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# *Thermoanaerobacterium thermosaccharolyticum* $\beta$ -glucosidase: a glucose-tolerant enzyme with high specific activity for cellobiose

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## Abstract

**Background:**  $\beta$ -Glucosidase is an important component of the cellulase enzyme system. It does not only participate in cellulose degradation, it also plays an important role in hydrolyzing cellulose to fermentable glucose by relieving the inhibition of exoglucanase and endoglucanase from cellobiose. Therefore, the glucose-tolerant  $\beta$ -glucosidase with high specific activity for cellobiose might be a potent candidate for industrial applications.

**Results:** The  $\beta$ -glucosidase gene *bgl* that encodes a 443-amino-acid protein was cloned and over-expressed from *Thermoanaerobacterium thermosaccharolyticum* DSM 571 in *Escherichia coli*. The phylogenetic trees of  $\beta$ -glucosidases were constructed using Neighbor-Joining (NJ) and Maximum-Parsimony (MP) methods. The phylogeny and amino acid analysis indicated that the BGL was a novel  $\beta$ -glucosidase. By replacing the rare codons for the N-terminal amino acids of the target protein, the expression level of *bgl* was increased from 6.6 to 11.2 U/mg in LB medium. Recombinant BGL was purified by heat treatment followed by Ni-NTA affinity. The optimal activity was at pH 6.4 and 70°C. The purified enzyme was stable over pH range of 5.2–7.6 and had a 1 h half life at 68°C. The activity of BGL was significantly enhanced by Fe<sup>2+</sup> and Mn<sup>2+</sup>. The  $V_{max}$  of 64 U/mg and 120 U/mg were found for p-nitrophenyl- $\beta$ -D-glucopyranoside ( $K_m$  value of 0.62 mM) and cellobiose ( $K_m$  value of 7.9 mM), respectively. It displayed high tolerance to glucose and cellobiose. The  $K_{cat}$  for cellobiose was 67.7 s<sup>-1</sup> at 60°C and pH 6.4, when the concentration of cellobiose was 290 mM. It was activated by glucose at concentrations lower than 200 mM. With glucose further increasing, the enzyme activity of BGL was gradually inhibited, but remained 50% of the original value in even as high as 600 mM glucose.

**Conclusions:** The article provides a useful novel  $\beta$ -glucosidase which displayed favorable properties: high glucose and cellobiose tolerance, independence of metal ions, and high hydrolysis activity on cellobiose.

**Keywords:**  $\beta$ -glucosidase, Glucose tolerance, *Thermoanaerobacterium thermosaccharolyticum*, Over-expression, Phylogeny

## Introduction

Cellulosic biomass is the most abundant renewable resource on earth, whose natural degradation represents an important part of the carbon cycle within the biosphere [1].  $\beta$ -Glucosidase (EC 3.2.1.21) is a glucosidase enzyme that acts upon  $\beta$  1–4 bonds linking two glucose or glucose-substituted molecules. It is an important component of the cellulase enzyme system. The limiting

step in the enzymatic saccharification of cellulosic material is the conversion of short-chain oligosaccharides and cellobiose, which was resulted from the synergistic action of endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91), to glucose, a reaction catalyzed by  $\beta$ -glucosidases [2]. It is well established that cellobiose inhibits the activities of most cellobiohydrolases and endoglucanases [3].  $\beta$ -glucosidases reduce cellobiose inhibition by hydrolyzing this disaccharide to glucose, thus allowing the cellulolytic enzymes to function more efficiently [4,5]. Furthermore,  $\beta$ -glucosidase is used as a flavor enzyme to enhance the flavor of wine, tea and fruit juice [6,7]. In fruits and other plant tissues many secondary metabolites, including flavor

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compounds, are accumulated in their glucosylated form [8,9]. Because  $\beta$ -glucosides constitute the majority of the known glycoconjugated flavor compounds,  $\beta$ -glucosidases play an important role in flavor liberation from these precursors. Therefore, producing high-activity and glucose-tolerant  $\beta$ -glucosidase has become important.

Recently, the search for  $\beta$ -glucosidases insensitive to glucose has increased significantly, for these enzymes would improve the process of saccharification of lignocellulosic materials. A few microbial  $\beta$ -glucosidases have been reported to tolerate glucose [10-14]. For example,  $\beta$ -glucosidases from *Aspergillus tubingensis* CBS 643.92, *A. oryzae*, *A. niger* CCRC 31494, *A. foetidus*, and marine microbial metagenome displayed high inhibition constant by glucose ( $K_i$ ) of 600 mM, 1390 mM, 543 mM, 520 mM, and 1000 mM, respectively. But these  $\beta$ -glucosidases have considerably lower specific activity for cellobiose than for p-nitrophenyl- $\beta$ -D-glucopyranoside. Therefore, over-expression of thermostable  $\beta$ -glucosidase with high glucose tolerance and specific activity for cellobiose abilities will help shed light on degradation of cellulosic biomass.

Thermostable enzymes have several generic advantages, allowing a decreased amount of enzyme needed because of higher specific activity and elongated hydrolysis time due to higher stability. In addition, thermostable enzymes are generally more tolerant and allow more flexibility in process configurations [15,16]. Although some glucose-tolerant  $\beta$ -glucosidases from fungi and bacteria have been reported [10-14], the glucose-tolerant  $\beta$ -glucosidases genes have not been expressed and characterized from thermophilic bacteria. Bacterium *Thermoanaerobacterium thermosaccharolyticum* is a strict anaerobe that grows on wide range of hexose and pentose at temperature from 37°C to 75°C, which have attracted considerable interests to hydrogen production and thermostable enzyme production [17]. *T. thermosaccharolyticum* DSM 571 could utilize cellobiose, but the gene for  $\beta$ -glucosidase, the key enzyme in degradation cellobiose, was not reported in the Genbank (NC\_014410.1). Because the optimal growth temperature for *T. thermosaccharolyticum* DSM 571 was at 60°C, the thermostable  $\beta$ -glucosidase could have a considerable potential for industrial applications. Owing to the inherent difficulty of cultivation of *T. thermosaccharolyticum* DSM 571, it is difficult to obtain a sufficient amount of cells for large-scale enzyme production. For the production of the recombinant protein, genetic engineering is the first choice because it is easy, fast, and cheap.

