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In order to achieve the aims and objectives, the work has been divided into following parts:

3.1 Extraction and screening of L-asparaginase from *C. sativa*

3.2 Purification and kinetic characterization of L-asparaginase

3.3 Development of L-asparagine biosensor to monitor L-asparagine in leukemia cells

**Procurement of Chemicals**

All chemicals and reagents were procured from Hi-Media, SD fine chemicals, Sigma, Rankem etc.

**3.1 Extraction and Screening of L-asparaginase from *C. sativa***

**3.1.1 Estimation of Ammonia by Nessler’s Reagent Method**

Potassium tetraiodomercurate (II) is an inorganic compound containing potassium cations and tertiaiodomercurate (II) anion. It has mainly been used as Nessler’s reagent (Vogel *et al.*, 1979) which is an alkaline solution of potassium mercuric iodide represented as K$_2$HgI$_4$. The solution becomes deep yellow in the presence of ammonia. At still higher concentrations, a brown precipitate may form. A known volume of sample is treated with Nessler’s reagent to produce yellow color.

The reaction between ammonia and Nessler’s reagent is represented as following equation:

$$2K_2HgI_4 + NH_3 + 3KOH \rightarrow I-Hg-O-Hg-NH_2 + 7KI + 2H_2O$$

**3.1.1.1 Preparation of Nessler’s Reagent**

10g HgI$_2$ and 7g KI was dissolved in 25 ml of distilled water. In another beaker, dissolved 16 g NaOH in 50 ml distilled water. Both the solutions were mixed and the final volume was made to 100 ml.
3.1.1.2 Enzyme Assay

1.7 ml of L-asparagine (prepared in 1 M Tris HCl, pH 8.6) and 2 ml of 1 M Tris HCl were added to a test tube. 980 µl KCl buffer (pH 8.6) was added to that test tube. Then, 20 µl of enzyme was added and incubated at 37°C for exactly 10 min. In order to stop the reaction 0.1 ml of 1.5 M TCA (Trichloroacetic acid) was added to the mixture. Reaction mixture was clarified by centrifugation and 2.5 ml clear supernatant was mixed to equal volume of distilled water. To this, 0.5 ml Nessler’s reagent was added and incubated at room temperature for 10 min. Absorbance was taken at 480 nm and amount of ammonia released was determined using an ammonium chloride standard (10 ppm solution of ammonium chloride was prepared by dissolving 0.1 mg of NH₄Cl in 10 ml of distilled water).

Calculation of Enzyme Activity

\[
\text{Enzyme Activity} = \frac{\text{Micromoles of ammonia released}}{\text{Incubation time} \times \text{enzyme used}}
\]

3.1.2 Protein Estimation (Lowry et al., 1951)

Lowry’s method was used to prepare the standard curve for protein estimation.

The detailed procedure of Folin-Lowry method is as follow:

**Reagent A:** 0.5g Copper sulphate and 1.0g Sodium potassium tartrate in 100 ml distilled water.

**Reagent B:** 2%Sodium carbonate in 0.1 N NaOH.

**Reagent C:** Folin-Ciocalteau and distilled water were mixed (1:1).

**Reagent D:** A and B reagents were mixed in the ratio of 50:1.
Procedure

5ml of reagent D was added to different protein dilutions for 10 min. 0.5 ml of reagent C was added and incubated for 30 min at 37°C. Blue colour was appeared in the reaction mixture. The absorbance of the solution was determined at 660 nm.

3.1.3 Collection of the sample

*C. sativa* is an annual herbaceous plant found all over India. The plant was collected from different regions of North India. The plant parts i.e. leaves, stem and roots were separated and enzyme extraction was carried out (Table 3.1).

