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Original Article

Expression and characterization of a novel highly glucose-tolerant β-glucosidase from a soil metagenome

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A β-glucosidase gene *unbgl1A* was isolated by the function-based screening of a metagenomic library and the enzyme protein was expressed in *Escherichia coli*, purified, and biochemically characterized. The enzyme Unbgl1A had a K_m value of 2.09 ± 0.31 mM, and a V_{max} value of 183.90 ± 9.61 μmol min^{-1} mg^{-1} under the optimal reaction conditions, which were pH 6.0 at 50°C. Unbgl1A can be activated by a variety of monosaccharides, disaccharides, and NaCl, and exhibits a high level of stability at high concentration of NaCl. Two prominent features for this enzyme are: (i) high glucose tolerance. It can be tolerant to glucose as high as 2000 mM, with K_i = 1500 mM; (ii) high NaCl tolerance. Its activity is not affected by 600 mM NaCl. The enzyme showed transglucosylation activities resulting in the formation of cellotriose from cellobiose. These properties of Unbgl1A should have important practical implication in its potential applications for better industrial production of glucose or bioethanol started from lignocellulosic biomass.

Keywords β-glucosidase; glucose tolerance; metagenome

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Introduction

β-Glucosidase (β-glucoside glucohydrolase; EC 3.2.1.21) that catalyzes the hydrolysis of alkyl- and aryl-β-glycosides as well as di- and oligosaccharides, is an important industrial enzyme [1]. β-Glucosidases have been used in the production of soy isoflavones and flavonoids of *Ginkgo biloba*. Some β-glucosidases also have transglucosylation activities, which are responsible for the synthesis of gentiooligosaccharide [2,3]. Moreover, β-glucosidases are important components of the cellulase complex and key rate-limiting enzymes in cellulose degradation in the process of breakdown of cellulosic oligosaccharides into glucose [4].

Cellulose is the most abundant and cheapest renewable resource on the Earth, and plays a vital role in solving the energy and environmental crisis [5]. However, in most cases, cellulose cannot be used unless it is degraded into glucose. Cellulase is a class of enzymes that depolymerize cellulose to glucose, and it can be divided into three categories: (i) endoglucanase (EC 3.2.1.4), which randomly hydrolyzes long-chain cellulose to generate short-chain cellulose; (ii) exocellobiohydrolase (EC 3.2.1.91), which hydrolyzes short-chain cellulose to generate cellobiose; and (iii) β-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose to produce glucose and results in mitigating the product (cellobiose) inhibition on endoglucanase and exocellobiohydrolase [6–8]. During the whole reaction process, β-glucosidase is the rate-limiting enzyme, which is the bottleneck of glucose production by biological degradation [9]. Therefore, it is critical to develop a new type of β-glucosidase with high catalytic activity and glucose tolerance for industrial applications.

Most microorganisms in nature could not be purely cultured in the laboratory. Hence, metagenomic techniques are culture-independent methods that are developed to access the genetic resources of environmental microbial world [10]. The metagenomic approach has been used to clone enormous enzymes of industrial importance from the environment [11].

In the present study, using the activity screening strategy, the *unbgl1A* gene coding for β-glucosidase was discovered and isolated from the metagenomic library of enriched sugar soil, and then the enzymatic properties were characterized. To our knowledge, this is the first report of a metagenome-derived β-glucosidase isolated from the enriched sugar soil.

Materials and Methods

Metagenomic library construction

Soil samples were collected from the sugar refinery (Pumiao, China). DNA was extracted and purified based on

the method of Pang *et al.* [12]. A DNA library was constructed using the copy control Fosmid library product kit (Epicentre, Madison, USA) according to the manufacturer's instructions.

Library screening and gene cloning

Clones of the Fosmid DNA library were screened on Luria-Bertani (LB) agar plates supplemented with esculin hydrate and ferric ammonium citrate in order to detect β-glucosidase activity as described in the method of Eberhart *et al.* [13]. Clones of the Fosmid DNA library on LA plates [14] were screened by transferring them to the LA plates supplemented with esculin hydrate and ferric ammonium citrate and then incubated at 37°C for 15 h. The positive clones were digested with *Bam*HI and the fragments were sub-cloned into a pUC19 vector. The sub-cloned fragments were then sequenced from both ends by TaKaRa (Dalian, China).

DNA sequence analysis

Sequences assembly and open reading frame (ORF) prediction were carried out with Vector NTI program (Invitrogen, Carlsbad, USA). Sequence similarity searches were performed with the BLAST tools (www.ncbi.nlm.nih.gov). Protein domain analysis was performed with SMART tool (Simple Modular Architecture Research Tool) on SMART server (http://smart.embl-heidelberg.de). Alignment analysis was performed with Jalview using the online MUSCLE algorithm (http://www.ebi.ac.uk/Tools/muscle/). The phylogenetic trees were constructed and the figures were produced with Jalview.

Recombinant protein production and purification

The *unbgl1A* gene was amplified by polymerase chain reaction (PCR) with the following primers: sense primer, 5′-ACTCCATGGTG**CACCACCACCACCACCAC**ACCCACCCGCTCGACCGCACCG-3′ containing an *Nco*I site (underline) and His_6 tag at the 5′-end (bold), and anti-sense primer, 5′-CATAAGCTTCTACGCGGCCTTTTGCCCCCCG-3′containing a *Hin*dIII site at the 5′-end (underline). The conditions of the PCR were: 95°C for 2 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR product was digested with *Nco*I and *Hin*dIII, and then ligated directly into the pSE380 expression vector (Invitrogen) that was digested with the same enzymes. The resulting recombinant plasmid was transformed into *Escherichia coli* BL21. The transformants were screened by activity assay on LB agar plates with 0.1% esculin hydrate, 0.2% ferric ammonium citrate, 100 μg/ml ampicillin, and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and then digested with *Nco*I and *Hin*dIII to select the positive clones. The transformants were incubated in LB medium with ampicillin (100 μg/ml) at 37°C until $OD_{600} = 0.6$. Then, 1 mM IPTG was added to the broth and the incubation was continued at 37°C for another 10 h to induce the expression of Unbgl1A. Cells were harvested by centrifugation, washed twice with 100 mM phosphate buffer solution (pH 7.6), and lysed by sonication in 10 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The lysate was centrifuged at 13,400 *g* for 30 min at 4°C, and then purified by affinity chromatography using nickel−nitrilotriacetic acid (Ni−NTA; Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and mass spectrometry (MS). The protein purity was analyzed with high performance liquid chromatography (HPLC) using a 4.6 mm × 50 mm Agilent Macroporous Reversed-Phase C18 column (Santa Clara, USA). The 97% solvent A (water/0.1% trifluoroacetic acid) and 3% solvent B (acetonitrile/0.08% trifluoroacetic acid) were used as eluting buffer. The flow rate was 0.75 ml/min. The MS analysis was performed on the MALDI/TOF/TOF 5800 mass spectrometer (AB SCIEX, Foster City, USA) using sinapic acid as matrix.

