Chem. Res. Chin. Univ., 2018, 34(2), 212—220 doi: 10.1007/s40242-018-7408-7

# Characterization of a Thermophilic Monosaccharide Stimulated *β*-Glucosidase from *Acidothermus cellulolyticus*

LI Yuwei[1,2], BU Mingwei[1], CHEN Peng[3], LI Xiaohong[4], CHEN Changwu[4], GAO Gui[1], FENG Yan[1], HAN Weiwei[1] and ZHANG Zuoming[1*]

*1. Key Laboratory for Molecular Enzymology & Engineering, Ministry of Education, School of Life Science, Jilin University, Changchun 130012, P. R. China;*
*2. State Key Laboratory of Supramolecular Structure and Materials, College of Chemistry, Jilin University, Changchun 130012, P. R. China;*
*3. The Second Hospital of Jilin University, Changchun 130022, P. R. China;*
*4. College of Food Engineering, Jilin Engineering Normal University, Changchun 130052, P. R. China*

**Abstract** The gene(*ABK51908*) from *Acidothermus cellulolyticus* encodes a mature protein of 484 residues with a calculated molecular mass of 53.0 kDa. Sequence analysis revealed that the protein had 59% identity to the *β*-glucosidases CAA82733, which belongs to glycoside hydrolase family 1(GH1). We cloned and expressed the gene in *Escherichia coli* BL21-Gold(DE3). The recombinant protein(AcBg) had an optimal pH and temperature of 7.0 and 70 °C, respectively. The specific activities of AcBg under optimal conditions were 290 and 33 U/mg for *p*-nitrophenyl-*β*-*D*-glucopyranoside(*p*NPG) and cellobiose, respectively. AcBg hydrolyzed the oligosaccharide sequentially from the non-reducing end to produce glucose units according to the results of HPLC analysis. AcBg showed high salt tolerance and monosaccharide-stimulation properties. Its activity rose more than 2-fold when 5 mol/L NaCl/KCl were added. The activity of the *β*-glucosidase was remarkably enhanced in the presence of 0.2 mol/L *D*-glucose(increased more than 1.9-fold), 0.1 mol/L *α*-methyl-*D*-glucose(increased more than 1.4-fold) and 1.0 mol/L *D*-xylose(increased more than 1.9-fold). The catalysis kinetics and structural changes in various concentrations of glucose were determined. The results indicate that glucose reduces substrate affinity and causes conformational changes.

**Keywords** *β*-Glucosidase; Monosaccharide-stimulation; Salt tolerance; Thermophilic enzyme

## 1 Introduction

Cellulose is the most abundant carbohydrate in nature and can be hydrolyzed by cellulases into fermentable sugars[1,2]. The enzymatic conversion of cellulose is accomplished by the synergistic action of at least three types of cellulases, which are endoglucanases(E.C. 3.2.1.4), cellobiohydrolases or exoglucanases(E.C. 3.2.1.91) and *β*-glucosidases(E.C. 3.2.1.21)[3]. *β*-Glucosidase hydrolyzes cellobiose and other oligosaccharides into glucose[4]. This reaction is essential in the enzymatic hydrolysis of cellulose to eliminate the inhibition of cellobiose on endoglucanases and exoglucanases[5]. Therefore, *β*-glucosidase is considered to be the rate-limiting regulator in the degradation of cellulose[6]. In addition, *β*-glucosidases synthesize some chemical compounds, such as oligosaccharides, glycoconjugates and alkylglucosides[7,8]. A number of *β*-glucosidases have been utilized in different industrial fields, such as food, feed, textile, detergents, pharmaceutical and bioethanol conversion industries[9—12].

Up to this point in time, a number of *β*-glucosidases have been isolated and characterized from bacteria, fungi, plants and animals[10,13,14]. Some *β*-glucosidases exhibited favorable properties, such as broad substrate specificity[9,15], activation by cations[5,16] or anions[17], and transglycosylation activities[8]. Thermophilic enzymes often have advantages compared to their mesophilic counterparts[18] and several thermophilic *β*-glucosidases have been identified from thermophilic microorganisms. For example, the *β*-glucosidase from *Acidilobus saccharovorans* showed maximum activity at 93 °C and its half-life at 90 °C was approximately 7 h[19]. The *β*-glucosidase from the thermophilic fungus *Thermomucor indicae-seudaticae* N31 showed the highest catalytic activity at 75 °C and remained stable over the temperature range of 40—75 °C[20].

Glucose, an end-product cellobiose breakdown by *β*-glucosidase, often serves as an inhibitor of the catalytic process for most *β*-glucosidases[21,22]. A number of *β*-glucosidases that exhibit high glucose tolerance, and can even be stimulated by glucose, were recently discovered[14,23]. Interestingly, most glucose-tolerant *β*-glucosidases belong to the glycoside hydrolyses family 1(GH1), while GH3

*Corresponding author. E-mail: zmzhang@jlu.edu.cn
Received December 15, 2017; accepted January 25, 2018.
Supported by the National Basic Research Program of China(No.2012CB721003), the Sic-Tech Development Plan of Jilin Province, China(No.20170204040GX) and the Project of the Department of Education of Jilin Province, China(No.2016-438).

*β*-glucosidases are all inhibited by glucose. Thus, the discovery and mechanisms of glucose-tolerant *β*-glucosidases have received much attention in recent years. Giuseppe *et al*.[24] suggested that the entrance shape and electrostatic properties of the active site of *β*-glucosidases play key roles in glucose tolerance. It has recently been revealed that the glucose binding affinity/preference of several amino acids around the entrance and the middle of the substrate channel of *β*-glucosidases are regulated by the effects of glucose[25].

*Acidothermus cellulolyticus* 11B ATCC 43068, a thermophilic bacterium, was isolated from acidic hot springs in Yellowstone National Park, USA[26]. A number of thermophilic glycoside hydrolases from *A. cellulolyticus* have been characterized in recent years[27—29]. The recombinant proteins were all shown to have high thermostability and activity. In this paper, a *β*-glucosidase encoding-gene from *A. cellulolyticus* was cloned and expressed in *Escherichia coli*. The recombinant protein (AcBg) showed high thermostability and activity. Furthermore, the activity of AcBg was found to be stimulated by high concentrations of glucose, xylose and other monosaccharides. The mechanisms of glucose stimulation were also investigated by catalysis kinetics.

## 2 Materials and Methods

### 2.1 Strains, Vector and Materials

*A. cellulolyticus* 11B was cultured and DNA was isolated as previously described[28]. For cloning and expression of the AcBg gene(*ABK51908*), *E. coli* DH5*α*(Invitrogen, Carlsbad, CA, USA) and *E. coli* BL21-Gold(DE3)(Novagen, Madison, WI, USA) were used as the hosts for propagation and over-expression, respectively. The plasmid pET-20b(Novagen, Madison, WI, USA) was used as the expression vector. Restriction endonucleases, LA Taq DNA polymerase and DNA purification kits were obtained from TaKaRa(Dalian, China). T4 DNA ligase was purchased from NEB(New England Biolabs, USA). The enzyme assay substrates were all purchased from Sigma-Aldrich(St. Louis, MO, USA), unless otherwise stated. Regenerated amorphous cellulose(RAC) was prepared from Avicel® PH101(Fluka, Buchs, Switzerland) as described by Zhang[30]. All other chemicals were of analytical grade.

