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β -Galactosidase from *Lactobacillus brevis* PLA28: Purification, Characterization and Synthesis of Galacto-oligosaccharides

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Abstract

Objective: Purification and characterization of β-galactosidase from *Lactobacillus brevis* PLA28 were carried out and transgalactosylation activity was also studied for the synthesis of galacto-oligosaccharides (GOS).

Methods: β-Galactosidase was purified by using ammonium sulphate precipitation method and hydrophobic interaction chromatography. Reaction conditions were optimized for the assay of this β-galactosidase. GOS synthesis was carried out under the optimized reaction conditions in batch mode and the product formed was detected by thin layered chromatography (TLC).

Results: β-Galactosidase was purified to 6.6 fold with a yield of 6% and specific activity of 4 U/mg protein. Molecular weight of the purified β-galactosidase was found to be 45 and 60 kDa on SDS PAGE and 105 kDa on native PAGE. The temperature and pH optima of purified enzyme were 30°C and pH 6.5 respectively. The enzyme was found to be stable at 30°C for 6 h. V_{max} and K_M of the purified β-galactosidase were calculated to be 6.6 U/mg protein and 8.33 mM respectively. GOS synthesis was observed at pH 6.5, 30°C in 6-8 h of incubation by purified enzyme.

Conclusion: *Lactobacillus brevis* PLA28 β-galactosidase has exhibited capability for transgalactosylation reaction with lactose conversion to synthesize GOS.

Keywords: β-Galactosidase; *Lactobacillus brevis* PLA28; Galacto-oligosaccharide

Introduction

Lactic acid bacteria (LAB) constitute a diverse group of *lactococci*, *streptococci* and *lactobacilli*. Enzymes obtained from these bacteria (LAB) are extensively studied for their use in food based industries. Lactic acid bacteria are found widely in nature and predominates the habitat that is rich in carbohydrates, protein breakdown products and vitamins. The utilization of the milk sugar lactose is a primary function of *lactobacilli* and other LAB [1]. Moreover, they are commonly associated with food fermentation and play a significant role in enhance nutritional quality and digestibility of food.

β-Galactosidase derived from lactic acid bacteria are studied extensively because of safety aspects and application in food industries. It catalyzes the hydrolysis of β-1,4-D-galactosidic linkages and has been used in dairy industry as an important biocatalyst [2]. The sources of this enzyme are plants, bacteria, animal organs, yeasts and moulds. Bacteria are preferred sources of β-galactosidase due to ease in their handling, stability and high enzyme activity [3]. This enzyme is used for the conversion of cheese whey, a waste from dairy industry into different valuable products [4]. It catalyzes hydrolysis of milk sugar lactose and structurally related galactosides and transgalactosylation reactions in which lactose serve as galactosyl acceptors, yielding a series of di, tri-, and tetrasaccharides called galacto-oligosaccharides (GOS) [5]. Galacto-oligosaccharides along with the fructo-oligosaccharides are best studied prebiotics oligosaccharides that beneficially affect the host by selectively stimulating the growth and/or activity of probiotic bacteria in the colon.

The stability of GOS at high temperature and acidic conditions make them important in food and drink industries especially in confectionaries, acidic beverages and fermented milk products. Using β -galactosidases from isolates originated from fermented foods for the synthesis of prebiotic GOS is an interesting approach for the production of new carbohydrate-based functional food ingredients. The objective of present work was to purify β-galactosidase from *L. brevis* PLA28 and to study its transgalactosylation activity for the synthesis of GOS.

Materials and methods

Chemicals

All chemicals used in present study were purchased from Alfa Aesar, Johnson Matthey Company and Sigma (India). Man de, Rogosa and Sharpe (MRS) medium, Elliker HiVeg broth, and other media components were purchased from Himedia, Mumbai (India).

