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Cloning, purification and characterization of a thermostable β-galactosidase from *Bacillus licheniformis* strain KG9

F. Matpan Bekler[1], P. Stougaard[2], K. Güven[1], R. Gül Güven[3] and Ö. Acer[1]

[1] Biology Section, Science Faculty, Dicle University, 21280, Diyarbakir, Turkey
[2] Department of Plant and Environmental Sciences, University of Copenhagen, Denmark
[3] Science Teaching, Ziya Gökalp Education Faculty, Dicle University, 21280, Diyarbakir, Turkey

Corresponding author: Fatma Matpan Bekler, Biology Section, Science Faculty, Dicle University, 21280, Diyarbakir, Turkey. E-mail: fatmatpan@hotmail.com

Abstract

A thermo- and alkalitolerant *Bacillus licheniformis* KG9 isolated from Taşlıdere hot water spring in Batman/Turkey was found to produce a thermostable β-galactosidase. Phylogenetic analysis showed that the 16S rRNA gene from *B. licheniformis* strain KG9 was 99.9% identical to that of the genome sequenced *B. licheniformis* strain DSM 13. Analysis of the *B. licheniformis* DSM 13 genomic sequence revealed four putative β-galactosidase genes. PCR primers based on the genome sequence of strain DSM 13 were used to isolate the corresponding β-galactosidase genes from *B. licheniformis* strain KG9. The calculated molecular weights of the β-galactosidases I, II, III, and IV using sequencing data were 30, 79, 74, and 79 kDa, respectively. The genes were inserted into an expression vector and recombinant β-galactosidase was produced in *Escherichia coli*. Of the four β-galactosidase genes identified in strain KG9, three of them were expressed as active, intracellular enzymes in *E. coli*. One of the recombinant enzymes, β-galactosidase III, was purified and characterized. Optimal temperature and pH was determined to be at 60 ºC and pH 6.0, respectively. K_m was determined to be 1.3 mM and 13.3 mM with *o*NPG (ortho-nitrophenyl-β-D-galactopyranoside) and lactose as substrates, respectively, and V_{max} was measured to 1.96 µmol/min and 1.55 µmol/min with *o*NPG and lactose, respectively.

Key words: Thermophiles, *Bacillus licheniformis*, recombinant DNA, β-galactosidase, purification, enzyme activity.

Introduction

β-Galactosidase (β-D-galactoside galactohydrolase, lactase, EC 3.2.1.23) is an enzyme that hydrolyses terminally non-reducing β-D-galactose residues in oligo- and polysaccharides as well as in glycoproteins and glycolipids. The enzyme catalyses two main reactions: (a) hydrolysis of β-D-galactosides such as lactose to galactose and glucose (b) transgalactosylation in which the glycone moiety or galactose interact with acceptor molecules such as sugars and alcohols and synthesize sugar derivatives (1). At present, more than a hundred putative β-galactosidase sequences can be deduced from databases, and the sequences can be classified into four different glycoside hydrolase (GH) families GH-1, GH-2, GH-35, and GH-42, based on functional similarities (2, 3). Application of β-galactosidase in dairy and food processes has been known for a long time, and especially processes involving hydrolysis of lactose in order to enhance the digestibility of milk or to improve the functional characteristics of milk products have been investigated. Other applications of β-galactosidases involve transgalactosylation and other structural and functional modifications of food products, pharmaceutical and other biologically active compounds (4, 5), improving the sweetness and lactose crystallization (6), regaining whey (7), in production of fermented and alcohol free drinks, bakery (8), and use of the enzyme in ELISA assays in medicine (9).

β-Galactosidase is commonly found in a wide variety of microorganisms in Nature (10). When compared to other sources, β-galactosidases from bacteria, fungi and yeasts are more commercially important due to their easily controllable production and high yields. Bacteria from the genus *Bacillus*, *e.g. B. licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus subtilis*, have been studied thoroughly due to their potential for the production of a number of industrially important enzymes (11, 12). In particular, *B. licheniformis* has been reported to produce enzymes such as amylase (13-15), β-galactosidase (3), chitinase (16, 17), protease (18-20), glutaminase (21), keratinase (22), laccase (23), β-lactamase (24, 25), lichenase (26), lipase (27), mannanase (28), pectinase (29) and xylanase (30, 31), and for the production of some antibiotics such as bacitracin and lichenin (32). Some strains of *B. licheniformis* grow at elevated temperatures and they produce enzymes, which are active at high temperatures. The high temperatures may be more favourable in a number of industrial processes due to increased reaction velocity, high product yields and solubility of substrates and products, and reduced risk of contamination and product inhibition (33, 34). Furthermore, enzymes from thermophilic and thermotolerant bacteria are generally more stable at moderate temperatures and thermostable β-galactosidases have been used in dairy processes and in glycoconjugate synthesis (35).

