3. MATERIALS AND METHODS

3.1. CHEMICALS AND REAGENTS

Sephadex G-100 was purchased from MP Biomedicals. Nutrient broth, tris-HCL, EDTA, lysozyme, sucrose and sodium hydroxide were purchased from Himedia. Glutaraldehyde (grade 1.25%) was purchased from Sigma chemicals and methyl parathion was purchased from Shri Ram Fertilizers & Chemicals. Single walled carbon nanotubes were gifted by Professor Neeraj Dilbaghi, Department of Bio & Nanotechnology, GJUS&T, Hissar. All other chemicals were of analytical reagent grade unless otherwise stated. Double distilled water (DDW) was used throughout the experiments. Silver wire and PVC (Polyvinyl chloride) beaker were bought from local market.

3.2. INSTRUMENTS AND EQUIPMENTS

Electrochemical measurements were performed on a potentiostat (PGSTAT12/30/302, Autolab) with a three electrode system composed of a platinum (Pt) wire as an auxiliary electrode, Ag/AgCl reference electrode and carbon nanotubes paste based working electrode. UV spectrophotometer (Shimadzu corporation, Japan), temperature controlled water bath (SunRon), digital pH meter (EUTECH), refrigerator (LG), microwave oven (LG), rotatory incubator shaker (HICON), magnetic stirrer (HICON), refrigerated centrifuge (SIGMA), sonicator (ChromTech) and deep freezer (Voltas). The morphological studies were carried out with scanning electron microscope (SEM) (Model Joel JSM-6510, Japan) at Department of Chemistry, M. D. University, Rohtak, India.

3.3. MICRO-ORGANISM

The lyophilized sample of bacteria Brevundimonas diminuta (MTCC 3361) was purchased from IMTECH, Chandigarh.
3.4. REVIVAL AND GROWTH OF BACTERIA

Lyophilized sample of *B. diminuta* was mixed with 5 ml of nutrient broth (pH 8.0) in a test tube to make a bacterial suspension. The conical flask (250 ml) containing 100 ml nutrient broth (pH 8.0) was inoculated with 0.5 ml of bacterial suspension. The culture media was incubated in a rotatory incubator shaker at 200 rpm for 24 hours at 30˚C. Growth of bacteria was checked by taking O.D. at 600 nm.

3.5. EXTRACTION & PURIFICATION OF OPH FROM *Brevundimonas diminuta*

3.5.1. Extraction of crude OPH

i. *B. diminuta* cells were harvested by centrifugation at 8000 rpm for 10 minutes.

ii. The cells were suspended in 7.5 ml of 50 mM sodium phosphate buffer pH 8, containing 0.75 M sucrose and incubated at 20 °C for 10 min.

iii. 20 µl of 10 mg/ml lysozyme solution was added and again incubated for 10 min.

iv. Then the solution was diluted 3-fold with 1.5 mM K-EDTA and incubated for 1 hr (Herzlinger et al., 1984). Spheroplasts formation was checked microscopically.

v. Spheroplasts were harvested by centrifugation at 10000 rpm for 5 min.

vi. The spheroplasts pellet was resuspended in 20 ml of 50 mM sodium phosphate buffer (pH 8) containing 500 mM NaCl and incubated on ice for 15 min with constant stirring (Bernhardt and Boer, 2003). This step significantly enhanced the release of membrane-bound OPH enzyme into solution.

vii. Spheroplasts were removed by centrifugation at 10000 for 5 min.

viii. The supernatant containing enzyme was collected and considered as crude enzyme solution.

ix. Enzyme activity was monitored by enzyme assay. To concentrate the protein content of crude solution, sample was lyophilized using a lyophilizer machine. To perform the purification of OPH enzyme, lyophilized sample of crude solution was
dissolved in minimal volume of Tris-HCl buffer (50 mM, pH-8). The crude enzyme solution was subjected to chromatography.

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. Then 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 mM tris-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.

Then each fraction obtained above was tested for enzyme activity and protein concentration. The active fractions were pooled together. Then these 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 sometime. 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-Sephrose 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.

### 3.6. ENZYME ASSAY

The assay of purified organophosphorus hydrolase was carried out by method described by Chaudhary *et al.* (1988) with slight modification. The 0.1 ml of OPH solution was mixed with 2.9 ml of 50 mM sodium phosphate buffer (pH-8.0) containing 0.1 ml 10 mM OP pesticide as substrate (Table 4) and incubated for 10 min at 37° C. The activity was assayed by measuring the formation of 4-nitrophenol at 410 nm for 2 min at 37° C.

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

**Table 4:** Reagents used for enzyme assay.

One unit of OPH activity is defined as the amount of enzyme liberating 1 μmol of 4-nitrophenol per minute at 37° C (Wu *et al.*, 2004).
3.6.1. Reagents used for assay of purified OPH enzyme:
Following reagents were prepared:

3.6.1.1. Reagent A: 50 mM sodium phosphate buffer
Dissolve 0.71 gm of Na₂HPO₄ and 0.6 gm of NaH₂PO₄ in 50 ml of distill water, adjust the pH to 8.0 and make the final volume 100 ml by adding distill water.

