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USE OF MESOPOROUS MnO_2 AS A SUPPORT FOR IMMOBILIZATION OF LIPASE FROM *Candida rugosa*

Article Highlights

- We used a new mesoporous carrier for immobilization of lipase from *Candida rugosa*
- The amount of enzyme loading on support was reached to 700 units per 1 g of support
- The thermal resistance of the enzyme was enhanced by immobilization
- The physical adsorption of enzyme to the support was strong

Abstract

In this study, immobilization of lipase from Candida rugosa on mesoporous manganese dioxide by adsorption was carried out and the effect of three immobilization variables including temperature, process time and enzyme/support ratio on immobilization efficiency were studied. The characteristics of synthesized MnO_2 and lipase-bound MnO_2 were investigated by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FT-IR) methods. The porous property of the support particles was also studied by X-ray diffraction (XRD) and Brunauer, Emmett and Teller (BET) measurements. The thermal stability of immobilized lipase was determined to be better than that of free enzyme. Also the operational stability of lipase-bound MnO_2 was studied and showed an almost strong attachment of enzyme to support. The Michaelis-Menten kinetic parameters (K_m and V_{max}) were also determined for both free and immobilized lipases. It was observed that there is an increase of the K_m value (672.96 mg/ml) and a decrease of the V_{max} value (130.99 U/mg) for the immobilized enzyme comparing with the corresponding values of the free lipase.

Keywords: immobilization, Candida rugosa *Lipase, physical adsorption, mesoporous manganese dioxide.*

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are largely used as biocatalysts in biotechnology and modern chemistry [1]. These enzymes are also capable of catalyzing several reactions like esterification [2], transesterification (acidolysis, interesterification and alcoholysis), aminolysis, oximolysis, thiotransesterification and ammoniolysis in anhydrous organic solvents [3]. Considering the current high cost of lipases, it is necessary to use immobilization processes to make large-scale utilization of enzymatic reactions economically possible. Immobilization of enzymes can also change their secondary structure leading to modification of their activity [4], better operation control, easier product recovery and flexibility of reactor design [5]. Aside from the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography and biosensors. Therapeutic applications are also foreseen, such as the use of enzymes in extra-corporeal shunts [6]

Lipases can be immobilized on various supports by physical adsorption, covalent binding, ionic interactions or by entrapment [7]. Among these methods, adsorption of lipase onto insoluble support is the most commonly used approach because of its easy use and low cost. The forces between the support and the enzymes in this method include hydrogen bonding, van der Waals forces and hydrophobic interactions

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[8]. Lipase of *Candida rugosa* was adsorbed on different types of supports including synthetic or natural polymers [8-11], bentonite [12], kaolin [13], acrylic resins and textile membranes [14], glass beads [15] and magnetic beads [16].

Mesoporous MnO_2 is made up of nanoparticles with a large specific surface area that makes the high load of enzyme possible. There are certain requirements for the supports used in immobilization process that should be fulfilled to increase the efficiency of the process, such as large internal surface and high superficial density, and easy recovery of support at the end of process [17], which are all satisfied by utilizing MnO_2 as an immobilization host [18]. These characteristics have led MnO_2 nanoparticles to be successfully used in the fabrication of electrochemical biosensors [18,19]. In our previous work, MnO_2 nanoparticles were also successfully used as a support for immobilization of glucose oxidase [20].

In this study, lipase from *C. rugosa* was immobilized on mesoporous MnO_2 particles by physical adsorption. The manganese dioxide particles were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The adsorption of lipase onto MnO_2 particles was confirmed by Fourier transform infrared spectroscopy (FT-IR). The porous property of the support particles was also studied by X-ray diffraction (XRD) and Brunauer, Emmett and Teller (BET) measurements. The effects of three main immobilization variables (time, temperature and enzyme/support ratio) on process efficiency were studied. The properties of the immobilized lipase such as thermal and operational stability were also investigated.

EXPERIMENTAL

Materials

Lipase from *Candida rugosa* type VII (700 U/mg), sodium dihydrogen phosphate, di-sodium hydrogen phosphate, $MnCl_2$, $KMnO_4$ and CCl_4 in the analytical grade were all purchased from Sigma Chemical Co.

