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*Full Length Research Paper*

# Isolation, Purification and Characterization of a Thermostable β-Galactosidase isoform from *Erythrina indica* Seeds

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**β-galactosidase (EC 3.2.1.23) is widely distributed in plant tissues and is known to be implicated in hydrolyzing terminal non reducing beta D-galactosyl residues from polysaccharides. 50g *Erythrina indica* seeds were pulverized and β-galactosidase was extracted and purified in several protein purification steps involved ammonium sulphate fractionation (Am-SO4), gelfiltration, acidification and finally chromatography on cationic ion exchanger. Thus obtained pure enzyme was then characterized with respect to its optimum pH and temperature, effect of metal ions, kinetics parameters and glycoprotein nature. β-galactosidase was purified to apparent electrophoretic homogeneity to 459-fold with comparatively high specific activity (133 Unit/mg). On gelfiltration the estimated native enzyme molecular weight was 66kDa whereas 47 and 32 kDa were obtained for the subunits by SDS-PAGE. The enzyme is a glycoprotein existed in at least three isoforms. It had optimum pH at around 4.5 and 60 $^{o}$C as favorable temperature at which it was stable for up to 30 minutes. Metals ions like $Fe^{+2}$, $Zn^{+2}$, $Mg^{+2}$, , $Mn^{+2}$, and $Ca^{+2}$, had no clear effect on enzyme activity, on the other hand, enzyme activity was fully abolished when incubated with heavy metals like Hg. β-galactosidase had 2.8 and 27 for $k_m$ and $V_{max}$ values, respectively. The purified enzyme exhibited remarkable thermal stability and could, therefore, be good candidate in food industry and biotechnology applications.**

**Keywords:** *Erythrina indica*; legumanacae; seeds; β-galactosidase isoform; Characterization.

## INTRODUCTION

The importance of glycosidases in the metabolism of carbohydrates in plants, particularly in storage organs such as seeds, is well acknowledged. The process of fruit development, ripening, softening and maturation are complex outcome involve many enzymes that work synergistically on hydrolyzing cell wall pectin and other complex carbohydrate (Hösel and Conn, 1982). Among several hydrolytic enzymes which are endorsed to perform functions related to loosening cell wall structure is β-galactosidase (Ali et al., 1995) (Konozy et al., 2012). Beta-galactosidases (EC 3.2.1.23) are ubiquitous widely distributed in nature, being found and thoroughly studied and purified from microbial, plant and animal kingdom(Nikolajczyk and O'Rand, 1992; Pal *et al.*, 2013.), respectively. On Biotechnology and food industry the enzyme finds great attention in the dairy technology where its being used to remove lactose from dairy products for lactose intolerance people. In addition, the enzyme is also used in improvement of quality parameters such as texture,

flavor and taste in dairy technology (Fernandez-Garcia et al., 1998; Husain, 2010).

*Erythrina indica* or *variegata* is a showy, spreading tree legume with brilliant red blossoms. Commonly known as the 'Indian coral tree' in Asia or 'tropical coral' in the Pacific, this highly valued ornamental has been described as one of the gems of the floral world. From our laboratory, several studies, on this species protein and enzymes purification and characterization have been carried out (Konozy *et al.*, 2002; Kestwal *et al.*, 2007; Konozy and Bhide, 2012).

The aim of the present investigation was to purify and characterize Beta-galactosidase from the seeds of *Erythrina indica* and therefore pave way for further studies on the enzyme's structural and molecular characterization.

## Experimental

### *Chemicals*

*Erythrina indica* season fresh good quality seeds were collected from the Botanical garden, Pune University, Pune, India. Gel filtration molecular weight marker kit (MWGF70- IKT 098k6082) was purchased from Sigma-Aldrich, USA. Sephadex G-100 was from Pharmacia; Uppsala, Sweden. Biogel-100 was generously donated by the late Dr. Khan M. I., NCL, Pune. All other materials and chemicals were either of analytical grade or highest grade available.

