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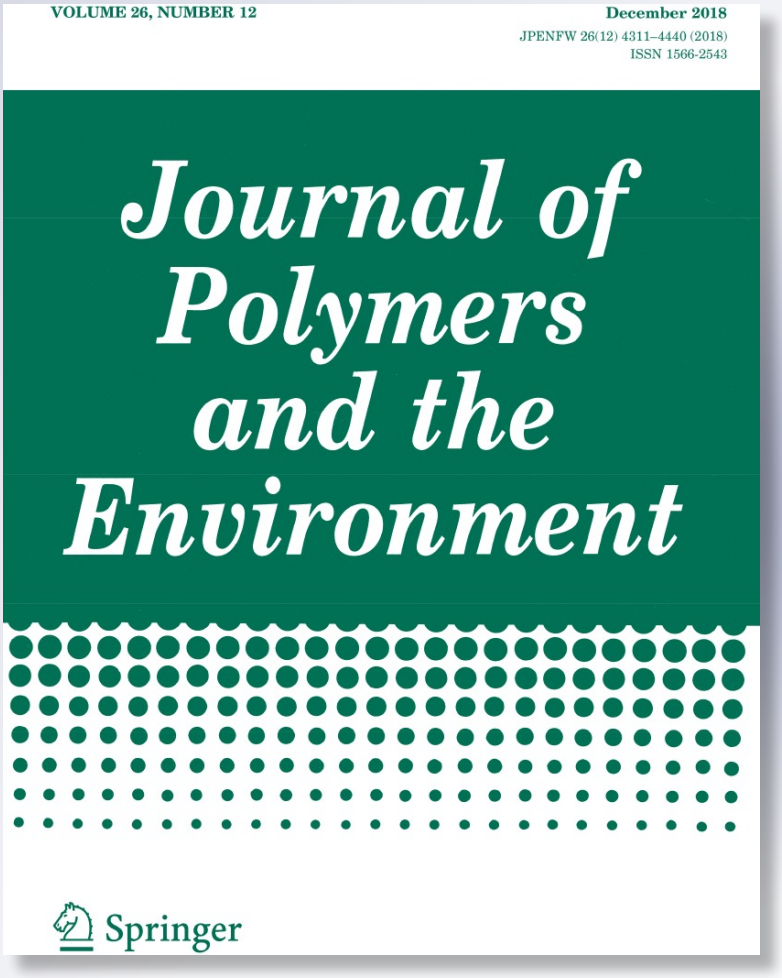

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ORIGINAL PAPER

Synthesis of Polymeric Matrices for Adsorption and Purification of Endoglucanase

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Abstract
Chitosan (CHS), chitosan–Eudragit® EPO (CHS–EPO) and chitosan beads partially cross-linked with glutaraldehyde (CHS–GLUT) were obtained in order to improve the adsorption selectivity of endoglucanase from a fungal culture obtained under SSF by *Aspergillus niger* using soybean harvest residues as support. The matrices synthetized were characterized in terms of physical and chemical changes. Fourier-transformed infrared spectroscopy with attenuated total reflectance device (FTIR-ATR) was employed to verify the chemical changes on the CHS matrix after the synthesis of CHS–GLUT and CHS–EPO. Scanning electron microscopy (SEM) was performed to compare the surface morphology of the polymeric beads. Two variables, purification factor and yield percentage of the adsorption process, were analyzed using a bifactorial ANOVA considering the matrix–time first order interaction. SEM results exhibited greater surface roughness in the CHS–GLUT and CHS–EPO matrices which may enhance endoglucanase adsorption. FTIR-ATR results confirmed an effective chemical modification of the CHS matrix after crosslinking with GLUT and corroborated the efficiency of the synthesis of the CHS–EPO matrix by the presence of chemical groups of the EPO polymer. An endoglucanase purification factor close to 9 was achieved with the CHS–GLUT matrix and a yield percentage of 60% was obtained with the CHS–EPO matrix. Bifactorial ANOVA results showed the matrix–time interaction to be significant for both variables. The CHS–GLUT matrix with low crosslinking times and the novel CHS–EPO matrix could be included in the bioseparation stage of endoglucanase using a simple and a low-cost method such as batch adsorption.

Keywords Polymeric matrices · FTIR-ATR · SEM · Endoglucanase · Adsorption

Introduction

Cellulase, an enzymatic complex composed of by endoglucanase, exoglucanase and β-glucosidase, is the third largest industrial enzyme sold worldwide and one of the most demanded enzymes in volume [1]. It is used in various industrial activities such as oil extraction, the production of bioenergy from lignocellulosic biomass and the manufacture of animal feed and bakery products. Endoglucanase has a major role in hydrolyzing the internal glycosidic bond of cellulose chains, an essential step in lignocellulosic biomass conversion into glucose monomers. It is used in various industrial processes such as the processing of fruit juice and beer and in animal feed production. However, the obtention of the enzyme poses a challenge from the economic point of view. The bioseparation stage for the recovery of the enzyme can represent between 50 and 80% of the total production costs [2, 3].