Preparation of mesoporous MnO_2

Mesoporous MnO_2 was prepared according to the method described in our previous work [20]. 80 ml CCl_4 and 30 ml of 1 M $MnCl_2$ solutions were added to a container and made a biphasic system. 40 ml of 0.5 M $KMnO_4$ was added by two drops per second to the CCl_4 layer and upon reaching the interface, a brown material appeared at the interface. After 48 h, the brown MnO_2 was collected and washed with distilled water and ethanol for several times. The material was then dried at 150 °C for 12 h, and stored for future use [21].

Immobilization method

0.5 g of MnO_2 was dispersed in 10 ml of enzyme solution in 0.1 M sodium phosphate buffer, pH 7, and put into the shaker for a definite time at constant temperature. The resulting lipase-bound MnO_2 particles were separated by centrifuging and the supernatant was taken for activity analysis. The lipase-bound particles were washed with distilled water for several times to ensure that all the unbound lipases were washed off. The enzyme loading on support was calculated by the following equation [22]:

$$U_{\text{imm.}} = \frac{U_{\text{i}} - U_{\text{f}}}{m} \quad (1)$$

where $U_{\text{imm.}}$ is the enzyme loading on support (U/g), U_{i} is the activity of enzyme in the attachment solution at the beginning of process and U_{f} is the activity of supernatant at the end of process, and m is the mass of support (g).

Characterization of biocatalyst

The morphology and size of samples was studied by scanning electron microscopy (SEM) Leo 1455 VP model with 10 kV voltage, and by transmission electron microscopy (TEM) using the Philips CM120 at 100 kV. The FT-IR spectra of mesoporous MnO_2, free lipase and lipase-bound MnO_2 was recorded by a Tensor 27 Model (Bruker German) spectrophotometer using KBr pellets. The X-ray diffraction (XRD) pattern of the mesoporous MnO_2 was determined using a D5000 diffractometer (Siemens German) with Cu K_α radiation source (λ = 1.54056 Å). The surface area and pore volume of the prepared MnO_2 were obtained through the Brunauer-Emmett--Teller (BET) with a Micromeritics Gemini 2375 (USA) analyzer.

Enzyme activity assay

The hydrolytic activity of free and immobilized lipase was tested by olive oil hydrolysis [23]. The activity assay was carried out with reaction mixture containing 4 ml of olive oil and 5 ml of phosphate buffer (0.1 M, pH 7) in a 25 mL flask. The reaction was accomplished by adding a predetermined amount of free enzyme solution (1 mL) or supernatant (1 mL) or immobilized enzyme (0.1 g) to the mentioned reaction medium and shaking the flask at a constant temperature of 37 °C in a shaking incubator for 30 min. The liberated fatty acids were measured by titrating the flask content with 50 mM NaOH using a phenolphthalein indicator. One unit of lipase activity was

defined as the amount of enzyme that liberates 1 μmol fatty acid per minute under the assay conditions [24]. All the activity assay experiments were repeated 3 times and the average amounts were used in calculations.

RESULTS AND DISCUSSION

Characterization of synthesized mesoporous MnO_2 particles

The morphology of MnO_2 samples was studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as shown in Figure 1. According to the figure, the average size of particles is about 1 μm and the porous property of MnO_2 is obvious. Also from TEM image, the size of MnO_2 particles is 38 nm length and 10 nm width.

Figure 2 shows the FT-IR spectra of lipase, the lipase-bound MnO_2 and the uncoated MnO_2. As it can be seen in spectra of MnO_2 particles, adsorption bands at 521 cm^{-1} and 602 cm^{-1} are related to Mn-O, and a band at 3360 cm^{-1} indicates tension vibration of -OH with Mn atoms [20,25]. For the pure lipase, Amide I band corresponding to -CO carbonyl stretching mode of the peptide, is present in the region of 1700-1600 cm^{-1} [26]. This band consists of a group of overlapped signals, which contain information on secondary protein structure of the enzyme. Bands centered at around 1547 cm^{-1} are assignable to the amide II band, which is an out-of-phase combination mode of the NH in plane bend and the CN stretching vibration with smaller contributions from the CO in plane bend and the CC and NC stretching vibrations [27]. Finally, a set of bands can be distinguished in

