Revista Mexicana de Física S **58** (2) 47–51 DICIEMBRE 2012

Immobilization and characterization of the Candida rugosa lipase enzyme on magnetic particles

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Recibido el 25 de junio de 2010; aceptado el 12 de octubre de 2010

Magnetic nanostructures have gained a remarkable interest in the last years both for basic research and applied studies. The use of magnetic nanostructures has been verified in biochemistry, biomedicine, and waste treatment among other fields. Their large magnetic moments gave them a broad range of applications which allow them to be transport and driven by external magnetic fields. The magnetic structures have also a great potential in biotechnological processes taking into account that they can be utilized as a carrier for enzymes in different biocatalytic transformations. In this work, the lipase enzyme Candida Rugosa was covalent bound to coated magnetic particles via glutaraldehyde activation in order to form a biocatalytic system. The analyses of XRD patterns indicated that the resultant magnetic particles were Fe_3O_4 with a mean diameter of 13 nm. The immobilized enzyme had a good behavior in the esterification reaction of oleic acid with butyl alcohol.

Keywords: Magnetic particles; immobilized lipase; enzyme activity; esterification.

Las nanoestructuras magnéticas han ganado considerable interés en los últimos años en estudios de investigación básica y aplicada. El uso de nanoestructuras magnéticas ha sido verificado en los campos de la bioquímica, biomedicina, tratamiento de aguas entre otros. Su gran momento magnético les da un amplio rango de aplicaciones las cuales permiten ser transportadas y dirigidas por campos magnéticos externos. Las estructuras magnéticas han tenido gran potencial en procesos biotecnológicos que las toman en cuenta ya que pueden ser utilizadas como portadores de enzimas en diferentes transformaciones biocatalíticas. En este trabajo, la enzima Candida Rugosa fue covalentemente unida a partículas magnéticas recubiertas usando activación con glutaraldehído con el fin de formar un sistema biocatalítico. El análisis de los patrones de difracción de rayos X indica que las partículas magnéticas resultantes fueron Fe_3O_4 con un diámetro medio de 13 nm. La enzima inmovilizada fue estudiada en la reacción de esterificación de ácido oleico con alcohol butílico, presentado un buen desempeño catalítico.

Descriptores: Particulas magnéticas; lipasa inmovilizada; actividad enzimática; esterificación.

PACS: 75.75.-c; 75.75.Cd; 75.60.Ej; 75.70.Cn; 75.47.Lx.

1. Introduction

Magnetic microparticles and nanoparticles are widely studied for their applications in different fields like physics, biochemistry, biology and medicine among others. Such applications include protein immobilization, diagnostic imaging, immunoassays, magnetic cell separation and purification, controlled drug delivery and targeting [1-3].

On the other hand, the use of biocatalysts for transforming fats, oils, partial glycerides and fatty acids into higher-value-added derivates is well documented [4-8]. One area of interest is the utilization of immobilized lipases for catalyzing the synthesis of simple esters from vegetable oils [9-11] and other agricultural lipid feedstocks [8,12].

Some efforts have been made on immobilization of lipase on modified ferromagnetic nanoparticles surface by polymer such as poly(ethylene glycol) and its copolymer with maleic acid [13,14] and in magnetic sol-gel matrices [15-18]. The former immobilization method seems to be more attractive because using magnetic nanoparticles as support yields a sufficiently large specific surface area for enzyme binding.

This work has as a main purpose demonstrate the results of Candida Rugosa lipase immobilization, characterizing the biocatalyst obtained and evaluating the system in butyl oleate production.

2. Experimental

Chemicals: Iron(III) chloride hexahydrate ($FeCl_3$) and iron(II) chloride tetrahydrate ($FeCl_2$) were the products of Carlo Erba and J.T. Baker, respectively. Tetraethyl orthosilicate ($Si(OC_2H_5)_4$) and [3-(2-aminoethylamino)propyl]trimethoxysilane ($(CH_3O)_3Si(CH_2)_3NHCH_2CH_2NH_2$) were purchased from Fluka. Sodium hydroxide (NaOH), iodine (I_2) and bromo (Br) were purchased from Merck. Ethyl acetate ($CH_3COOC_2H_5$) was the product of Riedel de Haën. Methanol (CH_3OH) was the product of FisherChemicals. Sodium fluoride (NaF) was the product of AnalaR. Ethyl alcohol (CH_3CH_2OH) and oleic acid ($CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$) were the product of Carlo Erba. Potassium hydroxide (KOH), Phenolphthalein solution indicator, 2% in ethanol ($C_{20}H_{14}O_4$), sodium thiosulfate ($Na_2S_2O_3$) and potassium iodide (KI) were obtained of Mol Labs. Butyl alcohol ($CH_3(CH_2)_3OH$), hexane ($CH_3(CH_2)_4CH_3$) and chloroform ($CHCl_3$) were obtained of Mallinckrodt. Glutaraldehyde solution, 50%

