Characterization of bioassay principle biology essay

Science, Biology

Chapter IIIMaterials and MethodAll the chemicals used were of analytical grade, commercially available from Himedia, Sigma, Merck etc. otherwise mentioned. Withania somnifera plants were collected from different locations of Punjab and adjoining areas for the extraction of enzyme. Each experiment was run in triplicate.

3. 1Enzyme extraction (Scopes, 2004)

Enzyme was extracted from the fruits of Withania somnifera. Different extraction methods were applied to get a high amount of enzyme. These are as follows:

3. 1. 1Grinding with chilled buffer

10 g of fruits of Ashwagandha were washed with distilled water. Then grinded in chilled pestle and mortar by adding 20 ml chilled 0. 01 M Na-borate buffer (pH 8. 6). The grinded mixture was centrifuged at 8000 rpm for 10 mins at 4°C. Supernatant was again centrifuged at 8000 rpm for 10 mins at 4°C. This supernatant was used as crude enzyme.

3. 1. 2 With homogenizer

10 g of fruits of Ashwagandha were washed with distilled water to remove the impurities and homogenized with double volumes of 0. 01 M Na-borate Buffer (pH 8. 6), centrifuged and the supernatant was separated. This was designated as the crude enzyme. The residue was re-extracted with 0. 01 M Na-borate buffer (pH 8. 6). All the steps were carried out at 4°C.

3. 1. 3 Using liquid Nitrogen

10 g of fruits of Ashwagandha were washed with distilled water to remove the impurities and were taken out in a chilled pestle and liquid nitrogen was added to it. The solid thus formed was crushed with a mortar. The step was repeated again and the final pellet thus obtained was dissolved in 20 ml of 0. 01 M Na-borate buffer (pH 8. 5). The contents were centrifuged at 8000 rpm for 5 mins at 4°C. The supernatant was designated as crude enzyme.

3. 1. 4 with sea sand

Before using sea sand, it was sterilized with acid and base treatment. First, the sea sand was treated with strong HCl and washed with distilled water for several times. Secondly, it was treated with strong NaOH and again washed with distilled water for several times. The sand was air dried and then it was ready to use. 10 g of fruits of Ashwagandha were washed with distilled water to remove the impurities and taken out in a chilled pestle and 1 g chilled treated sea sand was added to it. This mixture was crushed with a mortar. The step was repeated again and the final pellet thus obtained was dissolved in 20 ml of 0. 01 M Na-borate buffer (pH 8. 5). The contents were centrifuged at 8000 rpm for 5 mins at 4°C. The supernatant was again centrifuged at 8000 rpm for 5 mins at 4°C. The final supernatant was designated as crude enzyme.

3. 2 Characterization of Bioassay principle

3. 2. 1 Estimation of ammonia by Nessler's reagent test (Riley, 1953)

Principle- Nessler's reagent is an alkaline solution of potassium mercuric iodide (K2HgI4). The reaction between Nessler's reagent and NH3 may be represented as: 2K2[HgI4] +NH3 +3KOH I-Hg-O-Hg-NH2 + 7KI +2H2OA known amount of sample was treated with Nessler's reagent which produces a yellowish brown colour. The intensity of the colour is directly proportional to the amount of ammonia originally present. The standard curve was constructed to determine the concentration of ammonia produced in the reaction.

Procedure

For the preparation of 10 ppm solution of ammonia, 0. 297 mg ammonium chloride was dissolved in 10 ml of deionised water. To 5 ml of different concentrations of ammonia (1 to 10 ppm), 0. 5 ml Nessler's reagent was added and incubated at 37oC for 10 mins. After that absorbance was taken at 480 nm for each concentration and a graph was plotted between concentration of ammonia and absorbance.

3. 2. 2Enzyme assay (Meister et al., 1956)

The enzyme activity of the crude enzyme was detected by adding the following reagents in the mentioned proportions. 1. 7 ml L-asparagine (10 mM), 0. 2 ml of 0. 05 M Tris – HCl (pH 7. 6), 20 µl of the enzyme and 980 µl of 0. 01M Sodium Borate buffer (pH 8. 6) were mixed and incubated at 37˚C for 10 mins. The reaction was stopped by adding 0. 1 ml of 1. 5 M TCA. Then it was centrifuged and to 2. 5 ml of the supernatant was added 2. 5 ml of deionized water. 0. 5 ml of Nessler's reagent was then added and incubated at 37˚C for 10 mins. Absorbance of the test sample versus the respected blank was taken at 480 nm. Determined the micromoles of ammonia released from an ammonium chloride standard curve. One Unit of enzyme is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute into product.

3. 3 Optimization of Fruit stage

The fruits of Ashwagandha at different ripening stages were collected. These stages were green (young fruits), red (half ripened) and dry (fully ripened). Then crude enzyme was extracted from each stage by optimized method (Sea sand method).

3. 4 Cytological studies (Singhal and Kumar, 2008)

Materials for cytological studies from the wild plants were collected from different locations of Punjab and adjoining areas; Jalandhar, Patiala, Hoshiarpur, Amritsar, Pathankot, Mohali, Chandigarh, Panchkula, Solan and Sunam (Table No. 3. 1). The voucher specimens are deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN). For meiotic chromosome counts, floral buds of suitable sizes were fixed in Carnoy's fixative (6 Alcohol: 3 Chloroform: 1 Acetic acid v/v) for 24 hours. After 24 hours, the fixed materials were transferred to 90% alcohol and kept in the refrigerator. Smears of pollen mother cells were made in 1% acetocarmine using the standard acetocarmine technique. Meiotic preparations were made permanent after grading through a mixture of acetic acid and alcohol and then through absolute alcohol before mounting in euparal. A number of freshly prepared and permanent slides were carefully examined to determine the chromosome number at different stages.

