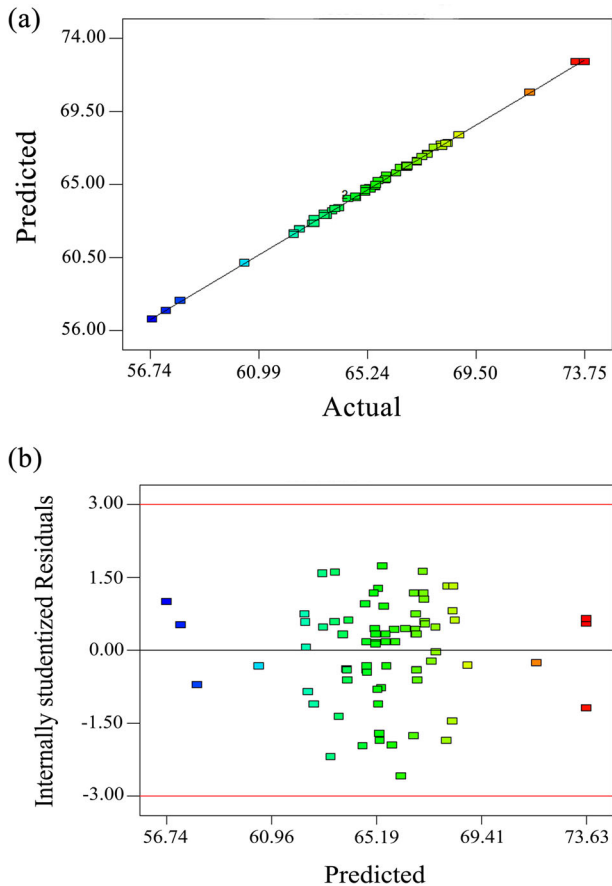


Fig. 3 **a** Predicted specific activity of immobilized lipase vs. experimental-specific activity. **b** Studentized residuals vs. predicted response by final reduced cubic model

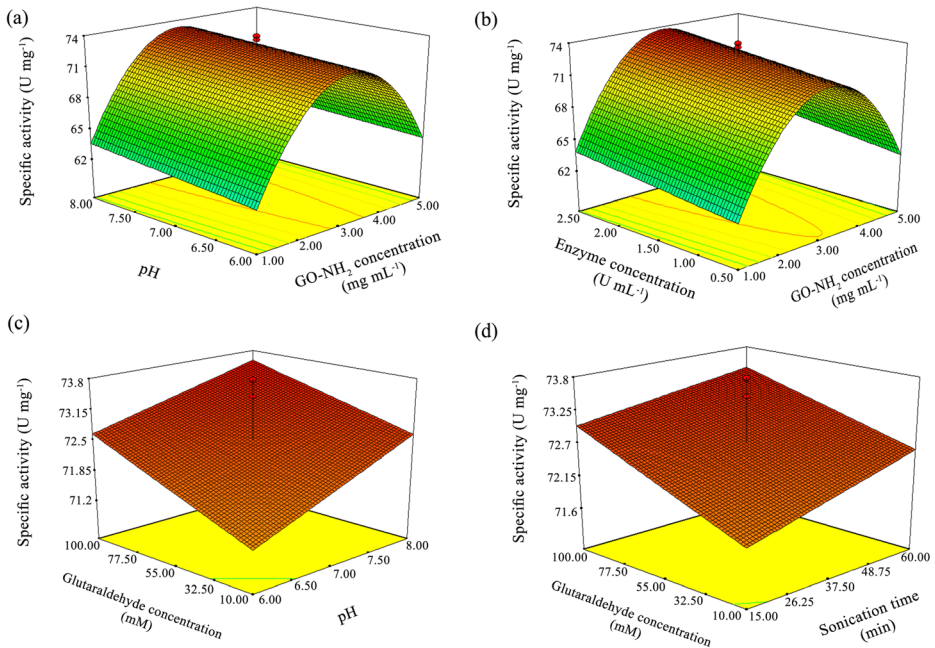


Fig. 4 Response surface plots of the impact of various factors on specific activity of the immobilized lipase. **a** GO-NH₂ concentration and pH, **b** GO-NH₂ concentration and enzyme concentration, **c** pH and glutaraldehyde concentration, and **d** sonication time and glutaraldehyde concentration. Other factors have constant level (their center points)

was detected with the increase of pH (8.0) and glutaraldehyde concentration (100 mM). The specific activity of the immobilized *BaL* was 71.36 U mg⁻¹ while the pH (6.0) and glutaraldehyde concentration (10 mM) were in their low levels. Neutral pH has been identified as the best pH for immobilization of enzymes [40, 41]. However, in some cases, it was found that doing the process at alkaline pH values increased the enzyme-support interaction, because the action of protein nucleophilic groups improved [40, 41]. The pH and buffer concentration affect the ionic nature of the lipase molecules [40]. The enzyme polarity may decrease at high pH and buffer concentration and could enhance the lipase immobilization [40, 42]. Huang and Cheng [42] described the influence of pH on the immobilization procedure of lipase from *Penicillium expansum*. The activity of immobilized lipase on ceramic foam was improved with the rise of the pH values (6–10), and the maximum activity is observed at pH 8.0 [42]. Glutaraldehyde cross-linking usually occurs at neutral or basic environments than in acidic environments [20, 43, 44].

Figure 4d shows the effect of sonication time, glutaraldehyde concentrations, and their interaction on the specific activity (U mg⁻¹) of immobilized *BaL*. Maximum specific activity of immobilized *BaL* was obtained at high sonication time (60 min) and high glutaraldehyde concentration (100 mM). Asmat et al. [23] assessed the impact of various concentrations of glutaraldehyde on the activity of immobilized lipase. They found that 2.5% (v/v) of glutaraldehyde in the reaction mixture was the most suitable concentration for the immobilization. A concentration that is higher than optimum can inactivate the enzyme due to the weakening of enzyme flexibility. At low concentration of glutaraldehyde, the cross-linkage may be not performed [23]. Among the different cross linkers, glutaraldehyde was mostly used due to its small size and penetration to the inside of the protein, as a result of their easy reaction with the amino groups of protein [44, 45].

