Chapter 3.
Materials and methods

The motive of the study was to develop an eco-friendly and cost effective technology for disposal of some toxic industrial wastes. Experiments were conducted to establish the efficiency of vermicomposting over traditional aerobic composting for recycling nutrients from the wastes of three industries and that of vermicompost as a biofilter for remediation of their waste waters.

3.1 *Eisenia fetida*:

Young non-clitellate earthworms were procured from the vermifarm of the Guru Nanak Dev University, Amritsar.

3.2 Industrial wastes:

Phytotoxic solid wastes of three industries were selected for their bioremediation using exotic earthworms *Eisenia fetida*. Effluents of these industries were also collected for their purification with the help of vermicompost filters.

3.2.1 Beverage Industry:

Biosludge and effluent were procured from a beverage industry in Jandiala, Amritsar (Plate 1).

3.2.2 Distillery Industry:

Biosludge and effluent were procured from a distillery industry in Khasa, Amritsar (Plate 2).

3.2.3 Pulp and Paper mill:

Biosludge and effluent were procured from a Pulp and Paper mill in Jandiala, Amritsar (Plate 3).

3.3 Cattle dung:

Fresh cattle dung was procured from a dairy farm situated in the vicinity of Guru Nanak Dev University, Amritsar (Plate 4).
3.4 Experimental Design

The experiments were carried separately for the solid wastes (sludges) and effluents.

3.4.1 Solid wastes:

The experiments were performed in rectangular plastic trays (28x 23x 6 cm) in triplicate under the sheds of the vermifarm of the University in two phases.

3.4.1.1 First phase:

In this phase experiments were conducted to find the proportion of a particular sludge to be mixed with cattle dung for supporting maximum population build up of worms and giving a product with best physico-chemical characteristics. Wastes from the three industries were mixed with cattle dung in various proportions and subjected to Vermicomposting (Set 1, with worms) and traditional aerobic composting (Set 2, without worms). 2 kg mixture was added in each tray, these were watered regularly and covered with hessian cloth to reduce evaporation and maintain moisture at 60-70%. The mixtures were turned over manually every 24 h for 21 days for removal of volatile toxins if any and then 20 young non clitellate worms of almost same weight were released in each tray of set 1. Data were recorded for percent mortality, growth rate, clitellum development, cocoon production, rate of hatching and number of hatchlings at 15 day intervals through 150 days. Earthworms, cocoons and hatchlings were sorted out by hand from the feed mixtures for recording their numbers and weights. In set 2 microbes were allowed to play their role for aerobic degradation of waste. For maintaining aerobic conditions the trays were given a turning every alternate day throughout the course of the study. The time taken for degradation of the wastes was recorded for both the sets. At the end of the experiment the products of vermicomposting and composting were sieved and dried in an oven for 36 h at 60 °C, packed in polythene bags and stored in a dry cool place for chemical analysis.

3.4.1.2 Second phase:

In this phase experiments were conducted to find out the appropriate weight of worms/Kg selected mixtures of each waste for the production of best quality product in the shortest possible time. Two mixtures/sludge giving maximum population buildup of earthworms were selected from the set 1 and subjected to vermidgradation
with 0, 7.5, 12.5 and 25 g worms/kg mixture. Time required for complete degradation of the waste was recorded along with physico-chemical characteristics of the products.

3.4.2 Effluents:

Vermicompost was used as a biofilter for purification of the effluents. The experiments were conducted in glass columns (38 cm l x 3.2 cm b) for removal of suspended and dissolved solids present in the effluents.

3.4.2.1 Effluent:

100 ml effluent was passed at a rate of 10 drops per minute through filters made of cattle dung vermicompost (0.23 mm particle size). For BOD, COD, pH, EC, TDS and TSS 50g vermicompost was used and for transition metals 20, 50 and 100g vermicompost was used. A comparison was made for these parameters in the effluent before and after filtration to evaluate the efficiency of vermicompost for improvement of the quality of effluents.

3.5 Physico-chemical analysis

For physico-chemical characterization of biosolids, colour, texture, pH, EC organic carbon, nitrogen, phosphorous, potassium, sodium and transition metals (Cu, Fe, Mn and Zn) were estimated at the beginning and end of the experiment. Physico-chemical characterization of the initial and final effluent was done by measuring colour, pH, EC, TDS, TSS, BOD and COD.

3.5.1 pH

5 g air dried sample was dissolved in 50 ml distilled water (1:10 w/v) and shaken on an orbital shaker for 40 minutes. Then supernatant was taken and pH of the supernatant was recorded by using pH meter model "Equip-tronics-614-A".

