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Asparagine Based Plant Biosensor for Leukemia

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L-asparagine is the most abundant metabolite for the storage and transport of nitrogen that is utilized in protein biosynthesis. Asparagine hydrolysis in plants is catalyzed by asparaginases with no homology to the bacterial-type enzymes. The enzyme L-asparaginase was extracted from leaves of *Citrus limon*. Immobilization of enzyme by physical entrapment method has been done. Different immobilization techniques like immobilization with calcium alginate, agar, agarose, polyacrylamide gel and gelatin were used. Among all these techniques, calcium alginate immobilization method was better one for construction of novel biosensor. The asparagine concentrations ranging from 10^{-1} to 10^{-9} M were studied. The level of asparagine in leukemic blood was 10^{-2} M and that in normal blood was in the range of 10^{-7} – 10^{-5} M. Plant Biosensors possess advantages such as reliability, sensitivity, accuracy, ease of handling, and low-cost compared with conventional detection methods.

Keyword: Biosensor, L-asparaginase, Immobilization, Polyacrylamide.

1. Introduction

L-asparaginase (EC 3.5.1.1) is an essential tetrameric protein in the treatment of childhood lymphoblastic leukaemia and lymphosarcoma[1]. Leukemic cells are deprived of an enzyme asparagine synthetase so they are dependent on the availability of extra-cellular asparagine[2]. Asparagine synthetase, encoded by a single gene located on human chromosome 7q21.3 is the only enzyme available for the synthesis of asparagine[3]. Leukemic cells are restricted in their protein synthesis following exposure to L-asparaginase. The enzyme hydrolyzes the deamidation of L-asparaginase to form aspartic acid and ammonia[4]. Lang[5] was the first to detect asparaginase activity in beef tissues and results were confirmed by Furth and Friedmann[6]. Kidd[7] reported antitumour properties of Guinea pig serum and the factor responsible for antilymphoma activity was L-asparaginase[8]. L-asparaginases derived from different biological source (*Escherichia coli* and *Erwinia carotovora*) possess antitumor activity particularly against acute lymphoblastic leukemia (ALL)[9]. Other microorganisms such as *Aerobacter*, *Aspergillus, Bacillus*, *Proteus Pseudomonas*, *Vibrio Serratia*, *Xanthomonas*, *Photobacterium*[10, 11, 12] have a potential for asparaginase production. However, most widely the purified enzyme from *E. coli* and *Erwinia* sp.[13] has been reported to be used as anti-tumor and anti-leukemia agent. In plants, L-asparagine is the major nitrogen storage and transport compound and also reported to be accumulated under stress conditions[14]. The presence of an amidase in barley roots capable of hydrolyzing L-

asparagine and its distribution was well studied by Grover and Chibnall[15]. L-asparaginase has also been reported to be produced from *Lupinus luteus* and *Dolichos lab lab* seedlings[16]. *Capsicum annum* and *Tamarindus indica* has been reported to have appreciable amount of L-asparaginase[17] and was also detected in young leaves of *Pisum sativum*[18]. The expression, purification and catalytic activity of *Lupinus luteus* asparagine beta-amidohydrolase was reported by[19]. L-asparaginase activity has been found in the soil of roots of *Pinus pinaster* and *Pinus radiate* due to ectomycorrhizal fungi in the wheat belt of Western Australia by Bell and Adams[20]. Cho *et al.*[21] isolated the low temperature inducible cDNA sequence that encodes L-asparaginase extracted from soybean leaves and expressed in *E. coli* and this asparaginase has been reported to have 3 times increased activity. There was screening of germinating and non-germinating seeds of five Egyptian cowpea cultivars. In all cultivars, L-asparaginase specific activity was higher in germinating seeds[22]. Plant-based biosensors offer a good alternative as compared with conventional biosensors based on isolated enzymes from different sources. Fabrication of asparaginase biosensor is a promising technology to detect L-asparagine. Earlier, *Allium sativum* L. peroxidase biosensor was immobilized on chitosan matrix for monitoring hydrogen peroxidase[23]. Polyphenol oxidase isolated from banana tissue was incorporated into polypyrrole matrix to make a biosensor for the analysis of acetaminophen in biological samples[24]. An enzyme diamine oxidase was characterized from *Lathyrus sativus* and evaluated as biocatalytic component of an electrochemical biosensor for the determination of biogenic amines in wine and beer samples[25]. An amperometric oxalate biosensor was formed by immobilizing a barley root oxalate oxidase onto gold-nanoparticle-porous $CaCO_3$ microsphere hybrid[26]. For the detection of target DNA fragment from the transgene cauliflower mosaic virus 35S (CaMV 35S) promoter a novel fluorescent biosensor was constructed[27]. The current study presents the development of a novel, diagnostic and cost-effective plant L-asparaginase based biosensor. Color visualization approach has been optimized to monitor asparagine levels. Storage stability of immobilized plant L-asparaginase was studied.

