Chapter III: Materials and Methods

This chapter outlines the experimental approach used to investigate the degradation of two specific nitro-PAHs (1-nitropyrene and 2-nitrofluorene) and the role of selected bacterial strains, native plants, and biostimulants in the process. The following sections provide a detailed description of the chemicals, materials, and methodologies employed in this study.

3.1. Chemicals and Reagents

3.1.1. Analytical Standards and Solvents

1-nitropyrene and 2-nitrofluorene, used as target contaminants in the degradation study, were obtained from Sigma-Aldrich, India. High-performance liquid chromatography (HPLC)-grade solvents, including acetonitrile, dichloromethane, n-hexane, acetone, and ethyl acetate**,** were also procured from Sigma-Aldrich, India, to ensure high-purity sample preparation and accurate chromatographic analysis.

3.1.2. General Chemicals

Analytical-grade reagents, including KCl, $CaCl_2 \cdot 2H_2O$, methyl red indicator, Kovac's reagent, ammonium molybdate, ammonium metavanadate, 5-sulfosalicylic acid, Taq DNA polymerase, universal bacterial primers (27F and 1492R), trichloroacetic acid, ammonium acetate, and hydroxylamine hydrochloride, were sourced from Sigma-Aldrich, India. Other essential reagents, including KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $FeSO_4 \cdot 7H_2O$, $FeCl_2$, NH_4NO_3, $(NH_4)_2HPO_4$, $KMnO_4$, $MgSO_4$, Na_2HPO_4, NaOH, Triton X-100, HCl, sodium citrate, methanol, picric acid, sodium carbonate, ferrous ammonium sulfate, anhydrous sodium sulfate, indole acetic acid, sodium thiosulfate, agar, casein hydrolysate, oxidase discs, ammonium sulfate, Tris-Cl, sodium malonate, bromothymol blue, sulfanilic acid, α-naphthylamine, pectin, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), bis-acrylamide, acrylamide, Tris base, Coomassie Brilliant Blue R-250, glycine, glycerol, catechol, β-mercaptoethanol, congo red, casein, sodium phosphate dibasic, and o-nitrophenyl-β-D-galactopyranoside, as well as analytical reagent-grade lactose, were procured from Sisco Research Laboratory (SRL), Mumbai, India.

3.1.3. Microbiological Media and Culture Components

For bacterial culture and biochemical assays, nutrient agar, beef extract, yeast extract, NaCl, tryptic soy broth, peptone, minimal salt medium, Simmons citrate agar, MR-VP broth, trypticase soy agar, Barritt's reagents A and B, urea, phenol red, hydrogen peroxide, Pikovskaya's agar and broth, tricalcium phosphate, tryptophan, glucose, sucrose, Chrome Azurol S, and McFarland's turbidity standard were purchased from HiMedia, Mumbai, India. Polytetrafluoroethylene (PTFE) filters and a Gram staining kit were procured from the same company HiMedia, Mumbai. Hexadecyltrimethylammonium bromide (HDTMA) was procured from Sigma-Aldrich, India.

3.1.4. Acids and Sequencing Reagents

Analytical-grade nitric acid, hydrochloric acid, and sulfuric acid were obtained from ThermoFisher Scientific, India. SureExtract PCR cleanup/Gel extraction kit was obtained from Genetix.

3.1.5. Other Materials

Farmyard manure was procured from Safex Bio-Organic Pvt. Ltd. Autoclavable polyethylene bags (12 × 24 cm) were purchased from Tarsons. DNeasy PowerSoil Pro Kits were obtained from Qiagen, while 0.2 μm nylon syringe filters and Whatman No. 1 filter papers were procured from Sigma-Aldrich, India. All chemicals and reagents were of high purity, ensuring reliability and accuracy in the experimental procedures.

3.2. Isolation and Identification of Potential nitro-PAHs Degrading Rhizobacteria and nitro-PAHs Accumulating Plants

3.2.1. Site Description

This study was conducted in Borholla, Upper Assam (26°45' latitude and 94°37' longitude), which falls under the Titabar Subdivision of Jorhat District, Assam. Plant species and soil samples were collected from the periphery of ONGC GGS (Oil and Natural Gas Corporation Limited Group Gathering Station) in Borholla using standardized methods (Fig. 3.1). The soil in this region is light-colored, slightly coarse in texture, and contains minimal gravel in the B horizon. It is classified as Dystric Eutrochrepts, indicating moderate fertility and acidic conditions (ICAR-NBSS&LUP, 2013). The area receives annual rainfall ranging from 700 to

1500 mm. The climate is highly humid, with summer temperatures reaching up to 38 °C and winter temperatures dropping as low as 6 °C (Sarma et al., 2019).

This region has a high concentration of oil drilling sites, many of which are located near tea gardens and rice fields. Additionally, human settlements are close to these drilling sites. ONGC has been actively engaged in crude oil extraction and transportation in this area since 1976 (Sarma et al., 2016).

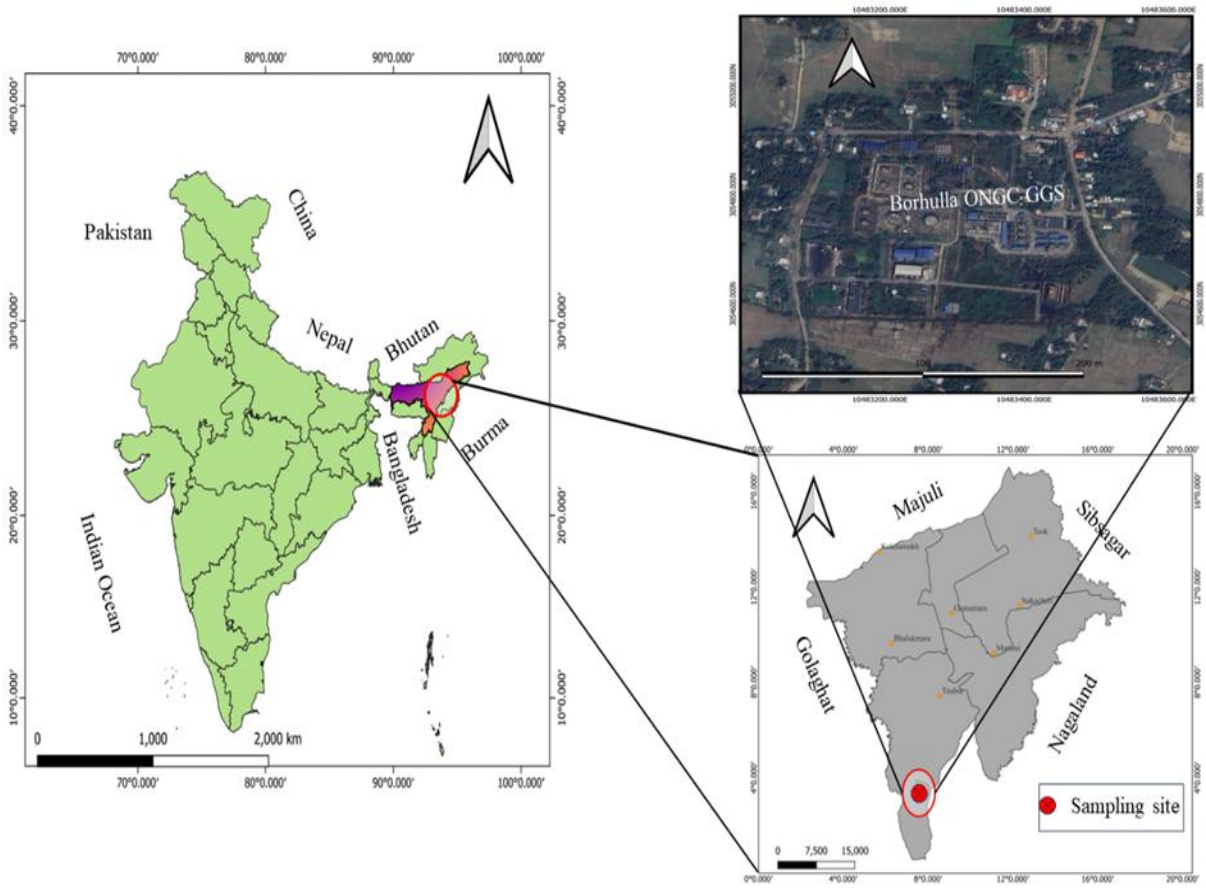

Fig. 3.1. Study site – ONGC GGS Borholla, Assam, India (26°45' N latitude and 94°37' E longitude), where plants and soil samples were collected.

