Journal of Molecular Catalysis B: Enzymatic 63 (2010) 11–16

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Identification of a metagenome-derived β-glucosidase from bioreactor contents

Chengjian Jiang[a,*], Zhen-Yu Hao[a], Ke Jin[a], Shuang-Xi Li[a], Zhi-Qun Che[a], Ge-Fei Ma[a], Bo Wu[a,b,**]

[a] Guangxi Key Laboratory of Subtropical Bioresources Conservation and Utilization, The Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering, College of Life Science and Technology, Guangxi University, 100 Daxue East Road, Nanning, Guangxi, 530004, People's Republic of China
[b] College of Chemistry and Ecology Engineering, Guangxi University for Nationalities, 188 Daxue East Road, Nanning, Guangxi, 530006, People's Republic of China

ARTICLE INFO

Article history:
Received 6 September 2009
Received in revised form
22 November 2009
Accepted 23 November 2009
Available online 27 November 2009

Keywords:
Metagenomic library
Uncultured sludge microorganisms
β-Glucosidase
Functional characterization

ABSTRACT

A novel β-glucosidase gene designated as *bgl1T* was cloned by function-based screening of a metagenomic library from uncultured microorganisms in contents of a bioreactor. The gene has an open reading frame of 1860 base pairs and encodes a 620 amino acid polypeptide with a predicted molecular mass of about 65 kDa. The deduced amino acid sequence comparison and phylogenetic analysis indicated that Bgl1T and other putative β-glucoside-specific II ABC subunit components, were closely related. Functional characterization with a high performance liquid chromatography method demonstrated that the recombinant Bgl1T protein hydrolyzed D-(+)-cellobiose to glucose. The maximum activity for Bgl1T protein occurred at pH 7.0 and 37 °C using *p*-nitrophenyl-β-D-glucoside as the substrate. The putative β-glucosidase had an apparent K_m value of 1.45 mM, a V_{max} value of 20.5 U/mg, a k_{cat} value of 1370/min and a k_{cat}/K_m value of 943/mM/min. The biochemical characterization of Bgl1T protein indicated its potential applications for better industrial production of glucose or ethanol by biological processes under moderate conditions.

1. Introduction

β-Glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) catalyze the hydrolysis of β-glucosidic linkages of various oligosaccharides and glycosides to form glucose and a shorter/debranched (oligo)saccharide or aglycone [1]. For most bioconversion processes, β-glucosidase is a key rate-limiting enzyme for the cellulose-hydrolyzing system in bacteria and fungi [2]. It is one of the three enzymes in the complete cellulase system composed of β-1,4-endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase [3]. Thus, β-glucosidase plays an important role in the utilization of biomass. It has also attracted considerable attention in the food industry for its potential applications in various biotechnological processes, such as hydrolysis of bitter compounds during fruit-juice extraction, release of aromatic compounds from flavorless precursor glucosides [3], and removal of bitter components of citrus products, and detoxification of cassava [4]. However, at present, its use in industrial cellulose hydrolysis for ethanol production is probably its primary biotechnological importance [5].

β-Glucosidases are commonly found in both prokaryotic and eukaryotic organisms. Based on amino acid sequence similarities, β-glucosidases have been classified in family 1 and family 3 of the glycoside hydrolase families [2,6]. At present, many β-glucosidases have been purified and characterized from cultured bacteria and fungi [2–8]. Recently, some β-glucosidases have been identified from uncultured samples including wetlands [9], alkaline polluted soils [10], contents of rabbit cecum [11], the cow rumen [12], and the large bowel of mice [13]. Nonetheless, detailed biochemical characterizations of these metagenome-derived β-glucosidases are currently lacking.

In the present study, a cosmid library was constructed from a metagenome derived from sludge samples from a bioreactor. Through enzyme function-based screening of a DNA library, a gene encoding a novel β-glucosidase (named Bgl1T) was isolated. The deduced amino acid sequence had moderate similarity to the known β-glucosidases in the Genbank database. Biochemical analysis of the overexpressed recombinant protein revealed that Bgl1T catalyzed hydrolysis of D-(+)-cellobiose to form glucose. To our knowledge, this is the first report of a metagenome-derived β-glucosidase isolated from the sludge content of a bioreactor.