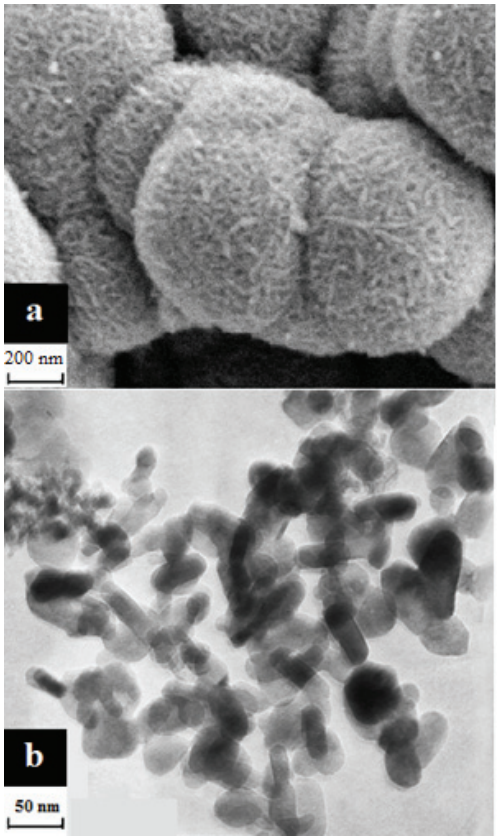


Figure 1. SEM (a) and TEM (b) images of the mesoporous MnO_2 samples.

the region of 1400-1200 cm^{-1} due to amide III mode. This mode is assigned to the in-phase combination of the NH deformation vibration with CN, with a minor contribution of CO and CC stretching [27]. The presence of amide I and amide III band of lipase enzyme in MnO_2 particles can be clearly discerned by com-

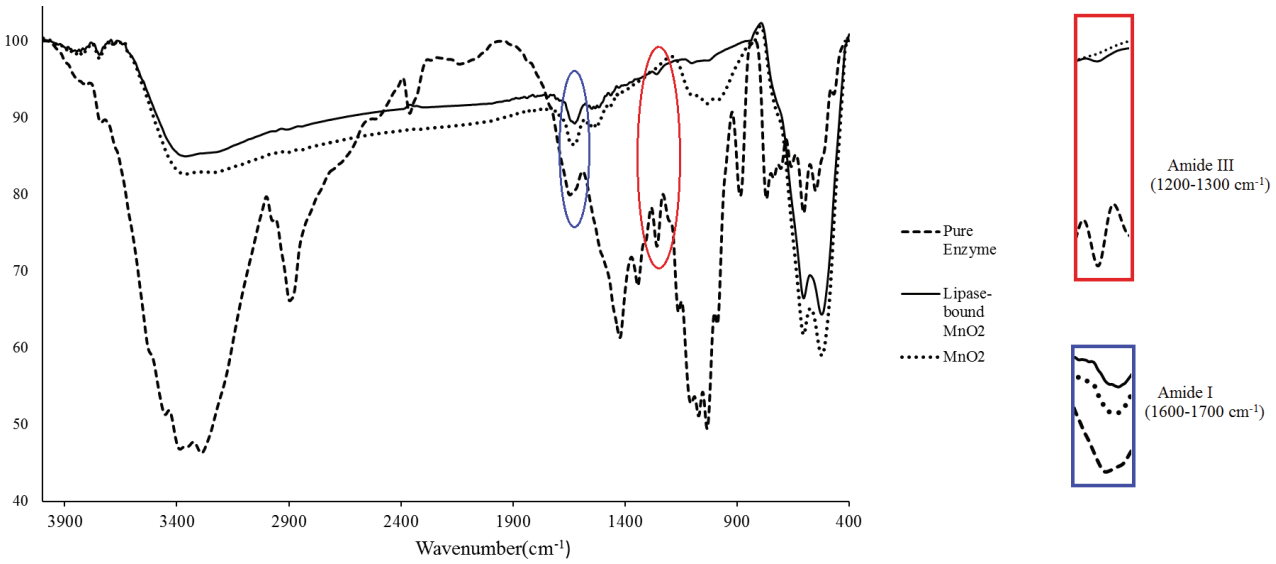


Figure 2. FT-IR Spectrum of pure lipase (blue), lipase-bound MnO_2 (black) and MnO_2 (red).

