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FABRICATION OF CHITOSAN ALGINATE NANOPARTICLE BASED FIBRE OPTIC BIOSENSOR

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ABSTRACT: In this work, a highly sensitive and selective nanoparticle-based fluorometric biosensor has been reported for the detection of asparagine levels. The L-asparaginase loaded chitosan alginate nanoparticles of size 396 nm with 600 µL of L-asparaginase (44.4 IU/mg) showing 83.5% entrapment efficiency was fabricated *via* ionic gelation method. In the presence of enzyme L-asparaginase (biocomponent) the substrate L-asparagine gets hydrolyze into L-aspartate and ammonium ions. The ammonium ions released were detected by a change in fluorescence intensity of pH-sensitive dye (Rhodamine 6G) immobilized along with L-asparaginase loaded chitosan alginate nanoparticles on the fiber optic probe. The developed fiber-optic biosensor under optimal conditions showed sensitivity in a detection range of 10^{-10} to 10^{-1}M concentration with a detection limit as low as 0.04×10^{-10} M within a response time of 2 min. The fabricated biosensor demonstrated good performance analysis in terms of reversibility, reproducibility, selectivity, and stability. The developed biosensor has the potential to be used for the determination of L-asparagine in the diagnosis of leukemia, and in the food industry, it can be utilized for the determination of the quality of food.

INTRODUCTION: The amino acid asparagine acts as a vital dietary requirement for the production of body's proteins, enzymes, and muscle tissue [1, 2]. Cells require asparagine as their nutritional requirement for their growth. Unlike normal cells, which can synthesize asparagine from L-asparagine synthetase, leukemic cells lack this enzyme and rely upon free circulating asparagine [3]. Thus, the monitoring of asparagine levels in leukemia patients would help in the detection and treatment of leukemia. L-asparaginase also acts as a quality insurance parameter in food [4].

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L-asparaginase undergoes degradation during the preparation process of foods like during cooking, storage, or on the application of acid-based food additives (like citric acid, malic acid, etc.). According to the literature reports, about 87% loss in total free amino acids with a major decrease in asparagine content on the storage of fruit juices at 37 °C has been reported [5]. Asparagine quantification also acts as a freshness indicator for vegetables [6]. Also, asparagine is the chief amino acid that forms acrylamide in baked food by reacting with reducing sugars at high temperatures.

L-asparaginase is also employed in the baking industry to treat the fried and baked foods containing a high level of starch and asparagine to avoid the formation of acrylamide (a mutagen) [7]. The concern over the acrylamide level in food brought asparagine monitoring to the forefront.

Laboratory scale analysis of asparagine was found to be expensive and time-consuming. With the aid of biosensor technology, asparagine can be analyzed within a few seconds and is more cost-effective in comparison to conventional analytical methods [8]. For asparagine biosensor fabrication, the principle involves hydrolysis of the asparagine into L-aspartate and ammonia by immobilized L-asparaginase as a biocomponent. For the development of fiber optic asparagine biosensor, the ammonia is detected as ammonium ions by the pH-sensitive indicator, which changes its fluorescence spectra upon deprotonation. The change in fluorescence is detected by the fluorescence-based optical transducer.

Although L-asparaginase is a considerable enzyme with a broad range of applications, there are still factors limiting its utilization as a biocomponent in biosensor development. The commercially available L-asparaginase is of bacterial origin (*E. coli* and *Erwinia chrysanthemum*) and is associated with hypersensitivity reactions and allergies. Other factors like low storage stability, effective enzyme loading, and unstable reproducibility of enzyme-based biosensors act as a hindrance in the development and commercialization of biosensors with the enzyme as biocomponent [9].

Various strategies have been studied to avoid the side effects and to improve the half-life of the enzyme. Chemical modification of L-asparaginase with polyethylene glycol [10], poly-(D, L-alanine) [11], and dextran [12] have been reported to reduce antigen reactivity. Further, the immobilization of enzymes in polymeric matrices improved the stability of L-asparaginase [13]. The choice of immobilization matrix depends on the type of enzyme, the intended application, and its compatibility with the enzyme of interest. L-asparaginase has been immobilized by various chemical and physical methods using natural or synthetic carriers. Organic, inorganic, and hybrid carriers have been reported for immobilization of L-asparaginase [13]. The non-toxic, biocompatible and biodegradable properties of natural organic matrices favored their utilization in immobilization of L-asparaginase [14]. Moreover, organic matrices showed higher enzyme loading ability due to their reactive functional groups through which they easily couple with enzymes [15, 16]. In recent years, the application of nano-technology for immobilization of enzymes further improved enzyme activity by increasing the surface to mass ratio of active enzyme and reducing the enzyme diffusion problem [17, 18, 19]. Nanoparticle preparation method depends on the physic-chemical characteristics of the polymer and enzyme/drug to be loaded. Various strategies have been studied for the development of polymeric nanoparticles like ionotropic gelation method, microemulsion method, polyelectrolyte complex method, emulsion solvent evaporation method, emulsion solvent diffusion method, complex coacervation method, co-precipitation method, and self-assembly method [20, 21].

