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TEOS hydrosol gel-chitosan matrix based biosensor for monitoring asparagine in various fruit juices

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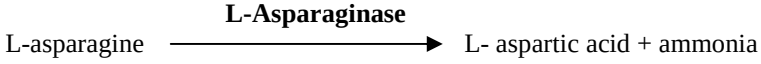
ABSTRACT

The biosensor for monitoring asparagine levels in Fruit juices was developed in present research work. L-asparaginase (biocomponent) has been extracted from Withania somnifera (L) Dunal, and immobilized into TEOS hydrosol gel-chitosan matrix. Kinetic characterization of both native and immobilized enzyme was compared. Response time studies had been carried out by Phenol Red indicator coimmobilized with asparaginase and colour visualization strategy has been optimized for a range of asparagine concentrations. The detection limit of asparagine achieved was 10^{-10}–10^{-1} M. Furthermore, the developed biosensor was applied on different fruit juices for detection of asparagine levels. Biocomponent was found to be stable for 32 days in Na borate buffer (pH 8.5) at 4°C and it is reliable for fruit juices.

Keywords: L-asparaginase, biosensor, hydrosol-gel, chitosan, *Withania somnifera* (L) Dunal.

INTRODUCTION

Asparagine is the chief amino acid that forms acrylamide in baked food by reacting with reducing sugars at high temperature. This reaction is called Millard Reaction (figure 1) i.e. amino acids and sugars give new flavours at high temperature [1]. Acrylamide is highly carcinogenic and should be removed from food. L-asparaginase is used for lowering the acrylamide level by hydrolysing the free asparagine into aspartate and ammonia and thus, the reaction limits the amount of asparagine to be converted into acrylamide [2]. Asparagine is required for the development of brain and it regulates the equilibrium of central nervous system [3]. It can also be a quality insurance parameter in fruit juices. Babsky *et al.* [4] studied that storage of juices at 37°C caused an 87% loss in the total free amino acids and major decrease was record in asparagine contents. Its analysis at laboratory scale is found to be time consuming and expensive. With the aid of biosensor technology asparagine can be analysed within few seconds and it is cheaper than conventional analytical methods. For Asparagine biosensor, the principle involves immobilization of L-asparaginase which hydrolyses the asparagine into aspartate and ammonia. Ammonia increases the pH of the reaction which changes the colour of Phenol red indicator (Light orange to pink).



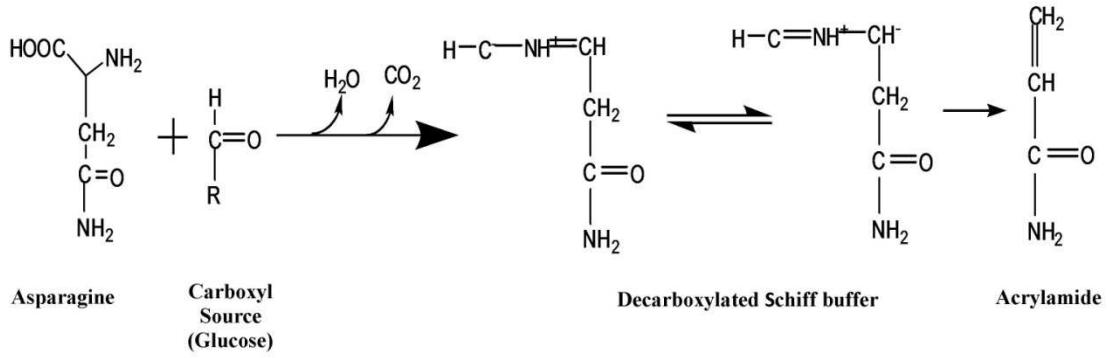


Figure 1: Formation of Acrylamide from Asparagine

L-Asparaginase (E.C. 3.5.1.1) acts as an anti-leukemic agent by inhibiting the protein synthesis via depletion of asparagine in leukemic cells. It is accessible as a drug under the trade name of " Elspar and Kidrolase". It is also used in food industry to reduce the formation of acrylamide from the baked product under the brand name of "Acrylaway and PreventAse" [5]. There is a wide range of sources of L-asparginase i.e. bacteria, algae, fungi, plants etc. Among plants, Ashwagandha ,*Withania somnifera* (L.) Dunal, also known as the Indian Ginseng, is considered as a good source [6]. This plant is one of the most widely used herbs in ayurvedic medical system because it has anti-inflammatory [7], anticancer [8], antistress, immunomodulatory [9] and cardiovascular activities [10]. It is more commonly found in Africa and Indian subcontinents. In India, it grows in dry parts of tropical and subtropical regions**.** Keeping in view the significance of monitoring asparagine in food, the current work deals with the development of a biosensor for determining asparagine in fruit juices. In previous study for comparison of cytotypes of *W. somenifera*, tetraploid plants revealed maximum L-asparaginase activity than diploids [11] from which enzyme was extracted and used as biocomponent in present study.

