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ENTRAPPED AMYLASES – VERSATILE BIOCATALYSTS FOR LIFE SCIENCES APPLICATIONS

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ABSTRACT

Enzyme protein structure preservation is a major purpose for biotechnology industry. A very efficient method to avoid denaturation, to reduce the enzyme stability, is immobilization in porous supports. The entrapment of enzymes consists in blocking the protein macromolecules of the enzymes in the pores of the support. Considering that no chemical bonds are formed between the enzyme molecules and the three-dimensional network, the change in the conformation of the enzyme is minimal. A proper three-dimensional network suitable for enzyme entrapment can be obtained by the sol-gel method. Microbial α -amylase obtained by submerged fermentation of Bacillus amiloliquefaciens DSM 7 cells were used in immobilization. The microbial preparation with α -amylase activity was entrapped in different sol-gels formed by polymerization of tetraalcoxysilanes, alginate gel and mixed silica-alginate gels. The influence of the type of alkoxysilane precursor on the catalytic activity of the entrapped α -amylase is discussed versus stability of the enzyme after immobilization. The immobilization efficiency of enzymes in single and mixed gels are studied comparatively. Our results showed that even enzymes with -amylase, can be entrapped in porous matrices and used with good results in the biotransformation of their substrates.

Keywords: α -amylase, Bacillus amiloliquefaciens, entrapment, stabilization.

INTRODUCTION

Enzymes are very efficient biocatalysts due to their high chemo-, enantio- and regioselectivity. This great specificity makes enzymes increasingly used at the industrial level, replacing chemical processes with enzymatic ones. Despite the efficiency of enzymes as biocatalysts, they present the disadvantage of instability to changes in the reaction environment (pH, temperature, organic solvents, etc.) and the difficulty of separation after the reaction is finished. Immobilization in/on insoluble supports proves to be a promising way to increase the operational and time stability of enzymes, also ensuring their easy separation [1-3]. Depending on the immobilization method and the nature of the support, the immobilized enzyme is protected from the denaturing agents that could change the conformation of the protein molecule of the enzyme, including its active site, which can lead to the loss in enzyme activity [4]. Based on the easy separation of immobilized enzymes, they present another important advantage for industrial processes, the possibility of reuse in several production cycles, which brings economic advantages [5].

α- Amylase is a hydrolase that catalyses starch hydrolysis, acting inside the macromolecule (endoenzyme). The hydrolysis products are shorter-chain polymers, glucose and maltose [6]. In the biotechnological industry amylases have applications in numerous fields, from food and feed to detergent, textile and paper industry, also in medicinal and clinical chemistry [7]. To improve amylases activity for industrial applications, the enzymes can be used in immobilized form [6]. Organic, inorganic or hybrid polymers are successfully used as matrix for enzymes entrapment in the pores of the biomaterial [8].

Alginate, a natural organic polymer was used for different enzymes immobilization, including α- amylase with very good results in starch hydrolysis [9-11]. Synthesis of biocompatible supports for the labile enzymes molecules by sol-gel process by using alkoxysilane precursors (TEOS, TMOS) has been done for several

enzymes, including α- amylase [12, 13, 6, 8]. The mild conditions required the sol-gel process (most important being the room temperature) and the light interaction between the enzyme blocked in the pores and the polymer network affects very little the structure of molecules and the enzymes can retain their native catalytic properties [3].

The aim of this work was to immobilize *Bacillus amiloliquefaciens* DSM 7 α- amylase in organic alginate gel, inorganic TEOS, TMOS and THEOS silica gels and also in hybrid alginate/TEOS and alginate/TMOS silica gels.

MATERIALS AND METHODS

Materials

Soluble potatoes starch, maltose, (+)-glucose, hexane, Folin-Ciocalteus phenol reagent, bovine serum albumin (BSA) and tetraethoxysilane (TEOS), tetramethoxysilane (TMOS), 3,5-dinitrosalicylic acid (DNS), alginic sodium salt were purchased from Merck. Tetrakis (2-hydroxiethyl) orthosilicate (THEOS) and potassium sodium tartrate tetrahydrate were from Sigma - Aldrich. Ethylene glycol (EG) was from Scharlau, ethanol and $CaCl_2$ sicc. from Chimopar. The *Bacillus amyloliquefaciens* DSM 7 strain was purchased from DSMZ Germany. All the other chemicals were obtained from local suppliers or were commercially available reagent grade products and were used without further purification.

Immobilization Methods

The enzymatic preparation with α -amylase activity was used in immobilization as lyophilized powder [14, 15]

The α-amylase entrapment in sol-gels based on TEOS, TMOS and THEOS precursors was made according to the methods described in our previous work [14, 15] and in the work of Shchipunov et al. [16].