The ionotropic gelation method also known as polyelectrolyte complexation involves the interaction of cations/anions with the ionic polymer to generate a highly cross-linked structure [22, 23]. The formation of nanoparticles using the ionotropic gelation method does not involve the use of harsh chemicals and oil bases. Another advantage of the ease of manipulating the degree of cross-linking and therefore buffering activity adds over the simplicity of the method and thus makes it a method of choice over other techniques [24, 25].

The present study is focused on the development of fiber optic asparagine biosensor with L-asparaginase as the biocomponent immobilized in polymeric nanoparticles. Polymeric matrices were evaluated as L-asparaginase carriers for nano-particle formulation. The L-asparaginase loaded nanoparticles were prepared by ionotropic gelation method, and the formed nanoparticles were characterized to evaluate the preferable polymeric matrix. The morphology, structural characterization, entrapment efficiency, and storage stability of formed nanoparticles were also systematically investigated.

MATERIALS AND METHODS:

Materials: All the chemicals and reagents used were of analytical grade.

L-asparaginase Extraction: Various medicinal plants were screened for L-asparaginase and the highest activity was attained in *Phyllanthus emblica* leaves [26]. *Phyllanthus emblica* leaves (1gm) were washed with distilled water and crushed and homogenized with 0.1M KCl buffer

(pH 8.6). The homogenized mixture was then centrifuged at 8000 rpm for 20 min at 4 °C and filtered. The supernatant thus obtained was considered as a crude enzyme.

L-asparaginase Purification: Purification of the L-asparaginase enzyme was further performed sequentially according to steps given by scopes [27].

Ammonium Sulfate Precipitation and Dialysis: The crude enzyme extract was precipitated by successively varying the ammonium sulfate saturation from 20% to 100% at 4 °C, with mild stirring followed by 12 h incubation. On the occurrence of precipitates, the sample was centrifuged for 20 minutes (5000 rpm at 4 °C). The pellets obtained were resuspended in 0.01M Sodium Borate buffer and were poured in an activated dialysis membrane tube. Dialysis was performed overnight against 0.01M Sodium Borate buffer at 4 °C. Enzyme activity and protein content of the dissolved pellet and supernatant was measured after the dialysis.

Q-Sepharose Strong Anion Exchange Chromatography: Q-Sepharose chromatographic column (1.2 × 8.2 cm) pre-equilibrated with 0.01M sodium borate buffer (pH 8.6) was prepared. The dialyzed fraction was loaded onto the prepared column at a flow rate of 1 ml min^{-1}. To remove the unbound proteins, the column was washed with 0.01M sodium borate buffer (pH 8.6). The elution was carried out by linearly raising the NaCl salt gradient from 0.1M-0.6 M and 1.5 mL fractions were collected at a flow rate of 1 mL min^{-1}. The collected fractions were assessed for enzyme activity and protein concentration.

Molecular Weight Determination: The molecular weight and purity of collected fractions with the highest specific activity were determined by running the fractions along with the protein marker onto the Polyacryl Amide Gel (10%) as a supporting medium. Electrophoresis was carried out at 100 V and the proteins progressed through the gel was tracked using bromophenol blue as tracking dye. Subsequent analysis was performed by staining the gel with coomassie brilliant blue.

Screening of Matrices for Nanoparticle Formulation: The individual effect of each matrix and multivalent cations on L-asparaginase activity was determined to screen the best matrix for nanoparticle formulation **Table 1**. 1 mL of polymeric solution (0.2% (w/v)) and 1 mL multivalent cations solution (0.1% (w/v)) were prepared from stocks. 1mL of purified L-asparaginase was added to each solution, and both mixtures were gradually shaken for 15 min. The percentage of preserved activity was calculated by Nesseler's Method for both solutions against the untreated L-asparaginase activity (taken as 100%).

TABLE 1: POLYMERIC MATRICES AND MULTI-VALENT CATIONS SCREENED FOR NANOPARTICLE FORMULATION

Natural Polymers		Multivalent cations (for cross-linking)
Cationic polymers	**Anionic polymers**	
Chitosan	Alginate	Sodium Tripolyphosphate
Gelatin	Pectin	Calcium Chloride
Dextran	Hyllauronic acid	Calcium Carbonate
		Aluminium, Zinc

Preparation of Blank Nanoparticles: Chitosan nanoparticles were prepared by ionotropic gelation method which is based on ionic interaction among oppositely charged polymers leading to cross-linking. Sodium alginate (0.3%, w/v) and calcium chloride (0.2%, w/v) solutions were prepared in distilled water as stock solutions. The pH of the sodium alginate solution was adjusted to 5. Chitosan was dissolved in distilled water containing 1% acetic acid to a concentration of 0.05% (w/v) as a stock solution. Various concentration of aqueous calcium chloride (0.5-2 mL) was added dropwise to 10 mL aqueous sodium alginate (0.3%) while stirring for 30 min, and each variation was checked for the development of microgel. After that various volume of chitosan solution (0.5-2 mL) was added into the resultant calcium alginate microgel and stirred for an additional 1 h.

