4. MATERIALS AND METHODS

4.1 SELECTION OF STUDY SITES AND SURVEY ON PESTICIDE USE PATTERN

The cotton, groundnut and vegetables cultivated lands of three different talukas viz. Rajkot, Gondal and Jetpur were selected as study sites. The criteria used to select these study sites were that the sites had extensive cultivation of cotton, groundnut and vegetables, and the known history of repeated use of endosulfan and chlorpyrifos as pesticides.

The qualitative as well as quantitative surveys were performed on the use-pattern of various pesticides as plant protection chemicals. The survey is based on the information provided by 30 randomly selected farmers from each of the three talukas selected as study sites. The qualitative survey was performed to determine the extent of different formulations of pesticides being used under different crops. Since the different formulations of pesticides differ in their potential of bioaccumulation, persistence and environmental hazards, hence it becomes important to know the formulations of pesticides being used under the current agricultural practices. Also, the quantitative survey was performed on the use-pattern of pesticide to determine the volume of a particular pesticide being consumed in the selected study sites.

4.2 COLLECTION OF SOIL SAMPLES

The soil samples were collected from three different talukas (administrative blocks) of Rajkot district of Gujarat State. The soil samples were collected from Rajkot taluka, Gondal taluka and Jetpur taluka. The samples were collected from agricultural fields growing mainly cotton, groundnut and vegetables. A total of 30 composite soil samples were collected from 30 different agricultural lands (10 random samples from the same site) having history of repeated endosulfan and chlorpyrifos applications. The details of site specific information are described in the section 1.13.

The soil samples were collected by using auger up to a depth of 15 cm. The collected samples were air dried, ground, passed through 2 mm sieve and stored in the sealed plastic bags at room temperature. These stored samples were used for further experimentation.
4.3 PESTICIDES, CHEMICALS AND REAGENTS

Based on the use-pattern of the pesticides in the Rajkot district of Gujarat State, two pesticides were selected in the present study. One pesticide was a technical grade of endosulfan (96%), a cyclodiene organochlorine and the other was chlorpyrifos (97%), an organophosphorus. Both of these pesticides were purchased from the local pesticide suppliers in Rajkot district of Gujarat State. The details of these pesticides are described in the sections 1.6 and 1.7.

The analytical grade chemicals and reagents used in this work include Hydrogen peroxide, Sodium hydroxide, Potassium dichromate (1N), Diphenylamine indicator (0.02%), Phenolphthalein indicator, Ortho-phosphoric acid (85%), Ammonium thiocyanate, Sulphuric acid, Agar agar, etc. from Hi-media, and Glacial acetic acid, Hydrochloric acid, Sodium chloride, Ferrous ammonium sulfate (0.5N), Silver nitrate, Sodium fluoride, etc. from Qualigens. The details of other chemicals and reagents used in this work are as mentioned in the respective sections.

4.4 CULTURE MEDIA USED

The different culture media viz. Nutrient Broth, Luria Bertani (LB) and M9 were used during the present study. The LB medium was prepared by mixing tryptone 10g, sodium chloride 10g and yeast extract 5g in 1L distilled water, and the medium pH was adjusted in the range of 7.0 – 7.2. For semi-solid LB medium, 1.5% agar was added while for LB broth agar was not added to the medium. Then the medium was autoclaved at 121°C temperature, 15 psi pressure for 20 minutes.

The M9 medium was prepared by adding di-sodium hydrogen phosphate 6g, potassium dihydrogen phosphate 3g, sodium chloride 0.5g and ammonium chloride 1g in 1L distilled water and the medium pH was adjusted in the range of 7.2 – 7.4. For semi-solid M9 medium, 1.5% agar was added while for liquid medium agar was not added to the medium. Then the medium was autoclaved at 121°C temperature, 15 psi pressure for 20 minutes. To this medium after autoclaving, filtered sterilized 10ml glucose (20%), and separately autoclaved 2ml magnesium sulfate (1M) and 0.1ml calcium chloride (1M) were added.
4.5 STERILIZATION OF MEDIA, SOLUTIONS AND APPARATUS

In the present work, media and solutions were sterilized by autoclaving at 121°C temperature, 15 psi pressure for 20 minutes. The glassware and other apparatus were sterilized in an oven at 180°C for an hour. After sterilization, the media and solutions were cooled to room temperature and then stored under refrigeration for their subsequent use. The sterilized glassware were stored separately in an oven at 60°C and cooled to room temperature before their subsequent use.

4.6 ISOLATION AND SCREENING OF PESTICIDE-RESISTANT BACTERIA

The enrichment culture technique was used for the isolation of bacterial strains capable of utilizing endosulfan and/or chlorpyrifos as a sole source of carbon and energy. Pesticide mixed media were prepared by thoroughly mixing different volumes of technical grade pesticides with 100 ml media (LB, M9 and N-broth) as a sole carbon source, when the media were about to solidify (45-50°C). Different concentrations ranging from 5 to 200 mg/L of endosulfan and chlorpyrifos were added and checked for the growth of bacteria.

One gram of each soil sample was suspended in 9 ml of distilled water and kept at room temperature for 24 hours. On the next day, 500 μl of the supernatant was spread on insecticide containing N-agar, LB and M9 petridishes, for the isolation of pesticide resistant bacteria. Solid N-agar, LB and M9 media were prepared, in which pesticide instead of glucose was added at a final concentration of 100mg/L. The samples were spread on N-agar, LB and M9 media containing different concentrations of ES and CP separately and the petridishes were incubated at room temperature and 37°C.