paring the spectra of lipase-bound MnO_2 with the one of uncoated MnO_2 in the region of about 1650 cm^{-1} and 1269 cm^{-1}, respectively (Figure 2). As it can be seen the band shape in lipase-bound MnO_2 is different from that of pure lipase, which indicates that some changes have occurred in the conformation of the enzyme after adsorption to the support. These conformational changes could be attributed to the loss of α-helix structure and probable rising of β-sheet and/or self-aggregates [28].

The N_2 adsorption-desorption measurement using liquid N_2 (temperature of -196 °C) was performed to determine the mesoporosity and textural property of the MnO_2 sample (Figure 3a). Comparing the obtained isotherm to IUPAC classification proves that synthesized MnO_2 is of type IV with strong affinities [29-31]. S_{BET}, the surface area of the sample calculated from the nitrogen isotherm using the BET method, was 176.93 m^2/g and its total pore volume was 0.47 ml/g at P/P_0 = 0.99.

The X-ray diffraction pattern of the MnO_2 which was recorded at 2θ = 10-70° is illustrated in Figure 3b. Five distinct peaks are seen at 2θ values of: 25.1, 32.0, 37.1, 42.5, and 57.1°. A peak at 2θ = 32° related to γ-MnO_2 (JCPDS 14-0644), peaks at 2θ 25.1 and 37.1° represent α-MnO_2 (JCPDS 44-0141), and peaks at 2θ 42.5 and 57.1° illustrate β-MnO_2 (JCPDS 24-0735) [32,33]. So the synthesized MnO_2 crystalline state is a combination of α, β and γ-MnO_2.

The spacing distance between crystals was calculated from the Scherer equation:

$$d_c = K\lambda/(b_{hkl}\cos\theta) \quad (2)$$

where d_c (nm) is the spacing distance, θ is the Bragg angle, K is the constant of diffraction, λ (1.54056 nm) is the X-ray wavelength and b_{hkl} is the peak width at the half-maximum, corrected for instrument broadening. The distance (nm) between adjacent pore centers for all the phases was calculated according to following equation:

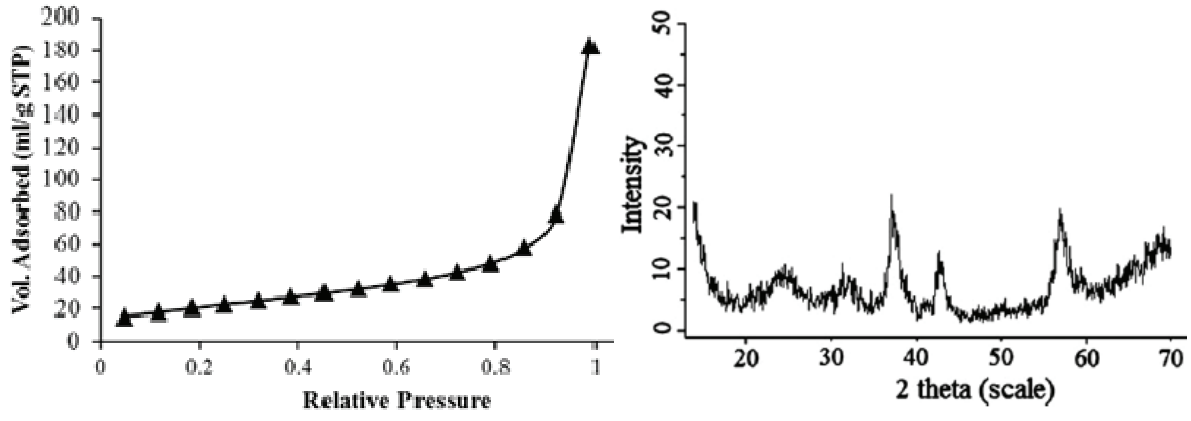
$$a = 2d_c/3^{1/2} \quad (3)$$

The d_c and a values of diffraction peaks are listed in Table 1. From this table, it can be seen that the probable pore size of the synthesized MnO_2 was approximately in the range 9-12 nm, indicating that the pore size was in the mesoscale range. These properties indicate that MnO_2 has enough specific surface area and suitable pore size.

