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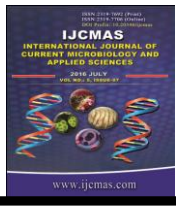

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β-Galactosidase from *Enterobacter cloacae*: Production, Characterization and Purification

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A B S T R A C T

The objective of the present study was to characterize an intracellular β-galactosidase from *Enterobacter cloacae* St. SJ 6. The yield of the enzyme obtained by disrupting the cells using ultrasonication is found to be better compared with other methods. Maximum production of the enzyme is noted in the medium composed of (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 2% , pH 7.0±0.2; using 1.5% cell suspension on 72 h cell growth at 30°C.Tryptone enhanced the production enzyme. The obtained K_m and V_{max} values were 7.92 mM and 134.3µMole/min/mg, respectively. The enzyme has been purified 12.48 fold by Sephadex G-100 chromatography. The optimum pH and temperature for maximum activity of the purified enzyme are found to be 9.0 and 50°C, respectively. Ca^{2+}, Co^{2+}, mercaptoethanol, polyethyleneglycol, sodium azide and CTAB stimulated the activity of the enzyme.

Introduction

β-D-Galactosidase or lactase is one of the most industrially important enzymes and finds widespread application in pharmaceutical, food and dairy industries. A large portion of global population cannot digest lactose, which is the main carbohydrate constituent of milk due to the presence of insufficient amount of this lactase in the intestine causing health problems (Shukla, 1975). In ice cream and condensed milk industries, high amount of lactose also causes crystallization problem. The presence of high amount of lactose in whey causes environmental pollution due to its high biochemical oxygen demand (BOD) (Becerra *et al*., 2001; Gonzalez-Siso, 1996). High BOD value of whey must be reduced to permissible limit before discharging into sewage system. One of the possibilities of reducing the BOD value is to produce some valuable products such as baker's yeast, alcohols etc. from whey which requires hydrolysis of lactose. All these create high demand of β-galactosidase in industry (Giacomini *et al*., 1998; Husain, 2010).

β-Galactosidase can be obtained from a variety of sources such as microorganisms,

plants and animals but their properties differ depending on the sources (Chakraborti *et al*., 2003;. Panesar *et al*., 2006) Different methods are available for disintegration of microorganism to isolate intracellular enzymes and their purification as relatively pure enzymes will be needed for a number of the applications (Fonseca *et al*.,2002; Ganeva *et al*., 2001; Kula *et al*., 1987; Nagy *et al*., 2001).

This paper describes certain parameters required for the production of crude enzyme along with nutritional requirements of the selected strain followed by purification of the enzyme and determination of some physicochemical properties of the purified enzyme.

Materials and Methods

Chemicals and reagents

All chemicals used in the present study were purchased from E.Merck, Germany. Ingredients of microbiological media were procured from Himedia, India. DEAE – Sepharose and Sephadex chromatography columns were obtained by Sigma Aldrich.

Microorganism and medium

Enterobacter cloacae St SJ 6, isolated from soil and identified in our laboratory (Ghatak *et al*., 2010) was used for the present study. The organism was maintained in the medium composed of (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 , pH 7.0±0.2, by monthly sub culturing at 30^{o}C for 24-48 h and stored at 4^{o}C.

Production of β-Galactosidase enzyme

Inoculum and fermentation medium was composed of the above mentioned ingredients except agar. Inoculum was prepared by transferring a loopful culture of *E. cloacae* from agar slant to 20 ml inoculum medium in 100 ml Erlenmeyer flask and incubated for 24 h at 30^{o}C with shaking (120 r.p.m). 1 ml of the inoculum was added to 50 ml fermentation medium in 250 ml Erlenmeyer flask and incubated for 72 h, other conditions remaining the same. The cells were harvested by centrifugation at 5000 r.p.m, for 20 min at 4^{o}C, washed twice with phosphate buffer (pH 7.0) and used for intracellular enzyme isolation.

Intracellular enzyme extraction

Different chemical and physical methods were employed to disrupt *E. cloacae* cells to isolate the intracellular enzyme; all operations were carried out at chilled conditions.

Treatment with Solvent and Grinding with abrasives (Numanoglu *et al*., 2004): Hervested cells (1.1±0.41gm wet cell/50 ml) of *E. cloacae* were grinded with 10 g powdered alumina and 0.1 ml ice-cold toluene-acetone mixture (9:1) in a morter-pestle with gradual addition of 10 ml phosphate buffer followed by centrifugation at 4^{o}C (5,000 r.p.m, 10 min) and the clear supernatant was used for enzyme assay.