MATERIALS AND METHODS

All the chemicals used were of analytical grade, commercially available from Himedia, Sigma, Merck etc. The green fruits of *W. somnifera* plants were collected from outside of Punjabi University, Patiala. Sample plant was submitted in Herbarium of Department of Botany, Punjabi University, Patiala and got the accession No. PUN 57618.

Extraction of enzyme

L-asparaginase was extracted from green fruits of *W. somnifera*. For enzyme extraction fruits were washed with distilled water and crushed with sterilized chilled sea sand at 4^{o}C. The same amount of sodium borate buffer (pH 8.6) was added to the finely crushed mixture and then centrifuged at 8000 rpm for 10 min at 4^{o}C. The supernatant was collected and used for measuring enzyme activity and used as biocomponent to develop the biosensor.

L-asparaginase assay

1.7 ml of 0.01M L-asparagine (prepared in 0.05M Tris-HCl) and 0.2 ml 0.05M Tris-HCl were added to a test tube. To this, 20µl enzyme (diluted to 1ml with Na borate buffer) was added and incubated at 37 ^{0}C for exactly 10 minutes and reaction was stopped by adding 0.1ml of 1.5 M Tri Chloroacetic Acid. Reaction mixture was clarified by centrifugation and 2.5 ml clear supernatant was mixed to equal volume of de-ionized water. To this, 0.5ml Nessler's reagent was added and incubated at room temperature for 10 minutes. Absorbance was taken at 480 nm and amount of ammonia released was determined using an ammonium chloride standard curve.

Immobilization of Biocomponent

Biocomponent was immobilized into TEOS hydrosol gel-chitosan based technique. Sol-gel was solidified by chitosan. This method was based on modification of Alqasaimeh *et al.* [12] method.

Preparation of Sol-gel solution

7.5ml TEOS and 0.2ml 0.1M HCl were added in a closed vessel and made the volume 10ml with distilled water. After that 0.1 ml of 1% chitosan solution was added. Vessel was closed tightly and placed on magnetic stirrer for 2 to 3 hours or till the solution became clear. This solution was stored at 4°C for further use.

Immobilization
200 µl of sol-gel solution in 3ml glass vessel was poured. The vessel was wrapped with parafilm to make it air tight and placed at room temperature for 24 hours. Then 50µl enzyme (0.63 IU), 45µl sol-gel solution and 5µl of phenol red indicator were mixed together and layered on solidified sol-gel solution. Again it was wrapped with parafilm for another period of 12 hours. Phenol red concentration was 4mg per 4ml of 1:1 ratio of distilled water and alcohol. After that it was ready for detection.

Kinetic properties
Kinetic properties were studied for both free as well as immobilized enzyme. Km and Vmax were evaluated by Lineweaver-Burk double reciprocal plot. Effect of different pH (6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5) and temperature (25^oC, 30^oC, 35^oC, 37^oC, 40^oC, 45^oC and 50^oC) was also studied.

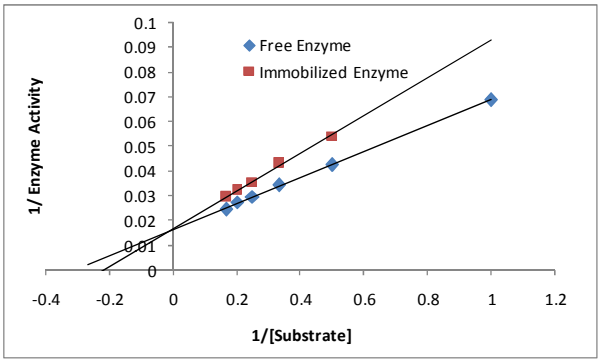
Analysis of Asparagine
Different concentrations of asparagine (10^{-1} to 10^{-10} M) were prepared in 50mM Tris HCl (pH 7.6). 100 µl of each concentration was added to each vessel in which enzyme was immobilized and the response time of colour change from light orange to pink was noted.

