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Gene Cloning and Molecular Characterization of a *β*-Glucosidase from *Thermotoga Naphthophila* RUK-10: an Effective Tool for Synthesis of Galacto-oligosaccharide and Alkyl Galactopyranosides

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Abstract A novel thermostable *β*-glucosidase(Tnap0602) with *β*-galactosidase activity was cloned from *Thermotoga naphthophila* RUK-10 and overexpressed in *Escherichia coli* BL21(DE3) with the aid of pET28b(+) vector. The recombinant *β*-glucosidase was purified to homogeneity by heat precipitation and Ni^{2+}-affinity chromatography. The molecular weight of the recombinant enzyme was estimated to be 51 kDa by SDS-PAGE analysis. The optimum temperature for the hydrolyses of *p*-nitrophenyl-*β*-*D*-glucopyranoside and *o*-nitrophenyl-*β*-*D*-galactopyranoside by the recombinant *β*-glucosidase were both above 95 °C, and the corresponding optimum pH value was found to be the same as 7.0. Thermostability studies show that the half-lives of the recombinant enzyme at 75, 80, 85 and 90 °C are respectively 84, 32, 14, and 3 h, and it is quite stable in a pH range of 5.0—10.0. The K_m and V_{max} values of the recombinant *β*-glucosidase for the hydrolysis of *p*NPGlu at 80 °C are 0.127 mmol/L and 18389.1 μmol·min^{-1}·mg^{-1}, the corresponding values are 0.625 mmol/L and 6250 μmol·min^{-1}·mg^{-1} for the hydrolysis of *o*NPGal, respectively. The enzyme also display the hydrolysis activity for lactose and cellobiose. Galacto-oligosaccharide and alkyl galactopyranosides could be synthesized from Tnap0602 when lactose was used as the transglycosylation substrate, indicating that the thermostable *β*-glucosidase could be a candidate for industrial application.

Keywords *β*-Glucosidase; Galacto-oligosaccharide; Alkyl galactopyranoside; Thermostablility; *Thermotoga naphthophila*; Transglycosylation

1 Introduction

β-Glucosidases(*β*-*D*-Glucoside glucohydrolases; EC 3.2.1.21), which catalyze the hydrolysis of *β*-1,4 glycosidic bond presented in alkyl-, aryl-*β*-glucosides as well as short-chain oligosaccharides, are widely distributed in all types of living organisms ranging from bacteria to plants. They normally play important roles in diverse biological processes, such as the degradation of polysaccharides, cellular signaling and host-pathogen interactions[1]. Up to now, a number of *β*-glucosidases have been isolated, cloned and characterized, many of which have potential advantages in industrial application, especially in cellulose degrading multienzymatic system. Cellulose, which is an unbranched bio-polymer of glucose units linked by *β*-1,4-glycosidic bonds, is the most abundant renewable resource in nature. Thus, it is important to hydrolyze cellulose into glucose because the process both provides energy resource and prevents environment pollution. *β*-Glucosidases can split short-chain ologosaccharides and cellobiose into glucose together with endoglucanases(EC 3.2.1.4) and cellobiohydrolases(EC3.2.1.91), thus convert complex cellulose into monomeric glucose for further utilization[2]. Not only do they possess hydrolysis activity, but also *β*-glucosidases have synthetic activity to transfer glycosyl groups to alcohols, saccharides, dopamine and so on, resulting in the formation of alkyl-glycosides, oligosaccharides and dopamine-glycosides, which can be used as non-ionic surfactants, probiotics and therapeutic agents[3—5].

β-Glucosidases can be classified into glycoside hydrolase (GH) families 1, 3 and 9 on the basis of sequence similarities in the CAZY database(http://www.cazy.org/Glycoside-Hydrolases.html). Not only do they have *β*-glucosidase activity, but aslo most of *β*-glucosidases from family 1 also possess significant galactosidase activity[2]. *β*-Galactosidases(*β*-*D*-galactoside galactohydrolase or lactase, EC 3.2.1.23) catalyze the hydrolysis of *β*-1,4-*D*-galactosidic linkages, including those of lactose,

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oligosaccharides and polysaccharides. These enzymes are widely distributed in nature, and found in plants, animals as well as numerous microorganisms. Based on the hydrolytic and transglycosylation activities they possess, *β*-galactosidases have two main biotechnological uses in the dairy industry: the removal of lactose from milk for lactose-intolerant people and the production of galacto-oligosaccharides(GOS) from lactose as probiotic food stuffs[6,7]. In addition, the lactose hydrolysis also has many other applications, such as the improvement of the unpleasant sandy texture of the ice cream and other refrigerated dairy products resulted from the low solubility and crystallization of lactose at a high concentration. Usually, GOSs formed by the enzymatic treatment of lactose have a degree of polymerisation(DP) from 2 to 10, with a terminal glucose. The component in the final products, linkage between the galactose units and the productivity strongly depend on the enzyme source as well as the specific reaction conditions. For their probiotic properties and their use as low-calorific sweeteners, GOSs have earned much more interest especially in food and drink industries. GOSs are stable in acidic environments and resistant to high temperatures, and they are nondigestible carbohydrates for they do not serve as the substrates of hydrolytic enzymes in upper digestive tract, but GOSs can be fermented by colonic microbiota, thus selectively benefiting the host and promoting the healthy balance. The injection of GOSs also attributes to the improvement of mineral bioavailability, and the stimulation of immune system and lipid metabolism[8].

