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RICE HUSK SILICA-COATED MAGNETIC PARTICLES AS A LOW COST SUPPORT MATERIAL FOR CRUDE *Pseudomonas Aeruginosa* LIPASE IMMOBILIZATION

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ABSTRACT

In this research, lipase produced from the bacteria *Pseudomonas aeruginosa* was immobilized on rice husk silica-coated Fe_3O_4 nanoparticles. The process included the following steps: preparation of magnetic ferosoferic oxide nanoparticles (NP), coating NP with silica from rice husk ash, activation with glutaraldehyde and covalent immobilization of lipase on the support. The synthesis of the nanoparticle was followed by characterization through FT-IR, XRD, and FE-SEM. The hydrolysis kinetics (using 4-Nitrophenyl palmitate as a substrate) of the immobilized lipase followed Michaelis-Menten model with a V_{max} and a K_m value of 4.0 $mM.s^{-1}$ and 0.63 mM, respectively. The immobilized lipase showed better tolerance to extreme temperature and pH compared to free lipase. About 60% of enzyme remained immobilized after five cycles of reuse and 68.13% of it were stable until 49 days. Thus the silica-coated Fe_3O_4 nanoparticles appeared to be a potential support material for lipase immobilization applications.

Keywords: *Core-shell magnetic nanoparticles; Pseudomonas aeruginosa; immobilized lipase; rice-husk silica.*

1. INTRODUCTION

In the field of biotechnology, lipases (EC 3.1.1.3) have got vast applications in biocatalysis. They belong to serine hydrolases and can catalyze both the hydrolysis of fats/oils and the biosynthesis of esters of glycerol or other alcohol with long chain fatty acids. Their use in the oleo chemical industry for the modification of fats and oils is particularly attractive [1]. Unfortunately, lipases showed instability during the commercial preparations of triglycerides, the products of triglyceride peroxidation being the main cause for inactivation [2]. Aldehydes, in particular, seem to affect the lipase stability to a great extent. The interaction with the lipase involves specific residues of the protein such as lysine, cysteine and histidine in the formation of covalent bonds. Due to lipase's non-toxic and eco-friendly nature, its catalytic use is a clean technology. It is crucial to focus on finding solutions to issues like the high cost of enzymes, methanol's inhibition, and lipase activity fatigue because these could complicate efforts to deploy enzymatic biodiesel synthesis on an industrial scale in the near future. [3].

Several approaches can be applied to improve the enzyme stability. Most important approaches include: enzyme modification and enzyme immobilization. In the second approach, the enzyme molecules are attached onto/incorporated into larger structures through encapsulation, adsorption or covalent binding [4, 5]. Modification of enzyme means the change of enzyme structure by means of covalent reactions. A functional group or polymer can be added on the surface of an enzyme molecule to change its surface properties that may improve its stability. Recently, enzymes are being immobilized on nanostructured materials

to improve their catalytic efficacy, because nanoparticles with high surface-area/volume ratios can more effectively enhance enzyme loading [6, 7]. However, there are some limitations in the recovery of nanomaterials immobilized enzyme. Use of magnetic nanoparticles can help overcome this problem. The magnetic nanoparticle-loaded enzymes can easily be recovered by applying magnetic field. This could reduce operating expenses and increase product quality. [8].

This work aimed to demonstrate the potential of using low cost support material in lipase immobilization. Rice husk ash is an abundant waste with high silica content having a promising application as support material. Silica is an amorphous inorganic polymer composed of silanol groups (Si-OH) distributed on its surface, making silica as hydrophilic support. In this study, surface modification was done using glutaraldehyde as a crosslinker for immobilization of enzymes in which the amino group of enzyme was expected to form a Schiff base with the glutarldehyde. First, SiOx-coated magnetic microspheres were produced. In order to make the enzyme more comfortable in a new environment, glutarldehyde was added to the Fe3O4/SiOx surface to prepare the macromolecule initiator.Then *Pseudomonas aureginosa* lipase (IPAL) was immobilized on glutarldehyde modified Fe_3O_4/SiO_x surface and investigated its efficiency in biodiesel production.

