3.1 Chemicals & Reagents

EDTA, NaCl, Lysozyme, tris-HCl, SDS, CHES and zinc chloride were purchased from Himedia Lab. Pvt. Ltd. (Mumbai). Sodium phosphate monobasic, sodium phosphate dibasic, acrylamide, bis-acrylamide, glycine, ammonium per sulphate, Coomassie brilliant blue, nafion and thioglycerol were obtained from Sisco Research Laboratories (Mumbai). Sephadex G-100 was purchased from MP Biomedicals (France). DEAE-Sepharose was purchased from Sigma Chemical Co., USA. Multi Walled Carbon Nanotubes (MWCNTs), Carboxylated Single Walled Carbon Nanotubes (cSWCNTs) and nafion were purchased from SRL. Gold Nanoparticles (AuNPs) used during the study were synthesized at Nanomaterial Synthesis Lab, Department of Bio & Nano Technology, Guru Jambheshwar University of Science & Technology, Hisar. Gold (Au) wire and Paraffin oil were purchased from Qualigens. All the chemicals used during the study were of analytical grade.

3.2 Instrumentation

Different instruments used during the study include UV-Vis Spectrophotometer (UV 2450, Shimadzu, Japan). Digital pH meter (EUTECH), Refrigerator (LG), Microwave oven (LG), Rotatory incubator shaker (Sanyo MLR 350, HICON, Japan), Magnetic stirrer (Tarsons), Refrigerated centrifuge (4K-15, SIGMA, Germany), Deep freezer (Voltas), Auto pipettes (Agile, India). Particle Size Analysis (ZS90, Malvern Instruments, Malvern, UK), X-ray diffraction (Mini Flex Desk Top X-Ray Diffractometer), Scanning Electron Microscopic (*Zeiss* EVO 40), Transmission Electron Microscopy (Model JEM-2100F JEOL), Fourier transform infrared spectrophotometer (Varian 7000) and Autolab Potentiostat/Galvanostat (PGSTAT 302 N, ECO Chemie, Netherland).

3.3 AChE Purification

3.3.1 Plant Material

In the present study, seeds of *P. vulgaris* (*Dwarf bean*) were obtained from the local market, Rohtak. Mature dry seeds were germinated and hypocotyls were used as explants.

3.3.2 Sterilization and Germination of *Phaseolous vulgaris*

The *P. vulgaris* seeds obtained from local market were surface sterilized by washing with 70 % for 2 min followed by washing with 0.1% mercuric chloride supplemented with 0.01 % Tween 20 for 1 min. The sterilized seeds were washed with double distilled water with at least five times for 15 min in order to completely remove the sterilizing agents. The

seeds were then transferred on the basal medium Murashige and Skoog (MS) (1962) for germination. The MS medium used was devoid of sucrose and solidified with 0.8 % agar in 175 ml tissue culture vessels (Sigma- Aldrich). The culture vessels with the seeds were transferred to Sanyo MLR 350 incubator at 25°C for 12 h photoperiod and light intensity (100 µmole $m^{-2}s^{-1}$). All subsequent cultures were incubated under the same conditions unless specified.

3.3.3 Enzyme Extraction & Purification

The *P. vulgaris* seedlings were harvested after 8 days and the hypocotyls were excised as explant. The above excised explants were homogenized in potassium phosphate buffer (10 mM, pH 7) supplemented with EDTA (10 mM) and ammonium sulphate in the ratio of 1:4 (W/V). After homogenization slurry obtained was left in dark for 2 h and temperature maintained at 10ºC. The homogenized slurry was filtered using a nylon net with pore size 60 µm. The resulting extract was subjected to centrifugation at 30,000 x g in refrigerated centrifuge and maintained at 4ºC for 20 min. This procedure was repeated and supernatant was collected. Supernatant from all the centrifuge tubes was pooled together. The above pooled supernatant was saturated with ammonium sulphate (70-80%) and left overnight in cold in order to precipitate the proteins. This was centrifuged at 40,000 x g at 4ºC for 30 min and resuspended in 5ml of 10 mM PBS (phosphate buffer saline) pH 7.5. The above obtained material was subjected to dialysis using PBS (pH 7.5). The precipitates obtained during dialysis were removed by centrifugation (6000 x g, 30 min, 4ºC) and supernatant was collected.

