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Biochemical properties of a novel glycoside hydrolase family 1 β-glucosidase (PtBglu1) from *Paecilomyces thermophila* expressed in *Pichia pastoris*

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ABSTRACT

A novel β-glucosidase gene (*PtBglu1*) from the thermophilic fungus, *Paecilomyces thermophila*, was cloned and expressed in *Pichia pastoris*. *PtBglu1* contained an open reading frame of 1440-bp nucleotides and encoded a protein of 479 amino acids which showed significant similarity to other fungal β-glucosidases from glycoside hydrolase (GH) family 1. The recombinant β-glucosidase (PtBglu1) was secreted at high level of 190.2 U mL^{-1} in high cell density fermentor (5 L). PtBglu1 was purified to homogeneity, and was found to be a glycoprotein with molecular mass of 56.7 kDa. The purified PtBglu1 showed optimum catalytic activity at pH 6.0 and 55 °C. The enzyme exhibited broad substrate specificity with highest activity toward *p*NP-β-D-glucopyranoside, followed by *p*NP-β-D-galactopyranoside and cellobiose. The K_m values for *p*NP-β-D-glucopyranoside, cellobiose, gentiobiose and salicin were 0.55 mM, 1.0 mM, 1.74 mM and 6.85 mM, respectively. These properties make PtBglu1 a potential candidate for various industrial applications.

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1. Introduction

β-Glucosidases (EC 3.2.1.21) catalyze the selective cleavage of β-glycosidic bonds from the non-reducing ends in their substrates, and some of them are also capable of forming long polymeric assemblies (Hong, Tamaki, & Kumagai, 2007; Ketudat & Esen, 2010). They are a very relevant group of enzymes because of the diversity of biological roles in which they are involved and their generalized occurrence in all sorts of living organisms (Bhatia, Mishra, & Bisaria, 2002). β-Glucosidases have received a great deal of attention due to their extensive potential applications in different industrial fields such as food, feed, textile, detergents, pharmaceutical and bio-ethanol conversion industries (Bhatia et al., 2002; Jeya et al., 2010; Zhou et al., 2012).

As a major group among glycoside hydrolase (GH) enzymes, β-glucosidases have been mainly classified into GH family 1 (GH1) and GH family 3 (GH3) based on their amino acid sequence identities (Henrissat et al., 1996). Generally, GH1 β-glucosidases are mainly from archaebacteria, plants and mammals, which usually show β-galactosidase activity, while GH3 β-glucosidases are mainly from bacteria, fungi and yeast (Bhatia et al., 2002; Ketudat & Esen, 2010). To date, a number of β-glucosidase genes have been isolated and characterized from various fungi such as *Trichoderma reesei* (Murray et al., 2004), *Humicola grisea* (Takashima, Nakamura, Hidaka, Masaki, & Uozumi, 1999), *Piromyces* sp. (Harhangi et al., 2002), *Talaromyces emersonii* (Collins et al., 2007), *Thermoascus aurantiacus* (Hong et al., 2007), *Stereum hirsutum* (Nguyen et al., 2010) and *Neosartorya fischeri* (Kalyani et al., 2012), while most of which belong to GH3. There are only few reports on the gene cloning of GH1 β-glucosidases from fungi such as *H. grisea* (Takashima et al., 1999), *Piromyces* sp. (Harhangi et al., 2002) and *N. fischeri* (Kalyani et al., 2012; Ramachandran et al., 2012). More recently, β-glucosidases from thermophilic fungi have attracted research interest due to the potential applications associated with their unusual inherent biochemical properties (Haki & Rakshit, 2003). However, other than *T. emersonii* (Collins et al., 2007), no reports on the gene cloning of GH1 β-glucosidases from thermophilic fungi are available till now. The genus *Paecilomyces* is known to be good enzyme producer which has been reported to produce several kinds of hydrolytic enzymes, such as chitinase (Khan, Williams, Molloy, & Nevalainen, 2003), protease (Khan et al., 2003) and tannase (Jr, Macedo, & Macedo, 2011). However, so far there is no report on the GH1 β-glucosidase from this species.

Paecilomyces thermophila J18, as a thermophilic fungus, can produce a series of lingocellulosic enzymes (Yang et al., 2006). In our previous study, a novel GH3 β-glucosidase from *P. thermophila* was purified, characterized and applied in the production of free isoflavone from soya bean (Yang, Wang, Yan, Jiang, & Li, 2009).

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Here, we report the cloning and expression of a GH1 β-glucosidase gene from *P. thermophila*. The recombinant enzyme was purified and its biochemical properties were further investigated. This study is the first report on the cloning, expression and properties of GH1 β-glucosidase from *P. thermophila*.

2. Experimental

2.1. Strains, plasmids and growth conditions

P. thermophila J18 used in this study was deposited in China General Microbiological Culture Collection Center (CGMCC, accession No. AS3. 6885). *Escherichia coli* strain JM109 (Stratagene, California, USA) was used for propagation of plasmids. *Pichia pastoris* GS115 was used for protein expression. The *P. pastoris* expression kit was obtained from Invitrogen (San Diego, California, USA). Restriction endonucleases and T4 DNA ligase were purchased from England Biolabs (Ipswich, Massachusetts, USA). The pMD18-T simple vector system and KOD-Plus DNA polymerase were the products of TaKaRa (Dalian, China).

P. thermophila J18 was grown in the culture medium at 50 °C for 4 d in a shaker with rotation speed of 200 rpm. The medium contained 2% (w/v) corncob xylan, 0.5% barley β-glucan, 1% tryptone, 1% yeast extract, 0.03% $MgSO_4{\cdot}7H_2O$, 0.03% $FeSO_4$ and 0.03% $CaCl_2$. Genomic DNA of *P. thermophila* J18 was isolated with a Fungal DNA Midi Kit (Omega Bioteck, Doraville, Georgia, USA). Total RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, California, USA), and mRNA was purified using an Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany).

2.2. Cloning of the full-length β-glucosidase cDNA

To clone the *PtBglu1* gene, degenerate primers PtBglu1CP1 (GGCCCTTCCATCTGGGAYACNTT) and PtBglu1CP2 (GGTGCCGTTCTCGGTGACRTADATYTTNGG) were designed based on the conserved sequences of known fungal β-glucosidases using the CODEHOP algorithm (Rose et al., 1998). Genomic DNA of *P. thermophila* J18 was used as template for polymerase chain reaction (PCR) amplification. PCR conditions were as follows: a hot start at 94 °C for 5 min, 10 cycles of 94 °C for 30 s, 61–55 °C for 30 s and 72 °C for 2 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min. The PCR product was purified, ligated to pMD18-T vector and sequenced.

