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L-Asparaginase from Novel Source- *Solanum nigrum* and Development of Asparagine Biosensor

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Abstract
Asparagine regulates the equilibrium of the central nervous system and is required for the development of brain. On the other hand, Asparagine forms acrylamide in baked food by reacting with reducing sugars at high temperature (Millard reaction), which is highly carcinogenic. Storage of fruit juices at 37 °C causes a major decrease in the content of asparagine, therefore, it can also be used as quality insurance parameter in fruit juices. Due to these significances of monitoring asparagine, the current work deals with the development of a biosensor for determining asparagine in fruit juice and comparison of various plants for L-asparaginase. Among these plants *Solanum nigrum* have higher titer of enzyme and it is novel source of L-asparaginase. For the development of the biosensor, L-asparaginase is co-immobilized with Phenol red in TEOS-Chitosan disc and fiber optic spectrophotometer is used as a transducer. The developed fiber optic asparagine biosensor has a very good response time of 5 min. with 10^{-1} to 10^{-10} M linear range of detection. The developed biosensor was applied on various fruit juices.

Keywords: *Solanum nigrum*, biosensor, immobilization.

1. Introduction

L-asparaginase is an enzyme of high therapeutic value due to its use in leukemia treatment. A number of bacteria produce L-asparaginase, but not all of these enzymes have anti-tumour properties. The variation in anti-tumor activity has been related to the affinity of the enzyme for its substrate and the clearance rate of the particular type of enzymes. Commercially used enzymes are obtained from *E. coli* and *Erwinia carotovora* [1]. ELSPAR, ONCASPAR, ERWINASE & KIDROLASE are the brand names of L-asparaginase which are used as medicines. It is also used in the food industry as a mean to reduce the formation of acrylamide from the baked product under the brand name of "Acrylaway and Preventase" [2]. 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 [3]. There are a wide range of L-asparaginase sources such as bacteria, fungi, yeast, actinomycetes, algae and plants. Broome in 1963 found that the serum of guinea pigs contain L-asparaginase which has the antitumor factor [4]. However, commercial production of L-asparaginase was possible only after the work of Mashburn and Wrist on [5]. They produced L-asparaginase from *E.coli* which inhibited tumors in mice. In the present study, some germinated seeds of Fabaceae and fruits of some plants of Solanaceae were compared for L-asparaginase. Out of these plants, *Solanun nigrum* showed maximum titer of L-asparaginase. Extracted Enzyme from *S. nigrum* is used as biocomponant for asparagines biosensor

Biosensor is an analytical device which can detect the analyte by changing biological signals into process able signals. A biosensor has two main components i.e. the biocomponent and the transducer. The biocomponent is a biological material, such as an antibody, a membrane receptor, a single stranded nucleic acid, an enzyme or even a whole cell, which is usually immobilized on the surface of a suitable transducer. In this system, the biological material is responsible for a specific recognition of the analyte at the transducer surface and the transducer for the conversion of said recognition event into a processable signal. In present study the biocomponent is L-asparaginase and the transducer is a fiber optic spectrophotometer. For the asparagine biosensor, L-asparaginase is co-immobilized with phenol red which hydrolyzes asparagine into aspartate and ammonia. This ammonia increases the pH which inhance the colour of phenol red and the increased absorbance is monitored by the fiber optic spectrophotometer (Figure 1).

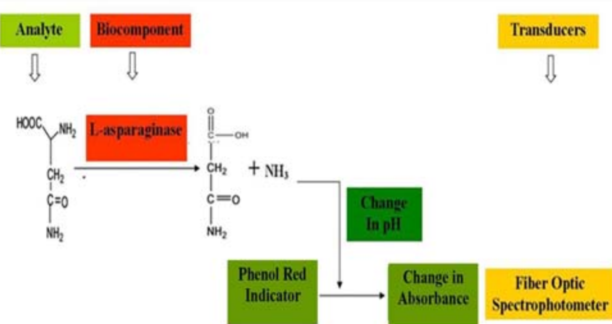

Fig 1: Schematic Representation of Asparagine Biosensor.