3.3.4 Gel Filtration

The Sephadex G-100 beads were incubated for 72 h at room temperature for swelling. A glass column of diameter 2.5 cm with a sintered plate near one end was used for gel filtration. The column was washed thoroughly with distilled water, dried and set on a clamp stand. The outlet was closed and column was loaded with Sephadex G-100 slurry. The gel was allowed to settle down in the column and more gel was added by disturbing the upper layer of gel in column upto a height of 25cm. 10 mM potassium phosphate buffer (pH 7) was subjected to flow through the column at a flow rate of 0.5 ml per min. The column was allowed to run till pH of outgoing buffer reached 7. 3 ml of the supernatant collected above (ammonium sulphate precipitated enzyme) was loaded onto the sidewalls of column with the help of pipette without disturbing the upper layer of column. The column was run in the same

buffer until one void volume was passed. Fractions (each of 3ml) were collected until two more void volumes of the elution buffer were passed. To determine void volume of the column, the height and diameter of column was noted and void volume was calculated using the formula:

$$\textbf{Void Volume} = 1/3\pi r^2 h$$

Where, r = radius of the column used, h = height upto which gel is filled in column.

Each fraction obtained above was tested for acetylcholine esterase activity and protein concentration. The active fractions were pooled together. The active fractions were further subjected to ion exchange chromatography.

3.3.5 DEAE-Sephacel column chromatography

Preparation of ion-exchanger

The DEAE-Sephacel was washed several times with distilled water to make the gel free of alcohol. A glass column of 1.5 cm diameter having sintered plate at its lower end was fixed on a burette stand and its outlet was closed. The gel was stirred gently with glass rod and added slowly into the column along the walls of the column with help of glass rod and was allowed to settle. The outlet of the column was opened and buffer flow rate adjusted to 0.5 ml/min. Column equilibration was done using 0.01M potassium phosphate buffer having pH 6.7 at a flow rate of 0.5 ml/min until pH and the ionic strength of both the incoming and outgoing buffer were same. The height of running column was finally 20 cm.

Loading of sample

In order to load the sample, the input of the eluent buffer was first stopped and the eluent buffer was allowed to drain to the bed surface. The pooled enzyme obtained from Sephadex G-100 chromatography were loaded onto the gel surface slowly along the sides of the walls of the column with the help of pipette and allowed to collect over the gel. The column was then connected for elution and the enzyme was eluted with 0.1-0.6 M linear gradient. Linear gradient of KCl was achieved with 200 ml of 0.02 M potassium phosphate buffer (pH 6.7) containing 0.1 M KCl in mixer and 200 ml of 0.02 M potassium phosphate buffer (pH 6.7) containing 0.6 M KCl. These buffers were transferred to aspirator bottles connected at the bottom and open to the atmosphere and mixing in buffer chamber was done using a magnetic stirrer. Rate of elution flow was adjusted at 10-12 drops/min. 3 ml of fractions were collected in each tube and every tube was analysed for acetylcholinestrase

activity and protein content. Fractions possessing highest specific activity were collected and used as purified enzyme in the further experimental work.

3.3.6 Determining the Activity and Protein Content

3.3.6.1 Assay for AChE

100 µL of AChE obtained after above step was diluted by mixing in 200 µL of the double distilled water (DDW). 12.5 mM acetyl thiocholine chloride (ATCI) was mixed with sodium phosphate buffer (100 mM, pH 7) followed by incubation at 30ºC for 5 min. 200 µL of the above obtained solution was added in diluted enzyme and further incubated at 30ºC for 90 min. The reaction mixture along with 100 µL of DTNB (100 mM sodium phosphate buffer, pH 7) was taken in fresh vial and final volume was adjusted to 2 ml by addition of 1400 µL of sodium phosphate buffer (100 mM, pH 7). Spectrophotometric measurements were recorded at 412 nm after 5 min along with the blank (reaction mixture without enzyme and the total volume compensated with the sodium phosphate buffer). Thionitrobenzoic acid imparts yellow colour and show activity at 412 nm.