The optimum specific activity of immobilized lipase from the model was determined at 3 mg mL⁻¹ for GO-NH₂ concentration, 50 mM of buffer concentration, pH 7.0, 60 min sonication time, 100 mM glutaraldehyde concentration, 25 U mL⁻¹ for enzyme concentration, and 8 h of immobilization time at 4 °C within the experimental region. The maximum specific activity of immobilized *BaL* experimentally was found to be 81.60 ± 0.28 U mg⁻¹, which was very close to the predicted amount (82.16 ± 0.35 U mg⁻¹). Verification of the immobilization conditions was well suggested that the resulted model from D-optimal design could be applied to explain the relationship between the variables and the response for immobilization of *B. atrophaeus* lipase on amine-modified graphene oxide. Maximum immobilization yield ($89.74 \pm 1.1\%$) and efficiency ($607.6 \pm 5.2\%$) were determined for immobilization of *B. atrophaeus* lipase in point prediction.

Characterization of the Immobilized *BaL*

The FTIR spectrum of the immobilized *BaL* (Fig. 5a) confirmed the existence of useful groups in the enzyme structure which might be involved in the covalent binding of the enzyme to GO-NH₂. The peaks at 3383, 3256, and 2926 cm⁻¹ corresponded to the linkage of the free COO⁻ group of lipase with the NH₂ group of support [23, 31] (Fig. 5b). Furthermore, the peak at 1636 cm⁻¹ could be ascribed to the C=O vibrations of the carboxyl and carbonyl functional groups on the GO-NH₂ and the presence of amide linkage between glutaraldehyde with GO-NH₂ and *BaL* [23, 31]. In addition, the peak at 1448 cm⁻¹ indicated that the enzyme was covalently bounded to GO-NH₂ via amide linkage [31] (Fig. 5b).

The thermal decomposition process of the immobilized and free *BaL* was surveyed by TGA technique (Fig. 5a) from which it was obvious that the free *BaL* showed faster decomposition compared with the immobilized *BaL*. The TGA profile of the immobilized *BaL* displayed two major weight loss steps from 20 to 250 °C and from 280 to 320 °C. Major weight loss (up to 80%) occurred below 300 °C (Fig. 5a) which indicated that the immobilized *BaL* was thermally more stable than its free form.

EDX elemental mapping was also used to probe the elemental distribution and structure of the GO-NH₂ (Fig. 6a) and the immobilized *BaL* (Fig. 6b). Elemental EDX analysis of GO-NH₂ showed the atoms of support including C, O, N, and Si (Fig. 6a) which displays the functionalization of APTES, especially by detecting Si atom [31, 46]. The elemental mapping of the immobilized *BaL* indicated the existence of atoms including C, N, O, Si, P, and S (Fig. 6b). The special presence of sulfur and phosphorous atom can endorse the effective immobilization of lipase on the support [31, 46].

pH and Thermal Stability

The achieved results of pH alterations (4–11) on the relative activity (%) of the free and immobilized *BaL* are shown in Fig. 7a. The maximum activity of free *BaL* was observed at pH 9.0 while the immobilized *BaL* on GO-NH₂ exhibited its maximum relative activity at pH 10.0 (*p* value < 0.05). Generally, the relative activity (%) of immobilized *BaL* was significantly higher than that of free *BaL* (*p* value < 0.05). The immobilized *BaL* exhibited 57.58, 75.76, and 60.6% relative activity after 120 min incubation at pH 7, 8, and 11, respectively, while the free *BaL* retained 52, 68, and 40% of relative activity following incubation at the same pH values. These results were in agreement with the study of Khoobi et al. [47], who assessed a wider range of relative activity for immobilized lipase of *T. lanuginose* on the synthesized

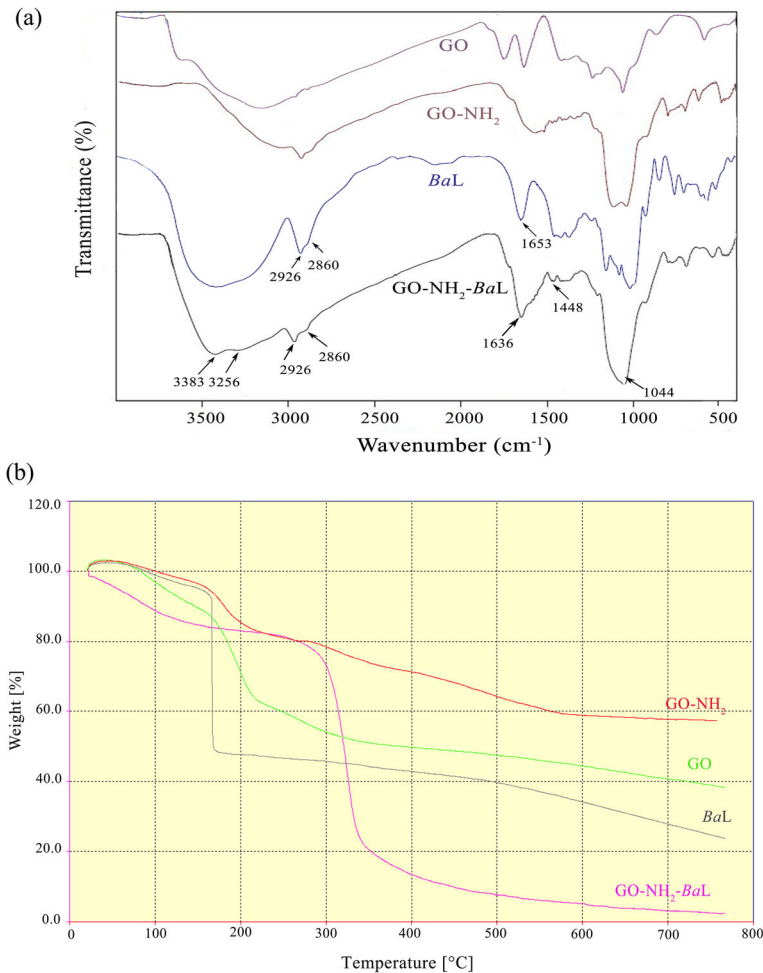


Fig. 5 **a** The FTIR spectra of graphene oxide (GO), amine-functionalized graphene oxide (GO-NH₂) nanosheets, BaL (free lipase), and GO-NH₂-BaL (immobilized lipase). **b** TGA profiles of graphene oxide (GO), amine-functionalized graphene oxide (GO-NH₂), BaL (free lipase), and GO-NH₂-BaL (immobilized lipase) under nitrogen atmosphere

nanocomposites compared with free lipase [47]. Asmat et al. [23] immobilized *Aspergillus niger* lipase on facile-synthesized polyaniline-coated silver-functionalized graphene oxide nanocomposites. The free and immobilized lipases showed their maximum activities at pH 7 and 8, respectively. The immobilized lipase showed the stability in the pH range of 7.0–9.0 [23].