Conventional methods for endoglucanase separation are size-exclusion chromatography, ion-exchange chromatography, precipitation with ammonium sulphate and affinity chromatography [4–6]. However, chromatography is a cost-ineffective method and, in addition, ammonium sulphate cannot be disposed of. Hence, it is important to develop a process for endoglucanase separation which reduces both cost and environmental impact.

Protein adsorption in batch systems is an interesting method of protein purification. This process has the

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advantages of being simple to operate and cost-effective. Due to the leading role of the adsorbent materials in the process, the synthesis of low-cost adsorbents with high adsorption capacity and selectivity has become the main objective in this area of research. Although activated carbon has been used for cellulase adsorption [7]; this adsorbent material is expensive. Thus, the search for low-cost alternatives has been the goal of specialists in the area.

Chitosan is one of the most abundant biopolymers in nature [8]. It is a partially *N*-deacetylated derivative of chitin, which is commonly found in the exoskeleton of crustaceans and cell walls of some fungi [9, 10]. Chitosan presents several advantages given that it is biocompatible, bioactive, biodegradable and contains polycationic properties [11, 12]. In addition, it is a low cost material because it comes from a natural source [13, 14]. Moreover, chitosan can be altered by chemical or physical methods to synthetize chitosan derivatives. Chemical modification improves mechanical resistance, prevents chitosan dissolution in acidic solutions and increases adsorptive selectivity [15].

The properties of a polymer can be modified by derivatization or copolymerization [16]. Chemical crosslinking can intensify the strength of the matrix by forming a spatial grid structure [13]. Crosslinking with glutaraldehyde (GLUT) is a typical example of chemical-mediated structural modification. GLUT crosslinking occurs through a Schiff's base reaction between the aldehyde ends of the crosslinking agent and the amine moieties of chitosan to form imine functions. This crosslinking, which is a simple and cheap method, increases the matrix resistance to microbiological biochemical and biochemical degradation [17]. Previous works [18, 19] have carried out the adsorption of metals on chitosan beads and cross-linked chitosan beads. Crosslinking was performed during a reaction time of 24 h as it is usually done. These long periods of times that make the process more expensive and difficult especially when scaling. However, crosslinking depends on glutaraldehyde concentration, time of contact, temperature and pH [20], which can be modified so as to reduce times and costs and even improve the selectivity by the adsorption of the enzyme.

Eudragit polymers are copolymers derived from esters of methacrylic and acrylic acid available in different ionic forms. Methacrylate copolymers manufactured under the mark Eudragit have been used in the production of micro- and nanosized particles, granules, tablets, as coatings. Some authors have used Eudragit L-100 to immobilize endoglucanase. Zhang et al. have covalently immobilized cellulase on Eudragit L-100 [21, 22] but this method has the disadvantage of not being able to recover the free enzyme from the polymer and it can also decrease the enzymatic activity [23]. However, there are no reports on the application of Eudragit EPO (a cationic polymer consisting of 1:2:1 ratio of methyl methacrylate, *N,N*-dimethylaminoethyl methacrylate, and butyl methacrylate monomers) in adsorption processes for the purification of cellulase.

The purpose of the present work is to explore novel sorbents for the adsorption and the purification of endoglucanase from a culture from *Aspergillus niger*. In order to accomplish this, chitosan beads were synthetized and chemically modified performing a crosslinking of low intensity GLUT and hybrid beads prepared with CHS and EPO were also synthetized in order to evaluate and compare the parameters of the purification process of all matrices obtained. The matrices synthetized were characterized in terms of physical and chemical changes. Fourier-transformed infrared spectroscopy with attenuated total reflectance device (FTIR-ATR) was employed to monitor chemical changes. The surface morphology of polymeric matrices was monitored by using scanning electron microscopy (SEM). Two variables, the purification factor and the yield percentage of the adsorption process, were analyzed using a Bifactorial ANOVA considering the matrix–time first order interaction in the model.

Materials and Methods

Materials

Chitosan (CHS) was purchased from Sigma (USA) with a minimum deacetylation degree of approximately 75%. All other reagents were of analytical grade.

Preparation of CHS Matrix

A solution of 2% w/v was prepared by dissolving chitosan in an acetic acid solution 5% v/v. The solution was kept under stirring during 1 day to ensure complete solubilization and then stored at 4 °C.

This solution was transferred to a syringe of 50 mL and allowed to fall drop-wise into a solution of 1 M NaOH in constant orbital agitation at 120 rpm. The beads were washed with distilled water to remove NaOH residues. Finally, the CHS beads were stored in distilled water at 4 °C.

