Purification, characterization, and gene cloning of a new cold-adapted β -galactosidase from *Erwinia* sp. E602 isolated in northeast China

Yu Xia,*†† Lili He,† Jingjing Mao,† Peipei Fang,† Xiaoyuan Ma,*†† and Zhouping Wang*††1

*State Key Laboratory of Food Science and Technology,

†School of Food Science and Technology, and

‡Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, Jiangnan University, Wuxi 214122, China

ABSTRACT

β -Galactosidases are widely used in industry for elimination of lactose from milk products. A new β -galactosidase was obtained from bacterial strain *Erwinia* sp. E602, newly isolated in northeast China. The enzyme was purified with the methods of ammonium sulfate fractionation, ion exchange, and gel filtration chromatography for further study of the enzymatic characteristics. The purified enzyme had a molecular weight of near 110 kDa. The optimum reaction temperature and pH of this enzyme was determined to be 40°C and 7.0, respectively, indicating that this enzyme was a mesophilic neutral β -galactosidase. Furthermore, the enzyme retained near 10% of the activity at 0°C, which also suggested its cold-adapted property. Kinetics of the β -galactosidase was studied, and the K_m (Michaelis constant) and V_{max} (maximum enzymatic reaction rate) of this enzyme were 0.21 mmol/L and 263.16 μ mol/mg per minute, respectively. The effects of metal ions on the enzymatic activity and the lactose hydrolysis efficiency in milk, as well as its trans-glycosylation activity, were studied in this work. The β -galactosidase coding gene was cloned to be a 3-kb length fragment, which shared at most 81% of identity with the published sequences in NCBI Blast database (<https://blast.ncbi.nlm.nih.gov>). Results in this work suggested it is a new β -galactosidase and it has potential to be used in dairy and food processing.

Key words: β -galactosidase, *Erwinia*, isolation and purification, enzymatic characteristics

INTRODUCTION

β -Galactosidase (EC 3.2.1.23) is a kind of glycoside hydrolase that can hydrolyze the β -1,4-D-galactosidic linkage in lactose. This enzyme is applied in the dairy industry for elimination of milk lactose to prevent the

symptom of lactose-intolerance in human beings (Vincent et al., 2013). This enzyme is also widely used in other food industrial areas (Panesar et al., 2010); for example, the production of galacto-oligosaccharides (GOS) using β -galactosidase appeared to be attractive in recent years (Lee et al., 2017). In the dairy industry, β -galactosidases used for lactose hydrolysis are mainly mesophilic enzymes of microbial origin, such as those from yeasts and fungi (Panesar et al., 2010), thus lactose hydrolysis treatments are usually carried out at moderate temperatures, which might cause undesired microbial contamination during the time of processing (Katrolia et al., 2011).

In recent years, cold-adapted enzymes have generated high interest in the dairy industry because they have some advantages over mesophilic enzymes. For example, such enzymes can catalyze reactions at lower temperatures, which might avoid loss of nutrition in foodstuffs and eliminate microbial spoilages of foods to a great extent (Ramli et al., 2011). Furthermore, the cold-adapted enzymes have relative high stability and catalytic efficiency at lower temperatures, reducing the energy cost of the processing and unexpected side effects as well as the property of rapid inactivation at moderate temperatures (Borchert et al., 2017). The number of studies on cold-adapted β -galactosidases continues to increase. Many of these enzymes are derived from cold-area deep-sea microorganisms, such as *Pseudoalteromonas* sp., *Paracoccus* sp., and *Rahnella* sp. (Cieřliński et al., 2005; Wierzbicka-Woř et al., 2011; Fan et al., 2015).

In the current study, we report a new β -galactosidase that has relatively good activity at low temperatures. This enzyme was obtained from the newly isolated *Erwinia* sp. strain E602, which was extracted from frozen soil in the northeast area of China. The β -galactosidase was obtained by fermentation of the strain E602 and purified by salting-out, dialysis, ion-exchange chromatography, and gel filtration chromatography. We investigated the enzymatic activity and properties, as well as the ability for hydrolysis of lactose in milk, in this work.

