3. MATERIAL AND METHODS

3.1. Chemicals and reagents used

Nutrient broth, methyl-parathion, sucrose, 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. c-MWCNTs were synthesised at Department of Bio & Nanotechnology, Guru Jambheshwar University, Hisar. Silver wire was purchased from local market. All the chemicals were of analytical grade. Double distilled water was used for making solutions.

3.2. Instrumentation

Cyclic voltammetry (CV) and other amperometric detections were performed on a potentiostat (PSTAT mini 910, Metrohm, Switzerland) with a three electrode system composed of a platinum wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and working electrode. Ultrasonication was performed on ChromTech Ultrasonic Liquid Processor. The morphological characterization of ZnONPs was done by transmission electron microscopy (TEM) (JEM-2100F microscope) at AIRF, JNU, New Delhi. Fourier transform infrared (FTIR) spectroscopy was performed with Varian 7000 FT-IR spectrometer (AIRF, JNU, New Delhi). X-ray diffraction (XRD) studies of ZnO NP and c-MWCNT were carried out using X-ray diffractometer at Department of Physics, M.D.U. Rohtak. Shimadzu cooperation UV 2450 spectrophotometer was used for spectrophotometric measurements.

3.3. Microorganism

Lyophilised culture of *Brevundimonas diminuta* was obtained from MTCC, Chandigarh (India).
3.4. Revival and Growth of Bacteria

Lyophilised culture of *Brevundimonas diminuta* was revived in nutrient broth. Nutrient broth with pH 7.4, 8, 9 was prepared in two sets. Firstly one set of flasks was inoculated with revived bacterial culture 1ml/l. Then to the second set flasks of nutrient broth was supplemented with 200µl of 25mM methyl-parathion/200ml, after that the broth was inoculated with the revived bacterial culture 1ml/l. Then the culture was kept in shaker incubator at 30°C for 24 hours. After 24 hours culture was obtained and its growth was checked at 600nm. A<sub>600</sub> was obtained more than 1 for all fractions of culture and it was observed that the flasks carrying methyl-parathion showed 25-30% more growth than the culture without methyl-parathion. Maximum growth was obtained at pH 9. Bacterial culture carrying methyl-parathion was further used as a source for extraction of OPH.

3.5. Extraction and purification of OPH from *Brevundimonas diminuta*

3.5.1. Extraction of OPH

Bacterial culture grown in methyl-parathion was split into various samples of 30 ml each. Then the cells were harvested by centrifugation at 8000rpm for 10 minutes. Supernatant was discarded and the cell pellet was suspended into the buffer. To each fraction of cells 2ml of 10mM Tris-HCl pH-8 and 3 ml of 75mM sucrose was added. It was incubated at 20°C for 10 min. Then 10µl of lysozyme (10mg/ml) was added and again incubated for 10 min. Then the suspension was diluted 3 times by 1.5 mM EDTA and an additional incubation for 1 hour was given. Spheroplasts were harvested by centrifugation at 10000 rpm for 5 minutes and treated with 350mm NaCl with continuous stirring and kept in ice for 1 hour (Bernhardt *et al.*, 2003). NaCl treatment facilitated the release of membrane bound organophosphorus hydrolase. Then the suspension was recentrifuged at 8000 rpm for 15 minutes to separate the spheroplasts and free enzyme in the suspension. Spheroplasts settled down and OPH remained in supernatant. Supernatant was then concentrated by lyophilisation. Then the crude enzyme was subjected to Sephadex G-100 column (1.5×30 cm) for gel permeation chromatography. The active fractions were pooled and run in DEAE-Sepharose column (2.5×12.5 cm) for further purification. The enzyme was eluted with a NaCl gradient.
Material and Methods

solution (.01-1 M) (Liu et al., 2004). The eluted fractions were then electrophoresed on a 12% SDS-PAGE gel for authenticating the purity of enzyme.

