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Biochemical characterization, partial purification, and production of an intracellular beta-galactosidase from *Streptococcus thermophilus* grown in whey

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ABSTRACT

Beta-galactosidase is one of the important commercial enzymes having several applications in food and pharmaceutical industry. In dairy industry, β-galactosidase has been used to prevent crystallization of lactose, to improve sweetness, to increase the solubility of the milk product. Moreover, it has been used to produce low lactose containing food products for low lactose tolerance people and for the utilization of whey, which would otherwise be an environmental pollutant. Based on its importance, the present research was aimed to isolate and purify β-galactosidase from Streptococcus thermophilus by fermentation process. The enzyme was purified by ammonium sulphate precipitation, dialysis, gel filtration chromatography using Sephadex G-100, and SDS-PAGE and some properties of the purified enzyme like pH, temperature optima and kinetic parameters were determined. Isolate A5 showed highest productivity of 7.76 U/ml with a protein content of 67 µg/mL, pH and temperature optima at pH 7.2 and 40°C. The apparent V_{max} and K_m values were found to be 2.8 IU/mL and 3.05 mM, respectively. Specific activity and fold purification of beta-galactosidase was found to be 119.38 & 1.13, respectively. These characteristics of isolated β-galactosidase showed that it could be a promising candidate for various industrial as well as biotechnological applications.

Keywords: Beta-galactosidase, fermentation, *Streptococcus thermophilus*, Whey.

ABBREVIATIONS:
SDS-PAGE = Sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis
ONPG = O-Nitrophenyl-Beta-D-Galactopyranosidase
Km = substrate concentration
V_{max} = maximal velocity
ONP = Ortho-nitrophenol
ST agar = Streptococcus thermophilus agar

INTRODUCTION

Many of the bio products of the biotechnology industry are proteins which are of interest because of their enzymatic activities, specific recognition, inter-actions or other therapeutic actions[6]. β-Galactosidases (EC 3.3.1.23) which is commonly known as lactase was found to be widely distributed in animals, plants and numerous microorganisms i.e., bacteria, fungi, archaea and yeast [40].

β-Galactosidases are well known biocatalyst to catalyze hydrolytic and transgalactosylation reactions. The hydrolytic activity contributed its application in the food industry for reducing the lactose content in milk almost for decades[27], while the transgalactosylation activity (i.e. which can oligomerise galactosides) has been used to synthesize di, tri, or higher galacto-oligosaccharides (GOS) [41]. GOS also has wide spread application as a prebiotic food ingredient for a variety of foods because of its ability to promote the growth and establishment of bifidobacteria in the human intestine and to inhibit potentially harmful gut bacteria such as clostridia and Bacteriodes species [10,29].

It was estimated that over 70% of the world's human adult population reported to have lactose intolerance or lactose malabsorption as a major health problem [15], resulting from absent or reduced β-galactosidase activity in the small intestine and results in higher concentrations of indigested lactose. Fermentation of indigested lactose by colonic microflora in the large intestine causes abdominal pain, gas, nausea and diarrhoea[1]. The extent of these symptoms is variable and indeed most individuals can tolerate a moderate amount of lactose in their diet [24].

Besides milk, the main source of lactose is cheese whey, which is a primary by-product of cheese manufacturing. About 9 L of whey stream are generated during the production of 1 kg of cheese, amounting to over 160 million tons of whey produced worldwide each year. Whey's organic load is high (biochemical oxygen demand of 30–50 g/L and chemical oxygen demand of 60–80 g/L), mainly because of the lactose content, which together with the high volumes to which it is generated makes cheese whey a quite concerning environmental issue, and solutions for its valorization are strongly required[13]. In this regard, lactose hydrolysis by β-galactosidases again plays an important part by broadening whey's applications. This, however, creates a potential market for the application of β-galactosidase.

Amongst the β-galactosidases thus far studied, the *Escherichia coli* enzyme has been well explored essentially due to its use as a molecular genetics tool, following the discovery of the lactose operon [11]. But its industrial use is hampered by the fact that it is not considered safe for food applications. Nevertheless; it is commercially available for analytical purposes [32]. Therefore, selection of microorganisms which are safe for human use and are capable of producing high levels of β-galactosidase becomes an urgent and attractive task.

