3. MATERIAL AND METHODS
MATERIAL AND METHODS

3.1 Pesticides

Following pesticides were used for the present study. Their purity, water solubility and source etc. are mentioned below:

Table 7

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Molecular weight</th>
<th>Purity</th>
<th>Melting point</th>
<th>Water solubility</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tetramethyl thiuram disulfide (TMTD)</td>
<td>240.44</td>
<td>98%</td>
<td>155-156°C</td>
<td>Insoluble</td>
<td>Fluka, AG Switzerland</td>
</tr>
<tr>
<td></td>
<td>( \text{H}_3\text{C}) ( \text{S}) ( \text{S}) ( \text{CH}_3 ) ( \text{N}) ( \text{C}) ( \text{S}) ( \text{S}) ( \text{C}) ( \text{N}) ( \text{CH}_3 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 1-naphthylmethyl-Carbamate (Sevin)</td>
<td>201.22</td>
<td>Technical</td>
<td>145°C</td>
<td>0.1%</td>
<td>Union Carbide of India Ltd.</td>
</tr>
<tr>
<td></td>
<td>( \text{OC}) ( \text{NCH}_3 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The stock solutions of the pesticides were prepared in Dimethyl sulfoxide (DMSO, Reidel) which was having no effect on the soil organisms at the concentration used. Sterile solutions of the pesticides were prepared separately by filter sterilization through sintered glass filter (G-5).

3.2 Type and preparation of soil

Composite soil samples of alluvial sandy loam, typical of north India were taken from near the department of Microbiology, PU Chandigarh. Soil has no previous history of pesticide treatment. Upper 15cm soil particles that passed through a 2 mm sieve were stored in a humid atmosphere (Rh 60-80%) at 20°C for a maximum of 7 days.
quantities of soil were removed as required. Drying of soil was prevented for increased carbon dioxide production which results on remoistening (Wilkinson et al., 1964). Quantities of soil mentioned in all experiments are on dry weight basis. The weight of soil used in the experiment was corrected for the amount of moisture present in soil. For example, a 300 g soil sample on dry weight basis was equivalent to 360 g if the sample had 20 percent of moisture.

3.3 Physical and chemical analysis of soil

Freshly procured samples of soil were analysed for physical and chemical characteristics. Mechanical analysis were determined by hydrometer method (Jackson, 1967); pH with a systronics analog pH meter and organic matter by chromic acid titrations (Jackson, 1967). Cation exchange capacity, available phosphorus, total nitrogen and nitrate and ammonium nitrogen were determined by the procedures outlined by Jackson (1967). The physical and chemical characteristics of the soil used are given in table 8.

Table 8. Characteristics of soil used

<table>
<thead>
<tr>
<th>Determination</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water holding capacity</td>
<td>44.5%</td>
</tr>
<tr>
<td>Cation exchange capacity</td>
<td>24 meq. 100 g⁻¹</td>
</tr>
<tr>
<td><strong>Mechanical analyses</strong></td>
<td></td>
</tr>
<tr>
<td>Sand</td>
<td>52%</td>
</tr>
<tr>
<td>Silt</td>
<td>28%</td>
</tr>
<tr>
<td>Clay</td>
<td>20%</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
<tr>
<td><strong>Nitrogen</strong></td>
<td></td>
</tr>
<tr>
<td>Ammonium</td>
<td>14.5 ppm</td>
</tr>
<tr>
<td>Nitrite</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate</td>
<td>6.5 ppm</td>
</tr>
<tr>
<td>Organic matter</td>
<td>0.65%</td>
</tr>
<tr>
<td>Phosphate phosphorus</td>
<td>2 ppm</td>
</tr>
<tr>
<td>— No NO₂⁻ detected</td>
<td></td>
</tr>
</tbody>
</table>
3.4 Mixing of pesticide in soil

The desired concentrations of pesticides (TMTD or Sevin) were mixed with soil properly before transferring to pots. The incubation temperature for the studies on soil biological process was $28\degree \pm 2\degree C$. For bacterization experiment, the soil and sand were mixed before filling into the pots. The soil was also amended with 1 per cent CaCO$_3$ and 0.05 per cent dipotassium hydrogen phosphate.

3.5 Effect of pesticides on soil microbial population

Varied concentrations of pesticides (TMTD, Sevin and both in combination) were properly mixed with the soil and incubated at $28 \pm 2\degree C$. Composite soil samples were taken after every week up to six weeks and soil microbial population was calculated as soil bacteria, soil fungi and soil actinomycetes. Experiment was performed in triplicates.

3.5.1 Soil bacteria

Soil bacteria were calculated by inoculating different dilutions of soil samples on soil extract agar (modified). To prepare 1000 ml of soil extract, 500 g soil was used in 1l tap water, mixed well to disperse and steamed in an autoclave for 1 hour at 121$\degree$C. The suspension was suction filtered using large Buchner funnel and Whatman filter paper No. 5. The filtered extract was made up to 11 with tap water, added 15 g agar and pH adjusted to 6.8-7.0 with IN HCL or 1N NaOH. The medium was modified by addition of 0.25 g dipotassium hydrogen phosphate ($K_2HPO_4$) and 5.0 g yeast extract (Parkinson et al., 1971). The soil samples were spread plated on the above said agar medium and incubated at $28 \pm 2\degree C$. Samples for bacterial counts were assayed after 4-5 days incubation.
The dilution that yielded 30-300 colonies/plate was selected and after enumerating the counts on those plates, exact count was calculated by multiplying with the dilution factor.