The enzyme entrapment protocol in alginate pearls and hybrid alginate-sol gels followed the protocols described the Konsoula et al. [17].

The alginate pearls containing α-amylase was covered with sol-gels according to method of Won et al. [18].

The immobilization yield was defined

Immobilization yield (%) = $100.U_{tot(im)}/U_{tot(i)}$,

where:

$U_{tot(im)}$ = activity of immobilized enzyme (U/mg)·x total weight of immobilized enzyme (mg),

$U_{tot(i)}$ = activity of native enzyme (U/mL)·x total volume of native enzyme used for immobilization (mL);

Cyclic Use of Immobilized Amylase

500 mg immobilized alginate and TEOS/EG enzyme and 5 mL starch 1% in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 4.6, 25ºC, were reused in 6 cycles of 30 min. At the end of the cycle's samples were withdrawn and the amylase activity was assayed. After each cycle the starch solution was replaced with a fresh one.

Assay of A-Amylase Activity

The enzymatic activity was assayed by Sumner method [19]. As reagent 3,5-dinitrosalycilic acid (DNS) was used. The reducing sugars resulted after reaction were measured spectrophotometrically at 540 nm (by using PG Instrument T60U Spectrophotometer at room temperature, based on a standard curve), against a blank. One unit of α-amylase activity is defined as the amount of enzyme that hydrolyses starch liberating 1 $\mu mol_{maltose} \cdot mL^{-1} \cdot min^{-1}$, at 25ºC.

Assay of Protein Content

The protein content was measured spectrophotometrically by using Folin-Ciocalteu reagent [20]. The absorbance was measured at 660nm, as standard bovine serum albumin (BSA) was used.

All the enzyme activity and protein concentration data are an average of at least two parallel assays.

RESULTS AND DISCUSSIONS

The α-amylase formed by fermentation of Bacillus amyloliquefaciens DSM 7 cells was lyophilized and used in immobilization. The α-amylase activity of the lyophilized preparation was 274 μmol/min·g and the protein content 292.51 mg_{BSA}/g.

α-Amylase entrapment was done in (1) silica gels matrices synthetized by using TEOS, TMOS and THEOS as precursors in the sol-gel process, (2) Ca-alginate gels, (3) hybrid gels – a) mixture of sol based on TMOS/TEOS and alginate solution, b). Ca-alginate gels containing the enzymes molecules was impregnated with silica gel based on TEOS or TMOS sol (Figure 1 and 2).

