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Immobilization of β-glucosidase onto a mesoporous silica support: physical adsorption and covalent binding of the enzyme

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Abstract: In this study, the immobilization of *β*-glucosidase onto mesoporous silica support by physical adsorption and covalent binding was investigated. The immobilization was performed onto micro-sized silica aggregates with an average pore size of 29 nm. During physical adsorption, the highest yield of immobilized *β*-glucosidase was obtained with an initial protein concentration of 0.9 mg mL^{-1}. The addition of NaCl increased 1.7-fold, while the addition of Triton X-100 decreased 6-fold adsorption yield in comparison to the one obtained without any addition. Covalently bonded *β*-glucosidase, *via* glutaraldehyde previously bonded to silanized silica, had a higher yield of immobilized enzyme as well as higher activity and substrate affinity in comparison to the one physically adsorbed. Covalent binding did not considerably change pH and temperature stability of the obtained biocatalyst in range of values that are commonly used in reactions in comparison to the unbound enzyme. Furthermore, covalent binding provided a biocatalyst that retained over 70 % of its activity after 10 cycles of reuse.

Keywords: *β*-glucosidase; immobilization; physical adsorption; chemical binding; mesoporous silica.

INTRODUCTION

The enzymatic modification of cellulose is a field of great interest in many processes due to the numerous applications of one of its degradation products, glucose. A cellulose polymer is degraded to glucose through the cooperative actions of a complex of cellulolytic enzymes. Cellobiohydrolases hydrolyze the cellulose polymer from the ends, releasing cellobiose as the product, while endoglucanases randomly hydrolyze the internal *β*-1,4-linkages creating more free ends for the action of cellobiohydrolases.[1] Finally, *β*-glucosidases hydrolyze the cellobiose to glucose. Cellobiohydrolases and endoglucanases are often inhibited

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by cellobiose, making *β*-glucosidases the key enzyme for efficient hydrolysis of cellulose in terms of avoiding decreased hydrolysis rates over time due to cellobiose accumulation.[2,3]

However, the cellulases complexes from the main fungal producers normally contain amounts of *β*-glucosidase that are not sufficient to hydrolyze all the released cellobiose.[3,4] Thus, during enzymatic hydrolysis of cellulose in order to prevent limitation of the overall hydrolysis rate by accumulated cellobiose, *β*-glucosidase often must be additionally supplemented to the reaction mixture.[5] However, enzyme cost is one of the major obstacles in the path of large-scale commercialization of the enzymatic hydrolysis of cellulose. Enzyme recovery and recycling, which is achievable by immobilization, is one of the most important and effective ways of increasing the efficiency of an enzymatic hydrolysis process.[6] With respect to cellulose hydrolysis, immobilization of *β*-glucosidase might be especially beneficial to process economy, considering the necessity for its addition to regular complex cellulases.

The variety of enzyme immobilization methods can be reduced to two main approaches, *i.e.*, physical adsorption and covalent binding with a carrier, both of which have advantages and shortcomings. Physical adsorption is simple to perform, cheap and tends to be less destructive to the enzyme than chemical binding, but interactions formed between the enzyme and the supports are unstable. In covalent binding, the bonds between the carrier and enzyme are very strong resulting in a highly stable conjugate, but, very often, the enzyme activity drops considerably.[5] Many supports have been tested for their ability to immobilize *β*-glucosidase, of which porous silica, the most commonly used material for enzyme immobilization, was proven to be suitable for the immobilization of *β*-glucosidase.[5,7]

The aim of this study was to investigate the immobilization of a new commercial *β*-glucosidase, which is part of the Novozymes Cellulosic Ethanol Enzyme Kit (Novozymes, Denmark), onto a mesoporous silica support using physical adsorption and covalent binding. The biocatalysts obtained by physical adsorption and covalent binding were compared with respect to the immobilization yield, activity and substrate affinity. Then, in next set of experiments, the biocatalyst that provided the best results for these parameters was tested for stability and reusability in a real system with carboxymethyl cellulose as the substrate.