Table 3. 1: Difference location from Withania somnifera Plants

LocationAltitudeNo. of plantsMohali316 meters8Solan1580 meters7Sunam (Sangrur)231 meters8Amritser232 meters10Jalandhar228 meters15Patiala255 meters17Hoshiarpur296 meters9Panchkula365 meters6Total

80

3. 5 Comparison of cytotypes of Ashwagandha for L-asparaginase activity

Enzyme was extracted from different cytotypes of W. Somnifera with optimized extraction method and enzyme activity was calculated. Among these cytotypes, the one with maximum enzyme activity was used for further studies.

3. 6Immobilization of crude enzyme and seminquantitative approach of biosensing of asparagine.

3. 6. 1Gelatine method

Dissolve 1. 0 g gelatine in 10 ml of water to prepare a 10% aqueous solution. Heat the solution gently to facilitate the dissolution process. 20 µl of enzyme (0. 3 U) and 2 ml of hardening solution (20% Formaldehyde, 50% Ethanol, 30% Water) was added followed by 10 µl of phenol red indicator to the solution. Then the mixture was poured into a mold and allowed to freeze at -

20 ° C for 4 hours to facilitate the gel formation. When the gel was set, it was raised to room temperature. Then the gel was cut into small blocks of approximately 3 mm per side (Alteriis et al. 1985). 12 blocks were prepared from gel. These blocks were put into 0. 1M L-asparagine and the response time was noted for change in colour of blocks from partly orange to dark purple.

3. 6. 2Agarose method

Agarose solution (1. 5%) was prepared in 25 mM Tris- acetate buffer (pH 7. 2) containing 2 mM CaCl2 by heating for 10 minutes. Then 20 µl enzyme (0. 3 U)/10 ml above solution were added followed by 10 µ l phenol red indicator. The mixture was poured into petriplate and allowed to solidify. The solidified gel was cut into small pieces of 1. 0 X1. 0 cm (Prakash et al. 2007). 15 pieces of agarose were prepared. The gel pieces were put into 0. 1M L-asparagine and the response time was noted for change in colour of small pieces from partly orange to dark purple.

3. 6. 3Agar method

A solution of 4% agar was prepared. Boiled and allowed to cool at 45 – 50°C. 20 µl enzyme (0. 3 U) and 10 µl phenol red indicator was added to the solution. It was then mixed thoroughly and poured into petriplate and allowed to solidify. The gel was then cut into square cakes of 1. 0X1. 0 cm with the help of knife or spatula (Mahajan et al., 2010). 10 square cakes were prepaed from agar. Then the cakes were put into 0. 1M L-asparagine and the response time was noted for change in colour of cakes from partly orange to dark purple.

3. 6. 4Calcium alginate beads

10ml Slurry of 3% sodium alginate was prepared. To this slurry, 20 µl of the enzyme solution (0. 3 U) and 10 µl of phenol red indicator were added. This solution was then poured drop wise through a glass syringe into a beaker containing 0. 075 M chilled CaCl2 with gentle stirring on a magnetic stirrer. Orange coloured beads (partly orange colour of the beads was due to phenol red indicator) were made with the help of 2. 5 ml syringe without needle (Johnsen and Flink, 1986). Approximately 40 beads were prepared from this slurry. The beads were hardened by placing for half an hour at room temperature. These all beads were then put into 0. 1M L-asparagine solution. The response time for change in colour of beads from partly orange to bright purple was noted.

3. 6. 5 Immobilization of enzyme with TEOS hydrosol gel-chitosan based technique.

Sol-gel was solidified by chitosan. This method was based on modification of Alqasaimeh et al (2007) method. 7. 5ml TEOS and 0. 2ml 0. 1M HCl were added in a closed vessel and the final volume was made to 10ml with distilled water. After that, 0. 1 ml of 1% chitosan solution was added. Vessel was closed tightly and placed on magnetic stirrer for 2 to 3 hours or till the solution became clear. This solution was stored at 4°C for further use. 200 µl of the above solution was poured into a 3ml glass vessel. The vessel was wrapped with parafilm to make it air tight and placed at room temperature for 24 hours. Then 50µl enzyme (0. 63 IU), 45µl sol-gel solution and 5µl of phenol red indicator were mixed together and layered on the solidified sol-gel solution. Again it was wrapped with parafilm and kept at room temperature for another period of 12 hours after which it was ready for detection. The concentration of phenol red used was 4mg per 4ml of 1: 1 ratio of distilled water and alcohol. Different concentrations of asparagine (10-1 to 10-10 M) were prepared in 50mM Tris HCl (pH 7. 6). 100 µl of each concentration was added to each vessel in which enzyme was immobilized and the response time of colour change from light orange to pink was noted.

3. 7Development of Colorimetric biosensor for asparagine.

3. 7. 1 Immobilization of enzyme with TEOS hydrosol gel-chitosan based technique.