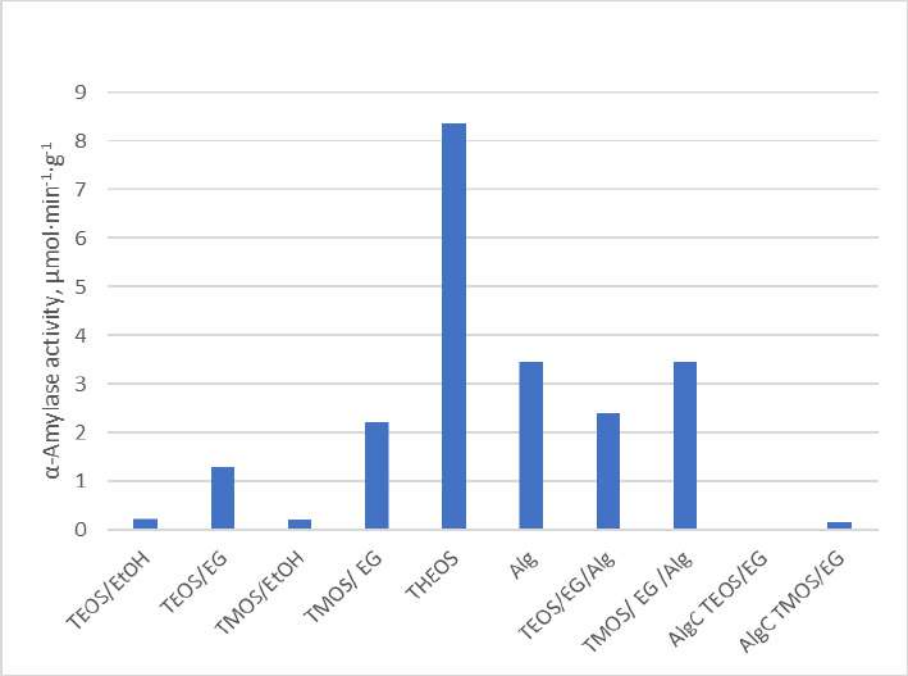

Figure 1: Entrapment of α-amylase in silica, Ca-alginate and hybrid gels

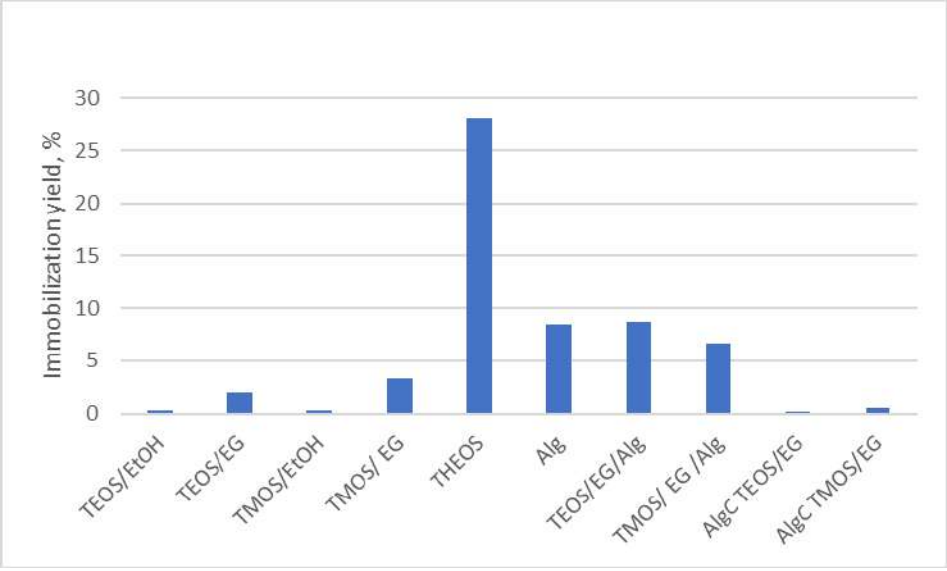

Figure 2: Immobilization yield of entrapped α-amylase in silica, Ca-alginate and hybrid gels

The classic sol-gel method, as it was described by Reetz and his collaborators [12, 13] uses TMOS and TEOS as precursors and EtOH as solvent in hydrolysis-condensation reactions. In figure 1 and 2 can be seen that the activity and immobilization yield of α-amylase entrapped in gels obtained by this method is much lower than the activity of enzyme entrapped in Ca-alginate beds. This can be due to the alcohol used and resulted from the hydrolysis-condensation reactions during the sol-gel process, being known that the alcohol may have negative effects on the enzyme molecules.

α-Amylase is a very fragile enzyme, being very sensitive to the increase in temperature and to the organic compounds in the reaction medium, like EtOH. Ethylene glycol (EG) is more protective with the enzyme and it was tested as cosolvent for sol synthesis based on TMOS and TEOS. The use in our experiments of EG as cosolvent was inspired by another precursor THEOS which is industrially prepared using EG and which allowed much higher enzymatic activities. The results obtained by us are follows the same trend with that published by Shchipunov and coworkers [16]. The enzyme immobilized in gels TMOS and EG and TEOS and EG, prepared by us, shown much higher activities and immobilization yields compared to the classic gels obtained with EtOH.

The alcohols used in sol-gel process may affect not only the enzyme molecules, but also the structure of the gels. The gel based on THEOS cannot be dried to the powder stage like the gels obtained from TMOS and TEOS. So that it does not have a very strong constricting effect on the enzyme molecules. TMOS and TEOS gels, especially those in which EtOH was used, can be dried to a constant mass, and by drying the pore size is greatly reduced. This negatively affects the enzymatic activity, especially since amylase has a macromolecular substrate.

The differences between the enzymatic activity measured in silica gel and alginate gel are due to the structure of alginate gel that is very different, being used in wet forms.

In case of combined method, the best results were obtained for the mixed sol. The Ca-alginate impregnated with silica gel did not give better results that the TEOS/ethylene glycol protocol.

Our results prove that the immobilization support has an important influence on the activity of enzymes and they are consistent with those obtained by Uzun and Yildirim Akatin when immobilized α-amylase in chitosan and alginate matrices. Also, the substrate specificity of α-amylase immobilized in the two matrices was different. The highest activity was measured for both of the entrapped enzymes for starch as substrate, but the α-amylase catalyzed the hydrolysis reactions of other substrates at different ratios. The Ca-alginate immobilized enzyme had a higher activity then chitosan immobilized enzyme when maltose and maltotriose were used as substrate, and lower in case of substrate glycogen and amylopectin [9].