The thermal stability pattern of free and immobilized BaL is shown in Fig. 7b. High relative activity of free BaL is exhibited at an optimum temperature of about 70 °C, and lipase activity significantly decreased at higher temperature (p value < 0.05) (Fig. 7b). The immobilized BaL on GO-NH₂ significantly showed the highest relative activity at 80 °C (Fig. 7b), and alteration of temperature to 90 °C significantly decreased the lipolytic activity to 68.18% of the maximum (p value < 0.05). Jafarian et al. [21] reported that the immobilized *C. rugosa* lipase on modified graphene oxide nanosheets have more thermal stability than the free lipase. Zhang

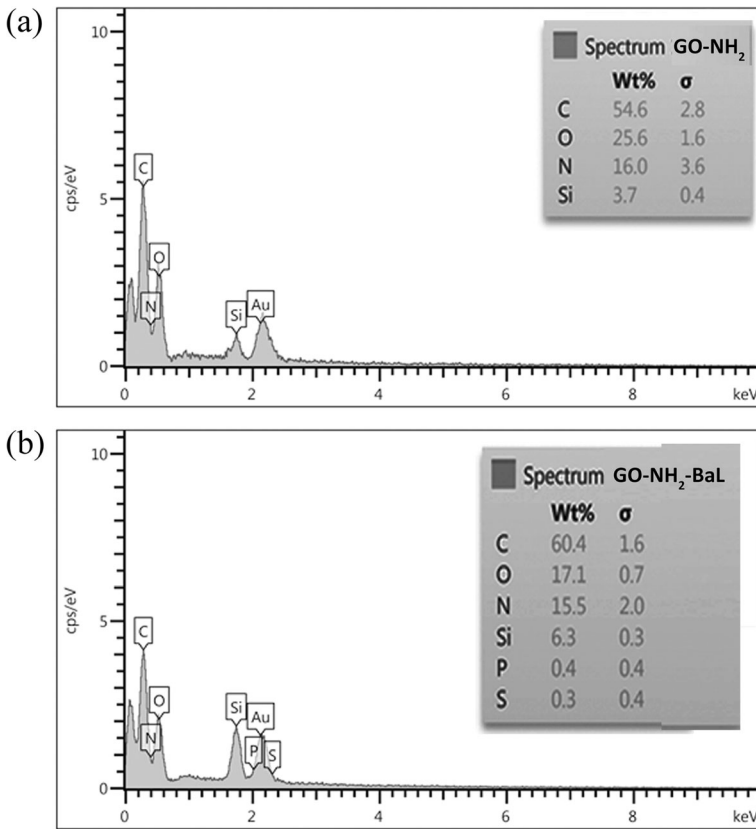


Fig. 6 **a, b** EDX analysis of amine-functionalized graphene oxide (GO-NH₂) and immobilized lipase on amine-functionalized graphene oxide (GO-NH₂-BaL)

et al. [13] immobilized lipase from *C. rugosa* onto a carrier, graphene oxide decorated with ZnO nanoparticles. They reported that the free lipase lost 64% of their initial activity when incubated at 50 °C for 30 min. Nevertheless, the immobilized lipase on support maintained 63% of its primary activity after 180 min of incubation at 50 °C [13]. These results were in accordance with the results of Asmat and Husain [48] who reported the enhanced thermal stability of lipase activity when immobilized on novel fabricated nanocellulose-fused polypyrrole/graphene oxide nanocomposite. Cui et al. [49] investigated the thermal tolerance of free and immobilized enzymes onto *N*-succinyl-chitosan beads. They found that the activities of both free and immobilized lipases reduced significantly with enhancing temperature. However, the immobilized lipase had a greater thermal stability than the free lipase.

Enzyme Stability in Organic Solvents

To survey the organic solvent stability of the free and immobilized BaL, both were individually incubated in ten different organic solvents and the obtained results are presented in Fig. 7c. The immobilized BaL significantly exhibited improved solvent stability to all used organic solvents compared with its free form especially in xylene, toluene, acetonitrile, *n*-hexane, and 2-propanol (360.84, 229.73, 194.59, 189.19, and 158.38%, respectively) (*p* value < 0.05).

