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## Purification and biochemical characterization of L-methioninase from Fenugreek

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**Abstract**

L-methioninase (METs) catalysez the disintegration of methionine, an essential amino acid into ammonia, α-Ketoglutarate and methanethiol. L-methionine has a central role in the metabolism of all macromolecules, control of gene expression, cytoprotection and membrane integrity. This enzyme has been extensively studied from a wide range of organisms, including plants, terrestrial and marine microbes. **Aim of the work:** The aim of the present study was to produce, purify, immobilized and comparing the characteristics of the free and immobilized L-methioninase from Fenugreek. **Material and methods:** L-methioninase was purified using ammonium sulphate precipitation, DEAE-cellulose and Sephadex G200. The final specific activity was 34.5 units $mg^{-1}$ protein and 61.3-fold. The enzyme was immobilized on both Ca-alginate and chitosan. Also, the effect of treatment with three phytohormones including jasmonic acid, gibberellic acid and kinetin was studied. **Results:** The results in the present investigation exhibited that chitosan was better than Ca-alginate in the immobilization efficiency. Increasing the time of immobilization elevated the immobilization efficiency with Ca-alginate and chitosan. The enzyme was reused seven cycles and after 7 cycles it retained appreciable activity particularly with chitosan. The enzyme was desorbed highly from Ca-alginate than chitosan**.** The optimum pH values were 8 and 9, respectively for the free and the immobilized enzyme. The optimum temperatures were 35 and 40ºC, for the free and immobilized L-methioninase respectively. Treatment with the three phytohormones activated the enzyme with different rates**. In conclusion**: We can conclude that L-methioninase was purified from fenugreek plants with appreciable specific activity. The enzyme was immobilized on Ca-alginate and chitosan. The later was being the best support. The immobilized enzyme was reused seven cycles with maintaining considerable activities.

*Keywords:* Type your keywords here, separated by semicolons ;

## 1. Introduction

L-methioninase (EC 4.4.1.11) is also known by methionine- gamma lyase, methioninase, methionine lyase, and methionine demethylase. This enzyme is a member of the aspartate aminotransferase family [1-3]. Several reports are on the production of L-methioninase from Arabidopsis plants. Constituent expression of *Arabidopsis thaliana* L-methioninase in aerial organs and roots shows that has housekeeping function in plants [4-6]. Production of L-methioninase was noticed also in plant cell suspension cultures from *Catharanthus rose* and from *Cucumis melo* . [7-9] identified a functional L-methioninase in potato, an important crop species and proposed a more universal role of L-methioninase in plant methionine catabolism.

L-methionine degradation products such as methanethiol and 2-oxobutyrate are used for the synthesis of S-methyl L-cysteine and isoleucine, respectively. L-methioninase is involved in the alternative reverse-trans sulfuration pathway, in which L-methionine is metabolized to cysteine, not via cystathionine [10]. Immobilization is a technical process in which enzymes are fixed to or within solid supports, creating a heterogeneous immobilized enzyme system. The Immobilized form of enzymes mimics their natural mode in living cells, where most of them are attached to cellular cytoskeleton, membrane, and organelle structures [11-12]. The aim of the present work was to isolate and immobilize L-

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methioninase from fenugreek then studying some biochemical characteristics.

## 2. Materials and Methods

### 2.1. Seed germination and growth conditions

1. Seeds of Fenugreek were germinated according to [13]. The seeds of surface were sterilized in 10 % sodium hypochloride for 10 min, soaked in running tap-water for 24 h, and then germinated between paper towels, moistened with distilled water in sterilized plastic trays. The trays were covered and incubated in the dark at 25° C for 48 h. The germinated seeds with well-grown roots were then supported on plastic bowls containing 0.2 mM $CaCl_2$ solution. $CaCl_2$ solution was continuous and vigorously aerated. Seeds were grown for further 10 days in light at 25° C. The leaves of 10-days old plants were collected and kept on ice to be used for enzyme extraction immediately.