Effect of different variables on immobilization efficiency

Enzyme/support ratio

The lipase from *C. rugosa* was immobilized onto mesoporous MnO_2 particles by physical adsorption method. This process was carried out under different immobilization variables to determine the optimum condition. Various amounts of enzyme/support ratios of the range 5.9-174 mg/g with temperature and time set at 15 °C and 3.5 h, respectively, were tested and the experimental results are shown in Figure 4a. It can be seen that by increasing the enzyme/support ratio to 120 mg/g, the amount of enzyme adsorbed on



(a) (b)

Figure 3. Nitrogen adsorption isotherm of the synthesized MnO_2 (a), and XRD pattern of the mesoporous MnO_2 (b).

Table 1. Crystalline XRD properties of the synthesized MnO_2

Deffraction angle (2θ / °)	Intensity	Average size of MnO_2 crystal d_c, nm	Distance between adjacent pore centers, a / nm
37.10	25	10.52	12.15
43.12	15	11.83	13.66
56.68	24	7.59	8.77

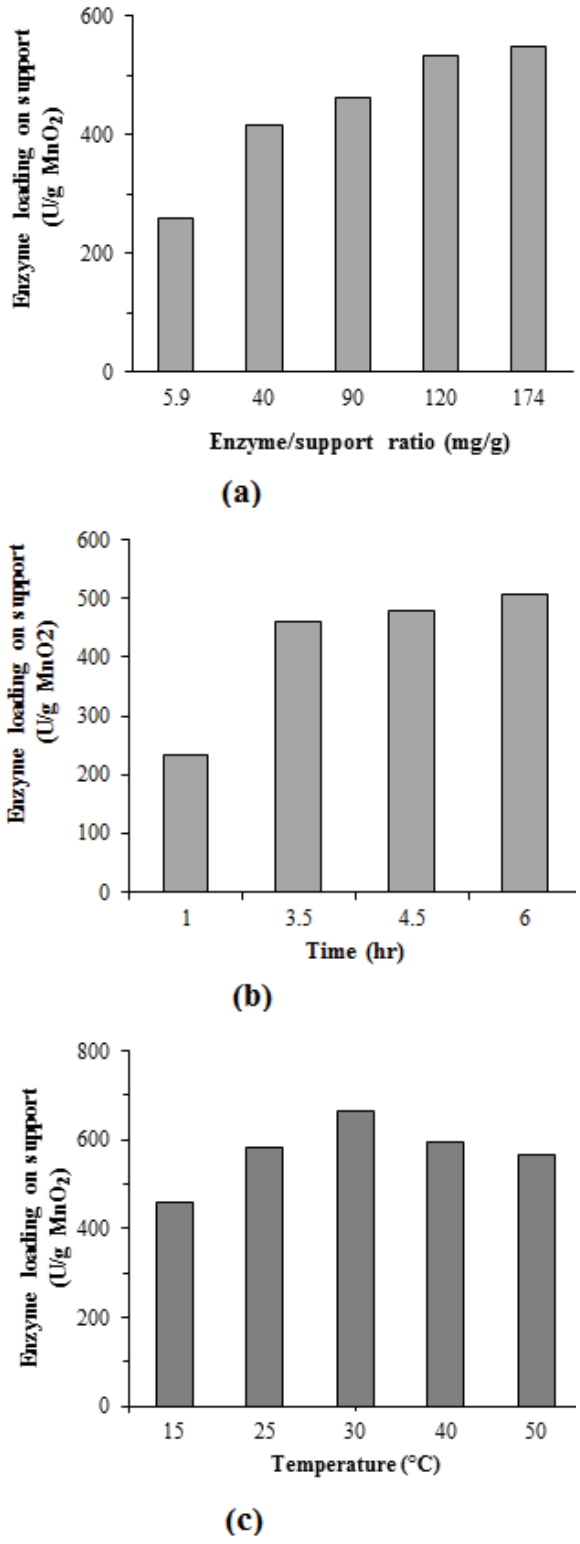


Figure 4. The effect of enzyme/support ratio (a), immobilization time (b) and temperature (c) on enzyme loading on MnO_2.