The petridishes were observed on the next day to till fourth day for the appearance of resistant colonies. The M9 medium plates containing separately endosulfan and chlorpyrifos concentration ranging from 5 to 200 mg/L were streaked with pesticide resistant colonies and incubated at 37°C for 7 days. The concentration at which the isolate failed to grow even after 7 days of incubation was considered as minimum inhibitory concentration (MIC) for the given bacterial isolates.
4.7 PURIFICATION OF POTENTIAL BACTERIAL ISOLATES

A single isolated colony of the pesticide degrading bacteria was picked up with the help of sterilized wire loop and was streaked on LB agar medium. Each isolated strain was streaked at least 3 to 4 times on LB agar plates for purification. After the purified isolates were obtained, they were re-streaked on M9 agar medium containing pesticides (endosulfan and chlorpyrifos) for confirmation of isolates. The single colony of bacterial strain was inoculated in 100mL LB broth, incubated at 37°C then used for further characterization of isolates. The isolated and purified bacterial strains were stored under refrigeration after preparing slants.

4.8 DETERMINATION OF BACTERIAL POPULATION IN SOIL

The population of selected bacterial isolates in terms of colony forming units (CFUs) was determined using viable plate count technique. This technique assumes that each colony is derived from an individual cell and that the incubation conditions allowed the recovery of all cells present. On solid media after incubation, the resultant colonies may be counted.

The soil samples, from which potential bacterial strains were isolated, were used for determining bacterial population per gram of soil. One gram of soil was properly dissolved in 9ml of sterile distilled water and diluted to $10^{-3}$ and $10^{-5}$ using the sterile distilled water. From these two dilutions, 0.1 ml portion was used to spread the prepared plates. The plates were incubated at room temperature for 48 hours.

A fresh pipette was used for each dilution and the work was performed in the laminar air flow cabinet, under sterile condition. The N-agar plates supplemented separately with endosulfan (10 mg/L), chlorpyrifos (10 mg/L) and endosulfan plus chlorpyrifos (10 mg/L of each) were used. Also, N-agar plates not supplemented with any pesticide were used as control to determine the total bacterial count in the untreated soil. The bacterial cells visible to the naked eyes were counted in terms of CFUs. All the plating was performed in triplicates and results were represented as mean. The viable count was obtained from this value by reference to the serial dilution used.
4.9 CHARACTERIZATION AND IDENTIFICATION OF BACTERIAL ISOLATES

**Colony Morphology**

For the selected bacterial isolates, colony morphology was observed by growing them on N-agar plates. Morphological characters viz. size, shape, surface, opacity, texture, elevation and pigmentation were determined by visual observation as well as by using light trans-illuminator and microscopy.

**Gram Staining**

The Gram staining technique was used for differentiation between gram positive and gram negative bacterial strains according to Benson (1994). A drop of sterile distilled water was placed on a neat and clean glass slide, and a single isolated colony of 24 hours old culture was mixed in it. The smear was made by spreading the culture. This smear was air dried and fixed by rapidly passing the slide three times over the flame. It was then inundated with crystal violet (2 g crystal violet, 9.5 mL ethanol and 0.9 g ammonium oxalate per 100 mL distilled water) for 2 minutes and then washed off with distilled water for 5 seconds. Then gram’s iodine solution (20 g KI and 10 g I₂ in 100 mL distilled water) was added to the smear and the glass slide was left for one minute. This step was followed by the application of decolorizing agent (ethanol), a single wash for few seconds. Decolorizing agent was immediately washed with distilled water and the smear was covered with safaranine (1.25 g safaranine and 10 mL 95% ethanol in 90 mL distilled water) for 30 seconds. The slide was washed with distilled water; air dried and was observed under the microscope.

**Growth on McConkey Agar**

The single colony of each isolate was streaked on Mac Conkey agar (5%) plates and incubated at 37⁰C overnight (Cheesbrough, 1993). Appearance of growth after 24 hours indicates lactose fermenting capability of the isolate.

**Starch Hydrolysis**

The single colony of each isolate was streaked on starch hydrolysis media plates (prepared by mixing 0.3 g soluble starch per 100 mL of nutrient agar and autoclaving the medium) and incubated at 37⁰C overnight. Next day, Gram’s iodine
solution was poured on these plates and the plates were kept for one hour at room
temperature (Benson, 1994). Appearance of clear area around the growth showed
positive results, while blue color indicated that starch is not hydrolyzed.

**Motility Test**

Motility of the organisms was checked with hanging drop method. A
suspension in sterile distilled water was made for each bacterial isolate and glass slide
with pit was used to hang the drop of bacterial suspension using cover slip. Motility of
the organisms was observed under the microscope.

**Oxidase Test**

A few drops of freshly prepared oxidase reagent (0.1g tetramethyl-p-
phenylenediamine dihydrochloride in 10 mL distilled water) were added on a piece of
filtered paper in a clean Petri plate. Using a glass rod, a colony of the isolate was
smeared on the filter paper and was observed for any change in the color of the
reagent (Benson, 1994).

**Catalase Test**

A thick growth of test culture was immersed in 3ml of 3% H₂O₂ solution with
the help of a sterile glass rod. Active bubbling within a few seconds showed a positive
catalase activity, otherwise the test is negative.

**Coagulase Test**

On a clean glass slide, 2 drops of 0.85% saline solution were taken and the test
tube culture was smeared on one of them. Then a single drop of human blood plasma
was added to both the drops and the slide was observed under a dissecting microscope
for rapid agglutination of plasma (Cheesbrough, 1993).