Microorganism and culture conditions

The LAB isolate *L. brevis* PLA28 was obtained from Research Lab II of Himachal Pradesh University (Shimla, India). It was isolated from *chhang*, a barley based traditional alcoholic beverage of Ladakh, India

and identified by sequence analysis of the amplified chromosomal 16S rDNA. GenBank accession number for the 16S rRNA gene sequence of *L. brevis* PLA28 is KJ722776. The bacterial culture was grown in Elliker HiVeg broth (pH 6.8) at 30°C for 36 h in an incubator shaker. The cells from the culture were harvested by centrifugation at 10,000 g for 10 min at 4°C and washed twice with 50 mM sodium phosphate buffer (pH 7.0). After two washings with the same buffer, the cells were finally suspended in buffer and stored at 4°C and these cells were termed as resting cells.

β-Galactosidase assay

β-Galactosidase activity was estimated according to the method described by [6]. Specific activity of β-galactosidase was expressed as micromoles of oNP (o-nitrophenol) released per milligram of protein per min under the assay conditions. Protein concentration in the sample was determined by the method of [7], using bovine serum albumin as standard.

Purification of β- galactosidase

L. brevis PLA28 was cultured in 2 L Elliker broth at 30°C for 36 h and resting cells were prepared as described above. Cells of *L. brevis* PLA28 (450 mg dry cell weight) were suspended in sodium phosphate buffer (50 mM, pH 7) containing 1 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethanesulfonyl fluoride), 1 mM EDTA and disrupted by six successive disruption cycles with 0.5 mm glass beads using Bead Beater™ (BioSpec Products, Inc.) on ice and the cell debris was removed by centrifugation at 10,000 g for 20 min at 4°C. Supernatant was collected and termed as cell free extract (cell lysate) which was stored at 4°C for further purification steps.

Ammonium sulphate precipitation

The crude extract of *L. brevis* PLA28 was precipitated at 4°C using ammonium sulphate saturation (30-40%). The resulting precipitates were suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA and 1 mM PMSF and dialyzed overnight against the same buffer at 4°C. The ammonium sulfate fractionate (ASF) having β-galactosidase activity was further used for purification.

Hydrophobic interaction chromatography

The dialyzed enzyme preparation was applied to phenyl sepharose column (15 cm x 1.5 cm) and pre-equilibrated with 1 M NaCl/KCl and sodium phosphate buffer (pH 7, 50 mM). Protein was eluted with a linear gradient of NaCl (0.1 M-1.0 M in 50 mM buffer, pH 7 containing 1 mM DTT and 1 mM EDTA) at a flow rate of 0.5 ml/min and fractions of 2 ml were collected.

Determination of molecular weight of β-galactosidase of *L. brevis* PLA28

Molecular weight of the purified β-galactosidase was determined by SDS (sodium dodecyl gel electrophoresis) and Native-PAGE.

Characterization of purified enzyme

The activity of purified β-galactosidase assayed in various buffer systems, i.e., citrate buffer (pH 3-6), borate buffer (pH 5-7), potassium phosphate buffer (pH 6-8), tris-HCl buffer (pH 6-9), sodium carbonate buffer (pH 9-10), sodium phosphate buffer (pH 6.5-7.5) and borax buffer (pH 9-10) of 0.1 M. The effect of buffer molarity (0.025-0.2 M), substrate concentration (0.002-0.06 M), temperature (25-50°C) and incubation time (5-30 min) on the activity of the purified β-galactosidase were explored. Thermal stability of the β-galactosidase was also studied at temperatures 30°C, 35°C, 45°C, and 55°C for 6 h.

Synthesis of galacto-oligosaccharides using β-galactosidase of *L. brevis* PLA28

Synthesis of galacto-oligosaccharides (GOS) was carried out in discontinuous mode at 30°C using purified β-galactosidase from *L. brevis* PLA28 according to the method described by Iqbal et al. [8] .The synthesized product formed was analyzed by thin layer chromatography according to the procedure of Iqbal et al. [8].

