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β-D-Galactosidase from *Enterobacter cloacae:* Production and Some Physicochemical Properties

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Abstract A bacterial strain isolated from soil and identified as *Enterobacter cloacae* had been found to be capable of producing both intra and extracellular β-D-galactosidase.The intracellular enzyme was thermostable and its optimum temperature, pH and time for enzyme—substrate reaction were found to be 50 °C, 9.0 and 5 min respectively, using ONPG as substrate. The maximum β-galactosidase production in shake flask was achieved at 30 °C, pH 7.0, incubation time 72 h using 50 ml medium in 250 ml Erlenmeyer flask. Only Mg^{2+} stimulated the activity of enzyme. Cetyl trimethyl ammonium bromide showed stimulatory effect on catalytic activity of the enzyme whereas EDTA inhibited enzyme activity. The enzyme retained its activity upto 55 °C after incubating at that temperature for 1 h.The maximum activity of crude intracellular enzyme was 14.35 IU/mg of protein. The K_m and V_{max} values of β-galactosidase using ONPG as substrate at 50 °C were 2.805 mM and 37.45×10^{-3} mM/min/mg, respectively.

Keywords β-Galactosidase · *Enterobacter cloacae* · Intracellular · Production · Thermostable

Introduction

β-Galactosidase (EC 3.2.1.23) hydrolyzes milk sugar lactose into glucose and galactose and also catalyzes the synthesis of different galactosides. Among few industrially important enzymes, β-galactosidase finds wide application in three main areas, health, food technology and environment [1]. Regarding health issues, large number of population all over the world suffers from lactose intolerance syndrome after consumption of milk due to insufficient

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amount of β-galactosidase in the small intestine to hydrolyze the disaccharide lactose. As a result, unhydrolyzed lactose arrives in the large intestine resulting in several disorders such as flatulence, abdominal bloating, cramps and fermentative diarrhea [2]. This causes large scale production and use of β-galactosidase to reduce the lactose content of milk [3]. Further in Food technology sector, high lactose content in milk leads to the production of inferior quality icecream and condensed milk due to crystallization of lactose. This problem can easily be overcome by hydrolyzing lactose using the enzyme β-galactosidase prior to condensation of milk [2] Moreover glucose and galactose are much sweeter than lactose [4]. The use of β-galactosidase for hydrolysis of lactose has opened up new possibilities for sweetmeat industries, resulting in improved dairy products and processes [5-7]. Regarding environmental issues, lactose present in whey permeates is mainly responsible for pollution due to its high biochemical oxygen demand value. β-Galactosidase can be employed to obtain glucose and galactose from lactose for subsequent production of different useful substances(ethyl alcohol, antibiotics, etc.) simultaneneously reducing the B.O.D. value of whey. β-Galactosidase of different physicochemical properties can be obtained from diverge varieties of sources such as plants , animals and microorganisms [7, 8]. As a result of commercial interest different microorganisms have been screened to assess for possible source of β-galactosidase. High optimum temperature and thermostability are important properties not only in food processing but also in other biotechnological application [9]. This manuscript describes properties of a thermostable β-galactosidase producing strain from soil and preliminary characterization of the crude enzyme.

Materials and Methods

Microorganism and Medium Composition

β-Galactosidase producing *Enterobacter cloacae* isolated from soil in our laboratory was used in the present study. The organism was maintained in the medium [10] containing the following constituents (g/L): Lactose, 15; KH_2PO_4, 1; KCl, 0.5; $NaNO_3$, 2; $MgSO_4$, $7H_2O$, 0.5; $FeSO_4$, $7H_2O$ 0.01; Malt extract, 1; Yeast extract, 1; Agar, 20. pH7.0±0.2. The strain was subcultured at regular interval of time of 30 days. The compositions of inoculum and fermentation medium were same except using agar to solidify the medium.