Sol-gel was solidified by chitosan. This method was based on modification of Alqasaimeh et al (2007) method. 7. 5ml TEOS and 0. 2ml 0. 1M HCl were added in a closed vessel and the final volume was made to 10ml with distilled water. After that, 0. 1 ml of 1% chitosan solution was added. Vessel was closed tightly and placed on magnetic stirrer for 2 to 3 hours or till the solution became clear. This solution was stored at 4°C for further use. 200 µl of the above solution was poured into a 3ml glass vessel. The vessel was wrapped with parafilm to make it air tight and placed at room temperature for 24 hours. Then 50µl enzyme (0. 63 IU), 45µl sol-gel solution and 5µl of phenol red indicator were mixed together and layered on the solidified sol-gel solution. Again it was wrapped with parafilm and kept at room temperature for another period of 12 hours after which it was ready for detection. The concentration of phenol red used was 4mg per 4ml of 1: 1 ratio of distilled water and alcohol. Different concentrations of asparagine (10-1 to 10-10 M) were prepared in 50mM Tris HCl (pH 7. 6). 100 µl of each concentration was added to each vessel in which enzyme was immobilized and the response time of colour change from light orange to pink was noted. C: UsersuserAppDataLocalMicrosoftWindowsTemporary Internet FilesContent. Word2012-06-15 17. 46. 32. jpg

Fig 3. 1: Immobilization of enzyme with TEOS hydrosol gel-chitosan based technique.

3. 7. 2Application of developed biosensor on fruit juices

To the above immobilized enzyme 100 µl of different fruit juices were added and response time was observed. Response times were compared with standard to get the concentration of asparagine in each juice.

3. 8 Estimation of protein.

The protein was quantified by method of Lowry et al. (1951). PrincipleThe phenolic group of tyrosine residue in a protein produces a blue colour with Folin-Ciocalteau reagent which consists of tungstate, molybdate and phosphate ions. Reagents: The following reagents were prepared. Solution A: 2. 0% Sodium carbonate in 0. 1 N sodium hydroxide. Solution B: 0. 5% Copper sulphate in 1. 0% sodium potassium tartarate. Solution C: Mixing 50 mL of solution A and 1 mL of solution ‘ B’ (Prepared fresh). Solution D: Folin-Ciocalteau’s phenol reagent and distilled water in 1: 1 ratio (Prepared fresh). Procedure: 5 mL of solution C was added to 1 mL of properly diluted sample. It was mixed and allowed to stand for 10 mins at room temperature. Subsequently 0. 5 mL of solution D was added and further kept at room temperature for 25 mins. Optical density of the resultant solution was measured at 660 nm using spectrophotometer. Bovine serum albumin was used as standard.

3. 9Purification of L-asparaginase (Scopes, 2004)

3. 9. 1Ammonium sulphate precipitation for the purification of L-asparaginase

3. 9. 1. 1Principle: Ammonium sulphate precipitation is a method used to purify proteins by altering their solubility. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i. e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein.

3. 9. 1. 2Procedure

The crude enzyme was mixed with different amounts of solid ammonium sulphate to get 20% saturation and then successively raised to 100% saturation. The amount of ammonium sulphate was calculated for each % saturation according to the below mentioned equation. All the steps were carried out at 4°C under mild stirring conditions. After this, the sample was kept for 12 hours at 4°C without stirring. When the precipitation occurred, the sample was centrifuged for 20 minutes (5000 rpm at 4°C) and pellet was dissolved in phosphate buffer saline. Enzyme activity and protein content of the dissolved pellet and supernatant was quantified after the dialysis. The fraction with 40-60 % saturation was observed to have maximum specific enzyme activity.

3. 9. 2Dialysis

3. 9. 2. 1Principle

Dialysis is a process in which small, unwanted molecules are separated from macromolecules in solution by a semi-permeable membrane. Dialysis is based on diffusion, a process in which molecules randomly move in solution from areas of higher to lower concentration until equilibrium is reached. The sample is fixed in dialysis membrane tube and placed in 200 times buffer solution. Sample molecules are larger than the membrane pores so that they are retained in the tube but smaller molecules and buffer salts may pass freely through the membrane, as a result of which the concentration of small molecules in the sample is reduced.

3. 9. 2. 2Activation of dialysis membrane

Dialysis membrane was washed with deionised water. Boiled for 1 hour in 100mM $NaHCO_3$, 10mM EDTA-Na salt (pH 7. 0) solution with mild agitation. After boiling, the tube was washed with deionised water several times.

3. 9. 2. 3Procedure

One end of the tube was tightly fixed with tag. 2 ml of each fraction of ammonium sulphate precipitation was poured in tube through the open end and this end was tightly fixed with tag. Tightly fixed tube was suspended in

200 ml 0. 01M Na Borate buffer for six hours on mild stirring at 4°C. After six hours, buffer was replaced with fresh 200 ml 0. 01M Na Borate buffer and kept for six hours on mild stirring at 4°C. The sample was removed from the tube, poured into vials and stored at 4°C.

3. 9. 3Gel permeation chromatography for the purification of L-asparaginase

3. 9. 3. 1Principle: Gel Permeation Chromatography separates molecules on the basis of their size. This differs from other separation techniques which depend upon chemical or physical interactions to separate molecules. The molecules can be separated on the basis of difference in their size by passing them through column packed with gel. The smaller analytes can enter the pores more easily and therefore spend more time in these pores, increasing their retention time. Conversely, larger analytes spend little time in the pores and are eluted quickly. The gel consists of an open cross linked three dimensional molecular network cast in bead form for easy column packing and optimum flow characteristics. The pores within the beads are of such size that some are not accessible by large molecules, but smaller molecules can penetrate through them (Plummer, 1988).

