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*Full Length Research Paper*

# Purification and characterization of α-galactosidase from *Lactobacillus acidofillus*

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**α–Galactosidase (α-D-galactoside galactohydrolase [EC 3.2.1.22]) was obtained from *Lactobacillus acidofillus* which was grown in modified de Man, Rogosa and Sharpe (MRS) medium, supplemented with raffinose. α-Galactosidase was released from the cells by ultrasonic treatment, then precipitated by ammonium-sulfate and further purified with Sephadex G-200 and DEAE cellulose chromatography with a 18.5-fold increase in specific activity and 28% recovery. $K_m$ and $V_{max}$ for this enzyme was determined by p-nitrophenyl-α-D-galactoside as substrate, to be about 0.47 mM, and 17.54 μmol/min per mg of protein, respectively. Maximum enzymatic activity occurred at pH 5.5 and temperature at 45°C. The enzymatic activity was retained at least for 30 min, at temperatures of 25 - 55°C, but there was inactive temperature at about 60°C. Galactose was able to decrease the enzyme activity by a factor of 63%. Among the sugars tested, fructose, glucose, sucrose, lactose and mannose reduced the enzyme activity only slightly (less than 10% of the control). A strong inhibition of α-galactosidase activity was found in the presence of 0.1 mM HgCl.**

**Key words:** α-Galactosidase, enzyme purification, *Lactobacillus acidofillus*, kinetic studies.

## INTRODUCTION

The enzyme α–galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) hydrolyses terminal, non-reducing α-D-galactose residual in the α-galactosides, as well as galactose oligosaccharides such as melibiose, raffinose and stachyose (Dey and Pridham, 1972). α-Galactosidase is not synthesized by humans, and thus the presence of these oligosaccharides could hinder digestion and cause flatulence, since they are utilized by the gas-generating intestinal microorganisms. α-Galactosidase can be used to clear these oligosaccharides and upgrade the nutrition of legume food (Thananunkul et al., 1976). Many attempts have been made to reduce these anti-nutritional factors by soaking and germination (Sugimoto and Van Buren, 1970).

Enzyme treatment with microbial α-galactosidase would be promising for the elimination of these oligosaccharides (Thananunkul et al., 1976). Lactobacillus species have been found in large numbers as part of the intestinal flora of humans and other animals, where they are thought to increase resistance to common intestinal disorders, especially those with a microbial pathogenesis, for example, gastroenteritis (Casas and Dobrogosz, 2000). They can achieve this by fortifying the normal micro-flora either through their fermentation products or by the production of glycosidases, which degrade carbohydrates, thereby supplying energy for the growth of other bacteria (Sandine, 1979).

Today, industrial strategies focus on the selective stimulation of growth and activity of Lactobacilli in the intestine by supplementation of the food with specific and non digestible carbohydrates (Tzortzis et al., 2003). α-Galacto-oligosaccharides, particularly raffinose and

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**Abbreviations: MRS,** de Man, Rogosa and Sharpe medium; **SDS,** sodium dodecylsulphate.

stachyose, have been described as prebiotic substrates promoting the growth of probiotic bacteria in the colon (Benno et al., 1987).

α-Galactosidase may have great potential in various applications. This enzyme can be used to clear α-galactooligosaccharides and upgrade the nutrition of legume food (Thananunkul et al., 1976). Fabry's disease of humans is due to a deficiency of thermolabile lysosomal α-galactosidase A, which consuming this enzyme will improve disease (Ulezlo et al., 1982). In the sugar industry, this enzyme can also degrade the raffinose in molasses and thus increase the yield of crystallized sugar (Linden, 1982). Type B erythrocytes, which contain 3-O-α-D-galactopyranoside, can be transformed into type O erythrocytes by exposure to α-galactosidase (Ulezlo et al., 1982). α-Galactosidase may be used in the future for such medical purposes as enzymotherapy.

To the best of our knowledge, there is no report on the purification of α-galactosidase from *Lactobacillus acidophilus* to date. Therefore, the purpose of this paper is to present information on the purification and characterization of α-galactosidase from *L. acidophilus*.