Asparagine (also known as asparamide) is a α-amino acid found in many proteins. It is closely related to amino acid aspartic acid into which it is easily hydrolyzed [6]. Asparagine is the most important amino acid which is required for the development of brain and it regulates the equilibrium of central nervous system because it plays an important role in coupling Cl^-binding to concentrative neurotransmitter uptake [7]. 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 2.) i.e. amino acids and sugars give new flavours at high temperature [8]. Acrylamide is highly carcinogenic and should be removed from food. It can also be a quality insurance parameter in fruit juices because Babsky *et al.* [9] 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. Keeping in view the significance of monitoring asparagine, the current work deals with the development of a biosensor for determining asparagine in fruit juice.

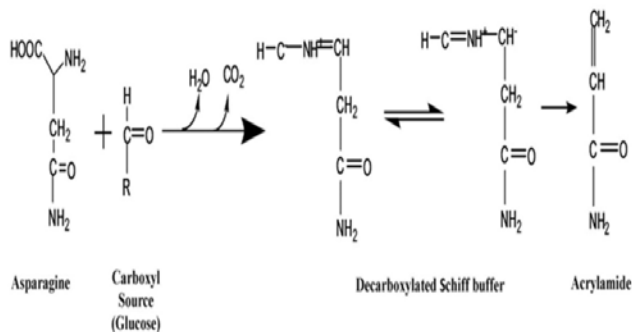

Fig 2: Formation of Acrylamide from Asparagine

2. Material and methods

Fresh samples of *Solanum nigrum* were collected from dry and shady areas of different localities of Patiala. The fruit of *Capsicum annuum, Solanum lycopersicum* were collected from the fields of Patiala. The fruit of *Piscum sativum* was collected from the market of Patiala. Cereals (*Lens culinaris, Vigna acontifolia, Vigna radiate and Phaseolus vulgaris*) were collected from the market of Patiala. All the chemicals and reagents were analytical grade and procured from Hi media, Sigma and SD fine chemicals.

2.1 Extraction of crude enzyme from fruits of various plants

For enzyme extraction the fruits from plants were collected and washed with distilled water. Then they were crushed at 4°C and centrifuged at 8000 rpm for 10 minutes at 4 °C. The supernatant thus obtained was taken as the crude extract [10] Estimation of ammonia by Nessler's reagent.

2.2 Extraction of crude enzyme from germinated cereals (Cereals are *Lens culinaris, Vigna acontifolia, Vigna radiate, Phaseolus vulgaris*):

For enzyme extraction the germinated seeds cereals, these seeds were dipped in distilled water for overnight. After that water was drained and cereals were covered with wet clothes until they germinated. Then they were crushed at 4 °C and centrifuged at 8000 rpm for 10 minutes at 4 °C. The supernatant thus obtained was taken as the crude extract. Then enzyme activity of each extract was calculated

2.3 Enzyme assay

1.7ml of L-asparagine (prepared in 0.1M tris HCL) and 0.2 ml of 0.1M Tris HCL were added to a test tube. To this, 0.980ml KCL buffer (pH 8.6) was added. To this, 20µl of enzyme was added and incubated at 37 °C for exactly 10 minutes and reaction was stopped by adding 0.1ml of 1.5M Tri Chloroacetic acid (TCA). Reaction mixture was clarified by centrifugation and 2.5ml clear supernatant was mixed to equal volume of distilled water. To this, 0.5ml Nessler's reagent was added and incubated at room temperature for 10 minutes. Absorbance was taken at 480nm and amount of ammonia released was determined using an ammonium chloride standard.

2.4 Comparison of plants for L-asparaginase activity

L-asparaginase activity of all extracts of plants and germinated seeds were calculated as said above methods. Then compare the activity all the plants.