2. Materials and Methods

2.1 Crude Extract Preparation

All the chemicals and reagents used in this study were of analytical grade (Hi-Media Laboratories Pvt. Ltd., India.). The plant, *Citrus limon* designated as lemon was collected from different regions of Punjab, India. Fresh leaves of lemon were taken to extract the enzyme. Leaves were washed with distilled water and homogenized with 3 volumes of 0.15M KCl and centrifuged at 8000 rpm for 20 minutes at 4 ^{0}C. The supernatant was separated out; this was designated as crude extract[18] and further used for the development of biosensor. In biosensor construction strategies, L-asparaginase was co-immobilized with calcium alginate, agar, agarose, polyacrylamide gel and gelatin as described by Kumar *et al.*[28].

2.2 Immobilization techniques include the following

2.2.1 Calcium Alginate beads

Slurry of 3% sodium alginate with 20 µl of the enzyme solution (0.5 U) was formed and 10 µl of bromothymol blue indicator were added to this slurry. This solution was then poured drop wise through a glass syringe into a beaker containing 0.075 M chilled $CaCl_2$ with gentle stirring on a magnetic stirrer. Pale color beads were made with the help of 2.5 ml syringe without needle following the method of Johnsen and Flink[29]. Harden the beads by placing it for half an hour at room temperature. 5 to 6 beads were put into varying concentrations of L-asparagine (10^{-10} -10^{-1} M) solutions. The response time for change in color of beads from pale to dark blue was noted.

2.2.2 Agar Method

A solution of 4% agar was prepared, boiled it and allowed to cool at 40 - 45 ^{o}C. 20 µl enzyme (0.5 U) and 10 µl bromothymol blue indicator was added to the solution. Mixed it thoroughly and poured it into 90 mm petriplate and allowed to solidify. The gel was then cut into square cakes of

1.0 x 1.0 cm with the help of knife as described by Mahajan *et al.*[30]. Then cakes were put into varying concentration (10^{-10}–10^{-1} M.) of L-asparagine and the response time was noted for change in color of cakes from pale to dark blue.

2.2.3 Agarose Method

Agarose solution (1.5%) was prepared in 25 mM Tris- acetate buffer (pH 7.2) containing 2 mM $CaCl_2$ and dissolved by heating. Then 20 µl enzyme (0.5 U) solution was added followed by 10 µl bromothymol blue indicator. Solution was poured into 90 mm petriplate and allowed it to solidify. The gel was then cut into small pieces of 1.0 X 1.0 cm[31]. Put the gel pieces into varying concentration of L- asparagine (10^{-10}–10^{-1} M) and the response time was noted for change in color of small pieces from pale to dark blue.