3.5.2. Purification of OPH

3.5.2.1. Gel filtration:

The Sephadex G-100 beads were kept for 72 hours 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 erect on a clamp stand. Then the outlet was closed and column was loaded with Sephadex G-100 slurry which was kept for swelling up to a height of 25 cm. The gel was allowed to settle down in the column and more gel was similarly added without disturbing the upper layer of gel in column up to a height of 25 cm. Then, 50 mMtris-HCl buffer, pH 8, was run into column at a flow rate of 0.5 ml per minute. The column was allowed to run till pH of outgoing buffer was 8. Then, crude solution 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 the one void volume was passed. Fractions (each of 1 ml) 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:

\[
\text{Void Volume} = \frac{1}{3} \pi r^2 h
\]

Here, \( r \) = radius of the column used, \( h \) = height up to which gel is filled in column.

Each fraction obtained above was tested for enzyme activity and protein concentration. The active fractions were pooled together. After that all the active fractions were subjected to ion exchange chromatography.

3.5.2.2. DEAE-Sepharose column chromatography:

Preparation of ion-exchanger - DEAE-Sepharose was washed several times with distilled water. A glass column of 1.5 cm diameter having sintered plate at its lower end was fixed erect 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. The gel was allowed to settle for some time. The outlet of the column was opened and allowed the buffer to flow at the rate of 0.5 ml/min. The column was then equilibrated with 50 mM tris-HCl buffer pH 8 at a flow rate of 0.5 ml/min, until the 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- The pooled active fractions obtained from Sephadex G-100 chromatography were loaded onto the DEAE-Sepharose column equilibrated with 50 mM tris-HCl buffer pH 8. The column was eluted with 10 to 1000 mM linear gradient of NaCl (Liu et al., 2004). The flow rate of the elution was maintained at 10-12 drops/min and the fractions of 1 ml each were collected. Each fraction was monitored for OPH activity and protein content. The fractions with high specific activity were pooled and treated as purified enzyme.

3.5.3 Enzyme assay

Enzyme activity was calculated by measuring the formation of p-nitrophenol at 410 nm. Methyl-parathion was hydrolysed to p-nitrophenol and its formation was measured spectrophotometrically. 1 mM methyl-parathion in 50 mM CHES buffer at pH 9 was treated with 10µl of enzyme extract. Whole set up was incubated for 10 minutes and then OD was taken at 410 nm by SHIMADZU UV-VIS spectrophotometer (Roger et al., 1999).

Unit of OPH is defined as the number of µmoles of p-nitrophenol produced per unit time at 37°C (Wu et al., 2004).

\[
\text{Methyl-parathion} + \text{H}_2\text{O} \xrightarrow{\text{OPH}} \text{4-nitrophenol} + \text{diethyl phosphate} \quad (\lambda = 410 \text{ nm})
\]

3.6. Determination of protein content

The protein content in enzyme preparation was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard protein.

3.6.1. Principle: Peptide bonds in the polypeptide chain react with copper sulphate in alkaline medium to give a blue colour complex. In addition tyrosine and tryptophan
residue of protein cause reduction of phosphomolybdate and phosphotungstate of Folin Ciocalteau (FC) reagent by tyrosine and tryptophan residues present in protein to give bluish colored complex which enhance the sensitivity of this method. This method (Lowry’s method) is most commonly used for determination of protein concentration because it is highly sensitive. However this method is interfered by several compounds like EDTA, carbohydrate, K⁺, NH₄⁺, thiol reagent like β-mercaptathanol, phenol. These compounds interfere in color development and therefore should be removed from sample to get better result.