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¹Corresponding author: wangzp@jiangnan.edu.cn

MATERIALS AND METHODS

Materials and Reagents

Medium components, such as tryptone and yeast extract, were purchased from Oxoid (Thermo Fisher Scientific, Waltham, MA). Ortho-nitrophenyl- β -D-galactopyranoside (**ONPG**) and dithiothreitol were purchased from Sangon Biotech (Shanghai, China). Isopropyl- β -D-thiogalactopyranoside and 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (**X-gal**) were purchased from Sigma-Aldrich (Shanghai, China). Acetonitrile was purchased from Sigma-Aldrich. Molecular weight protein marker was purchased from Thermo Fisher Scientific. Protein determination kit was purchased from Zoman Biotechnology (Beijing, China). Glucose determination kit was purchased from Rongsheng Biotech (Shanghai, China). The restriction enzymes and polymerase were purchased from New England Biolabs (Beijing, China). Other reagents were of analytical reagent grade. The *Escherichia coli* hosts DH5 α and BL21(DE3) were stocks in our laboratory. The commercial β -galactosidase derived from *Aspergillus oryzae* was purchased from Jiangsu Ruiyang Biotech Co. Ltd. (Wuxi, China). The milk used for the lactose hydrolysis experiment was fresh milk taken from the local dairy farm (Wuxi, China).

Strain Isolation and Identification

A sample of frozen soil was taken from a suburban forest farm in Qiqihar (Heilongjiang Province, China) and used for screening of microbial strains, which produced cold-adapted β -galactosidase. The medium (tryptone 15.0 g/L, soya peptone 5.0 g/L, K₂HPO₄ 2.5 g/L, lactose 2.5 g/L, yeast extract 3.0 g/L, NaCl 5.0 g/L, agar 15.0 g/L, pH 7.3–7.5; **TSA**) was used for isolation of the target strains. A 10.0-g sample of the soil was added to 100.0 mL of TSA liquid and cultivated at 12°C with shaking at 200 rpm. After 48 h of cultivation, the culture was 10-fold diluted and plated on the surface of TSA medium supplemented with lactose (2.5 g/L, as an inducer for β -galactosidase production) and X-gal (1.0 mg/mL), followed by incubation at 4 to 12°C for 72 h. Blue colonies that appeared on the plates were all picked up and inoculated in TSA liquids supplemented with lactose (2.5 g/L) for further investigation of β -galactosidase activities at different temperatures. The strain with highest β -galactosidase activity at 10°C were selected as the target strain of this work and named as E602. The cell and colony morphology of the target strain was determined by Gram staining and microscopy. For identification of the target strain in a molecular approach, the whole genomic DNA of strain E602 was extracted using the

cetyl trimethylammonium bromide method (Healey et al., 2014). The 16s rDNA sequence was then amplified by PCR using the whole genomic DNA as the template. The primers for this experiment were 27 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1391 reverse (5'-GACGGGCGGTGTGTRCA-3'). The amplified strand was subjected to DNA sequencing in Biosune Biotechnology Co. Ltd. (Shanghai, China) for further analysis. The online sequence alignment program NCBI BLASTN2.2.26+ (Zhang et al., 2000) was used for comparing and analyzing the sequence identity with the data of bacterial 16S ribosomal RNA. A phylogenetic tree was inferred using the alignment data with software MEGA7 (Tamura et al., 2004; Kumar et al., 2016). For identification of the target strain with physiological and biochemical experiments, utilization of various carbon sources and acid production results from various sugars were tested according to the methods previously described (Hauben and Swings, 2005).