3. 9. 3. 2Procedure

The ammonium sulphate fraction with maximum specific activity (40-60%) was applied to Sephadex G-75 (Pharmacia, Uppsala, Sweden) column, pre-equilibrated with 0. 01M Sodium Borate buffer (pH 8. 5) at 4oC. Dimensions of the column were 1. 2 X 9. 2 cm. The sample was eluted with 0. 01M Sodium Borate buffer (pH 8. 5) at a constant flow rate of 1 ml/min and fractions of 1. 5 ml were collected. The fractions were assayed for protein concentration and enzyme activity. Fractions having higher specific activity were pooled and used for ion exchange chromatography.

3. 9. 4Ion exchange chromatography

Chromatography is the most commonly and widely used means of purifying proteins and separating small molecules. Ion exchange chromatography requires that a protein contains a net ion charge under experimental conditions. As a result, the protein will displace a lower molecular weight ion from an ion exchange matrix and become bound. Separation of proteins by ion exchange chromatography requires differential binding of proteins to ion exchange matrix by electrostatic forces. After proteins are applied to an ion exchanger, those proteins which have no affinity for the matrix are removed during washing of the column. Then, the adsorbed proteins are removed in an elution step by raising counter ion (salt) concentration. In stepwise elution, salt concentration is increased in distinct steps. A gradient elution utilizes a gradient maker to establish a smooth (continuous) increase in salt concentration. Ion exchange separation is carried out mainly in column packed with an ion exchanger. There are basically two types of ion exchangers: Cationic exchanger: posses negatively charged groups. These will attract positively charged cations. e. g. SP- Sepharose. Anionic exchanger: Strong Anion exchangers contain strongly ionized groups, such as -NR3. The strong ion exchange resins are completely ionized and exist in the charged form except at extreme pH values e. g. Q-Sepharose.—NR3OH

—+NR3 + OH-Q-Sepharose was used as a purification support as a strong anion exchanger. C2H5QAE —CH2CH2— +N—CH2—CH(OH) —CH3C2H5

Figure 3. 2: Functional group of quaternary aminoethyl strong ion exchanger

3. 9. 3. 2 Procedure

Q- Sepharose fast flow supplied by Pharmacia is a strong anion exchanger. Strong exchangers provide equal levels of separation with greater reproducibility. Suspension of Q- Sepharose dissolved in a buffer was poured gently in column (1. 2 x 8. 2 cm) through the sides avoiding bubble formation. The Q- Sepharose was allowed to settle to make a bed and then the column was washed with 0. 01M sodium borate buffer (pH 8. 6) for equilibration. The sample from gel filtration step was loaded onto the column at a flow rate of 1 ml min-1. The column was washed with 0. 01M sodium borate buffer (pH 8. 6) to remove the unbound proteins. The elution was carried out by increasing salt gradient (NaCl, 0. 1M-0. 6 M). The fractions (1. 5 ml) were collected at flow rate of 1 ml/min. The fractions were evaluated for enzyme activity and protein concentration. The fractions possessing maximum asparaginase activity were pooled for further analysis.

3. 9. 4 PAGE and SDS-PAGE (Laemmli, 1970)

Electrophoresis is the study of movement of charged molecules in an electric field with help of supporting medium like gel made up of polyacrylamide for proteins. In electrophoresis techniques, the molecules move according to their charge and size. If the biological samples are treated so that they have a uniform charge, electrophoretic mobility then depends primarily on size. In

SDS PAGE, the sample is treated with detergent sodium dodecyl sulfate (SDS) and a reducing agent mercaptoethanol (b ME). SDS disrupts the secondary, tertiary and quaternary structure of protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. 1. 4grams of SDS binds per gram of protein. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds. Separating (4x) gel buffer: Tris-HCl (18. 3 g) was dissolved in 100 ml of deionised water and pH was adjusted to 8. 8 with 1N HCl. Stacking (4x) gel buffer: Tris-HCl (6. 055 g) was dissolved in 100 ml of deionised water and pH was adjusted to 6. 8 with 1N HCl. Acrylamide-bisacrylamide (30%): Acrylamide (29. 2 g) and bisacrylamide (0. 8 g) was dissolved in 100 ml of deionised water. Sample buffer: The sample buffer was prepared by mixing the following components. Tris HCl buffer (pH 6. 8): 0. 4 mlSDS (10%) : 2. 5 ml2-Mercaptoethanol: 0. 4 mlGlycerol : 2. 0 mlBromophenol blue : 0. 002 gDeionised water : 4. 7 mlFor PAGE, 2-mercaptoethanol was not added in the sample bufferRunning buffer: The electrode buffer was prepared by mixing the following components. Tris HCl : 6. 05 gGlycine : 28. 8 gSDS : 2. 0 gDeioised water: 2. 0 LpH : 8. 3Separating gel (10%): The solution for separating gel was prepared as under. MilliQ-water: 17. 2 mlAcrylamide (29. 2%) : 13. 3 mlbisacrylamide (0. 8%)4x Separating gel buffer: 10 mlSDS (10%) : 0. 8 mlGlycerol (10%) : 1. 75 mlTEMED : 20 µlAmmonium persulphate (2%): 0. 6mlStacking gel (3%): The solution for stacking gel was prepared as under. Deionised water: 6. 3 mlBisacrylamide (30%): 2. 0 ml4x Stacking gel buffer : 2. 5 mlSDS (10%) : 0. 2 mlGlycerol (10%): 0. 30 mlTEMED : 10 µlAmmonium persulphate (2%): 0. 13ml3. 9. 4. 1Procedure: Loading sample was prepared by mixing the protein sample with sample buffer in equal ratios and for SDS-PAGE it was heated in a boiling water bath for 2-3 mins but in case of PAGE sample was not heated. The gels were run at 100 V and stained with silver staining method.