Preparation of CHS-Eudragit® EPO Matrix

The CHS-Eudragit® EPO (CHS–EPO) matrix was prepared by dissolving 1.25 g of CHS and 0.35 g of Eudragit® EPO in 50 mL of 1% v/v acetic acid. It was left under stirring all night until its complete dissolution. Once dissolved, the mixture was dripped with a 50 mL syringe in a 2 M NaOH solution under constant orbital stirring at 120 rpm. The beads were kept in 1 M NaOH solution for 1 week at 4 °C. They were then stored in 0.1 M NaOH at 4 °C.

Preparation of CHS Matrix Crosslinked with Glutaraldehyde

Glutaraldehyde cross-linked CHS (CHS–GLUT) matrices were prepared by placing CHS 2% w/v beads with Glutaraldehyde 0.5% w/v for 30 min. The incubation was carried out in an ice bath, with constant orbital agitation of 90 rpm. They were then thoroughly washed; first, with ethanol and, finally, several times with distilled water to remove excess Glutaraldehyde. The beads were stored in distilled water at 4 °C until use.

Matrix Characterization

The synthesized matrices CHS, CHS–GLUT, CHS–EPO were lyophilized in a Model L-T8 Lyophilizer (Rificor) under vacuum at a temperature of − 40 °C.

Scanning Electron Microscopy

The lyophilized matrices were analyzed using a scanning electron microscope FEI QUANTA 200 F Feg at 12 keV under low vacuum at 0.35–0.50 mbar with a large field detector (LFD).

Fourier Transformed Infrared Spectroscopy

FTIR-ATR spectra of the lyophilized matrices were obtained using a Fourier transform infrared spectrometer, IR-FT Prestige-21 Shimadzu with ATR Smart Omni-Sampler accessory. Spectral scanning was performed in a wavenumber range of 4000–650 cm^{-1} at 4 cm^{-1} resolution.

Microorganism and Culture

Strains of *A. niger* NRRL3 were provided by the culture collection of the Agricultural Research Service, USDA. The microorganism was activated in potato-glucose agar medium at 30 °C for 5 days. The spores were collected with sterile distilled water under gentle agitation and quantified by microscopic counting using a Thoma chamber.

Endoglucanase Production by *A. niger* Under SSF

A fungal culture in a solid medium was carried out in 250 mL Erlenmeyers flasks, where 20 g of wastes from the soybean crop were placed and 50 mL of the culture medium was added. They were sterilized in an autoclave and allowed to cool. Then, they were inoculated with 1.2×10^6 conidia mL^{-1}. They were homogenized and incubated at 30 °C for 3 days.

The medium was prepared with the following composition (g L^{-1}): $(NH_4)_2SO_4$ (1.4); KH_2PO_4 (2.0); $MgSO_4 \cdot 7H_2O$ (0.5); $CaCl_2$ (0.4); $FeSO_4 \cdot 7H_2O$ (0.006); $MnSO_4 \cdot H_2O$ (0.002); $ZnSO_4$ (0.0014); $CoCl_2 \cdot 6H_2O$ (0.02); Urea (0.3); yeast extract (0.25).

The enzymatic extract was obtained from the culture in a solid medium by adding 60 mL of 50 mM acetate buffer (pH 5.00), were added to each flask. The liquid obtained was filtered on a fine pore metal sieve and finally centrifuged at 10,000 rpm for 10 min.

Determination of Endoglucanase Activity and Total Proteins

Assay for the activity of endoglucanase was performed as follows:

Carboxymethylcellulose (CMC, 1%) solution was prepared in 50 mM sodium citrate buffer (pH 5.30) and incubated with supernatant samples of batch adsorption at 50 °C for 10 min. Then, 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added and maintained at 100 °C for 10 min and absorbance was measured at 560 nm. The reducing sugar concentration generated from the enzymatic reaction was then used to calculate endoglucanase activity. A calibration curve of glucose was carried out under the same experimental conditions as the samples.

The total protein concentration was estimated by the Warburg and Christian method [24].

Adsorption and Elution of Endoglucanase from Fungal Culture

The adsorption experiments were carried out in flasks containing 0.50 ml crude enzyme solution, 1 g matrix and 9.5 ml of distilled water. The mixture was stirred at 120 rpm at room temperature for 120 min. The enzymatic activity and the concentration of total proteins in the supernatant were determined at different times. After the adsorption, the matrix was washed with 10 mL of distilled water and maintaining with constant orbital agitation at 120 rpm for 15 min. The desorption of the enzyme was performed by increasing the ionic strength of the medium. For this purpose, 10 mL of 250 mM NaCl was added to the previously washed matrix. The enzymatic activity and the concentration of total proteins in the supernatant were determined at different times of 15, 30, 60, 90 and 120 min. During the course of the desorption, the system was maintained in constant orbital agitation at 120 rpm.

The experiments were carried out in triplicate.

