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Cloning and biochemical characterization of a novel GH1 β-glucosidase from thermophilic bacterium, *Anoxybacillus flavithermus* DSM 2641^T

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β-glucosidases belong to the class of glycoside hydrolases (GHs) and can mediate the cleavage of β-1-4 glycosidic bonds in alkyl or aryl-β-D-glucosides, amino, cyanogenic glucosides, and oligo- or disaccharides. The gene encoding β-glucosidase of *Anoxybacillus flavithermus* DSM2641^T was subcloned and expressed in *E. coli* BL21 Star(DE3) cells with pET100 Directional TOPO Expression Kit. The recombinant AFBGL was purified by a Ni-NTA affinity chromatography. The enzyme was 53 kDa and had 5.13 × 10^5 µmol/min/mg protein of V_{max} and 422.3 µM of K_m towards p-nitrophenyl β-D-glucoside as a main substrate. The optimum temperature and pH of the purified enzyme were 65°C and 6.8, respectively. We also determined kinetic parameters of AFBGL against the other artificial substrats, pNPC, pNPX, and oNPG. The enzyme had the V_{max} and K_m for 5.13 × 10^5 µmol/min/mg protein and 0.42 mM for pNPG, 0.007 µmol/min/mg protein and 0.19 mM for pNPC, 0.01 µmol/min/mg protein and 11.97 mM for pNPX, 0.06 µmol/min/mg protein and 0.134 mM for oNPG, respectively. AFBGL's substrate affinity was found to be more directed towards oNPG. Although 0.5 mM Mg^{2+} increases the enzyme's activity 1.6-fold, higher concentrations of Mg^{2+} and the presence of K^+, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Al^{3+} decreases its activity. AFBGL retained 50% of the initial activity towards pNPG for 25 min and the activity was not detected after 225 min. According to amino acid motifs, AFBGL belongs to family 1 of glycosylhydrolase and 4/7 super family. So, AFBGL is a member of GH1, as the amino acid sequence contained a GH1 specific domain.

Keywords: β-glucosidase, *Anoxybacillus flavithermus* DSM 2641^T, GH1, Thermophilic

Cellulose is the most abundant renewable organic material produced photosynthetically and can be obtained from agricultural waste materials. It consists of β-1, 4-glycosidic residues. Cellulases are responsible for bioconversion of biomass especially by microorganisms and used in most common industrial applications such as in animal feeds, textile industry, detergents, paper industry, and food industry[1,2]. Cellulases hydrolyse β-1, 4-D-glucan bonds in cellulose and produce cellobiose, glucose, and oligosaccharides. There are three major types of cellulolytic enzymes. 1, 4-β-D-glucan cellohydrolase, (exoglucanases, cellobiohydrolase, EC 3.2.1.91), endo-β-1, 4-glucanase, (1, 4-β-D-glucan glucanohydrolase, EC 3.2.1.14), and β-glucosidase, (β-D-glucoside glucohydrolase, EC 3.2.1.21)[2]. β-glucosidases (β-glucoside glucohydrolases, EC 3.2.1.21) are important enzymes responsible for the hydrolysis of β-glycosidic linkages in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glycosides, and oligo- or disaccharides[3-6]. These cleavage by enzymes play important roles in metabolism that controls, for examples, breaking and reassembling of carbohydrates, the processing of various lipid and proteins containing oligosaccharides, and the formation of cell walls[4]. β-glucosidase hydrolyses oligosaccharides to glucose as well. β-glucosidase is responsible for beta-glucan synthesis during cell wall conformation, cell wall lignification, hormone activation, pigmentation, and aromatic compounds release in plant, and glycolipid and glycoside mechanisms in animals, especially the hydrolysis of glucosyl ceramides in human and other mammals[3,7]. Better understanding of these mechanisms by β-glucosidase in plants is important for agricultural applications. According to classifications of CAZY (http://www.cazy.org/Glycoside-Hydrolases.html, 2017), the number of glycoside hydrolases in the family (without non-classifieds) is 145. Glycoside hydrolases are also classified by a classification system

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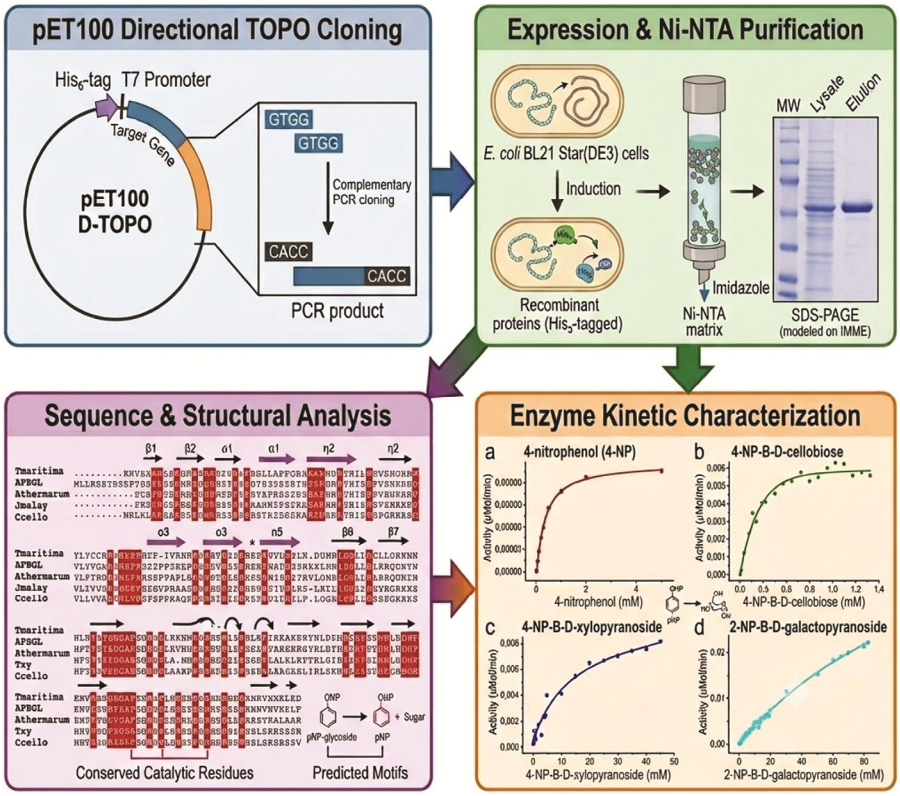

Graphical abstract

based on amino acid sequence and structural similarity[3,8,9]. In this system, amino acid sequence homology and conserved domains are highlighted. The enzymes with the same amino acid sequence and conserved motifs are grouped into the same family. According to amino acid sequences, β-glucosidases have been classified into GH1, -3, -5, -9, and -30 of glycosyl hydrolase families. Families having a similar catalytic structure and conserved catalytic amino acids are also referred as clans. Thus, GH1, -5, and -30 are in the clan A because all have (β/α)8-barrel structures. Clan A has the largest number of families[3,7]. In addition to characterization studies of β-glucosidases, it is important to determine their inhibitors. Because other than the above-mentioned relations, β-glucosidases are included in some metabolic disorders such as viral and bacterial infections, diabetes, and cancer. Therefore, finding out their inhibitors are gaining importance as they open areas for the possible treatment of these diseases for human and crops[6].