1 g of support reaches to 530 U. Further increasing of this ratio to 174 mg/g accounts to loaded enzyme addition of only 20 U. This means that by introducing 45% more enzymes to the attachment solution, the amount of enzyme loading on support was only increased by 3.7%. It can be predicted that by increasing enzyme/support ratio, there would be no significant raise in immobilization amount due to the saturation of active sites of support by enzyme.

Immobilization process time

The effect of immobilization process time on the efficiency of immobilization of lipase from *C. rugosa* was studied by carrying the process with enzyme/ /support ratio of 90 mg/g at 15 °C for different immobilization time of 1-6 h. The results are shown in Figure 4b. It can be seen that by increasing immobilization process time from 1 to 3.5 h, the amount of enzyme loaded on support is doubled. However, by continuing the immobilization process for another 2.5 h, the amount of enzyme loading on support was increased by only 8.7%. Considering the energy consumed during this additional time of 2.5 h and insignificant amount of enzyme loading gain of 8.7%, it is not reasonable to continue the process over 3.5 h. Also by increasing immobilization time, the protein conformation of lipase might denature and result in activity decrease and limitation of immobilization process [34].

Immobilization process temperature

The amount of heat energy that is introduced to the immobilization system is an important parameter. Increasing the temperature improves the accessibility and mobility of enzyme, but over a certain temperature, it would lead to the possible inactivation of enzyme by thermal denaturation. Therefore the immobilization process was carried out with enzyme/support ratio of 90 mg/g for 3.5 h at different temperatures ranging 15-50 °C, to find the optimum temperature. The results are shown in Figure 4c. It can be seen that the highest enzyme loading on support was achieved by running the immobilization process at 30 °C.

Effect of temperature on activity of free and immobilized enzymes

The effect of temperature on activity of both free and immobilized lipase was studied in the range of 25-60 °C as shown in Figure 5. It is obvious that the activity of free lipase is very dependent on temperature and the maximum amount of activity is observed at approximately 35-40 °C, while it shifts to a wider range of 35-45 °C for the immobilized lipase. The increase in optimum temperature range might be a result of reduced conformational flexibility that demands higher activation energy for the molecule to reorganization the proper conformation for binding to substrate [35]. One of the main advantages of enzyme immobilization is the mentioned increase in its

stability due to restricted conformational mobility of the protein molecule. Comparing the rate of activity loss for immobilized lipase from *C. rugosa* to the corresponding profiles of other carriers like magnetic microspheres [36] and sol-gel support [1] shows that lipase-bound MnO_2 retains 10 and 30% more activity at 60 °C, respectively.

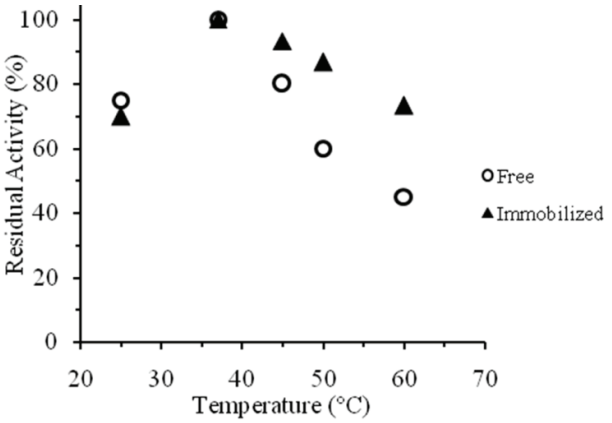


Figure 5. The effect of temperature on the relative activity of the free and immobilized enzymes.

