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Purification and characterization of a novel β-galactosidase from *Bacillus* sp MTCC 3088

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An extracellular β-galactosidase which catalyzed the production of galacto-oligosaccharide from lactose was harvested from the late stationary-phase of *Bacillus* sp MTCC 3088. The enzyme was purified 36.2-fold by $ZnCl_2$ precipitation, ion exchange, hydrophobic interaction and gel filtration chromatography with an overall recovery of 12.7%. The molecular mass of the purified enzyme was estimated to be about 484 kDa by gel filtration on a Sephadex G-200 packed column and the molecular masses of the subunits were estimated to be 115, 86.5, 72.5, 45.7 and 41.2 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point of the native enzyme, determined by polyacrylamide gel electrofocusing, was 6.2. The optimum pH and temperature were 8 and 60°C, respectively. The Michaelis–Menten constants determined with respect to *o*-NO_2-phenyl-β-D-galactopyranoside and lactose were 6.34 and 6.18 mM, respectively. The enzyme activity was strongly inhibited (68%) by galactose, the end product of lactose hydrolysis reaction. The β-galactosidase was specific for β-D anomeric linkages. Enzyme activity was significantly inhibited by metal ions (Hg^{2+}, Cu^{2+} and Ag^{+}) in the 1–2.5 mM range. Mg^{2+} was a good activator. Catalytic activity was not affected by the chelating agent EDTA. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 58–63.

Keywords: purification; extracellular β-galactosidase; *o*-NO_2-phenyl-β-D-galactopyranoside; lactose; *Bacillus* sp

Introduction

β-Galactosidase (EC 3.2.1.23) catalyzes not only hydrolysis of β-D-galactopyranosides, such as lactose, but also a trans-galactosylation reaction that produces galacto-oligosaccharides [21]. The enzyme is useful in the dairy and medical industries for prevention of lactose crystallization in frozen and condensed milk products. The enzyme is also used for the reduction of water pollution caused by whey from cheese and for treatment of milk for lactose-intolerance [12,28]. Recently, galacto-oligosaccharides, enzymatic transgalactosylation reaction products from lactose, have become of interest for human health. Galacto-oligosaccharides are recognized as a growth-promoting factor for intestinal bifidobacteria [20,27], which are helpful for maintenance of human health [11,16]. Development of an efficient and inexpensive method for the production of galacto-oligosaccharides is highly desirable. The purification of β-galactosidase from microbial sources are described [1–6,8,9,13,17,18,21,23–26]. Although many microbial β-galactosidases have been studied [1], very few studies have been reported on the thermostable β-galactosidase [8,9,19,21,22]. Recently Ohtsu *et al* [19] purified and characterized a thermostable β-galactosidase from *Thermus* sp A4. Vetere and Paoletti [30] isolated and characterized three β-galactosidases from *Bacillus circulans*. In view of the potential uses of this enzyme, it is desirable to study this enzyme from different microbial sources. In this study, we have described the purification and characterization of an extracellular thermostable β-galactosidase from a *Bacillus* sp.

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Materials and methods

Chemicals

o-NO_2-phenyl-β-D-galactopyranoside (ONPG), EDTA and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) were obtained from Sigma Chemical Company (St Louis, MO, USA). Q-Sepharose and Sephadex G-200 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of analytical grade.

Bacterial strain and preparation of enzyme

Bacillus sp MTCC 3088 was isolated from the water of a hot spring (Manikaran, India) and maintained on nutrient agar plates containing 1% (w/v) lactose. The medium used for production of β-galactosidase had the composition (g L^{-1}): lactose, 10; meat extract, 15; biopeptone, 5; yeast extract, 0.5; and sodium chloride, 1.5. The pH of the medium was adjusted to 7. The seed culture was prepared by inoculating a single colony from the maintenance plate into the culture medium and incubated in a rotary shaker (37°C, 200 rpm) for 18 h. This inoculum (10%, w/v) was used for production of enzyme in 500-ml Erlenmeyer flasks containing 100 ml medium and incubated in a temperature-controlled (37°C) shaker at 200 rpm for 9 days. The culture broth was centrifuged at 19 000 × *g* at 4°C for 15 min and the supernatant was assayed for enzyme activity and used for purification and characterization. β-galactosidase activity was assayed according to the method of Nagano *et al* [18] using ONPG as the substrate. One unit of enzyme

activity is defined as the amount that liberated 1 nmole *o*-nitrophenol per min.

