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OVEREXPRESSION, PURIFICATION AND CHARACTERIZATION OF *Aspergillus niger* BETA-GLUCOSIDASE IN *Pichia pastoris*

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ABSTRACT

This study describes the expression of β-glucosidase (BglA) from *Aspergillus niger* in *Pichia pastoris*, a methylotrophic yeast strain, under the regulation of an alcohol oxidase promoter. The heterologous expression of BglA was optimized in a shake flask. Optimal conditions were achieved using an initial cell density (OD_{600}) of 4-5 and an inducer concentration of 2.5% methanol for 72 hours. A recombinant protein with a molecular weight of ~116 kDa was produced. This recombinant BglA has optimal activity at 60°C in sodium acetate buffer at pH 4. This enzyme is stable between pH 3.0-6.0 and retained more than 50% of its maximum activity at pH 6.0 after incubation at 60°C for 30 min. However, it lost almost 80% of its maximal activity at pH 7.0 under the same conditions. A thermostability assay of this enzyme revealed that BglA is relatively stable up to 60°C. This enzyme retained 50% of its original activity at 60°C but was completely inactive after incubation at 70°C for 30 min. BglA showed highest activity and specificity towards the synthetic substrate *p*-nitrophenol-β-D-glucopyranoside with a specific activity of 347.62 U mg^{-1} and a specificity constant of 466.19 mL $mg^{-1}s^{-1}$. BglA had a specific activity of 6.2 U mg^{-1} and a specificity constant of 6.01 mL $mg^{-1}s^{-1}$ for cellobiose.

Key words: *Aspergillus niger*, β-glucosidase, *Pichia pastoris*, heterologous expression

INTRODUCTION

Lignocellulosic materials have long been recognized as a potential renewable source of mixed sugars for bioconversion into fuels and other chemicals. In response, extensive research and development efforts over the past few decades have focused on converting cellulosic biomass, a sustainable energy source, into bio-ethanol, a petroleum substitute. Several fungal genuses, such as *Aspergillus*, *Trichoderma* and *Phanerochaete*, have been extensively studied for their ability to break down this complex substrate by utilizing cellulase enzymes (Himmel *et al.*, 2007). Three types of cellulase enzymes, endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21), are needed to effectively decompose crystalline cellulose to glucose (Dashtban *et al.*, 2010).

β-glucosidase is an important enzyme in the cellulase complex. The enzyme not only plays a role in decomposing cellobiose or cello-oligosaccharides but also in helping the overall process of cellulose hydrolysis because β-glucosidase is responsible for removing cellobiose, the main cellulase inhibitor, by converting it to glucose (Sun and Cheng, 2002). Thus, the use of β-glucosidase in the cellulase enzyme complex for cellulose degradation is one way to reduce the accumulation of cellobiose and increase the effectiveness of the hydrolytic process (Zhao *et al.*, 2013). It is important to characterize different β-glucosidases, as they may demonstrate unique properties and may be suitable for specific industrial applications. Understanding the properties of different β-glucosidases is also important when designing a pool of enzymes tailored for the hydrolysis of a specific substrate (Farinas *et al.*, 2010). In this work, we produced a recombinant *A. niger* β-glucosidase in a methylotrophic yeast strain, *P. pastoris*, and characterized the

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biochemical properties of the enzyme. The long-term objective is to utilize the recombinant enzyme to hydrolyze locally produced lignocellulosic biomass.

MATERIALS AND METHODS

Heterologous protein expression

The *bglA* gene from *A. niger* encoding for β-glucosidase has previously been cloned into the pPICZαC vector and transformed into *P. pastoris* strain X33 (Kamaruddin *et al.*, 2008). The expression of the recombinant protein in *P. pastoris* was carried out as described by Al-Rashed *et al.* (2010) with some modifications. Transformants harboring multiple copies of the expression cassette were used to inoculate 100 mL of BMGY medium [(1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6, 1.34% (w/v) yeast nitrogen base without amino acids, 4×10^{-5}% (w/v) biotin and 1% (v/v) glycerol)] in a 1-L Erlenmeyer flask. The cells were cultivated with rotary shaking at 30°C and 250 rpm until the OD_{600} reached 4-5. All cells were harvested by centrifugation at 3000 × *g* for 5 min at room temperature. The cell pellets were suspended in 20 mL of buffered methanol complex medium [BMMY medium : (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6, 1.34% (w/v) yeast nitrogen base without amino acids, 4×10^{-5} % (w/v) biotin and 0.5% (v/v) methanol)] and cultivated for 3 days. Absolute methanol was added to a final concentration of 2.5% every 24 hr to maintain induction. The culture supernatants were collected and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, USA). Secreted protein was analyzed using both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a plate assay that relied on MuGlc (Sigma, USA), a UV-stimulated fluorescent substrate.