**Urease Test**

The slants of Christian urea agar (0.1 g peptone, 0.5 g sodium chloride, 0.2 g
di-calcium hydrogen phosphate, and 600μl phenol red in 100mL distilled water, pH
adjusted to 6.8-6.9 and then 2 g agar was added and medium was autoclaved). After
autoclaving, 1 mL filter sterilized 10% glucose and 10mL filter sterilized 20% urea
added to the medium. It was readily poured into sterilized test tubes to make slants
which were streaked with the bacterial isolates and incubated at 37\(^\circ\)C overnight (Benson, 1994). Appearance of pink color showed positive test, otherwise the test is negative.

**Methyl Red Test**

The medium was prepared by dissolving 5 g peptone and 5 g K\(_2\)HPO\(_4\) in 1L distilled water, pH adjusted at 7.6 and poured in test tubes. The medium was sterilized by autoclaving at 121\(^\circ\)C, 15 psi for 20 minutes. Then 0.25 mL glucose solution was added in 5 mL medium in each tube. The above media were inoculated with respective isolates and incubated at 37\(^\circ\)C for 48 hours. Then 5 drops of methyl red (0.1 g methyl red and 300 mL ethanol in 100 mL distilled water) were added and mixed. The bright red color indicated the positive test, otherwise the test is negative (Cheesbrough, 1993).

**Citrate Utilization**

The Simmon’s citrate medium was prepared [NaCl 500mg, MgSO\(_4\) 200mg, NH\(_4\)H\(_2\)PO\(_4\) 100mg, K\(_2\)HPO\(_4\) 100mg, trisodium citrate 500mg and agar 2g, 0.2% bromothymol blue 4 mL, poured in test tube (5ml each), autoclaved], inoculated with respective bacterial isolate and incubated at 37\(^\circ\)C overnight (Benson, 1994). The change of color of medium from green to blue indicates positive result.

**Nitrate Reduction**

The protease-peptone-yeast extract-broth was prepared by dissolving 2g protease peptone, 1g yeast extract, 0.5g NaCl in 100 mL distilled water. Then 0.5 mL potassium nitrate solution (2%) was added and pH adjusted at 7.4. Then 5 mL medium was poured in each test tube and autoclaved at 121\(^\circ\)C, 15 psi for 20 minutes. This medium was inoculated with 50\(\mu\)l log phase culture of each bacterial isolate and incubated at 37 \(^\circ\)C for overnight (Benson, 1994).

After overnight incubation, 1 mL reagent-1 (0.5g sulphanillic acid and 30 mL glacial acetic acid in 100 mL distilled water) and 0.25 mL reagent-2 (0.2g Cleve’s acid in 120 mL distilled water, warming in water bath and then finally adding 30 mL glacial acetic acid to it) were added and the appearance of red color showed a positive result.
**Voges-Proskauer (V.P.) Test**

The medium for V. P. test was prepared by dissolving 0.5g peptone, 0.5g di-potassium hydrogen phosphate in 100 mL distilled water, pH adjusted at 7.6 and then 0.5g of dextrose was added. The 5 mL medium was added to a test tube and autoclaved at 121°C, 15 psi for 20 minutes. The medium was inoculated with respective bacterial isolate and incubated at 37°C for overnight. Next day, 1 mL 40% KOH and 3 mL 5% α-naphthol were added. The tubes were aerated for 30 minutes and the appearance of red colour indicates positive test.

**Indole Test**

The test medium was prepared by dissolving 2g peptone and 0.5g NaCl in 100 mL distilled water, pH adjusted at 7.4. The medium was poured in test tubes and autoclaved at 121°C, 15 psi for 20 minutes. The bacterial isolate was inoculated in the medium and incubated at 37°C for overnight. Next day, 500μl of Kovac’s reagent (isomyl alcohol 150 mL, p-dimethyl-aminobenzylaldehyde 10g, conc. HCl 50 mL) was added and shaken gently. The development of red color in the upper layer showed the positive test.

**H₂S Production Test**

The medium for H₂S production test was prepared by dissolving 3g lab lameco powder, 3g yeast extract, 20g peptone, 5g NaCl, 10g dextrose, 0.3g ferric citrate, 0.3g sodium thiosulphate, 0.05g phenol red in 1L distilled water, pH adjusted between 7.2-7.6, followed by addition of 12g agar. This medium (6ml) was taken in each test tube, autoclaved at 121°C, 15 psi for 20 minutes and slanted. The bacterial isolates were streaked on these slants and incubated overnight at optimum temperature of the isolate. Blackening of medium indicated H₂S production by the isolate.

**Sporulation Test**

The bacterial isolates were grown at 37°C on LB-agar medium for one week. The smear was prepared from this culture, air dried and fixed by passing on the flame. The smear was covered with malachite green (5%) solution for 3-4 minutes, by heating the slide with continuous steaming. Then, slide was washed with distilled water, air dried and examined under the microscope at 100x, oil immersion objective. The presence of green colored oval or spherical bodies indicated a positive result.
**Acid Release from Sugars**

This test is used to differentiate organism that ferment a particular sugar, consequently acid and/ or gas may be produced. The basal medium was prepared by dissolving 1g peptone and 0.5g NaCl in 100 mL of distilled water and 1.25 mL of bromothymol blue (0.2% w/v) as indicator (prepared by dissolving 0.1g bromothymol blue in 2.5ml of 0.1 mol/L (0.1N) NaOH + 47.5 mL of sterile distilled water). This medium was divided into different test tubes, 5ml each. A pair of test tubes was used for one isolate (for each sugar), one for oxidation and gas production and one for fermentation. These test tubes were autoclaved at 121°C, 15 psi for 20 minutes. After cooling, 250μl of 10% (w/v) filter sterilized respective sugar (glucose, lactose, sucrose) was added into basal medium. The bacterial isolate (50 μl, log phase culture in peptone water) was inoculated at the bottom of the test tube.