3. 9. 4. 2Silver Staining Method

Silver staining Method (Oakley et al., 1980) is very useful as it has 100-fold greater sensitivity over dye, up to nanogram levels.

3. 9. 4. 2. 1Principle:

Silver staining is the most sensitive colorimetric method for detecting protein in gel. Silver nitrate is present in +1 oxidation state, which is soluble and white in colour. In the presence of formaldehyde, Ag+ is reduced to metallic silver at pH-12, which is an insoluble black form and visible after the development is complete. Silver ions interact and bind with certain functional groups of protein. Ag+ ions make strong interactions with carboxylic acid groups of Asp and Glu, imidazole of His, sulfhydryls of Cys, and amines of Lys. Various sensitizer and enhancer reagents are used to enhance the specificity and efficiency of silver-ion binding to proteins and reduction of silver to metallic silver.

Materials:

Composition of silver staining solutions are given in Table 3. 2

Table 3. 2: Composition of silver staining solutions.

Solutions

Components

Amount

Fixing solution

Glutaraldehyde (25%)200 µlFormaldehyde (37%)10 µlEthanol400 mlWater600 ml

Washing solution

Ethanol400 mlDistilled Water600 ml

Silver nitrate solution

Silver Nitrate0. 1 gDistilled Water100 ml

Sensitizing solution

Sodium thiosulphate40 gDistilled Water200 ml

Developer solution

Sodium Carbonate5 gFormaldehyde40 µlDistilled Water400 ml

Stopping solution

Acetic Acid5 mlDistilled Water95 ml

Procedure

Gel was removed from cassette and placed in 200 ml of fixing solution for 10 minutes to fix the protein, and for removal of extra detergent and ions. Rinsed in 200 ml of 40% Ethanol solution for 20 minutes to remove remaining ions and detergent. Placed the gel in 100 ml of sensitizing reagent for 2 minutes. Rinsed thrice with 200 ml of distilled water for one minute each. Placed in 100 ml of 0. 1 % Silver nitrate solution for five minutes. Rinsed with 200 ml of distilled water for one minute. Placed the gel in 100 ml of developing solution for one minute, poured off immediately and replaced with 100 ml fresh developing solution. Reaction was stopped in 5 % acetic acid for 5 minutes. Wet gels were stored in 0. 03 % sodium carbonate.

3. 10 Kinetic characteristics of L-Asparaginase:

Kinetics of enzyme is generally done in order to study the mechanism for determining the rate of reaction and how it changes in response to change in the experimental parameters like pH, temperature, and substrate concentration (Nelson et al., 2007).

3. 10. 1 Effect of Temperature on activity of purified enzyme:

Each enzyme has a temperature range in which it shows maximum activity to increase the rate of biological reactions. Above the limited range of increase in the temperature, the enzymatic activity is reduced due to denaturation of protein structure. Therefore, temperature optimization was done in order to determine the optimum temperature at which the enzyme shows maximum activity. Enzymatic activity of asparaginase was studied at different temperatures i. e 25°C, 30°C, 32°C, 35°C, 37°C, 40°C and 45°C.

3. 10. 2 Effect of pH on activity of purified enzyme:

pH plays a vital role in the enzymatic activity. Generally the enzymes are active in a limited pH range. Change in pH of enzyme results in decrease in its activity due to ionization and deionization of the acid or basic group involved in the active site of the enzyme molecule (Frankenberger et al. 2002). Enzymatic activity of asparaginase was studied at different pH i. e. 6, 6. 5, 7, 7. 5, 8, 8. 5, and 9.

3. 10. 3 Km and Vmax of purified enzyme

The rate of enzymatic reactions increases with the increasing substrate concentration for a given amount of enzyme. At a certain point, any further increase in substrate concentration does not increase the reaction rate. This is because at higher concentration the active sites of the enzyme molecules are saturated with the substrate. Different concentrations of substrate L-asparagine was used (1-10mM). A Lineweaver-Burk plot was plotted between reciprocal of enzyme activity 1/(V) Vs reciprocal of substrate concentration 1/(S) and from the plot, values of Km and Vmax were calculated. Km value is half of the substrate concentration at which enzyme shows its maximum activity.

3. 11Immobilization of Purified enzyme.

The purified enzyme was immobilized by hydrosol-gel techniques. Tetraethyl orthosilicate (TEOS) was used for hydrosol-gel and it was solidified with various biopolymers i. e. chitosan, dextran, gelatine, agar, agarose and acacia gum. TEOS on acid or base hydrolysis form hydrosol-gel. The solidification of sol-gel was based on modification of Alqasaimeh et al. (2007) method.

Figure 3. 3: Hydrolysis of TEOS

3. 11. 1 TEOS with Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-linked D-glucosamine and N-acetyl-D-glucosamine.(Figure 3. 4)C: UsersuserDesktopchitosan. gif

Figure 3. 4: Structure of Chitosan

Solutions4. 5 mL TEOS0. 1 mL 0. 01 M HCl1. 4 mL 0. 5 % Chitosan SolutionAll above solutions were mixed in air tight vial and kept on vigorous string at room temperature for 2 to 3 hours or till solution became clear. This solution was stored at 4°C for further use. 600 µL TEOS-chitosan solution was thoroughly mixed with 200 µL 0. 01M sodium borate buffer. 60 µL of the above solution was thoroughly mixed with 20 µL of purified L-asparaginase (5. 6 IU) and poured in an air tight container, sealed and placed at room temp. for 24 hours for solidification (Figure 3. 5)C: UsersuserAppDataLocalMicrosoftWindowsTemporary Internet FilesContent. Word2012-03-15 17. 57. 28. jpg