3.2.2. Screening and Selection of nitro-PAHs Accumulating Plant Species

In this study, potential plant species at the study site were selected using standard field ecological methodology described by Sarma et al. (2017) to ensure a robust and scientifically valid approach. 30 quadrats (1 m x 1 m) were randomly placed in the sample area. Key vegetative metrics, such as frequency, relative frequency, relative density, and the Importance

Value Index (IVI), were calculated. The plant species found within the quadrats were identified using the Flora of India and verified against existing herbarium records at the Botanical Survey of India to ensure accuracy and authenticity. The species with the highest IVI scores were chosen for further examination. These plant species were then investigated further for their ability to accumulate nitro-PAHs, highlighting their potential value in the environmental remediation approach.

The plant species were transplanted into a greenhouse that provided a controlled environment. The soil used was a well-drained mixture of loam and sand, with a pH level ranging from 5 to 7. To improve the soil's fertility, water retention, and texture, 2.5 kg/m² of farmyard manure was added one month before transplantation. These measures ensured that the plants remained healthy and free from physical ailments. No inorganic fertilizers were used during the experiment. The plants were then carefully moved to the greenhouse, which offered optimal conditions for growth and monitoring. To avoid any environmental changes that could impact plant development, the greenhouse maintained consistent temperatures, humidity levels, and light exposure.

3.2.3. Pot Experiment for Quantitative Estimation of nitro-PAHs Accumulation

Two-kilogram capacity earthen pots were used for the experiment, which were sterilized with ethanol and UV radiation before the experiment. Subsequently, each pot was then filled with 1 kg of sterilized soil spiked with 25 mg/kg of the respective nitro-PAHs. The filled pots were stored at 4 °C for 60 days to allow for complete acetone evaporation. The soil used in this study was collected from Titabor, India and its physicochemical parameters were investigated following the methodology of Pawar et al. (2009). Before filling in the sterilized pots, the soil was sieved through a 2 mm sieve and sterilized in a vertical autoclave (Optics Technology) for 1 hour at 121 °C for two consecutive days (Singha & Pandey, 2020). To confirm the efficacy of soil sterilization, 1 g of soil from each pot was diluted in sterile distilled water (10^{-8}) and plated on a nutrient agar medium at 15-day intervals. Following 42 hours of incubation at 32 °C, colony-forming units per gram (cfu/g) were measured. Throughout the testing period, no microbial growth was observed, validating that the soil employed in the pot experiment was effectively sterilized.

Plant species that were previously maintained in the greenhouse were transplanted into pots spiked with nitro-PAH and allowed to grow for 60 days. The plants were nurtured within a greenhouse under regulated conditions, including a 25-28 °C temperature range, a photoperiod of 16 hours of light and 8 hours of darkness, and systematic watering to maintain soil moisture (Singha & Pandey, 2020).

The accumulation of 1-nitropyrene and 2-nitrofluorene in the root tissues was determined using the methodology outlined by Yang et al. (2024), with slight modifications. Initially, 5 mg roots were thoroughly cleaned with tap water and ethyl acetate and then dried at 45 °C for 48 hours. The dried root samples were put in 15 mL polypropylene centrifuge tubes containing 2 mL of HPLC-grade acetonitrile and 1 g of NaCl. The mixtures were mixed in the vortex for 5 minutes and then shaken overnight in an orbital shaker incubator (Optics technology, Model No: OT-INS-61/9). Following that, the samples were sonicated in an ultrasonic bath sonicator (Athena Technology, Model No: ATS-1) for 1 hour. The resulting solutions were then centrifuged in a micro-refrigerated centrifuge (Remi, Model No: CM-12 plus) at 7000 rpm for 10 minutes. From the supernatant, 1 mL of the extract was transferred into a 4 mL centrifuge tube containing 0.15 g of anhydrous $MgSO_4$, 0.1 g of primary secondary amine (PSA) sorbent, and 0.05 g of C18. The mixture was vortexed for 1 minute and the supernatant was collected followed by centrifugation at 7000 rpm for 5 minutes. A 100 µL aliquot of each sample (2 mL total volume) was diluted in microcentrifuge tubes with 900 µL acetonitrile.

Simultaneously, the mobile phase was prepared by mixing acetonitrile and Milli-Q water in an 85:15 ratio and degassed using an ultrasonic bath sonicator for 15 minutes. The standard solutions were prepared by dissolving 1 mg of 1-nitropyrene and 2-nitrofluorene in 1 mL of acetonitrile to obtain a stock solution. This stock solution was then diluted with acetonitrile to prepare working standard solutions at concentrations of 5, 10, and 20 µg/mL. 20 µL of each prepared standard solution was injected into the High-performance liquid chromatography (HPLC) equipped with a UV detector-2489 (Waters Corporation, USA). The instrument was fitted with a ThermoFisher Scientific's C18(2) column (4.6 × 250 mm, 5 µm). Following the standard solution injection, the prepared samples were injected into the HPLC. During HPLC analysis, the column temperature was maintained at 40 °C, and the injection volume was set at 20 µL. The detection wavelength was fixed at 254 nm to ensure precise measurement of the target compounds. The standard calibration curves were constructed for 1-nitropyrene and 2-nitrofluorene using the standard concentrations and their corresponding retention areas

obtained from the HPLC spectra. Finally, the concentration of nitro-PAHs (mg/L) in the samples was determined by correlating their retention areas with the calibration curve.

3.2.4. Isolation of Potential nitro-PAHs Degrading Rhizobacteria

The soil was collected aseptically from the root zones of four selected plant species using a sterilized knife and transferred to sterile autoclavable polyethylene bags (12 × 24 cm). To avoid contamination, the samples were wrapped, appropriately labeled, and kept in an icebox at 4 °C and transported to the laboratory. Furthermore, all handling procedures were performed using sterile gloves to avoid external contamination. The soil samples were then dried in the shade, sieved through a 2 mm sieve, and stored at 4 °C. Soil (0.1 g) from each sample was gradually diluted in sterile water until a 10^{-8} dilution. 200 µL of this dilution was inoculated over sterile nutrient agar plates. Simultaneously, the stock solution was prepared by dissolving nitro-PAHs (1-nitropyrene and 2-nitrofluorene) into acetone [(25 mg/L (w/v %)] and stored at 4 °C for further use. After inoculation, the nutrient agar plates were sprayed with a 2% stock solution of nitro-PAHs (these nitro-PAHs serve as the carbon source) and incubated at 32 °C for 42 hours. Following the incubation period, the cfu/g of all samples were recorded.

The bacterial colonies showing a clear zone indicating their ability to catabolize nitro-PAHs were further isolated from nitro-PAHs nutrient agar plates. These colonies (standardized to a concentration of 50,000 cells using McFarland's turbidity standard by measured at 600 nm using a UV-visible spectrophotometer (Shimadzu, Model No: UV-1900i) were inoculated into nitro-PAHs-PBYS medium. The composition of the nitro-PAHs-PBYS medium was slightly modified from the methodology outlined by Sarma et al. (2019) and included the following components (per liter): 25 mg of respective nitro-PAHs, 2.5 g of beef extract, 50 g of NaCl, 1 g of yeast extract, 1 g of NH_4NO_3, 5 g of peptone, 0.5 g of KH_2PO_4, 0.01 g of $FeCl_2$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of KCl, 0.2 g of $CaCl_2 \cdot 2H_2O$, and 1 g of K_2HPO_4, with the pH adjusted to 7.0. Two different bacterial strains exhibiting strong tolerance and high biomass production in the nitro-PAHs-PBYS medium were selected for further study. The bacterial biomass (grams of dry cell weight per liter) was calculated using a UV-visible spectrophotometer at 600 nm, using the equation: Biomass = 0.5413 × absorbance (Moscoso et al., 2012).

To investigate the growth dynamics of selected bacterial strains, 1-nitropyrene, and 2-nitropyrene were spiked in PBYS medium to prepare nitro-PAHs spiked PBYS medium at concentrations of 12.5 mg/L, 25 mg/L, 37.50 mg/L, and 50 mg/L. The bacterial colonies were standardized to a concentration of 50,000 cells using McFarland's turbidity standard to ensure

consistency across all samples. These bacterial suspensions were then inoculated into the nitro-PAHs-PBYS medium and cultured at 37 °C for 7 days at 200 rpm. Before inoculation, the medium was left uncovered in a laminar flow cabinet for 12 hours to let the acetone evaporate. The bacterial biomass (grams of dry cell weight per liter) in the flasks was recorded at the end of the incubation period. Abiotic control flasks containing nitro-PAH spiked PBYS medium but no bacterial inoculation were prepared and incubated under the same conditions. The complete experiment was carried out three times to ensure reliability and accuracy.