Analysis of Purification Process

The purification factor and recovery of endoglucanase were determined.

The purification factor (PF) was defined as the number of times that the specific activity increases in each fraction. The PF was calculated by comparing the endoglucanase specific activity in the desorbed fraction with the endoglucanase specific activity in the initial sample as follows:

$$PF = \frac{SAd}{SAc}$$

where SAd is the endoglucanase specific activity in the desorbed fraction and SAc is the endoglucanase specific activity initial sample. Endoglucanase specific activity was calculated as the endoglucanase activity divided by total protein concentration.

Percentage of recovery (%R) represents the percentage of retained activity with respect to the initial sample and it was calculated as the endoglucanase activity in the desorbed fraction divided by the endoglucanase activity in the initial sample as follows:

$$\%R = \frac{Ad}{Ac}$$

where Ad is the endoglucanase activity in the desorbed fraction and Ac is the endoglucanase activity in the initial sample.

Statistical Analysis

Purification factors and yield percentages were analyzed using a Bifactorial ANOVA, considering the matrix–time first order interaction model. In all cases, the assumptions required were verified graphically and by hypothesis testing in each case (Normality: Anderson–Darling test; Homoscedasticity: Levene test). Then, the treatment means were pairwise compared by Tukey's multiple comparisons method, using the lsmeans procedure of the homonymous package [25] of the statistical software R 3.2.3 [26]. A p value < 0.05 was regarded as significantly different.

Zymogram Analysis

SDS–PAGE was performed in a 15% (w/v) polyacrylamide gel according to the method described by Laemmli [27]. For visualization of endoglucanase activity bands on the gel, the method of Medve et al. was used with some adjustments [28]. The zymogram analysis of endoglucanase was carried out by adding 0.15% (w/v) CMC into the polyacrylamide gel. Subsequently SDS–PAGE, the gel was washed twice at room temperature with a solution made of 50 mM acetate buffer and 25% isopropanol (pH 4.80). Endoglucanase was renatured in a 50 mM acetate buffer (pH 4.8) containing 5 mM β-mercaptoethanol by stirring the gel overnight at 4 °C. Then, the gel was immersed in a 50 mM acetate buffer (pH 4.80) for 2 h and incubated at 50 °C for another 2 h. The gel was stained in 0.1% (w/v) Congo Red for 30 min and destained with 1 M NaCl. Endoglucanase activity was detected as clear bands against a red background.

Results and Discussion

Matrices Characterization

Matrices Surface Morphology

SEM was carried out to compare the surface morphology of the matrices. Figure 1 shows the SEM images of the CHS, CHS–GLUT, CHS–EPO matrices. According to SEM images, after chemical modification of chitosan matrices, CHS–GLUT, CHS–EPO matrices exhibited their surface modified especially regarding their roughness. This fact evidences the chemical modification of the CHS matrix.

According to Fig. 1a, the CHS matrix has a smooth and non-porous surface. On the other hand, the CHS–GLUT matrix has a grooved surface and the CHS–EPO matrix exhibits a rough surface. Other authors have reported that an increased roughness at the matrix surface could contribute to increased adsorption of biomolecules [29].

Fourier Transformed Infrared Spectroscopy

FTIR-ATR spectra of synthesized chitosan matrices were obtained in order to verify chemical modifications on the CHS matrix after the synthesis of CHS–GLUT and CHS–EPO (Fig. 2).

The spectrum of the CHS matrix shows the classical peaks assigned to the structure of the polysaccharide. The main signals are: an intense and wide band at 3360 cm^{-1}, which is attributed to the stretching vibrations of the NH group, which overlaps with the peak at 3440 cm^{-1}, corresponding to the presence of –OH groups; a peak at 2870 cm^{-1}, associated with the symmetrical and asymmetric stretching vibrations of the pyranose ring; a peak at 1655 cm^{-1} corresponding to axial C=O stretching in amide group; a signal at 1560 cm^{-1} attributed to NH-bending vibration in the amide group; a peak at 1415 cm^{-1}, corresponding to the vibration intensity of OH in the pyranose ring. The peak located at 1377 cm^{-1} corresponds to the symmetrical deformation of the CH_3 group. Finally, the region between 1150–900 cm^{-1} belongs to the vibrations of the C–O and C–O–C groups of the glycosidic bond which are characteristic signals of chitosan [30, 31].