Zymogram analysis

The zymogram analysis of Unbgl1A was determined by non-denaturing PAGE according to a published method [15] with minor modifications. The non-denaturing PAGE was performed with a 10% (w/v) acrylamide resolving gel and a 4% stacking gel at 100 V for 3 h. The gel was washed with distilled water three times and then was divided into two parts. One was prepared as a zymogram by incubating with 0.1% esculin hydrate and 0.25% ferric ammonium citrate at 37°C for 1 h; the other identical gel was stained with Coomassie Brilliant Blue R-250.

Enzyme assay

Enzyme activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG; Sigma, St Louis, USA) as a substrate according to Odoux *et al.* [16], with minor modifications. Briefly, 0.02 μg of suitably diluted enzyme was added to 130 μl of 100 mM citrate-phosphate buffer (pH 6.0) containing 2.5 mM of *p*-NPG. The reaction was carried out at 50°C for 15 min and stopped with the addition of 70 μl of 0.4 M Na_2CO_3. The release of *p*-nitrophenol (*p*-NP) was measured at 420 nm. Enzyme assays were performed under this condition, unless otherwise indicated. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-NP from *p*-NPG per minute. Specific activity was expressed in units of active enzyme in milligram of protein.

The profiles of activity versus pH and activity versus temperature were determined using the standard assay method. To measure the effect of pH on the activity of Unbgl1A, the enzyme activity was assayed in the range of

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pH 3.6−8.0 (100 mM citrate-phosphate buffer). To measure the effect of temperature on activity of Unbgl1A, the enzyme activity was assayed at various temperatures (25−70°C) for 15 min with *p*-NPG as the substrate in 100 mM citrate-phosphate buffer (pH 6.0).

Substrate specificity of the β-glucosidase was measured by incubating the purified enzyme in 100 mM citrate-phosphate buffer (pH 6.0) containing 2.5 mM aryl-glycosides (*p*-NP-β-D-glucopyranoside, *p*-NP-α-D-glucopyranoside, *p*-NP-β-D-cellobioside, *p*-NP-β-D-xyloside, and *p*-NP-β-D-galactoside, all from Sigma), or 0.2% (w/v) saccharides (cellobiose, sucrose, trehalose, lactose, cellulose, and maltose, all from Sigma) at 50°C for 15 min. The released glucose was determined using the glucose oxidase peroxidase reagent from Sigma. The activities were calculated from the liberated *p*-NP and the released glucose.

Various metal compounds (NaCl, KCl, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$, and $FeCl_3$), chelating agent (ethylenediaminetetraacetic acid (EDTA)), and surfactant (SDS) were added to optimal reaction systems, respectively, to investigate their effects on enzyme activity. The concentrations of metal ions, EDTA, and SDS were 5 mM, 5 mM, and 1% (w/v), respectively.

The effect of mono- or disaccharides on the enzyme activity was determined by using standard enzyme assay conditions in the presence of D-fructose, xylose, D-galactose, L-arabinose, lactose, maltose, or sucrose at concentrations ranging from 5% to 20% (w/v). The K_i value of glucose was defined as the amount of glucose required for inhibiting 50% of the β-glucosidase activity and was taken as the average value of two separate experiments performed in duplicate.

Enzyme kinetic parameters of Unbgl1A were obtained by measuring the rate of hydrolysis of *p*-NPG at various concentrations (1−10 mM) at 50°C for 15 min in 100 mM citrate-phosphate buffer (pH 6.0). The enzyme concentration was 15 μM and the enzymatic kinetic parameters, K_m and V_{max}, were determined from the Lineweaver−Burk plots using the computer program enzyme kinetics.

The product of the enzyme reaction was further analyzed by HPLC using the NH_2-Spherisorb column (0.25 cm × 30 cm) in Agilent 1100 system equipped with an evaporative light scattering detector. Acetonitrile/water (70 : 30) was used as the solvent, and the flow rate was set at 1 ml/min.

Statistical analysis

All of the experiments were carried out in triplicate. The data of tables were analyzed in a Microsoft excel spreadsheet where the average and standard error were determined. In addition, other experimental graphs were generated using the software GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). The error bars represent the standard deviation.

Results

Gene cloning and molecular analysis

A Fosmid library containing about 90,000 clones was constructed with the soil sample from the sugar refinery. From this metagenomic DNA library, a clone, named FBG1, was isolated by using the function-based screening strategy that allowed strong expression of β-glucosidase activity. As shown in Supplementary Fig. S1, a black spot surrounded the positive clone FBG1 against a plain background after 20 h at 37°C. The identified subclone, containing an ORF of 1410 bp, was studied in detail and named *unbgl1A*. This gene has been deposited in GenBank with the accession number JX566949. The ORF of the *unbgl1A* gene has 1410 nucleotides, with a G−C content of 72.25%. BLASTX analysis based on the GenBank database revealed that the deduced protein sequence of Unbgl1A had the highest homology to the β-glucosidase of *Streptomyces avermitilis* MA-4680 (identities 87%, positives 93%). The product of the *unbgl1A* gene has 469 amino acids, with a relative molecular weight of 51,669.5 Da and a theoretical isoelectric point of 5.45. Structural analysis using the SMART revealed that it contains a domain common to the glycosyl hydrolase family 1 (GH1).

Figure 1 shows the multiple alignments of the deduced amino acid sequence of Unbgl1A with related β-glucosidases from GH1, revealing the existence of several conserved regions. **Figure 2** presents the phylogenetic tree, showing the relationship between Unbgl1A and other β-glucosidases based on amino acid sequences. The phylogenetic tree also revealed that Unbgl1A is most closely related to β-glucosidase from *Thermus nonproteolyticus* HG102.

Expression and purification of Unbgl1A

The recombinant Unbgl1A was expressed in the *E. coli* BL21 strain. The soluble recombinant protein was purified with Ni−NTA by one-step performance. **Figure 3(A)** shows a major protein band with molecular weight of ∼52 kDa detected by SDS−PAGE.

The molecular weight of the purified Unbgl1A protein was analyzed by MS. **Figure 4(A)** shows the presence of a strong peak at 52,276 *m/z* and a weak peak at 26,172 *m/z*. The peak at 52,276 *m/z* represents the monomeric Unbgl1A protein, since the molecular weight measured from MS matches the calculated size deduced from the amino acid sequence of a single protein chain. The molecular weight of the Unbgl1A protein is 52,276 Da. Another peak at 26,172 *m/z* was originated from doubly charged ions of the monomeric protein.

The protein purity of Unbgl1A was determined by HPLC [**Fig. 4(B)**]. The retention time of a strong peak is at 9.7 min.

