CHAPTER 3: MATERIALS AND METHODS

3.1. Chemicals and media

3.1.1. Chemicals

Imidacloprid (analytical grade), imidacloprid (commercial grade), HPLC-grade water, Acetonitrile (HPLC grade), MilliQ water, TE buffer, TAE buffer, Phosphate buffer. All the chemicals used in the study are of analytical grade.

3.1.2. Media

3.1.2.1. Nutrient broth (NB)

Nutrient Broth is a general-purpose liquid media containing yeast extract, peptone and sodium chloride for the growth of a wide variety of microbes that do not require specific nutrients. The Nutrient broth media was used for the isolation of bacteria from the soil samples. 13 g of nutrient broth powder is added to 1 L of distilled water, mixed thoroughly and sterilized in autoclave at 121°C for 15 minutes.

3.1.2.2. Nutrient agar media (NAM)

In addition to the components of nutrient broth, nutrient agar media contains agar as a solidifying agent. This media was used in the present study for obtaining pure isolates of the bacteria and storing bacteria on plates. 28 g of nutrient agar powder is suspended in 1 L of distilled water, mixed and autoclaved.

3.1.2.3. Mineral salt media (MSM)

MSM is a liquid media used for the isolation, enrichment or growth of specific types of bacteria. This media does not contain any carbon, so a suitable source of carbon is added prior to culturing. The media composition used in the study is as follows: KH_2PO_4 (1 g/L), $K_2HPO_4.2H_2O$ (1 g/L), $MgSO_4.7H_2O$ (0.3 g/L) NaCl (0.5 g/L), $(NH_4)_2.SO_4$ (0.3 g/L), and trace elements [$CaCl_2$ (2 g/L), $MnSO_4.H_2O$ (3 g/L), $FeSO_4$ (0.25 g/L), $ZnSO_4$ (0.05 g/L), $MgSO_4$ (1 g/L)]. The media pH was adjusted to 7 ± 0.2 and autoclaved at 121°C for 15 min for sterilization.

3.1.2.4. Luria-Bertani media (LB media)

It is a comparatively simple medium for bacterial growth. For the preparation of 1 liter of LB media, 10 g tryptone, 5 g yeast extract and 10 g NaCl are mixed together and pH is adjusted to 7.0, followed by sterilization of the media using an autoclave. In the present study the media was used for culturing luminescent bacteria *Photorhabdus luminescens* subsp. akhurstii.

3.1.2.5. Dulbecco's Modified Eagle Medium (DMEM)

It is an extensively used basal medium for promoting the growth of various types of mammalian cells. It contains an abundant amount of vitamins and amino acids. It is abundant in vitamins and amino acids, among other nutrients. The sodium bicarbonate buffer system used by DMEM enables the preservation of physiological pH in an environment with 5-10% CO_2. Supplementing DMEM with fetal bovine serum (FBS) is common. The ingredients of DMEM include 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. It can be used for cell culture viability and growth.

3.2. Analytical techniques and instruments

3.2.1. UV-visible spectrometry

UV-visible spectroscopy is a fundamental technique in analytical chemistry, offering insights into the electronic structure of molecules and aiding in quantitative analysis. By measuring the absorption of ultraviolet and visible light by chemical compounds, researchers can gather valuable information about the nature of the molecules being studied. In UV-visible spectroscopy, when a molecule absorbs UV or visible light, it undergoes electronic transitions, where electrons are excited from a ground state to higher energy levels. This absorption of light corresponds to the energy required to promote electrons to higher energy levels, often associated with specific molecular orbitals. The resulting spectrum reveals peaks at wavelengths where absorption occurs.

Quantitative analysis using UV-visible spectroscopy relies on the Beer-Lambert law, which relates the absorbance of light by a solution to its concentration and the path length of the light through the solution. By measuring the absorbance of a sample at a specific wavelength and comparing it to a calibration curve generated from known standards, the concentration of the analyte in the sample can be determined. This technique finds wide application in various fields, including pharmaceuticals, environmental monitoring, and biochemical analysis, owing to its sensitivity, simplicity, and versatility. To calibrate a UV-visible spectrophotometer, the instrument was warmed up. The wavelength settings were adjusted using standards to ensure accuracy. Standard solutions of known concentration were prepared, their absorbance was measured and a calibration curve was plotted. A validation test was performed after calibration and results were documented to ensure traceability.

In the present study, a UV-visible spectrophotometer (ELICO SL 159) was used to estimate the optical density (OD) of the bacterial culture. Samples were collected after the bacterial growth in culture media and centrifuged at 6000 rpm for 10 minutes. The supernatant was collected and filtered using a 0.22 µm syringe filter and absorbance was measured at 600 nm. The bacterial biomass concentration was estimated using a calibration curve produced earlier.

3.2.2. TOC (Total Organic Carbon) analyzer

A TOC analyzer was used to determine the total organic carbon of the samples. TOC is the measure of organic carbon, inorganic carbon and total carbon that is found in an organic compound. TOC analyzer works by oxidizing the organic compound to a form that can be quantified. The majority of TOC analyzers change all the carbon present into carbon dioxide, then use an NDIR (Non-dispersive infrared) detector designed specifically to measure CO_2. The CO_2 must be swept through the detector by a carrier gas in order to perform NDIR detection.

The instrument records these measurements and converts them into TOC concentrations using calibration curves established with known standards. Calibrating a TOC analyzer involves several key steps to ensure accurate measurements of organic carbon content in the samples. Initially, all necessary equipment, including TOC standards, calibration solutions, deionized water, and cleaning materials, are gathered. The analyzer is then powered on and allowed to warm up.

The process begins by measuring the baseline TOC level using a blank sample (deionized water), to establish a reference point. Next, a series of TOC calibration standards covering the expected range of organic carbon concentrations are prepared, ensuring they are traceable to certified reference materials. Each calibration standard is introduced into the analyzer, and the TOC content is measured, with readings recorded. The entire calibration process, including standards used, readings, adjustments, and verification results, is documented to maintain traceability and quality control. The resulting TOC concentration provides valuable insight into the organic carbon content of the water sample.

TOC analysis was carried out by injecting 500 µl the sample at 850ºC in a TOC analyzer (Model No.: Multi N/C 2100, Analytic Jena, Germany). The readings were obtained as total carbon and inorganic carbon. The organic carbon was obtained as the difference between the total carbon and inorganic carbon present in the sample.