According to Fig. 2, a remarkable reduction in the intensity in the band at 3360 cm^{-1} corresponding to the NH groups and a decrease in the signal at 1100 cm^{-1}, corresponding to aliphatic amino groups, are observed after modification chemistry of the CHS matrix to synthesize the

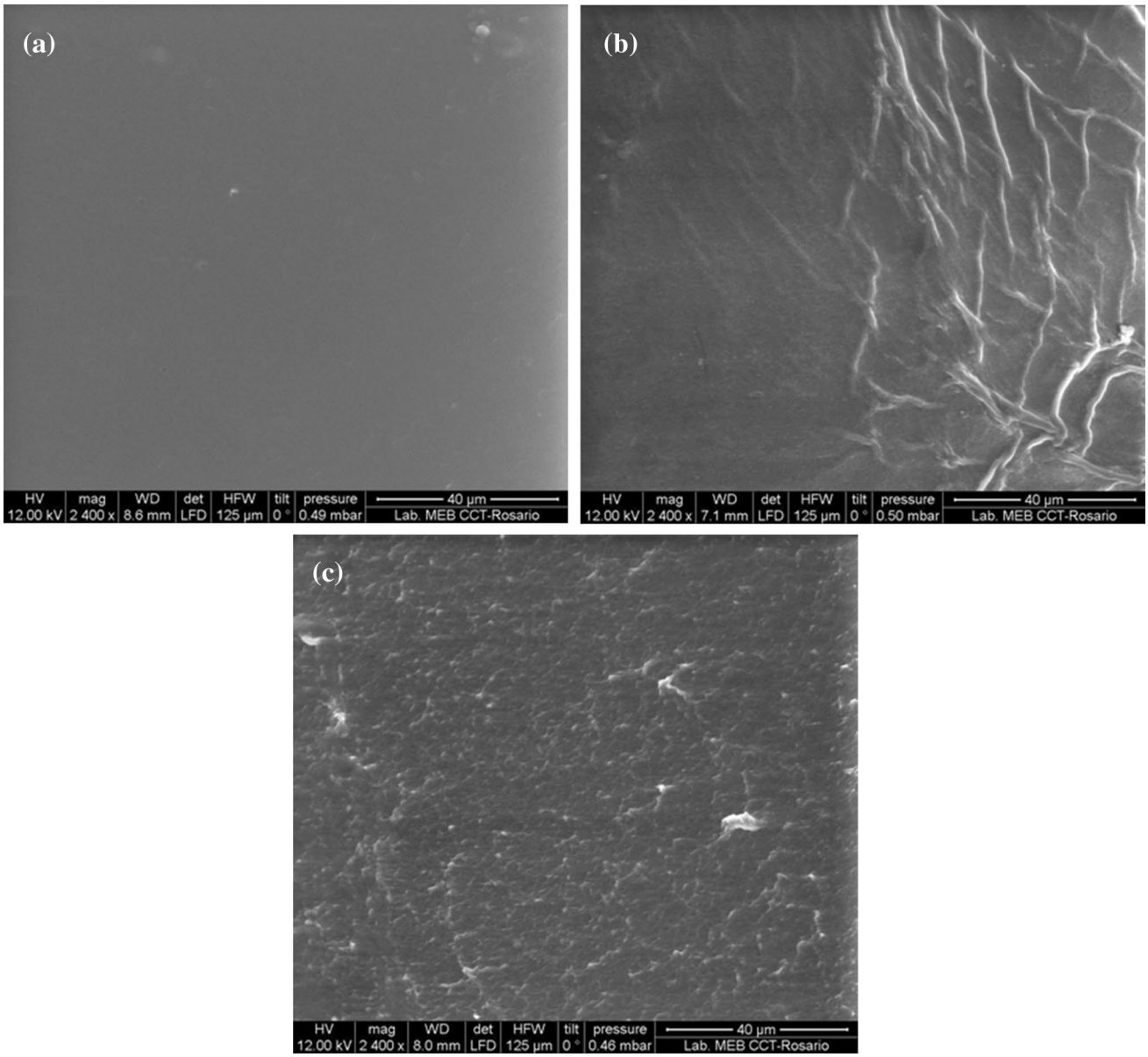

Fig. 1 SEM of the matrices of CHS (**a**), CHS–GLUT (**b**), CHS–EPO (**c**)

CHS–GLUT matrix. This result is consistent with the fact that the crosslinking of chitosan by glutaraldehyde occurs mainly through the amino groups of chitosan [15, 32]. In addition, after the crosslinking reaction, a new peak arises at 1529 cm^{-1} suggesting an interaction of the $–NH_2$ groups of CHS with the –CHO groups of GLUT [17].

On the other hand, the matrix CHS–EPO presents a band at 1728 cm^{-1} which is a characteristic signal of the Eudragit® EPO, pertaining to vibrations of C=O in the ester group. Besides, the non-ionized dimethylamine groups of the EPO have a signal at 2823 cm^{-1} which overlaps with the 2870 cm^{-1} band as shown in Fig. 2. These facts confirm the presence of EPO after the synthesis of the matrix [33].

Evaluation of Chitosan Matrices for Adsorption of Endoglucanase from Fungal Extract

Chemically modified chitosan matrices were synthetized in order to enhance the selectivity of endoglucanase adsorption.