2. MATERIALS AND METHOD

2.1. Materials.

Sodium hydroxide, citric acid, chloride acid, iron(II) chloride tetrahydrate, iron (III) chloride hexahydrate, 2-propanol, ethanol, methanol, acetone, ammonium sulfate, magnesium sulfate, glucose, calcium chloride, sodium dihydrogen phosphate, potassium phosphate, glutaraldehyde, ammonia, gum arabic, tris-HCl buffer, phosphate buffer, acetone, and hexane were obtained from Merck. *Pseudomonas a eruginosa* ATCC315442 MBL USA was supplied by Lonza Bioreseach Team Sdn. Bhd. Rice husk was collected from rice mills in Kedah, Malaysia. Olive oil was purchased from Basso Oil Product.

2.2. Synthesis of rice husk silica-coated Fe_3O_4

2.2.1 Preparation of rice husk silica

Rice husk was cleaned to eliminate stems, leaves, gravel and other materials, then washed. Moist husk was dried in the oven for 2 h at 70 °C and then burnt into ashes at 700 °C. Afterwards, The ash was sieved through a 200 mesh sieve after being macerated in a porcelain mortar. Following that, 10 grams of ash were cleaned with 60 milliliters of 0.1 M HCl, neutralized with distilled water and rinsed with deionized water. Furthermore, dried the clean ash in an oven at 100 °C for 2 h. Sodium silicate was prepared by mixing 10 g rice husk ash with 80 ml of 3 N NaOH into a plastic container with stirring for 3 h at 90 °C. The mixture was suction filtered to obtain sodium silicate.

2.2.2 Preparation of iron oxide magnetic nanoparticles.

Magnetic nanoparticles were prepared by co-precipitating Fe^{2+} and Fe^{3+} ion in NH_4OH following the method of Xie and Ma [9] with some modifications. A 4.4 g of $FeCl_3.6H_2O$ and 1.98 g of $FeCl_2.4H_2O$ were dissolved in 61 ml of deionized water. The solution was purged with nitrogen for 30 min, and then 143 ml of 0.7 M NH_4OH added drop wise into the solution. During the additional of NH_4OH, the solution changed color from the original orange to dark brown and then to black. The solution was heated at 80 °C for 30 min, the precipitates were filtered and washed several times with distilled water and ethanol, and dried under vacuum at room temperature.

2.2.3. Silica coating of magnetic nanoparticles

Coating of the nanoparticle was performed by taking after the strategy of Sun et al. [10] with a few adjustments. The nanoparticle was suspended in 0.1 M HCl (2 mg/ml) and the pH was balanced to 3. Citric acid (1 M) was included to the suspension (with 5% molar proportion of Fe) beneath magnetic stirring. After 4 h the solution was washed 3 times with water by magnetic decantation and balanced to unique volume with refined water. At that 2 ml of magnetic solution was ultrasonically scattered in outright ethanol, 120 µl sodium silicate solurion (from rice husk ash) was included to the methanol solution beneath mechanical mixing. After 4 h, 4 ml water, 1.4 ml NH4OH and 50 µl sodium silicate was sequentially included to the reaction. The hydrolysis of sodium silicate was carried out beneath mechanical blending for 12 h; the ultimate item was washed with water.

2.3. Microbial lipase production

The bacterial strain *Pseudomonas aeruginosa* was utilized for lipase generation. Around 2% v/v of the seed culture was utilized to vaccinate a few 250 ml Erlenmeyer jars, each containing 150 ml of a medium composed of (g/L):1% glucose, 0.1% KH_2PO_4, 0.5% NH_4Cl, 0.24% $(NH_4)_2SO_4$, 0.01% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 1% emulsified olive oil. The inoculated media were cultured for 96 hours at 37 °C and 180 rpm in a shaker incubator. Growth was measured using a spectrophotometer to measure absorbance at 600 nm, and the optical density was converted to colony forming units (CFU). The contents of each flask were then centrifuged at 12,000 rpm for 15 minutes at 4 °C to produce crude lipase.

2.4. Immobilization of lipase

About 100 mg of Fe_3O_4 nanoparticles were combined with different concentrations (in weight percent) of glutaraldehyde as a coupling reagent, and the reaction was allowed to proceed at room temperature for 2 hours in order to prepare reactive magnetic support with glutaraldehyde groups on their surface. Following a magnetic decantation separation, glutaraldehyde-activated particles were then rinsed with distilled water. The mixture was then agitated (at 180 rpm) at room temperature for 1–7 hours with varied quantities of the crude lipase (555–3360 U/mL in phosphate buffer at pH 7.5). Following the completion of the process, the precipitates were collected and washed three times with phosphate buffer before the unbounded enzyme was extracted in a magnetic field (50 mM, pH 7.5).