### 2.2 Gene Cloning

DNA manipulations were carried out as described previously[31]. *A. cellulolyticus* 11B genomic DNA was used as the template for PCR amplification. The AcBg gene was obtained by PCR amplification using the following primers: 5′-CGACTT***CATATG***ACACAAATCGAAGAGCG-3′(forward) and 5-ACCG***CTCGAG***TCAGGGCGCCGCGATCGTGTTC-3 (reverse). The restriction endonuclease sites for *Nde* I and *Xho* I are indicated in bold italics. The PCR conditions were the same as those described by Chen *et al.*[32], except for the annealing temperature, which was changed from 58 °C to 53 °C. The amplified PCR products were purified and digested with *Nde* I and *Xho* I restriction enzymes and then ligated to the vector pET-20b using T4 DNA ligase. The recombinant plasmid (*pAcBg*) was then transformed into *E. coli* DH5*α* and grown in a Luria-Bertani(LB) medium containing 50 μg/mL ampicillin. After the confirmation of the correct sequence(Comate Bioscience Co., Ltd., Changchun, China), the *pAcBg* plasmid was extracted and transformed into *E. coli* BL21-Gold(DE3).

### 2.3 Expression and Purification of *β*-Glucosidase

*E. coli* BL21-Gold(DE3) cells carrying *pAcBg* were grown to an $OD_{600}$ of 0.5—0.6 in an LB medium containing 50 μg/mL ampicillin at 37 °C and 200 r/min. Expression was induced by adding 1 mmol/L isopropyl-*β*-*D*-1-thiogalactopyranoside (IPTG), with an inducing temperature and time of 15 °C and 20 h, respectively, and the stirring speed was decreased to 120 r/min. The cells were then harvested by centrifugation at 4 °C for 30 min at 4000 r/min resuspended in buffer A(50 mmol/L Tris-HCl and 300 mmol/L NaCl, pH=7.9) and then disrupted by ultrasonic treatment at 4 °C. The crude extract was incubated at 60 °C for 30 min and the cell debris and the denatured proteins were removed by centrifugation at 12000*g* for 15 min at 4 °C. The supernatant was subjected to a Ni-NTA column chromatography(Qiagen, Valencia, CA, USA) with resin that had been previously equilibrated with buffer A. Elution was carried out with a linear gradient of imidazole in buffer A and the fractions with *β*-glucosidase activity were pooled. The pooled fraction was dialyzed against 50 mmol/L Tris-HCl(pH=7.9) buffer and loaded onto a HiTrapQ ion-exchange column(Amersham Biosciences). The proteins were then eluted with a linear gradient of 0—1 mol/L NaCl in 50 mmol/L Tris/HCl(pH=7.9) over 20 column volumes. Fractions with enzyme activity were dialyzed against 50 mmol/L Tris/HCl(pH=7.9) and concentrated by ultra-filtration. Purity of the protein sample was checked by 0.12 g/mL sodium dodecyl sulphate-polyacrylamide gel electrophoresis(SDS-PAGE) and the protein bands were visualized using Coomassie Brilliant Blue R-250 staining. Protein concentration was measured using the Bradford assay[33] with bovie serum albumin as the standard.

### 2.4 Enzyme Activity Assay

*β*-Glucosidase activity was determined by using 5 mmol/L *p*-nitrophenyl-*β*-*D*-glucopyranoside(*p*NPG) as a substrate. The reaction was performed at 70 °C for 1 min in 50 mmol/L sodium phosphate buffer(pH=7.0). The reaction was terminated by adding 200 μL of $Na_2CO_3$(1 mol/L) and placing on ice. The optical density was detected at 405 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmol of *p*-nitrophenol per min under the assay conditions. The molar extinction coefficient of *p*-nitrophenol at pH=9.5 was 18500 L $mol^{-1}$ $cm^{-1}$[34].

The kinetic parameters of AcBg were determined using *p*NPG as a substrate. The reaction mixture(950 μL) containing *p*NPG ranging from 0.166 μmol/L to 0.334 μmol/L was pre-incubated at 70 °C for 3 min and the reaction was initiated by the addition of AcBg(0.32 μg). The absorbance of the mixture at 405 nm was continually measured and the amount of *p*-nitrophenol produced was calculated using a molar extinction coefficient of 7250 L $mol^{-1}$ $cm^{-1}$ at pH=7.0[35]. The kinetic

constants($K_m$ and $V_{max}$) of AcBg toward *p*NPG were calculated using Lineweaver-Burk plot[35]. The effect of glucose on $K_m$ and $V_{max}$ of AcBg was also examined, where glucose was added in the reaction mixture to a final concentration of 0.2 or 1.0 mol/L.

### 2.5 Enzyme Characterization

To determine the *β*-glucosidase activity at different pH values, the reactions were performed at 70 °C in the indicated buffer for 5 min. The effect of temperature on *β*-glucosidase activity was determined in 50 mmol/L phosphate buffer (pH=7.0) by the incubating reaction mixtures at different temperatures ranging from 30 °C to 80 °C. For the measurement of thermostability, the enzyme was incubated at 70 or 75 °C for various lengths of time. The residual activity of enzyme was then determined by a standard method.

### 2.6 Effects of Metal Ions, Chemical Reagents and Sugars on *β*-Glucosidase Activity

The reaction mixture containing 50 mmol/L sodium phosphate(pH=7.0), *p*NPG(5 mmol/L) and the respective reagents was pre-incubated at 70 °C for 5 min. The reaction was initiated by adding purified and diluted AcBg and incubated at 70 °C for further 1 min. The reaction was terminated by the addition of 200 μL of $Na_2CO_3$(1 mol/L) and placed on ice. The optical density of the reaction mixture was measured at 405 nm with denatured enzyme(boiled for 5 min) as a blank control. The activity of AcBg without added reagents was considered as 100%. Sodium phosphate buffer was replaced with 50 mmol/L Tris-HCl(pH=7.0) when determining the effect of the following metal ions(5 mmol/L as the final concentration of metal ions): $Mg^{2+}$, $Ca^{2+}$, $Al^{3+}$, $Fe^{2+}$, $Mn^{2+}$, $Ba^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$. The concentration of $Na^+$ or $K^+$ ranged from 0 to 5 mol/L. The effect of chemical reagents(sodium dodecyl sulfate, ethylene diamine tetraacetic acid and *D*,*L*-dithiothreitol) on *β*-glucosidase activity was determined by adding them individually into the reaction mixture at a final concentration of 1.0%. The effect of monosaccharide, disaccharide and glycoside derivatives on enzyme activity was tested using 100 mmol/L *D*-galactose, *D*-rhamnose, *D*-arabinose, *D*-xylose, *D*-glucose, *D*-ribose, *L*-arabinose, *D*-fructose, *D*-mannose, *D*-trehalose, lactose, cellobiose, maltose, sucrose, *D*-mannitol, *D*-sorbitol, *α*-*D*-glucose-1-phosphate, *D*-galacturonic acid and *α*-methyl-*D*-glucose. The effects of *D*-glucose and *D*-xylose on the activity were also measured at a final concentration ranging from 0 to 2 mol/L and 0 to 4 mol/L, respectively.