* Corresponding author. Tel.: +86 771 3239283; fax: +86 771 3239403.
** Corresponding author at: Guangxi University for Nationalities, 188 Daxue East Road, Nanning, Guangxi, People's Republic of China. Tel.: +86 771 3239283; fax: +86 771 3239403.
E-mail addresses: jiangcj0520@gmail.com (C. Jiang), wubogxu@yahoo.com.cn (B. Wu).

1381-1177/$ – see front matter.
doi:10.1016/j.molcatb.2009.11.009

2. Materials and methods

2.1. DNA manipulation and protein analysis

All DNA manipulations, including cloning and subcloning, transformation of *Escherichia coli* cells, and PCR were performed according to standard techniques [14] or following the manufacturer's instructions, unless indicated otherwise. Protein preparation and analysis, including protein extraction from *E. coli*, protein quantification, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were performed as described in the standard protocols [15].

2.2. Sludge metagenomic DNA preparation and purification

Sludge samples were collected from a biogas reactor located in WuMing county of Guangxi Province, South China (23°10′N, 108°17′E). This particular biogas reactor has been running successfully for over 30 years. The metagenomic DNA was extracted from the sludge samples following the direct lysis method of Roh et al. described [16], with some modifications. Briefly, the DNA pellet from 5 g of sludge sample was washed twice with 75% ethanol, air dried, and dissolved in 1× TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM Na_2EDTA). To remove contaminants, the crude metagenomic DNA extract was loaded onto a column (200 mm × 10 mm) containing Sephadex G200 and acid-washed polyvinylpolypyrrolidone (PVPP) at a ratio of 50:1. Finally, DNA was eluted from the column with TE buffer and further purified by electroelution, as described [14].

2.3. Construction and screening of the cosmid library for β-glucosidase genes

A metagenomic library was constructed using the pWEB::TNC Cosmid Cloning Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the purified DNA was end repaired with T4 DNA polymerase and T4 polynucleotide kinase to generate blunt ends. End-repaired DNA was separated on a low-melting-point agarose gel (FMC Corporation, Philadelphia, PA, USA). The DNA fragments between 40 and 50 kb were recovered and ligated into the cosmid vector pWEB::TNC that had been linearized at the unique SmaI site and dephosphorylated. The ligated products were packaged and infected into *E. coli* EPI100. After overnight growth on LB agar plates containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml), white colonies harboring plasmids bearing inserts were collected and used to construct the metagenomic library. The library was stored at −80 °C until screening.

Colonies of the library were replica plated onto Luria–Bertani (LB) agar plates supplemented with ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Esculin hydrate and ferric ammonium citrate (Sigma, St. Louis, MO, USA) were used to detect β-glucosidase activity according to the method described by Kwon et al. [17]. The plasmids of the positive clones were retransformed into *E. coli* DH5α to confirm that the activities were due to the cloned DNA.

2.4. Identification of β-glucosidase genes

To identify the β-glucosidase genes carried on recombinant plasmid pGXN10, the target plasmids were nested-deleted from one end by *in vitro* Tn5 transposition (pWEB::TNC Deletion Cosmid Transposition Kit; Epicentre, Madison, WI, USA) according to the manufacturer's protocol. The plasmid with the smallest insertion expressing β-glucosidase was sequenced from priming sites on the vector adjacent to the deletion start sites using the following primer (5′-TGTGAAATTTGTGATGCTATTGCT-3′) provided by the manufacturer. Complete coverage of the sequences for the target gene was obtained by primer-walking sequencing from both strands.