2.5. Enzyme assay

Using the substrate 4-nitrophenyl palmitate (pNPP), the activity of free and immobilized Pseudomonas aeruginosa lipase was measured using a spectrophotometric assay. The buffer solution, which contains 0.1 mM Tris-HCl, pH 8.0, 0.4 % (w/v) triton X-100 and 0.1 % (w/v) gum arabic, was combined with one volume of a solution of pNPP (5 mM) to create the substrate solution. Then, 100 L of each type of lipase—free and immobilized—was added to 3.4 mL of substrate solution individually. The lipase activity was measured at 410 nm after 5 min of incubation. The quantity of enzyme that released 1 mol of pNP every minute was considered one unit of activity. By assessing the enzyme's residual activity after 30 min of incubation at a given temperature and pH, thermal and pH stability was assessed. In the pNPP concentration range of 0.1-0.5 mM, the kinetic characteristics of free and immobilized enzyme were calculated.

2.6. Characterization

By using FE-SEM, magnetic nanoparticle size and shape could be seen (JSM-7800F) and RigakuD/max-3B X-ray diffractometer using a Cu K diffractometer, XRD measurements were taken. The FT-IR (PerkinElmer Spectrum 100 FT-IR Spectrometer) spectra of magnetic nanoparticles and lipase-bound nanoparticles were obtained using the KBr pellet method.

3. RESULTS AND DISCUSSION

3.1 Lipase production

Pseudomonas aeruginosa was used for lipase production in batch culture over a period of 96 h at temperature 37 °C, pH 7.5, and agitation at 180 rpm. After 36 hours of incubation, the

lipase activity reached its peak; however, after 42 hours, the activity started to decline. The usual lipase activity of the culture media's supernatant was determined to be 15.3 U ml-1 under ideal culture conditions (Fig. 1).

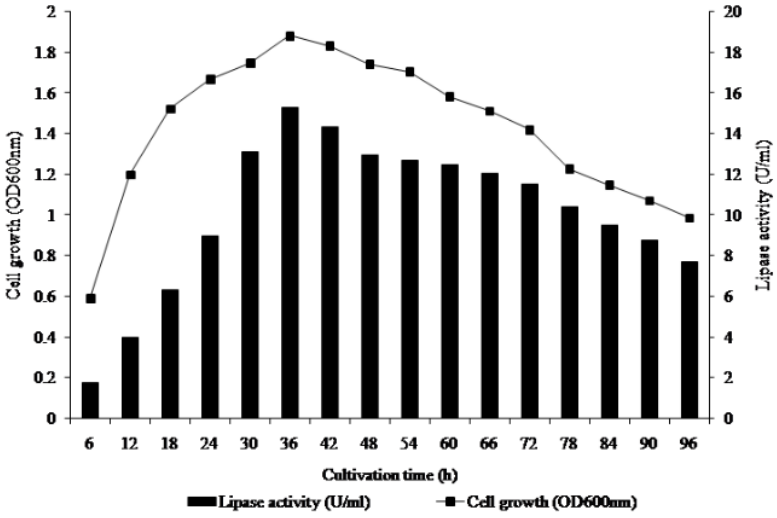

Fig. 1. The impact of culture time on Pseudomonas aeruginosa's ability to produce lipase.

3.2. Preparation of support material

Preparation of the silica support material from rice husk ash as sodium silicate took place through the chemical reaction as shown in Eq. (1).

$$SiO_2 + 2NaOH \rightarrow Na_2SiO_3 + H_2O \qquad (1)$$

Magnetic nanoparticles (Fe_3O_4 or γ-Fe_2O_3) were prepared by using co-precipitation method in the inert atmosphere in an aqueous medium with sufficient amount of nitrogen gase bubbling through the solution to protect against the critical oxidation of the magnetic nanoparticles, and also to reduce the particle size, which is represented by the following chemical reactions (Eq. 2-3).

$$FeCl_3 + FeCl_2 + 5NH_4OH \xrightarrow{H_2O} Fe(OH)_3 + Fe(OH)_2 + 5NH_4Cl \qquad (2)$$

$$2Fe(OH)_3 + Fe(OH)_2 \xrightarrow{\Delta 60^{\circ}C} Fe_3O_4(\text{magnetite}) + 4H_2O \qquad (3)$$

In the third step, magnetic nanoparticles were coated with sodium silicate by hydrolysis and condensation of sol gel precursor. The hydrolysis used citric acid at pH 5.0 to prepare surface-modified magnetic nanoparticles. The specific interactions between magnetic 12 and silica could be intermolecular covalent bond formation (Si-O-Fe), electrostatic interaction between negatively charged Si-O terminal and positively charged on the particle surface, or hydrogen-bonds between hydration layer of silanol groups and the particles surface [11]. It might be a suitable approach for the interaction of magnetic NP and silica by magnetic dipole attraction through the silica shell [10].