MATERIALS AND METHODS

β-*Glucosidase and enzyme assay*

In this study *β*-glucosidase NS22118 from Novozymes Cellulosic Ethanol Enzyme Kit, a kind gift of Novozymes (Novozymes, Denmark), was used for the immobilization experiments. The protein concentration in the enzyme solution was 26.7 mg mL^{-1}, while its activity was 558 U mL^{-1}, which were determined as described below.

A sample of enzyme solution or obtained biocatalyst (support with immobilized enzyme) was added to 1 mL of 50 mM citrate buffer (pH 5.0) containing 2 mM 4-nitrophenyl-β-D-glucopyranoside.[3] The reaction mixture was incubated for 10 min at 50 °C. After addition of 2 mL Na_2CO_3 solution (1 M), the absorbance was read at 405 nm. A standard curve was obtained from the released *p*-nitrophenol. One unit of enzyme activity was defined as 1 mM *p*-nitrophenol produced per minute under the assay conditions and it is expressed per mL of enzyme solution or per gram of material containing the immobilized enzyme.

The protein concentration of enzyme preparation and solutions (supernatant obtained after separation of the support) were determined by the Bradford method using bovine serum albumin as the standard.[8]

The experimental results are the mean value of at least three measurements (the accuracy was ±5 %) on a minimum of three replicas for every experimental point.

Support material

Mesoporous silica particles were used as the support for β-glucosidase immobilization. The materials were synthesized from highly basic sodium silicate solutions of three different SiO_2 concentrations as reported by Filipović *et al*.[9,10] Sulfuric acid was slowly added into the well stirred sodium silicate solutions at 90 °C to precipitate silica particles. Finally, the pH was adjusted at value of 4 to prevent dissolution process. The white precipitated powders were washed with distilled water, separated from liquid phase by filtration and finally dried at 120 °C for 1 day.[9] The obtained silica powders were calcined at 500 °C for one hour in order to remove the synthesizing template.

The particle size was measured by dynamic light scattering on Zetasizer Nano ZS, (Malvern Instruments, UK). The specific surface area (according to the BET method), pore size distribution (according to the BJH method) and pore volume of the as-synthesized particles were measured by low temperature nitrogen adsorption using a Autosorb-3B instrument (Quantachrome, USA). The size and morphology of the particles were examined using a scanning electron microscope (JEOL JSM 6460 LV, Japan).

Immobilization of β*-glucosidase*

In the experiments of physical adsorption of β-glucosidase onto a silica support, 25 mg of silica and 4 mL of a β-glucosidase solution in 50 mM citrate buffer, pH 4.7 were used. Adsorption was conducted at the room temperature for one hour under magnetic stirring. The experiments were performed with the aim of determining the influence of the initial enzyme concentration, in the range 0.54–2.70 mg mL^{-1}, on the physical adsorption of β-glucosidase. In order to investigate the influence of poly(ethylene) glycol-*p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100) or NaCl on the physical adsorption of β-glucosidase, the immobilization was conducted under the previously mentioned conditions at an initial enzyme concentration 0.9 mg mL^{-1}, in 50 mM citrate buffer containing 0.1 vol. % Triton X-100 or 0.8 M NaCl.

The chemical immobilization of β-glucosidase onto the silica support was conducted through covalent bonding as reported by Weetall.[11] The support material was first functionalized through silanization with a 0.5 vol. % solution of 3-amino-propyltriethoxysilane (APTS) for 3 h at 75 °C.[11] Subsequently, the material was washed three times with distilled water and dried for 15 h at 105 °C. Activation of functionalized material was conducted with 2.5 vol. % solution of glutaraldehyde in sodium phosphate buffer (pH 7.0) for 45 min at 20 °C and washed three times with distilled water. Finally, immobilization of β-glucosidase (protein concentration 20 mg mL^{-1} in 0.01 M phosphate buffer, pH 6.8) was realized through contact of the enzyme with functionalized and activated support (0.1 g) for 15 h at 20 °C.[11]