2.5. Overexpression and purification of the recombinant β-glucosidase

The sequence of *bgl1T*, excluding the sequence encoding the N-terminal signal peptide, was amplified by polymerase chain reaction (PCR) using pGXN10 as the template, using the following primers: sense primer 5′-CCG**GAATT**CTATGGAATATCAAGCACTGGC-3′ (containing an EcoRI site at the 5′ end) and antisense primer 5′-AA**CTGCAG**CTGATGGCTGACGCAAAGTA-3′ (containing a PstI site at the 5′ end). Amplified DNA was digested with EcoRI and PstI prior to its ligation into the vector pETBlue-2 (Qiagen, Valencia, CA, USA) digested with the same enzymes, resulting in the plasmid pGXN10A. The recombinant plasmid, pGXN10A, then was transformed into *E. coli* Tuner (DE3) pLacI (Qiagen, Valencia, CA, USA). His-tagged Bgl1T was expressed and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

2.6. Identification of the hydrolysis product

The hydrolysis product of the putative β-glucosidase was identified by high performance liquid chromatography (HPLC). D-(+)-Cellobiose (Sigma, St. Louis, MO, USA) was used as the substrate for characterization of the β-glucosidase. The enzymatic reaction mixture contained 10 mM D-(+)-cellobiose, 0.2 M Na_2HPO_4-citric acid buffer, pH 7.0, 1.0 mM $MgCl_2$, and approximately 20 μg purified recombinant protein. The reaction was conducted in a total volume of 2.0 ml at 35 °C for 15 min. Upon termination of the reaction with 2.0 ml 1 M Na_2CO_3, the residual protein was removed by centrifugation through a membrane (Vivaspin 500, Vivascience, Littleton, MA, USA). The filtered reaction sample was separated on a Fast Carbohydrate Cartridge Column (150 mm × 4.6 mm, Waters, USA) eluted with double distilled H_2O and acetonitrile (15:85, v/v) as the mobile phase at a flow rate of 1.0 ml/min and detected using a Waters R-401 differential refractometer [10,18].

2.7. Physico-chemical characterization of recombinant β-glucosidase protein

Unless otherwise specified, β-glucosidase activity was assayed at 40 °C by incubating an enzymatic reaction mixtures (1 ml) containing 20 μg of suitably diluted enzyme with 2 mM *p*-nitrophenyl-β-D-glucoside (Sigma, St. Louis, MO, USA) in 50 mM Na-phosphate buffer (pH 7.0) for 15 min. The reaction was stopped by adding 600 μl of 0.4 M Na_2CO_3 and the *p*-nitrophenol (*p*NP) released was determined by reading the absorbance at 420 nm. One unit of β-glucosidase activity (U) is defined as the amount of enzyme that will hydrolyze 1 μmol *p*NP-β-D-glucoside per minute under the experimental conditions described above [19]. The profiles of activity versus pH and activity versus temperature were determined using standard assay methods. To measure the effect of pH on the activity of Bgl1T protein, the enzyme activity was assayed in the range of pH 4.5–9.0 (50 mM Na-phosphate buffer) and pH 8.6–10.0 (0.1 M glycine-NaOH buffer). For the effect of temperature on activity of Bgl1T protein, the enzyme was assayed at various temperatures (20–60 °C) for 15 min, using *p*NP-β-D-glucoside as the substrate in 50 mM Na-phosphate buffer (pH 8.0). Various metal compounds (NaCl, KCl, LiCl, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$, KNO_3), chelating agents (ethylenediaminetetraacetic acid, EDTA), and surfactant (SDS) were added to optimal reaction systems to investigate their effects on enzyme activity. The con-

centrations of metal ions, EDTA, and SDS used were 1 mM, 2 mM, and 1% (v/v), respectively.

2.8. Enzyme kinetic assays

Enzyme kinetic parameters of Bgl1T were obtained by measuring the rate of hydrolysis of *p*NP-β-D-glucoside at various concentrations (0.1–10 mM) at 37 °C for 15 min in 50 mM Na-phosphate buffer (pH 7.0). The enzymatic kinetic parameters, K_m and V_{max}, were determined from Lineweaver–Burk plots using the Enzyme Kinetics computer program [20].