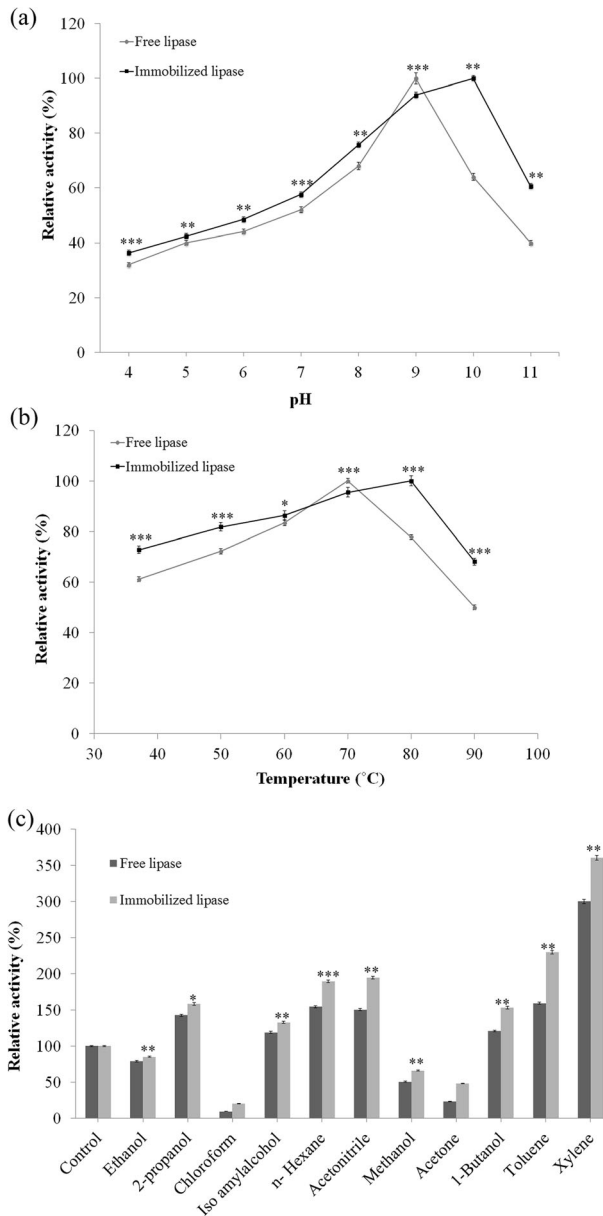


Fig. 7 **a** Effect of pH on the relative activities of free lipase and immobilized lipase. **b** The thermal stability of free and immobilized lipase. **c** Solvent stability of free and immobilized lipase in the presence of various organic solvents. Note that the maximum relative activity of both lipases considered as 100%. Significance was evaluated after ANOVA analysis of the obtained results (* p value < 0.05)

Likewise, incubation with 1-butanol showed 153.24% of relative activity for immobilized *BaL*, while the free *BaL* exhibited nearly 120.78% of its activity. In general, *BaL*'s stability in the presence of organic solvents was found to be considerable [32], and immobilization of enzyme increased this stability. Similar results were also reported in the literature by Asmat

et al. [50] which used an innovative support, polypyrrole-methylantranilate-functionalized titanium dioxide nanocomposite, for immobilization of lipase from *Rhizopus oryzae*. They displayed enhanced activity of the immobilized lipase against organic solvent (150 and 125% in acetone and isopropanol, respectively) as compared with its free form. In the study performed by Hermanová et al. [15], it was found that among the tested organic solvents, the higher activity of immobilized lipase from *R. oryzae* onto the graphene oxide nanosupport occurred in non-polar solvents. All four applied solvents in the study of Patel et al. [51] positively affected the esterification reaction of immobilized lipase from *C. rugosa* on exfoliated graphene oxide. They found that the free and immobilized lipase showed esterification percent of 56 and 82, respectively, in the presence of cyclo-octane [51].

Enzyme Stability in Metal Ions, Enzyme Inhibitors, and Detergents on *BaL* Activity

As presented in Table 4, all of the tested heavy metal ions significantly enhanced the immobilized *BaL* activity (p value < 0.05) compared with the free *BaL*. As presented in Table 4, Ca^{2+} (relative activity of $159.09 \pm 0.8\%$), Mg^{2+} (relative activity of $150 \pm 0.7\%$), and Zn^{2+} (relative activity of $131.81 \pm 1.6\%$) significantly improved the immobilized *BaL* activity (p value < 0.05). The free *BaL* activity significantly declined with adding each of the heavy metal ions (except for calcium, $135.83 \pm 1.2\%$) into enzyme solutions and pre-incubating the enzyme in their presence (p value < 0.05) (Table 4).

EDTA (as chelating agent), Triton X-100, Tween-80, Tween-40 (as non-ionic surfactants), as well as SDS (as anionic surfactant) significantly decreased the immobilized *BaL* activity to 94.09 ± 1.2 , 67.72 ± 0.8 , 60 ± 1.3 , 16 ± 1.8 , and 8.63 ± 1.2 , respectively (p value < 0.05) (Table 4).

Table 4 Obtained results of relative activity (%) of the free and immobilized lipase in the presence of metal ions, enzyme inhibitors, and detergents

Metal ions	Final concentration (mM)	Relative activity (%)	
		Free lipase	Immobilized lipase
Control	—	100	100
Mg^{2+}	5	$99.5 \pm 1.5^{**}$	$150 \pm 0.7^{**}$
K^{+}	5	90.83 ± 1.6	99.09 ± 2.3
Cu^{2+}	5	$60.83 \pm 0.8^{**}$	$80 \pm 2.2^{**}$
Ca^{2+}	5	$135.83 \pm 1.2^{**}$	$159.09 \pm 0.8^{**}$
Na^{+}	5	$79.16 \pm 1.8^{**}$	$95.45 \pm 1.5^{**}$
Zn^{2+}	5	$96.16 \pm 1.4^{***}$	$131.81 \pm 1.6^{***}$
Fe^{3+}	5	$61.16 \pm 1.2^{**}$	$69.09 \pm 0.8^{**}$
Mn^{2+}	5	79.16 ± 1.5	80.90 ± 1.8
Enzyme inhibitors			
EDTA	2.5	$68.18 \pm 0.9^{**}$	$94.09 \pm 1.2^{**}$
Detergents			
Triton X-100	1	54.63 ± 1.5	67.72 ± 0.8
Tween-80	1	$51.54 \pm 2.2^{**}$	$60 \pm 1.3^{**}$
Tween-40	1	$13.63 \pm 1.2^{**}$	$16 \pm 1.8^{**}$
SDS	1	$3.54 \pm 0.9^{**}$	$8.63 \pm 1.2^{**}$