In this paper, we report the phylogenesis analysis, cloning, over-expression, and detailed biochemical characterization of the  $\beta$ -glucosidase from *T. thermosaccharolyticum* DSM 571. The favorable properties make the  $\beta$ -glucosidase a good candidate for utilization in biotechnological applications.

## Results

### Cloning and sequence analysis of *bgl*

By analysis of the genome sequence of *T. thermosaccharolyticum* DSM 571, a protein (Tthe\_1813), defined as  $\beta$ -galactosidase in Genbank, consists of a 1,329-bp fragment encoding 443 amino acids, which belonged to family 1 of the glycoside hydrolases. It shares the highest sequence similarity of 66% with the  $\beta$ -glucosidases from *Thermoanaerobacter mathranii* (Genbank No. YP\_003676178.1) and *Thermoanaerobacter pseudethanolicus* ATCC 33223 (Genbank No. YP\_001665894.1), which were revealed by whole-genome sequencing but has not been biochemically characterized. Alignment of the BGL cluster with several representative members of GH1 indicated that they share similar blocks. The catalytic proton donor, Glu<sup>135</sup> and Glu<sup>351</sup> in BGL are well conserved among all GH1 proteins (Figure 1). The sequence around Glu<sup>351</sup> in BGL is [LYT-NGAA], which is consistent with the consensus pattern of PS00572. The results indicated that the protein (Tthe\_1813) could be a novel  $\beta$ -glucosidase. Then the DNA fragment of a protein (Tthe\_1813) gene was amplified from genomic DNA of *T. thermosaccharolyticum* DSM 571, and ligated to pET-20b at *Nde* I and *Xho* I sites to generate plasmid pET-20-BGL.

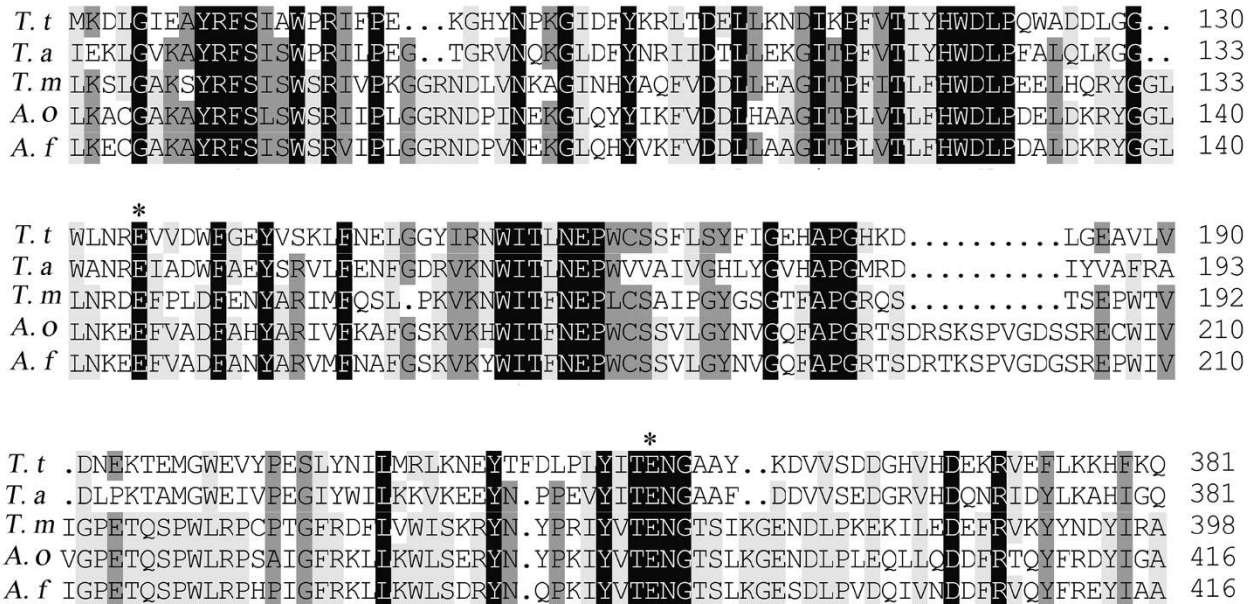
### Over-expression of BGL

In order to increase the expression level of BGL in *E. coli*, site-directed mutagenesis were designed and performed to optimize codons of BGL for *E. coli* expression system. pET-20-BGLII was obtained from pET-20-BGL in which the rare codons for the N-terminal amino acid residues were replaced by optimal codons in *E. coli* without and change of amino acid sequence (Figure 2), so pET-20-BGLII encodes the same  $\beta$ -glucosidase as that encoded by the wild-type gene. The  $\beta$ -glucosidase activity expression from pET-20-BGLII was 7.5 U/mL (11.2 U/mg total of cell protein) and was estimated to be about 30% of the total protein, which was about 1.7 times higher than the expressed from pET-20-BGL (Figure 3, lane 2 and 3).

### Purification and Characterization of recombinant BGL

The protein in the cell-free extract was purified to gel electro homogeneity after a heat treatment and a Ni-NTA affinity. The final preparation gave a single band on SDS-PAGE gel and the molecular mass of the enzyme was estimated to be 52 kDa (Figure 3, lane 4).

The biochemical properties of BGL were investigated by using the purified recombinant BGL. The optimal pH of the BGL was determined to be 6.4 (Figure 4a), while the  $\beta$ -glucosidase activity was higher than 50% of the maximum activity at the pH range from 5.6 to 7.2. The enzyme was stable for about 1 h at pH 5.6 to 8.0 at 60°C in the absence of the substrate (Figure 4c). The optimal temperature of



**Figure 1 Multi-alignment of BGL with some GH1 family members.** Sequence alignment was performed by using Clustal X2.0. The active sites are indicated as\* on the top of the alignment. *T. t*: *T. thermosaccharolyticum* DSM 571 (YP\_003852393.1), *T. a*: *Trichoderma atroviride* (EHK41167.1), *T. m*: *Thermotoga maritima* (Q08638.1), *A. o*: *Aspergillus oryzae* (BAE57671.1), *A. f*: *Aspergillus fumigatus* (XP\_752840.1).