### 2.7 Substrate Specificity

The specificity of purified AcBg towards various *p*-nitrophenyl(*p*NP) *β*-glycosides was determined by a standard method as mentioned above. In this case, the following substrates were used at a concentration of 5 mmol/L: *p*NPG, *p*NP-*β*-*D*-galactopyranoside(*p*NPGal), *p*NP-*α*-*D*-glucopyranoside(*p*NP-*α*-G), *p*NP-*β*-*D*-mannopyranoside (*p*NPMan), *p*NP-*β*-*D*-xylopyranoside(*p*NPXyl), *p*NP-*β*-*L*-arabinopyranoside(*p*NPAra), *p*NP-*N*-acetyl-*β*-*D*-glucosaminide (*p*NPGNAc), and *p*NP-*β*-*D*-cellobioside(*p*NPC). The activity of AcBg towards cello-oligosaccharides(DP 2—6), and disaccharides(maltose, sucrose, lactose and trehalose) was measured using a final concentration of 1 mmol/L for each compound. The generated glucose was determined using glucose assay kit(Changchun Huili Biotech Ltd., China). In assays for activity against polysaccharides(starch, CMC and RAC), the reaction mixture containing 0.01 g/mL of the respective substrate was incubated at 70 °C for 10 min and the same volume of DNS reagent[36] was added to stop the reaction. The reducing end generated from the polysaccharides by the enzyme was measured using the DNS method with glucose as a standard. In these assays, one unit of *β*-glucosidase activity was defined as the amount of enzyme required to release 1 μmol of glucose or reducing sugars per min under the test conditions.

The hydrolytic products resulted from AcBg activity on cellopentose were assessed by HPLC. The reaction mixtures (100 μL) containing 5 mmol/L substrate and 0.1 μg of enzyme were incubated at 70 °C for different time and terminated by boiling for 5 min. After centrifugation, the products were analyzed using a Waters 600 HPLC system(Waters Corporation, Milford, MA, USA) fitted with an XAmide column(4.6 mm× 250 mm, 5 μm particle size, Acchrom, Beijing, China). The mobile phase was acetonitrile/water[65/35(volume ratio), 0.22 μm filtered and degassed] with a flow rate of 1.0 mL/min at 25 °C.

### 2.8 Conformation Assay

The effect of glucose on the conformation change of AcBg was determined using circular dichroism(CD) and intrinsic fluorescence spectrum at room temperature. Purified AcBg was incubated in 50 mmol/L sodium phosphate(pH=7.0) containing various concentrations of glucose at 4 °C overnight. The above mixture was incubated at room temperature for 1 h before analysis. For CD spectra recordings, a Jasco J-810 spectrometer (Jasco Inc., Japan) was used with a 10 mm sample cell. The CD spectrum of AcBg was acquired between 200 nm and 250 nm, in triplicate. The fluorescence intensity of AcBg was monitored on an RF-5301PC spectrophotometer(Shimadzu, Kyoto, Japan) with 4 mm spectral bandwidths using a 0.1 cm×1.0 cm matched quartz cuvette. Excitation spectra at 280 nm were used and the emission spectra were acquired by scanning between 300 nm and 400 nm.

## 3 Results and Discussion

### 3.1 Cloning and Sequence Analysis of ABK51908

The gene *ABK51908* from the genome of *Acidothermus cellulolyticus* 11B, which encodes a 478-residue protein (Acel_0133) with a calculated mass of 53.0 kDa, was predicted to be a *β*-glucosidase belonging to glycoside hydrolase family 1(GH 1)(http://www.cazy.org). As predicted by the SignalP 4.0 program(http://www.cbs.dtu.dk/services/SignalP), no putative signal peptides were present in this protein, indicating that the *ABK51908* encoded an intracellular *β*-glucosidase. The

*ABK51908* gene was amplified by PCR from the genomic DNA of *A. cellulolyticus* 11B and inserted into plasmid pET-20b. After verifying the sequence, the reconstructed plasmid(*pAcBg*) was isolated from *E. coli* DH5*α* and transformed into *E. coli* BL21-Gold(DE3).

The protein sequence of Acel_0133, named AcBg, was compared with other characterized *β*-glucosidases of the GH1 family using BLAST[37] and a multiple alignment was performed using Clustal X. AcBg showed a relatively high identity to the other *β*-glucosidases, for example 59% identity to CAA82733, 54% to AAZ549, 52% to AAA25311, and 49% to AAA23091(Fig.1). The two active site residues, Glu178 and Glu382(AcBg numbering), which act as the acid-base and nucleophilic amino acid, respectively, are all conserved in these five *β*-glucosidases. Two substrate binding motifs, the (I/V)-(T/X)-E-N-G and T-(F/I/L)-N-E, are all highly conserved among GH1 family members[38]. Miao *et al.*[39] recently identified the substrate-binding residues in BglU from *Micrococcus antarcticus*. Comparably, the residues of AcBg and other four *β*-glucosidases that interact to the first monosaccharide from non-reducing end are well-conserved, while some of residues that bind to the second monosaccharide are not, for example, Asn235, Gln259 and Asn310(AcBg numbering). Furthermore, the six residues that bind to the first nonreducing monosaccharide are also well-conserved[38]. The sequence homology and identity of key residues supported the notion that the *ABK51908* gene encoded a *β*-glucosidase.