Treatment with detergent and solvent (Numanoglu *et al*., 2004; Bansal *et al*., 2008): Hervested *E. cloacae* cells (1.2 ± 0.29 gm cells / 50 ml) were treated with 0.01% SDS and 200µl chloroform using chilled Z buffer (Na_2HPO_4, $7H_2O$, 0.8g; NaH_2PO_4, H_2O, 0.28g; 1 M KCl, 0.05 ml; 1M $MgSO_4$, 0.05ml; β-mercaptoethanol, 0.135ml, per 50 ml, pH 7.0) . The suspension was centrifuged at 4^{o}C, 5,000 r.p.m for 10 min & the clear supernatant was used for enzyme assay.

Treatment with detergent and abrasives: 1 ml of 1% CTAB was added to 50 ml cell

suspension contains 1.25± 0.24 gm wet cell/ 50 ml and incubated for 3 h at 30°C with mild shaking, then it was centrifuged and the cell pellet was grinded using sea- sand with gradual addition of phosphate buffer (pH 7.0). This was centrifuged and the supernatant was assayed.

Use of abrasives (Bansal *et al*., 2008)*:* 1.19±0.36 gm wet cell of *E. cloacae* in 50 ml was suspended in 10 ml phosphate buffer (pH 7.0) and disrupted by adding equal volume of glass beads and stirring vigorously in a vortex. Cell debris along with glass beads were removed by centrifugation and the supernatant was used for enzyme assay.

Utrasonication (Becerra *et al*., 2001; Georis *et al*.,2000;): Harvested *E. cloacae* cells (1.2±0.28 gm wet cell) suspended in 50 ml phosphate buffer (pH 7.0) were sonicated for 30 min in a Takashi Ultrasonicator (Takashi Electric Co. Limited, Tokyo, Japan) under chilled condition (4°C) with an interval of 5 min. This was centrifuged at 4°C (10,000 rpm) for 15 min. The clear supernatant was used for intracellular β-galactosidase assay.

Enzyme assay

Immediately after cell disintegration, the activity of intracellular β-Galactosidase was determined using a substrate orthonitrophenyl- β-D-galactopyranoside (ONPG) as described by a method of Onishi and coauthor (Onishi *et al*., 1995). 1 unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmole of orthonitrophenol (ONP) per min under standard assay conditions. Each experiment was performed in triplicate. Protein was estimated following the method described by Lowry using bovine serum albumin as the standard (Lowry *et al.,* 1951).

Effect of carbon and nitrogen sources on enzyme production

To the basal medium used for β-galactosidase production, a given carbon compound is added in 1.5% concentration. 13 different carbohydrates other than lactose were tested. Different organic nitrogen (N_2 level 0.165gm per lit) and inorganic nitrogen (N_2 level 33 mg/100ml) sources were also determined by adding them separately to the basal medium. All flasks containing media were autoclaved accordingly.

Determination of V_{max} and K_m

V_{max} and K_m values for β-galactosidase were determined using the Lineweaver-Burk plot. This investigation was carried out by using ONPG in the concentration range: 1.25, 2.5, 4.0, 5.0, 6.0 mM.

Purification of enzyme

Enterobacter cloacae intracellular β-galactosidase was purified from the culture filtrate by ammonium sulphate precipitation followed by dialysis. Saturation from 20 - 80% was achieved for proteins to precipitate. Each precipitate was collected by centrifugation. The fractions showing maximum activity were dissolved in minimum amount of 0.2 M Glycine-NaOH buffer (pH 9.0) and dialyzed against the same buffer overnight using a magnetic stirrer and changing the buffer solution twice during the dialysis.

The dialyzed protein was collected by centrifugation. Purification was further achieved by gel filtration chromatography on a DEAE- Sepharose FF column (25x2cm, Sigma –Aldrich) equilibrated with 0.25 M NaCl/0.05 M sodium phosphate buffer (pH 7.0). Fractions of 2.0 ml were collected at a flow rate 0.5 ml /min using a fraction

collector. β-galactosidase activity and protein content were determined for collected fractions. The active fractions were combined and further purified by Sephadex G-100 (20X1 cm) columns. Elution was performed by applying a linear salt gradient (0 ~ 0.5M NaCl n 50 mM sodium phosphate buffer (pH 7.0). Fractions (2.0 ml each) were again collected at a flow rate 0.5 ml/min and analyzed for protein concentration and β-galactosidase activity.