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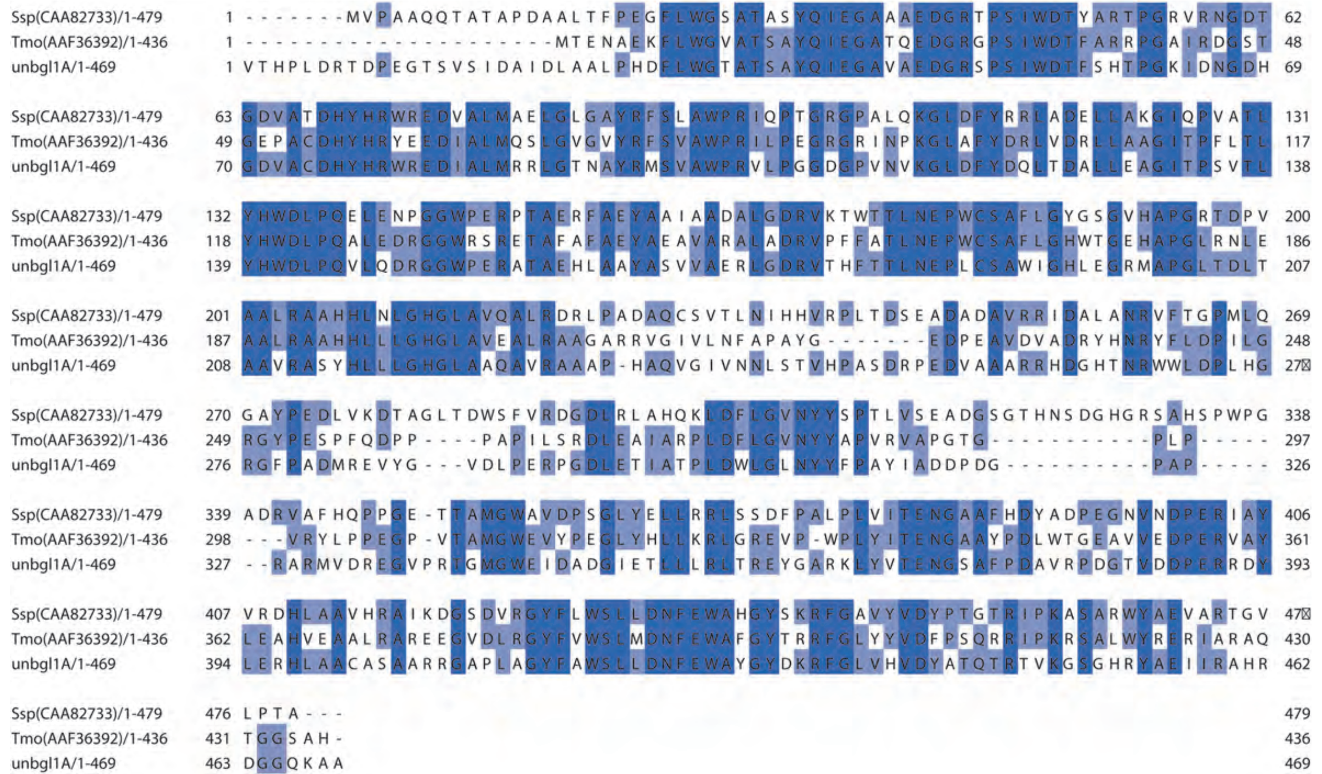

Figure 1 Unbgl1A sequence alignment with related β-glucosidase from GH1 The sequences were identified as follows: Ssp β-glucosidase from *Streptomyces* sp. (CAA82733); Tmo β-glucosidase from *T. nonproteolyticus* HG102 (AFF36392).

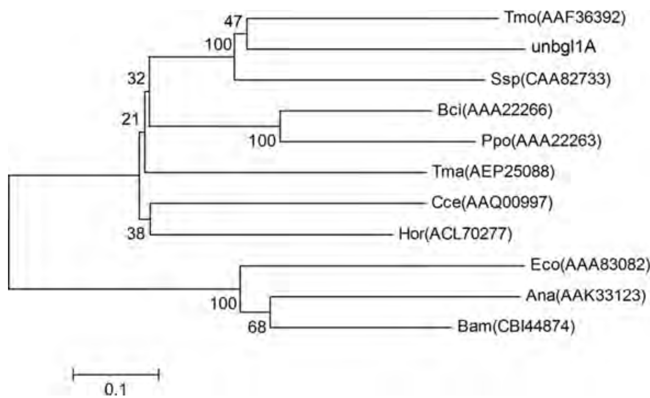

Figure 2 Phylogenetic relationship of Unbgl1A with other β-glucosidase The sequences were identified as follows: Ssp β-glucosidase from *Streptomyces* sp. (CAA82733); Tmo β-glucosidase from *T. non-proteolyticus* HG102 (AFF36392); Bci β-glucosidase from *Bacillus circulans* sub sp. Alkalophilus (AAA22266); Ppo β-glucosidase from *Paenibacillus polymyxa* (AAA22263); Tma β-glucosidase from *Thermotoga maritima* MSB8 (AEP25088); Cce β-glucosidase from *Clostridium cellulovorans* (AAQ00997); Hor β-glucosidase from *Halothermothrix orenii* H168 (ACL70277); Ana β-glucosidase from *Actinomyces naeslundii* (AAK33123); Bam β-glucosidase from *Bacillus amyloliquefaciens* DSM 7 (CBI44874).

The area of main peak was 94.7%, and the rest of the cumulative area of four small peaks was 5.3%. Results showed that the purity of the Unbgl1A protein was 94.7%.

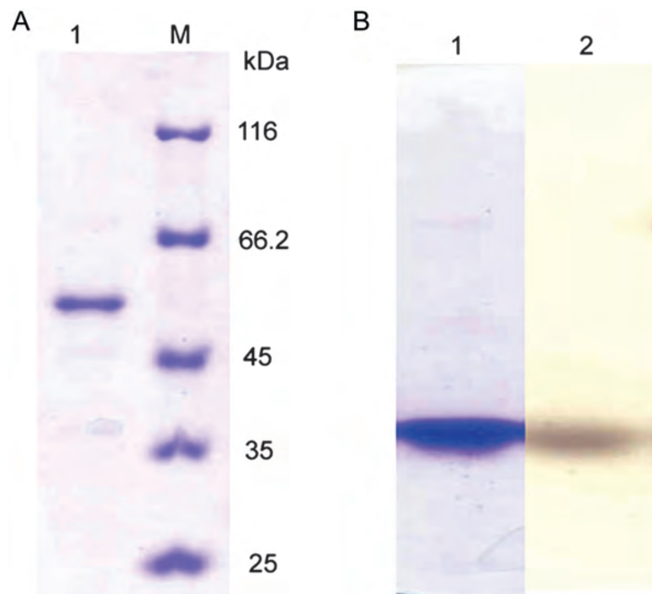

Figure 3 PAGE analysis of recombinant Unbgl1A (A) Purified recombinant Unbgl1A was detected by SDS–PAGE. Lane M: protein molecular weight marker. Lane 1: purified Unbgl1A. Recombinant protein was purified with Ni–NTA chromatography. (B) Purified recombinant Unbgl1A was analyzed by non-denaturing PAGE and zymogram. Lane1: recombinant Unbgl1A stained using Coomassie Brilliant Blue R250. Lane 2: recombinant Unbgl1A incubated with 0.1% esculin hydrate and 0.25% ferric ammonium citrate.