Experiments were performed in triplicates

*Significance was attained after ANOVA with Dunnett's T3 post hoc test

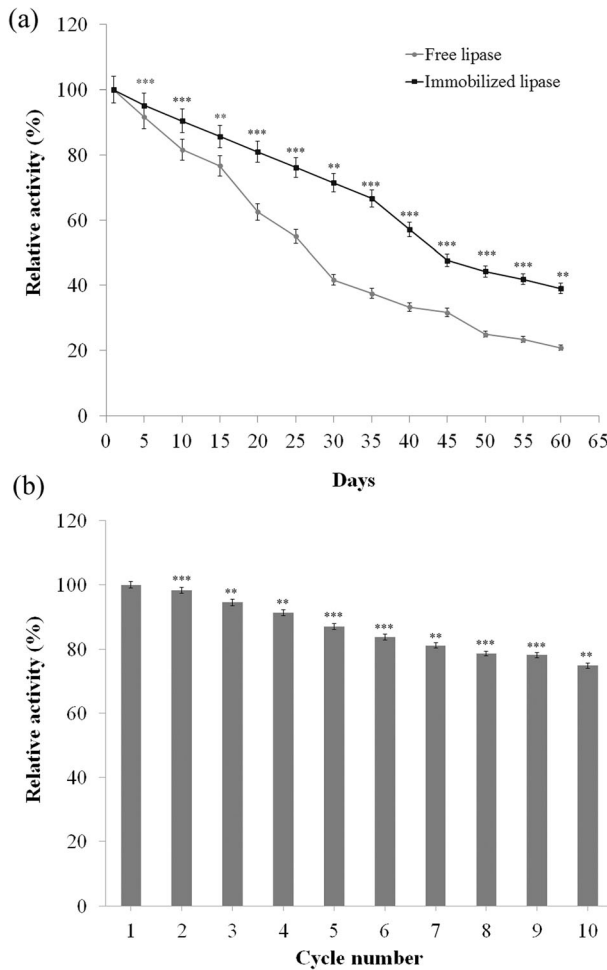


Fig. 8 **a** The storage stability of immobilized and free lipase. The enzyme was stored at 25 °C for different time. **b** The reusability of immobilized lipase on GO-NH₂ for 10 cycles; **p* value < 0.05, significant values

Storage Stability and Reusability of the Immobilized *BaL*

Stability of the immobilized enzymes under long storage is one of the main aspects in using immobilized enzymes in biotechnological and industrial processes [21, 52]. The storage stabilities of free and immobilized *BaL* were also investigated (Fig. 8a). The free and immobilized *BaL* were kept at 25 °C for a 60-day period. The relative activity of the free *BaL* significantly reduced much faster than that of the immobilized *BaL* under similar reaction conditions (*p* value < 0.05). The relative activity of immobilized *BaL* was about 71.4% after 30 days while that of the free *BaL* was about 41.6%. The free *BaL* lost about 80% of initial activity after 60 days while the immobilized *BaL* retained 40% of its initial activity after 60 days of storage at 25 °C. Limited conformational changes of the immobilized *BaL* together with inhibition of auto-digestion as a result of covalent attachment of enzyme to the support assisted the immobilized *BaL* to exhibit higher relative activity compared with that of the free *BaL* [2, 21].

Reusability is one of the major features of immobilized enzymes which reduced the cost of the industrial application of biocatalysts [2, 38, 47]. The *BaL* immobilized on the GO-NH₂ was stable for 10 cycles of application (Fig. 8b). The results showed a significant decline in the activity of immobilized *BaL* (25%) after 10 cycles (p value < 0.05) which might be ascribed to the inactivation of the enzyme. The strong interactions between lipases and supports significantly improved the enzyme reusability in covalent attachment [38, 52]. Zhang et al. [13] evaluated the GO/ZnO composites for immobilization of lipase from *C. rugosa* and showed that 90% of the initial activity can be recovered after reusing the immobilized lipase for 14 times. Xie and Huang [16] also immobilized *C. rugosa* lipase onto graphene oxide Fe₃O₄ nanocomposite. They reported that the immobilized lipase could be reused for five times without significant loss of its activity.

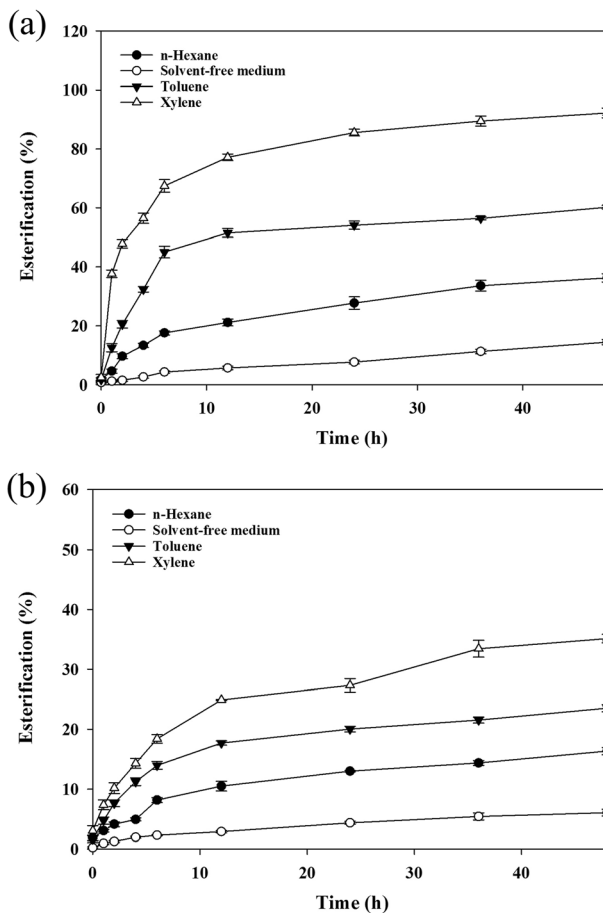


Fig. 9 Lipase-catalyzed synthesis of pentyl valerate by immobilized lipase (a) and free lipase (b), in aqueous and non-aqueous media. Data are shown as mean $\pm$ SD ($n = 3$)