In this report, a β-galactosidase producing, thermotolerant *B. licheniformis* was isolated from a hot spring in Batman in Turkey. Four β-galactosidase genes were isolated and one gene was expressed in *E. coli*. The recombinantly produced enzyme was purified and characterized, and showed to be active at elevated temperatures.

Materials and methods

Bacterial strains, plasmids and growth media

The thermotolerant *B. licheniformis* strain KG9 strain was isolated from Taşlıdere hot water spring in Batman in Turkey (38°13′17″N, 41°16′17″E). The physicochemical properties of the water in the hot spring were: Temperature 78 °C, pH 6.7, salts Ca^{2+}>Na^{+}>K^{+} and SO_4^{2-}>Cl^{-}>HCO_3^{-}. *Escherichia coli* TOP10 [F^{-} *mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL*(Str^R) *endA1* λ^{-}] (Invitrogen) was used for gene cloning and expression of recombinant β-galactosidase. Plasmid pUC18dLacZ with deleted α-fragment part of *lacZ* was used for expression of recombinant β-galactosidase as described by Schmidt and Stougaard (36). *B. licheniformis* and *E. coli* were cultured in Luria Bertani or Lysogeny Broth (LB) (37) at 55 °C and 37 °C, respectively. When necessary, LB was supplemented with ampicillin (Ap; 100 µg/ml), 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal; 40 µg/ml), isopropyl-beta-D-thiogalactopyranoside (IPTG; 0.6 mM).

DNA techniques and gene cloning

Nucleotide sequencing was carried out by GATC-Biotech/Germany (http://www.gatc-biotech.com). Analysis of DNA sequences including multiple sequence alignments in Clustal W was in the CLC Workbench v. 4.0 (CLC bio, Denmark) and Blast™ analysis (38) was carried out at www.ncbi.nlm.nih.gov/blast.

Genomic DNA extraction, PCR mediated amplification of the 16S rRNA gene, and purification of the PCR product was carried out as described previously (39). Purified PCR product was sequenced using the CEQ™ DTCS-Quick Start Kit (Beckmann Coulter) as according to the manufacturer's protocol. Sequence reactions were electrophoresed using CEQ™8000 Genetic Analysis System. For construction of the phylogenetic dendrogram operations of the PHYLIP (Phylogeny Inference Package) were used: pairwise evolutionary distances were computed from percent similarities by the correction of Jukes and Cantor and the phylogenetic tree was constructed by the neighbour-joining method based on the evolutionary distances values (40).

Genomic DNA for isolation of β-galactosidase genes was isolated by conventional phenol-chloroform extraction methods (41). The β-galactosidase genes were amplified by PCR. The DNA sequences of the four PCR primer pairs were based on the sequences of putative β-galactosidase genes in the genome sequenced *B. licheniformis* strain DSM 13 (GenBank accession no. NC_006270). Primers for amplification of the *β-gal*I gene were cccggggatccgATGAAGATGAACGGAAAGC (5' primer) and gcatgcctgcagTCATTTATTCTCTGATAACA (3' primer), for the *β-gal*II gene cccggggatccgATGGTTAAACCGTATCCCCCG (5' primer) and gcatgcctgcagCTATGCCTTATGGCTTCTC (3' primer), for the *β-gal*III gene cccggggatccgATGAAGATGAACGGAAAGC (5' primer) and gcatgcctgcagTCATTTATTCTCTGATAACA (3' primer), and for the *β-gal*IV gene cccggggatccgATGCCAAAAATTTATACGAC (5' primer) and gcatgcctgcagCTAATTCTTTTGCTTTTAC (3' primer), *BamH*I and *Pst*I sites are underlined, β-galactosidase encoding sequences in capitals. PCR was performed with the four primer pairs, the corresponding DNA fragments were fractionated by agarose gel electrophoresis, and the purified DNA fragments were digested with the restriction enzymes *BamH*I and *Pst*I prior to ligating into the plasmid pUC-18dLacZ similarly restricted with *BamH*I and *Pst*I. The ligation mixtures were transformed into chemical competent *E. coli* TOP 10 cells (Invitrogen) and transformants were selected on LB agar plates supplemented with ampicillin, X-gal, and IPTG at 37 °C overnight. Next day, blue colonies were selected and sequenced to confirm the DNA sequence. DNA sequencing was carried out by GATC Biotech (gatc.biotech.com). *E. coli* TOP 10 cells carrying the *β-gal*III gene in plasmid pUC-18dLacZ were selected for production of recombinant β-galactosidase.