The inoculated medium of one tube was covered with 10 mm deep layer of sterile liquid paraffin for excluding oxygen to check the fermenting ability of the isolate for a particular sugar. The gas production is revealed by the formation of a void in the inverted (dipped) vial of the Durham tube. The test tubes were incubated at 37°C overnight and up to one week for the confirmation of test result. The appearance of yellow color in open tube showed acid production and green in covered tube (with paraffin layer) indicates organism is oxidative whereas, yellow color in both tubes indicates production of acid and organism is fermentative in nature. The presence of green or blue color in both tubes indicated no carbohydrate utilization and no acid production (Benson, 1994).

**4.10 PHYSICOCHEMICAL PROPERTIES OF SOILS**

The important physicochemical properties, viz. bulk density, porosity, soil moisture, soil pH, electrical conductivity, organic carbon, organic nitrogen and available phosphorus, of the soils used for the screening of bacterial isolates were determined. The details of these methods are as described in the following sections.

**Bulk Density and Soil Porosity**

The bulk density (g/cc) measurement is a valuable tool for understanding soil processes such as heat, water and nutrient exchange. Bulk density includes both the volume of the solid (mineral and organic) portion of the soil and the spaces where air
and water are found. Bulk density often depends on texture, structures and organic matter status of soil.

The Bulk Density of soil was measured by taking an undisturbed block of soil. This block of soil was dried at 105°C for 24 hours. The dried sample was then weighed in an electronic balance. The exact volume of soil was determined by measuring the cylinder volume. The calculation of cylinder volume was done by using the following equation which takes into account the height (h) up to which the soil is occupying the cylinder and the inner radius (r) of the cylinder. The calculation is done using the following equation:

\[
Volume \ of \ Soil \ Core = \pi r^2 h
\]

Bulk Density is thus calculated as:

\[
\text{Bulk Density} = \frac{\text{Weight of oven dried soil (g)}}{\text{Volume of soil core (cc)}}
\]

By knowing both the bulk density and particle density the amount of pore space or porosity of the soil can be calculated using the following equation:

\[
\text{Porosity (\%)} = 1 - \frac{\text{Bulk Density}}{\text{Particle Density}} \times 100
\]

Where, Particle Density refers to the ratio of mass of dry soil to volume of air dried soil.

**Soil Moisture**

When moist soil is heated at 105°C for about 48 hours, only the water, which had been absorbed or held within the soil, pores, is evaporated. There is no loss of water of crystallization and the oxidation of organic matter does not occur at this temperature. The soil moisture (%) was measured by gravimetric method.

Air dried 100 g soil sample was taken in a pre-weighted container and subjected to oven drying at 105°C for 48 hours. Then the soil sample was reweighted to account for changes in the weight due to moisture evaporation.
The Calculations for soil moisture is done by the following formula:

\[
\text{Soil moisture (\%)} = \frac{\text{Wet mass} - \text{Dry mass}}{\text{Dry mass} - \text{Container mass}} \times 100
\]

**Soil pH**

Traditionally soil pH is measured in a soil paste prepared by the addition of a dilute CaCl\(_2\) solution with an appropriate electrode. While achieving a pH measurement of the soil is relatively easy, interpretation of its affect on microbial processes is difficult. This is because concentrations of cations adsorbed to the surfaces of the negatively charged soil colloids are 10–100 times higher than those of the soil solution.

In the present work, for the measurement of pH, the dried soil sample was grounded finely using mortar and pestle and the coarse fraction was separated out using 2 mm sieve. The soil sample (10g) was mixed with boiling distilled water (40 mL) in the ratio of 1:5 (The International standard NF ISO 10390, 1994). Then the entire mixture was kept on an oscillating table for 5 minutes to secure proper agitation. The mixture was then allowed to settle and decanted to obtain the clear layer of water. By using a standard digital pH meter, the pH of the clear layer was measured three times and an average of these three readings was taken to minimize the error.

**Soil Electrical Conductivity (E.C.)**

For the measurement of E.C., the dried soil sample was grounded finely using mortar and pestle and the coarse fraction was separated out using 2 mm sieve. The soil sample (10g) was mixed with boiling distilled water (40 mL) in the ratio of 1:5 (The International standard NF ISO 10390, 1994). Then the entire mixture was kept on an oscillating table for 5 minutes to secure proper agitation. The mixture was then allowed to settle and decanted to obtain the clear layer of water. By using a standard \textit{HACH} sensor (conductivity meter), the E.C. the clear layer was measured three times and an average of these three readings was taken to minimize the error. The electrical conductivity of soil sample was recorded in mS/cm.
**Soil Organic Carbon and Nitrogen**

For the measurement of soil organic carbon and nitrogen, first of all standardization of ferrous ammonium sulphate solution was performed. Potassium dichromate (1N, 10mL) solution was taken in a 250 mL conical flask and 20mL of concentrated H$_2$SO$_4$ was slowly added to it. This solution was continuously swirled and after 10 minutes allowed to cool down. Then 200 mL of distilled water was added, followed by addition of 10mL of ortho-phosphoric acid and 1 mL indicator solution, followed by vigorous mixing. Then ammonium ferrous sulphate was titrated from the burette while continuously shaking the flask. The color change from blue to green indicates end point. This data is used as a blank reading.

