

Original Research Article

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Response of Free and Immobilized Uricase from Fenugreek Leaves to various treatments and Digestive Enzymes

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ABSTRACT

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Uricase (Urate: oxygen oxidoreductase (EC 1.7.3.3) was isolated from 15-day old *Trigonella foenum-graecum* L. seedlings. Kinetin, gibberellin (GA3) and benzylaminopurine induced the enzyme and kinetin was the best inducer. The polyols including mannitol, sorbitol, glycerol, erythritol and xylitol expressed appreciable thermostability for uricase at 60 °C. Xylitol was better than trehalose as stabilizer at 60 °C. The enzyme was then purified using 80% ammonium sulfate, DEAE-cellulose, and Sephadex G-200. The final specific activity was 80 units mg⁻¹ protein. The purified uricase was immobilized on alginate (entrapped) and alginate with glutaraldehyde activity (cross-linked). The cross-linked method was better than entrapped method. The V_{max} values were 15.6, 13.8 and 15.2 units mg⁻¹ protein for the free, entrapped and cross-linked enzyme, respectively. The K_m values were 0.072, 0.092 and 0.084 mM, respectively. The optimal pH was 8.5 for the free, entrapped, and cross-linked enzyme. The optimal temperature was 35 °C, for free and entrapped but it was 45 °C for cross-linked uricase. Uricase exhibited appreciable resistant digestion by pepsin and trypsin.

Introduction

Uric acid (2, 6, 8-trihydroxypurine) is the final product of purine metabolism in human body. The high concentration of uric acid in the blood caused hypertension and cardiovascular diseases (Abbas, 2016). Therefore, uric acid detection method is very important to develop.

Microorganisms, animals and higher plants are capable of producing uricase on its own, but human being cannot produce uricase because of the mutation in the fifth exon of uricase gene (Nanda *et al.*, 2012).

In higher plants, purine degradation has been studied particularly in legumes which grow symbiotically with nitrogen-fixing bacteria, where degradation leads to ureides (allantoin and allantoic acid) which play an important role in nitrogen mobilization in many nodulated plants (Atkins, 1991).

Uricase (EC 1.7.3.3, UC) is an enzyme belonging to the class of the oxidoreductases and catalyzes the oxidation of uric acid to allantoin, and thus plays an important role in the purine degradation

pathway. Uricase in its conjugated form can be used as a therapeutic enzyme for the treatment of hyperuricemia and gout (Nancy *et al.*, 2006).

Uricase in its conjugated form can be used as a therapeutic enzyme for the treatment of hyperuricemia and gout (Nancy *et al.*, 2006). Furthermore, immobilized uricase can be used as a uric acid concentrations (Ademek *et al.*, 1989).

Uricase was found in cotyledons (Theimer and Beevers, 1971) and soybean leaf (Christensen and Jochimsen, 1983). This work aimed to isolate, purify, immobilize uricase from *Trigonella* leaves compare the characteristics of free and immobilized uricase.

Materials and Methods

Plant Material and Germination

Pure strain of *Trigonella foenum-graecum* L was obtained from Egyptian Ministry of Agriculture. Seeds were germinated according to El-Shora (2002) and 15-day old seedlings were collected and leaves were used for the present investigation.

Enzyme Extraction

Leaves (10gm) of *Trigonella foenum-graecum* L. were suspended in 50 ml of prechilled 20 mM phosphate buffer (pH 7.0) and the mixture was blended in warren blender and stirred. The resulting homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant constitutes the crude enzyme extract which was used for subsequent analysis (El-Shora, and Abo-Kassem, 2001).

Assay of Uricase

Uricase activity was assayed according to

Klose *et al.*, (1978). The reaction mixture contained 2ml of 10mM uric acid in 100mM sodium borate buffer (pH 8.5), 0.2 ml of 50 mM 4-aminoantipyrine, 0.2 ml of 15 U/ml peroxidase from horseradish, 0.2 ml of 2 % phenol, 0.2 ml of 100mM sodium borate buffer (pH 8.5) and 0.2 ml of enzyme solution to a final volume of 3.0 ml. The mixture was incubated at 30°C for 10 min and the increase in absorbance was measured spectrophotometrically at 505 nm. One unit of uricase is defined as the amount of enzyme that produces 1.0 mole of H₂O₂ per minute under standard assay conditions.