After each immobilization reaction, the silica support (with immobilized *β*-glucosidase) was separated through centrifugation at 14,000 rpm for 5 min (Mini Spin Plus, Eppendorf) and the protein concentration in the supernatant was measured in order to calculate the amount of *β*-glucosidase immobilized on the silica support (q_i / mg *β*-glucosidase g^{-1} dry silica) using the following equation:

$$q_i = \frac{V(c_0 - c_s)}{w} \quad (1)$$

where V is the volume of the aqueous phase (mL), c_0 and c_s (mg *β*-glucosidase mL^{-1} solution) are the concentrations of *β*-glucosidase initially added and in the supernatant obtained after immobilization, respectively, and w is the weight of dry silica (g).

Stability and reuse

Effect of pH on the relative stability of the covalently bonded *β*-glucosidase was investigated during 1 h exposure to pH values in the range 2–9 (Britton–Robinson buffer), while effect of temperature was investigated during 1 h exposure to temperatures in the 20–80 °C for both the free and immobilized enzyme.

The reusability of the covalently bonded *β*-glucosidase was investigated by performing successive reactions with the biocatalyst (for 30 min each). The biocatalyst was supplemented to a cellulase complex (Celluclast 1.5 L, Novozymes) and used for the degradation of a 2 vol. % solution of carboxymethyl cellulose (as substrate) in 50 mM citrate buffer (pH 4.7) at a temperature of 40 °C under gentle mixing (50 rpm).

RESULTS AND DISCUSSION

Physical adsorption of β*-glucosidase*

In order to determine the most suitable silica support for the adsorption of *β*-glucosidase mesoporous silica supports with different characteristics were used. The mesoporous silica supports were synthesized as described earlier[9,10] and their characteristics regarding pore size, specific surface area and total pore volume were determined.[10] The tested silica particles were micro-sized aggregates (Fig. 1). The pore size distribution of the silica supports donated as Snv-90, Sns-90 and Snn-90, with an average pore size of 29, 24 and 16 nm, respectively, are presented in Fig. 2.

The results obtained for the amounts of enzyme physically adsorbed onto the different silica supports are shown in Fig. 3. The highest amount of adsorbed *β*-glucosidase was obtained with the silica support having the largest average pore size (29 nm),[10] which also had the widest pore size distribution (Fig. 2). It was found that *β*-glucosidase from *Aspergillus niger* has an unsymmetrical, so-called tadpole-like shape with a diameter of 15 nm (*i.e.*, maximum diameter) and a radius of gyration of 4.2 nm.[12] Accordingly and depending of the molecule orientation, it might be that the enzyme requires more space for entrance into the pores than is defined by its diameter. As result, the highest amount of adsorbed enzyme was obtained on the support with the largest investigated pore size and this material was used for the further experiments. In all experiments of physical

adsorption, the conditions with respect to the pH value of the reaction were chosen to prevent electrostatic repulsion between silica and the enzyme (pH 4.7).

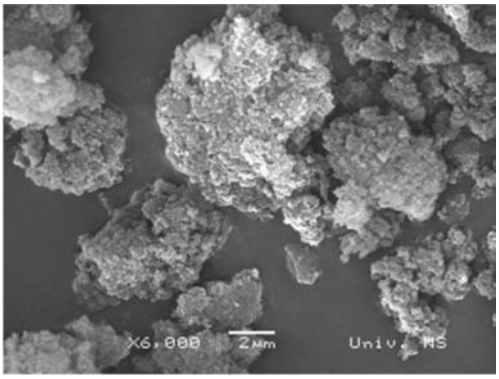

Fig. 1. Scanning electron micrograph of the mesoporous silica aggregates.

