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Single-step purification and characterization of an extreme halophilic, ethanol tolerant and acidophilic xylanase from *Aureobasidium pullulans* NRRL Y-2311-1 with application potential in the food industry

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ABSTRACT

An extracellular xylanase from *Aureobasidium pullulans* NRRL Y-2311-1 produced on wheat bran was purified by a single-step chromatographic procedure. The enzyme had a molecular weight of 21.6 kDa. The optimum pH and temperature for xylanase activity were 4.0 and 30–50°C, respectively. The enzyme was stable in the pH range of 3.0–8.0. The inactivation energy of the enzyme was calculated as 218 kJ mol^{-1}. The xylanase was ethanol tolerant and kept complete activity in the presence of 10% ethanol. Likewise, it retained almost complete activity at a concentration range of 0–20% NaCl. In general, the enzyme was resistant to several metal ions and reagents. Mg^{2+}, Zn^{2+}, Cu^{2+}, K^{1+}, EDTA and β-mercaptoethanol resulted in enhanced xylanase activity. The K_m and V_{max} values on beechwood xylan were determined to be 19.43 mg ml^{-1} and 848.4 U ml^{-1}, respectively. The enzyme exhibits excellent characteristics and could, therefore, be a promising candidate for application in food and bio-industries.

1. Introduction

Xylan is the principal structural polysaccharide in plant cell walls, accounting for approximately one-third of all renewable organic carbon on the earth (Collins, Gerday, & Feller, 2005). The xylan backbone basically consists of *β*-1,4-linked D-xylose units. It can include different substitutions, such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl and glucuronic acid residues. Complete breakdown of xylan requires the synergistic action of several hydrolases due to its highly complex structure. Enzymes included in the hydrolysis of xylan are: (i) endo-1,4-β-xylanase, (E.C.3.2.1.8), (ii) β-xylosidase (E.C.3.2.1.37), (iii) α-glucosiduronase (E.C.3.2.1.139), (iv) α-L-arabinofuranosidase (E.C.3.2.1.55), (v) acetyl xylan esterase (E.C.3.1.1.72), (vi) ferulic acid esterase (E.C.3.1.1.73), (vii) *p*-coumaric acid esterase (E.C.3.1.1.x) (Beg, Kapoor, Mahajan, & Hoondal, 2001). Among those enzymes, the most important hydrolase is endo-1,4-*β*-xylanase (EC 3.2.1.8) that degrades the xylan backbone randomly to liberate xylooligomers which in turn can be further hydrolysed to xylose by β-xylosidase (Motta, Andrade, & Santana, 2013).

In recent years, xylanases have received a great deal of attention due to their potential industrial applications. Xylanases find applications in a wide range of industrial processes. They are mainly used in food, feed, pulp and paper industries. Xylanases have applications in different sectors of the food industry. Utilization of xylanases in bread-making significantly improves the desirable texture, loaf volume and shelf life of the bread (Dutron et al., 2004). Xylanases together with pectinases, carboxymethylcellulases and amylases are used for clarification of juices (Motta et al., 2013). They are also used in the brewing industry in order to improve filtration efficiency (Wang et al., 2016). Xylooligosaccharides obtained by enzymatic hydrolysis of xylan are important ingredients for the development of novel functional foods due to their prebiotic effects. Xylanases in synergism with several other enzymes, such as mannanases, ligninases and glucanases, can be used for the generation of biological fuels from lignocellulosic biomass. Incorporation of xylanase as a supplement in feed formulation results in induced intestinal viscosity of animals, thereby improving both weight gain and feed conversion efficiency (Beg et al., 2001).

The extensive application of xylanase requires development of cost-effective strategies for its large scale recovery and purification. Utilization of crude enzyme preparations for some industrial processes might be beneficial, such as bioethanol production and waste treatment, where synergistic effects of hydrolases are usually desirable for complete hydrolysis of the biomass. However, a high degree of purity is a prerequisite for the food and pharmaceutical industry. The presence of interfering enzyme activities (e.g. proteases that may degrade the enzyme of interest) and undesirable

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secondary metabolites in crude enzyme preparations hinders their application in such industries where high purity is required.

The industrial production of xylanolytic enzymes is based on microbial biosynthesis. To date, several xylanase producing strains have been described. However, commercial xylanase production is mainly carried out by *Trichoderma* sp. and *Aspergillus* sp. (Kulkarni, Shendye, & Rao, 1999). It has been proven that certain strains of yeast like fungi *Aureobasidium pullulans* produce an extremely high level of cellulase free extracellular xylanase(s) (Li, Zhang, Dean, Eriksson, & Ljungdahl, 1993). Purification and partial characterization of some of the xylanases from different strains of *A. pullulans* have also been performed (Leathers, 1989; Li et al., 1993). Unlike previous studies, in the present study, a single-step chromatographic purification technique was successfully applied to capture the xylanase from the culture supernatant. A single-step purification strategy helps to decrease the downstream processing cost, thereby widening the industrial applications of the enzymes.