Screening of β-Galactosidase Producing Organism

β-Galactosidase producing organisms were isolated from soil using plate and dilution technique [11]. Each isolate was tested for its intracellular β-galactosidase activity.

Inoculum was prepared by growing the organism in 50 ml medium in 250-ml Erlenmeyer flasks incubated at 30°C for 24 h in a rotary shaker (120 rpm). One milliliter of the inoculum was transferred to the 50 ml fermentation medium in 250-ml Erlenmeyer flask and incubated under the conditions stated above. At the end of the incubation cells were separated from the fermented broth by centrifuging at 6,000 rpm for 10 min; washed with 0.2 M phosphate buffer (pH6.5), finally resuspended in the same buffer for intracellular enzyme assay.

Assay of β-Galactosidase

To determine the intracellular β-galactosidase activity, buffer suspended cells were sonicated for 30 min in a Takashi Ultrasonicator (Takashi Electric Co. Limited, Tokyo, Japan) under ice-cold

condition (4°C) with an interval of 5 min after each 5 min operation and again centrifuged at 4°C, 10,000 rpm in a Sorvall RC-5B centrifuge for 15 min. The clear supernatant was used for intracellular β-galactosidase assay using orthonitrophenyl-β-D-galactopyranoside (ONPG) as substrate as described by the method of Onishi et al. [12]. Absorbance was measured at 420 nm. Orthonitrophenol (ONP) was used as standard.1 unit of enzyme activity was defined as the amount of enzyme required to produce 1 micromole (1 μmol) of ONP per minute under standard assay conditions. Each experiment was performed in triplicate.

Protein Estimation

Protein was estimated by the method of Lowry et al. [13] with bovine serum albumin as the standard.

Results and Discussion

Screening of β-galactosidase Producing Organism

Twenty six strains having intracellular β-galactosidase activity were isolated from soil. Among these one isolate (AGS12) was selected for further studies as it showed the highest β-galactosidase activity (specific activity 14.35±1.02 IU/mg of protein).

Taxonomical Studies

The organism was identified on the basis of cultural and biochemical characteristics (Table 1) following Bergey's Manual of Determinative Bacteriology [14]. 16S rRNA sequence analysis was done by Bangalore Genei, India and The Phylogenic tree is shown in Fig. 1.

On the basis of 16S rRNA sequence analysis (Gene Bank Accession number EU779827) (Fig. 1) the isolate AGS12 has been identified as *E. cloacae* St SJ 6.

Fermentative Parameters for the Production of β-Galactosidase

E. cloacae St SJ 6 strain exhibits a specific pattern of growth shown in Fig. 2. Cells were in log phase after 9 h of growth and reached stationary phase after approximately 12 h of growth. Both intracellular and extracellular enzyme productions were demonstrated at regular interval of time. Significant amount of extracellular enzyme appeared only after 13 h of fermentation, whereas intracellular enzyme production was low initially and increased with time up to 72 h and then decreased. After 72 h, the enzyme might be excreted in the fermentation medium due to lysis of the cell thus causing increase in extracellular enzyme production (Fig. 2). Figure 3 shows that maximum enzyme production was obtained using 24 h inoculum. Since inoculum volume influences fermentation, the experiment was conducted using inoculum (2.5 mg/ml inoculum density) ratio (1–3%). It was noted that maximum enzyme production was obtained using 1.5% inoculum volume (data not shown). β-Galactosidase production by *E. cloacae* in relation to aeration rate was studied using different volumes of medium in 250-ml Erlenmeyer flask, keeping the other fermentation parameters at optimum level. Maximum production was noted when 50 ml medium was taken in 250-ml Erlenmeyer flasks (Fig. 4). At higher medium volume due to improper aeration, maximum biomass production could not be achieved.

Table 1 Taxonomical characteristics of the selected strain AGS12.