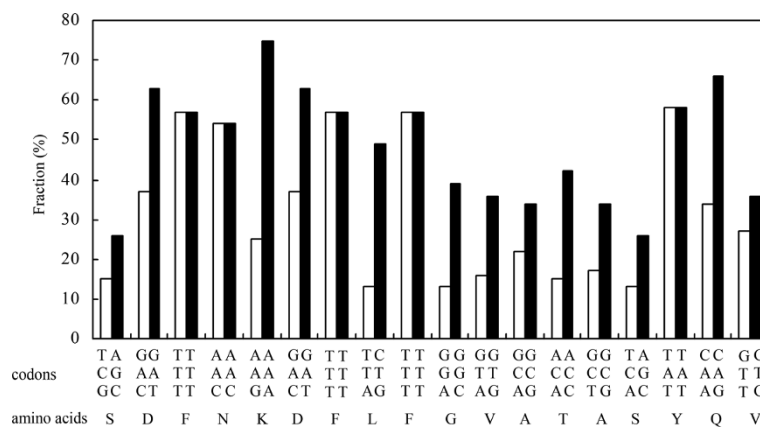
the BGL was 70°C, which the β-glucosidase activity was higher than 40% of the maximum activity at the temperature range from 45 to 75°C (Figure 4b). Thermostability assays indicated that its residual activity was more than 80% after being incubated at 60°C for 2 h (pH 6.4, Figure 4d).

The effects of metal ions and some chemicals on the enzyme activity were shown in (Table 1). In various assays, the enzyme activity was significantly enhanced by Fe<sup>2+</sup>, or Mn<sup>2+</sup>, and completely inactivated by Zn<sup>2+</sup>, Cu<sup>2+</sup>, Ag<sup>2+</sup>, or Hg<sup>2+</sup>. The effects of Mg<sup>2+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>, Li<sup>2</sup>, or

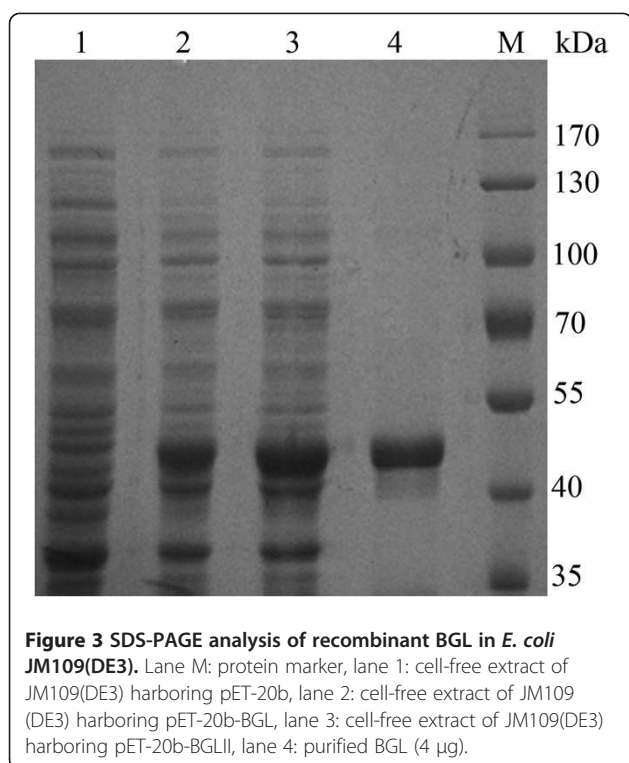
EDTA (10 mM) on the enzyme activity were not so significant.

#### Effect of glucose on BGL activity and substrate specificity

The enzyme was able to hydrolyze p-nitrophenyl-β-D-glucopyranoside, cellobiose, and p-nitrophenyl-β-D-galactopyranoside, while no activity was detected upon p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-β-D-xylopyranoside, maltose, CMC, and sucrose. p-nitrophenyl-β-D-Galactopyranoside was hydrolyzed at 40% of that of p-nitrophenyl-β-D-glucopyranoside. The dependence of the



**Figure 2 The codons for the amino acid between the 1<sup>st</sup> and 19<sup>th</sup> which were subjected to site-directed mutagenesis.** Original sequence of the BGL (open square); optimal sequence of the BGL (filled square).



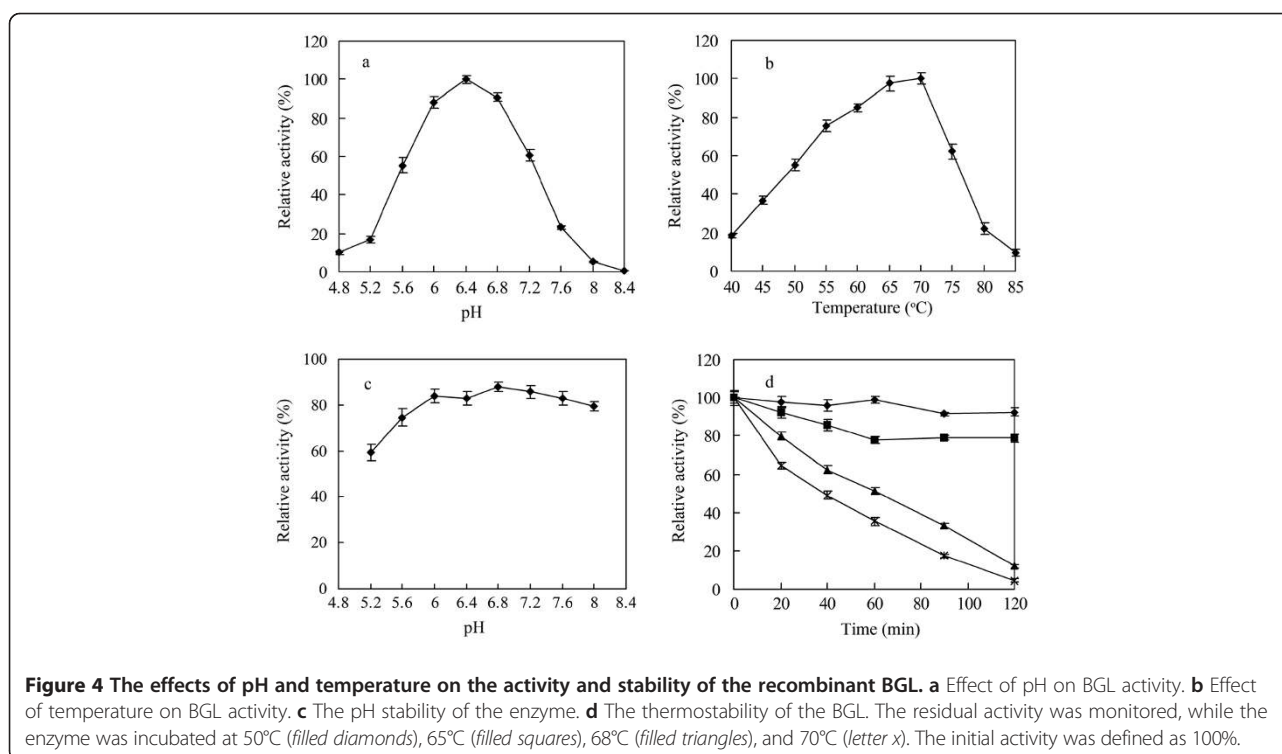
rate of the enzymatic reaction on the substrates concentration followed Michaelis-Menten kinetics, with  $K_m$  and  $V_{max}$  values of 0.62 mM and 64 U/mg for p-nitrophenyl-β-D-glucopyranoside, and for cellobiose 7.9 mM and 120 U/mg