The scheme for nanoparticle preparation is shown in **Table 2**. The suspension obtained was equilibrated overnight to achieve uniform particle size. After 24 h, the suspension was centrifuged (25,000×g, 25 °C for 30 min) and the pellets obtained were suspended in distilled water and dispersed by sonication, to determine the particle size and polydispersity index using zeta sizer (Malvern Instruments, Worcestershire, UK).

TABLE 2: SCHEME FOR NANOPARTICLE FORMULATION

Chitosan (0.05%)	Sodium Alginate (0.3%)	Calcium Chloride (0.2%)
0.5ml	10ml	0.5ml
1ml	10ml	1ml
1.5ml	10ml	1.5ml
2ml	10ml	2ml

Preparation of L-asparaginase loaded Nanoparticles: The stable and suitable ratio of polymers and multivalent cation was selected during the preparation of blank nanoparticles. Further, various concentrations (200µL-1000µL) of purified L-asparaginase enzyme (44.4 IU/mg specific activity) were mixed with calcium chloride (2 mL) solution, and the mixture was added dropwise to aqueous sodium alginate (0.3%) while stirring for 30 min. After 30 min, 1.5mL of chitosan solution (0.05%, w/v) was added dropwise in enzyme loaded calcium alginate pre-gel and stirred for 1 h. The resultant solution was kept overnight and then each solution was centrifuged at 25,000×g, 4 °C for 30 min. To determine the entrapment efficiency, the amount of free enzyme in each supernatant was analyzed. Further, the pellets were dissolved and dispersed in distilled water to conclude the particle size and polydispersity index using a zeta sizer.

Characterization of L-asparaginase loaded Nanoparticles:

Entrapment Efficiency Estimation: To determine the entrapment efficiency of the nanoparticles, the amount of free enzyme in the supernatant was measured by Nessler's Reagent. The L-asparaginase entrapment efficiency was calculated using the following equation**:**

Entrapment Efficiency (%) = Total Protein used in formulation-Free protein / Total protein used in formulation × 100

Morphology and Structure Characterization: The morphology and particle size measurements of the nanoparticles were performed by SEM. Nanoparticles separated from suspension were dried by freeze dryer, and the molecular interactions involved in the formation of nanoparticles were characterized by the FTIR spectrometer.

Effect of pH on Enzyme Activity and Stability: The activities of the immobilized and free L-asparaginase were evaluated at different pH values in the range between pH 5 and 10 adjusted with Tris–HCl (0.1 M). In the case of pH stability experiment, the immobilized and free enzymes were incubated for 24 h at 4°C ± 1°C at different pH values (pH 5 to 10).

Effect of Temperature on Enzyme Stability: Thermostability studies were carried out by pre-incubating the immobilized and free L-asparaginase at different temperatures (20 °C to 50 °C) for 60 min. The percentage of residual activity was determined and calculated based on the untreated control activity, which was taken as 100%.

Fabrication of Fiber Optic Biosensor: Fiber optic asparagine biosensor was fabricated based on the principle of the breakdown of L-asparagine into aspartate and ammonia by the action of L-asparaginase. The ammonium ions released were detected by a change in fluorescence intensity of pH-sensitive dye (Rhodamine 6G). Thus, the quantitative detection of ammonia with the relative change in fluorescence intensity allowed the detection of asparagine concentration**.** 2% Rhodamine 6G was prepared in **a** 1:1 ratio of water, and ethanol was analyzed for its emission spectrum. All further readings for fluorescence experiments in terms of intensity counts were taken at this wavelength.

A 20µL solution containing the L-asparaginase loaded chitosan alginate nanoparticles (the activity of 44.4 IU/mg) along with 2% Rhodamine 6G (20µL) were then immobilized on a nylon membrane (0.2 µm pore size) incised in the form of disc and attached to the common end of a bifurcated optical fiber bundle and kept at a right angle to the lamp. The other end of the optical fiber probe was attached to the detector. 20µL of asparagine solution (10^{-1} M) was poured onto the nylon membrane containing enzyme loaded chitosan alginate nanoparticles. The reaction was constantly recorded for 10 min, and the intensity count was recorded at an interval of 1 min, and the response time for the reaction to be completed was acquired. For the construction of the standard curve, 20µL of varying concentrations of L-asparagine (10^{-1} M to 10^{-10} M) were placed on the nylon membrane disc, containing L-asparaginase loaded chitosan alginate nanoparticles and

fluorescent dye and the change in intensity count with respect to response time were recorded. Further, the performance analysis of the fabricated biosensor was checked in terms of repeatability, reproducibility, and selectivity. L-asparaginase loaded chitosan alginate nanoparticles were dissolved in sodium borate buffer (0.01M) pH 8.6 and stored at 4 ± 1 °C to determine the storage stability. At the time interval of 24 h, sampling was done without replacement for the determination of enzymatic activity.

RESULTS AND DISCUSSION:

L-asparaginase Extraction and Purification: Crude L-asparaginase from *Phyllanthus emblica* leaves showed enzyme activity 20.3IU/mL and a specific activity of 5.2 IU/mg. Purification of the L-asparaginase enzyme was performed using various chromatographic techniques like ammonium sulfate precipitation, dialysis, and ion exchange chromatography. The purified enzyme after ion-exchange chromatography showed higher specific activity of 44.4 IU/mg. About 63.5% of the original enzyme activity was recovered with a purification fold of 8.5.

