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Activity and stability of immobilized Candida rugosa lipase on chitosan coated Fe_3O_4 nanoparticles in aqueous and organic media

Mohamed A. Abd-Elhakeem[1], Ahmed M. Elsayed[2], Taher A. Alkhulaqi[1]

1. College of Biotechnology, Misr University for Science and Technology, Giza, Egypt

mohamed.abdelhakim@must.edu.eg

2. Research and Development Center, Misr University for Science and Technology, Giza, Egypt

ahmed.elsayed@must.edu.eg

3. College of Biotechnology, Misr University for Science and Technology, Giza, Egypt

Dr.taher_biotech@hotmail.com

ABSTRACT

Fe_3O_4 (magnetite) nanoparticles were prepared by coprecipitation method, coated by chitosan and functionalized by glutaraldehyde. Lipase enzyme from *Candida rugosa* was immobilized on the prepared particles via cross linking reaction. Synthesis steps and characterization were examined by XRD, TEM, and FTIR.

The immobilization conditions were 10 mL of phosphate buffer (0.1 M, pH 6.5) containing 30 mg of functionalized magnetic chitosan nanoparticles and 2.0 mg·mL^{-1} of lipase, immobilization temperature of 4 ℃ and immobilization time of 1 h. Under these conditions, lipase was successfully immobilized with loading capacity of 87 mg/g.

The immobilized enzyme showed good operational and storage stability, where it remained stable after 30 days of storage at 4◦C.and retained about 61% of its initial activity after twenty repeated uses. Finally enzymatic catalyze synthesis of butyl and hexyl oleate at 40 ◦C with shaking (200 rpm) was realized in *n*-hexane and confirmed by GC analysis.

Indexing terms/Keywords

Lipase; magnetic nanoparticles; chitosan; immobilization

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1. INTRODUCTION

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is regarded as one of the most important and widely used enzyme in industrial and analytical chemistry applications [1-3]. Lipase catalyzes the hydrolysis of triacyl-glycerol into glycerol and fatty acids in aqueous media and the reverse reaction (formation of ester) in organic solvents [4].

In industry the free enzymes are generally unfavorable because it is difficult to recover for reuse, and it has low stability [5].

Enzyme immobilization is an efficient technique to enhance the enzyme stability and to allow its reuse. So many immobilization methods have been reported in the literatures with different carriers such as glass beads, alginate beads, activated carbons and others [6-9]. Magnetic support was firstly applied in enzyme immobilization in 1973 which allow the magnetic separation of the immobilized enzyme from the reaction system [10]. In the magnetic separation there is no need for expensive and time consuming techniques like chromatography, filtration, and centrifugation.

With arising of nanotechnology, Fe_3O_4 (magnetite) nanoparticles are widely used in enzyme immobilization due to its biocompatibility, high surface area to volume ratio [11].

Magnetite nanoparticles **(MNP)** are commonly superficially modified by coating with organic, inorganic or polymers. Polymeric coatings are characterized by better dispersion and stability in solvents than inorganic or small organic compounds [12]. For instance, chitosan is the partially deacetylated derivative of chitin (2-amino-2-deoxy-(1 → 4)-β -D-glucan). The presence of amino groups allows the conjugation with proteins via cross linking agents such as glutaraldehyde, N-(3-dimethylaminopropyl)-N/ethylcarbodiimide (EDC), or N-hydroxysuccinimide (NHS) [5]. Chitosan as immobilization support is characterized by widely commercial availability, non-toxicity, and low cost [13]. In the present study lipase from *Candida rugosa* was immobilized on chitosan coated magnetite nanoparticles. Then activity, reusability and stability in both aqueous and organic solvents were investigated.

2. MATERIALS AND METHODS:

2.1. Materials:

Iron (II) chloride tetrahydrate, iron (III) chloride hexahydrate were purchased from Merck (Germany). Candida rugosa lipase (> 2U/mg), chitosan (low molecular weight), glutaraldehyde, 4-nitrophenyl palmitate, and Bradford reagent were purchased from Sigma–Aldrich (USA). Acetic acid and sodium hydroxide were purchased Fluka (India).