Expression of B. licheniformis β-galactosidase

E. coli TOP10 cells carrying the pUC18dLacZ plasmid with the *β-gal*III gene were grown in 100 mL LB medium containing 100 µg/mL ampicillin at 37 °C overnight. The cultures were induced by adding IPTG and incubated further at 20 °C for 20 h. Subsequently, the induced cells were precipitated by centrifugation (8,200 g, 30 min, 10 °C) and the pellet was resuspended in 1 mL of 0.1 M phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7.6). The cells were lyzed by bead beating in a FastPrep instrument (Bio101/Savant Instruments, Holbrook, NY) at speed 5.5 for 3 times 25 sec. The samples were cooled on ice in-between beating cycles. The lysates were centrifuged at 10,000 g for 25 min at 4 °C, and supernatants containing the β-galactosidase enzyme were transferred to clean tubes. These crude extracts were used for subsequent analyses together with purified enzyme, cf. below.

β-galactosidase assay

100 µl of enzyme were incubated at 60 °C for 10 min with 50 µl of 60 mM *o*-nitrophenyl-*β* -D-galactopyranoside (*o*NPG, Sigma, in 50 mM sodium phosphate buffer (pH 6.0) . The enzyme reaction was terminated by the addition of 500 µl of 2 M sodium carbonate (Na_2CO_3) and the absorbance was measured at 420 nm. Enzyme activity was expressed as *o*-nitrophenol (*o*NP) units liberated, where one unit (U) is defined as the amount of enzyme that released 1 µmol of *o*NP from *o*NPG per minute under the assay conditions (60 °C, pH 6.0). An extinction coefficient (420 nm, pH 10) for ONP of 4,3834 M^{-1} cm^{-1} was used to calculate the specific activity. The protein content was determined by the Lowry method (42) using bovine serum albumin (BSA) as a standard. Specific activity is the amount of enzyme activity per milligram of protein (micromoles of product formed per minute per milligram of protein, or units per milligram).

Protein purification

Recombinant β-galactosidase was purified by chromatography on diethylaminoethyl-cellulose (DEAE-cellulose) and *p*-aminobenzyl-1-thio-β-D-galactopyranoside agarose (PABTG-agarose).

Step I: Crude extract and ammonium sulphate precipitation:

E. coli TOP10 cells carrying the pUC18dLacZ plasmid with the *β-gal*III gene were grown in 900 mL LB medium containing 100 µg/mL ampicillin at 37 °C over-

night and the cultures were then induced by adding IPTG and incubated at 20 °C for 20 h. Intracellular β-galactosidase was obtained from recombinant *E. coli* by Fast-prep method as described above. The crude extracts were separately precipitated by ammonium sulphate added slowly over period of time on ice with a constant stirring up to a final concentration of 70% (w/v). The centrifuged precipitate (8,200 ×*g*, 20 min, 4 °C) was dissolved in 0.1 M sodium phosphate buffer (pH 6.0), and dialyzed overnight against the same buffer. Dialyzed samples were applied to a stirred ultrafiltration cell (PBGC membrane, Millipore).

Step II: DEAE-cellulose chromatography:

The extracts from the previous step were applied onto a column (1.5 X 30 cm) of DEAE-cellulose previously equilibrated with 0.1 M sodium phosphate buffer (pH 6.0). An elution programme was performed with a linear gradient of 0.1–1.0 M NaCl in the same buffer at a flow rate of 3 mL/min. The enzyme containing fractions were pooled, concentrated by ultrafiltration, and then dialysed overnight against sodium phosphate buffer (pH 6.0).

Step III: Affinity chromatography:

The active pool from DEAE-cellulose chromatography was applied onto a column (PABTG-agarose) equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer until the flow-through was complete. A linear gradient of 0.1–1.0 M NaCl in sodium phosphate buffer was applied at a flow rate of 0.2 mL/min (3 mL fractions). The column was washed with 10 mM and 100 mM sodium borate buffers (pH 6.0) applied at a flow rate of 1 mL/min (1 mL fractions). The fractions were measured at 280 nm for determination of amount of protein and enzyme assays were measured at 420 nm. The active pools, which did not bind to the ligand on the affinity column, were dialysed in sodium phosphate buffer and concentrated by ultrafiltration.

