

FORMULATION OF BIOFERTILIZER WITH HEAVY METAL TOLERANT STRAINS OF *RHIZOBIUM* AND *BACILLUS*

PROJECT WORK

Submitted in partial fulfilment of the requirements for the award of a Bachelor of
Science degree in Microbiology

By

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INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

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This is to certify that this Project Report is the bonafide work of **PARTHIBAN K (41300027), KAMALA GOWTHAM T (41300017)**, who carried out the project entitled "**FORMULATION OF BIOFERTILIZER WITH HEAVY METAL TOLERANT STRAINS OF RHIZOBIUM AND BACILLUS**" under my supervision from **January 2024 to April 2024**

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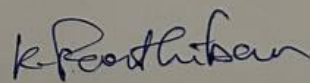
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DECLARATION

We, Parthiban K (41300027), Kamala Gowtham T (41300017), hereby declare that the Project Report entitled "FORMULATION OF BIOFERTILIZER WITH HEAVY METALTOLERANT STRAINS OF *RHIZOBIUM* AND *BACILLUS*" done by us under the guidance of Dr. Indumathi S M at SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY is submitted in partial fulfilment of the requirements for the award of Bachelor of Science in Microbiology.

DATE: 26/4/2024

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ABSTRACT

Biofertilizers have been a major component in the field of agriculture and their use and need is always an increasing factor. However, the Biofertilizers themselves must be dealt with many efficacies' issues, one such problem is the presence of heavy metals (trace amount) in soil in which eventually reduce the efficiency of the biofertilizers. In order Biofertilizers are an important part of agriculture, and demand for them is only going to grow. However, there are a few efficacy issues with biofertilizers itself. One such issue is the presence of heavy metals in soil in trace amounts, which eventually lowers the biofertilizers' efficiency. To address this issue in the future manufacture of biofertilizers, we have investigated and experimented. By adding or employing strains of *Rhizobium* and *Bacillus* that are tolerant of heavy metals, which can withstand certain conditions and aid in the growth of target plants in both controlled and harsh environments to overcome such problem in the upcoming biofertilizers production was conducted in the study. These strains help in promoting the growth of the desired plants in some of the extreme conditions as well as in controlled environments and also helped in the biocontrol of certain diseases which can affect the whole system of the plant. Meanwhile the bacterial cells are also subjected to heavy metal resistance test in no time with adding the trace amounts of inorganic salts in it. The very first objective of our study is to obtain appropriate strains of *Rhizobium* and *Bacillus* (heavy metal tolerant) from various sources which involves soil sample (various site) and the MTCC. The acquired bacterial strains are subjected to 16s rRNA sequencing to ensure the credibility of the bacterial culture. Furthermore, the bacterial cells are grown in specified media and various biochemical tests were carried out to ensure the characteristics of the cells. The main objective of the study is to incorporate the bacterial cells into carrier media by solid state fermentation via specialized containers.

Keywords: *Rhizobium*, *Bacillus*, Biofertilizers, Vermicompost, Coir pith, MTCC, 16s rRNA

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LIST OF ABBREVIATION

MR	Methyl red test
VP test	Voges Proskauer test
CFU	Colony forming units
MTCC	Microbial type culture collection
YEMB test	Yeast extract mannitol broth
SSF	Solid state fermentation

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CHAPTER 1

INTRODUCTION

1.1 BIOFERTILIZER

In recent years, the environmental impact of conventional fertilizers has raised significant concerns, prompting researchers to explore sustainable alternatives that mitigate soil degradation and pollution. Among these alternatives, biofertilizers have emerged as promising solutions for promoting plant growth while reducing environmental harm. This introduction will explore the significance of biofertilizers, the role of heavy metal-tolerant strains of *Bacillus* and *Rhizobium*, the utilization of carrier media such as vermicompost and coir pit, and the process of solid-state fermentation in biofertilizer formulation

1.2 IMPORTANCE OF BIOFERTILIZER

Biofertilizers have gained significant attention in recent years due to their potential to revolutionize agricultural practices and address environmental concerns associated with conventional chemical fertilizers. These eco-friendly alternatives offer numerous benefits that contribute to sustainable agriculture and soil health. One of the key advantages of biofertilizers is their ability to improve soil fertility and structure. Unlike chemical fertilizers, which often lead to soil degradation and loss of beneficial microorganisms, biofertilizers enhance soil health by promoting microbial activity and organic matter decomposition. This results in improved soil structure, water retention, and nutrient cycling, ultimately leading to better crop yields and long-term sustainability (Smith *et al.*, 2019).

Furthermore, biofertilizers play a crucial role in reducing the environmental impact of agricultural practices. Chemical fertilizers are known to contribute to nutrient runoff, groundwater contamination, and greenhouse gas emissions, posing significant threats to ecosystems and human health. In contrast, biofertilizers promote nutrient cycling and minimize nutrient leaching, thus mitigating environmental pollution and conserving natural resources (Yadav *et al.*, 2020).

Another significant benefit of biofertilizers is their cost-effectiveness and scalability. Unlike chemical fertilizers, which often require extensive manufacturing processes and transportation, biofertilizers can be locally using inexpensive raw materials and simple production techniques. This makes them accessible to small-scale farmers and reduces dependence on external inputs, ultimately leading to lower production costs and improved economic viability (Kumar et al., 2020). Moreover, biofertilizers offer a sustainable solution to the growing demand for food production while reducing reliance on synthetic inputs. With global population expected to reach 9 billion by 2050, there is an urgent need to increase agricultural productivity while minimizing environmental degradation. Biofertilizers provide a viable alternative to chemical fertilizers, offering a sustainable approach to meeting food security goals and ensuring the long-term viability of agricultural systems (Subramaniam et al., 2017). In addition to their agronomic benefits, biofertilizers also contribute to climate change mitigation by reducing greenhouse gas emissions associated with conventional farming practices. Chemical fertilizers are major sources of nitrous oxide emissions, a potent greenhouse gas that contributes to global warming and ozone depletion. By promoting biological nitrogen fixation and nutrient recycling, biofertilizers help reduce the need for synthetic fertilizers and mitigate their environmental impact (Ramesh et al., 2019).

1.3 ROLE OF *BACILLUS* IN PLANT GROWTH

Bacillus species, particularly *Bacillus subtilis* and *Bacillus megaterium*, are well known for their beneficial effects on plant growth and development. These bacteria colonize the rhizosphere and rhizoplane of plants, forming symbiotic relationships that promote nutrient uptake, disease resistance, and stress tolerance. One of the primary mechanisms by which *Bacillus* species enhance plant growth is through the production of plant growth-promoting substances. These include hormones such as auxins, cytokinin's, and gibberellins, which regulate various aspects of plant growth and development, including root elongation, shoot growth, and flowering. Additionally, *Bacillus* species produce siderophores, which chelate iron and make it more available to plants, thus alleviating iron deficiency stress (Kumar et al., 2019).

Furthermore, *Bacillus* species exhibit antagonistic activity against plant pathogens, thereby enhancing plant health and reducing the incidence of diseases. These bacteria produce antimicrobial compounds such as antibiotics, lytic enzymes, and volatile organic compounds that inhibit the growth of pathogenic fungi, bacteria, and nematodes. By suppressing pathogens, *Bacillus* species protect plant roots from infection and promote overall plant vigor and productivity (Santoyo *et al.*, 2012). In addition to their direct effects on plant growth, *Bacillus* species also play a role in improving soil fertility and structure. These bacteria are capable of solubilizing insoluble nutrients such as phosphorus and potassium, making them more available to plants. They also enhance soil aggregation and organic matter decomposition, leading to improved soil structure, water retention, and nutrient cycling. As a result, *Bacillus*-inoculated soils exhibit higher levels of fertility and productivity compared to non-inoculated soils (Bashan *et al.*, 2014). Moreover, *Bacillus* species have been shown to enhance plant tolerance to abiotic stresses such as drought, salinity, and heavy metal toxicity. These bacteria produce osmolytes and stress-responsive proteins that protect plants from water loss, ion toxicity, and oxidative damage under stressful conditions. By improving plant stress tolerance, *Bacillus* species help plants maintain growth and productivity even in challenging environments (Nadeem *et al.*, 2021).

1.4 ROLE OF *RHIZOBIUM* IN PLANT GROWTH

Rhizobium, a genus of soil bacteria, forms symbiotic relationships with leguminous plants, exerting profound effects on plant growth, soil fertility, and ecosystem sustainability. This section explores the multifaceted role of *Rhizobium* in promoting plant growth and enhancing soil health. *Rhizobium*'s most notable contribution to plant growth lies in its ability to fix atmospheric nitrogen through symbiotic nitrogen fixation.

Within root nodules formed by *Rhizobium-legume* symbiosis, nitrogenase enzymes convert atmospheric nitrogen into ammonia, a readily available nitrogen source for the host plant. This nitrogen input significantly enhances plant growth and

development, particularly in nitrogen-deficient soils, while reducing the need for synthetic nitrogen fertilizers (Gage, 2004).

Moreover, *Rhizobium* colonization of plant roots triggers physiological and biochemical changes in the host plant that further promote growth and development. *Rhizobial* infection induces the formation of root nodules, specialized structures where nitrogen fixation occurs, and stimulates the production of phytohormones such as cytokinins and gibberellins, which regulate root and shoot growth. Additionally, *Rhizobium* symbiosis enhances nutrient uptake, water absorption, and stress tolerance in leguminous plants, leading to improved crop yields and resilience to environmental stresses (Ghosh *et al.*, 2021).

In addition to nitrogen fixation, *Rhizobium* species contribute to soil fertility and ecosystem sustainability through several mechanisms. *Rhizobium*-legume symbiosis enhances soil nitrogen availability and promotes the growth of Non leguminous plants through nitrogen cycling and organic matter decomposition. Furthermore, *Rhizobium* inoculation improves soil aggregation, structure, and water-holding capacity, leading to enhanced soil fertility, erosion control, and carbon sequestration (Bashan *et al.*, 2014). Furthermore, *Rhizobium legume* symbiosis enhances soil microbial diversity and activity, creating a conducive environment for beneficial soil organisms and promoting nutrient cycling and soil organic matter turnover. *Rhizobium*-mediated increases in soil biological activity and diversity contribute to soil health and ecosystem resilience, buffering against environmental stresses and promoting sustainable land management practices (Zahra *et al.*, 1999).

1.5 IMPORTANCE OF *BACILLUS* AND *RHIZOBIUM* BASED BIOFERTILIZERS

Rhizobium and *Bacillus*-based biofertilizers represent promising alternatives to conventional chemical fertilizers, offering numerous benefits for sustainable agriculture, soil fertility, and environmental conservation. This section examines the importance of biofertilizers derived from *Rhizobium* and *Bacillus* species and their implications for agricultural sustainability.