Production and Purification of β -Galactosidase from *Erwinia* sp. E602

The strain E602 was streaked on TSA medium at 26°C for 24 h. A single colony of the strain was inoculated in 5.0 mL of TSA liquid and cultivated with shaking at 26°C for 12 h. The strain E602 was activated on TSA medium at 26°C for 24 h. A single colony of the strain was inoculated in 5.0 mL of TSA liquid and cultivated with shaking at 26°C for 12 h. Aliquots were taken from the culture and inoculated at a ratio of 1% (vol/vol) into 100.0 mL of TSA liquid in a flask and cultured at 26°C for 15 h. The secondary seed was then inoculated at a ratio of 1% (vol/vol) into 5.0 L of TSA liquid supplemented with lactose (2.5 g/L) in a fermentor (New Brunswick Scientific, Edison, NJ) and fermented at 26°C for 18 h with the agitation speed of 200 rpm.

The culture cells of strain E602 were then harvested and subjected to centrifugation at 4°C and 10,000 $\times g$ for 30 min on a Beckman Avanti J-26xp centrifuge (Beckman Coulter Inc., Fullerton, CA) and then treated with sonication. The supernatant was treated with 20% ammonium sulfate saturation and then centrifuged at 4°C, 10,000 $\times g$, for 10 min. After centrifugation, the sediment was discarded and the supernatant was brought to 40% ammonium sulfate saturation, followed by centrifugation at 4°C, 10,000 $\times g$, for 10 min. The sediment was collected and dissolved in 10.0 mmol/L of PBS (pH 7.0), and dialyzed at 0°C for 12 h. The resultant solution was subjected to anion exchange chromatography, followed by gel filtration chromatography.

The anion exchange chromatography process was performed on an AKTA purifier system (GE Health-

care, Little Chalfont, UK) using a HiPrep 16/10 DEAE column and equilibrated with 20.0 mmol/L of Tris-HCl (pH 7.0). The target enzyme was eluted by a solution (containing 2.0 mol/L of NaCl and 20.0 mmol/L of Tris-HCl, pH 7.0) at gradients (vol/vol) of 10, 15, 27, 33, and 40%. The elution speed was 5.0 mL/min. Gel filtration chromatography process was also performed on AKTA purifier system using a HiPrep 16/60 Sephacryl S-100 column (GE Healthcare). The β -galactosidase was eluted in 50.0 mmol/L of PBS (150.0 mmol/L of NaCl, pH 7.0) at a speed of 0.5 mL/min.

Enzymatic Activity Assay

The β -galactosidase activity was determined as previously described (Xia et al., 2010) with some modifications. The 5.0-mL substrate solution (ONPG, 0.5 mg/mL, pH 7.0) was mixed with 0.9 mL of PBS (pH 7.0) and the mixture was kept in water bath at the optimum temperature of the enzyme for 30 min. The 100.0 μ L of purified enzyme (0.02 mg/mL) was then added to the mixture and reacted for 10 min. After that the reaction mixture was added to 75.0 mg/mL of Na_2CO_3 solution for termination of the reaction. The reaction mixture was then held at 25°C for 15 min, followed by testing of the absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μ mol of substrate ONPG per minute. The specific activity of β -galactosidases was expressed in units per milligram of enzyme protein. This method was used for all the enzyme properties determination, except for special parameters mentioned in context.

Determination of Properties of β -Galactosidase

The effects of temperature on enzymatic activity were determined at temperatures ranging from 0 to 60°C. The thermostability of the enzyme was tested by incubation of the enzyme at temperatures ranging from 10 to 50°C for 6 h. The effects of pH on enzymatic activity were determined at 40°C in buffers that ranged in pH from 4.0 to 9.0. The pH stability profile of the enzyme was determined by incubation of this enzyme at 4°C for 1 h in PBS buffers (pH 4.0–9.0), followed by activity determination at 40°C under standard conditions. The effects of these factors were evaluated by determination of the residue enzymatic activities and illustrated as the relative activities.

The effects of several chemical reagents on enzyme activity were determined by assaying the residue activities after incubating the enzyme in 1.0 to 10.0 mmol/L of the reagents (dissolved in PBS, pH 7.0) at 25°C for 20 min. The cations tested were Li^+ , Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} , and Pb^{2+} . The thiol reagents

2-mercaptoethanol and dithiothreitol, the enzyme inhibitor p-chloromercuribenzoic acid, and the chelating agent EDTA were also examined. Relative activity was estimated in above experiments by comparison to the activity obtained in control.

