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Production and Partial Characterization of a Novel β-galactosidase from a Newly Isolated *Bacillus polymyxa*

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Bacillus polymyxa, isolated from the waters of the hot spring (Manikaran, India), has a great potential for the production of a novel extracellular β-galactosidase. Optimization of culture conditions and media components revealed that meat extract is essential for the production of β-galactosidase. The organism produced a significant level of enzyme activity (12.6×10^3 IU/mL) at the later stages of growth (16 d) in shake flasks. An initial culture showed that pH of 7 and temperature of 37°C were optimum conditions for growth and enzyme production by *B. polymyxa*. Among the various carbon and nitrogen sources used, lactose and meat extract were the most effective for enzyme yield. The specific growth rate of the organism was found to be 0.01/h in the bioreactor with an optimized medium. The enzyme had an optimum pH of 7 and an optimum temperature of 60°C. The enzyme was highly stable at 50°C with a half-life of 17 d. At 60°C, the thermo stability of β-galactosidase was less than that at 50°C. At higher temperatures (65 and 70°C), the enzyme was not stable. The Michaelis-Menten constants were determined with respect to o-nitrophenyl-β-D-galactopyranoside and lactose were 4.72 and 36.31 mM, respectively. The inhibition constant for galactose was 2.83 mM. Lactose (10-50 g/L) and whey (lactose concentration, 18 g/L) were hydrolyzed with expedient efficiencies with this enzyme (activity 4.247×10^3 IU/mL). The activation and deactivation energies of the partially purified enzyme were 6.30 and 20.6 J/mol, respectively. Different metal ions (Cu^{2+}, Fe^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+} and Ag^{+}) inhibited the enzyme activity in a 2.5 to 25 mM concentration range. The chelating agent, EDTA, did not affect catalytic activity.

INTRODUCTION

The hydrolysis of lactose is of interest from a nutritional, technological and environmental viewpoint. β-D-galactoside galactohydrolase (EC. 3.2.1.23), which converts lactose to its constituents, glucose and galactose, by hydrolyzing β-1,4-D-galactosidic linkages, is widely distributed in nature [1-4]. β-galactosidases are used to prevent lactose crystallization in dairy food products, in order to get lactose free milk for those with lactose intolerance and for cheese whey utilization to control environmental pollution [5,6]. For industrial use, β-galactosidases, from microbial sources like bacteria, yeast and fungi, are found to be the best [4,7]. The fungal species, which have been most frequently used, are *A. niger* and *A. oryzae* [8-10]. However, fungal fermentation has its own disadvantages. Most work has been carried out on β-galactosidases produced by *Kluyveromyces lactis* and *Kluyveromyces fragilis* [11-13]. Although β-galactosidase has been commercially available for many years, little information has been published in literature, regarding its production from a bacterial source. To use this enzyme efficiently, Hirata et al. [14] enhanced fifty times higher production by cloning the β-galactosidase gene of *Bacillus stearothermophilus IAM11001 (ATCC 8005)* into *Bacillus subtilis*. Machida et al. [15] showed the over-production of β-galactosidase in active form by an *Escherichia coli* system, co-expressing the chaperonin GroEL/ES. In view of the tremendous industrial potential of β-

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galactosidase, it is desirable to study this enzyme from different microbial sources. The culture conditions and the nature of nutrients in the culture media are known to affect the growth and enzyme production by various microorganisms [16-18]. In the present study, a bacterium, *Bacillus polymyxa*, with hyper-producing β-galactosidase has been isolated from the extreme environment of a hot spring. This paper describes the effect of culture conditions and media components on the production of β-galactosidase and the partial characterization of this enzyme from this organism.

MATERIALS AND METHODS

Chemicals

o-Nitrophenyl-β-D-galactopyranoside (ONPG), EDTA and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside) were purchased from the Sigma Chemical Company (MO, USA). All other chemicals used were of an analytical grade.