### *Protein Extraction and Fractionation*

*Erythrina indica* seeds were ground to fine powder, defatted and extracted for protein as stated by Konozy *et al.,* (Konozy *et al*., 2002) . In brief: 50g *Erythrina indica* seeds were soaked overnight in warm water, outer shells were peeled off, softened seeds cotyledons were mixed in fruit mixer and defatted by n-butanol-1 (5mL/1g powder); lipid and pigments layer was removed by centrifugation. Protein was precipitated with chilled acetone, the precipitant was filtered through cheesecloth, completely dehydrated with several folds of chilled acetone and left to dry at room temperature. Thus obtained powder is termed Acetone Dried Powder. The acetone dried powder was then extracted for 3h with 10mM sodium phosphate buffer pH 7.2 prepared in 0.145M NaCl. Extract was filtered through cheesecloth, turbid supernatant was centrifuged at 6000*rpm* for 45 min at 10 $^{o}$C, clear supernatant was collected and denoted as Fraction A (FrA). Protein fractionation of FrA was done by addition of 80% of solid ammonium sulfate (Am-SO4). Precipitated protein by salting out was dissolved in minimal amount of 0.145M NaCl, dialyzed exhaustively against the same saline to remove excess of Am-SO4. The fraction obtained by 80% Am-SO4 were preserved at -20 $^{o}$C till further use .

### *Protein quantification*

Protein contents were determined either by UV at $280_{nm}$ or colorimetrically as performed by Lowry *et al* (Lowry *et al*., 1951) using Bovine Serum Albumin (BSA) as the standard.

### *Purification of β-galactosidase*

Fractions rich in β-galactosidase activity obtained upon fractionation of FrA on gel filtration matrix on Bio-gel P-100 column, were pooled, pH of the solution was adjusted to 5.0. Solution was centrifuged to remove precipitated proteins and clear supernatant was dialyzed against Tris-HCl buffer pH 7.2 and this was loaded on DEAE-cellulose column equilibrated with Tris-HCl buffer pH 7.2. Washings basically rich in β-galactosidase activity were pooled, dialyzed exhaustively against distilled water, lyophilized to dryness.

### *Native molecular weight determination on gelfiltration*

The molecular weights of β-galactosidase was estimated by gel elution on Sephadex G-100, in 0.145 M, NaCl as described by konozy (Konozy, 1999).

The molecular weights were calculated by plotting a graph of log MW x $10^4$ Vs Ve/V0. Where: Ve: is the volume at which the protein peak is obtained.

The following protein molecular weight markers were initially used to calibrate the column: Bovine serum albumin-(BSA), ovalbumin, pepsin, trypsin and cytochrome C.

### *Purity confirmation*

Purity of β-galactosidase was assessed on native electrophoresis essentially as described (Williams and Reisfeld, 1964).

V0: Void volume, as determined by using Blue Dextran 2000.

### *Subunits molecular weight estimation by SDS-PAGE*

This was done essentially according to the procedures of Laemmli (Laemmli, 1970). The electrophoresis was carried out on 8% acrylamide gel with Tris glycine buffer pH 8.3, at a constant current.

### *Preparation of ConA-Seralose 4B affinity resin*

Coupling of ConA to Seralose 4B was done essentially as previously shown (Konozy and Bhide, 2012). 10mg protein were loaded onto ConA-Seralose 4B column, recycled several times to ensure maximum retention, unbound proteins were washed off till $OD_{280}$ < 0.02. Bound enzyme

was eluted with 50 mM mannose. Fractions of 2mL were collected at a flow of 1mL/2min.

***Influence of pH***

50 µL of the enzyme (~10 Units) was incubated with 200 µL of buffers of different pH ranging between pH 3.0 to pH 7.2 for 30 min. and 250 µL of *p*. nitrophenyl galacotside was added. Reaction was arrested after suitable time period by addition of 1 ml of borate buffer-pH 9.5, 0.1 M. The released *p*-nitrophenol was monitored spectrophotometerically at 405 min.