**Table 1** Effects of cations and reagents on purified BGL activity

Cation of reagent <sup>a</sup>	Residual activity (%)
Control	100
Fe <sup>2+</sup>	172
Mg <sup>2+</sup>	104
Zn <sup>2+</sup>	7
Mn <sup>2+</sup>	223
Ca <sup>2+</sup>	108
K <sup>+</sup>	101
Al <sup>3+</sup>	43
Li <sup>+</sup>	110
Cu <sup>2+</sup>	2
Hg <sup>2+</sup>	0
Co <sup>2+</sup>	37
Ag <sup>2+</sup>	19
EDTA (10 mM)	102

<sup>a</sup> Final concentration, 1 mM or as indicated. Values shown are the mean of duplicate experiments, and the variation about the mean was below 5%.

under optimal conditions. The effects of the substrate, cellobiose (290 mM), on the enzyme activity were not significant. The  $K_{cat}/K_m$  value for cellobiose  $13.3 \text{ mM}^{-1} \text{ s}^{-1}$  was less than the β-glucosidase from *A. oryzae*, but the activity of β-glucosidase from *A. oryzae* was inhibited by cellobiose, and rapidly decreased above 50°C (Table 2). Furthermore, the enzyme activity was enhanced by the concentrations of glucose below 200 mM, and the enzyme activity was





**Table 2 Characteristics of glucose-tolerant  $\beta$ -glucosidases from *T. thermosaccharolyticum* DSM 571 and other microorganisms**

Strain	$K_m$ (mM)		$V_{max}$ (U/mg)		$K_i$ for glucose (mM)	Cellobiose inhibition (%)	$K_{cat}/K_m$ (mM <sup>-1</sup> s <sup>-1</sup> ) for cellobiose	Optimal Temp (°C)
	pNPG <sup>a</sup>	Cellobiose	pNPG	Cellobiose				
<i>T. thermosaccharolyticum</i>	0.63	7.9	64	120	600	No effect	13.3	70
Uncultured bacterium [13]	0.39	20.4	50.7	15.5	1000	ND <sup>b</sup>	0.65	40
<i>Debaryomyces varrijiae</i> [18]	0.77	57.9	668	84.3	439	ND	2.43	40
<i>A. oryzae</i> [19]	0.55	7	1,066	353	1,390	50	36.1	50
<i>A. niger</i> [12]	21.7	ND	124.4	ND	543	ND	ND	55
<i>A. tubingensis</i> [10]	6.2	ND	28.4	0.32	600	ND	ND	60
<i>Candida peltata</i> [21]	2.3	66	108	8.5 <sup>c</sup>	1400	No effect	0.1 <sup>c</sup>	50
<i>Scytalidium thermophilum</i> [20]	0.29	1.61	13.27	4.12	>200	ND	1.7	60

<sup>a</sup> pNPG: p-nitrophenyl- $\beta$ -D-glucopyranoside.

<sup>b</sup> ND: not determined.

<sup>c</sup> It was calculated by the data based on the reference.

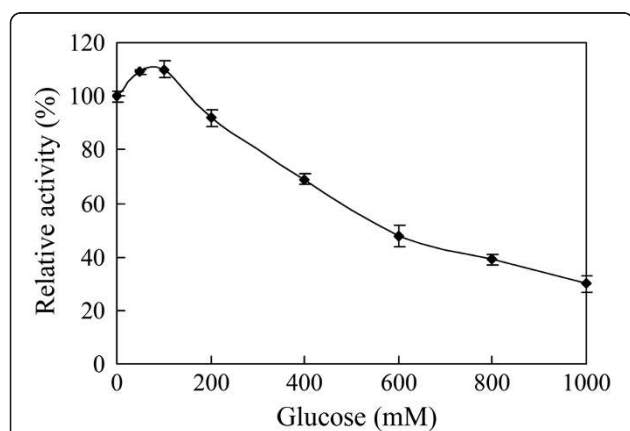
increased 110% when adding 100 mM glucose into reaction mixtures (Figure 5). When glucose was increased, the enzyme activity of BGL was gradually inhibited, with a  $K_i$  of 600 mM glucose (Figure 5). The properties of the glucose-tolerant  $\beta$ -glucosidase from other microorganisms are summarized in Table 2. As Table 2 shows, these enzymes have many distinct features, especially in their catalytic properties [12,13,18-21].