Microorganism and Cultivation Conditions

The microorganism studied in this report was screened from the waters of the hot spring (Manikaran, India) and maintained on nutrient agar plates containing 1% lactose. The organism used for the production of β-galactosidase was identified as *Bacillus polymyxa* by the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The organism was deposited under the accession no. MTCC 3088. The medium used for the production of β-galactosidase had the following composition (g/L): 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. Different sets of 500 mL Erlenmeyer flasks, each containing 100 mL of production medium, were inoculated with 10 mL inoculum and incubated in a temperature-controlled (37°C) shaker at 200 rpm. Duplicate flasks were taken out each day and processed for growth and enzyme activity and residual substrate concentration. Different carbon sources (lactose, glucose, sucrose, fructose, maltose and starch) and organic nitrogen sources (yeast extract, meat extract, tryptone, beef extract, soyatose, malt extract, soyabean meal, soyapeptone, casein and glycine) were used at 10 and 15 g/L concentration, respectively. The growth and production of β-galactosidase by *B. polymyxa* were carried out in a 6.2 L bioreactor (LKB Bromma, Sweden,). The fermentation was carried out with varied concentrations (10, 20 and 30 g/L) of glucose and 0.5% meat extract, instead of 1.5% as used in the shake flasks.

Assay Methods

β-galactosidase activity was assayed according to the method of Nagano et al. [19] by using ONPG as substrate. The reaction mixture contained 2 mL ONPG (2 mM in 0.1 M potassium phosphate buffer pH 7) and 1 mL appropriately diluted enzyme solution. The reaction was allowed to proceed for 20 min at 60°C and stopped by adding 2 mL of 1 M Na_2CO_3 solution. The absorbance was measured at 416 nm. Appropriate enzyme and substrate blanks were included. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 nmol o-nitrophenol per min. Further, in order 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). The thermostability and pH stability of β-galactosidase activity were examined by incubating the enzyme solution at different temperatures (50, 55, 60, 65 and 70°C) and pHs (3-10) and assaying the enzyme activity at a regular interval of time. Extracellular protein was measured using Lowry's method, with bovine serum albumin as the standard. Total reducing sugar concentration was measured as an equivalent of glucose by using the dinitrosalicylic acid method.

Determination of Kinetic Constants

Kinetic constants of β-galactosidase were determined with respect to its artificial and natural substrates ONPG and lactose, respectively, at 60°C. ONPG, at concentrations of 0 to 10 mM in a 0.1 M phosphate buffer (pH 7), containing 100 μL of enzyme solution, was used. The reaction mixture was incubated at 60°C for 20 min and the release of o-nitrophenol was measured. Lactose, at concentrations of 0 to 200 mM in a 0.1 M, phosphate buffer (pH 7) containing 1.2 mL enzyme solution, was used. Aliquots (0.5 mL) were removed and the reaction was stopped by boiling the reaction mixture for 5 min. The release of glucose was measured by the glucose oxidase/peroxidase method. The kinetic constants of β-galactosidase were determined using lactose and galactose as the substrate and inhibitor, respectively. One unit of β-galactosidase activity was defined as the amount of enzyme, which released 1 μmol of glucose per min at 60°C. K_m and V_{max} values against ONPG and lactose were calculated by using Lineweaver-Burk plots. For determining the galactose inhibition constant, lactose and galactose solutions were used at a concentration ranging from 0-200 and 0-50 mM, respectively, in a 0.1 M phosphate buffer (pH 7), containing 1 mL of enzyme solution.

Hydrolysis of Lactose and Whey

β-galactosidase with varied activity was added to different concentrations (10 - 50 g/L) of lactose for hydrolysis in 250 mL flasks containing 50 mL of reaction mixture. The final concentration of phosphate buffer was 0.1 M (pH 7). The flasks, in duplicate, were incubated at 60°C (200 rpm). Aliquots (0.5 mL) were removed at regular intervals and the glucose produced was measured by the glucose oxidase/peroxidase method. Finally, the percentage of hydrolysis was calculated in terms of the percentage of maximum efficiency. Whey was obtained from a local dairy plant. The chemical composition of the whey was: lactose (total reducing sugar) 18 g/L and protein 0.438 g/L (pH 7). The sterile whey was used for hydrolysis by β-galactosidase in a 250 mL flask, containing 35 mL of whey and the reaction mixture volume was made up to 70 mL by enzyme solution, distilled water and a phosphate buffer. The final concentration of phosphate buffer was 0.1 M (pH 7). The flasks, in duplicate, were incubated at 60°C in a shaker (200 rpm). Aliquots (0.5 mL) were removed at regular intervals and the glucose produced was measured by the glucose oxidase/peroxidase method.