Kinetic Analysis of β -Galactosidase

The kinetic parameters (the Michaelis constant, K_m , and the maximum enzymatic reaction rate, V_{max}) of the β -galactosidase were calculated from Lineweaver-Burk plots using ONPG as substrate. The ONPG concentration ranged from 0.8 to 10.0 mmol/L. For analysis of the inhibition effects of substrate-like reagents on the β -galactosidase, the ONPG solutions were added with lactose, galactose, and glucose at final concentrations of 10.0, 100.0, and 100.0 mmol/L, respectively. All the experiments above were carried out at 40°C and pH 7.0.

Hydrolysis of Lactose in Milk by β -Galactosidase

Fifteen units of the purified β -galactosidase was added to 5 mL of fresh milk (containing 5% (wt/vol) lactose). The solution was incubated at different temperatures ranging from 4 to 40°C for 12 h. One hundred microliters of the aliquots was withdrawn every 2 h from the incubation and the β -galactosidase was denatured by adding 10% trichloroacetic acid. After centrifugation at 4°C, $10,000 \times g$ for 15 min, the supernatants were subjected to glucose concentration analysis to evaluate the hydrolysis ratio of milk lactose; every test was carried out in 3 separate trials.

Trans-Glycosylation of Lactose by β -Galactosidase and the GOS Assay

Forty-five units of the purified β -galactosidase was added to 5.0 mL of lactose solution (300 mg/mL). The solution was incubated at 40°C for 12 h. After that, 0.5 mL of the aliquot was withdrawn and incubated in boiling water for 2 min for inactivation of the enzyme, followed by detection of GOS with LC-MS.

Cloning of Coding Sequence for β -Galactosidase from *Erwinia* sp. E602

The whole genomic DNA of strain E602 was extracted using the cetyl trimethylammonium bromide method (Healey et al., 2014). The DNA extracted was partially digested with the restriction enzyme *Sau*3AI. The DNA fragments in the *Sau*3AI digestion were recovered by gel recovery with lengths of 2 to 4 kb and then subcloned to plasmid pUC19 and transformed *E. coli* host DH5 α . About 1,000 positive transformants

(out of about 3,500 colonies) of the subcloning were picked and sequenced, in which the insertion fragments of about 3 kb were picked out for open reading frame (ORF) analysis. The ORF of about 3 kb in length were then amplified respectively with PCR and cloned to the inducible expression plasmid pET28a(+) with restriction site *Nco*I and *Xho*I. The recombinant plasmids were then transformed *E. coli* host BL21(DE3) and the transformants were plated on Luria-Bertani (LB) agar plates supplemented with kanamycin (50.0 μ g/mL), isopropyl- β -D-thiogalactopyranoside (1.0 mmol/L), and X-gal (1.0 mg/mL). The blue colonies were picked up for sequencing and verification of insertion sequences.

RESULTS AND DISCUSSION

Isolation and Identification of β -Galactosidase-Producing *Erwinia* sp. E602

The isolation of the β -galactosidase-producing strain was carried out by blue colony testing of the diluted samples plating on the selective agar plates, followed by retesting of the enzyme activity of the colonies and the strain identifications. A total of 80 microbial isolates that showed blue colonies on TSA plates supplemented with lactose and X-gal were picked up for β -galactosidase assays; 15 isolates produced β -galactosidase active at low temperatures (0–12°C). The strain with the highest β -galactosidase activity at 10°C was named E602. Cells of strain E602 were rod-shaped, 0.5 to 1.0 μ m wide and 1.0 to 2.0 μ m long, occurring singly or in pairs, gram-negative, and nonendospore-forming. This strain showed milky white colony with a little metallic sheen on TSA medium and it had a growth temperature range of 4 to 30°C with optimum growth at 26°C.

