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Immobilization and characterization of bovine liver catalase on eggshell

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Abstract: Bovine liver catalase immobilized on eggshell particles was characterized and the reusability of the immobilized catalase was investigated in a batch type reactor. For immobilized catalase onto ground eggshell (ICATG), the optimum initial amount of catalase was 85 mg g^{-1} of eggshells, the optimum pH was 6.0 (75 mM citrate buffer) and the temperature was 30 °C. The V_{max} and K_m values of ICATG were determined as 29.1±1.2 U/mg of protein and 41.9±2.7 mM, respectively. The reusability of ICATG was tested and the remaining activity of ICATG was found to be 73 % of the initial activity after 80 cycles of batch operation. The amount of catalase bound onto the carrier was estimated by using the results of induced coupled plasma measurements. The catalytic efficiencies (k_{cat}/K_m) of free catalase and ICATG were found to be 1.4×10^6 and 2.8×10^3 dm^3 s^{-1} mol^{-1}, respectively. Catalase immobilization onto eggshell is economic and has good reusability. Hence, it can be concluded that eggshell is an efficient carrier for immobilizing catalase.

Keywords: catalase; eggshell; immobilization; glutaraldehyde; induced coupled plasma.

INTRODUCTION

Catalase (EC 1.11.1.6) is an abundant enzyme in nature decomposing hydrogen peroxide to water and molecular oxygen. Catalase from bovine liver (M_r = 240 kg mol^{-1}) is composed of four tetrahedrally arranged identical subunits. Each subunit consists of a single polypeptide chain, which associates with a prostetic group, ferric protoporphyrin IX. Immobilized catalase has useful applications in various industrial fields for the removal of hydrogen peroxide used as oxidizing, bleaching or sterilizing agent and in the analytical field as a component of hydrogen peroxide or glucose biosensor systems.[1–7] Catalase has been immobilized on numerous carrier materials, such as magnesium silicate, magne-

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tite, eggshell membrane, eggshell, gelatin, starch cellulose acetate, starch polycaprolactone blends, chitosan, cellulose, alumina, and agarose covalently; bioskin, kaolin and bentonite by adsorption; polyacrylamide gels and poly(isopropylacrylamide-co-hydroxyethyl methacrylate) (PNIPAM/HEMA) copolymer hydrogel by entrapment.[1,3–5,8–16]

In the present study, bovine liver catalase was immobilized onto hen eggshell by crosslinking with glutaraldehyde. Eggshell being an abundantly available and non-biodegradable waste product, unlike other supports which are expensive but commonly used, was selected as carrier.[17] Hen eggshell is a porous ceramic material mainly composed of calcium carbonate, known as calcite which is a more stable polymorph at room temperature. It has good mechanical strength and it is resistant to microbial attacks.[18]

In this paper, the immobilization of catalase onto eggshell and the characteristics of the immobilized catalase are reported.

EXPERIMENTAL

Materials

Hydrogen peroxide (aqueous solution, 30 % w/w), sulfuric acid (96.0 % w/w) were obtained from Merck AG (Darmstadt, Germany). Bovine liver catalase with a specific activity of 3090 U/mg of solid, glutaraldehyde solution (aqueous solution, 50 % w/w) and all other chemicals were obtained from Sigma (St. Louis, MO). Hen eggs were purchased from a local market.

Catalase immobilization

The hen eggshell was manually stripped from eggshell membrane after the albumen and yolk had been removed. Then eggshells were ground into pieces as small as possible in a mortar, kept in boiling water for 15 min, washed several times with acetone and dried in an oven at 60 °C. This type of carrier was considered as ground carrier. The immobilization method described by Chatterjee[9] was used with minor modifications. Briefly, 1.0 g of dried carrier was added to 8.5 ml of catalase solution in a final concentration of 5.0, 10.0, 15.0, and 20.0 mg ml^{-1} in 0.020 M, pH 6.8, potassium phosphate buffer and stirred for 15 min. Glutaraldehyde was added slowly with shaking to a final concentration of 1.2 % (w/v) to the mixture and the mixture incubated at 5 °C for 4 h. The resulting immobilized catalase (ICATG) was washed with the buffer until no catalase and glutaraldehyde were detected in the wash solution (filtrate). The ICATG samples were kept overnight at 5 °C in an incubator and then stored in closed glass tube.