#### Analysis of cellobiose degradation

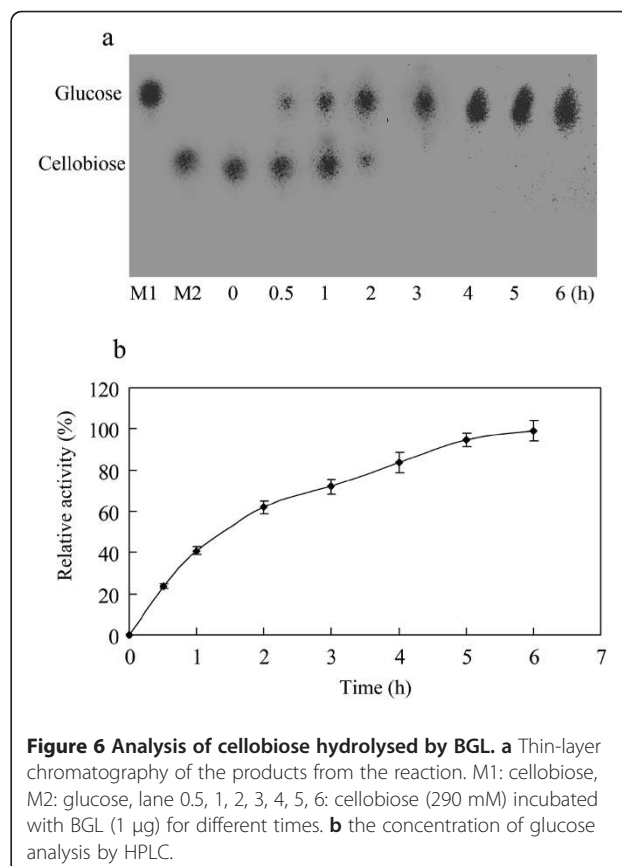
Production of glucose from 290 mM cellobiose (10%) by the purified BGL was examined. Even if the final concentration of glucose in reaction reached about 580 mM, cellobiose (290 mM) was found to be degraded completely (Figure 6a, b). At the beginning of the reaction, the  $K_{cat}$  was 67.7 s<sup>-1</sup> within one hour at 60°C which was identical to the theoretical value. During the whole degradation process, the  $K_{cat}$  was 28.2 s<sup>-1</sup>.

#### Phylogenies analysis of BGL

To gain insights into the evolutionary relationship among  $\beta$ -glucosidases, we constructed the phylogenetic trees of 40 candidate sequences using the NJ method and the MP method respectively, both supporting almost the same topology. The results revealed the presences of five well-supported clades: Clade II was GH1  $\beta$ -glucosidases from fungi, and Clade III was the GH3  $\beta$ -glucosidases from bacteria, and Clade IV was the GH3  $\beta$ -glucosidases



**Figure 5 The effects of glucose on BGL activity.** Influence of glucose on enzyme activity with p-nitrophenyl- $\beta$ -D-glucopyranoside as the substrate.



**Figure 6 Analysis of cellobiose hydrolysed by BGL.** **a** Thin-layer chromatography of the products from the reaction. M1: cellobiose, M2: glucose, lane 0.5, 1, 2, 3, 4, 5, 6: cellobiose (290 mM) incubated with BGL (1  $\mu$ g) for different times. **b** the concentration of glucose analysis by HPLC.

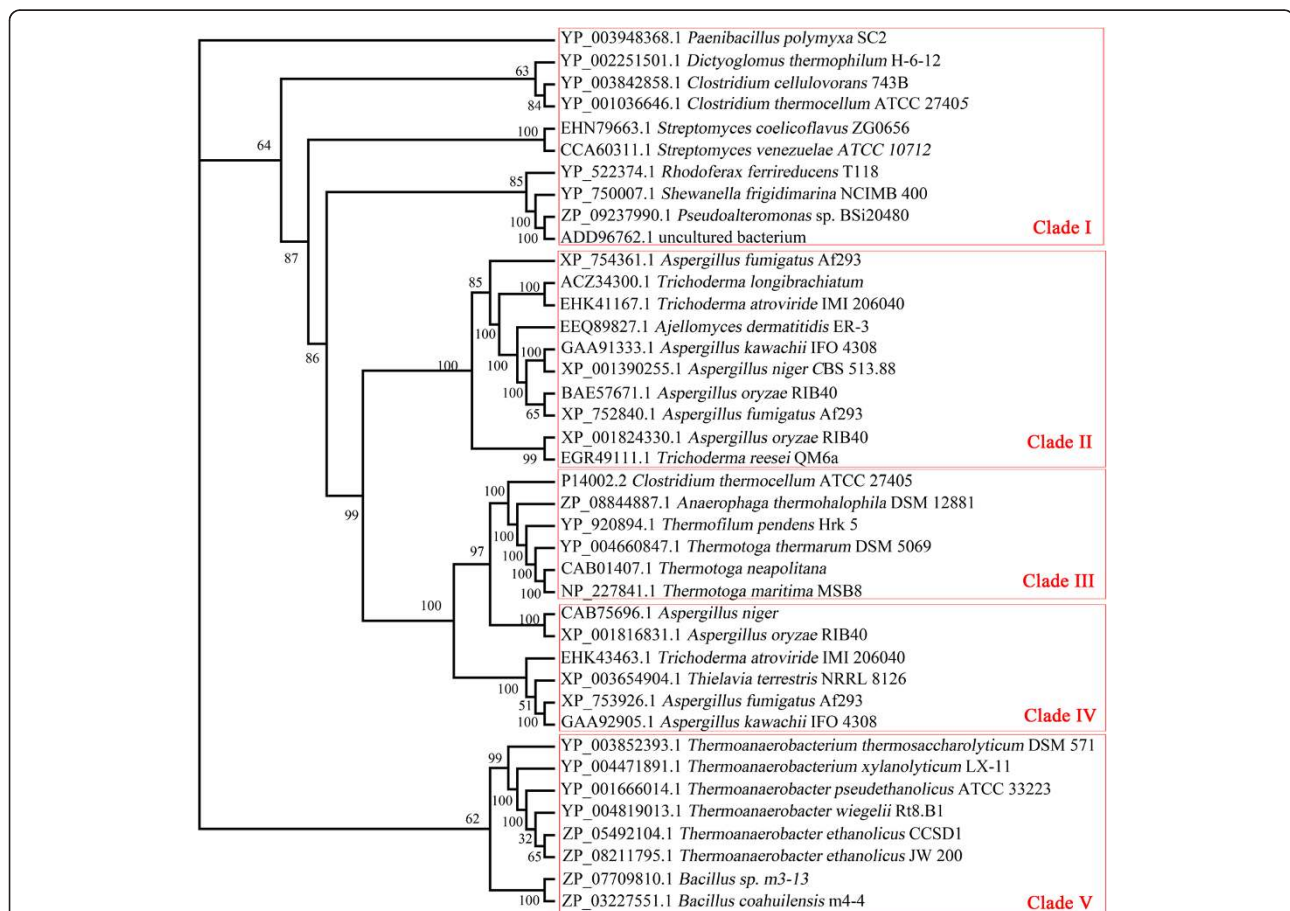
from fungi. The GH1  $\beta$ -glucosidases from bacteria was divided into two clades: Clade I mainly contained mesophilic bacteria; Clade V mainly contained thermophile, which is formed by further divided into two subclasses, of which one contains all thermophile, and the other *Bacillus* GH1  $\beta$ -glucosidases. Clade II and clade III had a relatively close relationship, and the GH1  $\beta$ -glucosidases from thermophile were distant from the other clades (Figure 7).