Polyacrylamide Gel Electrophoresis

To determine the purity and the molecular weight of β–galactosidase, SDS- PAGE was carried out following the method of Laemmli (1970). After electrophoresis, gels were stained with 0.25 % Coomassie Brilliant Blue R-250. Enzyme molecular weight was estimated by comparing the mobility of the enzyme with those of standard proteins. Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kDa), Lactate dehydrogenase (140 kDa) were included as molecular weight markers.

Determination of reaction pH and pH stability of the purified enzyme

The enzyme-substrate reaction was carried out at different pH ranges viz. 6.0- 7.5, 7.5-8.5, 8.0-9.5 using 0.2 M Phosphate, 0.2 M Tris-HCL and 0.2 M glycine –NaOH buffers respectively at 50 ° C for 5 min. The pH stability of the purified enzyme was determined by incubating the enzyme with 4 ml of 0.2 M glycine-NaOH buffer of pH (8.0-9.5) for 1 h and then preservation stability was determined by the standard assay method.

Determination of reaction temperature and temperature stability

The enzyme-substrate reaction was carried out at various temperatures viz. 30,35,40,45,50,55,60^{o}C with partially purified β-galactosidase in 0.2 M glycine-NaOH buffer (pH 9.0) for 5 min with ONPG as substrate. The purified enzyme was incubated at different temperatures (20-60 °C) for 1 h at pH 9.0 using the same buffer. The residual activities were evaluated according to standard assay procedure.

Effect of metal ions and reducing agents

To investigate the effect of metal ions on the activity of purified β–galactosidase, each metal salt (as chloride or sulfate) was added to the reaction mixture at 0.5, 1.0, 5.0 and 10 mM final concentration. Similarly, reducing agents such as mercaptoethanol, polyethyleneglycol, sodium azide and CTAB were tested at 1.0, 5.0 and 10 mM final concentrations. Activity was measured using standard assay method.

Results and discussion

Cell disruption

Different methods have been employed to release intracellular enzymes. Among them, cell disruption with sonication showed the best result with specific activity 14.03±2.04IU/mg (Table 1). In the present study, the enzyme β-galactosidase from *E.cloacae* is found to be intracellular. Isolation of enzyme requires cell disintegration. The shock waves of sonicator disrupt cells present in suspension.

Effect of superior carbon and nitrogen sources for fermentative production of β-galactosidas

Enzyme production depends on the composition of the growth medium which is not necessesarily the same that supports the best growth. Carbon source of the medium greatly influences β-galactosidase

production (Hsu *et al.*, 2005). A number of carbon sources at a concentration of 1.5% (other than lactose) were used in the fermentation medium keeping other parameters same.

Lactose (1.5%) was found to be the superior carbon source for β-galactosidase production from *Enterobacter cloacae* (Fig.3). Different organic (N_2 level 0.165gm per lit) and inorganic (N_2 level 33 mg/100ml) nitrogen sources were tested (El-hofi *et al.*, 2010). Sodium nitrate at 0.2% concentration was found to be the best inorganic nitrogen source having specific activity 14.07±1.76 IU/mg. Enzyme production was found to be high in presence of some amino acids viz. glycine & glutamic acid (0.495gm nitrogen/L) (Table 2). Amino acids used in production medium can often be used as growth enhancer.

Determination of V_{max} and K_m

V_{max} and K_m values for β-galactosidase were determined using the Lineweaver-Burk plot. K_m and V_{max} for enzyme substrate reaction were 7.92 mM and 134.3µmole/min/mg respectively (Fig.2).

Purification of β-Galactosidase

The enzyme was purified 12.48 fold with 15.78 % recovery. Purification process increased the specific activity from 12.24 to 152.83 IU/mg (Table 3). One prominent band having molecular weight 340kDa was appeared (Fig.3).

