ISSN 2249-4073
Current Trends in Biotechnology and Chemical Research

Received : August 01, 2011; Accepted : September 15, 2011

Lactose hydrolysis by β - galactosidase from a newly isolated bacterial strain *Bacillus sp.* NV1

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Abstract

β-Galactosidase (EC 3.2.1.23) was produced from mesophilic bacterial strain *Bacillus* sp. NV1 from local milk plant. The enzyme was partially purified using ammonium sulphate (20-70%). The optimum temperature of β-galactosidase was 40°C and pH range was 6-7. The enzyme was quite stable and lost 40% activity in 6 h at 50°C with $t_{1/2}$ of 3 h at 55°C. The enzyme was slightly inhibited by Fe^{3+} and Cu^{2+} and activated by Na^{+}, Ni^{+}, Ca^{+} and Zn^{+}, Co^{2+} with K_m of 20.83 mM and V_{max} of 204.08 U/ml. The partially purified enzyme hydrolyzed 98% of lactose (5%) within 12 h of incubation thereby making this enzyme useful for various industrial purposes.

Keywords: β-Galactosidase, lactose hydrolysis, inhibition, whey

Introduction:

Lactose, a disaccharide, is a major component of milk (3-8% of mammalian milk) and other dairy products (70-80% of solid component of whey). Due to low solubility direct utilization of lactose is limited. Persons with little or no intestinal β- galactosidase, would suffer from a variety of lactose intolerance related gastro-intestinal symptoms [1]. Removal of milk from diet can lead to deficiency of important components including calcium, vitamin D and riboflavin. Lactose, a major by-product of cheese industry also causes many pollution problems. Acid hydrolysis of lactose is possible under extreme temperature and pH. Further a number of undesirable products can be formed during the process which causes damage to vessel material. Therefore, β- galactosidase, which hydrolyzes non- reducing terminal of β- galactose residues from lactose, is the ultimate choice for hydrolysis of milk and whey.

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β- galactosidase (E.C 3.2.1.23) is widely distributed in microorganisms [2-3], plants [4], and animals [5-6]. β-galactosidase is involved in various biological processes including lactose utilization in body [7], development and ripening of fruits [8-9] and also as a tool for organic chemistry [10-12]. Product of hydrolysis i.e. glucose and galactose are sweeter, more soluble and lead to reduction in freezing point of mixture, thereby, less prone to crystallization [13]. These are easily absorbed and metabolized. Decreased viscosity of hydrolyzed sugar syrups is also advantageous in handling and transporting such products [14-15]. Lactose hydrolysis in whey results in useful sweet syrups which are used as important components in dairy, confectionary, baking and soft drink industry [16].

β-galactosidase also helps in production of galactosylated products like galacto-oligosaccharides, which are growth stimulators for bifidobacteria in the lower part of human intestine and prevent growth of harmful bacteria [17-19]. Galacto-oligosaccharides, being water soluble and mildly sweeter, are not utilized by mouth micro flora and decrease the chance of caries. These oligosaccharides are low calorie sweetener for diabetic persons. Currently, a number of oligosaccharides are added as the pure compounds to dairy products. Biosensor systems are also developed using β-galactosidase and glucose oxidase as biological active materials [20]. Number of studies involving microbial β-galactosidase have been reported [21-25]. In order to search for an enzyme with greater potential for lactose hydrolysis, the mesophilic β-galactosidase was isolated, produced and characterized from strain NV1 has been carried out.

Material and methods

Chemicals

All chemicals used for the present study were of analytical grade and obtained from Sigma Chemicals (St. Louis, USA) and SD Fine chemicals (Mumbai). Media and its component were purchased from Himedia Ltd. (Mumbai).

Screening of β-galactosidase producing microorganism

Various soil and water samples were collected from the local milk plant and screened for -galactosidase production. The samples were plated onto lactose (1%) agar plates having X-gal. Plates were incubated at 40^0C for 24 h. Colonies showing β-galactosidase activity with a deep blue green appearance caused by the released dye 5 bromo 4 chloro indigo were selected [26].

Production of β-galactosidase by strain NV1

Production of β-galactosidase from strain NV1 was carried out in a medium containing 1% lactose in Luria Broth at 40^0C. The pH of the medium was adjusted to 7.0. The shaking speed was maintained at 150 rpm. Duplicate samples were taken out at different time intervals and checked for growth and enzyme activity and residual substrate concentration. The pellet obtained after centrifugation at 12,000 rpm for 40 minutes was discarded and supernatant was assayed for β-galactosidase activity. The enzyme was partially purified with ammonium sulphate precipitation (20-70% saturation) and used as source of crude enzyme.