3.3.6.2 Protein Determination

Total protein content of the purified enzyme was estimated in sample as described by Lowry et al., (1951) using the following reagents:

A. Folin-Ciocalteu (FC) reagent (2N)

   This reagent was diluted with distilled water to 1.0 N before use.

B. Alkaline reagent

   i. Two percent sodium carbonate (Na_2CO_3) in 0.1 N NaOH.

   ii. One percent copper sulfate ($CuSO_4.5H_2O$) in distilled water.

   iii. Two percent sodium potassium tartarate in distilled water.

   iv. Mix solution ii and solution iii in 1:1 ratio.

Procedure

To 1.0 ml of properly diluted sample, 5 ml of freshly prepared alkaline reagent was added. The contents were mixed thoroughly and allowed to stand for 10 min. then 0.5 ml of 1.0 N FC reagent was added and mixed immediately. The tubes were incubated in a water bath at 30ºC for 10 min. the intensity of the developed colour was measured at 412 nm using spectrophotometer (UV 2450, Shimadzu, Japan). The protein in the sample was extrapolated

from standard curve prepared similarly by using different concentrations (100-600 µg/ml) of bovine serum albumin as standard as shown in Figure 23.

Purified protein reacts with Folin Ciocalteau (FC) reagent resulting in the formation of yellow colored complex. FC is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric *in vitro* assay. This color formation is a result of reaction between the peptide part of protein with Copper (Alkaline) followed by reduction reaction in which tyrosine and tryptophan reduces phosphomolybdate.

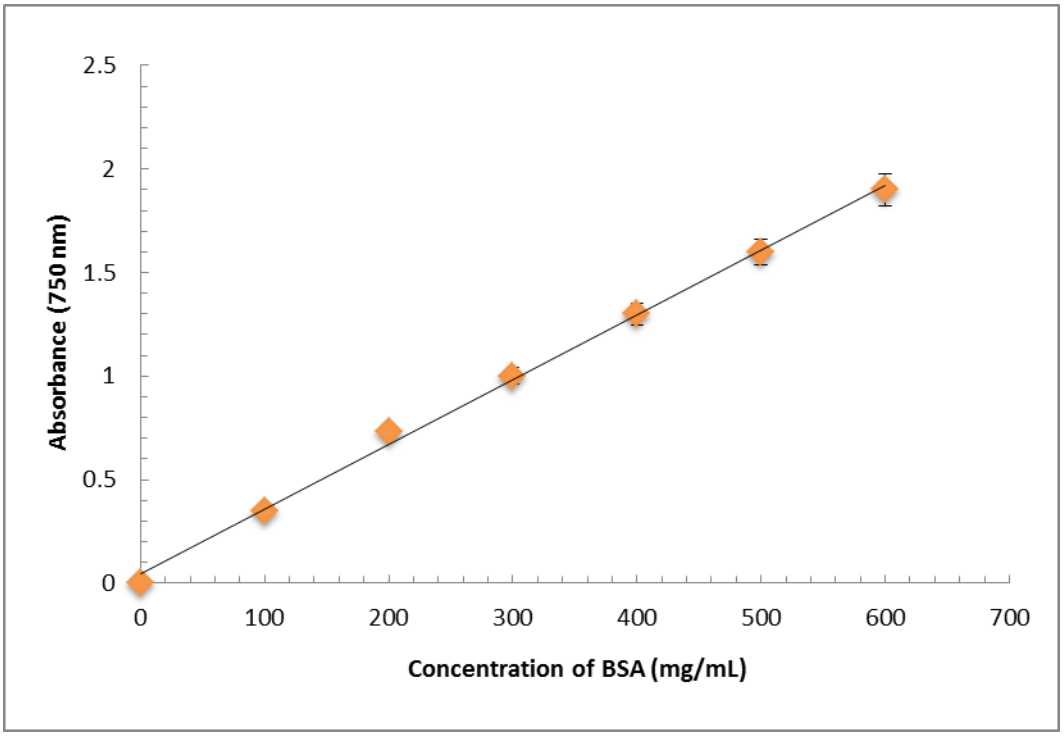

Figure 23: Standard curve of bovine serum albumin (BSA) using Lowry's method

3.3.7 Polyacrylamide gel electrophoresis of purified AChE

3.3.7.1 Reagent Preparation

All the reagents were prepared in double distilled water and stored at 4ºC in amber colored bottle.