***Influence of temperature ,***

50 µL of the enzyme (~10 Units) was incubated with 200 µL of 0.2 M citrate puffer pH 4.5, at different temperatures ranging between 25 to 70$^{o}$C for 10 min. 250 µL of substrate was added. The reaction mixture was incubated for suitable time period and then arrested by addition of 1 ml 0.1 M borate buffer pH 9.5. The released *p*-nitrophenol was monitored spectrophotometerically at 405 nm.

***Influence of substrate concentration***

50 µL of the enzyme (~ 50 Units) solution was incubated with 200 µL of 0.1 M citrate buffer pH 4.5, to this mixture different concentrations of the corresponding pseudo-substrate ranging between 1 mM to 7 mM were added, test mixtures were incubated for suitable time period, the reactions were arrested by addition of 1 ml 0.1 M borate buffer pH 9.5. The released *p.* nitrophenol was measured spectrophotometerically at 405 nm.

***Influence of metal ions on enzyme activity***

Three mM solutions of metal ions namely Zn, Mg, Mn, Hg. Ca and Cu ions were prepared from their corresponding salts. The assay was done by incubating 50 uL of each metal ion solution with a suitably diluted enzyme (~ 10 Units) for suitable time period under the standard conditions of pH and temperature. The substrate was added, and the reaction mixture was re-incubated for suitable time period. The reaction was arrested by addition of 1 ml 0.1 M borate buffer pH 9.5.

## RESULTS AND DISCUSSION

***Purification of the enzyme***

During our preliminary work with *Erythrina indica* seeds extract, we noticed that seeds extract is very rich with pigments and fat. These contaminants interfered with accurate quantification of protein as well as enzymes assay. Therefore, rigorous initial treatment of seeds extract with defatting solvent was indispensable prior to any further biochemical work. Thus n-butanol treatment was used for depigmentation and defatting, which resulted in a reasonably, fat and pigment free seeds extract that enabled satisfactory determination of β-galactosidase activity. Purification of the enzyme from its crude extract on the affinity resin of Concanavalin A (ConA) was initially tried. Though the enzyme activity was detectable upon elution of the affinity resin with Con A haptenic sugar mannose, the amount of retained enzyme was very little (figure 1). Therefore, the search for alternative methods for purification of β-galactosidase in reasonable quantities to enable further biochemical studies was must. Loading of FrA on Biogel P-100 allowed excellent fractionation of protein in general and β-galactosidase in particular. Three discrete protein peaks were obtained (figure 2a and b), upon testing these peaks fractions for beta-galactosidase activity, it was observed that most of the enzyme activity was falling between protein peak 1 and 2 (figure 2a).

β-galactosidase rich fractions were pooled and dialyzed exhaustively against 50 mM acetate buffer, pH 5.0. This step resulted to precipitation of several proteins with concomitant 163-fold purification. The clear supernatant obtained upon centrifugation was dialyzed against 10 mM Tris-HCl buffer pH 7.2 and loaded onto the cationic ion exchanger DEAE cellulose. All proteins got retained on DEAE cellulose whereas β-galactosidase was obtained in in the flow through in electrophoretically pure form with 460-fold purification (Table 1). The purity of the preparation was assessed by native gel electrophoresis which resulted in a single sharp band, indicating homogeneity of the protein (figure 3A).

***Native and subunit molecular weight determination***

On loading pure β- galactosidase on Sephadex G-100 that was previously calibrated against standard protein markers, 66kDa was obtained for the enzyme as native molecular weight, whereas on SDS-PAGE under reduced condition resulted 47 and 32kDa, indicating the heterodimeric nature of the enzyme (figure 3B). Our SDS-PAGE results for the subunits molecular weights is in agreement with the enzyme purified from chick pea where 48 and 37 kDa were reported for the heterodimeric β-galactosidase (Kishore. D. and Kayastha. A. A., 2012).