3.2.3. Biochemical Oxygen Demand (BOD)

BOD is the amount of oxygen needed for the organic components in the effluent to oxidize biologically. It is ascertained by monitoring the variation in dissolved oxygen in a sample over a period of five days. After five days of incubation at 20°C, the sample is taken out of the incubator to determine the final DO concentration. The initial DO content of a specific sample volume is recorded. A greater BOD indicates that more oxygen is needed, which suggests that the water quality is low. Low BOD suggests that the water is purer since it shows that less oxygen is removed from the water.

To estimate the BOD, biologically treated as well as untreated imidacloprid-contaminated samples were collected and magnesium sulfate was added to the samples to inhibit microbial activity. Initially, the sample is collected and neutralized with sodium thiosulfate. The initial dissolved oxygen (DO) is measured using the Winkler titration method. The sample was diluted and multiple 300 ml BOD bottles were filled with the diluted sample. These bottles were incubated in the dark at 20°C for five days. After incubation, the final DO was measured. BOD was calculated by subtracting the final DO from the initial DO.

3.2.4. Chemical Oxygen Demand (COD)

The COD determines the amount of oxygen needed for the inorganic and organic components of the effluent to oxidize chemically. Since practically all organic substances can oxidize to carbon dioxide under acidic conditions when exposed to a strong oxidizing agent, COD is determined. A potent oxidant, such as potassium dichromate or potassium permanganate, is commonly used to assess COD in acidic environments. A known excess concentration of the oxidant is added to the sample. The amount of oxidant that remains in the solution after the oxidation process is finished is measured to find out how much organic material is present in the sample. This is often achieved by titrating using an indicator solution. The amount of oxygen utilized per liter of solution is measured in milligrams per liter of COD.

For COD estimation, 100 ml of the sample was collected and digested by adding potassium dichromate solution and sulfuric acid containing silver sulfate as catalyst and refluxing the mixture for two hours. After cooling, the mixture was transferred to a conical flask, and ferroin indicator was added. The sample was then titrated with ferrous ammonium sulfate (FAS) until a color change

from blue-green to reddish-brown was observed, and the volume of FAS used was recorded. A blank sample with distilled water was also prepared to determine the blank COD value.

3.2.5. Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a laboratory process that quickly amplifies a particular segment of DNA. The process involves denaturation, where DNA strands separate; annealing, where primers bind to the DNA template; and extension, where DNA polymerase synthesizes a complementary strand. These steps are repeated in cycles, resulting in exponential DNA amplification. PCR requires a DNA template, primers, DNA polymerase, nucleotides, and a buffer. It is widely used in DNA analysis, cloning, sequencing, and diagnostics due to its ability to selectively amplify specific DNA sequences. Since the 16S rRNA gene is found in all bacteria and contains highly conserved sequences, it is frequently used for identification of different bacteria. By analyzing the 16S rRNA nucleotide sequence and comparing it to a database containing known sequences, bacteria can be identified.

DNA amplification was done using PCR (BIO-RAD T100 Thermo Cycler) in a 50-µL reaction mixture containing 20 pmol of universal primers (8F-5′ AGAGTTTGATCCTGGCTCA3′ and 1492 R-5′GGT TACCTTGTTACGACTT3′) each, 20 ng of template DNA, and 25 µL PCR Master Mix. The thermocycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C (1 min), 50°C (1 min), 72°C (1 min); and final extension at 72°C for 10 min.

3.2.6. High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is an analytical technique for separating, identifying, and quantifying specific components in mixtures. It involves a stationary phase within a column, a mobile phase that carries the sample through the column, and differential interactions between the sample components and the stationary phase. As the components move through the column, they separate based on their properties. Detection and analysis of the separated components are performed, generating a chromatogram for quantification. HPLC can only be used to analyze compounds that are dissolved in solvents. Compounds dissolved in a liquid sample can be separated using HPLC, which also enables qualitative and quantitative analysis of each component in the sample.

The equipment used for quantification of imidacloprid was a high-performance liquid chromatograph (Shimadzu, Japan), comprising of a binary pump and photodiode array (PDA) detector. The C18 analytical column was operated isocratically at room temperature with a flow rate of 1 ml/ min using 80:20 (v/v) acetonitrile and HPLC-grade water. The compounds were detected using UV spectroscopy at 270 nm, by injecting 25 µl of samples. Appropriate calibration standards were used for the quantitative analysis of the samples. The LC solutions software was used to collect and process data.

3.2.7. Gas Chromatography-Mass Spectroscopy (GC-MS)

Gas Chromatography-Mass Spectrometry (GCMS) was used for the analytical determination of pesticide concentration. When a mixed solution sample is injected into the GC system, the compounds in the sample, including the solvent components, are heated and vaporized within the sample injection unit. In a GC system, the mobile phase, also referred to as the carrier gas, always flows in a sequential manner from the sample injection unit to the column and, ultimately, to the detector. After being vaporized in the sample injection unit, the target components are transported to the column by the carrier gas. Once the mixture of compounds is in the column, it separates into its constituent parts, and the detector records the quantity of each compound in the sample. The detector produces an electrical signal corresponding to the concentration of each compound, which is then transmitted to a data processing unit. The data acquired facilitates the identification and quantification of the compounds present in the sample.

The samples were collected and centrifuged at 13000 rpm for 10. The supernatant was collected for GC-MS analysis. 8 µl of the sample was injected into Shimadzu GC-MS-QP2010 Plus for analysis. Helium was used as a carrier gas; initial column oven temperature 50ºC, then raised to 125ºC at 25ºC/min and finally increased to 300ºC at 10ºC/min and held for 15 min. The injection temperature was 250ºC, the injection mode was split-less, and the column flow rate was 1.70 ml/min. DB-5MS analytical column was used. The ion source temperature was 200oC, and the interface temperature was 280ºC. Compounds were identified using NIST08s and Wiley7 library.

3.3. Methodology

3.3.1. Sample collection

Soil samples were collected from agricultural fields (Latitude 25.25797, Latitude 82.9876) of Banaras Hindu University (BHU), Varanasi. The agricultural fields have a history of being exposed

to different agrochemicals. Various herbicides and pesticides are applied depending on the crops or vegetables grown in the particular fields. The soil sample was collected from the 10-15 cm upper layer of the field and immediately taken to the laboratory for further analysis. The samples were air-dried and sieved to remove debris, plant residues, and particles larger than 0.5 mm. The samples were examined for physicochemical properties before being enriched with imidacloprid. A map of the sample collection site has been presented in Figure 3.1.

