MATERIALS AND METHODS

CHAPTER 3
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3.1 Soil sample collection and preparation

Roughly 120 soil specimens were sterilely gathered from different agricultural areas in Uttarakhand. Each sample, weighing around 500 grams, was obtained using a sterilised spatula and then placed in dry, uncontaminated polythene bags. The bags were marked and secured with a rubber band alongside other necessary tools. After exposure to air for a week, these samples were crushed and put through a sieve. After being screened, the soils were used in an experiment to isolate actinomycetes [132].

3.2 Isolation of Actinomycetes

Five grams of the collected soil samples were suspended in 50 millilitres of standard saline solution (NaCl-0.85g/L). The soil suspension was shaken for 3 minutes at a speed of 200 revolutions per minute while it was incubated at a temperature of 28 degrees Celsius in an orbital shaker incubator. After serially diluting soil in YIM6 Starch- casein medium, *actinomycetes* were isolated using spread plate techniques [133].

Different mixtures of salts, including Sodium Chloride (NaCl) between 100 to 150 grams, Potassium Chloride (KCl) 20 grams, Magnesium Chloride ($MgCl_2$) 30 grams, Magnesium Sulphate ($MgSO_4$) 5 grams, Dipotassium Phosphate (K_2HPO_4) 1 gram,

along with starch 20 grams, and casein or milk powder at 10 grams. All of the media, as mentioned above, had a pH between 10 and 12. Nalidixic acid (25-50 g/liter) was included in the media. *Actinomycetes* growth was observed on separate plates incubated at 28 degrees Celsius for 25-35 days. These are discussed below:

3.2.1 Aerial Mass Color

Mycelia are recorded as white, grey, red, green, blue, or violet, depending on whether or not they are actively sporulating. When the colour of the air mass is halfway between two sets of colours, data for both are stored. Both colour series are recorded if the strain studied has intermediate shades in its aerial mass colour.

3.2.2 Melanoid Pigments

The classification relies on the dispersion of melanoid pigments. (i.e., pigments with shades of brown, black, light green, and other colours) throughout the medium. Melanoid pigment creation (+) and non-creation (-) are used to categorise the strains.

3.2.3 Reverse Side Pigments

The strains were divided into two categories depending on their ability to generate specific pigments on the underside of the colony. These categories were labelled as distinctive (+) and non-distinctive or missing (-). If a pigment with low intensity, like yellow, olive, or yellow-brown, is detected, it is allocated to the second group (-).

3.2.4 Soluble Pigments

The strains have been divided into two classes, namely positive (+) and negative (-) producers, depending on their ability to create soluble pigments apart from melanin. The observed colours included orange, blue, yellow, green, red, and violet.

3.2.5 Spore Chain Morphology

The strains are classified into distinct "sections" in spore chain morphology. As per the classification system by Shirling and Gottlieb in 1966, the genus Streptomyces is divided into three sections, specifically recti-flexibiles (RF), retina-culiaperti (RA), and spirales (S). There are instances when a strain might produce spore chains belonging to two sections, S and RA, which would be annotated as SRA.

3.2.6 Reproductive Structure Surface

A scanning electron microscope is advised to determine spore morphology and surface characteristics. Leveraging cross-hatched cultures designed for viewing through light microscopy can assist in accomplishing this goal. The cleaning of the electron grid and the subsequent application of adhesive tape onto its surface are considered essential. The process involves the precise placement of fully developed spores of the particular strain onto the surface of the adhesive tape, followed by applying a layer of gold coating for 30 minutes. Subsequently, the sample should be scrutinised utilising an electron microscope at diverse magnification levels. The reproductive structures' outlines are distinguished by their spiny, smooth, warty, and hairy features.

3.3 Screening of isolates for PGPR traits

The PGPR traits of actinobacterial isolates were examined for the following

3.3.1 IAA production

The quantitative production of indole acetic acid (IAA) was evaluated using the Salkowski method [134]. The Actinobacterial cultures thrived in a liquid medium of Luria broth at a temperature of 36±2 °C. 500 µl of 48-hour actinobacterial cultures were incorporated into 50 ml of Luria Bertani (LB) broth that has 0.1% DL tryptophan in it. This concoction was situated in a refrigerated incubator shaker, calibrated to 30±0.1°C and 180 revolutions per minute, and left undisturbed in darkness for 48 hours. Subsequently, the bacterial cultures that had reached maturity were centrifugated for 10 minutes at a rotational speed of 10,000 revolutions per minute while ensuring a constant temperature of 4°C. The colorimetric assay was employed to quantify the quantity of indole-3-acetic acid (IAA) generated in the supernatants. A volume of 1 mL of supernatant was introduced into the Salkowski reagent. The preparation of the Salkowski reagent requires the precise combination of 1 millilitre of a 0.5 molar solution of ferric chloride ($FeCl_3$) with 50 millilitres of a 35% solution of perchloric acid ($HClO_4$). It's crucial to quantify each component to achieve reliable outcomes precisely. Then, introduce 100 millilitres of a 10-millimolar orthophosphoric acid solution and add 2 millilitres of the Salkowski reagent. The mixture should then react at a temperature of 28 degrees Celsius for 30 minutes. Following the conclusion of the incubation period, the test tubes exhibited

an identifiable pink colouration, indicative of the synthesis of indole-3-acetic acid (IAA). IAA quantification was performed by measuring the absorption of the pink colour at a wavelength of 530 nm using a UV/VIS spectrophotometer after 30 minutes [135]. The data revealed the existence of three distinct levels of indole-3-acetic acid (IAA) production, namely the lowest level (+), a moderate level (++), and highest level (+++).