Figure 3. 5: Preparation of TEOS-Chitosan Discs

3. 11. 2 TEOS with Xanthan gum

Xanthan gum is a polysaccharide used in thickening of foods. It consists of linear (1-4) linkage of β-D- glucose and has three sugar side chains on alternate glucose molecules (Figure 3. 6). C: UsersuserDesktopXanthan. jpg

Fig 3. 6: Structure of Xanthan gum

Solutions4. 5 mL TEOS0. 1 mL 0. 01 M HCl1. 4 mL 0. 3 % Xanthan Gum SolutionAll above solutions were mixed in an air tight vial and kept on vigorous stirring at room temperature for 2 to 3 hours or till solution became clear. This solution was stored at 4°C for further use. 600 µL TEOS-xanthan gum solution was thoroughly mixed with 200 µL 0. 01M sodium borate buffer. 60 µL of above solution was thoroughly mixed with 20 µL enzyme (5. 6 IU) and poured in air tight container, sealed tightly and placed at room temp for 24 hours for solidification.

3. 11. 3 TEOS with Gelatin

Gelatine is a polypeptide, hydrolysed form of collagen. It is used in the manufacture of candiesSolutions4. 5 mL TEOS0. 1 mL 0. 01 M HCl1. 4 mL 1 % Gelatin SolutionAll above solutions were mixed in air tight vial and kept on vigorous stirring at room temperature for 2 to 3 hours or till solution became clear. This solution was stored at 4°C for further use. 600 µL TEOS-gelatine solution was thoroughly mixed with 200 µL 0. 01M sodium borate buffer. 60 µL of the above solution was thoroughly mixed with 20 µL enzyme (5. 6 IU) and poured in air tight container, sealed tightly and placed at room temp for 24 hours for solidification.

3. 11. 4 TEOS with Agar

Agar is a polysaccharide of a disaccharide composed of D-galactose and 3, 6-anhydro-L-galactose (Figure 3. 7). Agar is used as a medium for culturing bacteria and cellular tissues. It is also used in the preparation of some desserts in Japan and other Asian countries. The gels produced with agar have a crispier texture than the desserts made with animal gelatine.

Agarobiose

Figure 3. 7: Structure of Agar

Solutions4. 5 mL TEOS0. 1 mL 0. 01 M HCl1. 4 mL 1. 5 % agar SolutionAll above solutions were mixed in air tight vial and kept on vigorous string at room temperature for 2 to 3 hours or till solution became clear. This solution was stored at 4°C for further use. 600 µL TEOS-agar solution was thoroughly mixed with 200 µL 0. 01M sodium borate buffer. 60 µL of above solution was thoroughly mixed with 20 µL enzyme (5. 6 IU) and poured in air tight container, sealed tightly and placed at room temp for 24 hours for solidification.

3. 11. 5 TEOS with Acacia gum

It is a complex polysaccharide contains sugar monomers galactose, arabinose, rhamnose and glucoronic acid in a ratio of 3: 3: 1: 1.

Figure 3. 8: Structure of Acacia gum

Solutions4. 5 mL TEOS0. 1 mL 0. 01 M HCl1. 4 mL 1 % Acacia gum SolutionAll above solutions were mixed in air tight vial and kept on vigorous string at room temperature for 2 to 3 hours or till solution became clear. This solution was stored at 4°C for further use. 600 µL TEOS-acacia gum solution was thoroughly mixed with 200 µL 0. 01M sodium borate buffer. 60 µL of above solution was thoroughly mixed with 20 µL enzyme (5. 6 IU) and poured in air tight container, sealed tightly and placed at room temp for 24 hours for solidification.

3. 12Development of Ion Selective Electrode (ISE) based Potentiometric Biosensor

3. 12. 1Ion Selective Electrode

An ion selective electrode generates a difference in electrical potential between itself and a reference electrode. The output potential is proportional to the amount or concentration of the selected ion in solution. The measured electrode potential, E, is related to activity of an ion species by Nernst Equation. $E = E0 + 2.3\ RT / nF \log ACTIVITY$Where, E0 = a constant for a given cellR = the gas constantT = the Temperature in Kelvinn = the ionic chargeF = the Faraday constantAnd the expression RT / nF is termed the Slope Factor. For example, when measuring Ammonium ions, (i. e. n = +1), the slope factor at 25°C has a value of 56 ± 2 mV. This is termed the Ideal Slope Factor, and means that for each tenfold change in ammonium concentration, an ideal measuring system will sense a mV change of 56 ± 2 mV. The measurement of slope factor gives an indication of the performance of the electrode system. Ionic strength is an important parameter in ion measurement; it expresses the concentration of all ions present in the solution both as concentration, molarities and charge. Ion Strength Adjuster (ISA), also called electrolyte is needed in order to give the sample and the standard solution a high and constant ionic strength. It does not interfere with the measurement but compensate for the differences between the activity and concentration. Contrary to colorimetric or spectrophotometric techniques for elemental analysis, the measurement through ISE is uninterrupted by colour, turbidity or particle in suspension in the sample.

3. 12. 1. 1Calibration of NH_4^+ISE:

Calibration of ISE was done by firstly activating ISE in 10 ppm solution of NH_4^+ overnight. Then the ISE tip was washed with deionised water and again kept in 10 ppm NH_4^+ for 10 mins and change in potential was recorded until a stable reading. The same process was repeated for 10 fold higher concentrations and difference in the potential of two concentrations was calculated. According to the standard method, the difference, also called the slope of the electrode should be 56 ± 2 mV. The mentioned difference was obtained for proper calibration of electrode and then it was used for further work.