Purification of recombinant β-glucosidase

Crude extracts of recombinant BglA were purified using ion exchange chromatography and gel filtration. Crude extracts of BglA were loaded onto a HiTrap Q HP column (GE Healthcare, Sweden) pre-equilibrated with 50 mM phosphate buffer (pH 5.7). The elution was performed with a gradient of 0-0.5 M NaCl in 50 mM phosphate buffer (pH 5.7). Fractions containing β-glucosidase activity were pooled and concentrated through Amicon Ultra Centrifugal Filter Devices with a molecular weight cutoff of 50,000 (Millipore, USA). The fractions were further chromatographed on a Superdex 200 10/300 GL (GE Healthcare, Sweden) column using 50 mM phosphate buffer (pH 6.0) as the elution buffer. Detection of β-glucosidase activity was carried out using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate for the enzymatic reaction. Active fractions were pooled and concentrated by ultrafiltration. The purity of the enzyme was monitored by SDS-PAGE.

Biochemical characterization of BglA

The amount of recombinant BglA produced was measured using the Bradford method (Bradford, 1976). Enzymes were assayed using pNPG and cellobiose (Sigma, USA) as substrates. The pNPG assay was carried out by adding approximately 10 µL of purified BglA to 90 µL of reaction mixture containing 5 mM pNPG in 50 mM sodium acetate buffer (pH 4.0). The reaction mixture was incubated at 60°C for 30 min and terminated by the addition of 100 µL of 1 M sodium carbonate. The release of *p*-nitrophenol was monitored at A_{405}. One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1.0 µmol of *p*-nitrophenol per minute under standard assay conditions. The cellobiose assay was carried out by adding approximately 10 µL of purified BglA to 900 µL reaction mixture containing 5 mM cellobiose in 50 mM sodium acetate buffer (pH 4.0). The reaction mixture was incubated at 60°C for 30 min. The amount of glucose released was measured with a Glucose (GOD-PAP) Kit (Roche Diagnostic, Switzerland) as described in the manufacturer's instructions. One unit of enzyme activity was defined as the amount of enzyme that releases 1.0 µmol of glucose per minute under standard assay conditions.

The optimum BglA temperature was measured by running the pNPG assay at 30, 40, 50, 60 and 70°C. Temperature stability was assessed by incubating the enzyme at 20, 30, 40, 50, 60 and 70°C for 30 min, and residual activity was determined using standard assay conditions. The pH optimum was determined by running the standard assay at different pH values (i.e., pH 3-8), while pH stability was assessed by incubating the enzymes over a pH range from 3 to 8 at 60°C for 30 min. Residual activity was determined using standard assay conditions. The buffers used were sodium acetate (pH 3-5) and potassium phosphate (pH 6-8). The Michaelis-Menten constant (K_m) and maximum velocity of substrate hydrolysis (V_{max}) were determined from a Lineweaver-Burk plot with various substrates over a range of concentrations at 60°C for 30 min. The substrates used were cellobiose and pNPG (Sigma, USA). Enzyme activity was assessed using the standard method. The catalytic constant (K_{cat}) and the specificity constant (K_{cat}/K_m) were calculated. All experiments were carried out with three technical replicates and two biological replicates.