2.2.4 Polyacrylamide Method

A 10 % acrylamide and bis-acrylamide solution (9% acrylamide and 1% bis- acrylamide) was prepared in 0.1 M phosphate buffer (pH 7.0). 20µl of enzyme (0.5 U) was co-immobilized with 10µl of bromothymol blue indicator. 0.5 g of ammonium per sulphate and 50 ml TEMED were added to the above solution. After gentle stirring, the solution was poured into petriplate. After solidification the gel was cut into square blocks of 1.0 X 1.0 cm[32]. The asparagine concentrations studied were in the range 10^{-10}–10^{-1} M and color change was noted.

2.2.5 Gelatin Method

10% aqueous solution of gelatin was prepared and solution was heated to facilitate the dissolution process. Approximately 20 µl of enzyme (0.5 U) and 2 ml of hardening solution (20% Formaldehyde, 50% Ethanol, 30 % Water) were added followed by 10 µl of bromothymol blue indicator to the solution. Then poured it into a plastic mold and allowed to freeze at -20 ºC for 4 hours to facilitate the gel formation. Set gel was warmed at room temperature and then cut into small blocks of approximately 3 mm per side[33]. Put these blocks into varying concentration of L-asparagine (10^{-10}–10^{-1} M) and the response time was noted for change in color of blocks from pale to dark blue.

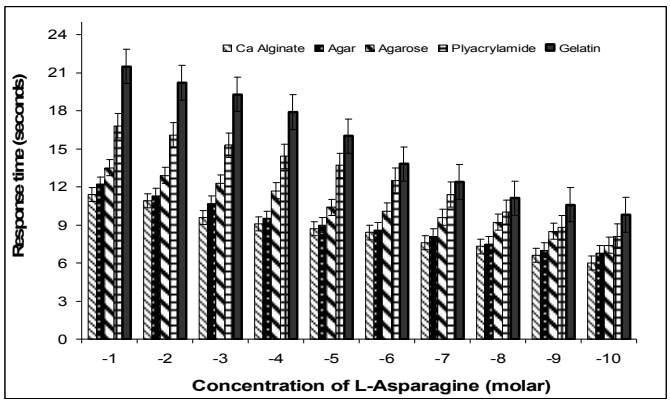
2.2.6 Storage Stability

To know the storage stability of biocomponent, calcium alginate beads, agar cakes, agarose pieces, polyacrylamide gel pieces and gelatin gel blocks were kept in refrigerator. The activities of immobilized biocomponents were checked.

3. Results

3.1 Calcium Alginate beads

For calcium alginate beads, visual color change was observed. The Figure-1 shows comparison of color of beads before and after the reaction. Detection limit of L-asparagine achieved was 10^{-10}–10^{-1} M. For concentration level of 10^{-1} M L-asparagine, response time detected was 11.4 seconds and for the concentration level of 10^{-2}-10^{-10} M L-asparagine, response time detected was in the range of 6-11 seconds (Graph-1). Response time decreases with decrease in concentration of L-asparagine indicating more of NH_4^+ ion produced after hydrolysis.



Graph 1: Performance of biosensor with L-asparagine standards bromothymol blue

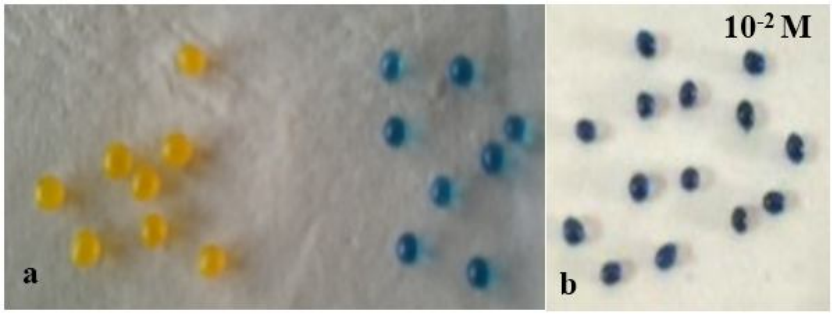


Fig 1(a): Comparison of color of calcium alginate beads with (Before and after the reaction**) (b):** Leukemic Blood Sample (10^{-1}-10^{-10} M) using different matrix

Response time for change in color of beads was 8 seconds for normal serum sample. The L-asparagine concentration level was 10^{-5} M. For leukemic serum samples, response time for change in color of beads till blue color appears was 9-11 seconds corresponding to 10^{-3}-10^{-2} M L-asparagine.