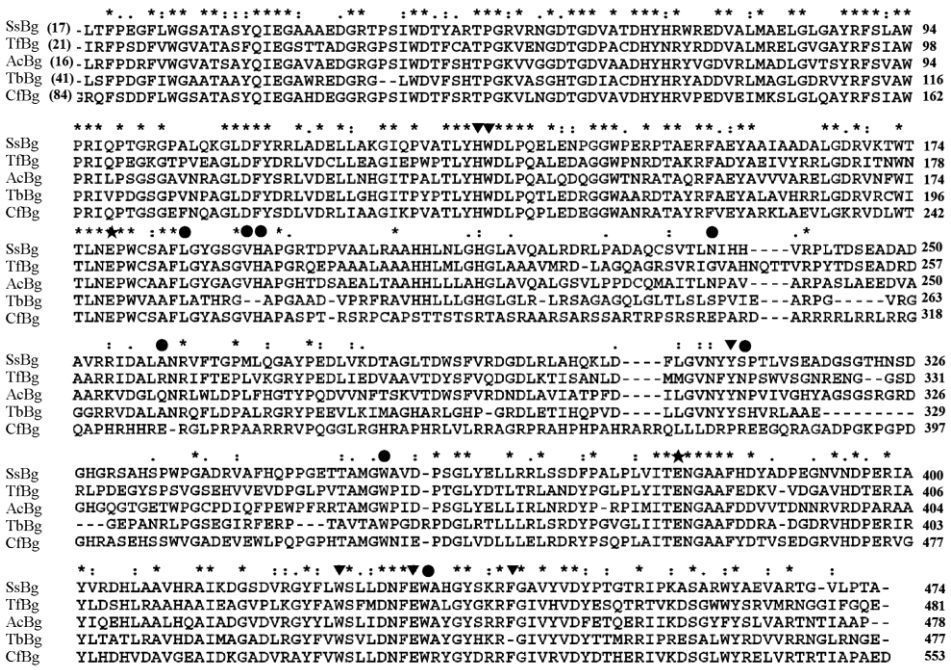


**Fig.1 Multiple alignment of AcBg with characterized *β*-glucosidases from bacteria**

Sequence alignment was performed with Clustal X1.8. The active site residues, Glu178(acid-base) and Glu382(nucleophilic), for AcBg are indicated by a star on the top of the sequence. The residues binding to the first and the second monosaccharide from non-reducing end are indicated by a solid triangle and circle, respectively. Identical, highly conserved and weakly conserved residues are indicated by "*", ":" and ".", respectively. SsBg: CAA82733 of *Streptomyces* sp.; TfBg: AAZ54975 of *Thermobifida fusca*; AcBg: ABK51908 of *Acidothermus cellulolyticus* 11B; TbBg: AAA25311 of *Thermobispora bispora*; CfBg: AAA23091 of *Cellulomonas fimi*.

## 3.2 Production and Purification of AcBg

The *ABK51908* gene encoding AcBg was expressed in *E. coli* BL21-Gold(DE3). As the produced protein was only found in inclusion bodies when the expression performed at 37 °C, the induction temperature was decreased to 15 °C and the incubation time was increased to 20 h. As indicated in Fig.2, the production level of soluble protein was still quite low. The crude extract was treated at 60 °C for 30 min and then centrifuged. The recombinant protein, AcBg, containing a C-terminal polyhistidine(6×His) tag, was further purified by affinity chromatography using Ni-NTA resin. After dialysis, the eluted fractions containing *β*-glucosidase activity were purified by ion exchange chromatography on a HiTrapQ ion-exchange column. Fractions with enzyme activity were pooled, dialyzed and concentrated to 1 mg/mL. The homogeneity and purity of the

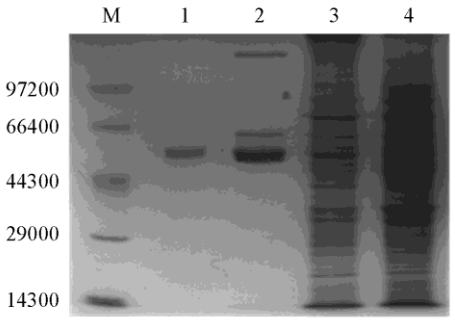


**Fig.2 SDS-PAGE of AcBg**

Lane M: protein marker; lane 1: the enzyme after ion-exchange chromatography, eluted in 50 mmol/L Tris-HCl buffer, 0.3 mol/L NaCl; lane 2: the enzyme after Ni-NTA affinity chromatography, eluted in 50 mmol/L Tris-HCl buffer, 0.3 mol/L NaCl, 0.05 mol/L imidazole, pH=7.9; lane 3: the protein after heat treatment at 60 °C for 30 min; lane 4: crude extract of *E. coli* BL21(DE3) with 1 mmol/L IPTG induction.

recombinant AcBg were analyzed by SDS-PAGE(Fig.2). The molecular weight of the recombinant AcBg was determined to be approximately 54 kDa by ImageJ(http://rsb.info.nih.gov/ij/), which agreed with the predicted molecular weight of the mature protein.

### 3.3 Characterization of AcBg

The properties of purified recombinant AcBg were determined using *p*NPG as the substrate(Fig.3). The optimum temperature for activity is found to be 70 °C[Fig.3(A)]. The activity of AcBg maintains more than 80% at 70 °C for 2 h，but decreases sharply at 75 °C. AcBg exhibits the highest *β*-glucosidase activity at around pH=7.0 and exhibits 80% of its maximum activity between pH=6.0 and pH=8.0[Fig.3(B)]. The recombinant AcBg presents a specific activity of 290 U/mg under the optimum conditions(70 °C, pH=7.0)(Table 1). The thermostability of AcBg was determined by incubating the protein at 70 and 75 °C for various time[Fig.3(C)]. The values of the half life of AcBg are 8 h at 70 °C and 60 min at 75 °C. The other glycoside hydrolyases that have recently been characterized from *A. cellulolyticus* were all shown to have high thermostability and activity[27—29].

In the past two decades, a number of thermophilic *β*-glucosidases from thermophiles have been characterized. In comparison, the *β*-glucosidase from hyperthermophilic archaea showed higher thermo stability than those from bacteria or fungi[40]. For instance, the *β*-glucosidase from the hyperthermophile *Pyrococcus furiosus* had an optimal activity at 105 °C with a half-life of 85 h at 100 °C[19]. The properties of thermostable *β*-glucosidases are highly suitable for the requirement of lignocellulose degradation[41]. In this study, AcBg showed high thermostability combined with glucose- enhancement activity, which give AcBg a high potential for use in industrial applications.

