Egypt. J. Chem. **Vol. 63,** No. 11 pp. 4463 - 4468 (2020)

Egyptian Journal of Chemistry
http://ejchem.journals.ekb.eg/

Enhanced Biological Activity of *Candida rugosa* Lipase via Immobilization on Magnetic Nanoparticles

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Abstract

Magnetic nanoparticles Fe_3O_4 were synthesized by the co-precipitation method. Magnetic nanoparticles were activated and modified with (3-aminopropyl) triethoxysilane (APTES) and glutaraldehyde (GA), respectively. Then, these nanoparticles were characterized by transmission electron microscope and Fourier transform infrared. *Candida rugosa* lipase (CRL) was successfully immobilized on GAMNPs by the chemical method via forming a covalent bond between a functional group on an enzyme protein molecule and a reactive group on the surface of solid support by chemical interaction. CRL-Fe_3O_4 displayed an elevate recovery rate of upward to 1348%, which was a 13-fold improvement in its free one. Compared to a free enzyme, the pH and thermal properties of the immobilized lipase were also increased. This study clearly indicates that GAMNPs could be deemed good support for the immobilization of enzymes.

Keywords: Immobilization, *Candida rugosa*, magnetic nanoparticles, enzymatic properties

1. Introduction

The activities of enzymes have been identified and exploited since ancient times. They are biocompatible, biodegradable and are derived from renewable resources[1]. Several enzymes have been used in different industries as industrial biocatalysts such as food, dairy, pharmaceutical, detergent, textile, bioenergy, and cosmetics[2]. Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) were gained considerable importance. They are used in most of the fields mentioned for enzyme applications. This high interest in lipases is mainly due to their features regarding enantioselectivity, regioselectivity and broad substrate specificity[3]. In spite of the advantages of lipase, it is very sensitive to the environmental conditions, for example, showed very low activity and reduced stability in strong acid, alkali, heat, organic solvents, mechanical force and other conditions; also, lipase is costly and difficult to retrieval from the reaction system and recycle, these problems are caused the high cost of the products, thus restricting the application of lipase in the industrialization[4]. The immobilization of enzymes is a typical solution for the height price of enzymes and other problems mentioned previously[5].

To date, various types of materials, such as synthetic polymers[6], natural polymers[7] and inorganic materials[8] have been utilized to immobilize enzymes and to some degree enhance their catalytic efficiency. In recent years, there has been widespread use of many types of nanostructured materials, including nanorods, nanotubes, nanowires, nanorings [9]. The advantage of employing these nanoscale composites for immobilization is that they decrease the hindrance to transfer and optimize the usable surface area, raising the successful loading of enzymes. *Candida rugosa* lipase dropped nearly 85% of its original activity following immobilization on glutaraldehyde-activated nanofibrous membranes [10]. Li et al. [11] lost nearly 73.3% of the initial activity for nuclease P1 when immobilized it on glutaraldehyde cross-linking macroporous absorbent resin. The decline of activity through immobilization is likely as a result of limitations of mass transfer following immobilization or inactivation of the enzyme during immobilization. To overcome this

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Receive Date: 15 March 2020, Revise Date: 06 April 2020, Accept Date: 27 April 2020
DOI: 10.21608/EJCHEM.2020.25662.2512
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problem, magnetic nanoparticles (MNPs) depend on iron oxides have been extensively studied due to their characteristic features that improve the performance of biocatalysts , *i.e*., High surface properties, decrease transfer resistance and, ease separates from mix reaction using a piece of the magnet [12]. Magnetic nanoparticles are an extremely valuable substrate for the attachment of homogeneous organic and inorganic catalysts [13]. Besides, this technique has been applied in numerous industries such as anti-cancer drugs supports[14], magnetic resonance imaging (MRI)[15] and, biosensors[16].

The common enzymatic strategies in immobilization mainly comprised entrapping and adsorption that is physical strategy, which adsorbent or enter the enzyme into the carrier without to need for chemicals materials, conversely, a chemical strategy included cross-linking and covalent bonding which are used chemical substances. [17]. Hence, covalent bonding has drawn increasing attention because it has the benefit of strong interactions between the enzyme and the carrier which prevent enzyme leakage [18].