The full-length cDNA sequence of *PtBglu1* was obtained by 5′ and 3′ RACE (rapid amplification of cDNA ends) using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, California, USA), following the manufacturer's instructions. PCR was performed with the following primer pairs: PtBglu1GSP1 (GGCCGATGGAATTGCCGAACTTGTC) and 10× Universal Primer A Mix (UPM) for first PCR, Nested Universal Primer A (NUP, BD Biosciences, US) and PtBglu1NGSP1 (TTGCTGCCTTTGACCAGGGCGATT) for 5′ RACE, and PtBglu1GSP2 (GACCGACTCCCCACCTTCACCGACG) and 10× UPM for 3′ RACE. The obtained PCR product was purified, cloned and sequenced. The cDNA sequence of PtBglu1 from *P. thermophila* J18 was deposited in the GenBank nucleotide sequence database with accession No. HM036350. The coding region of *PtBglu1* was amplified from the *P. thermophila* J18 genomic DNA using the specific primers PtBglu1DNAF (ATGACCACCGCAACTCTGCCG) and PtBglu1DNAR (TCACTCCTTGCGAATGTACTTTTC). The DNA product was purified and cloned into a pMD18-T vector, and transformed into *E. coli* JM109 for sequencing. The gene and deduced amino acid sequence were analyzed and compared with sequence databases in NCBI using available online tools (http://www.expasy.ch/tools/).

2.3. Construction of expression vector in P. pastoris

The coding region of *PtBglu1* without the signal peptide-coding sequence was amplified by PCR using primers PtBglu1SnaBIF (AAGCTTTACGTAATGACCACCGCAACTCTGCC) and PtBglu1AvrIIR (AAGCTTCCTAGGTCAATGATGATGATGATGCTCCTTGCGAATGTACTT). PCR amplification was carried out using the KOD-Plus DNA polymerase at an annealing temperature of 65 °C. The PCR product was purified and cloned into pPIC9K vector between *Sna*BI and AvrII sites, yielding the recombinant plasmid pPIC9K-PtBglu1. The recombinant plasmid was linearized with restriction endonuclease *Sac*I, and then transferred to *P. pastoris* GS115 competent cells by electroporation.

The His^+ transformants were selected based on their resistance to Geneticin 418 (G418, Life Technologies, Gaithersburg, Maryland, USA). After incubating the plates for 2 days at 30 °C, the His^+ transformants were scored and suspended in sterile water and 10^5 cells were coated on yeast extract peptone dextrose (YPD) plates containing G418 at a final concentration of 0.5–4.0 mg mL^{-1}, and then the putative multi-copy transformants were selected. To differentiate Mut^+ from Mut^s, one patch of GS115/His^+ Mut^+ β-gal and GS115/His^+ Mut^s Albumin were used as positive control and negative control, respectively. His^+ transformants scored as Mut^+ phenotype based on rapid growth on minimal methanol medium (MM) plates were then used to express *PtBglu1* gene.

2.4. High-level expression of PtBglu1 in P. pastoris

The recombinant *PtBglu1* was expressed in *P. pastoris* GS115 cells. A single colony was inoculated in 10 mL buffered minimal glycerol complex medium (BMGY) in a 100-mL shake flask. The cells were grown at 30 °C in a shaking incubator (250 rpm) until the cell density reached an OD_{600} of 2.0–6.0, then the cells were collected and re-suspended in 50 mL BMMY medium (same composition as BMGY but with 0.5% methanol instead of glycerol) in a 500 mL shake flask and then further incubated under the same conditions. Methanol (100%) was added every 24 h to ensure a final concentration of 0.5% to maintain induction. After 3 days of cultivation, the cultures were centrifuged at 10,000 × *g* for 10 min, and the supernatant was used as the crude enzyme.

High-level expression of *PtBglu1* was performed using high cell-density fermentation according to the *Pichia* Fermentation Process Guidelines (Version B, 053002, Invitrogen). The positive strain with the highest activity was cultured in shake flask at 30 °C with rotation speed of 250 rpm until the cell density reached an OD_{600} of more than 10.0, and then the culture was inoculated into a 5-L fermentor containing 2.0 L BSM medium with 8.7 mL PTM_1 trace salts. The fermentation temperature, pH and agitation rate were maintained at 30 °C, pH 4.0 and 700 rpm, respectively. The initial dissolved oxygen (DO) level was maintained at >20% air saturation. After the initial glycerol was depleted, fed-batch fermentation was started by supplementing glycerol at a flow rate of 36.8 mL h^{-1} for 6 h. Then, the methanol containing PTM_1 trace salts (pH 6.0, 12 mL L^{-1}) was supplemented, increasing the flow rate from 7.2 mL h^{-1} at initial stage to 21.8 mL h^{-1} over a 4-h period. Samples withdrawn every 12 h were analyzed by SDS-PAGE, enzyme activities, protein concentrations and wet cell weights were also determined.

2.5. Enzyme assay and protein determination

β-Glucosidase activity was determined at 55 °C using 5.0 mM *p*NP-β-D-glucopyranoside (*p*NPG) as substrate in 50 mM citrate buffer (pH 6.0). After 10 min of incubation, the reaction was stopped by adding three volumes of saturated sodium tetraborate solution, and the absorbance at 405 nm was measured (Peralta, Kadowaki, Terenzi, & Jorge, 1997). One unit of enzyme activity was defined as

the amount of enzyme that is required to liberate 1 μmol *p*NP per minute under the above conditions.

Protein concentrations were measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin (BSA) as the standard. Specific activity was expressed as units per milligram protein.

2.6. SDS-PAGE, zymogram analysis and molecular mass determination of PtBglu1

SDS-PAGE was performed using 12.5% (w/v) separating gel according to the method of Laemmli (1970). Proteins in the gel were stained by Coomassie brilliant blue R-250, and the molecular mass was estimated with reference to broad range molecular mass protein standards. The protein standards used were phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

A zymogram of PtBglu1 was produced by incubating the SDS-PAGE gel in 0.5 μM 4-methylumbelliferyl-β-D-glucuronide hydrate solution at 50 °C for 0.5 h, after washing the gel with 25% isopropanol and 50 mM phosphate buffer (pH 6.0), respectively (each for 3 times). The β-glucosidase location on the gel was then visualized under UV light at 260 nm.

Native molecular mass of PtBglu1 was estimated by gel filtration with a Sephacryl S-200 HR column pre-equilibrated with 50 mM phosphate buffer (pH 7.2) containing 50 mM NaCl.