3.2.5. Compatibility Assessment and Biodegradation Efficiency of nitro-PAH-Degrading Rhizobacteria

The compatibility of the bacterial strains was performed following the methodology proposed by Irabor and Mmbaga, (2017). In brief, each bacterial strains were cultured individually in a nutrient broth medium overnight at 28 °C and 200 rpm. Following that, 100 µL of the test bacterial strains BG034 and BG05 were dispersed individually on the surface of nutrient agar plates with a population of around 10^8 cfu/mL. Filter paper discs with a diameter of 1 cm (Whatman number 1) were put on the spread plate. Each disc was inoculated with a nutrient broth culture of the strain being evaluated for compatibility. The plates were subsequently incubated at 28 °C for 4 days and their growth pattern was monitored at 24-hour intervals.

The isolated bacterial strains were again cultured on nutrient broth at 32 °C for 12 hours at 200 rpm. The bacterial pellets were separated through centrifuging at 8000 rpm for 10 minutes at 4 °C and rinsed three times with sterile distilled water. These pellets were then suspended in a minimal salt medium. The bacterial cultures were adjusted to 50,000 cells using McFarland's turbidity standard. Each bacterial suspension (10 mL) and co-inoculum (5 mL of bacterial suspension A and 5 mL of bacterial suspension B) were inoculated into nitro-PAHs-PBYS medium containing 25 mg/L of nitro-PAHs (1-nitropyrene and 2-nitrofluorene separately) as a carbon source. Before bacterial inoculation, the medium was placed in a laminar cabinet for 12 hours to complete the evaporation of acetone. The flasks were then incubated at 37 °C and 200 rpm after inoculation. At intervals of 24, 48, and 72 hours, 15 mL of culture media were collected and centrifuged at 8000 rpm for 15 minutes at 4 °C. The supernatant was mixed with an equal amount of acetone and hexane (20:80 v/v) solvent for liquid-liquid extraction. Anhydrous sodium sulfate (Na_2SO_4) was employed to remove leftover water, and the samples were filtered before being analyzed by HPLC. The HPLC instrument operating conditions and sample injection procedure were maintained as described in Section 3.1.3. The nitro-PAH

concentration (mg/L) was determined from the HPLC spectra and their biodegradation percentage was determined using the following formula:

Degradation % = $\frac{Initial\ concentration - Final\ concentration}{Initial\ concentation}$ ×100% (Sarma, et al., 2019)

Furthermore, the kinetics of chemical degradation were assessed after calculating the compound concentration from their respective HPLC spectra. The data were evaluated using three kinetic models—zero-order, first-order, and second-order—with their respective integrated mathematical formulations:

For, Zero-order: $[A] = -kt + [A]_0$

First-order: $ln[A] = -kt + ln[A]_0$

Second-order: $\frac{1}{[A]} = kt + \frac{1}{[A]o}$ (Pyun & Lipschitz, 1976)

Where, $[A]_0$ is the initial concentration, $[A]$ is the final concentration at time t, *ln* is the natural logarithm, *k* is the rate constant, and t is the time

To determine the most suitable model, graphical representations were created by plotting concentration versus time for zero-order, *ln*[Concentration] versus time for first-order, and $\frac{1}{Concentration}$ versus time for second-order, with the model demonstrating the highest coefficient of determination (R^2) deemed the most fitting (Das et al., 2010; Kokkinidou, 2008). A statistical analysis was performed utilizing regression equations to calculate R^2 values. The ideal model was used to determine the rate constant (*k*) and half-life ($T½$) providing insights into the compound's degradation rate and behavior under the given experimental circumstances.

3.2.6. Maintenance and Storage of Bacterial Isolates

All the pure isolates obtained were grown on nutrient broth and stored at -20 °C in 40% glycerol for long-term storage. During experimentation, cultures were revived on nutrient agar and stored at 4 °C for short-term storage. Every week, cultures were transferred on fresh nutrient agar plates, and fresh cultures were revived from the stock cultures and stored at -20 °C. The stocks were maintained at IBT Hub (DBT) at N N Saikia College, Titabor, Assam.

3.2.7. Biochemical Characterization

The isolates of pure culture were carried out by a series of biochemical characterizations to evaluate their metabolic capabilities, and are cited below.

3.2.7.1. Gram Staining

Clean slides were sterilized using 70% alcohol. A drop of distilled water was mixed with a small quantity of the bacterial culture to create a smear. An inoculating loop was then used to spread the smears evenly, and the slides were heat-fixed. Next, the slides were treated with crystal violet solution for 1 minute, rinsed with distilled water, and allowed to air dry. Following this, an iodine solution was applied to the slides for another minute, after which it was drained, and the slides were washed with 95% ethanol. To complete the staining process, the smears were exposed to safranin for 30 seconds, rinsed again with distilled water, and left to air dry. Finally, the slides were examined under a light microscope, and the results were recorded (Erkmen, 2021).

3.2.7.2. Citrate Utilization

To further characterize the bacterial isolates, citrate utilization was tested. In this test, oxaloacetate was used as a carbon source to evaluate the ability of isolated bacteria to metabolize citrate. When the bacteria metabolized citrate and converted it to oxaloacetate, the pH of the medium increased, resulting in a bright blue color. Simmons citrate agar slants were streaked with pure cultures of bacterial strains and then incubated for 72 hours at 30 °C. After incubation, the appearance of a bright blue color in the tubes indicated a positive result (MacWilliams, 2009).

3.2.7.3. Methyl Red Test

The Methyl Red test was conducted to determine the bacterial isolates to produce stable acid end products from glucose fermentation. A pure bacterial culture was prepared in trypticase soy broth and incubated at 30 °C for 18 hours. Subsequently, 1 mL of the prepared inoculum was added to the MR-VP broth, which was then incubated for 48 hours at 37 °C to allow the bacteria to ferment glucose and produce acid. After incubation, a few drops of Methyl Red indicator were added to the broth. A red color indicated a positive Methyl Red test, confirming acid production. Conversely, a yellow or orange color upon the addition of the indicator

indicated a negative Methyl Red test, suggesting no significant acid production (Ali et al., 2022).

3.2.7.4. Voges Proskauer Test

To further evaluate the ability of isolated bacterial strains to produce acetoin, the Voges-Proskauer (VP) test was performed. Individual bacterial strains were inoculated into MR-VP broth with 18-hour-old pure cultures grown on trypticase soy agar. The broth cultures were then incubated at 37 °C for 48 hours. After incubation, 2.5 mL of MR-VP broth, 0.6 mL of Barritt's reagent A (5% α-naphthol), and 0.2 mL of Barritt's reagent B (40% KOH) were mixed. The tubes were gently shaken for 1 minute to ensure that the medium was properly exposed to oxygen. The tubes were then allowed to stand for 30 minutes. The presence of red color in the tubes indicates a positive Voges-Proskauer test (Ali et al., 2022).

3.2.7.5. Urease Test

To further evaluate the ability of the bacterial isolates to hydrolyze urea, the urease test was performed. Bacteria in the logarithmic growth phase were inoculated into test tubes with a nutrient broth medium. The nutrient broth medium was supplemented with 2% (w/v) urea and 0.012% phenol red. Following that, all of the test tubes were incubated for 7 days at 30 °C. The appearance of pink color in the test tubes after incubation indicated a positive result for urea hydrolysis. This color shift is usually caused by the alkaline products of urea hydrolysis (Brink, 2010).

3.2.7.6. Catalase Test

To further evaluate the enzymatic activity of the isolated bacteria, the catalase test was conducted. A volume of 1 mL hydrogen peroxide (H_2O_2) was added immediately to 18-hour-old pure culture strains that had been cultivated on a nutrient agar slant. Those tubes were then placed against a shadowy area, and carefully observed for emerging bubbles. The formation of effervescence with the quick emergence of bubbles indicates a positive response. On the other hand, the absence of bubble formation indicates a catalase-negative response, denoting that the organism lacks the catalase enzyme required to hydrolyze (Reiner, 2013).