Thus, in the present work, magnetic nanoparticles were prepared and then used as a carrier for immobilized of C. *rugosa* lipase via covalent bonding method. The ideal condition of immobilization and, morphological traits, in addition, enzymatic properties were studied.

2. Materials and methods

2.1 Materials

Candida rugosa lipase (CRL, EC 3.1.1.3) was gained from Sigma Aldrich (Copenhagen, Denmark). Coomassie brilliant blue G-250, KOH, and bovine serum albumin (BSA) were bought from Shenshi Chemical Industry (Wuhan, China). APTES, glutaraldehyde and with extra reagents of analytical grade such as aqueous ammonia ($NH_3.H_2O$), ferrous chloride tetrahydrate (Fe $Cl_2.4H_2O$), hexahydrate ferric chloride (Fe $Cl_3.6H_2O$), ethanol, acetone, and olive oil were purchased from Sinopharm Chemical Reagent Company (Shanghai, China).

2.2. Methods

2.2.1 Preparing of MNPs

The technique of co-precipitation was used to prepare magnetic nanoparticles according to [19, 20]. Below aerobic circumstances, the Fe2+ can be readily oxidized to Fe3+. Therefore, we have to attempt to raise the oxygen in the reaction system. Accordingly, the entire reaction is beneath anaerobic conditions. Briefly, It took 500 mL of a flask, added a specific percentage of the content quantity ($FeCl_2$. $4H_2O$ and $FeCl_3$. $6H_2O$), then applied 150 mL of deionized water (DW) to dissolve it and permeate nitrogen to protect the oxidized ferrous ions. 25% of NH_3. H_2O was slowly added to the above solution and stirred vigorously at 70 °C to reaches pH about 10. Then, quickly heated to 80°C and retained for 30 minutes. Fe_3O_4 nanoparticles formed were magnetically recovered and rinsed with deionized water three times to remove excess ammonia.

2.2.2 Activation and modification of surface MNPs

In 2 mL of ethanol (95%), 100 mg of Fe3O4 nanoparticles were dispersed by sonication equipment. After that 60 µL of (APTES) was placed in this mixture. The combination of the reaction was shaking speed at 200 rpm for 24hr. Magnetic separation separated the supernatant and washed the precipitates with ethanol and distilled water. Then 4 mL of glutaraldehyde (10%) was placed to the APTES-coated MNPs precipitates and the interaction remained at room temperature for 3 h. After 3 h the magnetic field separated the glutaraldeh yde activated particles and then washed with distilled water[21].

2.2.3 Immobilization of Lipase

Covalent bonding combined lipase molecules with carriers. CRL were distributed in the phosphate buffer with GMNPs. The immobilized enzyme was washed with buffer three times to discarded surplus unbound lipase. The residual protein content in the supernatant was evaluated employing the Bradford protein assay using bovine serum albumin (BSA) as the standard protein to determine the quantity of immobilized enzyme[22].

2.2.4 Activity assay for immobilization CRL

The activity for immobilized and free lipases was tested via hydrolysis process according to[23]. 5 mL 0.1 M phosphate buffer, 4 mL olive oil emulsion, a specific amount was added to the reaction system from the immobilized and free enzyme. Continuous shaking reactions at 200 rpm were conducted at 40 ° C for 30 min. After that, added 15 mL of stop solution to terminate the interaction. As a pH indicator was used Phenolphthalein solution (0.05%, w/v). (0.05 M) NaOH for determining the quantity of lipase that catalyzes the hydrolysis of olive oil the free fatty acids, consequently the activity of enzyme was determined. Activity Unit (U) was expressed as[24] .

$$\text{Specific activity (U/g protein)} = \frac{\text{initial activity}}{\text{protein content of immobilized lipase}}$$

$$\text{Immobilization efficiency (\%)} = \frac{\text{immobilized protein}}{\text{total loading protein}} \times 100\%$$

$$\text{Activity recovery (\%)} = \frac{\text{activity of immobilized lipase}}{\text{total activity of free lipase}} \times 100\%$$

2.2.5 Optimization conditions for immobilization of CRL

Immobilization conditions should always be investigated and improved to keep the original activity of enzyme and to reach higher efficiency of immobilization. The effect of CRL loading (25–50 mg), time (30–180 min), pH value (6-8.5) and, temperature (20–45 ° C), on an activity recovery, specific activity, and efficiency of immobilization were studied.