A. Acrylamide/Bis-Acrylamide (29.2:0.8)

A mixture of acrylamide (29.2 g) and bis-acrylamide (0.8 g) were dissolved in 100 ml of distilled water and stored under dark at 4ºC.

B. Resolving Gel Buffer (pH 8.8)

Tris Buffer (1.5 M)

18.17 g of Tris was added in 75 ml double distilled water and pH was adjusted to 8.8 using 6 N HCl and final volume was adjusted to 100 ml.

C. Stacking Gel Buffer (pH 6.8)

0.5 M Tris Buffer

Dissolve 3.0 g Tris in 40 ml distilled water and adjust pH 6.8 with 6 N HCl. Finally volume was adjusted to 50 ml using double distilled water.

D. Electrophoresis Buffer (pH 8.3)

Three g Tris, 14.4 g Glycine, 1.0 g Sodium Dodecylsulfate (SDS) were dissolved properly in 1000 ml of double distilled water and stored at 4ºC.

E. Ammonium per sulphate Solution (10 %)

Dissolve 0.15 g in 10 ml distilled water. This solution was prepared fresh.

F. SDS Solution (10%)

Dissolve 1.0 g SDS in 10 ml distilled water. This solution was prepared fresh every time.

G. 6 N Hydrochloric Acid (HCl)

Dissolve 26.48 ml of HCl in 50 ml distilled water.

H. Preparation of Sample Buffer (pH 6.8)

(i) Glycerol 20%: 2 ml for 10 ml sample, (ii) Bromophenol Blue 0.2%: 20 mg, (iii) SDS 4%: 400 mg, (iv) Tris Base 100 mM: 1 ml (pH 6.8), (v) 2 Mercaptoethanol 2%: 200 mg.

I. Staining Solution (for 100 ml)

(i) Methanol 45%: 45 ml, (ii) Acetic Acid (Glacial) 10%: 10 ml (iii) Coomassie Brillient Blue (R 250) 0.02%: 20 mg. Make final volume upto 100 ml with distilled water.

J. De-staining Solution

It was prepared fresh by dissolving methanol (4.5%) and glacial acetic acid (10%) in distilled water in 1:1 ratio.

K. Preparation of Resolving gel (12%)

It was prepared by mixing distilled water, 0.4 ml of 30% Acrylamide, 1.5 M Tris (pH 8.8) (2.5 ml), 0.1 ml of 10% SDS, 0.1 ml of 10% APS and 5µl of TEMED to raise the final volume to 10 ml.

L. Preparation of Stacking gel (4%)

It was prepared by mixing distilled water (6.8 ml), 30% Acrylamide mixture (1.7 ml), 1.0 M Tris (pH 6.8) (1.25 ml), 10% SDS (0.1 ml), 10% APS (0.1 ml), TEMED (0.01 ml).

M. Preparation of Sample Buffer

It was prepared by mixing 5.0 ml distilled water, 1.0 ml of 0.5 M Tris HCl buffer (pH 6.8), 0.8 ml of 20% glycerol, and 0.2 ml of 0.05% bromophenol blue.

N. Sample Preparation

Enzyme and the sample buffer mixed in a ratio of 1:4 and the sample was boiled for 3 min.

3.3.7.2 Procedure

The glass slabs were washed and dried properly and were sealed with wax and fixed on the vertical casting tray. The resolving gel was poured upto a height of 6 cm with the help of pipette between the fixed glass slabs and allowed to polymerize for 15 to 20 min at room temperature. Upon polymerization, the stacking gel was poured in the same way upto the top of the glass slabs and a comb was inserted quickly before the polymerization for the purpose of sample loading. After polymerization the glass slabs were taken out carefully from the gel casting tray and fixed on the vertical assembly for SDS PAGE. The running buffer was then poured in lower and upper chamber of assembly. After polymerization, the comb was carefully removed. Sample wells were rinsed with electrode buffer and electrode buffer was added. The protein markers and the samples (20 µl) were loaded in each well and electrophoresis was carried out at 15 mA/ 60 V for first 30 min. followed by 15 mA/ 100 V constant current till the dye approached the end of the resolving gel.