3. Results and discussion

3.1. Construction of the metagenomic library and screening of the library for β-glucosidase gene

The approach of generating a metagenomic library from soils or sediments that are known to harbor a high level of microbial diversity has been used successfully to find a wide variety of novel catalytic proteins and secondary metabolites [21]. The metagenomic DNA in the current study was directly extracted from samples of sludge from a bioreactor. The constructed library was expected to contain a genome pool of all the microorganisms in the reactor sludge, including those of uncultivable bacteria. A cosmid library of ca. 30,000 clones was constructed using the metagenomic DNA isolated from the bioreactor contents. Restriction analysis of randomly chosen recombinant plasmids revealed a high level of diversity of the foreign DNA fragments found in pWEB::TNC. *Bam*HI digestion of plasmids from 14 randomly selected recombinants resulted in insert sizes ranging from 20 to 60 kb on agarose gels (data not shown), with an average size of ca. 35 kb. The library possessed a capacity of approximately 1.05×10^9 bp of insertion DNA. One metagenomic DNA library clone, named pGXN10, that expressed strong β-glucosidase activity was isolated using a function-based strategy. The positive clone pGXN10 is surrounded by a black spot against a plain background after 24 h at 37 °C, indicating active β-glucosidase. The positive rate of β-glucosidase activity in the library was approximately 1/30,000, because sludge metagenomic DNA originated from thousands of different types of microorganisms [22,23]. A recent paper reported that the positive rate of cellulase in a cosmid library constructed from soil samples was approximately 1/70,000 [24], which is somewhat lower than the final results obtained in the current study.

3.2. Sequence analysis of the cloned β-glucosidase

The plasmid from this positive clone of pGXN10 was extracted, subcloned, and sequenced by primer-walking. The insert DNA sequence had a length of 3041 base pairs (bp), and it had the highest identical (79%) with a DNA fragment (GenBank accession no. CP000653.1) from the genome of *Enterobacter* sp. 638 at DNA level in the database. However, it shared moderate homology with the other known putative β-glucoside-specific IIABC subunit components at amino acids level, according to the BLASTx program. Based on the sequence similarity results, we considered that the cloned gene on pGXN10 could be a novel β-glucosidase gene and named it *bgl1T*. The gene had an open reading frame of 1860 bp and the deduced peptide was predicted to have a molecular mass of ~65 kDa and an isoelectric point of 5.69. The *bgl1T* nucleotide sequence has been deposited in the GenBank under accession number GQ507800. When the deduced amino acid sequence of Bgl1T was searched against the NCBI and Expasy databases, it was found that Bgl1T had the highest identical (85%) and similar (92%) with β-glucoside-specific PTS system components IIABC (GenBank accession no. YP_001177456) from *Enterobacter* sp. 638. Others β-glucosidases include a cellobiose-specific PTS permease (GenBank accession no. AAB51563) from *Klebsiella oxytoca* (78% identical and 87% similar), β-glucoside-specific PTS system components IIABC subunits (GenBank accession no. YP_001336226) from *Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 (75% identical and 86% similar), a β-glucoside transport protein (GenBank accession no. ABG73228) from *Klebsiella aerogenes* (75% identical and 86% similar), a putative cellobiose-specific PTS permease (GenBank accession no. YP_002920438) from *K. pneumoniae* NTUH-K2044 (75% identical and 86% similar), and a β-glucoside-specific phosphotransferase enzyme IIA component (GenBank accession no. ZP_04635638) from *Yersinia intermedia* ATCC 29909 (63% identical and 77% similar).

Multiple alignments of the deduced amino acids of Bgl1T with the most homologous β-glucosidase proteins (above 60% similarity) (NCBI database) are presented in Fig. 1. Amino acid sequence comparison revealed that the deduced Bgl1T peptide shared the conserved amino acid residues with other known β-glucosidases. Fig. 2 presents the phylogenetic tree, showing the relationship with other β-glucosidases, based on amino acids. The phylogenetic tree also revealed that Bgl1T was most closely related to PTS system beta-glucoside-specific EIIBCA component from *Cronobacter turicensis* and it clustered together with other β-glucosidases from several bacterial species, including *Pectobacterium atrosepticum* and *Yersinia frederiksenii*.