Results

Purification

The purification of β-galactosidase from *L. brevis* PLA28 was achieved in two steps summarized in Table 1. This enzyme was purified by applying ammonium sulphate precipitation method and hydrophobic interaction chromatography. It was purified to 6.6 fold with a yield of 6% (Table 1). Specific activity of the purified enzyme was found to be 4.0 U/mg protein. The purified β-galactosidase of *L. brevis* PLA28 consists of two distinct protein bands, which confirmed the dimeric nature of this enzyme. SDS-PAGE analysis of the β-galactosidase showed protein bands of 45 and 60 kDa while in native PAGE a single band of 105 kDa was observed (Figures 1 and 2).

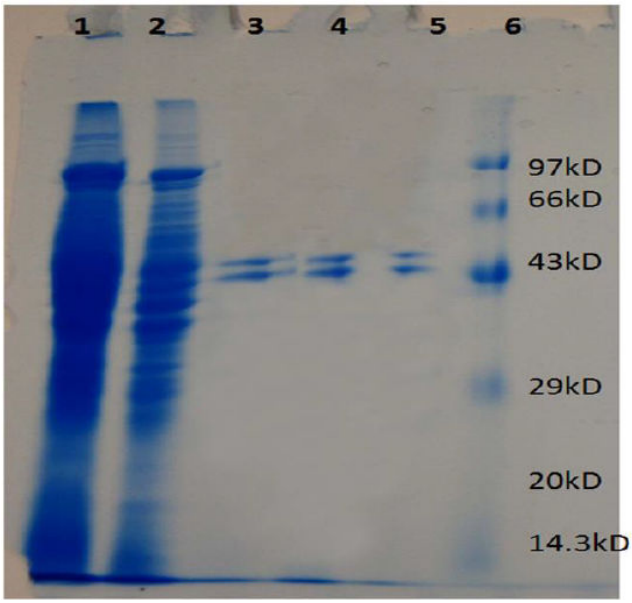

Figure 1: SDS-PAGE of purified β-galactosidase from *L. brevis* LA-63. lane 1: crude, lane 2: dialyzed sample, lane 3, 4, 5: purified samples, lane 6: marker.

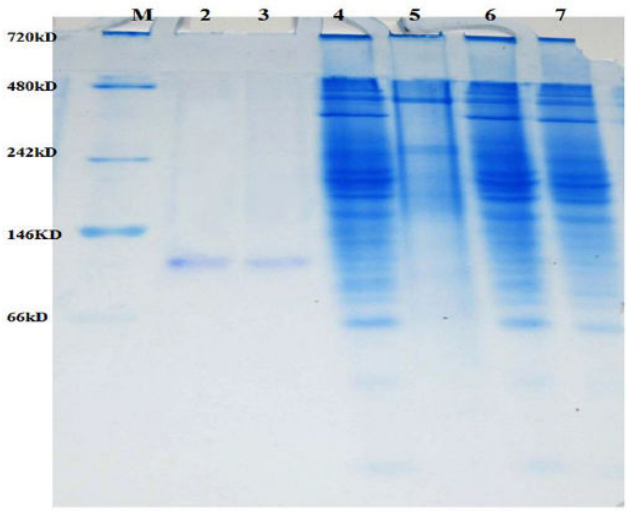

Figure 2: Native-PAGE analysis of purified enzyme on 8% acrylamide gel, lane 1: Marker, lane 2 and 3: purified fractions lane 4 and 5: dialyzed sample, lane 6, 7: crude.

Purification steps	Vol. (ml)	Protein(mg/ml)	Total protein (mg)	Specific activity (U/mg protein)	Total activity(U)	Yield (%)	Fold purification
Cell free extract	20	2	40	0.6	24	100	1
Ammonium sulphate precipitation	4	0.9	3.6	1.4	5.04	21	2.3
Hydrophobic interaction chromatography	4	0.1	0 .4	4	1.6	6	6.6

Table 1: Purification summary of β-galactosidase of *L. brevis* PLA-28.

Characterization of purified enzyme

Buffer pH and molarity

Temperature

Optimum temperatures for the hydrolysis of ONPG was found to be 30°C with specific activity of 4.3 U/mg protein, although this enzyme was quite active at 25-50°C (Figure 3).

