Cloning, Purification, and Characterization of a Thermostable β-Glucosidase from *Thermotoga thermarum* DSM 5069

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A 56-kDa β-glucosidase (TthBgl) derived from *Thermotoga thermarum* DSM 5069 was expressed and purified from *Escherichia coli* BL21 (DE3). The purified enzyme showed hydrolytic activity towards only *p*-nitrophenyl-β-D-glucopyranoside among the synthetic glycosides tested. The pH maximum was 5.0, and under the conditions tested, maximal activity was at 85 ºC, and pH stability occurred from 5.0 to 6.0. After being incubated at 80 ºC for 120 min, TthBgl retained 80% of its original activity. The β-glucosidase had no apparent requirement for metal ions or other co-factors, but its activity was significantly inhibited by 0.1% SDS and 1mM Cu^{2+}, in which only 3% and 10% residual activity was maintained, respectively. The V_{max} of TthBgl was 8.79 U mg^{-1} for *p*-nitrophenyl-β-D-glucopyranoside, while the K_m was 2.41 mM. The Enzyme activity was gradually inhibited by the addition of glucose, but remained approximately 50% of its original value in 500 mM glucose. 789.25 mg/L glucose was released from cellobiose by the incubation of 0.2 U/mL TthBgl for 9 h at 75 ºC. According to a phylogenetic analysis, TthBgl belongs to the glycosyl hydrolase family 3 (GH3).

Keywords: GH3 β-glucosidase; Thermotoga thermarum DSM 5069; Thermostability

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INTRODUCTION

Cellulose, a polymer of glucose units joined together by β-1,4-glycosidic bonds, is the most abundant and renewable lignocellulosic biomass resource on earth (Denman *et al.* 1996). The degradation of cellulose requires the synergistic action of several types of enzymes, including endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Yan *et al.* 2012). After the actions of endoglucanase and cellobiohydrolase, β-glucosidase (Bgl) removes glucose from either non-reducing cellobiose or cellotriose (Wu *et al.* 2013). Because of its contribution to the hydrolysis of cellulose, Bgl has attracted significant attention for use in a wide range of industrial processes, such as improving the flavor of wine and clarifying juice in beverage and food production (Ketudat Cairns and Esen 2010; González-Pombo *et al.* 2011).

Glycoside hydrolases are classified into families based on their amino acid similarities. Of the 99 families classified, Bgls belong to families GH1, GH3, GH5, GH9, GH30, and GH116 (http://www.cazy.org/fam/acc_GH.html). In general, Bgls from thermophiles or hyperthermophiles are much more stable than those from mesophiles because their special structures allow them to maintain enzymatic activity at high

temperatures. In addition to increasing the hydrolysis reaction rate, high temperature can also reduce the risk of contamination (dos Santos *et al*. 2011; Shi *et al*. 2014). Therefore, thermostable Bgls deriving from hyperthermophiles have great potential in industrial processes. For example, in the industrial production of paper and pulp, it is commonly accepted that a preliminary hemicellulose extraction stage produces a certain amount of solubilized glucan that is useless for pulping and papermaking processes. The high temperature waste containing hemicellulose and cellulose can be further processed into various end-products, such as bioethanol for energy purposes. Thus, enzymatic hydrolysis at high temperature not only can save energy for cooling the samples down, but also can decrease the substrate viscosity, a factor that inhibits the hydrolysis of biomass at high-solids loadings (Loaiza *et al.* 2015).

GH3 β-glucosidases have been purified and characterized from many organisms, including bacteria, eukaryotes, and archaea (http://www.cazy.org/fam/acc_GH.html; Roy *et al*. 2005; Kudou *et al*. 2014). *Thermotoga thermarum* 5069 is a hyperthermophilic bacterium that grows at 80 ºC; it was isolated from continental solfataric springs at Lac Abbe in Djibouti, Somalia, Africa (Windberger *et al*. 1989). In this study, a thermostable β-glucosidase from *Thermotoga thermarum* 5069 was cloned, expressed, and characterized.

EXPERIMENTAL

Materials and Methods

Bacterial strains, plasmid, and growth conditions

Escherichia coli Top10 and *E. coli* BL21 (DE3) were used as cloning and expression hosts, respectively. Both were grown at 37 ºC in Luria-Bertani medium (LB) containing ampicillin (100 μg/mL). The plasmid, pET-20b (Novagen, Darmstadt, Germany), was used as the cloning and expression vector.

Plasmid construction

A DNA fragment of about 1500 bp (GenBank Protein No. WP_013931664) from *Thermotoga thermarum* DSM 5069 was amplified by PCR using the following primers: *TthBgl*-1, 5'-GGAATTCCATATGACGCTTTCGGAGGTTGTTG-3', and *TthBgl*-2, 5'-CCGCTCGAGCCTTAACACCTCCACCGGCAGT-3'. Restriction sites for *Nde*I and *Xho*I are underlined. The 1.5-kb PCR product was digested with *Nde*I and *Xho*I (Takara, Dalian, China) and ligated into the *Nde*I- and *Xho*I- linearized expression vector, pET-20b, to produce the recombinant plasmid pET-20b-*TthBgl*.