Alkyl glycosides, which belong to a group of newly nonionic surfactant, have drawn a lot of attentions in the researches of enzymatic synthesis recently. Owing to their low surface tension, high activity, strong detergency, low skin irritation, good biodegradability and environmental friendliness, alkyl glycosides have been widely applicated in many areas, such as detergent, food and cosmetic industries. Alkyl glycosides can be synthesized by chemical and enzymatic methods. Compared with the chemical methods, enzymatic-catalyzed synthesis of alkyl glycosides could be carried out under milder reaction conditions and the product has good regioselectivity and stereoselectivity[9]. The alkyl glycosides with transglycosylation could be synthesized by two steps: in the first step the substrate binds to a pair of carboxylic acids to form a covalent galactosyl-enzyme intermediate; in the second step, a nucleophile attaches to the anomeric centre to yield an alkyl glycoside[10].

The current biotechnological interest in enzymes has been shifted to *β*-glucosidases from thermophilic organisms. With the outstanding thermostability, thermophilic *β*-glucosidases are active at conditions under which mesophilic enzymes are normally denatured. Thus, temperatures of enzyme reactions could be increased that results in the increase of substrate solubility and initial reaction rate, and also the inhibition of microbial contamination.

We cloned a new gene Tnap0602 corresponding to a putative *β*-glucosidase from *Thermotoga naphthophila* RUK-10, a hyperthermophilic bacterium with optimum cell growth at 80 °C. The gene was expressed in *Escherichia coli* BL21(DE3) in a soluble form with a His-tag at the N-terminus. The recombinant enzyme was purified *via* column chromatography. The molecular weight, biochemical properties and transglycosylation activity were determined. The recombinant *β*-glucosidase belongs to GH family1, and *β*-galactosidase activity was also found.

2 Materials and Methods

2.1 Materials

All the restriction enzymes and protein molecular weight standards were purchased from Takara Biotechnology Co., Ltd.(Dalian, China). T_4 DNA ligase, *EasyTaq* DNA polymerase and protein molecular weight standards were supplied by Beijing TransGen Biotech Co., Ltd.(Beijing, China). The glucose oxidase kit was purchased from Changchun Huili Biotech Co., Ltd.(Changchun, China). All the other chemicals were of reagent grade that were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd.(Beijing, China). *p*-Nitrophenyl-*β*-*D*-glucopyranoside(*p*NPGlu) and *o*-nitrophenyl-*β*-*D*-galactopyranoside(*o*NPGal) were purchased from Sigma(St. Louis, MO, USA). Silica gel plates were supplied by Branch of Qingdao Haiyang Chemical Co., Ltd.(Qingdao, China). AxyPrep DNA gel extraction kit was provided by Axygen Biotechnology Ltd.(Hangzhou, China).

2.2 Bacterial Strains and Plasmids

The genomic DNA of *Thermotoga naphthophila* RUK-10 (Japan Collection of Microorganisms, JCM10882) was used as polymerase chain reaction(PCR) template, and *Escherichia coli* strain JM109 was used for DNA manipulation and amplification. *Escherichia coli* strain BL21(DE3) was used for the expression of *β*-glucosidase. The plasmid pET-28b was used as the DNA cloning and expression vector.

2.3 Cloning and Expression Vector Construction of *β*-Glucosidase Gene from *Thermotoga naphthophila*

The gene Tnap0602 encoding *β*-glucosidase(1341 bp) from *Thermotoga naphthophila* was amplified by PCR with *EasyTaq* DNA polymerase. Based on the DNA sequence of the *β*-glucosidase from *Thermotoga naphthophila* reported in GenBank(accession number CP001839.1), two oligonucleotides, 5′-forward primer containing a restriction site for *Nde*I(5′-CTGACACATATGAACGTGAAAAAGTTCCCT-3′) and 3′ reverse primer with a *Ecor*RI site(5′-CTGCAC GAATTCTTAATCTTCCAGACTG-3′), were designed, respectively. The amplified DNA fragment was purified by virtue of AxyPrep DNA gel extraction kit and digested with both *Nde*I and *Eco*RI endonucleases. The digested DNA fragment was purified and inserted into the pET-28b plasmid digested with the same restriction enzyme by means of T4 DNA ligase kit. *Escherichia coli* strain JM109 was transformed with the ligation mixture and plated on Luria- Bertani(LB) agar containing 10 mg/mL kanamycin. The kanamycin-resistant transformants were screened by colony PCR and the recombinant

pET-Tnap0602-28b was confirmed by endonucleases digestion and sequencing.

2.4 Expression of *Thermotoga naphthophila* *β*-Glucosidase Gene in *E. coli* as a His-tagged Recombinant Enzyme

A fresh clone of *Escherichia coli* BL21(DE3) harboring the pET-Tnap0602-28b vector was grown in a 1 L of LB medium containing 10 mg/mL kanamycin with agitation at 37 °C. Isopropyl-*β*-*D*-thiogalactopyranoside(IPTG, 0.4 mmol/L) was added to the culture at an optical density(OD) of 1.0, determined at 600 nm, and the medium was further incubated under agitation at 150 r/min and 25 °C for 12 h. The induced cells were harvested by centrifugation and washed once with 50 mmol/L sodium phosphate buffer(pH=7.0).

