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Synthesis and characterization of chitosan membranes functionalized with amino acids and copper for adsorption of endoglucanase

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ABSTRACT

Chitosan membranes were obtained and functionalized with amino acids and copper in order to improve adsorption selectivity of endoglucanase. The membranes were characterized by Fourier-transformed infrared spectroscopy with attenuated total reflectance device (FTIR-ATR) to monitor chemical changes. Scanning electron microscopy (SEM) was performed to compare the surface morphology of the membranes. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were carried out in order to analyze thermal stability of functionalized membranes and to identify the differences between functionalized chitosan membranes before and after endoglucanase adsorption. SEM results proved that functionalization had occurred since the surface of the chitosan membrane was modified. FTIR-ATR results confirmed an effective chemical modification of chitosan membranes with amino acids and copper and corroborated endoglucanase adsorption. The characteristic parameters of DSC and TGA also evidenced endoglucanase adsorption. The use of functionalized membranes as adsorbents increased 40-fold the percentage of endoglucanase adsorption as compared to unmodified membranes. Thus, chitosan membranes functionalized with amino acids and copper may represent a novel, low-cost adsorbent to be used in endoglucanase purification from complex systems.

1. Introduction

Cellulase represents approximately 20% of the enzyme global market and it is the world's third largest industrial enzyme by dollar volume [1]. The increasing demand for cellulase is due to its use in the detergent, textile, pulp and paper, medical and pharmaceutical industries [2]. The cellulase system is composed of three enzymes: endoglucanase, exoglucanase and β-glucosidase which acting together, catalyze the hydrolysis of cellulose [3]. Endoglucanase is the most important component for cellulose degradation [4]. However, the high cost of this enzyme represents a challenge to industrial applications. Bioseparation for the recovery of the endoglucanase can account for 50–80% of overall production costs [5].

Conventional methods for endoglucanase separation are ion-exchange chromatography, affinity chromatography, size-exclusion chromatography and precipitation with ammonium sulphate [6–10]. However, chromatography is an expensive method of separation and ammonium sulphate is not environment-friendly; thus, it is necessary to design and develop a process for cellulase recovery which reduces cost and environmental impact.

The adsorption of proteins in batch systems represents an attractive method of protein purification. These processes are cost-effective and simple to operate [11,12]. As a result, the development of low-cost adsorbents with high adsorption capacity and selectivity has been a great challenge. Previous studies have carried out adsorption of cellulase from *Aspergillus niger* on commercial activated carbon [13]; however, activated carbon is considered to be an expensive adsorbent [14], and, therefore, the production of low-cost alternatives has been the aim of researchers in the area.

Chitosan is a linear polysaccharide composed of chains $\beta(1 \rightarrow 4)$ D-glucosamine and *N*-acetyl-D-glucosamine [15,16]. It is produced by deacetylation of chitin, which is a structural element in the exoskeleton of crustaceans and one of the most widely available natural polymers in nature [17,18]. Chitosan exhibits beneficial chemical and biological properties since it is bioactive, biocompatible, biodegradable and contains polycationic properties [19]. In addition, chitosan is economically attractive as it comes from a natural source [20]. Chitosan can be modified by physical or chemical methods to prepare chitosan derivatives. It can be used to synthetize membranes, gel beads or fibers, depending

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on its final application [21,22]. Chemical modifications are performed in order to prevent chitosan dissolution in acidic solutions and to improve mechanical resistance and adsorptive selectivity [23,24].

Recently, there has been an increasing interest in polymeric membranes for protein separation [25,26]. Polymeric membranes with functional groups improve adsorption capacity and efficiency. The development of new chemically modified polymeric membranes has been of great scientific concern due to the key role of the adsorbent in the adsorption process [27]. Previous works have reported that chitin matrix modified by L-cysteine has significantly increased the adsorption capacity by introducing free amino groups [28]. In addition, amino acids have been reported to exhibit strong interactions with metals which is important in protein binding [29,30].

Moreover, it has been shown that transition metal ions present affinity for proteins. Hari et al. [31] reported a selective adsorption of human IgG on cellulose membranes immobilized with copper ions.

The aim of this work was to prepare and modify chitosan membranes by functionalizing them with amino acids and copper in order to characterize the functionalized membranes and evaluate the batch adsorption of endoglucanase from *Aspergillus niger*. Chitosan membranes were characterized in terms of physical properties and chemical changes. In order to accomplish this, Fourier-transformed infrared spectroscopy with attenuated total reflectance device (FTIR-ATR), differential scanning calorimetry (DSC) and thermal gravimetric analyses (TGA) were employed. Moreover, the surface morphology of chitosan membranes was monitored by using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

Chitosan (CHS) in powder form was purchased from Sigma (USA). The CHS source is shrimp shells and has a minimum deacetylation degree of approximately 75% and a molecular weight of 340 g/mol, determined by gel permeation chromatography. Endoglucanase (EG) was purchased from Megazyme (Ireland). All other reagents were of analytical grade. All solutions were prepared with deionized ultrapure Milli-Q water.