For identification of this strain, the coding sequence of its 16S rRNA was amplified by PCR from the genomic DNA of strain E602. The 1.5-kb amplified fragment was subjected to BLAST alignment with bacterial 16S rDNA sequence online (<http://blast.ncbi.nlm.nih.gov>). The phylogenetic tree (Figure 1) was obtained by using the neighbor-joining algorithms on software MEGA7. The 16S rDNA analysis results suggested that strain E602 belong to the genus *Erwinia*. To identify strain E602 to species, the physiological and biochemical experiments were done to test the utilization of various carbon sources and acid production from various sugars. The results were listed in Table 1. According to the diagnostic characteristics published by previous studies (Hauben and Swings, 2005; Thapa et al., 2012), the strain E602 was suggested to be *Erwinia rhapontici*. This species is a conditional pathogenic ice-nucleation bacterium to plants, and it is rarely reported in China (Wang et al., 2017). As the phylogenetic relationship

between E602 and *E. rhapontici* was not so obvious, strain E602 still cannot be finally determined to be *E. rhapontici* species. Nevertheless, this work might be the first detailed enzymatic study on β -galactosidase obtained from genus *Erwinia*.

Production and Purification of β -Galactosidase from *Erwinia* sp. E602

We cultivated strain E602 with induction of lactose for production of β -galactosidase. The enzyme produced was first treated with ammonium sulfate precipitation and then the target enzyme was further purified by 2-step separations: anion exchange chromatography and the gel filtration chromatography (results listed in Supplemental Figures S1 and S2; <https://doi.org/10.3168/jds.2018-14605>). The elution corresponding to the single peak was collected for β -galactosidase enzymatic activity assay. The purification procedures and results of the enzyme were summarized in Table 2, which depicted the specific activities and the purification folds in the steps. After these purification procedures, the final enzymatic activity reached 177.00 U/mg of protein with purity more than 100 fold of the original sample. The enzyme samples of every purification step were subjected to 12% SDS-PAGE analysis and the final purified β -galactosidase showed a single band of about 110 kDa (Figure 2).

Temperature and pH Profiles of β -Galactosidase

The effect of temperature on the enzymatic activity was studied at temperatures ranging from 0 to 60°C at pH 7.0. The purified enzyme showed the maximum activity at 40°C (Figure 3A), which is similar to the optimum reaction temperature of the cold-adapted β -galactosidase from *Pseudoalteromonas* sp. 22b (Turkiewicz et al., 2003). Furthermore, the enzyme in this work retained near 10% of the activity at 0°C and about 15% of the activity at 10°C (Figure 3A), which suggested cold-adapted property of this enzyme. Thermostability of the enzyme was determined by incubation at 10, 20, 30, and 40°C for 6 h (Figure 3B). Results showed that more than 85% of the enzymatic activity remained after 6 h of incubation at 10°C, and about 85% remained after 1 h of incubation at 40°C. The enzymatic half-life of the β -galactosidase at 10, 20, 30, 40°C were 13, 11, 8, and 7 h, respectively. The thermostability of the enzyme at 50°C was also tested (figure not shown) and the half-life of the enzyme at this temperature was 2 min. After incubation of the enzyme at 50°C for 15 min, the enzyme was almost entirely inactivated.

Figure 1. The phylogenetic tree of *Erwinia* sp. strain E602 based on 16S rDNA gene sequences. This tree was obtained using software MEGA7 (Kumar et al., 2016) by the Neighbor-Joining method. Numbers at branch nodes indicate bootstrap values of 1,000 trials (only bootstrap values above 50% were shown). Bar = 0.005 substitutions per nucleotide position.