2.7. Purification of the recombinant PtBglu1

The crude enzyme solution was dialyzed against buffer A (50 mM pH 8.0 phosphate buffer) overnight, and then applied to a Ni-IDA agarose column (1.0 cm × 10 cm) pre-equilibrated with buffer B (buffer A containing 500 mM NaCl and 10 mM imidazole). After binding for 30 min, the column was washed with buffer B followed by buffer C (buffer A containing 500 mM NaCl and 20 mM imidazole). Finally, the absorbed proteins were eluted with buffer D (buffer A containing 500 mM NaCl and 50 mM imidazole). Flow rates in each elution were adjusted to 1.0 mL min^{-1}. The homogeneity of the fraction with enzyme activity was monitored by SDS-PAGE.

2.8. Biochemical characterization of the purified PtBglu1

Optimum pH of the purified PtBglu1 was determined by measuring its activity in different buffers (50 mM) with pH varying from 2.5 to 11.0, and pH stability was determined by measuring its residual activity after it was incubated with different pH buffers for 30 min at 45 °C. Optimum reaction temperature of the enzyme was determined by measuring its activity at 30–70 °C, and thermostability was determined by measuring its residual activity after treatment at 30–70 °C for 30 min in 50 mM citrate buffer (pH 6.0).

2.9. Substrate specificity of the purified PtBglu1 and kinetic parameters

Substrate specificity of the purified PtBglu1 was determined by measuring the enzyme activity using different *p*NP-glycosides, disaccharides, oligosaccharides and polysaccharides as the substrates. The reactions were carried out in 50 mM pH 6.0 citrate buffers at 55 °C for 10 min with the *p*NP-glycosides (5 mM) or the substrate concentration of 1% (w/v) for others. The amount of *p*NP released from *p*NP-glycosides was determined by the standard enzyme assay. The glucose released from disaccharides and oligosaccharides was measured using a GOD-POD assay kit (Kangtai, Beijing, China). The reducing sugars released from polysaccharides were analyzed according to the DNS method (Miller, 1959). Enzyme units were defined as the amount of the enzyme that is required to produce 1 μmol of *p*NP or glucose or reducing sugar per minute under the above assay conditions. One unit of enzyme activity towards gluco-disaccharides such as cellobiose, sophorose and gentiobiose was defined as the amount of enzyme that is required to form 2 μmol of glucose per minute. The kinetic parameters of PtBglu1 toward different substrates were determined by measuring the enzyme activities with the substrate concentrations ranging from 0 to 20 mM in 50 mM citrate buffer (pH 6.0) at 55 °C for 2 min. The K_m and V_{max} values were calculated using "GraFit" software.

2.10. Hydrolysis properties of PtBglu1

The hydrolytic properties of PtBglu1 were evaluated by incubating 10% (w/v) of individual cello-oligosaccharide or 1% of polysaccharide with 10 U mL^{-1} of purified PtBglu1 in 50 mM citrate buffer (pH 6.0) at 55 °C for 6 h. Samples withdrawn at different time intervals were analyzed. Glucose released was qualified by a GOD-POD assay kit. Other hydrolytic products were qualitatively analyzed by thin-layer chromatography (TLC) on Kieselgel 60 plates (Merck, Germany) with a butan-1-ol–acetic acid–water (2:1:1, v/v) solvent system. The plates were developed with one run followed by heating for few minutes at 130 °C in an oven after spraying the plates with a methanol–sulfuric acid mixture (95:5, v/v).

3. Results

3.1. Cloning and sequence analysis of PtBglu1 from P. thermophila

A 1200-bp gene fragment was obtained by PCR using degenerate primers PtBglu1CP1 and PtBglu1CP2 designed from other fungal β-glucosidase sequences in the GeneBank database, with *P. thermophila* J18 genomic DNA as template. This sequence was subsequently used to design RACE primers to obtain full-length coding region of the gene. Primers corresponding to the regions containing the putative start and stop codons deduced from the 5′ and 3′ RACE PCR products were then used to amplify full-length *PtBglu1* sequence from first-strand cDNA and genomic DNA of *P. thermophila*. The predicted product of the *PtBglu1* insert revealed a single open reading frame (ORF) of 1440-bp (Fig. 1) encoding a polypeptide of 479 amino acid residues with a calculated molecular mass of 54,997 Da without predicted signal peptide, and a deduced isoelectric point (p*I*) of 5.41. The putative protein sequence contained one possible *N*-glycosylation site. Sequence analysis showed that the full-length *PtBglu1* gene is interrupted by four putative introns (64, 55, 68 and 61 bp).

The deduced amino acid sequence of the *PtBglu1* showed high homology with some GH1 β-glucosidases from other fungi, such as *T. emersonii* (AAL89551.2, Collins et al., 2007) (82%), *Talaromyces stipitatus* (EED22235.1) (79%), *Penicillium marneffei* (EEA26357.1) (78%), *N. fischeri* (EAW22388.1, Kalyani et al., 2012) (74%) and *Aspergillus fumigatus* (EAL90802.1) (74%). The nucleotide sequence of the *PtBglu1* gene was deposited in the GenBank database under the accession number HM036350.

3.2. Expression and purification of PtBglu1 in P. pastoris

A secreted expression plasmid, pPIC9K-PtBglu1 was successfully constructed by subcloning the *PtBglu1* gene into pPIC9K vector under the control of the *AOX*1 promoter, and then it was transferred to *P. pastoris* GS115 competent cells. A positive strain which secreted high level of recombinant β-glucosidase in flask culture was isolated, and then subjected to high cell-density fermentation. The maximum β-glucosidase activity of 190.2 U mL^{-1} with