**Table 3.1: Screening of *C. sativa* from different regions of North India**

<table>
<thead>
<tr>
<th>Location (Area) of Plant Collection</th>
<th>Altitude (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Punjab</strong></td>
<td></td>
</tr>
<tr>
<td>Ropar</td>
<td>262m</td>
</tr>
<tr>
<td>Patiala</td>
<td>250m</td>
</tr>
<tr>
<td>Ludhiana</td>
<td>244m</td>
</tr>
<tr>
<td>Amritsar</td>
<td>234m</td>
</tr>
<tr>
<td>Barnala</td>
<td>233m</td>
</tr>
<tr>
<td>Jalandhar</td>
<td>228m</td>
</tr>
<tr>
<td>Abohar</td>
<td>186m</td>
</tr>
<tr>
<td><strong>Haryana</strong></td>
<td></td>
</tr>
<tr>
<td>Ambala</td>
<td>264m</td>
</tr>
<tr>
<td>Yamunanagar</td>
<td>255m</td>
</tr>
<tr>
<td>Karnal</td>
<td>252m</td>
</tr>
<tr>
<td>Jind</td>
<td>235m</td>
</tr>
<tr>
<td>Sonipat</td>
<td>224m</td>
</tr>
<tr>
<td>Kaithal</td>
<td>220m</td>
</tr>
<tr>
<td>Hisar</td>
<td>215m</td>
</tr>
<tr>
<td><strong>Location (Area) of Plant Collection</strong></td>
<td><strong>Altitude (m)</strong></td>
</tr>
<tr>
<td><strong>Himachal Pradesh</strong></td>
<td></td>
</tr>
<tr>
<td>Shimla</td>
<td>2444m</td>
</tr>
</tbody>
</table>
3.1.3.1 Optimization of Extraction Buffer

The optimization of buffer solution for the extraction of L-asparaginase from the leaves of *C. sativa* was done by preparing different buffers at different concentrations. The buffer solutions used are Sodium borate, Potassium chloride and Tris-HCl with different concentrations (0.15, 0.1, 0.05, 0.02 and 0.01M) at pH 8.
3.1.3.2 Extraction of Enzyme

The collected plant parts were washed thoroughly with tap water then with distilled water. Each plant part was separately homogenized with two volumes of 0.1M KCl buffer (pH 8.6) at 4°C. The extract of leaves, stem and roots were filtered to remove the cell debris then centrifugation was done at 8,000 rpm for 10 min. The protein estimation and enzyme activity determination was done using supernatant (crude enzyme).

3.1.4 Micropropagation of C. sativa

For micropropagation initiation of aspetic cultures, the explants (nodal segment) of C. sativa were collected from selected regions (Shimla and Solan) that were showing highest enzyme activity. The basal MS medium as described by Murashige and Skoog (1962) was employed for the culture of C. sativa. A variety of growth regulators i.e. BAP (6-benzyl aminopurine), Kinetin (N6-furfuryladenine), IBA (Indol-3-butyric acid) and NAA (α-Naphthaleneacetic acid) were also added. The concentrated stock solutions of all the components were prepared individually or in combinations and stored under refrigeration.

Appropriate concentration of the stock solutions were added for the preparation of medium in distilled water. The pH was adjusted to 5.8 by using 1N NaOH or 1N HCl and agar 0.7 % (w/v) was used for medium preparation for culture initiation and establishment. Before autoclaving, the media was poured into washed culture bottles (40 ml). These bottles containing media were autoclaved at 121°C for 15 min at a pressure 15-psi and shifted to the growth room.

3.1.4.1 Surface Disinfection of the Explants

The explants i.e. nodal segments were cut into small pieces of 4-5 cm in size. These explants were put in bottles that were covered with net and washings were given for 40
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min under tap water for the removal of the sand particles. It was further dipped in liquid
detergent and again washed in running tap water for another 15 min to take away
detergent. The explants were dipped in 0.1 % bavistin for 30 min. Then these were taken
out from the bavistin solution using forceps and washed thoroughly in sterilised distilled
water under laminar air flow. The explants were treated with 0.1% HgCl₂ solution with a
few drops of Tween-20 for 3-4 min. The explants were given 4-5 washings with
sterilized distilled water so that traces of HgCl₂ were removed.