### 2.2 Enzyme extraction

The enzyme was extracted from the leaves as described by [13]. The tissue was ground with a pestle and mortar at 0-4 ºC using 50 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 5,000 rpm for 15 min, and the supernatant was collected, and represented the crude enzyme extract.

### 2.3. Purification of L-methioninase

The crude extract obtained by the extraction method was treated with solid ammonium sulphate (85 % saturation). The pellets obtained was suspended in the minimum volume of 0.1 M phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer. The dialyzed preparation was centrifuged at 5,000 rpm for 20 min and then poured on to DEAE-cellulose column (2×10 cm), equilibrated with 0.1M phosphate buffer pH (7.0) containing 0.2 M NaCl. To remove non-specially bound protein, the column was washed with equilibrating buffer. L-methioninase was eluted from the column by a steep gradient of 0.1 to 0.5 M NaCl in equilibrating buffer at flow rate of 0.5 ml $min^{-1}$. The active fractions were pooled and dialyzed over night against 30 mM phosphate buffer pH (7.0). The eluted enzyme from DEAE-cellulose column was loaded onto Sephadex G-200 column (2×25 cm). The active fractions were collected and considered as the pure enzyme.

### L-Methioninase Assay

The activity of L-methioninase was estimated by measuring the amount of ammonia liberated from L-methionine spectrophotometrically following Nesslerization according to [14].

### 2.4 Determination of protein content

Protein content was determined according to [15]. Five ml of diluted Coomassie-Brillant blue G-250 were mixed with 1 ml of enzyme extract and the mixture was put for one min in dark and the absorption was measured spectrophotometrically at 595 nm. Bovine serum albumin (BSA) was used as standard.

### 2.5. Determination of Molecular Weight by Gel Electrophoresis

The homogeneity of purified L-methioninase was checked using dissociating polyacrylamide gel electrophoresis (SDS-PAGE) and was carried out according to a protocol proposed by [16].

### Immobilization of L-methioninase on chitosan beads

Chitosan beads were mixed with L-methioninase solution 2 mg/ml in 150 mM potassium phosphate buffer (pH 7.0) for 2 h with slight stirring and washed at 4 °C then, the beads dried at room temperature and stored at 4°C. The activity of immobilized enzyme was estimated by subtracting the activity of in the supernatant after immobilization from the added activity on chitosan bead.

### Immobilization of L-methioninase on alginate bead

This method of immobilization was initially adopted from that reported by [17]. The pure L-methioninase was mixed with 40 ml sodium alginate (3 % w/v). The resulting solution was placed in a separating funnel and suspended over a beaker contained 200 ml of 3% w/v $CaCl_2$. The alginate bead was prepared by gentle dropping of the solution through a 200 µl ependorf tip into $CaCl_2$ solution at a rate of 30 drops $min^{-1}$. After for 4 h gentle stirring the bead was filtered out of the $CaCl_2$ solution and washed using the same buffer. The activity of L-methioninase immobilized was assayed.

## 3. Results and discussion

### Purification of L-methioninas

The purification of L-methioninase was carried out by ammonium sulphate precipitation (85%), DEAE-Cellulose and Sephadex G-200. The results in Table 1 show that the enzyme was purified 61.3-fold with 34.5 $Umg^{-1}$ protein. [18] purified L-methioninase 2.55-fold from *Stryptomyces*.

