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Characterization of a Thermophilic and Inhibitor-Tolerant GH1 β-Glucosidase Present in a Hot Spring

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Abstract: β-glucosidase is a key enzyme in the degradation of lignocellulosic biomass, which is responsible for the conversion of oligosaccharides from cellulose hydrolysates to glucose. However, its required high temperatures and the presence of inhibitors have limited its use in industry. In this study, a new β-glucosidase gene, named *thbg2*, was obtained from the metagenome Ruidian Hot Spring, Tengchong City, Yunnan Province, southwestern China. The gene was synthesized, cloned, heterologously expressed, and enzymatically characterized. Its optimum temperature and pH were 60 °C and pH 5.6, respectively. ThBg2 exhibited more than 60% relative activity in temperatures ranging from 40 °C to 70 °C and across a pH of 4.0–6.6. It maintained 100% relative activity after incubation at either 50 °C for 24 h or 60 °C for 12 h and more than 80% residual activity after incubation at pH 4.0–6.0 for 24 h. Moreover, it maintained more than 80% relative activity in the presence of heavy metal ions, ethanol, SDS etc. Furthermore, glucose yields from corn stalks increased by 20% after ThBg2 (0.05 mg/mL) was added to the commercial cellulase reaction system. Overall, this work identified a thermophilic and inhibitor-tolerant β-glucosidase with potential applications in commercial lignocellulose utilization and the bioenergy industry.

Keywords: hot spring; metagenome; thermophilic; heavy metal ion tolerant; glucose tolerant; β-glucosidase

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1. Introduction

Cellulose is an important component of lignocellulose and is widely found in agricultural residues such as straw, rice, wheat, and corn as well as bagasse [1]. The utilization and transformation of cellulose are of great significance in solving the world's energy crisis as well as food shortage, environmental pollution, and other global problems [2]. The conversion of lignocellulosic biomass to biofuels is now being proposed as a sustainable and alternative energy option to sustain global energy demand [3]. The conversion of cellulose to biofuels depends on the synergistic hydrolysis of enzymes, which include endo-beta-1,4-glucanase (EC 3.2.1.4), exo-beta-1,4-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) [4]. Although β-glucosidase does not act directly on cellulose, it can eliminate the inhibitory effect of cello-oligosaccharides on endo-beta-1,4-glucanase and exo-beta-1,4-glucanase. These effects are of great importance in cellulose degradation [4,5]. Therefore, β-glucosidase is considered to be the rate-limiting enzyme for cellulose degradation.

β-glucosidase is broadly found in nature [6,7]. It catalyzes the cleavage of glycosidic bonds in cellulose, oligosaccharides and either alkyl or aryl β-glucosides [8]. Currently, β-glucosidases are mainly derived from culturable microorganisms, such as *Saccharomyces* [9],

Aspergillus niger [10], *Clostridium thermocellulosum* [11], and *Bacillus* sp. CGMCC 1.16541 [12]. In most industrial applications, high temperatures and strong acids are required pretreatment steps. Given these requirements, thermophilic and acidophilic enzymes have historically been more advantageous. However, many reported β-glucosidases have some disadvantages, including poor thermal stability and low enzymatic activity [13]. Therefore, there has been increased demand for new thermophilic and thermostable β-glucosidases options.

More than 99% of known microorganisms have not been cultured purely with current laboratory culture techniques [14]. In this case, metagenomic technology is sufficient to avoid the limitations of pure cultures and provide an effective way to exploit uncultured microbial functional genes. Importantly, this approach has now been successfully used to identify novel biocatalytic enzyme genes from uncultured microbial communities in a variety of environmental samples [15,16]. For example, many new β-glucosidase genes were obtained by metagenomic approaches [17–19], which have included novel β-glucosidases and genes highly tolerant to ethanol and glucose from termite gut [20], soil [21], cattle rumen [22], compost [23], etc.

As a typical high-temperature environment, hot springs contain a rich and diverse resource of thermophilic microorganisms and thermophilic enzymes [24]. With more than 50 volcanoes and 140 geothermal zones, Tengchong is rich in geothermal resources [25,26]. The aim of this study was to mine new thermotropic β-glucosidases from Tengchong hot springs. Here, a novel β-glucosidase gene (*thbg2*) was detected in the metagenome of the Ruidian Hot Spring in Tengchong City, Yunnan Province, China. The recombinant protein ThBg2 was next expressed and purified. Its enzymatic properties were investigated to provide a thermophilic, Cu^{2+}-, SDS-, and ethanol-tolerant β-glucosidase with potential applications in commercial lignocellulose utilization and the bioenergy industry.

2. Materials and Methods

2.1. Sample Collection and Metagenomic DNA Extraction

Samples were collected from the Ruidian Hot Spring in Tengchong City (25.439621° N, 98.460999° E), Yunnan Province, southwestern China. The surface temperature of Ruidian Hot Spring was around 63 °C with a pH of 7.6. Metagenomic DNA was extracted with the Power Soil Kit (MOBIO DNeasy PowerSoil Kit, New York, NY, USA) according to the manufacturer's instructions.

2.2. Metagenomic Sequencing and Synthesis of β-Glucosidase Gene

Metagenomic sequencing was performed using a HiSeq 2500 instrument at GENWIZ, Suzhou, China. The IMG server (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi) (accessed on 1 April 2019) was used to investigate all resulting sequences. To further detect the potential functions of individual genes and ORFs, KEGG [27], COG [28], and Pfam [29] were used. Based on this functional prediction, a β-glucosidase gene sequence—*thbg2*—was obtained from the metagenomic database. The ThBg2 gene was synthesized and cloned to the pUC18 vector by Guangzhou Tanjin Biotechnology Co. (Guangzhou, China). The gene was codon-optimized for expression in *E. coli*. (Supplementary Figure S1). The nucleotide sequence of *thbg2* was submitted to GenBank (NCBI) under accession number OP880884.

