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Enhancing purification of chitosanase from *Metarhizium anisopliae* by expanded bed adsorption chromatography using Doehlert design

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ABSTRACT

The use of expanded bed adsorption with three different adsorbents Streamline (SP XL, DEAE and PHENYL) to purify the chitosanase from *Metarhizium anisopliae* strain with and without clarification using Doehlert design for the optimization of the experiments has been investigated. Chitosanases are enzymes that hydrolyze chitosan, a carbohydrate, resulting oligosaccharides that have many biological remarkable activities, such as anti-cancer, anti-HIV and anti-oxidants activities. The superficial velocity was evaluated in five levels, from 40 to 260 cm h^{-1}, while the type of adsorbent, a qualitative variable, was assessed at three levels, saving materials and time according to the Doehlert design. The results showed that it is possible to purify chitosanase from *M. anisopliae* by expanded bed adsorption. Chitosanase purification factor ranged from 2 to 3.1-fold and yield ranged from 27.7 to 45.3%. The highest enzyme activity in the elution was 0.06 U/mL values which were higher than or close to those reported. The molecular mass of chitosanase was about 45 kDa using SDS-PAGE.

1. Introduction

Chitosanases (EC 3.2.1.132) represent a class of hydrolytic enzymes that catalyze the β-(1→4) glycosidic bond hydrolysis of chitosan, a linear polysaccharide composed of β-(1→4)-linked D-glucosamine residues, to produce glucosamine oligosaccharides, called chitooligosaccharides (Wang et al., 2008; Xia et al., 2011).

Chitosan is known to exhibit a wide variety of physiological activities such as antibacterial (He et al., 2011), antioxidant (Rao et al., 2011) and antimicrobial activity (Xiao et al., 2011), however the high molecular weight and water insolubility of chitosan are disadvantageous for various applications, and currently chitosan has attracted interest when converted to chitooligosaccharides because they are not only water-soluble and of low molecular weight but they also have many remarkable biological activities, such as non-toxic and biocompatible anti-cancer activity, including anti-metastatic effects (Shen et al., 2009), anti-HIV (Artan et al., 2010), hepatoprotective (Senevirathne et al., 2011), antioxidant (Santana et al., 2014), hypocholesterolemic, antimicrobial, immunostimulating, antitumor, accelerating calcium and iron absorption and anti-inflammatory activities (Xia et al., 2011).

These chitooligosaccharides are traditionally processed by chemical reaction in the industry that have many problems such as difficulty in controlling the hydrolysis reaction, producing a large amount of short-chain and low income oligosaccharides, high cost of separation, and also environmental pollution, since it is an acidic hydrolysis. However, obtaining these chitooligosaccharides by hydrolysis with chitosanase has become an important alternative in recent years, with its advantages in terms of environmental compatibility, low cost and reproducibility (Xia et al., 2011; Artan et al., 2010). The enzyme purified by expanded bed adsorption in this study was the chitosanase produced by *Metarhizium anisopliae*, which is an entomopathogenic filamentous fungus used in biological control (Santi et al., 2010), and belongs to the order *Moniliales*, family *Moniliaceae* (Assis et al., 2010).

Enzymes play a central role in the biotechnology industry and for most applications they need to possess high degree of purity (Chase, 1994). Conventional purification techniques for bioproducts recovery, however, are typically carried out using packed bed chromatography, which is widely criticized because many liquid streams in downstream processing contain suspended solids, making them particularly difficult to process until removal of the

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particulates has taken place (Chang and Chase, 1996). Hence, the best option for successful initial recovery of a protein from raw materials containing large amounts of cells or cellular debris is the direct adsorption of protein (Sousa Junior et al., 2015).

Expanded bed adsorption chromatography (EBAC) or simply expanded bed adsorption (EBA) allows direct purification of the bioproducts from unclarified feedstocks where the additional solid–liquid separation and concentration of bioproducts are not necessary prior to the purification processes, and as a result of this fast operation biodegradation or proteolysis of the target products can be circumvented (Yap et al., 2010). Many enzymes have been purified with expanded bed adsorption, such as alkaline lipase (da Silva Padilha et al., 2009), penicillin G acylase (Pinotti et al., 2009), alcohol dehydrogenase (Hidayat et al., 2004) and xylanase (Silvino Dos Santos et al., 2002).

Thisresearchalso used a Doehlert matrix for planning the experiments, and Doehlert design is a planning method that allows multivariate optimization of variables (Ouejhani et al., 2008), defined as a second-order design (Ferreira et al., 2004). A second-order design, as Doehlert design, not only determine the influence of the independent variables to be optimized on the response, but also enable the response function to be obtained and optimized (Ferreira et al., 2002). Doehlert design is the most efficient of the three (Doehlert Design (DD); Central Composite Design (CCD) and Box-Benhken Design (BBD)) as shown in Table 1 (Ferreira et al., 2004).