2.2. Preparation of Fe3O4 nanoparticles:

The magnetite (Fe_3O_4) nanoparticles were prepared by a chemical co-precipitation of Fe (III) and Fe (II) ions. 500 mL of FeCl2 (0.2 mol/L) and 500 mL of FeCl3 (0.3 mol/L) aqueous solution were mixed under the presence of N_2 gas. When the solution was heated up to 60 ◦C, NH_4OH (25% w/v) was dropped into the mixture with vigorous stirring. The black precipitate that formed was collected by magnetic separation, washed several times with deionized water and finally dried at room temperature.

2.3. Preparation of chitosan coated Fe3O4 nanoparticles:

Chitosan solution was prepared by dissolving 0.5 g of chitosan in 50 ml 2% (v/v) acetic acid. 10 ml of this solution was used to suspend 30 mg of MNP. The mixture was vigorously stirred for 30 minutes then 2.5 ml NaOH (1N) was added drop wisely to precipitate the coated MNP. The particles were magnetically separated, washed several times by deionized water and suspend in 10 ml deionized water.

1% (v/v) of glutaraldehyde was added to the suspension with continuous stirring at room temperature for 4 hours. The particles were recovered by applying a magnet, washed and dried.

2.4. Immobilization of enzyme:

0.3g Functionalized MNP was mixed with 10 ml of (2mg/ml in 0.1 M phosphate buffer pH 6.5) Candida Rugosa lipase. The mixture will stirred for several minute and allowed to stand for 24 h at 4oC. The particles were separated by applying outer magnetic field and washed several time by buffer solution.

The amount of immobilized lipase linked with the modified MNP was determined by the measuring of the initial concentration of lipase and its concentration in a supernatant after immobilization using the Bradford protein assay method. A calibration curve constructed with lipase solution of concentration (0.5–2 mg/mL) was used in the calculation of enzyme concentrations. All data used for calculation are average of triplicate of the experiments.

2.5. Characterization:

Structure and size of crystals were determined by XRD on a Rigaku MINIFLEX II X-ray diffractrometer using Cu Kα radiation (λ = 1.540562). TEM observation was performed on a microscope (JEOL JAM-2100-HR-EM). Fourier transform infrared spectroscopy (JASCO model 6100 FT- IR spectrophotometer) was used to study chemical bonds between native and coated magnetic particles.

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2.6. Enzyme assay:

Activity of the free or immobilized lipase was assayed using 0.5% (w/v) p-nitrophenyl palmitate in isopropanol as substrate. Fifty milligram of immobilized lipase was suspended 3 ml of 0.05 M phosphate buffer solution (pH 7.0) contain 0.1 ml triton. The mixture was added to 0.1 ml of p-nitrophenyl palmitate (p-NPP) solution and the solution was incubated at 30 ◦C for 10 min, the absorbance at 410 nm was measured with a UV/VIS spectrophotometer (Shimadzu UV1800). One unit (U) of enzyme is defined as the amount of enzyme which liberates 1 mmol p-nitrophenol per minute under assay conditions.

2.7. Operational and storage stabilities:

Free and immobilized enzymes were stored at 4 ◦C in 0.05 M phosphate buffer (pH 7.0) for 30 days. The storage stability of enzymes was determined by periodical measurement of the activity at regular time intervals. The residual activities were calculated as percentage of the initial activity.

The operational stability (reusability) of immobilized lipase was studied by detecting the residual activity for many cycles. At the end of each cycle, the immobilized lipase was completely washed to remove any residual substrate. Then they were reintroduced into fresh reaction medium and the supernatant was assayed for activity.