RESULTS AND DISCUSSION

The expression system in *P. pastoris* was regulated by a methanol-inducible promoter (*AOX1* promoter). BglA was successfully expressed when the media was supplemented with 2.5% (v/v) methanol. SDS-PAGE analysis revealed the appearance of recombinant protein with a molecular weight of ~116 kDa (Fig. 1A). This value is slightly higher than the predicted molecular weight for BglA. The higher apparent mass can potentially be attributed to glycosylation resulting from expression in the *P. pastoris* system. BglA was expressed in *P. pastoris* at a final titer of 87 mg L^{-1}. These data are consistent with the *P. pastoris* expression levels of *A. niger* endoglucanase (40 mg L^{-1}) (Quay *et al.*, 2011) and *Volvariella volvacea* endoglucanase (65-100 mg L^{-1}) (Ding *et al.*, 2002). Subsequently, recombinant BglA was successfully purified using two-step purifications. The Coomassie-stained SDS-PAGE indicated that purified BglA formed a single band with a molecular weight of ~116 kDa (Fig. 1B).

Purified BglA was characterized by several methods. BglA exhibited maximal activity on pNPG at pH 4.0 in sodium acetate buffer (Fig. 2). Activity declined rapidly in alkaline pH; BglA retained only 40% of its activity at pH 7.0. A pH stability study revealed that BglA was stable in acidic pH and retained more than 75% of its maximum activity at pH 6.0 after incubation at 50°C for 30 min. The stability decreased in the alkaline pH range. These data indicate that BglA works better in acid, which is consistent with most fungal cellulases (Table 1). However, this property often limits their application under neutral or alkaline conditions. BglA has optimal activity at 60°C in sodium acetate buffer pH 4. The optimal activity for BglA is consistent with β-glucosidases from other organisms in genus *Aspergillus* as described in Table 1. For the stability profile at different temperatures, BglA is stable under a short temperature range between 20°C-50°C with 80% residual activity. There was no detectable activity when incubated at 70°C.

Table 1 compares the biochemical characteristics of recombinant BglA from *A. niger* with several recombinant β-glucosidases from other fungi. Although all β-glucosidases were derived from different fungal sources, they bear similar biochemical characteristics. All β-glucosidases harbor the general characteristics of fungal cellulases and are active at acidic pHs (4-6). *A. niger* β-glucosidase has the highest specific activity against a synthetic substrate (i.e., pNPG) out of all tested enzymes. In addition, β-glucosidases from the genus *Aspergillus* have the same characteristics with molecular sizes exceeding 100 kDa and optimum temperatures of 60°C.

The specificity of the enzyme toward certain substrates can be determined from the K_{cat}/K_m value. The K_{cat}/K_m (catalytic efficiency), often referred to as a specificity constant, is a useful index for comparing the relative rates of an enzyme acting on alternative, competing substrates (Pham *et al.*, 2011). A higher specificity constant denotes that the enzyme more strongly prefers that substrate. In this study, the specificity constant of BglA is higher for the synthetic substrate (i.e., pNPG) than for the natural substrate (i.e., cellobiose). Specificity constants for pNGP and cellobiose are 466.19 mL $mg^{-1}s^{-1}$ and 6.01 mL $mg^{-1}s^{-1}$, respectively. Thus,