2.2.6 Measurement

The morphological and dimensions of the composites were conceived by Transmission electron microscope (TEM) H-7000FA (Hitachi, Japan). A drop of ethanol suspension containing the specimens was added to copper grids coated with carbon and dried at room temperature. In transmission mode, using the KBr pellet technique, Fourier transform infrared spectroscopy (FT-IR, VERTEX 70, Germany) with a length of 400−4000 cm has been used −1, the information of functional groups and chemical bonds in the particle was collected.

2.2.7 Enzymatic Properties of the Immobilized CRL

An enzyme's stability is critical to its practical uses. To study the stability of the immobilized CRL, its activity was determined at different pH values and temperatures for 1 h after incubation.

3. Results and Discussion

3.1 Synthesis and characterization of CRL-Fe_3O_4

After the synthesis of Fe_3O_4, then vacuum dried. CRL immobilized on Fe_3O_4 (Figure 1). The Transmission electron microscopy (TEM) investigations monitored the morphological variations before and after immobilization as shown in Figure (2 a,b). As a result of the immobilization CRL-Fe_3O_4, the particles resort to agglomerate.

FT-IR spectra were also used to observe Fe_3O_4, CRL and CRL-Fe_3O_4 are displayed in Figure 3. A notable characteristic absorption at 580 cm^{-1} attributed to Fe-O bond of magnetite[25]. The C–H stretching vibrations of Fe_3O_4, CRL and CRL-Fe_3O_4 are marked at 2865 and 2925 cm^{-1}. The 900–1150 cm^{-1} absorption band is due to the Si-O bond

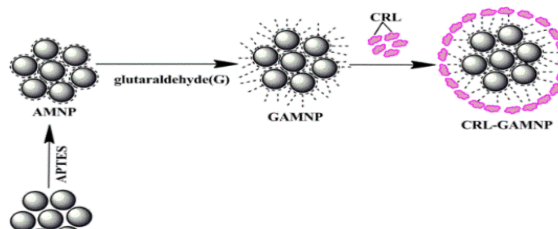

Figure 1. Vacuum dried. CRL immobilized on Fe_3O_4

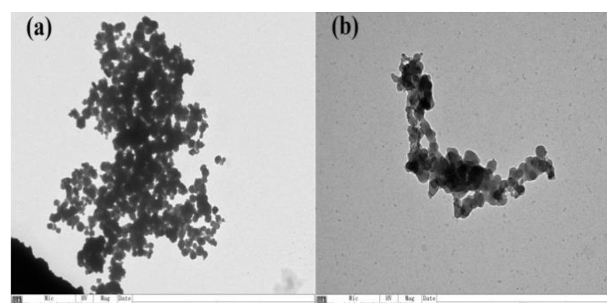

Figure 2. TEM images: (a) Fe_3O_4; (b) CRL-Fe_3O_4.

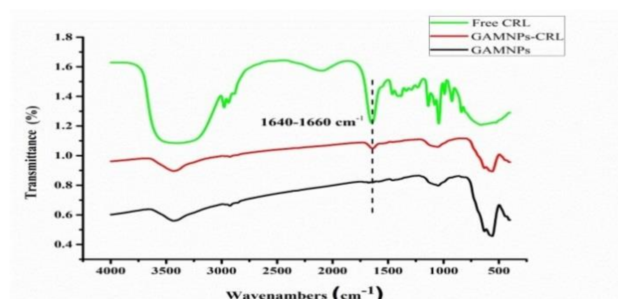

Figure 3. Samples spectra of Fourier transforming infrared (FT-IR), GAMNPs; GAMNPs-CRL and CRL

vibration of Fe_3O_4. Bands at 1640-1660 cm−1 define the enzyme amide I and II, referring respectively to the stretching C = O and N – H[26] .