One of the primary advantages of *Rhizobium* and *Bacillus*-based biofertilizers is their ability to enhance nutrient availability and plant growth through biological

nitrogen fixation and nutrient solubilization. *Rhizobium-legume* symbiosis enables plants to access atmospheric nitrogen, reducing the need for synthetic nitrogen fertilizers and minimizing nitrogen pollution of water bodies and air. Similarly, *Bacillus* species solubilize insoluble nutrients such as phosphorus, potassium, and iron, making them more available to plants and improving nutrient uptake efficiency (Bashan *et al.*, 2014).

Moreover, *Rhizobium* and *Bacillus*-based biofertilizers contribute to soil health and fertility by promoting beneficial soil microorganisms, enhancing soil structure, and increasing organic matter content. *Rhizobium-legume* symbiosis stimulates microbial activity and diversity in the rhizosphere, leading to improved soil structure, water retention, and nutrient cycling. *Bacillus* species produce extracellular enzymes and organic acids that facilitate organic matter decomposition, humus formation, and nutrient mineralization, enriching soil fertility and supporting sustainable crop production (Ghosh *et al.*, 2021).

In addition to their agronomic benefits, *Rhizobium* and *Bacillus*-based biofertilizers offer environmental advantages by reducing the environmental footprint of agricultural practices. By promoting biological nitrogen fixation and nutrient cycling, biofertilizers decrease the reliance on synthetic fertilizers, which are energy intensive to produce and contribute to greenhouse gas emissions. Furthermore, biofertilizers mitigate nutrient runoff and leaching, reducing water pollution and eutrophication of aquatic ecosystems, thus preserving water quality and biodiversity (Zahran *et al.*, 1999).

Furthermore, *Rhizobium* and *Bacillus*-based biofertilizers have been shown to enhance crop productivity, improve soil fertility, and increase farmer income in various agroecosystems worldwide. Field trials and on-farm demonstrations have demonstrated the efficacy of biofertilizers in increasing crop yields, improving nutrient use efficiency, and reducing production costs. Farmers adopting biofertilizer-based practices have reported higher profits, reduced input costs, and improved soil health and crop quality, leading to sustainable intensification of agriculture and enhanced livelihoods (Ghosh *et al.*, 2021).

Moreover, *Rhizobium* and *Bacillus*-based biofertilizers offer a viable solution to the challenges of climate change, soil degradation, and food security facing agricultural systems worldwide. Effects of heavy metals in agriculture soil.

1.6 EFFECTS OF HEAVY METALS IN AGRICULTURE SOIL

Heavy metals, such as cadmium, lead, mercury, arsenic, and chromium, are naturally occurring elements that can accumulate in agricultural soils through various anthropogenic activities, posing significant risks to ecosystem health, food safety, and human well-being. This section explores the adverse effects of heavy metals in agricultural soil and their implications for sustainable agriculture and environmental management.

One of the primary concerns associated with heavy metal contamination in agricultural soil is its impact on soil fertility and plant productivity. Heavy metals can interfere with essential soil processes, such as nutrient cycling, microbial activity, and soil structure, leading to decreased soil fertility and impaired plant growth. These metals can inhibit enzyme activity, disrupt nutrient uptake, and alter soil pH, thus affecting plant physiology and metabolism. Consequently, crops grown in metal-contaminated soils may exhibit stunted growth, reduced yields, and nutritional deficiencies, compromising food security and economic livelihoods (Kabata Pendias *et al.*, 2011).

Furthermore, heavy metals in agricultural soil can pose significant risks to human health and food safety through the accumulation of toxic metals in food crops and the food chain. Plants grown in metal-contaminated soils can absorb and accumulate heavy metals in their tissues, particularly in edible parts such as roots, stems, leaves, and grains. Consuming crops contaminated with heavy metals can lead to chronic health effects, including neurological disorders, kidney damage, carcinogenicity, and developmental abnormalities, posing serious risks to public health and well-being (Alloway *et al.*, 2013).

Moreover, heavy metal contamination in agricultural soil can exacerbate environmental pollution and degradation through leaching, runoff, and groundwater contamination. Metals such as cadmium and lead are highly persistent in the environment and can accumulate in water bodies, posing risks to aquatic

ecosystems and biodiversity. Furthermore, heavy metals can contaminate surface water and groundwater sources, jeopardizing drinking water quality and availability for humans and wildlife (Kabata Pendial *al et.*,2011).

Addressing heavy metal contamination in agricultural soil requires integrated and sustainable approaches that prioritize soil remediation, pollution prevention, and agricultural best management practices. Strategies such as soil testing, phytoremediation, soil amendments, and crop rotation can help mitigate heavy metal risks and restore soil health and productivity. Furthermore, regulatory measures, policy interventions, and public awareness campaigns are essential for promoting responsible land use, pollution control, and sustainable agriculture practices that safeguard human health and environmental integrity (Alloway *al et.*, 2013).

The adverse effects of heavy metals in agricultural soil pose significant challenges to sustainable agriculture, food security, and environmental sustainability. Addressing heavy metal contamination requires collaborative efforts from governments, industries, farmers, and researchers to implement effective soil management practices, mitigate pollution risks, and safeguard human health and ecosystem integrity in a changing world.

1.7 ROLE OF *RHIZOBIUM* AND *BACILLUS* IN HEAVY METALS

Rhizobium and *Bacillus* species play pivotal roles in mitigating heavy metal contamination in soil through various mechanisms, offering sustainable solutions for soil remediation and environmental management. This section examines the diverse strategies employed by *Rhizobium* and *Bacillus* to reduce heavy metal levels in soil and their implications for sustainable agriculture and ecosystem health.

One of the primary mechanisms by which *Rhizobium* and *Bacillus* species mitigate heavy metal contamination in soil is through the process of microbial assisted phytoremediation. *Rhizobium*-legume symbiosis facilitates the uptake and accumulation of heavy metals by leguminous plants, which act as hyperaccumulators capable of accumulating high concentrations of metals in their tissues. *Rhizobium* inoculation enhances plant growth and metal uptake, promoting

phytoextraction of heavy metals from soil and their sequestration in aboveground plant biomass (Ghosh et al., 2021).

Similarly, *Bacillus* species contribute to heavy metal remediation in soil through various mechanisms, including metal sequestration, immobilization, and detoxification. *Bacillus* species produce extracellular polymeric substances (EPS) that bind heavy metals and form complexes, reducing their bioavailability and mobility in soil. Furthermore, *Bacillus*-mediated precipitation of heavy metal ions into insoluble forms, such as metal sulfides or hydroxides, immobilizes metals in soil and reduces their leaching and plant uptake (Glick, 2010).

In addition to phytoextraction and immobilization, *Rhizobium* and *Bacillus* species enhance heavy metal tolerance and detoxification in plants through the production of metal chelators, antioxidants, and stress-responsive proteins. *Rhizobium*-legume symbiosis induces plant defense mechanisms against heavy metal stress, such as metallothionein synthesis, glutathione metabolism, and antioxidant enzyme activity, which mitigate metal-induced oxidative damage and enhance plant survival in contaminated soils (Ghosh et al., 2021).

Furthermore, *Rhizobium* and *Bacillus*-mediated rhizoremediation processes enhance soil microbial activity and diversity, promoting metal biotransformation, biodegradation, and mineralization. *Rhizobium*-legume symbiosis stimulates microbial biomass and activity in the rhizosphere, leading to increased microbial degradation of organic matter and metal-complexing compounds. *Bacillus* species produce enzymes such as metallothionein, metalloenzymes, and metal resistant proteins that facilitate metal detoxification and degradation, thus enhancing soil microbial communities' resilience to heavy metal stress (Glick et al., 2010).

Moreover, *Rhizobium* and *Bacillus*-mediated biostimulator and bioaugmentation strategies enhance the effectiveness of other soil remediation techniques, such as phytoremediation, chemical oxidation, and electrokinetic remediation.

Rhizobium legume symbiosis enhances plant growth and metal uptake, while *Bacillus* inoculation improves soil fertility, microbial activity, and metal tolerance, synergistically enhancing the efficiency of remediation processes and promoting soil recovery and ecosystem restoration (Ghosh et al., 2021).

Rhizobium and Bacillus species play critical roles in reducing heavy metal contamination in soil through various mechanisms, including phytoextraction, immobilization, detoxification, and microbial remediation. Harnessing the BioRemedial potential of Rhizobium and Bacillus offers sustainable solutions for soil remediation, environmental restoration, and ecosystem sustainability in heavy metal-contaminated environments.

1.8 FACTORS INVOLVES IN FORMULATION OF BIOFERTILIZER

Formulating biofertilizers involves a multifaceted approach that considers various factors, including microbial selection, carrier materials, nutrient content, production methods, and quality control measures. This section explores the key factors involved in the formulation of biofertilizers and their implications for enhancing microbial efficacy, nutrient delivery, and agronomic performance. The selection of appropriate microbial strains is crucial for the effectiveness of biofertilizers. Microorganisms such as *Rhizobium*, *Bacillus*, *Azotobacter*, and *Azospirillum* are commonly used in biofertilizer formulations due to their ability to fix nitrogen, solubilize nutrients, and promote plant growth. The selection criteria include strain compatibility, effectiveness in local soil conditions, competitiveness, and tolerance to environmental stresses (Jha *et al.*, 2020).

Carrier materials serve as a medium for microbial inoculants, providing protection, nutrients, and a stable environment during storage and application. Common carrier materials include peat, vermiculite, perlite, compost, and various organic residues. The choice of carrier material depends on factors such as microbial viability, shelflife, ease of application, cost-effectiveness, and compatibility with target crops and soil types (Saravanan *et al.*, 2017). Biofertilizers should contain adequate nutrients to support microbial growth and activity, as well as to meet plant nutritional requirements. Nutrient content varies depending on the microbial strain and target crop. Key nutrients include nitrogen, phosphorus, potassium, micronutrients, and growth-promoting substances such as hormones and vitamins. Formulations may also include organic additives and bio-stimulants to enhance nutrient availability and plant-microbe interactions (Singh *et al.*, 2015).

Various production methods are employed to manufacture biofertilizers, including solid-state fermentation, liquid fermentation, and carrier-based inoculation. Solid state fermentation involves growing microorganisms on solid substrates such as agricultural residues, whereas liquid fermentation utilizes liquid media for microbial cultivation. Carrier-based inoculation involves impregnating carrier materials with microbial inoculants to enhance shelf-life and field efficacy (Bashan *et al.*, 2014).