3.3. Overexpression and purification of recombinant Bgl1T protein

In order to characterize the function of Bgl1T, we subcloned the gene in frame with a six-histidine tag sequence into expression vector pETBlue-2 and expressed it in *E. coli* Tuner (DE3) pLacI. Initial analysis with crude cell lysates showed that the bacteria containing recombinant plasmid pETBlue-2-*bgl1T* produced a substantial amount of the expected recombinant protein, while this protein was not detectable in cultures of the bacteria containing the empty vector pETBlue-2.

The recombinant Bgl1T protein was purified with Ni-NTA Magnetic Agarose Chromatography, followed by the removal of degraded and/or nonspecifically bound polypeptides with gel filtration chromatography (GFC). As shown in Fig. 3, the recombinant Bgl1T protein was purified to homogeneity. The recombinant protein eluted from the column had a molecular weight of ~66 kDa, which is consistent with the theoretical mass of 65 kDa.

3.4. Functional characterization of recombinant Bgl1T protein

We determined the product of enzymatic cellobiose hydrolysis by HPLC. The retention time of enzymatic product was 4.75 min and that of the substrate peak (D-(+)-cellobiose) was 8.48 min. The enzymatic product peak eluted earlier than the substrate peak and matched the retention time (4.57 min) of a glucose standard. These results suggested that Bgl1T protein catalyzed the formation of glucose from D-(+)-cellobiose. The functional characterization of Bgl1T may provide new insights into the relationship between the sequence, structure, and activity of the known β-glucoside-specific PTS system component IIABC subunits [25].

3.5. Physico-chemical characterization of recombinant Bgl1T protein

To determine the optimal pH for this putative β-glucosidase, we measured the enzymatic activity of the purified Bgl1T protein at various pH values from pH 4.0 to 9.5 using *p*NP-β-D-glucoside as the substrate. The data, presented in Fig. 4A, demonstrated that Bgl1T