Determination of Protein Concentration

Protein concentration was determined according to Bradford (1967) with BSA as standard.

Effect of Phytohormones

The 7-day old seedlings were treated with 100 µmol of kinetin, GA₃ and benzylaminopurine for 72 h followed by enzyme assay.

Effect of Polyols and Sugars

The thermostability of purified free enzyme at 60 °C was investigated in presence of 10mM of mannitol, sorbitol, erythritol, sucrose, glucose, and 10% glycerol.

Purification of Uricase

The purification of uricase was done through three steps including ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Ammonium sulfate was added to the crude uricase until it reached to 80 % saturation. Precipitation of the protein was carried out by leaving the solution for 24 h at 4 °C. A column of DEAE-(diethylaminoethyl) cellulose was prepared

and the desalted uricase was poured on the surface of column (2 x 50 cm). The elution of sample was carried out with 100mM phosphate buffer (pH 6.0). A column of Sephadex G-100 (3 x 50 cm) was prepared and the eluent from the previous step was added. Elution was then carried out by 100mM phosphate buffer (pH 8.0). A total of 20-fractions of three-ml each were collected. The enzyme activity was assayed and the protein estimation was carried out. Fractions containing enzyme activity were pooled and used for enzyme assay.

Immobilization of Uricase

The immobilization of uricase by entrapment was carried out according to El-Shora *et al.*, (2015). The pure enzyme was added to 50 ml sodium alginate (3 % w/v). The solution was placed in a separating funnel and suspended over a beaker containing 150 ml CaCl₂. The alginate bead was prepared by gentle dropping of the alginate solution through a 200 μ l Eppendorf tip into CaCl₂ solution (30 drops min⁻¹). After 2h of gentle stirring the bead was filtered out of the CaCl₂ solution and thoroughly washed. The activity of the immobilized uricase was assayed. Cross-linking method for uricase immobilization was carried out by addition of 5 % (v/v) glutaraldehyde.

Effect of Uric Acid on Uricase Activity

The activity of the two forms of immobilized uricase from *Trigonella* leaves were determined at various concentrations of uric acid (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2). Lineweaver –Burk plot of uricase was carried out by plotting 1/v against 1/s and v_{max} and K_m were calculated.

Effect of Ph on Uricase Activity

The activity of free, entrapped and cross-

linked uricase was determined at pH values 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.

Effect of Temperature on Uricase Activity

The activity of free, entrapped and cross-linked uricase was determined at various incubation temperatures 25, 30, 35, 40, 45, 50, 55 and 60 °C.

Effect of Digestive Enzymes on Uricase Activity

The activity of free and immobilized uricase by cross-linking was measured after incubation with 200 units of trypsin or pepsin for various time intervals (20,40, 60, 80 and 100 min).

Desorption of Entrapped and Cross-linked Enzyme

Both entrapped and cross-linked uricase was treated with various concentrations (0.3, 0.6,0.9, 1.2, 1.5, 1.8 and 2 mM) of sodium dodecylsulphate (SDS) followed by measuring the desorptivity.

All the data in the present study are expressed as mean \pm SE obtained from three measurements.

Results and Discussion

The results in Fig. 1 reveal that uricase was induced by kinetin, gibberellic acid (GA3) and benzylaminopurine. GA3 activated other enzymes including phosphoenolpyruvate carboxylase (Bihzad and El-Shora, 1996), sucrose phosphate synthase (Kaur *et al.*, 2000), NADH-glutamate synthase (El-Shora, 2001), acid phosphatase (El-Shora and Metwally, 2009) and glutaminase (El-Shora and El-Naqeb, 2014). Kinetin induced carbonic anhydrase (Shah, 2008).