3.1.4.2 Inoculation of Explants

Fresh cuts were given to the exposed ends after disinfection. The explants were then
vertically inoculated on variously supplemented media. Then the mouth of the culture
bottle was quickly flamed and tightly capped and sealed with cling film. All the cultures
were maintained at controlled temperature of 25 ± 2°C. The intensity of illumination was
50 µ mol/ m²/s at the level of cultures. Light regime was followed by 16 hrs of light and
8 hrs of darkness. For shoot proliferation only Solan region culture were taken.

3.1.4.3 Shoot Proliferation

MS medium for shoot proliferation was supplemented with a range of concentrations of
BAP (0 to 2.5 mg/l) and Kinetin (0.1 mg/l). After 15 days of incubation the newly
formed shoot buds were cut out from the propagated explants and further inoculated on
the similar media to enhance the number of shoots for further work.

3.1.4.4 Root Induction

Newly formed shoots 4-5cm in length were cut off from the media with parent explants
and shifted onto rooting media. Elongated shoots were rooted on 3 types of rooting
media, one basal MS medium devoid of hormone and other MS media supplemented
with (0-5mg/l) IBA and one basal media supplemented with (0-5mg/l) of NAA. Three -
five shoots per culture and 5 replicates were taken per treatment. Data was recorded after fifteen days of culture.

3.1.4.5 Acclimatization of Plantlets

After culturing for 15 days on rooting media, these plantlets then transferred to poly bags for hardening before transfer to soil. For hardening of plants, the micro-propagated plantlets were deflasked with a forcep to prevent any damage to newly formed roots. The plantlets were added in luke warm water to remove any adhering medium from the surface of the plantlets followed by washings with bavistin 0.1% (w/v) to prevent fungal infection. The newly developed plants were then washed with tap water. After washing plantlets were shifted to poly bags containing soil: vermicompost in the ratio of 1:1. The poly bags containing C. sativa plantlets were kept inside a polyhouse with 80% humidity and 31°C for 10 days. The hardened plants were then transferred to shade-house with indirect sunlight and less humidity level for another 20 days. Here, plants were watered twice a day to prevent wilting (if any). The plantlets were then transplanted in field. These micropropagated plantlets were then used for purification of L-asparaginase.

3.2 Purification and Kinetic Characterization of the enzyme

3.2.1 Purification of L-asparaginase

The crude extract of leaves was further purified. The protocol for purification includes various steps consecutively like Ammonium Sulphate Precipitation, Gel Filtration Chromatography, Ion Exchange Chromatography and PAGE.

3.2.1.1 Ammonium Sulphate Precipitation for the Purification of L-asparaginase (Scopes, 1994)

Salting out of protein using high concentration of salt is one of the commonly used techniques in enzyme purification. It largely depends upon the hydrophobicity of the protein (Scopes, 1994).
The samples were mixed with varied amounts of ammonium sulphate to get 20% and later 100% saturation. Mild stirring of the sample was done at 4°C for 1 hr. The sample mixture was kept for 14 hr at 4°C, followed by centrifugation for 20 min (5,000 rpm at 4°C). The pellet from each saturation fraction was dissolved in 1 M KCl buffer. Quantification of protein content and enzyme activity of supernatant and dissolved pellet was done. The maximum specific enzyme activity was observed in the fraction having 40-60% saturation.

The ammonium sulphate added to reach a predetermined concentration was analysed from the formula given below:

\[ g = \frac{533 (S_2 - S_1)}{100 - 0.3 S_2} \]

\( g \) stands for gram

\( S_1 = \% \) saturation to acquire it to \( S_2 \% \) saturation

**Dialysis (Swain et al., 1993)**

The process of separating molecules in the basis of their diffusion rates through semipermeable membrane (such as dialysis tube) is known as dialysis. Diffusion can be defined as the thermal, random movement from higher concentration of molecules to a lower concentration of molecules till equilibrium is attained (Swain et al., 1993).

**Activation of dialysis membrane**

Dialysis membrane was washed with distilled water and boiled for 1 hr in a solution of 100 mM NaHCO\(_3\) and 10 mM EDTA-Na salt (pH 7.0) with a mild agitation. The tube was washed with deionised water several times.