3.2.7.7. Gelatine Test

Furthermore, the isolated bacteria were assessed for gelatinase activity. The bacterial isolates were placed in nutrient agar test tubes containing 5 mL of gelatin. These tubes were then incubated for 48 hours at 30 °C to allow bacterial colonies to proliferate. After adequate development, the test tubes were refrigerated at 4 °C for 1 hour. Bacterial isolates that contained the gelatinase enzyme revealed the ability to break down the protein in the medium, causing the tubes to remain in a liquid state. The presence of liquid in these tubes was viewed as a positive indicator of gelatinase activity (dela Cruz & Torres, 2012).

3.2.7.8. Oxidase Test

Following the gelatine test, the oxidase test was performed using the Filter Paper Spot Method. Well-isolated bacterial colonies from freshly cultured plates (18-hour-old) were transferred onto a small piece of filter paper using a sterile loop. The bacterial smear is then treated with 1 or 2 drops of 1% Kovács oxidase reagent. Color changes were observed to dark purple within 5 to 10 seconds. Positive delayed oxidase findings occur when the color turns purple in 60 to 90 seconds (Shields & Cathcart, 2013).

3.2.8. Genomic DNA Extraction and 16S rDNA Sequencing

Genomic DNA was isolated from pure bacterial cells using Qiagen's DNeasy PowerSoil Pro Kits (Cat. no. 47014) according to the manufacturer's instructions. The 16S rDNA region, a widely recognized marker for bacterial identification, was amplified via polymerase chain reaction (PCR) in master cycler (Eppendorf) with Taq DNA polymerase and universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), which are designed to target conserved areas of the bacterial 16S rDNA gene. The amplified fragments were purified using SureExtract PCR cleanup/Gel extraction kit and sequenced on an ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific). The results sequences were compared to those in the NCBI database using the Basic Local Alignment Search Tool for Nucleotide sequences (BLASTn) to determine closely related species. The phylogenetic tree was constructed by using the software MEGA (Molecular Evolutionary Genetics Analysis), Version 11, and the Neighbor-Joining technique. The 16S rDNA gene sequence acquired in this study was submitted to the NCBI GenBank database and assigned an accession number.

3.2.9. *In Vitro* Assessment of Plant Growth-Promoting Traits

3.2.9.1. Phosphate Solubilization Assay

Tricalcium phosphate ($Ca_3(PO_4)_2$) was used as the insoluble phosphate source to test phosphate solubilization. Pikovskaya's agar medium containing bromophenol blue was spot-inoculated with the isolated strains. Following that, the plates were incubated at 30 °C for 96 hours to allow bacteria to grow. The emergence of a distinct yellow halo surrounding the colony indicated the presence of a zone of phosphate solubilization. This halo creation showed that organic acids were produced as a route for phosphate solubilization (Pande et al., 2017). The halo diameter around the colony and the colony diameter were measured, and the solubilization index (PSI) was calculated according to the following formula:

$$PSI = \frac{\text{Colony diameter} + \text{Holozone diameter}}{\text{Colony diameter}}$$ (Pande et al., 2017).

For the quantitative assessment, 10 mL of Pikovskaya's broth containing 5000 mg/mL of tricalcium phosphate was inoculated with 0.1 mL of bacterial culture which was adjusted to a concentration of 2×10^8 cfu/mL. The cultures were incubated at a temperature of 30 °C for 96 hours. Following the incubation period, the supernatant was obtained by centrifuging the culture at 10,000 rpm for 20 minutes. The resultant supernatant was subsequently filtered through a 0.45 µm membrane filter paper. 0.1 mL of the resultants filtered was mixed with 0.25 mL of Barton's reagent [25 g of ammonium molybdate ($(NH_4)_2MoO_4$) was dissolved in 400 mL of distilled water (Solution A). Separately, 1.25 g of ammonium metavanadate (NH_4VO_3) was dissolved in 300 mL of boiling distilled water, cooled, and combined with 250 mL of concentrated nitric acid (HNO_3) (Solution B). Solutions A and B were then mixed, and the final volume was adjusted to 1000 mL with distilled water]. The final test volume was adjusted to 5 mL by adding distilled water. After 10 minutes, the optical density was measured at 430 nm using a UV-visible spectrophotometer. The phosphate concentration in the samples was determined by extrapolating the absorbance values from a standard calibration curve.

The standard curve was prepared by preparing a stock phosphorus solution by dissolving 0.02195 g of monopotassium phosphate (KH_2PO_4) (pre-dried at 60 °C for 1 hour and cooled in a desiccator) in 100 mL of distilled water. A working solution was subsequently prepared by

diluting 15 mL of this stock solution to a final volume of 25 mL with distilled water. Various aliquots (ranging from 0.1 to 1 mL) of the working solution were combined with 0.25 mL of Barton's reagent, and the total volume was adjusted to 5 mL with double-distilled water. After 10 minutes, the absorbance at 430 nm was measured, and a standard curve was plotted by correlating the absorbance with phosphate concentrations. All experiments were performed in triplicate, and the data were expressed as mean values (Pande et al., 2017).

3.2.9.2. Siderophore Production and Quantification

A loopful of bacterial culture was inoculated onto a sterile Chrome Azurol S (CAS) agar medium and incubated at 30 °C for 96 hours. The medium surrounding the bacterial growth underwent a dramatic color change. A change in color from blue to orange or yellow around the colonies indicated the production of siderophore (Himpsl & Mobley, 2019).

The CAS shuttle assay was used to determine the quantitative amount of siderophores. For this assay, 1 mL of each culture supernatant was mixed with 1 mL of CAS reagent, which included 10 mM HDTMA, 1 mM $FeCl_3$ solution, and 2 mM CAS solution. The absorbance was then measured at 630 nm using a UV-visible spectrophotometer. A reference sample was prepared by mixing 1 mL of uninoculated broth with 1 mL of CAS reagent. The siderophore activity was represented as a percentage of siderophore units, determined using the following formula:

$$\text{Percentage of siderophore units} = \frac{\text{Absorbace of reference} - \text{Absorbance of sample}}{\text{Absorbance of reference}} \times 100$$ (Srivastava et al., 2022).

3.2.9.3. Ammonia Production Assay

Each strain was tested for its capacity to produce ammonia in peptone water. This method for evaluating ammonia output was based on the process developed by Cappucino & Sherman, (1992). Overnight broth cultures containing about 3 x 10^8 cfu/mL were added to 10 mL of peptone water. These tubes were then incubated at 30 °C for 96 hours to allow bacteria to proliferate. Following incubation, 1 mL of Nessler's reagent was added to each tube. The color shift from brown to yellow was recorded as a positive outcome, showing ammonia formation in the medium and the optical density of the samples was measured at 450 nm using a UV-visible spectrophotometer. The ammonia concentration was determined by referencing a

standard calibration curve prepared using ammonium sulfate in the range of 500–2500 µM (Cappucino & Sherman, 1992).

3.2.9.4. Indole Acetic Acid (IAA) Production Assay

Following the HCN test, the isolated bacterial strains were tested for indole acetic acid (IAA) production to evaluate their potential for auxin biosynthesis. The production of IAA has been observed using the methodology proposed by Mu'minah et al. (2015). 1 mL of chosen bacterial cultures was suspended in 100 mL of nutritional broth supplemented with tryptophan at a concentration of 0.1g/L. These cultures were then allowed to incubate at 30 °C for 96 hours. After incubation, the bacterial cells were extracted by centrifugation at 1000 rpm for 5 minutes. After that, 1 mL of Salkowski's reagent (50 mL 35% H_2SO_4, 1 mL of 0.5 M solution of $FeCl_3$) was added to 4 mL of supernatant. The reaction solution was then incubated at 30 °C for 30 minutes. Afterward, the tubes were examined for the appearance of a pink color, which indicated a positive IAA test (Mu'minah et al., 2015). The samples' optical density was measured at 530 nm with a UV-visible spectrophotometer after 30 minutes of pink color development. The quantity of IAA was evaluated by comparing the absorbance results to a standard calibration curve prepared using pure IAA, within the range of 10–100 µg/mL.