All lignocellulosic biomass containing cellulose, and lignin must be digested into their components for industrial usages. Many enzymes have been developed for this reason. One of the best ways to reduce costs and improve performance in the production of cellulases is the use of thermophilic sources with recombinant technology. Thermozymes are enzymes from thermophilic microorganisms. They are stable at high temperature and have exclusive durability for chemical conditions and pH stability. Therefore, they are preferred in many industrial applications. Their specific features benefit them in these applications to provide, for example, reduced microbial contamination risk, lower viscosity, and improved substrate solubility. Thermostable degradation enzymes, *i.e.*, β-glucosidase, amylase, xylanase, glucose isomerase, and cellulase etc., are good for food, chemical, pharmaceutical, paper industries, and wastewater refining systems. With these features plus lower cost, thermozymes are preferred in most applications[4,10-12].

Anoxybacillus flavithermus DSM 2641^T is a thermophilic bacterium with optimum growth conditions of 55-60°C[12]. This bacterium is facultatively aerobic heterotroph. *Anoxybacillus* species have become very popular in industrial research[13-20]. In this research, lipase, β-glucosidase, pullunase, α-amylase, glucose isomerase *etc*. were isolated and characterized from *Anoxybacillus* species. Therefore, to evaluate the industrial use of β-glucosidase from *A. flavithermus* DSM 2641^T, we

aimed to investigate its biochemical properties by cloning and expressing the relevant gene.

Materials and Methods

Microorganism, chemicals and other materials

Anoxybacillus flavithermus DSM 2641^T and *E. coli* DH5α were obtained from our laboratory stocks. *E. coli* BL21Star^{TM}(DE3) was obtained from Invitrogen. Modifying enzymes such as DNA polymerase, DNA ligase and restriction endonucleases, and dNTPs were purchased from ThermoScientific (USA). A purification kit for PCR products or DNA restriction fragments from agarose gels was obtained from Wizard® SV Gel and PCR Clean–Up System (Promega Co., Medison, USA). Plasmid and genomic DNA isolation kits were purchased from Thermo Sci. (GeneJet Plasmid Miniprep Kit) and Promega Co. (Wizard® Genomic DNA Purification Kit), respectively. All other chemicals and reagents were obtained from Sigma & Aldrich Inc. (St. Louis, MO, USA) and Merck GmbH (Germany). All primers for PCR were synthetized by Macrogen Inc. (Amsterdam, Netherlands).

Strains and their growth conditions

Anoxybacillus flavithermus DSM 2641^T was cultivated at 55°C in Luria-Bertani (LB) broth medium. The host cells, *E. coli* DH5α for cloning and BL21Star^{TM} (DE3) for expression studies were grown in LB medium with suitable antibiotics (ampicillin, 100 µg/mL and kanamycin, 30 µg/mL) at 37°C.

Cloning of *AFBGL* gene

Genomic DNA isolation from *A. flavithermus* DSM 2641^T was performed by Promega Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's protocol. To obtain *Afbgl* gene by PCR from *A. flavithermus* DSM 2641^T, we designed a pair of primers (forward, AfbglF: 5′-CACCATGGTAACGAAAAAATTCG-3′ and reverse, AfbglR: 5′-CTCGAGTTGATCAATTAAT CC-3′, restriction sites were underlined, *Nco*I and *Xho*I, respectively) according to NCBI GenBank data of β-glucosidase gene from *A. flavithermus* WK1 (NCBI Reference Sequence: NC_011567.1). The PCR was achieved by iProof High Fidelity DNA polymerase (BioRAD). The PCR conditions were as follows: one cycle of denaturation at 95°C for 3 min, 20 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 min, extension for 72°C for 1 min, and final extension at 72°C for 10 min. After gel extraction with Wizard® SV Gel and PCR Clean-Up System (Promega Co., Medison, USA), the 1386 bp PCR product of *Afbgl* was ligated into pET100/D-TOPO vector (pET100 Directional TOPO Expression Kit, Invitrogen) according to manufacturer's protocol. This vector was transformed into chemically competent *E. coli* DH5α host cells. The confirmed recombinant plasmid by restriction digestion, PCR, and DNA sequencing, was introduced into *E. coli* BL21Star^{TM} (DE3) for expression.

Expression and purification of recombinant enzyme (AFBGL)

One colony was inoculated into 3 mL LB broth containing ampicillin (100 µg/mL) and incubated overnight at 37°C with shaking at 150 rpm. A sufficient aliquot of the overnight culture was added into 400 mL LB medium with 100 µg/mL ampicillin and incubated at the same conditions. When the optical density at 600 nm of the cultures reached 0.6, cells were induced with 1 mM IPTG. After 4 h, the pellet of the cultures was obtained by centrifugation at 8000 rpm for 10 min at 4°C. The pellet was resuspended in 5 mL lysis buffer (500 µL 50 mM Tris pH 6.8, 10 µL DTT, 500 µL 5 M NaCl) and disrupted by sonication (30% amplitude, 20'' ON/OFF, 4 min) on ice. Then, the cell lysate was centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was incubated at 60°C for 30 min to remove host cells proteins. After the centrifugation at 14000 rpm for 15 min, the supernatant was filtered and passed through a Ni-NTA column (Bio-RAD). The affinity chromatography conditions follow binding buffer (100 mM HEPES, pH 7.5 with 10 mM imidazole), two times with washing buffer (100 mM HEPES, pH 7.5 with 20 mM imidazole), and elution buffer (100 mM HEPES, pH 7.5 with 200 mM imidazole) were performed, respectively. The protein was dialyzed against the 100 mM HEPES buffer, pH 7.5 to remove the imidazole and then was checked by SDS-PAGE (12% *w/v*). Then, the protein concentration of the dialyzed sample was determined by the Bradford methods[21].

Enzyme assays

Enzyme activity was determined using pNPG according to Park *et al*.[5]. As distinct from this method, we used pre-incubation temperature as 70°C. The color change in the assay was read at

405 nm by a microplate reader (Spectramax M5, Molecular Devices, CA, USA). A standard graph was obtained with pNP in the same assay conditions. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of pNP per min at 65°C.

Characterization of AFBGL

Effects of pH and temperature on AFBGL activity

The pH and temperature effects on the activity were determined in the pH range of 3.0-10.0 and 40-90°C, respectively. The buffers were Mcilvaine buffer (3.0-8.0) and glycine-NaOH (8.4-10.0).

Effect of various metal ions, chemicals, and substrates on enzyme activity

The effect of various metal ions was investigated with different concentration of K^+, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Al^{3+}. In addition, the effect of some chemicals such as detergent, solvents, reducing agents on the hydrolysis of the pNPG was investigated with different concentrations or percent of 2-propanol, 2-butanol, ethanol, EDTA, DTT, beta mercaptoethanol, TritonX-100. Substrate specificity was determined using other chemical (artificial) substrate of the enzyme such as 4-nitrophenyl-β-D-cellobiose (pNPC), 2-nitrophenyl-β-D-galactopyranoside (oNPG), and 4-nitrophenyl-β-D-xylopyranoside (pNPX).

Determination of kinetic parameters

The K_m and V_{max} values of the hydrolysis of pNPG by the enzyme, AfBGL, were determined using 0.01-20 mM substrate. After 250 μL of Mcilvaine buffer at pH 6.8 including the substrate was preincubated at 65°C for 5 min, the reaction was started by 5 μL (20 ng) of the enzyme solution. After 30 min, the reaction was stopped by adding 500 μL of 1M Na_2CO_3. The kinetic values were determined from the results using Lineweaver-Burk plots. In this study, in addition to pNP-β-D-glucopyranoside (pNPG) as the main substrate, pNP-β-D-cellobioside (pNPC), oNP-β-D-galactopyranoside (oNPG) and pNP-β-D-xylopyranoside (pNPX) were used as other artificial substrates.

Thermal stability of AFBGL

To determine thermal stability of the recombinant enzyme, the standard reaction described above was used with standard substrate, pNPG, at various time at 65°C. The enzyme half-life values were calculated from the graph of the residual activity *vs* time.