Enzyme assay

β-Galactosidase activity was assayed spectrophotometrically at 420 nm using ortho nitrophenyl β-D-galactopyranoside (ONPG) as the substrate [27]. The assay mixture was composed of 2 ml of ONPG and 1.0 ml of apparently diluted enzyme solution. The mixture was incubated for 30 minutes at 40^0C and reaction was stopped by the addition of 2 ml of 0.5 M Na_2CO_3.

One Unit (nanokats) of β-galactosidase is defined as amount of enzyme required to release 1 nmol of orthonitrophenol (ONP) per second under specified condition.

Estimation of extra cellular protein

Protein estimation was done using method of Lowry *et al.* [28]. Calibration curve from standard BSA solution (0.02-0.2 mg) was used to measure protein concentration.

Characterization of β-galactosidase

Effect of pH on activity and stability of β–galactosidase

Relative enzyme activity of strain NV1 was measured at different pH values with 5 mM ONPG using 50mM citrate (pH 3-5), Sodium phosphate (pH 6-8) and Glycine-NaOH (pH 9-10) buffer. Optimum pH was studied using standard assay conditions. For pH stability studies, relative activity (%) of β-galactosidase was estimated at different pH values 5-10 after incubating the enzyme in different buffers for 24 h. Samples were withdrawn and residual β-galactosidase activity was measured with standard assay conditions.

Thermal stability and temperature optimum studies

The activity of the β-galactosidase was determined by incubating the reaction mixture at different temperatures in the range of 30-80^0C. To determine the enzyme stability with changes in temperature the enzyme was incubated at different temperature (37- 55^0C) and relative activities were estimated.

Effect of metal ions on the β-galactosidase activity

The effect of metal ions like K^+, Na^+ Li^+ Ca^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+} each 5 mM, were determined by adding them into the reaction mixture and incubating for 30 minutes at 40^0C before the addition of substrate (ONPG) followed by measurement of relative enzyme activity.

Enzyme kinetics

The K_m and V_{max} values of the β-galactosidase were determined for the substrate ONPG. Substrate hydrolysis was determined from 1- 15 mM of ONPG at 40^0C and pH 7. The kinetic constant was obtained from the slope of the intercept of the regression line of Lineweaver -Burke plot.

Lactose hydrolysis

Different amount (10-25 U) of β- galactosidase enzyme was incubated with 5% lactose at optimum temperature (40^0C) and pH 7.0 and residual glucose concentration was determined by Glucose Trinder Kit no. 135-100 (Sigma). The percentage hydrolysis of lactose was calculated in terms percentage of maximum efficiency.

Results and discussion

Screening of β-galactosidase producing organism and production of enzyme

A large number of soil and water samples were screened for presence of β-galactosidase producing organisms. Eight strains were selected on the basis of β-galactosidase production as indicated by intensity of formation of blue green colonies. Of these, one isolate NV1 was selected on the basis of characteristic and yield of β-galactosidase (Figure 1). This strain was maintained on medium containing 1% lactose. Bacterial isolate NV1 is Gram positive rod, catalase positive, able to grow at temperature 20-50^0C (Table 1), utilize lactose, ONPG, xylose, maltose, sucrose, esculin and sucrose. The selected bacteria showed variable sensitivity to various antibiotics but resistance to bacitracin and nalidixic acid (Table 2). Figure 2 shows the course of cultivation of NV1 for production of β-galactosidase. The maximum cell growth corresponding to optical density of 4.7 was observed at 18 h and then started decreasing. The increase of β-galactosidase activity started within 3 h and reached

maximum secretion of 45.8 U/ ml in 24 h due to release of enzyme at the later stages of growth by cell lysis. Hsu et al. [22] studied the culture conditions of growth and found maximum production of 36.7 U/ml in 10 h of fermentation at pH 6.5 and temperature of 37°C

Effect of pH on enzyme activity

Figure 3 shows that the optimum pH for β-galactosidase activity was 6-7 at 40°C. NV1 enzyme was sensitive to acidic conditions as indicated by 60 % and 94% loss in activity at pH 5.0 and 4.0 respectively. This pH optimum under the conditions used was the same as reported for β-galactosidase from other mesophilic microorganisms [29]. The purified β-galactosidase from *Alicyclobacillus acidocaldarius* subsp. *rittmannii* had optimum activity at pH 6.0 [30], while Li et al. [31] reported pH 5.5 to be optimum for enzyme from *Thermotoga maritima.* Loss of 35% NV1 β-galactosidase was observed in buffer of pH 8.0. The enzyme retains its original activity at pH 6-7 after 24 h of incubation. An exceptional property of NV1 β-galactosidase was its stability at pH 3-5 although crude enzyme was not active in acidic pH. Only 4 % activity loss was observed at pH 3 after 24 h of incubation. The neutral pH optimum is very much suitable for the application of β-galactosidase in different fields including application in food industry [32]. At pH 8-9, 80% of activity was obtained as compared to control. Although there might be some interaction of enzyme molecule with the buffer components, the NV1 β-galactosidase was stable over wide pH range (3-9) (Figure 3). β-galactosidase from *Thermotoga maritima* having >90% stability with pH range of 4.5-9.7 was obtained [31]. Rhimi et al. [23] reported β -galactosidase retaining 90% of the activity for 32 h at pH 5.5 and more than 50% at pH 5.0-6.0. At pH 4.5 half life of 24 h was observed.