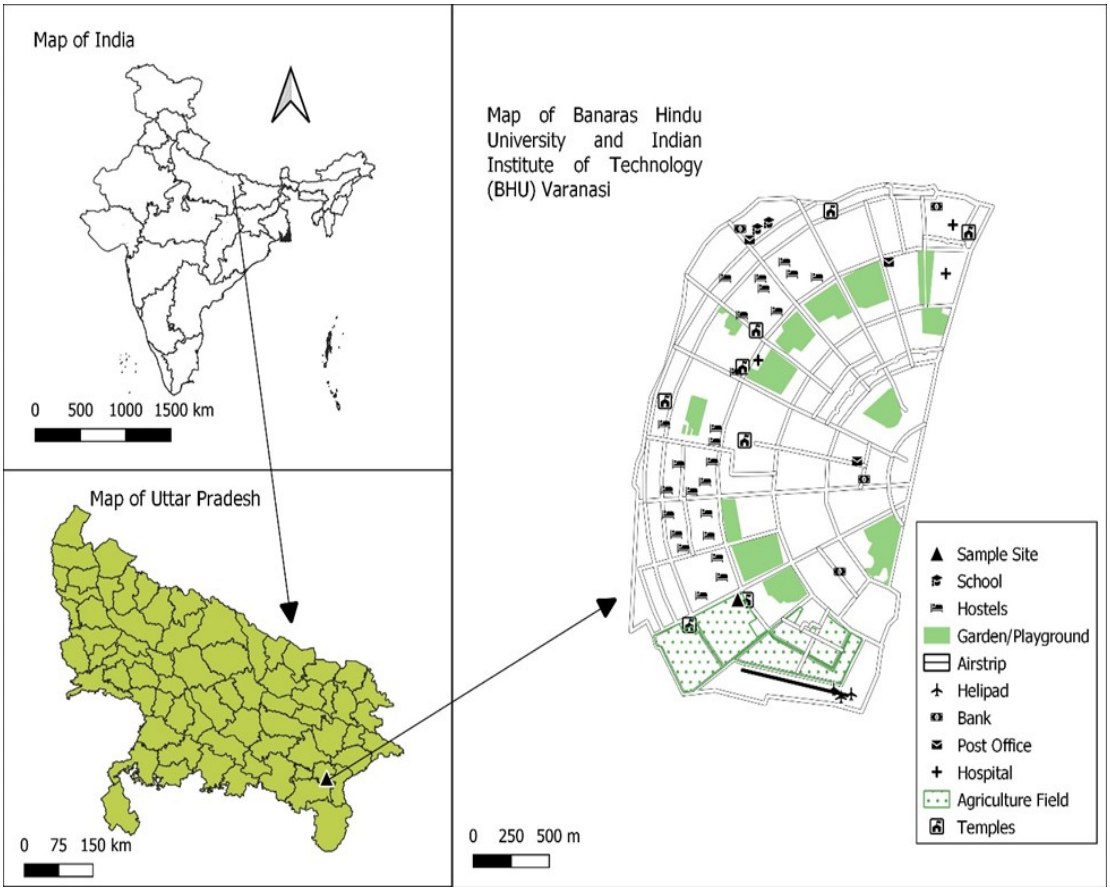

Figure 3.1: Map of sampling site

3.3.2. Isolation of Imidacloprid degrading bacteria

The bacteria were cultured from the collected soil samples using Nutrient broth. Serial dilution of the soil sample was done. Serial dilution of the sample before culturing is an important step. The collected soil sample was serially diluted by adding 9 ml of distilled water and 1 gram of the soil sample. For an even distribution of soil in the water, one gram of soil was combined with nine milliliters of sterile water and shaken. After that, 1 ml of the solution from one test tube was

transferred to another test tube along with 9 ml of sterile water. This results in a dilution of 10^{-1}. Similarly, dilutions up to 10^{-6} were prepared following the same pattern.

1 ml of the diluted sample was added to 100 ml sterilized nutrient broth and incubated for 24 hours. A mixed bacterial culture was obtained after 24 hours, which was used as inoculum for the isolation of imidacloprid-degrading bacteria using MSM, with imidacloprid as a source of carbon and glucose as co-substrate. The concentration of glucose was decreased gradually and imidacloprid was used as the sole source of carbon after 20 days. The enrichment process was carried out for 45 days. After the enrichment process, imidacloprid-degrading bacteria were cultured on nutrient agar plates. The plates were incubated at 35±2°C for 48 hours. Bacterial growth could be observed on the plates after 48 hours. The isolated bacterial colonies were further sub-cultured.

Morphologically different colonies were identified and sub-cultured to obtain pure strains. Pure bacterial colonies were obtained through the streak-plate technique, using nutrient agar and MSM agar as media. Imidacloprid degrading capability of individual isolates was examined, and the most effective bacterial species were selected for further studies. The insecticide at varying doses was used for bacterial growth to find the maximum concentration of imidacloprid tolerance of the bacteria. The isolated bacterial cultures were inoculated into different sets of sterilized MSM broth (25 ml) spiked with varying concentrations of imidacloprid (50, 100, 150, 200, 250, and 300 ppm) and incubated at 35±2°C. The control flask was uninoculated. The growth of bacteria was assessed using absorbance at 600 nm. The imidacloprid concentration that sustained the maximum growth was recorded. The bacteria were identified as *Tepidibacillus decaturensis* strain ST1, *Klebsiella variicola* strain ST3, *Brevibacillus borstelensis* strain ST4 and *Bacillus licheniformis* strain ST5.

3.3.3. Identification of imidacloprid-degrading bacteria

The pure colonies were identified morphologically for their size, and shape, Gram staining ability, and biochemical tests. The genomic DNA of isolated bacteria was extracted. The spectrophotometric quantification of the DNA sample was done at wavelengths of 260 and 280 nm, with the assumption that one absorbance unit at 260 nm wavelength is equivalent to 50 µg DNA per ml (Gallagher 2017). Agarose gel electrophoresis was used to evaluate the quality and purity of the DNA. Amplification of the collected DNA was carried out using PCR. The amplified PCR products were outsourced for sequencing (Bioraj Laboratories, Nagpur). 16S rRNA sequencing and phylogenetic analysis were done for molecular identification of the isolate. To identify the

organism, the nucleotide sequences were used in a BLAST analysis against the NCBI database. Phylogenetic trees were constructed by using MEGA (version 10.2.5) software. GenBank accession number was assigned for 16S rRNA gene sequences of the isolate.