### Immobilization of L-methioninase

The immobilization of the enzyme was tested on chitosan and ca-alginate (Table 2). The

immobilization efficiency value was 67.4% and 88.0% on calcium alginate and chitosan, respectively. The immobilization of enzyme causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability toward denaturation by raising the temperature [19]. It should be noted that the biocompatibility of the support could also play an important role on stabilization of enzyme confirmation. Among the main reasons for enzymes immobilization is the anticipated increase in its stability to different deactivating force due to restricted conformational mobility of the molecules following immobilization. Thus, the immobilized enzyme could work in the harsh environmental conditions with less activity loss compared to the free counterpart [20-21]. The free enzyme cannot be used to catalyze more reactions because the free enzyme cannot be recovered from the reaction mixture but the process of immobilization can make it feasible [22]. However, the loss of activity of some immobilized enzymes in repeated use is famous phenomenon [23], and this loss of activity is likely a result of desorption of the enzyme. The support of the immobilization generally has a protective effect at high temperature at which deactivation occurs. The conformational flexibility of the enzyme is affected by immobilization. Enzyme immobilization causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation by raising the temperature [19].

**Effect of time course of immobilization on immobilization efficiency of L-methioninase**

The effect of time course of immobilization of L-methioninase on the immobilization efficiency was investigated through various time interveals (10, 20,30,40,50, and 60 min). The results in Fig. 1 indicate that increasing the time of immobilization increased the immobilization efficiencey.

**Desorption of immobilized L-methioninase**

Desorption of L-methioninase was carried out using SDS and the results in Fig. 2 reveal that desorption of the enzyme immobilized on alginate was higher than that for the enzyme immobilized on chitosan. This indicates that cross-liking method of enzyme immobilization is better than entrapment where the enzyme could be freed quickly. SDS is known as denaturant of proteins, it could unfold most protein by interactions between the charged head groups of SDS and the positively charged amino acid chains of the proteins [24].

**Reusability of immobilized L-methioninase**

The reusability of the immobilized enzyme was carried out for 7 cycles (Fig. 3). It was apparent that the enzyme could be used several times with maintaining appreciable activity particularly that immobilized on chitosan. However, the activity of immobilized enzyme decreased gradually throughout the seven cycles. The reduction was more apparent with Ca-alginate. The observed decrease during the use of immobilized enzyme is famous phenomenon [23]. The reduction could be due to protein degradation, protein deactivation and physical loss of the alginate-bound protein. The decreased activity of L-methioninase may also be due to the hydrophobic characteristics of sodium alginate. The size of the pores gradually becomes larger after repeated use, resulting in increased leakage of the enzyme and reducing the activity [25].

**Effect of pH on the activity of free and immobilized L-methioninase**

This experiment aimed to investigate the influence of pH on the activity of free and immobilized L-glutamase . Therefor, various pH values were tested (5, 6, 7, 8, 9 and 10). The results in Fig. 4 reveal that in case of the free L-methioninase the activity increased gradually up to 8.0 which seems likely to be the optimal pH for the free enzyme. After pH 8.0, the activity declined gradually to reach 11.0 $Umg^{-1}$ protein at pH 10.0. However, in case of the immoilized enzyme the optimal pH was 9.0 for the entrapped and cross-linked enzyme and declined at pH 10.0 .

Extreme of the pH scale may cause denaturation and the following some of the possible effects that might be happened by change in pH value. Firstly, at extreme pH it can bring about changes in protein structure that can irreversibly alter the stability of the enzyme. Secondly, it changes the ionization of the enzyme-substrate complex. Thirdly, it changes the ionization of the substrate, which may affect the binding of the substrate to the enzyme. Fourthly, it changes the ionization of various groups of the enzyme molecule [26]. The inhibitory effect of higher acidic and basic pH on the enzyme activity may suggest the change on enzyme ionization state, modifying its surface changes, dissociation of subunits, thus disrupting the enzyme-substrate intermediate.

**Effect of temperature on the activity of free and immobilized L-methioninase**

The effect of different incubation temperatures on the activity of free and immobilize enzyme on alginate as well as on chitosan was investigated. The various temperatures were 20, 25, 30, 35, 40, 45, 50, 55 and 60ºC.The results in Fig. 5 show the activity for the free enzyme increased gradually untill it reached 40 ºC after which the activity declined contiously and reached 5.0 $Umg^{-1}$ protein at 60 ºC. However, for the immobilized enzyme on alginate or chitosan the optimal activity was shifted to 45 ºC after which ther was gradual decline in the activity with increasing the

temperature. At 60 ºC the activity was 17.2 $Umg^{-1}$ protein for the enzyme immobilized on chitosan and 15.0 $Umg^{-1}$ protein for immobilized enzyme on alginate.