Effect of temperature of β-galactosidase activity

The β-galactosidase activity of NV1 was studied in the temperature range of 30-80°C (Figure 4). Maximum activity was obtained at 40°C, thereafter β-galactosidase enzyme activity decreased progressively with increase of temperature. 97% of β-gal activity was obtained at incubation temperature of 35°C. At 45°C, reduction of 1.5 % activity as compared to optimum was observed. Lu et al. [24] isolated *Enterobacter cloacae* B5 from soil sample showed β-galactosidase activity at 35°C. Crude β-galactosidase studies of the thermal stability at different temperature (Figure 5) shows the activity of β-galactosidase from strain NV1 retaining 98.6% activity after 6 h of storage. At 48 h of incubation, the enzyme retained 85% of activity at 37°C after 48 h of incubation. Enzyme NV1 lost 40% activity in 360 min at 50°C with $t_{1/2}$ of 12 h was observed (data not shown). At 55°C, the enzyme activity declined sharply with $t_{1/2}$ of 3 h. Mutated and wild type β-galactosidase from *L. delbrueckii* subsp. *bulgaricus* had stability at 40°C and 45°C for 16 h respectively but showed half life of 360 min and 180 min at 50 and 55°C respectively [23].

Kinetics parameters

The K_m and V_{max} value of -galactosidase activity from strain NV1 was 20.83 mM and 204.08 U/ml respectively, thereby indicating the weak binding of substrate (Figure 6). The K_m value for ONPG range from 1.7 to 11.3 mM had been reported for β-galactosidase from different microbial sources [32-36].

Effect of metal ions on enzyme activity

β-Galactosidase from strain NV1 was activated significantly by Zn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Li^{+}, where as certain transition metal ions Fe^{3+}, Cu^{3+} slightly inhibited. Hg^{2+} strongly inhibited the β-galactosidase activity (Table 3).

Table 1. Morphological and biochemical characterization of strain NV1

Test	Result	Test	Result
Gram stain	+ ve	Catalase test	Positive
Shape	Rods	Oxidase test	Negative
Colony characteristics	White, Circular, Flat, Elevation with entire margin	Hydrogen Sulfide (H_2S) production	Negative
Growth temperature	20-50 °C	Indole Test	Negative
Growth at different pH	4.0-9.0	Methyl Red test	Positive
Growth at different NaCl concentrations	1-7%	Voges-Proskauer test	Negative

Table 2. Carbohydrate utilization and antibiotic sensitivity of strain NV1

(a) Carbohydrate utilization test

Test	Result	Test	Result
Lactose	+	Xylitol	-
Xylose	+	ONPG	+
Maltose	+	Esculin	+
Fructose	+	D-Arabinose	-
Dextrose	+	Citrate	-
Galactose	+	Malonate	-
Raffinose	+	Sorbose	-
Trehalose	+	Ribose	-
Melibiose	+	Rhamnose	-
Sucrose	+	Cellobiose	-
L-Arabinose	+	Melezitose	-
Mannose	-	Sorbitol	-
Inulin	-	Mannitol	-
Sodium gluconate	-	Glucosamine	+
Glycerol	+	Dulcitol	-
Salicin	+	Inositol	-
-methul-D-glcoside	-	.methyl-D-mannoside	-

(b) Antibiotic susceptibility test

Antibiotic	Zone of inhibition (mm)	Antibiotic	Zone of inhibition (mm)
Ampicilin (30mcg)	17	Streptomycin (10mcg)	21
Bacitracin (10mcg)	0	Tetracyclin (30mcg)	35
Clindamycin (02mcg)	18	Novobiocin (30mcg)	20
Erythromycin (15mcg)	34	Penicillin G (10mcg)	19
Kanamycin (30mcg)	24	Polymixin B (300U)	12
Nalidixic acid (30mcg)	0	Rifampicin (05mcg)	27