2.3. Sequence Analysis of β-Glucosidase Gene

DNA and protein sequences were compared using BLASTx and BLASTp programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 April 2019), respectively. The molecular weight of protein ThBg2 was predicted using EXPASY (https://web.expasy.org/protparam/, accessed on 1 April 2019). The multiple alignment of protein sequences closely related to ThBg2 was performed, and phylogenetic analysis was performed using MEGA 7.0 [30].

2.4. Culture Medium

Luria broth (LB) medium was comprised of 0.5% yeast extract, and 1% tryptophan, 1% NaCl at pH 7.4. Kanamycin was used as an antibiotic, and its final concentration was 50 μg/mL. The LB solid medium was supplemented with 2% agar.

2.5. Gene Amplification and Construction of Recombinant Vector

Functional genes were amplified by PCR using the above synthetic genes as templates by primers (ThBg2-F: CATGGATCCATGAACAAATACCACTTCCCG and ThBg2-R: TGCAAGCTTTCACTCGTTCAGGCCGTTGT). The underlined part of the sequence indicates the restriction sites for *EcoRI* and *Hind III* (Thermo Fisher Scientific, New York, NY, USA), respectively. Amplification was performed using the high-fidelity DNA polymerase Gold Medal Mix (Beijing Prime Tech Biotechnology Co., Beijing, China). The PCR program consisted of pre-denaturation at 94 °C for 4 min, followed by 32 cycles, denaturing at 94 °C for 30 s, annealing at 55 °C for 35 s, extending at 72 °C for 90 s, and undergoing a final extension phase at 72 °C for 5 min. Identification was performed using 1.0% agarose gel electrophoresis. The PCR products were then observed under UV light (Supplementary Figure S2), and the correct size bands were cut off and recovered by gel recovery using the DNA Gel Recovery Kit (Beijing Tsingke Biotech Co., Ltd., Beijing, China).

The above PCR product and vector pET-28a(+) were double-digested using restriction endonucleases (*EcoRI* and *Hind III*) according to the manufacturer's instructions. The resulting digested products were then recovered. The digested PCR product and vector were ligated by T4-DNA ligase at 25 °C for 2 h, and the ligated product was introduced into *E. coli* DH5α using the Ca^{2+} chemical transformation method by heat shock at 42 °C for 60 s. The positive clones were obtained by colony PCR and sequencing. The positive recombinant vector was extracted using a plasmid extraction kit from the positive clone, named pET-28a-*ThBg2*.

2.6. Heterologous expression of ThBg2

The recombinant vector pET-28a-*ThBg2* was transformed into *E. coli* BL21 (DE3) to obtain the recombinant strain *E. coli* BL21 (DE3)-*ThBg2*. A single colony was picked from the LB medium plate containing 50 μg/mL kanamycin and inoculated in 10 mL LB medium containing 50 μg/mL kanamycin. It was cultured overnight at 37 °C and 220 rpm for overnight incubation. The above strain was incubated in 200 mL LB medium containing 50 μg/mL kanamycin at 1% inoculum, 37 °C, and 220 rpm for about 2 h. Until $OD_{600} \approx 0.6$, IPTG was added to a final concentration of 0.2 mM to induce the expression of the recombinant β-glucosidase. Cultures were incubated at 25 °C and 220 rpm for 8 h. Finally, cultures were centrifuged at 4 °C and 7000 rpm for 20 min to obtain *E. coli* cells.

2.7. Purification of Recombinant Proteins

E. coli cells were resuspended in centrifuge tubes with PBS solution (pH 7.6) containing 10 mM imidazole and placed in an ice-water mixture for 30 min with ultrasonic crushing, followed by centrifugation using a high-speed refrigerated centrifuge at 4 °C and 7000 r/min for 20 min. After centrifugation, the supernatant was collected. Purification was conducted using an Ni-NTA column. The Ni-NTA column was equilibrated with five times the column volume of equilibration solution (PBS solution with 10 mM imidazole, pH 7.6). The supernatant mentioned above was added to the Ni-NTA column for loading and repeated once, after which the column was washed with 10 times the column volume of the equilibration solution. The target protein was eluted with three times the column volume of the eluent solution (PBS solution containing 250 mM imidazole, pH 7.6), and the protein solution was collected in 1.5 mL centrifuge tubes with 1.0 mL per tube. The protein concentration was estimated using Bradford reagent, using bovine serum albumin as standard. The absorption value was measured at OD_{595} and analyzed using SDS-PAGE electrophoresis, after which the target protein was purified (The protein ladder was from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China).

2.8. Enzyme Assay of β-Glucosidase

A commercially available glucose oxidase-peroxidase assay kit was used in this study (BioSino Bio–Technology & Science Inc., Beijing, China). The β-glucosidase activity was determined using cellobiose as a substrate. Briefly, 20 μL of purified enzyme solution (0.142 mg/mL) was added to 180 μL of buffer containing 1% (*w*/*v*) cellobiose, and the reaction was performed at the optimum temperature for 15 min and terminated by freezing at −80 °C for 2–3 min. Then, 10 μL of the reaction mixture was added to a 96-well culture plate with 200 μL of Glucose Oxidase-Peroxidase Assay Kit buffer, incubated at 37 °C for 10 min, and the absorption value was measured at 492 nm using an enzyme marker. One unit (U) was defined as the amount of enzyme required to release 2 μmol of glucose from cellobiose per minute. All reactions were performed in triplicate.

