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Immobilization of Commercial Cellulase and Xylanase by Different Methods Using Two Polymeric Supports

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Abstract

Industrial applications require enzymes highly stable and economically viable in terms of reusability. Enzyme immobilization is an exciting alternative to improve the stability of enzymatic processes. The immobilization of two commercial enzymes is reported here (cellulase and xylanase) using three chemical methods (adsorption, reticulation, and crosslinking-adsorption) and two polymeric supports (alginate-chitin and chitosan-chitin). The optimal pH for binding was 4.5 for cellulase and 5.0 for xylanase, and the optimal enzyme concentrations were 170 µg/mL and 127.5 µg/mL respectively, being the chitosan and the ideal support. In some cases, a low concentration of crosslinking agent (glutaraldehyde) improved stability of the immobilization process. Biotechnological characterization showed that the reusability of enzymes was the most striking finding, particularly of immobilized cellulase using glutaraldehyde, which after 19 cycles retained 64% activity. These results confirm the economic and biotechnical advantages of enzyme immobilization for a range of industrial applications.

Keywords

Cellulase, Crosslinking-Adsorption, Immobilization, Reticulation, Xylanase

1. Introduction

Enzymes have enormous potential as industrial catalysts, largely because they are substrate-specific and easy to produce. Their use is becoming increasingly widespread, especially in the biological and chemical industries.

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Hydrolytic enzymes, for example, are widely used in the textile, pulp and paper industries [1]. The engineering of enzymes for this purpose has been hailed as one of the most exciting, complex and interdisciplinary goals of biotechnology. Despite their large-scale commercial availability, however, these biocatalysts still have certain drawbacks, chief among which is their extremely limited reusability [2]. Enzyme immobilization is a technology aimed at enhancing the stability of enzyme-related processes [3], with a view to enabling continuous processing through the reuse of enzymes [4].

Immobilized enzymes can be defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously" [5]. In bioaffinity immobilization, the enzyme/protein is immobilized via bioaffinity interactions [6]. Immobilization enables continuous economic operation, automation and the recovery of product with a high degree of purity [7]. For that reason, there is a growing industrial demand for immobilized biocatalysts.

Immobilized cellulase and xylanase are widely used in the biotechnology industry, among other things for clarifying juices and wines, for extracting plant oils and coffee, for the bioconversion of agricultural waste [8], and for improving the digestibility of animal feed ingredients [9]. A major application at present is in the biodegradation or bioconversion of cellulose- and hemicellulose-containing materials to monomeric sugars [10]. Agricultural waste rich in lignocellulosic material could be used in manufacturing a whole range of commercial products including ethanol [11], organic acids [12] and, if the process were economically competitive, other chemical products [13].

The present study investigated the immobilization of cellulase and xylanase on a range of supports, analyzing the behavior of various biochemical parameters and comparing it to that of the native enzymes.

2. Materials and Methods

2.1. Materials

Chitin from lobster shells (degree of deacetylation = 10% [14], average particle size = 30 μm) was obtained from Empresa Mario Muñoz (Havana, Cuba). Chitosan was obtained by alkaline deacetylation of chitin [15]; the degree of deacetylation was 90% [14]. Sodium alginate extracted from *Laminaria hyperborea* was purchased from BDH (Poole, UK). Molecular weight was 1.97×10^5.

Commercially-available soluble xylanase (Pentopan Mono BG) and cellulose (Celluclast BG) were purchased from Novozymes (Denmark). Glutaraldehyde (50%, w/v) was obtained from SIGMA (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of Carriers

Alginate-Chitin: 600 mg of alginate was dispersed in 60 ml of potassium-phosphate buffer (pH 6.0) to which 150 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was then added. The reaction was maintained at room temperature for 1 h with continuous stirring. Activated alginate was mixed with 3 g of a chitin solution (w/v), dissolved in 30 mL distilled water and stirred for 16 h at 25˚C.

Chitosan-Chitin: 1 g of chitin was dispersed in 10 mL distilled water, and glutaraldehyde was added to a final concentration of 5% (v/v). The reaction was maintained at 25˚C for 4 h with continuous stirring. The solid was collected by centrifugation, washed several times with distilled water until no aldehyde was detected in waste, and finally suspended in 25 mL of distilled water. This volume of activated chitin was mixed with 10 mL of chitosan (1% chitosan solution (w/v)) which was previously dissolved in 3% acetic acid (v/v), and finally the support chitin-chitosan was stirred for 4 - 6 h to room temperature. $NaBH_4$ was then added to a final concentration of 200 mM, and the solution was stirred for 16 h. The solid was collected by filtration and washed several times with distilled water.