2.8. Biocatalysis in organic solvent

An esterification reaction was performed between oleic acid and two alcohols 1-butanol or 1-hexanol 40oC as follow: an equimolar (0.5 mmol) mixture of oleic acid, 1-butanol or 1-hexanol was added to 30 mg immobilized lipase dispersed in 1 ml hexane. 20 mg of molecular sieve (3A˚) was added for the adsorption of water generated during esterification. Samples taken at different time intervals were analyzed for residual 1-butanol or 1-hexanol by gas chromatography (HP 6890 equipped with a flame ionization detector and a ZB1, 30m x 0.25 mm x 0.25 μm column). The analysis conditions were set as follows: oven temperature: 100°C to 300°C with 10°C/min heating rate, injector temperature 275°C, detector temperature 300°C, carrier gas (helium) flow 1.5 mL/min). The formation of esters was confirmed GC (HP-5890) equipped with HP-5972 mass spectrometer under the following conditions: inlet temperature: 300˚C, mobile phase helium, flow rate: 1ml/min., oven temperature program: initial temperature 80˚C, 10˚C/min, up to 280˚C for 10 min. HP-innowax column, 30m X 0.25 I.D. MS detector temperature: 300˚C. The fragmentation pattern of the obtained mass spectra was analyzed by Wiley7N mass library.

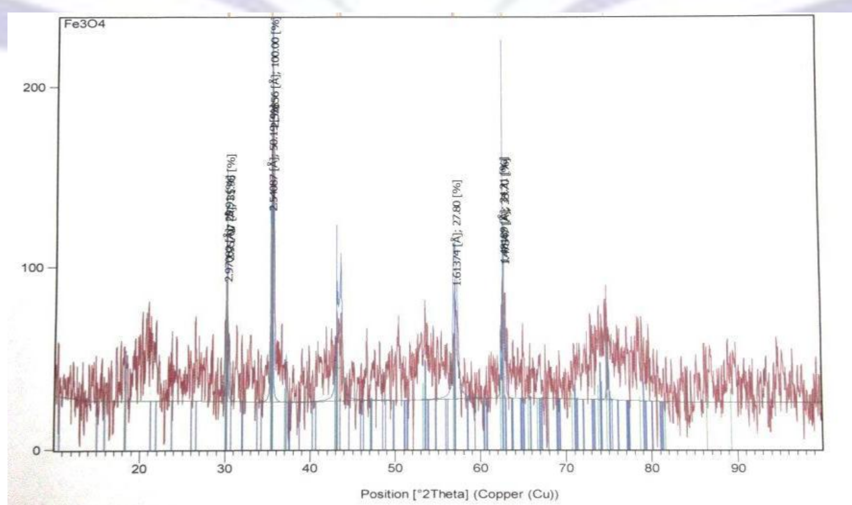
3. RESULTS AND DISCUSSION:

3.1. Preparation and characterization of the Fe3O4 and chitosan coated Fe3O4

Although, various methods have been reported for synthesis of MNP in the literature, the chemical co-precipitation method, as a simple and fast method was used in this study. The drawback of this method is the possibility of formation of maghemite Fe2O3 beside the magnetite Fe3O4 [14]. Under our conditions magnetite is the only mineral phase formed in two mineral forms cubic and orthorhombic as confirmed by The X-ray diffraction analysis (Fig.1). X-ray diffraction of the synthesized particle shows the characteristic peaks of magnetite. The average grain size was calculated by using data from their XRDPs and the well-known Scherrer equation, which is represented as:

$$d = K \lambda / \beta \cos \theta$$

Where d is the average diameter of grain in nm; K is the dimensional factor (0.9); λ is the X-ray wavelength (0.154 nm); β is the line broadening at half the maximum intensity in radians and Ө is the Bragg´s angle. Calculated values of average diameter of grain were about 16.5 nm.