3.2 Influences of Immobilization Parameters on the Enzyme Activity

As is expected, activity recovery and efficiency of immobilization were influenced by conditions of immobilization. The maximum activity recovery was 698%, at CRL concentration of 30 mg in the range of 20 to 45 mg CRL, and the efficiency of immobilization was constantly reduced at amounts greater or lesser than 30 mg (Fig. 4a). High enzyme loading concentrations can result in multilayer adsorption of the hydrophobic structure, which reduces the surface area also limits diffuse increase. As assumed that elevate enzyme concentration results in a higher rate of reaction from the reaction kinetics

aspect, the comparable boost in viscosity for height enzyme concentration may reduce the mixing competence of the CRL and MNP reaction mixture[27]. Such two impacts competed with each other and it appears that if the enzyme load is to height, the kinetic benefit from a higher concentration of enzyme is diminished by the poor efficiency of mass transfer, resulting in reduced enzyme activity.

The pH value is a significant factor in many reactions as well as in enzyme immobilization. As illustrated in Fig. 4b, an acidic condition is helpful to Schiff base interaction between GAMNPs and lipase. Therefore, at lower pH levels, the activity recovery and immobilization efficiency was improved to the extent 970%. Also, a basic buffer appeared adverse to the activity of the enzyme, while acidic conditions had a positive impact on the activity of the enzyme, with the highest value pH 6.5. The obvious explanation was that the variation in the reaction system pH would influence the activity of the enzyme's specific group (i.e., carboxylic groups, COOH) and thus minimize CRL adsorption on the supporting material [28, 29].

As shown in Figure 4c, in the first 1.5 h of immobilization time, the efficiency of immobilization and enzyme activity gradually rose. The recovery of activity subsequently achieved the highest value of 1154% then, decreasing when the time of immobilization surpassed1.5 h, while immobilization efficiency was slightly increased to 2.5 h.

The immobilization temperatures ranging from 20 to 45 ° C, the efficiency of immobilization continuously improved. At the same time, despite the high temperature, activity recovery increased, resulting in a maximum activity recovery of 1348% at 35 ° C. Afterward, the recovery of activity began to diminish due to lipase thermal deactivation (Figure 4d). Therefore, 35 ° C was chosen as the optimal temperature for immobilization. The sensible explanation is the low temperature enhances enzyme features, such as stability and conformation, and prevents denaturation of protein in solution buffer that accordance by Liu et al[30], which stated an increase in enzyme reaction rate with a rise in temperature overall to a specific level, and then the rising temperature will induce denaturation of protein and therefore reduces the rate of reaction.

3.3 pH and Thermal Properties of immobilized CRL

Figure 5a shows the free and immobilized CRL pH stability. The relative activity showed a similar pattern, as can be seen, whereas the activity of the immobilized CRL decreased more gradually and to a lesser degree than free CRL. For the immobilized enzyme, in the range of pH 5.5~7, the remaining enzyme activity continued with simple changed. In the acid and alkaline environment, the activity of the