The recombinant enzyme did not bind to the affinity adsorbent on the column as the enzyme was eluted from the column at very low NaCl concentrations. Therefore, a preparative electrophoresis step was included in order to obtain pure enzyme.

Step IV: Preparative electrophoresis

Preparative gel electrophoresis was utilised to obtain pure enzyme. The enzyme solution obtained from affinity chromatography was resolved by native PAGE, and the enzyme band corresponding to β-galactosidase was excised from the gel. The gel slice was manually ground up in 0.1M sodium phosphate (pH 6.0) buffer and then eluted from the gel, as described by Fogel and Sypherd (43) and Galvani et al. (44). The enzyme purity was then checked on both native and SDS-PAGE (see below).

Electrophoretic analysis

The enzymatically active pools from the various steps of purification and the crude preparation were analysed by polyacrylamide gel electrophoresis (PAGE) (45). The PAGE was performed under mild denaturing conditions (0.01% sodium dodecyl sulphate (SDS) using two parallel continuous 7% gels. After electrophoresis, the protein bands were detected either by staining with Coomassie Brilliant Blue (CBB) R-250 or by 6-bromo-2-naphthyl-galactopyranoside (BNG) staining for β-galactosidase activity using the methods described by Gul-Guven et al.(46).

Characterization of recombinant β-galactosidase

The optimum pH of the purified β-galactosidase activity was studied over a pH range of (sodium citrate buffer pH 4.0-6.0; sodium phosphate buffer pH 7.0–9.0; and glycine-NaOH buffer pH 10.0). For the measurement of pH stability, the purified enzyme was incubated at 60°C for 1 h in different buffers (sodium citrate buffer pH 4.0-6.0; sodium phosphate buffer pH 7.0–9.0; and glycine-NaOH buffer pH 10.0).

To investigate the effect of temperature, the purified β-galactosidase activity was tested at different temperatures between 30 and 90 °C for 15 min at pH 6.0. In order to determine enzyme thermostability, the purified enzyme was incubated at different temperatures (60 and 65°C) for different time intervals (15-120 min). The comparisons were made using the unheated crude enzyme activity as 100%. The remaining activity was measured under standard assay conditions.

The substrate specificity of the enzyme was determined by assaying the enzyme with 1.0-10.0 mM *o*NPG substrate in 0.1 mM sodium phospate buffer (pH 6.0) at 60 °C for 10 min. The enzyme activity was measured spectrophotometrically at 420 nm, as described earlier. For lactose hydrolizis, enzyme was incubated with 1.0-200.0 mM lactose substrate in 0.1 mM sodium phospate buffer (pH 6.0) at 60 °C for 10 min. The reaction was terminated by boiling for 15 min, after which the amount of glucose released was measured. The absorbance at 505 nm was converted to glucose concentration, using a standard glucose curve. One unit of enzyme activity is defined as the amount of enzyme needed to produce 1 lmol of glucose per minute under the defined conditions. The K_m and V_{max} values were calculated from a Lineweaver–Burk plot.

Results and Discussion

The *B. licheniformis* KG9 used in this study was isolated from Taşlıdere hot water spring of Batman/Turkey and deposited as DSM 18503 (Leibniz Institute-DSMZ-German Collection of Microorganisms and Cell Cultures; Braunschweig/Germany). The thermo-alkali tolerant strain KG9 was found to grow at temperature of 30-55 °C and pH of 5.0-10.5. The isolate from the spring water was confirmed to be a member of the species *B. licheniformis* by using biochemical, physiological, morphological and 16S rRNA gene sequence analysis (47). Phylogenetic analysis showed that the *B. licheniformis* strain KG9 16S rRNA gene sequence was 99.9% identical to *B. licheniformis* DSM 13 (Fig 1). The genome sequence of strain DSM 13 has previously been determined and revealed a number of genes encoding putative enzymes of biotechnological importance (48). Since *B. licheniformis* strain KG9 displayed a high similarity to *B. licheniformis* strain DSM 13, the genome sequence of strain DSM 13 was used as template for a search for genes encoding putative β-galactosidases. Analysis of the strain DSM 13 genome sequence revealed four potential β-galactosidase genes. The genes were used as templates for synthesis of PCR primers, and the corresponding four β-galactosidase genes in *B. licheniformis*