Glutaraldehyde was detected according to Boratynski and Zal.[19] 200 µl of filtrate or a glutaraldehyde standard solution (8.0×10^{-6}–2.5×10^{-4} M) was mixed with 1.0 ml of phenol reagent (40 µl of an aqueous 5.0 % phenol solution was added to 10 ml of 70 % perchloric acid) and incubated at room temperature for 15 min. Absorbance was measured *vs*. the phenol reagent at 479 nm with a UV/Vis spectrophotometer.

The amount of catalase as a metalloenzyme in the filtrate was determined by induced coupled plasma (ICP, Varian Liberty Series II) using bovine liver catalase as the protein standard. Briefly, the filtrate was concentrated using a concentrator until the water had almost evaporated. 4.0 ml of sulfuric acid was added to the concentrated filtrate, then 3.0 ml of an aqueous H_2O_2 solution (30 %) was added and the solution was diluted to 25 ml with distilled water. To produce a calibration curve, the same procedure was applied for different amounts of catalase (2.5–25.0 mg). The iron content of the solvents used was also measured. The amount

of immobilized catalase was estimated by subtracting the amount of catalase determined in the filtrate from the total amount of catalase used in the immobilization procedure.

Enzyme activity assay

The catalase activity was determined according to Lartillot,[20] which is a modification of the method described by Bergmeyer.[21] The catalase activity was measured spectrophotometrically at 240 nm using a specific absorption coefficient of 0.0392 $cm^2/\mu mol$ H_2O_2. The reaction mixture containing 2.5 ml of substrate made up of 10 mM hydrogen peroxide in a 50 mM, pH 7.0, phosphate buffer and 2.78×10^{-4} mg of free catalase or 5.0 mg of immobilized catalase was used unless otherwise mentioned. The reaction was performed at 25 °C for 2 min and stopped by adding 0.50 ml of 1.0 M HCl. Eggshells alone and denaturated ICATG, kept in a boiling water bath for 1 h, were also used as controls to determine whether they decompose hydrogen peroxide. The activity of the free catalase is given as U/mg of protein and the activity of immobilized catalase is given as U/g of carrier or U/mg of protein.

Effects of pH, buffer concentration and temperature on the activity of free and immobilized catalase

Effect of pH. The dependence of the activity on pH was assayed using 50 mM acetate buffer for pH 5.0 and 5.5, 50 mM citrate buffer for pH 6.0, 50 mM phosphate buffer for pH 6.5, 7.0, 7.5 and 8.0 and 50 mM borate buffer for pH 9.0 for both the free catalase and the ICATG.

Effect of buffer concentration. The dependence of the activity of free catalase and ICATG on ionic strength was assayed using 25, 50, 75 and 100 mM buffer at pH 7.5 and 6.0, respectively.

Effect of temperature. The effect of temperature on the activity of free catalase and ICATG were investigated in the temperature range 10–60 °C.

Thermal, storage and operational stabilities

Thermal stability. An estimation of the thermal stability was performed by measuring the residual activity of free catalase and ICATG exposed to temperatures of 30, 35, 40, 50 and 60 °C. Samples were taken at 1, 3, 7 and 15 h time intervals during incubation and the residual activities were measured.

Storage stability. Free catalase and ICATG were stored at 5 °C and room temperature and the residual activities were measured.