***Effect of pH on enzyme activity***

Due to the fact that almost all of the so far characterized β-galactosidases, regardless of their origin, are possessing acidic pH optima, we focused our study on pH between 3 to 5.5. The enzyme had a pH optima at around pH 4.5, lost total activity at pH 5.5 (figure 4). The enzyme from almond seeds exhibited a broad pH optimum between 4.5 to 5.5 (Pal *et al*., 2013.).

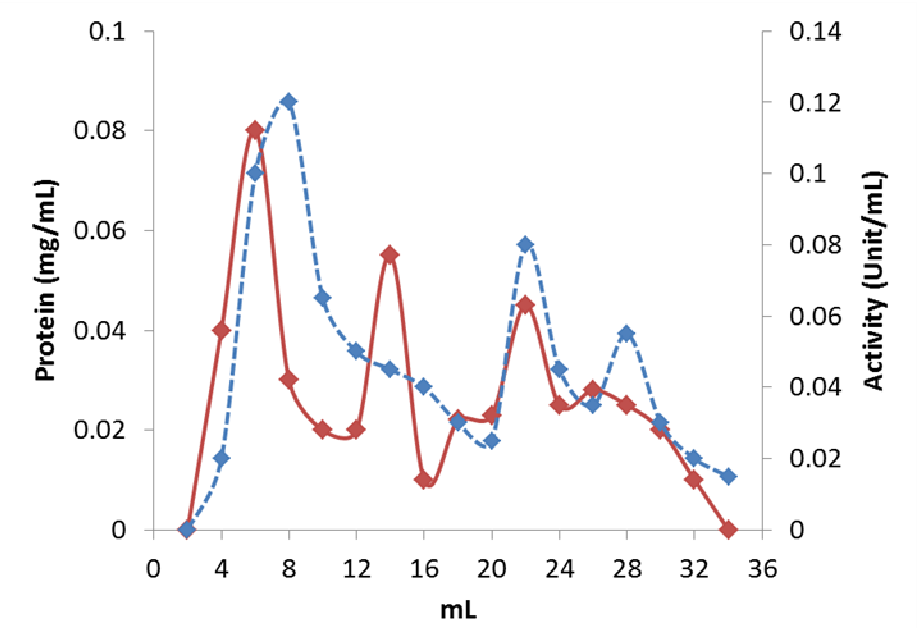


**Figure 1.** *Erythrina indica* seeds β-galactosidase fractions rich on the affinity matrix ConA-Seralose.
10 mg protein were loaded onto the affinity column and recycled several times to obtain maximum retention. Column was washed till free of unbound proteins. Bound protein was eluted by using 50 mM mannose. Fractions of 2mL were collected and monitored for protein content and enzyme activity.
Solid line: Protein, dashed line: β-galactosidase activity.