After the identification of the most efficient imidacloprid-degrading isolates, two consortia (CS1 and CS2) were prepared. Two different bacterial consortia were prepared (Consortium CS1 and Consortium CS2). In preparation for CS1, an equal volume of two isolates, i.e., Klebsiella variicola strain ST3 and Brevibacillus borstelensis strain ST4, were used, while for the preparation of consortium CS2, *Klebsiella variicola* strain ST3, *Brevibacillus borstelensis* strain ST4 and *Bacillus licheniformis* ST5 were used.

In the study, only two consortia were prepared. The selection of organisms for the consortia was based on their capacity to eliminate imidacloprid. Bacterial isolates incapable of effectively degrading imidacloprid were excluded from the consortia preparation. *Tepidibacillus decaturensis,* when used as a pure culture, degraded over 80% of imidacloprid. Even in the consortium, the degradation rate remained in the same range. Hence, it was not used in the consortium.

3.3.4. Optimization of biodegradation conditions

The optimum conditions for the degradation of imidacloprid were studied in the case of the most efficient bacterial isolate *Tepidibacillus decaturensis* as well as the consortium CS1 (consisting of *Klebsiella variicola* strain ST3 and *Brevibacillus borstelensis* strain ST4). In sets of experiments, the growth of most efficient bacteria and the consortium was studied by varying pH, temperature and shaking speed. Varying concentrations of imidacloprid from 50 to 300 mg/L have been considered. To assess the influence of pH and temperature on the biodegradation of imidacloprid, experiments were performed at varying temperatures (15, 25, 35, and 45°C) and pH (5, 6, 7, 8, and 9). Incubation conditions (shaking and static) were varied from 50 to 250 rpm. The studies were carried out in triplicate, and non-inoculated controls were maintained under the same conditions. The consortium was also exposed to different concentrations of imidacloprid to evaluate the maximum imidacloprid tolerance.

3.3.5. Imidacloprid biodegradation

3.3.5.1. Batch reactor

The analysis for degradation of imidacloprid was conducted in 250 mL flasks containing 100 mL of MSM broth, supplemented with increasing concentration of imidacloprid (50−250 mg/L). The inoculation of flasks was done with the most efficient bacterial strain as well as the consortium. As a control, non-inoculated flasks were used. The flasks were incubated for 8 days in a rotary shaker at 35±2 °C and 150 rpm. For analysis of the bacterial biomass and imidacloprid concentration, samples were collected at regular intervals (every 24 hours). The degradation of imidacloprid was quantified by using high-performance liquid chromatography (HPLC).

3.3.5.2. Stirred tank batch bioreactor

In the current investigation, a 3-L borosilicate glass stirred tank bioreactor (height, 22 cm; internal diameter, 13.2 cm) operating in batch mode with a working volume of 1.5 L was employed to degrade imidacloprid (Figure 3.2). The reactor was filled with MSM and 150 mg/L imidacloprid was added to it. Temperature was maintained at 35±2°C. Actively growing bacterial cells from 24-h cultures were introduced in the bioreactor as inoculum. Agitation was done using a Rushton impeller at 150 rpm, and air was supplied continuously at 0.5 L/min. The initial pH of the reactor was 7.0, and the reactor was operated for 30 days. pH and temperature were monitored throughout the operation.

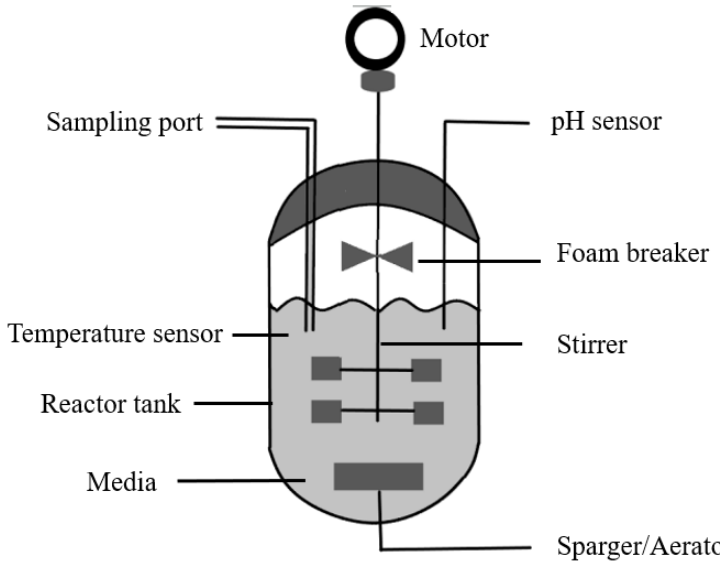

Figure 3.2: Schematic representation of stirred tank batch bioreactor

Two reactors under the same operating conditions were maintained as controls, one containing 1.5 L of MSM to assess the natural degradation of imidacloprid with time and the other with autoclaved bacterial biomass to evaluate the imidacloprid loss due to adsorption. Quantification of imidacloprid was done using HPLC.

3.3.5.3. Slurry reactor

Experiments on imidacloprid biodegradation were also conducted in soil slurry. The slurry was prepared in flasks by adding 30 g of sterile soil as well as unsterile soil separately and 70 ml of autoclaved distilled water. The resulting slurry from the sterile soil sample was autoclaved again. Slurries were spiked with imidacloprid (200 ppm) and incubated at 35±2°C in a shaking incubator at 120 rpm, for 10 days. Experiments were conducted in triplicate with the corresponding biotic and abiotic controls. Residual imidacloprid concentration and microbial growth were determined at regular intervals (every 24 hours) throughout the study.

3.3.5.4. Soil microcosm

A microcosm analysis was conducted to estimate the imidacloprid degrading capability of the most efficient bacterial isolate in soil under natural conditions. The collected soil sample was divided into two sets, one was sterilized and the other was unsterilized. From each set, four subsets containing 1000 g of soil sample were taken in four containers. 1000 g of dry soil was weighed and added to each container, and soil moisture was adjusted by using a mixture of imidacloprid and deionized water. The containers spiked with imidacloprid, without bacterial inoculation (sets 1 and 2) served as control. The treatments in sterile soil (A) and unsterile soil (B) were as follows: control (set 1): (Soil + Imidacloprid), control (set 2): (Soil + Imidacloprid + plant), treatment of soil with imidacloprid and bacteria but without plantation (set 3): (Soil + Imidacloprid + Bacteria), treatment of soil using combination of bacteria and plant (set 4): (Soil + Imidacloprid + Plant + Bacteria). A pictorial representation of the different sets and subsets has been given in Figure 3.3.