Expression and purification of TthBgl

The recombinant plasmid pET-20b-*TthBgl* was transformed into *E. coli* BL21 (DE3). The *TthBgl* gene was expressed in overnight cultures containing pET-20b-*TthBgl* in 200 mL of LB medium supplemented with ampicillin (100 μg/mL) and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation (10,000 × *g* for 10 min at 4 ºC), washed twice, and re-suspended in 5 mL of 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl Buffer (pH 7.9). The cell extracts after sonication were treated at 60 ºC for 30 min, cooled in an ice bath, and centrifuged (10,000 × *g* for 30 min at 4 ºC). The obtained supernatant was loaded onto a Ni-NTA affinity column (Novagen, Darmstadt, Germany). A gradient of 20 mM to 200 mM

imidazole was used for eluting the protein, and 1-mL fractions were collected for activity assays and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Enzyme assay

Standard β-glucosidase assays (200 μL) contained 20 mM *p*-nitrophenyl-β-D-glucopyranoside (10 μL) and diluted enzyme (10 μL) in 50 mM imidazole-potassium buffer. The reaction was incubated at 85 °C for 10 min and terminated by the addition of 0.6 mL of 1 M Na_2CO_3. The amount of *p*-nitrophenol released was determined by measuring the absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) per minute. The purified protein concentration was determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

Characterization of TthBgl

The optimal pH of TthBgl was analyzed in 50 mM imidazole-potassium buffer covering a pH range of 4.0 to 6.5 in increments of 0.5 at 80 ºC. The pH stability was examined by pre-incubating TthBgl for 1 h. The effect of temperature on enzymatic activity was determined by incubation at temperatures ranging from 70 ºC to 95 ºC for 10 min. TthBgl was pre-incubated at 75 ºC, 80 ºC, 85 ºC, and 90 ºC for 30 min, 60 min, 90 min, and 120 min, respectively. Thermostability assays were conducted by measuring residual TthBgl activity. The results were expressed as percentages of the original activity.

The substrate specificity of the enzyme was tested using the chromogenic *p*-nitrophenyl (*p*NP)-glycosides *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-β-D-xylopyranoside, and *p*NP-α-L-arabinofuranoside (Sigma, St. Louis, USA) as well as cellobiose (Aladdin, Shanghai, China). Enzyme activity was examined at 85 ºC and pH 5.0 for 10 min with *p*NPG from 0.2 mM to 1 mM to calculate kinetic parameters (V_{max} and K_m) and turnover number (k_{cat}) according to the Lineweaver-Burk method. The influence of various glucose concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mM) on β-glucosidase activity was assayed. The K_i value of glucose was defined as amount of glucose required for inhibiting 50% of the β-glucosidase activity and was given as the averages of three separate determinations.

The effects of metal ions and chemical reagents on TthBgl activity were also determined. Cu^{2+}, Co^{2+}, Al^{3+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} at final concentrations of 1 mM were mixed with the enzyme for 2 h, and TthBgl activity was measured. Chemical reagents, including Tween 80, Tris, SDS, and EDTA, were also mixed with the enzyme for 2 h at final concentrations of 0.05%, 0.05%, 0.1%, and 0.5 mM, respectively.

Hydrolysis of cellobiose and HPLC analysis of cellobiose product

The enzyme (0.2 U) was condensed using a SpeedVac concentrator (Thermo, Waltham, USA) and combined with 0.02 g of cellobiose in 50 mM imidazole-potassium buffer (pH 5.0) in a 2-mL bioreactor at 75 ºC. The enzymatic reaction was carried out for 1, 3, 5, and 9 h and stopped by cooling the reaction mixture to -20 ºC. Samples were centrifuged at 13,000 × *g* for 15 min to remove the protein. Then, 0.4 mL of supernatant was mixed with 1.2 mL ethanol and lyophilized, and the pellets were re-suspended in 400 μL of water. Separation and quantification of glucose were performed using a Prevail

carbohydrate column (4.6 × 250 mm, 5 μm) with high-performance liquid chromatography (HPLC) (model 1260, Agilent, Santa Clara, USA) with an evaporative light scattering (ELSD) detector (Alltech, Shanghai, China). Acetonitrile/water (75/25%, v/v) was used as the mobile phase with a flow rate of 0.8 mL/min.

Bioinformatics analysis

Amino acid sequence similarities were examined by means of the system BLAST (http://blast.ncbi.nlm.nih.gov/). Sequence alignment of several Bgls was performed using Clustal X2 (Larkin *et al*. 2007). The neighbor-joining (NJ) and maximum-parsimony (MP) trees were created in Mega 6 software (megasoftware.net) in order to postulate phylogenetic relationships of various Bgls from different organisms.

RESULTS AND DISCUSSION

Expression and Purification

A putative gene encoding β-glucosidase from *T. thermarum* was cloned and expressed. After purification via heat treatment and Ni^{2+} column affinity chromatography, the enzyme showed a single band on SDS-PAGE, with a molecular mass of approximately 56 kDa (Fig. 1), which is consistent with the theoretical value of 56,264 kDa, which had been separately determined with the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The monomeric mass of β-glucosidase in this study was similar to the value of a 56 kDa β-glucosidase from *Thermotoga lettingae* TMO (Compute pI/Mw tool), which showed a 63.35% sequence similarity with the TthBgl (described below). Its mass was lower than the 95 kDa GH3 β-glucosidase from *Thermotoga maritima* MSB8 (Gabelsberger *et al*. 1993), the 81 kDa GH3 β-glucosidase from *Thermotoga neapolitana* (Compute pI/Mw tool), the 77.8 kDa GH3 β-glucosidase from *Thermofilum pendens* (Li *et al*. 2013), and the 83.5 kDa GH3 β-glucosidase from *Talaromyces emersonii* (Collins *et al*. 2007).