2.5 Development of Fibre Optic Asparagine Biosensor

Fiber optic biosensor is a device in which immobilized enzyme kept in the tip of fibre optic sensing element. As the enzyme react with substrate, ammonia is produced which is detected with the help of sensing element [11]. It measured the amount of light absorbed by the sample which is directly proportional to the concentration of asparagine.

2.5.1 Absorbance Spectra of Phenol red.

Phenol red solution (4mg/4ml) was prepared in mixture of alcohol and water (1:1) and λmax was found by fiber optic spectrophotometer.

2.5.2 Immobilization of enzyme

The crude enzyme from *Solanum nigrum* was immobilized by hydrosol-gel techniques. Tetraethyl orthosilicate (TEOS) was used for hydrosol-gel and it was solidified with chitosan. TEOS on acid or base hydrolysis form hydrosol-gel. The solidification of sol-gel was based on modification of Alqasaimeh *et al.* [12] method. Immobilization of enzyme is defined as the immobilization of the enzyme molecule to the supporting medium. Stock solution of Sol gel was prepared by adding 8 ml of TEOS, 0.6 ml of 0.1M HCl and 2.8 ml of chitosan in a small closed vial and stirred for 2 to 3 hours until a clear solution was obtained. Took 600 µl from the above TEOS sol gel solution and mixed with 200 µl of sodium borate buffer (0.01M, pH 8.6). From this mixture took 100 µl and added in 100 µl of enzymatic solution containing 40 µl of phenol red solution. 10 µl of above solution was added on the transparency disc and allowed to incubate for 2 hours to immobilize on the disc in air tight container.

2.5.3 Optimization of response time.
For absorbance measurements, TEOS-chitosan disc was kept in the upper mirror position of fiber optic tip and dipped in the cell containing 10 ml 10^{-1} M L-Asparagine solution (Figure 3). The reaction was continuously monitored for 10 minutes and the absorbance were noted down at intervals of 1 minute at 538 nm. A response time of 5 minutes was found to be optimum for the enzymatic reaction to be completed.

Fig 3: Fiber optic spectrophotometer.

2.5.4 Construction of L-Asparagine Standard Reference Chart using fiber optic spectrophotometer.
10^{-10} to 10^{-1} M L-Asparagine solutions were prepared from the 10^{-1} M stock solution. The TEOS-chitosan matrix having enzyme was kept in the bottom mirror position of the tip of the fiber-optic cable supplied with the instrument for absorbance measurement. The tip was closed and dipped in 10 mL of 10^{-10} M L-asparagine solution and the subsequent absorbances were noted down. Same procedure was repeated with each dilutions and a plot was drawn between concentrations of asparagine and optical density.

2.6 Analysis of different fruit juices with Developed Biosensor
Grape juice, mango juice, orange juice, apple juice and litchi juice were purchased from the market and 10 times diluted with tris HCl. The TEOS-chitosan matrix (having enzyme and phenol red) was kept in the upper mirror position of the tip of the fiber-optic cable supplied with the instrument. The tip was closed and dipped in 10 mL of diluted fruit juices and optical density were noted down at 538 nm after 5 min. The optical density was then correlated with the L-asparagine standard reference chart and subsequently, the L-asparagine levels were calculated.

3. Results and discussion

3.1 Enzyme activity: The enzyme activity of plants and cereals listed below (Table 1.). Among the plants and cereals the highest enzyme activity is of *Solanum nigrum* 58.62 U/mg and the least enzyme activity shown by the cereal *Phaseolus vulgaris* 12.77 U/mg.