3.3.2 Solubilisation of phosphate

On Pikovskaya agar plates enriched with insoluble tricalcium phosphate (TCP), newly obtained strains of actinobacteria were spread using a loop. Subsequently, the plates were incubated for 120 hours within a temperature range of 28±2°C. The observation of a ring-like region encompassing the bacterial colonies indicates the effective dissolution of phosphate. The strength of phosphate solubilisers was assessed and categorised into three levels: "High" (+++), "Medium" (++), and "Low" (+).

3.3.3 Siderophores and HCN Production

A modified version of the chrome azurol sulphonate (CAS) assay was employed to qualitatively estimate the quantity of siderophore produced by the actinobacteria isolates. The presence of actinobacterial colonies results in the development of a distinct orange halo region surrounding them, which provides an indicative sign of siderophore production. Furthermore, an investigation was conducted to assess the capacity of each bacterial isolate to produce hydrogen cyanide (HCN). The filter paper underwent a noticeable change in colour from deep yellow to a reddish-brown hue, which indicates the production of hydrogen cyanide (HCN). Isolates with a high

concentration of siderophores and HCN (++), isolates with moderate attention to siderophores and HCN (++), and isolates with a low concentration of siderophores and HCN (+) were grouped based on the data.

3.3.4 Catalase activity

Actinobacterial cultures were cultivated in a nutrient agar medium for 48 hours, maintaining a temperature of 28°C. Following the designated time frame, the fully developed bacterial colonies were exposed to the introduction of 2-3 droplets of 3% hydrogen peroxide solution. This process was meticulously carried out on a sterile glass slide, employing a sanitary toothpick for thorough mixing. The emergence of oxygen bubbles or effervescence indicated the presence of catalase activity. The results were classified and documented as High, Medium, and Low Catalase producers (+++, ++, and +, respectively).

3.4 Screening of actinobacterial isolates for monocrotophos pesticide degradation

3.4.1 Primary Screening

3.4.1.1 Determination of zone of clearance of pesticide degradation

The study on actinobacterial strains for the decomposition of the monocrotophos pesticide was carried out as described in reference [136]. Each distinct actinobacterial sample was cultured in 100 ml of Mineral Salt Media (MSM) enriched with 25 ppm of MCP pesticide. These specimens were then placed in a rotary shaker set at 30°C, operating at 150 rpm for seven days. The growth trajectory

of the actinobacterial samples was tracked by assessing the optical density at various intervals using a UV-spectrophotometer. The samples were routinely shifted every 3 to 4 days or when an increase in cloudiness was detected. After several such transfers, a 0.1 ml aliquot of the culture medium was drawn and introduced into designated wells in pesticide agar plates. These plates underwent incubation at 30°C for a duration of 5 to 8 days. Strains capable of breaking down the pesticide were discerned by the emergence of transparent regions around the wells, signifying the degradation area.

3.4.1.2 Secondary Screening

3.4.1.2.1 Determination of pesticide degradation by culture streak technique

The capacity of the actinobacterial isolates to break down the pesticide was gauged by observing the clearance zone, a result of streaking the cultures on agar enriched with the pesticide [137].

3.4.1.2.2 Gene expression of potential Actinobacteria isolates within E. coli cells

The enzymes critical for the degradation of monocrotophos pesticides are identified as phosphodiesterase (PTE) or organophosphorus hydrolysae (OPH), distinctly regulated by the opd gene. This gene was discovered in promising actinobacterial strains that have shown the capacity to degrade monocrotophos pesticides (MCP).

The opd gene encoding PTE and OPH enzyme was isolated from promising actinobacterial strains. The identified gene, comprising 996 nucleotides, specifically TTGCAAN17TATACT, was combined with an appropriate plasmid vector, pBR322, through genetic engineering techniques. This recombinant DNA was subsequently introduced into E. coli cells to enhance the expression of the targeted genes and their respective proteins. Utilizing a liquid culture approach, these modified E. coli cells were used to degrade monocrotophos pesticides.

3.5 Microbial decomposition of monocrotophos pesticides

In the experiment, Monocrotophos pesticide (MCP) was introduced at a concentration of 10 µg/ml into various batches of nutrient broth. Following this, each batch underwent sterilisation and was subsequently inoculated with E. coli cells. After the inoculation, the broth was allowed to incubate for duration of 48 hours. To evaluate the extent of MCP degradation within the broth culture, techniques such as Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) were employed, in accordance with the procedures detailed in the description [139].