3. 12. 2Potentiometric Biosensor

L-asparaginase hydrolyses the L-asparagine into aspartate and ammonia. This ammonia is detected with ISE by change in potential.

3. 12. 2. 1Immobilization of biocomponent

Purified L-asparaginase was immobilized in TEOS-Chitosan disc as described earlier.

3. 12. 2. 2Response time

The potentiometric biosensor was developed by bringing the ISE in close proximity of the biocomponent in the form of enzyme immobilized on TEOS-chitosan matrix (hydrosol gel immobilization method as mentioned above). The ISE containing the matrix in the tip cover was dipped in 5 ml L-asparagine (100 mM) solution and the change in potential after every minute was recorded. The time at which the potential became stable was the response time of reaction.

3. 12. 2. 3Construction of L-Asparagine Standard Reference Chart using ISE:

10-10 to 10-1 M L-Asparagine solutions were prepared from the 10-1 M stock solution and pH of each solution was set to be 7. 6. 5 ml of each concentration of L-Asparagine solution was placed in a glass vial on a magnetic stirrer and initial reading of each solution was monitored with ISE. Purified asparaginase enzyme was immobilized in TEOS-chitosan matrix (hydrosol gel immobilization method as mentioned above). The ISE containing the matrix in the tip cover was dipped in 5 ml L-asparagine solution and initial reading of each solution was monitored. The change in potential (Δ mV) after 10 minutes was recorded for each dilution. Δ mV values were calculated after subtracting the final mV values from initial mV values for all concentrations. A graph of Δ mV reading of L-asparagine solution was plotted against the log of concentration.

3. 12. 3Application of the Developed Biosensor

3. 12. 3. 1Analysis of different fruit juices:

Grape juice, Pineapple juice, Orange juice, Guava juice, Apple juice and Lemon Juice were purchased from the market. Then the Enzyme TEOS-chitosan matrix was fixed in ISE tip cover dipped in the sample (5 ml) and initial reading was noted and after 10 minutes final mV reading of the juice sample was noted. The change in mV reading was noted down and correlated with the ΔmV reading of the L-asparagine standard reference chart and L-asparagine levels were calculated. 3. 12. 3. 2Testing of normal and leukemic blood samples: The work was carried out as per the guidelines of the Ethical and Biosafety Committee (ICEC/11/2011). Leukemia serum samples were collected from Max multispecialty hospital, Mohali, with prior information and consent of patients and administration. These were then analyzed for the L-asparagine concentration. Total five leukemia serum samples and two normal serum samples were analyzed. Purified enzyme based biosensor was used for the quantitative detection of asparagine levels. Serum samples were brought to normal room temperature. Then the Enzyme TEOS-chitosan matrix was fixed in ISE tip cover and dipped in normal serum sample. Initial mV value was noted and final mV was noted after response time (10 min). ΔmV was determined and serum asparagine concentration was determined from asparagine standard reference chart. Same procedure was repeated for each leukemia serum sample and normal serum samples and asparagine levels were evaluated. Table 3. 3: Details of Leukemia PatientsNameSexAgeTypeMunishmale11ALLPushpinder Kaurfemale37ALLSukhdevmale50CMLKritifemale29ALLNavtejmale15CLLTable 3. 4: Details of Normal Serum samplesNameSexAgeTypeAtulmale24normalMukulMale23normal

3. 12. 4 Reliability check of the developed Biosensor

To check the reliability of ISE and its use for quantitative estimation of L-asparagine in food sample, 2. 5 ml of sample (already analyzed by developed biosensor) was spiked with 2. 5 ml of same L-asparagine concentration solution. The Δ mV was studied for samples and L-asparagine concentration was calculated from the standard reference chart. It was compared with the values obtained previously by the biosensor for the samples analyzed.

3. 12. 5 Storage Stability of Biocomponent

To know the storage stability of the biocomponent, the TEOS-chitosan matrices were kept in 0. 1M sodium borate buffer on 4°C. The activities of the immobilized biocomponent were checked at various time intervals.

3. 13 Development of Fluorescence based Fibre Optic Biosensor

3. 13. 1Fluorescence Spectroscopy: Fluorescence is a spectrochemical method of analysis where the molecules of the analyte are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The light from the excitation source passes through a filter and strikes the sample. Some amount of light is absorbed by the sample as explained by Lembert beer's law i. e. when a beam of monochromatic radiation passes through any solution, the intensity of the beam reduces to some amount. Absorbance (A)= log Io/I= εclThe fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter and reaches a detector, which, for a fluorescence measurement is placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.

3. 13. 1. 1Principle of measurement:

The principle of fluorescence based Asparagine biosensor is the breakdown of L-asparagine into aspartate and ammonia by the action of L-asparaginase.

Production of ammonia whose detection as ammonium ions is done by the protonated pH sensitive indicator (Rhodamine 6G) which changes its fluorescence spectrum upon deprotonation. Upon exposure to ammonia, the fluorescence of the immobilized fluorophore based sol-gel matrix system decreases (due to formation of colourless non-fluorescent lactone), consistent with the dye becoming deprotonated with the formation of ammonium ions in the sol-gel matrix. Thus, the flourophore reacts quantitatively with ammonia resulting in change in fluorescence allowing the calibration of fluorescence as a function of pH alteration due to the production of ammonia. 3. 13. 1. 2Indicator Fluorescent Dye: For fluorescence asparagine biosensor, Rhodamine 6G was used as the indicator dye. Rhodamine dyes are used frequently in biotechnology applications such as fluorescence microscopy, flow cytometry and ELISA. The dye Rhodamine 6G has great photo stability, high quantum yield and low cost. The dye is a protonated pH sensitive indicator (INDH+) which changes its structure to form a colourless non-fluorescent lactone upon deprotonation when exposed to ammonia.