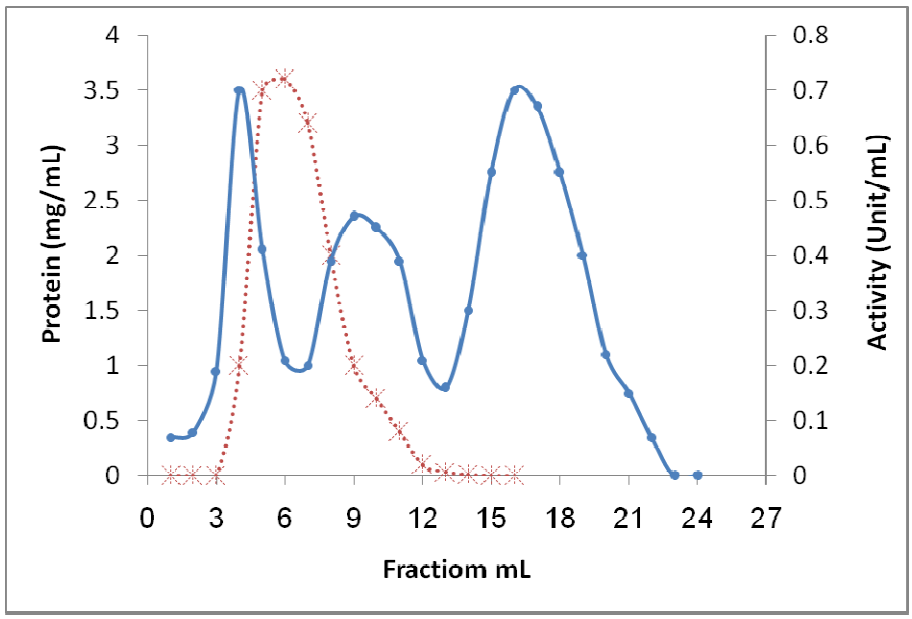


**Figure 2a:** Fractionation of *Erythrina indica* seeds crude extract on Biogel P100. 10 mg protein were loaded onto the column, fractions of 2 mL were collected at a flow rate of 1mL/2 min. Solid line represents protein, while dashed line represents β-galactosidase activity.

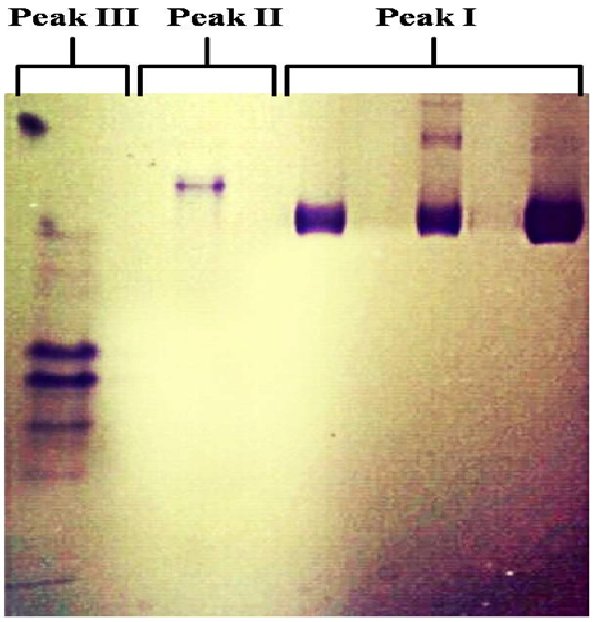


**Figure 2b:** *Erythrina indica* seeds FrA protein fractionation on Biogel P100.
Each peak fractions obtained on fractionation of *Erythrina indica* crude seeds extract on Biogel P-100 were separately pooled and loaded on SDS-PAGE. 20 µg protein were loaded and gel was stained with coomassie brilliant blue.

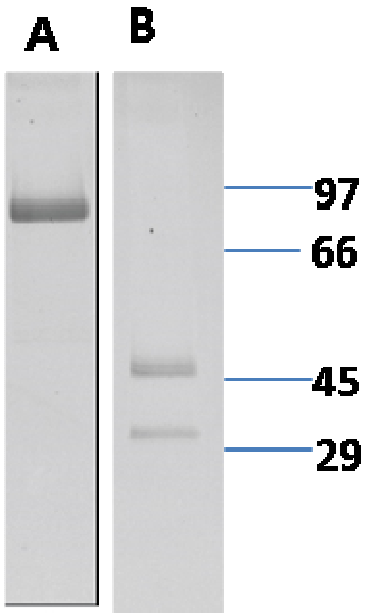


**Figure 3:**
A) Native-PAGE
B) SDS Polyacrylamide gel electrophoresis for pure β-galactosidase. Numbers to the right indicate molecular weight markers.
5µg protein were loaded and gels were stained with coomassie brilliant blue

**Table 1.** β-galactosidase purification stages.

| Stage | Total Protein (mg) | Activity (*unit/mL) | Specific Activity (Unit/mg) | Fold Purification |
|---|---|---|---|---|
| *Saline Extract* | 2070 | 2.6 | 0.3 | 1 |
| *Fraction A* | 432 | 19 | 1.5 | 5 |
| *Biogel P-100* | 7.2 | 23 | 28 | 97 |
| *pH 5 acidification* | 2.3 | 12 | 47 | 163 |
| *DEAE-cellulose* | 0.2 | 3 | 133 | 459 |

* 1 Unit of enzyme is defined as the amount of enzyme required to completely hydrolyze 1 µm of *p*-nitrophenyl phosphate per minutes.