Quality control measures are essential to ensure the effectiveness, safety, and consistency of biofertilizer formulations. Quality control protocols include microbial purity and viability testing, nutrient analysis, carrier material characterization, contamination screening, and product labeling. Quality assurance practices help maintain product integrity, meet regulatory standards, and build consumer trust in biofertilizer products (Kumar *et al.*, 2020). Environmental factors such as temperature, humidity, pH, and oxygen availability influence microbial growth and activity during biofertilizer production and storage. Optimal environmental conditions are essential for maintaining microbial viability, metabolic activity, and product stability. Controlled environment chambers, fermentation vessels, and storage facilities are used to regulate environmental conditions and optimize biofertilizer performance (Subramaniyam *et al.*, 2017).

CHAPTER 2

LITERATURE SURVEY

2.1 INTRODUCTION TO BIOFERTILIZERS AND BACTERIAL ISOLATES

Biofertilizers have emerged as sustainable alternatives to chemical fertilizers, leveraging the beneficial interactions between microorganisms and plants to enhance soil fertility and promote sustainable agriculture. Among these microorganisms, *Rhizobium leguminosarum* and *Bacillus amyloliquefaciens* have garnered significant attention for their multifaceted roles in plant growth promotion, nutrient cycling, and environmental remediation (Bashan & deBashan, 2010; Singh & Jha, 2016; Malhotra *et al.*, 2020).

2.2 RHIZOBIUM LEGAMINOSARUM

Rhizobium legaminosarum a gram-negative nitrogen-fixing bacterium, forms symbiotic associations with leguminous plants, facilitating nitrogen fixation and improving soil fertility. Through nodulation, *Rhizobium* establishes specialized structures on plant roots, known as nodules, where it converts atmospheric nitrogen into ammonia for plant uptake (Sessitsch *et al.*, 2013; Thilagar *et al.*, 2021; Masson Boivin *et al.*, 2009). Recent research has highlighted its potential in alleviating heavy metal stress in plants by mechanisms such as metal sequestration and chelation (Sessitsch *et al.*, 2013; Malik *et al.*, 2019).

2.3 BACILLUS AMYLOLIQUEFACIENS

Bacillus amyloliquefaciens, a versatile gram-positive bacterium, is known for its production of antimicrobial compounds, plant growth-promoting metabolites, and stress tolerance mechanisms. It enhances plant growth and soil fertility by promoting nutrient solubilization, root growth, and disease suppression (Meena *et al.*, 2012; Rajendran *et al.*, 2008; Choudhary *et al.*, 2021). Furthermore, *Bacillus amyloliquefaciens* exhibits remarkable tolerance to heavy metals and can actively participate in their detoxification and immobilization in contaminated soils (Meena *et al.*, 2012; Choudhary *et al.*, 2021).

2.4 INTERACTION BETWEEN BACTERIAL STRAIN AND HEAVY METALS

Both *Rhizobium leguminosarum* and *Bacillus amyloliquefaciens* possess diverse mechanisms for interacting with heavy metals in soil environments. These mechanisms include metal sequestration, chelation, reduction, and enzymatic detoxification, which collectively contribute to the alleviation of heavy metal toxicity in plants and soils (Ma *et al.*, 2016; Kumar *et al.*, 2011; Zaidi *et al.*, 2019).

Moreover, the synergistic interactions between these bacterial strains and heavy metals can enhance soil health and plant productivity in metal-contaminated environments (Zaidi *et al.*, 2019; Malik *et al.*, 2019).

2.4 USE OF CARRIER MEDIA IN BIOFERTILIZER

In biofertilizer production, the selection of an appropriate carrier medium is crucial for ensuring the viability and efficacy of microbial inoculants. Carrier media serve as matrices that provide a supportive environment for microbial growth, protect cells during storage and transportation, and enhance the delivery of beneficial microorganisms to the rhizosphere. Among the various carrier materials explored for biofertilizer formulation, vermicompost and coir pith have emerged as promising candidates due to their unique properties and potential benefits for soil health and plant nutrition (Gopal *et al.*, 2020; Singh & Sharma, 2019; Selvi *et al.*, 2019).

2.4.1 Vermicompost

Vermicompost, also known as worm castings or vermicast, is a nutrient-rich organic fertilizer produced through the decomposition of organic matter by earthworms (vermicomposting). It is characterized by its dark, granular texture and high content of humus, microbial biomass, and plant-available nutrients such as nitrogen, phosphorus, and potassium (Gopal *et al.*, 2020; Sharma & Garg, 2019). Vermicompost serves as an excellent carrier medium for biofertilizers due to its porous structure, which facilitates aeration and moisture retention, and its diverse microbial community, which includes beneficial rhizosphere microorganisms (Sharma & Garg, 2019; Selvi *et al.*, 2019).

Research studies have demonstrated the effectiveness of vermicompost as a carrier medium for various biofertilizer formulations, including those containing nitrogenfixing bacteria, phosphate solubilizers, and plant growth-promoting rhizobacteria (Gopal *et al.*, 2020; Saha & Mina, 2020). The incorporation of vermicompost in biofertilizers has been shown to improve soil fertility, enhance nutrient availability to plants, suppress soil-borne pathogens, and promote sustainable agricultural practices (Sharma & Garg, 2019; Selvi *et al.*, 2019).

2.4.2 Coir pith

Coir pith, also known as coconut coir dust or coir peat, is a renewable byproduct derived from the husks of coconut fruits. It is widely used in horticulture and agriculture as a soil amendment, substrate, and growing medium due to its excellent water retention capacity, aeration properties, and biodegradability (Singh & Sharma, 2019; Kamaraj *et al.*, 2020). Coir pith consists of lignocellulosic fibers and organic compounds such as lignin, cellulose, and hemicellulose, which contribute to its structural integrity and nutrient-holding capacity (Kamaraj *et al.*, 2020).

Studies have explored the potential of coir pith as a carrier medium for biofertilizer production, highlighting its suitability for supporting microbial growth, nutrient release, and plant-microbe interactions in soil environments (Singh & Sharma, 2019; Kamaraj *et al.*, 2020). The incorporation of coir pith in biofertilizers has been shown to enhance soil structure, moisture retention, and nutrient availability, leading to improvements in crop productivity, soil health, and environmental sustainability (Singh & Sharma, 2019; Kamaraj *et al.*, 2020; Elanchezhian *et al.*, 2021).

2.5 RECENT ADVANCES AND FUTURE DIRECTIONS

Recent advances in biofertilizer technology have focused on optimizing carrier media formulations, exploring innovative techniques for microbial inoculant encapsulation, and enhancing the efficacy and shelf life of biofertilizer products. Research efforts have also addressed the challenges associated with large-scale production, quality control, and field application of biofertilizers using vermicompost

and coir pith as carrier materials (Selvi *et al.*, 2019; Saha & Mina, 2020; Elanchezhian *et al.*, 2021).

Future research directions may involve the development of tailored carrier media blends, the integration of beneficial additives such as microbial consortia, plant growth regulators, and organic amendments, and the application of advanced biotechnological tools for biofertilizer optimization and customization (Selvi *et al.*, 2019; Saha & Mina, 2020; Elanchezhian *et al.*, 2021). Additionally, efforts to enhance the compatibility of carrier media with specific microbial strains, soil types, and cropping systems will be crucial for realizing the full potential of vermicompost and coir pith based biofertilizers in sustainable agriculture and environment managements.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To formulate biofertilizer with heavy metal strains of *Bacillus* and *Rhizobium*.

3.2 OBJECTIVES

- To obtain cultures from soil sample and maintain microbial culture purity by subculture.
- To grow the adequate bacterial cells by increasing the biomass of the bacteria of interest by fermentation method
- To use coir pit and vermicompost a carrier media for the incorporation of bacterial cells for sustainable growth rate.
- To store in dark environment and effectively maintain the moisture at 60%
- To check the efficacy of the biofertilizer by treating it with seeding and look its better growth.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS REQUIRED

Nutrient agar and YEMA media were bought from HIMEDIA used for culturing bacterial samples. Lignite, vermicompost, coir pit and plastic container were used for solid state fermentation. Methyl red reagent, MR-VP broth, alpha naphthalin, citrate agar, hydrogen peroxide, oxidase disk are used for biochemical test. PH paper is used for measuring the pH of the produced biofertilizer. Colony counter, swab and nutrient agar are used to measure the number of colonies produced in a culture. Ethanol and cotton are used for sterilization. Incubator is used for incubation.

4.2 METHODS

4.2.1 sample Collection

4.2.1.1 Collection Of Soil Sample

Collection of Soil Samples: Soil samples were collected from the vicinity of a coal crushing factory located in Periyapalayam to isolate *Bacillus* strains tolerant to heavy metals. Sampling sites were selected based on proximity to the factory and variations in soil characteristics. At each sampling site, soil samples were collected from multiple locations using a soil auger to a depth of insert depth. Care was taken to avoid surface debris and vegetation, and samples were collected in sterile containers to minimize contamination.

4.2.1.2 Collection Of Root Nodules

Rhizobium strains were isolated from root nodules of Canavalia plants growing near Sathyabama Institute of Science and Technology. Sampling was conducted during the appropriate season for legume growth to ensure active nodule formation. Canavalia plants were selected for sampling based on their abundance and proximity to the institute. Root nodules were carefully excavated from the root

system using sterile tools to avoid contamination. Nodules exhibiting healthy morphology and pink/red pigmentation were selected for further analysis.

4.2.2 Isolation Of Bacteria

4.2.2.1 Serial Dilution

To perform a serial dilution with 9 test tubes and 1g of soil in each tube, along with 9ml of distilled water, follow these steps. Begin by labelling each test tube with the desired dilution factor, starting from the highest to the lowest, such as 10^{-1} , 10^{-2} , and so on, up to 10^{-9} . Using a sterile pipette, add 1g of soil to the first test tube labelled 10^{-1} . Then, add 9ml of distilled water to the same test tube containing the soil. Mix the contents thoroughly to ensure uniform distribution of the soil particles in the water. Using a fresh pipette, transfer 1ml of the mixture from the first test tube to the second test tube labelled 10^{-2} . Add 9ml of distilled water to the second test tube and mix well. Repeat this process for the remaining test tubes, transferring 1ml of the diluted mixture from the previous tube to the next tube, each time diluting it further by a factor of 10. After completing the dilution series, mix the contents of each test tube thoroughly. Now, you have a series of dilutions ranging from 10^{-1} to 10^{-9} , each containing decreasing concentrations of soil particles. We can use these dilutions for various purposes, such as plating on agar plates for microbial enumeration or further analysis of soil properties. Ensure proper labelling and documentation of each dilution to track the dilution factors accurately. By following this procedure, we can systematically reduce the concentration of the soil sample in a stepwise manner, allowing for accurate analysis and experimentation.