It is possible that phytohormones can increase the enzyme activity through acting at the level of transcription (Hayat *et al.*, 2001). It is also possible that phytohormones can increase the enzyme activity through acting at the level of the stabilization of the transcripts.

All polyhydric alcohols particularly mannitol (Fig. 2) had a positive effect against thermal denaturation. According to literature, the protective effect of polyols depends on the increasing number of hydroxyl groups (Graber and Cobes., 1989). However, in the present experiment, the effect of the length of the polyhydric alcohol molecule was not clearly observed.

The phenomenon of protein stabilization by polyhydric alcohols may be explained by changes in the microenvironment of the enzyme, which result in a more rigid conformation of the enzyme structure. Probably, the benefit of polyols is related to the effect they promote by increasing the degree of organization of water molecules, which in turn, intensify the hydrophobic interactions among nonpolar groups. Hydrophobic interactions appear to be the single most important factor to stabilize protein structure, therefore making the protein more resistant to unfolding (Triantafyllou *et al.*, 1997).

Polyols are by far the best co-solvent for their ability of maintaining both solvophobic interactions and have the capability to form hydrogen bonds that support the native configuration of the protein stabilization (Bhosale *et al.*, 2016). Trehalose as disaccharide protected uricase (Fig.3) against heat inactivation but with higher stabilizing factor than mannitol. These are in harmony with those reported by El-Shora and Metwally (2009) for α -glucosidase enzyme. Trehalose probably protects proteins by stabilizing their most compact

structure, therefore reducing backbone movements away from the fully folded state (Nirmal and Laxman, 2014). The degree of structural protection conferred by sucrose correlates with the extent of hydrogen bonding potential and the polarization of the backbone permanent dipole which induce an increase in hydrogen bonding character of neighboring polar residues. In this regard Sola-Penna and Meyer-Fernandes, (1998) proposed that trehalose has a larger hydrated volume than other carbohydrates such as maltose, sucrose, and glucose facilitating preferential hydration and stabilizing proteins.

Enzyme purification techniques are required whenever commercial use of enzymes is made. The repeated estimation of enzymes is required at different steps of the purification process to find out the effectiveness of the process. Uricase in the present investigation was purified using 80 % ammonium sulfate, DEAE-cellulose and Sephadex G200 (Table 1) with specific activity of 80 units mg⁻¹ protein.

The immobilization of uricase on alginate (entrapped) or alginate plus glutaraldehyde (cross-linked) was carried out (Fig. 4). The cross-linking uricase exhibited 90 % immobilization whereas the entrapment showed only 70% immobilization. Enzyme immobilization causes an increase in enzyme rigidity which is commonly reflected by an increase in stability towards denaturation by raising the temperature (Chang and Juang, 2005).

The results in Fig. 5 reveal that there was a gradual increase in uricase activity with increasing uric acid concentration for free, entrapped and cross-linked uricase. Plotting $1/v$ against $1/s$, where v is the velocity of the reaction and s is the concentration of uric acid exhibited straight lines for the three forms of uricase (Fig. 6). The

intercept of $1/v$ represents the value of V_{max} and the intercept $1/s$ represent K_m value. The V_{max} values were 15.6, 13.8 and 15.2 units mg^{-1} protein for the free and cross-linked enzyme, respectively. The K_m values were 0.072, 0.092 and 0.084 mM, respectively. Several reasons can account for the variations of the k_m values and v_{max} values of the enzyme upon immobilization. Among the reasons are the interactions of the enzyme molecules with the bead surface. This interactions might have induced an inactive conformation to the enzyme molecules. It should be noted that the immobilization process does not control the proper orientation of immobilized enzyme on the support (Yildiz, 2005). This improper fixation and /or the substrate the change in the property of the active sites might hinder the active site for binding of the substrate to

the immobilized enzyme molecules.