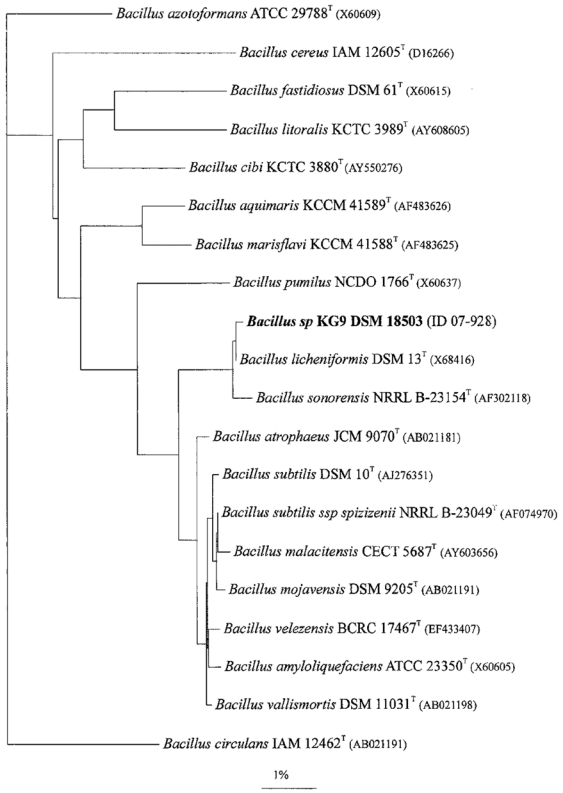

Figure 1. Full 16S rDNA sequence based phylogenetic neighbour joining tree showing the phylogenetic relationship of strain KG9 relative to the type strains of species in the genera *Bacillus* species. Bootstrap values (%) from 1,000 replicates are as shown.

strain KG9 were retrieved by PCR. Analysis of the derived amino acid sequences of the four β-galactosidases from strain KG9 showed a high degree of similarity to other β-galactosidases: All β-galactosidases from *B. licheniformis* strain KG9 showed 100% identity to similar enzymes in the *B. licheniformis* DSM 13 strain, confirming that strains KG9 and DSM 13 are closely related. The putative β-galactosidase β-GalII from strain KG9 was identical to the β-galactosidase GanA (accession no. YP_077685.1), the strain KG9 β-GalIII was identical to the YesZ β-galactosidase (accession no. YP_006712760.1), and the β-GalIV from strain KG9 was identical to a third β-galactosidase from *B. licheniformis* DSM 13 (accession no. YP_081350.1). The *B. licheniformis* DSM 13 β-galactosidase, LacA, identical to the β-GalII from *B. licheniformis* strain KG9, has been characterized previously by Juajun and co-workers (3). However, the β-GalIII in *B. licheniformis* DSM 13 which is identical to that of strain KG9 is not purified and characterised. Therefore, we focused on the isolation, purification and characterization of β-GalIII from *B. licheniformis* strain KG9.

Cloning and expression of a β-galactosidase gene from B. licheniformis KG9

β-galactosidase genes have been cloned in various microorganisms (3, 5, 35, 36, 49-54). Among these, genes that encode intracellular β-galactosidase from *B. licheniformis* strains have been cloned into *E. coli* by

Table 1. Purification steps of recombinant β-galactosidase.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	67.6	30613.2	453	1	100
Ammonium sulphate precipitation and dialysis	11.8	16569.3	1407	3.1	54.1
DEAE	5.2	13187.5	2514	5.6	43.1
Preparative electrophoresis	0.1	3481.9	23895	52.8	11.4