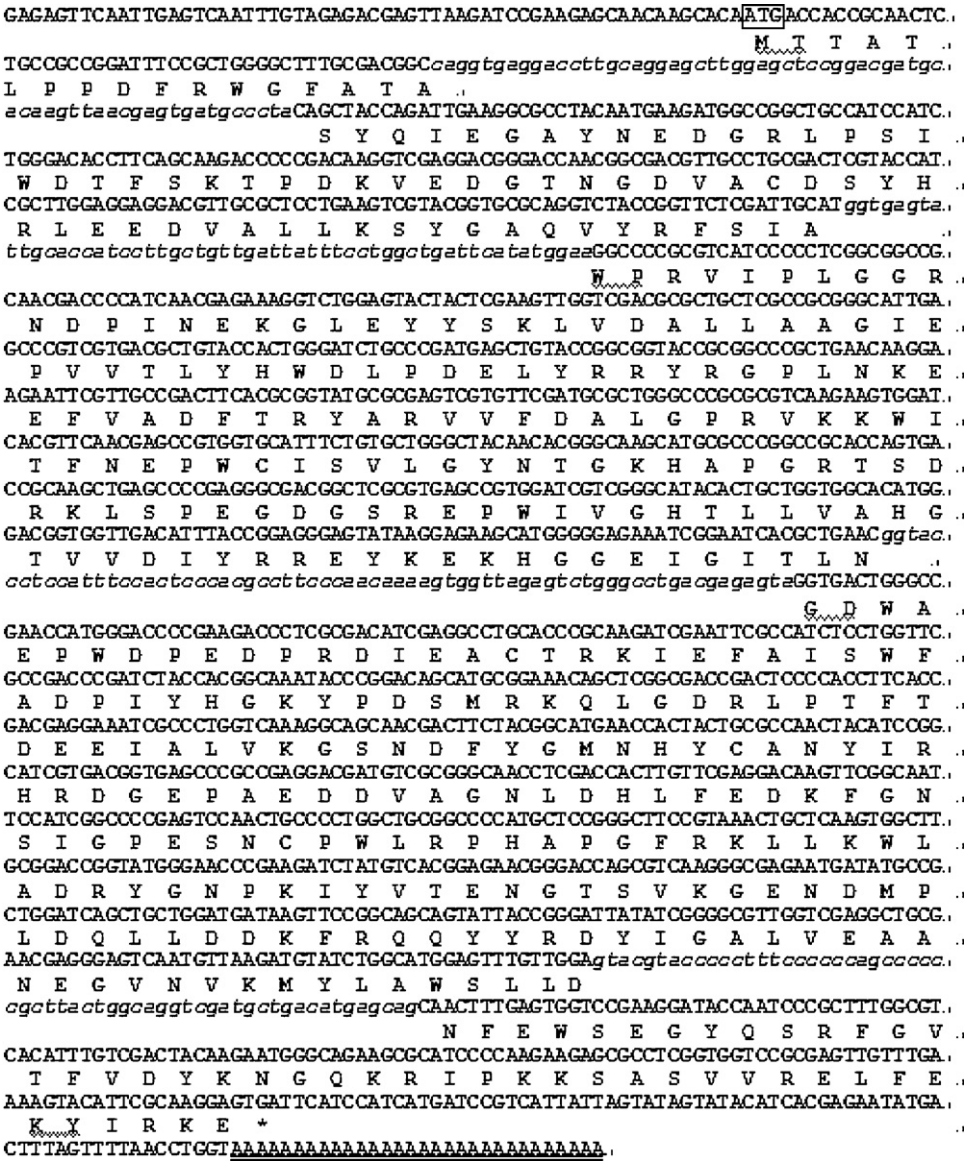

Fig. 1. Nucleotide sequence of *PtBglu1* from *Paecilomyces thermophlia* and deduced amino acid sequence of the gene product. Flanking regions and four introns are shown in lowercase letters while coding regions are shown in uppercase letters. Start codon (ATG) is boxed. Poly (A+) tail is double underlined. The nucleotide sequence reported here was submitted to Genbank under accession number HM036350.

secreted protein of 8.9 g L^{-1} was obtained after 144 h of induction by methanol. Also, the highest cell density of the fermentation broth with OD_{600} value of 644 was achieved (Fig. 2a). The recombinant PtBglu1 was purified to apparent homogeneity with a purification fold of 9.0 and a recovery yield of 85.2% (Table 1). The molecular mass of the enzyme was estimated to be 58.0 kDa and 56.7 kDa by SDS-PAGE (Fig. 2b), and gel filtration chromatography, respectively. Endo H treatment of PtBglu1 did not result in a protein band shift on SDS-gel (Fig. 2b).

3.3. Biochemical characterization of PtBglu1

The recombinant PtBglu1 was most active at pH 6.0 (Fig. 3a), and it exhibited good pH stability, since more than 80% of its activity remained after treatment in different buffers (pH 5.0–11.0) for 30 min (Fig. 3b). The optimal temperature of the purified PtBglu1 was found to be 55 °C (Fig. 3c), and the enzyme was fairly stable at this temperature. The residual activity reached up to 88% of its original activity after treatment at 55 °C for 30 min (Fig. 3d).

Table 1
Purification of the recombinant β-glucosidase (PtBglu1) expressed in *P. pastoris*.

Purification step	Total activity (U)[a]	Total protein (mg)[b]	Specific activity (U mg^{-1})	Purification factor (-fold)	Recovery (%)
Culture supernatant	9510	445	21.4	1	100
Ni-NTA	8102.5	42.0	192.7	9.0	85.2

[a] Enzyme activity was measured in 50 mM citrate buffer (pH 6.0) at 55 °C using 5 mM *p*NPG as the substrate.
[b] The protein was measured by the Lowry method (Lowry et al., 1951), using BSA as the standard.

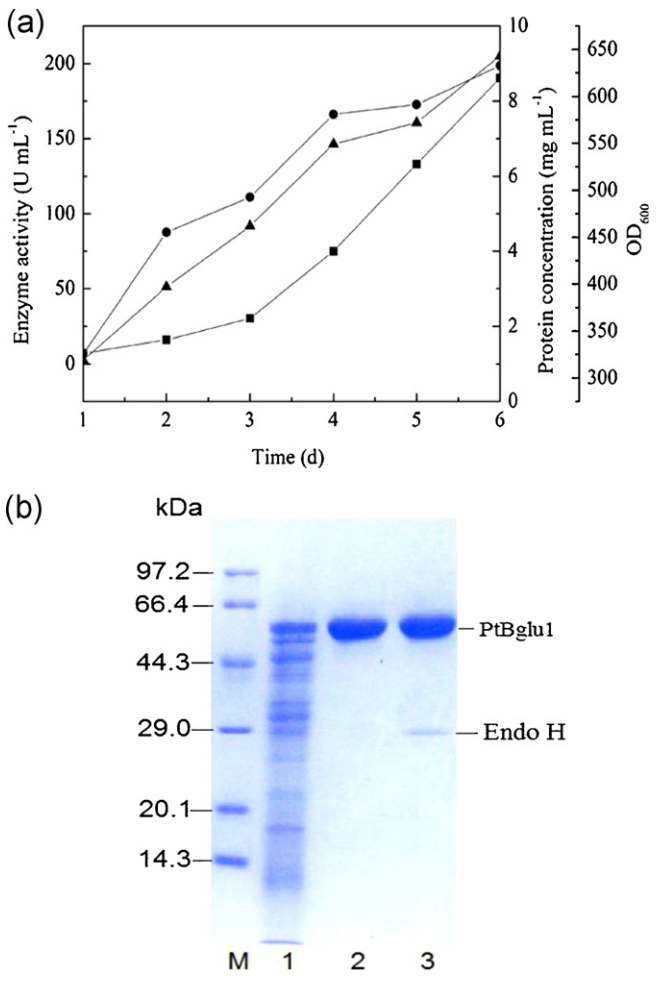

Fig. 2. Time course of PtBglu1 production expressed in *Pichia pastoris* by high density-fermentation (a) and SDS-PAGE of proteins during purification process of the recombinant PtBglu1 (b). (■) Enzyme activity, (●) protein concentration, (▲) OD_{600}. Lane M, standard protein molecular weight markers; lane 1, culture supernatant; lane 2, fraction after Ni-NTA; lane 3, PtBglu1 treated by Endo H.