Operational stability. To determine the operational stability, 1.0 g of ICATG was mixed with 5.0 ml of 10 mM hydrogen peroxide solution in a glass column reactor (diameter 1.1 cm and length 10 cm) for 2 min at room temperature. The solution was then immediately separated from the ICATG and the absorbance was measured at 240 nm. The same procedure using the same ICATG sample was repeated every 2 min eighty times.

Effect of immobilization on the kinetic constants

The effects of substrate concentration (2.5–25.0 mM H_2O_2) on the activities of free and ICATG were investigated. The Michaelis–Menten coefficients (K_m) and maximum velocities (V_{max}) were determined from Lineweaver–Burk plots and the catalytical efficiencies (k_{cat}/K_m) were calculated.

RESULTS AND DISCUSSION

Immobilization of catalase on eggshell

The activity of ICATG as a function of the total amount of catalase contacted with 1.0 g of ground eggshell during immobilization is shown in Fig. 1. On increasing the total amount of catalase used in the immobilization from 42.5 mg to

85 mg, the ICATG activity increased from 38 to 86 U/g of carrier. However, the ICATG activity decreased when the total amount of catalase used in the immobilization was increased above 85 mg. Therefore, the optimal initial amount of catalase for immobilization was determined as 85 mg/g of carrier.

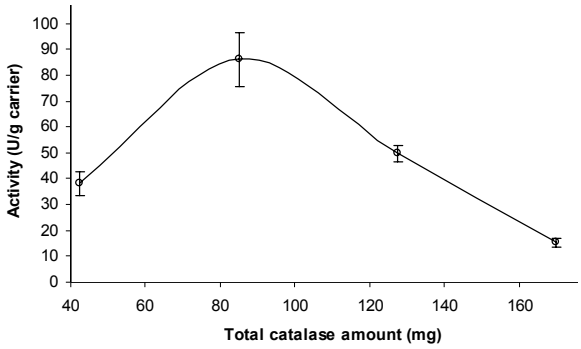

Fig. 1. Activity of ICATG as a function of the total amount of catalase contacted with 1.0 g of ground eggshell during immobilization.

In most of the immobilization studies, the enzyme load was measured by detecting the decreased amount of protein in the residual enzyme solutions. The biuret method, the Folin–Ciocalteu assay as modified by Lowry, the determination of Bradford with the dyestuff Coomassie brilliant blue or spectroscopic absorption measurements at 280 nm are frequently employed analyses. In the present study, it was not possible to obtain reproducible and reliable results for protein determination with the above-mentioned classical methods, probably due to the presence of glutaraldehyde in the filtrate, although in some studies it was reported that the protein content of the filtrate was determined using the Lowry method[9] and optical density measurement at 280 nm[17] even in the presence of glutaraldehyde. Therefore, the amount of catalase immobilized on the carrier was determined based on the iron content of catalase in the filtrate. The calibration curve for analysis of the iron content of the catalase solutions showed a linear correlation between the catalase concentration and the ICP iron signal, Fig. 2. In the present immobilization experiments, the amount of bound catalase ICATG was 82.3, mg/g of carrier, when 85 mg of total catalase was used per g of carrier.

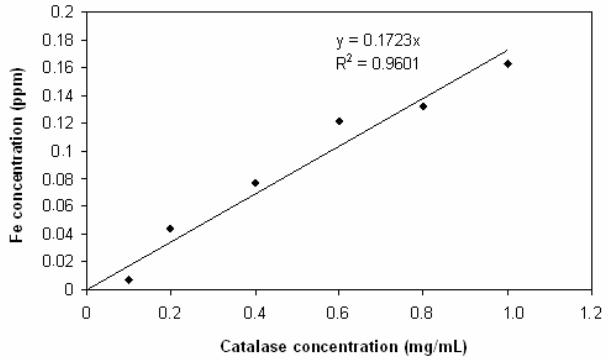

Fig. 2. The calibration plot for the determination of the amount of catalase using ICP. (The calibration plot shows the iron content of catalase *vs*. the concentration of bovine liver catalase).