## MATERIALS AND METHODS

### Microorganism and culture conditions

*L. acidophilus* PTCC1643 used in this study was obtained from the culture collection of the Iranian Research Organization for Science and Technology. The strain was selected according to its capacity to utilize melibiose and raffinose. The organism was grown on modified MRS-medium containing (g/l): meat extract, 5; yeast extract, 5; sodium acetate, 5 ; $K_2HPO_4$, 2; ammonium citrate, 2; $MgSO_4$, 0.1; and Tween 80, 1 ml; pH 6.5 (sterilized at 121°C, 15 min). This medium was used as basal medium for the assays. Raffinose was sterilized by filtration and added separately to the growth medium at a final concentration of 1%. Active culture was inoculated at 10% in MRS-raff broth and then incubated at 37°C for 18 h.

### α-Galactosidase assay

α-Galactosidase activity was routinely assayed by measuring the release of p-nitrophenol from p-NPG in McIlvaine buffer (pH 5.5) (Linden, 1982). The reaction mixture contains: 10 mM PNPG; 100 mM buffer McIlvaine pH 5.5, cell-free extract, 100 µl of the fractions obtained during purification; final volume, 0.2 ml. The mixture was incubated at the optimum temperature (50°C) for 10 min. The reaction was stopped by adding 1.3 ml 1 Mol. $Na_2CO_3$. The amount of PNP released was determined at 400 nm (Garro et al., 2004). One unit of enzyme activity was defined as the amount which released 1 µmol of p-nitrophenol per minute under the specified conditions.

Protein was determined according to Lowry et al. (1951) with bovine serum albumin as standard.

### Preparation of cell-free extracts

Cells obtained from fermentation medium were harvested by centrifugation (4000 g, 25 min, 4°C) and washed twice with cold McIlvaine buffer pH 5.5 ($C_6H_8O_7$,$Na_2HPO_4$) containing 0.1% (v/v) mercaptoethanol. The washed cells were suspended (20% w/v) in McIlvaine buffer (pH 5.5) and sonicated (Sonoplus HD 200, Bandelin Electronic GmbH) in an ice bath at 10 kHz for 5 min with a pause of 1 min. The cell debris was removed by centrifugation and the clear supernatant was used as the source of crude enzyme.

### Purification of α-galactosidase

The extract supernatant was concentrated by addition of solid ammonium sulfate 90% saturation. The mixture was shaken at 4°C. Precipitated proteins were collected by refrigerated centrifugation (14000 g for 20 min), resuspended and dialyzed overnight against the same buffer.

The dialyzed extract loaded on Sephacryl G-200 (Sigma) column (200 × 15 mm), the column was washed with 2 V equilibrated with 10 mM McIlvaine buffer pH 5.5. Elution was carried out at a flow rate of 0.5 ml/min and 3 ml fractions were collected. The enzyme was eluted and fractions containing the highest activity were pooled, and loaded on DEAE cellulose (Sigma) column (30 × 120 mm), equilibrated with 10 mM McIlvaine buffer pH 5.5 containing 0.15 M NaCl and 4 ml fractions were collected.

The proteins eluted from both columns were followed up by measuring the absorbance at 280 nm with a Shimadzu spectrophotometer UV-210A. α-Galactosidase activity was determined in all the fractions and those showing the highest enzyme activity were being pooled.

### Electrophoresis

Electrophoresis in denaturing conditions was carried out in the presence of sodium dodecylsulphate (SDS) at 10% concentration. The sample was treated according to Laemmli (1970) and heated at 100°C for 3 min; bromophenol blue was employed as a front run marker. The gels were run for 3 h at 20 mA and 100 V in an electrophoresis unit. After the run, they were stained for protein by Coomassie brilliant blue R-250 (Kapitany and Zabrowsky, 1973).

### Kinetic studies

The optimum pH for activity of the α-galactosidase was determined by varying the pH ranging from 3.0 - 8.0 and the optimum temperature was determined at varying the temperatures from 25 - 70°C. The pH and temperature stability was determined by incubiting the enzyme.

Enzyme assays were performed in the presence of various metal ions such as $Hg^{2+}$, $Mn^{+2}$, $Mg^{+2}$, $Fe^{+2}$, $Ca^{+2}$ and $Zn^{+2}$ (final concentration 10 mM) and in the presence of sugars such as galactose, fructose, glucose, sucrose, lactose and mannose (100 mM). Michaelis–Menton constant ($K_m$) and the reaction rate ($V_m$) for synthetic substrate pNPGal was carried out in the range of 0.1 to 10 mM.

