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# DEVELOPMENT OF ELECTROCHEMICAL BIOSENSOR FOR DETECTION OF ASPARAGINE IN LEUKEMIC SAMPLES

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**Keywords:**

*C. sativa*, immobilization, biosensor, Acute Lymphoblastic Leukemia, asparagine.

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**ABSTRACT:** Acute Lymphoblastic Leukemia (ALL) is one of the deadly diseases among children causing a number of deaths worldwide. It could be treated using chemotherapy, bone marrow transplantation or irradiation therapy but it may cause side-effects. Therefore, interest has arisen in the use of plants for treatment of ALL. *Cannabis sativa* is known to produce L-asparaginase which is used to treat ALL. L-asparaginase breaks down asparagine to aspartic acid and ammonia. Thus the cancer cells die due to asparagine starvation as they cannot synthesize asparagine on their own. L-asparaginase was extracted from *C. sativa* and immobilized to develop a biosensor for the detection of asparagine levels in leukemic serum samples. Out of the various immobilized techniques Polyvinyl Alcohol and hydrosol gel on nylon membrane were found to give the fastest response. Therefore, they were used to detect asparagine levels in leukemic serum samples. The concentration of asparagine in leukemic samples was $10^{-2}$ to $10^{-3}$ M and in normal samples it was $10^{-5}$ M.

**INTRODUCTION:** Acute Lymphoblastic Leukemia (ALL) is one type of cancer in which immature and malignant WBCs (White Blood Cells) of bone marrow multiply rapidly [1]. Chemotherapy, radiation therapy, bone marrow transplantation or treatment with enzymes is known to cure ALL. L-asparaginase is the first enzyme characterized for the treatment of cancer in humans. Based on the study of biochemical and crystallographic data, the asparaginase sequences can be divided into two different families. The bacterial-type L-asparaginase is related to first family and the second is plant-type L-asparaginase [2].

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Among the various sources of L-asparaginase *Cannabis sativa* is a potent source of L-asparaginase [3]. *C. sativa* is a wide plant found mainly in the regions with plenty of sunlight. It secretes two compounds namely THC (tetrahydrocannabinol) and cannabionoids which makes its use as a recreational drug. It is also used for the construction of biosensor for the detection of asparagine levels in leukemic serum samples. The first plant based biosensor was developed from immobilized slices of yellow squash tissue to detect atmospheric carbon dioxide [4].

Many other plant biosensors have been also constructed such as using banana [5], spinach [6], *Allium sativum* (garlic) for the detection of ammonia (using online gas analyser) [7], coconut [8], mushroom [9], palm tree [10], hydrogen peroxidase (immobilized on chitosan matrix) [11] and barley [12]. Biosensors for the detection of asparagine levels were developed using the extract of *Capsicum*

*annum* [13], *Citrus lemon* [14] and *Withania somnifera* [15]. Working with *C. sativa* our group earlier developed a biosensor in which phenol red dye was used as an indicator and visual approach was used to measure the response time [3]. In the present study we used spectroscopic analysis for measuring the asparagine levels in leukemic serum samples.

## MATERIALS AND METHODS:

The chemicals and reagents used in the study were of analytical grade and purchased from HiMedia Laboratories Pvt. Ltd., India.

### Preparation of crude extract:

The leaves from the plants were collected and then washed with distilled water. The leaves were homogenized with 0.15 M KCl buffer and centrifuged at 8000 rpm for 20 min at 4°C. The supernatant thus obtained was taken as the crude extract[16]. To check the presence of L-asparaginase in the crude extract, it was coimmobilized with phenol red indicator. Due to the deamination reaction in which L-asparaginase break down L-asparagine to aspartic acid and ammonia, the solution changed from partly orange to bright purple colored solution.

### Agarose:

1.5 % agarose solution was prepared in 25 mM Tris-acetate buffer of pH 7.2 containing 2 mM $CaCl_2$ by heating for 10 min. Then 20 µl of enzyme (0.5 U) (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 petri plates and allowed to solidify. The gel was cut into small pieces of 1.0 x 1.0 cm [17]. The pieces were put into varying concentrations of L-asparagine from $10^{-10}$ to $10^{-1}$ M. The change in pH was noted before and after the reaction.