3.3 Immobilization of lipase

Rice husk silica-coated Fe_3O_4 nanoparticles was used for lipase immobilization by covalent crosslinking between glutaraldehyde and an amino group of lipase at room temperature.

Furthermore, the effect of various concentrations of glutaraldehyde (0.5; 1; 1.5; 2; 3; 4; 5 wt.%) on the surface activation of support material was investigated. Fig. 2 shows the optimum concentration of gluteraldehyde for immobilization to be 1.5%. Increasing the amount of glutaraldehyde above this value reduced the lipase activity. This result is in full agreement with that reported by He et al. [11] and Lee et al. [12]. Glutaraldehyde facilitates the binding of the enzyme to the support via its aldehyde group and the amino group of lipase. Glutaraldehyde can influence the activity of lipase and can cause denaturation of enzymes due to attachment of excess gluteraldehyde with other amino groups of the enzyme at or near the active site. Excessive amount of glutaraldehyde may also undergo self-cross-linking, which might have a steric effect on lipase immobilization [9].

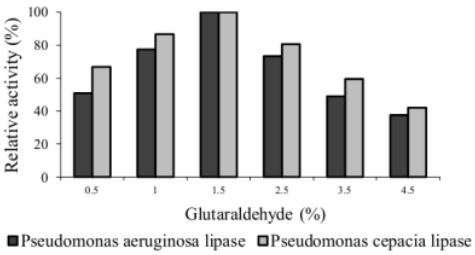

Fig. 2. Effect of glutaraldehyde concentration on the immobilization of lipase.

Effect of incubation time (1-6 h) on the activity of immobilized lipase was also studied. As shown in Fig. 3, With increasing incubation time, the relative activity changed, and 3 hours produced the highest activity. Thus, the optimal incubation time was considered to be about 3 h. The binding of enzyme to the support was limited when the incubation time was too short or too long [13]. The quantity of covalent interactions between lipase and carriers rises with the length of incubation. Too long incubation time may be attributed to decrease the lipase activity because the active site of lipase could get involved in covalent bond formation with the support [9].

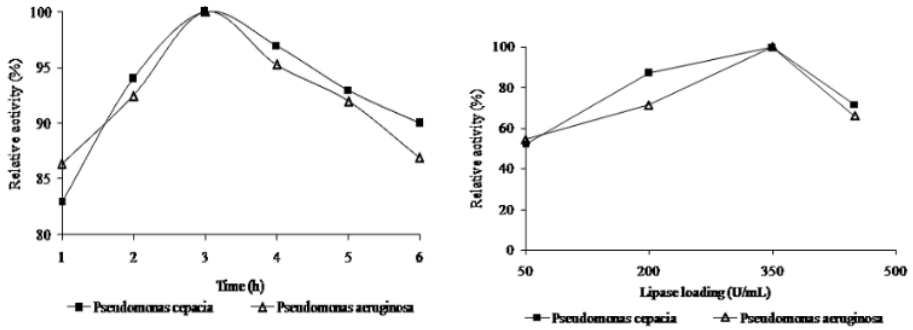

Fig. 3. (a) Effect of incubation time, and (b) lipase loading on the immobilize process. Immobilization condition: t = 3h, 100 mg of RHS-Fe_3O_4 in phosphate buffer (50 mM, pH 7.5), 180 rpm, and T = 25 °C.

This section employed various concentrations of crude lipase (50-450 U/mL) to immobilize on 100 mg of RHS-coated Fe_3O_4 nanoparticles. As seen in Fig. 3b, as the starting amount of lipase was raised, more immobilized lipase was produced, and the relative activity peaked at 350 U/ml. It appears that excessive enzyme molecule loading on RHS-Fe3O4 results in some undesirable protein-protein interactions, which therefore lowers the enzyme activity. [6].