RESULTS AND DISCUSSION

Screening and Culture Conditions of the β-galactosidase Producing Organism

Various soil and liquid samples were collected from a hot spring (Manikaran, India) and screened for β-galactosidase producing organisms. Screening was done on nutrient agar plates containing (g/L) lactose, 10; Triton X-100, 1 and X-Gal, 0.02. The β-galactosidase producing colonies developed a blue-green colour, due to the released dye i.e., 5-bromo-4-chloro indigo [20]. The selected strain was maintained at the same medium without X-Gal and Triton X-100. Varying growth temperatures, at 3°C intervals between 34 and 43°C, had little effect on cell mass and enzyme yield, although optima were observed at 37°C. This temperature was used for all subsequent experiments. Varying the initial cultivation pH of the medium between pH 6 and 8, with intervals of 0.5 units, was found to have an effect on both cell growth and enzyme production by *Bacillus polymyxa* (data not shown). The rate of growth of the organism was highest when the initial pH of the cultivation medium was adjusted to 6.5 and, at this pH maximum, cell mass was also obtained. Maximum β-galactosidase activity was obtained when the initial pH of the cultivation medium was adjusted to pH 7 (data not shown). It is, thus, evident that growth of cell mass and β-galactosidase production took place at two different pHs.

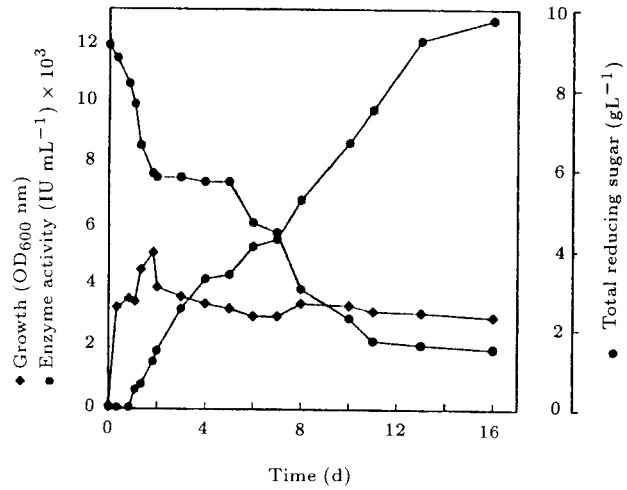

Figure 1. Course of cultivation of *Bacillus polymyxa* during the production of β-galactosidase.

Course of Cultivation in a Shake Flask

Figure 1 shows the course of cultivation of *Bacillus polymyxa* for the production of β-galactosidase in shake flask. In the early hours of fermentation, active cell growth took place up to nearly 2 d and then started decreasing. β-galactosidase activity started increasing after 1 d of fermentation and continued to increase up to 12 d. The cell mass concentration decreased up to 7 d of fermentation and then a slight increase in growth was observed. From 8 d onwards, the cell mass concentration started increasing with an increase in enzyme activity and a decrease in residual sugar concentration. It is well known that β-galactosidase breakes down lactose to glucose and galactose and, most probably, utilization of the hydrolyzed products of lactose started from 8 d onwards. It may be that secretion of β-galactosidase at the later stages of growth was higher compared to early stages of fermentation. Cell lysis may take place with the secretion of enzyme. Total residual sugar (as equivalent of glucose) concentration started decreasing from the very beginning. The rate of lactose utilization was highest during the exponential phase of the growth.

Effect of Carbon and Nitrogen Sources

Figure 2 shows the effect of different carbon sources on the production of β-galactosidase by *Bacillus polymyxa*. It is clear from the figure that the rate of enzyme production was faster with lactose than with any other carbon source used. *B. polymyxa* produced a low amount of β-galactosidase in the media containing starch or sucrose, in comparison to lactose, glucose, fructose and maltose. Brady et al. [21] also reported a higher level of β-galactosidase activity by *Kluyveromyces marxianus* IMB3, when 2% (w/v) lactose was used in the fermentation media. Lactose

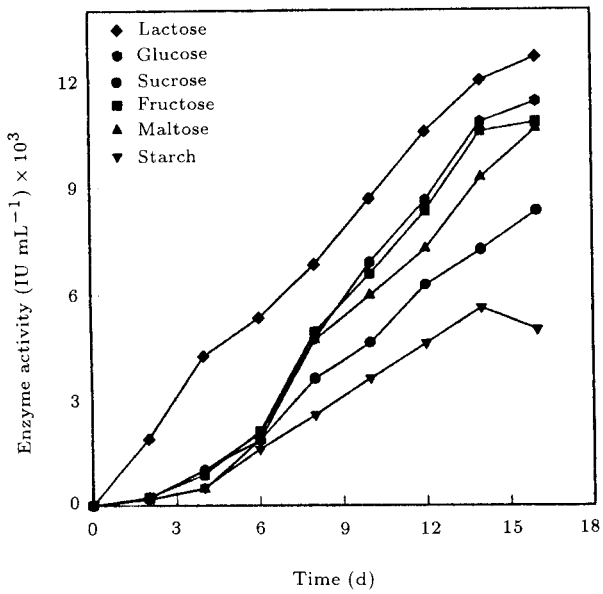

Figure 2. Effect of carbon sources on the production of β-galactosidase by *Bacillus polymyxa*.