The pH profiles of the enzyme were shown in Figure 4. The enzyme displayed its maximum enzymatic activity near pH 7.0 and retained more than 80% of its activity at the pH range of 6.5 to 7.5 (Figure 4A), which is a good property with respect to milk processing because

the pH of milk is close to 6.7. The pH stability assay showed that this enzyme was stable in the pH range of 5.0 to 8.0 (Figure 4B). The relative enzymatic activity remained about 55% at pH 4.0 and more than 80% at pH 9.0, which is slightly better than the native cold-

Table 1. Physiological and biochemical characteristics of *Erwinia* sp. E602

Characteristic	Result ¹
Motility on 3% agar	+
Growth temperature (°C)	4–30
Growth at 36°C	–
Growth in 5% NaCl	+
Pink pigment on YDC ²	–
Yellow pigment	–
Oxidase	–
Catalase	+
Urease	–
Pectinase	–
Anaerobic growth	+
H ₂ S production	–
Aerogenesis with glucose	–
Indol production	–
Casein hydrolysis	–
Gelatin liquefaction	–
Ortho-nitrophenyl-β-D-galactopyranoside utilization	+
VP test ³	–
Acid production from	Glucose, lactose, galactose, fructose, sucrose, maltose, mannose, cellobiose, gentiobiose, rhamnose, melibiose, trehalose, melizitose, melitose, D-arabinose, L-arabinose, ribose, D-xylose, L-fucose, D-fucose, D-turanose, D-lyxose, glycerol, esculin, arbutin, amygdalin, salicoside, inositol, mannitol, sorbitol, xylitol, n-acetylglucosamine, methyl α-D-glucoside, methyl α-D-mannopyranoside

¹– Negative; + Positive.
²YDC = yeast extract-dextrose-CaCO₃ medium.
³VP test = Voges-Proskauer test. The VP test is a microbiological test for acetoin production.

Table 2. Purification of β -galactosidase from *Erwinia* sp. E602

Steps	Total protein (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg)	Purification folds
Cell-free extract	3.50	6.12	1.75	1.00
Ammonium sulfate precipitation	0.66	2.22	3.36	1.93
Anion exchange	0.03	1.63	54.33	31.04
Gel filtration	0.02	3.54	177.00	101.14

adapted β -galactosidase from *Pseudoalteromonas* sp. 22b (Cieřliński et al., 2005).

A comparison in the specific activity, temperature, and pH profiles of this enzyme to those of the other cold-adapted β -galactosidases (including a commercial enzyme used in the local dairy plant) is listed in Table 3. Results showed that the β -galactosidase obtained in our work had similar properties to the other enzymes listed and was even slightly better than the commercial one.

Effects on Enzyme Activity of β -Galactosidase

According to the results listed in Table 4, the cations Li^+ , K^+ , and Mg^{2+} activated the enzyme activity whereas Cu^{2+} , Fe^{2+} , and Pb^{2+} inhibited the enzyme activity. Enzyme inhibitors displayed inhibition against the enzyme whereas thiol reagents revealed some pro-

motion effects. The chelating agent EDTA also inhibited the enzyme activity.

The enzyme kinetic parameters for ONPG and other substrate-like inhibitors were calculated from

Figure 2. The SDS-PAGE analysis of the purified β -galactosidase from different purification steps. In each sample lane, 10.0 μL of protein sample was added. Lane 1 = protein marker, lane 2 = cell-free extracts, lane 3 = fraction of $(\text{NH}_4)_2\text{SO}_4$ precipitation, lane 4 = fraction of AKTA DEAE column purification (GE Healthcare, Little Chalfont, UK), lane 5 = fraction of AKTA Sephacryl S-100 column purification.

Figure 3. Effects of temperature on enzymatic activity of β -galactosidase. The enzymatic activities were assayed according to the method described above using ortho-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate. (A) The optimum reaction temperature of the enzyme and (B) thermostability of the enzyme assayed at different temperatures. The relative activities were shown as the percentages of the values of each point to the maximum. The error bars represent the SD from 3 trials. $\blacktriangle$ = 10°C; $\blacktriangledown$ = 20°C; $\blacksquare$ = 30°C; $\bullet$ = 40°C.