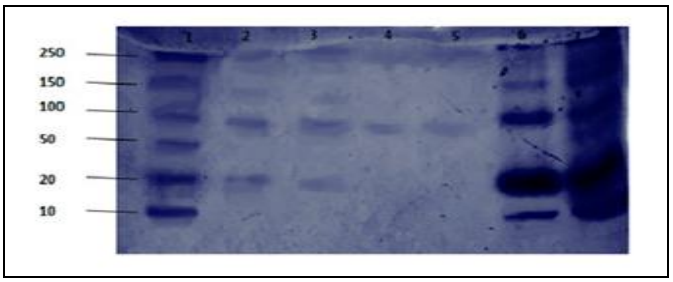

FIG. 1: MOLECULAR WEIGHT DETERMINATION OF L-ASPARAGINASE FROM *PHYLLANTHUS EMBLICA* LEAVES (1) BROAD RANGE (10-250 kDa) PROTEIN MARKER (2) & (3) AMMONIUM SULFATE PRECIPITATION (4) & (5) Q-SEPHAROSE ANION EXCHANGE CHROMATOGRAPHY (6) & (7) CRUDE ENZYME

Purification was examined by native Polyacryl Amide Gel Electrophoresis (PAGE) following coomassie brilliant blue staining **Fig. 1**. The single band in the electrophoresis study further confirmed the purity achieved through purification steps. The approximate molecular weight of the purified enzyme was found to be 85kDa, as determined by comparing it against the protein marker ladder on the gel. The findings are in comparison with L-asparaginase isolated from *Withania somnifera* with a molecular weight of 72 KDa [28] and from *Phaseolus vulgaris* seeds of molecular weight 79 KDa [29].

Screening of Matrices for Nanoparticle Formulation: Both polymeric matrices and multivalent cations have their characteristic charge and may likely affect L-asparaginase stability and activity. The behavior of L-asparaginase in the polymeric matrices (Chitosan, Gelatin, Dextran, Alginate, Pectin, and Hyaluronic acid) and cation solutions (Sodium Tripolyphosphate and Calcium Chloride) was individually investigated before the preparation of nanoparticles. The percentages of the preserved L-asparaginase activity with respect to untreated enzyme activity was found to be highest in chitosan (87.8%) followed by alginate (83.7%). Among the multivalent cation, calcium chloride had the least effect on L-asparaginase activity, with 93.5% of preserved L-asparaginase activity. Therefore, chitosan among the cationic polymers and alginate among the anionic polymers with calcium chloride as the cation (crosslinker) was selected for L-asparaginase embedded nanoparticle formulation.

Preparation of Blank Nanoparticles: For the preparation of chitosan alginate blank nanoparticles, optimization of chitosan, sodium alginate and calcium chloride concentrations was performed by varying their concentrations. With the sodium alginate solution at a lower concentration (0.3% w/v), macroscopic gel aggregates or microgels were formed instead of a continuous gel when calcium chloride was added at concentrations > 1.5mL of (0.2%) in absence of chitosan **Table 3**.

TABLE 3: MACROSCOPIC OPTIMIZATION CONDITIONS FOR NANOPARTICLE FORMULATION

Calcium Chloride (0.2%)	Sodium Alginate (0.5%)	Sodium Alginate (0.3%)			
	Without Chitosan	Without Chitosan	With Chitosan (0.05%) 1ml	With Chitosan (0.05%) 1.5ml	With Chitosan (0.05%) 2ml
0.5ml	Solution	Solution	Microgel	Aggregates	Aggregates
1ml	Solution	Solution	Microgel	Aggregates	Aggregates
1.5ml	Gel	Microgel	Microgel	Nanoparticle	Aggregates
2ml	Gel	Microgel	Microgel	Nanoparticle	Aggregates
2.5ml	Gel	Microgel	Microgel	Aggregates	Aggregates
3ml	Gel	Microgel	Aggregates	Aggregates	Aggregates

Below this concentration of calcium chloride, homogeneous solutions were obtained. When 1.5 mL chitosan (0.05%) was further added to the pre-gel/microgel, nanoparticle formulation was observed only when calcium chloride concentration was 1.5-2 mL (0.2%). Above 2 mL of calcium chloride (0.2%) aggregates were formed on the addition of chitosan.

Further, the optimization for calcium chloride concentration for nanoparticle formulation was performed using zeta sizer. At constant concentration of sodium alginate (0.3%) and chitosan (0.05%), smaller particle size of 274.1nm with 0.31 polydispersity index was obtained with 2 mL calcium chloride (0.2%) **Table 4**. The size of nanoparticles prepared was comparable to the related studies where chitosan alginate nanoparticles were prepared by the ionotropic gelation method [30-33].