**Procedure**

One end of tube was tightly fixed with a tag. To this, 2 ml of each fraction of dissolved ammonium sulphate pellet was poured through open end and then this end was also tightly fixed with a tag. Further tube was suspended in 200 ml of 0.01 M Na borate
buffer for 6 hrs on mild stirring at 4°C. After 6 hrs, buffer was replaced with fresh 200 ml of 0.01 M Na borate buffer and kept for 6 hrs on mild stirring at 4°C. Each sample was assayed for enzyme activity and protein content.

3.2.1.2 Gel Permeation Chromatography for the Purification of L-asparaginase (Plummer, 1988)

The molecules were separated on the basis of their size which passes through the gel packed column. The gel consisted of three dimensional molecular network (open cross linked) casted in the form of a bead to ensure easy column packing and optimum flow characteristics. Smaller molecules can penetrate the process large molecules are not accessible to the process (Plummer, 1988).

Sephadex G-100 is used as a molecular sieve in gel permeation chromatography. At a flow rate of 1 ml/min and temperature 4°C, jacketed column of 1.2 X 8.2 cm dimension was filled with Sephadex. Phosphate Buffer pH 8.5 (0.01M) was used for its equilibration. Enzyme solution (2 ml) after the dialysis step was loaded in the Sephadex G-100 column and elution was carried with Phosphate buffer (pH 8.5, 0.01M) and fractions of 2 ml each were collected. The protein content and enzyme of the samples were calculated. The enzyme rich fraction was later used in further purification treatments.

3.2.1.3 Q-Sepharose Strong Anion Exchange Chromatography for the Purification of L-asparaginase

The most widely used adsorbents are Ion exchangers. These exchangers attract proteins by electrostatic attractions by exploiting the net charge on protein at a given pH. Strong anion exchangers contain strongly ionized groups like -NR3 which exist in the charged form and are completely ionized except at extreme pH values:

\[-NR_3OH \longleftrightarrow ^+NR_3 + OH^-\]
Q-Sepharose was used as a purification support as a strong anion exchanger.

![Chemical structure of quaternary aminoethyl strong ion exchanger](image)

**Functional group of quaternary aminoethyl strong ion exchanger**

For the strong anion exchange chromatography a column (1.2 X 8.2 cm) was packed with Q-Sepharose. Phosphate Buffer of pH 8.5 (0.02M) containing 10 mM NaCl was used to equilibrate the column with flow rate 1 ml/min at 4°C. The fractions from the gel permeation containing maximum enzyme units were pooled, loaded in the column and eluted with phosphate buffer of pH 8.5 (0.01M) containing 0.1- 0.5 M NaCl in gradient. 2 ml fractions were collected. The protein and enzyme content of every fraction was calculated.

### 3.2.2 PAGE (PolyAcrylamide Gel Electrophoresis)

PAGE (Polyacrylamide Gel Electrophoresis) was performed as described by Bollag and Edelstein (1991) to verify the purity of the enzyme. In vertical moulds of plates the mixture was poured on the separating gel and comb was fixed in it. The gel was allowed to solidify for 30 min.

The comb was removed after the polymerization of stacking gel. Sample was prepared by heating in boiling water bath for 2-3 min. After the sample was cooled at 25°C, it was loaded in sample wells. Electrophoresis was carried out at 100V. The gel was taken out and washed with deionised water. Then silver staining was carried out.

### 3.2.2.1 Silver Staining Method (Oakley et al., 1980)

Silver staining Method is very useful as it has 100-fold greater sensitivity over dye, up to nano gram levels.
Principle

Silver nitrate is present in +1 oxidation state, which is soluble and white in color. In the presence of formaldehyde, Ag+ is reduced to Ag (oxidation state zero) at pH-12, which is an insoluble and visible black form.