2.2. Preparation of chitosan membranes

A solution of 2.5% w/v was prepared by dissolving 15 g of chitosan in 600 mL of acetic acid solution 3% v/v. The solution was kept under mechanical stirring at 3000 rpm and then stored at 4 °C.

The solution was spread on Petri dishes. Drying was carried out at 60 °C until the mass was 50% of initial mass. Chitosan membranes were immersed in 1 M NaOH for 24 h at 25 °C. After addition of 1 M NaOH, chitosan membranes have sufficient mechanical strength to be easily removed without breaking. Finally, they were washed exhaustively with Milli-Q water until all alkali was removed and stored in Milli-Q water at 4 °C.

2.2.1. Functionalization of chitosan membranes

Functionalization of chitosan membranes was performed following the method proposed by Sano and Murase [32].

Firstly, epichlorohydrin and the amino acid were mixed in a 1:1 proportion in 2 M NaOH for 4 h at 60 °C: 0.95 mL of epichlorohydrin was added to each flask containing 2.66 g of L-aspartic acid (Asp), 2.94 g of L-glutamic acid (Glu), 3.10 g of L-histidine (His), and 2.50 g of L-taurine (Tau), respectively. The solution was then cooled to 0 °C and NaOH was added under constant stirring. Chitosan membranes were added to the solution containing the amino acid and epichlorohydrin. The reaction was conducted at 65 °C for 16 h. Finally, the membranes were washed repeatedly with Milli-Q water in order to remove residues. The following membranes were obtained: CHS-Asp, CHS-Glu, CHS-His, CHS-Tau, respectively.

Functionalization with copper was carried out by placing the functionalized membranes with amino acids in 2.6 mM $Cu(NO_3)_2$ solution under constant stirring for 60 h. The membranes were preserved intact after shaking. Finally, the membranes were washed with Milli-Q water.

2.3. Endoglucanase assays

Assay for the activity of endoglucanase was performed as follows:

Carboxymethylcellulose (CMC, 1%) solution was prepared in 50 mM sodium citrate buffer (pH 5.3) and incubated with supernatant samples of batch adsorption at 50 °C for 10 min. After adding 1 mL of 3,5-Dinitrosalicylic acid (DNS) reagent, it was further incubated at 100 °C for 10 min and absorbance was read at 560 nm. The reducing sugar concentration generated from the enzymatic reaction was then measured and used to calculate endoglucanase activity.

2.4. Endoglucanase adsorption

Chitosan membranes were immersed in a 5% v/v endoglucanase solution. Adsorption was carried out for 3 h at 25 °C and 200 rpm. The membranes were separated from the solution.

The percentage of endoglucanase adsorption was calculated by comparing the final activity of the adsorption with the initial activity of endoglucanase as follows:

$$EG\ adsorption(\%) = (\ (Ai - Af\ )/Ai) \times 100$$

where *Ai* (U/L) and *Af* (U/L) are endoglucanase activities before and after adsorption experiments, respectively.

The experiments were carried out in triplicate.

2.5. Membrane characterization

All membranes, before and after adsorption, were lyophilized to remove the water present in the pores without destroying them. A Labcomco equipment (Freeze Dry System/Freezone 4.5, Brazil) was used under vacuum of 35×10^{-3} bar and at a temperature of −65 °C.

2.5.1. Scanning electron microscopy

The lyophilized membranes were analyzed by scanning electron microscopy. Samples were coated with a 200 Å - thick gold layer using an EMITECH K450 sputter coater (Kent, United Kingdom). Micrographs were obtained in a scanning electron microscope with Energy Dispersive X-ray detector, (Leo 440i, EDS 6070, SEM/EDS: LEO Electron Microscopy/Oxford Cambridge, England).

2.5.2. Fourier transformed infrared spectroscopy

FTIR spectra were obtained in ATR mode on a Nicolet 6700 Fourier transform infrared spectrometer (Thermo Scientific, Madison, USA) equipped with Smart Omni-Sampler. Spectral scanning was acquired in a wavenumber range from 4000 to 675 cm^{-1} at 4 cm^{-1} resolution.

2.5.3. Thermogravimetric analysis

TGA analyses were performed using a SHIMADZU TGA-50 M thermogravimetric analyzer (Kyoto, Japan) under a nitrogen atmosphere. Samples were placed on an alumina cell, and heated from 22 °C to 900 °C at a heating rate of 10 °C/min.

2.5.4. Differential scanning calorimetry

Lyophilized membranes (4–5 mg) were used in order to analyze thermal properties. Differential scanning calorimetry measurements were performed with a Mettler Toledo DSC1 (Zürich, Switzerland). Samples were heated from −80 °C to 350 °C at a heating rate of 10.0 K/min, under nitrogen atmosphere at a flow rate of 50 mL/min.