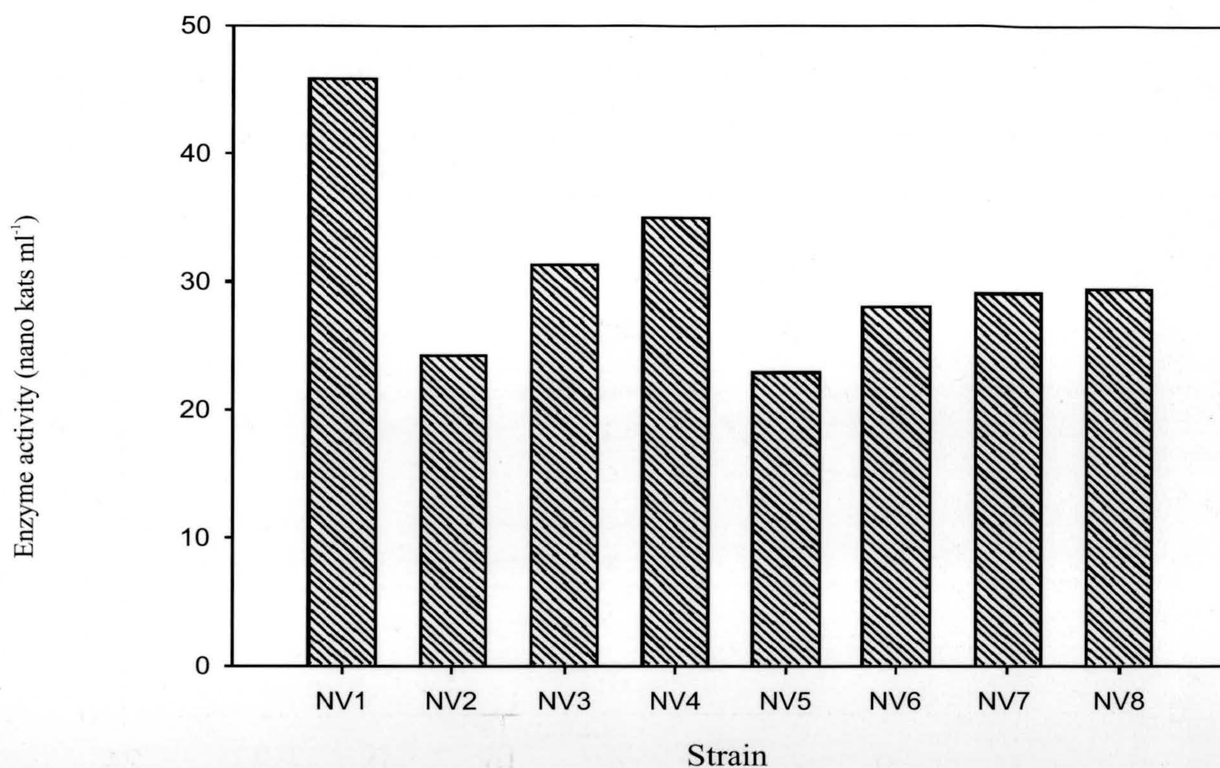

Figure 1. Production of β-galactosidase from various strains isolated

Table 3. Effect of metal ions on the activity of β-galactosidase activity from strain NV1

Metal ions (5mM)	Relative enzyme activity (%)
Na^+	157.5
Li^+	121.5
K^+	126.4
Co^{2+}	128.8
Ca^{2+}	137.7
Ni^{2+}	135.5
Zn^{2+}	124.1
Mg^{2+}	118.3
Mn^{2+}	102.9
$^{b}Cu^{2+}$	89.5
Fe^{3+}	65.4
Hg^{2+}	28.5
Control	100.0