The oven dried soil was ground, passed through 0.2 mm sieve and 0.5 gm of the soil sample was placed at the bottom of a 500 mL conical flask. Exactly 10 mL of 1N potassium dichromate solution was added and the flask was swirled gently to disperse the soil in the solution. Then 20 mL of concentrated sulphuric acid was added carefully from a measuring cylinder and allowed the flask to stand for 30 minutes. This was followed by addition of 200 mL of distilled water, 10 mL of orthophosphoric and 1mL indictor.

Finally, the content in the flask was titrated with ferrous ammonium sulphate solution till the colour changed from blue-violet to green, which indicated the end point of the titration. Also a blank without soil sample was run in parallel.

Soil organic carbon was calculated using following equation:

\[
\text{Organic Carbon} \% = \frac{0.03 \times (B - T)}{B \times S} \times 100
\]

Where,

- \( B \) = Volume of ferrous ammonium sulphate required for blank titration in mL.
- \( T \) = Volume of ferrous ammonium sulphate needed for soil sample in mL.
- \( S \) = Weight of soil sample in grams.
Soil organic nitrogen was calculated using following equation:

\[
\text{Organic Nitrogen (\%)} = 0.0862 \times \% \text{ Organic Carbon}
\]

**Available Phosphorus**

Phosphorus is extracted from the soil using Bray No. 1 solution as extractant. The extracted phosphorus is measured colourimetrically based on the reaction with ammonium molybdate and development of the ‘Molybdenum Blue’ colour. The absorbance of the compound is measured at 882 nm in a spectrophotometer and is directly proportional to the amount of phosphorus extracted from the soil (Bray, 1945). The available phosphorus in soil is expressed in mg/Kg of soil.

**4.11 DETERMINATION OF OPTIMUM GROWTH CONDITIONS**

There are many parameters which affect growth of bacterial isolates viz. temperature, pH, aeration, salt concentration, nutrient availability, radiation, presence of heavy metals, nature of carbon source, etc. Out of these, two important parameters viz. temperature and pH, affecting bacterial growth were considered.

For determination of optimum temperature, 100 mL N-broth was taken in 250 mL borosil make flasks and autoclaved at 121°C, 15 psi for 20 minutes. After cooling, each flask was inoculated with purified bacterial isolate in active log phase, in the proportion of 2% of medium. For each bacterial isolate, 5 sets of flasks were used in triplicates, and after inoculation, these flasks were incubated at five different temperatures, viz. 15, 25, 30, 37 and 45°C. The absorbance was taken at 600 nm after 48 hours of incubation and the data were used to plot a graph to determine the optimum growth temperature of the respective bacterial isolates.

To determine the optimum pH for the growth of bacterial isolates, 100 mL N-broth was taken in 250 mL flasks, pH adjusted to 5.0, 6.0, 7.0, 7.5, 8.0 and 9.0 respectively, and autoclaved at 121°C, 15 psi for 20 minutes. After cooling, each flask was inoculated with purified bacterial isolate in active log phase, in the proportion of 2% of medium. For each bacterial isolate, 6 sets of flasks were used each having the unique value of pH. The set of 6 flasks was inoculated in triplicates, and after
inoculation, these flasks were incubated at room temperature for 48 hours. The absorbance was taken at 600 nm and the data were used to plot a graph to determine the optimum growth pH of the respective bacterial isolates.

4.12 EXTRACTION OF PESTICIDE FOR ANALYSIS

The recovery of pesticide from N-broth and soil slurry is important to determine the extent of bioremediation by the bacterial isolates and mixed culture used separately. The extraction of both endosulfan and chlorpyrifos from N-broth and soil slurry was carried out using three solvents, viz. acetonitrile, chloroform and methanol.

For the extraction of pesticide from N-broth, an autoclaved broth was used, in which endosulfan (20 mg/L) and/or chlorpyrifos (20 mg/L) was/were added. After incubation period, 10 mL of broth was taken in a centrifuge tube and centrifuged at 4000 rpm for 20 minutes. Then 5 mL of supernatant was transferred in another tube and an equal volume of solvent (acetonitrile / chloroform / methanol) was added, shaken gently and allowed to settle for 30 minutes. The upper water layer was discarded and 2 mL of bottom layer containing the pesticide was taken in an eppendorf tube. The sample was stored at 4°C till analyzed by D-TLC and/or GC-MS.

For the extraction of pesticide from soil slurry, an autoclaved soil (20 g) was added in 200 mL of double distilled water in a sterile flask, and mixed vigorously to secure homogeneous medium. In this way, soil slurry was prepared, in which endosulfan (20 mg/L) and/or chlorpyrifos (20 mg/L) was/were added. After incubation period, 10 mL of soil slurry was taken in a centrifuge tube and centrifuged at 4000 rpm for 20 minutes. Then 5 mL of supernatant was transferred in another tube and an equal volume of solvent (acetonitrile / chloroform / methanol) was added, shaken gently and allowed to settle for 30 minutes. The upper water layer was discarded and 2 mL of bottom layer containing the pesticide (endosulfan and/or chlorpyrifos) was taken in an eppendorf tube. The sample was stored at 4°C till analyzed by D-TLC and/or GC-MS.
4.13 D-TLC ANALYSIS

A simple, rapid, and accurate method based on thin-layer chromatography (TLC) combined with image-analysis software has been used for the quantitative analysis of endosulfan and chlorpyrifos. The results obtained have been compared with those obtained from GC-MS. The percentage error between D-TLC and GC-MS ranged between ± 5.4% for ES and ± 6.2% for CP.