3.5.2 Electrical conductivity (EC)

5 g air dried sample was dissolved in 50 ml distilled water (1:10 w/v) and shaken on an orbital shaker for 40 minutes. Then supernatant was taken and EC of the supernatant was recorded using an “HM-Digital EC meter”. The results were expressed in mS/cm.
3.5.3 Nitrogen

The method of Bremner and Mulvaney (1996) was used for estimation of Total Kjeldhal Nitrogen.

3.5.3.1 Reagents

a) Digestion mixture:

It was prepared by mixing $K_2SO_4$, $CuSO_4$ and $SeO_2$ in the ratio of 10:4:1.

b) Boric acid indicator solution:

Twenty gram boric acid was dissolved in about 700 ml of hot distilled water. The cooled solution was transferred to one liter volumetric flask containing 20 ml of mixed indicator solution (prepared by dissolving 100 mg bromocresol green and 50 mg of methyl red in 100 ml of ethanol). After thoroughly mixing the contents of the flask, final volume was made to one liter with distilled water.

c) Sulphuric acid: Concentrated sulphuric acid (AR grade)

d) 0.01N HCl (for titration).

3.5.3.2 Digestion of sample:

a) 0.5g dried sample was taken in a 100 ml flask and 15 ml of digestion acid mixture (1g digestion mixture in 15ml concentrated sulphuric acid) was added to it.

b) Initially it was heated at low temperature until frothing stopped and then temperature was raised to boiling point.

c) Digestion was continued, until the contents became light yellow green. The flask was rotated intermittently.

d) The digest was cooled and final volume was made 50 ml with distilled water.

3.5.3.3 Distillation:

a) An aliquot of 10ml was taken from the digested sample and run in micro Kjeldhal apparatus with 10ml of 40% NaOH.

b) 5 ml boric acid indicator was taken into a flask and placed below the condenser taking care that the tip of the condenser dipped into it. After this distillation was started and about 50 ml condensate was collected in the flask. The flask was removed
before stopping the heat to prevent back sucking of the liquid. The indicator in condensate turned greenish-blue due to dissolution of ammonia, which was titrated with 0.01N HCl. At the end point the colour changed from greenish-blue to permanent light pink

**Calculation:**

\[
\% \text{ Nitrogen} = \frac{(a-b) \times N \times 1.4}{S}
\]

Where, 
- \(a\) = volume of HCl used for sample (ml),
- \(b\) = volume of HCl used for blank (ml)
- \(S\) = weight of sample taken (g)
- \(N\) = Normality of HCl
- 1.4 = multiplication factor

**3.5.4 Organic carbon (OC)**

Content of organic carbon was determined by the method of Nelson and Sommers, (1996). 0.5 g of air-dried sample (< 2 mm particle size) was taken in a pre-weighed China crucible. The sample was ignited in a Muffle furnace at 550 °C for 60 min. The furnace was allowed to cool and the ash produced was weighed. TOC was calculated with the help of the formula given below.

\[
\text{Ash} \% = \frac{\text{Wt. of sample after ignition}}{\text{Wt. of sample taken}} \times 100
\]

**Organic carbon percentage (%) = \frac{100 - \text{ash percentage}}{1.724}**

**3.5.5 Phosphorus**

Phosphorus was estimated by the method of John (1970).

**3.5.5.1 Reagents**

a) Stock Solution:

20 g ammonium molybdate was dissolved in 300 ml of distilled water. 450 ml of 10N H₂SO₄ was added to it slowly with constant stirring then 100 ml of 0.5%
antimony potassium tartarate was added. Final volume was made to one liter with distilled water and stored away from direct light in a dark colored glass bottle.

b) Working Reagent:

1.5 g ascorbic acid was added to 100 ml of stock solution. This reagent was always prepared fresh.

c) Standard solution:

Standard stock solution of 1000 mg/l (1000 ppm) was prepared by dissolving 0.439 g KH$_2$PO$_4$ in 100 ml of distilled water and standard curve was prepared in the range of 0.2, 0.4, 0.6, 0.8 and 1.0 µg P/ml.

d) Diacid mixture:

It was prepared by mixing concentrated nitric acid (HNO$_3$) and perchloric acid (HClO$_4$) in the ratio of 4:1 (V/V).