3.5.2 Soil fungi

Soil fungi were enumerated the same way as bacteria using the Martin's rose bengal medium (Martin, 1950). It contained 10.0 g glucose; 5 g peptone; 1.0 g KH$_2$PO$_4$; 0.5 g MgSO$_4$·7H$_2$O; 0.03 g rose bengal and 20 g agar in 1000 ml water. Autoclaved at 15 lbs and 121° C for 20 minutes, cooled to about 48° C and add 1 ml solution of streptomycin (0.3 g to 10 ml of sterile water) to a final concentration of 30 µg/ml. Similarly soil samples were spread plated under different dilutions, incubated at 28 ± 2° C for 7 days and numbers counted.

3.5.3 Soil actinomycetes

Various dilutions of the soil samples were prepared and spread plated on starch casein medium (Kuster and Williams, 1964; Williams and Davies, 1965). Combine 10.0 g of soluble starch, 0.3 g of vitamin free casein, 2.0 g KNO$_3$, 2.0 g NaCl, 2.0 g KH$_2$PO$_4$, 0.05 g MgSO$_4$·7H$_2$O, 0.02 g CaCO$_3$, 0.01 g FeSO$_4$·7H$_2$O and 20 g agar with 1 liter of distilled water. Adjust pH to 7.0 and autoclave. The plates were incubated for 15-20 days before enumerating soil actinomycetes.

3.6 Effect of pesticides on Nitrification

Diammonium hydrogen phosphate (200 ppm) was used as a substrate for nitrification in soil. Varied quantities of pesticides (TMTD, Sevin and their combination) were mixed with soil and incubated at 28 ± 2° C. Each study included appropriate control and studies were
carried out in triplicates. Soil samples were taken every week for nitrification. Nitrate-N was extracted by the methods of Jackson (1967) and estimated colorimetrically by the methods of Onken and Sunderman (1977).

3.7 Effect of pesticides on Ammonification

Soil was mixed with appropriate amount of peptone to give a nitrogen content of 800 ppm. Pesticide/s were added in defined concentrations and incubated at 28 ± 2°C. Soil samples were removed every week till six weeks. Ammonium-N was extracted (Jackson, 1967) and estimated (Onken and Sunderman, 1977).

3.8 Effect of pesticides on Symbiotic Nitrogen Fixation

The effect of different concentrations of TMTD, sevin and their combination (0, 100, 250 and 500 ppm) was studied on growth, total nitrogen and nitrogen fixation of soybean plants (Glycine max). The effect was also seen when plants were inoculated with specific Rhizobium sp. i.e. Bradyrhizobium japonicum (isolated from the root nodules of the plants). The experiment was done under pot house conditions, taking unsterile soil. Six plants were initially grown in each pot and later only best three were kept and rest were uprooted at sapling stage only. Experiments were conducted in triplicates. Plants were raised under constant moisture conditions and were uprooted after 60 days along with their roots and washed in running water. The roots were blot dried on a filter paper. The number of nodules in each plant was counted and detached for nitrogen fixation capacity. Nitrogen fixation capacity was measured as nitrogenase activity expressed as nmoles of ethylene formed per gm. per hr. on a Gas chromatograph
Using Porapak-R column. Plants were dried in an oven at a constant temperature of 60°C and dry weight of each plant was recorded. Total nitrogen of plants was determined by the conventional Kjeldahl's method (Jackson, 1967).

3.9 Effect of pesticides on soil enzymes

Soil was mixed with different concentrations of the pesticides (TMDT, Sevin and both) and inoculated as described earlier. Soil samples were removed from pots from the glass house at different intervals of time and the enzyme activities were determined as follows:

3.9.1 Amylase

(Hofmann and Hoffmann, 1966; Cole, 1977). Toluene (0.3 ml) was added to 2 gm. of air dried soil sample and shaken. Mixture was allowed to stand for 15 minutes. 5 ml. of sodium acetate buffer (0.1M, pH 5.0) containing 50 mg soluble starch was added to above mixture. The contents were incubated at 28°C for 24 h. 10 ml. of distilled water was added to the contents after incubation period and centrifuged at 12,000xg for 10 minutes. Reducing sugars were estimated from the supernatant by the method of Nelson (1944).

3.9.2 Invertase

(Hofmann and Hoffmann, 1966; Cole, 1977). Invertase was assayed in a manner identical to the amylase, except that 0.1 M sodium acetate buffer (pH 5.5) containing 18 mM sucrose was used and incubation period was 3 h.
3.9.3 Phosphatase (Tabatabai and Bremner, 1969)

To 1 g of soil was added 0.3 ml. toluene and 1 ml of 5 mM sodium-p-nitrophenyl phosphate in acetate buffer (pH 5.2) and the contents were incubated at 37°C for 1 h. After incubation, 4 ml distilled water, 10 ml Calcium chloride (0.5M) and 4 ml sodium hydroxide (0.5 M) were added and the whole mixture was filtered through Whatman filter paper (No. 42) after shaking for 1 minute and yellow color of p-nitrophenol released was estimated colorimetrically at 400 nm.

3.9.4 Dehydrogenase

(Casida et al., 1964; Kiss and Boaru, 1965) 3g of soil was taken in a screw capped test tube previously saturated with 0.5 ml of 3 % (W/V) solution of triphenyl tetrazolium chloride (TTC) and 1.5 ml distilled water. The contents were thoroughly shaken and incubated at 37°C for 24 h. Triphenyl formazan formed was extracted with methanol and estimated spectrophotometrically at 485 nm.