was the most effective carbon source for β-galactosidase synthesis, giving a maximum enzyme activity of 12600 IU/mL. Glucose may be used as an alternative carbon source for the production of β-galactosidase. Of the various organic nitrogen sources, meat extract was found to be most effective in promoting β-galactosidase synthesis (Figure 3). Maximum enzyme activity of 12600 IU/mL was obtained when meat extract was used in the medium. With tryptone or beef extract, a considerable amount of enzyme activity was also obtained. Soyabean meal and soyapeptone, which are cheaper nitrogen sources than peptone, were both satisfactory, but meat extract yielded more consistent and higher activity and was, therefore, used in subsequent experiments.

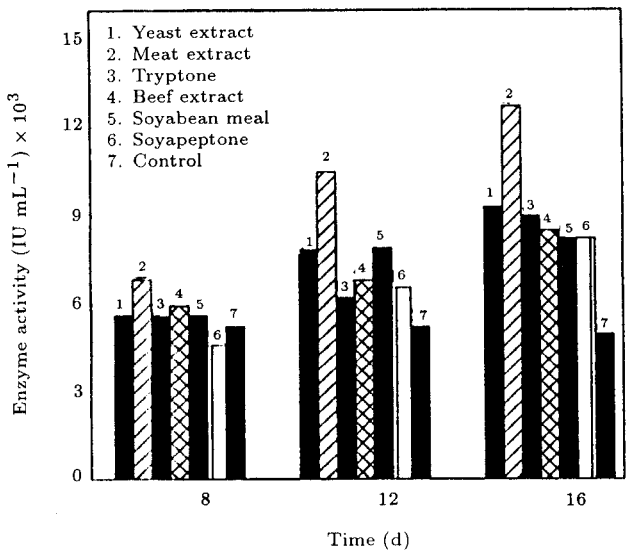

Figure 3. Effect of organic nitrogen sources on the production of β-galactosidase by *Bacillus polymyxa*.

Course of Cultivation in the Batch Reactor

The course of cultivation of *B. polymyxa* for the growth and production of β-galactosidase in a 6.2 L stirred tank reactor, with 10 g/L glucose, is shown in Figure 4. Although in shake flask studies, it was shown that lactose as a carbon source yielded maximum enzyme activity, due to easy availability and less expense, glucose was used as a carbon source in the reactor. It is evident from the figure that the growth of the organism took place from the very beginning, while production of β-galactosidase activity started from 40 h of cultivation. Residual glucose concentration became zero at 80 h of fermentation. The rate of consumption of glucose was very rapid during the initial phase of fermentation and then slowed down before becoming almost zero. It is interesting to note that the synthesis of β-galactosidase started immediately after glucose concentration reduced to zero in the medium. Increase of β-galactosidase activity after the maximum cell mass synthesis might be due to the release of enzyme from the cells of *B. polymyxa*, due to lysis. The specific growth rate of the organism was found to be 0.01/h. At this phase, the pH of the environment was alkaline (8.5) and was amenable to cell lysis.

The synthesis of β-galactosidase started only when the glucose concentration in the reactor became low, glucose being one of the products of β-galactosidase action on its natural substrate, lactose. As the glucose concentration in the reactor increased from 10 to 30 g/L, the enzyme synthesis delayed (data not shown). Most probably, this may be due to the repression of β-galactosidase synthesis by glucose.

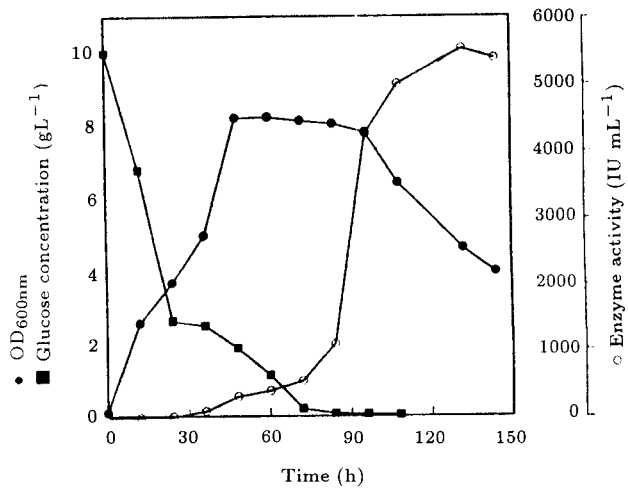

Figure 4. Course of cultivation of *Bacillus polymyxa* during the production of β-galactosidase in a stirred tank reactor (meat extract, 5 g/L; glucose, 10 g/L).