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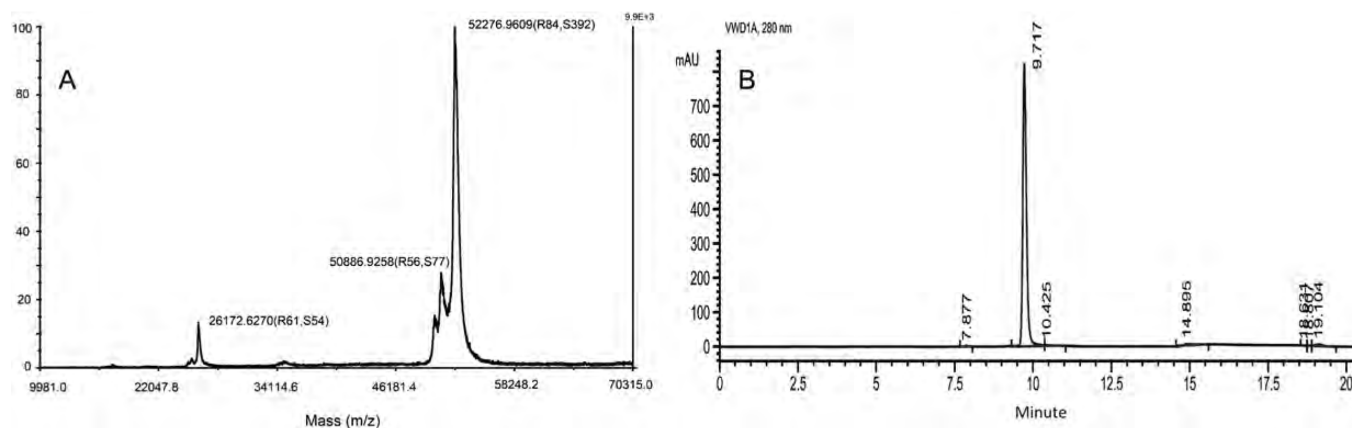

Figure 4 MS and protein purity analysis of recombinant protein Unbgl1A (A) The molecular weight of the purified Unbgl1A protein was analyzed by MS. (B) The protein purity of Unbgl1A was determined by HPLC.

Zymogram analysis

To verify that the purified proteins had retained proteolytic activity, a zymogram was performed. The zymogram showed that the single brown band of activity appeared on the gel. This result showed clearly that Unbgl1A was consisted of a single polypeptide, i.e. it was a monomeric enzyme [**Fig. 3(B)**].

The optimal pH and temperature of Unbgl1A

The activity of Unbgl1A at different pH (3.6−8.0) values was measured at 37°C using 2.5 mM *p*-NPG as the substrate. The optimal pH of the enzyme is around 6.0, because it showed maximal enzymatic activity [**Fig. 5(A)**]. Enzymatic activity remained at a high level with little change when the pH was between 5.0 and 7.0. However, the activity was dramatically decreased when pH value was below 4.0 or above 8.0. The optimal pH of Unbgl1A is similar to that of β-glucosidase purified from *Streptomyces* sp. QM-B814 [17].

Under the optimal pH condition (citric acid−disodium hydrogen phosphate buffer, pH 6.0), the activity of Unbgl1A at different temperatures (25−70°C) was measured. As shown in **Fig. 5(B)**, the enzyme activity of Unbgl1A was gradually increased when the temperature was elevated from 25 to 55°C, and reached the peak at 50°C, followed by a rapid decline of the activity. The activity remained only 40% at 60°C, and was fully abolished at 70°C. Therefore, the optimal temperature of the recombinant Unbgl1A was 50°C.

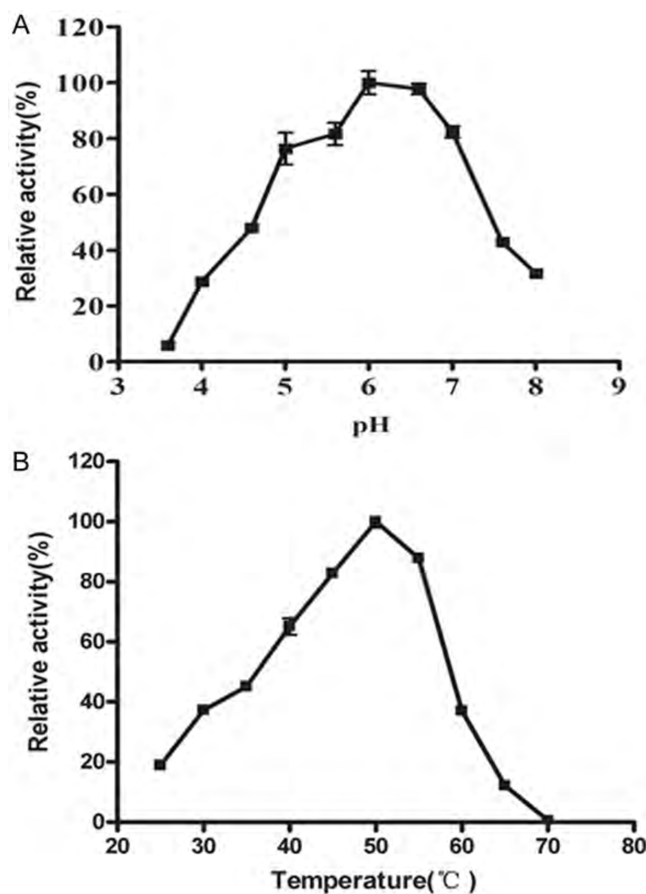

Figure 5 Effects of pH and temperature on the activity of recombinant Unbgl1A (A) Effects of pH on the activity of recombinant Unbgl1A. The enzyme activity was measured in 100 mM citrate-phosphate buffer (pH 3.0–8.0). (B) Effects of temperature on the activity of recombinant Unbgl1A. Experiments were conducted in a temperature range of 25–65°C, pH 6.0.