3.10 Isolation of pesticide degrading organisms

Soil samples were collected from different locations. Basic synthetic/salt medium (SM) was used for isolation and growth studies. It contained \( \text{K}_2\text{HPO}_4 \) (5.8 g/litre), \( \text{KH}_2\text{PO}_4 \) (4.5 g/litre), \( (\text{NH}_4)\text{}_2\text{SO}_4 \) (2.0 g/litre), \( \text{MgCl}_2 \) (0.16 g/litre), \( \text{CaCl}_2 \) (20 mg/litre), \( \text{NaMoO}_4 \) (2 mg/litre), \( \text{FeSO}_4 \) (1 mg/litre) and \( \text{MnCl}_2 \) (1 mg/litre) (Kilbane et al, 1982). Appropriate modification was made to the SM so that TMTD or sevin could serve as the sole source of carbon. To 50 ml of basic synthetic/salt medium (SM), 1 g of soil was added and incubated at 28°C ± 2°C for 7 days under
shaking conditions. 1 ml of suspension was transferred to fresh medium containing TMTD or sevin separately (200 ppm) and again incubated for 7 days under similar conditions. After six transfers in a similar way, the enriched population was plated on SM containing TMTD or sevin. the colonies that appeared were further evaluated in liquid broth and various isolated organisms were checked for their ability to degrade different pesticides. Sterile solution of the pesticide in DMSO (passed through a sintered glass filter grade G5) was added to the medium which gave a fine suspension of the pesticide.

The isolates degrading TMTD or Sevin were maintained on synthetic medium agar slants (Agar 2 %) containing respective pesticide and stored at 4°C. The organisms were subcultured every 30 days.

3.11 Identification of the isolates

The morphological, cultural and biochemical characteristics of the isolates were studied (Conn, 1957 and Skerman, 1967). The isolates were identified according to the schemes of Breed et al. (1974).

3.12 Estimation of pesticides

3.12.1 TMTD

TMTD was extracted from the medium or in soil by chloroform and estimated by the colourimetric method of Rangaswamy et al. (1970). To one ml of chloroform extract was added 2 ml. of freshly prepared cuprous chloride reagent (100 mg cuprous chloride in 20 ml of 0.1 N HCl; dilute the clear solution to 100 ml using 95 % ethanol). Dilute the contents to 5 ml by addition of 95% ethanol. Immediately read absorbance at 385 nm against the blank prepared in chloroform.
3.12.2 Sevin

Sevin was measured indirectly by measuring its immediate degradation product 1-naphthol by the method of Casida and Augustinsson (1959) and Lacoste et al. (1959). Add 2 ml of distilled water to 1 ml sample and then 2 ml of borate buffer (pH 10.0). Now add 0.5 ml each of 4-aminoatipyrine (0.2%) and potassium ferricyanide (0.8%). Allow the colour to develop for 6 minutes and measure the absorbance at 510 nm.

3.13 Growth characteristics of isolates and pesticide degradation

100 ml of liquid synthetic medium containing 200 ppm pesticide (TMTD or Sevin) was inoculated with pesticide degrading isolate (1 x 10^7 cells and incubated at 28° ± 2°C on a rotary shaker at 90 rpm. Control medium with 200 ppm pesticide (without inoculation) was also maintained under similar conditions. Representative samples were removed at different intervals. Residual pesticide was estimated and growth was determined by pour plate technique (in triplicate samples) and expressed as log of colony forming units (Log CFU).

3.14 Utilization of pesticides in synthetic medium as a source of carbon or supplemented with different compounds

To show the degradation of pesticides (TMTD or Sevin) by isolates; these were supplied as sole source of carbon and nitrogen in synthetic medium. A filter sterilized solution of pesticide was added to a final concentration of 200 ppm. Degradation was also studied by supplementing the medium with glucose or yeast extract. All other growth conditions were kept the same. Growth, residual pesticide and glucose utilisation were checked periodically. Glucose was estimated by Anthrone reaction (Mokrasch, 1954).
3.15 Degradation of pesticides in soil

500 ppm of TMTD or sevin was added to soil with proper mixing. The soil was inoculated with respective pesticide degrading organisms \((1 \times 10^6 \text{ cells.g}^{-1})\). A control of uninoculated soil was kept in each case. Percent pesticide degraded and total bacterial population (expressed as log CFU) was calculated after different days of incubation. Amount of pesticide recovered on 0 day of incubation was considered as 100%.

3.16 Effect of possible TMTD-degradation products on growth of TMTD degrading organism and TMTD degradation

100 ml of synthetic medium was supplemented separately with different concentrations of possible degradation products of TMTD (\(\alpha\)-ketoglutarate, 150 \(\mu\)g. ml\(^{-1}\); Methionine, 50 \(\mu\)g. ml\(^{-1}\); Dimethylamine, 50 \(\mu\)g. ml\(^{-1}\) and formaldehyde, 50 \(\mu\)g. ml\(^{-1}\)) and then inoculated with TMTD-degrading organisms \((1 \times 10^6 \text{ cells})\). The flasks were incubated at 28 \(\pm\) 2°C and samples were taken at different intervals to see growth. In another set, TMTD (200 ppm) was also added along with the degradation products. The flasks were similarly inoculated and incubated under set conditions. Residual TMTD and growth was determined at different time intervals.

3.17 Growth of TMTD degrading organism and production of enzyme degrading TMTD

TMTD degrading organism was grown in the synthetic medium in presence of TMTD (100 \(\mu\)g/ml) under shake conditions at 28 \(\pm\) 2°C. 100 ml of medium was inoculated with 2 ml of inoculum from 24 h old culture. The flasks were incubated for 72-80 h. The cultures were
centrifuged at 10,000xg for 20 minutes and cell free supernatant was collected for enzyme assays.