The optimal pH of uricase from *Trigonella* leaves was 8.5 (Fig. 7) for the free, entrapped, and cross-linked enzyme as indicated from. Ohe and Watanabe (1981) reported highest uricase activity from *Streptomyces cyanogenus* at 8.0. Huang and Wu (2004) reported that the highest activity of uricase was at pH values (pH 6-11) from *Candida subtilis*. Abdullah and Flayyih (2015) recorded pH optimal of 9.0 for the enzyme from *Pseudomonas aeruginosa*.

The optimal temperature of uricase (Fig.8) was 35 °C , for free and entrapped but it was 45 °C for cross-linked uricase. However, the optimal temperature of uricase was 45 °C for the enzyme from *Pseudomonas aeruginosa* (Abdullah and Flayyih, 2015).

Table.1 Purification of Uricase from *Trigonella foenum-graecum*

Purification step	Total protein (mg)	Total Activity (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Fold of purification
Crude extract	168	24	0.14	100	1
Ammonium sulfate (60-80 %)	121	22	0.18	92	1.29
DEAE-cellulose	80	18	0.22	75	1.57
Sephadex G-200	0.20	16	80	67	571

Fig.1 Effect of Phytohormones on Uricase Activity

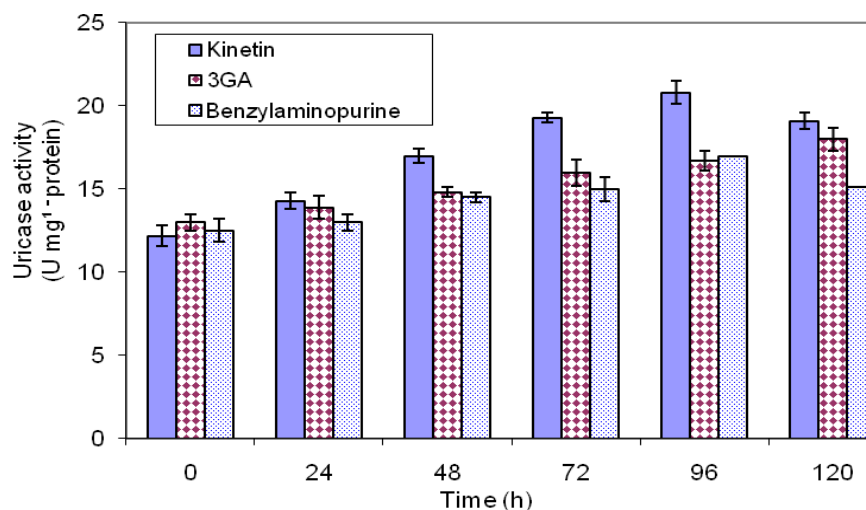


Fig.2 Effect of various Additives on Thermostability of Uricase at 60°C

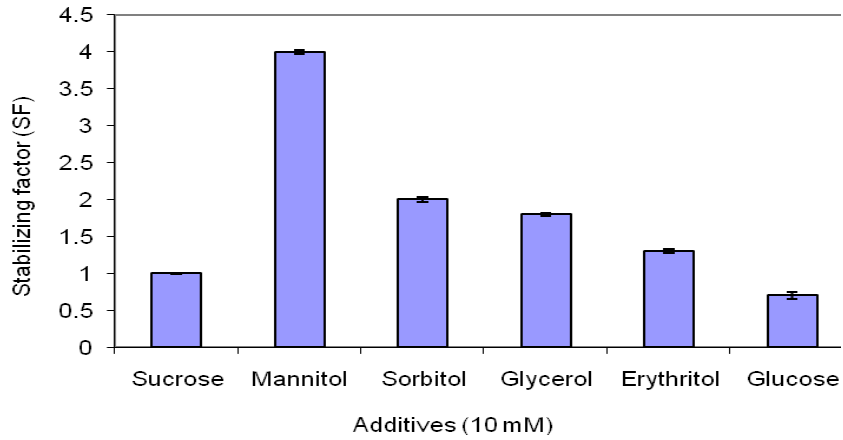


Fig.3 Effect of various Additives on Thermostability of Uricase at 60°C

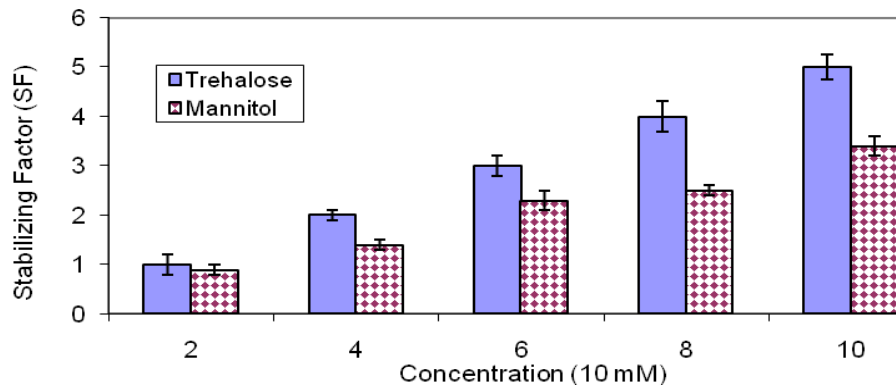