3.4 Physicochemical properties of nanomaterials and immobilized lipase

X-ray diffraction (XRD) was used to determine the crystal structure of the magnetic nanoparticles obtained by coprecipitation method, which are shown in Fig. 4A. There are various possible iron oxide formations, including magnetite (Fe_3O_4), hematite (α-Fe_3O_4), maghemite (γ-Fe_3O_4). The characteristic peaks appeared at 18.59° (111), 30.38° (220), 35.62° (311), 53.85° (422), 57.30° (511), 62.97° (440), 72.62° (533) [14, 15]. The pattern's determined d spacing is similar to magnetite's d spacing. Because of the presence of non-equivalent iron cations in two valence states Fe^{2+} and Fe^{3+}, Fe_3O_4 demonstrates interesting properties in the crystal structure [16]. This nanoparticle has small size and large surface area and easy to be recovered because of the nanoparticles has a large magnetic susceptibility.

Magnetite-silica core shell structures are shown in Fig. 4B. The characteristic peaks appeared at 17.78° (020), 22.92° (110) (400) (220), 36.19° (040) (002) (332), 62.77° (320) (152) (651). The broad peak at high angels (4B) at 2θ between 20° until 30° are attributing to the peak of the siliceous material. The d spacing calculated from the pattern is close to those of iron silicate, ferrosilite and skiagite, syn. According to Lei et al. [17], By adding a magnetic field, Fe_3O_4/SiOx, which has a high saturation magnetization, can be quickly and readily separated from the reaction medium. Silica coating renders the sol anionic within the working pH range and is comfortable for biomolecules. Silica could coat the surface of magnetite NP with a strong affinity for iron oxide [18]. Furthermore, mesoporous silica shell is capable for the derivation of numerous functional groups.

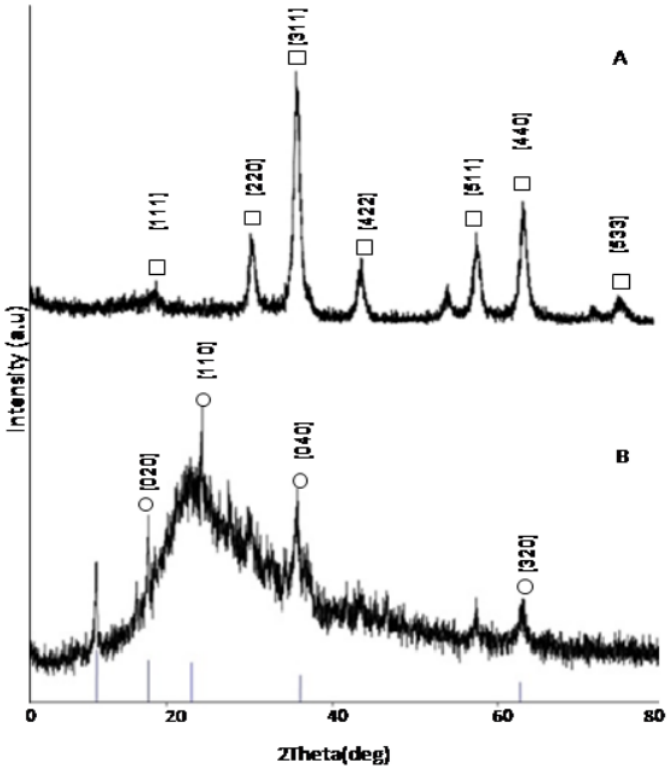

Fig. 4. XRD patterns of modified nanoparticles: (A) □ Fe_3O_4, (B) ○ SiO_2-Fe_3O_4

FT-IR spectroscopy was used to characterize the functionality of the resulting SiO_2/Fe_3O_4 nanoparticles. Fig. 5 shows the FT-IR spectra of the (A) Fe_3O_4 nanoparticles, (B) SiO_2-Fe_3O_4 nanoparticles and (C) immobilized lipase. The characteristic absorption peaks at 498, 585 and 586 cm^{-1} are attributed to the vibration of Fe-O bond structure (A), after SiO_2 is ated on the surface of Fe_3O_4 nanoparticles (B) and after immobilization of lipase (C). The main characteristic absorption bands such as δ(FeOH) deformation Si-OH stretching, Si-O bending, and Si-O-Si bending are demonstrated at 803–805 cm-1, 863–945 cm-1, and 1123–1123 cm-1, respectively. The common one with the broad bands at roughly 16 0 cm-1 and 3460 cm-1 is caused by the related water molecules' O-H bending and stretching. Peak at 420 cm-1 is indicative of lipase-nanoparticle surface bond g (C). Peaks between 2840 and 2975 cm-1 are indicative of C-H stretching vibration [19]. The sample exhibited the distinctive bands of both lipase and Fe3O4 after immobilizing lipase on the magnetic nanoparticles, but the characteristic peaks of lipase for the enzyme-bound nanoparticles are weak.