Bioinformatic analysis of AFBGL

Amino acid sequences of β-glucosidases from some thermophilic species from Firmicutes were compared by COBALT analysis from NCBI (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi). Phylogenetic relationships of AFBGL with them were calculated using Neighboor-Joining (NJ) methods by MEGA 6 program[22]. In addition, amino acid modeling was performed by comparing the amino acid sequences of thermophilic enzymes with those of *T. maritima* BGL (1OIM) using the ENDscript program[23].

Results

Cloning and expression of *Afbgl* gene

We obtained 1386 bp of *Afbgl* gene by PCR and cloned into pET100 Direction TOPO vector. DNA sequencing also confirmed this gene. The information of conserved domains of AFBGL and the alignments of amino acid sequences of BGL and phylogenetic analysis in some Firmicutes members had shown in (Figs. 1-3). p6xHis-*Afbgl* was constructed and successfully expressed in *E. coli* BL21Star™ (DE3) cells. After expression with IPTG, the cells were collected and sonicated. A heat treatment of cell extract at 60°C at 30 min eliminated most of host cell's proteins. Then a Ni-NTA affinity chromatography was used to purify the AFBGL, and dialysis was performed. SDS-PAGE analysis (12%) showed that the purified protein was homogeneous with a molecular mass of 53 kDa (Fig. 4).

Effect of pH and temperature on the activity of AFBGL

The optimal pH for AfBGL was 6.8 with pNPGal as a substrate. The enzyme shows 35% of the activity at pH 3.0 and pH range of 7.2-8.0 (Fig. 5a). The

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MLTKKFVGGLKMLQFPKDFMWGAATSSYQIEGTATGEEKIYSIWDHFSRIPGKVANGDNGDIAIDHYNRYVEDVSLMKTLHLKGYRFSTSWARLYS
GMPGKFSEKGLDFYKRLVNELLENDIEPMLTIYHWDMPQALQEKGGWENRDIVYYFQEYASFLYENLGDVVKKWITHNEPWVVTYLGYGNGEHAPG
IQSFKSFLAAAHHVLLSHGEAVKAFRSIGPKDGEIGITLNLTPGYAVDLKDERAVDAARKWDGFMNRWFLDPVFKGKYPTDMLEVYKDYLPDVYKE
GDLQTIQQPIDFFGFNYYSTATLKDWKKGEREPIVFEHVSTGRPVTDMNWEVNPNGLFDLLVRLKKDYGDIPLYITENGAAYKDFVNEDGKVEDDE
RITYIQEHLMACHRAIEQGVKLKGYYVWSLFDNFEWAFGYDKRFGIVYVDYETLERIPKKSALWYKETIMNNGLIDQ-
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Fig. 1 — Amino acid sequences of AFBGL. Bold letters indicate the motifs of family 1 β-glucosidases as active sites and all underlined letters indicate other conserved sequences in β-glucosidases. Double underlined was for active sites and underlined ones were for conserved sequences for AFBGL

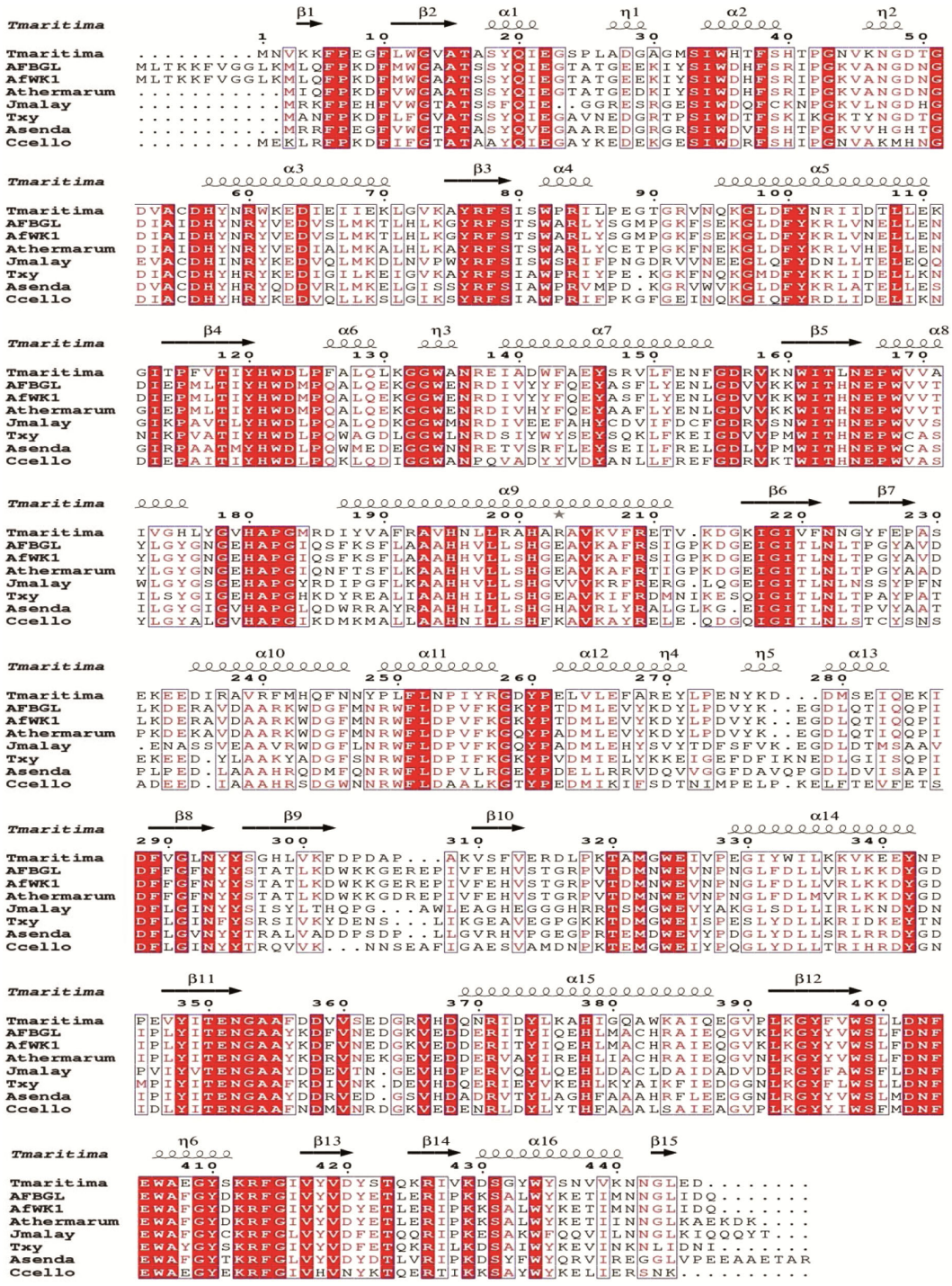

Fig. 2 — Amino acid sequence alignment of AFBGL with homologous GH1 beta-glucosidase enzymes. Secondary structural elements of *T. maritima* (1OIM) are shown at the top of the alignment. Tmaritima (*T. maritima* WP_004082398), AFBGL (this study), AfWK1 (*A. flavithermus* WK1, ACJ34717), Athermarum (*A. thermarum* WP_043965812), Jmalay (*J. malaysiensis* AJD92791), Txy (*Thermoanaerobacterium xylanolyticum*, WP_013788946), Asenda (*Alicyclobacillus sendaiensis*, WP_283204657), and Ccello (*C. cellulovorans*, WP_010076042). The modeling figure was generated using ENDscript[23] based on the *T. maritima* BGL (1OIM)

optimal temperature for AFBGL was 65°C. At the bacterial growth temperature (55°C), the enzyme shows 76% of the original activity. At 70°C, the enzyme lost 70% of the activity and after this point, the activity was decreased. At 90°C, the enzyme activity was completely inhibited (Fig. 5b).