Increase of the temperature results in increasing of the inherent energy of the enzyme system. Thus, more molecules obtain the necessary activation energy for the reaction to take place. However, there comes a point where the increase of the reaction due to the influence of the temperature on the activation of the enzyme molecule is equal to the reduction in the reaction rate due to the destruction of the tertiary structure [27-28]. At this point the enzyme activity is at its maximum and this temperature is known as optimal temperature. Generally, the chemical reactions whether exothermic or endothermic have to overcoming the energy of activation to take place. It is known that as the activation energy becomes greater, the more the heat which supports a successful reaction [29]. Any enzyme can lower the overall activation energy of the reaction by binding the reactants in the right orientation for the reaction to take place [30].

**Effect of phytohormones on the activity of free and immobilized L-methioninase**

The present results in Fig. 6 revealed that the phytohormones $GA_3$, kinetin and jasmonic acid activated L-methioninase activity. In support, $GA_3$ induced other enzymes such as phytase [31], phosphoenolpyruvate carboxylase [32], NADH-glutamate synthase [13] and phenylalanine ammonia lyase [33]. In addition, $GA_3$ increased the activities of sucrose synthase and sucrose phosphate synthase [34].

In conclusion, L-methioninase was purified from fenugreek plants with appreciable specific activity. The enzyme was immobilized on Ca-alginate and chitosan. The later was being the best support. The immobilized enzyme was reused seven cycles with maintaining considerable activities.

**Table1: Purification of L-methioninase**

| Purification step | Total activity (U) | Total protein (mg) | Specific activity ($Umg^{-1}$) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Homogenate | 228.0 | 575.0 | 0.40 | 1.0 | 100.0 |
| Ammonium sulphate precipitation (85%) | 160.0 | 240.0 | 0.67 | 1.68 | 70.2 |
| DEAE-Cellulose | 92.0 | 52.0 | 1.77 | 3.95 | 40.4 |
| Sephadex G-200 | 69.0 | 2.0 | 34.5 | 61.25 | 30.3 |

**Table 2: Immobilization of L-methioninase on calcium alginate and chitosan**

| Support | immobilization efficiency (%) |
|---|---|
| Ca-alginate | 67.4±1.2 |
| Chitosan | 88.0±2.1 |