Effects of sugars on Unbgl1A activity

Monosaccharides and disaccharides could enhance the activity of the recombinant Unbgl1A in a dose-dependent manner. The 5% of xylose, D-galactose, lactose, and maltose could increase the Unbgl1A activity by 50%, 40%, 30%, and 10%, respectively (**Fig. 6**); 20% of D-fructose and 10% of L-arabinose increased the Unbgl1A activity by 50% and 25%, respectively [**Fig. 6(A)**]; and 20% sucrose increased the Unbgl1A activity by 25% [**Fig. 6(B)**]. Krisch *et al.* [18] reported that L-arabinose and D-galactose inhibited the activity of zygomycete *Rhizomucor miehei* β-glucosidase,

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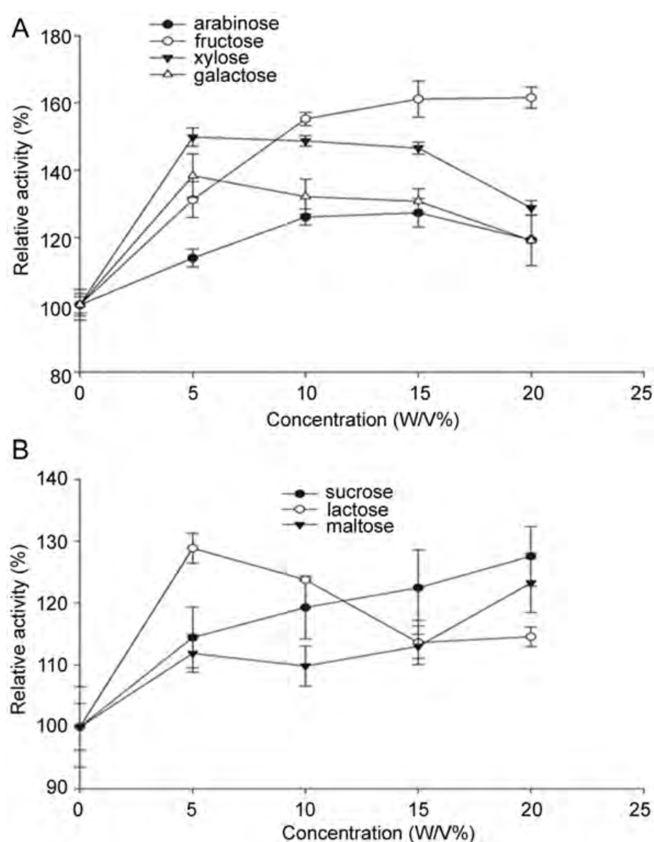

Figure 6 Effects of mono- and disaccharides on *p*-NPG hydrolysis by Unbgl1A (A) Effect of monosaccharide (L-arabinose, D-fructose, xylose, and D-galactose) on *p*-NPG hydrolysis by Unbgl1A. (B) Effect of disaccharides (sucrose, lactose, and maltose) on *p*-NPG hydrolysis by Unbgl1A. The activity measured without any additive (as control) was considered to be 100%.

resulting in more than 80% reduction in the presence of the highest sugar concentrations. In contrast, the enzyme activity was enhanced by up to 160% with addition of 15% (w/v) sucrose. According to these results, it can be hypothesized that mono- and disaccharides could probably act as glucose acceptors in a transglucosylation reaction, meaning that the enzymatic reaction can proceed in favor of product formation because the glucose was presumably consumed very fast in the transglucosylation step. **Figure 7** shows the Lineweaver–Burk plotting for Unbgl1A, from which our enzyme kinetic parameters were derived. Under the optimal conditions, Unbgl1A had a K_m value of 2.09 ± 0.31 mM and a V_{max} value of 183.90 ± 9.61 μmol min^{-1} mg^{-1}, when *p*-NPG was used as the substrate.

Effects of metal ions and other reagents

The effects of various metal ions, EDTA, and SDS on the hydrolysis activity of Unbgl1A under optimal conditions were shown in **Table 1**. The enzyme activity of Unbgl1A without metal ions was taken as 100%. Results showed that K^+, Mg^{2+}, Mn^{2+}, Fe^{3+}, and Ca^{2+} could inhibit Unbgl1A activities. Significant inactivation was observed by adding

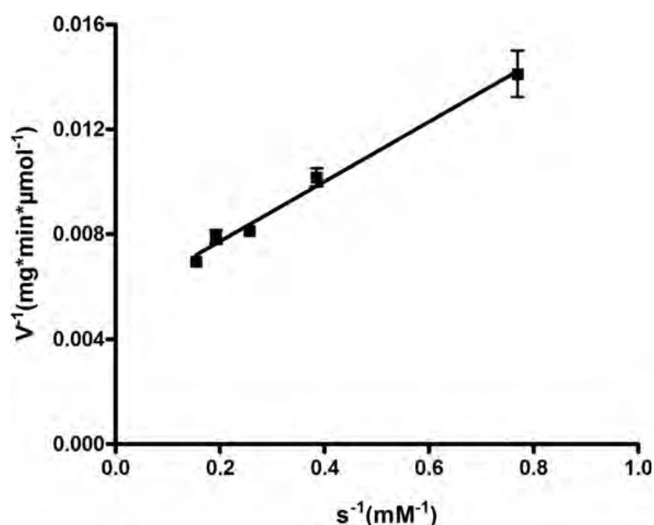

Figure 7 Lineweaver–Burk plot of Unbgl1A Unbgl1A had a K_m value of 2.09 ± 0.31 mM and a V_{max} value of 183.9 ± 9.61 μmol min^{-1} mg^{-1} in the presence of *p*-NPG.

Table 1 Effects of various metal ions, chelating agent, and surfactants on Unbgl1A activity

Reagents	Concentration	Relative activity (%)
Li^+	5 mM	212.0 ± 3.5
Na^+	5 mM	110.0 ± 4.7
K^+	5 mM	69.0 ± 0.9
Mg^{2+}	5 mM	40.0 ± 2.9
Mn^{2+}	5 mM	11.0 ± 1.1
Ca^{2+}	5 mM	3.0 ± 0.5
Zn^{2+}	5 mM	111.0 ± 5.7
Cu^{2+}	5 mM	114.0 ± 3.6
Pb^+	5 mM	88.0 ± 4.8
Fe^{3+}	5 mM	30.0 ± 3.2
Co^{2+}	5 mM	104.0 ± 2.1
EDTA	5 mM	90.0 ± 2.0
SDS	1% (w/v)	30.0 ± 5.6

The activity measured without any additive (as control) was considered to be 100%.

Ca^{2+}, Mn^{2+}, and SDS. Under optimal conditions, SDS could almost inactivate Unbgl1A, probably because SDS can destroy the 3D structure of Unbgl1A. In contrast, Li^+, Zn^{2+}, and Cu^{2+} could increase the Unbgl1A activity by 112%, 11%, and 12%, respectively, which is in contrast to previous reports [19,20], in which Cu^{2+} strongly inhibited β-glucosidase activity. EDTA slightly decreased the activity of Unbgl1A.