**3.6.2. Preparation of reagents:** The following reagents were used in the method -

- Reagent A: 2% sodium carbonate in 0.1N sodium hydroxide.
- Reagent B₁: 1% copper sulphate in distilled water. It was stored at 4°C.
- Reagent B₂: 2% sodium potassium tartarate. It was stored at 4°C.
- Reagent B: B₁+B₂ in 1:1 ratio. It was prepared fresh just before use.
- Reagent C: 50 ml of reagent A +1 ml of reagent B. It was freshly prepared.
- Reagent D: 1 part of Folin-Ciocalteau reagent (2N) +1 part of distilled water.
- Stock solution of BSA (1 mg/mL) was prepared in DDW.

**3.6.3. Procedure:** To 0.1 ml of protein solution, 0.4 ml of distilled water was added and then 5 ml of reagent C was added, mixed well and allowed to stand for 10 min at room temperature, 0.5 ml reagent D was added and mixed thoroughly with vortex mixer. The mixture was allowed to stand at room temperature for 30 min. The blank was prepared by mixing 0.5 ml distilled water, 5.0 ml of reagent C and 0.5 ml of reagent D. The mixture was allowed to stand for 30 min at room temperature. The intensity of color developed was measured in Spectronic-20D at 750 nm.

**3.6.4. Preparation of standard curve of BSA:** Different known concentrations of BSA were used to plot the standard curve between A₇₅₀ and different concentrations of BSA solution (Fig. 3). The amount of protein in enzyme solution was calculated from standard curve of bovine serum albumin.
Fig. 3. Standard curve of bovine serum albumin (BSA) using Lowry’s method.

3.7. SDS-PAGE of purified OPH

Slab gel electrophoresis of purified enzyme was carried out in 12% SDS-PAGE, using method by Laemmli (1970). The following reagents and gel were prepared.

A. Acrylamide/bis-acrylamide (30: 0.8): 14.6 g acrylamide and 0.4 gm N’N methylene bis acrylamide were dissolved in 20 mL DDW and final volume was made up to 50 mL. It was filtered and stored at 4°C in amber colored bottle in dark.

B. 1.5 M Tris-HCl buffer, pH 8.8: 18.15 g Tris base was dissolved in 40 mL DDW. The pH of the solution was adjusted to pH 8.8 with 1N HCl and final volume was made up to 100 mL with DDW and stored at 4°C.

C. 0.5 M Tris-HCl buffer, pH 6.8: 6.0 g Tris base was dissolved in 40 mL DDW. The pH of the solution was adjusted to pH 6.8 with 1N HCl and its final volume was made to 100 mL with DDW and stored at 4°C.

D. 10% Ammonium persulfate solution: It was prepared fresh every time before use. To prepare it, 10.0 g ammonium persulfate was dissolved in DDW and final volume was made 100 mL.
Material and Methods

E. **Running (electrode) buffer:** 3 g Tris base, 1 g sodium dodecyl sulfate (SDS) and 14.2 g glycine were dissolved in 1000 mL DDW. The pH of the solution was adjusted to pH 8.6.

F. **Tracking dye:** 0.05% bromophenol blue stain was prepared in 1.5 M Tris-HCl buffer, pH 8.5.

G. **10% Sodium dodecyl sulfate (SDS):** 10 gm of SDS dissolved in 50 ml of distilled water and make the final volume 100 mL.

I. **Preparation of separating gel:** It was prepared by mixing DDW (5 mL), 3.75 ml of solution B; 6.0 mL of solution A; .15 mL of solution D; 0.2 mL of 10% SDS solution and 20 μL TEMED (supplied in liquid form) to make 10 mL total volume.

II. **Preparation of spacer gel:** DDW (3 mL) was added into 1.25 mL of solution C, 0.67 mL of solution A, 5 μL of TEMED, 0.32 mL of 10% SDS solution and 0.25 mL of solution D.

III. **Preparation of sample buffer:** It was made by adding 4.0 mL of DDW, 1.0 mL of 0.5 M Tris-HCl buffer, pH 6.8, 0.8 mL of 20% glycerol, 1.6 mL of 10% SDS solution and 0.2 mL of 0.05% bromophenol blue (tracking dye).