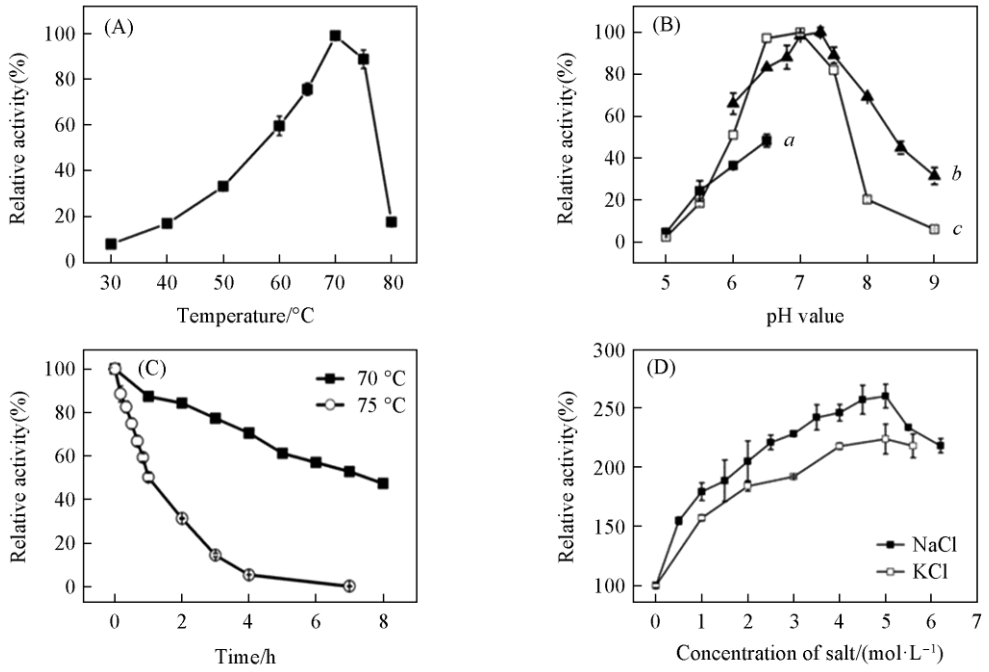


**Fig.3 Properties of recombinant AcBg**

(A) Effect of temperature on *β*-glucosidase activity; (B) effect of pH on the *β*-glucosidase activity: *a*. 50 mmol/L sodium acetate(pH=5.0—6.5); *b*. 50 mmol/L sodium phosphate(pH=6.0—9.0); *c*. 50 mmol/L Tris-HCl(pH=5.0—9.0); (C) thermostability of recombinant AcBg; (D) effects of salt(NaCl and KCl) on the activity of recombinant AcBg. Each value in the panels represents mean±SD(*n*=3).

The effects of divalent and trivalent cations on *β*-glucosidase activity are shown in Table 1. The enzyme activity is significantly enhanced by $Mg^{2+}$ and $Ca^{2+}$, but is inhibited by $Mn^{2+}$, $Fe^{2+}$, $Zn^{2+}$ and $Ni^{2+}$. $Ba^{2+}$, $Al^{3+}$ and $Co^{2+}$ have no obvious effects on enzyme activity. EDTA(0.3%), DTT(10 mmol/L), and SDS(0.1%) can be found to strongly reduce the enzyme activity to 39%, 11% and 0.6% compared to the control, respectively. While DMSO(10 mmol/L) has no effected, AcBg activity can be greatly enhanced by salt[Fig.3(D)]. Enzyme activities are increased to 260% and 220% in 5 mol/L NaCl and KCl solution, respectively. Enzyme activity is greater than twice as high under nearly saturated salt conditions. Recently, three *β*-glucosidases from *Streptomyces*[40], *Aspergillus niger*

**Table 1 Effects of cations and other reagents on the activity of AcBg**

| Reagent | Concentration | Relative activity(%) |
|---|---|---|
| None | — | 100±0.9 |
| $Mg^{2+}$ | 5 mol/L | 150±9.8 |
| $Ca^{2+}$ | 5 mol/L | 120±3.4 |
| $Ba^{2+}$ | 5 mol/L | 110±4.0 |
| $Al^{3+}$ | 5 mol/L | 110±4.5 |
| $Co^{2+}$ | 5 mol/L | 92±1.7 |
| $Ni^{2+}$ | 5 mol/L | 66±10 |
| $Zn^{2+}$ | 5 mol/L | 40±1.4 |
| $Fe^{2+}$ | 5 mol/L | 25±1.2 |
| $Mn^{2+}$ | 5 mol/L | 4.2±0.7 |
| DMSO | 10 mol/L | 99±5.2 |
| EDTA | 3 mg/mL | 39±4.7 |
| DTT | 10 mol/L | 11±1.9 |
| SDS | 1 mg/mL | 0.6±0.1 |

and *Bacillus* sp. SJ-10[42] were also reported to have salt tolerant activity. Bowers *et al.*[43] showed that salt induced p$K_a$ shifts and narrowed the pH profile of the *β*-glucosidase from Sigma-Aldrich. However, the pH profiles of salt tolerant *β*-glucosidases from *Bacillus* sp. SJ-10 and this work(data not shown) show that they can be not obviously affected by salts. Chamoli *et al.*[44] suggested that the interactions between the charged substrate and catalytic residues of *β*-glucosidase might be enhanced by salts and thus increased their activity. However, the mechanism of salt enhancement for activity remains unclear.

### 3.4 Substrate Specificity

As shown in Table 2, recombinant AcBg exhibits activity towards various substrates, including *p*-nitrophenyl(*p*NP) glycoconjugates, natural disaccharides and cello-oligosaccharides. *p*NPG, cellobiose and *p*NPGal(decreasing order) are the preferred substrates for AcBg. AcBg shows a high activity toward *p*NPG but lower activity with *p*NPGal. The activity of AcBg for other aryl-*β*-glycosides and aryl-*α*-glycosides is quite low. Furthermore, the activity of recombinant AcBg toward *α*-linked natural disaccharides(maltose, sucrose and trehalose) is also quite low. These results suggest that recombinant AcBg has a preference for hydrolyzing glucose and galactose in decreasing order, with a *β*-linked bond at the non-reducing end. The specificity of *β*-glucosidases for aryl-*β*-glycosides is a common property that has been described elsewhere[23]. However, AcBg presents unusual activity for some *α*-linked substrates. For example, the relative activities of AcBg for *p*NP-*α*-G and maltose are 8.5% and 29.6%, respectively, compared to their *β*-linked counterparts. Other microbial *β*-glucosidases commonly show negligible or no detectible activity towards *α*-linked substrates[23,43,45,46].

**Table 2 Substrate specificity of AcBg**

| Substrate | Specific activitiy/(U mg$^{-1}$) | Substrate | Specific activitiy/(U mg$^{-1}$) |
|---|---|---|---|
| *p*NPG | 290±13 | Cellobiose | 33±2.9 |
| *p*NPGal | 97±8.1 | Cellotriose | 26±3.3 |
| *p*NP-*α*-G | 25±2.6 | Cellotetraose | 28±1.3 |
| *p*NPMan | 15±5.5 | Cellopentose | 26±4.5 |
| *p*NPXyl | 10±3.5 | Cellohexaose | 17±3.2 |
| *p*NPAra | 6±2.9 | Maltose | 9.9±0.2 |
| *p*NPGNAc | 7.6±1.4 | Sucrose | 3.8±0.1 |
| *p*NPC | 5.7±3.2 | Lactose | Trace |