2.5 Enzyme Purification

The harvested induced cells were resuspended in 50 mL of buffer A(50 mmol/L sodium phosphate buffer, 0.4 mol/L NaCl, pH=7.0) and were disrupted by sonication. Cellular debris was removed by centrifugation(15000*g*, 15 min and 4 °C) to obtain the crude lysate. The crude extract was incubated in a water bath for 10 min at 80 °C to denature *E. coli* proteins. The extract was then centrifuged to obtain the heat-treated crude cell extract. The supernatant was loaded onto a Ni^{2+}-NTA agarose resin column equilibrated with buffer B(20 mmol/L sodium phosphate buffer, 0.4 mol/L NaCl, pH=7.0). The enzyme was eluted with a linear gradient of 0—200 mmol/L imidazole in buffer B. The molecular weight and purity were determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE), and the protein concentration was determined *via* the Bradford assay with bovine serum albumin(BSA) as the standard.

2.6 Assay of *β*-Glucosidase and *β*-Galactosidase Activities

β-Glucosidase and *β*-galactosidase activities were measured with *p*NPGlu and *o*NPGal as substrates on a UV-visible Spectrophotometer 2550(SHIMADZU) run with UVProbe 2.33 software. The absorbance change of 1 mL of the reaction mixture, which contained 0.5 mmol/L *p*NPGlu or *o*NPGal, 50 mmol/L sodium phosphate buffer(pH=7.0) and 20 μL of purified enzyme, was observed at 420 nm(A_{420}) and at 80 °C over 1 min. One unit of enzyme activity was defined as the amount of the enzyme that release 1 μmol of *p*NP or *o*-NP per minute under the defined conditions[11].

For the hydrolysis of lactose, 20 μL of purified enzyme (0.2 mg/mL) was added to a reaction mixture containing 5% lactose and 50 mmol/L disodium hydrogen phosphate-citric acid buffer(pH=5.5) to achieve a final volume of 1 mL. The reaction mixture was incubated at 85 °C for 30 min, the amount of glucose produced during incubation was determined using a glucose oxidase kit and observing absorbance alterations at 510 nm(A_{510}). One unit of enzyme activity was defined as the amount of the enzyme that liberates 1 μmol of glucose per minute under the defined conditions[12].

2.7 Effects of Temperature and pH on Enzyme Activity

The temperature dependence of enzyme activity was investigated by measuring the activity over a temperature range of 50—95 °C. For the hydrolyses of *p*NPGlu and *o*NPGal, the reactions were carried out in 50 mmol/L sodium phosphate (pH=7.0) with 20 μL of 0.02 and 0.2 mg/mL purified enzyme, respectively. To estimate its thermostability, the enzyme(0.2 mg/mL) was pre-incubated in 50 mmol/L sodium phosphate (pH=7.0) at 75, 80, 85 and 90 °C, respectively. Samples were withdrawn at certain time intervals, and the residual activity was measured by the standard assay method with *o*NPGal as the substrate. The optimum pH values for *β*-glucosidase and *β*-galactosidase activities were determined at 80 °C with 20 μL of 0.02 and 0.2 mg/mL purified enzyme, respectively. Two different 50 mmol/L buffer systems were examined: disodium hydrogen phosphate-citric acid buffer(pH=4.0—8.0) and oracic acid-potassium chloride-sodium hydroxide buffer(pH= 7.8—10.0). To determine pH stability, the enzyme(0.2 mg/mL) was incubated at 25 °C in buffer at various pH values. Residual enzyme activity was determined at various time intervals under the standard assay conditions with *o*NPGal as the substrate.

The optimum temperature dependence of the recombinant enzyme activily was measured over a temperature range of 50—95 °C for the hydrolysis of lactose. The optimum pH for lactose hydrolysis was determined at 85 °C. Two different 50 mmol/L buffer systems were examined: disodium hydrogen phosphate-citric acid buffer(pH=4.0—8.0) and boracic acid-potassium chloride-sodium hydroxide buffer(pH=7.8—10.0). The mixture was incubated at 85 °C for 30 min, then the concentration of glucose was determined *via* glucose oxidase kit.

2.8 Kinetic Analysis

Various concentrations of *p*NPGlu(0.008 to 0.05 mmol/L) and *o*NPGal(0.08 to 0.5 mmol/L) were used to determine the kinetic parameters of the enzyme. All the experiments were performed in 50 mmol/L phosphate buffer(pH=7.0) for 5 min at 80 °C. The kinetic parameters of the enzyme toward lactose hydrolysis were determined at 85 °C in disodium hydrogen phosphate-citric acid buffer(pH=5.5) for 30 min by varying the concentration of lactose(0.0125 to 0.05 mmol/L). The amount of enzyme in each experiment was 20 μL(0.2 mg/mL). K_m(mmol/L) and V_{max}(μmol·min^{-1}·mg^{-1}) values were calculated *via* Lineweaver-Burk Plot.