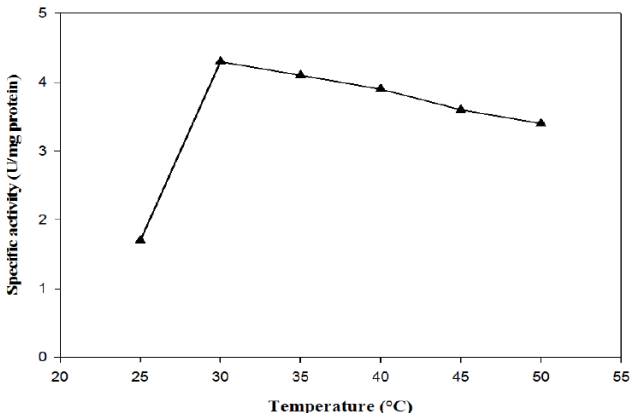

Figure 3: Effect of temperature on β-galactosidase of *L. brevis* PLA28.

Incubation time

To optimize the reaction time for assay of β-galactosidase, the reaction mixture was incubated at 30°C for 5-30 min. The highest specific enzyme activity (4.3 U/mg protein) was observed at 10 min of incubation.

Substrate concentration

The effect of substrate concentration on β-galactosidase activity revealed that the enzyme exhibited highest activity (4.6 U/mg protein) at 10 mM substrate concentration in the reaction. The activity of β-galactosidase remained constant when substrate concentration was increased above 10 mM (Figure 4).

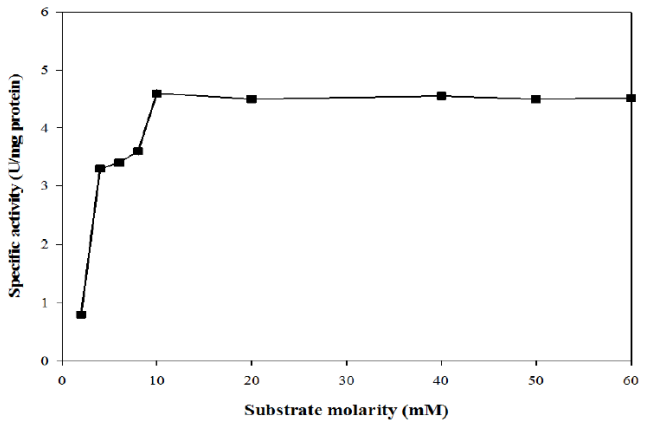

Figure 4: Effect of substrate concentration on β-galactosidase of *L. brevis* PLA28.

Thermal stability

Thermostability profile of β-galactosidase revealed no loss in catalytic activity of enzyme at 30°C and 35°C till 6 h whereas at 45°C the half life of enzyme was found to be 1.31 h while half life of the enzyme was recorded to be 30 min at 55°C.

Kinetic constants

The analysis of Lineweaver-Burk plot showed that β-galactosidase had K_M of 8.33 mM and V_{max} of 6.6 U/mg protein.

Synthesis of galacto-oligosaccharides using β-galactosidase of *L. brevis* PLA28

In order to assess the potential of β-galactosidase from *L. brevis* PLA28 for the synthesis of galaco-oligosaccharides (GOS), a discontinuous lactose conversion process was studied at 30°C, using an initial lactose concentration (50-200 mg/ml), enzyme (0.5-5.0 U/ml), and pH 6.5 at 30°C. Samples collected at regular intervals of time and analyzed by thin layer chromatography (TLC). The TLC chromatograms were developed for standards and the samples. The galacto-oligosaccharides (GOS) synthesis was recorded at 4-8 h of incubation. The TLC chromatogram demonstrated the transgalactosylation capability of β-galactosidase, as the group of sugars appeared at the position corresponding to oligosaccharides. The galacto-oligosaccharides (GOS) synthesis was observed with substrate of 200 mg/ml and enzyme of 5 U/ml at pH 6.5 at 30°C in 6 to 8th h of incubation.