3.2.9.5. Hydrogen Cyanide (HCN) Production Assay

Following Lorck's methodology, rhizosphere bacterial isolates were subjected to a screening process to evaluate their potential for hydrogen cyanide production. Specifically, the bacteria were spread onto modified nutrient agar slants after the nutrient broth was supplemented with 4.4 g of glycine per liter. In each tube, a Whatman No. 1 filter paper was soaked in a solution containing 2% sodium carbonate and 0.5% picric acid. These preparations were then incubated for 96 hours at a temperature of 30 °C. A color change from orange to red indicated the generation of hydrogen cyanide (Lorck, 1948).

A quantitative assessment of HCN was performed to evaluate extracellular and free non-complexed cyanide ions (CN^-) present in the culture medium using the Baumeister & Schievelbein (1971) methodology. The bacterial cultures were incubated for 96 hours in Luria-Bertani broth (Miller) at 30 °C, enhanced with 5 g/L glycine, and adjusted to a pH of 7.4, as this specific medium promotes HCN synthesis, thereby enabling the evaluation of its optimal production capabilities. The methemoglobin reagent was generated by dissolving 0.34% (w/v) hemoglobin in 4 mM $NaNO_2$ and allowing the solution to incubate for 10 minutes.

Subsequently, a phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) was added in equal proportions, followed by an additional incubation period at ambient temperature for 30 minutes. To create a quantification benchmark, a potassium cyanide (KCN) solution served as the standard. The standard calibration curve was plotted by measuring their optical density at 424 nm utilizing a UV-visible spectrophotometer at different concentrations of KCN (i.e. 0.4 M, 0.8 M, 1.2 M, 1.6 M, 2 M, and 2.4 M). All prepared samples were subjected to spectrophotometric analysis at 424 nm utilizing a UV-visible spectrophotometer, and their HCN concentration was measured using the standard calibration curve (Baumeister & Schievelbein, 1971; Rijavec & Lapanje, 2016).

3.2.9.6. The Triple Sugar Iron (TSI) Fermentation Test

TSI test was used to evaluate bacterial strains' fermentation capacity using three sugars: glucose, sucrose, and lactose. TSI agar medium was made with 0.1% glucose and 1% lactose and sucrose. The bacterial strains were placed in TSI agar medium tubes and cultured at 30 °C for 48 hours. Positive findings were seen when the medium became yellow, suggesting sucrose and lactose fermentation (Lehman, 2005).

3.2.10. Rhizobacterial Inoculation and Growth Assessment in Plants

The isolated bacterial strains were cultured on nutrient broth at 32 °C for 12 hours with continuous shaking at 200 rpm. Then, 10 mL of each bacterial culture, and the co-inoculum (consisting of 5 mL of bacterial suspension A and 5 mL of bacterial suspension B) were added to a new nutrient broth medium and cultured until reaching the exponential phase. The cells were then collected and rinsed three times with sterile water. A UV-visible spectrophotometer was used to adjust the absorbance of the cell suspensions to a range of 0.3 to 0.4 at 600 nm. Before conducting the pot experiment, the roots of the seedlings were carefully washed and dipped in a bacterial solution made in a beaker, achieving a final density of 4.7×10^{13} cfu/mL. The plants were nurtured within a greenhouse under regulated conditions, including a 25-28 °C temperature range, a photoperiod of 16 hours of light and 8 hours of darkness, and systematic watering to maintain soil moisture. Measurement of shoot length, and root length, of selected plant species were recorded at 7 days, 14 days, and 21 days intervals (Fig. 3.2).

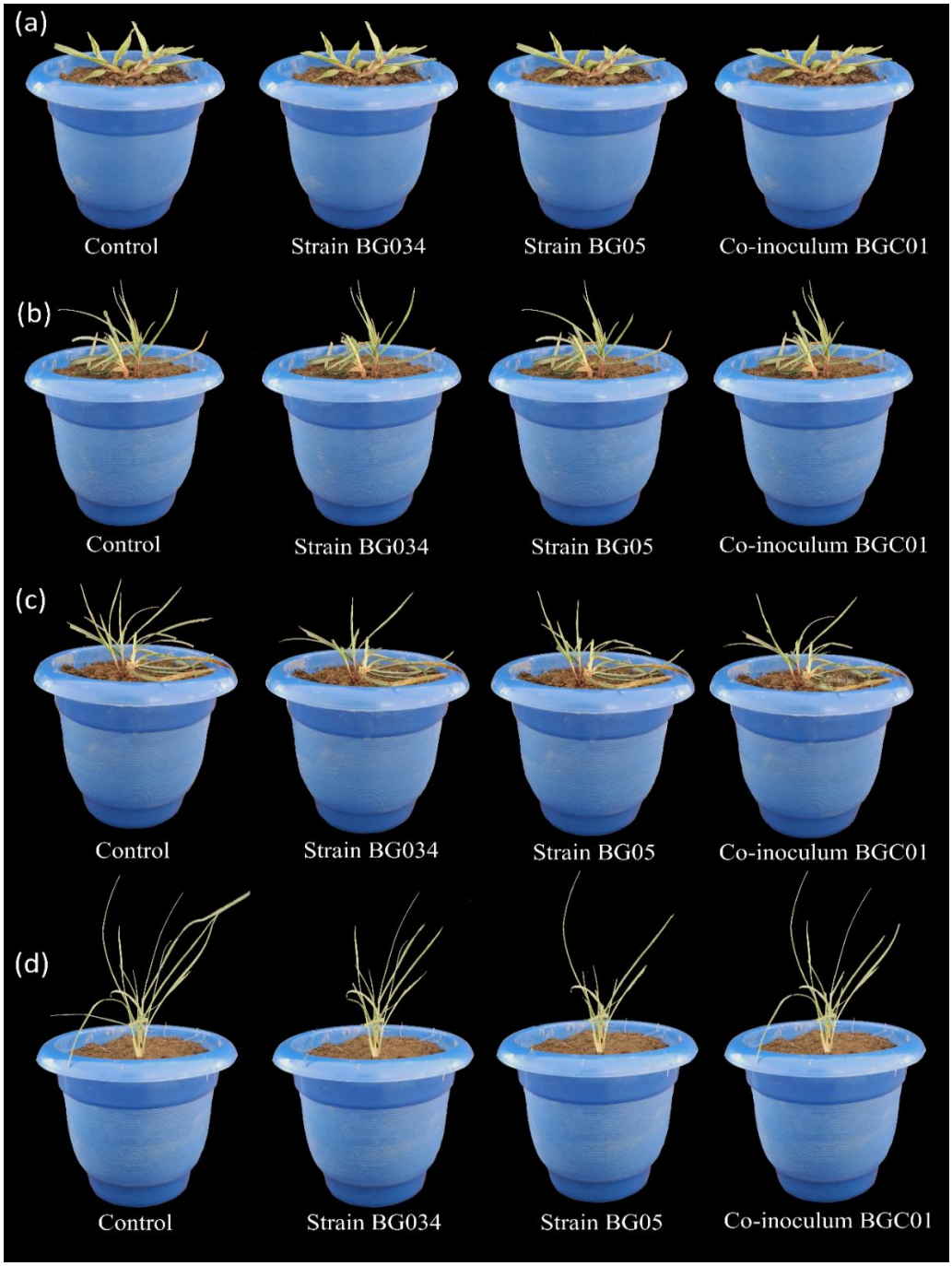

Fig. 3.2. Greenhouse experimental setup for evaluating the effects of isolated bacterial strains and their co-inoculum (BGC01) on the growth of (a) *Cyperus rotundus*, (b) *Cyperus esculentus*, (c) *Axonopus compressus*, and (d) *Imperata cylindrica*.

3.3. Preparation of Biostimulant from Agricultural Byproducts

Agricultural byproducts such as mustard oil cake and papain were selected for the preparation of biostimulant. The mustard cake was cleaned to eliminate contaminants, finely crushed, and combined with 1% papain derived from papaya latex, and then dried in a hot air oven to form

a powder. To activate the enzymatic activity of papain, the mixture was heated at 50 °C for 30 minutes. Following the enzymatic treatment, the mixture was centrifuged at 4000 rpm for 10 minutes to separate the biostimulant from the solid residue, yielding a pure product (Fig. 3.3) (Sarma & Joshi, 2022, AU Patent No. AU2021103319A4). The amino acid concentrations in the biostimulant were then determined using an amino acid analyzer (Sykam Germany, Model No: S 433).