2.9 Formation of Galacto-oligosaccharide and Alkyl Galactopyranosides

The synthesis of galacto-oligosaccharide was carried out at 70 °C with lactose as both the sugar donor and acceptor. The reaction mixture contained lactose(0.1 g) in 20 mmol/L sodium phosphate buffer(2.0 mL, pH=6.0) and the purified enzyme (3.5 mU). Samples were withdrawn at defined time intervals. The transglycosylation product was analyzed by thin-layer

chromatography(TLC). Reaction mixtures were loaded onto silica gel plates, resolved in a system containing butanol-propanol-ethanol-water(2:3:3:2, volume ratio) and dried. Plates were stained with *α*-naphthol(2.56 g/L) in an ethanol:sulfuric acid mixture(90:10, volume ratio). Carbohydrates were detected by heating at 100 °C for a few minutes.

The syntheses of alkyl galactopyranosides were carried out in an aqueous(sodium phosphate buffer, 20 mmol/L, pH=6.0) organic solvent mixture(9:1, volume ratio) with lactose(0.036 g) as sugar donor and *n*-butanol, *n*-hexanol and *n*-octanol as sugar acceptors, and 3.5 mU enzyme was added in the mixture to start the reaction. The reaction was performed at 75 °C in a total volume of 2 mL. Samples were withdrawn at defined time intervals. The reactions were analyzed by TLC with chloroform-methanol-acetic acid-water(12:6:1:1, volume ratio) as the developing solvent, and plates were stained with the method described above.

2.10 Purification and Identification of Galacto-oligosaccharide and Octyl Galactopyranosides

The aqueous phase of the GOS reaction was concentrated by rotary evaporation, and the residues of the reaction mixture were redissolved in 2 mL of mobile phase[methanol-chloroform-acetic acid-water(30:60:5:5, volume ratio)], and the galacto-oligosaccharide in the mixture was separated from other components by silica gel chromatography.

The octyl-*β*-*D*-galactopyranoside was purified as follows. The organic phase was separated from aqueous phase when the reaction was finished and concentrated by evaporation. The residues of organic phases were redissolved in acetone, and the octyl-*β*-*D*-galactopyranoside was separated by silica gel chromatography with acetone as the mobile phase.

The process was monitored by TLC. The collected fractions were evaporated to remove the mobile phase and purified galacto-oligosaccharide and octyl-*β*-*D*-galactopyranoside were finally obtained.

The purified galacto-oligosaccharide and octyl-*β*-*D*-galactopyranoside were analyzed by high performance liquid chromatography-electrospray tandem mass spectrometry (Agilent 1290-micrOTOF Q II, Bruker). And the purified galacto-oligosaccharide was further confirmed by ^{1}H NMR spectrometry(500 MHz, AVANCE III500, Bruker).

3 Results and Discussion

3.1 Gene Cloning and Enzyme Expression

A putative *β*-glucosidase gene of *Thermotoga naphthophila*(Tnap0602) was cloned and expressed in *E. coli* as a soluble recombinant protein with a hexa-histidine tag at the N-terminus. The recombinant vector was confirmed by sequencing from the 5′ T7 promoter to 3′ T7 terminator, and compared with the putative *β*-glucosidase gene from *Thermotoga naphthophila* reported in GenBank. The recombinant *β*-glucosidase gene encodes 466 amino acids with a presumptive molecular weight of approximately 53672.6 Da calculated by ProtParam(http://web.expasy.org/protparam/). Expression conditions were optimized by varying IPTG concentration (0.1—1 mmol/L) and induction temperature(25, 30 and 37 °C). A maximum expression level was obtained after incubation at 25 °C for 12 h with 0.4 mmol/L IPTG.

3.2 Enzyme Purification and Molecular Mass Determination

The recombinant *β*-glucosidase was purified by the method described in Section 2.5. *β*-Glucosidase was finally purified 11.99-fold(Table 1) and a single band was observed on SDS-PAGE(Fig.1). The molecular weight of the purified *β*-glucosidase was estimated to be approximately 51 kDa by SDS-PAGE and 90 kDa by non-denaturing gradient PAGE(Fig.2), indicating that the recombinant enzyme was a

Table 1 Purification of the recombinant *β*-glucosidase from *T. naphthophila**

Purified method	Total amount of protein/mg	10^{-3} Total activity/U	Specific activity/(U·mg^{-1})	Purification fold	Recovery(%)
Crude enzyme	655.68	155.98	237.88	1	100
Heat treatment	71.34	128.78	1805.17	7.59	82.56
Ni-NTA	8.21	23.413	2851.77	11.99	15.01

*Activity was measured in 50 mmol/L phosphate buffer(pH=7.0) at 80 °C with *o*NPGal as the substrate.

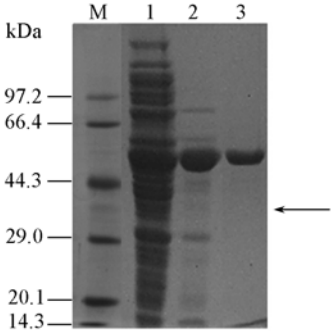

Fig.1 SDS-PAGE analysis of the recombinant enzymes

Lane M: recombinant molecular weight markers; lane 1: crude extract; lane 2: supernatant after heat treatment at 80 °C for 10 min; lane 3: the recombinant *β*-glucosidase purified by Ni^{2+}-NTA chromatography.