in H_2O ($OHC(CH_2)_3CHO$) was purchased from Sigma-Aldrich. Acetic acid (CH_3CO_2H) was obtained of J.T. Baker. Palm oil was obtained from Alianza Team. *Candida rugosa* Lipase was purchased from Sigma-Aldrich. Phosphate buffer solution (pH 7.0, 0.1M) was prepared with sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4).

Synthesis of magnetic particles (MP): Magnetite was made according to the method proposed by Zeng [18]. For the preparation of magnetic particles, a mixture of 500 mL of $FeCl_2$ (0.2 mol/L) and 500 mL of $FeCl_3$ (0.3 mol/L) aqueous solution was added to a flask that contained 5 g stearic acid. Then the mixture was stirred vigorously for 10 min, and 200 mL of NaOH aqueous solution (4 mol/L) was dropped into the flask. A black precipitate was obtained. The precipitate was washed immediately with ethyl acetate three times by magnetic separation. Finally, magnetic particles were filtrated and dried.

Preparation of coated magnetic particles (CMP10, CMP20, CMP30): Magnetic particles coating was accomplishes by suspending two grams of Fe_3O_4 powder in 100 mL of distilled water. A mixture of 5.0 mL of [3-(2-aminoethylamino)propyl]trimethoxysilane (AEPTS), methanol and NaF (1%) aqueous solution was stirring for 10 minutes and added to magnetic particles suspended in distilled water. The mixture was stirred for five minutes. Then 10 mL (20 or 30 mL) of tetraethyl orthosilicate (TEOS) was dropped slowly into the flask and stirred vigorously at room temperature for 24 hours. The precipitate was washed with ethanol four times, filtrated and dried. This procedure was repeated for two more preparations with 20 and 30 ml of TEOS.

Cross-Linking with glutaraldehyde (GMP): Cross-linking was carried by adding 100 mL of glutaraldehyde (10%) to 4.4 g of coated magnetic particles (CMP20), and stirring for 24 h at room temperature. The precipitate was washed with distillated water three times, filtrated and dried.

Immobilization of lipase: For binding of lipase, 700 mg of *Candida Rugosa* lipase was added to 100 mL of phosphate buffer solution and stirred until all the lipase was dissolved ($\sim$10 minutes). Later, 1 g of coated magnetic particles activated with glutaraldehyde (GMP) was added to the solution and stirred for 24 h at room temperature. The immobilized lipase was separated by centrifugation and washed twice by phosphate buffer. Finally, the immobilized lipase was storage.

Characterization: Structure and size of crystal was determined by XRD on a Rigaku MINIFLEX II X-ray diffractrometer using Cu Kα radiation (λ = 1.540562 ?). Fourier transform infrared spectroscopy (FT-IR, Perkin Elmer, model Spectrum BX) was used to study chemical bonds between native and coated magnetic particles. BET-surface-area analysis was accomplished with a Micromeritics ASAP 2020 unit, using nitrogen as the adsorbing gas. The magnetization vs. field cycles of the materials under study was obtained with a Vibrating Sample Magnetometer (VSM)-VersaLab, Quantum Design, Inc. The applied field was varied in the range -27 $\leq$ H $\leq$ 27 kOe. Data were obtained for consecutive H = 100 Oe steps, stabilizing H before each reading, at 300 K.

Activity Measurement: In order to determine the enzymatic activity, esterification of oleic acid with butyl alcohol was followed. Reaction required 1.57 mL of oleic acid, 48 mL of hexane and 400 mg enzyme. This mixture was prepared at 40°C. After preparing the enzyme solution, 0.46 mL de butyl alcohol were added. Both, oleic acid and butyl alcohol concentration in hexane were of 0.1N. Sampling was made by taking 1 mL of reaction mixture. For stopping the reaction, samples were heated up to 60°C for 1 minute and diluted with 4 mL of ethanol. The samples were titrated with alcoholic potassium (0.01 N) using phenolphthalein as indicator. The purpose of this titration was to measure the quantity of oleic acid esterificated. The activity is expressed by the amount of oleic acid esterificated by time unit. This procedure was made for both free and immobilized enzyme.