### Gelatin:

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 of enzyme (0.5 U), 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 h to facilitate its solidification. The gel was warmed to room temperature and cut into small pieces of 1 cm per side [18]. The pieces were put into varying concentrations of L-asparagine from $10^{-10}$ to $10^{-1}$ M and change in pH values was noted.

### Agar:

4% agar solution in distilled water was prepared by boiling and then cooling it to 45-50°C. 20 µl of enzyme (0.5 U) and 10 µl of phenol red indicator were added. The solution was then poured into the petri plates for solidification. The solidified gel was cut into small square pieces of approximately 1.0x 1.0 cm size [19]. Different concentrations of L-asparagine from $10^{-10}$ to $10^{-1}$ M were prepared. The cut pieces of gel were put in the varying concentrations of L- asparagine and the pH was noted.

### Calcium alginate:

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 U) and 10 µl of phenol red indicator. The prepared $CaCl_2$ 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 [20]. The beads were hardened by placing them at room temperature for nearly half an hour. Beads were put in each test tube containing different concentration of L-asparagine i.e. from $10^{-10}$ to $10^{-1}$ M. The change in pH values was noted.

### Hydrosol gel on nylon membrane:

A homogenous stock sol-gel solution was prepared by mixing 600 µl ethanol, 10 µl 5 mM NaOH, 50 µl tertraethyle orthro silicate (TEOS) and 60 µl 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 µl of enzyme and 10 µl of phenol red indicator. The membrane was dried at room temperature for 30 min. Varying concentrations of L- asparagine were poured on the nylon membrane and the pH was noted [21].

### PVA (Polyvinyl Alcohol):

Aqueous solutions of 3% Alginate (Alg) and 12.5% PVA was prepared separately using 0.03mol/L Tris

buffer (pH 8.0) at room temperature (25°C) for Alg and at 75°C for PVA, with continuous stirring. Alg and PVA solutions were mixed in the desired proportions at room temperature and stirred overnight. 20 µl of enzyme (0.5 U) and 10 µl of phenol red indicator were added into the Alg/PVA mixture, with constant stirring at 4ºC for 1 h. Chilled $CaCl_2$ solution (2%) solution saturated with boric acid was prepared.

The prepared $CaCl_2$ 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 taken in different concentrations of L-asparagine asparagine i.e. from $10^{-10}$ to $10^{-1}$ M, the change in pH and color was noted down.

**Application of the Developed Biosensor:**

**Testing of normal and leukemic blood samples:**

The immobilization techniques with which the response time was faster were chosen to detect asparagine levels in leukemic and normal 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 in all the samples with the response time for change in color of hydrosol-gel surface on nylon membrane and PVA-alginate beads with standard concentration levels from $10^{-9}$ – $10^{-1}$ M of L-asparagine.

**Storage Stability:**

To know the storage stability of the biocomponent, calcium alginate beads and polyacrylamide gel pieces were wrapped in a Whattmann filter paper soaked in $CaCl_2$ and kept in refrigerator. The hydrosol-gel membrane was wrapped in a dry whattmann filter paper and kept in refrigerator. The activities of the immobilized biocomponent were checked.

**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 Δ pH and time response was studied with the help of hydrosol gel on nylon membrane and PVA method. The results were comparable.

$$1/2x + 1/2y = X$$

Where, x= Serum sample

y= Synthetic sample of L-asparagine

**RESULTS AND DISCUSSION:**

**Immobilization Techniques:**

The enzyme was immobilized using different techniques as shown in **Fig. 1** and the change in pH (ΔpH) before and after the reaction with various asparagine concentrations is shown in **Fig. 2**.