Both carriers were collected by centrifugation, washed several times with distilled water and dried in vacuum drying apparatus until their use for immobilization by adsorption and reticulation (adsorption-crosslinking). The other immobilization method (crosslinking-adsorption) required additional preparation of carrier using a bifunctional reagent (glutaraldehyde), which can block amino groups and render polysaccharide structures (alginate or chitosan) more inert and resistant to the acid medium [16].

Preparation of crosslinked chitosan-chitin carrier: one gram of chitosan-chitin pre-treated carrier was added to 20 mL of two chosen glutaraldehyde concentrations (15% (v/v) and 0.5% (v/v)) and dissolved in 200 mM phosphate buffer (pH 7.0). The mixture was stirred in the dark at 25˚C for 1 hour (support activated with 0.5%

glutaraldehyde) or 15 h (support activated with 15% glutaraldehyde). The crosslinked chitosan carrier was centrifuged and washed several times with optimal immobilization buffer.

2.3. Optimal Conditions for Immobilized Enzymes

The methods employed for enzyme immobilization are outlined below. The first step was to optimize enzyme-support conditions.

2.3.1. Adsorption

For this method, 10 mL of support (alginate-chitin and chitosan-chitin) at a concentration of 0.03 g/mL were suspended in the relevant buffer (depending on the pH used) to a final volume of 40 mL. The following variables were optimized:

- pH: a fixed enzyme concentration was tested (170 µg/mL) with pH values ranging from 2.5 to 5.5 [17], using 50 mM citric acid/Na_2HPO_4 (pH 2.5) and 50 mM sodium acetate/acetic acid (pH 3.0 - 5.5). A total of 7 immobilization reactions were obtained per support and enzyme.
- Enzyme concentration: different concentrations of enzyme were tested (8.5 µg/mL, 17 µg/mL, 42.5 µg/mL, 84 µg/mL, 127.5 µg/mL, 170 µg/mL and 340 µg/mL), obtaining 7 immobilization reactions per enzyme at the optimal pH.

  Both assays were kept in darkness at 10˚C for 16 h, with continuous gentle stirring.
- Binding time: using the two optimized parameters (pH and enzyme concentration), the two enzymes were then tested over a range of enzyme-support binding times (20, 40, 60, 90, 120, 150, 180, 210, 210 and 240 min), yielding a total of 10 reactions for each enzyme.

2.3.2. Reticulation (Adsorption-Crosslinking)

After optimizing adsorption conditions for the chitin-chitosan support, immobilization was performed by reticulation, by adding glutaraldehyde to the chitosan-enzyme system at 5 different concentrations (from 0.125% to 1.5%) for 0.5 h [18].

2.3.3. Crosslinking-Adsorption

Having prepared the crosslinked chitosan carrier, immobilization by adsorption was repeated under previously-optimized conditions.

In all cases, after immobilization the suspension was collected by centrifugation and repeatedly washed with 50 mM of the buffer used for immobilization. Excess glutaraldehyde (reticulation method) or protein (all methods) was removed.

2.4. Measurement of Cellulase/Xylanase Activity, and Retained Protein

Once the enzyme was bound to the support, enzyme activity was checked. The reaction mixture comprised 100 µl of enzyme (cellulase or xylanase) solution and 400 µl of 1% (w/v) carboxymethylcellulose (CMC) or xylan substrate, respectively, in 50 mM acetate buffer (pH 5.5). The mixture was incubated for 30 min at 37˚C and reducing sugars were measured using the dinitrosalicylic acid method [19], using glucose and xylose, respectively, as standards. Assays were performed in triplicate. One unit of enzyme activity (U) was defined as the amount of enzyme required to release one µmol of glucose per minute under these assay conditions.

Native and immobilized enzyme activity recovery was calculated as: activity recovery (%) = (total activity of enzyme/maximum total activity of enzyme)*100%.

Absorbed protein was calculated as the difference between added protein and unbound protein detected in washing solutions after immobilization. Concentrations were calculated using the Bradford method [20], with bovine serum albumin (BSA) as standard.