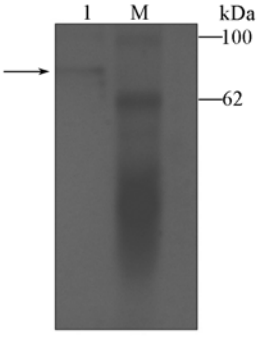

Fig.2 Non-denaturing gradient PAGE analysis of the recombinant *β*-glucosidase

Lane M: recombinant molecular weight markers; lane 1: the recombinant *β*-glucosidase purified by Ni^{2+}-NTA chromatography.

homodimer. *β*-Glucosidase from *Caldicellulosiruptor saccharolyticus* was also a homodimer with a molecular weight of 54 kDa of each subunit[13]. *β*-Glucosidase from *Talaromyces thermophilus* was a monomer with a molecular weight of 50 kDa[14], while *β*-glucosidase from *Pyrococcus furiosus* was a tetramer, each subunit has a molecular weight of (58±2) kDa[15].

The amino acid sequence of *β*-glucosidase from *Thermotoga naphthophila* has a similarity of 99% to that of *β*-glucosidase from *Thermotoga maritima* by BLAST(Basic Local Alignment Search Tool), which has already been cloned and characterized[16].

3.3 Effects of Temperature and pH

The effect of temperature on the enzyme activity was studied at temperatures ranging from 50 °C to 95 °C at pH=7.0. The optimum temperatures for *p*NPGlu and *o*NPGal hydrolyses by the purified *β*-glucosidase were found to be both above 95 °C(Fig.3). The optimum pH of *β*-glucosidase activity is 7.0 for both of *p*NPGlu and *o*NPGal hydrolyses[Fig.4(A) and (B)]. The optimum temperature and pH towards lactose hydrolysis by the recombinant enzyme were 85 °C[Fig.3(B)] and 5.5 [Fig.4(C)], respectively. Under the optimum conditions of lactose hydrolysis, the recombinant *β*-glucosidase showed an activity of 84%(27.96 U/mg) for the hydrolysis of cellobiose. *β*-Glucosidase is very sensitive to the change in pH. When *p*NPGlu and *o*NPGal were used as the substrates, the enzyme displayed the maximal activity in neutral pH environment and retained 80% of its activity at pH=6.5. As for the hydrolysis of lactose, the recombinant *β*-glucosidase showed the maximal activity in acid pH environment and retained 80% of its activity over a pH range of 5.0—6.5.

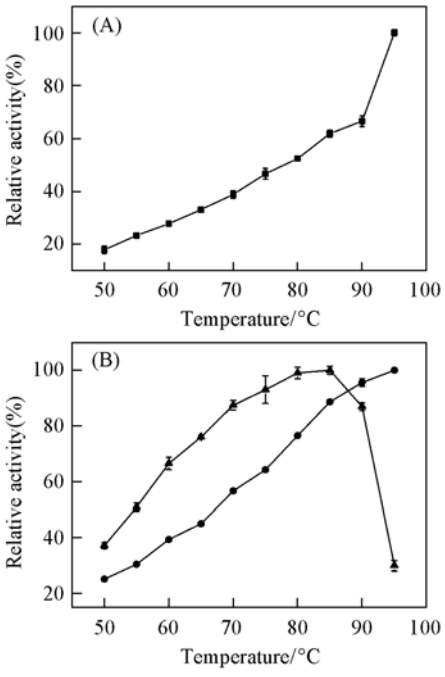

Fig.3 Effects of temperature on the *β*-glucosidase(A) and *β*-galactosidase(B) activities

The determinations were carried out in 50 mmol/L phosphate buffer (pH=7.0) with *p*NPGlu(■), *o*NPGal(●) or lactose(▲) as substrate.

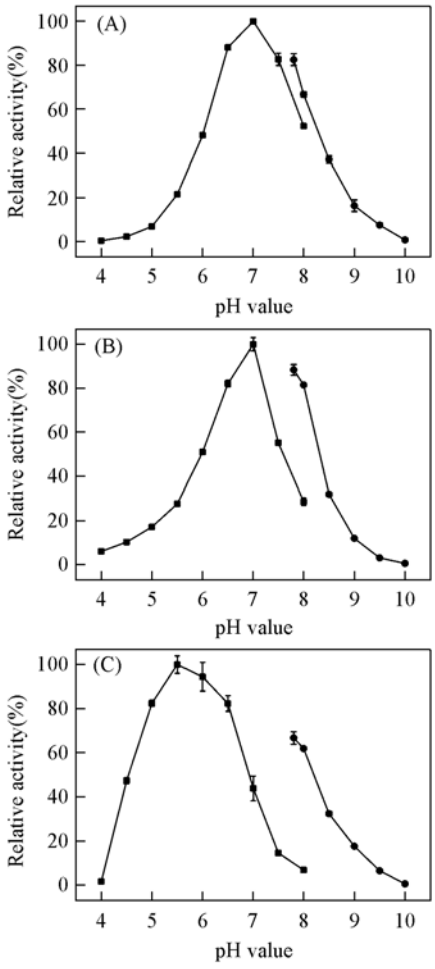

Fig.4 Effects of pH on the activity of recombinant *β*-glucosidase

The determination conditions were as follows: for the hydrolysis of *p*NPGlu(A) and *o*NPGal(B), at 80 or 85 °C, respectively; for lactose(C), 50 mmol/L disodium hydrogen phosphate-citric acid buffer(■) or boracic acid-potassium chloride-sodium hydroxide buffer(●).