A total of 24 treatments were included with 12 sets of sterilized and unsterilized soil samples each. The containers were watered regularly to maintain the moisture content of the soil. Imidacloprid was added to all of the containers in each set at a final concentration of 200 mg/kg. After mixing, a suspension of 24-h-old imidacloprid-degrading bacterial culture was added to the soil samples in sets 3 and 4. For plantation, *Cicer arietinum* seeds were surface sterilized with $HgCl_2$ (0.2%) for 5 min before being thoroughly washed with sterile distilled water. The losses other than

biodegradation were also noted. The experiment was conducted in triplicate in sterilized as well as unsterilized soil. Imidacloprid residue was extracted from each container separately after every 5 days and quantified using HPLC.

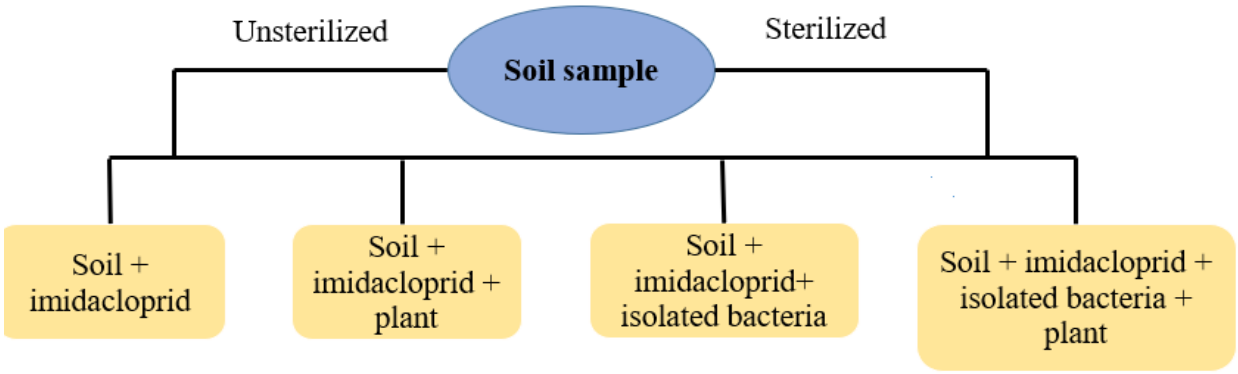

Figure 3.3: The sets of sterilized and unsterilized soil samples for microcosm study

To fit the experimental data of imidacloprid degradation kinetics in soil, the first-order kinetic equation and second-order kinetic equation were used:

$$C_t = C_o e^{-kt} \quad (3.1)$$

$$\ln C_t = \ln C_o - kt \quad (3.2)$$

where C_o is the initial concentration of imidacloprid in the medium, C_t is the concentration of imidacloprid at time t, k is the degradation rate constant (day^{-1}), and t is the degradation time. The biodegradation half-life ($T_{1/2}$) of imidacloprid was calculated as:

$$T_{1/2} = \frac{\ln 2}{k} \quad (3.3)$$

The natural logarithm values of C were plotted against time t to determine k. Residual imidacloprid concentration and % degradation at different time intervals were calculated with respect to imidacloprid residue on the initial day, after the application (100%). The slope of the line was used to calculate the k of each concentration.

The second-order kinetic equation can be expressed as follows:

$$\frac{1}{C_t} = \frac{1}{C_o} + kt \qquad (3.4)$$

3.3.6. Extraction and quantification of imidacloprid

Total imidacloprid residue in the soil, as well as uptake by the plant, was analyzed using HPLC. For determining residual imidacloprid concentration in slurry, plant parts, and soil, extraction was done. Residual imidacloprid from soil was extracted by adding 5 g of soil to 20 ml acetonitrile in a flask and shaken for 1 h. The mixture was then centrifuged and filtered. The filtrate was transferred in a round bottom flask, and it was dried in a rotary evaporator at 50 °C. The residue was dissolved in methanol and filtered by a 0.22 µm filter. Samples of imidacloprid were analyzed using HPLC.

For extraction of imidacloprid from plant parts, the plants were crushed and homogenized. A representative sample of the homogenized plant was extracted with 100 ml acetonitrile. The extract was filtered and salted out with sodium chloride, followed by drying. The dried fraction was dissolved in acetonitrile: water (4:1, v/v) and vortexed for 30 s. The supernatant was dried in a rotary evaporator. The residue was dissolved in methanol for further analysis.

Imidacloprid residue from liquid samples was extracted using acetonitrile and water in the ratio 80:20 (Baskaran et al., 1997). The sample was mixed with an equal volume of acetonitrile to extract residual imidacloprid, and then dried in a rotary evaporator. The residue was then dissolved in methanol and 0.22 µm syringe filter was used for filtration before analysis in HPLC.

3.3.7. Toxicity assessment

Although several chemical-biological processes have been demonstrated to have effective remediation potential for pesticide-contaminated samples, the toxicity level in the treated solution remains a concern (Chaturvedi et al., 2021). The samples were collected after bioremediation by the isolated bacteria and consortium, centrifuged at 8000 rpm for 10 min., and filtered through a 0.22 µm filter paper. The supernatant was separated in all the samples and sterilized for conducting phytotoxicity, bacterial-toxicity and cytotoxicity analyses. All the experiments were carried out in triplicates.

3.3.7.1. Phytotoxicity assay by germination assay

Phytotoxicity can be acute or chronic, depending on whether it causes the immediate death of the plant tissue or interferes with physiological functions that make the plant less productive. Studies on phytotoxicity are mostly focused on the impact on root elongation or plant growth as well as the prevention of seed germination. Assays for short-term phytotoxicity include testing on seed germination and survival as well as root and shoot length. Seed germination assay is quick, sensitive, effective and commonly used method to determine the toxicity of hazardous chemical compounds. Therefore, the toxicity of imidacloprid was assessed through seed germination of *Cicer arietinum*. It was used in the experiment since it can be grown in a shorter period and imidacloprid is used against pests in Cicer arietinum. The seed was chosen due to its easy availability in Northern India and its frequent usage as a test plant by researchers for toxicity assessments in the literature (Bhattacharya et al., 2012; Siddiqui & Abbas, 2021). The phytotoxicity caused by the presence of imidacloprid was compared with the phytotoxicity of the sample obtained after carrying out bioremediation.

