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Short Communication

High-level production of thermotolerant β-xylosidase of *Aspergillus* sp. BCC125 in *Pichia pastoris*: Characterization and its application in ethanol production

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HIGHLIGHTS

- ▶ Thermotolerant β-xylosidase from fungal origin was produced to highest yield.
- ▶ Enzyme efficiency (K_{cat}/K_m) toward *p*-nitrophenyl-β-D-xylopyranoside was superior.
- ▶ Simple enzyme production with high yield from *Pichia pastoris* was described.
- ▶ Synergism of xylanolytic activity contributed by β-xylosidase was demonstrated.
- ▶ Application in enzymatic conversion of xylan to ethanol was demonstrated.

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ABSTRACT

A gene coding for thermotolerant β-xylosidase from *Aspergillus* sp. BCC125 was characterized. The recombinant enzyme was expressed in methylotrophic yeast *Pichia pastoris* KM71 and especially high yield of secreted enzyme was obtained. β-xylosidase possessed high enzyme efficiency (K_{cat}/K_m = 198.8 mM^{-1} s^{-1}) toward *p*NP-β-D-xylopyranoside (*p*NPβX) with optimal temperature and pH for activity of 60 °C and pH 4.0–5.0, respectively. The identified β-xylosidase showed clear synergism with previously identified xylanase for hydrolysis of xylan *in vitro* as well as simultaneous saccharification and fermentation process (SSF) *in vivo* with *Pichia stipitis*.

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1. Introduction

The conversion of plant biomass, which comprises mostly of cellulose and hemicellulose, into its sugar components by enzymatic treatment is considered a sustainable technology with little effects on the environment. Hemicellulose contains β-1,4 xylan as a major component, with its main chain made of xylose monomer. The hydrolysis of xylan requires the cooperative action of xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). Xylanase catalyzes the endohydrolysis of the 1,4- β-D-xylosidic linkage randomly and gives rise to xylooligosaccharide and xylose monomer, while β-xylosidase completes the hydrolysis by cleaving off xylose monomer from the non-reducing end of xylooligosaccharide (Knob et al., 2010). Previously, the production of recombinant xylanase (*XylB*) by heterologous expression in the methylotrophic yeast *Pichia pastoris* was reported (Ruanglek et al., 2007). To obtain xylan-degrading enzymes available for studying lignocellulosic biomass degradation, β-xylosidase was sought from fungal collections in Thailand's BIOTEC Culture Collection (BCC), and the *Aspergillus* sp. BCC125 strain was identified as a strong producer of β-xylosidase. In this study, the recombinant β-xylosidase from *Aspergillus* sp. BCC125 was produced to very high yield using *P. pastoris* expression system and enzyme kinetic properties were characterized. The synergistic action of recombinant β-xylosidase and xylanase for xylan hydrolysis was demonstrated, as well as the synergism of both enzymes in ethanol fermentation in the presence of xylose fermenting yeast, *P. stipitis.*

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2. Methods

2.1. Fungal genomic DNA isolation and PCR cloning of β-xylosidase gene

Aspergillus sp. BCC125 was cultured in malt extract broth (MEB) at 25 °C for 3 days and the genomic DNA was extracted using a genomic DNA extraction kit (Promega). Primers were designed based on the nucleotide sequence of the *xlnD* gene coding for β-xylosidase from the *Aspergillus niger* (Genbank accession No. Z84377). The full-length *Aspergillus* sp. BCC125 β-xylosidase gene sequence has been submitted to the GenBank database under accession No. JX535027. Restriction sites for *Eco*RI and *Xba*I were added to the 5′ and 3′ primers (Forward primer -AGC*GAATTC***ATG**GCGCACTCAATGTCTCG and Reverse primer - ATC*TCTAGA*CTACTCCTTCCCCGGCCAC), respectively, to facilitate β-xylosidase gene cloning into yeast expression vector pPICZαA (Invitrogen). A putative signal peptide was predicted at the 5′end region (1–78 nt) by the SignalP 4.0 program (Petersen et al., 2011). Subsequently, the gene sequence encoding mature β-xylosidase, lacking its native signal peptide (79–2,415 nt), was ligated to yeast expression vector pPICZαA to create pPICZαA-β-xylosidase-MA (pXyloMA). The *P. pastoris* KM71 was transformed with pXyloMA plasmid and correct transformants identified by colony PCR.