Cello-oligosaccharides from *n*=2 to *n*=6, starch, CMC (carboxymethylcellulose) and RAC(recyclable amorphous cellulose) were used as substrates to analyze the activity of recombinant AcBg. The enzyme exhibited the highest activity for cellobiose among the tested oligosaccharides but had no activity toward starch, CMC and RAC. Interestingly, the decreasing activity of AcBg toward oligosaccharides was independent on the chain length of the oligosaccharides, as the specific activities of AcBg with cellotetraose and cellopentose were all slightly higher than cellotriose. Additionally, AcBg had the same level of activity toward cello-oligosaccharides from *n*=2 to *n*=5, but much lower activity toward cellohexaose. These results suggested that AcBg had a preference for hydrolyzing oligosaccharides less than 5 glucose units. The catalytic process of recombinant AcBg against cellopentose was analyzed by HPLC(Fig.4). Only glucose can be determined while no cellobiose or cellotriose are observed within the first 1 min of incubation. As the incubation time progresses, the main glucose concentration increases dramatically, however, a few of cellobiose, cellotriose and cellotetraose are also determined. These results indicate that the enzyme hydrolyzed glucose units from oligosaccharides with a sequential pattern. Combined with the results of *p*NPG hydrolysis assay, AcBg was suggested to sequentially hydrolyze the glucose unit from the non-reducing end of oligosaccharide. AcBg could hydrolyze cello-oligosaccharides in a stepwise manner, a property that has been commonly found in microbial *β*-glucosidases[23]. AcBg showed the highest activity toward cellobiose in cello-oligosaccharides, but the specific activity of AcBg toward these substrates(except for cellohexaose) was at the same level. This property was different to other *β*-glucosidases. The *β*-glucosidases(NfBGL595) from *Neosartorya fischeri* could not hydrolyze cello-oligosaccharides(*n*=2—4)[46]. The activity of the *β*-glucosidase(Bgl) from *Streptomyces* sp. increased with the chain length of soluble cellodextrins[47], while the *β*-glucosidase Ks5A7 showed lower activity toward cellopentose compared to cellotetraose and cellotriose[48]. Thus, it seems that there are no common rules on the relative activity of *β*-glucosidases toward soluble cellodextrins of different chain lengths.

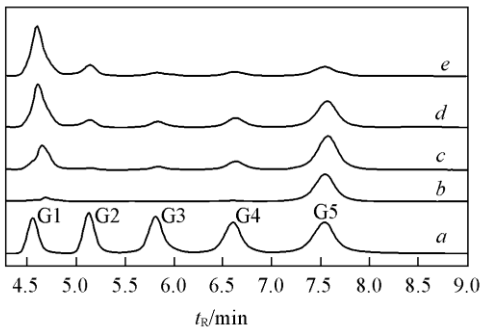


**Fig.4 HPLC analysis of AcBg hydrolysis products of cellopentose**

*a*. Standard oligosaccharides from Glc(G1) to cellopentose (G5); incubation time/min: *b*—*e*. 1, 30, 50, 90.

### 3.5 Effect of Sugars on the Activity and Conformation of AcBg

The effects of various monosaccharides(pentose and hexose), disaccharide and glycoside derivatives on the activity of recombinant AcBg were determined[Fig.5(A)]. The reaction mixture was composed of 50 mmol/L sodium phosphate(pH=7.0), substrate(5 mmol/L *p*NPG) and different sugars (100 mmol/L). The reaction was carried out by the addition of AcBg and then incubated at 70 °C for 1 min. The results indicate that most of the pentose do not affect the activity, except for *D*-xylose, which enhances the relative activity by 40%. *D*-Glucose and *D*-galactose of hexose also show increased activity of approximately 27% and 20%, respectively. Cellobiose can decrease the relative activity of AcBg by 40%.

*α*-Methyl-*D*-glucose enhances the activity(145%) while *D*-galacturonic acid inhibits the activity(27%). Because AcBg exhibits excellent glucose and xylose stimulation, the activities of AcBg supplemented with different concentrations of glucose and xylose were further investigated[Fig.5(B)]. AcBg shows the highest activity when supplemented with 0.2 mol/L glucose(192%) or 1.0 mol/L xylose(193%) and still maintains approximately 100% activity when the mixture contained 1.0 mol/L glucose(98%) or 3.0 mol/L xylose(112%). *β*-Glucosidases are often inhibited by glucose and act as rate-limiting component with endoglucanases and exoglucanases synergistically hydrolyzing the cellulose to fermentable sugars[49]. AcBg is found to be stimulated by glucose. The activity of *β*-glucosidases is remarkably enhanced in the presence of 0.2 mol/L *D*-glucose(rose more than 1.9-fold). Some *β*-glucosidases with high glucose tolerance or stimulation have also been characterized in recent years(Table 3). AcBg and other two *β*-glucosidases, from *T. thermarum* and an uncultured bacterium, show the highest glucose tolerance with a $K_i$ of 1500 mmol/L .

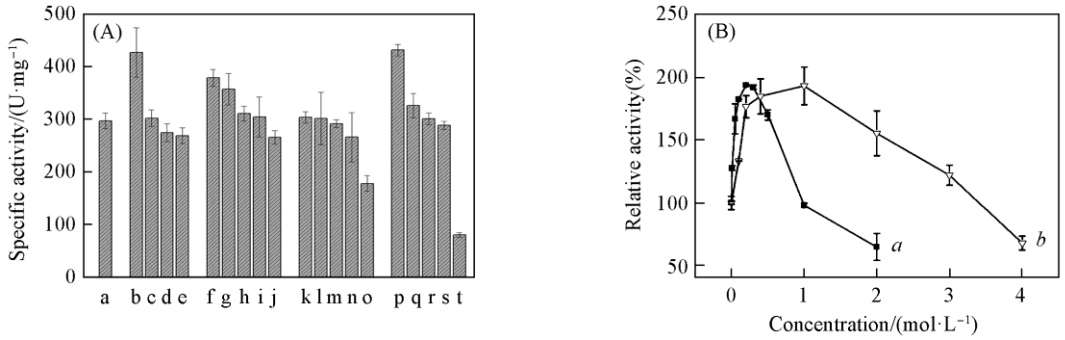


**Fig.5 Effects of various sugars on the activity of recombinant AcBg**

(A) Effects of monosaccharides, disaccharides and glycoside derivatives on the activity of AcBg. a. Control; b. *D*-xylose; c. *D*-arabinse; d. *D*-ribose; e. *L*-arabinose; f. *D*-glucose; g. *D*-rhamnose; h. *D*-galactose; i. *D*-mannose; j. fructose; k. lactose; l. maltose; m. *D*-trehalose; n. sucrose; o. cellobiose; p. methyl-*D*-glucose; q. *D*-sorbitol; r. *D*-mannitol; s. glucose-1-phosphate; t. *D*-galacturonic acid; b—e. pentose; f—j. hexose; k—o. disaccaride; p—t. derivant; (B) effects of glucose(*a*, 0—2 mol/L) and xylose(*b*, 0—4 mol/L) on the activity of AcBg. Each value in the panels represents mean±SD(*n*=3).