In this regard, Lactic acid bacteria (include a diverse group of lactococci, streptococci and lactobacilli) and bifidobacterium, which are generally recognized as safe (GRAS) organisms, have been regarded as good sources of β-galactosidases, especially for functional food applications[15]. Among lactic acid bacteria, yogurt bacteria (*Lactobacillus delbrueckii ssp. Bulgaricus* and *Streptococcus thermophilus*) are the highest β-galactosidase producers [39]. Yoghurt, a major food product with an increasing consumption rate due to its health promoting properties, is produced by the action of yoghurt bacteria and their metabolites [22]. *Streptococcus thermophilus* was of major importance for the food industry since it was massively used for the manufacture of dairy products and it was considered as the second most important industrial dairy starter after *Lactococcus lactis*. Nevertheless, over 10^{21} live cells *of Streptococcus thermophilus* ingested annually by the human population [8,38].

The objective of this study was to isolate β-galactosidase from *S. thermophilus* isolates and also to investigate fermentation factors which affect enzyme production by submerged fermentations in shake flasks using Acid whey as a media, as well as partial purification, and characterization of the crude enzyme extract. In addition, this work also aimed to propose a simple and economical system for β-galactosidase production by *S. thermophilus*.

MATERIALS AND METHODS

Enzyme source:
A total of 3 commercial brands of yoghurt used in the present study were purchased from different places of India like Pondicherry, Erode, Coimbatore, and Chennai. Nine samples from each brand of yoghurt used in the present study were named as follows, I brand - A1, A2, A3, A4, A5, A6, A7, A8, A9, II brand - N1, N2, N3, N4, N5, N6, N7, N8, N9, and III brand NE1, NE2, NE3, NE4, NE5, NE6, NE7, NE8, NE9 and stored at refrigerated temperature until used for studies.

Chemicals:
Acryl amide, ammonium per sulphate, Bisacrylamide, Mercaptoethanol, Bovine serum albumin, Bromophenol blue, Bromocresol purple, Coomassie Brilliant Blue G-250, MRS medium, and Mannitol were purchased from HI-

MEDIA (Mumbai, India). Sephadex G-100 and β-Galactosidase enzyme were obtained from Sigma-Aldrich, Germany. O-Nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma-Aldrich, Germany. *Streptococcus thermophilus* NCIM NO: 2412 strain was purchased from NCIM, Pune. Acid whey was obtained from MMD Dairy Institute, Tamilnadu, India. All other chemicals used for the experiments and analyzes were of analytical grade.

Isolation of *Streptococcus* Strains from yoghurt samples:
The media used for isolation of *S. thermophilus* was HYA agar which consists of following ingredients, Agar 15 g/L; Peptone 10 g/L; Beef extract 1 g/L; Lactose solution 10 ml; Galactose solution 10 ml and Glucose solution 10 ml were added to make total volume of 1L [4,28].

For the isolation two sets of Sterile HYA agar plates were used. The first sets of HYA agar plates were streaked with loopful of yoghurt sample directly from the container. Similarly another set of HYA agar plates were streaked with loopful of yoghurt diluted with saline. All the plates were incubated at 37°C for 24 hours. After the incubation, all the plates were observed for the formation of very small and white colonies. Before the experiment, the small white colonies obtained were activated by successive transfer in MRS broth and stored at 4-8°C and used for further studies.

Morphological and Biochemical characterization of isolated strains:
Morphological identification of isolated colonies was carried out by simple staining, Gram's staining, and motility testing by hanging drop method [37]. Biochemically the isolated strains were characterized by the following tests like catalase test, Fermentation tests for different sugars like lactose, glucose and mannitol were done by using lactose broth, glucose broth and mannitol broth respectively [16]. Identification tests of isolates were done using ST agar plates at 37°C for 24 hours[9]. After the incubation, the inoculated plates were observed for the presence of well developed yellow colonies which further confirms the isolated strains as *Streptococcus thermophilus*. Furthermore all the morphological characteristics of isolated strains were compared with standard strain of *Streptococcus thermophilus* obtained from NCBI, Pune.