Parameters	Characteristics
Cellular characteristics	
Morphology	Straight small rods, ~2.4 μm, occurring single, some in pairs, motile
Staining characteristics	Gram negative, spore former, spores are terminally located
Cultural characteristics	
Nutrient broth	
Stationary condition	Poor growth, no ring or pellicle formation
24 h	
48 h	Moderate growth, no ring or pellicle formation
Nutrient broth	
Shaking condition	
24 h	Moderate growth, turbidity, no ring formation, pellicle formation
48 h	Moderate growth, ring formation, off-white colour
Nutrient agar colonies	Convex upper surface, round, <1 mm in diameter, opaque, smooth, off-white with entire edge, glossy, no pigmantation
Physicochemical characteristics	
1. Growth at different temperatures	
10°C	Scanty
20°C	Moderate
30°C	Abundant
40°C	Moderate
50°C	Scanty
2. Growth on maintenance media (comprising lactose and mineral salts)	
a) In broth	
Stationary condition (24, 48, 72 h)	Scanty to none, no ring or pellicle formation
Shaking condition (48, 72 h)	Abundant , ring formation, pellicle formation, off-white coloration
b) In agar plates	Filiform, opaque, smooth surface, off-white, no pigmentation
3. Growth at Glucose-Peptone-Yeast extract broth with NaCl concentration	
2%	Abundant, ring formation
5%	Moderate, ring formation
7%	Moderate to scanty, ring formation
10%	None
4. Growth at different pH	
5.4	Moderate, poorly developed ring
6.8	Abundant, ring formation
9.6(with 6.5% NaCl)	Moderate, no ring formation

Table 1 (continued).

Parameters	Characteristics	
Biochemical characteristics		
1. Ammonia from arginine	Positive	
2. Arginine used as a sole c source	Positive	
3. Nitrate reduced to nitrite	Positive	
4. Hydrolysis of Starch	Negative	
5. Catalase test	Positive	
6. Hydrolysis of Urea	Positive	
7. Growth under anaerobic condition with/without Beef extract	Positive	
8. Voges-Proskeur reaction		
pH <6	Positive	
pH >7		
9. Litmus milk test	Negative	
10. Indole formation test	Negative	
11. Carbohydrate fermentation test	Acid formation	Gas formation
a. Glucose	(−)	(+ +)
b. Fructose	(−)	(+ +)
v. Sucrose	(+)	(+ +)
d. Starch	(+)	(+)
e. Lactose	(+)	(+ +)
f. Sorbitol	(−)	(+ +)
g. Mannitol	(+)/(−)	(+ +)
h. Xylose	(+)	(+ +)
i. Salicin	(+)	(+)
j. Galactose	(+)	(+)/(−)
k. Dulcit	(+)	(+)/(−)
12. ONPG test	ONPG broth turns orange on overnight incubation. The strain is capable of producing β-Galactosidase	
13. Hydrolysis of gelatin	Negative	

Influence of initial pH of the fermentation medium on β-galactosidase production was studied over pH values 6.0–8.0. The experiment could not be conducted below pH value 6.0 or above 8.0 due to inhibition of growth of the microorganism. Maximum production of β-galactosidase was observed when the initial pH of the production medium was adjusted to 7.0. Fermentation was carried out at different temperatures also, from 24–38 °C at pH7.0. There was a sharp rise in enzyme production with increase in temperature upto 30 °C. Above that, enzyme production was declined (Fig. 5).

Physico-Chemical Properties of the Crude β-Galactosidase

The *E. cloacae* β-galactosidase exhibits a hyperbolic response with increasing concentration of ONPG (1.25, 2.5, 4.0, 5.0, 6.0 mM), the assay substrate. The K_m and V_{max} values,