Effects of pH, buffer concentration and temperature on the activity of free and immobilized catalase

Effect of pH. The effect of pH on the activity of free catalase and ICATG was studied in the pH range 5.0–9.0. The relative activities at different pH values are shown in Fig. 3. Free catalase and ICATG showed their maximum activity at pH 6.0 and 7.5, respectively. Chatterjee[9] reported that eggshell bound goat liver catalase showed two pH optima at 6.4 and 7.6. However, in this study only one pH optimum was determined. The maximum activity of catalase shifted in the acidic direction (pH 6.0) after immobilization on eggshell, as compared with that of free catalase. The relative activities of free catalase and ICATG were 18 and 56 % of maximal activity, respectively, at pH 5.0 (50 mM acetate buffer).

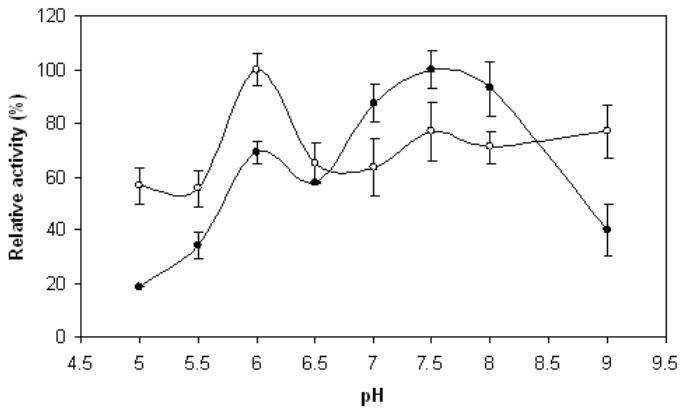

Fig. 3. Effect of pH on the activity of free catalase (●) and ICATG (○).

Effect of buffer concentration. The effect of buffer concentration (25, 50, 75 and 100 mM) on the activity of free catalase and ICATG were determined at pH 7.5 and 6.0, respectively. The results are presented in Fig. 4. The optimum buffer concentrations were determined as 50 mM and 75 mM for free catalase and ICATG, respectively. The ICATG activity was more significantly affected by the buffer concentration than that of free catalase. As the buffer concentration was increased gradually from 25 to 75 mM, the relative activity of ICATG also increased from 64 to 100 %. When the buffer concentration was increased from 75 to 100 mM, the relative activity of ICATG dramatically decreased from 100 to 33 %.

Effect of temperature. The effect of the temperature on the activity was investigated in the temperature range from 10 to 60 °C. The activities of free catalase and ICATG as a function of temperature are shown in Fig. 5, from which it can be seen that the maximum activities of free catalase and ICATG were 25 and 30 °C, respectively. At 60 °C, the relative activity of free catalase was only 13 %. However, the relative activities of ICATG at high temperatures (50–60 ºC) were higher than those of free catalase.

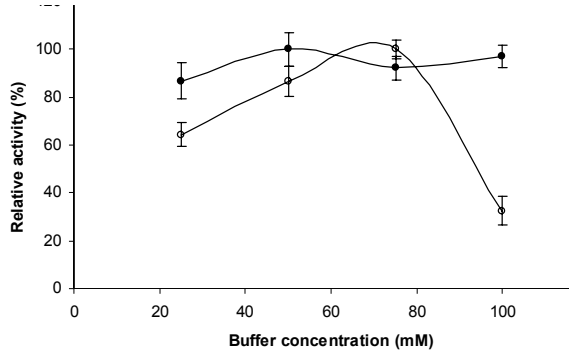

Fig. 4. Effect of buffer concentration on the activity of free catalase (●) and ICATG (○).

