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ENTRAPMENT OF MICROBIAL AMYLASES AND CELLULASES IN SILICA-GELS

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Amylases and cellulases are very useful hydrolases with extensive biotechnological applications. Effective bioconversion catalyzed by enzymes claims different kinds of stable and reusable biocatalysts. A good strategy to improve the stability of free enzymatic preparations could be immobilization in porous supports. The sol-gel process is a well-known method to obtain hybrid organic-inorganic biocompatible ceramic mesoporous materials under mild conditions. The aim of this study was to immobilize two enzymatic preparations from *Bacillus amyloliquefaciens* and *Trichoderma viride* with amylase and cellulase activity respectively, by entrapment in silica gel. The silica gels were obtained by using an organoalkoxisilane precursor tetrakis (2-hydroxiethyl) orthosilicate (THEOS). The activity and stability of native and entrapped enzymatic preparations were studied comparatively, as well as their kinetic behavior.

INTRODUCTION

Enzymes catalyze biochemical reactions being involved in the metabolism of all living organisms, and in the same time are useful biocatalysts with great specificity for industrial biocatalytic conversions.

Enzymatic bioconversion of starch and cellulose and also of their derivatives (for instance carboxymethyl cellulose (CMC)), to monomeric sugars, with significant commercial interest, has been intensively studied in the recent years to produce bioethanol and bio-based products, food and animal feeds, many valuable chemicals, pharmaceuticals and biosensors.[1-3]

Effective conversion of starch and cellulose to fermentable sugars requires enzymatic preparations with great stability for specific processes and high catalytic efficiency.[4] A good strategy to improve the low stability of free enzymatic preparations with amylase and cellulase activity could be immobilization in hybrid organic-inorganic porous supports, with high surface area and high pores volume.[5,1]

The sol-gel process is a well-known method to obtain biocompatible mesoporous materials under mild conditions. Bioimmobilization by entrapment in silica gels has been shown to improve enzyme activity and stability in aqueous and organic-aqueous media.[6-9]

Tetrakis (2-hydroxiethyl) orthosilicate (THEOS) is a completely water-soluble precursor that forms porous three dimensional networks in aqueous solution in the absence of added alcohol.[10] The alcohol needed by the poor-water soluble common tetraalcoxysilane precursors (tetramethoxysilane, TMOS and tetraethoxysilane, TEOS) can lead to partial denaturation of enzyme molecules. During the gelification process the enzyme is entrapped in silica materials without covalent binding to the matrix.[11-13]

RESULTS

Enzymatic preparations with amylase and cellulase activities were obtained by fermentation of a series of *Bacillus* and *Trichoderma* microbial

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cells, in order to select the strains producing the best enzymatic activity. Amylase production by five *Bacillus* strains and cellulase production by two *Trichoderma* strains are shown in Table 1 and Table 2 respectively.

In our immobilization work two microbial strains were used, an amylase producing bacterial *Bacillus amyloliquefaciens* DSM 7 strain and a cellulase (CMCase and cellobiase) producing fungal *Trichoderma viride* CMGB 1 strain. These enzymatic preparations were subsequently immobilized by entrapment in silica gel obtained using tetrakis (2-hydroxiethyl) orthosilicate (THEOS) as organoalkoxisilane precursor by sol-gel method. Enzyme activity and protein content of free and immobilized enzymes are given in Table 3.

Table 1

Selection of amylase producing *Bacillus* strains

Bacterial strain	**pH**	**Fermentation time h**	**Amylase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$**	**Protein content $mg_{BSA} \cdot mL^{-1}$**
Bacillus subtilis 222	8.00	72.00	0.92±0.18	1.89±0.16
Bacillus subtilis SML amy+	8.00	72.00	0.94±0.21	2.28±0.19
Bacillus subtilis 3218	8.00	72.00	2.03±0.28	2.34±0.22
Bacillus globigi CMIT 1.44	8.00	72.00	2.36±0.30	2.46±0.23
***Bacillus amyloliquefaciens* DSM 7**	**8.00**	**24.00**	**10.54**±1.32	**3.38**±0.29