3.2 Agar Method

Detection limit of L-asparagine achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M asparagine, response time detected 12 seconds and for the concentration level of 10^{-10}-10^{-2} M L-asparagine response time detected was in the range of 6-11 seconds (Graph-1). With decrease in concentration of asparagine, the response time for color change decreased. Visual color change was observed (Figure-2).

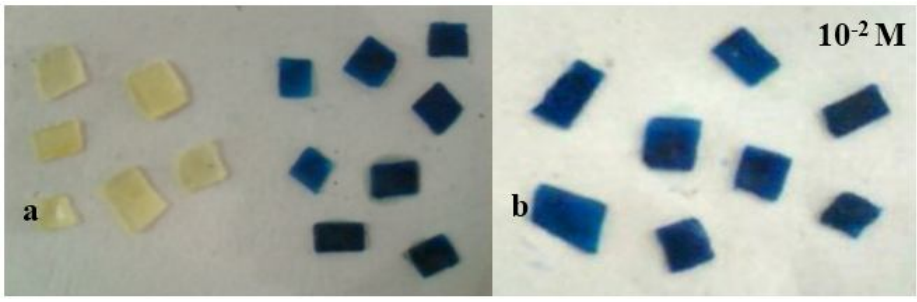


Fig 2(a): Comparison of color of Agar gel pieces with bromothymol blue (Before and after the reaction) (b): Leukemic Blood Sample

Response time for change in color of gel blocks was 9 seconds for normal serum sample. The L-asparagine concentration level was 10^{-5} M. For leukemic serum samples, response time for change in color was 10-12 seconds corresponding to 10^{-3}-10^{-2} M L-asparagine.

3.3 Agarose Method

For agarose method, visual color change was observed. The Figure-3 shows comparison of color of gel blocks before and after the reaction. Detection limit of L-asparagine achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M L-asparagine response time detected was 14 seconds and for the concentration level of 10^{-10}-10^{-2} M L-asparagine, response time detected was in the range of 7-13 seconds (Graph-1). The response time for change in color was found to be directly proportional to the asparagine concentration levels. Response time for change in color of gel pieces was 10 seconds for normal serum sample. The L-asparagine concentration level was 10^{-5} M. For leukemic serum samples, response time for change in color of gel pieces was 12-13 seconds corresponding to 10^{-3}-10^{-2} M L-asparagine.

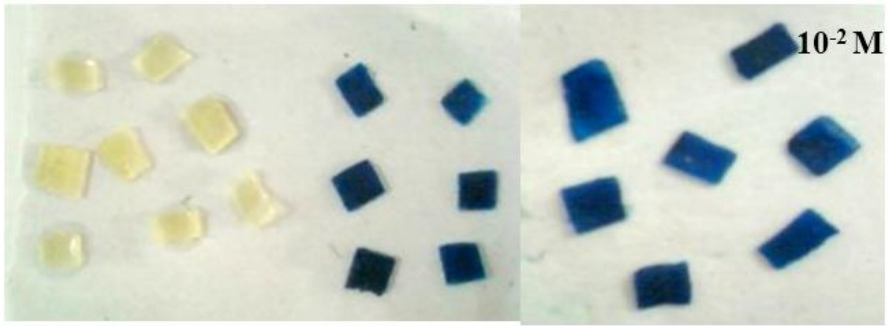


Figure 3(a): Comparison of color of agarose gel pieces with bromothymol blue (Before and after the reaction) **(b):** Leukemic Blood Sample