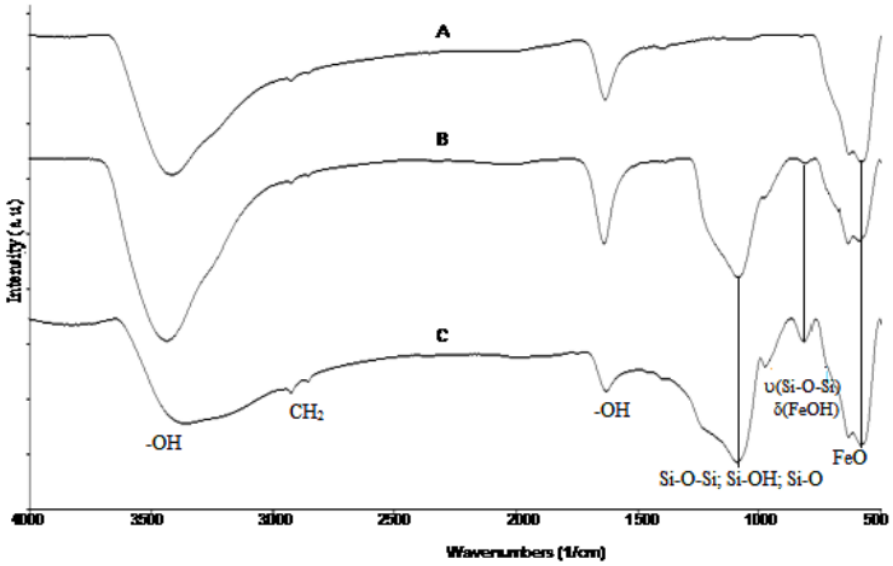

Fig. 5. FT-IR spectra of modified nanoparticles (a) Fe_3O_4, (b) silica-magnetic nanoparticles, (c) immobilized lipase

Since the covalent interaction between the lipase and carrier is of the same type as common protein bonds, there was no extra band in the spectra of the immobilized lipase. The amine groups of the enzyme and the support can react with glutaraldehyde to produce amide linkages that are similar to those found in free lipase. Lipase is thus successfully attached to the magnetic nanoparticles' surface.

The morphology of pure magnetic nanoparticle appeared to be spherical (Fig. 6a) with smooth surface. The core-shell nanoparticles were found to be textural [15] and the pore size measured as 4.5 nm. From FE-SEM result, Fe_3O_4 nanoparticles shell in spherical-magnet was calculated to be around 30-120 nm. Coating Fe_3O_4 nanoparticle shells with amorphous silica would result in smooth porous materials (Fe_3O_4-SiO_2), thereby increasing surface area (Fig. 6b). Furthermore, Fig. 6c shows the cluster of crosslinking agent glutaraldehyde at the surface of porous material interacting with lipase, and the immobilized lipase seemed to be fully bound on the surface of that porous nanomaterial (Fig. 6d).

3.5 Kinetics of immobilized lipase

The relationship between incubation pH (4-11) and the relative activity of free and immobilized *Pseudomonas aeruginosa* lipase (IPAL) was investigated at 30 °C for 30 min. As shown in Fig. 7 (a), the immobilized lipase exhibited broader range of pH compared to free lipase. The optimum pH for free lipase was found to be 8.0 whereas that of IPAL was 8.5. This indicates that no conformational changes affecting the pH stability of lipase occurred during immobilization. Similar results have been demonstrated in former literatures [20]. Protein is easy to undergo denaturation under extreme conditions of pH. Immobilization has improved catalytic activity probably by offering more stable environment to retain its required ionic state at or around the active site.