Table 2

Selection of cellulase (CMCase and cellobiase) producing *Trichoderma* strains

Strain	**pH**	**CMCase activity $mmol \cdot min^{-1} \cdot mL^{-1}$**	**Cellobiase activity $mmol \cdot min^{-1} \cdot mL^{-1}$**	**Protein content $mg_{BSA} \cdot mL^{-1}$**
***Trichoderma viride* CMGB 1**	**4.50**	1.79±0.16	0.72±0.11	**3.30**±0.27
Trichoderma longibrachiatum DSM 769	5.00	0.24±0.08	0.32±0.09	2.28±0.10

Table 3

Protein content and enzyme activity of free and immobilized preparations

***Bacillus amyloliquefaciens* DSM 7 amylase**

	Protein content $mg_{BSA} \cdot mL^{-1}$ $mg_{BSA} \cdot g^{-1}$	**Amylase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$ $\mu mol \cdot min^{-1} \cdot g^{-1}$**	**Immobilization yield[c] %**
Native			
Fermentation medium[a]	3.38±0.29	10.54±1.32	-
Lyophilized enzymatic preparation[b]	278.36±23.36	1196.00±10.87	-
Immobilized[b]			
Entrapment THEOS	3.56±0.28	7.34±1.21	45.08±5.13

***Trichoderma viride* CMGB 1 cellulase**

	Protein content $mg_{BSA} \cdot mL^{-1}$ $mg_{BSA} \cdot g^{-1}$	**CMCase activity $mmoli \cdot min^{-1} \cdot mL^{-1}$ $mmoli \cdot min^{-1} \cdot g^{-1}$**	**Immobilization yield[c] %**
Native			
Fermentation medium[a]	3.30±0.27	1.79±0.16	-
Lyophilized enzyme[b]	19.28±2.05	66.15±3.38	-
Immobilized[b]			
Entrapment THEOS	14.62±1.31	2.03±0.30	8.98±1.12
	Cellobiase activity $mmoli \cdot min^{-1} \cdot mL^{-1}$ $mmoli \cdot min^{-1} \cdot g^{-1}$	**Specific activity $U\ mg_{BSA}^{-1}$**	**Immobilization yield[c] %**
Native			
Fermentation medium[a]	0.72±0.11	0.22±0.04	-
Lyophilized enzyme[b]	23.65±2.79	1.23±0.09	-
Immobilized[b]			
Entrapment THEOS	2.26±0.33	0.16±0.02	5.87±1.04

[a]liquid, [b]solid, [c]Immobilization yield (%) = $100.U_{tot(im)}/U_{tot(i)}$, where $U_{tot(im)}$ = activity of immobilized enzyme (U/mg)·total weight of immobilized enzyme (mg), $U_{tot(i)}$ = activity of native enzyme (U/mL)·total volume of native enzyme used for immobilization (mL)

The influence of the ionic microenvironment and the heat energy around amylase and cellulase molecule, on the free and entrapped enzymatic preparation activity, was studied comparatively. The enzymes activities (amylase and CMCase activity) were determined in buffered solutions of pH range 2.2 – 8.0 (citric acid/Na_2HPO_4 buffer solution) and at temperatures ranging from 20 to 95ºC (as shown in Figs. 1 and 2).

The stability of entrapped enzymatic preparations with amylase and CMCase activity was tested for an hour in aqueous medium, at pH 2.6 and 37ºC. The residual activity vs. time dependence of the entrapped amylase and CMCase are shown in Fig. 3.

The enzyme-substrate affinity modification was investigated based on the kinetics of native and immobilized enzymes respectively, in the hydrolysis of starch in case of amylase, and in the hydrolysis of CMC in case of CMCase. The kinetic parameters of free and entrapped *Bacillus amyloliquefaciens* DSM 7 amylase and *Trichoderma viride* CMGB 1 cellulase obtained in the hydrolysis of starch and CMC-cellulose respectively are given in Table 4.

Enzymes stability was analyzed as well after two months of storage at 4ºC. The immobilized enzymatic preparations were kept at 4ºC and the stability was monitored in time. The enzymes activity was determined periodically. The residual amylase and CMCase activity of the immobilized enzymes in the absence of any preservation agent is shown in Table 5.