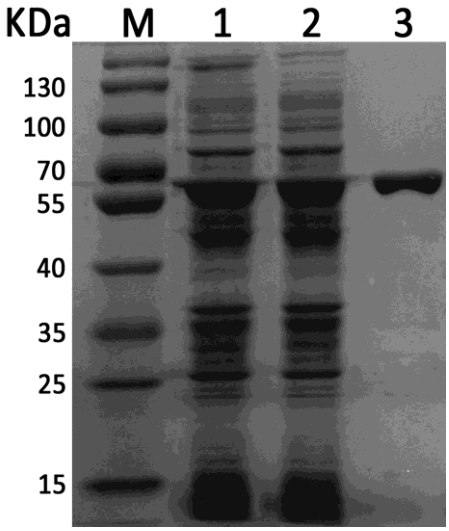

Fig. 1. SDS-PAGE analysis of purified TthBgl. Lane M: protein marker, Lane 1: culture supernatant of *E. coli* BL21 (DE3), Lane 2: culture supernatant treated at 60 ºC for 30 min, Lane 3: TthBgl purified by Ni^{2+} affinity column chromatography

Biochemical and Kinetic Parameters

The enzyme activity was examined over a pH range of 3.5 to 6.5 at 80 ºC. The maximum activity of TthBgl was recorded at pH 5.0 (Fig. 2a). TthBgl had a higher acid-tolerance than β-glucosidases from *T. maritima* MSB8 (pH 6.2) and *Thermotoga petrophila* RKU-1 (pH 7) (GenBank Protein No. ABQ46970) (Gabelsberger *et al*. 1993; Haq *et al*. 2012) but a similar optimal pH to β-glucosidase from *Pholiota adiposa* (Jagtap *et al*. 2013). The optimal temperature of TthBgl was 85 ºC (Fig. 2b), which is higher than the optimum for enzymes from *Actinosynnema mirum* (37 ºC), *Azospirillum irakense* (45 ºC), *Flavobacterium johnsoniae* (37 ºC), *Martelella mediterranea* (45 ºC), *Myceliophthora thermophile* (60 ºC), *Aspergillus niger* (70 ºC), and *Thermoascus aurantiacus* CBMAI-756 (75 ºC) (Rashid and Siddiqui 1997; Faure *et al*. 2001; Leite *et al.* 2008; Mao *et al*. 2010; Hong *et al*. 2012; Cui *et al*. 2013; Zhao *et al.* 2015). With good thermostability, the enzyme maintains its activity for a longer time, and the amount needed in the reaction is reduced (Pei *et al*. 2012; Shi *et al*. 2013). After pre-incubation at 80 ºC for 120 min, TthBgl retained 80% of the initial activity (Fig. 2c).