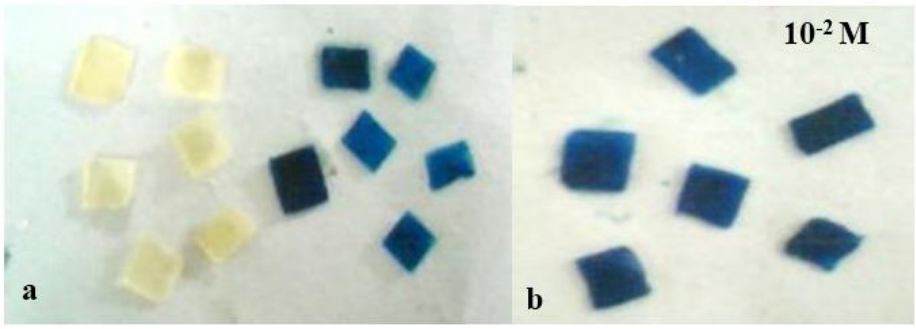


Figure 4(a): Comparison of color of Polyacrylamide gel pieces with bromothymol blue (Before and after the reaction) **(b):** Leukemic Blood Sample

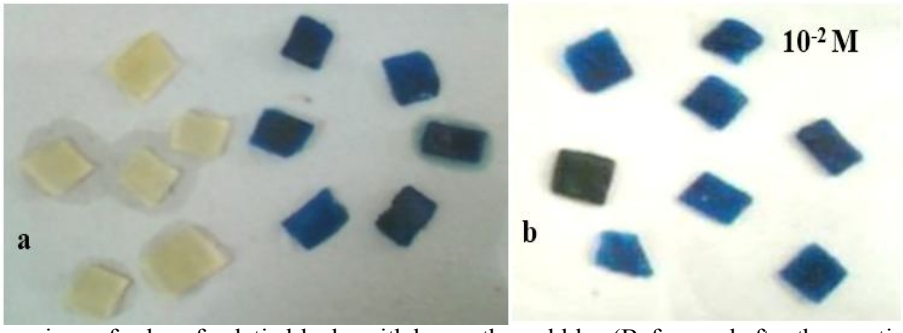


Figure 5(a): Comparison of color of gelatin blocks with bromothymol blue (Before and after the reaction) **(b):** Leukemic Blood Sample

3.4 Polyacrylamide Method

The Figure-4 shows the comparison of color of polyacrylamide gel pieces before and after the reaction. The same detection limit of 10^{-10} - 10^{-1}M was achieved. For L-asparagine concentration 10^{-1} M, the response time detected was 17 seconds. For the concentration range 10^{-10} - 10^{-2} M L-asparagine response time detected was in the range of 8-16 seconds (Graph-1). In this method also due to increased concentration of NH_4^+ ions produced after the reaction, the response time decreased with decrease in asparagine concentration.

Response time for change in color of gel blocks was 13 seconds for normal serum sample. The L-asparagine concentration level was 10^{-5} M. For leukemic serum samples, response time detected was 15-16 seconds corresponding to 10^{-3}-10^{-2} M L-asparagine

3.5 Gelatin Method

For gelatin method, visual color change was observed. The Figure-5 shows comparison of color of gel blocks before and after the reaction. Detection limit of asparagine achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M L-asparagine, response time detected was 21 seconds and for the concentration level of 10^{-10}-10^{-2} M L-asparagine, response time detected was in the range of 10-20 seconds (Graph-1). Response time decreased with decrease in concentration of asparagine indicating more of NH_4^+ ion produced after hydrolysis**.**

Therefore, these gel blocks have been used for subsequent experiments of optimization of the response time and monitoring asparagine levels in normal and leukemia serum samples. Response time for change in color of blocks was 16 seconds for normal serum sample. The L-asparagine concentration level was 10^{-5} M. For leukemic serum samples, response time for change in color of blocks was 19-21 seconds corresponding to 10^{-3}-10^{-2} M L-asparagine.