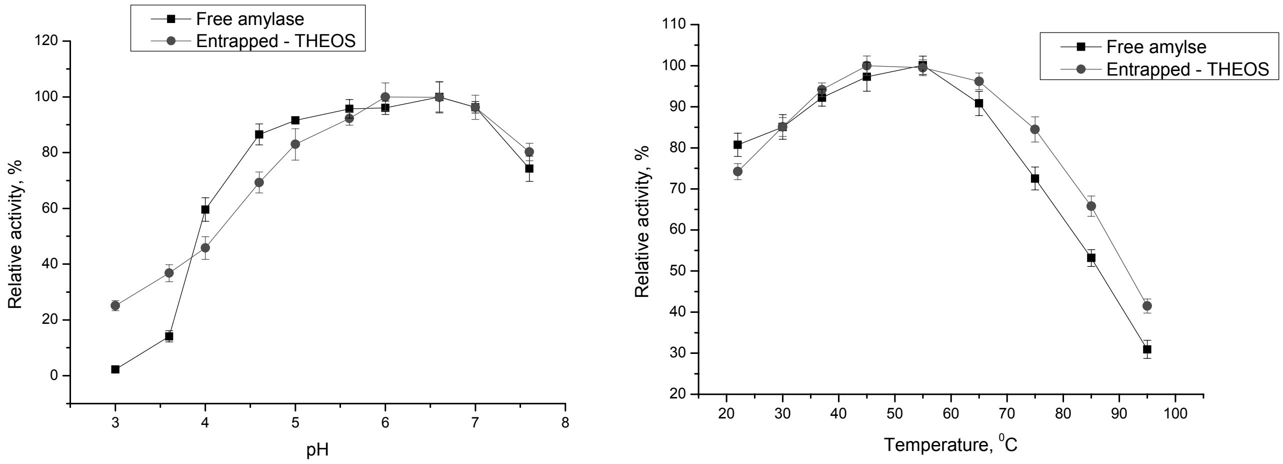

Fig. 1 – The effect of pH and temperature on the activity of immobilized and native amylase.

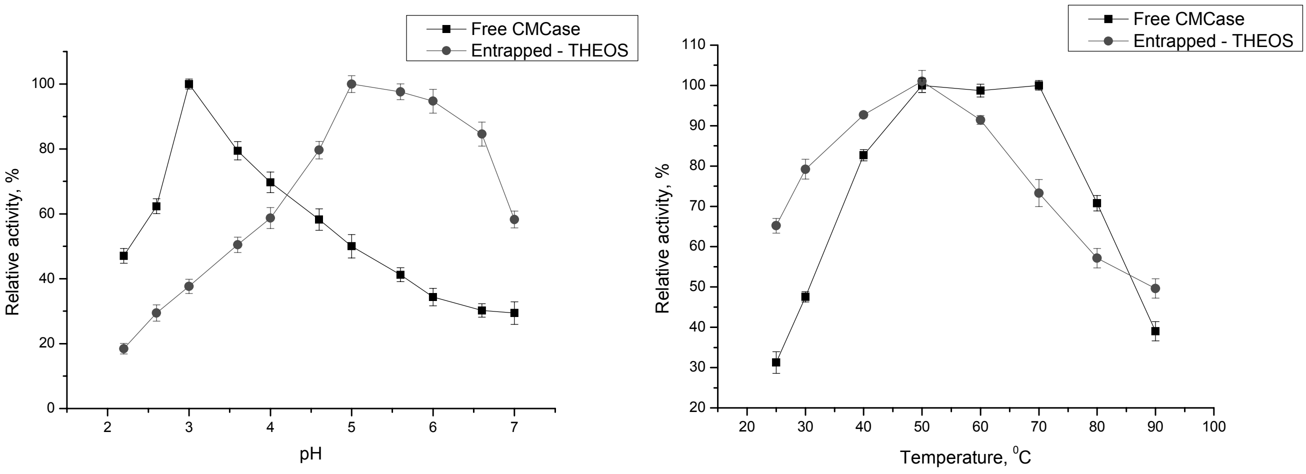

Fig. 2 – The effect of pH and temperature on the activity of immobilized and native cellulase (CMCase activity).

