Immobilized Microbial Cellulases in Organic-Inorganic Hybrid Materials

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Abstract
The *Aspergillus niger* cellulase was immobilized by entrapment in natrium alginate gels and in natrium alginate/silica gel hybrid materials. The silica gels were obtained using two different precursors, tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS), by sol-gel method. Comparing the enzymatic activities of the immobilized products, it was noticed that the CMCase activities obtained in natrium alginate were 1.12-1.17 times higher than those obtained by entrapment in mixed organic – inorganic gels, for similar enzyme loadings. The operational stability was tested in the substrate presence. The cellulase entrapped in the three types of organic-inorganic gel matrices mentioned above retains about 13% of its activity after 4 cycles. After one hour of storage at 37ºC, pH 3.0, the relative activity of the immobilized *Aspergillus niger* CMCase was more than 98% of initial.

Keywords: *Aspergillus niger,* Ca-alginate, cellulase, entrapment, sol-gel.

1. Introduction

Cellulases, a group of enzymes that sinergically can hydrolyze cellulose to glucose, are one of the largest global industrial enzymes because of their utilization in obtaining of bioethanol, feed additives and detergent enzymes and in cotton processing and paper treatment. There has been an increased interest in production of cellulases, as key resources in ethanol synthesis from lignocellulosic biomass, a renewable source of energy [1]. Most bacterial cellulases used in biotechnology are obtained from strains of *Trichoderma, Aspergillus, Penicillium, Fusarium, Humicola, Phanerochaete*, etc.
The performance of cellulase mixtures in biomass conversion processes depends on several of its properties like higher catalytic efficiency on insoluble cellulosic substrates, increased stability at temperature and pH, higher tolerance to inhibition. Improvement of cellulases activities and stabilities may be achieved by development of production technology [2,3] and/or immobilization of enzymes in order to obtain stable catalysts, highly active with a good specific activity and substrate specificity [4 - 6].
The aim of this study was to immobilize *Aspergillus niger* cellulase by entrapment in Ca-alginate and mixed Ca-alginate – silica gels matrixes. As precursors for the silica sols, two tetraalkoxisilanes, tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) were used, based on the sol-gel method. The activity and stability of the immobilized cellulases is discussed.

2. Materials and methods

Carboxymethyl cellulose (CMC), Folin-Ciocalteus phenol reagent, bovine serum albumin (BSA) and Na-alginate were purchased from Merck, 3,5-dinitrosalicylic acid (DNS), tetraethoxysilane (TEOS), tetramethoxysilane (TMOS) and *Aspergillus niger* cellulase were obtained from

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Fluka. All the other chemicals were obtained from local suppliers or were commercially available reagent grade products and were used without further purification.

A buffered enzymatic solution containing 40 CMCase units of *Aspergillus niger* cellulase in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 4.0 was stirred with 1.18 mL of 2% natrium alginate solution and dropped with a syringe needle in a 0.2 M $CaCl_2$ solution. The pearls (2 mm diameter) were kept in the $CaCl_2$ solution for 30 min., under low speed stirring, washed with distilled water to eliminate the $CaCl_2$, filtered at vacuum and used as wet gel [7].

The silica sols were prepared using Si-precursor and ethylene glycol in acid catalysis, HCl 1N (3.4:1:0.03, v/v) [8], kept for aging at 4°C for 60 days and used for enzyme entrapment. The natrium alginate 2% solution was partially replaced by sol, to obtain a solution with 4% sol in the final volume. Than the enzyme was immobilized and the pearls were treated as described previously.

The protein content was assayed according to the Lowry method, using the Folin-Ciocalteus phenol reagent and bovine serum albumin (BSA) as standard [9]. The CMCase activity was measured by UV-VIS spectrometry, according to the Petterson and Porath method, using CMC as substrate and DNS as reagent [10].