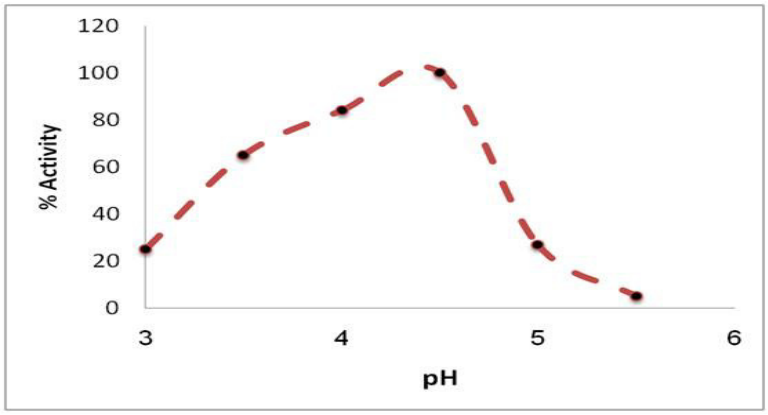


**Figure 4:** Effect of pH on enzyme activity

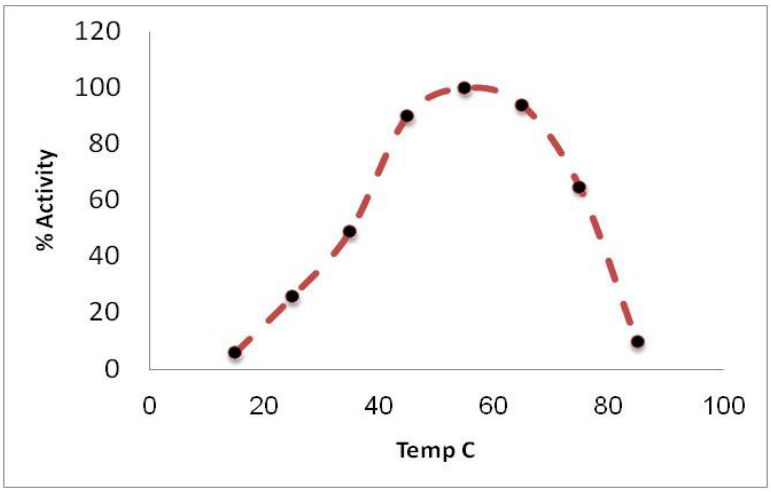


**Figure 5:** Effect of temperature on enzyme activity

### *Effect of temperature on enzyme activity*

The enzyme exhibited remarkable thermostability when challenged in temperatures ranging from 15 to 85 °C. β-galactosidase retained almost 100% of its original activity in temperature ranges from 45 till 60 °C. At 70 °C, for 30 minutes, the enzyme retained more than 60% of its original activity. However, at 80 °C the enzyme lost more than 90% of its original activity (figure 5). Again, our preparation share some similarities with chick pea (Kishore. D. and Kayastha. A. A., 2012). from one side and almond β-galactosidase (Pal et al., 2013.) from other side in which both remained stable till 60 °C, and therefore, authors of