2.9. Determination of Optimum Temperature and Thermostability of ThBg2

According to the activity assay, the purified β-glucosidase was measured at different temperatures (25–85 °C) under optimum reaction pH conditions. The optimum reaction temperature of ThBg2 was determined across a gradient of 5 °C increments. For the thermal stability analysis, the enzyme solution was incubated at different temperatures as follows: 50 °C, 60 °C and 70 °C for different times (0–24 h), to determine the temperature stability. To determine the residual activity of ThBg2, this analysis was conducted using a gradient of 30 min for each of the first 5 h, followed by 1 h for hours 6–12.

2.10. Determination of Optimum pH and pH Stability of ThBg2

The enzymatic reaction of β-glucosidase was conducted using buffers with different pH levels to determine its optimum reaction pH. Across this range, pH 3.0–8.0 citric acid-NaH_2PO_4 buffers and pH 8.0–10.0 glycine-NaOH buffers were used. The pH suitability results of the enzyme were determined at the optimum reaction temperature from the gradient of pH 3.0–10.0.

To conduct our pH stability analysis, pH buffers (pH 3.0–8.0 citric acid-NaH_2PO_4 buffers, pH 9.0–10.0 glycine-NaOH buffers) were used for pH stability analysis. The pure enzyme solution was mixed at a ratio of 1:2, respectively, and the positive control was diluted twice with pH 7.6 PBS solution without buffer treatment. All other conditions remained unchanged. The above enzyme solutions were then incubated at 4 °C for 12 h and 24 h before measuring the residual activity of ThBg2.

2.11. Effects of Metal Ions and Other Chemicals on the Activity of ThBg2

To determine the effects of metal ions and inhibitors on β-glucosidase, different metal ions (K^+, Mg^{2+}, Fe^{3+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ag^+, Mn^{2+}, Pb^{2+}, and Ni^{2+}) were separately added to the enzyme solution at 60 °C until the concentrations reached at 1 mM and 10 mM. The concentrations of different inhibitors [disodium ethylenediaminetetraacetate (EDTA), sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PSMF), and dithiothreitol (DTT)] added were 0.1% and 1%, and the concentrations of different organic solvents (isopropanol, methanol, ethanol, β-mercaptoethanol and Tween) were 1% and 10% for 10 min before the determination of residual enzyme activity. A reaction mixture without additives under standard conditions was used as a control (100%).

2.12. Effect of Glucose Concentration on Enzymatic Activity

Using p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate, 10 μg protein was added to a 200 μL reaction mixture containing 2.5 mM pNPG (Sigma, St. Louis, MO, USA) at pH 5.6 (citric acid-NaH_2PO_4 buffer). After a 5 min incubation at the optimal temperature, the reaction was stopped by adding 450 μL of (1M) Na_2CO_3. The release of p-nitrophenol was measured by monitoring absorption at 405 nm using p-nitrophenol (Sigma, USA) as the standard. To investigate the effect of D-glucose on the catalytic activity of ThBg2, varying concentrations of D-glucose (0–3M) were incorporated into the assay mixture—containing

1 mM pNPG—under standard conditions. For the control, the same reaction system was used, but no glucose was added.

2.13. Kinetic Parameters of ThBg2

Cellobiose concentration was determined under optimal assay conditions of 0.2–2%. The values of K_m and V_{max} were calculated according to the Michaelis–Menten equation.

2.14. Hydrolysis of Corn Stalks by ThBg2

Briefly, 10 g corn stover was sifted through 80 mesh and boiled for 30 min in 100 mL of hot water. The corn stalks were filtered through a paper filter and dried at 80 °C. Then, 0.2 g of corn stalks that had been pre-treated with hot water was added to 1 mL buffer (pH 5.6), to which 0.2 mg cellulase (Sangon Biotech, China), obtained from *Trichoderma reesei*, and/or 0.05 mg ThBg2, were added to the reaction system. The mixtures were then incubated at 50 °C, respectively. Samples were taken 0–8 h after the reaction began, with an hour interval between each sample. Glucose concentration in the reaction solution was determined using a Glucose Oxidase Assay Kit and according to the manufacturer's instructions (Abnova, Taiwan, China). The control condition was the reaction solution without added enzymes. Three biological replicates were performed for each group, and the average was used for all subsequent analyses.

2.15. Statistical Analysis

Unless otherwise stated, all assays were carried out in triplicate, and the average was used in all analyses. The results were analyzed using SPSS 20.0 and expressed as means ± SEM (standard error of mean). Statistical analyses were performed by using the T-test to compare the treated and untreated groups. In all comparisons, p values < 0.05 were considered statistically significant.

3. Results

3.1. Sequence Analysis

The theoretical molecular weight of ThBg2 predicted by EXPASY was 52 KDa. Sequence analysis revealed that ThBg2 contained a different catalytic module of glycosyl hydrolase family 1 (GH1) in the predicted enzyme protein. The protein sequence of ThBg2 had the highest amino acid sequence identity (89%) with the β-glucosidase of *Caloramator* sp. (NCBI: MBZ4663882.1) (Figure 1).

3.2. Expression and Purification of ThBg2

The β-glucosidase gene ThBg2 was successfully expressed in *E. coli* BL21 (DE3). The resulting recombinant protein with a His-tagged N-terminus was purified by Ni-NTA affinity chromatography. Using SDS-PAGE, the purified ThBg2 protein showed a single band with a molecular weight of appropriately 50 kDa by SDS-PAGE, which was consistent with its theoretical predicted molecular weight (52 kDa) (Figure 2).