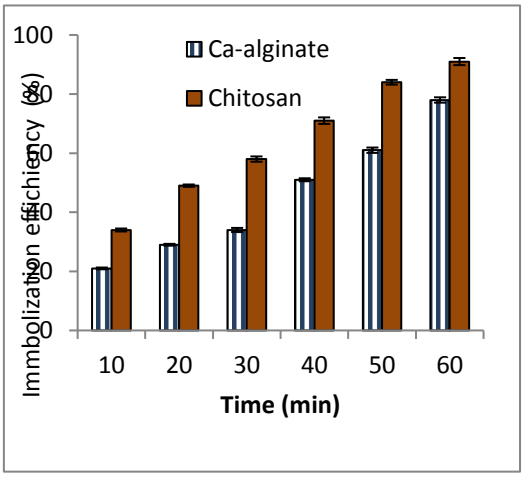


**Fig.1: Effect of time course on immobilization efficiency of L-methioninase from fenugreek**

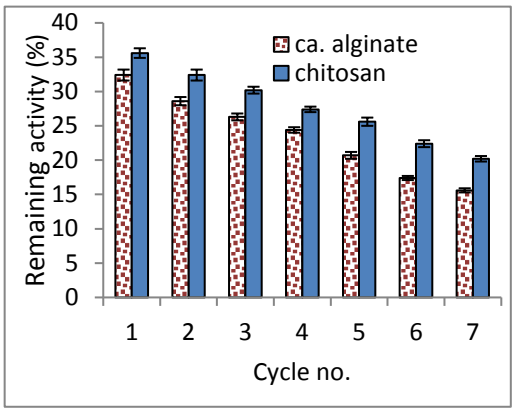


**Fig.2: Reusability of immobilized L-methioninase from Fenugreek**

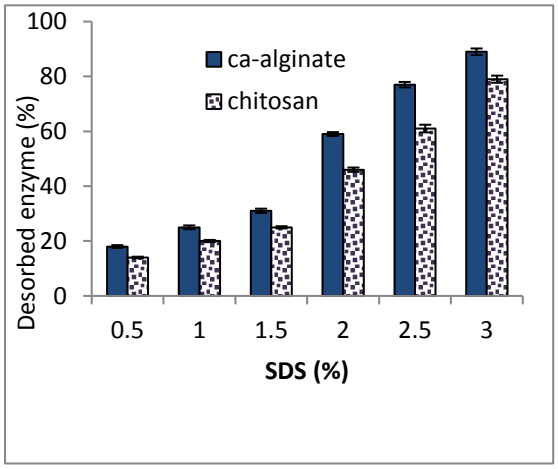


**Fig.3: Desorption of immobilized L- methioninase from Fenugreek by SDS**

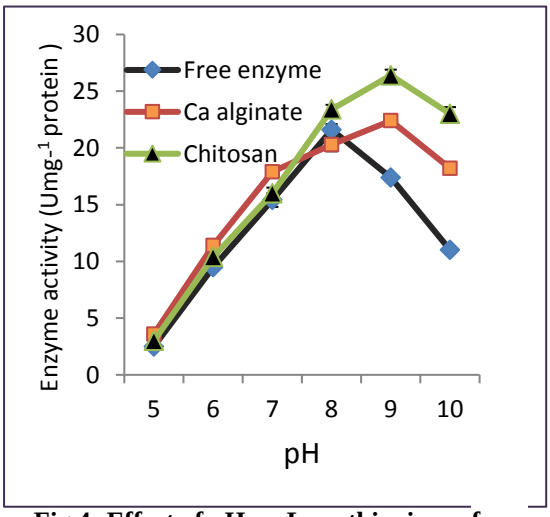


**Fig.4: Effect of pH on L-methioninase from Fenugreek activity**

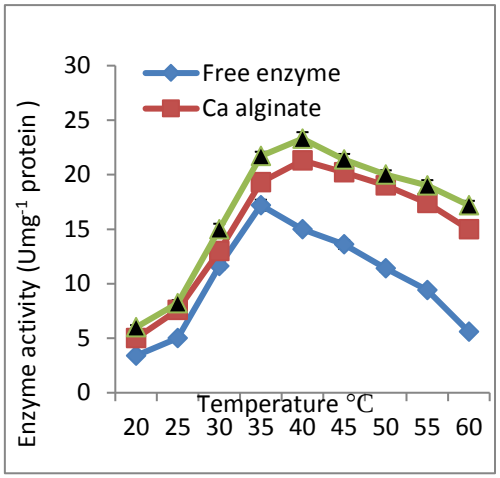


**Fig.5: Effect of temperature on free and immobilized L-methioninase from Fenugreek**

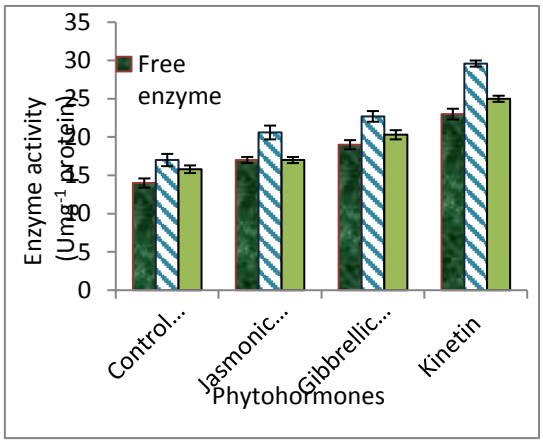


**Fig.6: Effect of plant growth regulators on L- methioninase from Fenugreek activity**

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**5. Declaration of interest**:

No conflicts of interest.