3. Results and discussion

Size and Structure of crystal (XRD): The structure of the synthesized magnetite particles can be identified by X-ray diffraction. Figure 1 shows the XRD patterns for native and coated magnetic particles. Six characteristic peaks for Fe_3O_4 (2θ = 30.0°, 35.4°, 43.0°, 53.5°, 57.0° and 62.6°), marked by their indexes ((220), (311), (400), (422), (511) and (440)), were observed for both samples. Due to coated with AEPTS-TEOS a characteristic broad peak of amorphous silica oxide ($2\theta \sim 23°$), was observed for coated magnetic particle. Using the peak at 2θ = 35.4° (Miller indexes (311)), the average size of magnetite particle synthesized by the method described in this paper is $\sim$ 13 nm. Compared with the reported values these magnetite particles have near size ($\sim$ 10 to 15 nm) [1,15].

Chemical bonds (FT-IR): Fig. 2 shows FT-IR spectra of native and coated Fe_3O_4 particles. It can be seen that, compared with the native sample, the coated Fe_3O_4 particles posses adsorption bands in 1068 cm^{-1} due to the stretching

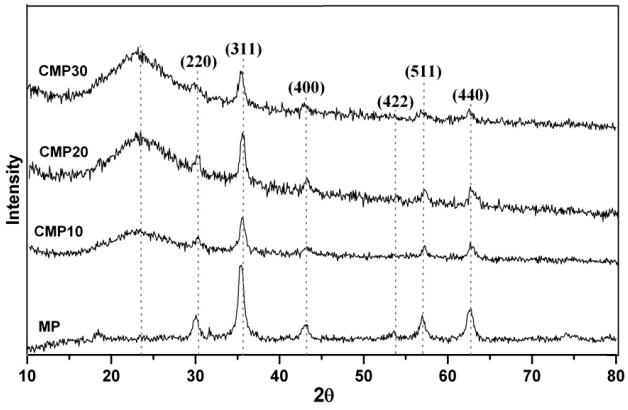

FIGURE 1. XRD patterns of magnetic particle native (MP) and coated (CMP10, CMP20, CMP30).

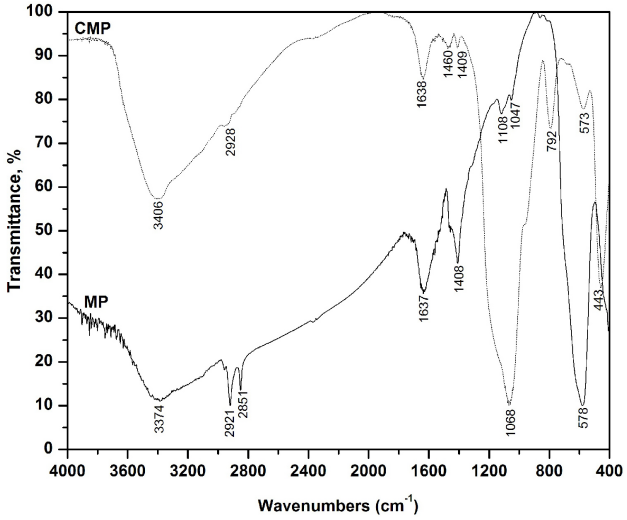

FIGURE 2. FT-IR spectra of the native and coated magnetic particles.

vibration of Si-O bond, band in 792 cm^{-1} due to the bending vibration of $–NH_2$ group. All these reveal the existence of AEPTS and TEOS.

In addition, in Fig. 2 (a) and (b) the absorption bands near 3400 and 1630 cm^{-1} refer to the vibration of remainder water in the samples, bands near 2920 and 2850 cm^{-1} due to stretching vibration of C-H bond, bands near 570 cm^{-1} due to stretching vibration of Fe-O, bands near 1400 cm^{-1} due to stretching vibrations of N-H.

The superficial area and average pore diameter for the synthesized materials can be observed in Table I. The superficial area of MP decrease drastically (90%) relative to CMP10, whereas the areas for CMP20 and CMP30 samples (450% and 250% respectively) were greater than that of original material (MP). The average pore diameter of CMP10 is the largest

Immobilization Efficiency: The amount of protein in immobilized lipase was measured by the Kjeldhal method, which calculates the amount of total nitrogen in a sample and it is multiplied by his respective factor for calculating the percentage of protein. The percentage of nitrogen in the support and immobilized lipase were 3.43 and 4.71, respectively. In order to calculate the protein content in the immobilized lipase is necessary to subtract the nitrogen percentage of support. Finally, the percentage of total nitrogen that has the immobilized lipase is 1.28, which corresponds to 8% protein (the factor to convert the total nitrogen to protein is 6.25).