IV. **Sample preparation:** Enzyme sample was mixed with sample buffer in 1:4 ratio.

V. **Procedure:** The glass slabs were sealed with white wax and separating gel was poured up to the height of 5 cm sandwiched between the vertical plates. It was allowed to polymerize for some time at room temperature. Then spacer gel solution was layered to the height of 3 cm and comb was fixed immediately so that well could be formed for sample application on polymerization. The spacer fixed on the lower side was removed. The lower and the upper chamber were filled with running buffer and gel plates were kept in lower chamber in such a way that no air bubble was formed between gel and buffer system. The comb was removed and enzyme sample (40 μL) and protein marker (50 μL) were loaded in well with the help of auto pipette. The apparatus was connected to electrophoretic power supply unit and run at 15 mA/40 V until the blue color approaches to the lower end of spacer gel. After that the voltage was increased to 100 V
Material and Methods

till the blue color reached near the bottom of the gel slab (approx. 2-3 h). The power supply was turned off and the gel slab was carefully removed from the glass plates.

VI. Staining of protein by Coomassie brilliant blue: To prepare the staining solution, Comassie brilliant blue 0.02% (w/v) was dissolved in a solution containing 45% (v/v) methanol and 10% glacial acetic acid (v/v) in 1:1 ratio. The gel was put into the staining solution for 30 min at 35°C.

VII. Destaining of protein: The destaining of the protein was done in a mixture of 10% methanol (v/v), 7% glacial acetic acid (v/v) in DDW in 1:1 ratio for 3 h with intermittent washing of the gel after every 30 min with the same mixture.

3.8. Kinetic properties of Organophosphorus hydrolase (OPH)

3.8.1. Effect of pH

To determine the optimum pH of free enzyme, pH of reaction buffer was varied from 6-10 using the following buffers: succinate buffer (pH-6.0, 6.5 and 7.0), phosphate buffer (pH-7.5, 8.0 and 8.5) and borate buffer (pH-9.0, 9.5 and 10.0) each at a final concentration of 0.1M.

3.8.2. Effect of incubation temperature

To determine the incubation temperature for optimum activity, the reaction mixture was incubated at different temperatures ranging from 20°C to 60°C at an interval of 5°C.

3.8.3. Effect of substrate concentration

The effect of substrate (Methyl-parathion) concentration on the enzyme activity was studied from 0.1µM to 600 µM in the reaction mixture at an interval of 50 µM.

3.8.4. Determination of $K_m$ and $V_{max}$

$K_m$ and $V_{max}$ values for free OPH enzyme was also calculated from Lineweaver-Burk plot between reciprocal of substrate concentration [1/S] and reciprocal of initial velocity of the reaction [1/V].
3.9. Synthesis of ZnO nanoparticles

ZnO particles were synthesised chemically in alcoholic media. 100 ml of 0.1 M NaOH was taken in a beaker then 1 ml of thioglycerol (10^{-3}M) was added slowly with continuous stirring. Then the solution was stirred for 1 hour. Then 20 ml of ZnCl\(_2\) (0.1M) was added drop wise and stirred continuously for 3-4 hours until a milky white solution was obtained (Ashtaputre \textit{et al.}, 2005). Then the size selective precipitation of nanoparticles was carried out using ethanol as non-solvent. Then the precipitates were filtered and washed with methanol and dried overnight at room temperature. Transmission electron microscopy (TEM) of the synthesised ZnO NP was done (at AIRF, JNU, New Delhi) to study the electronic structure and size of particles.