Fig(1) X-ray diffraction analysis of the prepared magnetite

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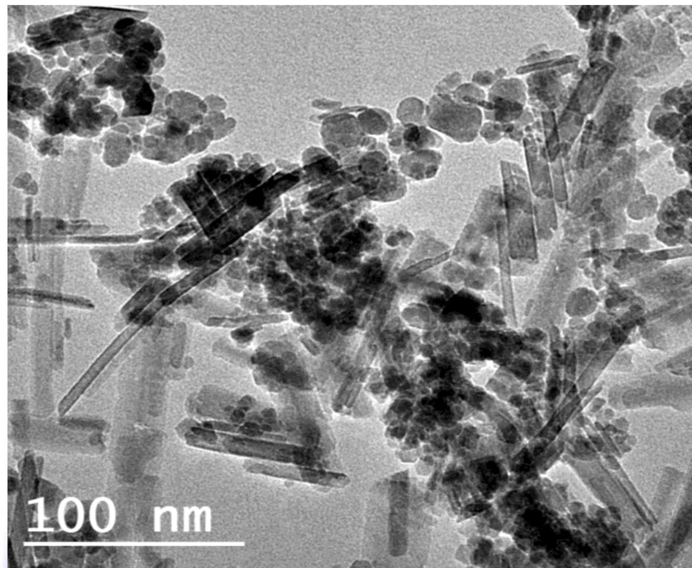
The TEM showed that the morphology of the obtained Fe2O3 nanoparticles are varied from random sphere-like to rod-like of average size 10-20 nm. (Fig. 2)



Fig(2) TEM images of magnetic nanoparticles

The successful formation of chitosan-Fe_3O_4 and Glutaraldehyde – chitosan linking was confirmed by the FTIR spectroscopy. The spectrum of nanoparticles coated only with chitosan shows peaks at 3439, 2936 and 1650 cm^{-1} and indicates the stretching vibrations of OH, aliphatic CH and amide group, respectively. The stretching vibrations of NH_2group of chitosan were masked by the OH group peak. The characteristic vibration peak at 1631 cm^{-1}increase with the C=N groups formation as a result of glutaraldehyde crosslinking reaction between amino groups of CS and carbonyl groups of aldehyde.

3.2. Immobilization efficiency and Enzyme assay:

The relation between immobilization conditions and enzyme activity has been investigated in many reports **[10-14]**. According to the literatures, we have chosen the best conditions without any modifications. Under these conditions, the covalent bonding between the modified magnetic nanoparticles and the amino group of lipase has taken place. The amount of the immobilized lipase was found to be **87 mg/g** of magnetic nanoparticles. The activity of the immobilized lipase was retained **86 %** of the added enzyme and then it was used for further investigation. The retention in activity has been reported after enzyme immobilization **[11-12]**. This may be due to modification in three-dimensional structure of the enzyme, which causes conformation change of the active center.

3.3. Storage and operational stability in aqueous media:

One of the key factors to be considered when using immobilized lipases is the ability to be stored without affecting the activity[15]. However the storage stability of the immobilized enzymes was clearly better than the free lipase. Where the immobilized enzyme retained 90% of its activity but the free enzyme retained 52% of its activity after 30 days (fig 4). The obtained results showed that through immobilization, the enzyme gained more stable character than the free one.

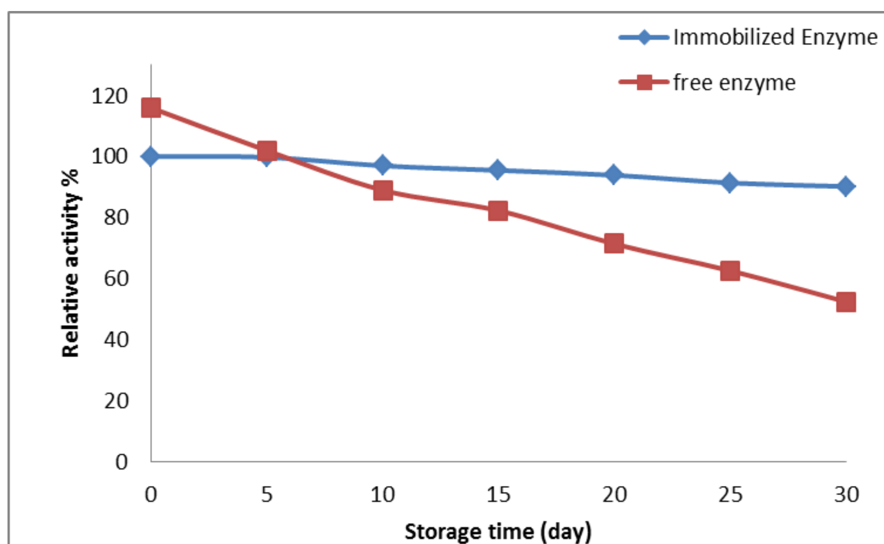


Fig (3) Storage stability of free and immobilized lipase over 30 days.