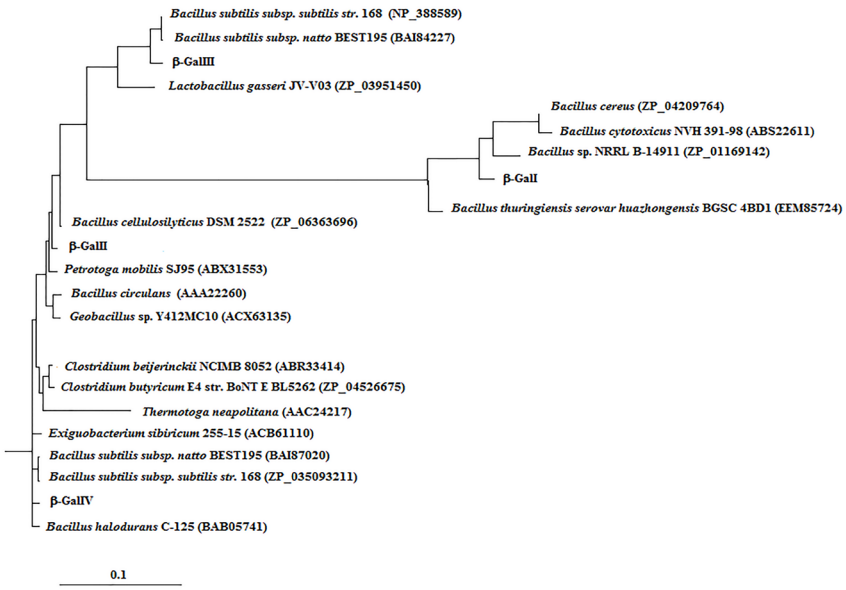

Figure 2. Phylogenetic tree of β-Galactosidases.

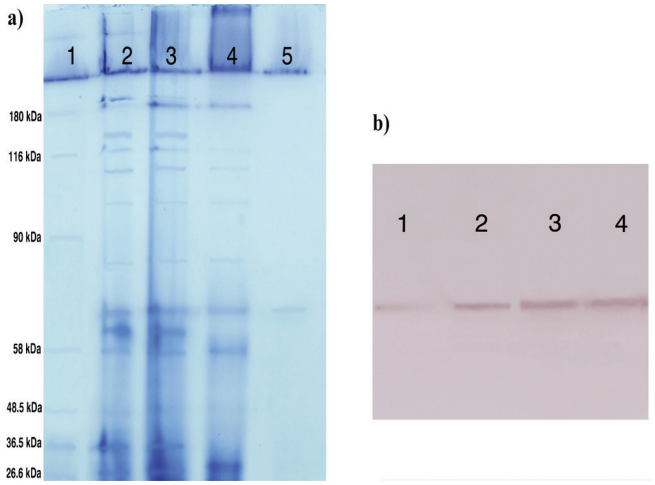

Figure 3. SDS-PAGE CBB-staining **(a)** BNG-staining **(b)** analysis of β-galactosidase from *B. licheniformis* KG9 overexpressed in *E. coli* TOP10. **3a:** Lane 1, molecular mass markers [Sigma SDS7B2: α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), lactoferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephospate isomerase (26.6 kDa)]; lanes 2, 3, 4 and 5 for CBB-staining of crude extract, partially purified recombinant β- galactosidase (ammonium sulphate precipitation/dialysis/ DEAE cellulose and preparative electrophoresis), respectively. **3b:** Lanes 1 and 2, 3 and 4 for BNG-staining of crude extract and partially purified recombinant β- galactosidase (ammonium sulphate precipitation/dialysis/DEAE cellulose and preparative electrophoresis), respectively.

Phan Trân et al. (50) and Juajun et al. (3).

Since the 16S rRNA gene sequence of the thermophilic *B. licheniformis* KG9 was very similar (99.9% identity) to that of *B. licheniformis* DSM 13, we also expected a high degree of sequence similarity between β-galactosidase genes. Therefore, we isolated putative β-galactosidase genes from *B. licheniformis* KG9 using PCR with primers based on the sequence of corresponding β-galactosidase genes from *B. licheniformis* DSM 13. Four different putative β-galactosidase genes (β-*gal*I, β-*gal*II, β-*gal*III, β-*gal*IV) were isolated and inserted into the pUC18dlacZ vector and transformed into *E. coli*.

Four putative β-galactosidase genes from *B. licheniformis* KG9 were isolated and DNA sequence analyses showed 100% identity to the corresponding sequences in *B. licheniformis* KG9. Sequence analysis showed that the β-*gal*I showed 42.9% identity to a β-*gal* from *Bacillus cereus*, the β-*gal*II showed 68.3% identity to a β-*gal* from *Bacillus circulans*, β-*gal*III displayed 68.8% identity to a β-*gal* from *Bacillus subtilis*, and β-*gal*IV showed 76.7% identity to a β-*gal* from *B. subtilis*. (Fig. 2).

Purification of the recombinant β-galactosidase

β-Galactosidase III, produced in *E. coli*, was purified by DEAE-cellulose and PABTG-agarose chromatography methods (Table 1). The results showed that the use of affinity chromatography was not suitable for the purification of the *B. licheniformis* KG9 β-galactosidase as it bound weakly to the column. While β-galactosidase belonging to GH-2 family can easily be purified by using PABTG-agarose affinity chromatography, GH-42 members cannot be purified easily using affinity chromatography. Hidaka et al. (55) hypothesized that the reason for poor binding to PABTG-agarose is the active centre of the enzyme is located in a large pocket in the protein structure. Thus, as the active centre is not on the outer surface and it cannot come in contact with the binding sites in affinity chromatography. The specific activity of the partially purified enzyme was determined and compared to that of the crude extract as shown in Table 1. The β-galactosidase preparation obtained from a large scale purification step had a specific activity of 26,185 U/mg protein at 60 °C using *o*NPG as substrate. The purification steps resulted in 52.8 fold purification and a yield of 11.4%.