The adsorption of enzymes on the surface depends on a combination of forces mainly electrostatic, van der Waals and hydrophobic interactions [34].

Since counter-ions can interfere with the adsorption process by interacting with the adsorbent support, no buffer solution was used [35–37].

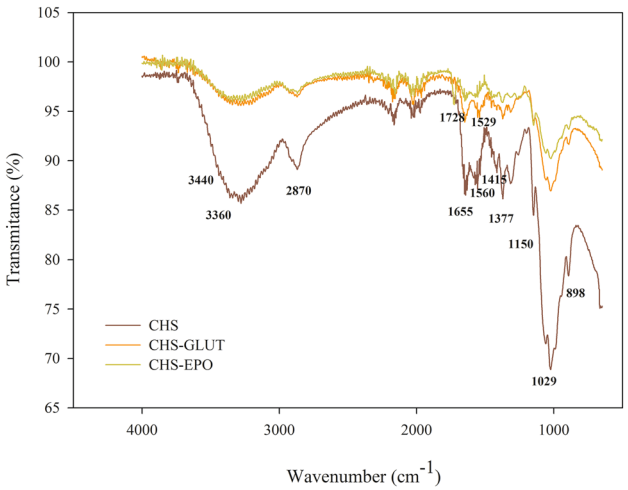

Fig. 2 FTIR-ATR spectra of chitosan matrices:CHS, CHS–GLUT, CHS–EPO

The pH values of the solutions were found to be within the 6.50–7.50 range, which is the range of optimal and stability endoglucanase pH [38, 39].

The adsorption and elution profiles of endoglucanase from fungal extract on the synthesized chitosan matrices were evaluated. Table 1 shows the results of adsorption and subsequent elution of endoglucanase from fungal extract from the surface of different chitosan matrices. A reduction in endoglucanase adsorption is observed after the crosslinking of the CHS matrix with glutaraldehyde. As observed in the previous FTIR results, there is a reduction in the free amino groups after the crosslinking of the CHS matrix with glutaraldehyde. Therefore, the adsorption of endoglucanase may occur through amino groups. This is in agreement with previous bibliography [37]. A similar finding has been reported for the adsorption of lipase of *A. niger* on chemically modified chitosan matrices [40].

Table 1 Adsorption and elution efficiencies of endoglucanase from fungal extract on different chitosan matrices at different times of process

Chitosan matrix	Time (min)	Adsorption (%)	Elution (%)
CHS	15	6.3 ± 0.6	29 ± 5
	30	23 ± 2	32 ± 1
	60	29 ± 4	43 ± 2
	90	42 ± 9	45 ± 2
	120	46 ± 5	53 ± 9
CHS–EPO	15	19 ± 6	88 ± 4
	30	37 ± 5	89 ± 9
	60	46 ± 4	96 ± 5
	90	54 ± 1	97 ± 8
	120	53 ± 5	89 ± 8
CHS–GLUT	15	5 ± 1	17.4 ± 0.4
	30	16 ± 2	51 ± 9
	60	20 ± 1	55 ± 7
	90	32 ± 6	88 ± 3
	120	36 ± 4	67 ± 8

On the other hand, an increase in endoglucanase adsorption was observed when the CHS–EPO matrix was used. The Eudragit® EPO polymer confers tertiary amines to the matrix, which could favor the interaction between the endoglucanase and the CHS–EPO matrix. In addition, the endoglucanase of *A. niger* presents 19 negatively charged residues at the working pH that could interact with the amino groups of CHS–EPO through electrostatic interactions [41].

Although the adsorption percentage using the CHS–GLUT matrix was lower than when the CHS matrix was used, the elution percentage of the enzyme doubled when the CHS–GLUT matrix was used.

The CHS–GLUT and CHS–EPO matrices reached the maximum elution percentages of the enzyme close to 90% at 90 min of the desorption. Thus, both matrices proved to be more selective for the endoglucanase enzyme, becoming more appropriate for use in a purification process.

Other authors [29] have studied the adsorption of cellulase isolated from *A. niger* using chitosan/alginate particles functionalized with epichlorohydrin as the adsorbent. They reported an enzyme elution percentage of only 30%, being one-third of the percentage obtained in this work.

Efficiency of the Endoglucanase Purification Process from a fungal culture

Figures 3 and 4 show the purification factors and yield percentages, respectively, at different desorption times using the synthesized matrices.