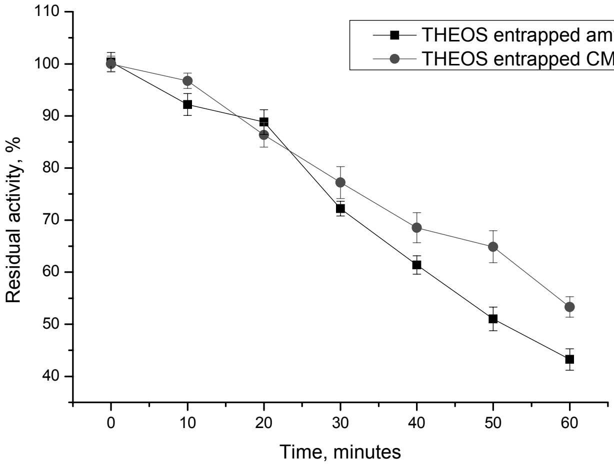

Fig. 3 – Stability of the immobilized enzymatic preparation at pH 3.0 and 37ºC.

Table 4

The kinetic parameters obtained from the Lineweaver – Burk linearization

Lineweaver – Burk linearization	***Bacillus amyloliquefaciens* DSM 7 amylase**		
Kinetics parameters	**K_m mg_{starch}/mL**	**V_{max} $\mu mol_{maltose}$/mL·min**	**$V_{max}.10^3/K_m$ $\mu mol_{maltose}$/mg·min**
Free amylase	12.28±1.11	0.82±0.09	66.86±5.83
Entrapped (THEOS) - amylase	20.94±2.38	0.99±0.12	47.28±4.86
Lineweaver – Burk linearization	***Trichoderma viride* CMGB 1 cellulase**		
Kinetics parameters	**K_m mg_{CMC}/mL**	**V_{max} $mmol_{glucose}$/mL·min**	**$V_{max}.10^3/K_m$ $\mu mol_{glucose}$/mg·min**
Free CMCase	6.10±0.55	0.95±0.09	155.73±16.21
Entrapped (THEOS) - CMCase	7.85±0.62	0.89±0.07	113.38±10.44

Table 5

Preservation stability of immobilized enzyme

Immobilized enzymes	**Residual enzymes activity %**		
	initial	**one month**	**two months**
Entrapped (THEOS) amylase	100.00	83.25±6.41	67.32±4.82
Entrapped (THEOS) CMCase	100.00	105.78±8.74	89.64±6.77

DISCUSSION

An important objective of any enzymes stabilization work should be to minimize costs. The enzyme could represent one of the main cost components and following this point of view we tested a series of microbial strains to produce the immobilized enzymatic preparations as cheap as possible. The best enzyme activities were obtained in case of *Bacillus amyloliquefaciens* DSM 7 strain (submerse mineral medium, stirred flasks) and *Trichoderma viride* CMGB 1 strain (solid state fermentation). The aqueous enzymatic preparations were concentrated from fermentation medium by liophylization and then the enzymes were entrapped in silica gel.

Our results show that both enzymes, but especially amylase, were successfully entrapped in THEOS based silica matrices. The mild condition required by the sol-gel synthesis allowed immobilization at a slightly lower pH and low temperature needed by enzymes to retain their biocatalitic activity. The good enzyme activities measured after entrapment could be explained by a minimal modification in conformational structure of enzyme. The entrapped amylase was obtained with an immobilization yield of 45%. In case of CMCase and cellobiase, the immobilization yield was 8.44%, at a lower initial enzyme loading of silica matrix than in case of amylase because of lower free enzyme activity (66.15 CMCase units/g lyophilized powder, 1196 amylase units/g lyophilized powder).

Optimum temperature and pH for the activity of free and entrapped amylase and CMCase were determined comparatively. The optimum pH of the free enzymatic preparation with amylase activity was 6.6, and at pH range 5 to 7 the enzyme retained more than 90% of its maximum activity. The highest entrapped amylase activity was achieved when the pH values of the environment were 6-7. The relative activity obtained for immobilized amylase at pH 2.6 was 10% higher than in case of free one. The enzymatic activity as a function of temperature had almost the same profile for free and immobilized amylase. Both of them preserved more than 90% of the maximum activity in 37-65ºC temperature range. At temperatures above 70ºC the activity of free and entrapped amylase decreased slowly.