Crude Enzyme Production using acid whey:
The isolated strains A5 from yoghurt which was characterized as *Streptococcus thermophilus* and also showed high yield used as inoculum for β-Galactosidase production using acid whey as media [3,26,34]. Acid whey was obtained from MMD Dairy Institute, Erode, Tamilnadu, India was used as media and it was deproteinized by heating at 90°C (pH 4.5) for 10 minutes. Then it was filtered through Whatman No: 1 filter paper to remove coagulated protein and adjusted to pH 7.0. It was then sterilized at 121°C for 15 minutes. The sterile whey was inoculated with 1% active culture of *Streptococcus thermophilus* strain A5 and incubated at 40°C for 24 hours.

At the end of incubation, cells were harvested by centrifugation at 15,000 rpm for 20 minutes. The pellets were washed twice with 0.1 M phosphate buffer (pH 7.0) solution and stored in 0.1 M phosphate buffer. Since β-galactosidase from *S. thermophilus* is an intracellular enzyme, sterilized sand were added to this cell suspension and triturated for an hour and was centrifuged. The supernatant represented the crude enzyme.

Protein Determination:
Protein content was determined according to the method of Lowry *et al* [25] using bovine serum albumin (BSA) as standard. The samples were read at 600 nm using a double beam UV – visible spectrophotometer (model SL 164, Elico, Hyderabad, India). The sample analyzes were performed against respective blank solutions. Protein concentration readings were taken in triplicate and an average value was used for the calculation.

Determination of β-Galactosidase Activity:
Preparation of ONP standard curve: ONP was an important compound as an indicator for β-Galactosidase activity. ONP (0.033384 g) was mixed with 1 ml 0.05 M phosphate buffer (at pH 7) and 3 ml ethanol. Final volume of the mixture was set to 4 ml [18]. This mixture (4 ml) was agitated until most of the solid particles were become soluble. This mixture was transferred into preheated water bath at 45 °C to solubilise ONP completely. This mixture was taken as ONP (60 mM) stock solution. From the stock solutions, several dilutions (0.006mM - 6mM) were prepared out by 0.05 M sodium phosphate buffer at pH 7. Each dilution from the stock solution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

Blank solution was prepared by mixing 3 ml alcohol and 1ml sodium phosphate buffer (0.05 M at pH 7). Absorption values were read against the blank solutions in UV spectrophotometer at 420 nm. A graph was plotted between concentration of ONP and absorbance values.

β–Galactosidase Assay:
β-Galactosidase activity was determined by release of O-nitrophenol from a 2.0 mM O-nitrophenyl-β-D-galactopyranoside (ONPG) in 0.01% (w/v) Bovine Serum Albumin (pH 6.0 at 37°C) as substrate solution [5,23,30]. To 0.5 ml of this solution, 0.3 ml of deionised water was mixed by inversion and equilibrated to 37°C. Then the reaction was initiated by the addition of 0.2 ml of enzyme solution and incubated at 37°C for 10 minutes. After 10min of incubation, the reaction was stopped using 4ml of 1M sodium carbonate solution. A blank containing 1ml of substrate solution was used to correct the thermal hydrolysis of ONPG. Absorbance was noted at wavelength of 420 nm.

One unit of enzyme activity (EU) will hydrolyze 1.0 micromole of o-nitrophenyl Beta-D-Galactopyranoside (ONPG) into o-nitrophenol and Beta-D-Galactose per minute at pH 6.0 at 37°C. Specific activity is defined as β-galactosidase units/mg of protein determined as by Lowry et al. 1951.

Partial Purification of Enzyme:
Partial Purification of β-Galactosidase was carried out by using the following methods, all steps involved were conducted at room temperature except when specified.

Ammonium sulphate precipitation:
The crude extract with β-galactosidase activity was precipitated by ammonium sulfate added slowly over period of time on ice with a constant stirring up to a final concentration of 70% (w/v) [2,30]. The centrifuged precipitate (8,000 rpm, 20min, 4°C) was redissolved in a small volume of 0.1 M phosphate buffer and used for further purification.

Dialysis:
Precipitate obtained 70% saturation of ammonium sulphate was dialyzed against 0.1 M phosphate buffer (pH 7) solution overnight [2,30]. Dialysis tubes, which were previously soaked in 1M Tris HCl buffer, were used for the dialysis of the precipitate. The precipitate was dissolved in 1M Tris HCl buffer and dialysed against the same buffer.