Characteristics of purified enzyme

Effect of pH

The enzyme-substrate reaction was carried out at different pH ranges viz. 6.0- 7.5, 7.5-8.5, 8.0-9.5 using three different buffers at 50 ° C for 5 min for each pH. The pH stability of the partially purified enzyme was also determined by a method described above. The optimum pH for maximum enzyme activity was found to be 9.0 and the enzyme was stable at pH range 8.0-9.0(Fig. 4). 50.06% of activity was retained at pH 9.5. Fernandes *et al*. described the properties of a psychrotrophic β-galactosidase from *Pseudoalteromonas* sp. having an optimum activity at pH 9.0 (Fernandes *et al.*, 2002)

Table.1 Extraction of β-galactosidase by different chemical and physical methods

Methods	Activity (IU/ml)	Specific activity(IU/mg)
Use of Abrasives		
a) *Glass beads*	20.8±1.5	4.58±0.22
b) *Sea sand*	39±1.67	11.2±0.46
Ultrasonication	48±2.014	14.03±2.04
Treatment with Solvent and grinding with abrasive *Toluene - Acetone- Alumina*	26.5±0.2	8.24±0.53
Treatment with Detergent & solvent *SDS-Chloroform-Mercaptoethanol*	25.73±0.61	10.65±0.71
Detergent treatment *CTAB*	39.2±3.01	12.37±0.865

Table.2 Effect of nitrogen sources on crude β-galactosidase production

Nitrogen sources	Growth (abs at 600nm)	Activity (IU/ml)	Specific activity (IU/mg)
Control	2.3	46.55±1.13	14.07±1.76
Organic			
Yeast extract	1.9	49.55±0.07	12.25±0.89
Malt extract	1.9	40.0±1.33	10.06±0.548
Tryptone	2.8	84.26±1.708	7.82±1.7
Peptone	1.8	41.1±3.38	7.35±2.294
Beef Extract	1.5	10.5±1.087	1.21±2.01
Casein Hydrolysate*	2.5	81.5±2.16	7.86±0.385
Inorganic			
$(NH_4)H_2PO_4$	1.4	28.14±1.13	5.78±1.25
$(NH_4)_2HPO_4$	1.2	17.76±0.62	1.75±0.845
$(NH_4)Cl$	0.9	10.52±1.06	2.04±0.91
$(NH_4)NO_3$	1.5	9.70±0.33	3.14±1.41
$(NH_4)_2SO_4$	1.1	10.525±2.081	6.21±1.632
Amino acids			
Glycine	2.8	72.49±0.58	19.97±1.4
Glutamic acid	2.3	68.33±1.32	16.66±0.083

*Interference with assay procedure

Table.3 Purification of β-galactosidase

Stage of purification	Total Protein(mg)	Total activity (IU)	Recovery (%)	Specific activity (IU/mg)	Purification (fold)
Crude extract	114.65	1403.7	100	12.243	1
Ammonium sulfate (40%) and Dialysis	9.63	968.1	68.96	100.53	8.213
DEAE-Sepharose	2.30	344.83	24.56	149.92	12.24
Sephadex G-100	1.45	221.6	15.78	152.83	12.48

Table.4 Effect of metal ions and reducing agents on purified enzyme

Metal ions/Reducing agents	Relative activity (%) at concentrations (mM)			
	0.5	**1.0**	**5**	**10**
Ca^{2+}	103.9	81.3	55.6	30.03
Co^{2+}	101.5	77.86	52.37	31.42
Mg^{2+}	81.6	90.88	59.7	29.85
Mn^{2+}	73.3	65.9	63.2	54.2
Sn^{2+}	24.9	21.4	18.53	16.6
K^{+}	35.46	37.62	29.38	26.35
Zn^{2+}	26.97	29.8	23.13	21.2
EDTA		47.04	92.7	36.42
SDS		91.89	102.6	89.7
Sodium azide		104	103.4	101.36
Mercaptoethanol		109.9	88.0	71.3
Polyetheleneglycol		105.1	63.1	62.43
CTAB		79.96	106.2	61.83