**Table 3 Characteristics of glucose-stimulated *β*-glucosidases**

| Strain | $K_i$/(mmol $L^{-1}$) | $K_m$/(mmol $L^{-1}$) | $V_{max}$/(U $mg^{-1}$) | Optimal temperature/°C | Ref. |
|---|---|---|---|---|---|
| *A. cellulolyticus* | 1500 | 0.4 | 290 | 70 | This paper |
| *T. thermarum* | 1500 | 0.59 | 142 | 90 | [16] |
| Uncultured bacterium | 1500 | 2.09 | 183.9 | 50 | [49] |
| *C. peltata* | 1400 | 2.3 | 221 | 50 | [50] |
| *A. oryzae* | 1360 | 0.55 | 3040 | 50 | [51] |
| *A. unguis* | 800 | 4.85 | 2.95 | 60 | [52] |
| *T. aotearoense* | 800 | 0.66 | 180.6 | 60 | [53] |
| *T. thermosacch* | 600 | 0.63 | 64 | 70 | [5] |
| *N. takasagoensis* | 600 | 0.67 | 8 | 65 | [14] |
| *A. niger* | 543 | 21.7 | 124.4 | 55 | [54] |
| *Streptomyces* sp. | >500 | 10.9 | 24.1 | 45 | [42] |

Stimulation of AcBg by such high concentration of various monosaccharides has rarely been reported. To reveal the mechanisms of stimulation by glucose, the kinetic parameters of AcBg were calculated at different glucose concentrations (Table 4). The kinetic parameters of AcBg toward *p*NPG were examined using different concentrations of glucose. The $K_m$ value for the hydrolysis of *p*NPG is 0.37 mmol/L, increasing approximately 10 and 13 fold when 0.2 and 1.0 mol/L glucose were added, respectively. The $V_{max}$ and $k_{cat}$ values of AcBg are all increased about 2.2 and 1.2 times when 0.2 and 1.0 mol/L glucose were added to the reaction mixture. The $k_{cat}/K_m$ value of AcBg toward *p*NPG decreases with increasing the glucose concentration. $k_{cat}/K_m$ value decreases about 4.5 and 11 fold when 0.2 and 1.0 mol/L glucose were added, respectively. $V_{max}$ of AcBg initially increases but decreases as glucose concentration further increases. Similar behavior was observed for other *β*-glucosidases, such as Bgl1A from *Halothermothrix orenii*[25]. In comparison, the $V_{max}$ value of many *β*-glucosidases decreased when the glucose concentration increased, such as for Bgl1B from *Paenibacillus polymyxa*[25]. The apparent $K_m$ of AcBg, on the other hand, increases with the addition of glucose, which is similar to those of the Bgl1A and Bgl1B.

**Table 4 Kinetic parameters of AcBg supplemented with different concentrations of glucose**

| *c*(Glucose)/ (mol $L^{-1}$) | $V_{max}$ / (μmol·$min^{-1}$) | $K_m$ / (mmol $L^{-1}$) | ($k_{cat}/K_m$)/ ( L $mmol^{-1}$ $s^{-1}$) |
|---|---|---|---|
| 0 | 95±1.4 | 0.4±0.04 | 72 |
| 200 | 210±4.2 | 3.5±0.08 | 17 |
| 1000 | 110±3.2 | 4.9±0.08 | 6.5 |

The conformational changes of AcBg in different concentrations of glucose were determined by circular dichroism(CD) spectra and intrinsic fluorescence spectroscopy. The CD spectra of AcBg in various concentrations of glucose are quite similar, indicating that the secondary structure of AcBg was not significantly affected by glucose[Fig.6(A)]. The intrinsic fluorescence spectra of AcBg were measured with various

concentrations of glucose[Fig.6(B)]. The emission maximum of AcBg can be found at 337 nm when 0, 0.1 and 0.2 mol/L glucose were added. The fluorescence intensity of AcBg decreases from 441.6 a.u.(0 mol/L glucose) to 437.7 a.u.(0.1 mol/L glucose) and 421.1 a.u.(0.2 mol/L glucose). However, a 2 nm blue-shift from 337 nm to 335 nm of the emission maximum can be observed when the concentration of glucose increases to 1 mol/L. At same time, the fluorescence intensity at the emission maximum increases to 433.5 a.u. Yang *et al.*[25] suggested that *β*-glucosidases have other glucose binding sites beyond the active site. Glucose binding to the active site reduces substrate accessibility and thus decreases enzymatic activity while binding to the other site may stimulate activity[25]. Souza *et al.*[11] showed an allosteric mechanism, in which the modulator binding site regulated the stimulation. In this study, the CD data of AcBg indicate that the secondary structure does not change when glucose was added. On the other hand, the conformation of AcBg as analyzed by intrinsic fluorescence changes when glucose was added. These results are quite similar to the results of other reports[11].

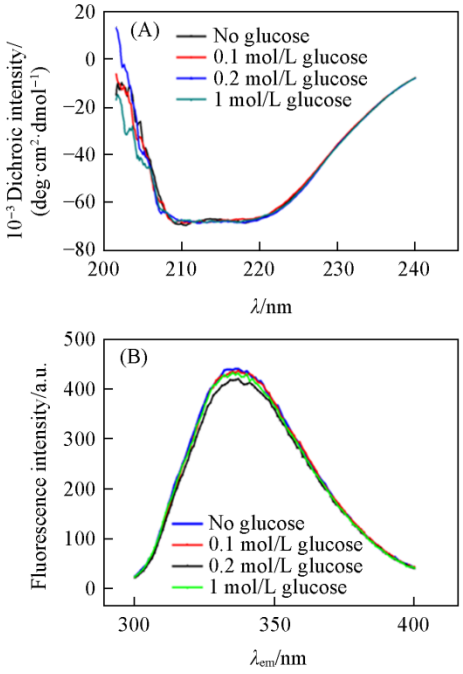


**Fig.6 Effects of different concentrations of glucose on dichroic spectra(A) and intrinsic fluorescence emission spectra(B) of recombinant AcBg**

## 4 Conclusions

We characterized a *β*-glucosidase(AcBg) from *A. cellulolyticus* 11B, which showed high thermostability and high salt-tolerance. AcBg had high activity toward a broad range of substrates. The activity of AcBg was stimulated by high concentrations of glucose, xylose and *α*-methyl-*D*-glucose.