3.3.7.3 Coomassie Brilliant Blue Staining

The glass plates were removed carefully from the SDS PAGE assembly. The spacers were separated from the both sides. The gel was fixed and stained overnight in Coomassie brilliant blue staining solution.

3.3.7.4 De-staining of the gel

The de-staining of the stained gel was done for 3 h with regular change of the de-staining solution every 30 min.

3.3.8 Kinetic properties of free acetylcholinestrase

3.3.8.1 Effect of pH

In order to determine optimum pH condition of free enzyme, the reaction mixture was adjusted to varying pH range from 6-10 using three buffers: sodium succinate (5.0 and 5.5), sodium phosphate (pH 6-7.5) and borate buffer (pH 8.0 to 10.0). The final concentration of buffers used was 50 mM.

3.3.8.2 Effect of temperature

In order to determine optimal incubation temperature for optimum activity of free enzyme, reaction mixture was incubated at a temperature range of 20^{o}C to 50^{o}C with 5^{o}C interval.

3.3.8.3 Effect of incubation time

Incubation time is the time required for the reaction to take place when the free enzyme is present with the substrate in the reaction mixture. Incubation time was studied from 2 min to 12 min with regular interval of 2 min.

3.3.8.4 Effect of substrate concentration

The substrate concentration plays an important role in determining the optimal enzyme activity. The substrate ATCI was used in order to determine the effect of substrate concentration on enzyme activity. Effect of ATCI concentration on enzyme activity was analysed up to 600 μM with an increment of 50 μM.

3.3.8.5 Determination of K_m and V_{max}

K_m and V_{max} of free AChE enzyme was determined by plotting Lineweaver-Burk plot with reciprocal of substrate concentration [1/S] *vs* reciprocal of initial velocity of the reaction [1/V].

3.4 Fabrication of Nafion/AChE-SWCNT/MWCNT-Au working electrode

3.4.1 Synthesis of Gold Nanoparticles (AuNPs)

AuNPs were synthesized by the method of Gole and Murphy (2004) with modifications. 100 ml of $HAuCl_4$ (0.01%) was heated at 100 ^{o}C & stirred continuously

followed by addition of sodium citrate (1%). Within 2-3 min the solution colour turned to wine red. The solution was heated for 4-5 min and allowed to cool at room temperature and stored at 4ºC till further use.

3.4.2 Characterization of synthesized AuNPs

The AuNPs were characterized by UV-Vis spectroscopy, Particle Size Analyser (PSA), X- Ray Diffraction (XRD) and Transmission Electron Microscopy (TEM). The analysis of optical absorbance activity of the NPs was carried out using spectrophotometer (UV 2450, Shimadzu, Japan) and the spectra of the sample was monitored from 350 to 750 nm at room temperature. The size of AuNPs was determined by particle size analyser (ZS90, Malvern Instruments, Malvern, UK) using water as dispersant; temperature 25.0 ºC, count rate 156.0 kilo counts per second (kcps), testing duration 70 seconds and the measurement position was 4.65 mm. The XRD spectrum of Au NPs was analysed using Mini Flex Desk Top X-Ray Diffractometer. The intensity was recorded using Cu Kα radiation and 2 Theta (θ) values were determined in the range from 35 to 70º. The shape and size of AuNPs was confirmed by TEM (Model JEM-2100F, JEOL). The sample was crushed properly and glycerol was used as dispersant. The analysis was done at 20 nm scale; the direct magnification parameter was 50000 X with HV of 200.0 KV.

3.4.3 Depositing MWCNTs/Au NPs onto Au Wire

Multi-walled carbon nanotubes (MWCNT) 50% along with AuNPs 30% were mixed with paraffin oil 20% to obtain the consistency of paste. The paste (MWCNT/AuNPs) was filled in plastic tube (1 cm long and 4 mm wide) and fine Au wire was inserted to obtain electrical contact. After solidification of the paste, plastic tube was carefully removed; this formed the core of working electrode. The MWCNT/AuNPs-Au core electrode was washed with double distilled water to remove unbound material and stored at 4ºC.