All as chloride except b as sulphate

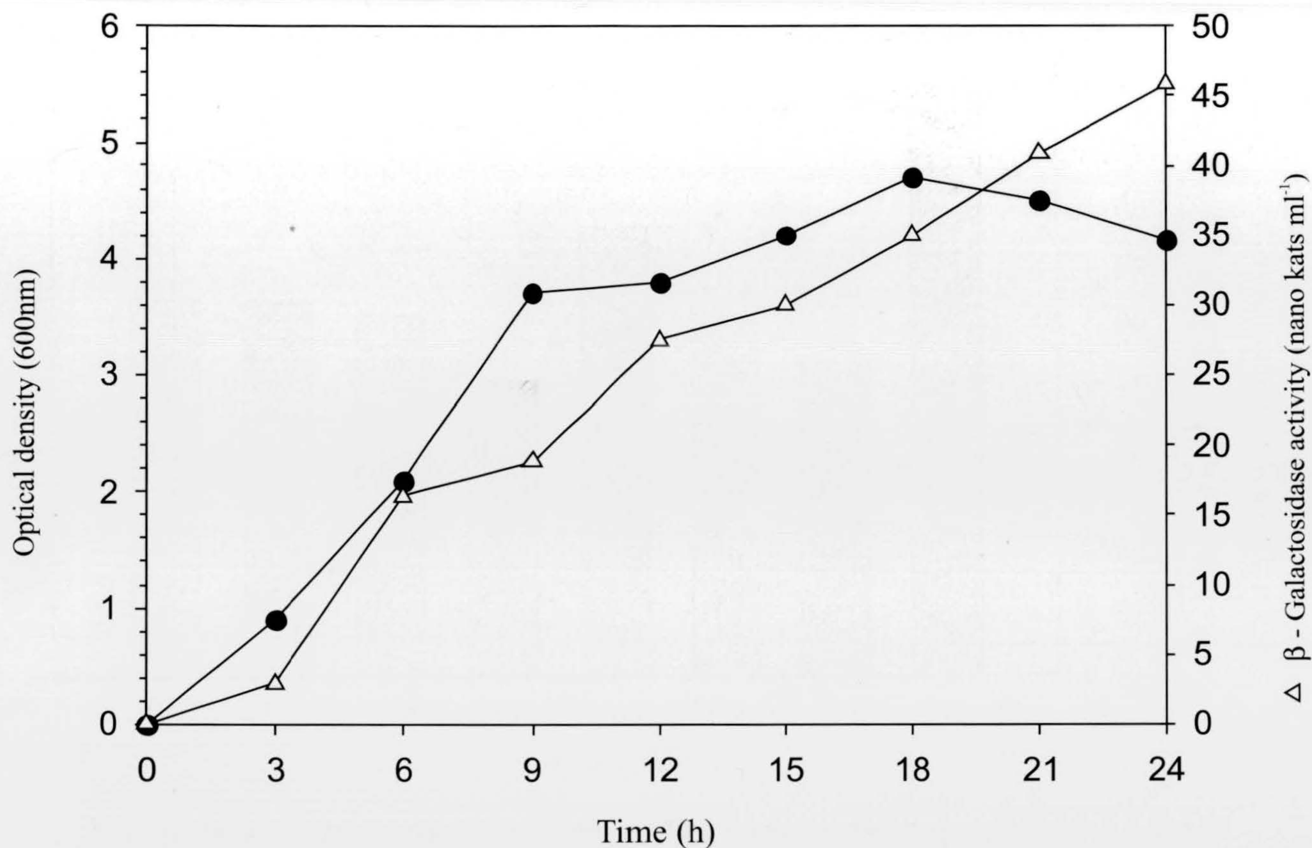

Figure 2. Time course of growth and β-galactosidase synthesis by bacterial isolate NV1