Materials

Composition of silver staining solution:

1. **Fixing solution: (40% Ethanol, 10% Acetic acid)**
   
   Ethanol : 200 ml
   
   Acetic acid : 50 ml
   
   Water : 250 ml

2. **Fixing/ sensitizing solution**
   
   Glutaraldehyde (25%) : 200 µl
   
   Formaldehyde (37%) : 10 µl
   
   Ethanol : 400 ml
   
   Water : 600 ml

3. **Washing solution**
   
   Ethanol : 400 ml
   
   Distilled Water : 600 ml

4. **Sensitizing solution**
   
   Sodium thiosulphate : 40 mg
   
   Distilled Water : 200 ml

5. **Stock silver nitrate solution:**
   
   Silver Nitrate : 0.1 gm
   
   Distilled Water : 100 ml

Make up and store in a dark glass bottle.
6. **Developer solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Carbonate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>40 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Make up just before use.

7. **Stopping solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>5 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>95 ml</td>
</tr>
</tbody>
</table>

**Precaution** - Wear gloves throughout this procedure.

**Procedure**

1. Placed the gel in 200 ml of 40 % Ethanol, 10 % acetic acid fixing solution for 10 min.
2. Rinsed the gel in 200 ml of distilled water for 10 min.
3. The gel was placed in 100 ml of Fixing or sensitizing solution for 5 min.
4. Rinsed the gel in 200 ml of 40% Ethanol solution for 20 min.
5. It was again rinsed in 200 ml of distilled water for 20 min.
6. Placed it in 100 ml of sensitizing reagent (0.2 % thiosulphate) for one min.
7. Rinsed it thrice with 200 ml of distilled water for one min each.
8. For 5 min it was placed in 100 ml of 0.1% silver nitrate solution.
9. Rinsed with 200 ml of distilled water for 1 min.
10. It was placed in 100 ml of developing solution until it turned yellow (approx. one minute), poured off immediately and replaced with 100 ml fresh developing solution. Developed until required degree of staining was obtained (approx. 15 min).
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11. 5% of acetic acid for 5 min was required to stop the reaction. Wet gels were stored in 0.03% sodium carbonate.

3.2.3 Kinetic Characterization of L-asparaginase

In the kinetic characterization of purified enzyme following parameters were studied:

1. Effect of pH on activity of purified enzyme
2. Effect of temperature on activity of purified enzyme
3. Substrate specificity and kinetic characterization of purified enzyme
4. Enzyme inhibition

3.2.3.1 Effect of pH on Activity of Purified Enzyme

Enzymes are active over a limited range of pH only. The change in the activity of enzyme with pH is because the protein, state of ionization of enzyme and other compounds of reaction mixture change (Plummer, 1988). Optimum pH of purified enzyme (after Q-Sepharose as strong anion exchange chromatography) was checked at different pH values (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0).

3.2.3.2 Effect of Temperature on Activity of Purified Enzyme

For the molecules to precede the reaction they must have energy of activation. The enzyme acts as catalysts and lowers the activation energy, resulting in a faster reaction. The maximum velocity (V) is plotted against temperature to calculate the activation energy of the enzyme catalyzed reaction. To detect the temperature optima of purified enzyme, enzyme activity was determined at various temperatures (25, 30, 35, 37, 40, 45, 50, 55 and 60°C).

3.2.3.3 $K_m$ and $V_{max}$ of Purified Enzyme

With the increased substrate concentration the rate of enzymatic reactions increases for a given amount of enzyme. But after a certain point, there is no increase in reaction rate with increase in substrate concentration. It is due to the fact that the active sites of
enzyme are saturated with substrate. Different concentrations of L-asparagine were used (2.5-20 mM) and Lineweaver-Burk plot was plotted between 1/V (enzyme activity) vs 1/[S] (substrate concentration). $K_m$ value is half of the substrate concentration at which enzyme shows its maximum activity.

3.2.3.4 Enzyme Inhibition

Many compounds react with enzyme and reduce its activity, such molecules are called enzyme inhibitors and phenomenon is known as enzyme inhibition. This property of enzyme is used in designing drugs, insecticides and biosensors. The rate of inhibition was checked by the use of inhibitors CuSO$_4$, HgCl$_2$, AgNO$_3$, FeCl$_2$, ZnSO$_4$, MgCl$_2$ and EDTA etc. on the purified enzyme. Concentration of each inhibitor used was 20 mM.