Reusability and operational stability of free and immobilized enzymes

Reusing the immobilized enzyme requires strong attachment of lipase to the support to assure activity retaining after several operations. To investigate whether the lipase-bound MnO_2 holds its catalytic activity during several operation, the immobilized particles were incubated at 37 °C in a flask containing 4 ml of olive oil and 5 ml of phosphate buffer (0.1 M, pH 7) for 30 min. The amount of lipase enzyme bound to the support is directly proportional to the yield of hydrolysis reaction in the flask. The biocatalyst particles were separated by filtration, and the flask content was analyzed by titrating. The separated lipase-bound MnO_2 particles were washed with phosphate buffer solution (0.1 M, pH 7) and used again to catalyze the aforementioned hydrolysis reaction. This procedure was repeated for 6 times and the results were presented in Table 2. After 6 times of reusing, reduction of activity was 30%. This would be caused by leakage of protein from support upon washing.

Table 2. Strength of lipase attachment to the MnO_2

Number of reusing	0	1	2	3	4	5	6
Residual activity, %	100	92.5	87.5	82.5	75	72.5	70

Thermal stability of free and immobilized enzymes

The thermal stability of immobilized enzyme is one of the important criteria for its application. The activity of immobilized enzyme must be more resistant than that of the free form against heat and denaturing agents. This was studied by incubating both free and immobilized enzyme in phosphate buffer (0.1 M, pH 7) at 50 °C. The activity of both free and immobilized enzyme decreased during the time, whereas the activity of lipase-bound MnO_2 decreased more slowly than the free one (Figure 6). It can be seen that the free enzyme lost its activity during 2.5 h of incubation period, but the activity loss of immobilized one is only 3% during the same time. Immobilized lipase was inactivated at a much slower rate than that of the free form, that the immobilized enzyme retains 71% of its initial activity after 14 h. This could be explained by the protein-surface interaction in immobilized enzyme that protects it from unfolding and prevents the conformation transition of the enzyme at high temperatures [31,37].

Kinetic parameters

The kinetics of simple enzyme catalyzed reactions can be determined by Michaelis-Menten equation. To determine the Michaelis-Menten kinetic para-

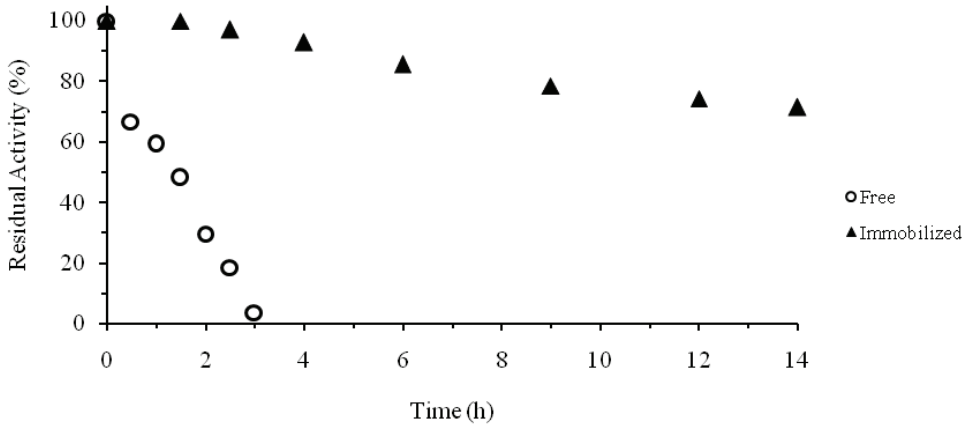


Figure 6. Stability of the free and immobilized lipase at 50 °C.

meters (K_m and V_{max}) for both free and immobilized lipase, different concentrations of olive oil over the range of 0.2–0.85 g/ml were used and the activities were measured. The parameters were calculated by the Lineweaver-Burk method as follows:

$$\frac{1}{v} = \left\{\left[\frac{K_m}{V_{max}}\right]\left[\frac{1}{S}\right]\right\} + \frac{1}{V_{max}} \quad (3)$$