## RESULTS

### Purification of α-galactosidase

### Step 1: Ammonium sulfate precipitation.

Solid $(NH_4)_2S0_4$ was added to cell-free extract with continuous stirring. By this step, the specific activity of the enzyme was increased about 1.4-fold, but it resulted in

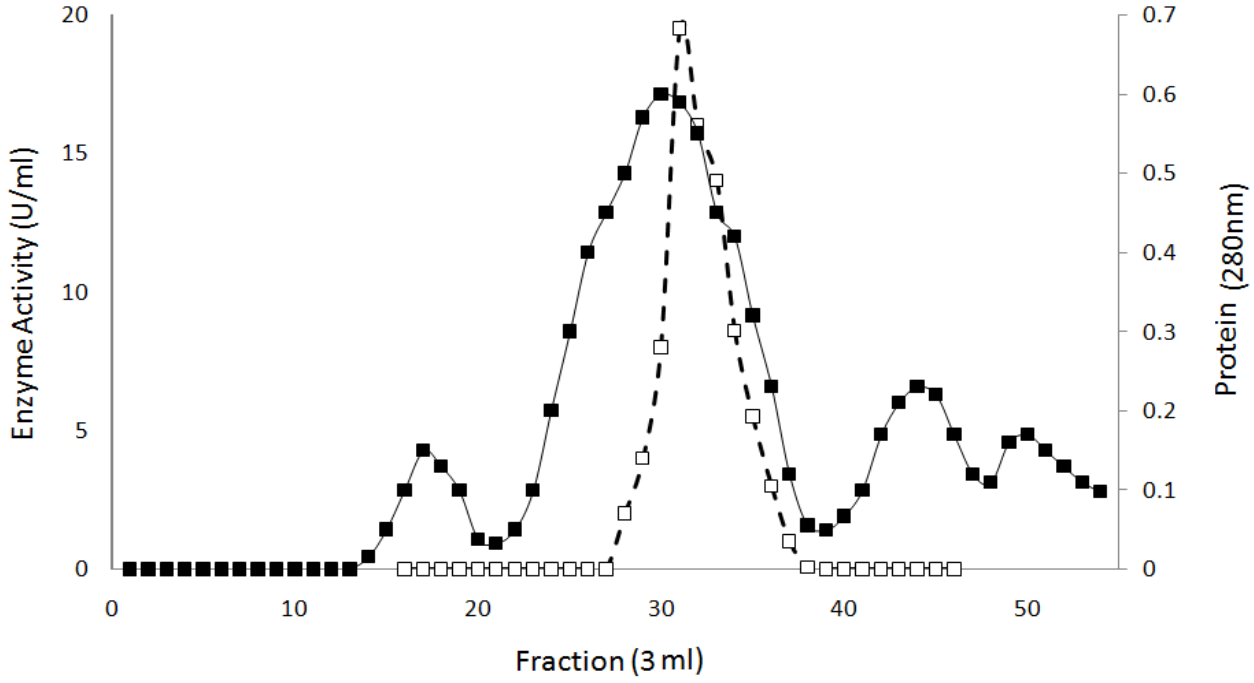


**Figure 1.** Profile of α-galactosidase from *L. acidophilus* in Sephadex G-200 (—□—), protein (280 nm) (—■— ) α-galactosidase activity ($\mu mol^{-1}min^{-1}mg^{-1}$).

**Table 1.** Summary of purification of intracellularα-galactosidase from *L. acidophilus.*

| Purification step | Activity (U) | Protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 5500 | 310 | 17.74 | 100 | 1 |
| Sulphate Ammonium per. | 4878 | 191 | 25.54 | 88.6 | 1.4 |
| G200-Sephadex | 2365 | 15.5 | 152.58 | 43 | 8.6 |
| DEAE-cellulose | 1536 | 4.7 | 326.8 | 27.9 | 18.4 |

about 12% less enzymatic activity.

### Step 2: Sephadex G-200 gel filtration

Figure 1 shows the elution profile in Sephadex G-200, the selection of the fraction eluted from the column was performed on the basis of the enzymatic activity and a single peak being observed. The fractions between 28 and 37 were pooled; then the total activity (2365 U) as well as the protein 15.5 mg were determined (Table 1).

### Step 3: DEAE-cellulose chromatography

Figure 2 shows the elution profile in DEAE-cellulose, the column was eluted with a linear gradient of NaCl. The active fractions were pooled with final specific activity of 326.8 U/mg protein. The enzyme was purified 18.4-fold with 28% yield (Table 1).