Fig.1 Effect of different carbon sources on production of crude β-galactosidase

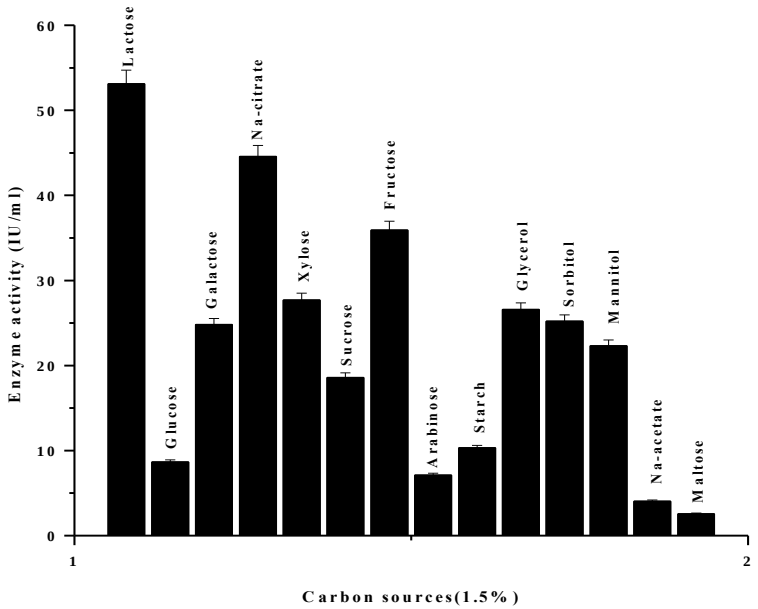


Fig.2 Line Weaver-Burk plot of β-galactosidase

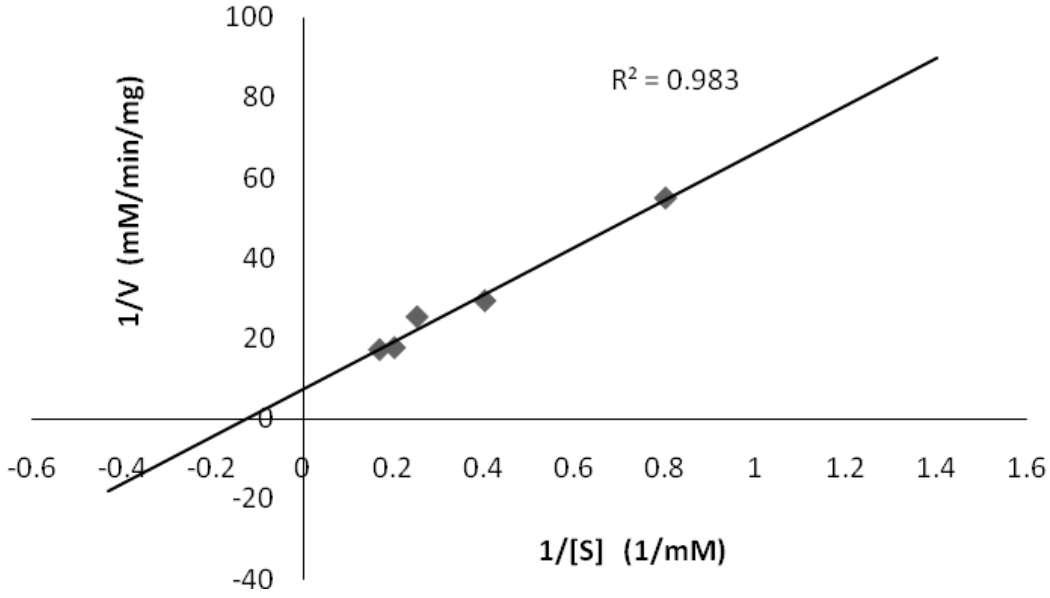
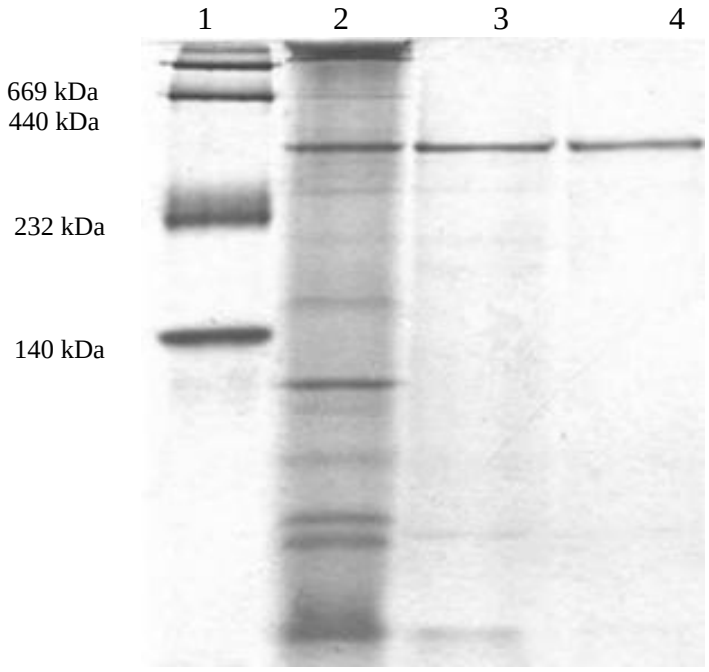