3.6.1.2. Reagent B: Substrate: (10 mM Methyl parathion)
0.263 gm of methyl parathion was dissolved in 100 ml of distill water. It was prepared freshly for all assays.

3.7. DETERMINATION OF PROTEIN CONCENTRATION

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

3.7.1. Principle: Peptide bonds in the polypeptide chain react with copper sulphate in alkaline medium to give a blue colored 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 conc. 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.

3.7.2. 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-Ciocalteu reagent (2N) + 1 part of distilled water.

Stock solution of BSA (1 mg/mL) was prepared in DDW.

3.7.3. 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.

3.7.4. Preparation of standard curve of BSA: Different known concentrations of BSA were used to plot the standard curve between $A_{750}$ and different concentrations of BSA solution (Fig. 10). The amount of protein in enzyme solution was calculated from standard curve of bovine serum albumin.

Fig. 10: Standard curve of bovine serum albumin (BSA) using Lowry’s method.
3.8. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED OPH

Slab gel electrophoresis of purified enzyme was carried out in 12.5% SDS-PAGE, using method of 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.

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 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.
3.9. KINETIC PROPERTIES OF FREE OPH ENZYME

3.9.1. 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.

3.9.2. 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 50°C at an interval of 5°C.

3.9.3. Effect of incubation time

Time of incubation was also studied from 2 min to 12 min at a regular interval of 2 min.

3.9.4. Effect of substrate concentration

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

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

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

3.10. COVALENT IMMOBILIZATION OF OPH ONTO INNER BOTTOM SURFACE OF PVC BEAKER

(a) Chemical modification of PVC surface (Hooda et al., 2009)

(i) Breaking of vinyl polymers:

Nitric acid being a strong oxidizing agent removes chlorine molecules from damaged end of polymer and introduces a double bond at the ends of these short-chain
polymers. The chemical reaction shown below randomly introduces nicks in long-chain polymer and generates free ends protruding from the polymer surface.

\[
[-\text{CH}_2-\text{CH (Cl)-CH}_2-\text{CH (Cl)-}] \text{ n-1}
\]

\[
\text{H}^+ + \text{NO}_3^- \xrightarrow{\text{Fuming}} \text{HCl}
\]

\[
[-\text{CH}_2-\text{CH(Cl)-CH=CH}_2]
\]

(ii) Modification of terminal protruding ends:

According to Markonikoff’s rule when the terminal active ethylene group was treated with methyl cyanide by nucleophilic addition mechanism for generation of terminal nitrile groups.

\[
[-\text{CH}_2-\text{CH(Cl)-CH=CH}_2]
\]

\[
\text{CH}_3^+ + \text{CN}^- \xrightarrow{\text{CN}^- -\text{ve part added to less hydrogen}} [-\text{CH}_2-\text{CH(Cl)-CH(CN)-C}_2\text{H}_5]
\]

(iii) Partial Hydrolysis of terminal nitrile group:

Partial hydrolysis of nitrile group was done by treating it with HCl to generate terminal amide groups.

\[
-\text{CH}_2-\text{CH(Cl)-CH(CN)-C}_2\text{H}_5
\]

\[
\text{Hydrolysis in presence of acids (HCl)}
\]

\[
-\text{CH}_2-\text{CH(Cl)-CH(C}_2\text{H}_5)\text{CO-NH}_2
\]
(b) Activation of PVC surface:

Glutaraldehyde, a bifunctional cross-linking agent, with aldehyde groups on both ends was allowed to react with the generated terminal amide groups of on PVC surface, this leads to activation of surface and ready for covalent coupling of enzyme.

\[
\text{-CH}_2\text{-CH(Cl)-CH(C}_2\text{H}_5\text{)CO-NH}_2 + \text{CHO(CH}_2)_3\text{CHO} \\
\text{ (Glutaraldehyde)}
\]

\[
\downarrow
\]

\[
\text{-CH}_2\text{-CH(Cl)-CH(C}_2\text{H}_5\text{)CO-N}=\text{CH-(CH}_2)_3\text{-CHO} \\
\text{ (Activated PVC surface)}
\]

(c) Immobilization of enzyme onto activated PVC surface:

Incubation of enzyme with activated PVC surface in the presence of phosphate buffer leads to effective collision between the amide of the enzymes and one free aldehyde group of glutaraldehyde present on activated PVC surface and results in formation of coupled covalent bond between the enzyme and PVC as described below:

\[
\text{-CH}_2\text{-CH(Cl)-CH(C}_2\text{H}_5\text{)(CO-N}=\text{CH-(CH}_2)_3\text{-CHO} + \text{NH}_2\text{-E} \\
\text{ (Activated PVC surface)} \quad \text{ (Free enzyme)}
\]

\[
\downarrow
\]

\[
\text{-CH}_2\text{-CH(Cl)-CH(C}_2\text{H}_5\text{)(CO-N}=\text{CH-(CH}_2)_3\text{-CH= N-E} \\
\text{ (Covalent coupled enzyme)}
\]
3.11. SCANNING ELECTRON MICROSCOPY OF PVC SURFACE

To confirm the immobilization of enzyme, the scanning electron microscopic (SEM) study of the surface of PVC beaker was carried out (before and after enzyme immobilization) at Department of Chemistry, M. D. University, Rohtak, India.