After the three purification steps, α-galactosidase from *L. acidophilus* had a molecular mass of 45 KDa, as determined by SDS-PAGE (Figure 3).

### Effects of pH and temperature

The enzyme activity at various pH and temperature is shown in Figures 4A and B, respectively. The optimum pH of α-galactosidase activity from *L. acidophilus* was determined to be 5.5, with PNPGal as substrate (Figure 4A; 10-min incubation at 50°C). It was stable at pH range of 4.0 to 6.0 (37°C, 1 h incubation) but very labile in acidic conditions (below pH 4.0).

The effect of temperature was determined at pH 5.5 between 25 and 70°C maximum activity occurring at 45°C. The enzyme activity increased with temperature to 55°C and then decreased suddenly (Figure 4B). It was stable up to 30 min at 55°C, but lost 90% of its original activity at 60°C for 30 min.

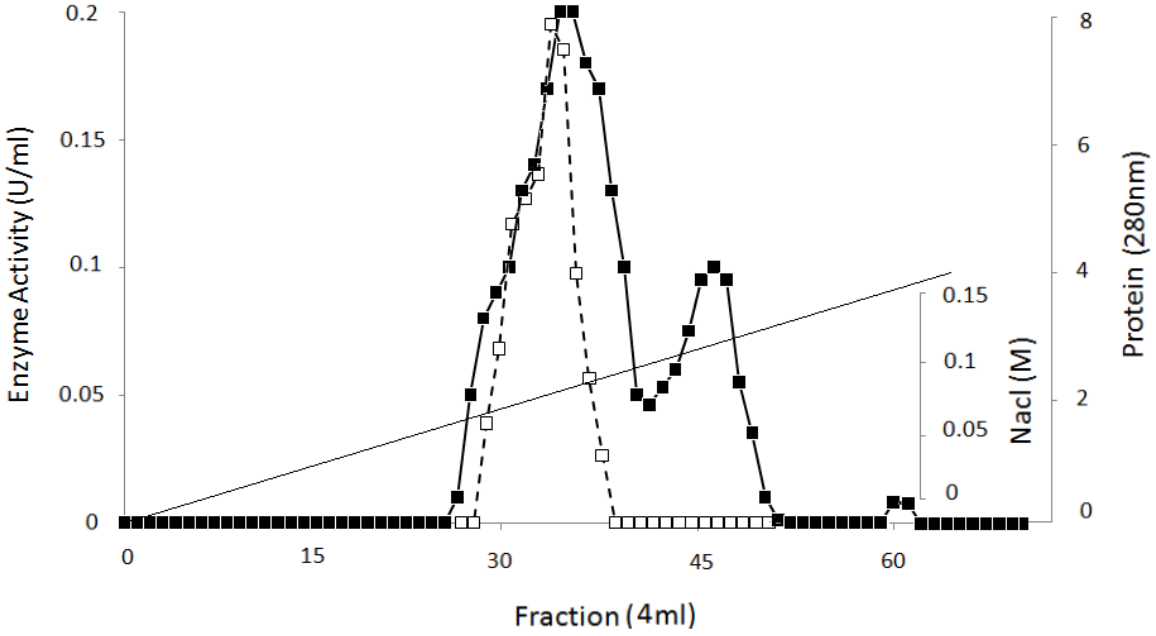


**Figure 2.** Profile of α-galactosidase from *L. acidophilus* in DEAE-Cellulose (—□— ), protein (280 nm) (—■— ) alpha-galactosidase activity (μmol$^{-1}$min$^{-1}$mg$^{-1}$), (— ) NaCl gradient.

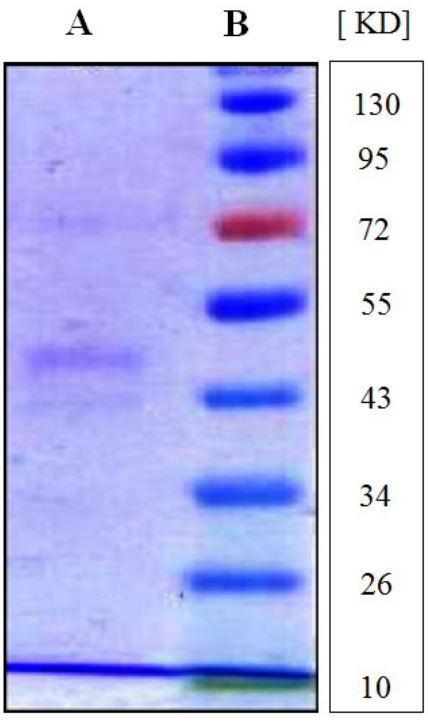


**Figure 3.** Electrophoresis of α-galactosidase under denaturing conditions (SDS-PAGE). Lane A: Purified α-galactosidase. Lane B: Fermentase standard marker (#SM067).