A plot of v against $1/S$ will give a straight line with the slope of K_m/V_{max} and an intercept on the ordinate at $1/V_{max}$ as shown in Figure 7. The Michaelis-Menten constants K_m and V_{max} were calculated as 131.93 mg/ml and 280.96 U/mg protein for free lipase, and 804.89 mg/ml and 149.97 U/mg support for immobilized lipase, respectively. The attachment of enzyme molecule to the support by means of non-covalent forces (hydrogen bonds, ionic, and van der Waals interactions) leads to a reduction of molecular mobility. Also, the immobilization of enzyme limits the accessibility of substrate to reach the active sites of enzyme. These all result in an increase of K_m value during the immobilization process. However, the aforementioned structural changes of lipase enzyme due to immobilization procedure improve the enzyme resistance to changes of temperature, pH and enhances its lifetime significantly [38]. As a result, the lower reaction rate of immobilized enzyme is still considered more economical.

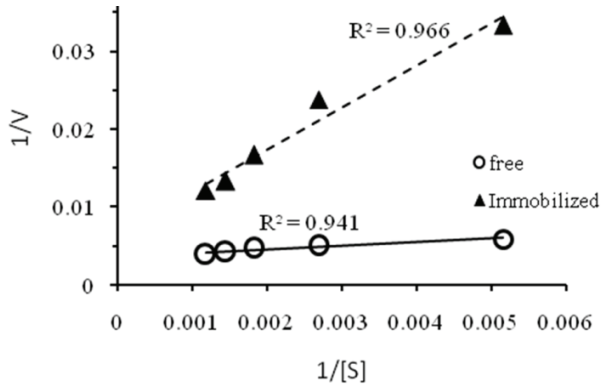


Figure 7. Lineweaver-Burke lines for free and immobilized lipase.

CONCLUSION

In the present work, lipase from *Candida rugosa* was immobilized on mesoporous MnO_2 by physical adsorption and the effect of different immobilization variables were studied. The best result of the immobilization process was achieved at enzyme/support ratio of 120 mg/g, immobilization temperature of 30 °C, and incubation time of 3.5 h. In addition, the immobilized enzyme exhibited better resistance to temperature inactivation and thermal stability than the free form. The reusability of the immobilized enzyme was studied, showing that it retained 70% of its initial activity after 6 times of reutilizing. By applying the Michaelis-Menten equation for the lipase catalyzed reaction, the K_m value for immobilized lipase was determined to be higher than that of the free one. This shows that the rate of immobilized-lipase catalyzed reaction is lower than free lipase. However, the thermal and operational stability of immobilized lipase makes the immobilized lipase more suitable for industrial and large-scale operations. Considering the simplicity and low cost of the immobilization process, it can be concluded that mesoporous MnO_2 is an efficient carrier for lipase immobilization.

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NAUČNI RAD

PRIMENA MEZOPOROZNOG MNO_2 KAO NOSAČA ZA IMOBILIZACIJU LIPAZE *Candida rugosa*

U ovom radu izvršena je imobilizacija lipaze Candida rugosa na mezoporoznom magnan--dioksidu adsorpcionom metodom. Ispitan je uticaj temperature, vremena trajanja procesa i odnosa enzim/nosač na efikasnost imobilizacije. Karakteristike sintetizovanog MnO_2 i MnO_2 vezanog za enzim su ispitane metodama skenirajuće elektronske mikroskopije (SEM), transmisione elektronske mikroskopije (TEM) i infracrvene spektroskopije sa Furijevom transformacijom (FT-IR). Poroznost nosećih čestica je ispitana difrakcijom X-zraka (XRD) i BET metodom. Utvrđeno je da je termalna stabilnost imobilisane lipaze bolja u odnosu na termalnu stabilnost slobodnog enzima. Na osnovu ispitivanja operacionih stabilnosti dokazana je jaka veza enzima i nosača. Određeni su kinetički parametri Mihaelis-Menten jednačine (K_m i V_{max}) za slobodnu i imobilisanu lipazu. Utvrđeno je da postoji povećanje vrednosti K_m (672,96 mg/ml) i opadanje vrednosti V_{max} (130,99 U/mg) kod imobilisanog enzima u odnosu na vrednosti kod slobodne lipaze.

Ključne reči: Imobilizacija, lipaza Candida rugosa, fizička adsorpcija, mezoporozni mangan-dioksid.