Partial Characterization of β-galactosidase

Effect of pH and Temperature

Figure 5a shows the pH optimum for β-galactosidase activity is 7. This pH optimum, under the conditions used, was the same as reported for β-galactosidase from other mesophilic microorganisms [22]. The enzyme lost 10% of its original activity at pH of 7.5 and, only 20% at pH of 8.5. The broad pH optima (6.5-7.5) is most suitable for the application of β-galactosidase in different fields, including application in the food industry. On the acidic side of the pH activity profile, the activity reduced and on the alkaline side, the enzyme was more stable. The enzyme was found to be very stable at the pH range of 5-10 (data not shown). The enzyme was stable for more than a month when kept at 4°C at pH of 5. On the alkaline side, the enzyme was found to be very stable at 4°C.

The effect of temperature on β-galactosidase activity was studied in the range of 20 to 70°C. The optimum temperature for maximum enzyme activity was 60°C (Figure 5b). The enzyme activity declined very sharply at temperatures above 60°C. The results in this paper corroborate earlier findings, in which optimal temperature and pH for β-galactosidase activity from mesophilic microorganisms were 55-65°C and 6-7, respectively [22,23]. The near complete inactivation of enzyme occurred at 70°C. From 30°C up to 60°C, the enzyme had a steep rise of activity. At 55 and 65°C, the enzyme exhibited 83% and 94% of its maximum activity, respectively. Figure 6 shows the thermostability of partially purified β-galactosidase at

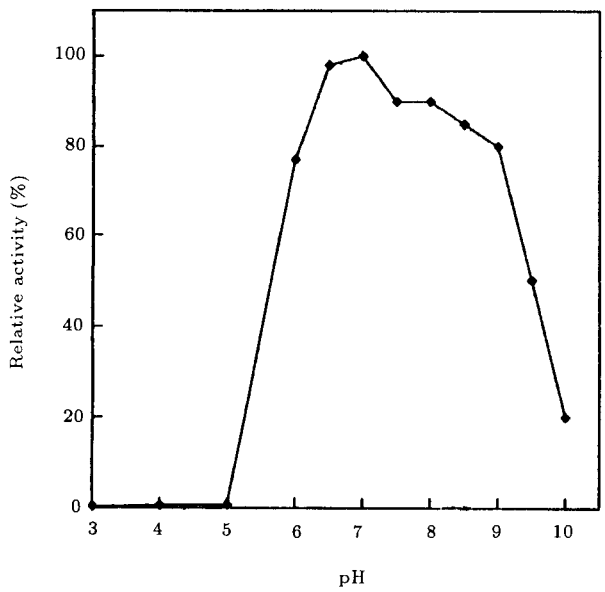

Figure 5a. Effect of pH on β-galactosidase activity of *Bacillus polymyxa* (enzyme activity was measured at different pHs (3-6) with ONPG as the substrate at an incubation temperature of 60°C).

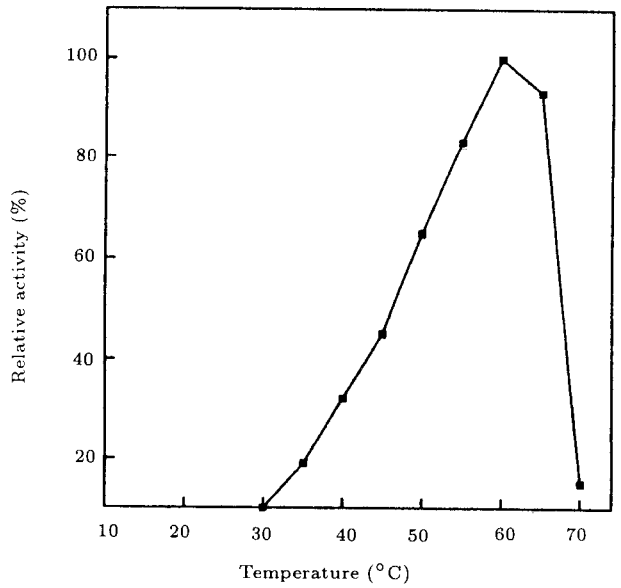

Figure 5b. Effect of temperature on β-galactosidase activity of *Bacillus polymyxa* (enzyme activity was measured at different temperatures (50, 55, 60, 65 and 70°C) with ONPG as the substrate at pH 7).