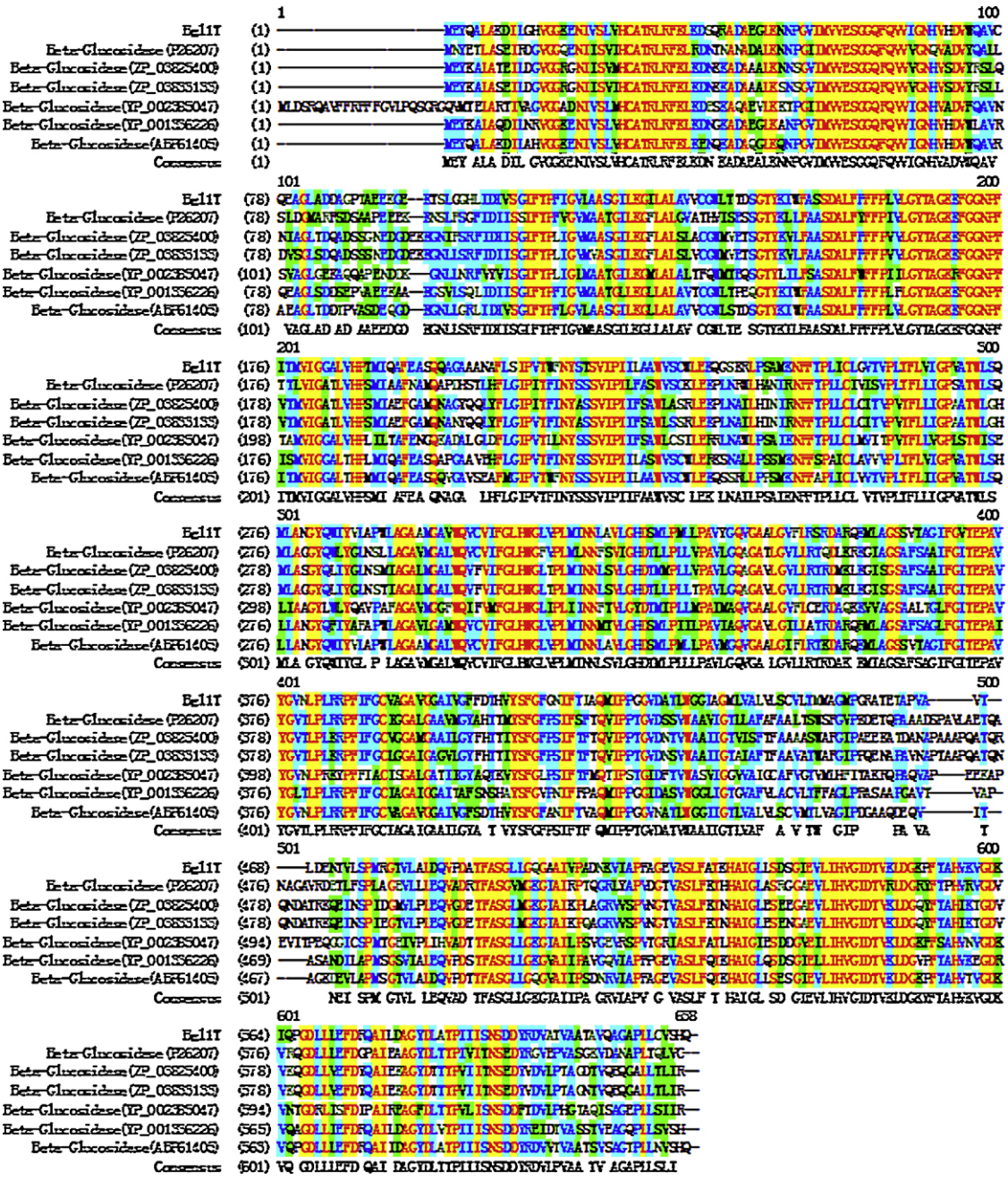

Fig. 1. Sequence alignment of Bgl1T protein with other β-glucoside-specific PTS system components. The proteins are identified by their GenBank accession number. Sequence similarity searches were performed with the BLAST 2.0 program. Amino acid sequence alignment of the target putative protein with homologous proteins was performed with the Align X program, a component of the Vector NTI suite (Informax, North Bethesda, MD, USA) using the blosum62mt2 scoring matrix.

protein was active at a pH range of 6.0–9.0. The optimal pH of the enzyme is around 7.0 because it achieves maximal enzymatic activity at this condition. Enzymatic activity remained at a high level when the pH was between 6.0 and 9.0. However, the activity of the recombinant enzyme was dramatically lost below pH 6.0 or above pH 9.0. The pH range of the recombinant enzyme was consistent with the reported properties of alkaline endoglucanase from *Bacillus circulans* [26] and metagenome-derived β-glucosidase from the contents of rabbit cecum [11].

To determine the optimal temperature for the enzymatic reaction of Bgl1T, its activity was measured at pH 7.0 in the temperature range from 20 to 60 °C. As shown in Fig. 4B, at a temperature lower than 30 °C, the enzyme showed 65% of its maximum activity. Bgl1T exhibited >70% of its maximum activity from 32 to 50 °C, and the enzyme reached its highest activity at approximately 37 °C. The temperature range of the recombinant enzyme was similar to that reported for a β-glucosidase from corn stover [27].

The effects of metal ions, EDTA, and SDS on the hydrolytic activity of Bgl1T protein were determined with *p*NP-β-D-glucoside as the substrate by measuring activity in the presence of these compounds. The enzyme activity of Bgl1T without added metal ions was taken as 100%. The presence of $CaCl_2$ and $MgCl_2$ significantly stimulated the enzyme activity to 142% and 136%, respectively, whereas $CuCl_2$, $MnCl_2$, $FeCl_2$ and $FeCl_3$ dramatically reduced the enzymatic activity to 73%, 63%, 41%, and 56%, respectively. In contrast, $ZnCl_2$, NaCl, KCl, LiCl, and KNO_3 had no significant effect on the enzymatic activity. The chelating agent EDTA and the anionic surfactant SDS reduced the activity to 63% and 50%, respectively. These results