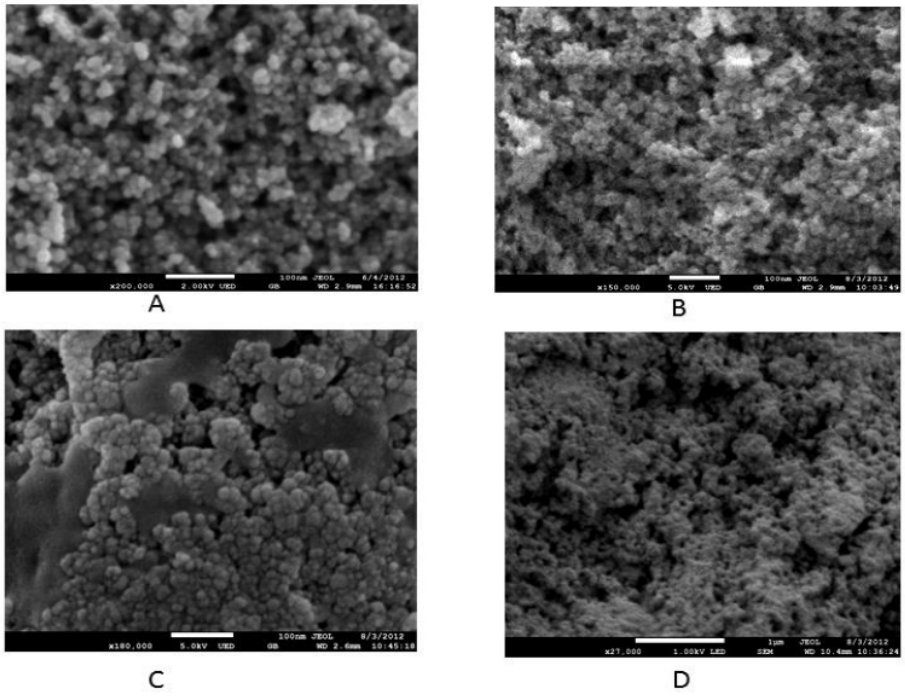

Fig. 6. FE-SEM image of (a) Fe_3O_4, (b) silica-magnetic nanoparticles, (c) silica magnetic-glutaraldehyde, (d) immobilized lipase

At constant pH levels of 8.0 and 8.5, respectively, the activities of free and immobilized lipase were evaluated at temperatures ranging from 30 to 70 C. The results are shown in Fig. 7(b); the optimal temperature of IPAL and free lipase are 40 and 50 °C, respectively. The activity of free lipase was almost completely lost at 60 °C whereas IPAL showed a broader range of temperature tolerance. Thus, when the temperature varied from 45 to 60 C, immobilized lipase was less sensitive to temperature variations than free lipase.

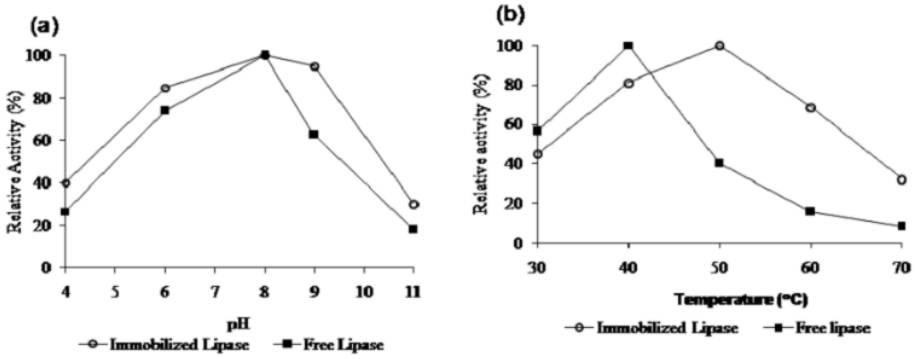

Fig. 7. (a) pH profile of free and immobilized lipase, (b) Temperature profile of free and immobilized lipase

To study the effect of lipase immobilization on rice husk silica-coated FeO_4 nanoparticles, kinetic constants (K_m and V_{max}) of free and immobilized lipase were determined. The results are demonstrated in Fig. 8, the K_m and V_{max} were 1.8 mM and 9.3 $mM.s^{-1}$, respectively for immobilized lipase, whereas those of free lipase were 1.5 mM and 6.4 $mM.s^{-1}$, respectively. The immobilized lipase demonstrated smaller V_{max} than free lipase. This might be due to some restrictions being imposed on the conformation of the active site of lipase upon immobilization. In accordance with Tran et al. [15] and Chen et al. [21], covalent binding may cause the structure of enzymes to become rigidified on the surface of support magnetic nanoparticles, inhibiting the active site of lipase and lowering lipase activity. Additionally, the orientation of the enzyme linked to the support may be hampered by the immobilization procedure.