2.5. Biotechnological Characterization of Free and Immobilized Enzymes

Having established the optimal conditions for immobilization, the influence of pH, temperature and storage stability on enzyme activity was determined; kinetic constants and degree of reusability were also investigated. To determine pH stability, cellulase and xylanase were incubated with CMC and xylan, respectively at 37˚C for 30

minutes in different buffers (100 mM citric acid/Na_2HPO_4 pH 2.0 - 2.5; 100 mM sodium acetate/acetic acid pH 3.0 - 6.0; 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 - 8.0).

Heat stability was determined by measuring residual activity after preincubating the enzymes for 10 minutes at different temperatures (from 40˚C to 90˚C at 5˚C intervals). Aliquots were chilled quickly and cellulase and xylanase activity was measured [17].

The kinetic properties V_{max} (μmol·min^{-1}·mg^{-1}) and K_m (mM) were determined from Michaelis-Menten plots of specific activities at 0.025% to 0.5% concentrations of specific substrate (CMC for cellulase and xylan for xylanase), and the rates were measured, ranging from 0.2 to 5 times the value of K_m. The values of V_{max} and K_m were determined by nonlinear regression [21].

Finally, the reusability of immobilized enzymes was determined by running consecutive cycles on CMC and xylan (cellulase and xylanase, respectively). All reactions were maintained at 37˚C for 30 minutes, applying in all cases the previously-identified optimal conditions. After each reaction, cellulase and xylanase were washed with the appropriate buffer. The activity of the immobilized enzyme was expressed as percentage residual activity.

2.6. Statistical Analysis

The statistical significance of the effect of free and immobilized enzymes in each assay, obtained in triplicate, was determined by one-way analysis of variance (ANOVA, version 12.9). Significant differences in results for each variable (pH, T, etc.) and each type of enzyme (unbound and immobilized) were also analyzed.

3. Results

In all cases, results were expressed as percentage relative activity, taking 100% as the maximum capacity for each assay.

3.1. Selection of Immobilization Method

Chitosan-chitin proved to be the best carrier for immobilizing both cellulase and xylanase. Results obtained on chitin-alginate were unsatisfactory, although xylanase kept linked at pH 2.5 to 4, the protein concentration of immobilized enzyme was very low; cellulase immobilization only was possible at pH 3, so this support was discarded in both cases. The best immobilization methods were adsorption and reticulation, while crosslinking-adsorption proved less effective with both enzymes (data not shown).

3.2. Optimal Conditions for Enzyme Immobilization

Conditions for the immobilization of commercial cellulase and xylanase are shown in **Figure 1**.

Testing the pH range from 2.5 to 5.5, maximum relative activity (100%) was achieved at pH 5.0 for xylanase and pH 4.5 for cellulase (in 50 mM sodium acetate/acetic acid buffer; **Figure 1(a)**).

The optimal enzyme concentration (tested over the range 8.5 to 340 μg/mL) was found to be 127.5 for xylanase and 170 μg/mL for cellulase (**Figure 1(b)**). The optimal enzyme-support binding time for immobilization proved to be 2 h for xylanase and 2.5 h for cellulase (data not shown).

Once these parameters had been optimized, they were applied for immobilization by reticulation with glutaraldehyde. The most effective glutaraldehyde concentration was 0.125; higher values prompted reduced activity, especially for cellulase (**Figure 1(c)**).

3.3. Biochemical Characterization of Free and Immobilized Enzymes

The pH stability of free and immobilized enzymes was determined by carrying out the enzyme assay at different pH values (pH 2.0 - 8.0). The optimum pH curves for both enzymes are shown in **Table 1(a)**. Xylanase displayed good stability over the pH range from 3.0 to 8.0; both native and immobilized enzyme achieved 100% activity at pH 6.0. By contrast, cellulase displayed good activity only at acidic pH values (from 2.0 to 4.0), with maximum activity at pH 3.0 (immobilized cellulase) and pH 4.0 (native cellulase). Higher pH values reduced activity in all three cases.