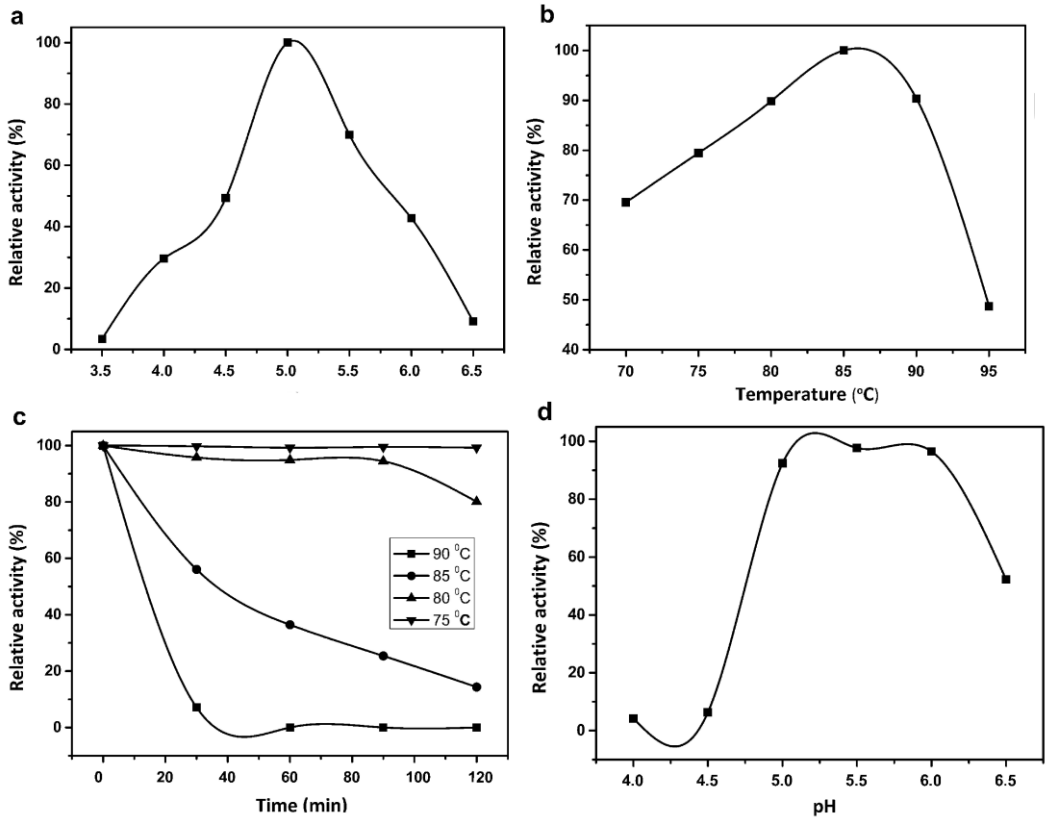

Fig. 2. Enzymatic properties of recombinant TthBgl. a: Optimal pH of TthBgl (80 ºC, pH 4.0 to 6.0 for 10 min). b: Optimal temperature (pH 5.0, 70 to 95 ºC for 10 min). c: Thermostability of TthBgl (pH 5.0, 75 to 90 ºC for 0, 30, 60, 90, and 120 min). d: pH stability (80 ºC, pH 4.0 to 6.5 for 1 h). The maximum activity was defined as 100% (a, b). The initial activity was defined as 100% (c, d). Data shown are from one typical experiment that was repeated three times, and the variation about the mean was below 5%.

β-glucosidase from *Thermofilum pendens* maintained 95% activity after incubation for 120 min at 90 ºC (Li *et al*. 2013), and the residual activity of His-tagged β-glucosidase from *T. maritima* is 75% after being incubated at 80 ºC for 60 min (Xue *et al*.

2009). The highly thermostable β-glucosidase from *T. petrophila* RKU-1 (ABQ46970) retains 80% activity after incubation for 120 min at 80 ºC (Haq *et al*. 2012). Therefore, compared with other thermostable Bgls in the GH3 family, TthBgl had a high thermostability at 80 ºC and pH stability at pH 5.0 to 6.0 with 90% remaining activity (Fig. 2d).

Kinetic parameters of TthBgl were obtained from Lineweaver-Burk plots. The results showed that its V_{max} and K_m were 8.79 U mg^{-1} and 2.41 mM (R^2 = 0.99), respectively, and k_{cat} was 8.25 s^{-1}.

In terms of substrate specificity, β-glucosidases are divided into three groups: those that hydrolyze oligosaccharides only, those with a strong affinity for aryl-β-glucose, and those able to hydrolyze multiple substrates (Zhao *et al*. 2013). When the substrate specificity was assayed with different substrates, TthBgl presented a strong affinity to *p*-nitrophenyl-β-D-glucopyranoside, confirming that this enzyme is a β-glucosidase (data not shown). Thus, TthBgl from *Thermotoga thermarum* DSM 5069 may belong to the second group, those having a strong affinity for aryl-β-glucose.

Bgl activity is known to be affected by many factors. Glucose concentration during the degradation of cellobiose or cellotriose inhibits enzyme activity via a feedback loop (Liu *et al*. 2011). Most Bgls that hydrolyze cellobiose are extremely sensitive to their own product, glucose (Saha and Bothast 1996), and in this study, TthBgl from *Thermotoga thermarum* DSM 5069 was no exception (Fig. 3). The relative activity of TthBgl decreased continuously as more glucose was added to the reaction. At 100 mM glucose, the relative activity declined steeply to around 75%, showing that TthBgl was sensitive to glucose. However, TthBgl still remained 50% of the original value in 500 mM, which is higher than the thermostable GH3 β-glucosidase from *Penicillium brasilianum* (2.3 mM), *Talaromyces emersonii* (0.245 mM), and the β-glucosidase III from *Aspergillus tubingensis* CBS 643.92 (470 mM) (Krogh *et al.* 2010; Murray *et al.* 2004; Decker *et al.* 2001). The reaction was conducted with *p*NPG as the substrate (Table 1).

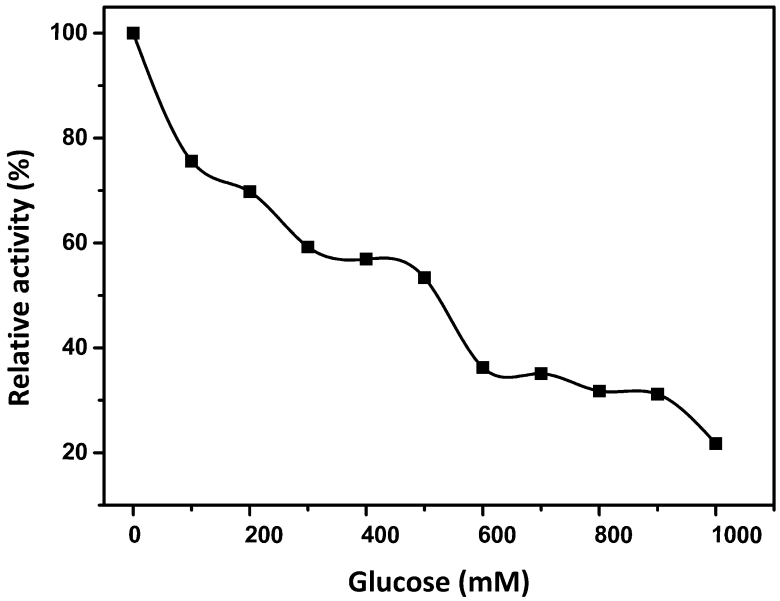

Fig. 3. Effects of glucose on TthBgl activity. The values are the mean of three separate experiments, and the standard deviations were below 5%.