3.10. Fabrication of PAA/OPH-ZnONP/c-MWCNT modified silver working electrode

Working electrode was prepared by mixing c-MWCNTs and ZnO NPs with paraffin oil in fixed proportion to obtain a consistent paste. Then the paste was filled in a plastic hollow tube (2cm x 4mm). An ultra-pure silver wire is inserted in the carbon paste. The Silver wire was cleaned with ethanol and ddH\(_2\)O by sonication before inserting into the carbon paste. Then the electrode was kept for drying and after drying it was immersed in OPH solution for 2 hrs. On immersing the electrode in enzyme solution, OPH will get bound on electrode surface. After immobilising enzyme whole electrode was layered with thin film of polyacrylamide (PAA). PAA layer will prevent the enzyme from leaching. Assembly of working electrode has been shown in figure 4.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Assembly of PAA/OPH-ZnONP/c-MWCNTs silver working electrode.}
\end{figure}
Material and Methods

3.11. Characterisation of carbon based working electrode

An XRD diffractometer was used to investigate the stability of c-MWCNTs decorated with ZnO NP. First the sample was ground and pressed into the sample holder to get a smooth plane surface and the diffraction pattern was recorded over a 2θ range of 10°-70°.

The fabricated electrode (ZnONP/c-MWCNTs/Ag electrode and OPH-ZnONP/ c-MWCNTs/Ag electrode was also characterized using Fourier transform infrared (FTIR). FTIR spectroscopy was performed in spectrometer to check the binding of OPH on ZnONPs/c-MWCNT/Ag electrode. For FTIR sample preparation was done in KBr.

3.12. Construction and Electrochemical studies of the biosensor

An amperometric OPH biosensor was constructed by using a conventional three-electrode system comprising Ag/AgCl (3M KCl saturated) electrode as reference electrode, Pt wire as counter-current electrode, and PAA/OPH-ZnONP/ c-MWCNTs/Ag electrode as working electrode connected through Potentiostat. Figure 5 shows the schematic representation of electrochemical detection of OP compounds

Electrochemical measurements were made through Potentiostat mini 910 with typical three electrode electrochemical cell. Cyclic voltammetric measurements were performed at room temperature. PAA/OPH-ZnONP/c-MWCNTs/AgE biosensor enzymatically hydrolysed the

![Schematic representation of electrochemical detection of OP compounds.](image)

Fig. 5. Schematic representation of electrochemical detection of OP compounds.
organophosphorus substrate methyl-parathion and produced p-nitrophenol. P-nitrophenol was then electrocatalytically oxidised vs Ag/AgCl reference electrode. The voltammogram was continuously recorded between −0.2 and +1.0 V at a scan rate of 50mV/s.

3.13. Kinetic properties of OPH biosensor

Effect of working conditions working potential, pH and temperature were studied for the present biosensor. Effect of substrate (Methyl-parathion) concentration and calculation of $K_m$ and $I_{max}$ were calculated from Double-reciprocal plot between reciprocal of substrate concentration $[1/S]$ and reciprocal of amount of the current $[1/I]$.

3.13.1. Effect of applied potential

The effect of applied potential was recorded from 0.0 V to+1.0 V with stepping potential 0.05V at each step.

3.13.2. Effect of pH

The effect of pH on the biosensor response in 0.1 M succinate buffer (pH-6.0, 6.5 and 7.0), phosphate buffer (pH-7.5, 8.0 and 8.5) and borate buffer (pH-9.0, 9.5 and 10.0) in 6.0–10.0 range was studied.

3.13.3. Effect of incubation temperature

To determine the incubation temperature for maximum activity, the reaction mixture was incubated at different temperatures ranging from 20°C to 60°C at a regular increase of 5°C. The response of biosensor was determined at these different incubation temperatures as described above.

3.13.4. Effect of substrate concentration

Effect of methyl-parathion concentration on the response of present method was studied up to 600 µM with an interval of 50 µM.
3.13.5. Determination of $K_m$ and $I_{max}$

A Lineweaver-Burk plot was made between reciprocal of substrate concentration ($I/[S]$) vs reciprocal of amount of current ($1/[I]$) measured. $K_m$ and $I_{max}$ were calculated from Lineweaver-Burk plot.