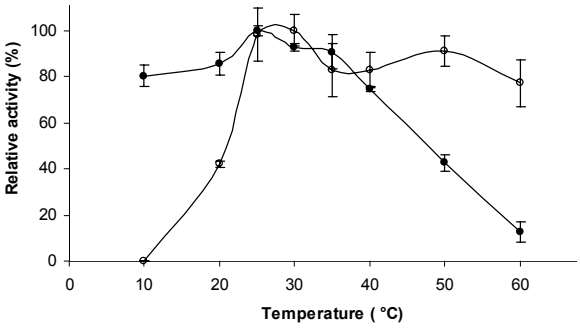

Fig. 5. Effect of temperature on the activity of free catalase (●) and ICATG (○).

Thermal, storage and operational stabilities

Thermal stability. The thermal stability studies were performed by measuring the residual activities of free catalase and ICATG after exposure to 5 different temperatures 30, 35, 40, 50 and 60 °C for 1, 3, 7 and 15 h. The residual activities of free catalase and ICATG are shown in Figs. 6a and 6b, respectively. The activities of the samples were determined under optimum conditions. Generally, as the pre-incubation time increased, the activities of free catalase and ICATG decreased at a constant test temperature. The residual activity of ICATG was 88 % of its original activity at 30 °C after 15 h pre-incubation time, as shown in Fig. 6a. The residual activities of ICATG at 50 and 60 °C were similar for the same incubation time. Free catalase did not show any activity after 15 h at 60 °C. Tükel and Alptekin[8] reported that catalase immobilized on florisil *via* glutaraldehyde showed no activity after 15 h at 60 °C. Betencor *et al.*[5] immobilized *M. lysodeikticus* catalase on agarose and they reported that the residual activity of the immobilized catalase was only 70 % of its initial activity after 10 h at 45 °C.

Storage stability. As shown in Fig. 7, ICATG stored at 5 °C was more stable than when stored at room temperature. After 36 days storage at room temperature, the residual activity of ICATG was 65 % of its initial activity while when stored at 5 ºC for the same time, the residual activity was 96 %. The free catalase completely lost its activity after storage for 11 days at both 5 °C and also at room

temperature. Çetinus *et al.*[22] immobilized catalase on glutaraldehyde-pretreated chitosan films. They reported that the free enzyme had retained about 50 % of its activity after 18 days, immobilized catalase stored wet about 50 % of its activity after 25 days and that stored dry about 50 % of its activity after 5 days at 5 °C. Arıca *et al.*[16] immobilized catalase on poly(2-hydroxyethyl methacrylate)-Cibacron Blue F3GA (poly HEMA-CB) and poly(2-hydroxyethyl methacrylate)-Cibacron Blue F3GA-Fe(III) (poly HEMA-CB-Fe(III)) derivatized membranes by adsorption. They found that the free enzyme lost all its activity within 20 days when stored at 4 °C. Immobilized preparations of poly HEMA-CB-CAT and poly HEMA-CB-Fe(III)-CAT lost 40 and 25 % of their activities during the same period at 4 °C. This decrease in activity was explained as a time-dependent natural loss in enzyme activity and this was prevented to a significant degree upon immobilization. Solas *et al.*[12] immobilized bovine liver catalase on bioskin by ionic adsorption. They reported that the free catalase lost its activity within 3 days and the immobilized catalase retained about 70 % of its activity for 16 days at room temperature.