Table 1. Characteristics of Recombinant β-glucosidase of *T. thermarum*

Property	Recombinant TthBgl
Optimum temperature	85 ºC
Optimum pH	5.0
Thermal stability (80 ºC)	2 h
Molecular weight)	56 kDa
V_{max}	8.79 U mg^{-1}
K_m	2.41 mM
k_{cat}	8.25 s^{-1}
K_i	500 mM

Because Bgls can be inhibited or activated by metal ions or chemical reagents, the influence of several cations and chemical reagents on TthBgl was also investigated (Table 2). The addition of cations and chemical reagents did not noticeably increase the activity of TthBgl. However, recombinant TthBgl was completely inhibited by 0.1% SDS and 1 mM Cu^{2+}. In most cases, the cation Cu^{2+} is an inhibitor of glycoside hydrolases (Shi *et al*. 2014). The glycoside hydrolases, *e.g*., β-xylosidase and α-L-arabinofuranosidase, from *Thermotoga thermarum* 5069 are also somewhat inhibited by Cu^{2+} (Shi *et al*. 2013, Shi *et al*. 2014). The enzyme was moderately inhibited by Co^{2+}, Al^{3+}, Zn^{2+}, and Tris, and its enzyme activity remained roughly constant after the addition of 0.5 mM EDTA, which confirmed that no metal ions were required in the reaction.

Table 2. Effects of Cations and Chemical Reagents on Purified TthBgl Activity

Cations/Chemical Reagents	Relative Activity (%)
None	100
Cu^{2+} (1 mM)	9.99 ± 0.44
Co^{2+} (1 mM)	80.92 ± 2.78
Al^{3+} (1 mM)	55.31 ± 3.74
Ba^{2+} (1 mM)	106.54 ± 4.16
Mn^{2+} (1 mM)	96.06 ± 2.35
Ni^{2+} (1 mM)	97.61 ± 1.60
Ca^{2+} (1 mM)	104.50 ± 2.70
Mg^{2+} (1 mM)	98.80 ± 2.53
Zn^{2+} (1 mM)	61.37 ± 1.96
Tween 80 (0.05%)	94.65 ± 3.09
Tris (0.05%)	81.63 ± 1.92
SDS (0.1%)	2.67 ± 0.53
EDTA (0.5 mM)	102.18 ± 0.84

Note: The activity of the enzyme without pre-incubation was defined as 100%. The experiment was performed in triplicate.

Release of glucose from cellobiose was also tested. A hydrolytic reaction was carried out by adding purified enzyme sample into mixtures at 75 ºC. This was done because TthBgl exhibited high thermostability at this temperature. The quantities of glucose released from cellobiose were 118.27 mg/L, 299.23 mg/L, 454.82 mg/L, and 789.25 mg/L for enzymatic reactions lasting 1, 3, 5, and 9 h, respectively.

Multiple Sequence Alignment and Phylogenetic Analysis of TthBgl

To further classify TthBgl, 22 candidate sequences were used to construct phylogenetic trees using the neighbor-joining (NJ) and maximum-parsimony (MP) methods (Fig. 4). Both methods exhibited similar topological structures (MP tree not shown). There were two well-supported clades in NJ trees, and each of them delegated a glycoside hydrolases family. Clade I was the GH3 β-glucosidase mainly from bacteria but also included archaea and fungi. Clade II was the GH1 β-glucosidase from bacteria and archaea. Although Bgls exist in families GH1, GH3, GH5, GH9, GH30, and GH116, only a few of them belonged to families GH5, GH9, GH30, and GH116. Hence, the phylogenetic tree did not present Bgls from these families mentioned above.