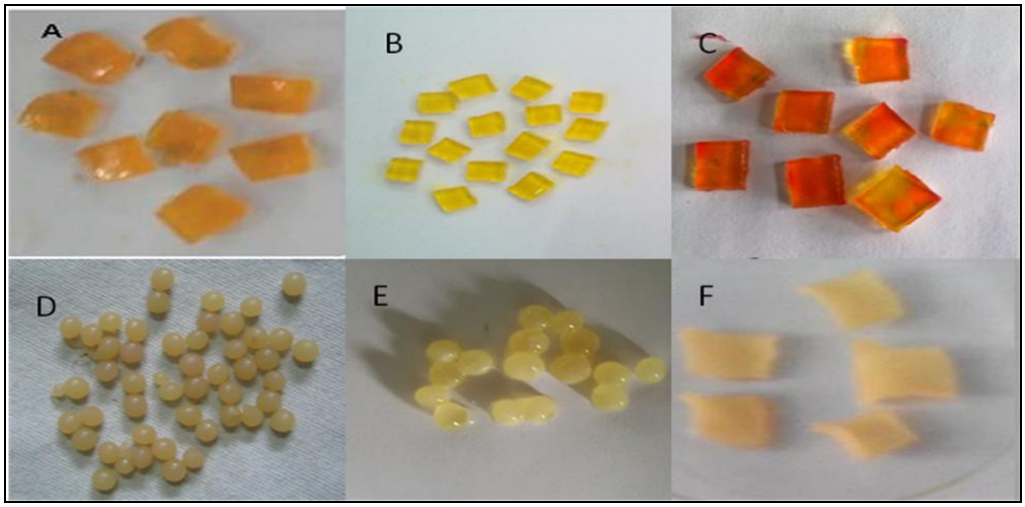


**FIG.1: IMMOBILIZATION OF ENZYME USING DIFFERENT IMMOBILIZATION TECHNIQUES: A. GELATIN, B. AGAROSE, C. AGAR, D. CALCIUM ALGINATE BEADS, E. PVA-ALGINATE BEADS, F. HYDROSOL GEL ON NYLON MEMBRANE.**

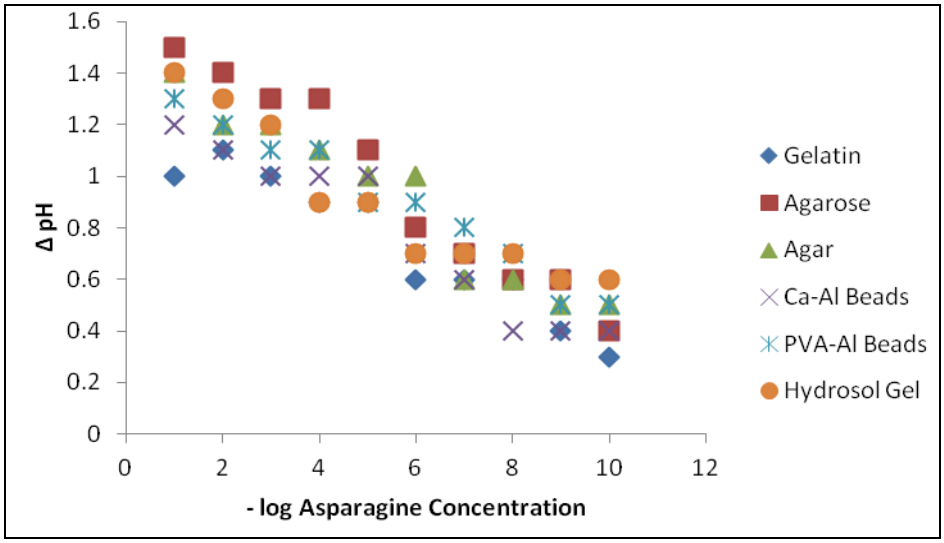


**FIG.2: ΔPH WITH VARIOUS CONCENTRATIONS OF ASPARAGINE**

**Application of developed biosensor:**

Out of all the above applied techniques, enzyme immobilized on PVA and hydrosol gel on nylon gave the best response time of 4.8 and 4.3 sec respectively therefore, it was used to detect asparagine levels in leukemic serum samples. The pH of leukemic samples was compared with the standard values (**Table 1**). Sodium saline (0.9 %) was used with the samples.