Discussion

The purpose of the present study was to purify, characterize and explore the potential of β-galactosidase from *L. brevis* PLA28 for the synthesis of GOS. It is interesting to study and characterize enzymes from microorganisms which are isolated from traditional fermented food products as use of such enzymes does not involve risk and are safe from health view point. Thus, Lactobacillus β-galactosidase originated from food products is safe for utilization in food industry as LAB are considered as GRAS (Generally regarded as safe). Vasiljevic and Jelen [9], reported that the enzyme derived from GRAS microorganisms e.g. LAB can be used without extensive purification. On the other hand, *Lactobacilli* are gaining a significant interest for its probiotic properties such as an improvement in lactose digestion and microbial balance in gastrointestinal tract which directly contribute benefit to host health [10,11].

β-Galactosidase from *L. brevis* PLA 28 is a heterodimer consisting of two subunits of approximately 45 and 30 kDa. In accordance with our result, majority of *Lactobacilli* β-galactosidases exist as hetrodimer (72 kDa and 35 kDa) that have been reported in *L. brevis* KB290 [12], Lactobacillus acidophilus R22 [6], Lactobacillus reuteri [13]. However, β-galactosidase from L. salivarius was reported to be a monomer of 30 kDa Bae while this enzyme exists as homodimer of 220 kDa [14] homotetramer of 257 kDa [15], in L. delbrueckii subsp. bulgaricus and L. helveticus respectively.

Hung and Lee [16], reported that the optimum pH for β-galactosidase enzyme from microbial sources range around pH 3.0-7.2. In this study, optimum pH was determined to be 6.5 pH while activity of this enzyme decreased gradually at pH values higher or lower than the optimum pH value. These results agree with the observation reported by Iqbal et al. [8], that the pH optimum of β- galactosidase from *Lactobacillus sakei* Lb790 was pH 6.5.

Optimum temperature for the hydrolysis of oNPG was found to be 30°C, although this enzyme was active at 25-50°C. The loss of activity of the enzyme at higher temperatures could be attributed to its unfolding and subsequent loss of active site [17]. Similar findings were reported by Ku and Hang [18-20]. The substrate optimum for *L. brevis* PLA 28 β-galactosidase was 10 mM. It was observed that the β-galactosidase activity remained constant when substrate concentration was increased above 10 mM, hence suggesting that this enzyme is not prone to inhibition by higher concentration of substrate [21], reported that β-galactosidase of Streptococcus thermophilus exhibited maximum activity of 24 mM (oNPG) as substrate. Kinetic constants KM and Vmax of purified β-galactosidases for oNPG was calculated to be 8.33 mM and 6.6 U/mg protein respectively. KM and V_{max} values have been reported in *Bacillus sp.* as (6.34 mM and 9351 IU ml-1), *Penicillium chrysogenum* (1.81 mM and 40 nkat mg^{-1}), *Thalassospira sp.* (1.2 mM and 1,645.66 U/ml) and *Lactobacillus pentosus* (1.67 mM and 304 μmol min^{-1} mg^{-1})[22-24,20].

Lactose utilization is an important biochemical characteristic of *lactobacilli* and other LAB (lactic acid bacteria) used in the fermentation of milk. β-Galactosidases play a crucial role in the metabolism of lactose in *lactobacilli* however, little attention has been paid to study its transgalactosylation activity for synthesis of GOS. GOS find their important application in the formulations of functional food because of their proven health benefits [3,25-29]. β-galactosidase

of *L. brevis* PLA28 catalyzed transgalactosylation reaction as reported earlier in case of β-galactosidase from Lactobacillus pentosus, Lactobacillus plantarum WCFS1, *L. sakei* Lb790[1,8], respectively.

Conclusions

The transgalactosylation activity of purified β-galactosidase from *L. brevis* PLA28 has potential application in GOS synthesis. Since *L. brevis* PLA28 has been isolated from fermented food, therefore its β-galactosidase has ample scope for synthesis of products in food and pharmaceutical industry.

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