Microbial Cellulases Immobilized in/on Porous Supports

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Abstract
Biodegradation of cellulose by enzymatic hydrolysis using cellulases has an important value in biotechnology and the immobilization of enzyme on inorganic materials is very useful in practical applications. Enzymatic preparations with cellulase and cellobiase activities from *Trichoderma viride* were liophylized from the culture medium and immobilized in/on porous matrices. The methods used for immobilization were physical adsorption on ceramics and entrapment in glass sol-gel matrices using as alkoxysilane precursors tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS). The immobilization efficiency of the solid enzymatic preparations was about 60%. The immobilized enzymatic preparations were used for hydrolysis of carboxymethyl cellulose (CMC) and cellobiose at different temperature and pH values. The resulted immobilized enzymes had the same optimum pH of 4.0 in the case of cellobiase substrate and a shifted optimum pH towards the less acid side (pH 5.0) in the hydrolysis of CMC. The optimum temperature of entrapped enzyme against CMC was shifted to a lower temperature (40°C) in comparison with the native one (60°C).

Keywords: cellobiase, cellulase, entrapment, physical adsorbtion, sol-gel, *Trichoderma*

1. Introduction

Cellulosolytic enzymes are involved in enzymatic hydrolysis of cellulose, one of the most abundantly occurring organic material that can be converted to products with significant commercial interest. Bioconversion of cellulose to monomeric sugars has been intensively studied in the recent years to produce bioethanol and bio-based products, food and animal feeds, many valuable chemicals [1, 9]. Effective conversion of cellulose to fermentable sugars requires, in one hand, different kinds of cellulases working sinergically and, in the other hand, enzymatic preparations with greater stability for specific processes, and higher catalytic efficiency on insoluble cellulosic substrates [2]. A good strategy to improve a low stability of free enzymatic preparations with cellulase activity could be immobilization in/on inorganic porous supports [3, 4].
The aim of this study was to determine the effects of the immobilization methods and the influence of environmental parameters on the cellulase activity.

2. Materials and methods

Carboxymethyl cellulose (CMC), cellobiose, glucose, Folin-Ciocalteus phenol reagent and bovine serum albumin (BSA) were purchased from Merck, 3,5-dinitrosalicylic acid (DNS), tetraethoxysilane (TEOS), tetramethoxysilane (TMOS) were obtained from Fluka. Ceramics and all the other chemicals were obtained from local suppliers or were commercially available reagent grade products and were used without further purification. The *Trichoderma viride* CMCB 1 strain was obtained from the Industrial Microbiology Laboratory of USAMVB Timisoara.

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The *Thricoderma longibrachiatum* DSM 769 strain was purchased from DSMZ Germany.
Microbial cells of *Thricoderma* were cultured in solid state fermentation [8].
The extraction of enzymatic preparation was done in distilled water (1:10) under magnetic stirring (75 minutes, 28°C, 150 rpm), after 168 hours of fermentation. The obtained extraction medium was filtered through gauze and centrifuged at 10000 rpm.
The enzymatic preparation with CMCase and cellobiase activity was liophylized from fermentation medium for 24 hours, at -56ºC and 26 mTorr (iLShin Europe Dry Freezer).
A buffered enzymatic solution (containing 3.5 CMCase units and 1.3 cellobiase units of lyophilized enzymatic preparation produced by *T. viridae* CMCB 1 in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 4.0) was stirred with the ceramic support (1 g) for one hour and a half and than filtered, washed with 3.5 mL distilled water and 3.5 mL acetone, dried at 4ºC over night and than assayed.
A sol-gel entrapment method was performed using a two steps procedure. The sols were prepared using Si-precursor and EtOH in acid catalysis, HCl 1N (3.4:1:0.03, v/v). Then the sol was mixed with buffered enzymatic preparation (3.5 CMCase units and 1.3 cellobiase units from lyophilized enzymatic preparation of *T. viridae* CMCB 1 in 0.1 M citric acid – 0.2 M Na_2HPO_4 buffer, pH 4.0) and NaF 1M (1:1:0.05, v/v). In all cases the gelation occurred in a few seconds. The gels were left overnight for aging (4°C) and washed with n-hexane [5].
The effect of temperature and pH on the activity of native and immobilized enzymes was investigated by DNS assay, measuring the glucose concentration in the medium at various temperatures (25-90ºC) 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. Enzymes stability was analyzed as well, after a month of storage at 4ºC.
The protein content was assayed according to the Lowry method, using the Folin-Ciocalteus phenol reagent and bovine serum albumin (BSA) as standard [6]. The endoglucanase 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 [7].