3.3 Development of L-asparagine Biosensor to Monitor L-asparagine in Leukemia Cells

3.3.1 Immobilization of purified enzyme

Purified enzyme (after Q-sepharose purification) was immobilized with covalent and gel entrapment methods. Former was carried out with the oxirane beads, while latter was carried in sodium alginate and polyacrylamide gel.

3.3.1.1 Covalent Immobilization of L-asparaginase in Oxirane Beads (Cusack and Beynon, 1989)

Covalent binding of enzyme was performed with the solid supports, which occurs through non-essential amino or carboxyl group of enzyme molecules and making them immobilized. The covalently couple biomolecules such as enzyme or antibodies or dissolved protein is either reacted with an activated water insoluble carrier or co-polymerized with reactive monomer. The reaction should involve only groups that are not essential for the biological activity of the biomolecules. Oxirane is formed by
copolymerization of methacyrlamide, methyl bisacrylamide, glycidal methacrylate and allyl glycidal ether (1-allyloxy-2,3-epoxypropane).

A slightly modified procedure given by Cusack and Beynon (1989) was used for covalent immobilization of the enzyme on oxirane beads.

Procedure

1. Oxirane beads (20 mg) were suspended in 5 ml of Phosphate buffer saline (PBS), pH 7.5 (50mM). To this solution purified enzyme (2.47± 0.05 IU) was added.

2. The reaction mixture was left overnight at 4°C with gentle shaking.

3. The beads carrying the bound enzyme were centrifuged for 30 min (4,000 rpm at 5°C) and washed thoroughly with PBS.

4. Incubation with 10% ethanolamine at pH 7.5 for 40 hrs at 4°C was given to block unreacted oxirane group.

5. The covalently immobilized oxirane L-asparaginase preparation was washed with PBS.

6. The beads were kept in PBS (0.1 M) at pH 7.2.

7. The beads were kept in dialysis membrane confined to a glass rod.

3.3.1.2 Gel Entrapment Methods for Immobilization of Purified Enzyme

The interstitial space of gel lattice can be used for immobilization of enzyme by polymerization of materials. Due to the entrapment the diffusion of biomolecules is prevented in the reaction mixture. In the gel entrapment techniques two different supports i.e. sodium alginate and polyacrylamide were used for immobilization.

3.3.1.2.1 Calcium Alginate Immobilization (Palmieri et al., 1994)

For the immobilization of purified enzyme the following procedure was employed (Palmieri et al., 1994):

1. 3% sodium alginate slurry was prepared with purified enzyme (2.46± 0.05 IU).
2. This slurry was then extruded drop wise through needle into beaker containing 100 ml (0.15 M) chilled calcium chloride with gentle stirring.

3. The beads were kept for 1 hr in calcium chloride for hardening under stationary conditions.

4. Beads were then washed with 50 mM Sodium acetate buffer (pH 5.6) for further use.

3.3.1.2.2 Polyacrylamide Gel Immobilization (Skryalbin and Koshcheenko, 1987)

The enzyme was entrapped in polyacrylamide using following procedure with slight modifications (Skryalbin and Koshcheenko, 1987):

1. A 10% acrylamide and bis-acrylamide solution (9% acrylamide and 1% bis-acrylamide) was prepared in 0.1 M phosphate buffer (pH 6.0).

2. Purified enzyme (2.45± 0.05 IU) and acrylamide solution was mixed.

3. In the above 0.5 ml (0.5%) ammonium persulphate was added.

4. 50µl (50%) TEMED (Tetramethylethylenediamine) was added and the contents were stirred gently and solution was poured in to the petriplate.

5. The gel was then cut into square blocks of 1.0 X 1.0 cm.

3.3.2 Kinetic Characterization of Immobilized Enzyme

Kinetic characterization of enzyme immobilized by oxirane beads, sodium alginate and polyacrylamide gel was done. After the immobilization, parameters like optimum pH, optimum temperature and kinetic characterization were studied.