3.17.1 Assay for enzyme degrading TMTD

The assay method is based on the conversion of TMTD by the enzyme and residual TMTD is estimated spectrophotometrically at 385 nm. The reaction mixture unless otherwise indicated contained 2 ml substrate (TMTD 0.5 mM in 0.1 M phosphate buffer, pH 7.0) 1.8 ml phosphate buffer (0.1 M, pH 7.0) and 200 µl of crude enzyme preparation (supernatant). The reaction was stopped by addition of chloroform (2 ml). The enzyme preparation boiled at 100°C for 15' was considered as control.

One unit of enzyme was defined as amount of enzyme required to degrade 1 µM of TMTD in 1 minute.

3.18 Assay for Protein concentration

The concentration of protein in whole of the studies was determined by the procedure of Lowry et al. (1951) taking crystalline bovine serum albumin as standard.

3.19 Growth of Sevin degrading organism and production of enzyme degrading sevin

Sevin degrading organism Pseudomonas cepacia was grown in the synthetic medium in presence of sevin (200 µg.ml⁻¹) under shaking conditions at 28 ± 2°C. 2 ml inoculum from 24 h old culture was inoculated in 100 ml of medium. The flasks were incubated for 96 h. The cultures were centrifuged at 10,000xg for 20 minutes. Cell pellet (1 g wet-weight) was suspended in 20 ml of 0.1 M phosphate buffer (pH=7.0) and
disintegrated on a sonicator at 10,000 mA for 20 min. at 0°C. The cell free extract was obtained by centrifugation at 30,000 x g for 30 min. at 0°C.

3.19.1 Assay of the enzyme degrading sevin

All assays were performed taking 3 ml of 0.2 mM aqueous solution of sevin, 1 ml of phosphate buffer (0.1 M, pH = 6.6) and 0.2 ml of enzyme preparation. The total volume of the reaction mixture was made up to 5 ml with distilled water. The reaction was allowed to go for 10 min. at 55°C. The reaction product was determined by modified 4-amino antipyrene method (Casida and Augustinsson, 1959, and Lacoste et al., 1959).

A unit of enzyme was defined as the amount of enzyme required to degrade 1 μM of sevin in 1 minute.

3.20 Factors affecting production of enzyme degrading TMTD

To maximise the production of the enzyme degrading TMTD various parameters were varied to study the effect on production of this enzyme as given below.

3.20.1 Incubation time and aeration

Organism was inoculated in the synthetic medium containing TMTD (200 μg/ml) and flasks were incubated at 28 ± 2°C under shake (90 rpm) as well as still conditions. Flasks were removed at different intervals of incubation viz. 2, 6, 10, 12, 24, 48, 72, and 96 hours and centrifuged at 10,000 xg for 20 minutes. Enzyme activity was assayed in cell supernatant (200 μl) under the conditions mentioned earlier.
3.20.2 Medium

Different media viz. Nutrient broth, synthetic medium (SM), SM + Glucose, SM+Glucose + Yeast extract, SM + TMTD, Nutrient broth + TMTD, SM + Glucose + TMTD and SM + Glucose + Yeast extract + TMTD were inoculated and incubated at 28 ± 2°C. Enzyme activity was assayed in the cell free supernatant obtained from different media.

3.20.3 Incubation temperature

Effect of different temperature of incubation i.e. 20, 28, 37 and 45°C on the production of enzyme degrading TMTD was studied by growing the organism at these incubation temperatures on rotary shaker. Enzyme activity was assayed by measuring the TMTD degradation.

3.20.4 pH

The production of enzyme was studied at different pH values viz. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5. Enzyme activity was assayed in the cell free supernatant by the similar procedure as described.

3.20.5 Inoculum size

Synthetic medium containing TMTD (200 µg/ml) was inoculated with different inoculum sizes ranging from 1% to 10%. The cultures were grown at 28°C for 96 h. The enzyme activity was assayed as described earlier.

3.20.6 TMTD concentration

TMTD concentration was varied in the production medium to see its effect on enzyme production. Different TMTD concentrations used were
0, 50, 100, 200, 250 and 500 μg/ml. Enzyme activity was assayed in cell free supernatant at all concentrations.

3.21 Factors affecting production of enzyme degrading sevin

All those parameters like incubation time and aeration, medium, incubation temperature, pH, inoculum size, pesticide concentration that were studied to maximize production of enzyme degrading TMTD were also studied for this enzyme similarly using same procedures.

3.22 Purification of enzyme degrading TMTD

The organism *Pseudomonas putida* capable of degrading TMTD was grown in synthetic medium containing TMTD (100 μg·ml⁻¹) at 37°C for 60 h an a rotary shaker (90 rpm) for the production of enzyme. The cultures were centrifuged at 10,000 x g for 20 minute in a refrigerated centrifuge at 4°C. The supernatant fluid was collected for enzyme studies and the purification of the enzyme was carried out according to the following scheme.

```
<table>
<thead>
<tr>
<th>60 h old Cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Centrifuge at 10,000 x g for 20 min at 4°C</td>
</tr>
<tr>
<td>Pellet (discarded)</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation (50% saturation)</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Centrifugation at 20,000 x g for 20 min</td>
</tr>
<tr>
<td>Supernatant</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation (70% saturation)</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>centrifugation at 20,000 x g for 20 min. ---</td>
</tr>
<tr>
<td>↓</td>
</tr>
<tr>
<td>Dissolved in TES-Tris buffer (pH=7.0)</td>
</tr>
</tbody>
</table>
```
Desalting through sephadex G-25 column equilibrated with TES-Tris buffer

Desalted preparation applied to mono cation exchange column (1X5 cm) equilibrated in TES-Tris buffer

Elution by a linear 0-0.5 M NaCl gradient in TES-Tris buffer (1.0 ml fraction collected).