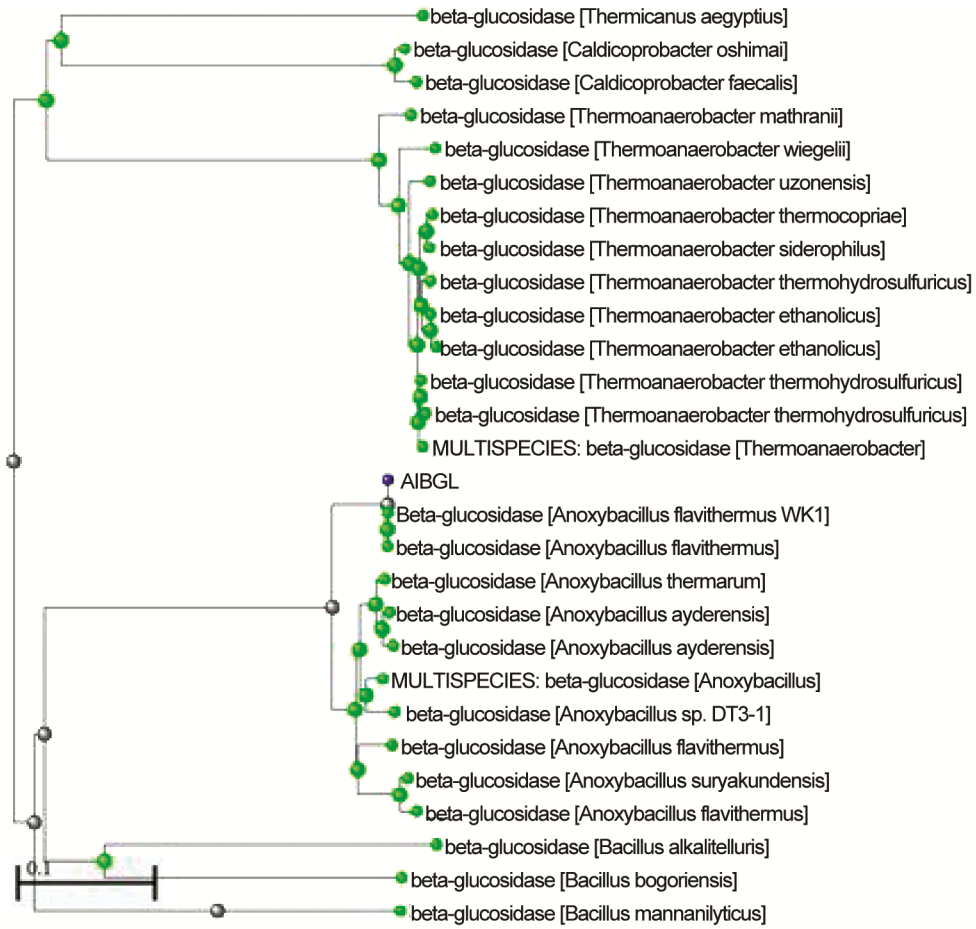

Fig. 3 — Phylogenetic analysis of AFBGL with other BGLs. The Neighboor-Joining (NJ) tree was constructed using MEGA 6 program to get the relationship of AfBGL and other enzymes in some Firmicutes samples

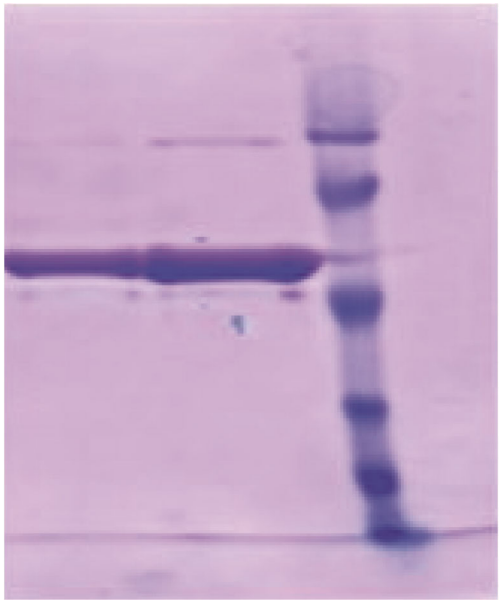

Fig. 4 — 12% SDS-PAGE profiles of AFBGL (Left to right: Ni-NTA purified AFBGL, heat shock treatment, and protein marker, Thermo Sci., USA)

Substrat specificity and determination of kinetic constants of chosen substrates

The enzyme was active on pNP-β-D-glucopyranoside (pNPG) as main substrate, pNP-β-D-cellobioside (pNPC), oNP-β-D-galactopyranoside (oNPG), and pNP-β-D-xylopyranoside (pNPX). The reaction kinetics of the AFBGL for four chosen substrates were determined from Lineweaver-Burk plots under optimal conditions. The enzyme had the V_{max} and K_m for 5.13×10^5 µmol/min/mg protein and 0.42 mM for pNPG, 0.007 µmol/min/mg protein and 0.19 mM for pNP-β-D-cellobioside, 0.01 µmol/min/mg protein and 11.97 mM for pNP-β-D-xylopyranoside, 0.06 µmol/min/mg protein and 0.134 mM for oNP-β-D-galactopyranoside, respectively (Fig. 6).

Effect of metal ions and other chemicals on the activity of AFBGL

We used different concentrations of the selected metal ions and some chemicals, *i.e.* detergents, reduced agents *etc.*, on the activity of AFBGL. All

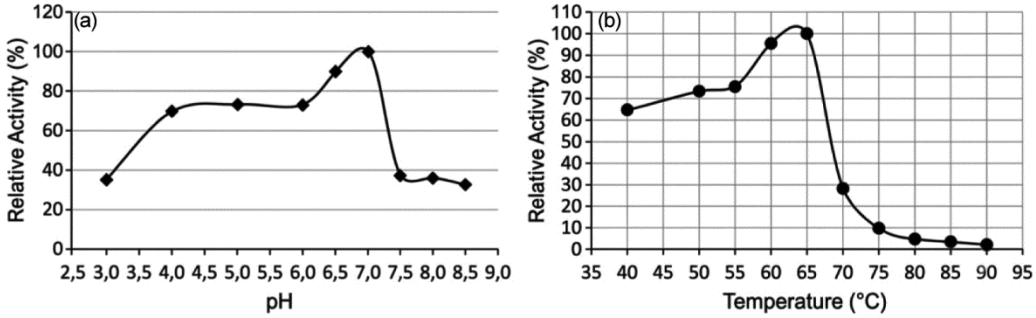

Fig. 5 — (a) Determination of optimal pH of AFBGL; and (b) Determination of optimal temperature of AFBGL