Application of developed biosensor
Constructed biosensor was applied to analyze L-asparagine concentrations in different fruit juice samples *i.e.* Orange, Apple, Grape and Guava juice. Reliability of the developed biosensor was checked with the spiked sample by standard addition method. Spiking was done by combining half volume of fruit juice (10^{-5}M) e.g. grape juice and half volume of synthetic Asparagine solution (10^{-5}M) and response time was observed by the developed biosensor. Bio-component (vessels in which enzyme was immobilized) was stored at 4°C and checked for activity at regular intervals for storage stability.

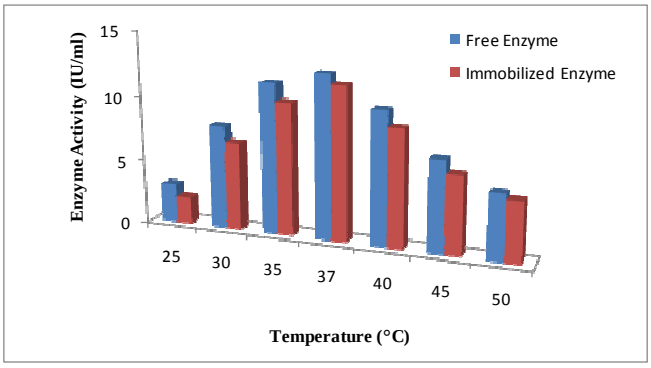
RESULTS AND DISCUSSION

L-asparaginase was extracted from fruits of *W. Somnifera* with 13 IU/mg activity and used for construction of biosensor.

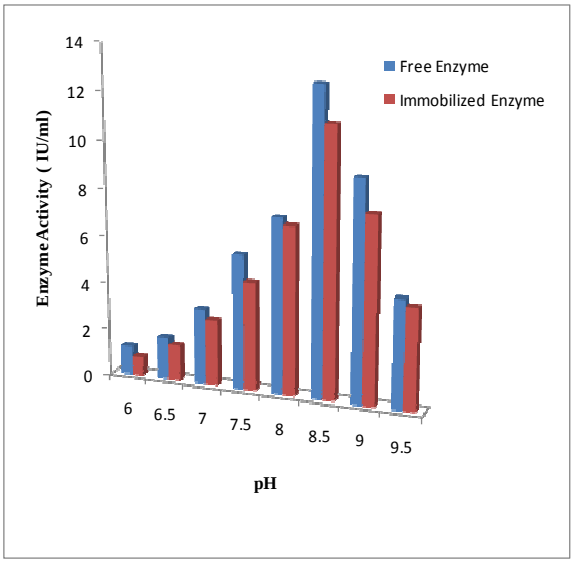
Kinetic properties
Comparisons of kinetic properties of free and immobilized enzyme have been done. Kinetic parameters Km and Vmax for free enzyme were found to be 3.34 mM and 62.5 µmol/min respectively. There is not much variation in Km and Vmax after immobilization of enzyme (Graph 1). Km and Vmax for immobilized enzymes were 4.1 mM and 58.6 µmol/min respectively. Optimum temperature (Graph 2) is 37°C for both conditions and optimum pH (Graph 3) is also similar for both states of enzyme (pH 8.5).



Graph 1: Comparison of Lineweaver-Burk Double reciprocal curve



Graph 2: Comparison of effect of temperature on native and immobilized enzyme.



Graph 3: Comparison of effect of pH on free and immobilized enzyme

Biosensor and application

The response time was observed for various concentrations of asparagine (10^{-10}M to 10^{-1}M) by developed biosensor (Table 1). The response time for change in colour was found to be inversely proportional to the asparagine concentration levels. With increase in concentration of asparagine, the response time for colour change decreases. Colour of biosensor was changed from light orange to pink after reaction (Figure 2). In comparison with the asparagine biosensors developed by Fraticelli & Meyerhoff [13], Wang *et al*. [14] and Verma *et al.* [15] asparagine range of 10^{-10}M could be detected while the earlier efforts could detect levels up to only 10^{-9}M.

Table 1: Response time for different concentrations of Asparagine.

