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Single-step purification of chitosanases from *Bacillus cereus* using expanded bed chromatography

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ABSTRACT

A chitosanase-producing strain was isolated and identified as *Bacillus cereus* C-01. The purification and characterization of two chitosanases were studied. The purification assay was accomplished by ion exchange expanded-bed chromatography. Experiments were carried out in the presence and in the absence of cells through different expansion degree to evaluate the process performance. The adsorption experiments demonstrated that the biomass does not affect substantially the adsorption capacity of the matrix. The enzyme bound to the resin with the same extent using clarified and unclarified broth (0.32 and 0.30 U/g adsorbent, respectively). The fraction recovered exhibited 31% of the yield with a 1.26-fold increase on the specific activity concerned to the initial broth. Two chitosanases from different elution steps were recovery. Chit A and Chit B were stable at 30–60 °C, pH 5.5–8.0 and 5.5–7.5, respectively. The highest activity was found at 55 °C, pH 5.5 to Chit A and 50 °C, pH 6.5 to Chit B. The ions Cu^{2+}, Fe^{2+} and Zn^{2+} indicated inhibitory effect on chitosanases activities that were significantly activated by Mn^{2+}. The methodology applied in this study enables the partial purification of a stable chitosanase using a feedstock without any pre-treatment using a single-step purification.

1. Introduction

Chitosan, a natural cationic polysaccharide, is produced by the deacetylation of chitin. The chitosan molecule has shown various properties such as functional, renewable, nontoxic and biodegradable biopolymer. In recent years, chitosan has been used as artificial skin, absorbable surgical suture, wound healing accelerator and also as chitoligosaccharides (COS) source [1]. The oligosaccharides from chitosan have many biological properties such as: antitumor [2,3], immunoenhancing [1], antibacterial [4,5], antidiabetic [6] and hypocholesterolemic [7]. These functions are dependent on oligosaccharide chemical structure and molecular size [1,8].

The COS is obtained mainly using chemical and/or enzymatic hydrolysis [8]. Enzymes are highly specific and act under mild conditions. Moreover, the use of enzymes allows the reaction control mainly product formation by adjusting concentration, pH, temperature and reaction time [9]. Thus, the enzymatic hydrolysis method has been proposed to be preferable for producing bioactive COS [10].

Chitosanases have previously been produced by a number of organisms, including fungi [2,11], plants [12], and bacteria [13,14]. The chitosanases produced by *Bacillus cereus* are currently investigated by several studies [15–20]. The process of purification of these enzymes normally involves ammonium sulfate precipitation, gel filtration and packed ion exchange chromatography [16,21–23]. These traditional methods imply in a large number of steps, extensive work time and scale up difficulties. Alternatively, the expanded bed adsorption (EBA) combines separation, concentration and capture of the target protein. This competitive biotechnological process has been proposed as a single-unit operation [24]. EBA allows the capture of target proteins from feedstock without any preclarification steps, which is helpful to industrial processes since it contributes to reduction of time and costs [25].

The aim of this present study is to evaluate chitosanase purification using an economic as well as a fast chromatographic method. Initially, the adsorption behavior of chitosanase onto Streamline-DEAE as well as biomass influence were either determined. After, a

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purification protocol was carried out in order to recover and purify the chitosanase.

2. Materials and methods

2.1. Materials

Chitosan (85% deacetylated; molecular weight: 90–190 kDa) acquired from Sigma–Aldrich Co. (Saint Louis, USA) was solubilized using 0.1 M HCl [14]. Streamline DEAE resin was purchased from GE healthcare (Uppsala, Sweden). BCA Protein Assay Kit was acquired from Pierce (Rockford, USA). PC33 Siemens Kit was provided by Maria Alice Hospital, Natal/Brazil. Buffer solutions and other chemicals were reagent grade and were used as supplied.

2.2. Isolation and identification of chitosanolytic bacterium

The microorganism was isolated from soil samples (Natal/Brazil S05°52′11″, Wo35°13′08.4″). Serial dilutions from soil samples were inoculated on plates containing peptone (6.0 g L^{-1}), chitosan (2.0 g L^{-1}), magnesium sulfate (0.5 g L^{-1}), potassium phosphate dibasic (1.0 g L^{-1}) and agar (15 g L^{-1}) and incubated at 30 °C for 2 days. A single colony showing prominent chitosanolytic activity was selected and subjected to morphological analyses, Gram-staining and pathogenicity test with PC33 Siemens Kit. Polymerase chain reaction (PCR) was performed to amplify part of the bacterial 16S rRNA gene to additional microbial identification. The forward and reverse primers were 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1525R (5′-AAG GAG GTG ATC CAG CC-3′), respectively. The PCR products were analyzed by electrophoresis on agarose gel. Subsequently, they were purified, quantified and used in sequencing reaction. Four sequencing reactions were run for each sample using the primers 518F (5′-CCA GCA GCC GCG GTA ATA CG-3′), 800R (5′-TAC CAG GGT ATC TAA TCC-3′) and 1492R (5′-TAC GGY TAC CTT GTTA CGA CTT-3′). The nucleotide sequence of the 16S rRNA gene of C-1 was determined by an ABI 3730 DNA Sequencer (Applied Biosystems, USA) and compared to16S rRNA sequences in a NCBI BLAST search.