Seeds of *Cicer arietinum* were purchased from a local market in Varanasi, India. Seeds that were healthy and of equal size were chosen and sterilization of the surface was done by washing thoroughly with distilled water, followed by sodium hypochlorite solution (1% v/v) for 10 min, then washed again with distilled water and dried at room temperature. Seeds were incubated for 24 h in all the samples, and distilled water served as control.

After 24 hours, seeds from all the samples were placed in Petri plates on double-layered filter papers that had been pre-wetted using distilled water. These were cultured for 7 days at room temperature with a constant supply of distilled water. All the experiments were performed in triplicates. Seed germination percentage (G %), shoot length (SL), root length (RL) and root shoot length ratio (RSL ratio) of the seeds was thoroughly examined. The germination percentage was calculated on the second day after sowing on filter paper by counting the number of seeds that germinated out of the total number of seeds treated. Each concentration was maintained in three replicates, with a control replicate, with each replicate containing 20 seeds per petri plate.

3.3.7.2. Phytotoxicity analysis using pot experiment

Pot experiments were also conducted to study the effect of varying concentrations of imidacloprid on plant growth using *Cicer arietinum* seeds. Sterilized *Cicer arietinum* seeds were planted in five

pots, each containing 1000 g of soil. Different concentrations of imidacloprid solution (150 ppm, 300 ppm, and 500 ppm) were prepared for the treatment of seeds. The treatments were conducted using distilled water (S1), treated sample (S2), 150 ppm (S3), 300 ppm (S4), and 500 ppm (S5). The pots were irrigated at regular intervals and incubated at 20-25ºC for 21 days. Morphological characteristics of *Cicer arietinum* seedlings were examined, including survival percentage (S%), seed germination percentage (G%), shoot length (SL), root length (RL), root shoot length ratio (RSL ratio), percentage phytotoxicity (P%), seedling vigor index (SVI), fresh root biomass, dry root biomass, and tolerance index (TI).

The number of germinated seeds was counted on the second day after sowing and the germination percentage was calculated (Akinci & Akinci, 2010; Scott et al., 1984). The ratio between the total number of survived seedlings and the total number of treated seeds was used to compute S% after 7 days of germination. On the seventh day, the lengths of the roots and shoots were measured, and their ratios were calculated. The germination (%) and seedling length are multiplied to determine the SVI of the seeds (Shahid et al., 2021).

Phytotoxicity evaluation of pesticide treatment was done by studying the germination index, % shoot and root elongation and was calculated according to Zayed et al. (2012) as follows:

$$\% \text{ germination} = \frac{Number\ of\ germinated\ seeds}{Number\ of\ planted\ seeds} \times 100$$

$$\% \text{ difference from control} = \frac{\%\ germination\ of\ control - \%\ germination\ of\ test\ solution}{\%\ germination\ of\ control} \times 100$$

$$\% \text{ shoot elongation} = \frac{Mean\ shoot\ length\ in\ treated\ seed}{Mean\ shoot\ length\ in\ control\ seed} \times 100$$

$$\% \text{ root elongation} = \frac{Mean\ root\ length\ in\ treated\ seed}{Mean\ root\ length\ in\ control\ seed} \times 100$$

Seed vigour index = Germination% × Plant total length

Vigour index 1 = Germination (%) × Average seedling length of 10 seedlings (cm)

Vigour index 2 =Germination (%) × Dry weight of 10 seedlings (mg)

$$\% \text{ phytotoxicity} = \frac{Shoot\ or\ root\ length\ of\ control - Shoot\ or\ root\ length\ of\ treatment}{Shoot\ or\ root\ length\ of\ control} \times 100$$

Tolerance index = $\frac{Mean\ length\ of\ longest\ root\ in\ the\ test\ solution}{Mean\ length\ of\ longest\ root\ in\ the\ control}$ x 100

Germination index = $\frac{\%\ seed\ germination\ X\ \%\ root\ elongation}{100}$

3.3.7.3. Stress tolerance index

The stress tolerance index is a valuable tool for assessing the high yield and stress tolerance potential of genotypes. The following formulae were used to calculate stress tolerance indices for various growth parameters (Patra et al., 2020; Wilkins, 1978):

Root length STI = $\frac{Root\ length\ of\ stress\ plant}{Root\ length\ of\ control\ plant}$ x 100

Shoot length STI = $\frac{Shoot\ length\ of\ stress\ plant}{Shoot\ length\ of\ control\ plant}$ x 100

Root fresh weight STI = $\frac{Root\ fresh\ weight\ of\ stress\ plant}{Root\ fresh\ weight\ of\ control\ plant}$ x 100

Shoot fresh weight STI = $\frac{Shoot\ fresh\ weight\ of\ stress\ plant}{Shoot\ fresh\ weight\ of\ control\ plant}$ x 100

Root dry weight STI = $\frac{Root\ dry\ weight\ of\ stress\ plant}{Root\ dry\ weight\ of\ control\ plant}$ x 100

Shoot dry weight STI = $\frac{Shoot\ dry\ weight\ of\ stress\ plant}{Shoot\ dry\ weight\ of\ control\ plant}$ x 100

3.3.7.4. Antioxidative enzyme assay

The effects of imidacloprid treatment on the activities of anti-oxidative enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) were examined using *Cicer arietinum* seedlings.

Catalase activity (CAT EC. 1.11.1.6): 100 mg of fresh leaf material was crushed in a pre-chilled mortar and pestle in 4 ml of extraction buffer (pH 7.0), and the mixture was then centrifuged for 15 minutes at 12,000 rpm. The supernatant was collected and mixed with H_2O_2 (0.005 M) in phosphate buffer (pH 7.0). After 5 minutes, the reaction was stopped by adding H_2SO_4 (2 N) and standardized against $KMnO_4$ (0.1 N). The catalase activity was determined by following the standard method.