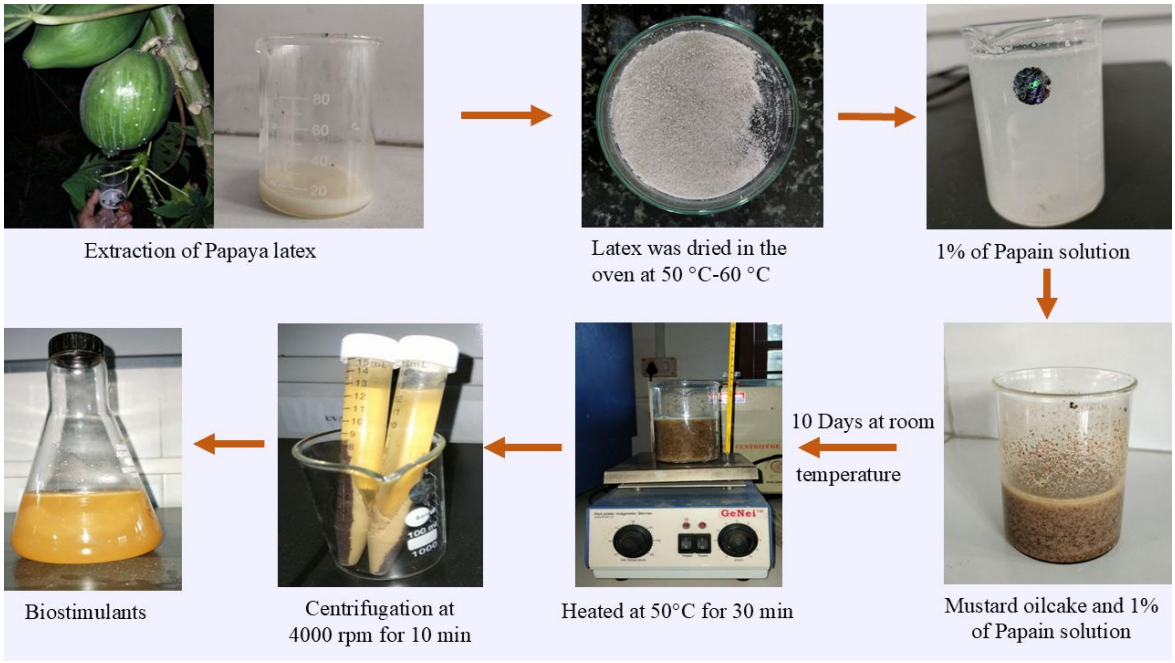

Fig. 3.3. Schematic illustration of the steps for biostimulant preparation.

3.3.1. Amino Acid Analysis Using an Amino Acid Analyzer

To analyze the sample using an amino acid analyzer. Firstly, 5-sulfosalicylic acid and 5% trichloroacetic acid (TCA) were added to the sample to precipitate proteins and peptides. The resulting mixture was thoroughly mixed and subjected to high-speed centrifugation at 10,000 rpm to separate the precipitated proteins and larger molecules from the supernatant. The supernatant contained the unbound amino acids of interest for analysis (Aristoy & Toldrá, 2015). Following protein removal, the supernatant was treated with 6N HCl to adjust the pH range between 1.8 and 2. Finally, the prepared sample was analyzed using an amino acid analyzer.

3.3.2. Greenhouse Experiment for Biostimulant Effectiveness

A greenhouse experiment was conducted to evaluate the effectiveness of biostimulant in promoting plant growth (Fig. 3.4). The plants used in the experiment had previously been grown in a greenhouse. Biostimulant was applied at various concentrations: 25%, 50%, 75%, and 100%. The plants were nurtured in a similar way mentioned earlier in the *in vivo* analysis of the PGP test. Key growth metrics, including shoot length, and root length, were measured at intervals of 7, 14, and 21 days.

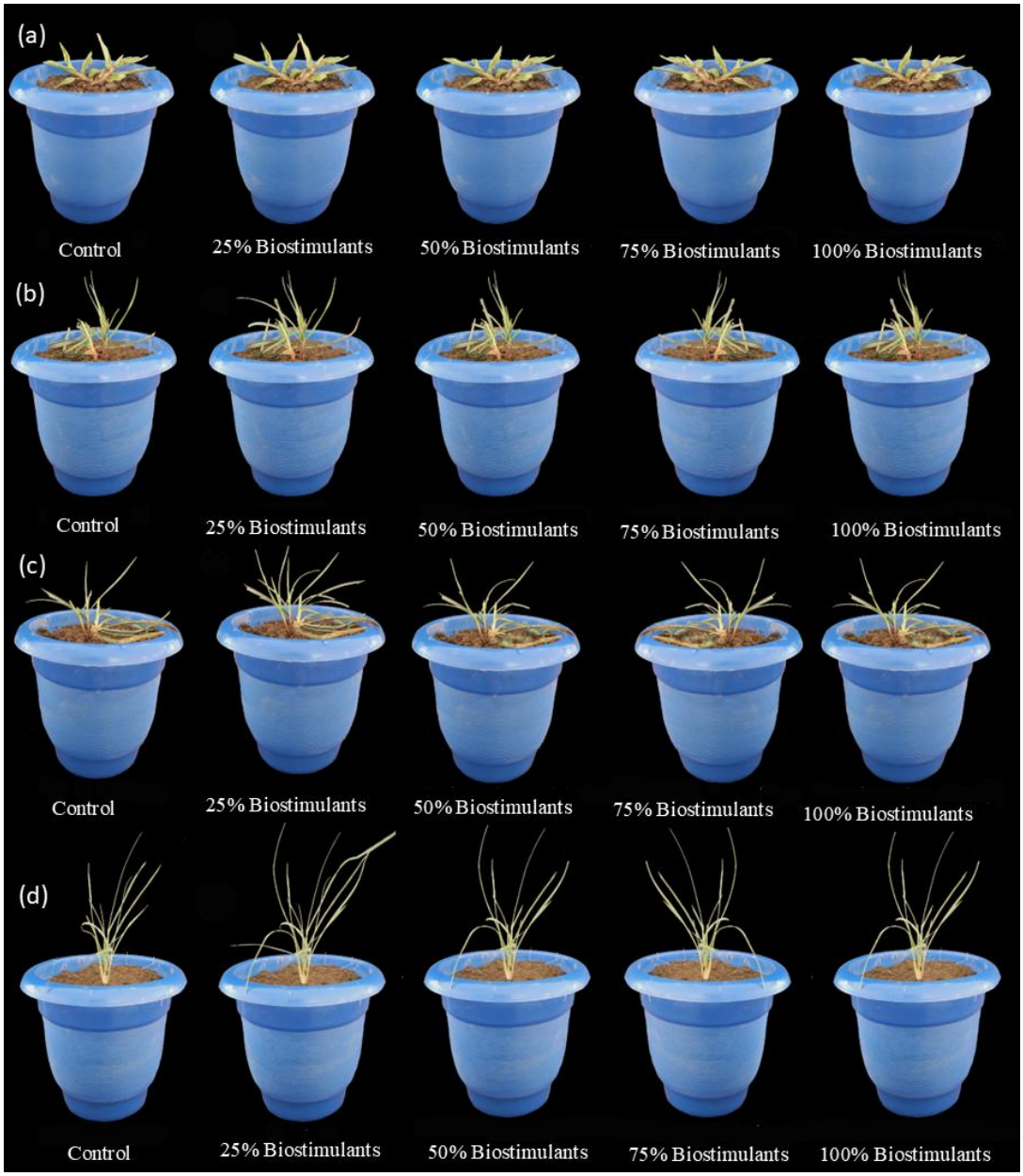

Fig. 3.4. Greenhouse experimental setup for evaluating the effects of the biostimulants on the growth of (a) *Cyperus rotundus*, (b) *Cyperus esculentus*, (c) *Axonopus compressus*, and (d) *Imperata cylindrica*.

3.4. Formulation of Plant-Bacterial Co-Inoculum

A plant-bacterial co-inoculum (BGCP01) was formulated by combining selected plant species and an isolated bacterial co-inoculum containing two isolated strains. The isolated bacterial species were grown in nutrient broth at 32 °C for 12 hours to ensure exponential growth of the strains. Simultaneously, the roots of the selected plant species were thoroughly washed. The bacterial co-inoculum was developed by combining equal volumes (5 mL) of the two bacterial suspensions in a beaker, resulting in a final concentration of 4.7×10^{13} cfu/mL. Washed plant roots were then dropped into the bacterial consortia overnight. After inoculating the plant roots with the bacterial co-inoculum, the plant-microbe co-inoculum was immediately transplanted into a prepared microcosm.