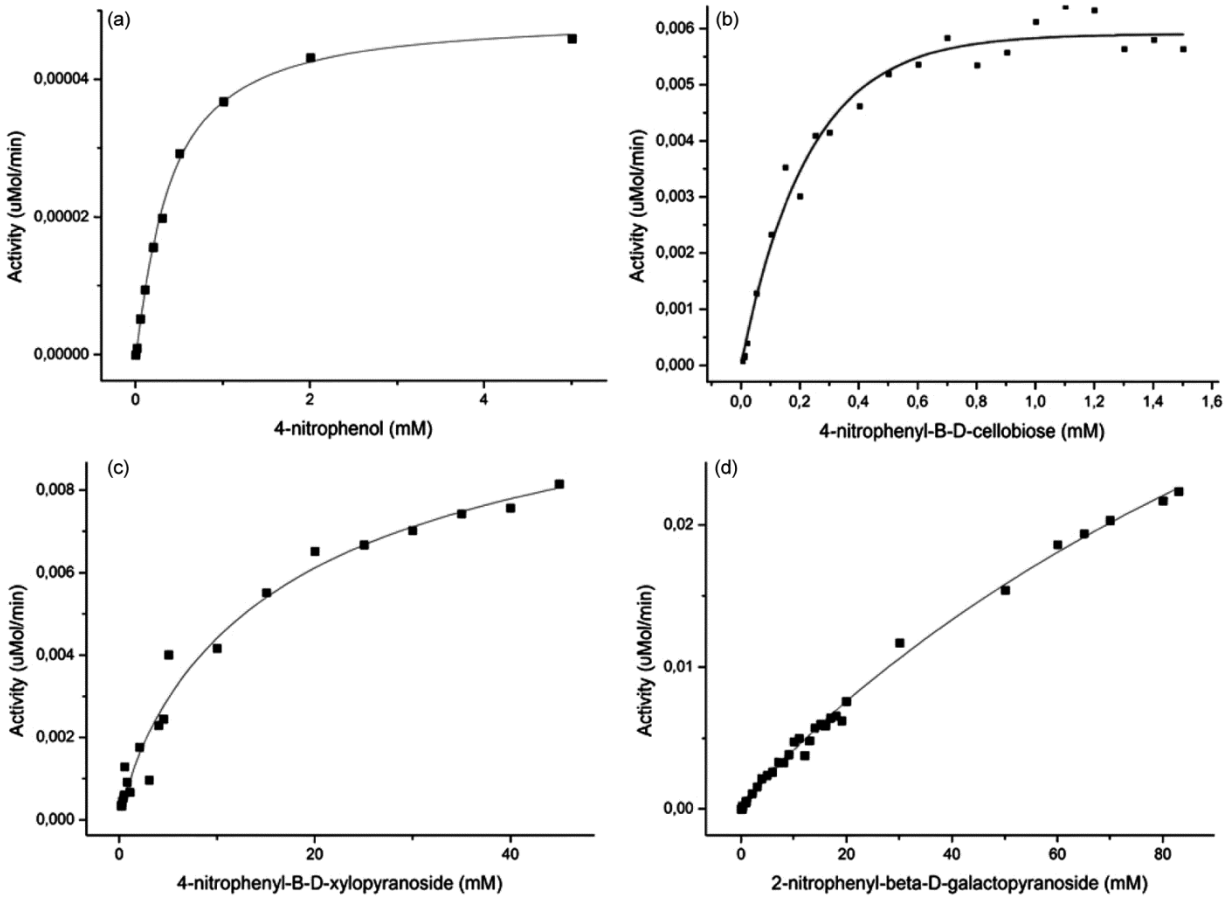

Fig. 6 — Lineweaver-Burk plots of several substrat of AFBGL under optimal conditions (a) pNPG (indicated with its product, 4-nitrophenol); (b) pNP-β-D-cellobioside; (c) pNP-β-D-xylopyranoside; and (d) oNP-β-D-galactopyranoside, respectively

of cations, the activity was only enhanced 1.6 times by the addition of 0.5 mM $MgCl_2$. $MnCl_2$, KCl, and $AlCl_3$ were reduced the activity but not inhibited completely for using concentrations. The other metal ions were completely inhibited the activity. 2-propanol, ethanol, and 2-butanol were reduced 90%, 90%, and 97% the activity by addition of 1%, 30%, and 1%, but completely inhibited by 20%, 30%, and 10%, respectively. Beta-mercaptoethanol and DTT as reduced agents reduced 97% of the activity by the addition of 0.1% and 1 mM but inhibited by the addition of 20% and 100 mM, respectively. 1 mM of EDTA was reduced 97% the activity and 150 mM of it was inhibited completely. 0.5% of TritonX-100 reduced 55% of the activity. All the results of metal ions and some chemicals on the activity of AFBGL were shown in (Figs. 7 and 8), respectively.

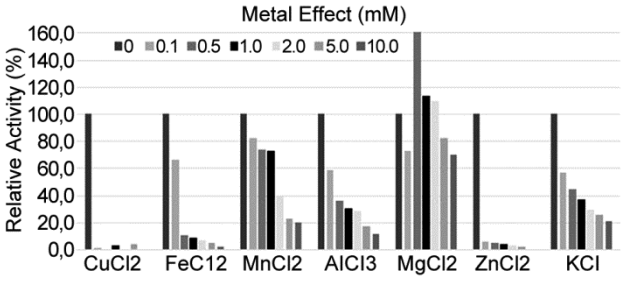

Fig. 7 — Metal ion effect on AFBGL activity

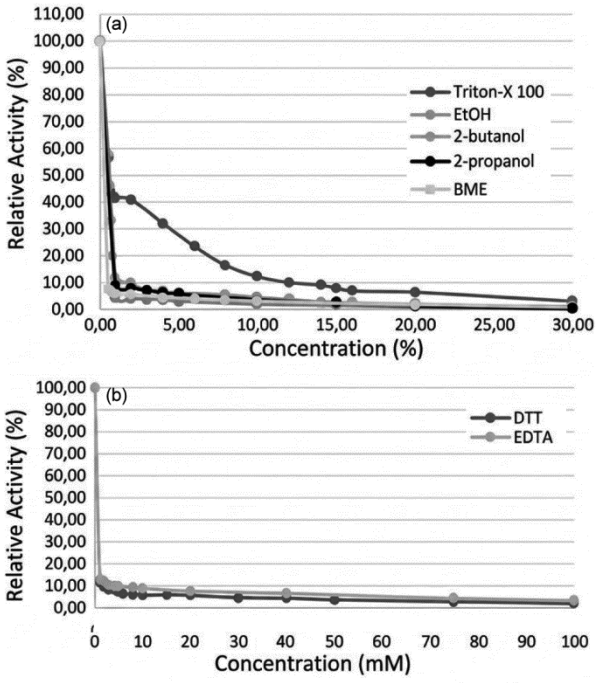

Fig. 8 — Effect of several chemical on AFBGL activity (a) 2-propanol, EtOH, 2-butanol, β-mercaptoethanol (BME), Triton X-100 with % concentration; and (b) DTT and EDTA with mM concentration, respectively.

Enzyme thermal stability

To examine the thermal stability of *A. flavithermus* DSM 2641^T β-glucosidase, the enzyme was preincubated with different temperatures. Results show that the AFBGL retained 50% of the initial activity towards pNPG after 25 min. After 225 min (3 h 45 min), the activity was not detected (Fig. 9). This suggests that although the stability level of AFBGL, and its contrasting characteristics with detailed thermophilic β-glucosidases, are modest, this does not strongly support considering AFBGL as thermostable despite its thermophilic origin.