Separation of fraction having enzymatic activity, pooled, concentrated and applied to sephadex G-200 column (1.5 x 80cm ) equilibrated in 0.15 M NaCl in TES-Tris buffer, pH 7.0, 2 ml fractions were collected.

3.22.1 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels (10%) according to the method used by Laemmli (1970). Gels were stained with 0.25% coomassie Brilliant Blue. Molecular weight markers used were lysozyme (14,400), trypsin inhibitor (20,100), carbonic anhydrase (30,000), egg albumin (43,000), bovine albumin (67,000) and phosphorylase b (94,000) for determining the molecular weight of the enzyme protein.

3.23 Enzyme kinetics

Standard assay conditions were used for enzyme degrading TMTD and various characteristics as pH, temperature optimum, reaction time, substrate specificity, michaelis' constant, reaction to enzyme inhibitors, metal ions and thermal inactivation were studied.

3.23.1 Temperature

The effect of temperature on enzyme activity was studied in the reaction mixture consisting of substrate (100 µg TMTD), enzyme (200 µl) and phosphate buffer (1.8 ml). Temperature at which enzyme reaction
proceeded was varied as 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75°C. TMTD degradation at the end of enzyme reaction was measured spectrophotometrically.

3.23.2 Reaction time

The effect of reaction time on the enzyme activity was studied by incubating the reaction mixture for different duration of time (1 to 30 minutes). The contents of the reaction mixture were kept same and enzyme activity was measured at the end of incubation time.

3.23.3 pH

Different buffer systems were used with different pH values (3-10) to study the effect of pH and buffer type on enzyme activity. Citrate-phosphate buffer (pH 3.0-8.0), phosphate buffer (pH 4.0-8.0), Tris-HCl buffer (pH 8.0-9.0) and Borate buffer (pH 10.0). Substrate concentration used was 100 μg (TMTD) and 200 μl of enzyme was used.

3.23.4 Substrate concentration

The activity of enzyme degrading TMTD was studied by determining the reaction rate at various concentrations of TMTD ranging from 20 μM to 120 μM (Michaelis and Menton, 1913). Other assay conditions were kept the same. The km value for the enzyme was determined by the double reciprocal plot method of Lineweaver and Burk (1934).

3.23.5 Metal ions and enzyme inhibitors

The effect of several metal ions and protein modifiers (enzyme inhibitors) was studied at varying concentrations on enzyme activity. The metal ion or enzyme inhibitor was mixed with the enzyme at the desired
concentration and incubated at room temperature (25°C) for 30 minutes. At the end of incubation enzyme activity was determined with standard assay procedure.

3.23.6 Thermal inactivation

The experiment was performed with same 0.2 ml of enzyme. The enzyme was added into 1 ml of distilled water in a screw capped tube and the tubes were kept in a water bath at constant temperatures and time intervals. The effect was studied at temperatures 50, 55, 60, 65, 70, and 75°C. After heating, the tubes were quickly transferred to ice-water bath and residual enzyme activity was assayed.

3.24 Purification of enzyme degrading sevin

The organism *Pseudomonas cepacia* was grown in the synthetic medium containing sevin (200 μg ml⁻¹) at 37°C for 72-80h on a rotary shaker (90 rpm) for the production of enzyme. The procedure for purification is outlined as under:

- 72 h culture of *P. cepacia* in synthetic medium with 200 μg ml⁻¹ sevin
- Centrifugation at 10,000 x g for 20 min at 4°C
- Cell pellet
- Supernatant discarded
- Sonication for 20 min at 10,000 mA (4 x 5 min. with 1 min. interval) at 0°C
- Centrifuged at 25,000 x g for 20 min. at 4°C
- Supernatant fluid
- Precipitate discarded
- Precipitated with MnCl₂ (2M) for 20 min.
The cell pellet was sonicated at 10,000 mA for 20 min. and cell free extract was subjected to MnCl₂ (2M) precipitation for 20 min. for each 19 ml of supernatant, 1 ml MnCl₂ solution was added. The clear solution was subjected to ammonium sulphate precipitations at 60 % saturation. The solution was stirred at 4°C for 30 minutes and then centrifuged at 20,000 xg for 20 min. at 4°C. The precipitate was redissolved in TES-Tris buffer [(10 mM Tris - 10 mM N-tris (hydroxy methyl) methyl-2-aminoethane sulfonic acid) mixed to obtain a pH of 7.0 at 25°C]. The solution was desalted by passing through sephadex G-25 column equilibrated with TES-Tris buffer. The desalted preparation was passed through sephadex G-200 using TES-Tris buffer equilibrated in 0.15 M NaCl (pH = 7.0) 2 ml fractions were collected.

3.24.1 SDS-PAGE

SDS-PAGE was carried out similarly as in the case of purification of TMTD degrading enzyme and same molecular weight markers were used (3.22.1).
3.25 Enzyme kinetics

Standard assay conditions were used for enzyme degrading sevin and various characteristics such as pH, temperature optimum, reaction time, substrate specificity, Michaelis' constant, reaction to enzyme inhibitors, metal ions and thermal inactivation were studied in a similar manner as in case of purified TMTD degrading enzyme (3.23).