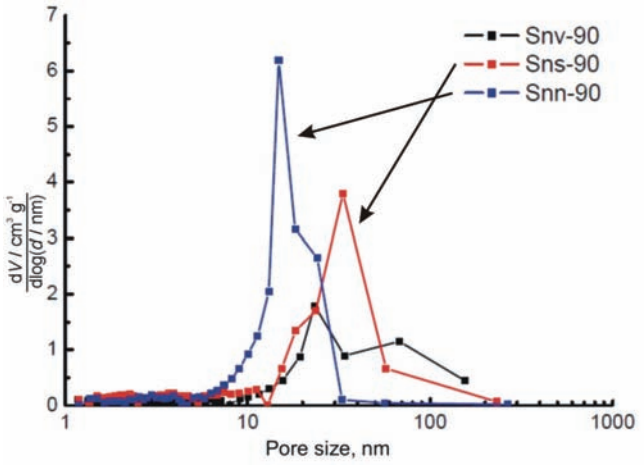

Fig. 2. Pore size distribution for tested silica particles (data for Snv-90 from the literature[10]).

Considering that the concentration gradient is an important driving force for physical adsorption, the influence of the initial β-glucosidase concentration on adsorption was investigated. In the investigated range of loaded enzyme, the highest amount of adsorbed β-glucosidase was obtained at a protein concentration of 0.9 mg mL^{-1} (data not shown), while higher values did not lead to an increase in the amount of adsorbed β-glucosidase. This might be due to saturation of the binding sites, suggesting that the available sites on the silica surface are the limiting factor for the adsorption, as was reported for other proteins.[13]

Physical adsorption provided a yield of immobilized enzyme of 20.1 mg of β-glucosidase per gram of silica support. The obtained biocatalyst had an activity of 7.65 U per gram of silica support and an affinity towards the substrate of 15.06 mM (Table I).

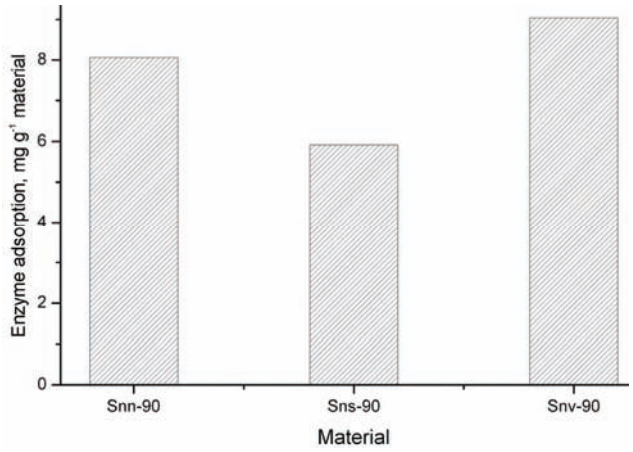

Fig. 3. Adsorption of *β*-glucosidase onto different silica supports.

TABLE I. Characteristics of physically adsorbed and covalently bonded *β*-glucosidase onto mesoporous silica support (pore size 29 nm)

β-Glucosidase	Physically adsorbed	Covalently bonded
Amount of adsorbed enzyme, mg g^{-1} material	20.10	42.00
Activity, U g^{-1} material	7.65	15.58
K_M / mM	15.63	11.06

Influence of Triton X-100 and NaCl on the physical adsorption of β*-glucosidase*

In order to reveal the mechanism of *β*-glucosidase adsorption onto the surface of silica, the effect of salt and detergent on the adsorption efficiency was investigated. The adsorption was performed in citrate buffer (pH 4.7) containing 0.8 M NaCl or 0.1 vol. % Triton X-100 and compared with adsorption in citrate buffer alone (Fig. 4). Presence of 0.8 M NaCl increased *β*-glucosidase adsorption by 1.7-fold in comparison to the adsorption yield obtained in its absence. On the other hand, the addition of 0.1 vol.% Triton X-100 decreased the *β*-glucosidase adsorption 6-fold in comparison to the adsorption in its absence (Fig. 4).