The calculated molecular weights of the β-galactosidases I, II, III, and IV using sequencing data were 30, 79, 74, and 79 kDa, respectively. Enzyme fractions from the purification were analysed on SDS-PAGE and the fractions from the last step showed one single band with an apparent molecular mass of ~75 kDa (Fig. 3), which is in agreement with the calculated results from the sequencing data.

Biochemical properties of the purified recombinant β-galactosidase

Effect of pH on the activity of recombinant β-galactosidase

The pH activity profile of recombinant *B. licheniformis* KG9 β-galactosidase III is shown in Figure 4. The optimum pH was found to be 6.0. Previous studies have shown similar optimal pH value of 6.0 for LacBI (accession number: U89996) from *B. licheniformis* (50), LacZ from *T. maritima* (35, 56) and BGalB (accession number: AAD35398) (35). Yuan et al. (53) have reported that β-galactosidase can be used in dairy products (pH 5.5-7.0) such as whole-fat milk (pH 5.8-

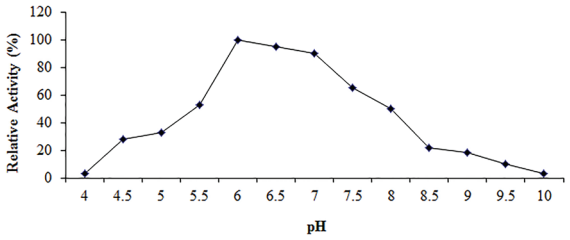

Figure 4. Effect of pH on activity of recombinant β-galactosidase. Optimum pH was determined under standard assay conditions using *o*NPG (pH 4.0–10.0). Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

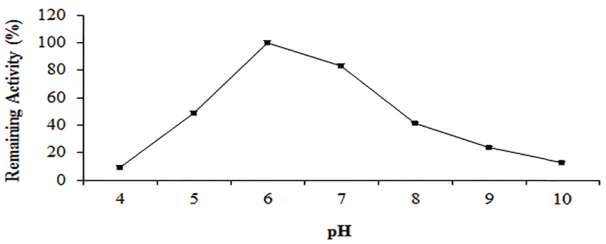

Figure 5. pH stability of recombinant β-galactosidase. The purified enzyme was incubated at 60 °C for 1 h in different buffers (sodium citrate buffer pH 4.0-6.0; sodium phosphate buffer pH 7.0–9.0; and glycine-NaOH buffer pH 10.0). The remaining proteolytic activity was measured under standard assay conditions.

6.0) or whey (pH 6.0) at neutral lactose hydrolysis. The enzyme activity remained about 70% when the enzyme was incubated between pH 6.0 and 10.0 at 60 °C for 1 h (Fig.5).

Effect of temperature on the activity of recombinant β-galactosidase

The recombinant *B. licheniformis* KG9 β-galactosidase III activity increased with temperature up to 60 °C, after which the enzyme activity decreased (Fig. 6). The enzyme is highly stable at 60 °C for 120 min (Fig. 7). The optimum temperature for the thermophilic recombinant *E. coli* β-galactosidase is comparable to those described for β-galactosidases of other thermophilic microorganisms. Di Laura et al. (57) have reported that the optimum temperature of purified β-galactosidase activity was also found to be 60 °C for the LacB from *A. acidocaldarius*.

Substrate specificity and kinetic parameters

K_m and V_{max} values of the enzyme depending on *o*NPG and lactose concentrations were calculated as 1.346 mM and 1.964 µmol/min (Fig. 8a) and 13.3 mM and 1.55 µmol/min, respectively according to the Lineweaver-Burk plot (Fig. 8b). The K_m value calculated from Linewever–Burk plot shows that the purified β-galactosidase has a strong affinity for *o*NPG compared to other members of GH 42 family; K_m value 6.34 mM for the thermostable β-galactosidase in *Bacillus* sp. MTCC 3088 (58) and K_m value 2.6 mM for β-galactosidase in *Bifidobacterium infantis* (6). Generally, the lactose hydrolysis of β-galactosidases which are classified as GH-42 family are weak and they prefer hydrolysing substrates