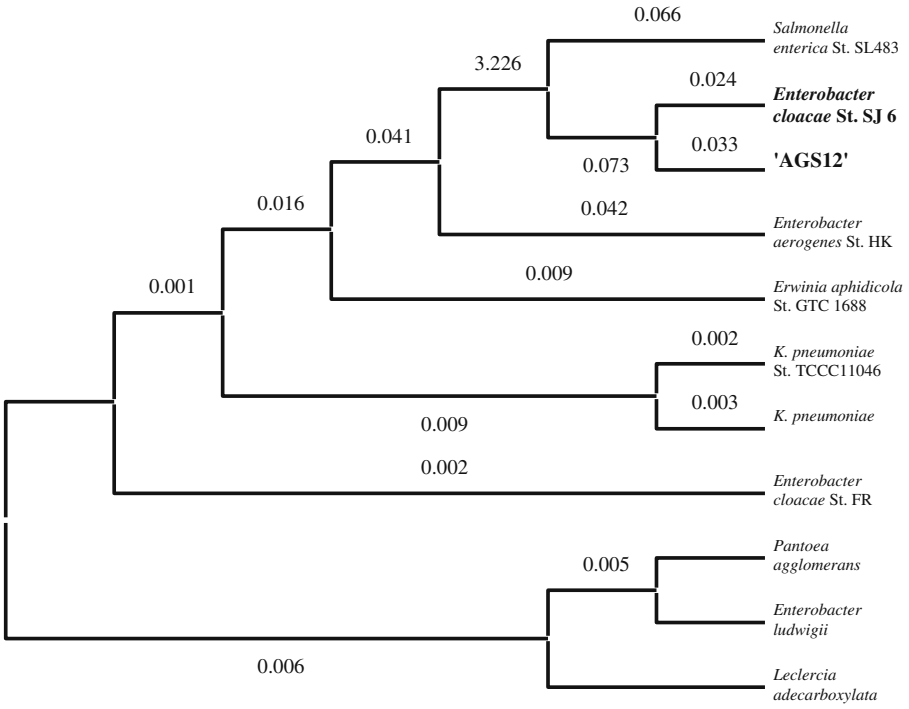

Fig. 1 Phylogenic tree (using neighbour-joining method)