2.3. Chitosanase production

Cells from the stock cultures were transferred to 50 mL aliquots of medium and dispensed into 250 mL Erlenmeyer flasks for the investigation of chitosanase production. Subsequently, they were incubated at 120 rpm for 72 h at 30 °C. The cultivation medium consisted of (g L^{-1}): peptone 6.0, chitosan 2.0, magnesium sulfate 0.5 and potassium phosphate dibasic 1.0. During cultivation, the culture broth was collected to measurement of chitosanase activity and protein content. Cell growth was monitored by extraction and quantification of total DNA content. The pellet originated from 3 mL of culture was used for DNA extraction using sodium dodecyl sulfate (SDS) and phenol/chloroform/isoamyl alcohol [26]. The culture broth was used for further purification by using expanded bed adsorption (EBA).

2.4. Hydrodynamic of the bed

The expansion characteristic of the bed was investigated with an initial settled bed height of 6.0 cm at 25 °C. The expanded bed height was visually checked as a function of incremental velocity ranging from 30 to 200 cm h^{-1}. The expansion degree to each velocity was registered and expressed as the ratio of the height of the expanded (H) to the settled (H_0) bed adsorbent. The flow rate was kept constant for 10 min in each step before the estimation of the bed height to ensure bed stabilization. The experiments were carried out using culture broth with cells and without cells. The cells were removed by centrifugation for 20 min (4 °C, 3000 rpm).

The empirical correlation of Richardson–Zaki [27] was applied to define the bed expansion characteristics:

$$u = u_T \cdot \varepsilon^n \quad (1)$$

u is the superficial velocity, ε is the bed voidage, u_T is the terminal velocity in an infinite medium and n is the exponent of Richardson–Zaki which expresses the flow regime. The settled bed voidage was considered to have a value of 0.4 according to [28]. All experiments were carried out in triplicate.

2.5. Operation of EBA

The purification assays were developed using EBA methodology. The streamline anionic exchanger resin containing the ligand Diethylaminoethyl (DEAE) and a homemade EBA column (2.6 cm × 30.0 cm) were used to EBA chromatography. Glass microspheres were added to enhance fluid distribution at the column inlet. The fluid was pumped using a peristaltic pump (model Perimax 12, Spetec). The vertical alignment of the column was guaranteed in all assays. All experiments were performed at room temperature.

The column with 6.0 cm settled-bed height of resin was equilibrated with 50 mM Tris–HCl pH 8.5 buffer (buffer A) to give a stable expansion degree of $H/H_0 = 1.5$. A volume of 150 mL culture broth was then loaded (flow-through step) onto column at 150 cm h^{-1} superficial velocity. After, the bed was washed with buffer A (120 mL). The elution was carried out by step-wise gradient mode with 50 mM Tris–HCl pH 8.5 buffer containing 0.3 M (buffer B), 0.7 M (buffer C), and 1 M (buffer D) NaCl. The both steps, washing and elution, were conducted at the superficial velocity of 100 cm h^{-1}. In all purification steps, several fractions were collected for protein quantification, enzyme assay and electrophoresis.

2.6. Dynamic binding capacity

The adsorption performance of the expanded bed was studied in an EBA column loaded with streamline DEAE to a settled bed height of 6.0 cm. The effect of microbial cells on the breakthrough curves behavior was studied. The bed was expanded to an expansion degree of 2.0 with buffer A at a superficial velocity of 150 cm h^{-1}. Subsequently, the unclarified and clarified feedstock was loaded. For clarified feedstock, the centrifugation was carried out for 20 min (4 °C, 3000 rpm). Then the protein fractions were withdrawn and assayed for protein concentration and chitosanase activity. The dynamic binding capacity of the bed was checked assuming that the enzymatic activity at the column exit reached 10% of the initial activity ($U/U_0 = 0.1$). This methodology was accomplished to avoid the loss of the target product in the flow-through.

The dynamic binding capacity, Q_B (U of absorbed enzyme per gram of settled adsorbent) was calculated as follows:

$$Q_B = \frac{U_0\left(V_f - V_m\right) - \int_{V_m}^{V_f} C dV}{m_{\text{ads}}} \quad (2)$$

U_0 is the initial enzymatic activity (U mL^{-1}), V_f is the final volume of the feed solution (mL), V_m is the volume at 10% breakthrough (mL) and m_{ads} is the adsorbent mass (g).

Three additional experiments at superficial velocity of 100, 150 and 200 cm h^{-1} (expansion degree of 1.2, 1.5 and 2.0, respectively) were performed using unclarified broth to estimate the effect of the expansion degree on the chitosanase breakthrough behavior.

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