### Kinetic studies and substrate specificity

The effect of substrate concentration on activity was examined by using p-nitro-phenylα-D-galactoside substrate. Lineweaver-Burk plots of the data showed an apparent $K_m$ of 0.47 mM and a Vmax of 17.54 μM ml$^{-1}$min$^{-1}$mg$^{-1}$ of protein for p-nitrophenyl-α-D-galactoside (Figure 5).

### Effect of sugars and metal ions

Table 2 shows the effect of cations and sugars upon the enzymatic activity. Galactose at 100 mM level showed more than 64% inhibition on α-galactosidase activity from *L. acidophilus*. Among the sugars tested, fructose, glucose, sucrose and lactose reduced the enzyme activity only slightly (less than 10% of the control).

The $Mn^{+2}$, $Mg^{+2}$ and $Zn^{+2}$ had no effect on the α-galactosidase activity; whereas, $Fe^{+2}$ and $Ca^{+2}$ slightly enhanced the enzyme activity. However, the enzyme activity was inhibited up to 95% addition of $Hg^{+2}$ (0.1 mM).

## DISCUSSION

Among the growing strains in MRS medium, enzyme activity was detected by p-nitrophenyl-α-D-galactoside as substrate. *L. acidophilus* PTCC1643 showed the highest

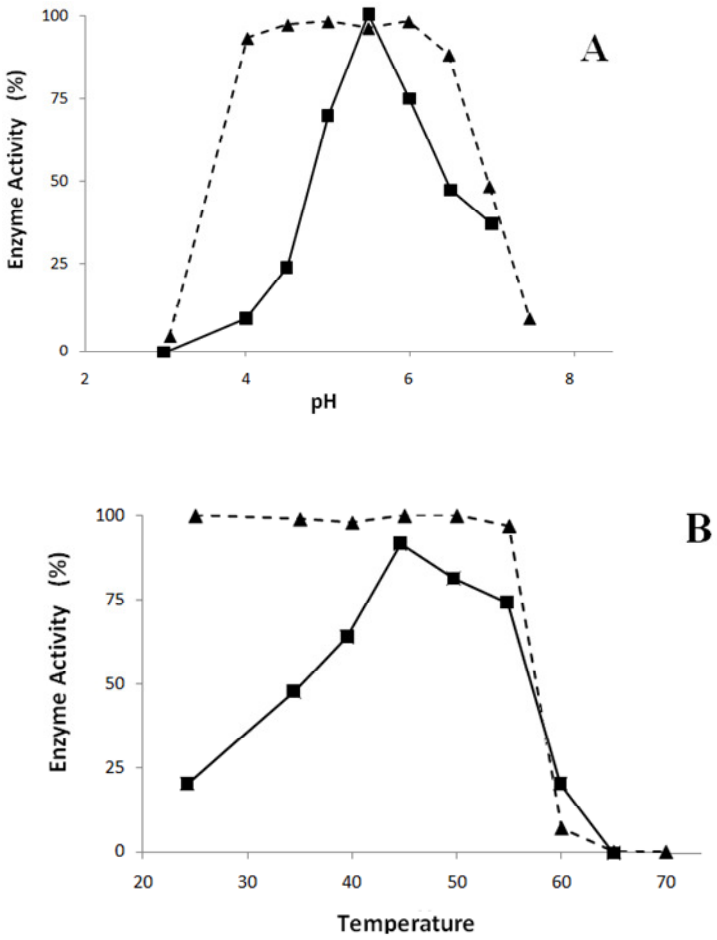


**Figure 4.** Effect of pH and temperature on the activity (—■—) and stability (—▲—) of *L. acidophilus* α-galactosidase.

