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Recombinant *Bacillus amyloliquefaciens* xylanase A expressed in *Pichia pastoris* and generation of xylooligosaccharides from xylans and wheat bran

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ABSTRACT

In this study, *BaxA* (GenBank: KM624029), which encodes the *Bacillus amyloliquefaciens* xylanase A (BaxA), was highly expressed in *Pichia pastoris* GS115 under the control of the AOX1 promoter. The recombinant xylanase, namely rePBaxA, was purified to homogeneity by using Ni-affinity resin and its molecular weight was 35.0 kDa. The optimum temperature and pH of rePBaxA were 50 °C and 5.0, respectively. The kinetic parameters Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) of rePBaxA were 5.41 mg/mL and 22.42 μmol/min/mL, respectively. High–performance liquid chromatography results showed that after 6 h of hydrolysis, rePBaxA released xylose–xylohexaose (X1–X6) mixture from beechwood and birchwood xylan, with xylobiose (X2) and xylotriose (X3) as the major products, respectively. The hydrolyates from oat spelt, wheat bran insoluble xylan and pretreated wheat bran by rePBaxA included X2–X6, with X6 having the highest concentration. The mode of action analysis revealed that rePBaxA was an *endo*-acting xylanase with transglycosylation activity. X2 might be the minimum oligomer hydrolyzed by rePBaxA. The pretreated wheat bran and wheat bran insoluble xylan could be directly hydrolyzed by rePBaxA. This study provided a basis for using agricultural waste by-products as substrates for manufacting value-added probiotics, namely, xylooligosaccharides.

1. Introduction

Xylan is a major structural component of plant cells and the most abundant natural hemicellulosic polymer. Endo-β-(1, 4)-xylanases (EC. 3.2.1.8, referred to as xylanase) hydrolyze linear polyxylose chains and are important enzymes involved in xylan degradation [1]. According to the sequence similarities and results of hydrophobic cluster analysis, xylanases are classified into families 10 (or F) and 11 (or G) of glycosyl hydrolases [2,3]. Xylanases are widely applied alone or in combination with other enzymes [4]. Xylanases are commercial applied in chlorine-free bleaching of wood pulp, juice clarification, brewer's spent grain saccharification, and production of xylooligosaccharides (XOs); these enzymes are also used as additives to animal feed and ingredient to improve the quality of baked products [5–11]. XOs have been proven to have important prebiotic properties and it has been added to infant milk powder, box-packed UHT pure milk, upmarket drinks, and et al. The fast-growing market for XOs speeds up processing xylan-rich materials into high-value products.

The family 11 xylanases have smaller molecular weight comparing with family 10 members, and most of them do not possess xylosidase and cellulase activity. So, the family 11 xylanases exhibit more advantage on diffusing in plant cell-wall structural polysaccharides for effective hydrolysis [2,3]. Prokaryotic bacteria belonging to *Bacillus* are used worldwide to produce hemicellulase, cellulase, proteinase, and other enzymes [12]. *Bacillus amyloliquefaciens* xylanase A (BaxA) belongs to family 11 xylanases and exhibits high homology with other enzymes in the family 11. The crystal structure of Baxa consists of a single domain of 16 β-strands and one α-helix [2]. BaxA, which has the potential in biotechnological application, is difficult to purify from source organisms and exerts low enzyme activity. To produce high amounts of xylanases and with the advent of genetic engineering and purification, hundreds

Abbreviations: BaxA, *Bacillus amyloliquefaciens* xylanase A; *baxA*, gene encoding BaxA; X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; HPLC, high–performance liquid chromatography; XOs, xylooligosaccharides; K_m, Michaelis–Menten constants; V_{max}, maximal activity.

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of xylanase genes have been cloned into homologous and heterologous hosts for expression [13]. In our previous study, the *baxA* gene (GenBank: KM624029), encoding BaxA, was cloned and expressed in *Escherichia coli* under the control of different promoters (*csp*A *lac* promoter and T7 *lac* promoter) [14]. The recombinant xylanase (reBaxA1) produced in *E. coli* BL21 with pET 30 (a)+ vector was inclusion body and did not show any xylanase activity. The recombinant xylanase (reBaxA2) expressed in *E. coli* BL21 with pCold TF vector (*csp*A *lac* promoter) was secreted into the culture medium and in the cytoplasm. However, the activity of reBaxA2 (2.63 U/mg) was low for industrial purposes.

In the last 30 years, the methylotrophic yeast *Pichia pastoris* has been used and proved to be an efficient host for expression of many recombinant proteins. *P. pastoris*, which shows clear genetic background and has inherent capability of introducing eukaryotic posttranslational modification, can be easily grown into high cell density in minimal media and secrete large-scale recombinant protein at low cost [15,16]. Many xylanase genes were cloned and successfully expressed in the yeast [17,18].

In this study, the *baxA* gene was successfully expressed in *P. pastoris* at high levels under the control of the AOX1 promoter. The recombinant xylanase, namely, rePBaxA, was purified and characterized in detail. Xylooligosaccharides released from different xylans and pretreated wheat bran was determined. Furthermore, the mode of action of rePBaxA was also investigated.