3.4.4 Fabrication of Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode

Carboxylated Single walled Carbon nanotubes (cSWCNTs) suspension (2 µL) was mixed with 2 µL of AChE. The above mixture was poured drop by drop onto the surface of electrode core MWCNT/AuNPs-Au and allowed to dry at room temperature. Finally, 1 µL Nafion (5%) was casted on the body surface which acts as binder to hold the AChE-SWCNT. On the core of working electrode the remaining solvent was allowed to evaporate. The resulting electrode fabricated was Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode. This fabricated electrode was refrigerated at 4 °C until used.

3.4.5 Characterization of working electrode

The working electrode was characterized using SEM, EIS and FTIR. Morphology of bare gold (Au) wire and nanomaterial based working electrode with immobilized enzyme was investigated by SEM. The electrodes were cut into small pieces transferred on specimen stub, and surface morphology examined by SEM. The EIS study of working electrode was carried out using bare Au wire; nanomaterial based working electrode without enzyme and working electrode with enzyme. The EIS studies were performed with Potentiostat with frequency ranging from 0.01 to 105 Hz, amplitude 5 mV and phosphate buffer (0.05 M, pH 7.5).

The FTIR spectrum of working electrode was also recorded at different stages of its fabrication. The deposited nanomaterial was scraped off from working electrode. The dry potassium bromide (KBr) was mixed with scraped material and ground properly. After grinding the mixture was pressed with mechanical press. Then resulting powder was kept in sample holder known as socket of FT-IR spectrophotometer (Varian 7000) and spectrum was recorded.

3.4.6 Electrochemical behaviour of working electrode

Electrochemical measurements were made using Potentiostat using Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au as working electrode, reference electrode (Ag/AgCl) and counter current electrode (Pt wire). Equilibration of working electrode was done at 0.4 V before each run until steady-state current was obtained at + 0.4V. The developed biosensor was then tested at + 0.4 V. Substrate Acetylthiocholine iodide (ATCI) (150 µL of 0.05 mM) was added into electrochemical cell containing 15 ml of sodium phosphate buffer (0.1 M, pH 7.4). ATCI was hydrolysed enzymatically to thiocholine. Further thiocholine was oxidised Vs Ag/AgCl reference electrode.

The oxidation current at anode was inversely proportional to toxic compound present and the time of exposure. Voltammetric measurements were monitored from −0.2 and +1.0 V with 50mV/s scan rate.

3.4.7 Optimization of developed amperometric method

For determination of optimum pH, reaction buffer was varied in pH range 5.0 to 9.0 with increment till 0.1 M final concentration. Three buffers were used for optimization including succinate buffer from pH 5.0 to 6.0, sodium phosphate buffer from pH 6.5 to 7.5 and borate buffer from pH 8.0 to 9.0.

Reaction mixture was subjected to temperature range from 10°C to 50°C for determination of optimum working temperature of this newly developed method. Effect of substrate (ATCI) concentration on biosensor working was also analysed in concentration range from 0 to 600 µM.

3.4.8 Determination of Organophosphorus compound by fabricated electrode

For determining concentration of organophosphorus compound, the Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode was first kept in phosphate buffer solution (0.1 M, pH 7.0) with two concentrations of methyl parathion (5µM and 10µM) as standard for 10 min and finally injecting it to electrochemical cell containing 15.0 mL sodium phosphate buffer (0.1 M, pH 7.0) along with ATCl (0.1 mM) to perform electrochemical analysis by cyclic voltammetry (CV). The methyl parathion inhibition (%) was determined using Equation 2. The peak current i_P (control) is the representation of ATCl on Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode (without pesticides); the peak current i_P (exp) is of ATCl on Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode with pesticide.

$$\text{Percent Inhibition} = \frac{i_{\text{p (control)}} - i_{\text{p (exp)}}}{i_{\text{p (control)}}} \text{ X } 100$$

Equation 2: Calculation of Percent Inhibition

3.4.9 Reactivation of immobilized AChE enzyme on electrode surface

After every use, Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode was subjected to cleaning using 0.1 M phosphate buffer (pH 7.5) for reactivation of surface of working electrode. This reactivation was done using 4.0 mM 2-PAM (5.0 mM) for 10 min. and electrochemical response was measured again. The reactivation efficiency (R%) was determined using Equation 3 given below:

$$\text{R (\%)} = 100 \text{ X } \frac{i_r - i_{\text{p, exp}}}{i_{\text{p, control}} - i_{\text{p, exp}}}$$

Equation 3: Calculation of reactivation of efficiency

where, i_r is the peak current of substrate (ATCl) on Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode with 2-PAM reactivation.