3.5.1 Assessment via High-Performance Liquid Chromatography

From the cultured supernatant, a 5-mL sample was taken, and upon extraction with ethyl acetate, a 4-mL filtrate was obtained. The HPLC (PerkinElmer 200 series, CT, USA) was employed to gauge the MCP concentrations at a wavelength of 276 nm. After the separation process, the filtrate was mixed with methanol in equal proportions. This mixture was then meticulously filtered through a 0.45-µm PVDF

membrane. For the analysis, it was introduced into an HPLC column (C18, 250×4.6 mm, 5 µm, Phenomenex, CA, USA). A precise 10 µL of the filtered sample was injected into the column, using a blend of 0.5% acetic acid and methanol (1:4 v/v) as the eluent, flowing at a consistent rate of 1 mL/min.

3.6 Actinobacterial Strain Formulations

Isolated actinobacterial strains were ingeniously used to produce a wide range of highly effective formulations. [140].

3.6.1. Granular Formulation Derived from Alginate-Kaolin (AL-KAO Composition)

To create the sodium alginate solution, 20 grams of dry sodium alginate was dissolved in an optimal volume of 10 millilitres of sterilised distilled water. The mixture was carefully added to a 1-litre volume of warm, agitated distilled water and left to mix within the stirring apparatus for 30 minutes, forming a uniform suspension. The alginate solution underwent sterilisation at a temperature of 121 degrees Celsius and a pressure of 101 kPa for 15 minutes. After thoroughly blending 20 grammes of the desiccated Actinobacterial isolate propagules with 20 grammes of the previously sterilised kaolin (aluminium silicate), the resulting mixture was incrementally introduced in 2-gram portions to a volume of 1 litre of sterile, distilled water. Additionally, the water was supplemented with four drops of Tween 20. The mixture was allowed to whirl for 10 minutes. When waiting for the sodium alginate solution and the alginate-kaolin-actinobacterial mixture to reach the desired state, the alginate-kaolin-actinobacterial mixture was agitated using a stirrer. With a spout at

the lower end and a T-valve outlet system, the droplet-forming apparatus connected to a 1-litre reagent bottle was built. This resulted in the formation of droplets. Before it was put to use, the entire device was put through a sterilisation process that lasted 15 minutes and was carried out at 121 degrees Celsius and 101 kPa. The sodium alginate solution and the kaolin mixture, which had been modified, were combined in equal proportions of 1:1 and introduced into the reagent bottle. The bottle was then stirred continuously. The suspension was concurrently dispensed through an Eppendorf pipette tip fitted with a T-10 valve, directing it into a sterile 0.1 M CaCl2 solution. Following this, the alginate-kaolin beads were allowed to rest in a newly prepared 0.1 M CaCl2 solution for a duration of 30 minutes. After incubation, the beads were separated using a sterile cheesecloth. These beads were then thoroughly washed with sterile distilled water at least three times. Their dry weight was ascertained by undergoing lyophilization at a temperature of -70 degrees Celsius. The freeze-dried mycobacterium formulation, henceforth termed as beads, was preserved in the absence of light at 0 degrees Celsius

3.6.2. Granular Formulation Derived from Flour-Kaolin (FLO-KAO formulation)

The flour and kaolin samples were subjected to different sterilisation processes at a temperature of 121°C and a pressure of 101 kPa for 15 minutes. Following the cooling process, a mixture was formulated by combining 200g of flour, 4g of kaolin, and 20g of inoculum. Subsequently, this blend underwent filtration using a sterile screen with 250 µm pores to guarantee the homogeneous dispersion of all constituents. To achieve a uniform mixture, 180 ml of sterile distilled water was

gradually added to this homogeneous blend with two drops of Tween 20. The mixture was continuously stirred until it reached a consistency similar to dough's. This dough was then freeze-dried at a temperature of -70 °C. The formulation is freeze-dried to maintain effectiveness and quality while extending shelf life, hereafter referred to as granules, stored in a dark environment at a temperature of 4°C.

3.6.3 Formulation of a Powder Composed of Calcium Carbonate and CMC (CC-CMC powder formulation)

The sterilisation of calcium carbonate and CMC material was carried out independently at 121 °C and 101 kPa for 15 minutes. After the completion of the cooling process, meticulous blending of CMC was carried out with 20 g of the inoculum. The resulting mixture was then passed through a sterile screen with a pore size of 250 µm to ensure the formation of a homogeneous blend. At a concentration of 90 kg, the calcium carbonate was added to the 10 kg carboxymethyl cellulose (CMC) mixture. The freeze-dried mixes (lyophilised) were after that dubbed as 'powder' and preserved in a dark environment at 4^0C. This procedure further assessed the longevity of the isolates in the materials and the powder's use in mitigating soil toxicity through bioremediation.

3.7 Statistical analysis

The data was evaluated using the SPSS 13.0 software suite, and the mean+SD value was taken wherever applicable.