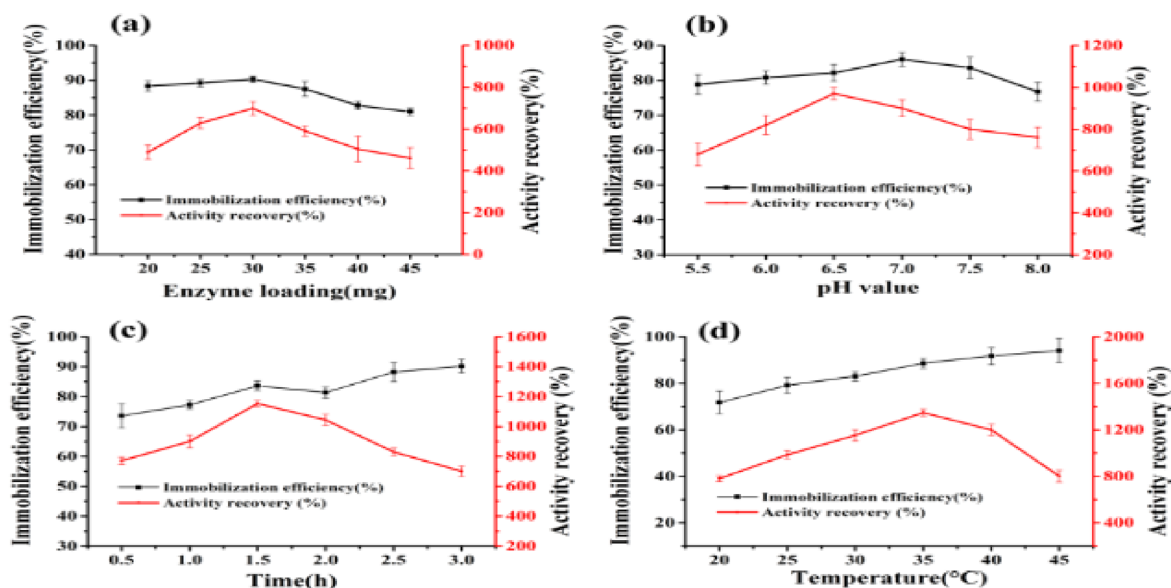

Figure 4. Effect of four variables on activity recovery and immobilization efficiency for lipase. (a) Enzyme loading; (b) pH value; (c) immobilization time; (d) immobilization temperature.

immobilized enzyme is also constant than that of the free one. When the pH value is 5, the free enzyme's remaining activity is 61.5 %, while the immobilized enzyme is 79.2 %, and the immobilized enzyme's remaining activity is 68.4 %, higher than that of the free enzyme when the pH value is 10. After immobilization, the high residual activity is maintained but under the influence of various pH values. The dissociation effects of the active centres on the proteins are different and their specific spatial conformations are altered resulting in a reduction in the catalytic activity [31].

As can be shown from the (Fig. 5b), the CRL-Fe_3O_4 is more stable to temperature changes from free enzymes. Free enzyme began to be strongly inactivated from 50°C, and when the temperature increased to 70°C, the remaining activity was just 31.2 %. In contrast, immobilized CRL began to inactivate at 60°C slowly. When the temperature was 70°C, the remaining activity of the immobilized enzyme was as high as 65.9 %. The increase in temperature stability of the immobilized enzyme is attributable to the rigidity of carrier GAMNPs that enhances the rigid structure of the lipase and prevents the enzyme from melting the chain or configuration at high temperatures, thereby delaying the decrease of the enzyme activity [32, 33].

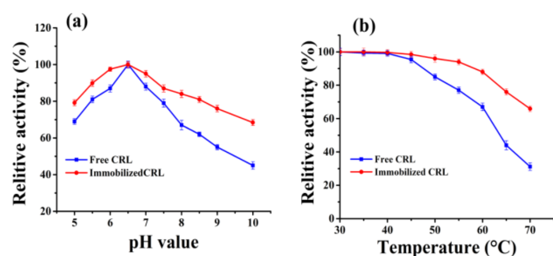

Figure 5. Influence of relative activity of GAMNPs-CRL. pH value (a) and Thermo-stability (b).

4. Conclusion

Fe_3O_4 was prepared in an aqueous solution and activated, then, used this carrier for immobilizing CRL. Additionally, we increased the recovery rate 13-fold by improving the conditions for the immobilization. The optimal conditions for immobilization were: lipase loading 30 mg, pH 6.5, 1.5 h, and 35 ° C. In enzymatic properties, the results revealed a significant increase in the temperature and pH stability of the immobilized enzyme comparable with the unbinding counterpart. The CRL-GAMNPs is promising as a nano-biocatalyst carrying out in many industries and in different fields.

Acknowledgment: The authors wish to thank the Environment and Water Directorate, Ministry of Science and Technology for their support in this work.

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