In the specific case of chromatographic systems the various advantages of using the Doehlert design was reviewed (Ferreira et al., 2007), and in addition, several studies have demonstrated the usefulness of Doehlert design (Ferreira et al., 2002; Re et al., 2010; Hammami et al., 2009).

In this work enhancing of chitosanase purification using expanded bed adsorption through a Doehlert design has been investigated as an important step in developing technology for future large-scale production of chitooligosaccharides.

2. Materials and methods

2.1. Chemicals

Commercial chitosan (85% deacetylated – POLYMAR/CE- Brazil) was used for the culture medium and as substrate for enzyme activity. D-Glucosamine (Sigma, MO/USA) was used as standard for measurement of chitosanase activity. All other chemicals were of reagent grade.

2.2. Microorganism

The fungus *M. anisopliae*, strain (CG374) used in this study was kindly provided by EMBRAPA Genetic Resources and Biotechnology (Brasilia/DF-Brazil).

2.3. Chitosanase production

The inoculum of *M. anisopliae* for enzyme production was done with 3.0 O.D. at 600 nm standardized spore samples, using a spectrophotometer (Thermospectronic Genesys 10 UV). The spores were collected from Potato Dextrose Agar (PDA) plate with strain growth of 5 days and suspended in sterile water. For the production of enzyme was used the following medium (gL^{-1}): chitosan 2.0, K_2HP0_4 1.0, $MgS0_4$ 0.5, KCl 5.0, yeast extract 3.0, peptone 5.0, $NaNO_3$ 2.0; $FeS0_4$ 0.01, pH of 6.5 in several 250 mL Erlenmeyer flasks containing 180 mL of culture medium and 20 mL of inoculum. The Erlenmeyer flasks were incubated for 24 h in a rotation incubator at 27 °C and 147 rpm. A portion of the broth was then used directly in the experiments of expanded bed adsorption without clarification, and another one was centrifuged at 3500*g* for 5 min and the supernatant was used to the expanded bed adsorption experiments, with measurement of enzyme activity of each sample. All determinations of this research were done in triplicate.

Table 1
Efficiency comparative of second order experimental designs Central Composite design (CCD), Box–Behnken design (BBD) and Doehlert design (DD)*, showing that for the same amount of variables Doehlert design has a higher efficiency requiring fewer experiments than the other two.

Number of variables	Number of coefficients	Number of experiments (*f*)			Efficiency (*p*/*f*)		
(*k*)	(*p*)	CCD	DD	BBD	CCD	DD	BBD
2	6	9	7	–	0.667	0.857	–
3	10	15	13	13	0.667	0.769	0.769
4	15	25	21	25	0.600	0.714	0.600
5	21	43	31	41	0.488	0.677	0.512
6	28	77	43	61	0.364	0.651	0.459
7	36	143	57	85	0.252	0.632	0.424
8	45	273	73	113	0.165	0.616	0.398

* Adapted from Ferreira et al. (2004).

2.4. Chitosanolytic activity

The enzyme activity, following methodology adapted from literature (Assis et al., 2010; Miller, 1959) was evaluated by determining the reducing sugars, generated by hydrolysis of 500 mL of chitosan solution dissolved in 0.1 N Hydrochloric acid, with pH adjusted to 6.5 and mixed with 500 mL of fermented broth. The reaction was performed for 30 min at 55 °C and terminated with 2.5 mL of dinitrosalicylic acid (DNS) and the mixture boiled for 10 min. Then it was cooled in an ice bath to room temperature (25 °C) and reducing sugars were quantified using a spectrophotometer at 600 nm and a standard curve with D-glucosamine. One unit (U) of chitosanase is defined (Li et al., 2008), as the amount of enzyme that is capable of releasing 1 μmol of reduced sugar equivalent to glucosamine per minute under the assay conditions, i.e. 55 °C and pH 6.5.

2.5. Protein determination

The determination of total protein was performed in accordance to the Sedmak and Grossberg Modified Method (Silvino Dos Santos et al., 2002), measuring absorbance at 595 nm/465 nm with bovine serum albumin (BSA from Sigma Aldrich-Ohio-USA) as the standard protein.

2.6. Purification factor and yield

Purification factor (PF) is defined as the mass (specific activity) of chitosanase pooled in the elution step divided by the mass (specific activity) of chitosanase in the feedstock, as long as yield (%) is defined as the concentration (i.e. activity) of chitosanase in the elution step divided by the concentration (activity) of the chitosanase in the feedstock (Santana et al., 2014).

2.7. Column

A house-made column (2.6 cm in inner diameter, 30.0 cm in height) was used in this work. It was fitted with an adjustable piston in order to minimize headspace over the fluidized bed. A perforated distributor was put at the bottom of the column. It consisted of a stainless steel perforated plate with five holes of