Enzyme purification

Nine-day-old broth was centrifuged (19 000 × *g*) at 4°C for 15 min. The pH of the supernatant was adjusted to 5 with 0.2 N acetic acid. The supernatant was precipitated with 1 M $ZnCl_2$ (final concentration 60 mM). The precipitated protein obtained by centrifugation (19 000 × *g*, 5 min) was dissolved in a minimum volume of 0.5 M EDTA and dialyzed against phosphate buffer (0.1 M, pH 7.5). The concentrated sample was applied to an anion-exchange column (Q-Sepharose, 2.5 × 10 cm). The column was previously equilibrated with 0.1 M phosphate buffer, pH 7.5. Elution of the column was performed with a linear gradient of NaCl (0–1 M) in phosphate buffer (0.1 M, pH 7.5) with a flow rate of 1 ml min^{-1} and 6-ml fractions were collected. The active enzyme fractions containing β-galactosidase were pooled and concentrated by lyophilization. The concentrated enzyme obtained from ion exchange chromatography was dialyzed against 0.1 M phosphate buffer, pH 7.5. The dialyzed enzyme solution was applied to Econo-pac methyl HIC cartridge (Bio-Rad, Hercules, CA, USA), which was previously equilibrated with 2 M ammonium sulfate in 0.1 M phosphate buffer, pH 7.5. Elution of the column was performed with a linear gradient (2–0 M ammonium sulfate) in 0.1 M phosphate buffer, pH 7.5. The flow rate was adjusted to 1 ml min^{-1} and 5-ml fractions were collected. The active enzyme fractions containing β-galactosidase activity were pooled and concentrated by lyophilization. The concentrated enzyme solution (0.4 ml) obtained from hydrophobic interaction chromatography and lyophilization was applied to a Sephadex G-200 column (1.5 × 45 cm) which was previously equilibrated with the 0.1 M phosphate buffer (pH 7) containing 0.15 M sodium chloride. Elution was performed at a flow rate of 0.5 ml min^{-1} (0.1 M phosphate buffer containing 0.15 M NaCl) and fractions (1 ml each) were collected. The isoelectric point of β-galactosidase was estimated on a Pharmacia multiphor system with isoelectric focusing polyacrylamide gel (5%, 0.2 mm thick) with a pH gradient of 3.5–9.5. The reference proteins used were amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β-lactoglobulin A (pI 5.1), carbonic anhydrase I (pI 6.6), carbonic anhydrase II (pI 5.9), myoglobin (pI 6.8, 7.2), lectin (pI 8.2, 8.6, 8.8) and trypsinogen (pI 9.3).

Characterization of purified β-galactosidase

Polyacrylamide gel electrophoresis was done on polyacrylamide slab gels with 0.1% sodium dodecylsulphate. The native gels were run without SDS at 15 mA for stacking and at 35 mA for resolving. The gels were then stained by Coomassie brilliant blue G-250. For activity staining of β-galactosidase, a 5% native polyacrylamide gel was used. The purified enzyme was mixed in a ratio of 1:1 with sample buffer (1 ml 0.5 M Tris buffer pH 6.8, 1 ml glycerol and 1 ml water containing 0.25 mg bromophenol blue). After completion of the run, the gel was incubated in X-gal which was previously dissolved in dimethylformamide and diluted to 0.02% by 0.1 M phosphate buffer (pH 7). After 4–6 h incubation, a blue-green band of β-galactosidase was found. To find out the optimum pH and temperature for enzyme activity, assays were carried out at different temperatures (20–70°C) and at different pHs by using sodium citrate (3–6), potassium phosphate (7–8) and glycine-NaOH buffers (8.5–10). Extracellular protein was measured using Lowry's method with bovine serum albumin as standard. A mixture containing purified enzyme solution, metal ions and EDTA (final concentration 1–25 mM) was incubated for 20 min at 60°C and the enzyme activity was checked. To see the effect of different sugars (4 mg ml^{-1}) on enzyme activity, the enzyme was incubated with different sugars for 20 min at 60°C. Enzyme activity was assayed under standard conditions and inhibition or activation was expressed as a percentage of the activity without effector (control).