Fig.3 Polyacrylamide GEL electrophoresis pattern of Enterobacter cloacae β-galactosidase



Lane 1: Markers; Lane 2: Dialyzed enzyme; Lane 3: DEAE-Sepharose; Lane 4: Sephadex G-100

Fig.4 Effect of reaction pH and storage pH on purified enzyme activity

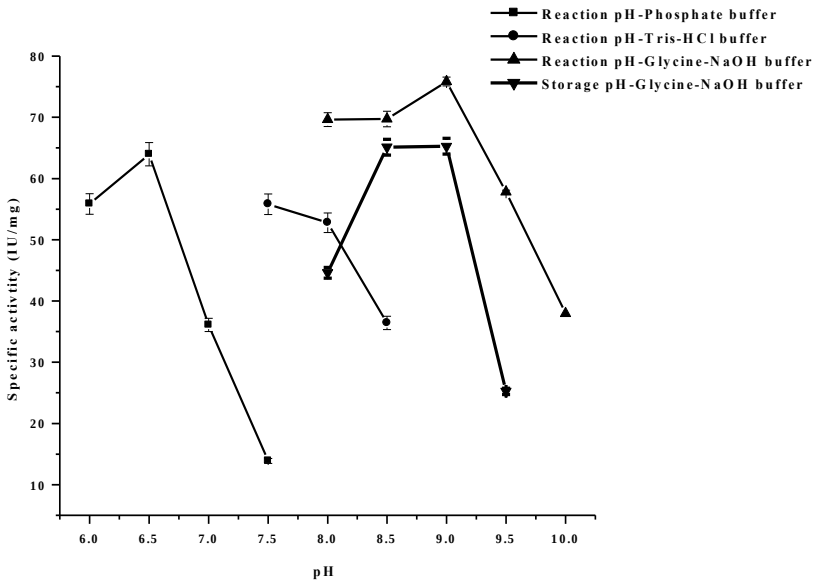
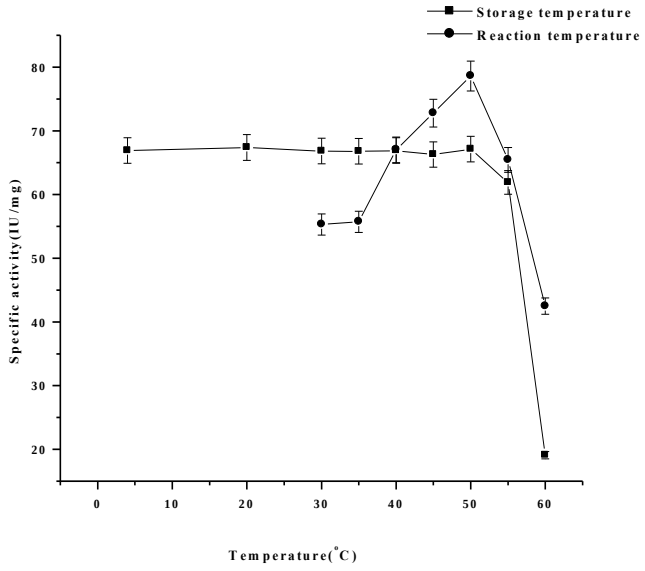


Fig.5 Effect of reaction temperature and storage temperature on purified enzyme activity



Effect of temperature

Activity of the purified enzyme was found to increase with increase in temperature, the optimum temperature upto 50°C and then it is decreased with increase in temperature (Fig.5) and the enzyme also showed stability upto 50°C. 94.7% activity was retained even at 55°C after 1 h incubation. At higher temperatures, structure of the enzyme would tend to be distorted owing to thermal deactivation. The enzyme purified from the

fungus *Beauveria bassiana* was optimally active at 50°C, but not stable at 60°C (Macpherson *et al.,* 1991).

Effect of metal ions and reducing agents

Ca^{2+} and Co^{2+} both at 0.5 mM , increased the activity of purified enzyme to 103.9 and 101.5%, respectively. Sodium azide stimulated enzyme activity at all three concentrations. β-mercaptoethanol and polyetheleneglycol, at 1.0 mM , SDS and CTAB at 5.0 mM stimulated enzyme's activity (Table 4).

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