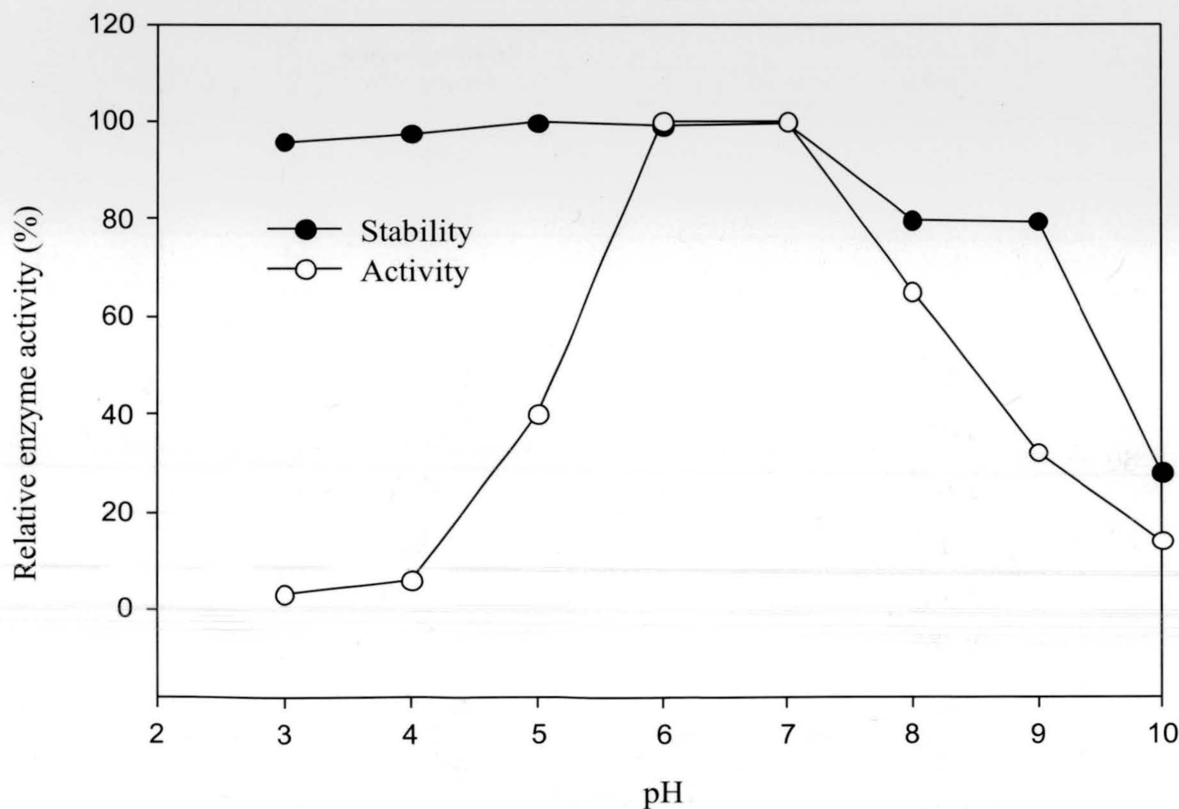

Figure 3. Effect of pH on the activity and stability (24 h) of β- galactosidase activity from strain NV1

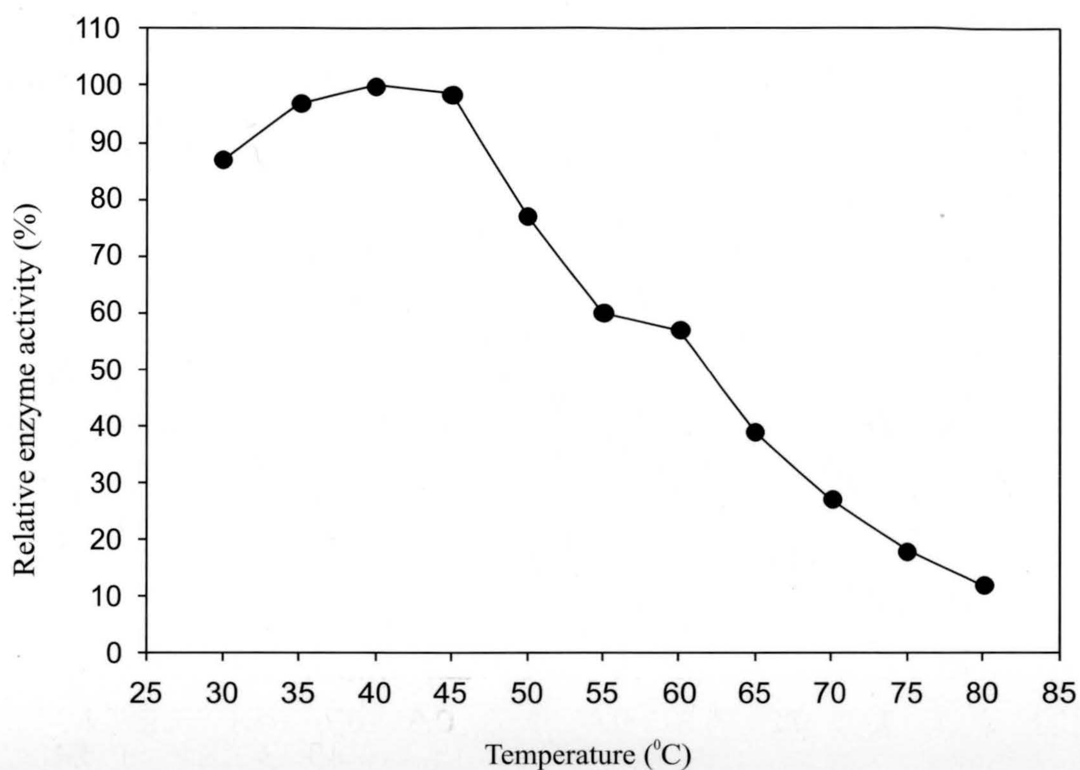

Figure 4. Effect of temperature on the activity of β-galactosidase activity from strain NV1