3.4. Substrate specificity and kinetic parameters

The activities of PtBglu1 on various substrates were examined (Table 2). Apparently, the enzyme exhibited broad substrate specificity, and the highest activity was observed with *p*NP-β-D-glucopyranoside as the substrate, followed by *p*NP-β-D-galactopyranoside (50.7%), cellobiose (33.8%) and lactose (20.7%), while it displayed relative low activity on *p*NP-α-D-glucopyranoside (3.4%), *p*NP-β-D-xylopyranoside (2.4%) and amygdalin (2.1%). It showed no activity toward carboxymethyl cellulose (CMC), birchwood xylan and sucrose. Unexpectedly, the enzyme even hydrolyzed polysaccharides such as lichenan, laminarin and barley-β-glucan, with 36.1%, 30.6% and 24.1% of relative activities on *p*NPG-β-D-glucopyranoside, respectively.

The kinetic parameters of the purified PtBglu1 were determined for several preferred substrates (Table 3). The K_m values toward *p*NP-β-D-glucopyranoside, cellobiose, gentiobiose, *p*NP-β-D-galactopyranoside, salicin and lactose were 0.55 mM, 1.0 mM, 1.74 mM, 5.3 mM, 6.85 mM and 26.1 mM, respectively.

3.5. Hydrolysis properties of PtBglu1

The hydrolysis properties of PtBglu1 toward polysaccharides and cellooligosaccharides were analyzed by TLC. Glucose was the major product released from barely β-D-glucan, lichenan and laminarin (Fig. 4), suggesting the action mode of the enzyme was *exo*-type. PtBglu1 could completely convert cellobiose, cellotriose and cellotetraose to glucose in 4 h (Fig. 4). In the initial stage (0–1 h) of the hydrolysis, some oligosaccharides with the degrees of polymerization higher than the responding substrates were observed, suggesting the enzyme had transglycosylation activity (Fig. 4). All the intermediate products were hydrolyzed to glucose finally with the extension of reaction time.

Table 2
Substrate specificity of the purified PtBglu1.

Substrate	Specific activity (U mg^{-1})[a]	Relative activity (%)
*p*NP-β-D-glucopyranoside	192.7	100
*p*NP-β-D-galactopyranoside	97.7	50.7
*O*NP-β-D-galactopyranoside	18.2	9.4
*p*NP-α-D-glucopyranoside	6.5	3.4
*p*NP-β-D-xylopyranoside	4.7	2.4
Cellobiose (Glcβ1-4Glc)	65.2	33.8
Cellotriose	58.2	30.2
Cellotetraose	48.7	25.3
Lactose (Galβ1-4Glc)	39.8	20.7
Salicin [2-(hydroxymethyl)phenyl-β-D-glucoside]	27.8	14.4
Amygdalin	4.1	2.1
Gentiobiose (Glcβ1-6Glc)	25.3	13.1
Lichenan	69.6	36.1
Laminarin	58.9	30.6
Barely-β-glucan	46.4	24.1

[a] The activity for *p*NP-β-D-glucopyranoside was defined as 100%. PtBglu1 did not act toward Avicel, CMC, filter paper, birchwood xylan, β-1,3-glucan, rutin, starch, pullulan, locust bean gum, maltose, xylobiose, sucrose, trehalose and raffinose.

4. Discussion

Although the physical and biochemical properties of β-glucosidases from various fungi have been studied extensively (Bhatia et al., 2002; Chen, Fu, Ng, & Ye, 2011; Hong et al., 2007; Krisch, Bencsik, Papp, Vagvolgyi, & Tako, 2012; Takashima et al., 1999), as far as we know this represents the first report on the cloning, expression and characterization of a novel GH family 1 β-glucosidase gene (*PtBglu1*) from the thermophilic fungus *P. thermophila*. PtBglu1 in the present study exhibited moderate homology to some GH1 β-glucosidases from other fungi such as *T. emersonii*, *T. stipitatus*, *P. marneffei*, *N. fischeri* and *Aspergillus fumigates* (82%, 79%, 78%, 74% and 74% identity, respectively). Thus, PtBglu1 is a new member of GH1 β-glucosidases.

A number of GH1 β-glucosidases have been isolated and characterized from plants (Chuenchor et al., 2011), bacteria (Fan, Miao, Liu, Liu, & Liu, 2011; Xu et al., 2010), and fungi (Collins et al., 2007; Harhangi et al., 2002; Kalyani et al., 2012; Nguyen et al., 2010; Takashima et al., 1999). In the present study, the β-glucosidase (PtBglu1) gene has been successfully expressed in *P. pastoris*, and the production of PtBglu1 reached 190.2 U mL^{-1} after 140 h of methanol induction, which is the highest value for GH family 1 β-glucosidases from fungi until date. This value is also much higher than most other fungal GH3 β-glucosidases (Chen et al., 2011; Hong et al., 2007).

The molecular mass of the purified PtBglu1 was estimated to be 58.0 kDa and 56.7 kDa by SDS-PAGE (Fig. 2b), and gel filtration chromatography, respectively, which indicates that the enzyme is a monomeric protein. The molecular mass is substantially lower than that of typical GH3 β-glucosidases, which range from about 70 to 100 kDa (Maekawa, Hayase, Yubisui, & Minami, 2006), while in agreement with those of many extracellular GH1 β-glucosidases

Table 3
Kinetic parameters for the purified PtBglu1.[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
*p*NP-β-D-glucopyranoside	328.8 ± 7.5	0.55 ± 0.03	5.5	9.96
*p*NP-β-D-galactopyranoside	325.0 ± 1.3	5.3 ± 0.06	5.4	1.02
Cellobiose	306.3 ± 6.3	1.0 ± 0.06	5.1	5.10
Lactose	149.0 ± 3.0	26.1 ± 1.15	2.48	0.095
Salicin	66.0 ± 0.4	6.85 ± 0.06	1.1	0.16
Gentiobiose	44.5 ± 1.1	1.74 ± 0.10	0.74	0.43

[a] Enzymatic reactions were carried out in 50 mM citrate buffer (pH 6.0) at 55 °C for 2 min.

from other fungal sources such as *T. reesei* (57 kDa, Takashima et al., 1999), *T. emersonii* (57.6 kDa, Collins et al., 2007) and *N. fischeri* (56 kDa, Kalyani et al., 2012).