Fig.4 Immobilization of Uricase by Entrapment and Cross-linked Methods

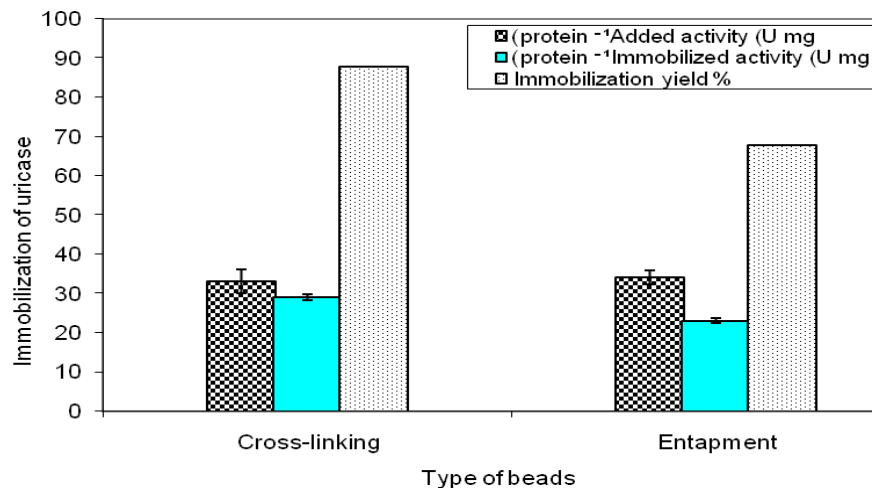


Fig.5 Effect of various Concentration of Uric acid on Uricase Activity

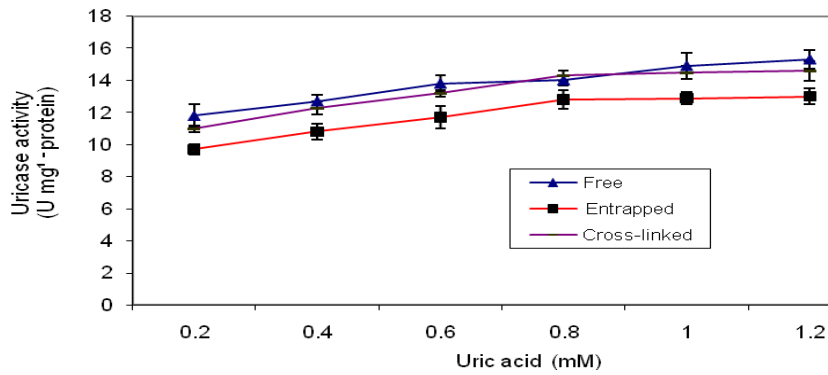


Fig.6 Lineweaver-Burk for Free, Entrapped and Cross-linked Uricase

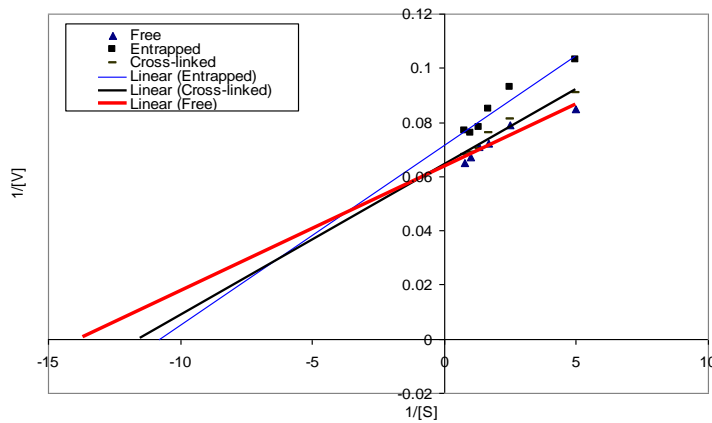


Fig.7 Effect of pH on the Activity of Free, Entrapped and Cross-linked uricase

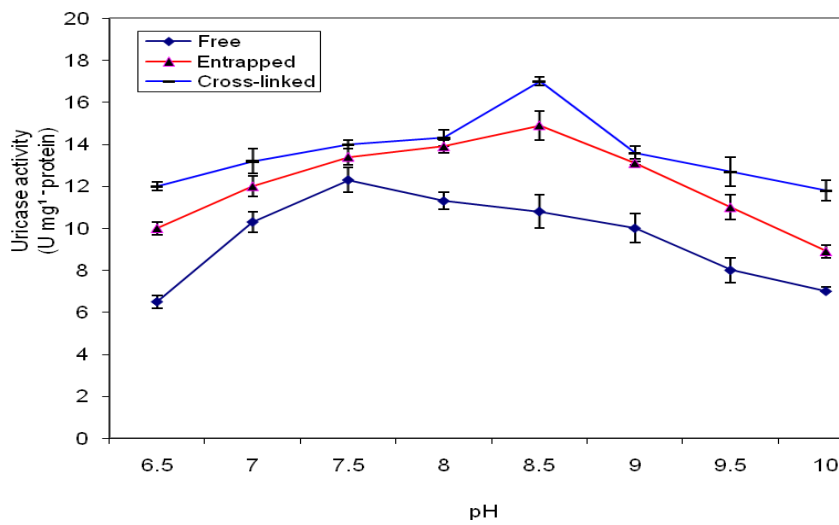


Fig.8 Effect of Temperature on Uricase Activity

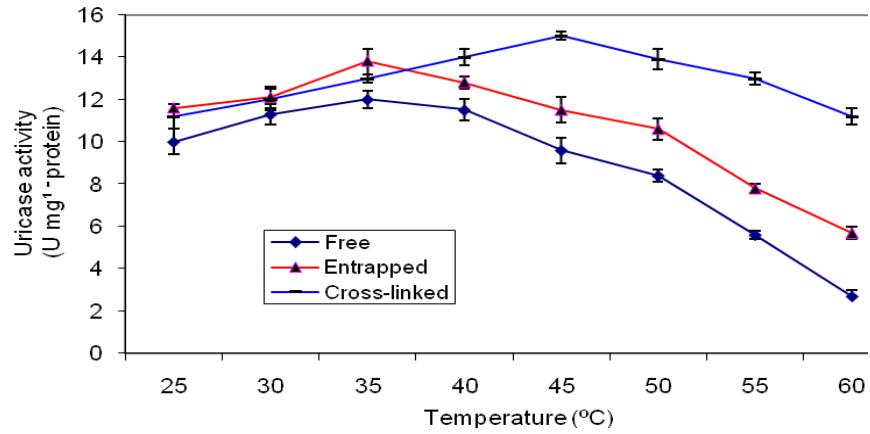


Fig.9 Reuse of Entrapped and Cross-linked Uricase

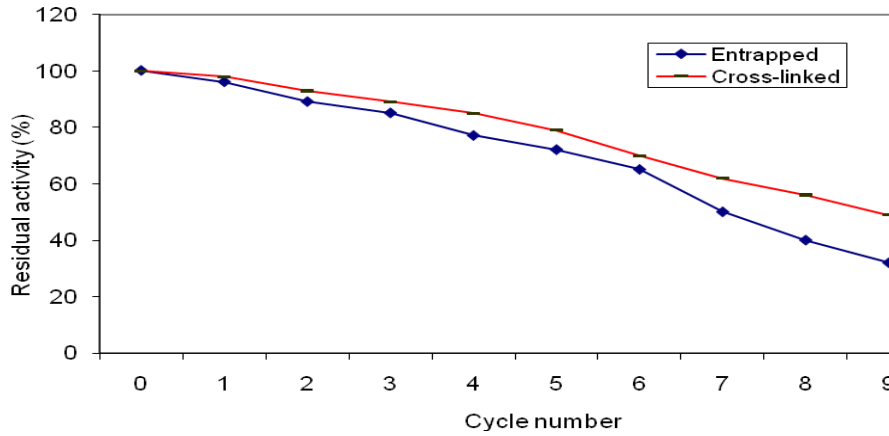


Fig.10 Effect of Pepsin on Uricase Activity.