3.3.2.1 Optimization of pH for the Catalytic Activity of Immobilized Enzyme

Various pH levels (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0) were studied to determine the optimum pH for the catalytic activity of immobilized enzyme.
3.3.2.2 Optimization of the Temperature for the Catalytic Activity of Immobilized Enzyme

Different temperatures (25, 30, 35, 37, 40, 45, 50, 55 and 60°C) were used to determine the optimum temperature for enzyme activity of the immobilized enzyme.

3.3.2.3 Kinetic Characterization

Different concentrations of substrate L-asparagine (2.5-20 mM) were used and Lineweaver-Burk plot between \(1/V\) (enzyme activity) \(\text{vs}\) \(1/[S]\) (substrate concentration) was plotted. From the graph \(K_m\) and \(V_{\text{max}}\) were calculated.

3.3.3 Strategies for the Development of L-asparagine Biosensor

The Bioassay Principle: L-asparaginase catalyses the conversion of L-asparagine into Aspartic acid and \(\text{NH}_4^+\) ions. The equation for the reaction is:

\[
\text{L-asparaginase} \quad \text{L-asparagine} \rightarrow \text{Aspartic Acid} + \text{NH}_4^+
\]

Biosensor construction strategies involve scrutiny of change in pH and mV due to production of \(\text{NH}_4^+\) ions by the enzyme action on different concentrations of L-asparagine.

3.3.3.1 Immobilization of the Biocomponent

The purified enzyme was immobilized by physical entrapment method. Phenol red was used as an indicator in a concentration of 4 mg/4 ml in ethanol and water mixture in 1:1. The response time with the change in color of immobilized enzyme, as well as pH change was noted. The following methods were used for the immobilization of the enzyme.

3.3.3.1.1 Agarose (Prakash et al., 2007)

1.5 % agarose solution was prepared in 25 mM Tris-acetate buffer (pH 7.2) containing 2 mM \(\text{CaCl}_2\) by heating for 10 min. The 20 µl of enzyme (0.5 IU) (per 10 ml of above solution) and 10 µl of phenol red indicator were added to the above solution. This was
followed by pouring the solution into the petriplates and allowed to solidify. The gel was cut into small pieces of $1.0 \, \text{cm}^3$. The pieces were put into varying concentrations of L-asparaginase from $10^{-10}$ to $10^{-1}$ M and initial and final pH was noted. The response time was noted for change in color of gel pieces from partly orange to dark purple (due to evolution of $\text{NH}_4^+$ detected by phenol red indicator co-immobilized with the enzyme).

3.3.3.1.2 Gelatin (Alteriis et al., 1985)

10 % aqueous solution of gelatin was prepared by dissolving 1.0 g gelatin in distilled water. The solution was heated mildly so that the gelatin dissolves properly. A hardening solution was prepared by mixing 20 % Formaldehyde, 50 % Ethanol and 30 % Water. After this 20 µl (0.5 IU) of enzyme, 2 ml of hardening solution and 10 µl of phenol red indicator were added to the gelatin solution. It was placed at -20°C for 4 hrs to facilitate its solidification. The gel was brought to room temperature and cut into small pieces of $1 \, \text{cm}^3$. The pieces were put into varying concentrations of L-asparaginase from $10^{-10}$ to $10^{-1}$ M. The change in pH values and response time was noted.

3.3.3.1.3 Agar (Mahajan et al., 2010)

4% agar solution in distilled water was prepared by boiling and then cooling it to 45-50°C. 20 µl of enzyme (0.5 IU) and 10 µl of phenol red indicator were added. The solution was then poured into the petriplates for solidification. The solidified gel was cut into small square pieces of approximately $1.0 \, \text{cm}^3$ size. Different concentrations of L-asparaginase from $10^{-10}$ to $10^{-1}$ M were prepared. The pieces were put into varying concentrations of L-asparaginase and initial and final pH was noted. The response time was also noted for change in color of gel pieces.