Generally, protein adsorption is the result of the action of electrostatic and van der Waals forces (between two dissimilar surfaces) and it would be expected that electrostatics play an important role in the adsorption process. However, the change of concentration of the supporting electrolyte may have surprising effects as recently demonstrated for cytochrome c[14] and lysozyme.[15] The increase of the adsorption upon NaCl addition might be explained by its influence on the charge on both the support and enzyme. Namely, the charge density on silica increases with increasing NaCl concentration while the effective charge on the surface of the enzyme might be strongly affected by the anion type and concentration (as shown for lysozyme).[15] As a result, the increase in ionic strength reduces the Debye length and consequently weakens the electrostatic attractive forces between

the protein and the silica-based surface. This favors van der Waals attractive interactions, which are stronger than expected because of the adsorption of Cl^- at the charged interface of the protein.[15]

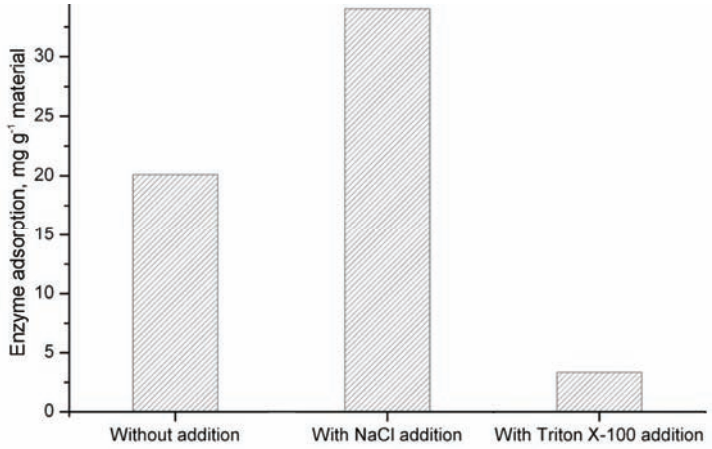

Fig. 4. Physical adsorption of β-glucosidase onto mesoporous silica support (pore size 29 nm).

On the other hand, in the presence of Triton X-100, the adsorption efficiency was reduced by more than six fold. This might be explained by the possible competition for regions where interactions with β-glucosidase might have occurred on the silica surface and additional physical hindrance of the silica surface by the formation of small aggregates, which are characteristic for the initial phase of detergent adsorption.[16] Furthermore, the presence of Triton X-100 might weaken van der Waals forces, which are one of the driving forces for β-glucosidase adsorption besides electrostatic interaction.[17]

Thus, presented results might indicate that physical adsorption of β-glucosidase onto silica support under investigated conditions (pH 4.7 and room temperature) could be result of synergistic effect of at least two types of interaction – electrostatic and van der Waals forces.

Covalent binding of β*-glucosidase*

Covalent binding is known to have the advantage of forming strong and stable linkages between an enzyme and the carrier, which consequently eliminates the loss of activity caused by enzyme leakage from the support.[5] Thus, with the aim of improving the characteristics and yield of bonded enzyme, covalent binding of β-glucosidase onto the silica support was performed. β-Glucosidase was covalently bonded onto silica material *via* glutaraldehyde, which had previously been attached to the functionalized silica support.

Covalent binding provided a yield of bonded enzyme of 42 mg per gram of silica support, which was 2.1-fold higher in comparison to the amount obtained

by physical adsorption (Table I). In addition, the covalently bonded *β*-glucosidase had a higher activity of enzyme per gram of material (15.58 U g^{-1}) in comparison to that physically adsorbed, while the biocatalyst obtained by covalent binding had a lower K_M value (11.06 mM), *i.e.*, a higher affinity towards the substrate (Table I). A similar yield of *β*-glucosidase covalently bonded onto silica gel was reported by Karagulyan *et al.*,[5] while Wang *et al.*[7] obtained a considerably higher yield with respect to the amount of bonded *β*-glucosidase, although its activity was similar to the that obtained in this study.