3.5.5.2 Procedure

a) 0.5 g dry material was taken in a 250 ml digestion flask and 15 ml of diacid mixture was added to it. The mixture was digested in a digestion chamber till it became colourless. The contents were diluted to about 30 ml with distilled water, filtered through whatman no.1 filter and transferred to a 50 ml volumetric flask, final volume was made 50 ml with distilled water.

b) From each flask, 1 ml aliquot was taken in a 50 ml volumetric flask and 5 ml of freshly prepared mixed reagent was added to it. Final volume was made 50 ml with distilled water. After 30 min, absorbance of the solution was measured at 880 nm using a UV-Visible spectrophotometer-117.

3.5.6 Potassium and sodium

Potassium and Sodium were measured according to APHA (1998) with the help of Systronics Flame photometer-117 in the diacid digest of the samples as in 3.5.5.2 (a).

3.5.6.1 Stock solution

Standard stock solution of 1000 mg/l (1000 ppm) Na and K was prepared by dissolving 0.2543 g of NaCl and 0.191g of KCl in 100 ml distilled water each and
standard curve was prepared in the range of 20, 40, 60, 80 and 100 mg/l for Na and K.

3.5.7 Transition metals

Contents of Fe, Cu, Mn and Zn in the compost were determined from the diacid digest of the samples (as in 3.5.5.2a) using Atomic Absorption Spectrophotometer (AAS) (Model Varian 20). Standard solutions were prepared with the nitrate salts of the selected transition metals.

3.5.8 Biological Oxygen Demand (BOD)

BOD was determined according to the Standard Methods for Examination of water and waste water (APHA, 1998).

3.5.8.1 Reagents

a) Phosphate buffer solution:

KH₂PO₄ (8.5 g), K₂HPO₄ (21.75 g), Na₂HPO₄·7H₂O (33.4 g) and NH₄Cl (1.7 g) were dissolved in about 500 ml distilled water and final volume was made to one litre. This reagent was discarded if there was any contamination.

b) Magnesium sulphate solution:

22.5 g MgSO₄·7H₂O was dissolved in distilled water and diluted to one litre.

c) Calcium chloride solution:

27.5 g CaCl₂ was dissolved in distilled water and diluted to one litre.

d) Ferric chloride solution:

0.25 g FeCl₃·6H₂O was dissolved in distilled water and diluted to one litre.

e) 1N. Acid and Alkali solution: for neutralization of caustic or acidic waste samples.

1) Acid solution: 28 ml concentrated sulphuric acid was added to distilled water slowly and the solution was continuously stirred. Final volume was made to one litre.

2) Alkali solution: 40 g Sodium hydroxide was dissolved in distilled water and diluted to one litre.

f) Sodium sulfite solution:
1.575 g Na$_2$SO$_3$ was dissolved in one litre distilled water. This solution is not stable so it was prepared freshly every time.

g) Ammonium chloride solution:

1.15 g NH$_4$Cl was dissolved in 500 ml distilled water.

h) Manganese solution:

182 g MnSO$_4$ was dissolved in 500 ml distilled water.

i) Alkali azide solution:

The solution was prepared by dissolving 125 g NaOH, 33.5 g NaI, 2.5 g NaN$_3$ in 500 ml distilled water.

j) Sodium thiosulphate solution:

This solution was prepared by dissolving 3.10 g Na$_2$S$_2$O$_3$ and 0.2 g NaOH in 500 ml of distilled water.

k) Starch solution:

1% starch solution was prepared by dissolving 1g starch powder in 100 ml distilled water.

3.5.8.2 Procedure:

20 ml sample was diluted to 1 litre in a BOD bottle and 1 ml each of MgSO$_4$, phosphate buffer, CaCl$_2$, and FeCl$_3$ were added to it. The BOD bottle was incubated for 5 days at 20°C.

3.5.8.3 Determination of initial Dissolved Oxygen (DO)

Initial DO was measured immediately after filling the BOD bottle with diluted sample. Brown precipitates appeared on addition of 1ml MnSO$_4$ and 1ml Alkali solution. After 15 minutes 1 ml concentrated H$_2$SO$_4$ was added to dissolve the precipitate. 1ml starch solution was added, the blue coloured solution was titrated with Na$_2$S$_2$O$_3$ till it became colourless.
3.5.8.4 Determination of final DO

Final DO was determined after 5 days incubation as per 3.5.8.3

Calculation:

\[ \text{BOD} \, 5\, \text{mg/l} = \text{DO}_{\text{initial}} - \text{DO}_{\text{final}} \times \text{Dilution} \]

3.5.9 Chemical Oxygen Demand (COD)

COD was determined according to the Standard Methods for examination of water and waste water (APHA, 1998).