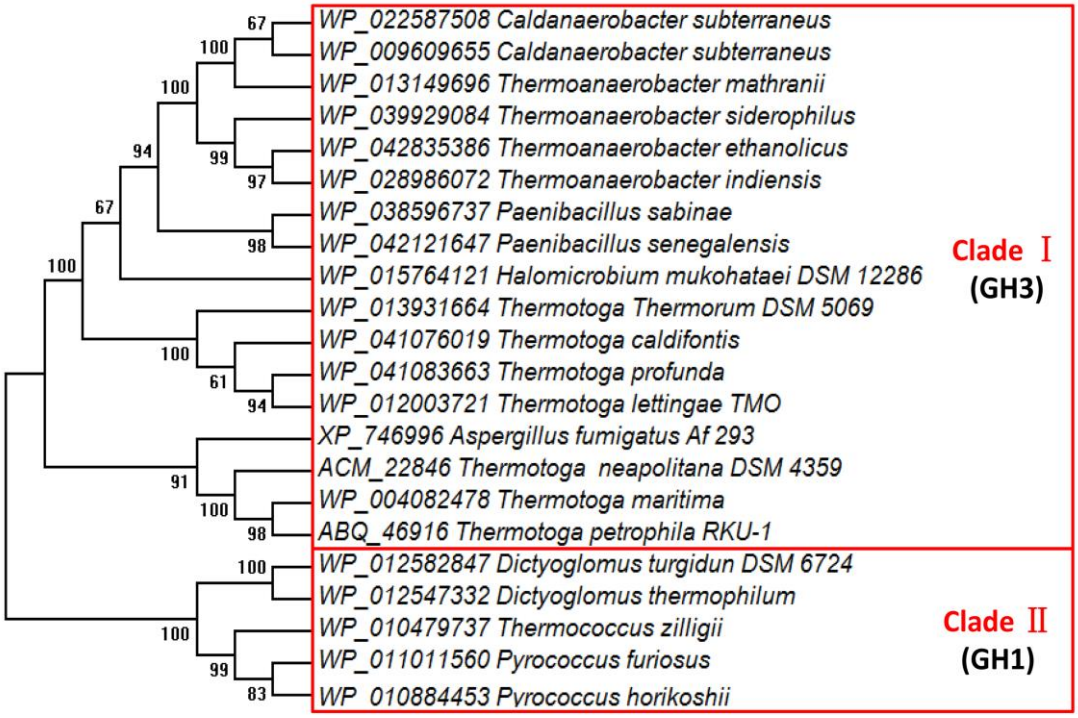

Fig. 4. Neighbor-joining (NJ) tree analysis of 22 β-glucosidase amino acid sequences from different organisms. Numbers on nodes correspond to percentage bootstrap values for 1000 replicates.

The amino acid sequence analysis indicated that TthBgl β-glucosidase belonged to the GH3 family. Through multiple sequence alignment using Clustal X2, TthBgl from *Thermotoga thermarum* possessed 65.07% sequence similarity with the β-glucosidase-related glycosidase from *Thermotoga profunda* (WP_041083663), 63.35% from *Thermotoga lettingae* TMO (WP_012003721), and 60.44% from *Thermotoga caldifontis* (WP_041076019). However, TthBgl β-glucosidase from *Thermotoga thermarum* shared only 13.72% sequence similarity with the Bgls from *Thermotoga maritima* MSB8

(WP_004082478) and *Thermotoga petrophila* RKU-1 (ABQ46916) and 13.32% similarity with the Bgl from *Thermotoga neapolitana* DSM 4359 (ACM22846). Therefore, the new GH3 TthBgl would be expected to exhibit some distinct properties. To further investigate the sequence similarity, the amino acid sequences of Clade I were aligned again. The exoglucanase from *Hordeum vulgare* (barley) was used as a structure-determined representative from the family. The equivalent amino acid residue in TthBgl is conserved, D254 (Fig. 5).

Fig. 5. Sequence alignment of the amino acid sequences of *Thermotoga thermarum* DSM 5069 with those of *Thermotoga lettingae* (*T. lettingae*), *Thermotoga profunda* (*T. profunda*) and *Thermotoga caldifontis* (*T. caldifontis*). The active sites are indicated as * on the top of the alignment.

The phylogenetic tree revealed that TthBgl is closely related to a β-glucosidase-related glycosidase from *T. lettingae* TMO. Unfortunately, the enzyme from *T. lettingae* TMO has not been characterized, and consequently, it was not possible to compare the enzymatic properties of these two enzymes. The tree also showed a distant relationship between β-glucosidases from *T. thermarum* DSM 5069 and *T. maritima* MSB8. For substrate specificity, TthBgl only hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside, while β-glucosidases from *T. maritima* MSB8 and *T. petrophila* RKU-1 (ABQ46916) hydrolyze several types of *p*NP-glycosides (Gabelsberger *et al*. 1993; Cota *et al*. 2015). In terms of K_m, TthBgl had a value of 2.41 mM for *p*NPG, which was lower than that of *T. petrophila* RKU-1 (ABQ46970) (2.8 mM) but higher than that of *T. maritima* MSB8 (0.43 mM) and *T. petrophila* RKU-1 (ABQ46916) (0.38 ± 0.03 mM) (Xue *et al*. 2009; Haq *et al*. 2012; Cota *et al*. 2015).

CONCLUSIONS

1. A β-glucosidase gene from *Thermotoga thermarum* DSM 5069, *TthBgl*, was cloned and sequenced. The recombinant protein was expressed, purified, and characterized for enzymatic properties.
2. Amino acid sequence analysis of TthBgl protein placed the enzyme in family 3 of the glycoside hydrolases. The recombinant enzyme possessed high thermostability and substrate specificity.

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REFERENCES CITED

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry* 72(1), 248-254. DOI: 10.1016/0003-2697(76)90527-3

Collins, C. M., Murray, P. G., Denman, S., Morrissey, J. P., Byrnes, L., Teeri, T. T., and Tuohy, M. G. (2007). "Molecular cloning and expression analysis of two distinct β-glucosidase genes, *bg1* and *aven1*, with very different biological roles from the thermophilic, saprophytic fungus *Talaromyces emersonii*," *Mycological Research* 111(7), 840-849. DOI: 10.1016/j.mycres.2007.05.007

Cota, J., Corrêa, T. L. R., Damásio, A. R. L., Diogo, J. A., Hoffmam, Z. B., Garcia, W., Oliveira, L. C., Prade, R. A., and Squina, F. M. (2015). "Comparative analysis of three hyperthermophilic GH1 and GH3 family members with industrial potential," *New Biotechnology* 32(1), 13-20. DOI: 10.1016/j.nbt.2014.07.009

Cui, C.-H., Kim, S.-C., and Im, W.-T. (2013). "Characterization of the ginsenoside-transforming recombinant β-glucosidase from *Actinosynnema mirum* and bioconversion of major ginsenosides into minor ginsenosides," *Applied Microbiology and Biotechnology* 97(2), 649-659. DOI: 10.1007/s00253-012-4324-5