3.3. Optimal Temperature and Thermal Stability of ThBg2

The ThBg2 showed the highest activity at a temperature of 60 °C and pH 5.6. Its enzymatic activity gradually decreased after 60 °C but it still had 50% of its enzyme activity at 85 °C (Figure 3a). The ThBg2 maintained stable enzyme activity at 50 °C and 60 °C and had high activity (close to 100%) after 5 h of treatment. The enzyme activity was maintained at 80% at 70 °C for 60 min of treatment, with good thermal stability (Figure 3c).

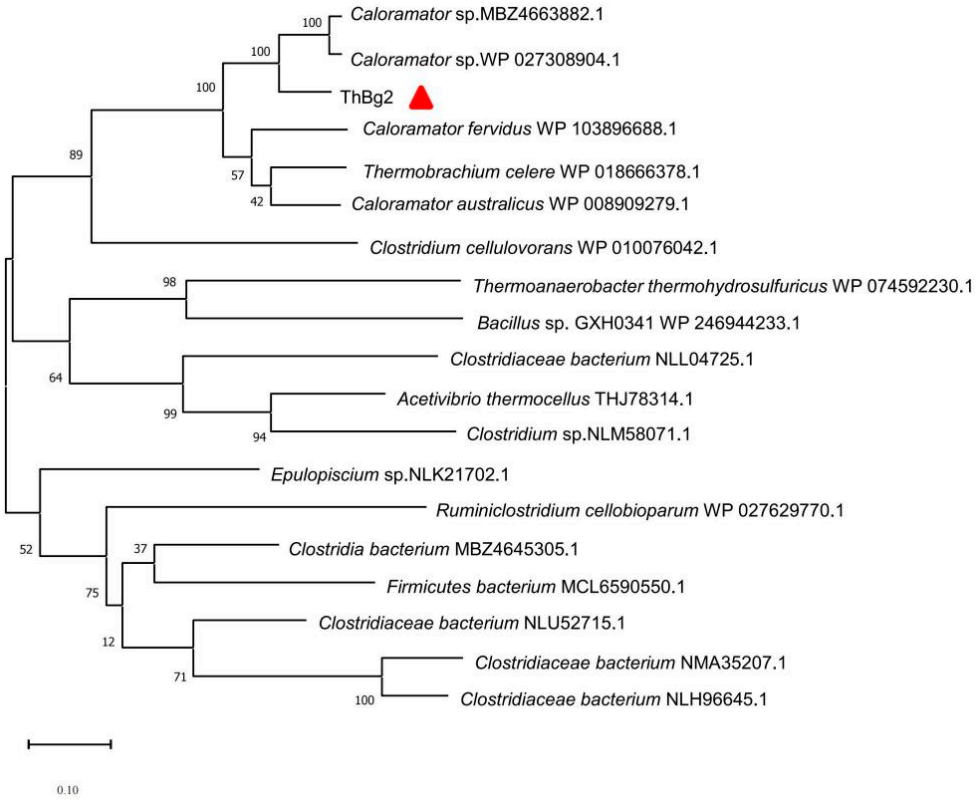

Figure 1. Phylogenetic dendrogram obtained by maximum likelihood analysis based on amino acid sequences, showing the phylogenetic position of ThBg2 related to β-glucosidases. Bootstrap values (expressed as a percentage of 1000 replications) are given at nodes.

3.4. Optimal pH and pH Stability of ThBg2

The ThBg2 exhibited the highest activity at pH 5.6 and the optimum temperature of 60 °C. β-glucosidase was able to maintain enzyme activity at 50% in the range of pH 3.5 to 7.0 (Figure 3b). It remained at approximately 100% after 24 h of treatment across a pH range of pH 3.0 to 10.0 (Figure 3d).

3.5. Effects of Metal Ions and Other Chemicals on the Activity of β-Glucosidase

The addition of metal ions did not significantly inhibit ThBg2 activity. Mg^{2+}, Ca^{2+} and Mn^{2+} showed no significant inhibitory effect on its enzymatic activity, with the enzymatic activity of ThBg2 remaining above 80%. Similarly, a Cu^{2+} concentration of 1 mM had no significant effect on its enzyme activity, which was maintained at approximately 60% at 10 mM (Figure 4a,b). This indicated that it had a certain tolerance to heavy metal ions.

However, four inhibitors did show some inhibitory effect on ThBg2 activity, but its activity remained above 50%. EDTA showed no significant inhibition of ThBg2 catalytic activity, and its activity remained above 80%. This indicated strong metal independence. Moreover, 1% SDS showed the strongest inhibition (Figure 4d), but enzyme activity remained at 50%. This was likely because SDS interfered with hydrophobic interactions and presented the original protein structure. The inhibition of β-mercaptoethanol was the most significant among the five organic solvents, while the other four that were inhibited did not show significant inhibition (Figure 4d). It is worth noting that when the ethanol concentration reached 10%, there was no significant effect on its enzyme activity.

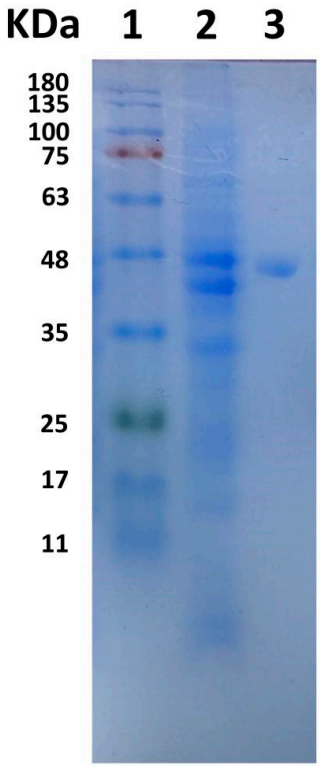

Figure 2. SDS-PAGE analysis of recombinant ThBg2 produced by *E. coli* BL21. Lane 1, protein molecular weight marker, mass indicated on the left; lane 2, total protein in IPTG-induced *E. coli* BL21/*pET28a-ThBg2*; lane 3, purified ThBg2.