Peroxidase activity (POX EC. 1.11.1.7)

100 mg of fresh leaf material was crushed in a pre-chilled mortar and pestle in 10 ml of extraction buffer (pH 7.0), and the mixture was then centrifuged for 15 minutes at 12,000 rpm. The supernatant (1 ml) was collected, and phosphate buffer (0.1 M, 5 ml), p-phenylenediamine (0.5% w/v) and H_2O_2 (0.01%, 1 ml) were added to it. The reaction was stopped by adding H_2SO_4 (5 N). The absorbance of the mixture was noted at 485 nm (Lück, 1965).

Superoxide dismutase (SOD) activity (SOD EC. 1.15.1.1)

100 mg of fresh leaf material was homogenized using phosphate buffer (pH 7.0), PVP and EDTA in a pre-chilled mortar and pestle and centrifuged at 12000 rpm for 15 minutes. The supernatant was added to tubes containing phosphate buffer (pH 7.8), methionine, nitroblue tetrazolium (NBT), riboflavin, and EDTA and exposed for 15 minutes in sunlight in order to complete the reaction. The absorbance of the mixture was noted at 560 nm, following the modified method of (Beauchamp and Fridovich, 1971).

3.3.7.5. Analysis of plant photosynthetic pigments

Estimation of plant photosynthetic pigments such as chlorophyll and carotenoids were done. The quantity of total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids was determined (Wellburn, 1994). Fresh leaves (1g) were collected and crushed in 80% (v/v) acetone (4 ml) to obtain a homogenous sample, followed by centrifugation at 15000 rpm at 4^{o}C for 30 minutes. The supernatant was collected, and absorbance was recorded at 645 and 663 nm using a UV-visible spectrophotometer. The chlorophyll a, b and total chlorophyll contents were determined using the method of (Jeffrey & Humphrey, 1975) as follows:

$CHL_A = 12.7OD_{663} - 2.69\ OD_{645}$,

$CHL_B = 22.9\ OD_{645} - 4.68\ OD_{663}$,

$CHL_T = CHL_A + CHL_B = 20.22\ OD_{645} + 8.02\ OD_{663}$.

where, CHL_A and CHL_B represent the contents of chlorophyll-a and chlorophyll-b respectively, CHL_T is the content of total chlorophyll. For estimation of carotenoid content, absorbance was noted at 480 and 510 nm.

3.3.7.6. Bacterial toxicity and bioluminescence inhibition

Photorhabdus luminescens subsp akhurstii was used in the present study to evaluate the acute and chronic toxicities of the biologically treated and untreated imidacloprid-contaminated samples. Lyophilized luminescent bacteria were obtained from NCMR (Pune, India) and grown in LB broth for conducting the toxicity assay. To assess the toxicity of the samples, a bacterial culture with optical density of one was used. 1 ml of culture of luminescent bacteria *P. luminescens* subsp akhurstii was added to 5 ml of each sample, i.e., T1 (control/distilled water), T2 (treated with isolate ST1), T3 (treated with consortium) and T4 (untreated imidacloprid-contaminated water) for the measurement of bioluminescence. % bioluminescence inhibition was calculated for all the samples using the following formula:

$$\%\ \text{bioluminescence inhibition} = \left(1 - \frac{\text{Sample intensity}}{\text{Control intensity}}\right) \text{x } 100$$

Using a Fluorescence spectrophotometer (Horiba, Model No.: PTI QuantaMaster™ 8000 series), the bioluminescence intensities of all the samples were measured in counts per second. The measurement of acute and chronic toxicities was done after 30 min and 24 hours of addition of luminescent bacteria to the samples.

3.3.7.7. Cytotoxicity assessment

To assess the quantitative and qualitative change in the cells on exposure to treated and untreated imidacloprid samples, MTT assay and DAPI staining tests were performed on mouse fibroblast cells, L929-RFP (red fluorescent protein). The cell proliferation of genetically modified L929 cells was monitored by exploring the fluorescence property (fluoresce red when excited at a green wavelength) of cells using a fluorescence microscope (Nikon Ti-U).

MTT assay: The MTT test is an indirect cytotoxicity test that evaluates in vitro cell growth and/or death in a cell culture. L929 cells were disaggregated with EDTA/trypsin, followed by seeding in 96-well plates at 10^4 cells per well. The cells were washed with PBS after 24 h of seeding and exposed to the samples T1, T2, T3 and T4. After incubation periods of 24 h and 48 h, 100 µL MTT solution, was added in each of the wells and incubated for 4 hours. 1% DMSO (dimethyl sulfoxide) was used as solvent control for all assays. Subsequently, solubilization of the formazan crystals was done in the wells for 15 min using 100 µL DMSO. The solution mixture was pipette-mixed and transferred to fresh wells. Measurement of optical densities of each well was done at 570 nm,

using a multimode microplate absorbance reader (Synergy H1 hybrid, Biotek, USA). All the experiments were conducted in triplicates. The absorbance values of samples were compared with those of the control after all values were corrected by subtracting the absorbance value of blank (DMSO only).

DAPI staining: DAPI is a blue fluorescent DNA stain is used to assess gross cell morphology and to determine the number of nuclei. For DAPI to enter cells and bind to the DNA, cells must be permeabilized and/or fixed. DAPI binds strongly to sections of DNA rich in A-T, and forms a fluorescent complex. Cells were obtained from IGIB (New Delhi, India) and grown in a humidified incubator 37°C with 5% CO_2 in complete growth medium containing DMEM supplemented with 1% antibiotic (100 µg/mL streptomycin and 100 nU/mL penicillin) and 10% FBS. The cells were sub-cultured till the flasks attained 75% cell density. The cells (1×10^5 cells/well) were further cultured for 12 hours in 12 well-plate, containing nutrient media. Distilled water (T1) was used as blank, sample treated with consortium (T2), sample treated with the most effecient bacterial isolate *Tepidibacillus decaturensis* strain ST1 (T3) and the prepared imidacloprid solution without exposure to bacterial remediation, referred as untreated sample (T4) were used in the study. The media was then discarded and a new medium containing four treatment groups (T1, T2, T3 and T4) were added to the culture plates in triplicate. 150 mg/L of imidacloprid solution was prepared and subjected to bioremediation using the bacterial consortium (prepared using two most efficient isolated imidacloprid-degrading bacterial species).

3.3.8. Metagenomic analysis

The soil sample collected from agricultural fields was divided into three parts, one part was left as such; in the second part, imidacloprid (150 ppm) was added to study the effect of imidacloprid on bacterial diversity in soil and the third was subjected to biodegradation after addition of imidacloprid (150 ppm) to study the change in bacterial diversity after biodegradation of imidacloprid. The three prepared samples were outsourced to Biokart, India for metagenomic sequencing, bacterial diversity and functional analysis.