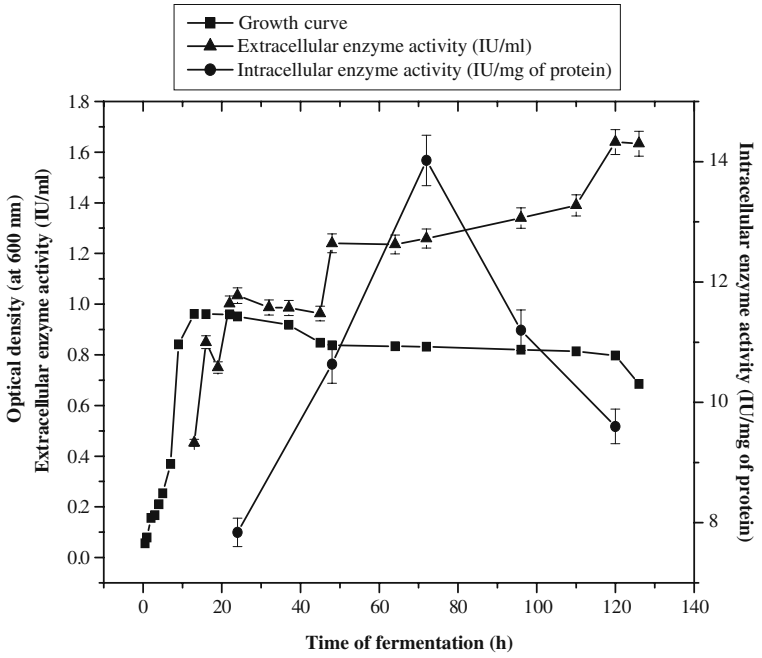

Fig. 2 Effect of fermentation time on enzyme production

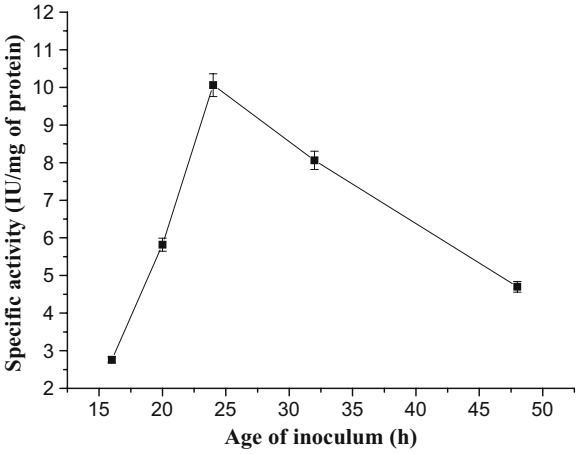

Fig. 3 Effect of age of inoculum on enzyme production

calculated using Lineweaver–Burk plot were 2.805 mM and $37.45X10^{-3}$ $mM^{-1}min^{-1}mg^{-1}$, respectively (Fig. 6) [15-17].The low K_m value suggests that the crude enzyme has high affinity for the substrate.

Effect of pH on β-galactosidase activity was studied over a pH range of 6.5–10.0 using 0.2 M phosphate buffer and 0.2 M Glycine–NaOH buffer. Maximum activity (39.89± 0.56 IU/ml) was noted at pH9.0. Further increase in pH led to decrease in enzyme activity. This corroborated the earlier findings of Fernandes et al. (2002) in case of β-galactosidase of a psychotrophic *Pseudoalteomonas* sp. [18]. pH9.0 was selected as optimum for further studies (Fig. 7).

β-Galactosidase activity of the cellular extract at different incubation temperatures viz., 35–60°C shows that the activity increased progressively with increase in temperature and maximum activity was noted (43.6±1.2 IU/ml) at 50°C. However, only a few reports are available where optimum temperature of β-galactosidase activity was noted at 50°C [19-21].Thermostability of crude β-galactosidase was also examined by incubating the enzyme at different temperature (4–70°C) for 1 h at pH9.0. At the end