Gel filtration:
The dialyzed enzyme solution was applied to a column (40 cm×1.5 cm) of Sephadex G 100 previously equilibrated with 0.1M phosphate buffer (pH 7). The samples were eluted with the same buffer at a flow rate of 3ml/hr and a total of 30 fractions were collected for determination of enzyme activity and protein content [2,30]. The fractions containing β-galactosidase activity were pooled and used for further studies.

SDS-PAGE:
The purity of enzymatically active pools from the various steps of purification and the crude preparation were analyzed by SDS-PAGE system using resolving gel (15%) and stacking gel (5%) [30,35]. After electrophoretic run, the proteins were stained by Coomassie brilliant blue and destained using methanol–water containing 10% acetic acid. Finally, the gel was observed in white illuminator for bands. A purple band on gel indicated the presence of enzyme. The molecular mass was estimated by comparing its mobility with those of the standard proteins.

Characterization of Partially Purified β-Galactosidase:
Effect of pH:
To determine the optimum pH, the β-galactosidase activity was studied within the range of pH from 4 to 8 [2]. Three different buffers were used; 0.05 M citrate buffer was used for pH range between 4 and 6.2, 0.05 M sodium phosphate buffer was used for pH range between 6.2 and 8, and 0.05 M tris-HCl buffer was used for pH 8. The enzyme activity was determined using the standard assay procedure described above.

Effect of Temperature:
To determine optimum temperature, the β-galactosidase activity of enzyme was measured at different temperatures 15 to 55°C at optimum pH 7.2 [2]. The enzyme activity was determined using the standard assay procedure described above.

Optimization of Substrate Concentration:
The enzyme solution was used to observe the effect of substrate concentration on β-galactosidase activity by using different concentrations of ONPG (1 to 35 mM in 0.1 M phosphate buffer at pH 7 [12,37].

Determination of K_m and V_{max} Value:
For the determination of K_m and V_{max} values, Line weaver-Burk double reciprocal plot was used [12,37]. A graph of $1/V_o$ versus 1/[S] was plotted to yield a straight line with an intercept on the Y-axis, that was, $1/V_{max}$ a slope = K_m/V_{max} and intercept on the negative side of the X-axis.

RESULTS

Isolation of *Streptococcus* Strains from yoghurt samples:
From the total 27 yoghurt samples used for isolation of *streptococcus* strains, 8 samples showed the growth of clear white colonies i.e., samples A1, A4, A5, and A6 from brand I, samples N5 and N6 from brand II and samples NE4 and NE5 from brand III. These colonies were isolated and subcultured in MRS broth for further studies.

Morphological and Biochemical characterization of isolated strains:
From the morphological characterization of isolated strains, isolates A4, A5, A6, N6, and NE5 were identified as spherical shaped, gram-positive, and non-motile, having characteristics of Streptococcus species. Isolates A1, N5, and NE4 were identified as rod shaped, gram-positive, and non-motile, having characteristics of Lactobacillus species.

Biochemical characterization was done only for the sample isolates A4, A5, A6, N6, and NE5, which were found to have characteristics of Streptococcus species. These five isolates gave negative results for catalase test and mannitol fermentation test and showed positive results for glucose fermentation test, lactose fermentation test, and ST agar test. So the five isolates A4, A5, A6, N6, and NE5 were subcultured in MRS broth and used for further studies. Results were shown in (Table-1).

Table 1: Biochemical Characterization of the yoghurt isolates

S.No.	Name of the test	Sample isolates				
		A4	A5	A6	N6	NE4
1	Catalase	-	-	-	-	-
2	Glucose fermentation	+	+	+	+	+
3	Lactose fermentation	+	+	+	+	+
4	Mannitol fermentation	-	-	-	-	-
5	ST agar test	+	+	+	+	+

Note: (+) indicates positive & (-) indicates negative

Crude Enzyme Production using acid whey:
The isolated strains A4, A5, A6, N6, and NE5 were undergone crude enzyme production studies using sterile acid whey as complete media. All the five isolates examined synthesized beta-galactosidase with yields ranging from 5.37 to 7.76 U/ml (Table-2). But the isolate A5 was selected for further studies because of high productivity (7.76 U/ml).

Table 2: Crude Enzyme Production using acid whey

S.No	Isolated Strains	β-galactosidase activity (U/ml)
1	A4	6.25
2	A5	7.76
3	A6	6.86
4	N6	5.98
5	NE5	5.37

Protein Determination:
The concentration of protein obtained from isolated strain A5 was found to be 67µg/ml.