Preparative TLC plates (20x20 cm aluminium plates precoated with thickness 0.05 mm silica gel GF254,) were purchased from Merck, Germany. The solvents used to prepare the mobile phase were of analytical-reagent grade. The standards (ES and CP) and samples were applied to the plates by means of the micro pipette and the application volume was 20 µl. Six spots per plate were applied 1cm from bottom edge and 15 mm apart. The plate was developed in a saturated glass chamber in solvent system Hexane – Chloroform – Acetone (9:3:1) in case of endosulfan and Hexane – Acetone (75:30) in case of chlorpyrifos. The migration distance was kept to 10 cm. After separation, the plate was air-dried for 20 minutes.

Evaluation of the developed TLC plates was performed by capturing the image of the developed spots on the TLC plate with a digital camera (Sony, Japan). Data acquisition and processing were performed using the TLC analyzer software installed in the IBM computer. The TLC system with UV – visualization chamber (with short and long wave facility) is as represented in the Figure-4.1.
4.14 GC-MS ANALYSIS

Mass spectrometry is a technique for converting molecules into ions and ion fragments and hence, deals with the study of the charged molecule and fragment ions produced from a sample exposed to ionizing conditions, and also of the relative intensity spectrum which results from the correlation of the ions with their mass to charge ratio, and designated as m/e.

Mass fragmentation pattern are usually characteristic of type of compound and provide detailed information regarding molecular structure. When organic substance in the gas or vapor state at the very low pressure $10^{-1}$ to $10^{-3}$ atmosphere are subjected to electron beam of 70 eV, they loss electrons and forms positively charged ion (cation) and fragments with different m/e values. They are known as ionic beam, which can be accelerated and deflected by magnetic or electrical field. The deflection depends on the mass, charge, velocity and magnetic field of fragments. If the charge, velocity, and magnetic field are constant then it depends upon only mass. Deflection is less for heavy particles and more for light particles. These cations are received by detector and produce mass spectrum, which is specific for the given organic compound like pesticide. The sample substance is bombarded with electrons of
energy of 15eV. The molecular ion produced by loss of single electron which is called as “molecular ion peak” or “parent peak”. The intensity of base peak is considered 100% and the height of the other peaks can be measured with respect to the base peak. The details of the parameters employed in the GC-MS analysis are as represented in the Table-4.1 to 4.5.

Table 4.1: Different parameters of Gas Chromatography (GC-2010)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column name</td>
<td>BPX5, 30m,0.25mm</td>
</tr>
<tr>
<td>Column oven temperature</td>
<td>180°C</td>
</tr>
<tr>
<td>Injection temp</td>
<td>260°C</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Split</td>
</tr>
<tr>
<td>Flow control mode</td>
<td>Pressure</td>
</tr>
<tr>
<td>Pressure</td>
<td>95.6 kpa</td>
</tr>
<tr>
<td>Total flow</td>
<td>30.9 mL/min</td>
</tr>
<tr>
<td>Column flow</td>
<td>0.90 mL/min</td>
</tr>
<tr>
<td>Linear velocity</td>
<td>36.4 cm/sec</td>
</tr>
<tr>
<td>Purge flow</td>
<td>3.0 mL/min</td>
</tr>
<tr>
<td>Split ratio</td>
<td>30.0</td>
</tr>
<tr>
<td>Equilibrium time</td>
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</tr>
</tbody>
</table>

Table 4.2: Value of different parameter of GC-MS-QP2010

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion source temp.</td>
<td>230</td>
</tr>
<tr>
<td>Interface temp</td>
<td>250</td>
</tr>
<tr>
<td>Solvent cut time</td>
<td>2 min</td>
</tr>
<tr>
<td>Detector gain mode</td>
<td>Absolute</td>
</tr>
<tr>
<td>Detector gain</td>
<td>0.90 kv</td>
</tr>
<tr>
<td>Threshold</td>
<td>100</td>
</tr>
</tbody>
</table>
### Table 4.3: The value of different parameters of MS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>1</td>
</tr>
<tr>
<td>Start time</td>
<td>3 min</td>
</tr>
<tr>
<td>End time</td>
<td>31.5 min</td>
</tr>
<tr>
<td>ACQ mode</td>
<td>Scan</td>
</tr>
<tr>
<td>Interval</td>
<td>0.50 sec</td>
</tr>
<tr>
<td>Scan speed</td>
<td>2000</td>
</tr>
<tr>
<td>Start m/z</td>
<td>25.00</td>
</tr>
<tr>
<td>End m/z</td>
<td>1000.00</td>
</tr>
<tr>
<td>Sample inlet system</td>
<td>GC</td>
</tr>
</tbody>
</table>

### Table 4.4: Temperature program used in GC-MS analysis

<table>
<thead>
<tr>
<th>Rate (°C/min)</th>
<th>Temperature (°C)</th>
<th>Hold time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>180</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>280</td>
<td>20</td>
</tr>
</tbody>
</table>

### Table 4.5: Pressure program used in GC-MS analysis

<table>
<thead>
<tr>
<th>Rate (°C/min)</th>
<th>Pressure (kPa)</th>
<th>Hold time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>95.6</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>300</td>
<td>26</td>
</tr>
</tbody>
</table>
4.15 BIOREMEDIATION CAPABILITY OF MONO- AND MIXED-CULTURES

For the measurement of degradation capability of selected bacterial isolates and/or mixed culture, the actively growing culture of respective bacterial isolates and/or mixed cultures were added either in the soil slurry or in the N-broth containing appropriate concentration of endosulfan and/or chlorpyrifos, and incubated at room temperature for 5, 10, 15 and 30 days. Then 20 mL of aliquots were taken out, pesticide was extracted and estimated by D-TLC and confirmed by GC-MS analysis.