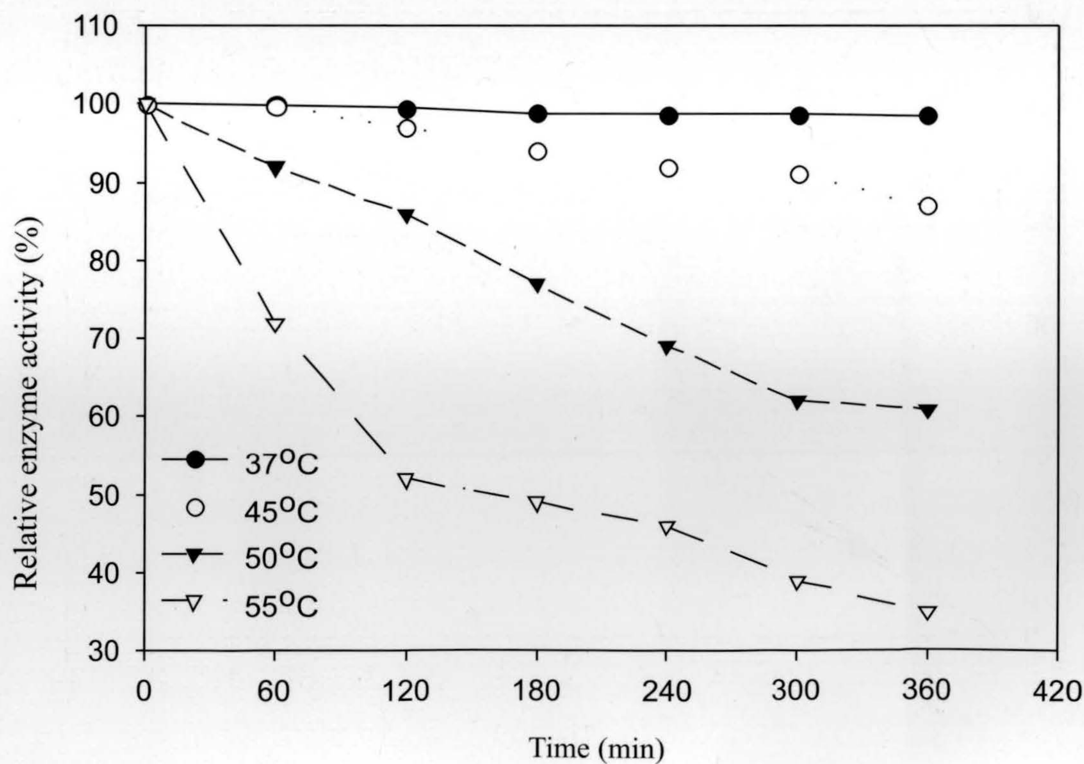

Figure 5. Effect of temperature on the stability of β-galactosidase activity from strain NV1

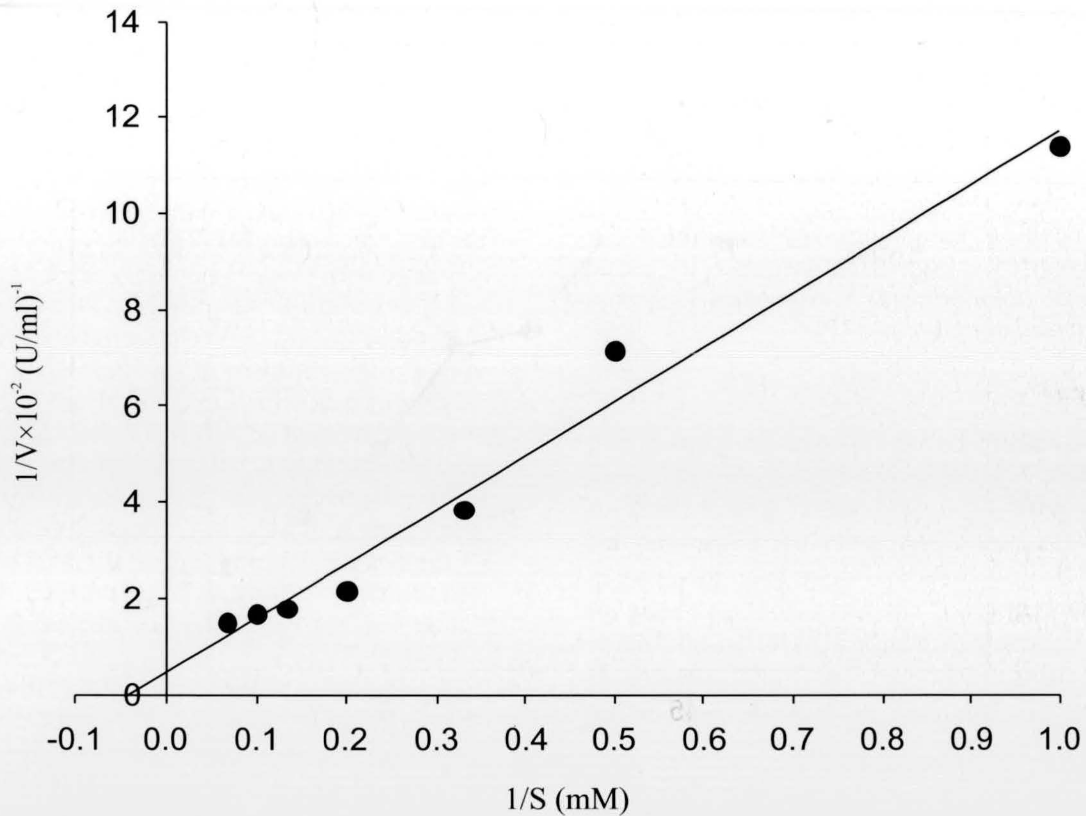

Figure 6. Lineweaver- Burk plot for partial purified β-galactosidase from strain NV1