2.2. β-xylosidase expression in P. pastoris KM71, activity assays and kinetic parameters determination

The *P. pastoris* KM71 transformants were cultured in buffered glycerol complex media (BMGY, Invitrogen) and induced for β-xylosidase expression in buffered methanol complex media (BMMY, Invitrogen) according to standard protocol (Invitrogen). β-xylosidase was collected on day-3 of induction. The secreted enzyme was used directly in the assays of β-xylosidase activity toward synthetic substrate, *p*NPβX (Sigma). The substrate-buffer mixture containing 200 μl of 5 mM *p*NPβX in 0.1 M HCl-acetate buffer pH 5 and 100 μl of appropriately diluted enzyme was incubated at 60 °C for 20 min and stopped by adding 1 ml of 1 M Na_2CO_3. The amount of released *p*-nitrophenol in the reaction was measured at OD_{405} nm using a microplate reader (PerkinElmer VICTOR). One unit of β-xylosidase was defined as the amount of enzyme that catalyzes the release of 1 μmol *p*-nitrophenol per min under the defined conditions. Michaelis constants (K_m) and maximum velocities (V_{max}) of β-xylosidase were determined from the plot of initial hydrolysis rate (V_i) as a function of substrate concentration (*p*NPβX) ranging from 0.01–15 mM. The values were estimated by fitting the data to the Michalis-Menten equation in the KaleidaGraph program (Synergy Software).

2.3. Effect of pH and temperature on β-xylosidase activity

The temperature optimum of β-xylosidase activity was investigated in the 30–80 °C range under reaction conditions as described above. The thermal stability test was carried out by pre-incubating enzyme at three selected temperatures (50, 60 and 70 °C) for 1, 2 and 3 h and the remaining enzyme activity after exposure was measured. The pH optimum was tested in various buffers with pH ranging from 2–11 (HCl-Acetate pH 2–5, Acetate pH 5–6, Phosphate pH 6–8, Tris–HCl pH 8–9 and Glycine-NaOH pH 9–11) at 60 °C. The pH stability test was performed by pre-incubating enzyme at 25 °C for 3 h using the same buffer set (pH 2–11) as described above. The remaining enzyme activity was then determined.

2.4. Substrate specificity of β-xylosidase

The activity towards selected *p*-nitrophenyl glycosides (Table 1.2), oligosaccharides (Cellobiose, Xylobiose and Xylotriose), and polysaccharide substrates (Birchwood and Oat spelt xylans) were investigated. The hydrolytic activity toward these selected substrates was evaluated using the method as described by Miller (Miller, 1959).

2.5. Synergism of xylanolytic activities of P. pastoris-expressed xylanase and β-xylosidase toward Birch wood xylan

The hydrolysis of Birch wood xylan (3 mg) was carried out at 50 °C for 15 min by single-enzyme treatment [containing 0.066 U xylanase (Ruanglek et al., 2007) or 0.3 U β-xylosidase] or a mixture of both enzymes (0.066 U xylanase plus 0.3 U β-xylosidase) in a 1 ml reaction containing 0.1 M Sodium acetate (pH 5.0). The production of reducing sugar was measured using the method described by Miller (1959).

2.6. Simultaneous saccharification and fermentation (SSF) by xylose fermenting yeast, P. stipitis BCC15191

Simultaneous saccharification and fermentation was carried out as described by Buaban et al., 2010. The carbon source (20 g L^{-1}) was derived from glucose, xylose or xylan. To initiate saccharification, xylanase (20 U g^{-1} substrate) and β-xylosidase (50 U g^{-1} substrate) were pre-incubated with the xylan substrate for 1 h at 50 °C. After 1-h saccharification, 0.5 ml of yeast inoculums (4.35×10^8 CFU ml^{-1}) in YPD was added into the saccharifying mixture. Fermentation was carried out at 30 °C for 72 h and the supernatant was collected to determine the amount of ethanol production by HPLC analysis (Buaban et al., 2010).

3. Results and discussion

3.1. Cloning and expression of the β-xylosidase from Aspergillus sp. BCC125 in P. pastoris

The β-xylosidase gene of *Aspergillus* sp. BCC125 containing an uninterrupted open reading frame of 2,415 nucleotides encoding a precursor protein of 804 amino acids in length was characterized. The nucleotide sequence showed 96% similarity to β-xylosidase of *A. awamori* strain X-100 (accession No. EU854433) and *A. niger* (accession No. AF108944) and 98% similarity in amino acid sequence to both strains (data not shown). Due to homology of amino acid sequence, the recombinant enzyme was classified to glycoside hydrolase family 3 (GH3). The mature gene of the identified β-xylosidase was then cloned into *P. pastoris* KM71. The selected transformant was shown to express β-xylosidase to a very high yield as compared to others with a specific activity of 156 unit mg^{-1} protein (Table 1.1). SDS–PAGE analysis of culture supernatant revealed a single protein band with an apparent molecular weight of approximately 130 kDa (Fig. 1). While the mature protein is predicted to be 85.6 kDa, the larger apparent (∼130 kDa) molecular weight is likely due to posttranslational glycosylation as generally found in most fungal β-xylosidase (Knob et al., 2010). For this recombinant β-xylosidase, there are 10 potential N-glycosylation sites predicted within the conserved Asn-Xaa-Ser/Thr sequence by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) (data not shown).

3.2. Enzyme kinetics and effect of pH and temperature on β-xylosidase activity

The secreted enzyme exhibited apparent K_m and V_{max} values of 1.7 mM and 211.5 μmol min^{-1} mg^{-1} protein, respectively. Its K_m was comparable to others, while enzyme recovery yield was very high due to no purification step involved (Table 1.1). In addition,

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