The endosulfan and chlorpyrifos degrading capability of the selected bacterial isolates and mixed cultures was computed and compared. The results were used to determine the most potential bacterial isolate in terms of degradation of endosulfan and chlorpyrifos respectively. Also, the bioremediation capability of mono- and mixed cultures was compared in terms of degradation of endosulfan and chlorpyrifos.

4.16 EFFECT OF TEMPERATURE ON PESTICIDE BIOREMEDIATION

The effect of temperature on pesticide bioremediation depends on the molecular structure of the pesticide. It is expected that the solubility of pesticide increases with the rise in temperature. It is also expected that the rise in temperature results in the stimulation of microbial activities. It is also found that the maximum growth and activity of microorganisms in soils occur between 25 to 35°C of temperature.

In the present study, the effect of temperature on bioremediation capability of six selected bacterial isolates and four different mixed bacterial cultures have been investigated separately. For this study, 90 mL of N-broth containing 5 mg each of endosulfan and chlorpyrifos, was inoculated with 10 mL of actively growing respective cultures and incubated at four different temperatures – 25, 30, 37 and 45°C respectively. After 5 days of incubation, 50 mL of aliquots were withdrawn from each culture vessel and the concentration of endosulfan and chlorpyrifos was estimated using the procedure described in the sections 4.12, 4.13 and 4.14.
4.17 EFFECT OF pH ON PESTICIDE BIOREMEDIATION

The pH of soil may affect pesticide degradation by altering the pesticide adsorption and also by influencing the microbial activity in the soil. Soil pH may also affect the mobility and bioavailability of pesticides. The effect of soil pH on degradation of a given pesticide depends greatly on whether the pesticide is susceptible to alkaline or acid catalyzed hydrolysis.

In this work, the effect of pH on bioremediation capability of six selected bacterial isolates and four different mixed bacterial cultures have been investigated separately. For this study, 90 mL of N-broth, adjusted at pH 6.0, 7.0, 7.5 and 8.0, containing 5 mg each of endosulfan and chlorpyrifos, was inoculated with 10 mL of actively growing respective cultures, respectively. After 5 days of incubation, 50 mL of aliquot was withdrawn from each culture vessel and the concentration of endosulfan and chlorpyrifos was estimated using the procedure described in the sections 4.12, 4.13 and 4.14.

4.18 EFFECT OF AGITATION ON PESTICIDE BIOREMEDIATION

The effect of agitation on the biodegradation of endosulfan and chlorpyrifos was investigated by maintaining static and shaking incubation conditions. The respective mono- and mixed-cultures were maintained to their respective optimum value of pH and temperature.

The effect of static vs. shaking conditions on bioremediation capability of six selected bacterial isolates and four different mixed bacterial cultures was determined separately. For this study, 90 mL of N-broth containing 5 mg each of endosulfan and chlorpyrifos was inoculated with 10 mL of actively growing respective cultures. After 5 days of incubation, 50 mL of aliquot was withdrawn from each culture vessel and the concentration of endosulfan and chlorpyrifos was estimated using the procedure described in the sections 4.12, 4.13 and 4.14.
4.19 EFFECT OF SOIL AMENDMENTS ON PESTICIDE BIOREMEDIATION

A soil amendment is any material added to a soil to improve its physical properties, such as water retention, permeability, water infiltration, drainage, aeration and structure. There are two broad categories of soil amendments: organic and inorganic. Organic amendments come from something that was alive. Inorganic amendments, on the other hand, are either mined or man-made. Organic amendments include sphagnum peat, wood chips, grass clippings, straw or crop residues, compost, manure, biosolids, sawdust and wood ash. Inorganic amendments include vermiculite, perlite, tire chunks, pea gravel and sand.

Organic amendments increase soil organic matter content and offer many benefits. Organic matter improves soil aeration, water infiltration, and both water- and nutrient-holding capacity. Many organic amendments contain plant nutrients and act as organic fertilizers. Organic matter also is an important energy source for bacteria, fungi and earthworms that live in the soil. Microbial activity is often stimulated by the addition of organic material to soil.

In the current work, the effect of organic amendments viz. compost, cow dung and crop residues, on bioremediation capability of six selected bacterial isolates and four different mixed bacterial cultures was studied separately. Two different sets of experiments were set off. In the first experiment, 100 g oven dried soil was used to prepare soil slurry, in which 20 mg each of endosulfan and chlorpyrifos was added and inoculated with 100 mL of actively growing respective cultures, without any incorporation of organic amendments. In the second experiment, the steps were same as that of first except incorporation of organic amendments, i.e. addition of 100 g of compost, cow dung or crop residues per kilogram of soil, separately along with the respective bacterial cultures. Both of these two systems were incubated at room temperature for 7 days. After incubation period, 20 g of sample was taken from each of the experimental systems, and the concentration of endosulfan and chlorpyrifos was estimated using the procedure described in the sections 4.12, 4.13 and 4.14.
4.20 EFFECT OF SOIL MOISTURE ON PESTICIDE BIOREMEDIATION

The effect of soil moisture on bioremediation capability of six selected bacterial isolates and four different mixed bacterial cultures was studied separately. Two different sets of experiments were set off. In the first experiment, 100 g oven dried soil containing 10 mg each of endosulfan and chlorpyrifos was inoculated with 100 mL of actively growing respective cultures. In the second experiment, 100 g oven dried soil containing 10 mg each of endosulfan and chlorpyrifos was inoculated with 100 mL of actively growing respective cultures along with the addition of one litre of distilled water (DW). Both of these two systems were incubated at room temperature for 5 days. Then 20 g of sample was taken from each of the experimental systems, and the concentration of endosulfan and chlorpyrifos was estimated using the procedure described in the sections 4.12, 4.13 and 4.14.