3.4.10 Pesticides Detection in vegetables and soil samples

In order to determine the presence of pesticides in vegetable samples such as onion, spinach and cabbage, the crushed sample (~50 g) was extracted with 10 mL phosphate buffer solution for 5 min followed by filtration. The resulting residue material was again washed with 10 mL of phosphate buffer. The above extracted solution was further used in testing real-sample with help of nanomaterial based working electrode.

Water samples used for drinking purpose were used directly for detection of organophosphorus compounds by the method as described by Kumaran et al. (1995) and with slight modification for analysing soil samples for the presence of the toxic organophosphorus compounds. The spiked samples were used in the present study. The samples collected from different parts of Haryana were oven dried at a temperature of 40 °C and were spiked by adding known concentration of pesticide. Pesticide (10 ml) stock solution (50 %) and 0.01 g of soil was taken in a 15 ml tube. The mixture in tube was mixed properly for 15 to 20 min and left overnight. The above mixture was sonicated for 4 minutes. Before sonication 100 mg of anhydrous sodium sulphate along with 5 ml of dichioromethane/acetone (2:1 v/v) were added in the sample left overnight. The above material in the test tube was shaken vigorously and then the sample was filtered. The remaining soil extracts were further washed using 10 ml of solvent. The test tube was then subjected to water bath for the solvent evaporation completely. 100 µL acetonitrile was added in the dried sample of soil and then the volume raised to 10 ml using working buffer. The resulting mixture was again sonicated for 2 min and then transferred in the electrochemical cell for determination of acutely toxic OP compounds.

3.4.11 Linearity and lower detection limit

A standard graph was plotted between linearity and minimum detection. LOD was calculated as three times the standard deviation for the smallest concentration of sample detected divided by the sensitivity (Sahin et al., 2011). The expression for calculating sensitivity (Equation 4) and LOD is shown in Equation 5.

$$\text{Sensitivity} = \sum_{i=1}^{n} \frac{\text{Current i}}{\text{Concentration i}}$$

Equation 4: Expression for calculating sensitivity

$$\text{LOD} = \frac{3 \text{ X SD of Smallest Sample Detected}}{\text{Sensitivity}}$$

Equation 5: Expression for calculating lower detection limit

3.4.12 Interference Study

The ionic effect of different heavy metals like Cd (II), Pb (II), Cu (II), Ni (II) and Zn (II) at 2 µM concentration was determined. The effect of electroactive species such as glucose, sucrose, uric acid and ascorbic acid were also monitored on the activity of working electrode.

3.4.13 Reusability and Storage stability

Nafion/AChE-cSWCNT/MWCNT/AuNPs-Au electrode was cleaned using sodium phosphate buffer (0.1 M, pH 7.0) every time after use. The fabricated enzyme electrode was stored at 4°C for 60 days and its response in accordance with activity was monitored. The response was monitored on every alternate day for 2 months in order to evaluate storage stability and reusability.

3.5 Statistical methods used

In order to evaluate the data obtained by present method, following statistical formulae were used:

Standard deviation (σ)

$$(\sigma) = \sqrt{\frac{\Sigma(X - \overline{X})^2}{(n-1)}}$$

where, X = each score; $\overline{X}$ = mean; n = number of samples

Standard error (SE)

$$\text{SE} = \frac{\sigma}{\sqrt{n}}$$

Coefficient of variation

$$\text{Coefficient of variation} = \frac{\sigma \text{ x } 100}{\alpha}$$

where, σ = SD; α = means of series

Correlation co-efficient (r) $$r = \frac{n\Sigma xy - \Sigma x\Sigma y}{\{n\Sigma x^2 - (\Sigma x)^2\}\{n\Sigma y^2 - (\Sigma y)^2\}}$$

where, x = value obtained by GC-MS method.

y = values obtained by present method.