**6. Data Availability:**

The data that supports this work is available upon reasonable request.

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**8. References**

1. Cooper, A. (1983). Biochemistry of sulfur-containing amino acids. *Annual Review of Biochemistry*, **52**(1), 187-222.
2. Sato, D., & Nozaki, T. (2009). Methionine gamma-lyase: the unique reaction mechanism, physiological roles, and therapeutic applications against infectious diseases and cancers. *IUBMB life*, ***61***(11), 1019-1028.
3. Suganya, K., Govindan, K., Prabha, P., & Murugan, M. (2017). An extensive review on L-methioninase and its potential applications. *Biocatalysis and Agricultural Biotechnology*, **12,** 104-115.
4. Goyer, A., Collakova, E., Shachar-Hill, Y., & Hanson, A. D. (2007). Functional characterization of a methionine γ-lyase in Arabidopsis and its implication in an alternative to the reverse trans-sulfuration pathway. *Plant and Cell Physiology*, **48**(2), 232-242.
5. Amir, R. (2010). Current understanding of the factors regulating methionine content in vegetative tissues of higher plants. Amino acids, **39**(4), 917-931.
6. Pratelli, R., & Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. *Journal of Experimental Botany*, **65**(19), 5535-5556.
7. Schwenn, J. D., Schriek, U., & Kiltz, H. H. (1983). Dissimilation of methionine in cell suspension cultures from Catharanthus roseus L. Planta, **158**(6), 540-549.
8. Gonda, I., Lev, S., Bar, E., Sikron, N., Portnoy, V., Davidovich-Rikanati, R., Lewinsohn, E. (2013). Catabolism of L–methionine in the formation of sulfur and other volatiles in melon (*Cucumis melo* L.) fruit. The Plant Journal, **74**(3), 458-472.
9. Huang, T., Joshi, V., & Jander, G. (2014). The catabolic enzyme methionine gamma-lyase limits methionine accumulation in potato tubers. Plant biotechnology journal, **12**(7), 883-893.
10. Hoffman, M., (1997). Methioninase: a therapeutic for diseases related to altered methionine metabolism and transmethylation: cancer, heart disease, obesity, aging, and Parkinson's disease. *Human Cell*, **10**, 69-80.
11. Homaei, A. (2015). Enzyme immobilization and its application in the food industry. *Advances in Food Biotechnology*, **9**, 145-164.
12. El-Shora, M. & El-Sharkawy, M. (2020). Tyrosinase from *Penicillium chrysogenum*: Characterization and application in phenol removal from aqueous solution. *The Journal of General and Applied Microbiology*, **66**(6), 323-329.
13. El-Shora, H. M. (2001). Properties and immobilization of urease from leaves of Chenopodium album (C3). *Botanical Bulletin of Academia Sinica*, **42**, 251-258.
14. Thompson, F., & Morrison, R. (1951). determination of organic nitrogen. *Analatical Chemistry*, **23**, 1153-1157.
15. Bradford M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*,**72**, 248–254.
16. Laemmli, UK. (1970). Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature*. **227**(5259), 680-685.
17. Foster, M., Dorrington, D. & Burton, G. (2003). Enhanced hydantoinase and N-carbamoylase activity on immobilization of *Agrobacterium tumefaciens. Biotechnology Letters.* **25**(1), 67-72.
18. Selim, H., Elshikh, H., Saad, M., Mostafa, E. & Mahmoud, A., (2016). Purification and characterization of a novel thermostable L-methioninase from *Streptomyces sp*. DMMMH4 and its evaluation for anticancer activity. *Journal of Applied Pharmaceutical Science.* **6**(7), 53-60.
19. Chang, M. Y. & Juang, R. S. (2004). Stability and catalytic kinetics of acid phosphatase immobilized on composite beads of chitosan and activated clay. *Process Biochemistry*. **39,**1087-1091.
20. Arıca, M. Y., Öktem, H. A., Öktem, Z., & Tuncel, S. A. (1999). Immobilization of catalase in poly (isopropylacrylamide-co-hydroxyethylmethacrylate) thermally reversible hydrogels. Polymer International, **48**(9), 879-884.
21. Bai, S., Thomas, C., Rawat, A. & Ahsan, F. (2006). Recent progress in dendrimer-based nanocarriers. Critical Reviews in Therapeutic Drug Carrier Systems. **23,** 437–495.
22. Romo-Sánchez, S., Camacho, C., Ramirez, L., & Arévalo-Villena, M. (2014). Immobilization of commercial cellulase and xylanase by different methods using two polymeric supports. Advances in Bioscience and Biotechnology, **5**(6), 517.
23. Arasaratnam, V., Galaev, I. Y. & Mattiasson, B., (2000). Reversibly soluble biocatalyst: optimization of trypsin coupling to Eudragit S-100 and biocatalyst activity in soluble and precipitated forms. Enzyme Microbial Technology. **27**, 254-263.
24. Virgen-Ortíz, J. J., Pedrero, S. G., Fernandez-Lopez, L., Lopez-Carrobles, N., Gorines, B. C., Otero, C., & Fernandez-Lafuente, R. (2017). Desorption of lipases immobilized on octyl-agarose beads and coated with ionic polymers after thermal inactivation. Stronger adsorption of polymers/unfolded protein composites. *Molecules*, **22**(1),91-99.
25. Verma, M. L., Chaudhary, R., Tsuzuki, T., Barrow, C. J. & Puri, M. (2013). Immobilization of *β*-glucosidase on a magnetic nanoparticle improves thermostability: application in cellobiose hydrolysis. Bioresource technology. **135**, 2-6.
26. Palmer, T. (1995). Understanding enzymes. John Wiley and sons, New York.Pp,55-71.