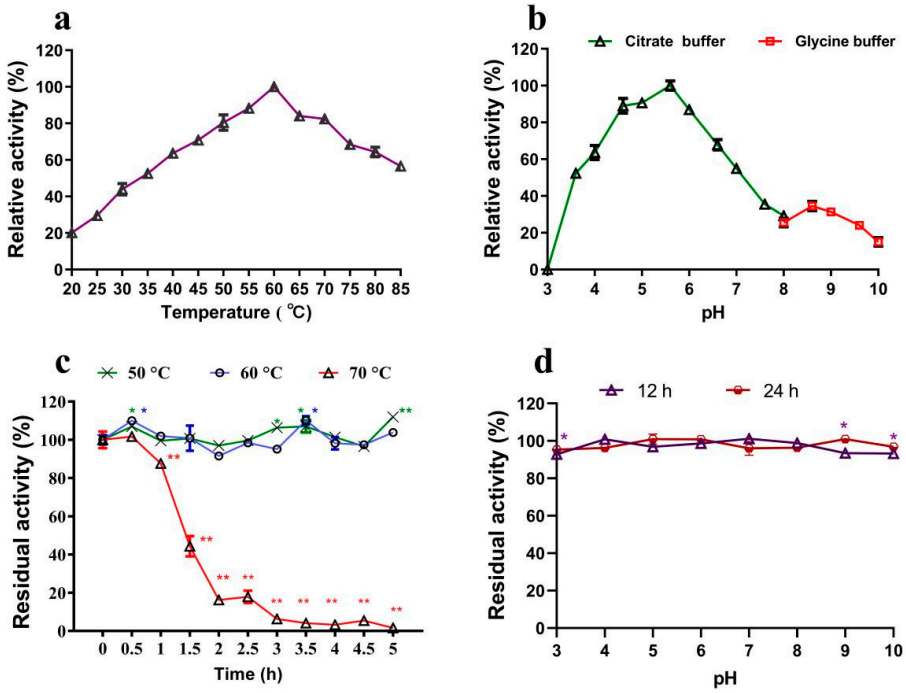

Figure 3. Effects of temperature and pH on the activity and stability of the recombinant ThBg2. (**a**) Temperature effect on the activity of ThBg2. (**b**) pH effect on the activity of ThBg2. (**c**) The effect of temperature on stability at different temperatures (50, 60 and 70 °C) for 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 h. (**d**) The effect of pH on stability. Values represent the mean of three biological replicates. Error bars represent the mean ± SEM of three biological replicates. ** $p < 0.01$, * $p < 0.05$. The primary activity was taken as 100%. 100% = 65 ± 2 U/mg.

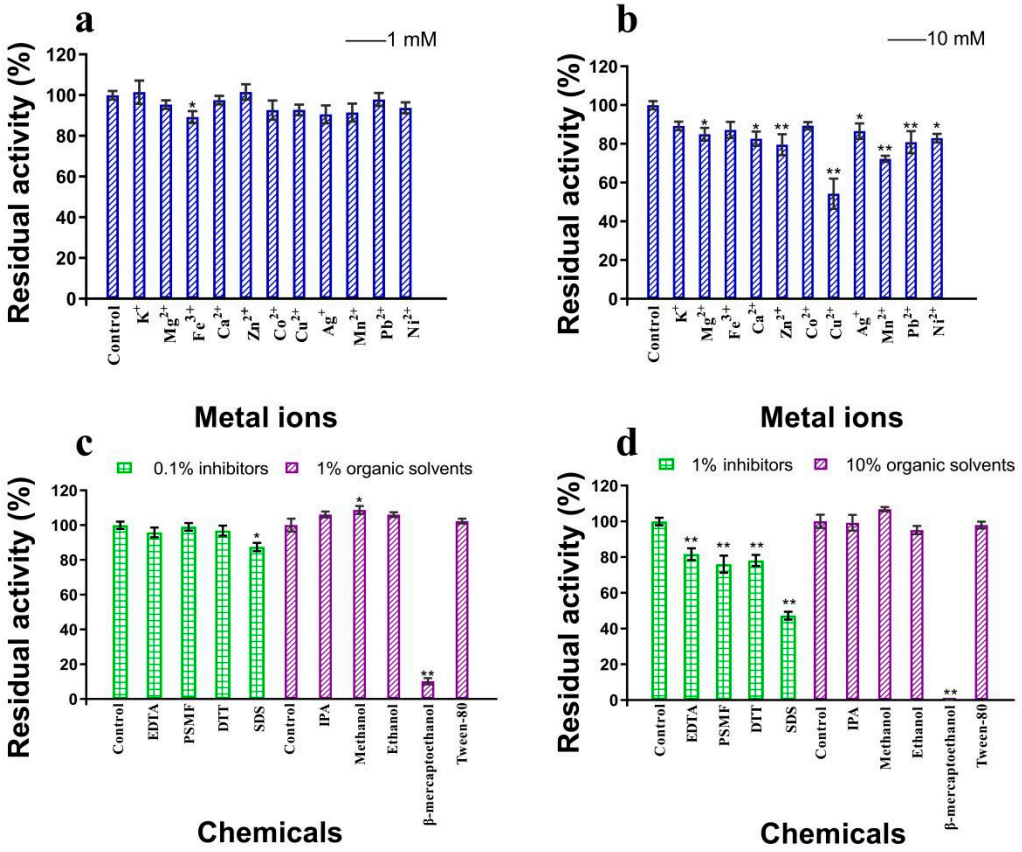

Figure 4. Effect of metal ions and chemicals on recombinant ThBg2 activity. (**a**) The effect of 1 mM metal ions on the activity of ThBg2. (**b**) The effect of 10 mM metal ions on the activity of ThBg2. (**c**) The effect of 0.1% inhibitors and 1% organic solvents on the activity of ThBg2. (**d**) The effect of 1% inhibitors and 10% organic solvents on the activity of ThBg2. Values represent the mean of three biological replicates. Error bars represent the mean ± SEM of three biological replicates. ** $p < 0.01$, * $p < 0.05$. 100% = 65 ± 2 U/mg.