TABLE 4: OPTIMIZATION CHARACTERISTICS FOR NANOPARTICLE FORMULATION USING ZETASIZER

Calcium Chloride (0.2%)	Sodium Alginate	Chitosan (0.05%) 1.5ml	Size (nm)	PDI
1.5ml	0.3%	0.05%	396±2	0.4
2ml	0.3%	0.05%	274.1±2	0.31

Each value is represented as means ± SD, Sample Size = 3

Preparation and Characterization of L-asparaginase loaded Nanoparticles: For the preparation of L-asparaginase loaded nanoparticles, the selected ratio of Sodium Alginate: $CaCl_2$: Chitosan ratio for blank nanoparticles with 274.1nm size was applied to optimize enzyme loading with various concentrations (200µL-800µL) of purified L-asparaginase enzyme with specific activity of 44.4 IU/mg. The entrapment efficiency for all concentrations of purified L-asparaginase was then compared to obtain the L-asparaginase loaded nanoparticles **Table 5**. The addition of 800 µL of purified enzyme led to the formation of insoluble precipitates, therefore excluded from studies. Entrapment efficiency and yield of the nanoparticles increased proportionally with an increase in the amount of enzyme loaded. The increase in enzyme concentration for nanoparticle loading does not show a sudden change in nanoparticle size. The final size of nanoparticle with 600 µL of L-asparaginase was 396 nm with 87.16 entrapment efficiency. Only 15% size enlargement on the loading of the enzyme in chitosan alginate nanoparticle was observed. The findings were comparable to a similar study of the immobilization of L-asparaginase in chitosan alginate nanoparticles of 340 nm with an entrapment efficiency of 70.6% [32].

TABLE 5: ENTRAPMENT EFFICIENCY AND YIELD OF CHITOSAN ALGINATE NANOPARTICLES ON DIFFERENT LOADINGS OF L-ASPARAGINASE

Purified L-asparaginase (µL)	Entrapment Efficiency (%)	Yield (%)
200	72.4	68.0
400	79.2	80.1
600	83.5	87.16
800	ND*	ND*

ND stands for Not Determined

The nanoparticle formation was confirmed by Electron microscopy analysis. Both blank and enzyme loaded nanoparticles were studied under Scanning Electron Microscopy (SEM). The particle size of blank and L-asparaginase loaded chitosan alginate nanoparticles were found to be in the range of 200-400 nm **Fig. 2**.

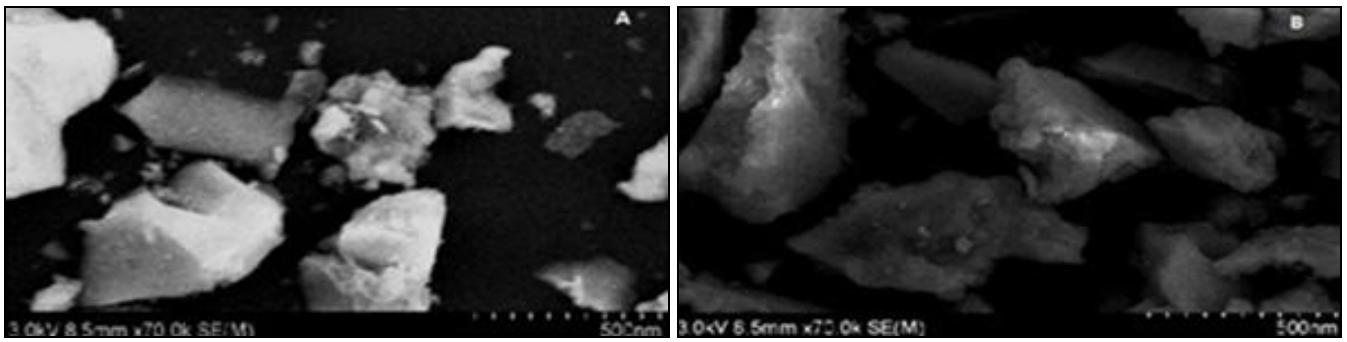

FIG. 2: A. CHITOSAN ALGINATE NANOPARTICLES; B. L-ASPARAGINASE LOADED CHITOSAN ALGINATE NANOPARTICLES

Fourier-transform infrared spectroscopy (FT-IR) was adopted to characterize the molecular interactions between chitosan alginate nanoparticle and enzyme (L-asparaginase). FT-IR spectra of blank and L-asparaginase loaded chitosan alginate nanoparticles are shown in **Fig. 3**.

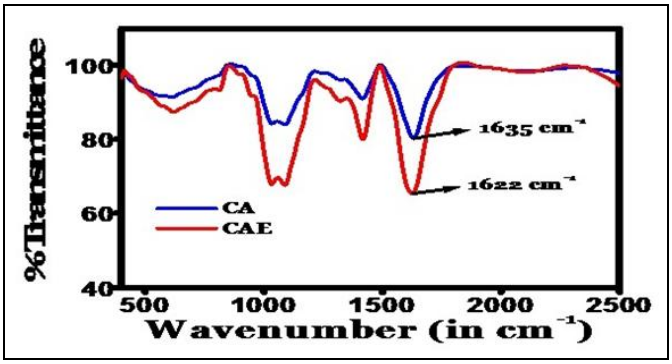

FIG. 3: COMPARATIVE FTIR SPECTRA OF CHITOSAN ALGINATE NANOPARTICLE-CA (BLUE) AND L-ASPARAGINASE LOADED CHITOSAN ALGINATE NANOPARTICLES-CAE (RED)

After loading of L-asparaginase in chitosan alginate nanoparticles, the –CO stretching vibration at 1635cm^{-1} shifted to 1622 cm^{-1}. This change in –CO stretching vibration indicates the reaction between Ca^{+2} ions and glucuronic acid residue of alginate forming an egg box-like structure where enzyme could be immobilized.