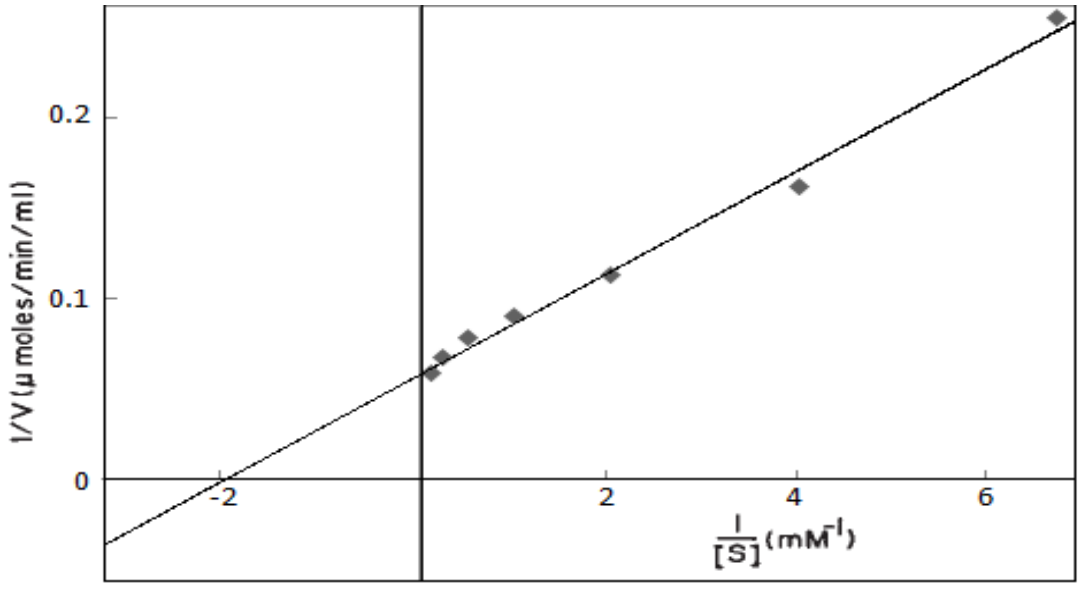


**Figure 5.** Lineweaver-Burk plot. Effect of p-nitrophenyl-α-p galactopyranoside (PNPG) concentrations on the α-galactosidase activity from *L. acidofillus*.

**Table 2.** Effect of sugars and metal ions on the activity of α-galactosidase. The enzyme (100 µl) was incubated with sugars and metal ions and the residual activity was determined.

| Sugars | Relative activity (%) | Mental ions | Relative activity (%) |
|---|---|---|---|
| Glucose | 89 | $FeSO_4$ | 100 |
| Galactose | 64 | $ZnSO_4$ | 98 |
| Fructose | 92 | $MnCl_2$ | 100 |
| Lactose | 88 | $CuCl_2$ | 110 |
| Manose | 100 | $CaCl_2$ | 103 |
| Xylose | 100 | $MgCl_2$ | 100 |
| Sucrose | 90 | $HgCl_2$ | 3 |

α-galactosidase activity with 11.3 U/ml of enzyme activity. α-Galactosidase from *L. acidophilus* was purified 18.4-fold by the sequential use of ammonium sulphate precipitation, anion-exchange chromatography and gel filtration. The final enzyme preparation was purified to give an overall yield of 27.9% of the initial α-galactosidase activity that hydrolyzed p-NPG at a specific activity of 326 U mg-1 protein.

Maximum enzymatic activity occurred at pH 5.5 and temperature of 45°C. These results concur with those of other α-galactosidases from *Lactobacilli* (Garro et al., 1997) and *Bifidobacteria* (Sakai et al., 1987). In contrast, α-galactosidase from fungi (Kotwal et al., 1998; Hin-chung et al., 1986) exhibit optimal activity at acidic pH values.

$K_m$ and $V_{max}$ for this enzyme was determined with p-nitrophenyl-α-D-galactoside as substrate, to be 0.47 mM, and 17.54 µmol/min per mg of protein. The $K_m$ determined for the enzyme under study is similar to the one reported by Tzortzis et al. (2003) for *Lactobacillus reuteri* ($K_m$ = 0.48 mM). However, it was lower than those found for the α-galactosidase isolated from other micro-organisms, using the same substrate (PNPG). According to the Km values reported by other investigators from *Citrobacter freundii* 5 mM (Lokage and Deepal, 2001) and *Bifidobacterium adolescentis* 0.9 mM (Susanne et al., 1999).