Discussion

The β-glucosidase gene from *A. flavithermus* DSM 2641^T has 1386 bp and 461 aa. This gene has 98%

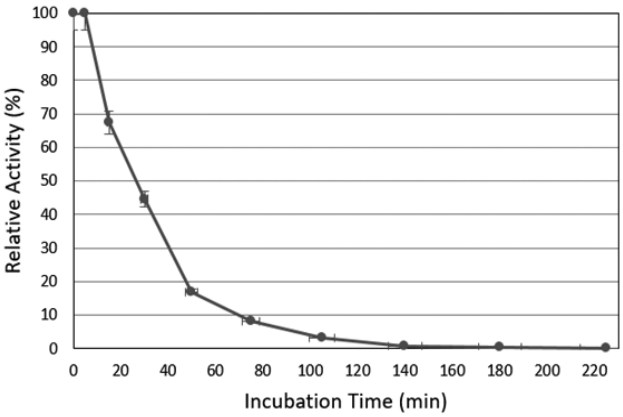

Fig. 9 — Thermal stability of AFBGL

similarity to *A. flavithermus* WK1 strain. Like other β-glucosidases, AFBGL has six conserved domain with a few modifications, SSYQI, YRFST, THNEP, FGFNYY, YITENG, and DNFEW, respectively. According to Burmeister *et al.*[24] and Sanz-Aparicio *et al.*[25], these sites are originally SAYQI, YRFSI, TFNEP, LGLNYY, YITENG, and DNFEW. The conserved domains, TFNEP and YITENG, indicate active sites of the enzymes. Similarly, Kaushal *et al.* identified β-glucosidase from a metagenomic source in an extremely hot (98°C) aquatic habitat in Chattisgarh, India, and comparative protein sequence and homology structure analyses determined this gene as a GH1 family β-glucosidase. Accordingly, multiple sequence alignment of BglM with homologous sequences revealed two conserved amino acid motifs, NEP and TENG. They stated that these two motifs are critical for substrate binding function in relation to β-glucosidase, and that the glutamate residues within these motifs are critical for catalytic function[26]. Similar results for AFBGL are shown in Figures 1 and 2. The conserved glutamate residue in these motifs is responsible for a potential acid/base catalyst and nucleophile of active sites[26,6]. And NEP and EFG motifs show the AFBGL belongs to family 1 of glycosylhydrolase and 4/7 super family. So, AfBGL is a member of GH1, as the amino acid sequence contained a GH1 specific domain. Wright *et al.*[27] and Trimbur *et al.*[28] showed Glu-358 and Asn-394 were responsible for nucleophile by region-directed mutation. In *A. flavithermus* BGL, these conserved residues were in 365th position of active sites for glutamine and 382nd position for asparagine. In addition, Asp-382 may play a role as an acid-base catalyst, protonating the leaving group and

Table 1 — Some properties of GH1 beta-glucosidases from various bacteria.

Organism	Temp[1] (°C)	pH[2]	MW (kDa)	K_m (mM)	V_{max} (µmol/min/mg protein)	Thermostability	References
A. flavithermus DSM 2641^T	65	6.8	53	0.42	5.13 x 10^5	$t_{1/2}$ 65°C= 25 min	This study
A. sp. DT3-1	70	8.5	53	0.22	923.7	$t_{1/2}$ 60°C= 24 h	17
A. flavithermus subsp. yunnanensis	60	7	52	0.29	771	$t_{1/2}$ 60°C= 10 h	14
Bacillus subtilis	60	6	53	0.82	55.6	$t_{1/2}$ 70°C= 45 min	34
Fusarium proliferatum	50	5	39.1	2.37	8.1 x 10^6	$t_{1/2}$ 60°C= 27.1h	35
Bacillus sp. ZJ1308	60	7	27	4.71	110.5	Retained 56% activity after incubation at 80°C for 30 min	36
Bacillus halodurans	45	8	51	4	-	$t_{1/2}$ 45°C= 1 h	37
Jeotgalibacillus malaysiensis	65	7	52	0.5	39.5	$t_{1/2}$ 65°C= 35 min	8
Thermotoga thermarum DSM 5069T	90	4.8	55	0.59	142	$t_{1/2}$ 90°C= 2 h $t_{1/2}$ 95°C= 50 min	32
Fervidobacterium islandicum	90	6-7	53	NA	NA	$t_{1/2}$ 90°C= 25 min $t_{1/2}$ 100°C= 15 min	33
Thermoanaerobacterium thermosaccharolyticum DSM 571	70	6.4	52	0.62	64	$t_{1/2}$ 68°C= 1 h	31

[1]Optimum temperature, [2]Optimum pH

subsequently deprotonating the water molecule as it attacks[28,29].

According to amino acid sequences of BGL from *Anoxybacillus* species on BLASTp analysis, AFBGL has identity and positivities of amino acid sequences (written in brackets) to *A. ayderensis*, *A. thermarum*, *A. gonensis*, and *A. suryakundensis* with 93% (96%), 92% (96%), 92% (96%), 91% (96%), respectively. The amino acid sequences of AFBGL have similarity and identity (written in brackets) of 100-96% (100-90%) from *Anoxybacillus* species, followed by 96% (60%) to *Bacillus alkalitelluris*, 96% (57%) to *Thermoanaerobacter thermohydrosulfuricus*, 97% (56%) to *Thermoanaerobacterium xylanolyticum*, 97% (55%) to *Alicyclobacillus sendaiensis*, 96% (55%) to *Jeotgalibacillus malaysiensis*, and 96% (53%) to *Clostridium cellulovorans*.

β-glucosidases are an enzyme that hydrolyses glycosidic bonds. Thus, they can be used for the release of monosaccharides from the oligosaccharides, for the release of glucose. In the past, detailed research has been carried out for fungal BGLs, especially because they are abundant in nature and naturally add cellulose to the nature[29]. Nowadays, due to their thermostabilities, the material has gained speed from thermophilic sources. Therefore, hyperthermophilic and thermophilic BGLs have been cloned, expressed, and investigated their biochemical futures[30,31]. These BGLs can potentially facilitate cellulose hydrolysis rates in biomass degradation. In addition, product formation can be increased by using thermostable BGLs in cellulose hydrolysis, and microbial contamination can be reduced as enzymatic hydrolysis can be carried out at a higher temperature[8]. GH1 β-glucosidases from various bacteria were characterized and show their biochemical futures in (Table 1).

The recombinant AFBGL is 53 kDa according to SDS-PAGE analysis. We know that class-I beta-glucosidases has 55 to 65 kDa with 447 to 527 aa[38]. We found the optimum pH and temperature of the recombinant enzyme were 6.8 and 65°C with pNPG. In BRENDA sources, we see that the optimal pH is ranged from 3.0 to 7.0, for example, 3.0 for *C. borealis*[37], 4.5 for *Fomitopsis palustris*[40], 5.2 for *Thermoascus aurantiacus*[41], 6.0 for *B. subtilis*[42], 6.5-7.0 for *Thermus thermophilus*[43], 8.5 for *Anoxybacillus* sp. DT3-1[25] etc. Similarly, optimal temperature of these enzymes is ranged from 32°C for *Penicillium pinophilum*[44] to 90°C for *Thermotoga maritima*[45]. Since the growth temperature of bacterial source, *A. flavithermus*, is 55°C, the optimal temperature of recombinant enzyme (65°C) was normal due to bacterial characteristics. Similarly, the optimum temperature for *Anoxybacillus* sp. DT3-1 has been recorded as 70°C[26].

In this work, we constructed pET100/Afbgl plasmid with 6xHis tagged for expression studies into *E. coli* BL21Star^{TM}(DE3) cells. The gene expression was

successfully achieved with 1 mM IPTG induction at 37°C for 4 h, then AFBGL was purified with Ni^{2+} affinity chromatography. The purified recombinant β-glucosidase had a specific activity of 5.13×10^5 µmol/min/mg against pNPG, but it had max activity of 0.05887 µmol/min/mg against oNPG. The substrate affinity of AFBGL is to oNPG. This affinity of oNPG is more than the enzymes of *B. subtilis*[42], *Thermoascus aurantiacus*[41], and *T. thermophilus*[43]. Although *Thermoanaerobacterium thermosaccharolyticum* DSM 571, with an optimum temperature of 70°C, showed 80% activity in 2 h at 60°C[31], *Thermotoga thermarum* DSM 5069^T showed 50% activity in 2 h at 90°C[32], *Anoxybacillus* sp. DT3-1 showed 50% activity in 24 h at 60°C (with an optimum temperature of 70°C)[16], *Anoxybacillus flavithermus* subsp. *yunnanensis* $E13^T$ has 50% activity in 10 h at 60°C14, AFBGL was found to have a half-life of only 25 min at its optimum temperature. While this result is like *Jeotgalibacillus malaysiensis* BGL (half-life: 35 min at 65°C)[8], it indicates that AFBGL has significantly lower thermostability compared to BGLs obtained from other thermophilic sources.