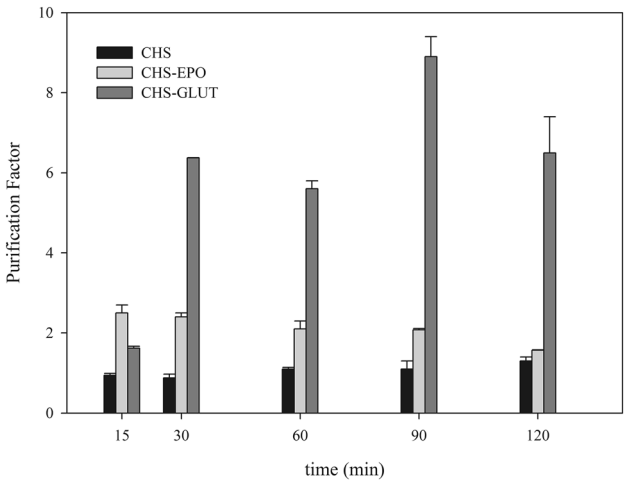

Fig. 3 Purification factors of the endoglucanase adsorption process at different desorption times

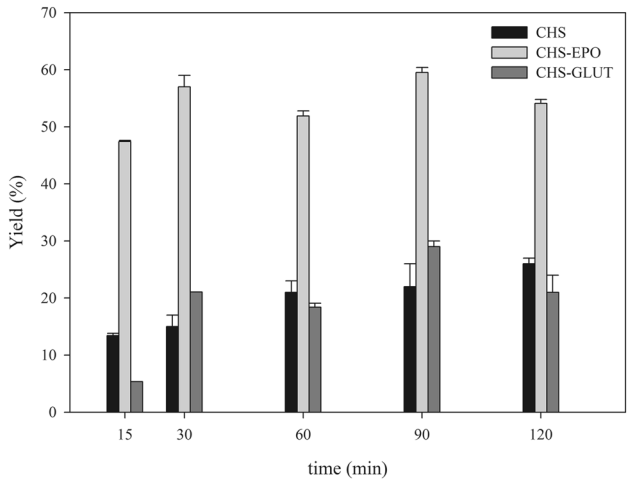

Fig. 4 Yield percentages of the endoglucanase adsorption process at different desorption times

The maximum purification factor obtained was close to 9 when the CHS–GLUT matrix was used and desorption was carried out at 90 min. However, the highest yield percentage, close to 60%, was reached when the CHS–EPO matrix was used and desorption was also performed at 90 min.

Previous research has purified endoglucanase from *Pseudomonas fluorescens* [42], where a purification fold of about seven by ammonium sulphate precipitation was obtained. Nevertheless, ammonium sulfate cannot be thrown into the environment. Besides, a purification fold of about 25 by ion exchange chromatography and percentages of yield of only 10% by gel filtration on Sephadex G-100 were reported. However, chromatography is an expensive method which is not suitable to be carried out on a large scale. Similarly, other authors [43] have purified endoglucanase from *Bacillus licheniformis* by successive steps of precipitation with ammonium sulfate and chromatographies, though poor yields close to 20% were achieved.

In this work, we synthesized a new matrix formed by a mixture of CHS–EPO as well as a matrix of CHS–GLUT with short crosslinking times. High purification values and yield percentages of endoglucanase from *A. niger* were obtained when CHS–EPO and CHS–GLUT were used respectively.

These performance parameters indicate that the CHS–GLUT and CHS–EPO matrices would be appropriate to be used in an endoglucanase purification process, either by using only one of them for a single step of purification or by making a combination of both. For example, CHS–EPO could be included in the first steps of purification and CHS–GLUT in the last stages, according to the requirements for the industrial use.

Statistical Analysis

Table 2 shows the Bifactorial ANOVA analysis (matrix, time) for the purification factor variable. According to Table 2, the matrix–time interaction was significant for the purification factor variable ($p < 0.001$).

According to the results of Tukey's multiple comparisons method (data not shown), no significant differences were found for the purification factor variable at 30, 60, 90, 120 min using CHS–GLUT matrix as adsorbent. However, there was a significant difference between 15 min of desorption and the rest of the times tested.

Moreover, significant differences were detected for the purification factor variable at 15 min of desorption between CHS matrix and the other ones. Besides, the purification factor between the three matrices showed a significant difference at 30, 60 and 90 min.

Table 3 represents the Bifactorial ANOVA analysis (matrix, time) for the variable percentage of yield.

As shown in Table 3, the matrix–time interaction was significant ($p = 0.0144$) for the variable yield percentage. Conforming to results of Tukey's multiple comparisons method, there were significant differences in the variable yield percentage between the CHS–EPO matrix and the CHS and CHS–GLUT matrices at all times studied.

Zymogram Analysis

Purification of endoglucanase from fungal extract after desorption from chitosan matrices was analyzed by zymogram.