Stability and reusability of covalently bonded β*-glucosidase*

Due to better immobilization yield and better characteristics of the biocatalyst obtained by covalent binding, such as higher enzyme activity per gram of material and lower K_M value, the biocatalyst was subjected to an investigation of its pH and temperature stability, as well as its reusability.

The stability of the covalently bonded *β*-glucosidase was investigated under different conditions with respect to temperature and pH. Within the investigated range of the temperatures (20–80 °C), covalent binding of *β*-glucosidase did not considerably affect the profile of temperature stability in comparison to that of the free enzyme (Fig. 5). On the other hand, at the screened values of pH (2–9), the stability profile of the covalently bonded enzyme was changed in comparison to that of free *β*-glucosidase (Fig. 6). However, the activity of the immobilized *β*-glucosidase was similar to that of the free enzyme in the pH range 4–5 that are commonly used in cellulase reactions (Fig. 6).

Recycling and reuse of an immobilized enzyme is one of the most important aims of its immobilization with respect to the economy of processes involving

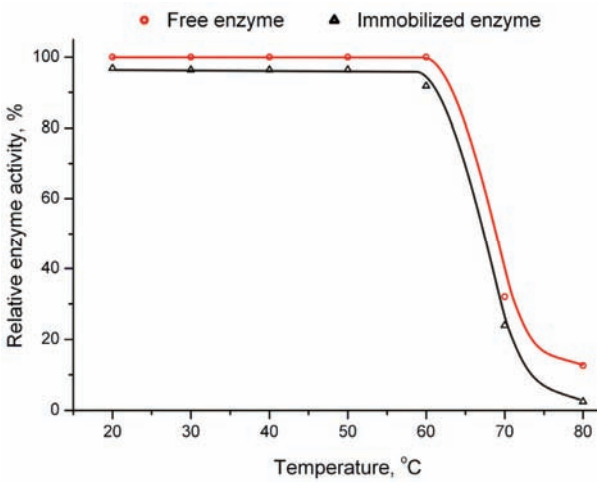

Fig. 5. Temperature stability of *β*-glucosidase covalently bonded onto mesoporous silica support (pore size 29 nm).

reactions with this kind of biocatalyst. Thus, the biocatalyst obtained by covalent binding of β-glucosidase onto silica was subjected to successive reactions (for 30 min each) with carboxymethyl cellulose as the substrate under mild conditions that were appropriate for the enzyme and at the same time not destructive for the material.[18]

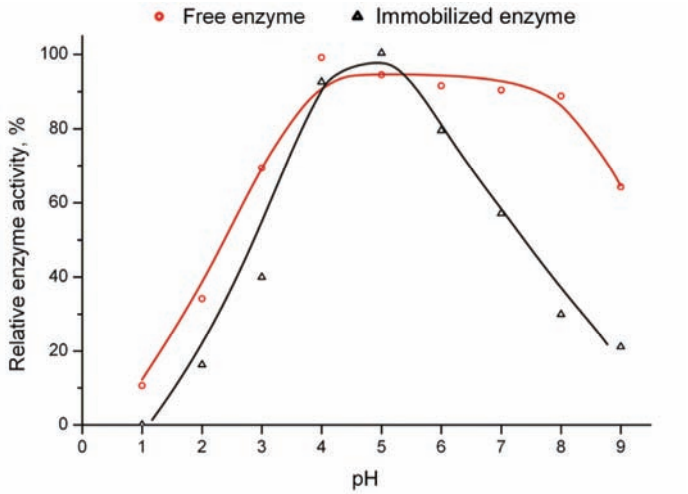

Fig. 6. pH stability of β-glucosidase covalently bonded to the mesoporous silica support (pore size 29 nm).