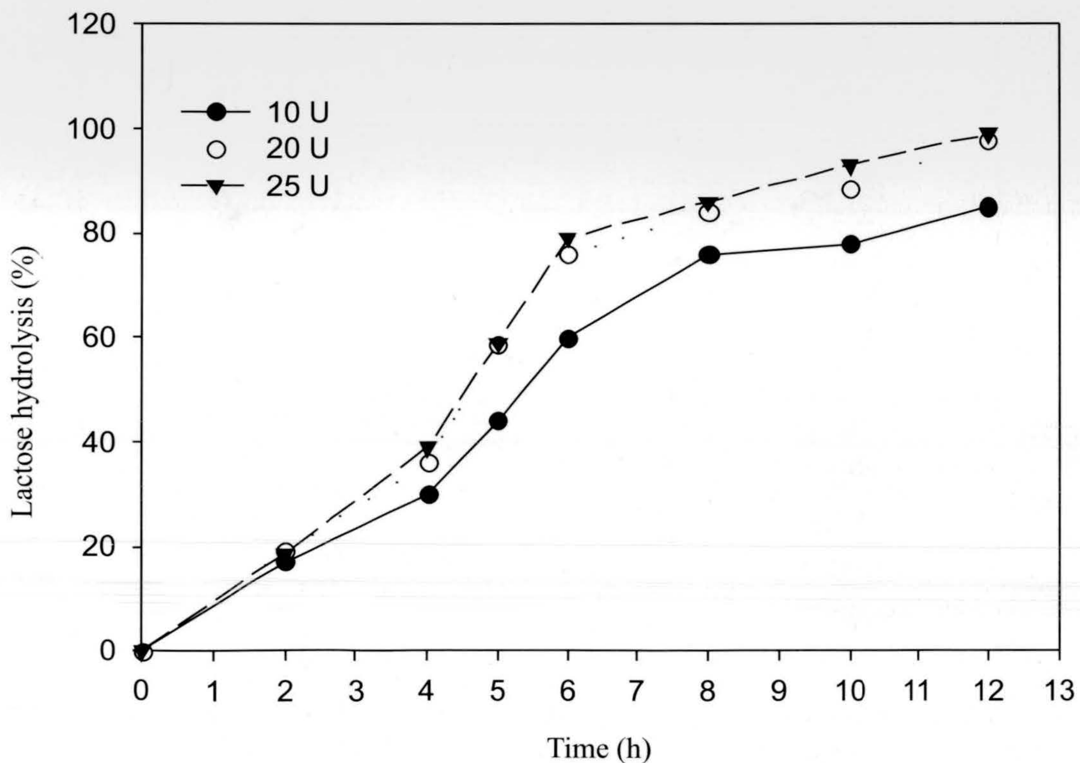

Figure 7. Effect of various concentration of β-galactosidase on lactose hydrolysis

Inhibition and activation by metal ions or other chemicals reagents has been studied in β-galactosidase from different sources [37-39]. Strong inhibition by transition metal ions had been observed with some bacterial β-galactosidase. *S. solfataricus* β-galactosidase had different characteristics and remained unaffected by bivalent metal cations [40]. Activation of enzyme activity by Na^+/K^+ may be due to complexes with metal ions. Mn^+ had no effect on NV1 β-galactosidase but it played activator role in *Lactobacillus bulgaricus* [23] and inhibitory action in *L. acidophilus* [41].

Lactose hydrolysis

Effect of enzyme activity on natural substrate lactose (5%) is shown in Figure 7. High level of hydrolysis of lactose was achieved with crude enzyme. About 90% of hydrolysis was obtained in 12 hr with 10 U of the enzyme, 20 U of enzyme hydrolyze 98% of sugar at temperature of 40°C which make the present enzyme potent candidate for milk and whey industry as nearly 70% of people suffer from either lactose intolerance or maldigestion. β-galactosidase from *K. fragilis* hydrolyzed 96% of lactose (5%) in 48 h [42].

Conclusion

The present NV1 strain produced β-galactosidase in short time period of 18 h. The enzyme has optimum temperature of 40°C and active at pH 6-7 with stability in acidic pH. The isolated enzyme carried out 98% hydrolysis in 12 h and is suitable for lactose hydrolysis of milk and whey at industrial level.

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