Entrapment in silica matrix of cellulase changed the optimum pH of CMCase from 3 to 5 and more than 85% of the peak activity was displayed in the pH range 5-7. The maximum CMCase activity of free enzymatic preparation was found at a temperature range of 60-70°C. Entrapment revealed a changing of optimal temperature to 50°C. The immobilized enzyme showed 65% and 50% of maximum activity at 25°C and 90°C, respectively.

A good enzyme stability at low pH values (pH 2-3) and below 40°C is very important from the viewpoint of application as feed additive. The enzymes performed a good stability at pH 2.6 and retained more than 40% and 50% of maximum amylase and CMCase activity respectively, after one hour of incubation at 37°C.

The kinetic constants of immobilized enzymes are usually different from those of the native form, as conformational changes, steric hindrances and partition and diffusion effects may occur simultaneously or separately on enzyme immobilization. The conformational effects may also change the affinity between enzyme and substrate.

The kinetic study revealed that both free and immobilized amylase and CMCase, follow a Michaelis–Menten type kinetics. In the case of the immobilized enzymes, the *apparent Michaelis constant*, K_m, was greater than that of the native one, as it was expected. K_m increased 1.7 times for the THEOS entrapped amylase and 1.3 times for the immobilized CMCase (Table 4). For the immobilized enzymes, V_{max}/K_m ratio indicated that immobilization decreased the catalytic efficiency by 1.4 times, for both enzymes. The maximum velocity changed insignificantly after immobilization.

Preservation stability is very important in industrial application of immobilized enzymes. Both amylase and cellulase present a good stability in time. The highest stability was obtained for *Trichoderma viride* CMGB 1 CMCase. After two months of storage at 4°C, entrapped CMCase has lost only 10% of initial enzyme activity. The slight increase in CMCase activity after one month could be explained by the slow drying of silica gel matrix containing the enzyme entrapped. The residual activity of *Bacillus amyloliquefaciens* DSM 7 amylase is 67% from initial enzyme activity. The high preservation stability of THEOS entrapped enzymes makes them to be appropriate preparations with amylase and cellulase activity in different applications.

EXPERIMENTAL

Soluble potatoes starch, maltose, carboxymethyl cellulose (CMC), cellobiose, glucose, Folin-Ciocalteu's phenol reagent and bovine serum albumin (BSA) were purchased from Merck, 3,5-dinitrosalicylic acid (DNS) and tetrakis (2-hydroxiethyl) orthosilicate (THEOS) were obtained from Fluka. All the other chemicals were obtained from local suppliers or were commercially available reagent grade products and were used without further purification.

The *Bacillus amyloliquefaciens* DSM 7 strain was purchased from DSMZ Germany. Microbial cells of *Bacillus amyloliquefaciens* were cultured under aerobic conditions (rotary shaker, 175 rpm), for 24 hours, at 37ºC, in Erlenmeyer flasks containing 50 mL mineral medium.[14] The purity, pH, amylase activity and protein content were monitored continuously. The enzymatic preparation with amylase activity was liophylized from fermentation medium for 24 hours, at -56ºC and 26 mTorr (iLShin Europe Dry Freezer).

The protein content was assayed according to the Lowry method, using the Folin-Ciocalteu's phenol reagent and bovine serum albumin (BSA) as standard.[15] The amylase activity was measured by UV-VIS spectrometry, according to the Sumner method, using DNS as reagent.[16] One unit of activity is defined as the amount of enzyme that hydrolyzes starch liberating 1 $\mu mol_{maltose} \cdot mL^{-1} \cdot min^{-1}$, at 25ºC.[17]

The *Trichoderma viride* CMGB 1 strain is preserved in the collection of industrial microorganisms of the Industrial Microbiology Laboratory of USAMVB Timisoara. Microbial cells of *Trichoderma* were cultured in solid state fermentation, for 168 hours, at 37ºC using wheat bran as carbon source.[11] The extraction of enzymatic preparation was done in distilled water (1:7) under magnetic stirring (75 minutes, 28ºC, 150 rpm). The extraction medium was initially filtered through gauze and filter paper and finally centrifuged (10 minutes, 4ºC, 10000 rpm). The enzymatic preparation with CMCase and cellobiase activity was lyophilized from extraction medium (24 hours, -56ºC, 26 mTorr (iLShin Europe Dry Freezer).