3.14. Evaluation of the present method for determination of OP compounds

The following parameters were studied in order to evaluate the newly developed method for determination of OP compounds employing enzyme electrode.

3.14.1. Linear working range and minimum detection limit

The linear working range and minimum detection limit were calculated by correlating the values with standard graph. Minimum detection limit is that concentration of the species which gives a minimum detectable signal.

3.14.2. Analytical recovery

To determine the reliability of the methods, different concentration of methyl-parathion (5, 10, 15, 20µM) was added to the samples and the mean analytical recovery of methyl-parathion was determined by the present method.

3.14.3. Precision and accuracy

To study the reproducibility of the present method, the level of OP compounds in samples was determined on same day (within batch) and in the same samples after storage at 4°C for one week (between batch), coefficients of variation (CVs) were calculated for the present method.

3.14.4. Correlation

In order to determine accuracy of present methods, the pesticides values in 10 water samples were determined by standard HPLC method (x) as well as by the present methods (y), the values obtained by both the methods were co-related using regression equation.
3.14.5. Effect of interfering substances

The amperometric response was also checked in the presence of potential interfering compounds glucose, fructose, sucrose, uric acid, ascorbic acid as well as metal ions (Zn (II), Cu (II), Cd (II), Ni (II) and Pb (II) each at a concentration of 2 μM.

3.15. Stability and reusability of biosensor

PAA/OPH-ZnONP/c-MWCNTs/Ag E biosensor was washed with phosphate buffer pH 7.5 every time after use. For checking its storage stability the biosensor was stored in refrigerator for 2 months. Its activity was checked on alternate days for determining its storage stability and reusability.

3.16. Application of the newly developed OPH based amperometric biosensor

Soil, water and food samples more prone to have OP compounds were collected from different places and used for OP determination. The soil matrix was placed in a tray and left to dry at about 35°C in oven. Then soil samples were suspended in test tubes containing phosphate buffer saline (PBS). The test tube was capped and the contents were mixed and left undisturbed overnight at room temperature. Then the supernatant was used for analysis of OP concentration.

The food samples i.e. apple, orange, grapes, okra and cabbage were washed with distilled water and chopped. 100 grams of each chopped sample were crushed in pestle mortar and homogenized with 50 ml of PBS (pH 7.0) by continuous stirring for 1 h at room temperature. The samples were filtered through filter paper and centrifuged at 5000 rpm for 10 min. Supernatant was collected and used for electrochemical measurements. Water samples taken from pond, canal, tap, ground and branded mineral water were also used for determination of OP compounds.

OP compounds present in the sample were hydrolysed to 4-nitrophenol by the OPH enzyme bound on the silver electrode. Optimum potential was applied to oxidize 4-nitrophenol and the response current generated was directly proportional to the OP compound concentration in the sample. Polyaclrylamide layer present on electrode prevent enzyme from leaching and improved the reusability and storage stability of the
biosensor. This electrochemical technique is employed to determine the concentration of OP compounds in real-samples.

3.17 Statistical methods used

To evaluate the data obtained by present methods following statistical formulae were used:

**Standard deviation (σ)**

\[
(\sigma) = \sqrt{\frac{\sum (X - \bar{X})^2}{n-1}}
\]

Where \(X\) = each score; \(\bar{X}\) = mean; \(n\) = number of samples

**Standard error (SE)**

\[
SE = \frac{\sigma}{\sqrt{n}}
\]

**Coefficient of variation**

Coefficient of variation = \(\frac{\sigma \times 100}{\alpha}\)

where, \(\sigma\) = SD; \(\alpha\) = means of series

**Correlation co-efficient (r)**

\[
r = \frac{n\sum xy - \sum x \sum y}{\sqrt{\{n\sum x^2 - (\sum x)^2\} \{n\sum y^2 - (\sum y)^2\}}}
\]

where, \(x\) = value obtained by HPLC method.
\(y\) = values obtained by present method.