Table 2 Bifactorial ANOVA for the variable purification factor

Source of variation	Degrees of freedom	Sum of squares	Square medium	F	p
Matrix	2	13.9107	6.9554	171.952	2.44×10^{-15}***
Time	4	1.6307	0.4077	10.079	5.33×10^{-5}***
Matrix:time	8	3.3632	0.4204	10.393	2.75×10^{-6}***
Error	25	1.0112	0.0404		

*$p \leq 0.05$
**$p \leq 0.01$
***$p \leq 0.001$

Table 3 Bifactorial ANOVA for the variable percentage of yield

SOURCE OF VARIATION	Degrees of freedom	Sum of squares	Square medium	F	p
Matrix	2	12581.1	6290.6	469.6893	$<2.2 \times 10^{-16}$***
Time	4	1020.8	255.2	19.0538	2.67×10^{-7}***
Matrix:time	8	331.4	41.4	3.0935	0.0144*
Error	25	334.8	13.4		

*$p \leq 0.05$
**$p \leq 0.01$
***$p \leq 0.001$

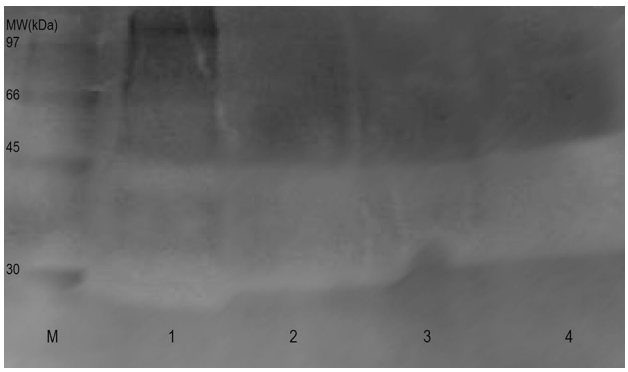

Fig. 5 SDS–polyacrylamide (15%) gel electrophoresis followed by staining for visualization of endoglucanase activity in the fungal extract and in the desorbed fraction at 120 min from CHS, CHS–EPO, CHS–GLUT. M, protein molecular weight markers. The molecular weight markers consist of: Phophorylase b (97 kDa); Albumin (66 kDa); Ovoalbumin (45 kDa); Carbonic anhydrase (30 kDa) Lane 1, fungal extract. Lane 2, desorbed fraction from CHS matrix. Lane 3: desorbed fraction from CHS–EPO. Lane 4: desorbed fraction from CHS–GLUT

Figure 5 shows the endoglucanase activity of the fungal extract and the desorbed fraction at 120 min from CHS, CHS–EPO, CHS–GLUT.

In the fungal extract, endoglucanase activity was observed in two major bands of 30 and 45 kDa; while at least one minor band with molecular weights higher than 45 kDa showed no endoglucanase activity. In the fractions desorbed at 120 min from CHS, CHS–EPO, and CHS–GLUT matrices, only the bands corresponding to a molecular-weight range from 30 to 45 kDa with endoglucanase activity were detected. These molecular weights agree with those reported for endoglucanases isolates from various sources [44, 45].

In this work, CHS, CHS–GLUT and CHS–EPO were synthetized, characterized in chemical and physical terms and evaluated according to the purification parameters of the enzyme endoglucanase, a glycoside hydrolase with different applications in industrial processes. Crosslinking chitosan-glutaraldehyde has been previously used to prepare chitosan membranes and chitosan beads [19, 32]. However, the crosslinking reaction was carried out with long reaction times, up to 24 h, which slows down the process. But in this work, a purification factor of endoglucanase close to 9 was reached with a crosslinking time of only 30 min. Moreover, a novel adsorbent, CHS–EPO, was synthesized and characterized. The results of the purification studies showed with this new matrix, the purification factor doubled and the yield percentages tripled, compared to the values obtained with the CHS matrix. In addition, these adsorption studies were carried out on a fungal extract obtained by SSF by *A. niger* from soybean harvest residues. The use of agro-industrial residues contributes to reducing environmental problems [46]. Besides, SSF is known to be a low-cost method for enzyme production and it offers various advantages such as lower energy demand, lower investment costs and greater yields [47].

The development of a bioprocess for the production and purification of endoglucanase using soybean harvest residues as support in SSF in the first stage and carrying out batch adsorption using the CHS–EPO and CHS–GLUT matrices in a second stage of bioseparation, would offer economic and environmental benefits for the industry.

Conclusions

In this work CHS, CHS–EPO and CHS–GLUT were obtained in order to compare their adsorption selectivity for the endoglucanase enzyme from a fungal culture obtained under SSF by *A. niger* by using soybean harvest residues as substrate.

FTIR-ATR studies evidenced the modifications of the CHS matrix. SEM results revealed an increase in the surface roughness of the CHS–GLUT and CHS–EPO matrices with respect to the CHS matrix. Using CHS–GLUT and CHS–EPO matrices high purification values and high yield percentages were obtained. respectively. In addition, as CHS–GLUT and CHS–EPO are easy-to-prepare matrices, they can be used in the bioseparation stage of endoglucanase for industrial uses. Besides, the batch adsorption process using CHS–GLUT and CHS–EPO may represent a selective, low-cost method for endoglucanase purification

from complex systems, allowing to develop a scaling of the process.

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