Characterization of Bacterial Siderophore in Plant Growth Promotion and Sustainable Agriculture

A Dissertation

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF

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In

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Submitted by

Under Supervision Of

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Gautam Buddha University, Greater Noida (U.P), June, 2022

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TRULY INDEBTED

To My Parents

Smt. Brijlata Sigh & Late Shri Ramjilal

For

Their Support and Blessings

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CANDIDATE'S SELF DECLARATION

I hereby declare that work embodied in the dissertation entitled, "**Characterization of Bacterial Siderophore in Plant Growth Promotion and Sustainable Agriculture**" by **Khushbu**, 20/MES/013 in the partial fulfilment of the requirements **for the award of the Degree of Master of Science in Environmental Science**, submitted to the Department of Environmental Science, School of Vocational Studies and Applied Sciences, Gautam Buddha University, Greater Noida, Uttar Pradesh is an authentic record of my original research work carried out **under the supervision of Dr. Shiv Shankar** I have followed research ethics to the best of my abilities. I have acknowledged all sources of information that I used in the dissertation. I have completed all pre-submission requirements as per the University rules.

20/MES/013

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GBU, Greater Noida June 2022

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ABSTRACT

The production and quality of food grains is declining globally, and the sustainability of the agroecosystem is deteriorating. The demand for chemical fertilisers is expected to increase to 200 million tonnes by the end of 2022. These chemical fertilisers have enhanced agricultural yield, but they have also increased pollution and put the ecological integrity of the global agroecosystem in danger. To lessen the environmental stress in the crop, safer and sustainable alternative to chemical fertilisers are required. Rhizosphere or "plant growth promoting rhizobacteria" (PGPR) interact positively with plant roots and aid in release of phytohormones, nitrogen fixation, mineral phosphate solubilization, siderophore production to lessen the negative effects of several stresses on plants. In the present study PGPR were isolate and cultivated in the lab. These bacterial strains were examined for siderophore production by qualitative and quantitative methods. The optimization of physical factors such as carbon, nitrogen, PH and temperature were also carried out. Application of PGPR in reducing environmental stress and plant growth was investigated. Results indicated the role of plant growth-promoting rhizobacteria in sustainable agriculture by reducing pollution and stress in the environment and improving the quality of crops.

Keywords: Plant growth promoting rhizobacteria (PGPR); Sustainable agriculture; agroecosystem; nitrogen fixation; siderophore.

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LIST OF ACRONYMS

Acronyms Used	Description
PGPR	Plant growth-promoting rhizobacteria
ePGPR	Extracellular plant growth promoting rhizobacteria
iPGPR	Intracellular plant growth promoting rhizobacteria
CAS	Chrome Azurol Sulphonate
RPS	Non-ribosomal peptide synthetase modular multienzymes
NIS	NRPS-independent
TBDR	TonB-dependent receptor
ABC	ATP-binding cassette
LMMOAs	low molecular mass organic acids
HPLC	High-performance liquid chromatography
Conc.	Concentration
G	Gram
i.e.	Id est (that is)
Kg	Kilo gram

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Introduction

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1.1. Sustainability of chemical fertilizers to promote plant growth in Agriculture

Unregulated use of fertilisers, especially those high in nitrogen and phosphorus, has significantly contaminated the soil, the air, and the water in the process of modern agriculture. The ecosystem is polluted and the soil microorganisms are negatively impacted by the over use of these chemicals **[Youssef MMA *et al.*,2014]**. Long-term use of these fertilisers frequently results in a drop in pH and exchangeable bases, rendering them inaccessible to crops and lowering crop output. Farmers have relied more and more on chemical sources of nitrogen and phosphate to solve this issue and increase plant yields. In addition to being expensive, the manufacture of chemical fertilisers depletes non-renewable resources, including the oil and natural gas used to make them, and presents risks to both people and the environment **[Joshi KK., 2006]**.

1.2. **Rhizosphere;** An Alternate Approach for Sustainable Crop Production

It is a common occurrence for rhizobacteria to promote plant development, and these rhizobacteria possess specific characteristics that contribute to this growth improvement. Extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) are two different types of plant growth boosting bacteria. In contrast to iPGPRs, which are typically found inside the specialised nodular structures of root cells, ePGPRs can exist in the rhizosphere, on the rhizoplane, or in the spaces between the cells of the root cortex. Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas, and Serratia are among the bacterial taxa that belong to the ePGPR. The iPGPR is a member of the Rhizobiaceae family, which also includes endophytes and Frankia species that can symbiotically fix atmospheric nitrogen with higher plants. Rhizobiaceae comprises Allorhizobium, Bradyrhizobium, Mesorhizobium, and Rhizobium.