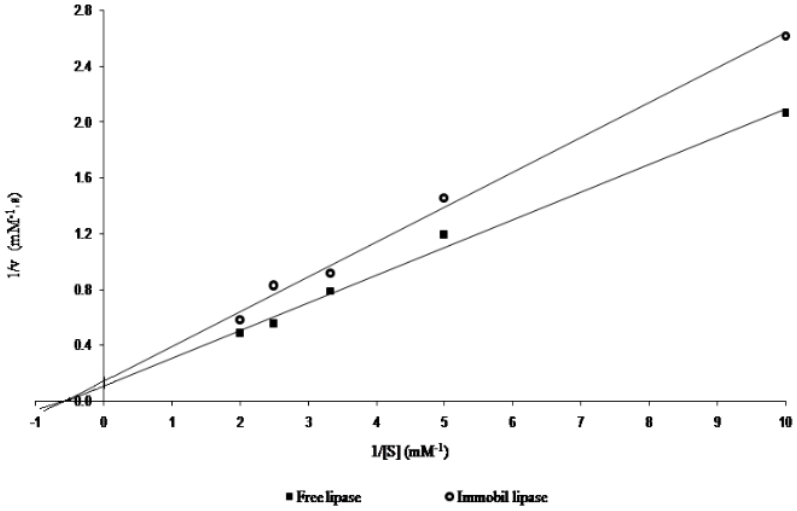

Fig. 8. Lineweaver-Burk plots for the hydrolysis of p-nitrophenylpalmitate by (■) free and (o) immobilized lipase

3.6 Reusability and thermal stability

Reusability is one of the relatively significant benefits of using immobilized lipase, particularly in the industrial sector. After one run of catalysis, the immobilized lipase was washed with phosphate buffer (0.1 M, pH 7.0), and its reusability was examined by reintroducing it into a brand-new p-NPP solution at 37 °C and measuring its activity. This process was repeated several cycles. The variation of the relative activity of IPAL after multiple-reuse is shown in Fig. 9(a). It was observed that after the 5th reuse the residual activity was still maintained at about 60% of the initial activity. Additionally, IPAL showed good stability and reusability. The gradual decrease of activity was considered as the denaturation and/ or partial leakage of enzyme from support during the process of reuse [22].

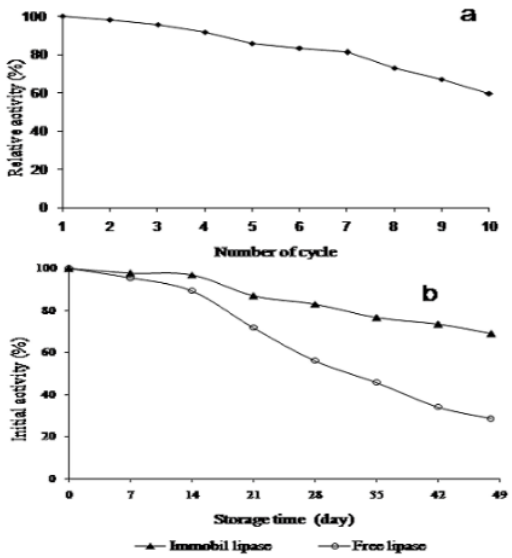

Fig. 9. (a) Relative activity during reuse of the immobilized lipase; (b) Storage stability of free and immobilized *Pseudomonas aeruginosa* lipase

For 49 days, free and immobilized lipase were maintained at 4 °C under identical circumstances, and their activities were recorded every seven days (Fig. 9b). Immobilized lipase had a slower rate of activity loss than free lipase at the same temperature [23]. Immobilized lipase preserved over 68.13 percent of its initial activity after 49 days of storage, but free lipase only kept 23.16 percent. The immobilized lipase was able to be stored and used for a longer period of time during the experiment than the free lipase, according to the data.

4. CONCLUSIONS

This article discussed a feasible technique for covalently immobilizing lipase on the surface of Fe3O4 nanoparticles coated with rice husk silica. The FE-SEM image and XRD pattern demonstrated that the silica-coated magnetic nanoparticles did not alter the properties of Fe3O4 and that their shells were spherical. The confirmation of the immobilization of lipase into nanoparticles was performed using the FT-IR spectrum. The results demonstrated that immobilization significantly increased lipase's thermal, pH, and storage stability. Additionally, kinetic studies found that immobilized lipase activity was efficiently stabilized. The immobilized lipase's hydrolysis kinetics, which used the substrate 4-Nitrophenyl palmitate, followed the Michaelis-Menten model, with a maximal reaction speed and a Michaelis constant of 4.0 mM.s-1 and 0.63 mM, respectively.

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