4.2.2.2 Pour plate method

First, prepare nutrient agar plates by sterilizing them through autoclaving or pressure cooking. Once sterilized, allow the agar to cool to a temperature where it can be handled safely without solidifying. Label the bottom of each agar plate with the corresponding dilution factor and any other relevant information. Pour a layer of nutrient agar into each labelled plate to cover the bottom, ensuring that there is

enough agar to form a solid layer once cooled. Using a sterile pipette or loop, transfer a small volume such as 0.1 ml (about 0 oz) of the diluted sample from each dilution tube onto the center of the solidified agar in the corresponding plate. Spread the inoculated sample evenly over the surface of the agar plate using a sterile spreader or glass rod. Be gentle to avoid damaging the agar surface.

4.2.2.3 Spread Plate Method

First, prepare nutrient agar plates by sterilizing them through autoclaving or pressure cooking. Once sterilized, allow the agar to cool to a temperature where it can be handled safely without solidifying. Label the bottom of each agar plate with the corresponding dilution factor and any other relevant information. Using a sterile pipette or loop, transfer a small volume (e.g., 0.1 ml) of the diluted sample from each dilution tube onto the center of a nutrient agar plate. Immediately after adding the sample, use a sterile spreader or glass rod to spread the inoculated sample evenly over the surface of the agar plate. Ensure that the entire surface of the plate is covered with the sample. Allow the agar plates to solidify completely at room temperature before incubating them upside down at the appropriate temperature for the growth of the target microorganisms. After the appropriate incubation period, observe the agar plates for the presence of microbial colonies. Count the colonies on plates with colony numbers ranging from 30 to 300 for accurate enumeration. Calculate the number of colonies forming units CFUs per ml of the original sample by multiplying the colony count by the reciprocal of the dilution factor and the volume plated. Record the results and calculate the average CFU for each dilution. By following this procedure using nutrient agar, we can effectively enumerate the viable microorganisms present in the soil sample at different dilutions.

4.2.2.4 Streak Plating

First, prepare nutrient agar plates by sterilizing them through autoclaving or pressure cooking. Once sterilized, allow the agar to cool to a temperature where it can be handled safely without solidifying. Label the bottom of each agar plate with the corresponding dilution factor and any other relevant information. Using a sterile inoculating loop, obtain a small sample of the diluted microbial culture from the

appropriate dilution tube. Starting from one edge of the agar plate, streak the inoculum onto the surface of the agar using a back-and-forth motion while gradually moving towards the opposite edge. Ensure that the streaks do not overlap. Sterilize the inoculating loop by passing it through a flame or dipping it into alcohol and flaming it before starting each new streak. Repeat the streaking process 2-3 times, rotating the agar plate approximately 90 degrees between each streak to ensure even distribution of the inoculum. After streaking, allow the agar plate to incubate upside down at the appropriate temperature for the growth of the target microorganisms. After the appropriate incubation period, observe the agar plate for the presence of isolated colonies. Colonies should appear as discrete, individual growths on the surface of the agar.

4.2.2.5 Isolation Of Bacteria From Soil Sample

Upon collection, soil samples from Periyapalayam were processed for the isolation of bacterial strains tolerant to heavy metals. The isolation procedure involved serial dilution of soil samples followed by spread plating onto selective media. Specifically, nutrient agar supplemented with heavy metals such as cadmium, lead, and zinc at concentrations known to induce heavy metal tolerance in *Bacillus species* was used. Plates were then incubated aerobically at insert temperature for insert incubation period] to encourage the growth of *Bacillus* colonies. Morphologically distinct colonies were selected for further characterization.

4.2.2.6 Isolation Of Bacteria From Root Nodules

Root nodules collected from Canavalia plants near Sathyabama Institute of Science and Technology were processed for the isolation of *Rhizobium* strains. After surface sterilization, nodule extracts were streaked onto Yeast Extract Mannitol Agar (YEMA) plates, a selective medium suitable for the growth of *Rhizobium* species. Additionally, pour plating was performed using YEMA medium to ensure efficient isolation of *Rhizobium* colonies. These plates were then incubated at insert temperature for insert incubation period under appropriate conditions for *Rhizobium*

growth and nodule occupancy. Colonies displaying characteristic morphology indicative of *Rhizobium* were isolated and subjected to further characterization.

4.2.3 Biochemical Tests

Biochemical tests are laboratory techniques used to identify and characterize microorganisms based on their metabolic properties. These tests are essential tools in microbiology for assessing the physiological capabilities of microorganisms and are widely employed in various fields, including clinical diagnostics, food safety, environmental monitoring, and research. In our project on the formulation of biofertilizer using *Bacillus* and *Rhizobium* strains, biochemical tests play a crucial role in evaluating the metabolic profiles of these microorganisms. By subjecting the isolated strains to a battery of biochemical tests, we can gain insights into their metabolic capabilities, which are essential for assessing their suitability for biofertilizer formulation. Biochemical tests are particularly useful for determining the enzymatic activities, sugar fermentation patterns, nitrogen-fixing abilities, and other metabolic traits of microorganisms. These tests provide valuable information about the microorganism's ability to utilize specific substrates, produce beneficial metabolites, and interact with their environment. The results of biochemical tests enable researchers to identify and select microorganisms with desired metabolic traits for various applications. In our project, we employ biochemical tests to assess the metabolic capabilities of *Bacillus* and *Rhizobium* strains isolated from soil samples collected near a coal crushing factory and root nodules of *Canavalia* plants. This characterization process allows us to identify strains with potential benefits for biofertilizer formulation, ultimately contributing to sustainable agricultural practices and environmental stewardship.

4.3.2.1 Indole test

The indole test is a biochemical test used to determine the ability of microorganisms to produce indole from the metabolism of tryptophan, an amino acid. Indole production is a characteristic feature of certain bacteria and can be useful in bacterial identification and classification. The indole test is based on the enzyme tryptophanase, which catalyses the hydrolysis of tryptophan to produce

indole, pyruvic acid, and ammonia. If a microorganism possesses tryptophanase, it will metabolize tryptophan in the growth medium to produce indole, which can be detected by adding a reagent that reacts with indole to form a colored compound. Inoculate the test organism onto a suitable growth medium containing tryptophan, such as Tryptone broth or Tryptone agar. Incubate the culture under appropriate conditions. Allow the culture to grow for a predetermined period, typically overnight, to allow sufficient time for tryptophan metabolism and indole production. After incubation, add Kovac's reagent to the culture medium. Kovac's reagent contains p-dimethylaminobenzaldehyde, which reacts with indole to produce a colored compound. Observe the culture medium for the development of a pink to red colour at the surface of the medium. A positive result indicates the presence of indole, indicating that the organism possesses tryptophanase and can metabolize tryptophan to produce indole. A positive indole test result is indicated by the formation of a pink to red colour within a few minutes after the addition of Kovac's reagent. A negative result is indicated by the absence of colour development. It's essential to handle Kovac's reagent with care as it contains hazardous chemicals. Additionally, some microorganisms may take longer to produce indole, so extended incubation times may be required for certain species.

4.3.2.2 MR test

The Methyl Red (MR) test is a biochemical test used to determine if an organism produces stable acidic end products, primarily lactic acid, acetic acid, and formic acid, from glucose metabolism. Here's an overview of the principle and procedure of the MR test. The MR test is based on the ability of certain bacteria to perform mixed acid fermentation, resulting in the production of large quantities of stable acidic end products. The pH indicator methyl red is added to the medium, which turns red in an acidic environment with a pH below 4.4. If the organism produces enough acidic end products, the pH of the medium will drop, causing it to turn red. Inoculate the test organism onto a suitable growth medium containing glucose, such as MR-VP broth. Incubate the culture under appropriate conditions for growth, typically at 35-37°C for 24-48 hours. After incubation, add a few drops of methyl red reagent to the culture medium. Methyl red reagent is composed of methyl red dye dissolved in 95% ethanol. Observe the colour change in the medium. A

positive result is indicated by a stable red colour, indicating the presence of acidic end products and mixed acid fermentation. A negative result is indicated by an orange or yellow colour, indicating the absence of sufficient acidic end products. A stable red colour indicates that the organism produces acidic end products from glucose fermentation, suggesting mixed acid fermentation. Orange or yellow colour indicates that the organism produces other fermentation products, such as neutral end products, indicating no mixed acid fermentation. It's essential to perform the MR test alongside other biochemical tests to accurately identify and characterize microorganisms.

4.3.2.3 VP test

The Voges-Proskauer (VP) test is a biochemical test used to detect the production of acetoin, a precursor of 2,3-butanediol, from glucose fermentation. The VP test is often performed alongside the Methyl Red (MR) test as part of the Voges Proskauer/Methyl Red (VP-MR) test to distinguish between different groups of enteric bacteria. Here's an overview of the principle and procedure of the VP test. The VP test detects the presence of acetoin, which is formed from the oxidation of 2,3-butanediol during glucose fermentation. Acetoin reacts with alpha-naphthol in the presence of potassium hydroxide (KOH) to produce a red complex, which is observed as a positive result for the VP test. Inoculate the test organism onto a suitable growth medium containing glucose, such as MR-VP broth. Incubate the culture under appropriate conditions for growth, typically at 35-37°C for 24-48 hours. After incubation, add equal volumes of alpha-naphthol reagent and KOH reagent to the culture medium. The alpha-naphthol reagent contains 5% alpha-naphthol in absolute alcohol, while the KOH reagent is a 40% aqueous solution of potassium hydroxide. Gently mix the reagents and the culture to ensure thorough mixing and interaction between the acetoin and the reagents. Allow the mixture to incubate at room temperature for 10-15 minutes. Observe the colour change in the medium. A positive result is indicated by the development of a pink to red colour within the medium. A positive VP test result indicates the presence of acetoin, suggesting a positive Voges-Proskauer reaction. Development of a pink to red colour within the medium indicates the presence of acetoin and a positive Voges Proskauer reaction. Absence of colour change indicates a negative Voges-Proskauer reaction. It's

essential to perform the VP test alongside other biochemical tests, such as the Methyl Red test, to accurately identify and characterize microorganisms.