β-galactosidase was assayed with respect to its artificial and natural substrates ONPG and lactose, respectively, at 60°C. ONPG was used at concentrations from 0 to 10 mM in 0.1 M phosphate buffer, pH 7, containing 100 μl enzyme solution. Lactose, at concentrations of 0–200 mM in 0.1 M phosphate buffer, pH 7, containing 1.2 ml enzyme solution was used. Aliquots (0.5 ml) were withdrawn and the reaction was stopped by boiling the reaction mixture for 5 min. The release of glucose was measured using GOD-POD [14]. One unit of β-galactosidase activity, with lactose as substrate, is defined as the amount of enzyme which releases 1 μmole glucose per min at 60°C. K_m and V_{max} values against ONPG and lactose were calculated using a Lineweaver–Burk plot.

Determination of molecular weights

(a) Relative molecular weight of native β-galactosidase: The native molecular mass of the purified enzyme was estimated by gel filtration chromatography on a Sephadex G-200 column (2.5 × 47.5 cm) purchased from Bio-Rad, USA and equilibrated with 0.1 M phosphate buffer (pH 7) containing 0.15 M NaCl. The distribution coefficient, K_{av} was determined for each protein as follows: $K_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e, V_0 and V_t are the elution volume, gel void volume and total volume of the column. The V_0 of the column was determined by elution volume of Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) through the column. The V_e for the each protein marker was estimated from the absorbance at 280 nm. The following protein standards (Pharmacia) were used to calibrate the column: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease (13.7 kDa). A plot of log (molecular mass) *vs* K_{av} was used to determine the apparent molecular mass of β-galactosidase from *Bacillus* sp.

(b) Relative molecular weights of β-galactosidase subunits: The molecular mass of the subunits was estimated by SDS-PAGE using a vertical gel electrophoresis system (model V-16, Gibco-BRL, Rockville, MD, USA). Purified enzyme was mixed in a ratio of 1:1 with sample buffer (10 ml glycerol, 5 ml 2-mercaptoethanol, 30 ml 10% SDS, 12.5 ml 0.5 M Tris buffer pH 6.8 and 100 mg bromophenol blue in a total volume of 100 ml). Reference pro-

teins (rabbit muscle myosin, 205 kDa; *E. coli* β-galactosidase, 116 kDa; rabbit muscle phosphorylase b 97.4 kDa; bovine albumin, 66 kDa; egg albumin, 45 kDa and bovine erythrocyte carbonic anhydrase 29 kDa) were used according to the instructions provided by the manufacturer (Sigma Chemical Company). High molecular weight kit, Cat. No. SDS-6H). Purified enzyme with sample buffer and reference proteins were boiled for 3–5 min and applied to the 10% SDS-PAGE with 5% stacking gel. After running the gel, the proteins were stained by Coomassie brilliant blue (acetic acid 100 ml, methanol 400 ml, water 500 ml and Coomassie brilliant blue 1 g) for 8–10 h.

Results and discussion

Purification of β-galactosidase

A typical purification of *β-galactosidase* from *Bacillus* sp MTCC 3088 is summarized in Table 1. The crude extract obtained after centrifugation, was subjected to fractional precipitation with $ZnCl_2$. This step yielded a 1.2-fold purification with a specific activity of 1441 units per mg protein. The $ZnCl_2$ precipitate was chromatographed on a Q-Sepharose column and β-galactosidase activity was eluted between 0.38 and 0.81 M NaCl. The fractions from the Q-Sepharose column with high β-galactosidase activity were pooled and concentrated by lyophilization. The Q-Sepharose step yielded a purification factor of 3.7 with a specific activity of 4504 units per mg protein while the percentage recovery was 42 (Table 1). It was subjected to further purification by hydrophobic interaction chromatography. The major peak on hydrophobic interaction column was eluted between 1.48 and 0.56 M ammonium sulphate. By this procedure β-galactosidase was purified approximately 10.2-fold from the culture filtrate with a specific activity of 12542 units per mg protein where the percentage recovery was 28.3 (Table 1). It was then subjected to further purification using a Sephadex G-200 column. The major peak was eluted with phosphate buffer (0.1 M, pH 7) containing 0.15 M NaCl. By this procedure β-galactosidase was purified approximately 36.2-fold from the culture filtrate with a specific activity of 44399 units per mg protein while the percentage recovery was 12.7 (Table 1).