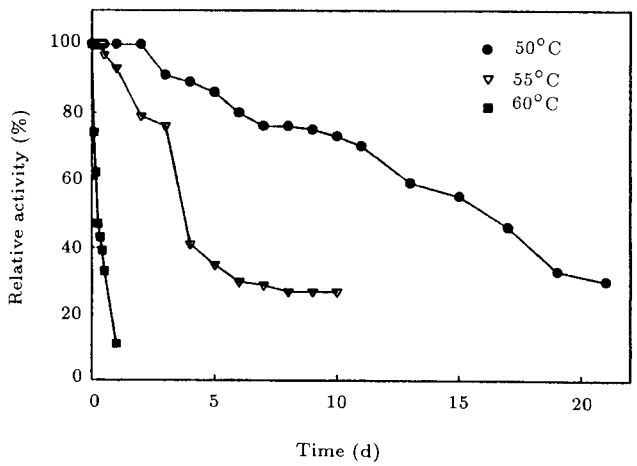

Figure 6. Thermostability of β-galactosidase activity of *Bacillus polymyxa* at various temperatures.

different temperatures. The half life of β-galactosidase at 50°C was 17 d. At this temperature, nearly 100% activity was retained up to 3 d of incubation, followed by a rapid decline. In this type of biphasic event, the first phase (a 3 d lag period) has an apparent half-life approaching infinity and the next phase appears to have a half-life of 12 d. The overall half-life of the enzyme was found to be 17 d, while the β-galactosidase produced by *Kluyveromyces marxianus* has a half-life of 5 min at 50°C [21]. At 55°C, the enzyme activity started declining from the very beginning up to the 2nd day of incubation and it lost only 5% of its maximum activity during this period. After that, a rapid decline of enzyme activity started and continued up to 8 d. At 55°C, the rate of enzyme

inactivation was higher compared to that at 50°C. At this temperature (55°C), the enzyme exhibited a half-life of 5 d. At 60°C, the rate of loss of enzyme activity was more, compared to that at 50 and 55°C. At 60°C, the enzyme had a half-life of 8 h. The activation and deactivation energies of the partially purified enzyme were 6.30 and 20.6 Joule/mol, respectively (data not shown).

Kinetic Studies

The kinetic parameters for β-galactosidase, such as maximum reaction velocity (V_{max}) and kinetic constant (K_m), were determined using an artificial (ONPG) substrate. The Lineweaver-Burk plots were used for the determination of kinetic constants. Under the conditions (60°C, pH 7), β-galactosidase exhibited Michaelis-Menten type kinetics. The kinetic constant (K_m), measured for o-nitrophenyl-β-D-galactopyranoside, was 4.72 mM and V_{max} was found to be 26313 IU/mL. The K_m value was less than that of (13.5 mM) alkalophilic and thermophilic *Bacillus sp.* TA-11 [22] and was higher than that of (2 mM) *Thermus aquaticus* [24]. No inhibition of enzyme activity was observed up to a 14 mM substrate (ONPG) concentration. The K_m and V_{max} values of β-galactosidase activity towards lactose are 36.31 mM and 656×10^{-5} IU/mL, respectively. Lineweaver-Burk plots show (Figure 7) that the hydrolysis of lactose in the presence of galactose at four different concentrations (10, 20, 30 and 50 mM) affect the apparent $K_{m'}$, but leaves the maximum velocity unchanged, typical of competitive inhibition. The rate equation is defined as:

$$V = V_{max}[lac]/(K_{mapp} + [lac]),$$

with:

$$K_{mapp} = K_m(1 + [gal]/K_{igal}).$$

[lac] and [gal] are lactose and galactose concentrations, K_{mapp} are the Michaelis-Menten constants determined at different galactose concentrations and K_{igal} is the galactose inhibition constant. A straight line is obtained between K_{mapp} and galactose concentrations, as shown in Figure 7 (inset), the slope of which gives the K_{igal} value. From the slope, the K_{igal} value was calculated to be 2.83 mM.