3. Results and discussion

In our study two fungal *Thricoderma* strains were tested. The best CMCase and cellobiase activities were obtained for the *Thricoderma viridae* CMCB 1 strain (Table 1). This enzymatic preparation was used in the subsequent immobilization studies.
The enzymatic preparation obtained by fermentation of a *Thricoderma viridae* CMCB 1 strain was immobilized using two methods, physical adsorbtion on a ceramic support and entrapment in silica gel obtained using two different precursors, tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS), by sol-gel method. The highest CMCase and cellobiase activities were found for the immobilized preparation obtained by physical adsorbtion on ceramic support. Comparing the enzymes activities of the immobilized products, it was noticed that the CMCase and cellobiase activities obtained by physical adsorbtion were 1.3-2 times greater than those obtained by entrapment, for similar enzyme loadings (Table 2, Table 3).
For comparison, the immobilization methods have been applied directly to the fermentation medium, after one month freezing. The results indicate (Table 4) lower CMCase activity than in case of lyophilized enzymatic powder, although the immobilization yields were comparable.

Table 1. Screening for CMCase and cellobiase producing *Thricoderma* strains

Strain	pH	CMCase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$	Cellobiase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$	Protein content $mg_{BSA} \cdot mL^{-1}$
Thricoderma viridae CMCB 1	4.5	0.113	0.125	3.30
Thricoderma longibrachiatum DSM 769	5.0	0.094	0.119	2.28

Table 2. Protein content and CMCase activity of the lyophilised and immobilized preparations

Enzymatic preparation of *Thricoderma viridae* CMCB 1		Protein content $mg_{BSA} \cdot mL^{-1}$ $mg_{BSA} \cdot g^{-1}$	CMCase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$ $\mu mol \cdot min^{-1} \cdot g^{-1}$	Immobilization yield[c] %
Native				
Fermentation medium[a]		3.30	0.113	-
Lyophilized enzymatic preparation[b]		19.28	17.33	-
Immobilized[b]				
Physical adsorbtion		2.75	1.38	59.65
Entrapment	TMOS	3.45	0.59	21.25
	TEOS	4.81	1.05	26.62

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

Table 3. Protein content and cellobiase activity of the lyophilised and immobilized preparations

Enzymatic preparation of *Thricoderma viridae* CMCB 1		Cellobiase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$ $\mu mol \cdot min^{-1} \cdot g^{-1}$	Specific activity	Immobilization yield[c] %
Native				
Fermentation medium[a]		0.125	0.04	-
Lyophilized enzymatic preparation[b]		6.50	0.34	-
Immobilized[b]				
Physical adsorbtion		0.65	0.24	47.13
Entrapment	TMOS	0.38	0.11	23.75
	TEOS	0.48	0.10	21.12

[a], [b], [c] – according to table 2

Table 4. Protein content and CMCase activity of the fermentation medium and immobilized preparations

Enzymatic preparation of *Thricoderma viridae* CMCB 1		Protein content $mg_{BSA} \cdot mL^{-1}$ $mg_{BSA} \cdot g^{-1}$	CMCase activity $\mu mol \cdot min^{-1} \cdot mL^{-1}$ $\mu mol \cdot min^{-1} \cdot g^{-1}$	Immobilization yield[c] %
Native				
Fermentation medium[a]		2.63	0.363	-
Immobilized[b]				
Physical adsorbtion		9.67	0.387	34.83
Entrapment	TMOS	4.76	0.24	18.52
	TEOS	5.72	0.52	20.87

[a], [b], [c] – according to table 2

The influence of some environmental parameters (pH, temperature) on the free and entrapped enzymatic preparation activity (using TEOS as precursor) and the stability of the immobilized product were studied (Figure 1, Figure 2, Table 5). The free enzymatic preparation had a maximum of CMCase activity at pH 3, and of cellobiase activity at pH 4. The optimal pH of the TEOS entrapped CMCase was shifted towards the less acid side (with aproximatively two point units) when compared with that of the native enzyme (Figure 1). In case of cellobiase activity the optimum pH was the same for both native and TEOS entrapped enzyme (Figure 2).