The metagenomics analysis of samples was done in the following 6 steps:

(i) DNA sample preparation

- DNA extraction
- DNA QC

(ii) Library preparation

- 16s amplification and Library preparation

(iii) Data generation

- Sequencing by 300 bp
- Raw data QC

(iv) Data analysis

- Data trimming
- Merge paired ends, chimera check
- OTU calling

(v) Statistical analysis

- Heat map
- Clustering
- Core microbiome

(vi) Additional analysis

- Comparison among the samples
- Prediction of metabolic pathways

DNA extraction from all three samples was performed using a commercially available QIAGEN DNA extraction kit. 50 g of soil from each sample was processed for DNA extraction. Extracted DNA from the samples was subjected to NanoDrop and GEL Check before being taken for PCR amplification: The NanoDrop readings of 260/280 at an approximate value of 1.8 to 2 are used to determine the DNA's quality. Gene amplification and sequencing were performed. The identification of microbiota present in the soil samples was done by targeting the V3-V4 region of 16S rRNA. The amplified 16s PCR Product is purified and subjected to GEL Check and Nanodrop QC. The NanoDrop readings of 260/280 at an approximate value of 1.8 to 2 are used to determine the quality of DNA.

The amplicons from each sample were purified with Ampure beads to remove unused primers and an additional 8 cycles of PCR was performed using Illumina barcoded adapters to prepare the sequencing libraries. Libraries were purified using Ampure beads and quantitated using Qubit dsDNA High Sensitivity assay kit. Sequencing was performed using Illumina Miseq with 2x300PE v3 sequencing kit. The databases used include SILVA / GREENGENES / NCBI. Each read was

classified based on % coverage and identity. The 16S workflow will be useful in identifying pathogens in a mixed sample or understanding the composition of a microbial community. The quality control check of raw sequence data coming from high throughput sequencing pipelines is done using FASTQC (version 11.2) and MULTIQC (version 1.9). MULTIQC consolidates FASTQC results into single report.

Trimming of adapters and low-quality reads was done by TRIMGALORE. The trimmed reads are further taken for the processing which such as merging of paired-end reads and chimera removal. Paired end reads obtained were further processed, and checked (for score distribution, base quality, average base content and GC distribution). Multiple filters were applied to generate high-quality reads. UCHIME algorithm was used to detect and remove chimera. The entire downstream analysis was done using QIIME program.

The Operational Taxonomic Unit (OTU) table gives an overall microbial community present in the given samples. From the generated OTU the stacked bar charts are pivoted based on taxonomic levels. All pre-processed reads obtained were pooled and clustered into OTUs. Sequences were assigned to taxonomy using a classifier trained on the NCBI 16S rRNA BLAST database. Based on reads and OTU distribution of class, order, phylum, family, and genus for each sample, the reads were categorized. OTU abundance calculation and estimation correction, which was achieved by QIIME / MOTHUR / KRAKEN / BRACKEN workflows. This workflow enables highly accurate investigations at the genus level. A heat map was constructed at the genus taxonomic level with the detailed view mode of <1500 features. The samples were clustered using the Ward cluster algorithm based on Euclidean distance measure. The dendrogram was constructed based on the distance measure of the Bray-Curtis Index with the Ward clustering algorithm.

The core microbiome refers to the set of taxa that are detected in a high fraction of the population above a given abundance threshold. The count data is transformed to compositional (relative) abundance to perform such analysis. The data was visualized with a sample prevalence of 20% and a relative abundance of 0.1 %. The 10 most prevalent genera in each of the samples were identified and the abundant features across the samples were noted. A comparative analysis of the genus present in the samples was done. The prediction was metabolic pathways was done using KEGG software.

3.3.9. Application of lyophilized and encapsulated bacteria

3.3.9.1. Lyophilization and encapsulation of bacteria

Lyophilization is a low-temperature dehydration process, typically used to preserve and extend the shelf-life of materials or make the material more convenient for transportation. Lyophilization works by freezing the material, and then placed under a vacuum, allowing the frozen water to sublimate.

The bacterial cells were grown in liquid media. For lyophilization of bacteria, the pre-cultured bacterial cells were revived in MSM with imidacloprid as a carbon source and glucose as a co-substrate and incubated for 48 hours. Subsequently, the culture volume was up-scaled to 1 L for lyophilization. On attaining the log phase, the cells were stored at -80ºC for 48 hours before carrying out the lyophilization process. The lyophilization process was carried out for 48 hours and stored at 4ºC for further studies.

The survival of cells was measured in terms of the colony forming units (CFUs) before lyophilization or after re-wetting of the lyophilized powder. Fresh and lyophilized cells, were plated onto agar plates for the determination of CFUs. The lyophilized cells re-suspended in 1 ml distilled water was equivalent to 1 mL of 1.0 OD_{600} nm fresh cells. The lyophilized cells when re-suspended rapidly formed a uniform suspension and were allowed to equilibrate for 5 min before the biodegradation assays were carried out.

To assess whether the lyophilized bacterial cells retained the ability to biodegrade imidacloprid and how these activities could alter throughout storage periods at 4°C, measurements of imidacloprid degradation rates by inoculum prepared from the stored cells were conducted. The stored cells were resuspended in a sterile saline solution (10 ml total volume). Sub-culturing of the cells was done and biodegradation studies were conducted. Further experiments were performed to evaluate the degradation capacity of bacteria after long-term storage. Lyophilized bacterial cells were revived after every 50 days and degradation experiments were performed. The study was carried out for 350 days. After carrying out the biodegradation experiment, the residual imidacloprid was extracted and estimated using HPLC.

3.3.9.2. Microcosm experiment

Imidacloprid biodegradation experiments were conducted in soil microcosms (1000 g of soil per microcosm) set up using agricultural soil. In the microcosm experiment, five distinct microcosms were prepared. Control microcosms, denoted as M1, were spiked with imidacloprid but not

bioaugmented. Biostimulation was implemented by adding urea in M2. Microcosm M3 involved bioaugmentation with lyophilized bacteria, while M4 was bioaugmented with encapsulated bacteria. Microcosm M5 comprised a combination of bioaugmentation and biostimulation. Each treatment was replicated in a minimum of two microcosms. The soil microcosm studies were conducted for 30 days. After degradation studies, imidacloprid residue was extracted and quantified using HPLC.