The operational stability was performed by incubating the immobilized cellulases (0.2 g in 5 mL CMC 1% solution, pH 4.0) at 37ºC for four cycles of 30 min. At the end of each cycle, samples were withdrawn and CMCase activity was assayed and than the CMC solution was replaced with a fresh one.

The stability of immobilized enzymes (0.2 g) in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 3.0 (3 mL) was studied for one hour, at 37ºC. Samples were withdrawn at every 20 minutes and CMCase activity was assayed.

3. Results and discussion

The *Aspergillus niger* cellulase was immobilized by entrapment in natrium alginate gel and in mixed natrium alginate/silica gels. The silica sols were obtained, according to the sol-gel method, using two different precursors, tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS). The highest CMCase activity was found for the immobilized preparation obtained by entrapment using natrium alginate. Comparing the enzymatic activities of the immobilized products, it was noticed that the CMCase activity obtained by entrapment in natrium alginate was 1.12-1.17 times higher than that obtained by entrapment in mixed organic – inorganic gels, for similar enzyme loadings (Table 1).

Table 1. Protein content and CMCase activity of the free and immobilized *Aspergillus niger* cellulase

Aspergillus niger cellulase	Protein content $mg_{BSA} \cdot g^{-1}$	CMCase activity $mmol \cdot min^{-1} \cdot g^{-1}$	Immobilization yield[a] %
Free enzyme	108.00	400.00	-
Immobilized			
Na-alginate	0.56	5.73	6.63
Na-alginate/TMOS	0.12	4.89	6.62
Na-alginate/TEOS	0.12	5.12	6.51

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

The operational stability was tested in 4 cycles of repetitive use of CMCase, in the presence of the substrate, CMC. Figure 1 shows the relative activity as a function of reuse number. The cellulase entrapped in the three types of organic-inorganic gel matrices retains about 13% of its activity after 4 cycles.

Enzymes stability was analyzed as well after an hour of storage at 37ºC, pH 3.0. The stability was monitored in time. Enzymatic activities were determined periodically (Table 2).

After one hour, the relative activity of the immobilized A. niger CMCase, for the all three gels was more than 98% of initial.

The stability results indicate that the immobilized CMCases are compatible with the animal digestion cycle and make the products useful as additives in feed biotechnology.

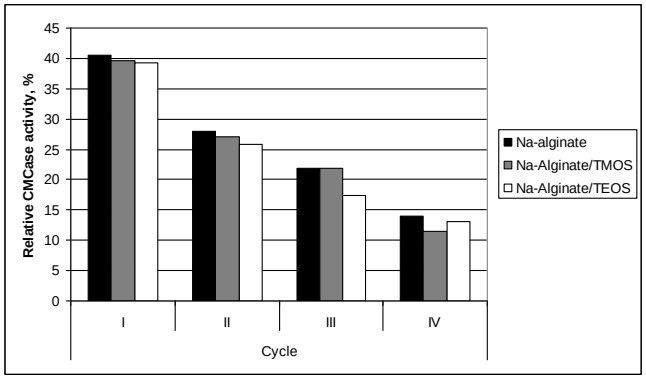

Figure 1. Cyclic use of CMCase entrapped in alginate beads, mixed alginate/TEOS and alginate /TMOS beds

Table 2. Stability of the immobilized cellulase at pH 3.0 and 37ºC

	Rezidual CMCase activity, %			
Immobilization matrix	Initial	20 minutes	40 minutes	One hour
Na-Alginate	100.00	99.45	98.21	98.03
Na-Alginate/TMOS	100.00	108.76	99.06	98.84
Na-Alginate/TEOS	100.00	112.18	99.76	99.18

4. Conclusions

A purified *Aspergillus niger* cellulase was immobilized by entrapment in pure natrium alginate beds and in two hybrid alginate – silica gels obtained by using alcoxisilanes TEOS and TMOS as SiO_2 source.

The immobilized microbial enzymatic preparations with CMCase activity can act on available substrates at low pH in mammalian stomach and can be used as feed additives. They are stable for a time sufficiently long to act in proximal segment of digestive tract.

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