Table 3. Comparison of temperature and pH profiles of β -galactosidase from *Erwinia* sp. E602 to those of others

Microbial origin	Specific activity ¹	Optimal reaction temperature	Relative activity at 0°C ²	Optimal reaction pH	Microbial accession number ³	Reference
<i>Erwinia</i> sp. E602	177.0 U/mg	40°C	About 10%	7.0	MG822787	This work
<i>Aspergillus oryzae</i>	31.6 U/mg ⁴	50°C	About 15%	5.0	N/A	Commercial product
<i>Pseudoalteromonas</i> sp. 22b	190.0 U/mg	40°C	About 10%	7.0	AF443784	Turkiewicz et al., 2003
<i>Arthrobacter</i> sp. 32c	155.9 U/mg	50°C	About 15%	6.5	FJ609656	Hildebrandt et al., 2009

¹Reported specific enzymatic activity after purification (U/mg of protein).

²Relative enzymatic activity ratios to the optimal value.

³Accession number for the enzyme producing microorganisms in NCBI databases (<https://www.ncbi.nlm.nih.gov/genbank/>).

⁴Specific enzymatic activity by the commercial product manual (U/mg of protein).

the Lineweaver-Burk plot (Figure 5). The K_m and V_{max} values for ONPG were 0.18 mmol/L and 263.16 μ mol/mg per minute, respectively. The substrate-like inhibitors, lactose (10 mmol/L), glucose (100 mmol/L), and galactose (100 mmol/L) were added to the substrate solution to determine the inhibition constant K_i values. Results showed that lactose and galactose had competitive inhibitor effects against ONPG, whereas glucose had little effect. The inhibitory activity of lactose was greater than galactose, which might be because lactose was the natural substrate of this enzyme. The K_i for lactose and galactose were 7.00 and 36.84 mmol/L, respectively.

Hydrolysis of Lactose in Milk and Trans-Glycosylation Test by β -Galactosidase

The lactose hydrolysis ability of the purified β -galactosidase from strain E602 was tested with fresh

Table 4. Effects of metal cations, EDTA, thiol reagents, and inhibitors on the β -galactosidase activity from *Erwinia* sp. E602

Item	Concentration (mmol/L)	Relative activity (%)
Control	0	100.0
Cations		
Li ⁺	10.0	131.3
Na ⁺	10.0	104.3
K ⁺	10.0	133.6
Mg ²⁺	10.0	124.8
Ca ²⁺	10.0	88.4
Zn ²⁺	5.0	28.7
Cu ²⁺	5.0	4.9
Fe ²⁺	5.0	32.5
Mn ²⁺	2.0	105.3
Pb ²⁺	2.0	2.8
Thiol reagents		
2-Mercaptoethanol	1.0	121.2
Dithiothreitol	1.0	124.5
Enzyme inhibitor		
p-CMB ¹	1.0	18.7
Chelating agent		
EDTA	1.0	74.3

¹p-CMB = p-chloromercuribenzoate.

Figure 4. The pH profile of the β -galactosidase. (A) Effect of pH on enzymatic activity of the β -galactosidase, assayed according to the method described in the Determination of Properties of β -Galactosidase section at 40°C. (B) The pH stability of the β -galactosidase, with enzymatic activities assayed after incubation of the β -galactosidase in PBS buffers (pH 4.0–9.0) at 4°C for 1 h followed by activity determination at 40°C under standard conditions. The error bars represent the SD from 3 trials.

Figure 5. Lineweaver-Burk plot of the enzyme and the effects of substrate-like inhibitors on the activity of β -galactosidase. The ortho-nitrophenyl- β -D-galactopyranoside (ONPG) concentration ranged from 0.80 to 10.0 mmol/L. All the experiments were carried out at 40°C and pH 7.0. $\blacktriangle$ = control; $\blacklozenge$ = glucose (100.0 mmol/L); $\blacksquare$ = galactose (100.0 mmol/L); $\bullet$ = lactose (10.0 mmol/L). The $1/V$ is the reciprocal of reaction speed V , and $1/S$ is the reciprocal of substrate concentration S .

milk at different temperatures (Figure 6). Results showed that the hydrolysis ratio of lactose in milk reached 60% after 12 h of hydrolysis at 30°C. When the reaction was carried out at 40°C, 8 h was needed to hydrolyze 50% of the lactose. The hydrolysis rate was slow at temperatures below 20°C, but the lactose hydrolysis ratio at 4°C still reached 5.4% after 12 h of reaction, indicating the hydrolysis reaction can be done during the milk cold-chain transportation and