In the industrial applications, the possibility of the reusable catalyst in reactions is an important aspect that should be taken into account in the design of new supports for enzyme immobilization. The chitosan coated MNPs can be effectively separated from the reaction mixture with the use of a magnet. The operational stability of the immobilized lipase was investigated for **20** consecutive cycles of p-nitrophenyl palmitate hydrolysis under the enzyme assay conditions.

As shown in Fig. [5], after 10th use, the residual activities for the studied particles were found about **86%** and at the end of the20th use, lipase activity had dropped to about **61.0 %** of its initial activity. The obtained results indicate that the lipase-immobilized chitosan nanoparticles can be used for 20 cycles with significant catalytic activity. The decreasing in the values of residual activity could be caused by the denaturation or the leak of lipase from the prepared magnetic supports and it is documented in the literatures **[12-15]**.

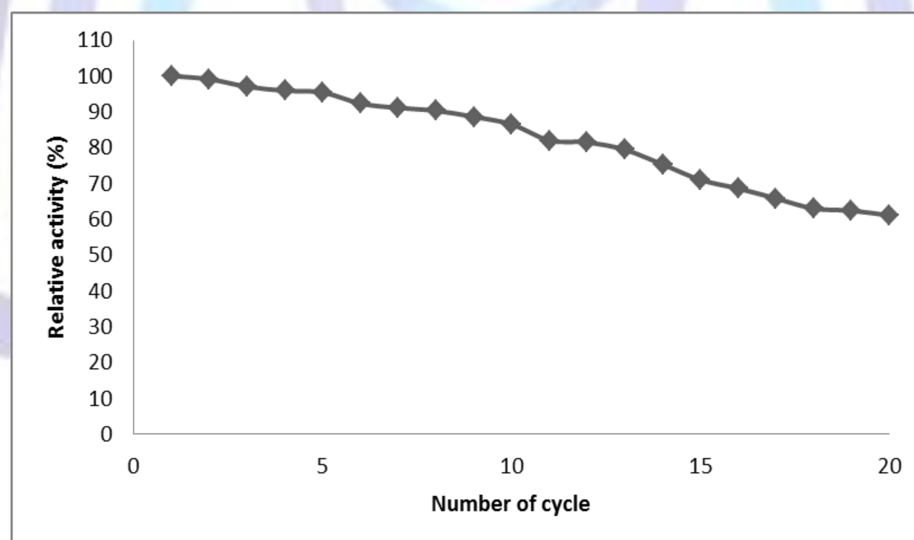


Fig (4) Operational stability of lipase immobilized on CMNP.

3.4. Biocatalysis in organic solvent:

Butyl and hexyl oleate are often used in cosmetic formulations, food industry and rubber manufacture [16]. The prepared nanoparticles have excellent dispersability in hexane and so hexane was used as a biocatalysis medium. The conversion yield of the starting materials to the corresponding ester was 82% after 48 h of reaction performance at 40°C. Mass spectrometry showed m/z values at 338, 366 correspond to molecular weights of butyl oleate and hexyl oleate respectively.

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4. CONCLUSION:

Lipase was successfully immobilized on chitosan coated magnetite via glutaraldehyde cross linking. The amount of immobilized enzymes, stability and reusability were investigated and the obtained results were in agreement with most published reports. It could be concluded that the immobilized lipase has good durability and reusability. Also we have introduced a green approach to synthesis of industrially important esters

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