3.6. Effect of Glucose Concentration on Enzymatic Activity

The relative activity of ThBg2 gradually decreased at a glucose concentration of 0–3M, and it still maintained a relative activity of more than 90% at a glucose concentration of 0.5 M. Moreover, it retained 30% of its initial activity at a concentration of 3M (Figure 5). These findings indicated that ThBg2 had good tolerance to glucose inhibition.

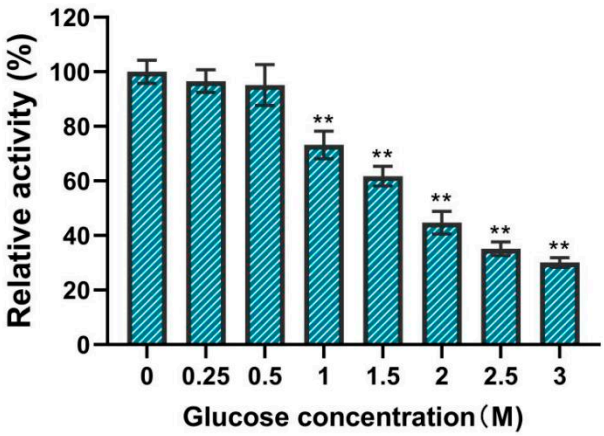

Figure 5. Effect of glucose on the activity of recombinant ThBg2. Values represent the mean of three biological replicates. Error bars represent the mean ± SEM of three biological replicates. ** $p < 0.01$. 100% = 65 ± 2 U/mg.

3.7. Kinetic Properties of ThBg2

The kinetic properties of ThBg2 were calculated based on the Michaelis Menten equation for cellobiose (Supplementary Figure S3). From these results, the K_m and V_{max} were calculated to be 1.47 mM and 161.3 μmol/min/mg at optimum temperature and pH, respectively.

3.8. Analysis of Hydrolysis of Corn Stalks by ThBg2

To evaluate the potential use of ThBg2 in lignocellulose degradation, the enzymatic hydrolysis of corn stalks was next performed. As shown in Figure 6, the glucose concentrations in the hydrolysis of corn stalks were tested. After adding the β-glucosidase ThBg2 to the commercial cellulase, the hydrolysis efficiency was significantly improved. The degradation rates increased by approximately 20% after 8 h. The above results suggest that ThBg2 has potential applications in lignocellulose degradation that works in synergistic cooperation with the commercial cellulase.

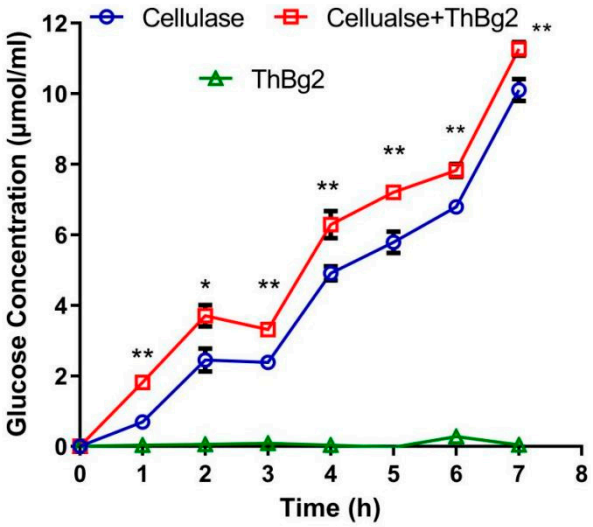

Figure 6. Cooperation of ThBg2 with the commercial cellulase in corn-stalk degradation. The green triangle shows the result of ThBg2 hydrolyzing corn stalks. The blue circle shows the results of commercial cellulase hydrolysis of corn stalks. The red rectangle represents the result of ThBg2 and commercial cellulase synergistic hydrolysis of corn stalks. Values represent the mean of three biological replicates. Error bars represent the mean ± SEM of three biological replicates. ** $p < 0.01$, * $p < 0.05$.

4. Discussion

In this study, the gene ThBg2 with β-glucosidase was detected from the microbial metagenome of the Tengchong Ruidian Hot Spring substrate in Yunnan Province. After detection, it was then cloned and expressed in *E. coli* BL21 (DE3) to obtain recombinant β-glucosidase ThBg2. The optimum reaction temperature of the enzyme was 60 °C, and it still maintained 50% activity at 85 °C. This indicates that it is a thermophilic enzyme. Residual activity remained at 100% after incubation at 50–60 °C for 12 h and remained at 100% after incubation at 60 °C for 24 h (Figure 3). These findings indicated that ThBg2 had very good thermal stability. The optimum temperature and thermal stability of ThBg2 were both higher and better than the following: Bgl7226 [20], a metagenome of bovine rumen microbes; r-Bgl66 [21], a metagenome of termite gut microbes; and unglu135B12 [22], a metagenome of mangrove sediment. Compared to mesophiles, thermophilic/thermotical enzymes increase reaction rates, reduce substrate viscosity, and reduce the risk of contamination [31].