3.12. PREPARATION OF CARBON NANOTUBES BASED WORKING ELECTRODE

Carbon nanotubes powder (1.0 g) and NH$_4$Cl (0.2 mg) were mixed with paraffin oil in a ratio to obtain the consistency of paste (Hooda et al., 2009). This paste was filled in a plastic hollow tube (2 cm length and 4 mm diameter) with one closed end. Electrical contact was obtained by inserting a silver wire into the carbon nanotubes paste. This formed the body of the working electrode. The surface of the electrode was washed with buffer and stored at 4°C when not in use.

3.13. ASSEMBLY OF OPH BIOSensor AND ELECTRO-ANALYTICAL MEASUREMENTS

An amperometric OPH biosensor was developed by using enzyme immobilized PVC reaction beaker along with the three electrodes i.e. carbon nanotubes paste as working electrode, Ag/AgCl electrode as reference and Pt wire as auxiliary electrode and connected through potentiostat/electrochemical analyzer. The electro-analytical measurements for OP pesticides were performed using a potentiostat/electrochemical analyzer. The cyclic voltammetric study of 4-nitrophenol was performed in sodium phosphate buffer (pH-8.0) of 20 mL working volume in a 30 mL PVC beaker reaction cell at room temperature. In the cyclic voltammetry experiments, the scan rate was 50 V/sec and the scan range was 0 V to 1.0 V.

3.14. KINETIC PROPERTIES OF OPH BIOSensor

The following kinetic properties of immobilized enzyme biosensor were studied: effect of pH, incubation temperature, time of incubation, effect of substrate (Methyl parathion) concentration and calculation of K$_m$ and I$_{max}$ from Line weaver - Burk plot.
between reciprocal of substrate concentration \([1/S]\) and reciprocal of amount of the current \([1/I]\).

### 3.14.1. 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.

### 3.14.2. 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.

### 3.14.3. Effect of incubation time

Time of incubation was also studied from 5 min to 20 min at a regular increase of 5 min.

### 3.14.4. Effect of substrate concentration

Effect of methyl parathion concentration on the response of present method was studied up to 500 µM with an interval of 50 µM.

### 3.14.5. 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.

### 3.15. EVALUATION OF THE PRESENT METHOD

The evaluation of present method was carried by studying its linearity, minimum detection limit, analytical recovery, precision and accuracy. The effect of interfering species on the response of biosensor was studied.
3.15.1. Linear working range and minimum detection limit

The linear working range and minimum detection limit were calculated by correlating the values with standard graph (Fig. 11).

3.15.2. Analytical recovery

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

3.15.3. Precision

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

3.15.4. Accuracy

To evaluate the accuracy of present method, the level of methyl parathion in 5 spiked water samples were determined by the present method and compared with those obtained by standard method.

3.15.5. Effect of interfering substances

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

3.16. REUSABILITY AND STORAGE STABILITY

Before reuse, working electrode was washed by washing buffer (0.01 M Phosphate buffer saline, pH-7.2 with 0.1% tween-20). The storage stability of the present biosensor was investigated over a period of two months, when reaction beaker was stored at 4°C containing reaction buffer (50 mM sodium phosphate buffer, pH-8.0). The response of present method was measured once in every 5 days.
3.17. APPLICATION OF NEWLY DEVELOPED OPH BIOSENSOR

The water and food samples prone to be contaminated with OP pesticides were collected. Water samples used for drinking purpose were employed directly for the study. The food samples i.e. vegetables and fruits (cauliflower, cabbage, grapes and apple) were bought from local market. The food samples were washed with distilled water and chopped. 100 grams of each chopped sample were crushed in pestle mortar and homogenize with 50 ml of PBS (pH 7.0) and stirred for 1 h at room temperature. The samples were filtered through filter paper and centrifuged at 8000 rpm for 8 min. Supernatant was collected and take 20 ml of supernatant for electrochemical measurements.

OP compounds present in the sample were hydrolyzed to 4-nitrophenol by the OPH enzyme immobilized onto PVC surface. Optimum potential was applied to oxidize 4-nitrophenol and the response current generated was directly proportional to the OP compound concentration in the sample. A standard curve was prepared between response current vs substrate concentration under their optimal assay condition (Figure 11). This electrochemical technique is employed to determine OP pesticide concentration in food and water samples.

**Fig. 11:** Standard curve between current response and substrate concentration.
3.18. STATISTICAL METHODS USED

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

**Standard deviation (σ)**

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

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)**

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

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