Characterization of β-galactosidase

The β-galactosidase was purified to homogeneity as indicated by native polyacrylamide gel electrophoresis with Coomassie brilliant blue 250 (Figure 1a). Enzyme-activity staining demonstrated a single band superimposable on that of protein staining as shown in Figure 1a. In Figure 1a, lane 1 shows a single band of β-galactosidase with activity staining, lane 2 shows the result of Coomassie blue staining. The use of X-gal as chromogenic substrate was more effective in demonstrating activity staining than ONPG, owing to poor yellow color resulting from ONPG hydrolysis. The relative native molecular weight of the purified enzyme was estimated by gel filtration chromatography on a Sephadex G-200 column. The molecular mass of the purified enzyme was about 484 kDa. This value is particularly high, but Ulrich *et al* [29] reported a molecular mass of 570 kDa for the β-galactosidase of *Thermus* T-2. Wide variations in molecular masses of β-galactosidase from microbial sources have been reported [1]. Among β-galactosidases from thermophiles, molecular masses of 150, 240, 440 and 700 kDa were reported by Berger *et al* [1]. The presence of isoenzymes has been noted for *Thermoanaerobacter* [13], *Bacillus subtilis* [23] *B. circulans* [17] and some *Bifidobacterium* species [1]. Hirata *et al* [10] described three isoenzymes for *B. stearothermophilus* having molecular masses of 120, 95 and 70 kDa, whereas *Kluyveromyces lactis* contained four isoenzymes with molecular masses of 630, 550, 41 and 19 kDa [15]. Variations have also been encountered with the β-galactosidase from *E. coli*, which is a tetramer composed of four identical subunits (135 kDa) with molecular masses of about 540 and 747 kDa for β-galactosidase from *E. coli* ML 308 [1].

The molecular masses of the subunits of β-galactosidase from *Bacillus* sp MTCC 3088 were estimated to be 115, 86.5, 72.5, 45.7 and 41.2 kDa by sodium dodecyl sulfate-

Table 1 Summary of purification of β-galactosidase activity of *Bacillus* sp MTCC 3088

Purification step	Total protein (mg)	Total activity ($IU \times 10^5$)	Specific activity ($IU\ mg^{-1}$)	Activity recovery (%)	Fold purification
Crude extract	2891	35.5	1227	100	1
$ZnCl_2$ precipitation	2112	30.4	1441	85.8	1.2
Q-Sepharose	331	15	4504	42	3.7
Hydrophobic interaction	80	10	12542	28.3	10.2
Sephadex G-200	10	4.5	44399	12.7	36.2

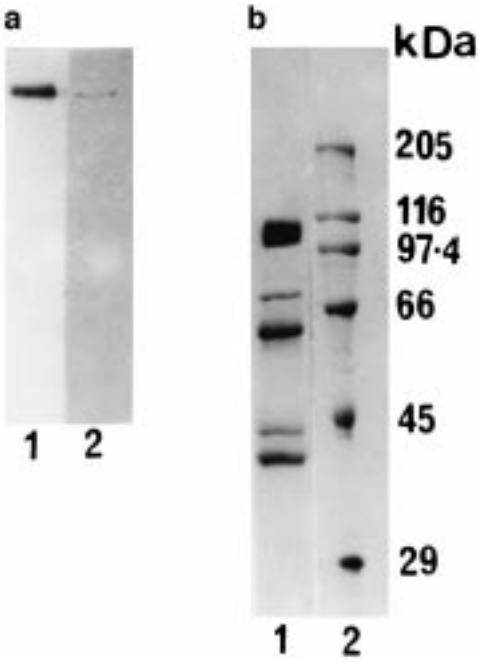

Figure 1 (a) Native PAGE pattern of β-galactosidase activity of *Bacillus* sp MTCC 3088. Lane 1, activity staining with X-gal; lane 2, Coomassie blue staining of purified enzyme. (b) SDS-PAGE pattern of purified β-galactosidase activity of *Bacillus* sp MTCC 3088. Lane 1, subunits of purified β-galactosidase; lane 2, marker proteins.