A homology-based model of the heat-resistant ThBg2 was generated using the SWISS-MODEL (https://swissmodel.expasy.orgprogram, accessed on 18 November 2022). The model with the highest degree of sequence identity (81.25%) and GMQE (0.98) was used

as the final template (Figure 7). The homologous model of heat-resistant ThBg2 clearly shows a typical TIM barrel-like structure, which consists of a peptide backbone with eight outer α helixes and eight inner parallel β chains. TIM barrel structures are common in known proteins, and the active site of enzymes with this structure is usually located at the c-terminus of the β chain. The formation of salt bridges within the β chain is thought to contribute to the stability of the entire protein structure [32]. β-linkage leads to different enzyme properties, one of which is heat resistance.

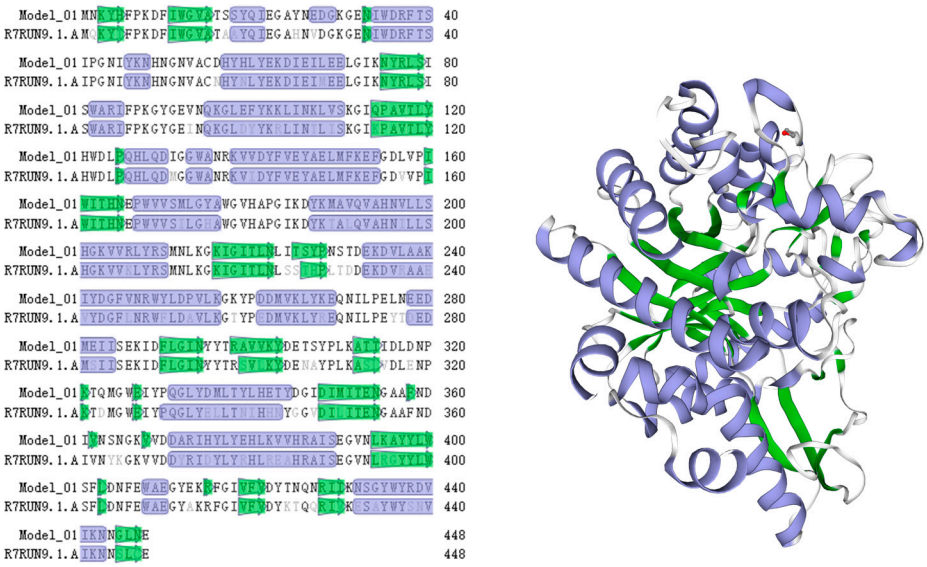

Figure 7. Amino acid sequence comparison of ThBg2 and homology models (**left**). The purple and green structures represent the α-helix and β-fold, respectively. ThBg2 homology model (**right**).

The optimum pH of ThBg2 was 5.6, and enzyme activity remained at 50% between pH 3.5–7. Across the pH range of pH 3.0–10.0, the enzyme activity remained close to 100%. This occurred even after 24 h of treatment, indicating a good pH tolerance and stability. This finding was similar to the results obtained by Suzuki and colleagues. Their work used the animal intestinal metagenomic library of active β-glucosidase and showed high activity and wide pH tolerance [26]. Most of the currently developed β-glucosidases have a pH close to neutral [33]. β-glucosidases that remain active over a wide range of pH values have been reported to be effective in improving the conversion of cellulosic materials to glucose. Thus, pH stability is another attribute that commercially focuses on improving β-glucosidase productivity [34,35]. Some relevant studies have shown that β-glucosidase—which has both thermophilic and acidophilic properties—has great value for industrial applications [36].

The inhibitory effect of metal ions on ThBg2 was not obvious, and its activity was maintained at more than 80%. When using Cu^{2+} as an inhibitor of β-glucosidase, ThBg2 enzyme activity decreased to 20% when the concentration of Cu^{2+} ($CuSO_4$) reached 15 mM (Figure 8). This indicated a certain tolerance to heavy metal ions. However, past work has shown that bgl enzyme activity of *Thermoanaerobacterium thermosaccharolyticum* origin was completely inhibited by 1 mM Cu^{2+} [37]. Similarly, PersiBGL1, a metagenome of sheep rumen microbiota, was inhibited to 11% enzyme activity when Cu^{2+} concentration was 5 mM [38]. As a kind of heavy metal ion, Cu^{2+} can bind with protein, causing it to lose structural characteristics and reducing biological activity, thus denaturing the protein. ThBg2 showed tolerance to Cu^{2+}, possibly due to its special surface charge structure, which reduced the influence of heavy metal ions on the biological function, structure and folding state of proteins, and reduced protein denaturation [39]. Given these past findings, ThBg2 has a better tolerance to Cu^{2+} and can avoid the inhibition of metal ions in industrial applications. The 1% negative surfactant SDS inhibited ThBg2 to some extent, but its enzymatic activity remained at approximately 50% and was more tolerable than the β-

glucosidase bglM derived from the extreme hot springs' metagenome [40]. Past work has shown that 1% SDS had greater inhibition on bglM enzymatic activity, leaving only 15% residual enzymatic activity [40].