3.6 Storage Stability

Biocomponent was found to be active. Biocomponent in immobilized calcium alginate beads, agar cakes, agarose pieces, polyacrylamide pieces and gelatin gel blocks was found to be stable for long time i.e. more than 3 months, 1 month, 20 days, 25 days and 9 days. Thus, immobilization in calcium alginate beads was considered most effective and reliable method.

4. Discussion

The developed *Citrus lemon* based plant biosensor using various immobilization techniques was able to detect asparagine levels from 10^{-10}-10^{-1} M. Further, it was used for detection of asparagine levels in normal and leukemia blood serum samples. Earlier *E. coli* K-12 aparaginase based asparagine biosensor as reported by Verma *et al.* [34] were able to detect the concentration only upto 10^{-9} M, whereas in present study concentration up to 10^{-2} M was detected successfully, which will be very useful in detection of low level of asparagine in leukemic patients. Among all the five immobilization techniques, calcium alginate bead method was found to be the best with least response time i.e. 11 seconds. In comparison with *W. somnifera* based asparagine biosensor developed by Kumar *et al.* [28] the minimum response time for change in color of calcium alginate beads was 11 seconds whereas in case of *Citrus lemon* response time for change in color of calcium alginate beads was observed to be 9 seconds in leukemic serum samples. The present study also shows the comparison of indicators used in different immobilization techniques. The response time with bromothymol blue indicator was better to that of phenol red as indicator Kumar and Walia [35]. Thus, the developed plant based biosensor is novel, inexpensive, easy to use, consistent, very rapid and capable to diagnose asparagine at nano level

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6. References

1. Schemer G and Holcenberg JS. Enzyme as drugs. Wiley Inter Science, New York. 1981; 455-473.
2. Asselin BL. The three asparaginases. Comparative pharmacology and optimal use in childhood leukemia. Adv Exp Med Biol. 1999; 457: 62-629.
3. Andrulis L, Argonza R and Cairney AE. Molecular and genetic characterization of human cell lines resistant to L-asparaginase and albizziin. Somat Cell Mol Genet 1990; 16:59-65.
4. Miller HK, Salser JS and Balis ME. Amino acid levels following L-asparaginase aminohydrolase (EC.3.5.1.1.) therapy. Cancer Research. 1969; 29:183–187.
5. Lang S. Uber desamidierung im Tierkorper. Beitr chem Physiol Pathol. 1904; 5:321-345.
6. Furth O and Friedmann. Uber die Verbreitung asparaginspaltender