The activities of Unbgl1A measured in the presence of 20–600 mM NaCl indicated that, below 600 mM, NaCl did not inhibit the activity of Unbgl1A [**Fig. 8(A)**]. To our surprise, NaCl in fact has very strong stimulating effect on Unbgl1A

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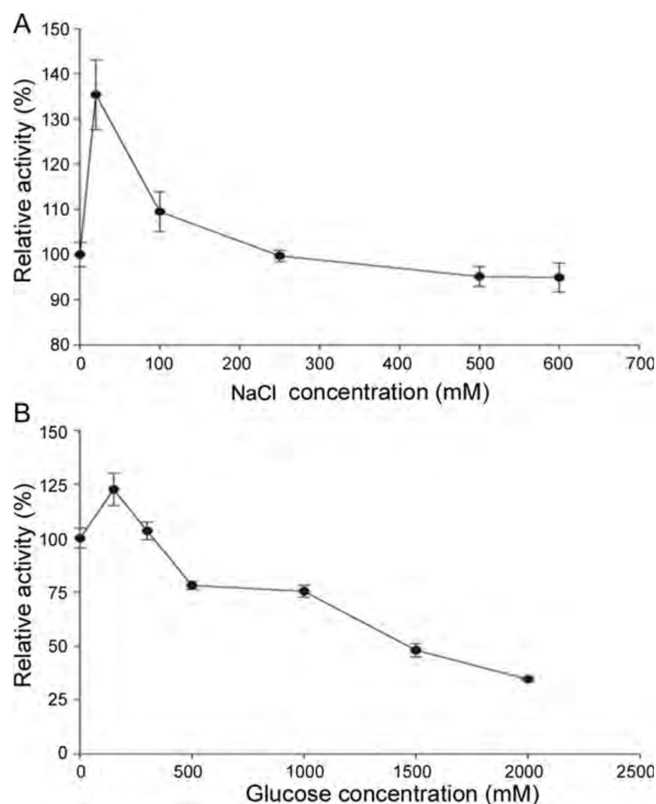

Figure 8 Effects of NaCl and glucose on Unbgl1A activity (A) Effect of NaCl on Unbgl1A activity. (B) Effect of glucose on Unbgl1A activity.

when its concentration is below 200 mM, and the strongest stimulating concentration is around 20 mM [**Fig. 8(A)**].

Substrate specificity and enzyme kinetics

Under optimal conditions, the Unbgl1A relative rates of hydrolysis on different substrates were measured (**Table 2**), including *p*-NP-β-D-galactopyranoside, *p*-NP-β-D-glucopyranoside, *p*-NP-β-D-glucopyranoside, *p*-NP-β-D-xylopyranoside, *p*-NP-β-D-cellobioside, and 0.2% of cellobiose, sucrose, trehalose, lactose, maltose, carboxy methyl cellulose (CMC), or xylan.

The enzyme was most active towards *p*-NPG and cellobiose, followed by *p*-NP-β-D-galactopyranoside and *p*-NP-β-D-cellobioside, which were 24% and 11.7%, respectively. The hydrolysis of substrates with a β-(1−4) glycosidic bond was much faster than those with α-(1−4) glycosidic bond. The enzyme could not hydrolyze sucrose, trehalose, CMC, or xylan. These observations indicated that Unbgl1A had strong activity to substrates with a β-(1−4) glycosidic bond, but had weak or no activity to substrates with other types of glycosidic bond. These results implied that Unbgl1A protein was a specific β-glucosidase.

Effect of glucose on enzyme activity

Different concentrations of glucose were added into the reaction system, and the activity was measured [**Fig. 8(B)**]. The concentrations of glucose below 300 mM stimulated slightly the *p*-NPG hydrolysis, reaching 120% at about 150 mM of glucose. When the glucose concentration was further increased, the enzyme activity of Unbgl1A was declined with a K_i of 1500 mM glucose, which was the highest K_i value for β-glucosidases known so far. This suggested that Unbgl1A was highly tolerant to glucose inhibition, which was desirable for practical applications in cellulose degradation.

Table 2 Substrate specificity assays

Substrate	Linkage of glycosyl group	Relative initial rate of hydrolysis (%)
Saccharides (2 mg/ml)		
Cellobiose	β-(1−4)Glc	100
Lactose	β-(1−4)Gal	9.5 ± 1.96
Maltose	α-(1−4)Glc	9.3 ± 0.68
Aryl-glycosides (2.5 mM)		
p-NPG	βGlc	100
p-NP-α-D-glucopyranoside	αGlc	7.9 ± 0.06
p-NP-β-D-cellobioside	βCel	11.7 ± 0.65
p-NP-β-D-galactopyranoside	βGal	24.2 ± 0.62
p-NP-β-D-xylopyranoside	βxyl	10.9 ± 0.57

Transglucosylation

The purified Unbgl1A was incubated with 35% (w/v) cellobiose with pH 6.0, at 37°C for 48 h. The equal volume mixture of inactivated Unbgl1A and cellobiose was used as the control (**Fig. 9**). Under this condition, cellobiose was hydrolyzed to produce glucose, and at the same time oligosaccharides (e.g. cellotriose) could be detected in the final product (**Fig. 9**), indicating that Unbgl1A possesses a transglucosylation activity that transfers a glucose unit to the receptor sugar (in this case, it is cellobiose). This result is consistent with the reported enzymes from *Stachybotrys microbispora* and zygomycete *R. miehei* [18,21]. Krisch and other groups showed that after 35% (350 mg/ml) cellobiose was mixed with β-glucosidases for 72 h, there was 86 mg/ml cellotriose generated [18,21,22]. Cellobiose may serve as the glucosyl donor in the transglucosylation by β-glucosidase. However, cellobiose could not serve as donor when β-glucosidase from *Trichoderma citrinoviride* was used [17].

Discussion

β-Glucosidase is an important component of the cellulase complex and a key rate-limiting enzyme in cellulose degradation. It is very sensitive to glucose inhibition [4]. β-Glucosidase hydrolyzes cellobiose to produce glucose,

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and the latter can be easily fermented by microbes to produce dozens of useful chemicals, such as ethanol [23]. At present, searching for novel β-glucosidases either with high glucose tolerance [24] or with ability to produce some specific medicines [25] has become a hot research spot, because this type of enzymes can diminish the inhibition of glucose and cellobiose generated during cellulose saccharification. It is reported that most of the microbial β-glucosidases are competitively inhibited by glucose and exhibit K_i values ranging from 100 to 500 mM [26,27]. However, some microbial β-glucosidases did show very high capacity for the glucose tolerance [24]. Till today, only a handful of microorganisms have been found to embody β-glucosidases showing high glucose tolerance with K_i values of more than 500 mM [27–31]. For example, β-glucosidases from marine microbial metagenome displayed high inhibition constant for glucose with K_i of 1000 mM [30], and only at glucose concentrations >300 mM, the β-glucosidase activity from *Streptomyces* sp. QM-B814 was slightly decreased [31]. Unbgl1A is a β-glucosidase with high glucose-tolerance ability with K_i for glucose as high as 1500 mM, which is the highest reported so far. The recombinant Unbgl1A is not only resistant to glucose inhibition, but also is activated by glucose at concentrations <300 mM. When glucose concentration was increased, the enzyme activity of Unbgl1A gradually declined, finally reaching a K_i value of 1500 mM. The high glucose tolerance of Unbgl1A enables the enzyme to survive at high concentrations of glucose during the process of cellulose saccharification, and thus to have great potential in biofuel production.