3.5.9.1 Reagents

a) Standard potassium digestion solution:

4.913 g K_2Cr_2O_7 previously dried at 103 °C for 2h, 167 ml conc. H_2SO_4 and 33.3 g HgSO_4 were mixed in 500 ml of distilled water. Cooled to room temperature, and diluted to 1000 ml.

b) Sulphuric acid:

This reagent was prepared by mixing Ag_2SO_4 in H_2SO_4 at the rate of 5.5 g Ag_2SO_4/litre H_2SO_4, allowed to stand for one to two days for dissolution of Ag_2SO_4.

c) Ferroin indicator:

1.485 g 1,10-phenanthroline monohydrate and 695 mg FeSO_4.7H_2O were dissolved in distilled water and diluted to 100ml.

d) Standard Ferrous ammonium sulphate titrant (FAS 0.1M):

39.2g Fe (NH_4)_2 (SO_4)_2.6H_2O was dissolved in distilled water, 20ml conc. H_2SO_4, was added to it, cooled and diluted to 1000 ml.

3.5.9.2 Procedure

2.5 ml sample solution, 1.5 ml digestion solution, and 3.5 ml sulphuric acid reagent were taken in a culture tube. For control, sample solution was substituted with same volume of distilled water. The mixture was autoclaved at 150 °C for 2 h. After cooling to room temperature 1 to 2 drops of ferroin indicator were added and it was titrated with FAS with constant stirring till a sharp colour change from blue green to reddish brown, within a few minutes blue colour appeared again.
Calculations:

\[
\text{COD mg/L} = \frac{(A - B) \times M \times 8000}{\text{Volume of Sample (ml)}}
\]

Where:

A = ml of FAS used for blank
B = ml of FAS used for sample
M = Molarity of FAS

3.5.10 Total Dissolved Solids (TDS)

TDS were determined according to Standard Methods for examination of water and waste water (APHA, 1998).

3.5.10.1 Procedure:

The sample was stirred on a stirrer, 100 ml sample was filtered through Whatman filter paper number 1, and the filtrate was transferred to a pre-weighed evaporating dish. The sample was evaporated to dryness at 180 ± 2°C, cooled in a desiccator and weighed. The increase in weight of the evaporating dish represented the total dissolved solids.

3.5.11 Total Suspended Solids (TSS)

TSS were determined according to Standard Methods for examination of water and waste water (APHA, 1998).

3.5.11.1 Procedure:

A well mixed sample was filtered through a weighed Whatman filter paper number 1 and the residue retained on the filter paper was dried to a constant weight at 103 to 105°C. The increase in its weight represented the total suspended solids.

3.6 Statistical Analysis

- One way ANOVA was used to calculate the differences among various mixtures. The data for numbers of worms, cocoons and hatchlings were subjected to square root transformation prior to analysis of variance.
Two way ANOVA was used to find the effect of the interaction between the proportion of sludge and weight of earthworms in the mixtures on the selected physico-chemical parameters of the products.

Response surface design was used for finding out the best expected concentration of a biosludge and cattle dung giving maximum number of worms, cocoons, hatchlings and worm biomass. The value of x was determined from the derived regression equation \( y = b_2 x^2 - b_1 x + a \) to know the appropriate concentration at which response will be maximum using the formula \( x = -b_1 / 2b_2 \).

Regression equation was derived for calculating weight of worms/Kg ideal feed mixture for its fastest degradation.

Pearson’s correlation coefficient was used to calculate the relationship between the concentrations and chemical parameters.

Student’s t-test was used to evaluate the differences between initial and final values of various chemical parameters.

Statistical analysis was done with the help of Minitab 14 computer software programme.

### 3.7 Langmuir Isotherm (Adsorption Isotherm)

The adsorption isotherms relate the adsorbate concentration in the bulk and the adsorbed amount at the interface. The simplest adsorption isotherm is based on the assumption that every adsorption site is equivalent and the ability of a particle to bind is independent of whether or not adjacent sites are occupied. Langmuir isotherm assumes that a monomolecular layer is formed when biosorption takes place and that there is no interaction between molecules (i.e. metals) adsorbed on adjacent binding sites.

The Langmuir isotherm is represented by the following equation:

\[
\frac{C_e}{(x / m)} = \frac{1}{ab} + \frac{1}{a} C_e
\]

Where \( x/m \) = amount adsorbed at equilibrium (mg/g)
a and b = Langmuir constant related to adsorption capacity and energy of adsorption

Ce = equilibrium concentration in mg/litre

The linear plot of \( \frac{C_e}{(x/m)} \) versus Ce shows that the adsorption obeys the Langmuir model and (a) versus (b) were determined from the slope.