The functional parameters of the native and TEOS/EtOH and THEOS entrapped enzymes were described in our previous work [14, 15]. The optimum pH was measured for native and entrapped enzymes in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer. The maximum amylase activity was found in the domain 4-7 for the free enzyme and 5.5-7 for the entrapped enzymes. In case of temperature study, the optimum was 40-55 for native amylase, and 30-70 for entrapped enzymes. The catalytic efficiency measured as $V_{max}\cdot 1000/ K_M$, $\mu mol_{glucose}.mg^{-1}\cdot min^{-1}$, as it was expected was lower for the entrapped enzymes for 1.4-1.8 times. But, the residual activity of entrapped amylase in a media with 2.6 pH, 37ºC, was 48-56%, which show a good improvement of its stability. The results are consistent with those obtained by Abu et al. for entrapped amylase on silica sol-gel, the entrapped enzyme retained more than 70% of its activity at different pH for 2 h incubation and more than 60% to 80% of its activity at 70°C [21]. Accordingly, it can be noticed that being a noninvasive method, the enzymes entrapment in gels, usually, does not alter significantly the most important operational properties.

The results obtained at cyclic reuse of amylase immobilized in alginate beads and TEOS/EG derived gels are presented in the figure 3.

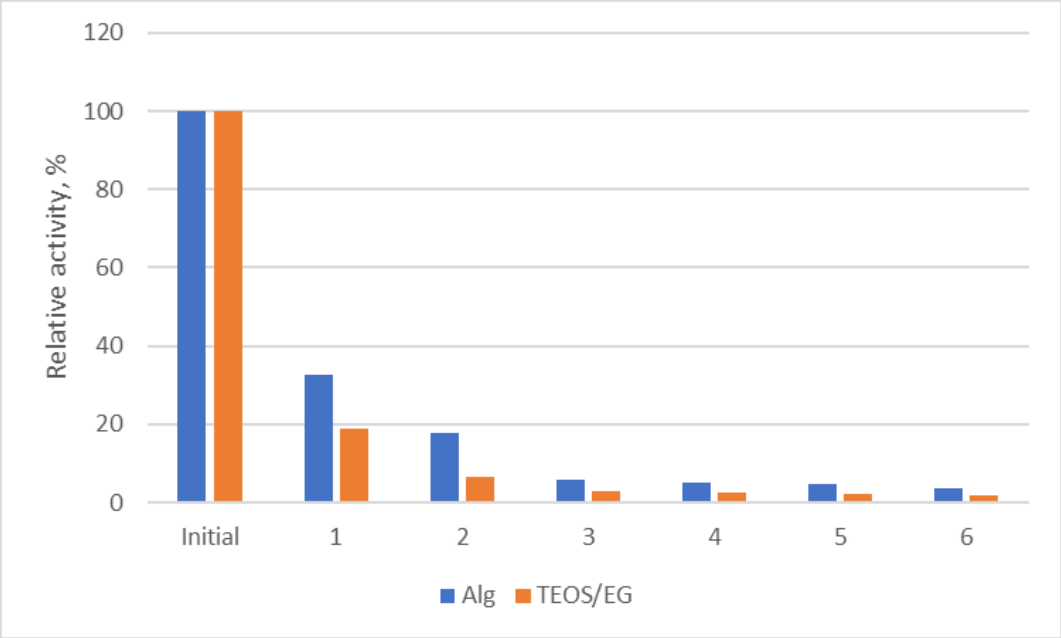

Figure 3: Cyclic use of amylase immobilized in alginate beads and TEOS/EG derived gels

After the first cycle of starch hydrolysis a falling down of the enzyme activity was observed. This decrease can be explained, on the one hand, by the inactivation of the enzyme, on the other hand, by the enzyme leaching from the polymer matrix. Starting with the third cycle, an operational stabilization is observed. In the first hour of use about 90% of the enzyme is released. These results make the products useful in different field of biotechnology. Our results are sustained by the study of Fernandez Caresani et al. which conclude that the α-amylase from *B. subtilis* and α-amylase from *A. oryzae* immobilized by sol–gel encapsulation method appear to be more stable enzymes, more robust and much more resistant during the starch hydrolysis process [6]. The immobilized amylase in Ca-alginate beds (5% concentration of Ca-alginate solution) was reused by Ningsih et al. for 15 times in starch hydrolysis and the relative activity was lower than 50% from initial [11]. Santos Silva et al. reported a residual activity of 13% after 5 cycles of reuse of α-amylase immobilized in ion exchange resin, Duolite® A-568, [22]. The results of Salem et al. demonstrated that immobilized microbial α-amylase on magnetic nanoparticles showed an improved storage stability and enhanced stability in several cycles of reuse [23]. The residual activity of the immobilized *α*-amylase enzyme from *Aspergillus fumigatus* after immobilized on a bentonite matrix after six cycles of reuse was 42% and its stability was increased [24].

CONCLUSIONS

Entrapment in silica matrices is an efficient method to immobilize biomolecules including the enzymes that have as substrate macromolecular compounds. The ethylene glycol was successfully used in sol-gel enzyme immobilization technology and plays three important roles: solubilization of precursor alkoxysilanes, active structural component and preserving agent for the enzyme. The immobilized *α*-amylase enzyme can be reused in starch hydrolysis applications.

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