Partial Purification and characterization of Enzyme:
The various purification steps are summarized in (Table-3).

Table 3: Partial purification of β-Galactosidase

S.No	Purification steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Enrichment (purity fold)
1	Crude enzyme	0.151	15.98	105.83	-
2	Ammonium sulphate fraction	0.088	9.86	112.05	1.06
3	Sephadex G-100 fraction	0.065	7.76	119.38	1.13

In the final step, the enzyme had a specific activity of 119.38 IU/mg and approximately 1.13 fold purity. (Figure-1) shows that the enzyme exhibited maximum activity at pH 7.2. (Figure-2) shows the optimum temperature was 40ºC. β-Galactosidase enzyme was purified by SDS-PAGE. The bands were compared with the standard β-Galactosidase enzyme (commercially produced from Escherichia coli) shown in (Figure-3).

Figure 1: Effect of pH

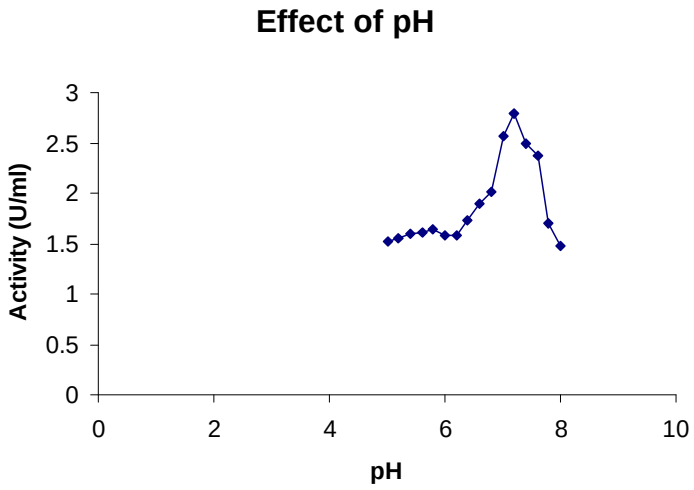


Figure 2: Effect of temperature

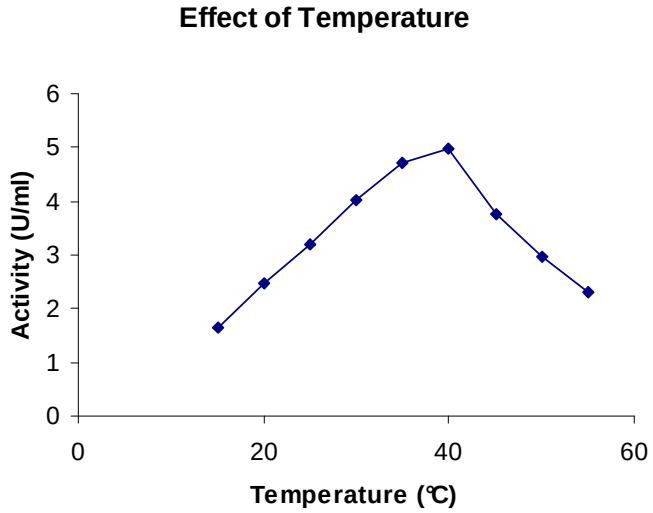
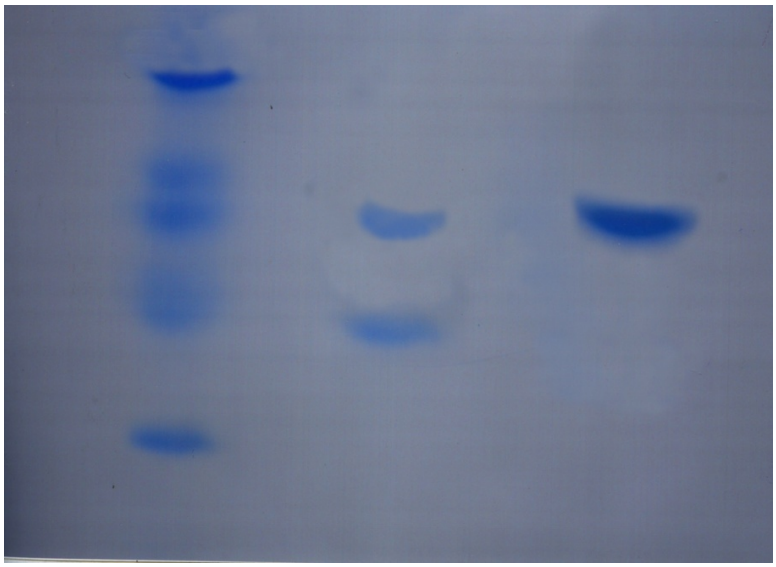