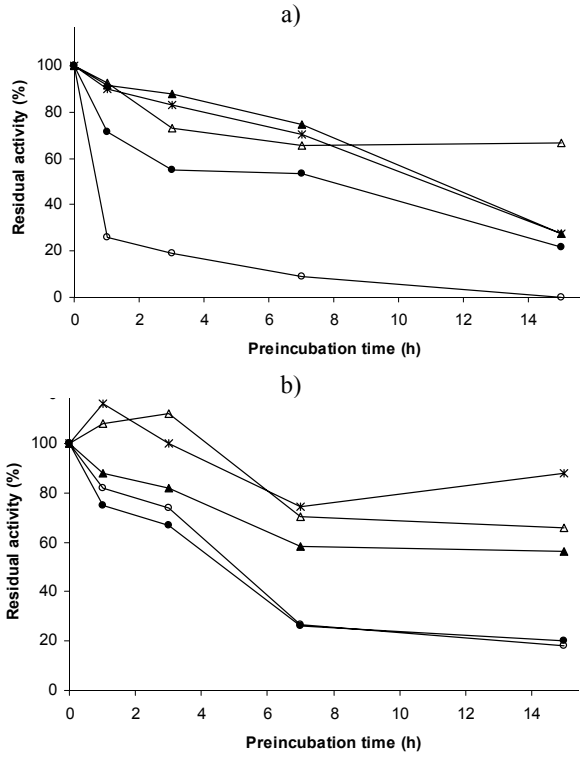

Fig. 6. Thermal stabilities of (a) free catalase and (b) ICATG at 30 (✳), 35 (▲), 40 (△), 50 (●) and 60 °C (○). The free catalase and ICATG activities were measured under their optimum conditions.

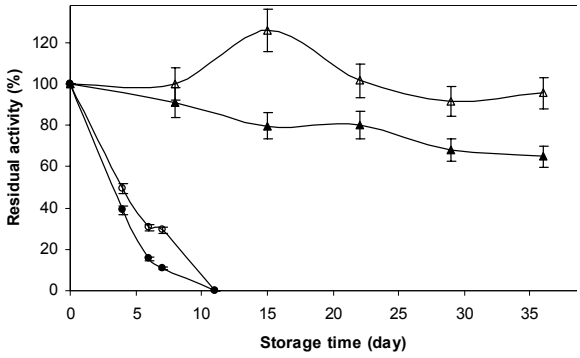

Fig. 7. Storage stabilities of free catalase at room temperature (●) and 5.0 °C (○) and ICATG at room temperature (▲) and 5.0 °C (△).

Operational stability. The operational stability of immobilized enzyme systems is very important for various biotechnological applications; an increased stability could make an immobilized enzyme more advantageous than its free counterpart. The operational stability of ICATG was determined and the results are presented in Fig. 8, from which it may be seen that the activity decays with increasing number of reuses. The remaining activity of ICATG was about 73 % of its initial value after 80 cycles of batch operation. Betancor *et al.*[2] immobilized catalase on dextrane and reported that the immobilized catalase had not lost its activity after 10 cycles. Tükel and Alptekin[8] reported that the remaining activity of catalase immobilized *via* glutaraldehyde on florisil was about 90 % and catalase immobilized *via* glutaraldehyde + spacer was about 30 % after 20 cycles of batch operation.

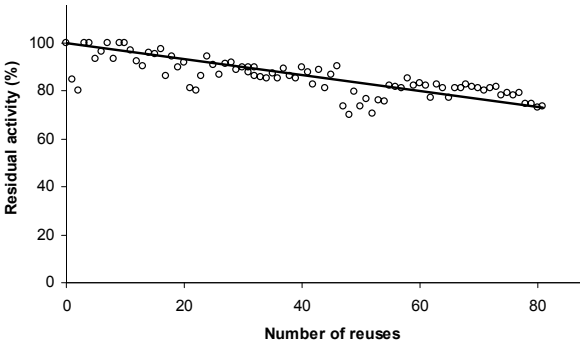

Fig. 8. The residual activity of ICATG in dependence on the number of reuses in a batch type reactor.