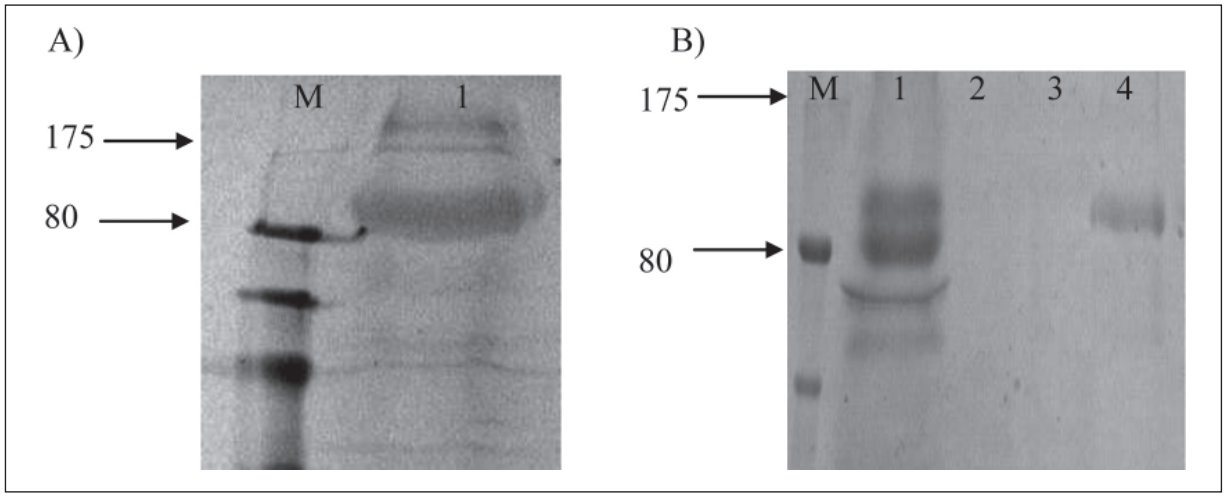

Fig. 1. A) SDS-PAGE of partially purified recombinant BglA with a molecular weight of ~116 kDa on a 12% polyacrylamide gel. M is Protein Marker Broad Range (NEB, UK). Lane 1: Recombinant BglA. B) SDS-PAGE of purified recombinant BglA on 12% polyacrylamide gel with molecular weight of ~116 kDa. M is Protein Marker Broad Range (NEB, UK). Lane 1: Crude extract of recombinant BglA, Lane 2- 4: Purified BglA enzymes from various collected fractions.

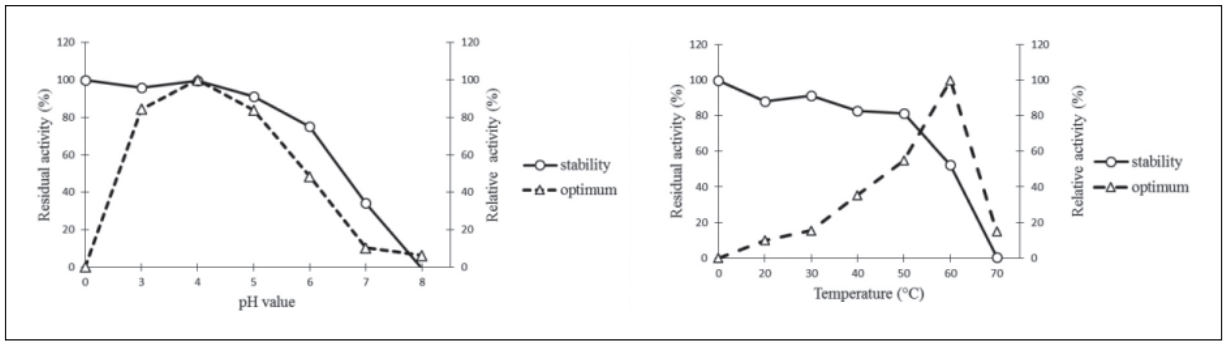

Fig. 2. A) Characterization of optimum pH and stability at various pH values for maximal activity of BglA. B) Characterization of optimum temperature and thermostability for maximal activity of BglA.

Table 1. Comparison of biochemical properties of recombinant BglA with other recombinant *Aspergillus* β-glucosidases produced in various hosts

β-glucosidase	Expression host	Optimum pH	Optimum temperature (°C)	Specific activity (U mg^{-1})	References
BglA *A. niger* strain ATCC 10574	*P. pastoris* X33	4.0	60	348 (pNPG) 6.2 (cellobiose)	This study
Bgl1 *A. niger* strain NL-1	*P. pastoris* GS115	4.0	60	270 (pNPG)	(Zhao *et al.,* 2013)
BG GH3 *A. oryzae*	*S. cerevisiae*	5.0	60	176 (pNPG)	(Langston *et al*., 2006)
Bgl1 *T. reesei*	*P. pastoris* GS115	5.0	70	197 (salicin)	(Chen *et al*., 2011)
BGLA *Phanerochaete chrysosporium*	*P. pastoris* strain KM71	4.5	30	52 (pNPG)	(Kawai *et al*., 2003)

BglA has a greater specificity toward pNPG than cellobiose. This study provides a useful recombinant β-glucosidase produced in an industrial suitable microorganism (i.e., *P. pastoris*). β-glucosidase can be used to improve the enzymatic conversion of cellulosic biomass to glucose and meet the industry's current demands.

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