**TABLE 1: RESPONSE TIME OF NORMAL AND LEUKEMIC SERUM SAMPLES USING PVA AND HYDROSOL GEL ON NYLON MEMBRANE**

| Samples | PVA-Al Beads | | Hydrosol Gel on Nylon Membrane | | Concentration of L-asparagine (M) |
|---|---|---|---|---|---|
| | Response Time (sec) | Δ pH | Response Time (sec) | Δ pH | |
| Normal | 8.8±0.02 | 0.9±0.02 | 8.9±0.01 | 0.9±0.01 | $10^{-5}$ |
| Normal | 8.7±0.02 | 0.8±0.02 | 8.8±0.03 | 0.8±0.02 | $10^{-5}$ |
| Leukemia-1 | 11.9±0.03 | 1.1±0.01 | 10.9±0.02 | 1.2±0.03 | $10^{-2}$ |
| Leukemia-2 | 11.7±0.02 | 1.1±0.03 | 10.7±0.02 | 1.1±0.01 | $10^{-3}$ |
| Leukemia-3 | 10.0±0.02 | 1.2±0.01 | 10.1±0.02 | 1.3±0.02 | $10^{-2}$ |
| Leukemia-4 | 11.6±0.01 | 1.1±0.02 | 10.5±0.01 | 1.2±0.02 | $10^{-2}$ |
| Leukemia-5 | 10.2±0.02 | 1.1±0.04 | 10.3±0.01 | 1.2±0.03 | $10^{-3}$ |

For leukemic samples the response time was 12 to 10 seconds The L-asparagine concentration was $10^{-2}$ to $10^{-3}$ M. The normal blood sample gave the response time of 8.8 and 8.7 seconds and the asparagine concentration was $10^{-5}$ M. Thus asparagine levels were found to be higher in leukemic cells as comparative to normal cells. Response time for change in color of the pieces, till purple color appears was 8.9 and 8.8 seconds for the normal blood sample. The asparagine concentration level was in the range of $10^{-5}$ M. Response time for change in color of the pieces, till purple color appears was approximately 11 seconds for the leukemic blood samples. The asparagine concentration level was in the range $10^{-2}$ to $10^{-3}$ M in leukemic cells. Therefore the leukemic samples had higher amount of asparagine.

**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 Δ pH and time response was studied with the help of hydrosol gel on nylon membrane and PVA method (**Table 2**). The results give comparable.

$$1/2x + 1/2y = X$$

Where, x= Serum sample

y= Synthetic sample of L-asparagine

**TABLE 2: RELIABILITY CHECK OF THE CONSTRUCTED BIOSENSOR (PVA-AL BEADS AND HYDROSOL GEL ON NYLON MEMBRANE)**

| Concentration + Serum Samples | PVA-Al Beads | | Hydrosol Gel on Nylon Membrane | |
|---|---|---|---|---|
| | Δ pH | Time Response (sec) | Δ pH | Time Response (sec) |
| $10^{-2}$ | 1.2±0.01 | 10.7±0.02 | 1.2±0.02 | 11.6±0.01 |
| $10^{-5}$ | 0.9±0.02 | 8.9±0.01 | 0.9±0.01 | 8.9±0.02 |

Hence, the developed biosensor is quite reliable and comparable. Hence, visualization approach coupled with the change in pH can be opted for monitoring L-asparagine concentration in blood samples of leukemia and normal samples.

**CONCLUSION:** The biosensor which was developed could monitor asparagine levels from $10^{-10}$ to $10^{-1}$ and was also used to detect asparagine levels in normal and leukemic serum samples. Different immobilization techniques were employed for the preparation of Asparagine biosensor. Out of all the constructed biosensors the best response time was given by PVA and hydrosol gel on nylon membrane. The biosensor is made extremely cost effective as it can detect asparagine rapidly and in minute quantities. The developed biosensor is not only novel but also rapid, easy, inexpensive and capable of use at nanolevel.

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