Figure 3: SDS-PAGE



Lane-1: crude enzyme
Lane-2: Enzyme partially purified by gel-filtration chromatography.
Lane-3: Standard Beta-Galactosidase enzyme

Determination of K_m and V_{max}:
The optimization of substrate concentration was performed using ONPG as substrate shown in (Figure-4). The optimum substrate concentration was found to be 24 mM. V_{max} and K_m were calculated from the Lineweaver-Burk reciprocal double plot (Figure-5). V_{max} was found to be 2.8 U/mL and K_m was found to be 3.05 mM.

Figure 4: Optimization of substrate concentration

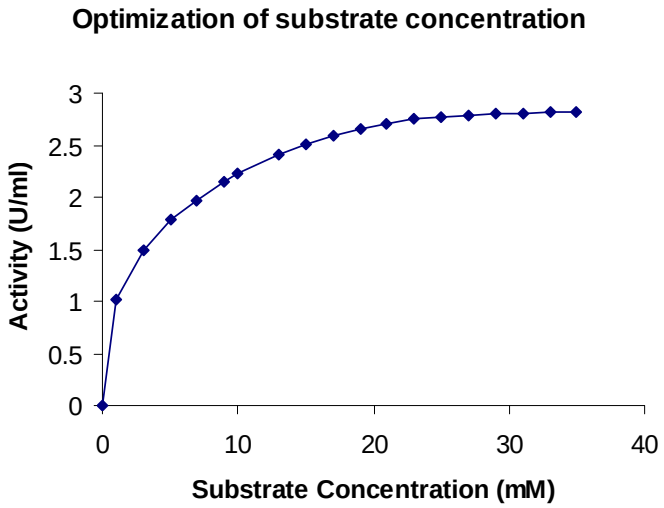
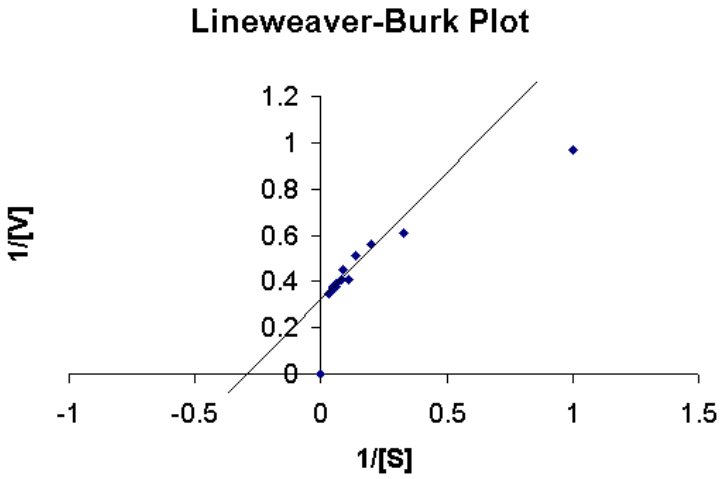


Figure 5: Lineweaver-Burk Plot



DISCUSSION

β-Galactosidase was an intracellular enzyme isolated from *Streptococcus thermophilus* grown in whey. One of the major obstacles to the whey utilization is lactose content, which causes crystallization at low temperatures, low sweetness, and poor digestibility when used as food. These problems can be solved if whey lactose is hydrolyzed to glucose and galactose.

Eight isolates were obtained from 27 samples of yoghurt and after morphological characterization five strains were found to have characteristics of Streptococcus species. Biochemical characterization was done for these five isolates and *Streptococcus thermophilus* strains were confirmed by ST agar identification test. *L. delbrueckii ssp. bulgaricus* either did not grow or formed tiny, white, cottony colonies that could easily be distinguished from *S. thermophilus* in ST agar.