Application of Immobilized *BaL* for the Esterification of Valeric Acid

A comparison of the esterification of valeric acid in both the organic and aqueous media using the free and immobilized *BaL* was evaluated (Fig. 9a, b). As shown in Fig. 9a, the formation of pentyl valerate was maximally obtained in the presence of immobilized *BaL* in the organic solvent. After replacing the aquatic medium (13.5% esterification yield) with xylene, toluene, and *n*-hexane, the esterification yield increased by 92.7, 60.8, and 35.0%, respectively, using the immobilized *BaL* after 48 h of incubation at 60 °C. The maximum esterification yield assisted by the free *BaL* (34.5%) was obtained after 48 h incubation in the existence of xylene (Fig. 9a). Furthermore, the esterification percentage was 22.9 and 17% in the presence of toluene and *n*-hexane, respectively, using free *BaL* (Fig. 9b). The achieved results of esterification studies (Fig. 9a, b) exhibited that replacement of aqueous reaction medium with organic solvents increased the esterification yield. Khoobi et al. [37] immobilized *Thermomyces lanuginosa* lipase on the polyethyleneimine-modified superparamagnetic Fe₃O₄ nanoparticles and synthesized the ethyl valerate in organic solvent and solvent-free media using the immobilized lipase. They found that the esterification percentages in *n*-hexane and solvent-free media were 72.9 and 28.9%, respectively. Asmat and Husain [48] synthesized a flavored compound, ethyl acetoacetate, using the lipase immobilized on nanobiocatalyst. They reported that the esterification percentages in solvent-free media and *n*-hexane were 27.5 and 75.5%, respectively. Raghavendra et al. [53] synthesized pentyl valerate using *C. rugosa* lipase immobilized in microemulsion based organogels. The maximum esterification percentages were 99 and 19% in the presence of immobilized enzyme and free enzyme, respectively. The highest ester production also occurred in cyclohexane (92%) medium during the esterification process [53]. In another study performed by Patel et al. [51], the potential application of immobilized lipase on ZnO bioconjugate into sodium bis(2-ethylhexyl) sulfosuccinate (AOT)-reverse micelles for pentyl valerate synthesis was demonstrated. They observed an esterification percent of 86 in *n*-hexane containing medium [54].

Conclusion

Immobilization of lipase from *Bacillus atrophaeus* FSHM2 (*BaL*) on amine-modified graphene oxide nanosheets was statistically optimized using fractional factorial design augmented to D-optimal design. The optimum conditions for immobilized lipase (specific activity of 81.60 ± 0.28 U mg⁻¹) were 3 mg mL⁻¹ for GO-NH₂, 50 mM for phosphate buffer, pH 7.0, 60 min sonication time, 100 mM glutaraldehyde, 25 U mL⁻¹ for *BaL*, and 8 h for immobilization time at 4 °C. The immobilized *BaL* showed a high relative activity at 80 °C and pH 10.0, while the maximum activity of free *BaL* was observed at 70 °C and pH 9.0. Moreover, the immobilized *BaL* onto GO-NH₂ retained about 80% of its initial activity after 20 days of storage and after 5 cycles of application. Pentyl valerate synthesis using the immobilized *BaL* in the organic solvents (xylene, *n*-hexane, and toluene) revealed 97.2, 60.8, and 35% production yield after 48 h of incubation.

Funding Information This work was financially supported by Pharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran. Research reported in this publication was also supported by Elite Researcher Grant Committee under award number from the National Institutes for Medical Research Development (987592), Tehran, Iran.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

- Forootanfar, H., Rezaei, S., Zeinvand-Lorestani, H., Tahmasbi, H., Mogharabi, M., Ameri, A., & Faramarzi, M. A. (2016). Studies on the laccase-mediated decolorization, kinetic, and microtoxicity of some synthetic azo dyes. *Journal of Environmental Health Science and Engineering*, 14, 7.
- Zhang, G., Ma, J., Wang, J., Li, Y., Zhang, G., Zhang, F., et al. (2014). Lipase immobilized on graphene oxide as reusable biocatalyst. *Industrial & Engineering Chemistry Research*, 53(51), 19878–19883.
- Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2015). Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. *Biotechnology Advances*, 33(5), 435–456.
- Choi, J.-M., Han, S.-S., & Kim, H.-S. (2015). Industrial applications of enzyme biocatalysis: current status and future aspects. *Biotechnology Advances*, 33(7), 1443–1454.
- Fernandez-Lafuente, R. (2010). Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst. *Journal of Molecular Catalysis B: Enzymatic*, 62(3–4), 197–212.
- Li, Q., Fan, F., Wang, Y., Feng, W., & Ji, P. (2013). Enzyme immobilization on carboxyl-functionalized graphene oxide for catalysis in organic solvent. *Industrial & Engineering Chemistry Research*, 52(19), 6343–6348.
- Guldhe, A., Singh, B., Mutanda, T., Permaul, K., & Bux, F. (2015). Advances in synthesis of biodiesel via enzyme catalysis: novel and sustainable approaches. *Renewable and Sustainable Energy Reviews*, 41, 1447–1464.
- Ameri, A., Shakibaie, M., Soleimani-Kermani, M., Faramarzi, M. A., Doostmohammadi, M., & Forootanfar, H. (2019). Overproduction of thermoalkalophilic lipase secreted by *Bacillus atrophaeus* FSHM2 using UV-induced mutagenesis and statistical optimization of medium components. *Preparative Biochemistry and Biotechnology*, 49(2), 184–191.
- Soflaei, S., Dalimi, A., Ghaffarifar, F., Shakibaie, M., Shahverdi, A. R., & Shafiepour, M. (2012). In vitro antiparasitic and apoptotic effects of antimony sulfide nanoparticles on *Leishmania infantum*. *Journal of Parasitology Research*, 2012, 756568.
- Gonçalves Filho, D., Silva, A. G., & Guidini, C. Z. (2019). Lipases: sources, immobilization methods, and industrial applications. *Applied Microbiology and Biotechnology*, 103(18), 7399–7423.
- Brady, D., & Jordaan, J. (2009). Advances in enzyme immobilisation. *Biotechnology Letters*, 31(11), 1639–1650.
- Min, K., & Yoo, Y. J. (2014). Recent progress in nanobiocatalysis for enzyme immobilization and its application. *Biotechnology and Bioprocess Engineering*, 19(4), 553–567.
- Zhang, S., Shi, J., Deng, Q., Zheng, M., Wan, C., Zheng, C., et al. (2017). Preparation of carriers based on zno nanoparticles decorated on graphene oxide (GO) nanosheets for efficient immobilization of lipase from *Candida rugosa*. *Molecules*, 22(7), 1205.
- Tang, A., Zhang, Y., Wei, T., Wu, J., Li, Q., & Liu, Y. (2019). Immobilization of *Candida cylindracea* lipase by covalent attachment on Glu-modified bentonite. *Applied Biochemistry and Biotechnology*, 187(3), 870–883.
- Hermanová, S., Zarevúcká, M., Bouša, D., Pumera, M., & Sofer, Z. (2015). Graphene oxide immobilized enzymes show high thermal and solvent stability. *Nanoscale*, 7(13), 5852–5858.
- Xie, W., & Huang, M. (2018). Immobilization of *Candida rugosa* lipase onto graphene oxide Fe₃O₄ nanocomposite: characterization and application for biodiesel production. *Energy Conversion and Management*, 159, 42–53.
- Akhond, M., Pashangeh, K., Karbalaei-Heidari, H., & Absalan, G. (2016). Efficient immobilization of porcine pancreatic α -amylase on amino-functionalized magnetite nanoparticles: characterization and stability evaluation of the immobilized enzyme. *Applied Biochemistry and Biotechnology*, 180(5), 954–968.
- Zniszczoł, A., Herman, A. P., Szymańska, K., Mrowiec-Białoń, J., Walczak, K. Z., Jarzębski, A., et al. (2016). Covalently immobilized lipase on aminoalkyl-, carboxy- and hydroxy-multi-wall carbon nanotubes in the enantioselective synthesis of Solketal esters. *Enzyme and Microbial Technology*, 87, 61–69.
- Tan, T., Lu, J., Nie, K., Deng, L., & Wang, F. (2010). Biodiesel production with immobilized lipase: a review. *Biotechnology Advances*, 28(5), 628–634.
- Dutta, N., & Saha, M. K. (2018). Immobilization of a mesophilic lipase on graphene oxide: stability, activity, and reusability insights. *Methods in Enzymology*, 609, 247–272.
- Jafarian, F., Bordbar, A. K., Zare, A., & Khosropour, A. (2018). The performance of immobilized *Candida rugosa* lipase on various surface modified graphene oxide nanosheets. *International Journal of Biological Macromolecules*, 111, 1166–1174.