2. Materials and methods

2.1. Materials

The *B. amyloliquefaciens* strain producing xylanases was screened and preserved by the Central Laboratory of Food Science Department, China JiLiang University. The pPICZαA vector, *P. pastoris* strain GS115, and Zeocin were from Invitrogen. Medium components were from Oxoid. The PCR kit, ligase, and restriction endonucleases were acquired from Takara Biotechnology Co., Ltd. Primers were synthesized by Sangni Biotechnology Co., Ltd. The protein molecular weight marker and antibodies used in Western blot analysis were supplied by Songon. High affinity Ni-charged resin was provided by GenScript. Beechwood, birchwood, and oat spelt xylan were purchased from Sigma–Aldrich. Xylose (X1) was obtained from Merck. Standard XOs (xylobiose-xylohexaose) were acquired from Megazyme. All other chemicals were of analytical grade.

2.2. Construction of expression plasmid and expression of baxA in P. pastoris GS115

The *baxA* gene was amplified from the recombinant pMD18T-*baxA* plasmid using specific primers of P1 (5′- CGGAATCCATGTTTAAGTTTAAAAAG-3′) and P2 (5′-CGGCGGCCGCCCACACTGTTACGTTTGAAC-3′) with the *Eco*R I and *Not* I recognition sites (underlined), respectively. The PCR parameters were as follows: denaturation at 94 °C for 2 min and 30 cycles of (1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C), followed by 10 min at 72 °C. The PCR product was separated via electrophoresis in 1.0% (w/v) agarose gel and then recovered. The recovered *baxA* was doubly digested by *Eco*R I and *Not* I and ligated into the pPICZαA vector. The recombinant pPICZαA-*baxA* plasmid was transformed into *E. coli* DH5α, and the transformants were screened with a low-salt LB agar plate containing 25 μg/mL zeocin [19]. Large amounts of pPICZαA-*baxA* plasmids were extracted using GenElute™ Plasmid Maxiprep Kit.

The pPICZαA-*baxA* plasmids were linearized with *Sac* I and transformed into competent *P. pastoris* strain GS115 with an electroporator (Bio-Rad Gene Pulser Xcell™) according to the manufacturer's protocol (operation voltage of 2000 V and operation time of 5 ms). The transformant products were spread on YPDS plates containing zeocin (100 μg/mL). After incubation at 30 °C for 60 h, colonies formed on the plates. A total of 165 colonies were tested for small-scale expression of recombinant xylanase in 5 mL of YPM medium (1% yeast extract, 2% peptone, 0.5% methanol). The positive clone (PPbaxA10, Mut$^+$) with the highest-level expression was used for large-scale expression and further studies.

2.3. Large-scale expression and purification of rePBaxA

The highest-expressing PPBaxA10 was grown in 500 mL of YPDS medium with constant shaking (250 rpm) at 30 °C for 24 h. Yeast cells were harvested and re-suspended in 500 mL BMMY medium in a 2000 mL baffled shake flask, and incubated at 30 °C for 96 h. For the induction of rePBaxA expression, methanol was added to the culture medium every 24 h, and the final concentration of the inducer was 0.5%.

The recombinant protein was purified by the following procedure: the culture supernatant of PPBaxA10 was concentrated by vacuum freeze-drying. The concentrated protein was mixed with high-affinity Ni^{2+}-charged resin and incubated in an ice bath with gentle inversion for 2 h. The slurry was transferred into the column (100 mm length and 10 mm diameter, GenScript). The mixture was washed with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, 8 bed volumes), and then the recombinant protein with 6*His tag was eluted from the resin with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0, 10 bed volumes). The elution was collected tube by tube (1.0 mL per tube) for subsequent analysis [14].

2.4. Activity, SDS-PAGE, and western blot assay of rePBaxA

Xylanase activity was assayed using 3, 5-dinitrosalicylic acid (DNS) with 0.5% (w/v) beechwood xylan as substrate [20]. Protein concentration was measured using Bradford method with bovine serum albumin as standard [21]. Activity unit was defined as the amount of xylanase that released 1.0 μmol of reducing sugar (D-xylose) from beechwood xylan within 1 min under the optimal conditions. The Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) of rePBaxA were determined from Linweaver–Burk plots of the initial velocities by using beechwood xylan as substrates (1, 3, 5, 7.5, and 10 mg/mL). For each assay, triplicate experiments were conducted and the mean value was obtained.

The samples (culture supernatants obtained from positive/negative clones induced by methanol for 96 h and purified rePBaxA) were subjected to SDS-PAGE and Western blot analysis. In the Laemmli system, the stacking and separating gels consisted of 5% and 15% polyacrylamide, respectively [22]. Proteins in the gel were visualized via Coomassie brilliant blue R-250 straining. The pPICZαA vector contained the sequences encoding polyhistidine (6*His) tag in the C-terminus. Thus, the anti-His monoclonal antibody and horseradish peroxidase-labeled goat anti-mouse IgG antibody were used in the Western blot assay of the recombinant fusion protein [14].

2.5. Optimum temperature and thermostability of rePBaxA

Enzyme from vacuum freeze-drying was dissolved in pure water. The effect of temperature on xylanase activity was determined from 30 °C to 90 °C. For thermal stability assay, the enzyme was treated with heat from 30 °C to 90 °C for 15 min and then placed

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