3.26.1 Detection and isolation of plasmid DNA

To isolate plasmid DNA from microorganisms, a modified method of Chaudhry and Huang (1988) was used. A cell pellet from 500 ml of culture was suspended in 5 ml of lysis buffer containing 20 mM Tris-HCl (pH 8.0), 0.4 M sucrose, and 10 mM EDTA (pH 8.0) in a centrifuge tube. The suspended cells were mixed with 5 ml of lysis buffer containing 5 mg of lysozyme per ml and 100 μg of RNase per ml and incubated at 37°C for 10 min. To the clear lysate, 20 ml of 0.2 M NaOH and 1.0 % SDS were added, mixed gently by inverting the tube and incubated in ice bath for 10 min. Chromosomal DNA was precipitated by adding 10 ml of cold 3 M sodium acetate solution (pH 4.5) and maintaining the mixture at 4°C for 30 min followed by centrifugation at 10,000 xg for 15 min. The supernatant was extracted with 5 ml chloroform-phenol (buffered with 100 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0) (1 : 1, v/v) and centrifuged. The clear aqueous top layer containing plasmid DNA was carefully transferred to a fresh centrifuge tube, extracted with 5 ml of cold ethyl ether, and mixed with iso-propyl alcohol (0.6 volumes) and incubated at RT for 20 min. Plasmid DNA was then obtained by centrifugation at 10,000 xg for 10 min., washed with 70 % ethanol and dried in vacuum. The dried DNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM
EDTA; pH 8.0). Restriction endonuclease digestion of plasmid DNA was performed by following the instructions of the manufacturer and the digests were analysed by electrophoresis on 0.7 % agarose gel in E buffer (Chaudhry et al., 1984).

3.26.2 Curing experiments

Pesticide degrading organisms were inoculated in LB broth containing varied concentrations of Acridine orange (filter sterilized) ranging from 0 µg/ml to 200 µg/ml with a step of 5 µg.

The experiment was put in triplicate tubes. The cultures were incubated at 28°C for 24-48 hours under shaking conditions and the minimum inhibitory concentration of acridine orange was calculated for both the organisms by evaluating growth at different concentrations.

Single colonies were inoculated in LB broth containing desired concentration of acridine orange (a concentration lower than MIC). The cultures were incubated for overnight growth at 28°C. Appropriate dilutions were spread on LB agar plates. Single colonies were picked up and tested for resistance to TMTD or sevin respectively.

3.27 Products of enzymatic hydrolysis of sevin

Purified enzyme preparation (2 mg protein) was incubated with 100 ml of phosphate buffer (pH 7.0, 0.1 M) containing sevin (0.2 mM) at 50°C for 10 min. The reaction mixture was extracted with one volume of ether. The ether extract was dried with anhydrous sodium sulphate and evaporated in vacuum. The ethereal residue was analysed by TLC in different solvent systems.
3.27.1 Identity of side chain of sevin

Sevin hydrolysis with purified enzyme produced 1-naphthol. Thus side chain (n-methylcarbamic acid) is released in the reaction mixture. This compound is highly unstable and decomposes to carbon dioxide and methylamine (Herrett, 1969). Methylamine detection was performed in the reaction mixture to confirm the release of N-methylcarbamic acid, which is one of the end products of N-methylcarbamic acid degradation. The assay system contained 60 ml of 0.2 mM aqueous solution of sevin, 20 ml of phosphate buffer (pH 7.0, 0.1 M), and enzyme (2 mg of protein) in distilled water to make 98 ml. The mixture was incubated at 30°C for 30 min. The mixture was not incubated at 50°C (optimum temperature for enzyme activity) since it caused loss of methylamine vapours. Reaction was terminated by adding 2 ml of 1N HCl. After estimating 1-naphthol, the reaction mixture was extracted thrice with equal volumes of dichloromethane to remove 1-naphthol and unhydrolysed sevin. Methylamine was concentrated and estimated by the procedure of Ormsby and Johnson (1950).

3.27.2 Thin Layer Chromatography of the Intermediate products of sevin degradation

The intermediate products of sevin degradation viz 1-naphthol, 1,2-naphthoquinone, salicylate, and catechol, were identified by chromatography in different solvent systems. Etheral extracts were chromatographed on 0.25 mm layers of silice Gel G (E. Merck) activated at 105°C for 30 min, however, for preparative TLC, 0.5 mm thick layers were used. The chromatograms were developed in different solvent systems to a level
of 15 cm from the point of application of sample. The solvent systems were:

1. Acetone : toluene : hexane (1 : 1: 3)
2. Acetone : hexane (1 : 5)
3. Chloroform : acetonitrile (4 : 1)
4. Benzene : dioxan : acetic acid (90 : 25 : 4)
5. Diethylether : hexane (4 : 1)

Detection reagents

Phenolic compounds were detected with methanolic para-nitrobenzene diazonium tetrafluoroborate (Bollag and Liu, 1971) or diazotized benzidine. Carbamate moiety was detected by spraying with 2 percent ninhydrin in pyridine (Oonnithan and Casida, 1968). Salicylate was detected by spraying with Trinder's reagent (Trinder, 1954).

3.27.3 Manometric experiments

1-naphthol, 2-naphthol, salicylate, salicylaldehyde, catechol, and cis, cis-muconic acid were checked for as possible intermediary metabolites of sevin by the method of Stanier (1947). Oxygen uptake experiments in the presence of these compounds were conducted in the conventional warburg constant-volume apparatus (Veb Glaswerk, Stutzerbach, East Germany) at 30°C according to Umbreit et al. (1964) using 13 ml single side arm flasks with air as gas phase. The main compartment of each flask contained 1 ml bacterial suspension (4 mg dry weight) in 0.1M phosphate buffer, pH 7.0, and 1 ml distilled water. Centre well contained 0.2 ml of 20 percent KOH with alkali resistant filter paper to absorb carbon dioxide. The side arm contained 1 ml of 1 mM solution of substrate in distilled water.
neutralized to pH 7.0. Endogenous oxygen uptake was measured in flask containing distilled water in place of substrate solution.