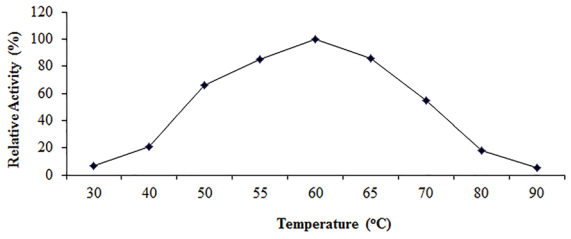

Figure 6. Effect of temperature on activity of recombinant β-galactosidase. Optimum temperature was determined under standard assay conditions using *o*NPG at temperatures ranging from 30 to 90 °C. Results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

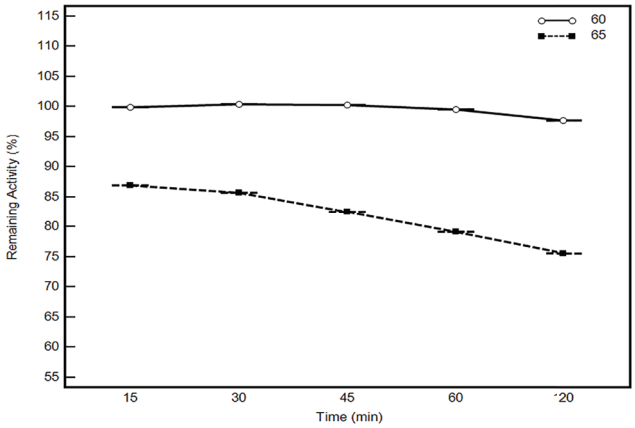

Figure 7. Effect of temperature on stability of recombinant β-galactosidase. The purified enzyme was incubated at different temperatures (60 and 65 °C) for different time intervals (15-120 min). The comparisons were made using the unheated crude enzyme activity as 100%. The remaining activity was measured under standard assay conditions.

such as *p*NPGal or *o*NPGal (35, 59).

Conclusion

In this study, a thermophilic and alkalitolerant *Bacillus licheniformis* KG9 isolated from a hot water spring was found to produce a high amount of thermostable β-galactosidase.

Four β-galactosidase genes with high similarity to β-galactosidase genes from *B. licheniformis* DSM 13 were isolated. One of the genes, *β-gal*III, was expressed in *E. coli* and recombinant enzyme was purified and characterized. The recombinant β-galactosidase displayed optimal activity at pH6 and at 60 °C and analysis of substrate specificity showed that the enzyme showed activity with lactose as substrate. Thus, the β-galactosidase from *B. licheniformis* KG9 may have an application potential within dairy processes.

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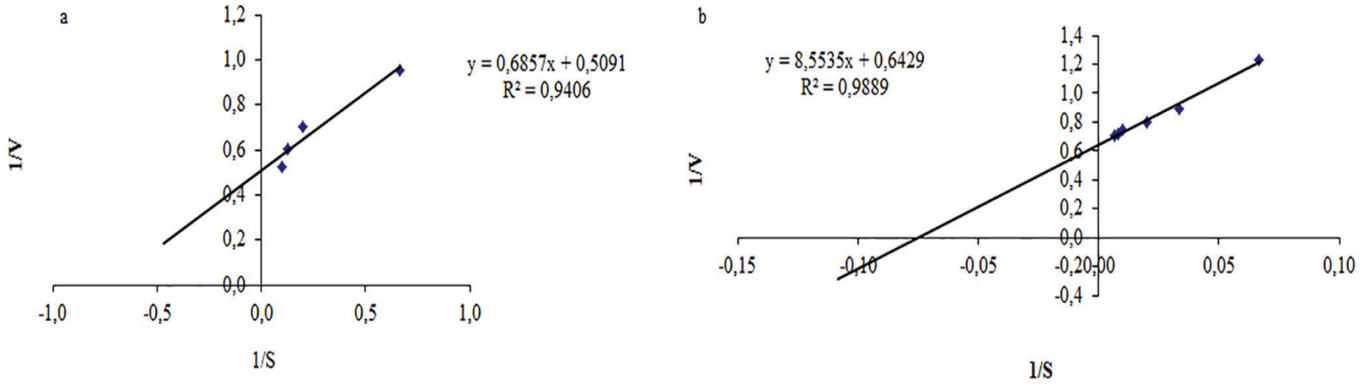

Figure 8. K_m and V_{max} values of the recombinant enzyme depending on *o*NPG **(a)** and lactose **(b)** concentrations (Linewever-Burk plot analysis).

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