Effect of pH on Free and Immobilized Enzyme Activity and Stability: L-asparaginase enzyme is generally active and stable at neutral and alkaline pH. The effect of pH on L-asparaginase activity and stability in free and immobilized preparations was studied in the range from 5 to 10. The enzymatic activity of both free and immobilized enzyme was optimal in pH 8.5 to 9.0, with a maximum pH of 8.5 for the free enzyme and 9 for the immobilized enzyme **Fig. 4A**. The pH stability **Fig. 4B** after 24-h incubation at 4°C ± 1°C showed that the free L-asparaginase retained 81% of its original activity at pH 8.5 whereas Immobilized L-asparaginase retained 98% of its original activity at pH 9.0.

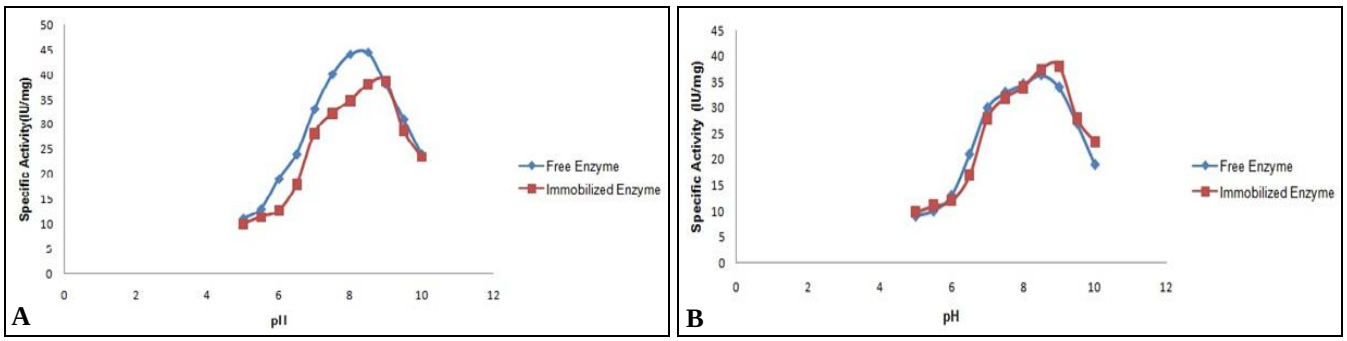

FIG. 4: A. EFFECT OF PH ON THE ACTIVITY OF FREE AND IMMOBILIZED L-ASPARAGINASE; B. EFFECT OF pH ON STABILITY OF FREE AND IMMOBILIZED L-ASPARAGINASE (AFTER 24-h INCUBATION AT 4°C ± 1°C). THE ACTIVITY WAS MEASURED AT STANDARD CONDITIONS

The Thermostability of the Free and Immobilized L-asparaginase: The thermostability of free and immobilized enzyme was measured at different temperatures ranging from 20-80 °C. The free and immobilized L-asparaginase was active at temperatures from 20 °C to 80 °C, with the highest stability at 35 °C and 40 °C, respectively. Both forms retained about 90% activity after 60 min of incubation (up to 50 °C), but the process of the loss of activity was faster for the free enzyme than immobilized enzyme when the temperature was increased beyond 50 °C **Fig. 5**. As a result, the immobilized L-asparaginase was more thermo-stable than the free enzyme.

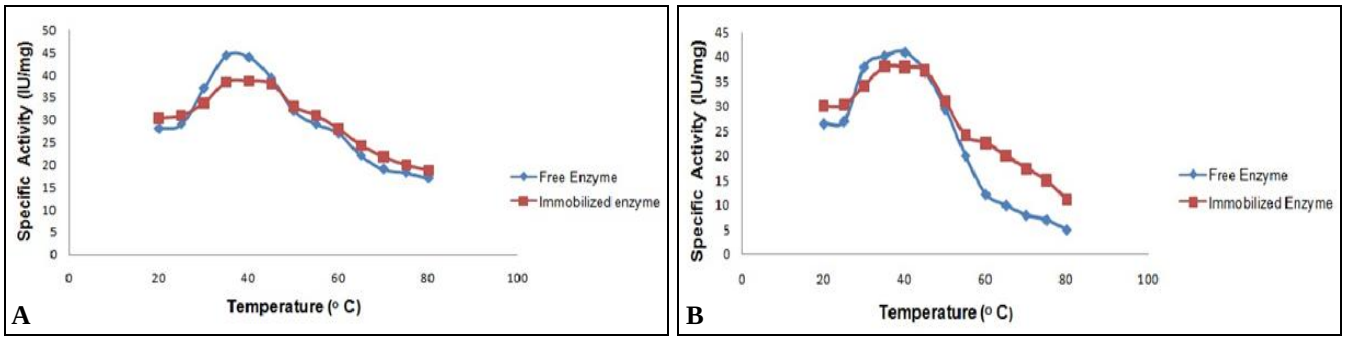

FIG. 5: A. EFFECT OF TEMPERATURE ON THE ACTIVITY OF FREE AND IMMOBILIZED L-ASPARAGINASE; B. EFFECT OF TEMPERATURE ON THE STABILITY OF FREE AND IMMOBILIZED L-ASPARAGINASE (AFTER 60 min INCUBATION). THE ACTIVITY WAS MEASURED AT STANDARD CONDITIONS