Concentration of asparagine (M)	Response time
Blank	No Colour Change
10^{-10}	7 min 15 sec ± 6sec
10^{-9}	5 min 35 sec ±7 sec
10^{-8}	3 min 51 sec ±5 sec
10^{-7}	2 min 12 sec ±6 sec
10^{-6}	1 min 54 sec ±3 sec
10^{-5}	57 ±4 sec
10^{-4}	38 ±2 sec
10^{-3}	18 ±2 sec
10^{-2}	8±1 sec
10^{-1}	3±2 sec

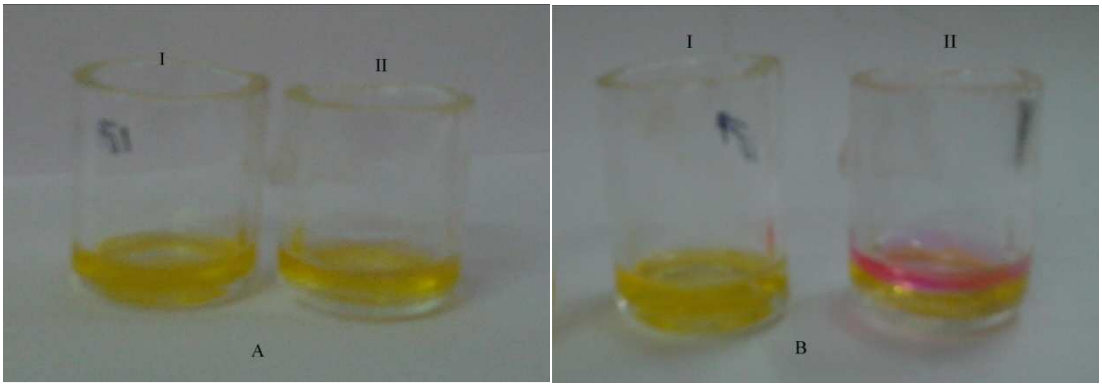
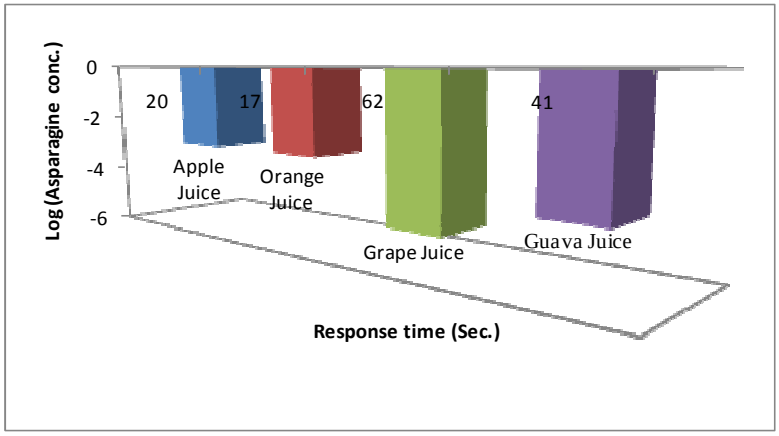


Figure 2 (A) Colour of the biocomponent before the reaction i.e. light orange. (I is blank and II is fruit juice sample.), (B) Colour of the biocomponent after the reaction changes from light orange to pink because of increase in pH of reaction by production of ammonia.

The constructed biosensor was applied on various fruit juices like guava, apple, orange and grapes for monitoring asparagine and their response times are shown in Graph 4. Among them Apple and orange juices have similar and higher asparagine contents. Grape juice has the lowest concentration of asparagine. Results of constructed biosensor are comparable with conventional methods found in literature. Asparagine in grape juice is present in traces [16]. Stability of biocomponent was checked at regular intervals and found to be stable for 32 days stored in Na borate buffer (pH 8.5) at 4°C. Reliability was checked by spiking asparagine in fruit juice sample and response time observed to be 58±3 sec comparable to the concentration of 10^{-5} M asaparagine. From this result it is clear that developed biosensor is reliable.



Graph 4: Response times for different Fruit juices.

CONCLUSION

L-asaparaginase was extracted from green fruits of *W. somnifera* with 13 IU/ mg activity and used for the construction of biosensor. Enzyme was immobilized on TEOS hydrosol-gel chitosan matrix and kinetic parameters were evaluated in comparison with free enzyme. The TEOS hydrosol-gel chitosan matrix was found to be suitable for the immobilization of L-asparaginase. Developed asparagine biosensor detects asparagine range up to 10^{-10}M and applied on fruit juices like orange (10^{-3} M), apple (10^{-3} M), grape (10^{-5} M) and guava (10^{-4} M) for monitoring asparagine. Among these juices orange and apple juices have more asparagine than grape and guava juices. Biocomponent was found to be stable for 32 days. The reliability of developed biosensor has been checked by spiking asparagine to fruit juice samples.

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