3.3.3.1.4 Calcium Alginate (Palmieri et al., 1994)

Chilled CaCl$_2$ solution (0.075 M) was prepared. The slurry was prepared by mixing 3 % sodium alginate with 20 µl of enzyme (0.5 IU) and 10 µl of phenol red indicator.
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The prepared CaCl\textsubscript{2} solution was placed on the magnetic stirrer and the slurry was added drop-wise. The orange colored calcium alginate beads were formed with the help of 2.5 ml syringe without needle. The beads were hardened by placing them at room temperature for nearly half an hr. Beads were put in each test tube containing different concentration of L-asparagine i.e. from $10^{-10}$ to $10^{-1}$ M. The response time and change in pH values was noted.

### 3.3.3.1.5 Polyvinyl Alcohol-alginate (PVA-alginate) (Bonilla et al., 2014)

Aqueous solutions of 3\% alginate and 12.5\% PVA was prepared separately using 0.03mol/l Tris buffer (pH 8.0) at room temperature (25°C) for alginate and at 75°C for PVA, with continuous stirring. Alginate and PVA solutions were mixed in the desired proportions at room temperature and stirred overnight. 20 \textmu l of enzyme (0.5 IU) and 10 \textmu l of phenol red indicator were added into the PVA-alginate mixture, with constant stirring at 4°C for 1 hr. Chilled CaCl\textsubscript{2} solution (2\%) solution saturated with boric acid was prepared. The prepared CaCl\textsubscript{2} solution was placed on the magnetic stirrer and the slurry was added drop-wise. The orange colored PVA-alginate beads were formed with the help of 2.5 ml of hypodermic syringe without needle. Washing was done twice with distilled water. Beads were put in different concentrations of L-asparagine i.e. from $10^{-10}$ to $10^{-1}$ M, the change in pH and color was noted.

### 3.3.3.1.6 Hydrosol Gel on Nylon Membrane (Wang et al., 2004)

A homogenous stock sol-gel solution was prepared by mixing 600 \textmu l ethanol, 10 \textmu l 5 mM NaOH, 50 \textmu l Tetra Ethyl Ortho Silicate (TEOS) and 60 \textmu l distilled water. This solution was kept overnight in the refrigerator at 4°C. On the nylon membrane of size 1x1 cm the stock sol-gel solution was applied followed by 20 \textmu l of enzyme (0.5 IU) and 10 \textmu l of phenol red indicator. The membrane was dried at room temperature for 30 min.
Varying concentrations of L-asparagine i.e. from $10^{-10}$ to $10^{-1}$ M was poured on the nylon membrane. The change in pH and color of hydrosol gel membrane was noted.

**3.3.3.2 Application of the Developed Biosensor**

(i) Testing of Normal and Leukemic Blood Samples

Hydrosol-gel membrane and PVA-alginate beads (Quantitative analysis) were added into normal and leukemic blood samples. Response time for color change of membrane and beads till purple color appears was noted. The detection range of L-asparagine levels in all the samples were elucidated by relating the response time for change in color of membrane and beads with the response time for change in color of hydrosol-gel membrane and PVA-alginate beads with standard concentration levels from $10^{-10}$ – $10^{-1}$ M of L-asparagine.

(ii) Storage Stability

To know the storage stability of the bio component, calcium alginate beads and polyacrylamide gel pieces were wrapped in a whatmann filter paper soaked in CaCl$_2$ and kept in refrigerator. The hydrosol-gel membrane was wrapped in a dry whatmann filter paper and kept in refrigerator. The activities of the immobilized biocomponent were checked at regular intervals.

(iii) Reliability Check for the Constructed Biosensor

Calculation of response times for change in color of $10^{-2}$ M and $10^{-5}$ M was done to check reliability of the developed biosensor, 25 µl of serum sample (leukemic and normal) was mixed with 25 µl of synthetic L-asparagine ($10^{-2}$ and $10^{-5}$). The $\Delta$ pH (change in pH) and time response was studied with the help of hydrosol gel on nylon membrane and PVA-alginate method.
\[
\frac{1}{2}x + \frac{1}{2}y = X
\]

Where, \(x\) = Serum sample, \(y\) = Synthetic sample of L-asparagine

\(X\) = Reliability factor