Hydrolysis of Lactose and Whey

The hydrolysis of lactose by β-galactosidase is shown in Figure 8a. In these experiments different concentrations (10-50 g/L) of lactose were used for hydrolysis with β-galactosidase (two different activities were used). In the case of 10 g/L lactose, 96% hydrolyzing efficiency was obtained at 48 h when enzyme activity was 4.268×10^3 IU/mL, while percentage of efficiency decreased when enzyme activity was just half of the former. The rate of hydrolysis is also different at different enzyme concentrations. A similar situation arose when the hydrolysis experiment was carried out with 20 g/L of lactose. As lactose concentration increased to 30 g/L, only 54% efficiency was observed with the

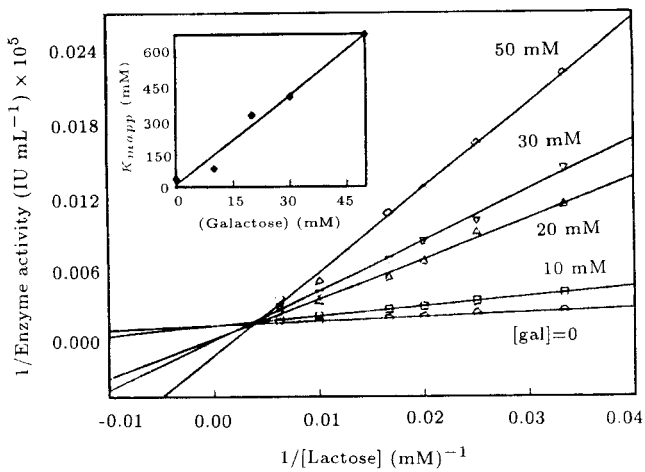

Figure 7. Lineweaver-Burk plot showing competitive inhibition by galactose. Determination of the Michaelis constant of activity towards lactose and galactose inhibition constant (inset). For the determination of galactose inhibition constant, galactose at a varied concentration (0-50 mM) was used in the reaction mixture and the activity was measured by the same method.

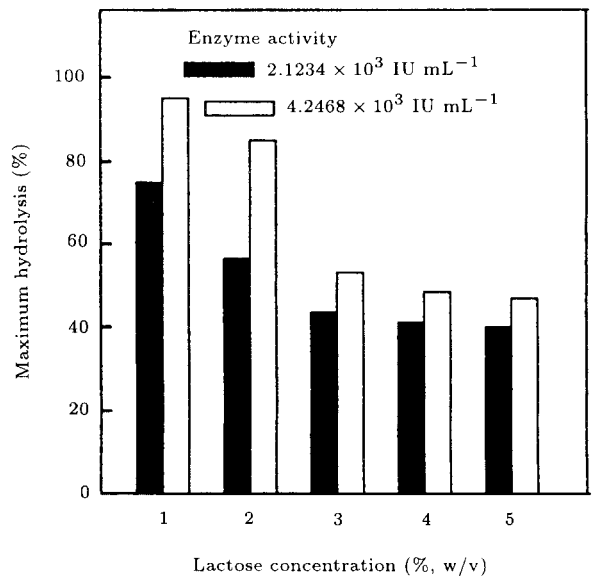

Figure 8a. Lactose hydrolysis by β-galactosidase of *Bacillus polymyxa* (hydrolysis experiments were carried out in 250 mL flask having 50 mL medium at 60°C and 200 rpm with two different concentrations of enzyme solution).

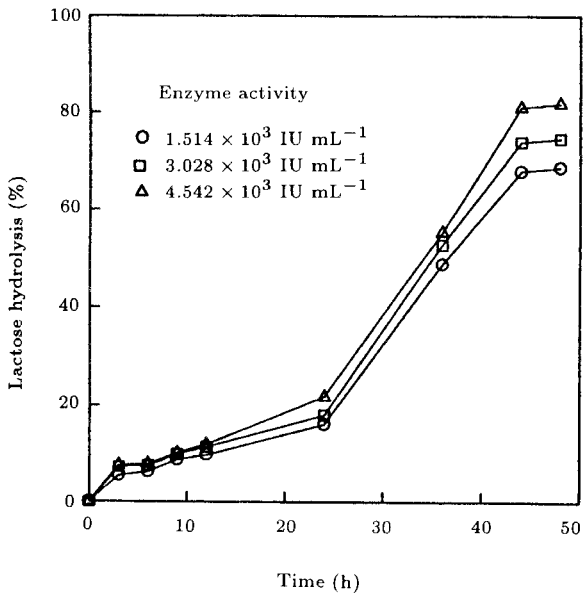

Figure 8b. Whey hydrolysis by β-galactosidase of *Bacillus polymyxa* (hydrolysis experiments were carried out in 250 mL flask having 35 mL sterile whey at 60°C and 200 rpm with varied concentrations of enzyme solution).