22. Tan, H., Feng, W., & Ji, P. (2012). Lipase immobilized on magnetic multi-walled carbon nanotubes. *Bioresource Technology*, 115, 172–176.
23. Asmat, S., Husain, Q., & Azam, A. (2017). Lipase immobilization on facile synthesized polyaniline-coated silver-functionalized graphene oxide nanocomposites as novel biocatalysts: stability and activity insights. *RSC Advances*, 7(9), 5019–5029.
24. Tian, S., Liu, Z., Shen, L., Pu, J., Liu, W., Sun, X., et al. (2018). Performance evaluation of mercapto functional hybrid silica sol–gel coating and its synergistic effect with f-GNs for corrosion protection of copper surface. *RSC Advances*, 8(14), 7438–7449.
25. Shao, Y., Jing, T., Tian, J., & Zheng, Y. (2015). Graphene oxide-based Fe₃O₄ nanoparticles as a novel scaffold for the immobilization of porcine pancreatic lipase. *RSC Advances*, 5(126), 103943–103955.
26. Amirbandeh, M., & Taheri-Kafrani, A. (2016). Immobilization of glucoamylase on triazine-functionalized Fe₃O₄/graphene oxide nanocomposite: improved stability and reusability. *International Journal of Biological Macromolecules*, 93(Pt A), 1183–1191.
27. Haghighi, N., Hallaj, R., & Salimi, A. (2017). Immobilization of glucose oxidase onto a novel platform based on modified TiO₂ and graphene oxide, direct electrochemistry, catalytic and photocatalytic activity. *Materials Science & Engineering C, Materials for Biological Applications*, 73, 417–424.
28. Mondal, M. K., Mukherjee, S., Saha, S. K., Chowdhury, P., & Sinha Babu, S. P. (2017). Design and synthesis of reduced graphene oxide based supramolecular scaffold: A benign microbial resistant network for enzyme immobilization and cell growth. *Materials Science & Engineering C, Materials for biological applications*, 75, 1168–1177.
29. Royvaran, M., Taheri-Kafrani, A., Landarani Isfahani, A., & Mohammadi, S. (2016). Functionalized superparamagnetic graphene oxide nanosheet in enzyme engineering: a highly dispersive, stable and robust biocatalyst. *Chemical Engineering Journal*, 288, 414–422.
30. Zhang, F., Zheng, B., Zhang, J., Huang, X., Liu, H., Guo, S., et al. (2010). Horseradish peroxidase immobilized on graphene oxide: physical properties and applications in phenolic compound removal. *The Journal of Physical Chemistry C*, 114(18), 8469–8473.
31. Badoei-dalfard, A., Karami, Z., & Malekabadi, S. (2019). Construction of CLEAs-lipase on magnetic graphene oxide nanocomposite: an efficient nanobiocatalyst for biodiesel production. *Bioresource Technology*, 278, 473–476.
32. Ameri, A., Shakibaie, M., Faramarzi, M. A., Ameri, A., Amirpour-Rostami, S., Rahimi, H. R., et al. (2017). Thermoalkalophilic lipase from an extremely halophilic bacterial strain *Bacillus atrophaeus* FSHM2: purification, biochemical characterization and application. *Biocatalysis and Biotransformation*, 35(3), 151–160.
33. Hummers Jr., W. S., & Offeman, R. E. (1958). Preparation of graphitic oxide. *Journal of the American Chemical Society*, 80(6), 1339–1339.
34. Akbari, T., Pourhajbagher, M., Hosseini, F., Chiniforush, N., Gholibegloo, E., Khoobi, M., Shahabi, S., & Bahador, A. (2017). The effect of indocyanine green loaded on a novel nano-graphene oxide for high performance of photodynamic therapy against *Enterococcus faecalis*. *Photodiagnosis and Photodynamic Therapy*, 20, 148–153.
35. Hasan-Beikdashti, M., Forootanfar, H., Safiarian, M., Ameri, A., Ghahremani, M., Khoshayand, M., et al. (2012). Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*. *Journal of the Taiwan Institute of Chemical Engineers*, 43(5), 670–677.
36. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254.
37. Khoobi, M., Motevalizadeh, S. F., Asadgol, Z., Forootanfar, H., Shafiee, A., & Faramarzi, M. A. (2015). Polyethyleneimine-modified superparamagnetic Fe₃O₄ nanoparticles for lipase immobilization: characterization and application. *Materials Chemistry and Physics*, 149, 77–86.
38. Rezaei, A., Akhavan, O., Hashemi, E., & Shamsara, M. (2016). Ugi four-component assembly process: an efficient approach for one-pot multifunctionalization of nanographene oxide in water and its application in lipase immobilization. *Chemistry of Materials*, 28(9), 3004–3016.
39. Amirkhani, L., Moghaddas, J., & Jafarizadeh, M. H. (2016). Optimization of *Candida rugosa* lipase immobilization parameters on magnetic silica aerogel using adsorption method. *Iranian Journal of Chemical Engineering*, 13, 19–31.
40. Aybastier, Ö., & Demir, C. (2010). Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on styrene–divinylbenzene copolymer using response surface methodology. *Journal of Molecular Catalysis B: Enzymatic*, 63(3–4), 170–178.
41. Yücel, Y. (2012). Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on olive pomace powder using response surface methodology. *Biocatalysis and Agricultural Biotechnology*, 1(1), 39–44.
42. Huang, L., & Cheng, Z.-M. (2008). Immobilization of lipase on chemically modified bimodal ceramic foams for olive oil hydrolysis. *Chemical Engineering Journal*, 144(1), 103–109.

43. Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2014). Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. *RSC Advances*, 4(4), 1583–1600.
44. Talekar, S., Joshi, A., Joshi, G., Kamat, P., Haripurkar, R., & Kambale, S. (2013). Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). *RSC Advances*, 3(31), 12485.
45. Ranjbari, N., Razzaghi, M., Fernandez-Lafuente, R., Shojaei, F., Satar, M., & Homaei, A. (2019). Improved features of a highly stable protease from *Pancreas vannamei* by immobilization on glutaraldehyde activated graphene oxide nanosheets. *International Journal of Biological Macromolecules*, 130, 564–572.
46. Heidarizadeh, M., Doustkhah, E., Rostamnia, S., Rezaei, P. F., Harzevili, F. D., & Zeynizadeh, B. (2017). Dithiocarbamate to modify magnetic graphene oxide nanocomposite (Fe₃O₄-GO): a new strategy for covalent enzyme (lipase) immobilization to fabrication a new nanobiocatalyst for enzymatic hydrolysis of PNPD. *International Journal of Biological Macromolecules*, 101, 696–702.
47. Khoobi, M., Khalilvand-Sedagheh, M., Ramazani, A., Asadgol, Z., Forootanfar, H., & Faramarzi, M. A. (2016). Synthesis of polyethylenimine (PEI) and β -cyclodextrin grafted PEI nanocomposites with magnetic cores for lipase immobilization and esterification. *Journal of Chemical Technology & Biotechnology*, 91(2), 375–384.
48. Asmat, S., & Husain, Q. (2018). Exquisite stability and catalytic performance of immobilized lipase on novel fabricated nanocellulose fused polypyrrole/graphene oxide nanocomposite: characterization and application. *International Journal of Biological Macromolecules*, 117, 331–341.
49. Cui, S., Zhou, Q. W., Wang, X. L., Yang, S. Q., Chen, K., Dai, Z. Y., et al. (2017). Immobilization of lipase onto N-succinyl-chitosan beads and its application in the enrichment of polyunsaturated fatty acids in fish oil. *Journal of Food Biochemistry*, 41(5), e12395.
50. Asmat, S., Husain, Q., & Khan, M. S. (2018). A polypyrrole-methyl anthranilate functionalized worm-like titanium dioxide nanocomposite as an innovative tool for immobilization of lipase: preparation, activity, stability and molecular docking investigations. *New Journal of Chemistry*, 42(1), 91–102.
51. Patel, V., Gajera, H., Gupta, A., Manocha, L., & Madamwar, D. (2015). Synthesis of ethyl caprylate in organic media using *Candida rugosa* lipase immobilized on exfoliated graphene oxide: process parameters and reusability studies. *Biochemical Engineering Journal*, 95, 62–70.
52. Khoobi, M., Motevalizadeh, S. F., Asadgol, Z., Forootanfar, H., Shafiee, A., & Faramarzi, M. A. (2014). Synthesis of functionalized polyethylenimine-grafted mesoporous silica spheres and the effect of side arms on lipase immobilization and application. *Biochemical Engineering Journal*, 88, 131–141.
53. Raghavendra, T., Panchal, N., Divecha, J., Shah, A., & Madamwar, D. (2014). Biocatalytic synthesis of flavor ester “pentyl valerate” using *candida rugosa* lipase immobilized in microemulsion based organogels: effect of parameters and reusability. *BioMed Research International*, 2014.
54. Patel, V., Deshpande, M., & Madamwar, D. (2017). Increasing esterification efficiency by double immobilization of lipase-ZnO bioconjugate into sodium bis (2-ethylhexyl) sulfosuccinate (AOT)-reverse micelles and microemulsion based organogels. *Biocatalysis and Agricultural Biotechnology*, 10, 182–188.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Affiliations

Atefeh Ameri¹ • Mojtaba Shakibaie^{1,2} • Mehdi Khoobi³ • Mohammad Ali Faramarzi⁴ • Alieh Ameri⁵ • Hamid Forootanfar^{2,6}

¹ Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran

² Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

³ Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran

⁴ Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran

⁵ Department of Medicinal Chemistry, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

⁶ Pharmaceutical Sciences and Cosmetic Products Research Center, Kerman University of Medical Sciences, Kerman, Iran