Fabrication and Performance Analysis of Asparagine Biosensor: Absorption maxima for indicator Rhodamine 6G were found to be 600.21 nm using fiber optic spectrofluorimeter. Response time was observed to be 2 min **Table 6**. In similar studies, biosensors for the detection of asparagine in fruit juices have been developed with a response time of 7 min [34] and 5 min [35].

TABLE 6: OPTIMIZATION OF THE RESPONSE TIME OF FIBER OPTIC BASED ASPARAGINE BIOSENSOR

Time (min)	Fluorescence Intensity Count
0	16700±33.9
1	16170±21.2
2	15900±20.1
3	15900±22.1
4	15900±20.9
5	15900±31.2

Each value is represented as means ± SD, Sample Size = 3

The sensitivity of the developed biosensor was investigated by varying concentrations of asparagine. The developed biosensor showed a linear correlation **Fig. 6** with regression equation y = 75.21x + 851 in the concentration range from 10^{-10} to 10^{-1}M. The detection limit obtained for the developed biosensor was as low as 0.04×10^{-10} M which is about two orders of magnitude lower than the detection limit of reported asparagine biosensors [35, 36, 37].

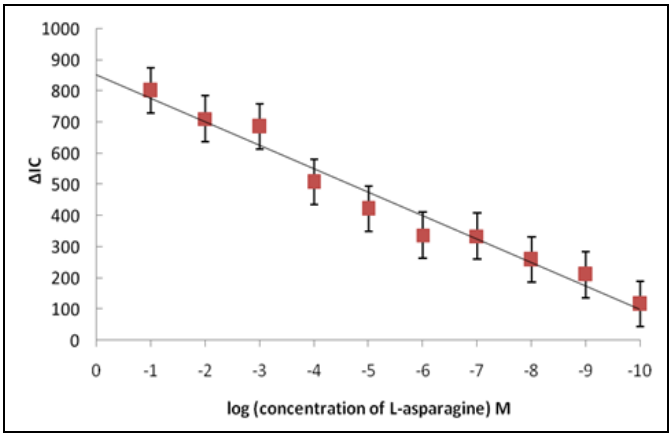

FIG. 6: STANDARD CURVE OF ASPARAGINE WITH FIBER OPTIC SPECTROPHOTOMETER

The repeatability of the fabricated biosensor was further evaluated by performing three experiments with the same biosensor system i.e. single nylon membrane containing L-asparaginase loaded chitosan alginate along with Rhodamine 6G dye immobilized on fiber optic probe. The results showed good repeatability performance for at least three consecutive experiments with the same biosensor system for asparagine concentration ranging from 10^{-10} to 10^{-1}M **Fig. 7A**.

Also, the reproducibility of the developed biosensor was examined to determine the consistency in the fabrication system of the biosensor. The same biosensor system was fabricated six times under the same conditions, and the fluorescence response for all six biosensors was checked against 10^{-1}, $10^{-5,}$ and 10^{-10} M asparagine concentration. As depicted in **Fig. 7B** the development of chitosan alginate nanoparticle-based asparagine biosensor is easy to reproduce as the relative standard deviation during the reproducibility of the biosensor was found to be less than 3%.

The selectivity of the developed asparagine biosensor was determined by investigating the effect of other amino acids and components present in fruit juice samples on the fluorescence signal. **Fig. 7C** indicates that the addition of other amino acids like L-proline, L-glycine, L-alanine, L-threonine, L- serine, L-glutamine, and other components like Fructose and Glucose, when added individually, did not significantly affect the measurement of 10^{-5} M of L-asparagine.

However, Citric acid showed a little interference by increasing the intensity count measurement of the sensor, which could be due to a change in the pH of the sample which can be overcome by diluting the sample.

Storage Stability of biocomponent (L-asparaginase loaded chitosan alginate nanoparticles) was checked at regular intervals and found to be stable for 72 days stored in sodium borate buffer (0.01M) pH 8.6 at 4°C **Fig. 7D**. The storage stability of L-asparaginase loaded nanoparticles was found to be higher than free L-asparaginase.

Similar studies with immobilized L-asparaginase has till now shown storage stability of 32 days when immobilized in TEOS-hydrosol gel-chitosan matrix [37]; 40 days when immobilized on to plastic chips of 5mm through hydro sol-gel approach [4]; 46 days when immobilized in chitosan alginate nanoparticles formed with TPP as crosslinker [32] and half-life of 6 days in conjugation with silica nanoparticles [38].