polyacrylamide gel electrophoresis (Figure 1b, lane 1). The native relative molecular mass of the purified enzyme was about 484 kDa by gel filtration chromatography. In comparison, the sum of the five subunits gave 360.9 kDa. Many β-galactosidases containing numerous subunits with various molecular masses have been described [1]. Dumortier *et al* [6] reported the native relative molecular mass of the purified β-galactosidase of *Bifidobacterium bifidum* was about 362 kDa by gel filtration chromatography; in comparison, the sum of the four subunits (163, 170, 178 and 190 kDa) was 690 kDa. Following isoelectric focusing gel electrophoresis, a single protein band with a pI of 6.2 was obtained (data not shown), which is within the range of values reported for β-galactosidases from other thermophilic bacteria [5,13,22]. Figure 2a shows the optimum pH for purified β-galactosidase to be 8. This pH optimum, under the conditions used, was the same as that reported for β-galactosidase from other mesophilic microorganisms. The enzyme lost 2% of its maximum activity at pH 7.5 and at pH 8.5, it lost only 24% of its maximum activity. The broad pH optima (7–8) is suitable for the application of β-galactosidase in different fields including the food industry. The optimum temperature for maximum enzyme activity was 60°C (Figure 2b). The enzyme activity declined very sharply above 60°C. Nearly complete inactivation occurred at 70°C. From 30°C to 60°C, the enzyme had a steep rise of activity. At 55 and 65°C, the enzyme exhibited 74 and 34% of its maximum activity, respectively.

Kinetic constants

Kinetic parameters like maximum reaction velocity (V_{max}) and kinetic constant (K_m) were determined for purified β-galactosidase with respect to its artificial and natural substrates ONPG and lactose, respectively, at 60°C by Lineweaver–Burk plots. Under the conditions (60°C, pH 7), β-galactosidase exhibited Michaelis–Menten type kinetics. The kinetic constant (K_m) measured for *o*-NO_2-phenyl-β-D-galactopyranoside was 6.34 mM and V_{max} was found to be 9351 IU ml^{-1} (Figure 3a). The K_m value was less than that of alkalophilic and thermophilic *Bacillus* sp TA-11 (13.5 mM) and was higher than that of *Bacteroides polypragmatus* (0.43 mM) and *Thermus aquaticus* (2 mM) [1,4,7]. No inhibition of enzyme activity was observed with ONPG levels up to 14 mM (Figure 3a). The K_m and V_{max} values of purified β-galactosidase activity towards lactose are 6.18 mM and 909×10^{-5} IU ml^{-1}, respectively (Figure 3b).

Substrate specificity

The relative hydrolytic activities with various *p*-NO_2-phenyl glycosides were compared by measuring ONPG