The CMCase and cellobiase activities were measured by UV-VIS spectrometry, according to the Petterson and Porath method, using CMC (CMCase activity) and cellobiose (cellobiase activity) as substrates and DNS as reagent.[18]

A sol-gel entrapment method was performed using a one step procedure by magnetically stirring of tetrakis (2-hydroxiethyl) orthosilicate (THEOS) with a buffered enzymatic solution (1:1.1, v/v). In all cases the gelation occurred in a few seconds. The gels were left overnight for aging (4°C), washed with n-hexane and then assayed.[19] A buffered enzymatic solution contained 28.7 amylase units/1 mL THEOS from lyophilized *B. amyloliquefaciens* enzymatic preparation and 13.5 CMCase units/1 mL THEOS and 9.86 cellobiase units/1 mL THEOS from lyophilized *T. viride* enzymatic preparation, respectively in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 4.6.

The effect of temperature and pH on the activity of native and immobilized enzymes was investigated by DNS assay, measuring the glucose concentration at various temperatures (20-95ºC) and in the presence of 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer ranged from 2.2 to 8 respectively, at room temperature.

Stability test – the immobilized enzymes (300 mg immobilized enzyme from *B. amyloliquefaciens* and *T. viride*, respectively) in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 2.6 (5 mL) were incubated at 37ºC for one hour. Samples were withdrawn at every 10 minutes and amylase and CMCase activities were assayed. Enzymes stability was analyzed as well, after two months of storage at 4ºC.

The kinetic study was carried out on the native enzymes and on the enzymes entrapped in silica gels obtained using THEOS as precursor. The substrates used were soluble starch, in case of amylase and carboximethyl cellulose in case of CMCase.

To determine the kinetic parameters, the substrate hydrolysis by amylase was performed for 20 minutes, at 25ºC, by varying the concentration of starch solution (2-8 mg/mL), with native (1 mL lyophilized enzymatic solution, 1mg/mL) and immobilized (100 mg) enzymes in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer solution, pH 4.6. Samples were taken at every 2 minutes; the absorbance was measured at 540 nm (reducing sugar assay with Sumner method) in a UV-VIS spectrophotometer (PG Instrument T60U Spectrophotometer, 37°C) comparatively with a control. The product concentration (glucose) was obtained from the calibration curve. The initial reaction rates were calculated from the d[glucose]/dt slope at all initial starch concentrations for the native and entrapped amylase. The data were fitted using the Lineweaver-Burk equation (linear regression).

In case of enzymatic preparations with CMCase activity the kinetic studies were performed following the same steps. The kinetic parameters were determined at 50ºC; the concentration of carboximethyl cellulose varied from 0.46 to 4.60 mg/mL, for both native (2 mL lyophilized enzymatic solution, 5 mg/mL) and entrapped (200 mg) enzymes in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer solution pH 4.6. The data were fitted using the Lineweaver-Burk equation (linear regression).

CONCLUSIONS

Tetrakis (2-hydroxiethyl) orthosilicate (THEOS) and sol-gel method have made possible the generation of hybrid organic-inorganic mesoporous tridimensional networks suited for entrapment of enzymes. The successful immobilization indicates that silica gels minimally affect the enzymes biocatalytic active centers. The kinetic parameters indicate that the immobilized enzymatic preparation shows less affinity for substrate, K_m being 1.7 and 1.4 times higher than that of the native enzyme.

The exogenous immobilized microbial enzymatic preparations with amylase and CMCase activity can act on available substrates at low pH in animal stomach and can be used as feed additives. They are stable for a time sufficiently long to act in proximal segment of digestive tract. The entrapped enzymatic preparations present an enhanced stability in time that makes them suitable for a lot of industrial applications.

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