27. Palmer, T. & Bonner, P. L. (2007). Enzymes: biochemistry, biotechnology, clinical chemistry. 2nd Edition, Woodhead Publishing, Cambridge.
28. El-Shora, H. M., Ibrahim, M. E. & Alfakharany, M. W. (2018). Activators and inhibitors of α-glucosidase from *Penicillium chrysogenum*. Annual Research and Review in Biology. **24**, 1-9.
29. EL-Shora, H. M., Abu-zied, A. M., Awadalla, O. A., Metwally, S. M. & EL-zawawy, N. A. (2021). Production, optimization and purification of L-methioninase from *Aspergillus flavipes* AUMC 1201. Plant Cell Biotechnology and Molecular Biology. **22**,312-326.
30. El-Shora, H.M. & Ali, A. S. (2016). Plant growth regulators induced urease activity in *Cucurbita pepo* L. cotyledons. Acta Biologica Hungarica. **67**, 53-63.
31. Gabard, K. A., & Jones, R. L. (1986). Localization of phytase and acid phosphatase isoenzymes in aleurone layers of barley. *Physiologia Plantarum*, **67**(2), 182-192.
32. Bihzad, M. A. & El-Shora, H. M. (1996). Phosphoenolpyruvate carboxylase from *Rumex dentatus*, a C3-plant. Journal of Plant Physiology. **149**,669-676.
33. El-Shora, H. M. (2002). Properties of phenylalanine ammonia-lyase from marrow cotyledons. *Plant Science*, ***162***(1), 1-7.
34. Kaur, S., Gupta, A. K. & Kaur, N. (2000). Effect of GA3, kinetin and indole acetic acid on carbohydrate metabolism in chickpea seedlings germinating under water stress. Plant Growth Regulation. **30**,61-70.