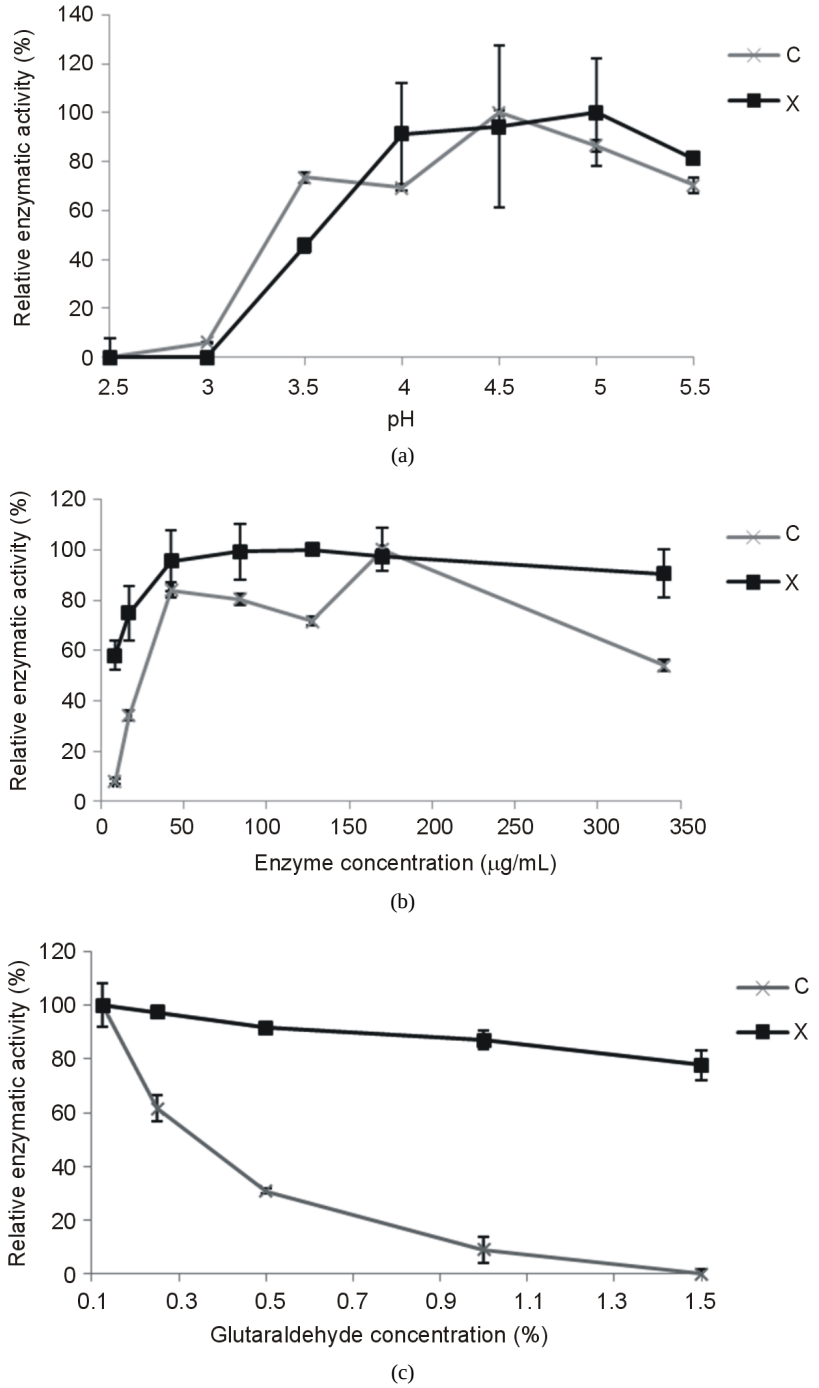


Figure 1. Effect of different conditions on the activity of immobilized cellulase (C) and xylanase (X). (a) Optimal pH; (b) Optimal enzyme concentration; (c) Optimal glutaraldehyde concentration. Relative activity was calculated by taking maximum enzyme activity as 100% in each case.

Enzyme heat stability is charted in **Table 1(b)**. Immobilization of cellulase, both by adsorption and by reticulation, prompted greater resistance to temperature increases. After 10 minutes at 75˚C, the activity of the native enzyme had dropped to 3.7%, whereas immobilized enzyme retained around 50% of its original activity. By contrast, native xylanase displayed greater heat resistance than immobilized enzyme at 75˚C, while the reverse was true at 55˚C (97.8% adsorption; 91.6% reticulation; 88.7% free).