INDH+ + NH3 IND + NH4+

The chemical structure of Rhodamine 6G is shown as below.

Figure 3. 8: Chemical structure of Rhodamine 6G (http://en. wikipedia. org/wiki/Rhodamine_6G)

3. 13. 1. 3Fluorescence Spectra of Dye: The fiber-optic fluorescence cable of the lamp is introduced and placed at right angles to that of the detector. One end of the fiber-optic fluorescence cable is connected to the detector and the

other end is at right angle to the lamp. The spectrometer was turned to Relative Irradiance mode. 2% Rhodamine 6G prepared in 1: 1 ratio of water and ethanol was taken in a cuvette and analyzed for its emission spectrum. A fluorescence maximum λmax of 600. 03 nm was found. The spectra were recorded in the spectrometer. All further readings for fluorescence experiments in terms of intensity counts were taken at this wavelength. 3. 13. 1. 4Immobilization of the biocomponent: Biocomponent was immobilized in TEOS-chitosan matrix. 70 µL TEOS-chitosan solution was poured in air tight container and sealed tightly. It was then placed at room temperature for 24 hours. Then 5µL of purified enzyme (1. 4 IU), 4µL TEOS-chitosan solution and 1µL of Rhodamine 6G (2%) were mixed thoroughly and poured onto the same container. Again it was sealed tightly and kept for another 12 hours at room temperature for solidification. After that small disc thus formed was removed from the container for analysis of asparagine or kept at 4oC for further use. The size of the biocomponent disc was apt for the tip of optic fiber. 3. 13. 1. 5Optimization of response time: For fluorescence measurements, TEOS-chitosan disc was kept in the bottom mirror position and 40µL of asparagine solution (10-1 M) was poured onto the disc. The reaction was continuously monitored for 10 minutes and intensity counts were noted down at intervals of 1 minute. A response time of 6 minutes was found to be optimum for the enzymatic reaction to be completed.

3. 13. 2Construction of L-Asparagine Standard Reference Chart using fiber optic spectroflourimeter:

10-10 to 10-1 M L-Asparagine solutions were prepared from the 10-1 M stock solution and. The TEOS-chitosan matrix having enzyme was kept in the bottom mirror position of the tip of the fiber-optic cable supplied with the instrument for fluorescence measurement. The tip was closed and 50 µL of 10-10 M L-asparagine solution was added on matrix and intensity counts were noted down. Same procedure was repeated with each dilution and a plot was drawn between concentrations of asparagine and intensity counts.

3. 13. 3Application of Developed Biosensor

3. 13. 3. 1Analysis of different fruit juices:

Grape juice, Pineapple juice, Orange juice, Apple juice and strawberry juice were purchased from market. The TEOS-chitosan matrix (having enzyme and Rhodamine 6G) was kept in the bottom mirror position of the tip of the fiber-optic cable supplied with the instrument on the bottom mirror of the fiber-optic cable for fluorescence measurement. The tip was closed and 50 µL of fruit juice was added on the matrix and intensity counts were noted down. The change in intensity count was noted down and correlated with the change in intensity count of the L-asparagine standard reference chart and L-asparagine levels were calculated. 3. 13. 3. 2Testing of normal and leukemic blood samples: The work was carried out as per the guidelines of the Ethical and Biosafety Committee. Leukemia serum samples were collected from Max multispecialty hospital, Mohali, with prior information and consent of patients and administration. These were then analyzed for the L-asparagine concentration. Total five leukemia serum samples and two normal serum samples were analyzed. Purified enzyme based biosensor was used for the quantitative detection of asparagine levels. Serum samples were brought to normal room temperature and pH of the samples was determined using pH strip. Then the Enzyme TEOS- chitosan matrix was fixed in fiber optic tip and 50µL normal serum sample was added on it. Initial intensity (Ic) counts were noted and final intensity counts were noted after response time (6 min). ΔIc was calculated and serum asparagine concentration was determined from asparagine standard reference chart. Same procedure was repeated for each leukemia serum sample and normal serum samples and asparagine levels were evaluated. Table 3. 5: Details of Leukemia Patients

Samples

Sex

Age

Type

RavinderM39CLLRajinder KaurF28ALLAvinash SinghM17ALLGurdev DhillonM48CLLJaikishanM21ALLTable 3. 6: Details of Normal Serum samplesNameSexAgeTypeMohitmale24normalSukhpreetMale23normal

3. 13. 3Validation studies of the developed Biosensor:

Reliability check of the developed fluorescence-based fiber optic biosensor was performed in food samples by taking 20 µl of the sample and spiked with 20 µl of asparagine of same concentration. The fluorescence intensity counts were recorded for evaluation of asparagine concentration from the standard chart. Readings from spiked juice samples were compared with their respective readings of juice sample obtained from the biosensor and T-test was used to ascertain statistically significant differences. 3. 13. 4Storage

Stability: The TEOS-chitosan discs (having enzyme and Rhodamine 6G) were stored in a refrigerator and used for fluorescence measurements at various time periods. Biosensor response at various time intervals was used to know the storage stability of the biocomponent.