Ten cycles of biocatalyst were performed and the obtained results are shown in Fig. 7. It could be noticed that after ten cycles of use, the covalently bonded β-

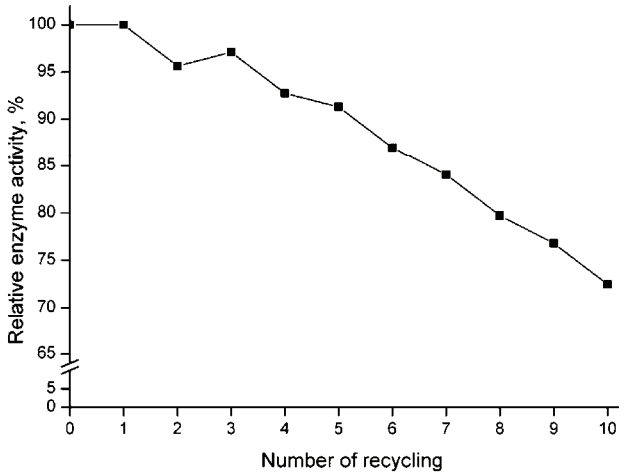

Fig. 7. Repeated use of covalently bonded β-glucosidase onto the mesoporous silica support (pore size 29 nm).

glucosidase had retained more than 70 % of its initial activity. Similar result within ten cycles of reuse of covalently immobilized *β*-glucosidase onto different silica-derived nanoparticles was previously reported.[7]

CONCLUSIONS

This paper presents preliminary results of an investigation of immobilization of a new commercial *β*-glucosidase (from Novozymes Cellulosic Ethanol Enzyme Kit). Considering process economy, immobilization of this *β*-glucosidase might be very beneficial because it is intended to be used for supplementation of cellulase complexes involved in the hydrolytic degradation of cellulose in (ligno)cellulosic biomass. The immobilization was performed on chosen micro-sized, mesoporous silica particles with an average pore size of 29 nm. Physical adsorption was conducted under conditions that prevented repulsion between the enzyme and support. The addition of NaCl increased, while the addition of Triton X-100 decreased the amount of physically adsorbed enzymes, indicating the mechanism involved in the process. Covalent bonding of *β*-glucosidase through silanization of the silica support and binding of enzyme *via* glutaraldehyde gave higher yields of bonded *β*-glucosidase 2.1-fold in comparison to that physically adsorbed. Furthermore, covalently bonded *β*-glucosidase had a higher activity (per gram of silica support) and higher affinity towards the substrate in comparison to the physically adsorbed enzyme, while it retained its thermal and pH stability in comparison to the free enzyme in the range of these parameters commonly used for cellulase reactions. Furthermore, the obtained biocatalyst retained high activity (more than 70 % of its initial activity) after ten times of recycling.

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И З В О Д

ИМОБИЛИЗАЦИЈА *β*-ГЛУКОЗИДАЗЕ НА МЕЗОПОРОЗНИ СИЛИКА НОСАЧ: ФИЗИЧКА АДСОРПЦИЈА И КОВАЛЕНТНО ВЕЗИВАЊЕ ЕНЗИМА

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У овом раду је испитана имобилизација *β*-глукозидазе на мезопорозном силика носачу применом физичке адсорпције и ковалентног везивања. Имобилизација је извршена на силика микро-агрегатима са просечном величином пора од 29 nm. Током физичке адсорпције највећи принос имобилизоване *β*-глукозидазе је добијен при почетној концентрацији протеина од 0,9 mg ml^{-1}. Додатак NaCl је 1,7 пута повећавао, а додатак детергента Triton X-100 6 пута смањио принос адсорпције у односу на принос остварен без додавања. Ковалентно везана *β*-глукозидаза, путем глутараледхида који је везан за претходно силанизовану силику, имала је виши принос имобилизованог ен-

зима, као и вишу активност и афинитет према супстрату у поређењу са физички везаном *β*-глукозидазом. У поређењу са слободним ензимом ковалентно везивање није значајно променило pH и температурну стабилност добијеног биокатализатора при вредностима ових параметра које се уобичајено користе у реакцијама. Осим тога, ковалентно везивање је обезбедило добијање биокатализатора који је задржао преко 70 % активнсоти након 10 циклуса узастопне употребе.

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