Decker, C. H., Visser, J., and Schreier, P. (2001). "β-Glucosidase multiplicity from *Aspergillus tubingensis* CBS 643.92: Purification and characterization of four β-glucosidase and their differentiation with respect to substrate specificity, glucose inhibition and acid tolerance," *Appl. Microbiol. Biotechnol.* 55, 157-163. DOI: 10.1007/s002530000462

Denman, S., Xue, G.-P., and Patel, B. (1996). "Characterization of a *Neocallimastix patriciarum* cellulose cDNA (*celA*) homologous to *Trichoderma reesei* cellobiohydrolase II," *Applied and Environmental Microbiology* 62, 1889-1896.

dos Santos, C., Squina, F., Navarro, A., Oldiges, D., Leme, A., Ruller, R., Mort, A., Prade, R., and Murakami, M. (2011). "Functional and biophysical characterization of a hyperthermostable GH51 α-l-arabinofuranosidase from *Thermotoga petrophila*," *Biotechnology Letters* 33(1), 131-137. DOI: 10.1007/s10529-010-0409-3

Faure, D., Henrissat, B., Ptacek, D., Bekri, M. A., and Vanderleyden, J. (2001). "The *celA* gene, encoding a glycosyl hydrolase family 3 β-glucosidase in *Azospirillum irakense*, is required for optimal growth on cellobiosides," *Applied and Environmental Microbiology* 67, 2380-2383. DOI: 10.1128/AEM.67.5.2380-2383.2001

Gabelsberger, J., Liebl, W., and Schleifer, K.-H. (1993). "Cloning and characterization of β-galactoside and β-glucoside hydrolysing enzymes of *Thermotoga maritime*," *FEMS Microbiology Letters* 109, 131-137. DOI:

10.1111/j.1574-6968.1993.tb06157.x

González-Pombo, P., Fariña, L., Carrau, F., Batista-Viera, F., and Brena, B. M. (2011). "A novel extracellular β-glucosidase from *Issatchenkia terricola*: Isolation, immobilization and application for aroma enhancement of white Muscat wine," *Process Biochemistry* 46(1), 385-389. DOI: 10.1016/j.procbio.2010.07.016

Haq, I., Khan, M., Muneer, B., Hussain, Z., Afzal, S., Majeed, S., Rashid, N., Javed, M., and Ahmad, I. (2012). "Cloning, characterization and molecular docking of a highly thermostable β-1,4-glucosidase from *Thermotoga petrophila*," *Biotechnology Letters* 34(9), 1703-1709. DOI: 10.1007/s10529-012-0953-0

Hong, H., Cui, C.-H., Kim, J.-K., Jin, F.-X., Kim, S.-C., and Im, W.-T. (2012). "Enzymatic biotransformation of ginsenoside Rb 1 and gypenoside XVII into ginsenosides Rd and F2 by recombinant β-glucosidase from *Flavobacterium johnsoniae*," *Journal of Ginseng Research* 36:418-424. DOI: 10.5142/jgr.2012.36.4.418

Jagtap, S. S., Dhiman, S. S., Kim, T.-S., Li, J., Chan Kang, Y., and Lee, J.-K. (2013). "Characterization of a β-1,4-glucosidase from a newly isolated strain of *Pholiota adiposa* and its application to the hydrolysis of biomass," *Biomass and Bioenergy* 54, 181-190. DOI: 10.1016/j.biombioe.2013.03.032

Ketudat Cairns, J., and Esen, A. (2010). "β-Glucosidases," *Cellular and Molecular Life Sciences* 67(20), 3389-3405. DOI: 10.1007/s00018-010-0399-2

Krogh, K. B., Harris, P. V., Olsen, C. L., Johansen, K. S., Hojer-Pedersen, J., Borjesson, J., and Olsson, L. (2010). "Characterization and kinetic analysis of a thermostable GH3 β-glucosidase from *Penicillium brasilianum*," *Appl. Microbiol. Biotechnol* 86, 143-154. DOI: 10.1007/s00253-009-2181-7

Kudou, M., Kubota, Y., Nakashima, N., Okazaki, F., Nakashima, K., Ogino, C., and Kondo, A. (2014). "Improvement of enzymatic activity of β-glucosidase from *Thermotoga maritima* by 1-butyl-3-methylimidazolium acetate," *Journal of Molecular Catalysis B: Enzymatic* 104, 17-22. DOI: 10.1016/j.molcatb.2014.02.013

Larkin, M.-A., Blackshields, G., Brown, N.-P., Chenna, R., McGettigan, P.-A., McWilliam, H., Valentin, F., Wallace, I.-M., Wilm, A., Lopez, R., Thompson, J.-D., Gibson, T.-J., and Higgins, D.-G. (2007). "Clustal W and Clustal X version 2.0," *Bioinformatics* 23, 2947-2948. DOI: 10.1093/bioinformatics/btm404

Leite, R. S. R., Alves-Prado, H. F., Cabral, H., Pagnocca, F. C., Gomes, E., Da-Silva, R. (2008). "Production and characteristics comparison of crude β-glucosidase produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulansI* in agricultural wastes," *Enzyme and Microbial Technology* 43, 391-395. DOI: 10.1016/j.enzmictec.2008.07.006