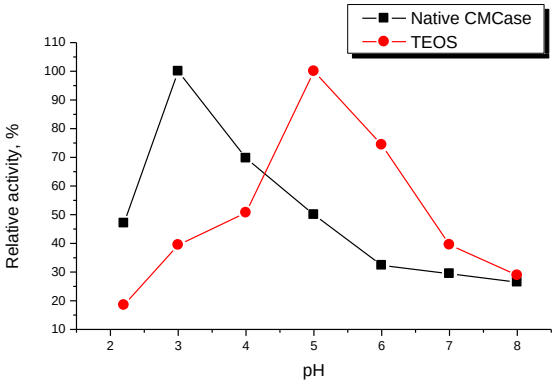

Figure 1. The effect of pH on CMCse activity of immobilized and native enzymatic preparation

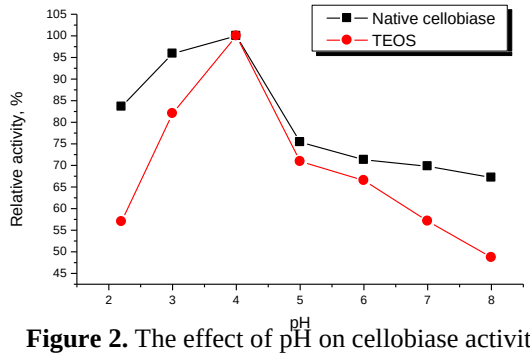

Figure 2. The effect of pH on cellobiase activity of immobilized and native enzymatic preparation

The optimum temperature of the free enzymatic preparation was 65-70ºC (Figure 3). The preparations immobilized by entrapment showed activities significantly greater than the native enzyme at temperatures less then the optimum. At 37ºC the residual activity of immobilized enzyme was more than 90% from the maximum (40ºC for the enzyme entrapped in TEOS).

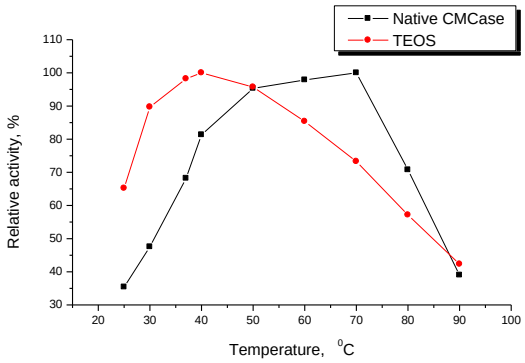

Figure 3. The effect of temperature on the activity of immobilized and native enzymatic preparation

Enzymes stability was analyzed as well after a month of storage at 4ºC. The immobilized enzymatic preparations were kept at 4ºC and the stability was monitored in time. Enzymatic activities were determined periodically (Table 5).

Table 5. Preservation stability of immobilized enzyme

Immobilization method	Rezidual CMCase activity %		
	initial	two weeks	a month
Physical adsorbtion	100	37.96	12.89
Entrapment (TEOS)	100	63.25	57.32
Entrapment (TMOS)	100	58.87	36,54

4. Conclusions

The culture broth and lyophilized enzymatic powder with CMCase and cellobiase activity obtained by *Thricoderma viridae* CMCB 1 strain cells fermentation was immobilized by physical absorbtion on ceramic support and entrapment in silica gel. Immobilization directly from the culture medium and after liophylization indicates almost similar values in case of immobilization yields.
Optimum pH of the entrapped enzymes increased by two units when compared to optimum pH of the native enzyme. The immobilization has led to enzymatic preparations with improved stability.

Acknowledgements

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