Figure 6. Milk lactose hydrolysis ratio by β -galactosidase; $\blacktriangledown$ = 4°C; $\blacktriangle$ = 10°C; $\blacklozenge$ = 20°C; $\blacksquare$ = 30°C; $\bullet$ = 40°C. The error bars represent the SD from 3 trails.

storage stages, which could realize parts of the lactose hydrolysis tasks. In consideration of the rapid enzyme inactivation at 50°C, lactose hydrolysis ability at lower temperatures is a good property for the actual lactose hydrolysis process. The hydrolysis reaction could be done at moderate temperatures and can be entirely terminated at pasteurization or UHT processes; thus, the hydrolysis ratio can be easily controlled to maintain uniform quality of the processed milk.

The purified β -galactosidase from strain E602 was used for *trans*-glycosylation reaction test with the substrate lactose and the GOS produced was assayed by LC-MS. The sample of lactose standard and the lactose hydrolysis solution were showed in Supplemental Figures S3 and S4, respectively (<https://doi.org/10.3168/jds.2018-14605>). The component that showed a peak at 12.46 min in Figure S4 was determined to be a kind of trisaccharide, and its molecular weight was 504 (Supplemental Figure S5; <https://doi.org/10.3168/jds.2018-14605>). These results showed that this β -galactosidase can effectively transform the lactose into GOS.

Gene Cloning of β -Galactosidase from *Erwinia* sp. E602

According to the SDS-PAGE analysis results, the β -galactosidase had a molecular weight of about 110 kDa (Figure 2), which corresponded to the DNA bases of about 3 kb. The partial restriction digestion library was constructed to probe the coding gene of β -galactosidase. The obtained coding sequences (ORF of about 3 kb in length) were verified by recombinant expression test on selective agar. The enzyme coding gene was obtained, sequenced, and submitted to GenBank (Accession number: MG822787). Results showed that the β -galactosidase coding gene, named as *lacZ* in this work, was 3,084 bp in length. Analyzed with NCBI Blast tool, this gene shared at most 81% of identity with other genes in the database, and its expressed AA sequence (putative) only shared 77% of identity with the β -D-galactosidase from *E. coli* K-12 substr. MG1655. These results indicated that this gene is entirely new; thus, the β -galactosidase obtained in our work is a new one.

CONCLUSIONS

Erwinia is a gram-negative bacterium that can be used for production of enzymes and additives for food industry (Ren et al., 2011); this genus is not pathogenic to animals and human beings. In this work, a β -galactosidase-producing strain, *Erwinia* sp. E602, was isolated from frozen soil in northeast China, which was surprising because the genus *Erwinia* is rarely reported

in China. The β -galactosidase produced by *Erwinia* sp. E602 was efficiently purified and comprehensively studied. The coding gene for this enzyme had 3,084 bp of length, and genetic analysis showed the gene and the enzyme were both entirely new. The optimum reaction temperature of the enzyme is 40°C and its optimum pH is 7.0. The thermostability of the enzyme was good from 10 to 40°C, and this enzyme was stable from the pH range of 5.0 to 8.0. This enzyme showed cold-adapted properties in reactions and it could be inactivated immediately at 50°C. The metal ions Li^+ , K^+ , and Mg^{2+} activated the enzyme activity and Cu^{2+} , Fe^{2+} , and Pb^{2+} inhibited the enzyme activity. The K_m and V_{max} of this β -galactosidase were 0.184 mmol/L and 263.16 $\mu\text{mol/mg}$ per minute, respectively, using ONPG as substrate. Other experimental results also indicated that this enzyme is proper for hydrolysis of milk lactose and production of low-lactose milk products. Trisaccharide was efficiently formed during the process of lactose hydrolysis so that this enzyme may also be applied for GOS production. In general, this new β -galactosidase has potential to be used in dairy and food processing.

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