4.3.2.4 Citrate test

The Citrate Utilization Test is a biochemical test used to determine if an organism can utilize citrate as its sole carbon source. It is often employed in the identification and differentiation of bacteria, particularly within the Enterobacteriaceae family. Here's an overview of the principle and procedure of the Citrate Test. The Citrate Utilization Test is based on the ability of certain microorganisms to utilize citrate as the sole source of carbon and energy for growth. The enzyme citrate-permease is required to transport citrate across the bacterial cell membrane. Once inside the cell, citrate is converted into pyruvate and carbon dioxide by the enzyme citrate lyase. The utilization of citrate causes an increase in alkalinity due to the production of ammonia, resulting in a rise in pH. Streak the test organism onto a citrate utilization medium such as Simmons citrate agar using a sterile inoculating loop. Incubate the inoculated agar plate at the appropriate temperature, typically at 35-37°C, for 24-48 hours. After the incubation period, observe the growth of the organism and any changes in the colour of the medium. Growth of the organism and a change in the colour of the medium from green to blue indicates citrate utilization. This colour change is due to an increase in pH caused by the production of ammonia. No growth of the organism or no change in the colour of the medium indicates the inability to utilize citrate. Growth of the organism and a change in the colour of the medium from green to blue indicates citrate utilization, suggesting a positive Citrate Test. No growth of the organism or no change in the colour of the medium indicates the inability to utilize citrate, suggesting a negative Citrate Test. Some organisms may take longer to show a positive result, so it's essential to incubate the plates for an appropriate duration.

4.3.2.5 Catalase test

The Catalase Test is a biochemical test used to determine if an organism produces the enzyme that catalyzes the breakdown of hydrogen peroxide into water and oxygen. This test is commonly used to differentiate between bacteria that

produce catalase (catalase-positive) and those that do not (catalase-negative). Here's an overview of the principle and procedure of the Catalase Test. Catalase is an enzyme produced by organisms that can catalyze the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). The reaction can be observed by the release of bubbles of oxygen gas when hydrogen peroxide is added to a bacterial colony or culture containing catalase. Place a small sample of the test organism on a clean microscope slide using a sterile loop or swab. Add a few drops of 3% hydrogen peroxide solution directly onto the bacterial colony or culture on the slide. Observe the reaction immediately. If the organism produces catalase, the addition of hydrogen peroxide will result in the rapid production of bubbles of oxygen gas. The presence of bubbles indicates a positive catalase reaction. Rapid production of bubbles upon addition of hydrogen peroxide indicates a positive catalase reaction and the presence of catalase enzyme. Absence of bubbles or a very slow production of bubbles indicates a negative catalase reaction and the absence of catalase enzyme. Rapid production of bubbles upon addition of hydrogen peroxide indicates a positive catalase reaction and the presence of catalase enzyme, suggesting a catalase-positive organism. Absence of bubbles or a very slow production of bubbles indicates a negative catalase reaction and the absence of catalase enzyme, suggesting a catalase-negative organism. It's essential to perform the Catalase Test on fresh cultures or colonies to obtain accurate results. Older cultures may give false negative results due to decreased enzyme activity.

4.3.2.5 Oxidase test

The Oxidase Test is a biochemical test used to determine if an organism produces cytochrome c oxidase, an enzyme involved in the electron transport chain of aerobic respiration. This test is particularly useful for differentiating between Gram-negative bacteria, as the presence or absence of cytochrome c oxidase varies among different species. Here's an overview of the principle and procedure of the Oxidase Test. Cytochrome c oxidase is an enzyme found in the inner mitochondrial membrane of eukaryotic cells and in the plasma membrane of aerobic bacteria. It catalyzes the transfer of electrons from cytochrome c to molecular oxygen, resulting in the reduction of oxygen to water. The oxidase test detects the presence of

cytochrome c oxidase by observing the oxidation of a redox indicator (tetramethyl-p-phenylenediamine) to a blue purple colour in the presence of the enzyme. Use a sterile swab or loop to transfer a small amount of the test organism from a fresh culture onto a filter paper or an oxidase test strip. Apply a drop of oxidase reagent (tetramethyl-p-phenylenediamine) directly onto the test area containing the bacterial cells. Observe the test area for a colour change within 10-30 seconds. A positive result is indicated by the development of a blue purple colour within the test area. Development of a blue purple colour within the test area indicates a positive oxidase reaction, suggesting the presence of cytochrome c oxidase. Absence of colour change, or the persistence of the original colour indicates a negative oxidase reaction and the absence of cytochrome c oxidase. Development of a blue purple colour within the test area indicates a positive oxidase reaction, suggesting the presence of cytochrome c oxidase and the organism's ability to perform aerobic respiration. Absence of colour change, or the persistence of the original colour indicates a negative oxidase reaction and the absence of cytochrome c oxidase, suggesting that the organism may utilize other terminal electron acceptors or is a facultative anaerobe. The Oxidase Test should be performed on fresh cultures or colonies to obtain accurate results. Older cultures may give false-negative results due to decreased enzyme activity.

4.3.2.6 Nitrite reduction test

The Nitrite Reduction Test is a biochemical test used to determine if an organism can reduce nitrate (NO_3) to nitrite (NO_2) or further to nitrogenous gases such as nitric oxide (NO), nitrous oxide (N_2O), or nitrogen gas (N_2). This test is often employed in the identification of bacteria, particularly within the Enterobacteriaceae family. Here's an overview of the principle and procedure of the Nitrite Reduction Test. The Nitrite Reduction Test detects the ability of microorganisms to reduce nitrate to nitrite or other nitrogenous gases through the activity of nitrate reductase enzymes. Nitrate (NO_3) serves as an electron acceptor in anaerobic respiration, and its reduction results in the production of nitrite (NO_2) or other nitrogenous gases, which can be detected by adding specific reagents. Inoculation: Inoculate the test organism into a nitrate broth medium containing potassium nitrate (KNO_3) as the sole source of nitrogen. Incubate the inoculated nitrate broth at the

appropriate temperature, typically at 35-37°C for 2448 hours. After incubation, add a few drops of sulfamic acid and alpha-naphthylamine reagents to the nitrate broth. These reagents react with any nitrite present in the medium to form a red colour. Observe the medium for the development of a red colour after the addition of the reagents. A positive result is indicated by the appearance of a red colour within a few minutes. If no red colour develops after the addition of sulfamic acid and alphanaphthylamine reagents, perform the zinc dust test as follows. Add a small amount of zinc dust to the medium and shake gently. If the medium turns red within a few minutes, it indicates that nitrate was reduced to nitrite and further to nitrogenous gases. The addition of zinc reduces any remaining nitrate to nitrite, which then reacts with the sulfamic acid and alpha-naphthylamine reagents to produce a red colour. If the medium remains colorless, it suggests that nitrate was not reduced by the organism. Development of a red colour in the medium after the addition of sulfamic acid and alpha-naphthylamine reagents indicates the presence of nitrite, suggesting a positive Nitrite Reduction Test. Absence of a red colour in the medium after the addition of reagents indicates the absence of nitrite, suggesting a negative Nitrite Reduction Test. By performing the Nitrite Reduction Test, microbiologists can identify bacteria capable of reducing nitrate to nitrite or further to nitrogenous gases, providing valuable information about their metabolic capabilities and aiding in microbial identification and classification.

4.2.4 Heavy Metal Tolerance Test

The Heavy Metal Tolerance Test is a microbiological assay used to determine the ability of microorganisms to tolerate and survive in the presence of elevated concentrations of heavy metals. Heavy metals such as cadmium, lead, mercury, arsenic, and chromium are known to be toxic to most living organisms, including bacteria. However, some microorganisms possess mechanisms to detoxify or resist the toxic effects of heavy metals, allowing them to thrive in contaminated environments. Here's an overview of the principle and procedure of the Heavy Metal Tolerance Test. The Heavy Metal Tolerance Test assesses the growth and viability of microorganisms in the presence of varying concentrations of heavy metals. Microorganisms with intrinsic heavy metal tolerance mechanisms, such as metal efflux pumps, metal-binding proteins, or enzymatic detoxification systems, will

exhibit greater growth or survival in heavy metal-containing media compared to sensitive organisms. Prepare nutrient agar plates or YEMA plates according to standard protocols. Supplement the agar plates with appropriate concentrations of zinc, lead, and cadmium solutions to achieve desired final concentrations, such as 1 mM, 5 mM, and 10 mM. Mix the heavy metal solutions thoroughly with the melted agar medium before pouring the plates. Inoculate the test microorganisms onto the heavy metal-supplemented agar plates using a sterile inoculating loop. Streak the plates to obtain isolated colonies. Incubate the plates at the appropriate temperature for the growth of the test microorganisms, typically at 35-37°C for 24-48 hours. After the incubation period, observe the growth of the microorganisms on the agar plates. Record any visible changes in colony morphology, such as size, shape, colour, and texture. Measure the diameter of bacterial colonies on the plates using a ruler or caliper. Compare the growth of microorganisms on heavy metal-supplemented agar plates with control plates without heavy metals. Increased growth or survival of microorganisms on plates containing heavy metals compared to control plates indicates heavy metal tolerance. Decreased growth or survival of microorganisms on plates with higher concentrations of heavy metals suggests sensitivity to heavy metal toxicity. Determine the minimum inhibitory concentration (MIC) for each heavy metal, which is the lowest concentration that inhibits visible growth compared to the control plates without heavy metals. Record and analyze the results, including MIC values and growth characteristics, to assess the heavy metal tolerance of the test microorganisms. Draw conclusions based on the observed heavy metal tolerance levels of the microorganisms and their potential applications in environmental remediation or bioremediation efforts.

4.2.5 16sRrna Sequencing

16S rRNA sequencing is a molecular biology technique used to identify and classify microorganisms based on the sequences of their 16S ribosomal RNA (rRNA) genes. These genes are present in the genomes of bacteria and archaea and are highly conserved across different species. However, they also contain variable regions that allow for the differentiation of closely related organisms. The principle of 16S rRNA sequencing is because the 16S ribosomal RNA (rRNA) gene is present in the genomes of bacteria and archaea and exhibits both conserved and

variable regions. This gene is essential for the translation of mRNA into proteins in the ribosome, and its sequence has evolved over time, resulting in regions that are highly conserved across different species as well as regions that are variable and unique to specific taxa. Specific regions of the 16S rRNA gene are amplified using polymerase chain reaction (PCR). Primers are designed to target conserved regions flanking variable regions of the gene. These conserved regions ensure that PCR primers can bind to a wide range of microorganisms, while the variable regions provide the genetic diversity necessary for distinguishing between different taxa. The amplified regions of the 16S rRNA gene are then subjected to DNA sequencing using Sanger sequencing or next generation sequencing (NGS) platforms. Sanger sequencing involves sequencing individual DNA fragments, while NGS allows for highthroughput sequencing of multiple samples simultaneously. The obtained sequencing data are processed and analyzed using bioinformatics tools and software. This involves quality control, trimming of sequencing adapters and lowquality bases, assembly of sequencing reads into contigs, and taxonomic classification of sequences using databases such as NCBI's BLAST or specialized 16S rRNA databases like SILVA or Green genes. Based on the similarity of the obtained sequences to known sequences in the databases, taxonomic assignments are made at various taxonomic levels (e.g., phylum, genus). This allows for the identification and classification of the microorganisms present in the sample. The results of the sequencing analysis are interpreted and reported, often including information on the diversity and composition of the microbial community, as well as the relative abundance of different taxa. Overall, 16S rRNA sequencing leverages the genetic information encoded in the 16S rRNA gene to identify and classify microorganisms present in a sample, providing valuable insights into microbial diversity, community structure, and ecological interactions.