The thermostability of *β*-glucosidase was examined by measuring the time course of its residual activity at different temperatures[Fig.5(A)]. The half-lives of the enzyme at 75, 80, 85 and 90 °C were found to be 84, 32, 14, and 3 h, respectively. The recombinant enzyme was very stable in a wide pH range of 5.0 to 10.0, retaining 90% of its activity at 25 °C after incubation for 24 h[Fig.5(B)].

The optimum temperature and pH for the hydrolyses of *p*NPGlu[Fig.4(A)] and *o*NPGal[Fig.4(B)] by *β*-glucosidase from *Thermotoga maritima* were 90 °C and 6.0—6.2, respectively[16], while in the present study, the recombinant *β*-glucosidase we cloned from *Thermotoga naphthophila* has a much higher optimum temperature(95 °C) and is more active in neutral pH for hydrolysis of aryl-*β*-glucoside. *β*-Glucosidases from *Periconia* sp[2] and *Caldicellulosiruptor saccharolyticus*[13] both have maximum activity at 70 °C, by comparison, the optimum temperature of *β*-glucosidase from *Pyrococcus furiosus*[15] is 102—105 °C. They both show the optimum activity in a pH range of 5.0—6.0. The optimum temperature for the hydrolyses of *p*NPGlu and *o*NPGal by *β*-glucosidase from *Thermotoga naphthophila* was much higher than most by the *β*-glucosidases reported so far.

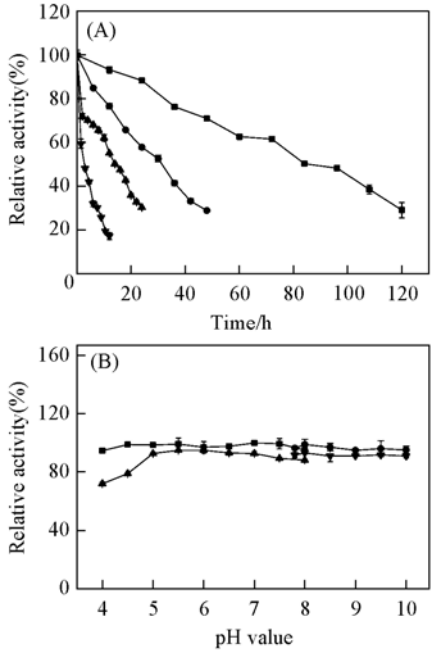

Fig.5 Thermostability(A) and pH stability(B) of recombinant *β*-glucosidase

The time course of change of residual activities of the enzyme with time was determined in 50 mmol/L phosphate buffer(pH=7.0) at 75(■), 80(●), 85(▲) and 90 °C(▼), respectively. Residual activities were measured by the standard assay method at different conditions: in disodium hydrogen phosphate-citric acid buffer after incubation for 1 h(■) and 24 h(▲) at 25 °C, and in boracic acid-potassium chloride-sodium hydroxide buffer after incubation for 1 h(●) and 24 h(▼) at 25 °C respectively.

Like *β*-glucosidase from *Caldicellulosiruptor saccharolyticus*[13], *β*-glucosidase from *Thermotoga naphthophila* also showed *β*-galactosidase activity, for both aryl-*β*-galactose and lactose. The optimum temperatures for the actives of *β*-galactosidases from *Sulfolobus solfataricus*[17], *Sterigmatomyces elviae* CBS8119[18] and *Pyrococcus woesei*[19] were 80, 85 and 93 °C, respectively, and the optimum pH values were 6.0, 4.5—5.0 and 5.4, respectively. The *β*-galactosidase activity of Tnap0602 studied in this work is among those of highly thermostable galactosidases.

The half-lives of the *β*-galactosidase from *Sulfolobus solfataricus*[17] were 91, 48, 35 and 2.6 h at 75, 80, 85 and 90 °C, respectively, while the half-lives of the *β*-glucosidase from *Caldicellulosiruptor saccharolyticus*[13] were 250, 24.3 and 0.4 h at 60, 70 and 80 °C, respectively. The most thermostable one reported so far is *β*-glucosidase from *Pyrococcus furiosus*[15] with the half-lives of 85 h at 100 °C and 13 h at 110 °C. The thermostability of *β*-glucosidase from *Thermotoga naphthophila* is comparable to those of other thermostable *β*-glycosidases.

3.4 Kinetic Parameters of the Enzyme

Enzyme kinetic parameters were calculated *via* Lineweaver-Burk plot of the Michaelis-Menten equations at different concentrations of substrate. The K_m values and the V_{max} values for the hydrolyses of *p*NPGlu and *o*NPGal at 80 °C were found to be 0.127 and 0.625 mmol/L and 18389.1 and 6250 μmol·min^{-1}·mg^{-1}, respectively. For the hydrolysis of lactose at 85 °C, the K_m and V_{max} values were 0.042 mmol/L and 28.027 μmol·min^{-1}·mg^{-1}, respectively(Table 2). Our study shows that the recombinant enzyme has a higher affinity for both *p*NPGlu and lactose, especially for lactose.