On the other hand, β-galactosidase detected in the culture of *Streptococcus thermophilus* strain A5 showed a specific activity of 119.38 U/mg protein which was significantly higher than that 6.48 U/mg protein detected in the culture of *B. longum* B6 [14]. Previously, β-galactosidase biosynthesis by bacteria, yeasts, and moulds has been reported by various investigators [17,31]. β-Galactosidase activity varied up to a maximum of 2.5 U/ml for *Lactobacillus* crispatus [21]. Therefore, β-galactosidase production by *S. thermophilus* A5 reported here is markedly higher than previously recorded across a wide range of microorganisms.

From the SDS-PAGE, it can be observed that, the majority of the low molecular weight proteins present in the crude extract were removed. The lane 2 sample showed two bands one of which corresponding the β-galactosidase in lane 3. Thus it was concluded that *Streptococcus thermophilus* isolated from yoghurt produced β-Galactosidase. Additionally, ONPG confirmatory test for β-Galactosidase was also done. The disc changed to yellow colour, which further indicated the presence of β-Galactosidase enzyme.

As the substrate concentration was increased above 3 mM rate of reaction also increased progressively up to 24 mM. When the concentration was increased above 24 mM the rate of reaction remained constant. Justifying that, at this concentration enzyme remained saturated with substrate and rate of reaction was found to be maximum. This showed that β-Galactosidase obtained from *S. thermophilus* followed the Michaelis-menten equation.

The optimum pH for the hydrolysis of ONPG by the investigated enzyme was 7.2. Similar results have been reported for several β-galactosidases from yeast and bacteria which have optimum pH in the range 6.5-7.5 [20,33]. The retention of about 70% of maximal activity at pH values of 5.0 and 7.4 indicates that the enzyme is suitable for hydrolysis of lactose in milk and sweet whey. The optimum temperature was found to be 40°C.

A K_m of 3.05 mM was calculated from a Lineweaver-Burk plot of activity as a function of ONPG concentration. The measured value was comparable to the K_m of β-galactosidases from *T. aquaticus* (4mM), *Sulfolobus solfataricus* (2.6mM) and *Pyroccocus furosus* (0.15mM) and shows that the enzyme from *S. thermophilus* has a strong affinity for the substrate [7,19,33].

CONCLUSION

The strong industrial interest in β-galactosidases arises not only from their ability to hydrolyze lactose into D-galactose and D-glucose, but also their transglycosylation activity. In this study, we have reported the purification and detailed characterization of β-galactosidase extracted from the probiotic food-grade *S. thermophilus* A5 strain, which is recognized as safe, thus promoting its application in the agrofood industry. This process appears highly attractive mostly due to the use of a cheap lactose source such as whey, a subproduct from the milk industry.

Furthermore, the isolated biocatalyst showed activity over a broad range of temperature and pH, which makes it an interesting candidate for various industrial as well as biotechnological applications. Finally, data presented here indicate that inexpensive and easily available material such as deproteinized milk whey can be effectively used as substrate for the production of β-galactosidase by *S. thermophilus* A5 which could otherwise be an environmental pollutant, thereby contributing to a reduction in the production cost of this enzyme as well as safest environment. To our knowledge, this is the first report concerning β-galactosidase production over low cost material.