Since we got negative results for *Rhizobium*. We ordered the strains from MTCC.

4.2.6 MTCC Culture Handling

Handling cultures from the Microbial Type Culture Collection (MTCC) follows standard microbiological practices with additional considerations for maintaining the

integrity and purity of the cultures. Here are some general guidelines for handling cultures from MTCC. Upon receiving cultures from MTCC, store them immediately according to the provided instructions. Typically, cultures are stored at recommended temperatures (20°C, -80°C, or as specified) to maintain viability. Follow any specific instructions provided by MTCC regarding storage conditions. If you need to subculture the culture for experimentation or propagation, carefully transfer a small amount of the culture to fresh growth medium using sterile techniques. Avoid repeated subculturing to prevent genetic drift or contamination. Check the purity of cultures by streaking them onto appropriate agar plates and observing colony morphology. Confirm that the colonies represent a single microbial species and are free from contaminants. Maintain accurate records of culture handling, including the date of receipt, storage conditions, subculturing history, and any observations made during handling. This documentation ensures traceability and facilitates reproducibility of experiments. Perform periodic quality control checks to verify the identity and purity of cultures. This may include biochemical tests, molecular identification methods (e.g., PCR, sequencing), or other assays specific to the microorganism.

4.2.6.1 Glycerol stock preservation

Glycerol stock preservation is a common method used to store bacterial cultures for long-term storage. The addition of glycerol helps protect the bacterial cells from damage during freezing and thawing processes. Prepare a stock solution of 40% glycerol by mixing 40 mL of glycerol with 60 mL of sterile water or buffer. Ensure thorough mixing to obtain a homogeneous solution. Prepare a fresh bacterial culture by growing the desired strain in a suitable growth medium under appropriate conditions. Ensure that the culture is in the logarithmic growth phase for optimal viability. Harvest the bacterial culture by centrifugation at a low speed (e.g., 3000 rpm for 5 minutes) to pellet the cells. Carefully resuspend the bacterial pellet in an equal volume of 40% glycerol solution. For example, if you have a 1 mL bacterial pellet, resuspend it in 1 mL of 40% glycerol solution. Mix the bacterial cells and glycerol solution thoroughly by gently vortexing or pipetting up and down. Ensure uniform distribution of glycerol throughout the suspension. Test tubes with the

bacterial strain name, date of preparation, and glycerol concentration (20%). Aliquot the resuspended bacterial culture into the labelled tubes. Each tube should contain a sufficient volume of the glycerol-cell suspension for multiple uses, typically 0.5 to 1ml. Place the labelled tubes containing glycerol stocks in an -80°C freezer for longterm storage. Avoid repeated freeze-thaw cycles, as they can reduce the viability of the bacterial cells. It's a good practice to store glycerol stocks in a dedicated freezer rack or box to prevent cross-contamination and facilitate organization. Maintain accurate records of the glycerol stock preparation, including the bacterial strain, date of preparation, glycerol concentration, and any relevant notes or observations. Update your laboratory inventory system or database to track the location and availability of glycerol stocks for future reference.

4.2.7 Formulation Of Biofertilizer

4.2.7.1 Preparation Of Carrier Media

Preparing carrier media for biofertilizer involves creating a suitable environment for the growth and proliferation of beneficial microorganisms. Coir pit and vermicompost are commonly used as carrier materials due to their organic nature and ability to support microbial growth. Here's how you can prepare carrier media using coir pit and vermicompost. If desired, sterilize the coir pit and vermicompost to eliminate any potential pathogens or unwanted microorganisms. This can be achieved by heating the materials in an autoclave or oven at high temperatures 121°C for 15-20 minutes. Break apart the coir pit into smaller pieces or fibers to create a loose, fluffy texture. This increases the surface area and aeration within the carrier media, facilitating microbial colonization. If using dry coir pit, hydrate it with sterile water or nutrient solution to achieve the desired moisture content. The moisture level should be sufficient to support microbial growth without becoming waterlogged. Ensure that the vermicompost is well aged and matured, as fresh compost may contain high levels of ammonia or phytotoxic compounds that can inhibit microbial activity. Break up any clumps or large particles in the vermicompost to create a uniform texture. This allows for better distribution of microorganisms throughout the carrier media. Combine the prepared coir pit and vermicompost in a mixing container at the desired ratio. The ratio of coir pit to

vermicompost can vary depending on factors such as nutrient content, porosity, and moisture retention. Aim for a well-balanced mixture that provides sufficient nutrients, moisture, and aeration for microbial growth. A common ratio is 1:1 equal part by volume, but this can be adjusted based on specific requirements or preferences. Gradually add sterile water or nutrient solution to the mixture while stirring until the carrier media reaches the desired moisture level. The media should be damp but not overly wet, with enough moisture to support microbial activity without excess water pooling at the bottom. Monitor the moisture content and adjust as needed to maintain optimal conditions for microbial growth. Store the prepared carrier media in a clean, airtight container or bag to prevent contamination and maintain its quality. Label the container with the contents, date of preparation, and any other relevant information. Keep the carrier media in a cool, dry place away from direct sunlight until ready for use.

4.2.7.2 Preparation Of Broth Culture

To prepare broth culture for infusing into carrier media, you'll first need to culture your desired microorganisms in a liquid growth medium. Once the broth culture is ready, you can mix it with the carrier media to inoculate it with beneficial microorganisms. Here's a general procedure for preparing broth culture and infusing it into carrier media for a 10 kg batch. To prepare 1 liter of broth, whether it's nutrient broth or YEM (Yeast Extract Mannitol) broth, you'll need to follow similar procedures with slight variations based on the ingredients and specific requirements of each type of broth. Here's how to prepare 1 liter of both nutrient broth and YEM broth. Dissolve 10 grams of beef extract, 5 grams of peptone, and 5 grams of sodium chloride in approximately 900 ml of distilled water in a clean and sterilized flask or container. Adjust the pH of the solution to around 7.0 using a pH meter or pH indicator strips. Use 1N hydrochloric acid (HCl) or 1N sodium hydroxide (NaOH) solution as needed. Add more distilled water if necessary to bring the volume to 1 liter. Mix the solution thoroughly to ensure complete dissolution of the ingredients. Sterilize the nutrient broth by autoclaving at 121°C for 15 minutes. Dissolve 10 grams of yeast extract and 10 grams of mannitol in approximately 900 ml of distilled water in a clean and sterilized flask or container. Adjust the pH of the solution to around 7.0 using a pH meter or pH indicator strips. Use 1N hydrochloric acid (HCl)

or 1N sodium hydroxide (NaOH) solution as needed. Add more distilled water if necessary to bring the volume to 1 liter.

Mix the solution thoroughly to ensure complete dissolution of the ingredients. Sterilize the YEM broth by autoclaving at 121°C for 15 minutes. After autoclaving, allow the nutrient broth and YEM broth to cool to room temperature in a sterile environment. Once cooled, the broth can be stored at room temperature for shortterm use or refrigerated at 4°C for longer-term storage.

4.2.7.3 Solid State Fermentation

Solid-state fermentation (SSF) is a microbial fermentation process that occurs on solid substrates in the absence or near-absence of free-flowing water. Unlike submerged fermentation, where microorganisms grow in a liquid medium, Solid state fermentation involves the cultivation of microorganisms on solid materials or substrates. Solid state fermentation is commonly used in various industries, including food and beverage, pharmaceuticals, biofuels, and bioremediation, to produce enzymes, organic acids, antibiotics, biofertilizers, and other bioproducts. Here's an overview of solid-state fermentation. In Solid state fermentation microorganisms grow and metabolize nutrients present in solid substrates, such as agricultural residues wheat bran, rice husk, lignocellulosic materials, Agroindustrial by-product's bagasse, corn cob sand synthetic polymers polyethylene. The solid substrate provides a physical support matrix for microbial growth, nutrient supply, and retention of moisture. Microorganisms secrete enzymes that degrade complex substrates into simpler compounds, which are then utilized for growth and metabolite production. The solid substrate is prepared by grinding, milling, or chopping it into smaller particles to increase the surface area and facilitate microbial colonization. The substrate may be pretreated to enhance its digestibility and nutrient availability for microorganisms. A microbial inoculum, usually in the form of spores, mycelia, or vegetative cells, is prepared from a pure culture of the desired microorganism. The inoculum is typically grown in submerged culture (liquid medium) and then inoculated onto the solid substrate. The prepared microbial inoculum is evenly spread or mixed with the solid substrate using aseptic techniques. The inoculated substrate is packed into fermentation vessels or trays to

create a uniform layer with optimal thickness for microbial growth. The inoculated substrate is incubated under controlled environmental conditions, including temperature, humidity, aeration, and pH, to promote microbial growth and metabolic activity. The fermentation process may last from several days to weeks, depending on the specific microorganism and desired product. During fermentation, various parameters such as temperature, moisture content, pH, and microbial growth are monitored to ensure optimal conditions for microbial activity and product formation.

Adjustments may be made as needed to maintain desired fermentation conditions. At the end of the fermentation period, the fermented solid substrate, known as the fermentation mass or fermented product, is harvested from the fermentation vessel. The product may undergo further processing steps, such as drying, milling, extraction, or purification, depending on the intended application. Utilization of inexpensive and renewable solid substrates. Reduced requirement for water and energy compared to submerged fermentation. Higher product yields and productivity due to higher cell densities and localized substrate concentrations. Production of diverse metabolites and enzymes with potential industrial applications. Are the advantages of solid-state fermentation.