The purified PtBglu1 showed optimal activity at pH 6.0 and 55 °C. The optimal pH is similar to that of most of the GH1 β-glucosidases from other fungi with pH optima around neutrality (pH 6.0–7.0) (Harhangi et al., 2002; Kalyani et al., 2012; Ramachandran et al., 2012; Takashima et al., 1999). The optimal temperature is higher than those of most other reported recombinant GH1 β-glucosidases both from bacteria (Fan et al., 2011; Spiridonov & Wilson, 2001; Xu et al., 2010) and fungi (Harhangi et al., 2002; Kalyani et al., 2012; Ramachandran et al., 2012; Takashima et al., 1999), with optimal temperature ranging from 35 °C to 50 °C. The purified PtBglu1 also exhibited good thermostability, with denaturing half-life of 1160 min at 50 °C. The high thermostability gives the enzyme many advantages in biotechnological applications, since a higher reaction temperature can not only improve the reaction rate, but also reduce the enzyme dosage.

GH1 β-glucosidases usually posses both β-glucosidase activity and β-galactosidase activity (Bhatia et al., 2002). The purified PtBglu1 in the present study showed high specific activity (97.7 U mg^{-1}) for *p*NP-β-D-galactopyranoside. Based on substrate specificity, β-glucosidases have been classified as (1) aryl β-glucosidases, which act on aryl-glucosides, (2) true cellobiases, which hydrolyze cellobiose to release glucose, and (3) broad substrate specificity β-glucosidases, which act on a spectrum of substrates (Bhatia et al., 2002). PtBglu1 from *P. thermophila* showed significant activity for both aryl-glucosides and cellooligosaccharides (Table 2), indicating that it belongs to broad substrate specificity β-glucosidases. The substrate specificity of PtBglu1 is similar to the GH1 β-glucosidase from the bacterium *Micrococcus antarcticus*, which can also hydrolyze aryl-glucosides as well as cellooligosaccharides (Fan et al., 2011), while it is different from *Sphingomonas pancimobilis* GH1 β-glucosidase, which showed no detectable activity towards substrates derived from cellulose hydrolysis and lactose (Marques, Coutinho, Videira, Fialho, & S.Á-Correia, 2003). Furthermore, the PtBglu1 in the present study is significantly different from GH1 β-glucosidases from other fungi, such as *S. hirsutum* (Nguyen et al., 2010) and *N. fischeri* (Kalyani et al., 2012), which also exhibited low or no activity for cellooligosaccharides. It should be noteworthy that the PtBglu1 in the present study can hydrolyze polysaccharides such as lichenan, laminarin

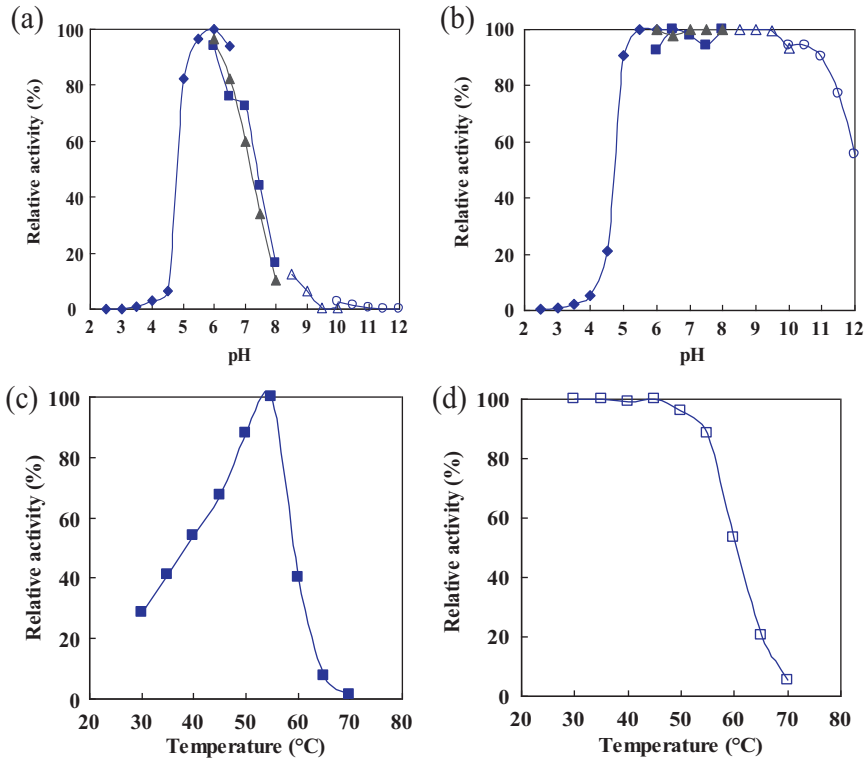

Fig. 3. Optimal pH (a), pH stability (b), optimal temperature (c) and thermostability (d) of the purified PtBglu1 from *P. thermophila*. The optimal pH of the enzyme was determined by measuring its activity at different pH in various buffers. For pH stability determination, the enzyme was treated at various buffers with different pH for 30 min, and then the residual activities were determined. The buffers used were (♦) citrate (pH 2.5–5.5), (■) phosphate (pH 5.0–7.0), (▲) MOPS (pH 6.0–8.0), (△) CHES (pH 8.0–10.0) and (○) glycine–NaOH (pH 10.0–12.0). The optimal temperature of the enzyme was determined by assaying its activity at 30–70 °C. For thermostability determination, the enzyme was treated at various temperatures (30–70 °C) for 30 min, and then the residual activities were measured.