## Discussions

A classification of glycoside hydrolases based on amino acid sequence similarities was proposed a few years ago, wherein  $\beta$ -glucosidases were mainly grouped into two superfamilies of glycoside hydrolases I (GH1), and GH3 [22]. Although, the amino acid sequence analysis indicated that BGL belongs to GH1, it shared the highest sequence similarity of 66% with the  $\beta$ -glucosidases from *Thermoanaerobacter mathranii* (YP\_003676178.1). Moreover, it shared only the 63% with the putative  $\beta$ -glucosidase (YP\_004471891.1) the *Thermoanaerobacterium xylanolyticum* LX-11, both

belonging to the genus *Thermoanaerobacterium*. The Phylogenies analysis showed that the BGL was distant with the glucose-tolerant  $\beta$ -Glucosidases from fungi and ADD96762.1 (Figure 7). The results indicated that the BGL could be a novel  $\beta$ -glucosidase with some different properties. On the other hand,  $\beta$ -Glucosidases may be divided into three groups on the basis of their substrate specificity. The first group is known as aryl- $\beta$ -glucosidases owing to strong affinity to aryl- $\beta$ -glucose. The second group consists of cellobiases that hydrolyze oligosaccharides only. The third group is broad specific  $\beta$ -glucosidases that exhibit activity on a wide range of substrates, and are the most commonly observed form of  $\beta$ -glucosidases [23]. The BGL, which was high affinity to p-nitrophenyl- $\beta$ -D-glucopyranoside, hydrolyzed cellobiose, p-nitrophenyl- $\beta$ -D-glucopyranoside, and p-nitrophenyl- $\beta$ -D-galactopyranoside, but not p-nitrophenyl- $\alpha$ -L-arabinofuranoside, p-nitrophenyl- $\beta$ -D-xylopyranoside, maltose, sucrose, and CMC. These results indicated that BGL belonged to the first group.

Enzymatic hydrolysis of cellulose is a complex process, the last step being a homogenous catalysis reaction



**Figure 7** The Neighbor-Joining (NJ) and Maximum-Parsimony (MP) trees results from analysis of  $\beta$ -glucosidases of 40 amino acid sequences. Numbers on nodes correspond to percentage bootstrap values for 1000 replicates.

involving the action of  $\beta$ -glucosidase on cellobiose. Cellobiose is a strong inhibitor of both cellobiohydrolases and endocellulases. Therefore,  $\beta$ -glucosidase with high tolerance for glucose has become heated in these fields. Fungi, especially *Aspergillus* species, are generally considered to be a good producer with high yield of  $\beta$ -glucosidases [24]. But the major  $\beta$ -glucosidases belonging to family 3 of the glycoside hydrolases (GH3) from *Aspergillus* species were subject to competitive inhibition of glucose to produce glucose, the  $K_i$  is generally 1–20 mM [10,14]. The minor  $\beta$ -glucosidases, which molecular weights are 40–50 kDa, exhibited a tolerance to glucose (Table 2). The effect of glucose on the BGL activity revealed that the enzyme is not only resistant to end-product inhibition, but is activated by glucose at concentrations from 0 to 0.2 M. Only two  $\beta$ -glucosidases, activated by glucose, have been reported from *Scytalidium thermophilum* and marine microbial (Table 2) [13,20].

Moreover, high specific activity for cellobiose and tolerance to substrate inhibition are other advantages for  $\beta$ -glucosidase in enzymatic hydrolysis of cellulose. Although, several  $\beta$ -glucosidases from a few fungi and bacteria show high glucose tolerant with  $K_i$  values of more than 200 mM, the  $V_{max}$  values of these enzymes for cellobiose were much lower than for p-nitrophenyl- $\beta$ -D-glucopyranoside. The  $V_{max}$  value of BGL for cellobiose was 120 U/mg, which was about 2 times higher than the  $V_{max}$  value of BGL for p-nitrophenyl- $\beta$ -D-glucopyranoside. To our knowledge, in only one other study have workers described the purification and characterization (from *A. oryzae*) of a  $\beta$ -glucosidase having such a high tolerance to glucose and high specific activity for cellobiose [19]. But the specific activity of  $\beta$ -glucosidase from *A. oryzae* for cellobiose was much lower than for p-nitrophenyl- $\beta$ -D-glucopyranoside (Table 2). The BGL was only the  $\beta$ -glucosidase been reported that it is not only resistant to glucose, but had higher specific activity for cellobiose than for p-nitrophenyl- $\beta$ -D-glucopyranoside. In addition, the BGL had high tolerance to substrate inhibition, cellobiose. The  $K_{cat}$  of BGL was  $67.7 \text{ s}^{-1}$  at  $60^\circ\text{C}$  and pH 6.4, when the concentration of cellobiose was 10% (Table 2).