4.2.8 Procedure

To conduct solid-state fermentation (SSF) using 10 kg of carrier media, 1 liter of nutrient broth, and 1 liter of YEM (Yeast Extract Mannitol) broth in a drum for 2 days, you'll need to follow a specific procedure tailored to your setup and requirements. Here's a general outline of the process. Mix the 10 kg of carrier media (coir pit and vermicompost) thoroughly to ensure uniform distribution of components. Hydrate the carrier media to the desired moisture content using water or nutrient solution. The moisture level should be sufficient to support microbial growth without excessive water accumulation. Place the hydrated carrier media in a clean and sterilized drum or fermentation vessel. Spread it evenly to create a uniform layer with optimal thickness for microbial colonization. Prepare the nutrient broth and YEM broth according to the previously outlined procedures. Inoculate the carrier media with the prepared broth cultures by evenly distributing them over the surface of the carrier media. Use sterile techniques to prevent contamination. Ensure that the broth

cultures are evenly mixed with the carrier media to facilitate uniform inoculation. Mix the inoculated carrier media thoroughly to ensure proper distribution of the broth cultures and uniform inoculation. Transfer the inoculated carrier media into the drum or fermentation vessel and seal it tightly to maintain optimal fermentation conditions. Incubate the drum or vessel containing the inoculated carrier media at the appropriate temperature and humidity for solid-state fermentation. The fermentation period is 2 days in this case. At the end of the fermentation period (2 days), harvest the fermented carrier media from the drum or fermentation vessel. Depending on the desired product or application, further process the fermented carrier media, such as drying, milling, or extracting bioactive compounds. Perform quality control checks on the fermented product to assess microbial viability, product yield, and purity. Analyse the fermented product for the presence of desired metabolites, enzymes, or bioactive compounds using appropriate analytical techniques. Store the fermented product in clean, airtight containers or bags to maintain its quality and shelf life. Apply the fermented product as a biofertilizer, soil amendment, or other agricultural inputs as needed, following recommended application rates and methods.

4.2.8.1 pH Identification

PH identification refers to the process of determining the pH value of a solution or a sample. PH is a measure of the acidity or alkalinity of a solution, and it is determined by the concentration of hydrogen ions (H^+) present in the solution. PH identification is essential in various fields, including chemistry, biology, environmental science, and food science, as it provides valuable information about the chemical properties and behavior of substances. Here's an overview of pH identification. pH is measured on a scale ranging from 0 to 14, where a pH of 7 is considered neutral, pH values below 7 indicate acidity, and pH values above 7 indicate alkalinity. pH identification relies on the use of pH indicators or pH meters to determine the pH value of a solution accurately. pH indicators are chemical compounds that change colour in response to changes in pH. They are often used in qualitative pH identification to visually assess the acidity or alkalinity of a solution based on the observed colour change. Common pH indicators include phenolphthalein, methyl orange, Bromothymol blue, and litmus paper. pH meters are electronic devices used for

precise and quantitative measurement of pH. They consist of a pH electrode that detects the concentration of hydrogen ions in the solution and converts it into a voltage signal, which is then displayed as a pH value on the meter's screen. pH meters are widely used in laboratory settings and industrial processes for accurate pH identification and monitoring. Obtain a small volume of the sample solution to be tested. Ensure that the sample is homogeneous and free from any visible particles or contaminants. Choose an appropriate pH indicator based on the expected pH range of the sample. Different pH indicators have different colour change ranges, so select one that matches the pH range of the sample. Add a few drops of the selected pH indicator to the sample solution. Stir or swirl the solution gently to ensure uniform mixing of the indicator. Observe the colour change of the solution. Compare the observed colour with the colour chart provided for the pH indicator to determine the approximate pH value of the sample.

4.2.9 Seed Treatment

Seed treatment with biofertilizer involves coating seeds with a biofertilizer formulation before planting. This process enhances seed germination, seedling vigor, and overall plant growth by providing beneficial microorganisms and nutrients directly to the seeds and the developing roots. Here's how seed treatment with biofertilizer is typically performed. If using a concentrated biofertilizer formulation, dilute it with water according to the manufacturer's instructions.

Ensure proper mixing to achieve a uniform solution. Place the seeds in a container large enough to accommodate them comfortably. Pour the prepared biofertilizer solution over the seeds, ensuring thorough coverage. Use a stirring rod or spoon to gently mix the seeds and biofertilizer solution, ensuring that all seeds are evenly coated. Place the seeds in a container large enough to accommodate them comfortably. Pour the prepared biofertilizer solution over the seeds, ensuring thorough coverage. Use a stirring rod or spoon to gently mix the seeds and biofertilizer solution, ensuring that all seeds are evenly coated. Allow the coated seeds to incubate for a short period to allow the biofertilizer to adhere to the seed surface. Optionally, spread the coated seeds in a thin layer on a tray or surface to air-dry for a short period. This step helps the biofertilizer adhere to the seed surface

more effectively. Plant the coated seeds immediately after treatment to prevent the biofertilizer from drying out or losing viability. Follow recommended planting depths, spacing, and planting practices for the specific crop being grown. Seed treatment with biofertilizer is typically performed just before planting to ensure maximum effectiveness. The biofertilizer formulation may contain beneficial microorganisms such as nitrogen-fixing bacteria, phosphate solubilizing bacteria, or mycorrhiza fungi, along with nutrients and growth promoting substances. Seed treatment with biofertilizer can improve seedling establishment, nutrient uptake, and crop productivity, especially under stress conditions or poor soil fertility. Follow recommended application rates and guidelines provided by the manufacturer of the biofertilizer formulation. Store any leftover biofertilizer solution according to the manufacturer's instructions for future use.

4.2.9 Root Dipping

Root dipping is a horticultural technique used to treat the roots of seedlings or transplants with a solution containing beneficial microorganisms, growth promoting substances, or protective agents before planting them in the soil. This method helps establish a symbiotic relationship between the roots and the applied solution, promoting root development, nutrient uptake, and overall plant health. Here's how root dipping is typically performed. Prepare the dipping solution by diluting the selected product biofertilizer, rooting hormone with water according to the manufacturer's instructions. Ensure thorough mixing to achieve a uniform solution. Pour the prepared dipping solution into a container large enough to accommodate the seedlings or transplants. Immerse the exposed roots of each seedling or transplant into the dipping solution, ensuring that the roots are fully submerged. Hold the seedlings in the solution for a few seconds to allow the roots to absorb the beneficial substances present in the solution. After root dipping, remove the seedlings or transplants from the dipping solution, allowing any excess solution to drain off. Place the treated seedlings or transplants on a tray or surface to air-dry briefly before planting. Plant the treated seedlings or transplants immediately in the desired location, following recommended planting depths and spacing for the specific crop or plant species. Root dipping is commonly used to inoculate seedlings with beneficial microorganisms such as rhizobia bacteria to

enhance nutrient uptake and promote plant growth. Root dipping can also involve the application of rooting hormones to stimulate root development and improve transplant survival. Follow recommended application rates and guidelines provided by the manufacturer of the dipping solution. Root dipping is often performed in combination with other planting practices, such as soil amendments, to optimize plant establishment and development.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 BACTERIAL ISOLATION AND SCREENING

5.1.1 Serial Dilution Of Soil Sample

Collected soil samples were done serial dilution the colonies were selected to plating.

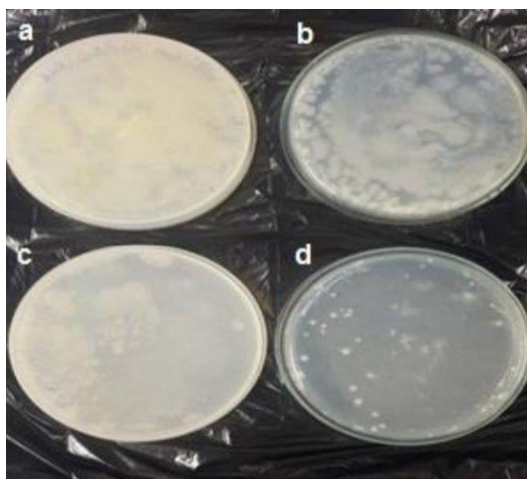


Fig 5.1 Serial dilution of soil sample

a) 10^{-5} dilution b) 10^{-6} dilution c) 10^{-7} dilution d) 10^{-8} dilution

5.1.2 Gram Staining

The gram staining was observed as positive for Bacillus and negative for Rhizobium.

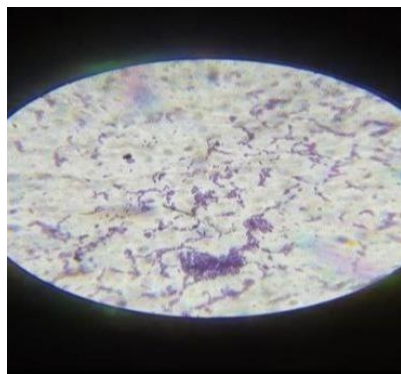


Fig 5.2 Gram positive Bacillus



Fig 5.3 Gram negative Rhizobium

5.1.3 Biochemical Test

Biochemical test such as indole production test, MR test, VP test, citrate test, catalases test, nitrate reduction test was and the results were identified as positive for MR test, catalase test, citrate test and nitrate reduction test and negative for VP test and indole production test.

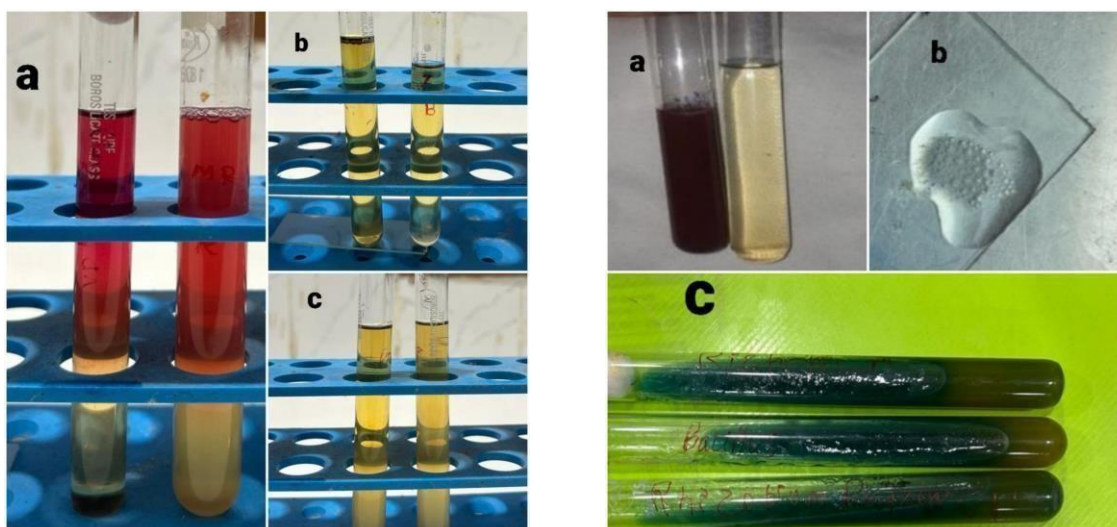


Fig 5.4 Biochemical test Fig 5.5 biochemical test a) MR test b) indole test c) VP test a) Nitrate reduction test b) catalase test c) citrate test

5.2 IDENTIFICATION OF EXACT GEANUS AND SPECIES OF THE ISOLATED BACTERIAL SAMPLE

5.2.1 16sRrna Sequencing

16sRrna sequencing shows positive result for Bacillus finding that the exact species is *Bacillus amyloliquefaciens*. And negative results for *Rhizobium*.