Metal ions play an important role in the catalytic effect of enzymes; therefore, the effect of metal ions on AFBGL activity was determined. According to An *et al*.[47], although Mg^{2+} ions are activators for β-glucosidases, AFBGL was 1.6 times more active in the presence of 0.5 mM Mg, but its activity was relatively reduced at 5 mM and higher concentrations. While other tested metal ions such as K^+, Fe^{2+}, Mn^{2+}, and Al^{3+} ions reduced activity, high concentrations of Zn^{2+} and Cu^{2+} completely inhibited the activity of the AFBGL recombinant enzyme. Zn^{2+} and Cu^{2+} generally activate some bacterial β-glucosidases[48,49]. The thermostable β-glucosidase from marine hyperthermophile bacterium, *Thermotoga neapolitana*, and from soft-rot fungus, *Humicola insolens,* are not affected by metal ions[5,50]. More or less similar results were obtained from the chemicals mentioned above on the activity of β-glucosidase obtained from *Anoxybacillus flavithermus* subsp. *yunnanensis* $E13^T$[14].

Using the alcohols, detergent, chelator, and other reduced chemicals in the activity reactions, the activity was reduced in low concentrations and then inhibited in high ones. Especially EtOH, 2-propanol, 2-butanol, beta-mercaptoethanol, DTT, and EDTA reduced the activity approximately by 90-97%. These results are like other thermophilic β-glucosidases, *i.e*., *T. thermophilus*[43], *Thermoascus aurantiacus*[40], but in the activity of thermophilic bacterium, *Thermophilum pendens*, the enzyme was 1.1-fold activated with 10% EtOH[49]. Exceptionally 2-butanol and 2-propanol were slightly activated the enzyme from *Thermoascus aurantiacus*[41]. BGLs can also be used in cellulosic ethanol production, but this requires tolerance to alcohol[26]. Although cellulose has not been tested as a substrate, its behavior towards ethanol (90% activity loss at 30% EtOH concentration) indicates that AFBGL cannot be used in this technology.

In this study, AFBGL was purified and biochemically characterized from *A. flavithermus* DSMZ 2641^T and expressed in *E. coli* cells. Structure and amino acid composition of AFBGL has been shown to completely coincide with the Firmicutes. Also, we show that AFBGL from *A. flavithermus* belongs to family GH1 and 4/7 super family. AFBGL can potentially facilitate cellulose hydrolysis rates in biomass degradation at higher temperatures without the risk of contamination. However, we recommend the application of region-specific mutations so that both the kinetic activity and, especially, the thermostability of the enzyme can be as high as other BGL enzymes. The addition of these features to the enzyme, along with its immobilization, could make it a candidate for industrial applications.

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Conflict of interest

All authors declare no conflict of interest.

References

1 Klemm D, Heublein B, Fink HP & Bohn A, Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angew Chem Int Ed Engl*, 44 (2005) 3358.

2 Sukumaran RK, Singhania RR & Pandey A, Microbial cellulases-Production, applications and challenges. *J Sci Ind Res*, 64 (2005) 832.

3 Singhania RR, Patel AK, Sukumaran RK, Larroche C & Pandey A, Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Biores Technol*, 127 (2013) 500.

4 Park NY, Cha J, Kim DO & Park CS, Enzymatic characterization and substrate specificity of thermostable β-glycosidase from hyperthermophilic archaea, *Sulfolobus shibatae*, expressed in *E. coli*. *J Microbiol Biotechnol*, 3 (2007) 454.

5 Park TH, Choi KW, Park CS, Lee SB, Kang HY, Shon KJ, Park JS & Cha J, Subtstrate specificity and

transglycosylation catalyzed by a thermostable β-glucosidase from *Pyrococcus horikoshii*. *Appl Microbiol Biotechnol*, 69 (2005) 411.

6 Bhatia Y, Mishra S & Bisaria VS, Microbial beta-glucosidases: cloning, properties, and applications. *Crit Rev Biotechnol*, 4 (2002) 375.

7 Cairns JRK & Esen A, β-Glucosidases. *Cell Mol Life Sci*, 67 (2010) 3389.

8 Liew KJ, Lim L, Woo HY, Chan K-G, Shamsir MS & Goh KM, Purification and characterization of a novel GH1 beta-glucosidase from *Jeotgalibacillus malaysiensis*. *Int J Biol Macromol*, 115 (2018) 1094.

9 Henrissat B & Davies GJ, Structural and sequence-based classification of glycoside hydrolases. *Curr Opin Struct Biol*, 7 (1997) 637.

10 Bruins ME, Janssen AE & Boom RM, Thermozymes and their applications. *Appl Biochem Biotechnol*, 2 (2001) 155.

11 Vieille C & Zeikus GJ, Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol Molec Biology Rev*, 65 (2001) 1.

12 Pikuta E, Lysenko A, Chuvilskaya N, Mendrock U, Hippe H, Suzina N, Nikitin D, Osipov G & Laurinavichius K, *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. *Int J Syst Evol Microbiol*, 50 (2000) 2109.

13 Coleri Cihan A, Karaca B, Ozel BP & Kilic T, Determination of the biofilm production capacities and characteristics of members belonging to Bacillaceae family. *World J Microbiol Biotechnol*, 6 (2017) 118.

14 Liu Y, Li R, WangJ, ZhangX, Jia R, Gao Y & Peng H, Increased enzymatic hydrolysis of sugarcane bagasse by a novel glucose- and xylose-stimulated β-glucosidase from *Anoxybacillus flavithermus* subsp. *yunnanensis* E13^T. *BMC Biochem*, 18 (2017) 4.

15 Acer Ö, Bekler FM, Pirinççioğlu H, Güven RG & Güven K, Purification and Characterization of Thermostable and Detergent-Stable α-Amylase from *Anoxybacillus* sp. *Food Technol Biotechnol*, 54 (2016) 70.

16 Chan CS, Sin LL, Chan KG, Shamsir MS, Manan FA, Sani RK & Goh KM, Characterization of a glucose-tolerant β-glucosidase from *Anoxybacillus* sp. DT3-1. *Biotechnol Biofuels*, 1 (2016) 174.

17 Kahar UM, Sani MH, Chan KG & Goh KM, Immobilization of α-Amylase from *Anoxybacillus* sp. SK3-4 on ReliZyme and Immobead Supports. *Molecules*, 9 (2016) 1196.

18 Karaoglu H, Yanmis D, Sal FA, Celik A, Canakci S & Belduz AO, Biochemical characterization of a novel glucose isomerase from *Anoxybacillus gonensis* G2(T) that displays a high level of activity and thermal stability. *J Mol Catal B-Enzym*, 97 (2013) 215.

19 Beris FS, De Smet L, Karaoglu H, Canakci S, van Beeumen J & Belduz AO, The ATPase activity of the G2alt gene encoding an aluminium tolerance protein from *Anoxybacillus gonensis* G2. *J Microbiol*, 49 (2011) 641.