Li, D., Li, X., Dang, W., Tran, P. L., Park, S.-H., Oh, B.-C., Hong, W.-S., Lee, J.-S., and Park, K.-H. (2013). "Characterization and application of an acidophilic and thermostable β-glucosidase from *Thermofilum pendens*," *Journal of Bioscience and Bioengineering* 115(5), 490-496. DOI: 10.1016/j.jbiosc.2012.11.009

Liu, J., Zhang, X., Fang, Z., Fang, W., Peng, H., and Xiao, Y. (2011). "The 184th residue of β-glucosidase Bgl1B plays an important role in glucose tolerance," *Journal of Bioscience and Bioengineering* 112(5), 447-450. DOI: 10.1016/j.jbiosc.2011.07.017

Loaiza, J. M., López, F., García, M. T., Fernández, O., Díaz, M. J., and García, J. C. (2015). "Selecting the pre-hydrolysis conditions for *Eucalyptus* wood in a

fractional exploitation biorefining scheme," *Journal of Wood Chemistry and Technology* 36, 211-223. DOI: 10.1080/02773813.2015.1112402

Mao, X., Hong, Y., Shao, Z., Zhao, Y., and Liu, Z. (2010). "A novel cold-active and alkali-stable β-glucosidase gene isolated from the marine bacterium *Martelella mediterranea*," *Applied Biochemistry and Biotechnology* 162(8), 2136-2148. DOI: 10.1007/s12010-010-8988-y

Murray, P., Aro, N., Collins, C., Grassick, A., Penttila, M., Saloheimo, M., Tuohy, M. (2004). "Expression in *Trichoderma reesei* and characterisation of a thermostable family 3 β-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*," *Protein Expression and Purification* 38, 248-257. DOI: 10.1016/j.pep.2004.08.006

Pei, J., Pang, Q., Zhao, L., Fan, S., and Shi, H. (2012). "*Thermoanaerobacterium thermosaccharolyticum* β-glucosidase: A glucose-tolerant enzyme with high specific activity for cellobiose," *Biotechnology for Biofuels* 5, 31. DOI: 10.1186/1754-6834-5-31

Rashid, M. H., and Siddiqui, K. S. (1997). "Purification and characterization of a β-glucosidase from *Aspergillus niger*," *Folia Microbiol* 42(6), 544-550. DOI: 10.1007/BF02815462

Roy, P., Mishra, S., and Chaudhuri, T. K. (2005). "Cloning, sequence analysis, and characterization of a novel β-glucosidase-like activity from *Pichia etchellsii*," *Biochemical and Biophysical Research Communications* 336(1), 299-308. DOI: 10.1016/j.bbrc.2005.08.067

Saha, B. C., and Bothast, R. J. (1996). "Production, purification, and characterization of a highly glucose-tolerant novel β-glucosidase from *Candida peltata*," *Applied and Environmental Microbiology* 62: 3165-3170.

Shi, H., Li, X., Gu, H., Zhang, Y., Huang, Y., Wang, L., and Wang, F. (2013). "Biochemical properties of a novel thermostable and highly xylose-tolerant β-xylosidase/α-L-arabinofuranosidase from *Thermotoga thermarum*," *Biotechnology for Biofuels* 6, 27-36. DOI: 10.1186/1754-6834-6-27

Shi, H., Zhang, Y., Xu, B., Tu, M., and Wang, F. (2014). "Characterization of a novel GH2 family α-l-arabinofuranosidase from hyperthermophilic bacterium *Thermotoga thermarum*," *Biotechnology Letters* 36(6), 1321-1328. DOI: 10.1007/s10529-014-1493-6

Windberger, E., Huber, R., Trincone, A., Fricke, H., and Stetter, K. (1989). "*Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs," *Archives of Microbiology* 151(6), 506-512. DOI: 10.1007/BF00454866

Wu, W., Kasuga, T., Xiong, X., Ma, D., and Fan, Z. (2013). "Location and contribution of individual β-glucosidase from *Neurospora crassa* to total β-glucosidase activity," *Archives of Microbiology* 195(12), 823-829. DOI: 10.1007/s00203-013-0931-5

Xue, Y., Song, X., and Yu, J. (2009). "Overexpression of β-glucosidase from *Thermotoga maritima* for the production of highly purified aglycone isoflavones from soy flour," *World Journal of Microbiology and Biotechnology* 25(12), 2165-2172. DOI: 10.1007/s11274-009-0121-4

Yan, Q., Hua, C., Yang, S., Li, Y., and Jiang, Z. (2012). "High level expression of extracellular secretion of a β-glucosidase gene (*PtBglu3*) from *Paecilomyces thermophila* in *Pichia pastoris*," *Protein Expression and Purification* 84(1), 64-

72. DOI: 10.1016/j.pep.2012.04.016

Zhao, J., Guo, C., and Tian, C. (2015). "Heterologous expression and chatacterization of a GH3 β-glucosidase from Thermophilic Fungi *Myceliophthora thermophila* in *Pichia pastoris*," *Appl Biochem Biotechnol* 177, 511-527. DOI: 10.1007/s12010-015-1759-z

Zhao, L., Xie, J., Zhang, X., Cao, F., and Pei, J. (2013). "Overexpression and characterization of a glucose-tolerant β-glucosidase from *Thermotoga thermarum* DSM 5069T with high catalytic efficiency of ginsenoside Rb1 to Rd," *Journal of Molecular Catalysis B: Enzymatic* 95, 62-69. DOI: 10.1016/j.molcatb.2013.05.027

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