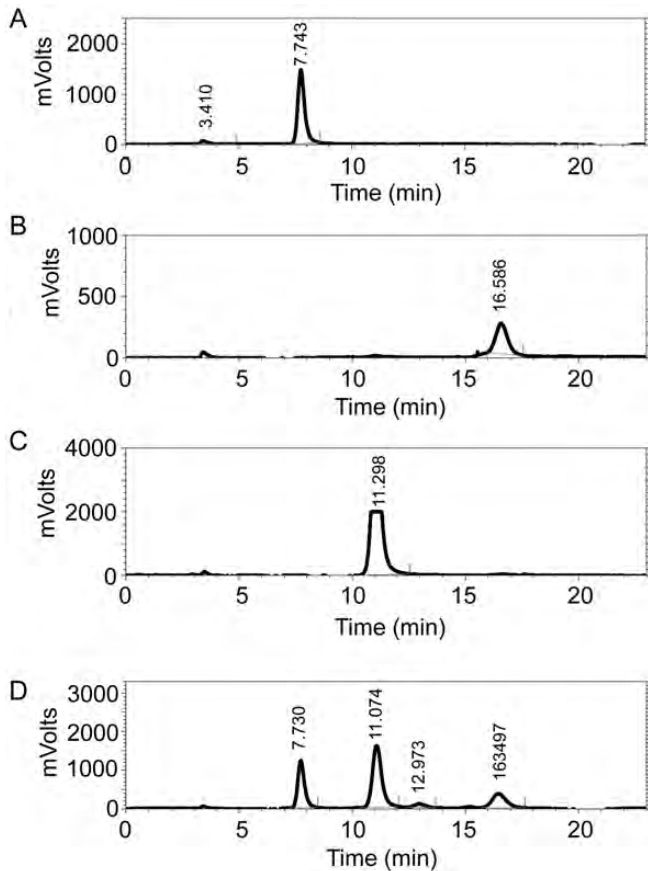

Figure 9 HPLC analysis of the hydrolysis products of cellobiose by Unbgl1A (A) Standard glucose. (B) Standard cellotriose. (C) Hydrolysis products of control. (D) Hydrolysis products of cellobiose by Unbgl1A.

The results of ion effects on Unbgl1A showed that this enzyme was significantly inhibited by Ca^{2+} and Mn^{2+}, and slightly stimulated by Zn^{2+} and Cu^{2+}, which differs from the situation in *Streptomyces* sp. QM-B814 β-glucosidase [31], where the enzyme was slightly stimulated by Ca^{2+} and Mn^{2+}, and rather strongly inhibited by Cu^{2+}. This could be related to its environmental origin since Unbgl1A is a metagenome-derived protein obtained from bare samples in the field, in which the genetic materials will be evolving to adapt to harsh conditions such as high concentration of metal ions. Unbgl1A shows a high tolerance to high concentration of NaCl. It was previously reported that 500 mM NaCl could decrease 14% activity of the β-glucosidase BglA, which was obtained from marine microbial metagenome [30]. In contrast, in the present

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Table 3 Comparison of glucose-tolerant glucosidase Unbgl1A and β-glucosidases from other microorganisms

Strains	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_i for glucose (mM)	References
Debaryomyces vanrijiae	0.77	668	439	[32]
Candida peltata	2.30	108	1400	[9]
A. oryzae	0.557	1063	1390	[33]
A. niger	21.70	124.4	543	[29]
Streptomyces sp. QM-B814	8.70	7.1	>500	[31]
Uncultured bacterium	0.39	15	1000	[30]
Uncultured bacterium	0.16	19.1	ND	[34]
Uncultured bacterium	0.19	4.75	ND	[35]
Uncultured bacterium	2.09	183.9	1500	This study

ND, not determined.

study, the activity of Unbgl1A was not affected by as much as 600 mM NaCl.

Table 3 shows a comparison of the properties of various β-glucosidases from a number of different sources. Unbgl1A shows very good kinetic characteristics, i.e. good conversion rate and high substrate affinity. With *p*-NPG as the substrate, Unbgl1A has a $V_{\max}$ of 183.90 ± 9.61 μmol min^{-1} mg^{-1} and a K_m of 2.09 ± 0.31 mM, which are much better than those of other β-glucosidases derived from metagenome by other research groups (**Table 3**). For instance, Jian and Wu [35] obtained a β-glucosidase from the metagenome, which showed a $V_{\max}$ of 4.75 μmol min^{-1} mg^{-1} and a K_m of 0.19 mM; and Kim *et al.* [34] obtained a β-glucosidase from the metagenome, which showed a $V_{\max}$ of 19.1 μmol min^{-1} mg^{-1} and a K_m of 19.1 mM.

To the best of our knowledge, the β-glucosidase from *Aspergillus oryzae* is so far the only one that shows comparable capacity in high tolerance to glucose and shows high enzyme activity for *p*-NPG [33]. In fact, the recombinant Unbgl1A has slightly higher tolerance to glucose than the β-glucosidase from *A. oryzae* (**Table 3**). In addition, the Unbgl1A also shows very good tolerance to high concentration of NaCl, and at the same time, this enzyme could be stimulated by a variety of monosaccharides, disaccharide, and again by NaCl. The mechanism of high glucose tolerance shown by Unbgl1A is not very clear at present. It can be speculated that Unbgl1A might transglycosylate any glucose to other sugar molecules (as acceptors), which relieves the effect of glucose on the enzyme. To prove this hypothesis, much work needs to be done. The first thing is to find the substrate/product-specific binding sites on Unbgl1A, and then mutagenesis should be brought to these sites to see whether the enzyme tolerance to high glucose concentration is affected or not.

In summary, we constructed a Fosmid library from enriched sugar soils and identified a novel β-glucosidase gene (*unbgl1A*) by function-based screening strategy. By detailed biochemical characterization of the recombinant β-glucosidase, we found that it is an excellent enzyme to be tested on cellulose hydrolysis. High glucose and high NaCl tolerances will probably make this enzyme to be the first choice of any attempt to utilize cellulosic feedstock to produce biofuels.

Supplementary data

Supplementary data are available at *ABBS* online.