ID [WYVBH8J4013](#) Search engine on 02/17/21 13:21 [Download All](#) 

Program BLASTN [Citation](#) 

Database Representative genomes (ref_protok_genomes) [See details](#) 

Query ID lcl|Query_3719255

Description SR417:RSR1_AG2.ab1

Molecule type dna

Query Length 729

Other reports [Distance tree of results](#) [MSA viewer](#) 

Organism only top 20 will appear

[+ Add sequence](#)

Percent identity to **E value** to **Query Coverage** to

[Filter](#) [Reset](#)

[Descriptions](#)

[Graphic Summary](#)

[Alignments](#)

[Taxonomy](#)

Sequences producing significant alignments

[Download](#)
[Select columns](#)
[Show 100](#)


☒ [select all](#) 103 sequences selected

Description	Identical Name	GenBank		Query Cover	E value	Pct. Ident	Acc. Len	Accession
		Max Score	Total Score					
Bacillus bacteriota HSCC_12613 (strain HSCC_13812C) sarr632.9, whole-genome shotgun, sequencing	Bacillus bacteriota	1309	1309	100%	0.0	95.96%	4692	NZ_LN1571002943.1
Bacillus velezensis strain J5259, chromosome, complete genome	Bacillus velezensis	1309	6779	100%	0.0	96.96%	400000	NZ_CP093676.1
Phycobactin synthase (b42_Ga034854_133, whole-genome shotgun sequence)	Phycobactin synthase	1304	1334	100%	0.0	96.73%	5025	NZ_LN84816100033.1
Nonacidobacterium strain KGC_C119017_KC_10127_Can0262, whole-genome shotgun, sequencing	Nonacidobacterium	1304	1334	100%	0.0	96.73%	1542	NZ_CP2731000022.1
Bacillus pasteurii strain HEP-4, 410911 (Bacillus pasteurii) contig_34, whole-genome shotgun	Bacillus pasteurii	1304	1334	100%	0.0	96.73%	4706	NZ_LN8021000003.1
Bacillus cereus strain A78.1, chromosome, complete sequence	Bacillus cereus	1204	12344	100%	0.0	96.73%	4140008	NZ_CP059550.1
Bacillus pasteurii strain A65.1, chromosome, complete sequence	Bacillus pasteurii	1304	1334	100%	0.0	96.73%	4247130	NZ_CP059550.1

5.2.2 MTCC Sample



Fig 5.7 culture form MTCC

5.3 HEAVY METAL TEST CFU TEST FOR RHIZOBIUM AND BACILLUS

Heavy metal test with zinc sulfate and lead nitrate is observed as positive due to the growth in media containing heavy metals.



Fig 5.9 Heavy metal test for *Bacillus amyloliquefaciens*

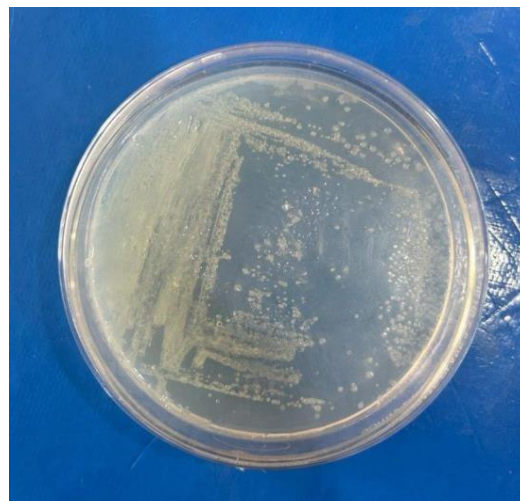


Fig 5.10 Heavy metal test for *Rhizobium leguminosarum*

5.3.2 Colony Forming unit Test

In this test the colony forming ability of the microbe infused in the biofertilizer is identified. CFU of bacillus is 42 and the CFU of Rhizobium is 36.



Fig 5.11 CFU test for *Rhizobium*

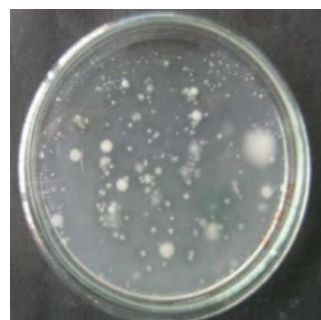


Fig 5.12 CFU test for *Bacillus*

5.3.3 Testing The Ability Of The Biofertilizer by Applying To Plants And Monitoring the Growth

CONTROL POTS

Day 1



Fig 5.13 Day 1 control pot

Day 2



Fig 5.15 Day 2 Control pot

Day 4



Fig 5.17 Day 4 Control pot

BIOFERTILIZER POTS

Day 1



Fig 5.14 Day 1 Biofertilizer pot

Day 2



Fig 5.16 Day 2 biofertilizer pot

Day 4



Fig 5.18 Day 4 Biofertilizer pot

CONTROL POTS
Day 6



Fig 5.19 Day 6 control pot

BIOFERTILIZER POTS
Day 6



Fig 5.20 Day 6 Biofertilizer pot

Day 8



Fig 5.21 Day 8 control pot

Day 8



Fig 5.22 Day 8 Biofertilizer pot

Day 10



Fig 5.23 Day 10 control pot

Day 10



Fig 5.24 Day 10 Biofertilizer pot

CONTROL POTS

Day 12



Fig 5.25 Day 12 control pot

BIOFERTILIZER POT

Day 12



Fig 5.26 Day 12 Biofertilizer pot

Day 14



Fig 5.27 Day 14 control pot

Day 14



Fig 5.28 Day 14 Biofertilizer pot

Day 16



Fig 5.29 Day 16 control pot

Day 16



Fig 5.30 Day 16 Biofertilizer pot

CONTROL POTS

Day 18



Fig 5.31 Day 18 control pot

BIOFERTILIZER POTS

Day 18



Fig 5.32 Day 18 Biofertilizer pot

Day 20



Fig 5.33 Day 20 control pot

Day 20



Fig 5.34 Day 20 Biofertilizer pot

Day 22



Fig 5.35 Day 22 control pot

Day 22



Fig 5.36 Day 22 Biofertilizer pot

CONTROL POT

Day 24



Fig 5.37 Day 24 control pot

BIOFERTILIZER POTS

Day 24



Fig 5.38 Day 24 Biofertilizer pot

Day 26



Fig 5.39 Day 26 control pot

Day 26



Fig 5.40 Day 26 Biofertilizer pot

Day 28



Fig 5.41 Day 28 Biofertilizer pot

Day 28



Fig 5.42 Day 28 Biofertilizer pot

CONTROL POT
Day 30



Fig 5.43 Day 30 Control pot

BIOFERTILIZER POTS
Day 30



Fig 5.44 Day 30 Biofertilizer pot

Day 32



Fig 5.45 Day 32 Control pot

Day 32



Fig 5.46 Day 32 Biofertilizer pot

Day 34



Fig 5.47 Day 34 Control pot

Day 34



Fig 5.48 Day 34 Biofertilizer pot

CONTROL POT

Day 36



Fig 5.49 Day 36 Control pot

BIOFERTILIZER POTS

Day 36



Fig 5.50 Day 36 Biofertilizer pot

Day 38



Fig 5.51 Day 38 Control pot

Day 38



Fig 5.52 Day 38 Biofertilizer pot

Day 40



Fig 5.53 Day 40 Control pot

Day 40



Fig 5.54 Day 40 Biofertilizer pot

CONTROL POT
Day 42



Fig 5.55 Day 42 Control pot

BIOFERTILIZER POTS
Day 42



Fig 5.56 Day 42 Control pot

Day 44



Fig 5.57 Day 44 Control pot

Day 44



Fig 5.58 Day 44 Biofertilizer pot

CHAPTER 6

SUMMARY AND CONCLUSION

In conclusion the biofertilizers created with *Bacillus amyloliquefaciens* and *Rhizobium leguminosarum* strains, using coir pith as carrier media, show potential to increase agricultural sustainability. The strong growth of shoots and roots as well as the good water-holding capacity of coconut peat point to their effectiveness in promoting plant development and conserving moisture. Additionally, seed germination rates of 80% indicate that the biofertilizers have positive effects on seed viability and early establishment of seedlings. These outcomes demonstrate how biofertilizers can replace chemical fertilizers thereby contributing towards all aspects of soil health, crop productivity and water use efficiency in agriculture. To fully exploit these advantages for sustainable food production and environmental protection, there is need to conduct more research on biofertilizer formulation as well as application methods. In summary, Biofertilizers become the solution to modern agriculture's problems and promise a path towards resilient farming systems that are environmentally friendly.

This study shows that biofertilizer promotes and improves the overall growth of the plant. It can be used as the best alternative for chemical biofertilizers due to its low cost of production and environmentally friendly.

Rate the efficiency of coir pith and vermicompost as carrier media for *Bacillus* and *Rhizobium* strains. Assess their potential to offer a suitable environment for microbial growth and survival. Consider how nutrient composition of the carrier media may regulate microbial activity and biofertilizer effectiveness. Coir pith and vermicompost are widely known for their high content of nutrients that can potentially elevate microbial performance.

Elaborate on the possibility of using both *Bacillus* and *Rhizobium* strains together. Both microorganisms are important in plant growth promotion and nutrient recycling, so combining their application could result in more substantial gains for agriculture. Stress the importance of heavy metal tolerant among these strains. With widespread heavy

metals contamination in agricultural soils, tolerant strains become sustainable alternatives to mitigate metal toxicity thereby enhancing plant health.

Highlight environmental advantage of using biofertilizers over chemical fertilizers. Biofertilizers made from naturally occurring microbes and organic carrier materials can help with soil health, reduce chemical inputs, as well as avoid environmental pollution. Investigating alternative carrier materials, studying microbial interactions in greater detail, or conducting field trials to evaluate long-term effectiveness are possible ways future research could optimize and further improve the biofertilizer formulation.

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ANNEXURE

ORIGINALITY REPORT			
18%	8%	10%	11%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS
PRIMARY SOURCES			
1	fastercapital.com Internet Source	1%	
2	Submitted to Howard University UG Student Paper	1%	
3	Rahul Prasad Singh, Priya Yadav, Indrajeet Kumar, Ajay Kumar, Rajan Kumar Gupta. "Precision biotechnology using beneficial microbes as a fundamental approach to the circular economy", Elsevier BV, 2024 Publication	1%	
4	Submitted to Mohave Community College Student Paper	<1%	
5	Submitted to Rutgers University, New Brunswick Student Paper	<1%	
6	Submitted to Oxnard College Student Paper	<1%	
7	Submitted to CSU, San Diego State University Student Paper	<1%	