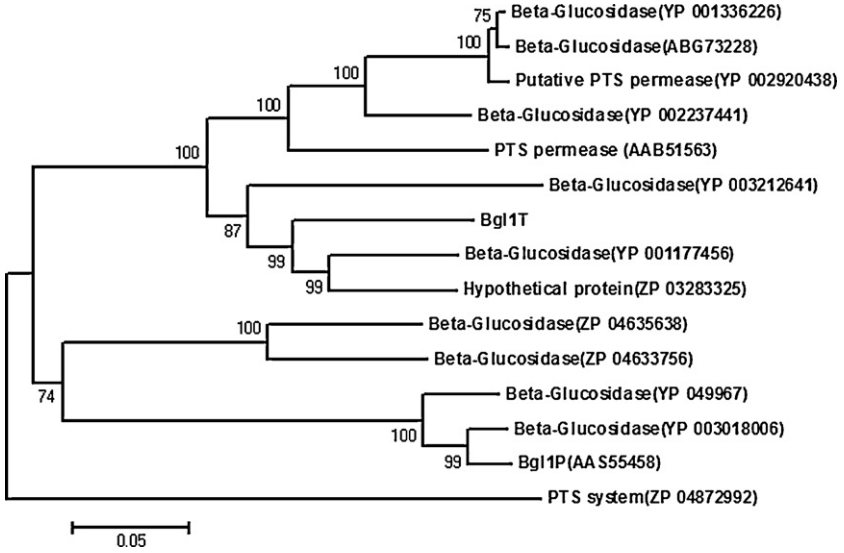

Fig. 2. Phylogenetic relationship of Bgl1T with related proteins. The proteins are identified by their GenBank accession number. Sequence alignment was performed by using ClustalW version 1.81 and the phylogenetic tree was constructed using the neighbor-joining method with Molecular Evolutionary Genetics Analysis 4.0 software (MEGA, Version 4.0) [28]. Boot-strapping value was used to estimate the reliability of phylogenetic reconstructions (1000 replicates). The numbers associated with the branches refer to bootstrap values (confidence limits) representing the substitution frequencies per amino acid residue.

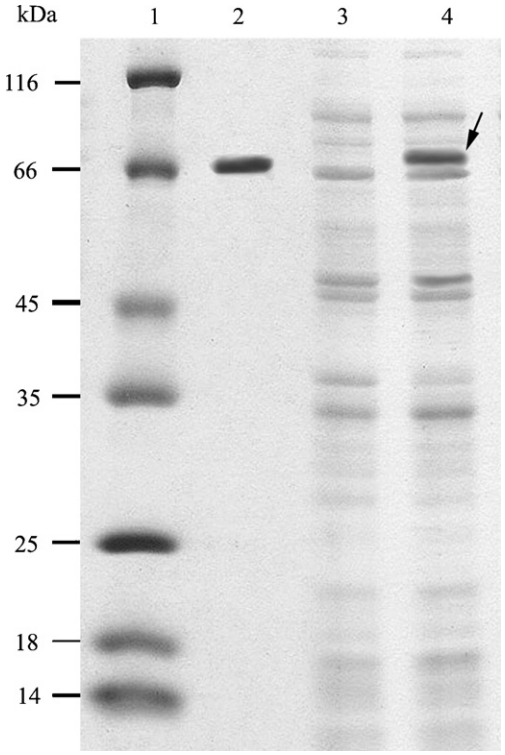

Fig. 3. SDS-PAGE analysis of Bgl1T. Proteins were separated by 12% SDS-PAGE and then stained with Coomassie brilliant blue G-250. Lane 1, protein molecular weight markers; lane 2, the purified target protein; lane 3, total protein of *E. coli* Tuner (DE3) pLacI harboring empty pETBlue-2 as control; lane 4, total protein of *E. coli* Tuner (DE3) pLacI harboring the recombinant *bgl1T* in pETBlue-2 induced by addition of 0.5 mM IPTG. The Bgl1T protein is indicated by the black arrow.