## References

[1] Zhang Y. H. P., Himmel M. E., Mielenz J. R., *Biotechnol. Adv.*, **2006**, *24*, 452
[2] Gao S., Gao R., *Chem. Res. Chinese Universities*, **2017**, *33*(1), 1
[3] Beguin P., *Annu. Rev. Microbiol.*, **1990**, *44*, 219
[4] Vinzant T., Adney W., Decker S., Baker J., Kinter M., Sherman N., Fox J., Himmel M., *Appl. Biochem. Biotechnol.*, **2001**, *91*, 99
[5] Pei J., Pang Q., Zhao L., Fan S., Shi H., *Biotechnol. Biofuels*, **2012**, *5*, 1
[6] Chen M., Zhao J., Xia L., *Carbohyd. Polym.*, **2008**, *71*, 411
[7] Brimer L., Cicalini A. R., Federici F., Petruccioli M., *Arch. Microbiol.*, **1998**, *169*, 106
[8] Pal S., Banik S. P., Ghorai S., Chowdhury S., Khowala S., *Bioresour. Technol.*, **2010**, *101*, 2412
[9] Bhatia Y., Mishra S., Bisaria V., *Crit. Rev. Biotechnol.*, **2002**, *22*, 375
[10] Choi I. S., Wi S. G., Jung S. R., Patel D. H., Bae H. J., *J. Wood. Sci.*, **2009**, *55*, 329
[11] Souza F. H. M., Meleiro L. P., Machado C. B., Zimbardi A. L. R. L., Maldonado R. F., Souza T. A. C. B., Masui D. C., Murakami M. T., Jorge J. A., Ward R. J., *J. Mol. Catal. B: Enzym.*, **2014** ,*106*, 1
[12] Zhou C., Qian L., Ma H., Yu X., Zhang Y., Qu W., Zhang X., Xia W., *Carbohyd. Polym.*, **2012**, *90*, 516
[13] Kong F., Yang J., Zhen Z., *Chem. Res. Chinese Universities*, **2015**, *31*(5), 774
[14] Uchima C. A., Tokuda G., Watanabe H., Kitamoto K., Arioka M., *Appl. Environ. Microbiol.*, **2012**, *78*, 4288
[15] Zhou J., Zhang R., Shi P., Huang H., Meng K., Yuan T., Yang P., Yao B., *Appl. Microbiol. Biotechnol.*, **2011**, *92*, 305
[16] Zhao L., Xie J., Zhang X., Cao F., Pei J., *J. Mol. Cat. B: Enzym.*, **2013**, *95*, 62
[17] Li Y. K., Chang L. F., Shu H. H., Chir J., *J. Chin. Chem. Soc.*, **1997**, *44*, 81
[18] Bhalla A., Bansal N., Kumar S., Bischoff K. M., Sani R. K., *Bioresour. Technol.*, **2013**, *128*, 751
[19] Gumerov V. M., Rakitin A. L., Mardanov A. V., Ravin N. V., *Archaea*, **2015**, *2015*, 978632
[20] De C. P. J., Leite R. S., Prado H. F. A., Bocchini M. D. A., Gomes E., Silva R., *Appl. Biochem. Biotechnol.*, **2014**, *175*, 723
[21] Chen L., Li N., Zong M. H., *Biochem.*, **2012**, *47*, 127
[22] Krogh K. B., Harris P. V., Olsen C. L., Johansen K. S., Hojer Pedersen J., Borjesson J., Olsson L., *Appl. Microbiol. Biotechnol.*, **2010**, *86*, 143
[23] Jabbour D., Klippel B., Antranikian G., *Appl. Microbiol. Biotechnol.*, **2012**, *93*, 1947
[24] Giuseppe P. O., Souza F. H. M., Zanphorlin L. M., Machado C. B., Ward R. J., Jorge J. A., Furriel R. P. M., Murakami M. T., *Acta Crystallogr. Sect. D: Biol. Crystallogr.*, **2014**, *70*, 1631
[25] Yang Y., Zhang X., Yin Q., Fang W., Fang Z., Wang X., Zhang X., Xiao Y., *Sci. Rep.*, **2015**, *5*, 17296
[26] Barabote R. D., Xie G., Leu D. H., Normand P., Necsulea A., Daubin V., Médigue C., Adney W. S., Xu X. C., Lapidus A., *Geno. Res.*, **2009**, *19*, 1033
[27] Alahuhta M., Adney W. S., Himmel M. E., Lunin V. V., *Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun.*, **2013**, *69*, 1335
[28] Wang J., Gao G., Li Y., Yang L., Liang Y., Jin H., Han W., Feng Y., Zhang Z., *Int. J. Mol. Sci.*, **2015**, *16*, 25080
[29] Zhang Q., Zhang W., Lin C., Xu X., Shen Z., *Prot. Exp. Purif.*, **2012**, *82*, 279
[30] Zhang Y. H. P., Cui J., Lynd L. R., Kuang L. R., *Biomacromol.*, **2006**, *7*, 644
[31] Mamiatis T., Fritsch E., Sambrook J., Engel J., *Molecular*

*Cloning——A Laboratory Manual*, Cold Spring Harbor Laboratory, Wiley Online Library, New York, **1985**

[32] Chen Y., Sun D., Zhou Y., Liu L., Han W., Zheng B., Wang Z., Zhang Z., *Int. J. Mol. Sci.*, **2014**, *15*, 5717

[33] Bradford M. M., *Plant*, **1976**, *168*, 214

[34] Rauscher E., Neumann U., Schaich E., Von Bülow S., Wahlefeld A., *Clin. Chem.*, **1985**, *31*, 14

[35] Lineweaver H., Burk D., *J. Amer. Chem. Soc.*, **1934**, *56*, 658

[36] Miller G. L., *Anal. Chem.*, **1959**, *31*, 426

[37] Altschul S. F., Madden T. L., Schäffer A. A., Zhang J., Zhang Z., Miller W., Lipman D. J., *Nucl. Acid. Res.*, **1997**, *25*, 3389

[38] Badieyan S., Bevan D. R., Zhang C., *Biochem.*, **2012**, *51*, 8907

[39] Miao L. L., Hou Y. J., Fan H. X., Qu J., Qi C., Liu Y., Li D. F., Liu Z. P., *Appl. Environ. Microbiol.*, **2016**, *82*, 2021

[40] Mai Z., Yang J., Tian X., Li J., Zhang S., *Appl. Biochem. Biotechnol.*, **2013**, *169*, 1512

[41] Voorhorst W., Eggen R., Luesink E. J., De Vos W., *J. Bacteriol.*, **1995**, *177*, 7105

[42] Lee J. M., Kim Y. R., Kim J. K., Jeong G. T., Ha J. C., Kong I. S., *Biopro. Biosys. Eng.*, **2015**, *38*, 1335

[43] Bowers E. M., Ragland L. O., Byers L. D., *Biochim. Biophy. Acta*, **2007**, *1774*, 1500

[44] Chamoli S., Kumar P., Navani N. K., Verma A. K., *Int. J. Biol. Macromol.*, **2016**, *85*, 425

[45] Ramachandran P., Tiwari M. K., Singh R. K., Haw J. R., Jeya M., Lee J. K., *Biochem.*, **2012**, *47*, 99

[46] Shin K. C., Oh D. K., *J. Biotechnol.*, **2014**, *172*, 30

[47] Pérez-Pons J. A., Rebordosa X., Querol E., *Biochim. Biophy. Acta*, **1995**, *1251*, 145

[48] Uchiyama T., Yaoi K., Miyazaki K., *Front. Microbiol.*, **2015**, *6*, 548

[49] Lu J., Du L., Wei Y., Hu Y., Huang R., *Acta. Biochim. Biophy. Sin.*, **2013**, *45*, 664

[50] Saha B. C., Bothast R. J., *Appl. Environ. Microbiol.*, **1996**, *62*, 3165

[51] Riou C., Salmon J. M., Vallier M. J., Günata Z., Barre P., *Appl. Environ. Microbiol.*, **1998**, *64*, 3607

[52] Rajasree K. P., Mathew G. M., Pandey A., Sukumaran R. K., *J. Ind. Microbiol. Biotechnol.*, **2013**, *40*, 967

[53] Yang F., Yang X., Li Z., Du C., Wang J., Li S., *Appl. Microbiol. Biotechnol.*, **2015**, *99*, 8903

[54] Yan T. R., Lin C. L., *Biosci. Biotechnol. Biochem.*, **1997**, *61*, 965