20 Baltas N, Dincer B, Ekinci AP, Kolayli S & Adiguzel A, Purification and characterization of extracellular α-amylase from a thermophilic *Anoxybacillus thermarum* A4 strain. *Braz Arch Biol Technol*, 59 (2016) e16160346.

21 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 (1976) 248.

22 Tamura K, Stecher G, Peterson D, Filipski A & Kumar S, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*, 30 (2013) 2725.

23 Robert X & Gouet P, Deciphering key features in protein structures with the new ENDscript server. *Nucl Acids Res,* 42 (2014) W320.

24 Burmeister WP, Cottaz S, Driguez H, Palmieri S & Henrissat B, The crystal structures of *Sinapis alba* myrosinase and a covalent glycosyl–enzyme intermediate provide insights into the substrate recognition and active-site machinery of an S-glycosidase. *Structure*, 5 (1997) 663.

25 Sanz-Aparicio J, Hermoso JA, Martinez-Ripoll M, Lequerica JL & Polaina J, Crystal structure of β-glucosidase A from *Bacillus polymyxa*: insights into the catalytic activity in family 1 glycosyl hydrolases. *J Mol Biol*, 275 (1998) 491.

26 Kaushal G, Rai AK & Singh SP, A novel beta-glucosidase from a hot-spring metagenome shows elevated thermal stability and tolerance to glucose and ethanol. *Enzyme Microb Technol*, 145, (2021) 109764.

27 Wright RM, Yablonsky MD, Shalita ZP, Goyal AK & Eveleigh DE, Cloning, characterization, and nucleotide sequence of a gene encoding *Microbispora bispora* BglB, a thermostable beta-glucosidase expressed in *Escherichia coli. Appl Environ Microbiol*, 11 (1992) 3455.

28 Trimbur DE, Warren RA & Withers SG, Region-directed mutagenesis of residues surrounding the active site nucleophile in beta-glucosidase from *Agrobacterium faecalis. J Biol Chem*, 15 (1992) 10248.

29 Rani V, Mohanram S, Tiwari R, Nain L & Arora A, Beta-Glucosidase: Key Enzyme in Determining Efficiency of Cellulase and Biomass Hydrolysis. *J Bioprocess Biotech*, 5 (2014) 197.

30 Olajuyigbe FM, Nlekerem CM & Ogunyewo OA, Production and characterization of highly thermostable β-Glucosidase during the biodegradation of methyl cellulose by *Fusarium oxysporum*. *Biochem Res Int*, (2016) 3978124.

31 Pei J, Pang Q, Zhao L, Fan S & Shi H, *Thermoanaerobacterium thermosaccharolyticum* β-glucosidase: a glucose-tolerant enzyme with high specific activity for cellobiose. *Biotechnol Biofuels*, 5 (2012) 31.

32 Zhao L, Xie J, Zhang X, Cao F & Pei J, Overexpression and characterization of a glucose-tolerant β-glucosidase from *Thermotoga thermarum* DSM 5069^T with high catalytic efficiency of ginsenoside Rb1 to Rd. *J Mol Catal B Enzym*, 95 (2013) 62.

33 Jabbour D, Klippel B & Antranikian G, A novel thermostable and glucose-tolerant β-glucosidase from *Fervidobacterium islandicum*. *Appl Biochem Biotechnol*, 93 (2012) 1947.

34 Chamoli S, Kumar P, Navani NK & Verma AK, Secretory expression, characterization and docking study of glucose-tolerant β-glucosidase from *B. subtilis*. *Int J Biol Macromol*, 85 (2016) 425.

35 Su J, Xu J, Yu H, He Y, Lu W & Lin G, Properties of a novel β-glucosidase from *Fusarium proliferatum* ECU2042 that converts ginsenoside Rg_3 into Rh_2. *J Mol Catal B*, 57 (2009) 278.

36 Xu Z, Zhang L & Yu P, Optimization of a heat-tolerant β-glucosidase production by *Bacillus* sp. ZJ1308 and its

purification and characterization. *Biotechnol Appl Biochem*, 63 (2016) 553.

37 Naz S, Ikram N, Rajoka M, Sadaf S & Akhtar M, Enhanced production and characterization of a β-glucosidase from *Bacillus halodurans* expressed in *Escherichia coli*. *Biochemistry*, 75 (2010) 513.

38 Kara H, *Beta Glukozidaz Enziminin Hidrofobik Etkileşim Kromotografisi İle Olea ropea Meyvesinden Saflaştırılması*, *Karakterizasyonu İle Bazı Pestisit Ve Ağır Metallere Karşı Afinitesinin Araştırılması*, Ph.D. thesis. Balıkesir University, Balıkesir, 2010.

39 Demirkan *G, Climacocystic borealis'den β-Glukozidaz Enziminin Saflaştırılması ve Karakterizasyonu*, Master's thesis, Karadeniz Technical University, Trabzon, 2009.

40 Yoon J, Kim KY & Cha CJ, Purification and characterization of thermostable β-glucosidase from the brown-rot basidiomycete *Fomitopsis palustris* grown on microcrystalline cellulose. *J Microbiol*, 1 (2008) 51.

41 Parry NJ, Beever DE, Owen E, Vandenberghe I, van Beeumen J & Bhat MK, Biochemical characterization and mechanism of action of a thermostable β-glucosidase purified from *Thermoascus aurantiacus. Biochem J*, 353 (2001) 117.

42 Kuo L & Lee K, Cloning, Expression, and Characterization of Two β-Glucosidases from Isoflavone Glycoside-Hydrolyzing *Bacillus subtilis* natto. *Natto J Agric Food Chem*, 56 (2008) 119.

43 Dion M, Fourage L, Hallet JN & Colas B, Cloning and expression of a β-glycosidase gene from *Thermus thermophilus*. Sequence and biochemical characterization of the encoded enzyme. *Glycoconj J*, 16 (1999) 27.

44 Joo AR, Jeya M, Lee KM, Lee KM, Moon HJ, Kim YS & Lee JK, Production and characterization of β-1,4-glucosidase from a strain of *Penicillium pinophilum*. *Process Biochem*, 45 (2010) 851.

45 Gabelsberger J, Liebl W & Schleifer KH, Purification and properties of recombinant β-glucosidase of the hyperthermophilic bacterium *Thermotoga maritima*. *Appl Microbiol Biotechnol*, 40 (1993) 44.

46 Nam KH, Sung MW & Hwang KY, Structural insights into the substrate recognition properties of β-glucosidase. *Biochem Biophys Res Commun*, 1 (2010) 1131.

47 An C, Lim WJ, Hong SY, Kim EJ, Shin EC, Kim MK, Lee JR, Park SR, Woo JG, Lim Y & Yun HD, Analysis of bgl Operon Structure and Characterization of β-Glucosidase from *Pectobacterium carotovorum* subsp. *carotovorum* LY34. *Biosci Biotechnol Biochem*, 11 (2014) 2270.

48 Nakkharat P & Haltrich D, Purification and characterisation of an intracellular enzyme with β-glucosidase and β-galactosidase activity from the thermophilic fungus *Talaromyces thermophilus* CBS 236.58. *J Biotechnol*, 3 (2006) 304.

49 Li D, Li X, Dang W, Tran PL, Park SH, Oh BC, Hong WS, Lee JS & Park KH, Characterization and application of an acidophilic and thermostable b-glucosidase from *Thermofilum pendens*. *J Biosci Bioeng*, 5 (2013) 490e496.

50 Meleiro LP, Zimbardi ALRL, Souza FHM, Masui DC, Silva TM, Jorge JA & Furriel JP, A Novel β-Glucosidase from *Humicola insolens* with High Potential for Untreated Waste Paper Conversion to Sugars. *Appl Biochem Biotechnol*, 2 (2014) 391.