The kinetic constant (K_m) and the maximum reaction rate (V_{max}) were obtained from Michaelis Menten plots. The kinetic behavior of cellulase and xylanase was modified by immobilization. K_m, a measure of the substrate's affinity for the enzyme, was higher for the immobilized enzymes than for their native counterparts (**Table 2**), while values for V_{max} were similar for both types.

The relative activity of the two immobilized enzymes over consecutive cycles of use is shown in **Figure 2**. In both cases, activity diminished with reuse. Xylanase could only be reused 8 times with both types of immobilization, achieving with the reticulated enzyme a maximum of 25% on the eighth cycle. By contrast, reticulation-immobilized cellulase retained up to 64% activity after 19 cycles, while the adsorption-immobilized enzyme displayed only 32% of its initial activity after the same number of cycles.

Table 1. (a) Effect of pH on xylanase and cellulase activity (%) using different buffers (pH 2.0 - 8.0); (b) Effect of temperature (40˚C - 90˚C) on xylanase and cellulase activity (%).

(a)

pH	FX	AX	RX	FC	AC	RC
2.0	1.5 ± 0.0^a	0.0 ± 0.0^a	0.0 ± 0.0^a	94.7 ± 2.9^a	87.8 ± 9.0^a	39.2 ± 0.0^b
3.0	75.3 ± 1.6^a	76.6 ± 3.6^a	76.3 ± 7.3^a	91.9 ± 5.1^a	100.0 ± 7.0^a	100.0 ± 11.0^a
4.0	87.9 ± 4.6^a	76.7 ± 10.0^a	80.5 ± 1.5^a	100 ± 9.0^a	88.1 ± 5.1^a	88.5 ± 10.0^a
5.0	95.3 ± 2.0^a	95.5 ± 3.0^a	97.0 ± 4.2^a	68.8 ± 7.0^a	55.5 ± 4.2^a	63.8 ± 9.0^a
6.0	100 ± 4.9^a	100 ± 6.0^a	100 ± 1.0^a	42.9 ± 1.1^a	62.7 ± 0.0^b	0.0 ± 0.2^c
7.0	86.9 ± 1.1^a	82.3 ± 4.7^a	81.8 ± 3.0^a	40.6 ± 0.3^a	11.9 ± 0.7^b	0.0 ± 0.0^c
8.0	80.1 ± 0.2^a	78.3 ± 2.3^a	78.8 ± 2.3^a	25.8 ± 0.3^a	4.3 ± 6.1^b	0.0 ± 0.0^b

(b)

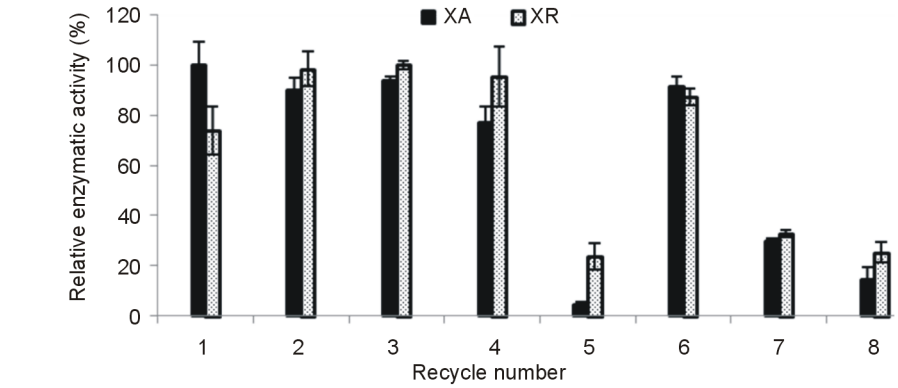
T^a	FX	XA	XR	FC	CA	CR
40	100.0 ± 4.1^a	100.0 ± 0.5^a	99.9 ± 0.9^a	97.0 ± 0.4^a	98.5 ± 1.5^a	89.2 ± 0.0^b
45	98.9 ± 3.8^a	99.6 ± 2.2^a	100 ± 3.6^a	98.9 ± 1.5^a	99.6 ± 1.2^a	90.0 ± 1.8^b
50	99.3 ± 1.4^a	99.9 ± 1.5^a	99.2 ± 1.4^a	100.0 ± 5.0^a	100.0 ± 4.0^a	90.2 ± 15.0^a
55	88.7 ± 1.5^a	97.8 ± 1.0^b	93.4 ± 3.0^a	96.1 ± 3.0^a	99.6 ± 3.0^a	87.3 ± 5.3^b
60	90.0 ± 3.7^a	88.0 ± 1.6^a	84.8 ± 2.6^a	96.3 ± 4.0^a	95.3 ± 2.6^a	100.0 ± 3.5^a
65	83.9 ± 5.0^a	79.2 ± 4.0^a	83.0 ± 1.5^a	92.1 ± 0.0^a	88.2 ± 3^a	90.3 ± 3.9^a
70	76.2 ± 4.9^b	73.8 ± 4.3^{a,b}	69.0 ± 4^a	70.2 ± 1.4^a	69.6 ± 2.8^a	78.3 ± 3.9^b
75	68.1 ± 2.7^a	39.3 ± 2.6^b	26.3 ± 3.5^c	3.7 ± 1.1^b	64.3 ± 8.0^a	46.8 ± 6.0^a
80	68.1 ± 2.4^b	19.9 ± 6.4^a	15.0 ± 4.8^a	0.0 ± 0.0^b	41.7 ± 6.0^a	51.1 ± 9.0^a
85	59.6 ± 2.2^b	14.2 ± 2.3^a	11.1 ± 1.6^a	0.0 ± 0.0^a	0.0 ± 0.7^a	0.0 ± 1.0^a
90	59.9 ± 1.7^a	10.6 ± 1.4^b	8.0 ± 1.0^c	0.0 ± 0.0^a	0.0 ± 0.7^a	0.0 ± 1.7^a