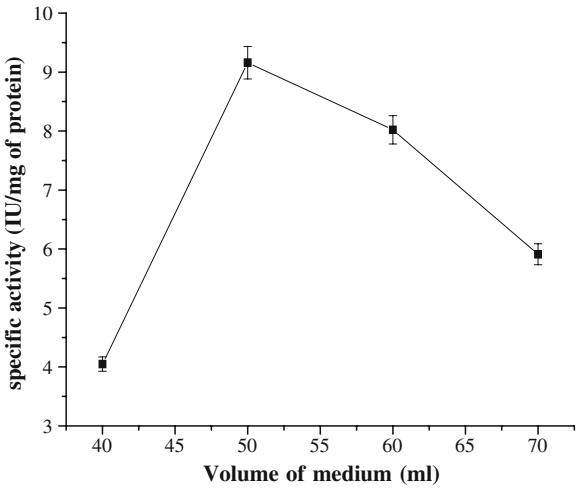

Fig. 4 Effect of volume of medium on enzyme production

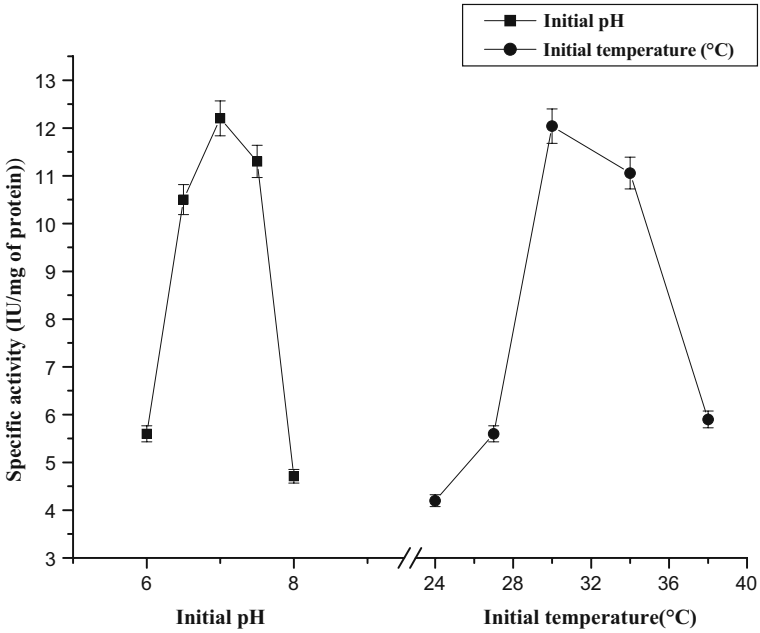

Fig. 5 Effect of initial pH and temperature on enzyme production

of the incubation period enzyme activities were determined according to the standard assay procedure. It was observed that the enzyme retained 92.05% of the activity even after incubating at 50 °C for 1 h (Fig. 8); activity decreased sharply with further increase in temperature.

Metal ions are required by most of the enzymes for their activity. So the effect of some metal ions on the activity of β-galactosidase was studied. The enzyme activity was enhanced in presence of 1 mM Mg^{2+}. Earlier it has been reported that β-galactosidase from *Kluyveromyces lactis* required Mg^{2+} for stability [22]; 0.5 mM and 5 mM Mg^{2+}, 0.5 mM and 1.0 mM Mn^{2+} shows little inhibition. The strong inhibitory effects of various metal ions Fe^{3+}, Sn^{2+}, Zn^{2+}, Cu^{2+}, K^{2+} suggests that the enzyme from *E. cloacae* St SJ 6 does not require any metal ion for its activity (Table 2). Chen et al. (2008) studied that almost all metal ions such as Fe^{3+}, Sn^{2+}, Zn^{2+}, Cu^{2+} except Mg^{2+} inhibited enzyme activity [17]. The