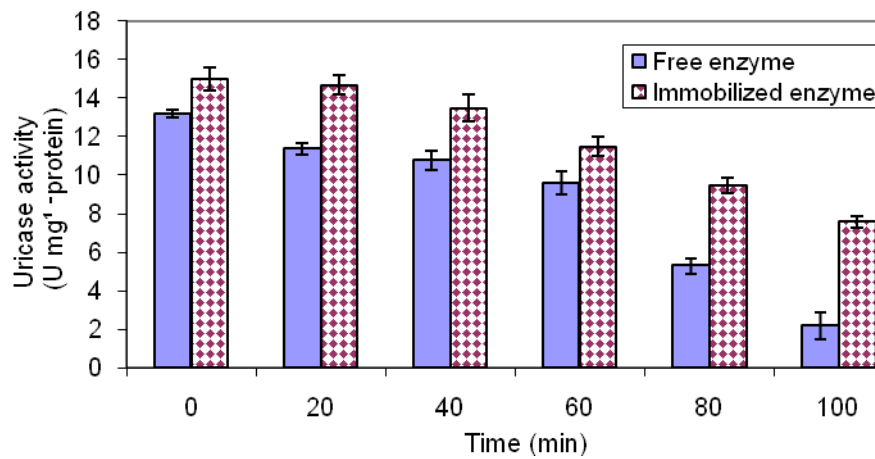


Fig.11 Effect of Trypsin on Uricase Activity

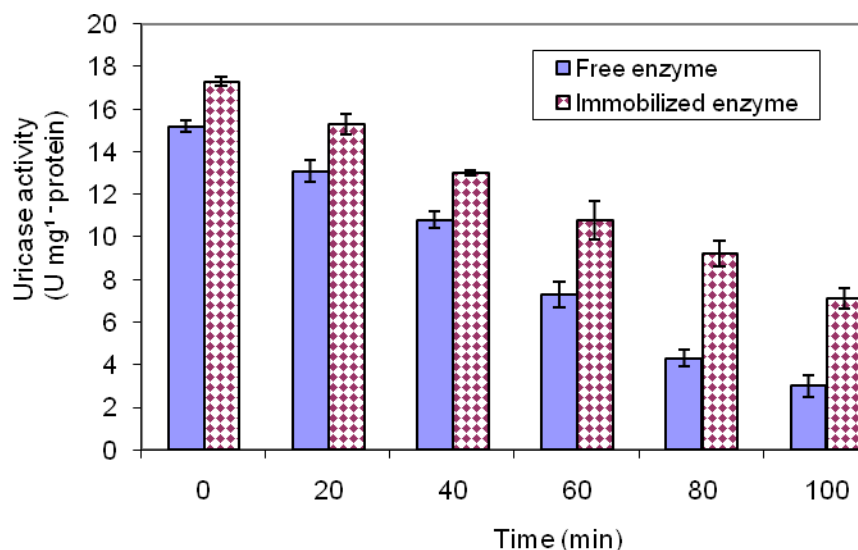
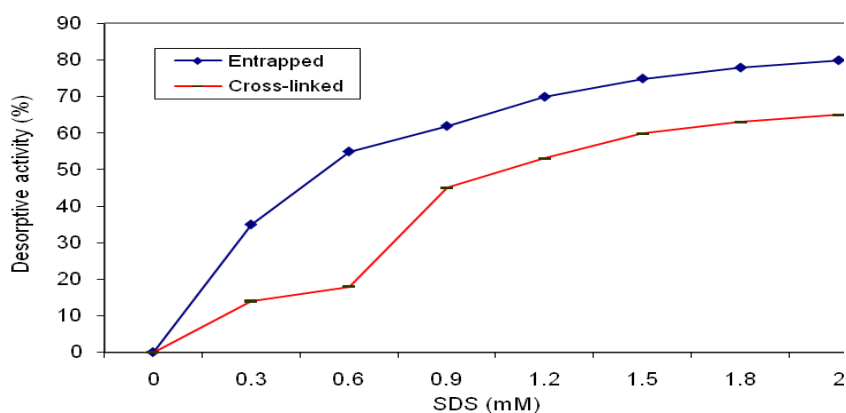


Fig.12 Desorptivity of Uricase by SDS



To investigate the possibility of reusing the immobilized enzyme by entrapment or cross-linking many times, the activity of the two forms of immobilized uricase was measured through successive ten cycles. The results in Fig. 9 indicate that uricase expressed appreciable activity throughout 9 cycles. The cross-linked uricase retained higher activity than entrapped enzyme throughout the ten cycles. The loss in activity upon reusing of immobilized preparation is a general observation. A lesser loss in activity in cross-linked enzyme preparation may be attributed to the cross-

linking of enzyme molecules by glutaraldehyde. Significant loss in activity of immobilized xylanases has been reported by many workers. Alginate entrapped lipase was active after 10 cycles without any loss in activity (Bhushan *et al.*, 2008).

The effect of proteolytic enzymes including pepsin (Fig.10) and trypsin (Fig. 11) on uricase activity was investigated at 200 units from each of the digestive enzymes. The results ross-linked urease was more resistant than the entrapment enzyme.