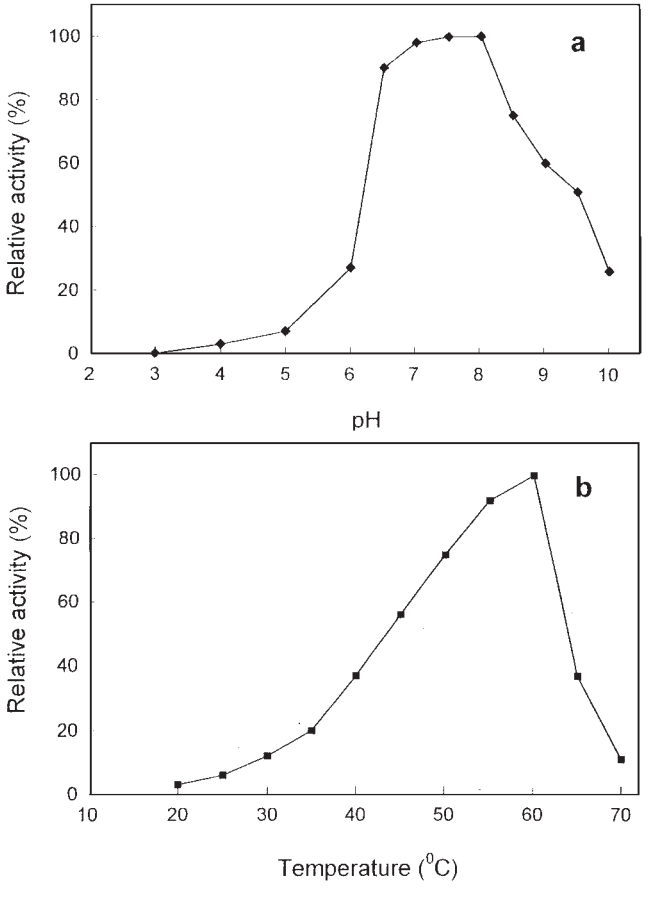

Figure 2 Effect of pH (a) and temperature (b) on β-galactosidase activity of *Bacillus* sp MTCC 3088.

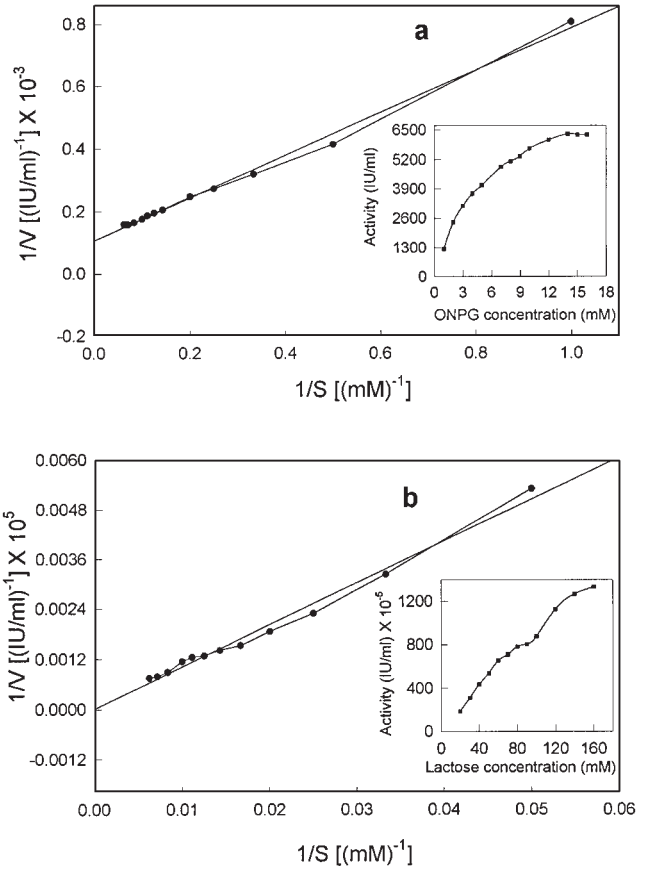

Figure 3 Lineweaver–Burk plot of β-galactosidase activity of *Bacillus* sp MTCC 3088. Insert: activity profile of β-galactosidase with respect to ONPG concentration (a) and with respect to lactose concentration (b).

Table 2 Substrate specificity of β-galactosidase activity of *Bacillus* sp MTCC 3088

Substrate	Hydrolytic activity (%)
o-NO_2-phenyl-β-D-galactopyranoside	100
p-NO_2-phenyl-β-D-galactopyranoside	95
p-NO_2-phenyl-α-L-arabinopyranoside	64
p-NO_2-phenyl-β-L-arabinopyranoside	0
p-NO_2-phenyl-α-D-mannopyranoside	0
p-NO_2-phenyl-α-L-fucopyranoside	0
p-NO_2-phenyl-α-D-galactopyranoside	0
p-NO_2-phenyl-α-D-glucopyranoside	0