3.5. Microcosm Soil Collection, Preparation, and nitro-PAHs Spiking

The soil for this experiment was collected from Titabor, India, the same site previously used for a pot experiment to quantitatively estimate nitro-PAH accumulation. The collected soil was sieved through a 2 mm sieve and sterilized in a vertical autoclave for 1 hour at 121 °C for two consecutive days. The soil's physicochemical parameters, such as organic carbon, nitrogen, phosphorus, potassium, and electrical conductivity, were initially evaluated following the methodology of Pawar et al. (2009). The soil was then artificially contaminated by nitro-PAHs, i.e. 1-nitropyrene and 2-nitrofluorene respectively. Each nitro-PAH was mixed over the soil at a concentration of 25 mg/kg. The soil was kept at 4 °C for 60 days to allow the complete evaporation of acetone. Later, the contaminated soil was transported to the microcosms (M2=1-nitropyene spiked soil, and M3= 2-nitrofluorene spiked soil) under greenhouse conditions (Fig. 3.5). The microcosms had an area of 1.11 m² and a depth of 15.24 cm. Similarly, a controlled microcosm (contaminated-free soil) was set up as a baseline control. To monitor microbial activity, cfu/g in the microcosmic soil before inoculation of prepared plants-bacterial-co-inoculum was determined on nutrient agar at 15-day intervals. However, no cfus were detected during this test period, hence no data could be provided. To enhance soil water retention, texture, and fertility, 2.5 kg/m² of farmyard manure was applied 30 days before the transplanted plants- bacterial co-inoculum (Sarma et al., 2024, IN Patent No. 554169).

Fig. 3.5. Experimental microcosms for phytoremediation studies before inoculation of plant-bacterial co-inoculum.

3.5.1. Microcosm Experiment Setup and Plant Transplantation

The previously formulated co-inoculum inoculated plant species were transplanted in prepared microcosm beds, having 24 plants organized in rows of 6 of each species per row. At a regular interval of 5 days, 75% biostimulant was sprayed over the microcosms. Two microcosms were set up for this experiment, one contaminated with 1-nitropyrene and the other with 2-nitrofluorene. In addition, a controlled microcosm was set up that did not contain the biostimulant and plant-bacterial co-inoculum (BGCP01). The plants were grown in the microcosm nurtured under regulated conditions, including a 25-28 °C temperature range, a photoperiod of 16 hours of light and 8 hours of darkness, and systematic watering to maintain soil moisture.

3.5.2. Nitro-PAHs Extraction, Purification, and GC-MS Analysis

Soil samples weighing 10 g were taken from each of the microcosms every 30 and 60 days for qualitative estimation of nitro-PAHs. These soil samples were mixed with 100 mL of dichloromethane (DCM) and stirred for an hour. A liquid-liquid extraction method, as described by Kootstra et al. (1995) with minor modification, was employed for this extraction. Each soil sample was extracted three times and then dehydrated with anhydrous sodium sulfate. The extract was filtered using 0.45 µm membrane filter paper, removing soil particles and bacterial cells. The filtrate was then submitted to GC/MS analysis (Agilent 7890B GC). The GC-MS

analysis was conducted using an Agilent 7890B GC system coupled with an Agilent 5977A MSD, utilizing an HP-5MS capillary column (30 m x 0.25 mm x 0.25 μm) with helium as the carrier gas at a flow rate of 1 mL/min. Samples were injected in split mode with a 10:1 split ratio. The oven temperature program started at 50 °C with a hold for 2 minutes, followed by an increase at 10 °C per minute to 150 °C, where it was held for 2 minutes. The temperature then increased at 5 °C per minute to 250 °C, with another 5 minutes hold, and finally, it was ramped at 10 °C per minute to 280 °C, holding for 5 minutes. The injector and transfer line temperatures were set at 250 °C and 100 °C, respectively. The mass spectrometry conditions included an ion source temperature of 230 °C, a quadrupole temperature of 150 °C, a mass range of 35-500 m/z, and a solvent delay of 3 minutes, with a scan rate of 1.562 scans per second. NIST20.1 library (2020) was then searched to compare the structures of the compounds with those of the NIST database. Compounds were then identified based on the retention times and mass spectra with already known compounds in the NIST library (C:\Database\NIST20.1).

3.5.3. Evaluation of Soil Physicochemical Properties

Soil samples were collected from each microcosm at 0, 30, and 60-day intervals. The soil samples' physicochemical properties were then determined using the methodology outlined below.

3.5.3.1. pH

To evaluate soil pH, 1 g of soil was mixed with 2.5 mL of distilled water, and allowed to settle for 30 seconds. Subsequently, the pH meter (Systronics, Model No: MKV1) was calibrated with buffer solutions at pH 4.0 and pH 7.0 for two-point calibration. Once calibrated, the electrode was dipped into the soil sample, and the pH of the sample was recorded.

3.5.3.2. Electrical Conductivity

To evaluate soil electrical conductivity 1 g of soil was mixed with 2.5 mL of distilled water, and allowed to settle for 30 seconds. The electrical conductivity of the sample was then determined potentiometrically using a conductivity meter (Systronics, Model No: 304).

3.5.3.3. Organic Carbon

The soil organic carbon was determined using the Walkley-Black chromic acid wet oxidation method (Pawar et al., 2009). The methodology includes sieving 2 g of soil using a 0.2 mm sieve, and mixing with 10 mL of potassium dichromate, followed by gently stirring. The

mixture was then carefully swirled with 20 mL of concentrated sulfuric acid, and the flask was left at room temperature for 30 minutes. A blank soil sample was also prepared in the same way. After cooling, 200 mL of distilled water and 10 mL of 85% orthophosphoric acid were added to the mixture and shaken well. Finally, ten drops of diphenylamine indicator were added, resulting in a violet color change (Pawar et al., 2009). Titrated the solution with 0.5 N ferrous ammonium sulfate until the color changed from violet to bright green. The amount of ferrous ammonium sulfate used for titration was measured for both the blank (A mL) and the sample (B mL).

The Organic carbon of the soil sample was calculated as follows:

$$\text{Walkey- Black organic carbon (\%)} = \frac{(A-B) \times 0.03 \times Normality\ of\ ferrous\ ammonium\ sulfate \times 100}{Wt.of\ soil\ (g)}$$

The Walkley-black titration method has a 76% recovery of soil organic carbon

Actual Organic carbon (%) = (Walkley Black Organic Carbon) × (100/70)

Organic Carbon (g/kg) = Actual organic carbon (%) × 10

3.5.3.4. Available Nitrogen

To determine the available nitrogen content, 20 g of air-dried soil that had been sieved were placed in a round-bottom flask. A few drops of distilled water were carefully added to ensure that the soil particles did not stick to the sides of the flask. To prevent bumping and frothing, 2 to 3 glass beads and 1 mL of liquid paraffin were added. Subsequently, 100 mL of freshly prepared potassium permanganate ($KMnO_4$) solution and 100 mL of freshly prepared sodium hydroxide (NaOH) solution were poured into the flask. The mixture was then distilled using a Kjel Plus auto distillation unit, and the 150 mL distillate was collected in a beaker with 20 mL of boric acid working solution. The same process was used to prepare a blank sample that did not contain soil. The distillate was then titrated with 0.02N H_2SO_4 until it changed color from green to red. The volume of H_2SO_4 used in titration was determined for both the sample (A mL) and the blank (B mL) (Pawar et al., 2009). The nitrogen content was then determined using the following formula:

$$N\% = \frac{(A-B)x\ Equivalant\ Weight\ of\ N\ x100\ g}{Weight.of\ soil\ sample} \text{ x} \frac{1}{100\ to\ convert\ N\ mg\ into\ g} \text{ x Normality of } H_2SO_4$$

$$N\% = (A - B) \text{ x Normality of } H_2SO_4 \text{ x } 0.014 \text{ x} \frac{100\ g}{Wt\ of\ the\ soil\ sample}$$

$$\text{Available N (kg/ha)} = N\% \text{ x} \frac{2240000}{100}$$

3.5.3.5. Available Phosphorus

The available phosphorus in the soil sample was determined by using a slightly modified Olsen et al. (1954) methodology. Initially, 2.5 g of soil were blended with 0.3 g of phosphate-free activated charcoal (AR grade). Following that, 50 mL of Olsen reagent was added, and the mixture was agitated at 180 rpm for 20 minutes. The resultant mixture was immediately filtered via Whatman No. 1 filter paper. 5 mL of the filtrate was mixed with 4 mL of a freshly prepared ascorbic acid and ammonium molybdate ($(NH_4)_6Mo_7O_{24}$) solution. This solution was carefully mixed and left for 30 minutes. The absorbance of the solution was then measured at 882 nm. A standard curve was constructed by using various KH_2PO_4 concentrations (0, 1, 2, 3, 4, and 5 ppm), with the extracting solution serving as a reference (Olsen et al.,1954; Pawar et al., 2009). The phosphorus content was calculated using the following formula:

$$\text{P (ppm)} = \frac{\text{GR} \times 50 \times 5}{\text{weight of soil}}$$

Where, GR = the concentration of P in the examined sample (determined using the standard curve).