Relative activity was calculated by taking the maximum activity of each free or immobilized enzyme as 100%. FX: free xylanase; XA: xylanase immobilized by adsorption; XR: xylanase immobilized by reticulation; FC: free cellulase; CA: cellulase immobilized by adsorption; CR: cellulase immobilized by reticulation. Different letters indicate significant differences (95% confidence) among three enzymes (free, adsorbed and reticulated).

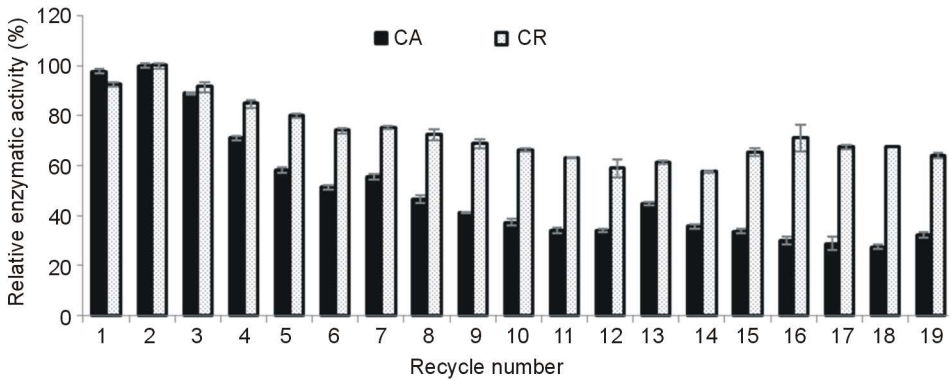
Table 2. Kinetic constants of native and immobilized cellulase and xylanase enzymes.

	Cellulase		Xylanase	
	$V_{máx}$^{b}	K_m^{a}	$V_{máx}$^{b}	K_m^{a}
Free	1.99	6.56	1.595	0.083
Immobilized by adsorption	1.74	12.0	1.66	0.115
Immobilized by reticulation	1.94	11.5	1.27	0.095

[a]Michaelis constant, K_m, was defined as concentration (mM) of substrate; [b]Maximum velocity, $V_{máx}$ refers to the substrate decomposition rate ($\mu mol \cdot min^{-1} \cdot mg^{-1}$).



(a)



(b)

Figure 2. Reusability of cellulase and xylanase immobilized by adsorption and reticulation. (a) XA: xylanase immobilized by adsorption; XR: xylanase immobilized by reticulation; (b) CA: cellulase immobilized by adsorption; CR: cellulase immobilized by reticulation. Data were expressed as means of three independent trials. Relative activities were calculated by taking maximum immobilized-enzyme activity as 100% in each case.