Through diversification and a focus on sustainable production systems, agriculture policy has significantly changed during the previous few decades. Since the term "rhizosphere" was coined to describe the comfortable environment of microorganisms near plant roots, rhizosphere study has produced a number of unexpected and intriguing research questions. Hiltner **[Hiltner L., 1904]** coined the phrase "rhizosphere" for the first time. The main

effects that the microorganisms in the rhizosphere have on plants now have crucial applications for protecting plant health in an environmentally friendly way **[Akhtar N., 2014].** These bacteria are frequently referred to as plant growth promotory rhizobacteria since they can affect plant growth **[Kloepper JW *et. al* 1980]**. To make the soil ecosystem active for nutrient turnover and durable for crop production, they are involved in a variety of biotic processes **(Sivasakhti S *et. al*., 2014)**. In recent years, PGPR has received a lot of attention because it can replace agrochemicals (fertilisers and pesticides) for the promotion of plant growth through a number of mechanisms, including the formation of soil structure, the decomposition of organic matter, the recycling of crucial elements, the solubilization of mineral nutrients, the production of numerous plant growth regulators, the degradation of organic pollutants, the stimulation of root growth, which is essential for soil fertility, and the biocontrol of soil and seed In bioremediation techniques **[Sagar S *et. al.,* 2012],** energy production procedures, and biotechnological Understanding plant growth-promoting rhizobacteria and their interactions with biotic and abiotic factors is crucial in industries including pharmaceuticals, food, chemical, and mining. Additionally, rhizobacteria that promote plant development can reduce the need for artificial fertilisers, which is advantageous for both the economy and the environment. They can also identify the optimum soil and crop management techniques to achieve more sustainable agriculture and soil fertility **[Maheshwari DK *et. al*., 2012]**. Extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) are two different types of plant growth boosting bacteria **[Viveros OM et al., 2010].** In contrast to iPGPRs, which are typically found inside the specialised nodular structures of root cells, ePGPRs can exist in the rhizosphere, on the rhizoplane, or in the spaces between the cells of the root cortex. Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas, and Serratia are among the bacterial taxa that belong to the ePGPR **[Ahemad M *et. al.,* 2014].** The iPGPR is a member of the Rhizobiaceae family, which also includes endophytes and Frankia species that can symbiotically fix atmospheric nitrogen with higher plants **[Bhattacharyya PN *et. al.,* 2012]**. Rhizobiaceae comprises Allorhizobium, Bradyrhizobium, Mesorhizobium, and Rhizobium.

1.3. **Plant Growth Promoting Rhizobacteria (PGPR): The Mechanism**

Production of secondary metabolites like siderophores is one of the primary mechanisms of PGPR in encouraging plant development **(Kloepper *et al.,* 1994).** Iron is abundant in soil,

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but because it often forms insoluble complexes, much of it is unavailable to plants or other living things. Thus, iron insufficiency is a significant global problem. By causing iron to be solubilized and chelated from organic or inorganic complexes present in soil, siderophores produced by PGPR aid in meeting the needs of plants for iron. Even at very low concentrations, microbial siderophores strongly chelate iron and promote iron uptake by producing a ferric-siderophore complex **(Gray and Smith., 2005)**

Thus, siderophores play a crucial function in supplying iron to other species, including humans, in addition to aiding in the growth of plants. Additionally, PGPR-produced siderophores aid in protecting plants from phytopathogens **(NK Arora *et al.,* 2017).** Due to iron hunger or due to competitive exclusion under iron-deficient environments, phytopathogens are suppressed in the rhizosphere by PGPR that produces siderophores. In addition to promoting plant development, siderophores are crucial in the bioremediation of heavy metals from contaminated sites because they bind to harmful metals like Cr3, Al3, Pb2, Cd2, Hg2, and others. Thus, by mobilising insoluble heavy metals, siderophore-producing microorganisms can be employed to detoxify heavy metal (**Ahmad *et al.* 2014).**