Organfermente. Biochem Z 1910; 26:435 - 440.
7. Kidd J. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. J Biol Chem 1953; 98:565.
8. Broome JD. Evidence that the L-asparaginase activity in guinea pig serum is responsible for its anti lymphoma effects. Nature (Lond.) 1961; 191:1114-1115.
9. Mashburn L, Wriston J. Tumour inhibitory effect of L-asparaginase from *Escherichia coli*. Arch. Biochem Biophy 1964; 104:450-452.
10. Peterson RE, Ciegler A. L-Asparaginase production by various bacteria. Appl Environ Microbiol 1969; 17:929–930.
11. Kafkewitz D, Goodman D. L-Asparaginase production by the rumen anaerobic *Vibrio succinogenes*. Appl Environ Microbiol 1974; 27:206–209.
12. Sarquis M, Oliveira E, Santos A and Costa G. Production of L-asparaginase by filamentous fungi. Mem Inst Oswaldo Cruz. 2004; 99:489–492.
13. Dejong PJ. L-Asparaginase production by *Streptomyces griseus*. Appl Environ Microbiol. 1972; 23:1163–1164.
14. Sieciechowicz KA, Joy KW and Ireland RJ. The metabolism of asparagine in plants. Phytochemistry 1988; 27:663–671.
15. Grover CE and Chibnall AC. The enzymic deamidation of asparagine in the higher plants. Biochem J. 1927; 21:857-868.
16. Lees EM, Blakeney AB. The distribution of asparaginase activity in legumes. Biochem Biophys Acta 1970; 215:145-151.
17. Bano M, Sivaramakrishnan VM. Preparation and properties of L-asparaginase from green chillies (*Capsicum annum* L). J Biosci 1980; 2:291-297.
18. Siechiechowicz K and Ireland R. Isolation and Properties of an Asparaginase from Leaves of *Pisum sativum*. Phytochem 1989; 28:2275.
19. Borek D, Michalska K, Brzezinski K, Kisiel A, Podkowinski J, Bonthron DT, Krowarsch D, Otlewski J and Jaskolski M. Expression, purification and catalytic activity of *Lupinus luteus* asparagines b-aminohydrolase and its *Escherichia coli* homolog Eur J biochem 2004; 271:3215-3226.
20. Bell TL and Adams MA. Ecophysiology of ectomycorrhizal fungi associated with Pinus spp. Plant Ecology. 2004; 171:35-52.
21. Cho CW, Lee HJ and Chung E. Molecular Characterization of the soybean L-asparaginase gene induced by low temperature stress. Molecules and cells. 2007; 23:280-286.
22. Ali EM. Purification and characterization of *Vigna unguiculata* cultivar asparaginase. J Biol Research 2009; 11:29 -36.
23. Ichi ES, Abdelghani A, Hadji I, Helali S, Limam F, Marzouki MN. A new peroxidase from garlic (*Allium sativum*) bulb: its use in H_2O_2 biosensing. Biotechnol Appl Biochem 2008; 51:33-41.
24. Rounaghi G, Kakhki RM. Preparation and electrochemical application of a new biosensor based on plant tissue/polypyrrole for determination of acetaminophen. Bull Mater Sci 2012; 35(5):811–816.
25. Fusco DM, Federico R, Boffi A, Macone A, Favero G, Mazzei F. Characterization and application of a diamine oxidase from *Lathyrus sativus* as component of an electrochemical biosensor for the determination of biogenic amines in wine and beer. Anal Bioanal Chem 2011; 401:707-716.
26. Chauhan N, Narang J, Shweta, Pundir CS. Immobilization of barley oxalate oxidase onto gold-nanoparticale-porous $CaCO_3$ microsphere hybrid for amperometric determination of oxalate in biological

materials. Clin Biochem 2012; 45:253-258.
27. Qiu B, Zhang YS, Lin YB, Lu YJ, Lin ZY, Wong KY and Chen GN. A novel liflower mosaic virus 35S promoter. Biosens Bioelectron. 2013; 41:168-171.
28. Kumar K, Katria M, Verma N. Plant asparaginase–based asparagine biosensor for leukemia. Artif Cells Blood Substit Biotechnol 2012; DOI:10.3109/10731199.2012.716062.
29. Johnsen A and Flink JM. Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells. Enz Microb Tech. 1986; 8:737-748.
30. Mahajan R, Gupta VK, Sharma J. Comparison and suitability of gel matrix for entrapping higher content of enzyme for commercial applications. Ind J Pharma Sci 2010; 72(2):223-228.
31. Prakash O, Puliga S and Upadhyay LSB. Immobilization of watermelon (*Citrus vulgaris*) urease in agarose gel for urea estimation. Biotech Biopro Engg 2007; 12:131- 135.
32. Skryabin GK and Koshcheenko KA. Immobilization of living microbial cells in polyacrylamide gel. Methods in Enzymology 1987; 198-216
33. Alteriis ED, Parascandola P, Salvadore S and Sardi V. Enzyme immobilization within insolubilized gelatin. J Chem Tech Biotechnol 1985; 35:60.
34. Verma N, Kumar K, Kaur G, Anand S. *E. coli* K-12 Asparaginase-Based Asparagine Biosensor for Leukemia. Artificial Cells, Blood Substitutes and Biotechnology, 2007; 35(4):449-456.
35. Kumar K and Walia S. L-Asparaginase Extracted From *Capsicum annum* L and Development of Asparagine Biosensor for Leukemia. Sensor and Transducer 2012; 144:192-200.