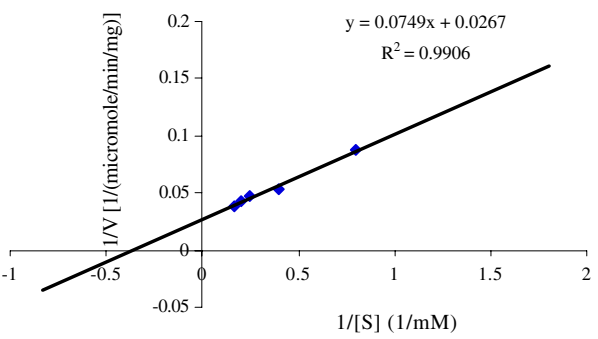

Fig. 6 Lineweaver–Burk plot of the *E. cloacae* β-galactosidase

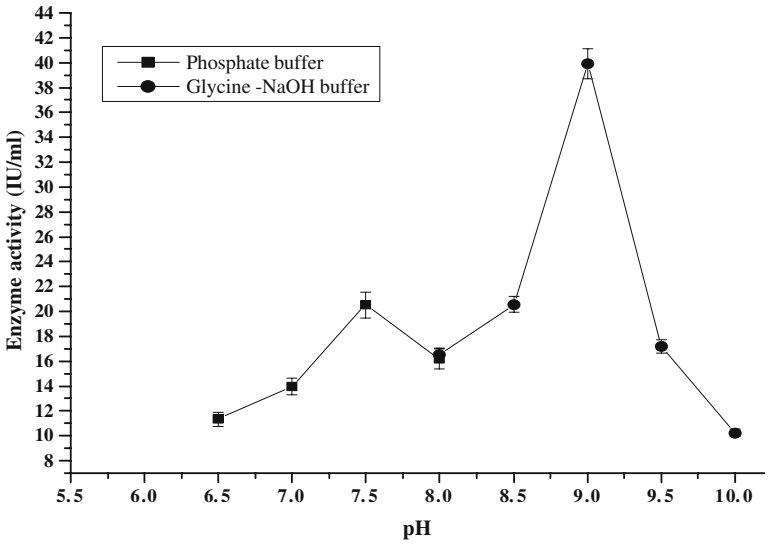

Fig. 7 Optimum pH of the crude β-galactosidase

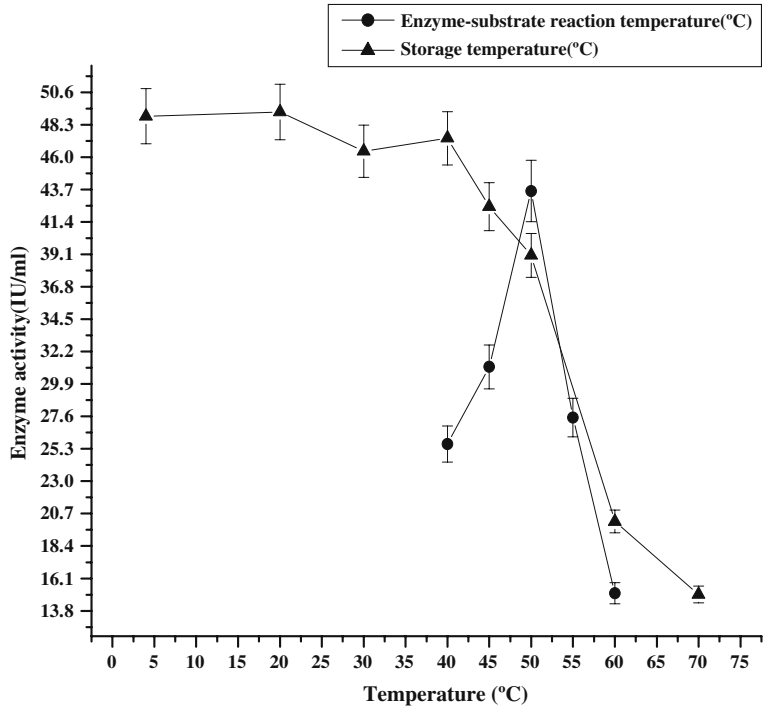

Fig. 8 Optimum temperature and thermostability of the crude β-galactosidase

Table 2 Effect of metal ions on β-galactosidase activity.

Metal ions	Enzyme activity (IU/ml) when metal ions concentrations (mM)			
	0	0.5	1.0	5
Control	42.36	–	–	–
Co^{2+}		35.09	34.83	3.51
Ca^{2+}		33.45	16.94	11.35
Cu^{2+}		1.12	0.84	0.334
Sn^{2+}		2.8	3.34	0.441
Zn^{2+}		3.12	0.86	0.238
Mg^{2+}		40.88	44.35	29.66
Mn^{2+}		41.12	37.62	21.39
Fe^{3+}		0.45	1.36	0.73
K^{+}		7.065	1.02	0.379

Data shown are average of four replicate experiments

inhibition by most of the metal ions may be attributed to the metal ion catalyzed oxidation-reduction reactions of some amino acid residues at the active sites resulting in decrease in the activity of the enzyme.

Different chemicals viz. Ascorbic acid, Sodium azide, Cetyl trimethyl ammonium bromide, Cysteine, EDTA, Polyethyleneglycol at different concentrations (viz. 1, 5 and 10 mM) were tested to study their influence on β-galactosidase activity. Amongst those CTAB (5.0 and 10 mM) showed stimulatory effects, on the other hand ascorbic acid, polyetheleneglycol, sodium azide, cysteine, EDTA inhibited β-galactosidase activity (Table 3). Cationic detergent CTAB might interact electrostatically with negatively charged amino acids of the side chain of protein or with the enzyme substrate reaction complex, resulting in either alteration of overall macromolecule charge or stabilization of the protein structure by promoting proper folding pattern to the protein moiety in the solution [23].