The chemical agents had various effects on the activity of BGL. The chelating agent EDTA displayed no influence on the  $\beta$ -glucosidase activity, indicating that the  $\beta$ -glucosidase is not a metalloprotein. However, the  $\beta$ -glucosidase activity was greatly stimulated by  $\text{Fe}^{2+}$  or  $\text{Mn}^{2+}$ , which implied that  $\text{Fe}^{2+}$  or  $\text{Mn}^{2+}$  is required for the maximal activity of BGL. These results distinguish BGL from the other bacteria  $\beta$ -glucosidases, on which  $\text{Ca}^{2+}$  show positive effects [13]. In practical applications, the high thermostability of the enzyme is desired because the longer active life means the less consumption of the enzyme. The BGL residual activity was more than 80%

after being incubated at  $60^\circ\text{C}$  for 2 h, and it in enzymatic hydrolysis of cellulose exhibited high activity in broad temperature, which could keep at high levels at temperatures from  $45$  to  $70^\circ\text{C}$ .

The properties of the BGL demonstrated a great potential of the gene in the genetic modification of strains for biomass degradation. Differences in codon usage preference among organisms lead to a variety of problems concerning heterologous gene expression, which can be overcome by rational gene design and gene synthesis. Protein with multiple repetitive rare codons especially within the first 20 amino acids of the amino terminus of the protein may significantly reduce the protein expression. Sometimes, it shuts down the expression completely. Since the rare codons of *bgl* from 1–20 amino acids were all changed into optimized codons, the activity of BGL was increased by about 70% (Figure 3). More optimization of codons for the other amino acid residues in the ORF of *bgl* may give further improvement in the gene expression levels.

## Conclusion

With this study, we successfully over-expressed the novel  $\beta$ -glucosidase (BGL) gene *bgl* from *T. thermosaccharolyticum* DSM 571 by replacing the rare codons with the optimal codons in *E. coli*. The Phylogenies analysis showed that the BGL had close relationship with the  $\beta$ -Glucosidases from thermophile, and was distant from the other glucose-tolerant  $\beta$ -Glucosidases. As compared on the enzyme properties, the BGL was higher tolerant to glucose and cellobiose, more efficient in hydrolysis of cellobiose, more thermal stability than  $\beta$ -glucosidases from other microorganisms. Thus, this study provides a useful novel  $\beta$ -glucosidase, which may be used to improve the enzymatic conversion of cellulosic to glucose through synergetic action.

## Materials and Methods

### Bacterial Strains, Plasmids, Growth Media

*Thermoanaerobacterium thermosaccharolyticum* DSM 571 was purchased from DSMZ (www.dsmz.de). It was grown anaerobically at  $60^\circ\text{C}$  as described previously [17]. *Escherichia coli* JM109 and JM109(DE3) was grown at  $37^\circ\text{C}$  in Luria-Bertani medium (LB) and supplemented with ampicillin when required. The expression vectors pET-20b (Novagen) were employed as cloning vector and expression vector.

### DNA manipulation

DNA was manipulated by standard procedures [25]. QIAGEN Plasmid Kit and QIAGEN MinElute Gel Extraction Kit (Qiagen, USA) were employed for the purification of plasmids and PCR products. DNA restriction and modification enzymes were purchased from TaKaRa (Dalian,

China). DNA transformation was performed by electro-  
 poration using GenePulser (Bio-Rad, USA). Site-directed  
 mutagenesis of genes and the modification of the plasmids  
 were performed by inverse-PCR followed by phosphoryla-  
 tion and self-ligation using T4 polynucleotide kinase and  
 T4 DNA ligase.

#### Plamid constructions

The  $\beta$ -glucosidase gene *bgl* was amplified from *T. ther-  
 mosaccharolyticum* DSM 571 genomic DNA by PCR  
 using primers *bgl*-1 and *bgl*-2 (Table 3), the PCR pro-  
 ducts were digested with *Nde* I and *Xho* I and inserted  
 into pET-20b at *Nde* I and *Xho* I sites, yielding the plas-  
 mid pET-20-BGL.

In order to improve the expression level of recombin-  
 ant BGL, the internal region from 1<sup>st</sup> to 19<sup>th</sup> amino acids  
 in open reading frame of *bgl* was mutated in situ by in-  
 verse-PCR to replace the rare codons with the optimal  
 codons of *E. coli*; the primers for the inverse-PCR were  
 designated as *bgl*-3 and *bgl*-4 (Table 3). Inverse-PCR  
 with primers was carried out using Pyrobest with pET-  
 20-BGL as template, generating the plasmid pET-20-  
 BGLII.

#### Expression and purification of BGL

Plasmids pET-20-BG and pET-20-BGLII were transformed  
 into *E. coli* JM109(DE3), and induced to expressed recom-  
 binant BGL by adding isopropyl- $\beta$ -D-thiogalactopyranoside  
 (IPTG) to final concentration of 0.8 mM at OD<sub>600</sub> about  
 0.7, and incubated further at 30°C for about 6 h.

One liters of the recombinant cells carrying pET-20-  
 BGLII were harvested by centrifugation at 5,000 g for  
 10 min at 4°C, and washed twice with distilled water,  
 resuspended in 50 mL of 5 mM imidazole, 0.5 mM  
 NaCl, and 20 mM Tris-HCl buffer (pH 7.9), and  
 French-pressured for three times. The cell extracts were  
 heat treated (60°C, 30 min), and then cooled in an ice  
 bath, and centrifuged (20,000 g, 4°C, 30 min). The  
 resulting supernatants were loaded on to an immobi-  
 lized metal affinity column (Novagen, USA), and eluded  
 with 1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl  
 buffer (pH 7.9). Protein was examined by SDS-PAGE  
 [26], and the protein bands were analyzed by density

scanning with an image analysis system (Bio-Rad, USA).  
 Protein concentration was determined by the Bradford  
 method using BSA as a standard.