Effect of immobilization on the kinetic constants

The K_m and V_{max} values for free catalase and ICATG were determined and are presented in Table I. from which it can be seen that the K_m value of ICATG was smaller than that of the free catalase. However, the V_{max} values and catalytic efficiencies of ICATG were smaller than those of the free catalase. The catalytic efficiencies (k_{cat}/K_m) of free catalase and ICATG were 1.4×10^3 and 2.9 $m^3\ s^{-1}\ mol^{-1}$, respectively. In a previous study,[8] it was found that the K_m value for catalase

immobilized *via* glutaraldehyde on florisil was appreciably higher (10 fold) than that of the free catalase and its V_{max} was about 1 % of the V_{max} of the free catalase. Çetinus *et al*.[22] immobilized bovine liver catalase on glutaraldehyde pretreated chitosan films. They found K_m = 25.16 mM and V_{max} = 24042 min^{-1} μmol H_2O_2/mg of protein for free catalase and K_m = 27.67 mM and V_{max} = 1022 min^{-1} μmol H_2O_2/mg of protein for immobilized catalase. Eberhardt *et al*.[5] immobilized *Aspergillus niger* catalase on cellulose *via* glutaraldehyde and reported K_m and V_{max} values of free and immobilized catalase as 0.02664±0.00674 M and 35120±6762 min^{-1} μmol/mg of enzyme and 0.115±0.0166 M and 3135±203.8 min^{-1} μmol/mg of enzyme, respectively. Thus, a compromise has to be made between immobilized catalase, which is less active but more stable, and free catalase, which although being more active is less stable.

TABLE I. Michaelis–Menten constant and maximal reaction rate values for free and immobilized catalase

Enzyme form	K_m / mM	V_{max} / U (mg protein)$^{-1}$	k_{cat} / 10^4 s^{-1}	$(k_{cat}/K_m)\times10^{-3}$ / m^3 s^{-1} mol^{-1}
Free catalase	49.0±1.6	1.7±0.05×10^4	6.8	1.4
ICATG	41.9±2.7	29.1±1.2	0.012	0.0029

CONCLUSIONS

In this study, the optimal initial amount of catalase for immobilization was determined as 85 mg/g of carrier. Reproducible and reliable results for protein determination could not be obtained with classical methods, probably due to the presence of glutaraldehyde in the filtrate. The amount of catalase bound on the carrier was estimated using the results of induced coupled plasma measurements. ICATG showed its maximum activity in 75 mM, pH 6.0, citrate buffer at 30 °C. The thermal stability of ICATG was higher than that of free catalase at 60 °C. Remaining activity of ICATG was about 73 % of the initial activity after 80 cycles of batch operation. Catalase immobilization onto eggshell is economic and has good reusability. Thus, it can be concluded that eggshell is an efficient carrier for immobilizing catalase.

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ИЗВОД

КАРАКТЕРИЗАЦИЈА КАТАЛАЗЕ ИЗ ЈЕТРЕ ГОВЕЧЕТА ИМОБИЛИСАНЕ НА ЉУСЦИ ЈАЈЕТА

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Окатрактерисана је каталаза из јетре говечета имобилисана на честицама љуске јајета, док је могућност поновног коришћења имобилисане каталазе испитивана у серијском реактору. За каталазу имобилисану на спрашеној љусци јајета (ICATG) оптимална почетна коли-

чина каталазе износи 85 mg/g спрашене љуске, оптимална pH вредност износи 6,0 (75 mM цитратни пуфер), а температура 30 °C. Одређене су вредности V_{max} и K_m за ICATG од 29,1± ±1,2 U/mg протеина и 41,9±2,7 mM, респективно. Иситивање могућности поновног коришћења ICATG указује на то да активност износи 73 % у односу на почетну активност након 80 циклуса узастопног коришћења. Количина каталазе везане за носач процењена је на основу резултата испитивања методом индуковане куповане плазме. Каталитичка ефикасност (k_{cat}/K_m) слободне каталазе и ICATG износе $1{,}4\times10^6$ и $2{,}8\times10^3$ $dm^3\ s^{-1}\ mol^{-1}$, респективно. Имобилизација каталазе на љусци јајета је економична са великом могућношћу поновног коришћења, па се закључује да је љуска јајета добар носач за имобилизацију каталазе.

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