The influence of sodium dodecylsulphate (SDS) on the desorptivity of the immobilized uricase either entrapped or cross-linked was carried out using various SDS concentrations. The results in Fig. 12 show that SDS was successful in desorption process of uricase than the entrapment or cross-linked. It was noticed that entrapped uricase was easier to be desorbed than the cross-linked enzyme throughout the various examined concentrations.

It is important to note that, after a number of reuse cycles, enzyme gets deactivated and it should be removed from the matrix. The studies performed on desorption were also meant to take into consideration the marginal amount of enzyme likely to be loosely entrapped.

It was found that SDS desorbed the enzyme from matrix even after cross-linking with glutaraldehyde. This might be due to the competition of anionic surfactant with enzyme surface bound to the matrix. Our results are in agreement with earlier reports which also provided similar suggestions (Ran *et al.*, 2010). Researchers have always paid attention to strongly cross-link the enzyme molecules in the polymer network with the help of linkers, aggregators, carriers, and composite materials (Torabizadeh *et al.*, 2014).

In conclusion, uricase expressed appreciable activity in leaves of *Trigonella* which is one of the important medical plants. Also, the enzyme was successfully immobilized by entrapment and cross-linking methods. In addition the immobilized enzyme was resistant to digestive enzymes including pepsin and trypsin. These characters provide the immobilized uricase an importance in medical application.

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