TABLE I. Summary of textural properties of the synthesized materials

Materials	Superficial Area BET (m^2/g)	Average Diameter of Pore (Å)[a]
MP	65.01	134.48
CMP10	6.91	140.37
CMP20	294.43	46.52
CMP30	162.08	100.06

[a] Taken from the adsorption isotherm

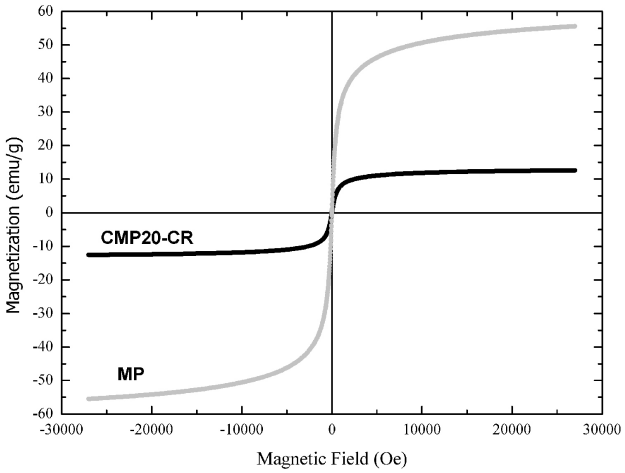

FIGURE 3. Magnetization versus H cycles.

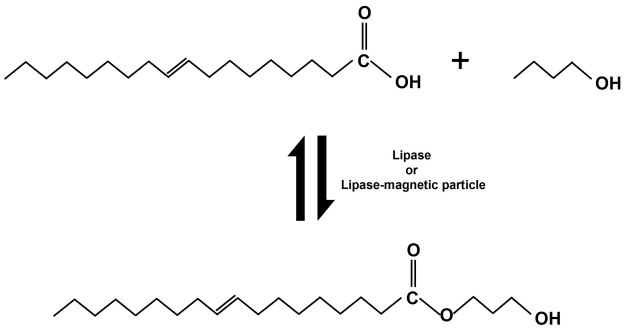

FIGURE 4. Reaction between oleic acid and buthanol.

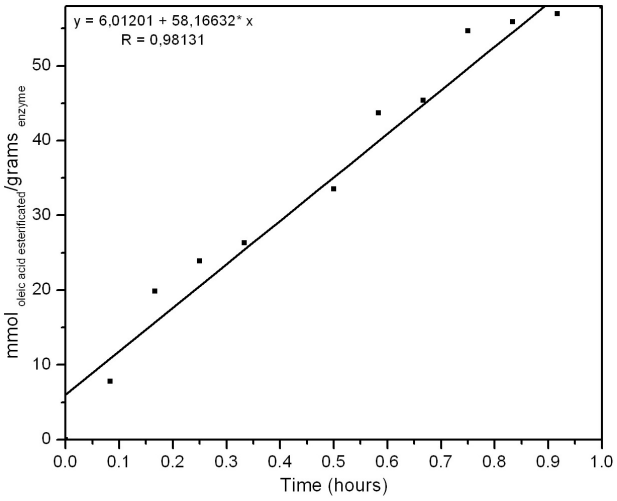

FIGURE 5. Activity for free enzyme.

Magnetic Behavior: The Fig. 3 shown the magnetization vs. H cycles at T = 300 K, for the samples MP and CMP20-CR. The samples exhibit zero remanence and coercitivity and do not reach saturation magnetization in the field range ana-