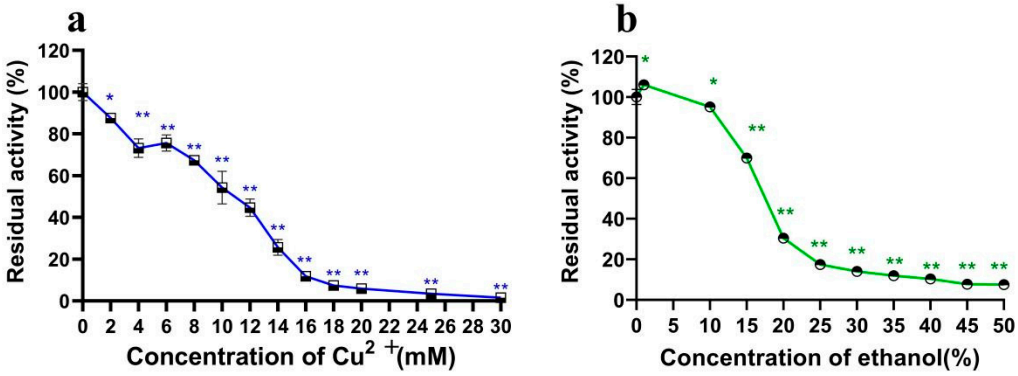

Figure 8. Effect of Cu^{2+} and ethanol on activity of recombinant ThBg2. (**a**) The effect of Cu^{2+} (0–40 mM) on the activity of ThBg2. (**b**) The effect of ethanol (0–50% mM) on the activity of ThBg2. Error bars represent the mean ± SEM of three biological replicates. ** $p < 0.01$, * $p < 0.05$. 100% = 65 ± 2 U/mg.

The organic solvent β-mercaptoethanol had the most significant inhibitory effect on ThBg2 enzyme activity. Its addition, it resulted in almost no enzymatic activity. Other than the absence of significant effects on their enzymatic activity, it is noteworthy that the effect of ethanol on β-glucosidase activity was crucial because these enzymes are present in high concentrations in many applications, such as simultaneous saccharification and fermentation processes [41–43]. Most β-glucosidases are inhibited in the presence of ethanol [44]. However, in the presence of ethanol, the activation of β-glucosidase activity has also been reported. For example, Alves et al. (2018) obtained a 1.7-fold increase in β-glucosidase activity from in the presence of 10% ethanol for Lfa2 [45]. Uchiyama et al. (2013) obtained a metagenomic β-glucosidase that was activated 1.16-fold by 10% ethanol [46]. ThBg2 also showed some tolerance to ethanol, having no effect on enzyme activity with ethanol at a concentration of 10%. Moreover, ThBg2 was maintained at 70% at an ethanol concentration of 15%. Considering that the final ethanol concentration in a conventional fermentation process is approximately 10–15% [47], ThBg2 can be used in the saccharification process for bioethanol production.

The Km value of ThBg2 is as low as 1.47 mM. However, the Km value for cellobiose has been previously reported to be 75.3 mM for Lfa2 of Atlantic soil metagenomic origin [45]. Moreover, bglM of hot spring metagenomic origin showed a Km value of 16.5 mM for cellobiose [39], indicating that ThBg2 had better substrate affinity.

Glucose, as the end product of cellobiose hydrolysis, inhibits beta-glucosidase activity, so the search for glucose-tolerant beta-glucosidases has been of great interest. ThBg2 has superior glucose tolerance than many previously reported beta-glucosidases, like those of metagenomic origin such as Unbgl1A [48], Tt-BGL from *Thermotoga thermarum* DSM 5069T [49], and DturβGlu from *Dictyoglomus turgidum* [50]. The superior glucose tolerance of ThBg2 may be related to the shape and electrostatic properties of the entrance to the active site [51], where the composition of amino acid residues improves glucose tolerance by enhancing the mobility of flexible loops around the active site [52]. In addition, its synergy with commercial cellulase also provides an alternative source for lignocellulose degradation. The glucose production did not continue to increase after 7 h, possibly because commercial cellulase may have been inactivated after incubation at 50 °C [53] although the ThBg2 was still highly active, and it required oligosaccharides, which were produced by commercial cellulase, as substrates to produce glucose. The residual activity of commercial cellulase was determined after incubation at 50 °C using 1% sodium carboxymethyl cellulose as a substrate. The results showed that it lost all activity after incubation at 50 ° C for 6 h

(Supplementary Figure S4). It is also suggested that only when used with thermophilic cellulases can the thermophilic advantages be fully realized.

5. Conclusions

In summary, a functional gene *thbg2* of a thermophilic, heavy metal ion-tolerant, ethanol-tolerant β-glucosidase was obtained from Yunnan Tengchong Ruidian Hot Spring using metagenomic techniques. This gene was then heterologously expressed in *Escherichia coli*. Its enzymatic properties were analyzed, and the results indicated that it is a thermophilic, acidophilic β-glucosidase with good thermal and pH stability and strong tolerance to inhibitors, such as heavy metal ions, glucose, and ethanol. This enzyme can be used in high-temperature, high-acidity and heavy metal-contaminated environments and has broad application prospects in the food, healthcare, feed, and medical industries.

6. Patents

The results of this research have been applied for the invention patent in the State Intellectual Property Office of China. Named: a thermophilic, heavy metal ion-tolerant β-glucosidase and its application. Patent No.: ZL 202210645225.7. Date of Authorization Announcement: May 19th, 2023. Certificate No.: 5981316.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15193389/s1, Figure S1: Gene Optimization of *thbg2*. Wild_type represents the original gene sequence of the *thbg2* from the metagenomic database. Optimized represents the optimized sequence of the *thbg2* based on the codon of *E. coli*; Figure S2: PCR amplification products; Figure S3: Lineweaver-Burk plot of ThBg2 β-glucosidase; Figure S4: The effect of temperature on stability of the commercial cellulase at 50 °C for 0, 1, 2, 3, 4, 5, and 6 h.

Author Contributions: Y.-R.Y. and Z.-H.L. conceived the study. Y.-Y.H. and P.W. were responsible for all PCR and gene cloning. P.W. and D.Z. cultured strains and collected samples. X.-C.W. and Q.-R.Z. purified the recombinant protein. Q.Z. measured enzymatic activity. H.-Z.Z. performed data analysis and phylogenetic mapping. Y.-Y.H., P.W., Y.-R.Y. and Z.-H.L. wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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