#### Determination of enzyme activities and properties

The reaction mixture, containing 50 mM imidole-  
 potassium buffer (pH 6.4), 1 mM p-nitrophenyl- $\beta$ -D-  
 glucopyranoside, and certain amount of  $\beta$ -glucosidase  
 in 0.2 mL, was incubated for 5 min at 70°C. The reac-  
 tion was stopped by adding 1 mL of 1 M Na<sub>2</sub>CO<sub>3</sub>. The  
 absorbance of the mixture was measured at 405 nm.  
 One unit of enzyme activity was defined as the amount  
 of enzyme necessary to liberate 1  $\mu$ mol of pNP per  
 min under the assay conditions.

The optimum pH for activity  $\beta$ -glucosidase was deter-  
 mined by incubation at 70°C for 5 min in the 50 mM  
 imidole-potassium buffer from pH 4.8 to 8.4. The  
 optimum temperature for the enzyme activity was deter-  
 mined by standard assay ranging from 45 to 85°C in the  
 50 mM imidole-potassium buffer, pH 6.0. The results  
 were expressed as percentages of the activity obtained at  
 either the optimum pH or the optimum temperature.

The pH stability of the enzyme was determined by  
 measuring the remaining activity after incubating the en-  
 zyme (0.1  $\mu$ g) at 50°C for 1 h in the 50 mM imidole-  
 potassium buffer from pH 5.2 to 8.0. To determine the  
 effect of temperature on the stability of BGL, the enzyme  
 (0.1  $\mu$ g) in the 50 mM imidole-potassium buffer (pH 6.4)  
 was pre-incubated for various times at 50°C, 65°C, 68°C  
 and 70°C in the absence of the substrate. The activity of  
 the enzyme without pre-incubation was defined as 100%.

The effects of metals and chemical agents on  $\beta$ -  
 glucosidase activity of purified enzyme (0.1  $\mu$ g) were  
 determined. Fe<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>, Al<sup>3+</sup>,  
 Li<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, and Hg<sup>2+</sup> were assayed at concentra-  
 tions of 1 mM in the reaction mixture. The chemical  
 agents EDTA (10 mM) were assayed. The enzyme was  
 incubated with each reagent for 10 min at 50°C before  
 addition of p-nitrophenyl- $\beta$ -D-glucopyranoside to initi-  
 ate the enzyme reaction. Activity was determined as  
 described above and was expressed as a percentage of  
 the activity obtained in the absence of the chemical  
 agents and metal cations.

The substrate specificity of the enzyme (0.1  $\mu$ g)  
 was tested by using following p-nitrophenyl- $\beta$ -D-  
 glucopyranoside, p-nitrophenyl- $\beta$ -D-xylopyranoside, p-  
 nitrophenyl- $\alpha$ -L-arabinofuranoside, maltose, sucrose,  
 and cellobiose. Kinetic constant of BGL was determined  
 by measuring the initial rates at various p-nitrophenyl- $\beta$ -  
 D-glucopyranoside concentrations (0.2, 0.4, 0.6, 0.8, 1, 2,  
 and 4.0 mM) or various cellobiose concentration (2, 4, 6,  
 8, 10, 12, 14, and 16 mM) under standard reaction condi-  
 tions. The *K<sub>i</sub>* value of glucose was defined as amount of  
 glucose required for inhibiting 50% of the  $\beta$ -glucosidase

**Table 3 Nucleotide sequences of used primers**

Primer	Nucleotide sequence
<i>bgl</i> -1	CCCCATATGTCGGACTTTAACAAGGAC
<i>bgl</i> -2	CCCCTCGAGAATGGTCCTAGTGGAATAAG
<i>bgl</i> -3	TTTGG <b>CGT</b> GG <b>CGAC</b> CG <b>CGAGCT</b> ATCAG <b>GTG</b> GAAGG TGCTTACAATGAGGA
<i>bgl</i> -4	<b>CAGAAA</b> AT <b>CT</b> TTGGT <b>AAAA</b> TC <b>GCT</b> CATATGTATATCT CCTTCTAAAG

The boldface italic nucleotides represented mutations for optimizing codons.



activity and was given as the averages of three separate experiments performed in duplicate.

### Phylogenies analysis of BGL

The codon usage preference of *E. coli* in translation initiation region of pET-20-BGL was analyzed by using codon usage tool (<http://gcua.schoedl.de/>). The potential ORF of *bgl* was searched using the ORF search tool provided by the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Database searching was performed with Blast at NCBI and against CAZy ([www.cazy.org](http://www.cazy.org)). The active site of the enzyme was analyzed with the prosite tool (<http://prosite.expasy.org/scanprosite>). The multiple sequence alignment tool Clustal X2.0 was used for multiple protein sequence alignment [27]. Sequences were further edited and aligned manually, when necessary, using the Mega 5 for editing. For phylogenetic analyses of conserved domains, sequences were trimmed so that only the relevant protein domains remained in the alignment [28]. Phylogenetic relationships were inferred using the Neighbor-Joining (NJ) and Maximum-Parsimony (MP) method as implemented in Paup 4.0 for the NJ and MP trees, the results were evaluated with 1000 bootstrap replicates [29]. The generated trees were displayed using TREEVIEW 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

### Analysis of cellobiose degradation

The cellobiose was treated with purified BGL, and the degradation was subjected to analysis of thin-layer chromatography (TLC) and HPLC. The reaction mixture (20  $\mu$ L) contained 290 mM cellobiose, and 1  $\mu$ g of BGL in 50 mM imidole-potassium buffer (pH 6.4). The reaction was performed for various times at 60°C, and stopped by heating for 5 min in a boiling water bath. After centrifuged for 10 min at 10,000 g, supernatants of the reaction mixtures were applied on silica gel TLC plates (60F254, Merck Co.). Sugars on the plates were partitioned with a solvent system consisting of *n*-butanol, acetic acid, and water (2:1:1, by vol/vol), and detected using the orcinol reagent [30]. The concentration of glucose was examined by HPLC on a carbohydrate analysis column (Waters Sugarpak1, USA) with water as a mobile phase.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

JP carried out the cloning and over-expression and drafted the manuscript. QP and SF helped to purify and characterize the BGL. LZ directed the over-all

study and drafted the manuscript. HS helped to perform phylogenies analysis of  $\beta$ -glucosidases. All authors read and approved the final manuscript.

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