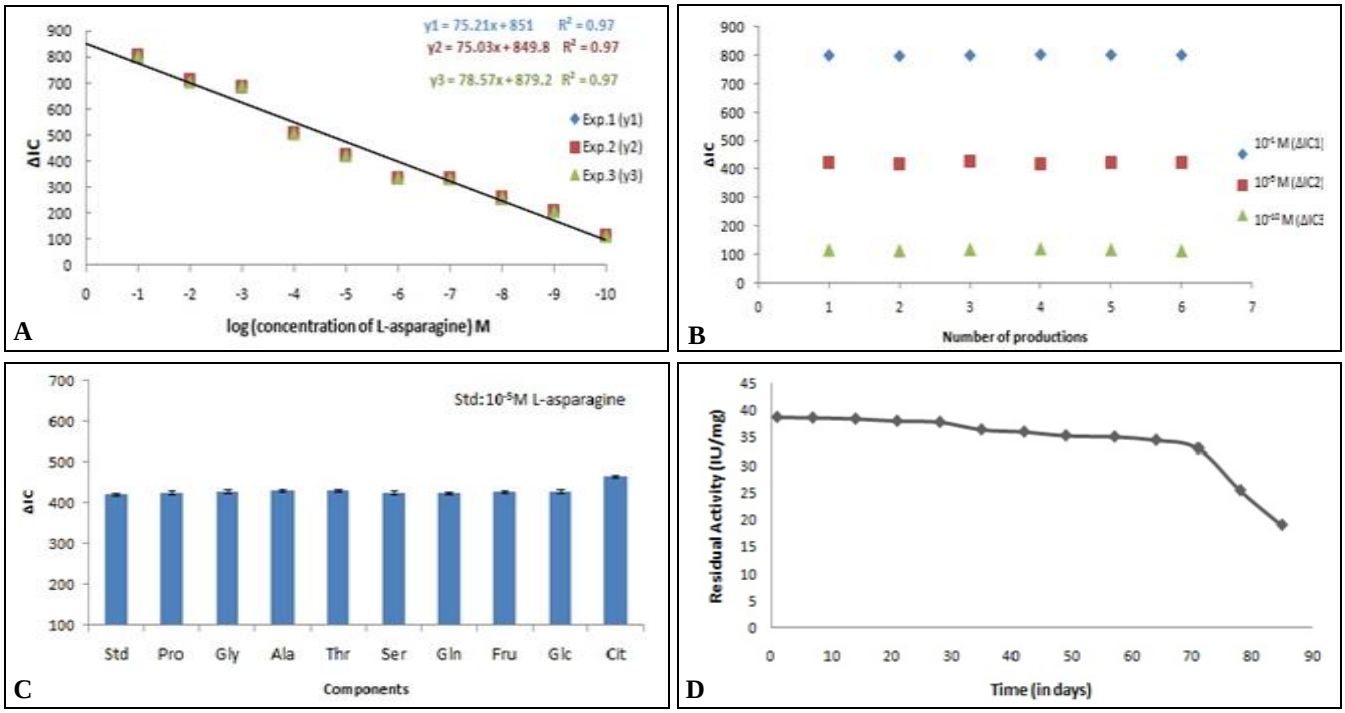

FIG. 7: A. CALIBRATION CURVE SHOWING THE BIOSENSOR REPEATABILITY FOR LEAST THREE CONSECUTIVE EXPERIMENTS WITH THE SAME BIOSENSOR SYSTEM FOR L-ASPARAGINE CONCENTRATION RANGING FROM 10^{-10} TO 10^{-1}M; B. REPRODUCIBILITY RESPONSE FROM SENSOR TO SENSOR FOR SIX BIOSENSOR SYSTEM AGAINST 10^{-1}, 10^{-5} AND 10^{-10} M L-ASPARAGINE CONCENTRATION; C. RESPONSE OF BIOSENSOR FOR SOLUTIONS OF 10^{-5}M L-ASPARAGINE (STD) AND 10^{-5}M OF EACH COMPONENT L-PROLINE (PRO), L-GLYCINE (Gly), L-ALANINE (Ala), L-THREONINE (Thr), L- SERINE (Ser), L-GLUTAMINE (Gln), FRUCTOSE (Fru), GLUCOSE(Glc) AND CITRIC ACID (Cit); D. VARIATION OF RESIDUAL ACTIVITY (IU/MG) OF DIFFERENT L-ASPARAGINASE LOADED CHITOSAN ALGINATE NANOPARTICLE AT DIFFERENT TIME INTERVALS (IN DAYS) SHOWING STORAGE STABILITY. THE ACTIVITY WAS MEASURED AT STANDARD CONDITIONS

CONCLUSION: A fiber optic asparagine biosensor with L-asparaginase (biocomponent) immobilized in polymeric nanoparticle was developed. L-asparaginase was immobilized in chitosan alginate nanoparticles formed by the ionotropic gelation method. The L-asparaginase immobilized in chitosan alginate nanoparticles showed more resistance to alkaline pH, high temperature and improved storage stability. These properties assisted in the development of a rapid, selective and stable biosensor. The fabricated biosensor proved to be a rapid approach for the detection of asparagine levels with a detection limit as low as 0.04×10^{-10} M and a response time of 2min. Further, the easy reproducibility of the proposed biosensor exhibits an advantage to its facile construction.

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