4.21 ENZYMATIC CHANGES DURING PESTICIDE BIOREMEDIATION

The enzymatic changes triggered during pesticide bioremediation were investigated by measuring the activity three enzymes, viz. cellulase, dehydrogenase and protease. The activity of the respective enzyme was measured in soil samples inoculated with selected bacterial mixed-cultures. The details of the enzyme extraction assays are as described subsequently.

Soil sample preparation for enzyme activity

Soil samples were collected from Rajkot, Gondal and Jetpur talukas of Saurashtra region of Gujarat. The collected soil samples were dried in sun light for 24 hours. Then soil was passed through 2 mm sieve and autoclaved at 121°C, 15 psi for 30 minutes. After cooling, one kilogram soil weighed and kept in plastic bags. Pesticide solution (endosulfan, chlorpyrifos and endosulfan + chlorpyrifos) at a concentration of 0, 5, 10 and 20 mg per 10g soil was added respectively, in triplicates. All bags were labelled according to pesticide solutions added and kept at room temperature, with alternate 12 hours light and 12 hours dark period. Suitable amount of water was sprayed at 3 days intervals and samples were analyzed for the activity of cellulase, dehydrogenase and protease on day 1, 7, 14 and 21.
**Cellulase activity**

Cellulase activity was measured using carboxymethyl cellulose powder as a substrate. The reaction mixture consisted of 0.2 ml culture filtrate, 0.5 ml of 2% carboxymethyl cellulose powder, 0.5 ml phosphate buffer (pH 5.0) and 0.8 ml distilled water, and incubated at 40°C for 30 minutes with shaking (130 rpm). The reaction was stopped by the addition of 1 ml of 0.3 % sodium dodecyl sulphate (SDS) to the reaction mixture and the treatment was preceded in boiling water for 10 minutes. After boiling the reaction mixture, the mixture was centrifuged at 800g for 5 minutes. The supernatant was utilized for the determination of the reducing sugars by measuring O.D. at 520 nm wavelength and cellulose as a standard. Cellulase activity is expressed as μg glucose formed/g dry weight of soil.

**Dehydrogenase activity**

To 3g air dried soil sample, 1mg glucose solution (30 mg/L) and 0.5 mL of a 3% solution of 2,3,5-triphenyltetrazolium chloride were added and the volume was made to 5mL by adding of 0.1M Tris buffer (pH 7.8). After incubating at 37°C for 24 hours, the formazan formed was extracted with 10mL ethanol and estimated spectrophotometrically at 485 nm. The concentration of formazan was calculated from its standard curve. The dehydrogenase activity is expressed as μg formazan formed/g dry weight of soil.

**Protease activity**

To 1 g of soil sample in a glass tube, 2.5 mL of Tris buffer (0.2 M, pH 8.0) and 2.5 mL of 2% Na-caseinate solution (20 g/L of DW) were added. The capped tubes were incubated in a water bath at 50°C for 2 hours. After incubation, remaining casein was precipitated with 5 mL 10% trichloroacetic acid. Then 1.5 mL of the solution was pipetted into a microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute. Then, 0.5 mL of clear supernatant was mixed with 0.75 mL Na₂CO₃ (1.4 M). This three-fold diluted Folin–Ciocalteu reagent was taken in a cuvette. After 5 minutes, the tyrosine concentration was measured colorimetrically at 680nm. For controls, same procedure was followed, except that the Na-caseinate was added after incubation and addition of trichloroacetic acid. The protease activity is expressed as μg tyrosine formed per g dry weight of soil.
4.22 DEVELOPMENT OF EFFECTIVE BIOTREATMENT PROCESS

Sustainable development requires the promotion of environmental management and a constant search for new technologies to treat a wide range of aquatic and terrestrial habitats contaminated by pesticides and other toxic recalcitrant compounds. Bioremediation is an eco-friendly and cost-effective alternative to physicochemical cleanup options. However, the strategy and outcome of bioremediation in open systems or confined environments depend on a variety of physicochemical and biological factors that need to be assessed and monitored. In particular, microorganisms are key players in bioremediation applications, yet their catabolic potential and their dynamics in situ remain poorly characterized. To perform a comprehensive assessment of the biodegradative potential of a contaminated site and efficiently monitor changes in the structure and activities of microbial communities involved in bioremediation processes, sensitive, fast and large-scale methods are needed.

To design and suggest a most effective and eco-friendly biotreatment process for the bioremediation of pesticide contaminated soil, the concept and principles of statistical analysis, mathematical modeling and simulation have been investigated. It is the known fact that the process of bioremediation is affected by a variety of physical, chemical and biological factors. Some of these factors are non-manageable viz. soil type and soil temperature, while some other factors can be managed easily or with some difficulty viz. soil pH, soil moisture and soil amendments. In this work, a correlation study between manageable parameters and bioremediation of endosulfan and chlorpyrifos have been investigated using SSP, MINITAB, MS-Excel and MATLAB.