REFERENCES

[1] Adam AC, Rubio-Texeira M, Polaina J, *Crit Rev Food Sci Nutr*, **2004,** 44, 553-7.
[2] Basha NS, Rekha R, Komala M, Ruby S, *Trop J Pharm Res*, **2009,** 8, 353-360.
[3] Basil JM, *Appl Environ Microbiol*, **1982,** 44(5), 1035-1038.
[4] Belma A, Yavuz B, *Turk J Vet Anim Sci*, **2004,** 28, 257-263.
[5] Borooah J, Leakback DH, Walker PG, *Biochem J*, **1961,** 78, 106-110.
[6] Chase HA, *Trends Biotechnol*, **1994,** 12, 296-303.
[7] Cowan DA, Daniel RM, Morgan HW, *Biotechnol Bioeng*, **1984,** 26, 1141-1145,.
[8] Crueger W, Crueger A, *Biotechnology: A Textbook of Industrial Microbiology*, (2nd Ed.), Panima Publishing Corporation, New Delhi/Bangalore, **2003,** pp 105-121.
[9] Dave RI and Shah NP, *J Dairy Sci*, **1996,** 79, 1529-1536.
[10] Fooks LJ, Fuller R, Gibson GR, *Int Dairy J*, **1999,** 9, 53-61.
[11] Fowler AV, Zabin I, *J Biol Chem*, **1978,** 253, 5521-5525.
[12] Graciette M, Flavio FM, Gisella MZ, *Acta Scientiarum*, **2003,** 25(1), 7-12.
[13] Guimaraes PM, Teixeira JA, Domingues L, *Biotechnol Adv*, **2010,** 28, 375-84.
[14] Hsu CA, Yu RC, Chou CC, *Int J Food Microbiol*, **2005,** 104, 197-206.
[15] Husain Q, *Crit Rev Biotechnol*, **2010,** 30, 41-62.
[16] Im WT, Lee ST, Yokota A, *J Gen Applied Microbiol*, **2004,** 50, 143-147.
[17] Itoh T, Suzuki M, Adachi S, *Agric Biol Chem*, **1982,** 46, 899-904.
[18] Kara F, *Release and characterization of beta-galactosidase from Lactobacillus plantarum*, M.Sc. Thesis, Middle East Technical University, **2004,** Turkey.
[19] Kengen SW, Luesink EJ, Stams AJ, Zehnder AJ, *Eur J Biochem*, **1993,** 213, 305-312.
[20] Khare SK, Gupta MN, *Biotechnol Bioeng*, **1988,** 31, 829-832.
[21] Kim JW, Rajagopal SN, *Folia Microbiol*, **2000,** 45, 29-34.
[22] Kristo E, Biliaderis CG, Tzanetakis N, *Int Dairy J*, **2003,** 13, 517-528.
[23] Kuby SA, Lardy HS, *J Am Chem Soc*, **1953**, 75, 890-896.
[24] Lifran EV, Hourigan JA, Sleigh RW, Johnson RL, *Food Aust*, **2000,** 52, 120-5.
[25] Lowry OH, Rosebrough NJ, Farr AL, Randell RJ, *J Biol Chem*, **1951,** 193, 265-275.
[26] Macris BJ, Markakis P, *Applied Environ Microbiol*, **1981,** 41(4), 956-958.
[27] Mahoney RR, *Food Chem*, **1998,** 63, 147-154.
[28] Mimi D, Gajaila G, Cotor G, Savu C, Gajaila I, *Bull USAMV Vet Medicine*, **2008,** 65(2), 253-255.
[29] Mitsuoka T, *J Ind Microbiol*, **1990,** 6, 263-268.
[30] Mukesh Kumar DJ, Suresh K, Saranya GM, Andal Priyadarshini D, Damodaran R, Kalaichelvan PT, *Europ J Exp Biol*, 2012, 2(3), 774-780.
[31] Nagy Z, Kiss T, Szentirmai A, Biro S, *Protein Exp Purif*, **2001,** 21, 24-29.

[32] Panesar PS, Panesar R, Singh RS, Kennedy JF, Kumar H, *J Chem Technol Biotechnol*, **2006,** 81, 530-543.
[33] Pisani FM, Rella R, Raia CA, Rozzo C, Nucci R, *Eur J Biochem*, **1990**, 187, 321-328.
[34] Rao MV, Dutta SM, *Applied Environ Microbiol*, **1997,** 34, 185-188.
[35] Sambrook J, David WR, *Molecular Cloning*, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, **2003,** pp A8.40-A8.51.
[36] Takeomi M, Takeshi H, Hiroki H, Satoshi A, Taichi U, *J Applied Glycosci*, **2005,** 52, 161-168.
[37] Ted RJ, Christine LC, *Laboratory Experiments in Microbiology*, (4th Ed.), The Benjamin/Cummings Publishing Company Inc USA, **1995,** pp 27-44.
[38] Trevan MD, Boffey S, Goulding KH, Stanbury P, *Biotechnology: The Biological Principles*, Tata McGraw-Hill Publishing Ltd, New Delhi, India, **2004,** pp 155-228.
[39] Vasiljevic T, Jelen P, *Innovative Food Sci Emerg Technol*, **2001,** 2, 75-85.
[40] Wallenfels K, Weil R, *Beta-Galactosidase*, Academic Press, New York, USA, **1972,** pp 617-663.
[41] Zarate S, Lopez-Leiva MH, *J Food Prot*, **1990,** 53, 262-268.