In our previous study, statistical optimization of xylanase production by *A. pullulans* NRRL Y-2311-1 utilizing wheat bran as the substrate in medium formulation was performed (Yegin, Buyukkileci, Sargin, & Goksungur, 2016). In the present study, a single step chromatographic procedure (ion exchange chromatography) was introduced for purification of *A. pullulans* NRRL Y-2311-1 xylanase produced under the optimum conditions. Biochemical and thermal characterization of the enzyme was performed. Kinetics of thermal inactivation and thermodynamic parameters were determined. Novel properties of the *A. pullulans* xylanase (e.g. extreme halophilicity and ethanol tolerance) were elucidated for the first time.

2. Material and methods

2.1. Materials

All of the microbial medium components were purchased from AppliChem GmbH (Darmstadt, Germany), except yeast nitrogen base which was obtained from Difco (BD Detroit, USA). Beechwood xylan was from BOC Science (Shirley, New York, USA) and protein marker (10–250 kDa) was from Bio-rad (Munich, Germany). All other chemicals used were of analytical grade and purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Wheat bran was obtained from local suppliers in Turkey.

2.2. Microorganism and propagation

A. pullulans NRRL Y-2311-1 was kindly supplied by the US Department of Agriculture, Agricultural Research Service. The strain was propagated on yeast-mould media (YM) containing (g/l): yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0 and agar 20.0. The YM plates were incubated at 24 °C for 3 days and sub-cultured every 3 weeks.

Pre-inocula were obtained from cultures grown on YM agar plates at 24 °C for 3 days. Two loops of *A. pullulans* cells were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of growth medium (pH 5.0) with the following composition (g/l): xylose 10.0, yeast nitrogen base, 6.7; asparagine 2.0, KH_2PO_4 5.0. The medium was sterilized at 121 °C for 15 min before inoculation. The pre-inocula were incubated at 28 °C and 150 rpm for 1 day on an orbital shaker.

2.3. Enzyme production in bioreactor system

Enzyme production was carried out in a Bio-Flo 110 Modular Benchtop Fermentor (New Brunswick Scientific Corporation, NJ, USA) with a 3.0 L vessel (1.5 L working volume). The system consisted of a glass vessel with 4 equally spaced vertical baffles. Agitation was carried out by dual Rushton-style impellers. The fermentation medium for enzyme production composed of (g/l): wheat bran 10.0, yeast extract 1.0, $(NH_4)_2SO_4$ 2.5, KH_2PO_4 5.0. The pH of the medium was adjusted to 4.24 with HCl and NaOH. The production medium and the reaction vessel were sterilized at 121 °C for 20 min. The bioprocess parameters were as follows: temperature, 30 °C; agitation, 200 rpm; aeration, 1.5 vvm and incubation time, 126 h. The production medium (1.5 L) was inoculated with 2% of the inoculum. At the end of the fermentation, the biomass and insoluble substrate (wheat bran) were removed by centrifugation (21500*g*) at 4 °C for 15 min and the supernatant was referred to the crude enzyme.

2.4. Enzyme assay

The xylanase activity test was performed according to the method of Bailey, Bieley, and Poutanen (1992) with slight modifications. Beechwood xylan was used as the substrate. In test tubes, 0.9 ml of substrate solution [0.5% (w/v) beechwood xylan prepared in 0.05 M citrate buffer, pH 5.2] and 0.1 ml of suitably diluted enzyme preparation was mixed thoroughly. The reaction mixture was incubated at 50 °C for 5 min. After incubation, the reaction was terminated by the addition of 1.5 ml 3,5-dinitro salicylic acid reagent and the content was boiled for 5 min followed by cooling in ice-cold water for 1 min. A control for each sample was run simultaneously that contained all the reagents where the reaction was terminated prior to the addition of the enzyme. The amount of reducing sugars released was estimated by measuring the absorbance at 540 nm. Xylose was used as the standard. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of xylose equivalent per min under the assay conditions. The xylanase activity was calculated using the following equation:

$$\text{Activity (U/ml)} = (X/150.13) \times (1/5) \times (1/0.1) \times D \quad (1)$$

where D is dilution factor and X is the amount of xylose in μg.

2.5. Protein determination

Total protein concentration was determined according to the modified Bradford method, using a Coomassie Plus™ Protein Assay Kit (Pierce, Fischer Scientific, Schwerte, Germany). Bovine serum albumin was utilized as the standard.

2.6. Enzyme purification

Twenty milliliters of crude enzyme preparation was freeze-dried and dissolved in 10 ml citrate buffer (20 mM, pH 5.0). The concentrated sample was subsequently desalted by manual PD-10 column (GE Healthcare, Munich, Germany). After that, 2 ml of the enzyme sample was subjected to ion exchange chromatography, which was performed on a SP Sepharose Fast Flow column (0.7 cm × 2.5 cm) with ÄKTA explorer system (GE Healthcare, Munich, Germany). The mobile phase consisted of Buffer A (20 mM citrate buffer, pH 5.0) and Buffer B (Buffer A containing 1 M NaCl). The column was equilibrated with 20 column volumes of Buffer A. The gradient was developed in 20 column volumes. The flow rate was 1 ml min^{-1}. The chromatographic fractions (1 ml) were analyzed for xylanase activity and the active enzyme fractions were pooled and utilized for further characterization studies.

2.7. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). 12.5%