Galactose was able to decrease the enzyme activity from *L. acidophilus* by a factor of 63%. It is an inhibitor of α-galactosidases which is very similar to the α-galactosidases from *Escherichia coli* (Kyu et al., 1983) and *Humicola* sp (Kotwal et al., 1998). The ion $Hg^{+2}$ is a strong inhibitor of α-galactosidases isolated from different sources. This fact suggests the possible presence of thiol groups at the catalytic site of the enzyme. These results concur with those of other α-galactosidases from *Lactobacillus fermentum* CRL 251 (Garro et al., 1997) and *Bifidobacterium breve* 203 (Sakai et al., 1987).

*L. acidophilus* has been found to be a rich source of α-galactosidase, which can be purified by simple purification steps. The enzyme may find application in food processing industries for the degradation of raffinose-family of oligosaccharides. Cloning of α-galactosidase gene in a suitable vector, followed by its expression in an appropriate host, would go a long way to meet the industrial requirement of this enzyme.

## REFERENCES


Benno Y, Endo K, Shiragami N, Sayama K, Mitsuoka T (1987). Effect of raffinose intake on human fecal microflora. Bifidobact Microflora. 6: 59-63.

Casas IA, Dobrogosz WJ (2000). Validation of the probiotic concept: Lactobacillus confers broad-spectrum protection against disease in humans and animals. Microbial Ecol. Health Dis. 12: 247-285.

Dey PM, Pridham JB (1972). Biochemistry of a- galactosidase. Adv. Enzymol. 36: 91-130.

Garro M, de Valdez GF, Oliver G, de Giori G (1997). Purification of alpha-galactosidase from Lactobacillus fermentum. J. Biotechnol. 45: 103-110.

Garro M, Graciela F, de Valdez GF, Graciela Savoy D (2004). alpha-Galactosidase Assay in Fermented Soymilk products. Environ. Microb. Methods Protocols, 16: 121-124.

Hin-chung W, Chien-A H, His-Lien Y, Wanchuang S (1986). Production, purification, and characterization of alpha-galactosidase from Monascus pilosus. Appl. Environ. Microbiol. 23: 1147-1152.

Kapitany RA, Zabrowsky EJ (1973). A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal. B Chem. 56: 361-367.

Kotwal SM, Gote M, Sainkar SR, Khan MI, Khire GK (1998). Production, purification and characterization of a constitutive intracellular a-galactosidase from the thermophilic fungus *Humicola* sp. Process Biochem. 33: 337-343.

Kyu Won K, Hyun JL (1983). Purification & characterization of alpha-galactosidase from E. coli . Korean Biochem. J. 16: 1-12.

Laemmli UK (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Linden JC (1982). Immobilized alpha-D-galactosidase in the sugar beet industry. Enzyme Microbiol. Technol. 4: 130-136.

Lokage MA, Deepal CM (2001). Kinetic study of extracellular alpha-galactosidase from Citrobacter frundii. J. Natl. Sci. Foundation Sri Lanka. 134: 97-106.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Sakai K, Tachiki T, Kumagai H, Tochikura T (1987). Hydrolysis of alpha-D-galactosyl oligosaccharides in soymilk by alpha-D-galactosidase of Bifidobacterium breve 203. Agric. Biol. Chem. 51: 315-332 .

Sandine WE (1979). Roles of Lactobacillus in the intestinal tract. J. Food Protect. 42: 259-262.

Sugimoto H, Van Buren JP (1970). Removal of oligosaccharides from soy milk by an enzyme from *Aspergillus saitoi*. J. Food Sci. 35: 655-660.

Susanne L, Winfried H, Stefan P (1999). alpha-Galactosidase of

Bifidobacterium adolescentis DSM 20083. Curr. Microbiol. 38: 101-106.
Thananunkul D, Tanaka M, Chichester CO, Li T (1976). Degradation of raffinose and stachyose in soybean milk by a-galactosidase from *Mortierella vinacea*. gel. J. Food Sci. 41: 173-175.
Tzortzis G, Jay M, Baillon G, Gibson R (2003). Rastall Synthesis of a galactooligosaccharides with alpha-galactosidase from *Lactobacillus reuteri* of canine origin. Appl. Microbiol. Biotechnol. 63: 286-292.
Ulezlo IV, Zaprometova M (1982). Microbial alphagalactosidase (a review). Appl. Biochem. Microbiol. 18: 1-12.