4. Discussion

In this study, two enzymes—cellulase and xylanase—were immobilized separately with a view to improving their performance in comparison with their native counterparts. Optimal conditions for the two enzymes were very similar. The most effective support proved to be chitosan, which has additional advantages including low cost, biocompatibility, good hydrophobicity, high porosity, and a large adhesion area. Moreover, its structure ensures minimal steric hindrance during immobilization [22]. Chitosan was also the most appropriate in other studies in our laboratory [23]. Also the optimal methods used for immobilization in both enzymes were adsorption and reticulation, while crosslinking-adsorption was rejected since showed the worst results. The glutaraldehyde concentration used to activate the support (0.5% and 15% (v/v)) could inhibit the enzyme, maybe in future works it could be tried the crosslinking-adsorption using lower concentration of glutaraldehyde.

Optimal pH was found to be 4.5 for cellulase and 5.0 for xylanase, reflecting the fact that polyanionic matrices give rise to the partitioning of protons between the bulk phase and the enzyme microenvironment, prompting changes in the optimal pH value. Changes depend on the immobilization method used, and on the structure and charge of the matrix [24].

With regard to biochemical properties, neither of the enzymes was affected by changes in pH, and there was no change in the pH response of the enzymes after immobilization. Xylanase, both free and immobilized, displayed good stability over the pH range from 3.0 to 8.0 and there was no significant differences between the adsorbed, reticulated and free xylanases as a function of pH. The same fact was observed by Pal and Khanum that reported that xylanase covalently immobilized on glutaraldehyde-alginate beads displayed behavior identical to that of the native enzyme at the same pH values [25].

Cellulase, both free and immobilized, proved stable only at acidic pHs. One-way ANOVA revealed significant differences between the three forms of cellulase at all pH values: at pH 3.0, free cellulase displayed the least hydrolysis, though it retained its activity slightly better than cellulase immobilized at pH 4.0. At neutral values (pH 6.0), reticulation-immobilized cellulase lost all activity, whilst free and adsorption-immobilized cellulase retained around 50% and 60%, respectively, of its initial activity. Similar findings are reported by Zhou [26] for cellulase immobilized on N-succinyl-chitosan. In all cases, changes in behavior as a function of pH may depend on the charge both of the enzyme and of the solid support.

Both xylanase and cellulase proved stable over the temperature range from 40˚C to 70˚C, and although statistical analysis revealed significant differences between formats over that range, for practical purposes these variations were negligible. For cellulase, both immobilization methods enhanced heat stability, with results significantly better than those of the native enzyme at temperatures of over 75˚C.

This enhanced thermostability is attributed to the covalent binding of cellulase to the copolymer [27]. In research using other matrices for immobilization [28] [29], enzymes did not remain stable over such a wide temperature range. Akkaya *et al*. [30] have reported that optimal temperatures for immobilized enzyme may be higher, lower or the same as for the native enzyme.

With regard to kinetic parameters, immobilization prompted an increase in the value of K_m, which might be due to changes in the accessibility of the substrate to the active sites of the enzyme caused by diffusional limitations, steric effects and enzyme structural changes following immobilization [31]. A similar increase has been noted in other studies [29]. Buchholz suggested that the increase in K_m upon immobilization of xylanase could be due to a conformational change in the enzyme resulting in lower affinity for the substrate [32].

Reusability is a major requirement for industrial enzyme applications. Xylanase displayed good activity up to its eighth reuse; after six consecutive cycles, the adsorption-immobilized and reticulation-immobilized enzyme retained 91% and 81%, respectively, of its initial activity. These findings are slightly better than those reported by Kapoor and Kuhad [33], who found that immobilized xylanase retained 70% of its initial activity.

Cellulase was reused up to 19 times, retaining good activity (32.09% for adsorbed enzyme, 63.8% for reticulated enzyme). These values are higher than those obtained by Wu *et al*. [34], who recorded only 36% residual activity after 6 reuses.

5. Conclusions

Two commercial enzymes, xylanase and cellulase, were immobilized on different supports using diverse chemical binding methods, in order to optimize their use in an industrial setting. The best results were obtained using chitosan as support and adsorption and crosslinking with glutaraldehyde for immobilization.

While biochemical analysis showed that both enzymes could be successfully reused, results for immobilized cellulase using glutaraldehyde as crosslinking agent were particularly striking: after 19 reuses, the enzyme retained 64% of its initial activity.

These results confirm the economic and biotechnical advantages of enzyme immobilization, especially regarding to the number of reuses, which open the possibility of different industrial applications.

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