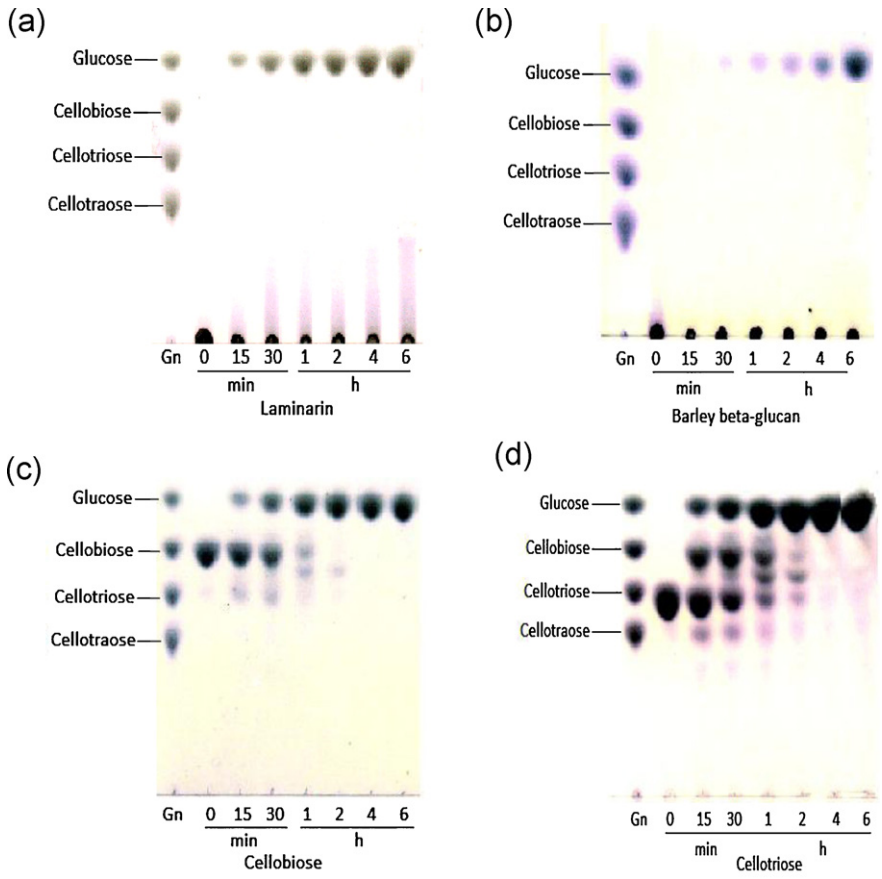

Fig. 4. TLC analysis of the hydrolytic products from polysaccharides and cello-oligosaccharides by the purified PtBglu1 of *P. thermophila*. Different substrates (1%, w/v) were hydrolyzed by 10 U mL^{-1} of purified PtBglu1 in 50 mM citrate buffer (pH 6.0) at 55 °C for 6 h. Samples withdrawn at different time intervals were analyzed by TLC.

and barely-β-glucan to release glucose, which is different from most other reported β-glucosidases (Jeya et al., 2010; Joo et al., 2009; Kalyani et al., 2012; Murray et al., 2004; Nguyen et al., 2010). PtBglu1 had a comparable K_m value of 0.55 mM for *p*NPG. This value is lower than that of the GH1 β-glucosidases from bacteria such as *M. antarcticus* (7 mM, Fan et al., 2011) and *Sphingomonas paucimobilis* (1.3 mM, Marques et al., 2003), and fungi including *N. fischeri* (2.8 mM, Ramachandran et al., 2012), *Piromyces* sp. E2 (1 mM, Harhangi et al., 2002) and *S. hirsutum* (2.5 mM, Nguyen et al., 2010). It is only slightly higher than that of a bacterial GH1 β-glucosidase (Spiridonov & Wilson, 2001) and a fungal GH1 β-glucosidase (Takashima et al., 1999) with the K_m values of 0.24 mM and 0.32 mM, respectively. In addition, the value is also lower than most GH3 β-glucosidases from other fungi, such as *Penicillium purpurogenum* KJS506 (5.1 mM, Jeya et al., 2010), *Fomitopsis pinicola* (1.8 mM, Joo et al., 2009) and *Daldinia eschsecholzii* (1.5 mM, Karnchanatat et al., 2007). The purified PtBglu1 showed relative low K_m values for other tested substrates ranging from 1.0 mM to 26.1 mM (Table 3) indicating that the affinities of enzyme to substrates are in the order of cellobiose > gentiobiose > *p*NP-β-D-galactoside > salicin > lactose. The broad substrate specificity and high affinity to substrates have the potential to enlarge PtBglu1's application. The properties of PtBglu1 against polysaccharides were investigated by thin layer chromatography (Fig. 4). During time course hydrolysis, only glucose was released from the substrate, suggesting that the action mode of PtBglu1 was exo-type. The purified enzyme also displayed transglycosylation activity in the hydrolytic process of cellobiose, cellotriose and cellotraose (Fig. 4). Some other fungal β-glucosidases also have been shown to form glycosidic linkages by transglycosylation action in the hydrolytic process (Joo et al., 2009; Murray et al., 2004).

In conclusion, a novel β-glucosidase gene, *PtBglu1*, from *P. thermophila* was cloned and successfully expressed in *P. pastoris* for the first time. The highest β-glucosidase production of 190.2 U mL^{-1} was obtained in a 5.0-L fermentor by high cell-density fermentation. The recombinant PtBglu1 was purified and characterized. The molecular mass of the enzyme was estimated to be 56.7 kDa by SDS-PAGE. The enzyme showed optimal activity at pH 6.5 and 55 °C. The enzyme exhibited broad substrate specificity, good thermal and pH stability. These properties make it potentially useful in a wide variety of industrial applications.

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References

Bhatia, Y., Mishra, S., & Bisaria, V. S. (2002). Microbial β-glucosidase: Cloning, properties, and applications. *Critical Reviews in Biotechnology*, *22*, 375–407.

Chen, P., Fu, X. Y., Ng, T. B., & Ye, X. Y. (2011). Expression of a secretory β-glucosidase from *Trichoderma reesei* in *Pichia pastoris* and its characterization. *Biotechnology Letters*, *33*, 2475–2479.

Chuenchor, W., Pengthaisong, S., Robinson, R. C., Yuvaniyama, J., Svasti, J., & Cairns, J. R. K. (2011). The structural basis of oligosaccharide binding by rice BGlu1 β-glucosidase. *Journal of Structural Biology*, *173*, 169–179.

Collins, C. M., Murray, P. G., Denman, S., Morrissey, J. P., Byrnes, L., Teeri, T. T., et al. (2007). Molecular cloning and expression analysis of two distinct β-glucosidase genes, *bg1* and *aven1*, with very different biological roles from the thermophilic, saprophytic fungus *Talaromyces emersonii*. *Mycology Research*, *3*, 840–849.