3.27.4 Hydroxylation of 1-Naphthol to 1,2-Dihydroxynaphthalene (1,2-DHN) and isolation of 1,2-Naphthoquinone

Cells were harvested from the logarithmic growth phase from sevin-salt medium and suspended in 1- or 2-naphthol-salt medium (cell density 2.0 at 600 nm), and incubated in a water bath at 30°C with occasional shaking. After 30 min. of incubation, 1,2-DHN was detected (Arnow, 1937).

1,2-Naphthoquinone was isolated from 1-naphthol-salt medium. Two litres of salt medium containing 125 μg of 1-naphthol per ml was inoculated with whole cells of the isolate (Cell density 2.0 at 600 nm) and incubated at 30°C in a water bath. When the test was highly positive for 1,2-naphthoquinone and negative for 1-naphthol, the cells were removed by centrifugation. The supernatant was extracted with three 500 ml quantities of acetone. Acetone extracts were combined, treated with anhydrous sodium sulphate, and dried under vacuum. The residue was recrystallised from benzene or alcohol and its melting point determined. Melting point of the mixture of the residue and authentic 1,2-naphthoquinone (Eastman Kodak, New York) was also noted. The residue was tested for all colour reactions characteristic of 1,2-naphthoquinone (Radt, 1952) such as green coloration with concentrated sulphuric acid and blue colour with ferroferricyanide solution. The residue was reduced with hydroiodic acid and tested for two hydroxal groups on an aromatic ring (Arnow, 1937, and Nair and Vaidyanathan, 1964).
3.27.5 Degradation of 1,2-DHN

Degradation of 1,2-DHN by 1,2-DHN oxygenase of the isolate was investigated by the procedure of Davies and Evans (1964). Four quantities of 1,2-DHN (25 mg each deposited on filter papers from an acetone solution of 1,2-DHN) were added to 40 ml of the enzyme solution (5 mg of protein per ml) obtained from 1-naphthol-grown cells in 400 ml of disodium hydrogen phosphate-potassium dihydrogen phosphate buffer (pH 6.5, 0.05M) containing ferrous sulphate (1.0 mM) at 30°C under shaking conditions. Five min. after the final addition of the substrate, the reaction mixture was processed according to Davies and Evans (1964) for the formation and isolation of perchlorate derivative of cis-o-hydroxybenzalpyruvate. Its melting point and colour reaction were determined (Davies and Evans, 1964).

3.27.6 Degradation of sevin and 1-naphthol to salicylate and isolation of salicylate

Salicylate was accumulated by whole cells of the isolate in the medium of Bushnell and Haas (1941) containing 0.5 g each of ferrous sulphate, and calcium chloride per litre (according to Klausmeier and Strawinski, 1957) in the presence of sevin or 1-naphthol. Two sets of flasks containing 150 ml of the modified salt medium having either 100 μg of 1-naphthol or 500 μg of sevin per ml were inoculated with a logarithmic phase culture of the isolate grown on sevin-salt medium to give an initial absorbance of 0.2 at 600 nm. The flasks were incubated at 28°C. Appropriate samples were drawn every 4 hrs and absorbance recorded at 600 nm. Simultaneously, sevin, 1-naphthol, and salicylate (Trinder, 1954) were estimated. The pH of the sample was also recorded.
For isolation of salicylate (from the degradation of 1-naphthol by the isolate) identical culture conditions were employed except that 1.5 litres of the medium was used. When the test for salicylate was maximum, cell and cell debris were removed by centrifugation. The supernatant was acidified with sulphuric acid and was extracted thrice with 300 ml aliquots of ether. The etheral extracts were pooled and extracted thrice with 25 ml aliquots of 10 percent sodium carbonate solution. Combined sodium carbonate extracts were acidified and extracted with ether. The ethereal extract was dried with anhydrous sodium sulphate, filtered, and evaporated to dryness. The residue was recrystallised from hot water and its melting point recorded. The melting point of the mixture of crystallised residue and authentic salicylic acid was also determined.

The TLC analyses of the crystallised-residue and authentic salicylic acid were performed in different solvent systems such as benzene, dioxan, acetic acid (90 : 25 : 4); diethylether, hexane (4 : 1) and chloroform, acetonitrile (4 :1).

3.27.7 Degradation of salicylate to catechol

Sevin degrading isolate was inoculated into salt medium containing 0.1 per cent salicylate as substrate. Atleast 2 g (wet weight) of cells were harvested per litre of medium after intermittently adding substrate. The cells were disrupted in an ultrasonicator. The ultra-sonic preparation was incubated in a shaker water bath in Tris-HCl buffer (pH 9) with salicylate as described by Yamamoto et al. (1965). At intervals, disappearance of salicylate was followed by Trinder’s method (1954). When about 50 per cent of salicylate had disappeared, the reaction mixture was precipitated with 6 ml of 4N HCl, and the precipitate was removed by centrifugation.
The residue was isolated and recrystallised as described by Yamamoto et al. (1965). The melting points of the residue, authentic catechol (BDH, India), and the mixture of the two were recorded. The TLC analyses of the residue and authentic catechol were performed in different solvent systems.

3.27.8 Degradation of catechol to cis-Muconic acid

Catechol 1,2-oxygenase (pyrocatechase, EC No. 1.99.2.2) was assayed by the procedure of Hegmeman (1966). The reaction mixture contained 4 μM of EDTA, 0.3 μM catechol, and 200 M of phosphate buffer pH 7.0 and cell-free extract (200 μg protein) of the isolate grown on catechol. The total volume of the reaction mixture was 3 ml. The absorbance was measured every 2 min. at 260 nm at 25°C with spectronic 21 spectrophotometer.