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References

1 Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Oonanant W, Bevan DR and Esen A, *et al*. Structural insights into rice BGlu1 beta-glucosidase oligosaccharide hydrolysis and transglycosylation. J Mol Biol 2008, 377: 1200–1215.
2 Kono H, Kawano S, Tajima K, Erata T and Takai M. Structural analyses of new tri- and tetrasaccharides produced from disaccharides by transglycosylation of purified *Trichoderma viride* β-glucosidase. Glycoconj J 1999, 16: 415–423.
3 Seidle HF and Huber RE. Transglucosidic reactions of the *Aspergillus niger* Family 3 β-glucosidase: qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. Arch Biochem Biophys 2005, 436: 254–264.
4 Bhatia Y, Mishra S and Bisaria VS. Microbial β-glucosidases: cloning, properties, and applications. Crit Rev Biotechnol 2002, 22: 375–407.
5 Suphavadee C, Tachaapaikoon C, Pason P, Kyu KL, Kosugi A, Mori Y and Ratanakhanokchai K. Isolation and characterization of endocellulase-free multienzyme complex from newly isolated *Thermoanaerobacterium thermosaccharolytium* strain NOI-1. J Microbiol Biotechnol 2011, 21: 284–292.
6 George SP, Ahmad A and Rao MB. Studies on carboxymethyl cellulose produced by an alkalothermophilic actinomycete. Bioresour Technol 2001, 77: 171–175.
7 Shin HJ and Yang JW. Galactooligosaccharide synthesis from lactose by *Penicillium Funiculosum* cellulase. Biotechnol Lett 1996, 18: 143–144.
8 Williams PJ, Christopher RS, Bevan W and Massy-Westropp RA. Studies on the hydrolysis of vitis vinifera monoterpenne precursor compounds and model monoterpene β-*D*-glucosides rationalizing the monoterpene composition of grapes. J Agric Food Chem 1982, 30: 1219–1223.
9 Saha BC and Bothast RJ. Production, purification, and characterization of a highly glucose-tolerant novel β-glucosidase from *Candida peltata*. Appl Environ Microbiol 1996, 62: 3165–3170.
10 Green BD and Keller M. Capturing the uncultivated majority. Curr Opin Biotechnol 2006, 17: 236–240.
11 Richardson TH, Tan X, Frey G, Callen W, Cabell M, Lam D and Macomber J, *et al*. A novel, high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable alpha-amylase. J Biol Chem 2002, 277: 26501–26507.
12 Pang H, Zhang P, Duan CJ, Mo XC, Tang JL and Feng JX. Identification of cellulase genes from the metagenomes of compost soils and functional characterization of one novel endoglucanase. Curr Microbiol 2009, 58: 404–408.
13 Eberhart B, Cross DF and Chase LR. β-Glucosidase system of *Neurospora crassa* I. β-Glucosidase and cellulase activities of mutant and wild-type strains. J Bacteriol 1964, 87: 761–770.
14 Sambrook J and Russell DM. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 2001.
15 Painbeni E, Valles S, Polaina J and Flors A. Purification and characterization of a Bacillus polymyxa beta-glucosidase expressed in *Escherichia coli*. J Bacteriol 1992, 174: 3087–3091.
16 Odoux E, Escoute J, Verdeil JL and Brillouet JM. Localization of β-*D*-glucosidase activity and glucovanillin in vanilla bean (*Vanilla planifolia* Andrews). Ann Bot 2003, 92: 437–444.
17 Chandra M, Kalra A, Sangwan NS and Sangwan RS. Biochemical and proteomic characterization of a novel extracellular beta-glucosidase from *Trichoderma citrinoviride*. Mol Biotechnol 2013, 53: 289–299.
18 Krisch J, Bencsik O, Papp T, Vagvolgyi C and Tako M. Characterization of a b-glucosidase with transgalactosylation capacity from the zygomycete *Rhizomucor miehei*. Bioresour Technol 2012, 114: 555–560.
19 Dekker RF. Kinetic, inhibition and stability properties of a commercial β-*D*-glucosidase (cellobiase) preparation from *Aspergillus niger* and its

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suitability in the hydrolysis of lignocelluloses. Biotechnol Bioeng 1986, 28: 1438–1442.

20 Namchuk MN and Withers SG. Mechanism of agrobaterium β-glucosidase: kinetic analysis of the role of non-covalent enzyme/substrate interactions. Biochemistry 1995, 34: 16194–16202.

21 Saibi W, Amouri B and Gargouri A. Purification and biochemical characterization of a transglucosilating β-glucosidase of *Stachybotrys* strain. Appl Microbiol Biotechnol 2007, 77: 293–300.

22 Smaali I, Maugard T, Ferid L, Legoy MD and Marzouki N. Efficient synthesis of gluco-oligosaccharides and alkyl-glucosides by transglycosylation activity of β-glucosidase from *Sclerotinia sclerotiorum*. World J Microbiol Biotechnol 2007, 23: 145–149.

23 Healy FG, Ray RM, Aldrich HC, Wilkie AC, Ingram LO and Shanmugam KT. Direct isolation of functional genes encoding cellulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. Appl Microbiol Biotechnol 1995, 43: 667–674.

24 Pei JJ, Pang Q, Zhao LG, Fan S and Shi H. *Thermoanaerobacterium thermosaccharolyticum* β-glucosidase: a glucose-tolerant enzyme with high specific activity for cellobiose. Biotechnol Biofuel 2012, 5: 1–10.

25 Wei Y, Zhao W, Zhang Q, Zhao Y and Zhang Y. Purification and characterization of a novel and unique ginsenoside Rg1-hydrolyzing β-*D*-glucosidase from *Penicillium sclerotiorum*. Acta Biochim Biophys Sin 2011, 43: 226–231.

26 Karnchanatat A, Petsom A, Sangvanich P, Piaphukiew J, Jwhalley JS, Reynolds CD and Sihanonth P. Purification and biochemical characterization of an extracellular β-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.Fr.) Rehm. FEMS Microbiol Lett 2007, 270: 162–170.

27 Mamma D, Hatzinikolaou DG and Christakopoulos P. Biochemical and catalytic properties of two intracellular β-glucosidases from the fungus *Penicillium decumbens* active on flavonoid glucosides. J Mol Catal B Enzym 2004, 27: 183–190.

28 Decker CH, Visser J and Schreier P. β-glucosidase multiplicity from *Aspergillus tubingensis* CBS 643.92: purification and characterization of four β-glucosidases and their differentiation with respect to substrate specificity, glucose inhibition and acid tolerance. Appl Microbiol Biotechnol 2001, 55: 157–163.

29 Yan TR and Liau JC. Purification and characterization of a glucose-tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. Biosci Biotechnol Biochem 1997, 61: 965–970.

30 Fang Z, Fang W and Liu J. Cloning and characterization of a β-glucosidase from marine microbial metagenome with excellent glucose tolerance. J Microbiol Biotechnol 2010, 20: 1351–1358.

31 Perez-Pons JA, Rebordosa X and Querol E. Properties of a novel glucose-enhanced β-glucosidase purified from *Streptomyces* sp. (ATCC 11238). Biochim Biophys Acta 1995, 1251: 145–153.

32 Belancic A, Gunata Z, Vallier MJ and Agosin E. β-Glucosidase from the grape native yeast *Debaryomyces Vanrijiae*: purification, characterization, and its effect on monoterpene content of a muscat grape juice. J Agric Food Chem 2003, 51: 1453–1459.

33 Riou C, Salmon JM, Vallier MJ, Günata Z and Barre P. Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. Appl Environ Microbiol 1998, 64: 3607–3614.

34 Kim SJ, Lee CM and Kim MY. Screening and characterization of an enzyme with β-glucosidase activity from environmental DNA. Microbiol Biotechnol 2007, 17: 905–912.

35 Jian C and Wu B. Characterization of a novel β-glucosidase-like activity from a soil metagenome. J Microbiol 2009, 47: 542–548.

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