Table 1: Comparision of enzyme activity of plants and cereals

S. NO	Plants and cereals	Family	Enzyme Activity(U/mg)
1.	*Solanum nigrum*	Solanaceae	58.62
2.	*Capsicum annuum*	Solanaceae	42.36
3.	*Pisum sativum*	Fabaceae	26.45
4.	*Solanum tuberosum*	Solanaceae	18.98
5.	*Lens culinaris*	Fabaceae	30.88
6.	*Vigna aconitifolia*	Fabaceae	22.78
7.	*Vigna radiata*	Fabaceae	17.36
8.	*Phaseolus vulgaris*	Fabaceae	12.77

3.2 Fibre Optic Asparagine Biosensor

3.2.1 Absorbance Spectra of Phenol red.
λmax of Phenol red solution by fiber optic spectrophotometer was found to be 538 nm. Therefore all the readings were taken on 538 nm.

3.2.2 Optimization of response time.
For the response time of biosensor reaction was continuously monitored for 10 minutes by spectrophotometer. After 5 minutes absorbance become constant as for the enzymatic reaction was completed so the response time of developed biosensor was 5 mins. Previously optical asparagine biosensor reported with a response time of 10 minutes [13] and Verma *et al*., [14] developed an asparagine biosensor with visual approach having response time from 7 min, 15 sec to 3 min, 2 sec for asparagines concentration 10^{-10} to 10^{-1} M

3.2.3 L-Asparagine Standard Reference Chart with fiber optic spectrophotometer.
Asparagine concentration was detected with immobilized enzyme on fiber optic tip and change in absorbance was studied with change in concentration from 10^{-1} to 10^{-10} M as shown in Fig 4. The detection range of the present study is also highly improved i.e. 10^{-10}M to 10^{-1}M than the previously developed optic asparagine biosensor. Kotzia and Labrou [13] developed the first optical biosensor with 10^{-6} M to10^{-1}M detection range.

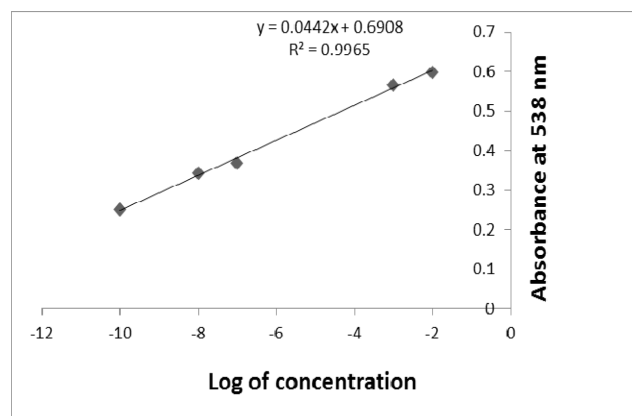

Fig 4: L-Asparagine Standard Reference Chart

3.3 Detection of asparagine in different fruit juices sample with developed Biosensor
Asparagine was determined in fruit juices with the Asparagine Fibre Optic Biosensor. Absorbances were noted down for, guava juice, apple juice, litchi juice, orange juice and mango juice as shown in Table 2. These values were compared with standard chart to get the asparagine concentration. Guava juice has the highest concentration of asparagine among these juices. Apple juice has the second highest asparagine concentration followed by orange juice and Litchi juice. Mango juice has the lowest asparagine concentration.

Table 2: Asparagine concentration in different juice sample

Juice sample	Absorbance at 538 nm	Asparagine concentration (M)
Guava	1.064	1.1×10^{-1}
Litchi	0.963	1.56×10^{-6}
Apple	0.764	1.86×10^{-2}
Orange	0.807	1.1×10^{-3}
Mango	0.169	1.05×10^{-11}

4. Conclusions
Different plants and germinated seeds were compare for L-asparaginase and a novel source of l-asparaginase was found with higher titer of enzyme i.e. *Solanum nigrum.* Crude L-asparaginase was successfully immobilized in TEOS -Chitosan discs. A fiber optic asparagine biosensor has been developed with a very good response time of 5 min. Linear range of detection was from 10^{-1} to 10^{-10} M. The developed biosensor was applied on various fruit juices and clinical samples. The developed biosensor has been applied for monitoring asparagine in fruit juices and is found to be simple, reliable and rapid.

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