same amount of enzyme. This might be due to the inhibition of β-galactosidase by galactose, a product of hydrolysis, or might be caused by the substrate. The inhibition of enzyme activity did not increase with the increase of substrate concentration from 30-50 g/L. Cavallie and Combes [25] reported the hydrolysis of lactose from *Kluyveromyces lactis*, where 9.2 mg/L lactose was completely hydrolyzed in 1 h at 37°C. They found that the glucose and galactose appeared rapidly, however, the glucose concentration was more than that of galactose within 30 min of incubation. The difference of concentration was explained by the formation of two by-products which, after 30 min of incubation, were consumed and galactose concentration reached the same as that of glucose. This behavior was explained to be due to transgalactosylation activity of β-galactosidase.

The hydrolysis of whey by β-galactosidase is shown in Figure 8b. It is observed from the figure that the rates of whey hydrolysis are not significantly affected by the amount of enzyme added. With 1.514×10^3 IU/mL enzyme activity, maximum percentage of hydrolysis obtained was 70%, while the percentages of hydrolysis (78 and 84%) were increased when 3.028×10^3 and 4.542×10^3 units of enzyme were used, respectively. In all cases, it was noticed that the rates of hydrolysis were less up to 25 h of incubation and then progressed with the higher rates. It seems that the hydrolysis of lactose in whey is biphasic in nature as, in the initial phase, the rates were comparatively slower than the later phase. In all cases, maximum hydrolysis took place at up to 44 h of incubation. It is also noticed from Figures 8a and 8b that by doubling and tripling the enzyme activity, the rates of hydrolysis did not increase proportionately.

Effect of Metal Ions and Chemicals

Table 1 shows the effect of metal ions and EDTA on β-galactosidase activity. Hg^{2+} almost completely inhibited enzyme activity at 1 mM, whereas Ag^{+} at this concentration showed 98% inhibition. Mg^{2+}, Ca^{2+} and Mo^{2+} showed very little inhibition, even at their very high concentration (20-25 mM). Zn^{2+} and Ni^{2+} at 20 mM concentration, showed 62% and 75% inhibition of enzyme activity, respectively. EDTA, at concentrations of up to 25 mM, did not show any inhibition of enzyme activity. Metal ion inhibition studies have also been carried out for β-galactosidase from other microbial sources. The strong inhibitory effect of most of the metal ions, except Mg^{2+}, Ca^{2+}, Mo^{2+} and no inhibition of activity by EDTA, suggested that the β-galactosidase from *Bacillus polymyxa* does not require metal ions for its activity. It is reported [25] that β-galactosidase from *Kluyveromyces lactis* required K^{+} and Mg^{+} for stability. Hg^{2+} was found to cause marked inhibition of β-galactosidase activity, irrespective of the sources as reported by various workers.

From the above findings, it is evident that β-galactosidase, from the newly isolated *Bacillus polymyxa*, has the tremendous potential of hydrolyzing lactose. The main advantage of this enzyme is its higher activity and stability at pH of 7, the natural pH of milk, compared to β-galactosidase from the *A. niger*. The other advantage is the stability at 50°C, which is more than that from *A. oryzae* [9,26].

Table 1. Effect of metal ions and EDTA on β-galactosidase activity.

	Percentage activity retained Concentration (mM)					
Metal Ions	**1**	**2.5**	**5**	**10**	**20**	**25**
Cu^{2+}	53	33	32	23	1	1
Zn^{2+}	60	95	98	94	38	7
Co^{2+}	97	90	92	82	82	42
Ni^{2+}	98	94	89	75	25	24
Mg^{2+}	93	92	92	94	93	99
Ca^{2+}	89	93	92	88	91	91
Mo^{2+}	91	91	94	91	93	92
Mn^{+2}	91	83	81	93	32	10
Hg^{2+}	1.08	1	1	3	1	1
Ag^{+}	2	ND	ND	ND	ND	ND
Fe^{2+}	89	87	87	13	2	0.27
EDTA	92	96	96	101	103	102

ND: Not detected, β-galactosidase activity not taken due to interference with assay procedure. The activity is expressed as a percentage of the activity level in the absence of chemical or metal ions.

Attempts to purify the enzyme are ongoing for the future application of this enzyme.

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