Two g (wet weight) of cells harvested from catechol salt medium were disrupted in an ultrasonicator. The ultra-sonic preparation, 2 mM of catechol, 0.16 mM of EDTA and 150 ml of M/15 phosphate buffer, pH 7.2, were together incubated aerobically at 30°C according to Grant (1970). When the catechol test was negative, the pH of the reaction mixture was lowered to 2.5 and the precipitate removed by centrifugation and extracted with ether. The combined ethereal extracts were dried with solid anhydrous sodium sulphate. The cream coloured residue was purified by basification, washing with ether and acidification (Elvidge et al., 1950). The melting points of the residue, and the admixture of residue with authentic cis, cis-muconic acid were recorded.

Cis, cis-Muconic acid was synthesized from re-distilled phenol by oxidation with peracetic acid (Elvidge et al., 1950). Catechol was determined by the method of Nair and Vaidyanathan (1964).
3.27.9 Degradation of cis-cis-Muconate to (+)-Muconolactone

Cell-free extract of catechol-grown cells of the isolate was assayed for lactonizing enzyme [EC No. 5.5.1.1. (+) - 4-carboxymethyl-4-hydroxy isocrotonolactone lyase (decycling)]. The assay was essentially that of Sistrom and Stanier (1954) as modified by Hegmeman (1966). The reaction mixture contained 200 μM of Tris-HCl buffer (pH 8.0), 0.3 μM cis, cis-muconic acid (sodium salt), 3.0 μM MnCl₂, and enzyme extract (200 μg of protein). The disappearance of cis, cis-muconate was followed after every 2 min. at 260 nm at 25°C with spectronic 21 spectrophotometer.

3.27.10 Degradation of catechol to β-keto adipic acid

Catechol grown cells (2 g wet weight) previously ultrasonicated were incubated on a rotary shaker water bath at 30°C with 2 mM of catechol in 100 ml of 0.1 M phosphate buffer of pH 7.2. When Rothera test (1966) was strongly positive and catechol test (Nair and Vaidyanathan, 1964) negative, the mixture was extracted several times with ether. Ethereal extract was dried and the residue was recrystallised from ethylacetate-light petroleum ether. The melting point of the crystals was recorded. The melting point of the admixture of recrystallised residue and authentic β-keto adipic acid (Sigma Chemical Co, USA) was also determined.

The residue was decarboxylated by heating above its melting point and the oily residue was processed for the preparation of 2,4-dinitrophenylhydrazone (Allen, 1930). The melting point of the derivative was determined.

3.28 Mutagenicity testing of pesticides

The standard mutagenicity test system developed by Maronn and Ames (1983) was carried out to study the mutagenicity of the pesticides.
used in the study, TMTD and sevin. Histidine negative *Salmonella typhimurium* tester strains TA 98, TA 98 NR and TA 98/1, 8-DNP₆ were used. For the actual assay, 25 and 100 µg of TMTD and sevin were dissolved in 0.1 ml of dimethylsulphoxide (spectroscopic grade), 0.1 ml of a 16 h bacterial culture (equivalent to 2-4 x 10⁸ cells) and 0.5 ml phosphate buffer pH 7.4, or S9 mix was mixed with top agar containing 0.1 µM histidine and 0.1 µM biotin and plated on minimal glucose agar plates according to the procedure. After incubating the plates for 2 days at 37°C the revertant colonies were counted. For each dose, the plates were set up in duplicate. The specific mutagenic activities were obtained from the slope of the linear section of the dose response curves. The spontaneous revertant frequencies in the absence (-) and presence (+) of S9 per plate were TA 98-18, +23; TA98NR - 31; and TA98/18 - DNP₆ - 14.

### 3.29 Reversal of the effect of pesticides on soil biological processes and soil enzymes

Different concentrations of both the pesticides TMTD and sevin; separately and in combination were mixed in soil as described earlier along with respective substrate for each process. In addition, the respective pesticide degrading organisms were also added to the soil (1 x 10⁶ cells. g⁻¹ soil). Soil samples were taken similarly at different intervals of time and estimation were done as described earlier under each soil biological process. Soil enzymes were also estimated accordingly. In case of symbiotic nitrogen fixation, the pesticide degrading cultures were added along with specific *Rhizobium* sp.
3.30 Determination of median effective dose (ED$_{50}$)

In the tests of pesticidal applications, for example, the distribution of tolerance concentration of the toxic-agent is seldom symmetrical, because all the organisms will be differently tolerant to pesticides. Thus if the effect is expressed in terms of logarithms of the concentrations instead of the absolute values, it will give a normal distribution and a graph of percentage responding against dose will give a steadily rising curve following a sigmoid pattern. The median effective dose that will produce a response in half the population is commonly referred to as ED$_{50}$, the more restricted concept of median lethal dose as the LD$_{50}$. ED$_{50}$ can usually be estimated more precisely than those for more extreme percentage levels.

ED$_{50}$ was calculated according to the method given by Finney (1977) by first plotting the percentages (percent kill) against log of concentrations. This gave a normalised sigmoid curve. The percentages were then transformed to probits (Y) according to the standard tables given by Fisher and Yates (1964) and plotted against log of concentrations which gave a straight line curve. ED$_{50}$ was estimated from the line as the dose at which Y=5 (equivalent to 50% effect).

3.31 Statistical analysis

The data pertaining to nitrification, ammonification, symbiotic nitrogen fixation and soil enzymes and reversal of the effect on these processes were subjected to statistical analysis by using Completely Randomized Block Design (Panse and Sukhatme, 1978). The combination of time of incubation and doses of pesticide were regarded as treatments.