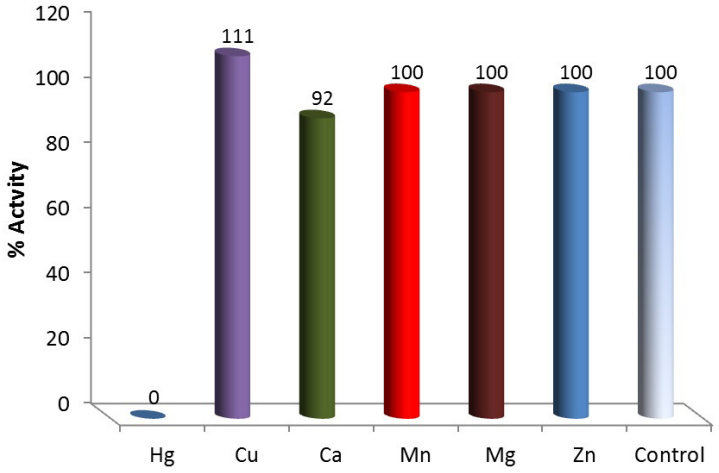


**Figure 6:** Effect of metal ions on enzyme activity

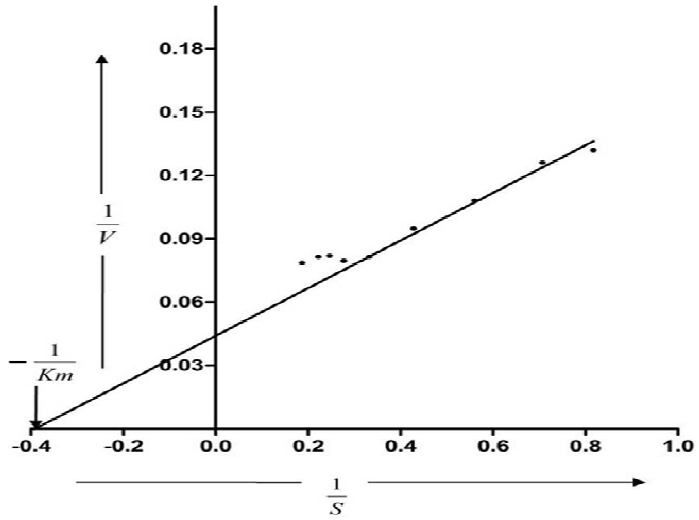


**Figure 7:** Effect of substrate concentrations on β-galactosidase activity

these papers recommended their possible applications in biotechnology applications.

### *Effect of metal ions on enzyme activity*

Incubating EDTA-treated enzyme with metal ion (3 mM) like Zn, Mn, Mg and Ca had no significant effect on enzyme activity. Slight increase in enzyme activity was observed with Cu ion. These results may indicate that either β-galactosidase is not a metaloprotein or the metal ion is deeply buried in the core side of the enzyme and inaccessible by chelating agent. Treatment of the enzyme with mercury totally abolished the enzyme activity (figure 6). Interestingly, in contrary to our results, incubation of apricot β-galactosidase with Ca ion resulted in enhancement of enzyme activity (Yossef and El Beltagey A., 2014).

### *The glycoprotein nature of the enzyme*

Loading of β-galactosidase on the affinity matrix, ConA-Seralose resulted in 100% retention of the enzyme. The absorbed enzyme could only be eluted from the column by using ConA haptenic sugar "D-mannose", in three distinct peaks (figure 1). This result besides it suggests the presence of enzymes in three isoforms, it may also conclude on the glycoprotein nature of the enzyme. Presence of β- galactosidase, in plant cell, in multiform is reported from varying plants such as apricot (Gulzar and Amin, 2012) tomato (Smith and Gross, 2000) and Avocado (Tateishi et al., 2001).

### *Effect of substrate concentration on enzyme activity*

Using the chromogenic pseudosubstrate *p*-nitrophenyl phosphate, 2.8 mM and 27 were obtained as $k_m$ and $V_{max}$ values for the enzyme, respectively (figure 7).

## CONCLUSION

In this investigation, β- galactosidase from *Erythrina indica* seeds was purified to apparent electrophoretic homogeneity in reasonably few steps. The enzyme exhibited astonishingly high thermostability which presents it as appropriate candidate for biotechnology applications as well as food industry.

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