Table 2 Kinetic parameters of the recombinant *β*-glucosidase from *T. naphthophila*

Substrate	K_m/(mmol·L^{-1})	V_{max}/(μmol·min^{-1}·mg^{-1})	k_{cat}/s^{-1}
*p*NPGlu	0.127	18389.1	15786.84
*o*NPGal	0.605	6250	5560.16
Lactose	0.042	28.03	24.06

The K_m value of the *β*-glucosidase from *Thermotoga naphthophila* for the hydrolysis of *p*NPGlu is comparative to that of the *β*-glucosidase from *Pyrococcus furiosus*(0.15 mmol/L)[15], but its V_{max} value is much higher. The K_m values of *β*-galactosidase from *Bacillus licheniformis*[20] and *Thermus* sp.IB-21(BgaA)[12] for the hydrolyses of *o*NPGal and *p*-nitriphenyl-*β*-*D*-galactopyranoside(*p*NPGal) were (13.7±0.1) and 0.41 mmol/L at 70 °C. For the hydrolysis of lactose, the K_m values were (169±0.8) and 42 mmol/L, respectively. *β*-Glucosidase from *Thermotoga naphthophila* has higher affinity for both *o*NPGal and lactose compared to most of reported *β*-galactosidases.

3.5 Synthesis of GOS and Alkyl Galactopyranosides

Transglycosylation activity was found when the recombinant *β*-glucosidase was incubated with lactose that acts as both glycosyl donor and acceptor, resulting in the production of GOS. The reaction mixtures were analyzed by TLC[Fig.6(A)].

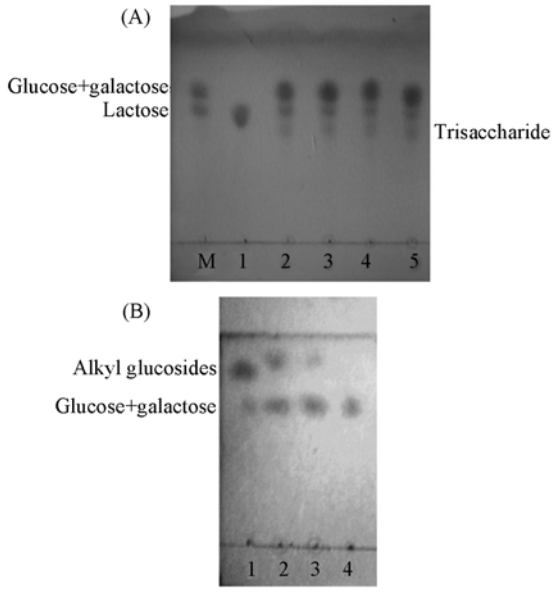

Fig.6 TLC analysis of transglycosylation reaction products

(A) Lane M: mixture of 1% galactose, glucose and lactose; lane 1: reaction mixture of 0 h; lane 2: reaction mixture of 24 h; lane 3: reaction mixture of 48 h; lane 4: reaction mixture of 72 h; lane 5: reaction mixture of 96 h. (B) lane 1: product of *n*-butanol reaction system (butyl-*β*-*D*-galactopyranoside, 24 h); lane 2: product of *n*-hexanol reaction system(hexyl-*β*-*D*-galactopyranoside, 24 h); lane 3: product of *n*-octanol reaction system(octyl-*β*-*D*-galactopyranoside, 24 h); lane 4: 1% galactose.

The only transglycosylation product is a trisaccharide and the most likely structure is *β*-galactosyl-lactose. In most cases, the form of the GOS products generated by transglycosylation reaction is a mixture of di-, tri- and even higher oligosaccharides[7,18,21,22]. It is interesting that transglycosylation reaction catalyzed by *β*-glucosidase from *Thermotoga naphthophila* generates a new single trisaccharide. The reason could be that the affinity of the enzyme for lactose is higher than that for other trisaccharide in the reaction system(data not shown). This phenomenon might be significant because the purification of GOS could be simplified. The transformation rates of transglycosylation reaction were 8.34% and 13.44% at 24 and 96 h, respectively.

Transglycosylation reaction was also found in aqueous organic solvent mixture when alcohol was used as the sugar acceptor. We explored the reactions between lactose and each of straight chain alcohols with different chain lengths, as shown in Fig.6(B). Our results indicate that the reaction velocity decreased as the length of carbon chain increased. The yield of butyl, hexyl and octyl galactopyranoside after 48 h were 9.68%, 8.23% and 1.29%, respectively. A similar result has been reported for the synthesis of alkyl glycosides with *β*-glucosidase[23].

HPLC-MS analysis shows that the peak of ion spectrum for the product is at *m/z* 527.1, which corresponds to the molecular weight of trisaccharide($M+Na^+$)[Fig.7(A)] and octyl-*β*-*D*-galactopyranoside($M+H^+$)[Fig.7(B)]. The trisaccharide was further confirmed by ^1H NMR analysis in D_2O solution, the anomeric signals at peak(*δ* 4.56—4.62, d) were assigned to H-1*β*. Peak at *δ* 4.36—4.41 was observed as overlapping signals due to H-1′, H-1″, and H-1‴.