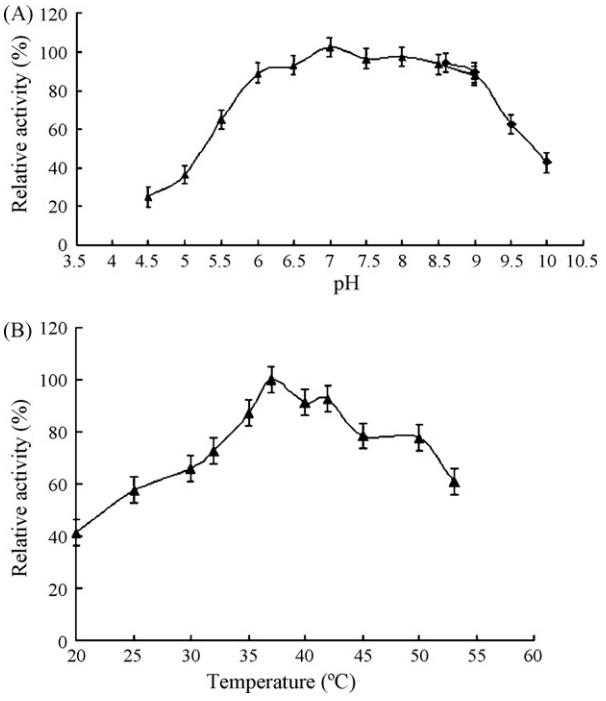

Fig. 4. (A) Effects of pH on the enzymatic activity of Bgl1T protein; the buffers used were Na-phosphate buffer (▲) (pH 4.5–9.0), glycine-NaOH buffer (♦) (pH 8.6–10.0). The relative activities are the enzyme activities at each pH divided by the maximal activity. (B) Effects of temperature on the enzymatic activity (▲) of Bgl1T protein. The relative activities are the enzyme activities at each temperature divided by the maximal activity.

Table 1
Purification of Bgl1T protein from *E. coli* Tuner (DE3) pLacI.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1030	4800	4.66	100	1.0
Ni-NTA	32.0	474	14.8	9.88	3.18
GFC	26.0	432	16.6	9.0	3.56

suggested that a $CaCl_2$ concentration of 10 mM was required for the optimal activity of Bgl1T protein. As shown in Table 1, the enzyme activity reached 16.6 U/mg protein using *p*NP-β-D-glucoside as the substrate under the optimal reaction conditions of pH 7.0, 37 °C, and 10 mM $CaCl_2$ for 15 min.

3.6. Enzyme kinetics

The kinetic parameters of the recombinant enzyme Bgl1T protein were determined using different *p*NP-β-D-glucoside concentrations as the substrate. The initial rate of the enzyme reaction was measured under the optimal reaction conditions. The reaction kinetic parameters of the purified enzyme were determined from double reciprocal Lineweaver–Burk plots. The putative β-glucosidase had an apparent K_m value of 1.45 mM, a V_{max} value of 20.5 U/mg, a k_{cat} value of 1370/min and a k_{cat}/K_m value of 943/mM/min. This V_{max} value for Bgl1T protein was in agreement with that recorded for β-glucosidase from uncultured microorganisms [9]. Knowledge of these properties of Bgl1T protein should allow better industrial production of glucose or ethanol by biological processes under moderate conditions.

4. Conclusions

We have identified a novel gene (*bgl1T*) that encodes an enzyme with β-glucosidase activity following a function-based screening of a metagenomic library from uncultured microorganisms. This appears to be the first study on the cloning and the characterization of a putative β-glucosidase gene from microbes in sludge samples from a bioreactor. Sequence analysis results demonstrated that Bgl1T protein was related to β-glucosidases. Characterization with HPLC confirmed that the recombinant Bgl1T protein could catalyze hydrolysis of D-(+)-cellobiose to form glucose. A more detailed biochemical characterization of Bgl1T is currently in progress. These results are a first step toward a better understanding of the properties of Bgl1T protein isolated from a bioreactor sludge metagenome.

Acknowledgements

This research was supported by the Open Research Fund Program of the Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering (Grant No. J0801).

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