hydrolysis (Table 2). The enzyme catalyzed not only the hydolysis of *o*-NO_2-phenyl-β-D-galactopyranoside but also those of *p*-NO_2-phenyl-β-D-galactopyranoside (95%) and *p*-NO_2-phenyl-α-L-arabinopyranoside (64%). No hydrolytic activity was observed against *p*-NO_2-phenyl-β-L-arabinopyranoside, *p*-NO_2-phenyl-α-D-mannopyranoside, *p*-NO_2-phenyl-α-L-fucopyranoside, *p*-NO_2-phenyl-α-D-galactopyranoside and *p*-NO_2-phenyl-α-D-glucopyranoside. Hydrolysis of different substrates (Table 2) was studied to determine the enzyme behavior towards β-D-anomeric linkages. The enzyme showed a strict specificity for this linkage, which has galactose or glucose in the glycone form. Moreover, specificity for the aglycone moiety has a drastic effect on the enzyme activity. Para-substitution on the phenolic ring of nitrophenol showed a positive effect on enzyme activity. Replacement of the substituted nitro phenolic group with a naphthyl group prevented enzymic activity. The effect of different carbohydrates on β-galactosidase activity is shown in Table 3. All sugars were used at a final concentration of 4 mg ml^{-1}. Catalytic activity was not affected by glucose, maltose, lactose, sucrose, starch, xylose, inositol and sorbitol. Enzyme activity was strongly inhibited by galactose. Product (galactose) inhibition has been described previously for the β-galactosidase from *Thermus* 4-1A [5]. However, an increase in β-galactosidase activity in the presence of glucose has been reported for *Thermoanaerobacter* [13] and *B. subtilis* [23]. The β-galactosidase from *S. solfataricus* was not inhibited by galactose or glucose [1] and the enzyme from *Crytococcus laurentii* was not inhibited by galactose [1]. The inhibition by glucose and galactose is believed to be due to their action as competitors at the catalytic site of the enzyme.

Table 3 Effect of different carbohydrates on purified β-galactosidase activity of *Bacillus* sp MTCC 3088

Carbohydrates (4 mg ml^{-1})	Relative activity (%)
Glucose	98
Galactose	32
Maltose	95
Lactose	98
Sucrose	100
Starch	100
Xylose	100
Inositol	101
Sorbitol	100
Fructose	101

Table 4 Effect of metal ions and EDTA on β-galactosidase activity of *Bacillus* sp MTCC 3088

Metal ions	Percentage activity retained at concentration (mM)					
	1	2.5	5	10	20	25
Cu^{2+}	9	6	ND	5	ND	ND
Zn^{2+}	95	85	84	46	34	ND
Co^{2+}	95	103	97	96	52	37
Ni^{2+}	56	58	52	36	16	12
Mg^{2+}	118	142	141	141	127	104
Ca^{2+}	104	111	109	97	79	57
Mo^{2+}	95	98	109	98	97	92
Mn^{2+}	88	114	95	73	24	48
Hg^{2+}	1	0	0	0	0	0
Ag^{+}	2	6	4	0	0	0
Fe^{2+}	88	94	83	28	ND	5
EDTA	96	99	101	100	100	98

Activity is expressed as a percentage of the activity in the absence of chemicals.
ND, not detected.

Effect of metal ions and EDTA

Table 4 shows the effect of metal ions and EDTA on β-galactosidase activity. Hg^{2+} and Ag^{+} almost completely inhibited activity at 1 mM. Ca^{2+} and Mo^{2+} showed no inhibition even at higher concentration (1–10 mM). Mg^{2+} at higher concentration (2.5–10 mM) increased enzyme activity by 41–42%. It has been observed that Cu^{2+} has a strong inhibitory effect on β-galactosidase even at a lower concentration, of 1 mM. Mn^{2+} also has an inhibitory effect at higher concentration (20 mM), while Fe^{2+} has no strong inhibition at concentrations up to 2.5 mM. Zn^{2+} and Ni^{2+} at 20 mM showed 66 and 84% inhibition of enzyme activity, respectively. EDTA at concentrations up to 25 mM did not inhibit enzyme activity. Metal ion inhibition studies have also been carried out for β-galactosidase activity from other microbial sources. The strong inhibitory effect of most of the metal ions except Mg^{2+} and Mo^{2+} and no inhibition of activity by EDTA suggested that the β-galactosidase from *Bacillus* sp MTCC 3088 does not require metal ions for its activity. It is reported [3] that β-galactosidase from *Kluyveromyces lactis* required K^{+} and Mg^{2+} for stability. Hg^{2+} was found to cause marked inhibition of β-galactosidase activity irrespective of the sources as reported by various workers.

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