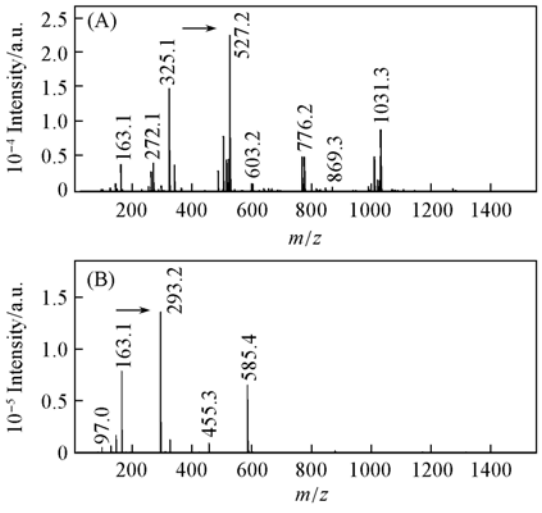

Fig.7 HPLC-MS analysis of transglycosylation products
(A) GOS; (B) octyl-*β*-*D*-galactopyranoside.

4 Conclusions

In the present work, the gene of a novel *β*-glucosidase from *Thermotoga naphthophila* has been cloned and expressed in *E. coli* successfully. Owing to its high thermostability, broad pH stability and high affinity towards substrates, *β*-glucosidase from *Thermotoga naphthophila* is found to be an efficient hydrolytic enzyme. Moreover, the recombinant enzyme possesses transglycosylation activity, which generates a trisaccharide and alkyl galactopyranosides with lactose as the sugar donor. The excellent thermostability, efficient hydrolytic and transglycosylation activity of this novel enzyme suggest that it will be a useful tool in industrial application, especially for the synthesis of trisaccharide and alkyl galactopyranosides, and the hydrolysis of cellobiose.

References

[1] Bhatia Y., Mishra S., Bisaria V. S., *Crit. Rev. Biotechnol*., **2002**, *22*, 375
[2] Harnpicharnchai P., Champreda V., Sornlake W., Eurwilaichitr L., *Protein Expr. Purif*., **2009**, *67*, 61
[3] Turner P., Svensson D., Adlercreutz P., Karlsson E. N., *J. Biotechnol*., **2007**, *130*, 67
[4] Krisch J., Bencsik O., Papp T., Vágvölgyi C., TakóM., *Bioresour. Technol*., **2012**, *114*, 555
[5] Sivakumar R., Divakar S., *Enzyme Microb. Technol*., **2009**, *44*, 33
[6] Mahoney R. R., *Food Chem*., **1998**, *63*, 147
[7] Urrutia P., Rodriguez-Colinas B., Fernandez-Arrojo L., Ballesteros A. O., Wilson L., Illanes A., Plou F. J., *J. Agric. Food Chem*., **2013**, *61*, 1081
[8] Macfarlane G. T., Steed H., MacFarlane S., *J. Appl. Microbiol*., **2008**, *104*, 305
[9] El-Sukkary M. M. A., Nagla A. S., Aiad I., El-Azab W. I. M., *J. Surfact Deterg.*, **2008**, *11*, 129
[10] Andersson M., Adlercreutz P., Patrick A., *Journal of Molecular Catalysis B: Enzymatic*, **2001**, *14*, 69
[11] Daabrowski S., Sobiewska G., Maciunska J., Synowiecki J., Ku J., *Protein Expression and Purification*, **2000**, *19*, 107
[12] Kang S. K., Cho K. K., Ahn J. K., Bok J. D., Kang S. H., Woo J. H., Lee H. G., You S. K., Choi Y. J., *J. Biotechnol*., **2005**, *116*, 337
[13] Hong M. R., Kim Y. S., Park C. S., Lee J. K., Kim Y. S., Oh D. K., *J. Biosci. Bioeng*., **2009**, *108*, 36
[14] Nakkharat P., Haltrich D., *J. Biotechnol*., **2006**, *123*, 304
[15] Kengen S. W. M., Luesink E. J., Stams A. J. M., Zehnder A. J. B., *Eur. J. Biochem*., **1993**, *213*, 305
[16] Gabelsberger J., Liebl W., Schleifer K. H., *Appl. Microbiol. Biotechnol*., **1993**, *40*, 44
[17] Kim Y. S., Park C. S., Oh D. K., *Enzyme Microb. Technol*., **2006**, *39*, 903
[18] Onishi N., Tanaka T., *Appl. Environ. Microbiol*., **1995**, *61*, 4026
[19] Dąbrowski S., Maciuńska J., Synowiecki J., *Mol. Biotechnol*., **1998**, *10*, 217
[20] Juajun O., Nguyen T. H., Maischberger T., Iqbal S., Haltrich D., Yamabhai M., *Appl. Microbiol. Biotechnol*., **2011**, *89*, 645
[21] Iqbal S., Nguyen T. H., Nguyen H. A., Nguyen T. T., Maischberger T., Kittl R., Haltrich D., *J. Agric. Food Chem*., **2011**, *59*, 3083
[22] Splechtna B., Nguyen T. H., Steinböck M., Kulbe K. D., Lorenz W., Haltrich D., *J. Agric. Food Chem.*, **2006**, *54*, 4999
[23] Kobayashi T., Adachi S., Nakanishi K., Matsuno R., *Journal of Molecular Catalysis B: Enzymatic*, **2000**, *11*, 13