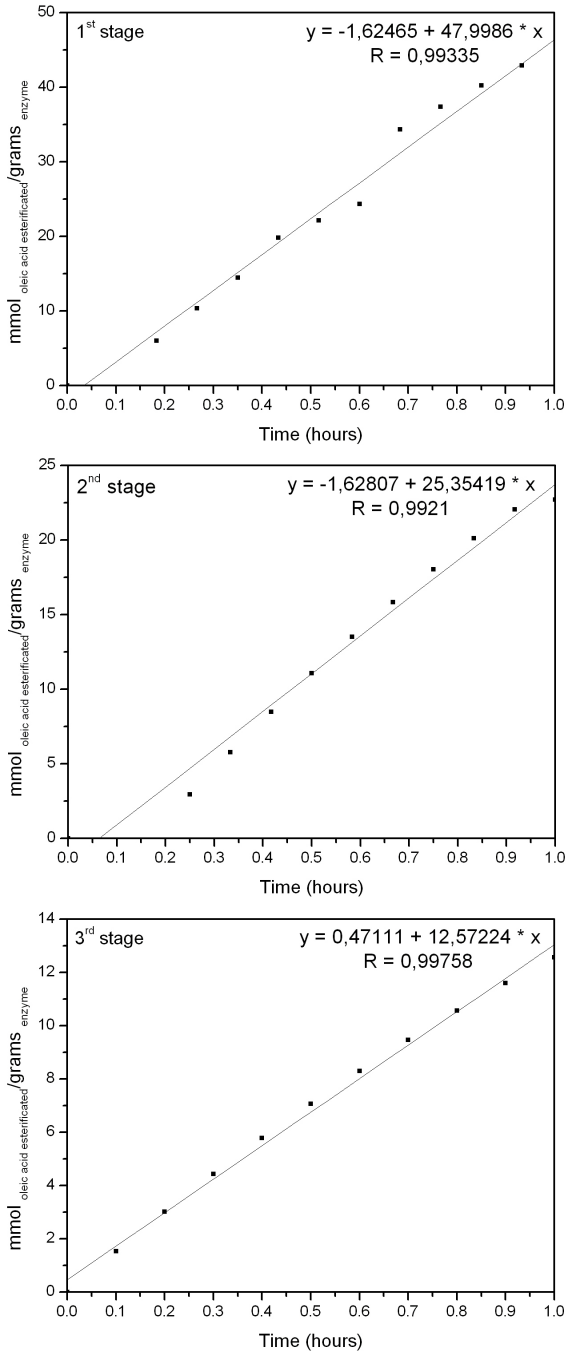

FIGURE 6. Activity for immobilized enzyme on magnetic carrier.

lyzed which suggests super paramagnetic behavior [19]. These results are according to magnetic silica reported by Ma *et al* [20]. The magnetization of CMP20-CR is smaller than MP, wich suggest that the cover with silica and immobilization procedures affect the magnetic response of the material due to non magnetic character of silica an lipase. However, the magnetization value of this material is near to the reported for core-shell magnetite structures [21-24].

Enzyme Activity: The Fig. 4 show the reaction followed for enzyme activity determination. In Fig. 5 is shown the milimoles esterificated of oleic acid by unit of time for free enzyme, considering the quantity of enzyme used. This adjustment to a straight line has a correlation coefficient of 0.963, which can be considered as a good adjustment. As it was said previously the slope of this straight line is the average enzymatic activity and for free enzyme is equal to 58.2.

TABLE II. Enzymatic activities average

Enzymes	**Activity average (mmol Ac. Ol./h*g$_{enz}$)**
Lipase *Candida Rugosa* free	58.2
Immobilized Enzyme (1^{st} Stage)	48.0
Immobilized Enzyme (2^{nd} Stage)	25.4
Immobilized Enzyme (3^{rd} Stage)	12.6

Figure 6 show the esterificated oleic acid (mmol/h) in immobilized enzyme considering the quantity of enzyme used. In this figure, three stages are observed, with stages means the reusability times that were proven in the immobilized enzyme with the same conditions of reaction, previously described.

When the esterification reaction is stopped, the immobilized enzyme is retired by the application of an external magnetic field, and it is washed with hexane for its reusability. This was repeated for stages two and three. For each stage, obtained correlation coefficients were 0.9, considered as a very good value for a straight-line regression. As it was said previously, the slope of these straight lines corresponds to the average enzymatic activities. For this case are 48.0, 25.4 and 12.6, respectively (Table II).

4. Conclusions

Lipase was directly bound to coated magnetic particles via glutaraldehyde activation. The analyses of XRD patterns indicated the resultant magnetic particles were Fe_3O_4 with a mean diameter of 13 nm. FT-IR spectra were utilized to prove the formation of Fe-O-Si chemical bonds and confirmed the coated of magnetic particle. With the characterization realized to the synthesized materials it is possible to conclude, what the best support is the CMP20. The immobilized enzyme had a good behavior in the esterification of oleic acid and their magnetic behavior is superparamagnetic according to VSM results due to magnetic support. Finally, we can ensure that it is a good catalyst, which can be reused and easily separated from reaction medium by the application of an external magnetic field and can be used in process that need to have the catalyst in the defined zone, achieved with its improve the yield in the process.

Acknowledgments

The authors thank the Direction of Research (DIMA) at National University of Colombia, Manizales Campus, for the economic support for this project.

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