Fan, H. X., Miao, L. L., Liu, Y., Liu, H. C., & Liu, Z. P. (2011). Gene cloning and characterization of a cold-adapted β-glucosidase belonging to glycosyl hydrolase family 1

from a psychrotolerant bacterium *Micrococcus antarcticus*. *Enzyme and Microbial Technology*, *49*, 94–99.
Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, *89*, 17–34.
Harhangi, H. R., Steenbakkers, P. J. M., Akhmanova, A., Jetten, M. S. M., Van der Drift, C., & dep Camp, H. J. M. (2002). A highly expressed family 1 β-glucosidase with transglycosylation capacity from the anaerobic fungus *Piromyces* sp. E2. *Biochimica et Biophysica Acta*, *1574*, 293–303.
Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J. P., & Davies, G. (1996). Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proceedings of the National Academy of Sciences of the United States of America*, *93*, 5674.
Hong, J., Tamaki, H., & Kumagai, H. (2007). Cloning and functional expression of thermostable β-glucosidase gene from *Thermoascus aurantiacus*. *Applied Microbiology and Biotechnology*, *73*, 1331–1339.
Jeya, M., Joo, A. R., Lee, K. M., Tiwari, M. K., Lee, K. M., Kim, S. H., et al. (2010). Characterization of β-glucosidase from a strain of *Penicillium purpurogenum* KJS506. *Applied Microbiology and Biotechnology*, *86*, 1473–1484.
Joo, A. R., Jeya, M., Lee, K. M., Sim, W., Kim, J. S., Kim, I. W., et al. (2009). Purification and characterization of a β-glucosidase from a newly isolated strain of *Fomitopsis pinicola*. *Applied Microbiology and Biotechnology*, *83*, 285–294.
Jr, J. V. M., Macedo, J. A., & Macedo, G. A. (2011). Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. *Bioresource Technology*, *102*, 7343–7348.
Kalyani, D., Lee, K. M., Tiwari, M. K., Ramachandran, P., Kim, H., Kim, I. W., et al. (2012). Characterization of a recombinant aryl β-glucosidase from *Neosartorya fischeri* NRRL181. *Applied Microbiology and Biotechnology*, *94*, 413–423.
Karnchanatat, A., Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A. J. S., Reynolds, C. D., et al. (2007). Purification and biochemical characterization of an extracellular β-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb: Fr) Rehm. *FEMS Microbiology Letters*, *270*, 162–170.
Ketudat, C. J. R., & Esen, A. (2010). β-Glucosidases. *Cellular and Molecular Life Sciences*, *67*(20), 3389–3405.
Khan, A., Williams, K., Molloy, M. P., & Nevalainen, H. (2003). Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. *Protein Expression and Purification*, *32*, 210–220.
Krisch, J., Bencsik, O., Papp, T., Vagvolgyi, C., & Tako, M. (2012). Characterization of β-glucosidase with transgalactosylation capacity from the zygomycete *Rhizomucor miehei*. *Bioresource Technology*, *114*, 555–560.
Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature*, *227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.
Maekawa, A., Hayase, M., Yubisui, T., & Minami, Y. (2006). A cDNA cloned from *Physarum polycephalum* encodes new type of family 3 β-glucosidase that is a fusion protein containing a calx-β motif. *International Journal of Biochemistry and Cell Biology*, *38*, 2164–2172.
Marques, A. R., Coutinho, P. M., Videira, P., Fialho, A. M., & S.Á-Correia, I. (2003). *Sphingomonas paucimobilis* β-glucosidase Bgl1: A member of a new bacterial subfamily in glycoside hydrolase family 1. *Biochemical Journal*, *370*, 793–804.
Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.
Murray, P., Aro, N., Collins, C., Grassick, A., Penttila, M., Saloheimo, M., et al. (2004). Expression in *Trichoderma reesei* and characterization of a thermostable family 3 β-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. *Protein Expression and Purification*, *38*, 248–257.
Nguyen, T. N. P., Lee, K. M., Lee, K. M., Kim, I. W., Kim, Y. S., Jeya, M., et al. (2010). One-step purification and characterization of a β-1,4-glucosidase from a newly isolated strain of *Stereum hirsutum*. *Applied Microbiology and Biotechnology*, *87*, 2107–2116.
Peralta, R. M., Kadowaki, M. K., Terenzi, H. F., & Jorge, J. A. (1997). A highly thermostable β-glucosidase activity from the thermophilic fungus *Humicola grisea* var. *thermoidea*: Purification and biochemical characterization. *FEMS Microbiology Letters*, *146*, 291–295.
Ramachandran, P., Tiwari, M. K., Singh, R. K., Haw, J. R., Jeya, M., & Lee, J. K. (2012). Cloning and characterization of a putative β-glucosidase from (NfBGL595) from *Neosartorya fischeri*. *Process Biochemistry*, *47*, 99–105.
Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovski, S., McCallum, C. M., & Henikoff, S. (1998). Consensus degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Research*, *26*, 1628–1635.
Spiridonov, N. A., & Wilson, D. B. (2001). Cloning and biochemical characterization of BglC, a β-glucosidase from the cellulolytic actinomycete *Thermobifida fusca*. *Current Microbiology*, *42*, 295–301.
Takashima, S., Nakamura, A., Hidaka, M., Masaki, H., & Uozumi, T. (1999). Molecular cloning and expression of the novel fungal β-glucosidase genes from *Humicola grisea* and *Trichoderma reesei*. *Journal of Biochemistry*, *125*, 728–736.
Xu, H., Xiong, A. S., Zhao, W., Tian, Y. S., Peng, R. H., Chen, J. M., et al. (2010). Characterization of a glucose-, xylose-, sucrose-, and D-galactose-stimulated β-glucosidase from the alkalophilic bacterium *Bacillus halodurans* C-125. *Current Microbiology*, *42*, 295–301.
Yang, S. Q., Wang, L. J., Yan, Q. J., Jiang, Z. Q., & Li, L. T. (2009). Hydrolysis of soybean isoflavone glycosides by a thermostable β-glucosidase from *Paecilomyces thermophila*. *Food Chemistry*, *115*, 1247–1252.
Yang, S. Q., Yan, Q. J., Jiang, Z. Q., Li, L. T., Tian, H. M., & Wang, Y. Z. (2006). High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. *Bioresource Technology*, *97*, 1794–1800.
Zhou, C., Qian, L., Ma, H., Yu, X., Zhang, Y., Qu, W., et al. (2012). Enhancement of amygdalin activated with β-D-glucosidase on HepG2 cells proliferation and apoptosis. *Carbohydrate Polymers*, *90*, 516–523.