Table 3 Effect of different reagents on β-galactosidase activity.

Reagents	Enzyme activity (IU/ml) at different concentrations of the reagents		
	1 mM	5 mM	10 mM
Control[a]	42.4		
Ascorbic acid	35.86	39.17	6.6
Polyetheleneglycol	30.90	37.21	36.8
NaN_3	31.21	35.55	30.5
CTAB	34.52	42.68	44.55
Cysteine	29.14	27.29	8.97
EDTA	23.14	17.87	–

Data shown are average of four replicate experiments

[a] Control flask received no reagents

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References

1. Neelkantan, S., Mohanta, A. K., & Kaushik, J. K. (1999). *Current Science, 77*, 143–149.
2. Shukla, T. P. (1975). *Critical Reviews in Food and Technology, 5*, 325–356.
3. Dziezak, J. D. (1991). *Food Technology, 45*(1), 78–85.
4. Lifran, E. V., Hourigan, J. A., Sleigh, R. W., & Johnson, R. L. (2000). New ways for Lactose. *Food Australia, 52*, 120–125.
5. Nijpels, H. H. (1980). *Enzymes in food processing* (Vol. Paper 5, pp. 89–104). London: Applied Science Publishers Limited.
6. Reed, G. (1975). *Enzymes in food processing*, 2nd edn. Academic Press, San Diego. pp. 83–391
7. Tanriseven, A., & Dogan, S. (2002). *Process Biochemistry, 38*, 27–30.
8. Panesar, P. S., Panesar, R., Singh, S., Kennedy, J. F., & Kumar, H. (2006). *Journal of Chemical Technology and Biotechnology, 81*, 530–543.
9. Chakraborti, S., Sani, R. K., Banerjee, U. C., & Sobti, R. C. (2003). *Scientia Iranica, 10*(3), 279–286.
10. Batra, N., Singh, J., Banerjee, U. C., Patnaik, P. R., & Sobti, R. C. (2002). *Biotechnology and Applied Biochemistry, 36*, 1–6.
11. Salle, A. J. (1974). *Text book of bacteriology* (7th ed., p. 274). New Delhi: McGraw Hill Publishing Co. Ltd.
12. Onishi, N., & Tanaka, T. (1995). *Applied and Environmental Microbiology, 61*(11), 4026–4030.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biological Chemistry, 193*, 265–275.
14. Holt, J. G., Krieg, N. R., Peter, H. A. S., & Bergy, D. H. (1994). Bergy's manual of determinative Bacteriology, 9th edn. Lippincott Williams & Wilkins, Philadelphia.
15. Kim, C. S., Ji, E.-S., & Oh, D.-K. (2004). *Journal of Applied Microbiology, 97*, 1006–1014.
16. Zhou, Q. Z. K., & Chen, X. D. (2001). *Biochemical Engineering Journal, 9*, 33–40.
17. Chen, W., Chen, H., Xia, Y., Tian, F., & Zhang, H. (2008). *Journal of Dairy Science, 91*, 1751–1758.
18. Fernandes, S., Geueke, B., Delgado, O., Coleman, J., & Hatti-kaul, R. (2002). *Applied Microbiology and Biotechnology, 58*, 313–321. doi:10.1007/s00253-001-0905-4.
19. Tomaska, M., Stredansky, M., Gemeiner, P., & Sturdik, E. (1995). *Process Biochemistry, 30*(7), 649–652.
20. Haider, T., & Hussain, Q. (2007). *Journal of the Science of Food and Agriculture, 87*(7), 1278–1283.
21. Numanoglu, Y., & Sungur, S. (2004). *Process Biochemistry, 39*, 703–709.
22. Pal, A., Pal, V., Ramana, K. V., & Bawa, A. S. (2009). *Journal of Food Science and Technology, 46*(3), 217–220.
23. Horowitz, P. M., & Tandon, S. (1967). *Journal of Biological Chemistry, 262*(10), 4486–4491.