P (kg/ha) = P (ppm) × 2.24

3.5.3.6. Available Potassium

To quantify potassium concentrations in soil, a standard curve was constructed using pure KCl in distilled water at various concentrations (0, 5, 10, 15, 20, and 25 µg K/mL). 10 g of air-dried soil sample was mixed with 50 mL of ammonium acetate ($NH_4CH_3CO_2$) and agitated for 30 minutes. The mixture was filtered with Whatman No. 1 filter paper (Pawar et al., 2009). The potassium concentration of the filtrate was determined with a flame photometer using the following formula:

K (ppm) = R (Reading from standard graph, µg K/ml in extract) × 5 × dilution factor

K (Kg/ha) = R × 5 × 2.24 × dilution factor

3.5.4. Analysis of Plant's Antioxidant Enzyme

The key antioxidative enzymes including peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT), were evaluated in four plant species exposed to 1-nitropyrene and 2-nitrofluorene contaminants, bacterial co-inoculum, and biostimulant in microcosms. Plants were grown in the microcosm nurtured within a greenhouse under

regulated conditions, including a 25-28 °C temperature range, a photoperiod of 16 hours of light and 8 hours of darkness, and systematic watering to maintain soil moisture. Sampling was done at 0, 30, and 60 days to assess enzymatic reactions over time. The detailed methodology for enzymatic analysis is cited below.

3.5.4.1. Peroxidase (POD)

The peroxidase activity was determined using the method described by Senthilkumar et al. (2021). 1 g of fresh plant leaves was crushed in 3 mL of 100 mM phosphate buffer. This mixture was then centrifuged at 1000 rpm for 15 minutes at 4 °C. The supernatant was extracted. A reference solution was prepared by mixing 30 µL of H_2O_2 solution, 50 µL of guaiacol solution, and 3.0 mL of phosphate buffer. To measure peroxidase activity, 100 µL of the extracted supernatant was added to the reference solution in a test cuvette. The absorbance of the mixture was then measured at 436 nm in a UV-visible spectrophotometer until it reached 0.050 (Senthilkumar et al., 2021).

Enzyme activity was then calculated using the formula:

$$\text{Enzyme activity (U/g F.W.)} = \frac{Vr \times Vt \times 1000}{\varepsilon \times l \times \Delta t \times 0.100}$$

Where, Vr = Reaction mixture volume (3.18 mL)

Vt = The volume of crude enzyme solution in the test cuvette (100 µL)

ε = The extinction coefficient at 436 nm (6.39 cm^2/µmol)

l= The light path (1 cm)

Δt = The time interval for the extinction increase to 0.100

0.100= One unit of POD defined as the amount of enzyme that increases 0.100 of absorbance at 436 nm/min.

F.W.=Fresh weight

3.5.4.2. Ascorbate Peroxidase (APX)

The activity of ascorbate peroxidase was determined using the methodology described by Senthilkumar et al. (2021). Initially, 1 g of fresh plant material was mixed with 10 mL of potassium phosphate buffer (0.1M, pH 7) containing 2 mM ascorbate. The plant material was crushed in the buffer and centrifuged at 10,000 rpm for 15 minutes. A 3 mL reaction mixture was prepared by mixing 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbic

acid, 0.1 mM H_2O_2, and 0.5 ml of enzyme extract. This combination was thoroughly mixed and kept at 25 °C for 5 minutes. The APX activity was determined by detecting the decrease in absorbance at 290 nm induced by ascorbic acid oxidation (Senthilkumar et al., 2021). The enzyme activity was measured as U/min/mg F.W. was calculated by following the formula

$$\text{Enzyme activity (U/min/mg) F.W.} = \frac{\Delta A290 \times Vr}{Vt \times \varepsilon}$$

Where, ΔA290= Change in the absorbance/min

Vr= Total volume of the reaction mixture in mL

Vt= Volume of the sample taken in mL

ε =Extinction coefficient of ascorbate 2.8 mM^{-1} cm^{-1}

3.5.4.3. Superoxide Dismutase (SOD)

To determine the SOD activity, 1 g of fresh plant material was crushed with 10 mL of 50 mM potassium phosphate buffer and centrifuged at 11,000 rpm for 10 minutes at 4 °C. A test cuvette was filled with 1.3 mL of sodium carbonate buffer, 500 µL of NBT, and 100 µL of Triton X-100. The UV-visible spectrophotometer was calibrated using a blank solution that included no enzymes or NBT. Another control, with NBT but no enzyme, was used as a reference. To start the reaction, 100 µL of hydroxylamine hydrochloride ($NH_2OH.HCl$) was introduced to the test cuvette. After a 2-minute incubation at room temperature, 70 µL crude enzyme extract was added. The absorbance at 560 nm was then measured every 15 seconds for 1–2 minutes (Senthilkumar et al., 2021).

The following formula was used to calculate the percentage of inhibition in the NBT reduction by SOD.

%Inhibition of NBT reduction by SOD =¼ Control OD -Treatment OD/Control ×100

In this assay, one unit of SOD activity is defined as the amount of enzyme required to achieve 50% inhibition of NBT reduction.

$$\text{The SOD units are calculated using the formula} = \frac{\%\, inhibition}{50}$$

To convert the SOD activity to units per gram (U/g), the activity is multiplied by a factor of 20

SOD Activity (U/g) F.W = SOD Units × 20 SOD Activity (U/g)

=SOD Units×20

3.5.4.4. Catalase

To determine catalase activity, 1 g of fresh plant leaves was blended with ice-cold phosphate buffer. The mixture was then centrifuged at 11,000 rpm at 4 °C for 10 minutes. The UV-visible spectrophotometer was calibrated at 240 nm using a 3 mL quartz cuvette containing 2 mL of phosphate buffer and 1 mL of 30 mM H_2O_2. In the experimental cuvette, 1 mL of phosphate buffer was mixed with 1 mL of prepared supernatant. 1 mL of 30 mM H_2O_2 was added to the cuvette to initiate the reaction, which was then quickly placed in the UV-visible spectrophotometer. The mixture was stirred using a glass rod and the absorbance was measured every 30 seconds for 3 minutes. A control was prepared by mixing 1.9 mL of phosphate buffer, 1 mL of 30 mM H_2O_2, and 0.1 mL of catalase solution in a cuvette. The blank was prepared with 2 mL of phosphate buffer and 1 mL of H_2O_2 (Senthilkumar et al., 2021). The rate of absorbance drop was used to determine catalase activity using the following formula:

$$U/\ mg = (A_0\text{-}A_{180})\times Vt/E_{240} \times d \times Vs \times Ct \times 0.001$$

Where, $A_0\text{-}A_{180}$ is the difference between the initial and final absorbance.

Vt is the total volume of the reaction (3 mL).

ε_{240} is the molar extinction coefficient for H_2O_2 at OD240 (34.9 $mol^{-1}1\ cm^{-1}$).

d is the optical path length of the cuvette (1 cm).

3.6. Statistical Analysis

The datasets were analyzed statistically, with each data point representing the average of three replicates. Error bars indicate standard deviations to account for variability. Statistical analyses were conducted using PAST 4.3 software, selecting appropriate tests based on data distribution. For comparisons involving multiple groups, a one-way ANOVA was used for normally distributed data to determine significant differences between group means. When the data were skewed, the Kruskal-Wallis test served as a nonparametric alternative. In comparisons between the two groups, a paired t-test was applied for normally distributed data, while the Mann-Whitney U test was used for skewed data. Statistically significant differences were defined as a *p*-value of ≤ 0.05.