Therefore, siderophore-producing bacteria have a wide range of applications, such as bioremediation, sustainable agriculture, biosensors, and even medicine. The chrome azurol sulphonate (CAS) assay described by Schwyn and Neilands is frequently used to detect the ability of microorganisms to produce siderophores (1987). Supernatants of microbial cultures are employed for the quantitative measurement of siderophore synthesis. Additionally, solid CAS agar media are employed for qualitative siderophore production detection. The siderophore and ferric complex of the CAS dye (CAS-iron-detergent complex) compete with one another for iron uptake in the CAS test. The media changes colour from blue to orange as a result of the siderophore's strong chelation of the iron from the iron-dye complex **(Louden *et al.,* 2011)**. Spectrophotometric estimate is used to determine how much siderophore is produced by bacteria. In this traditional procedure, the CAS reagent and microbial supernatant are combined, and the siderophore quantity is calculated by measuring the optical densities of each sample separately. This method, however, needs a lot of area, time, labour, and chemicals. In light of this, a significantly less expensive, time-consuming, and labor-intensive improved method of siderophore estimation was created. The classical Schwyn and Neilands (1987) method, which employed a 96-well microtiter plate and plate reader to allow the simultaneous screening of many PGP strains,

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served as the main inspiration for this quantitative method of siderophore production quantification.

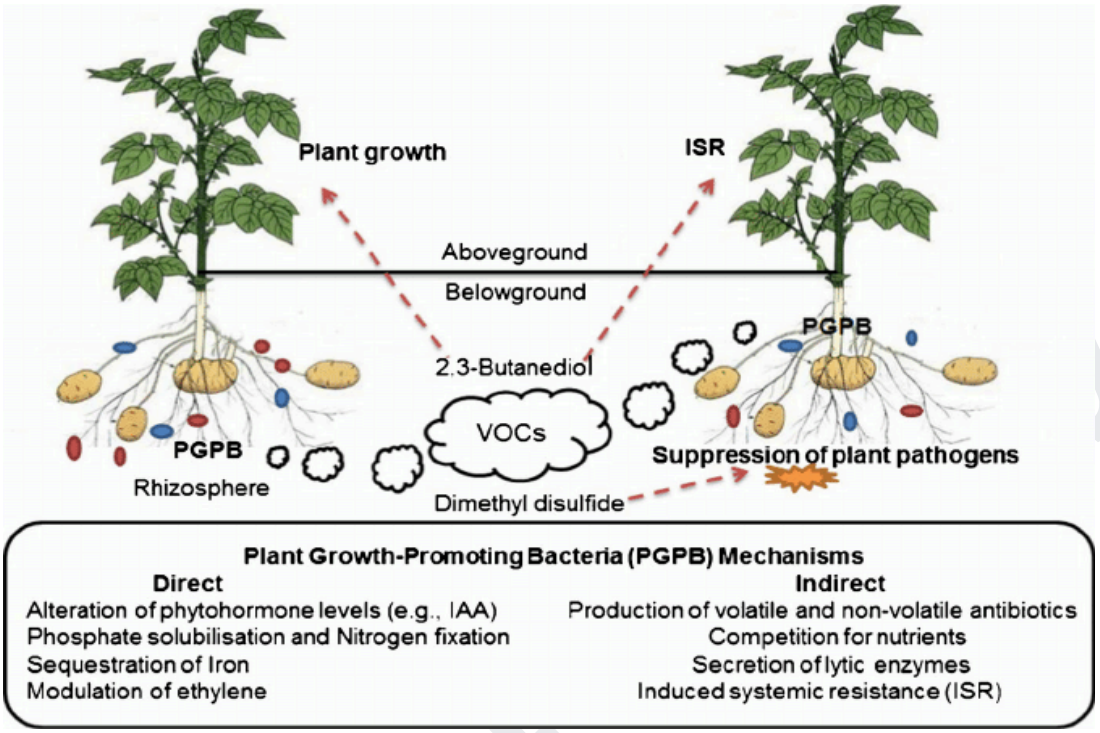

Fig.1 Mechanism of Plant- Growth-Promoting Bacteria (Velivelli, *et al*., 2014)

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1.4 Siderophore

Low molecular weight secondary metabolites called siderophores have the ability to chelate iron. These are substances that have side chains and functional groups on tiny peptidic molecules that have a high-affinity ligand to bind ferric ions and transport them through the cell membrane **(M. Ahemad *et. al.,* 2014).** On the basis of their structural characteristics, functional groups, and types of ligands, siderophores are produced by a variety of microorganisms and are divided into four main classes (carboxylate, hydroxamates, catecholates, and mixed type). It has been observed that a wide variety of bacterial and fungal taxa, including human diseases and environmental microbes like plant growth-promoting rhizobacteria (PGPR), create siderophores.

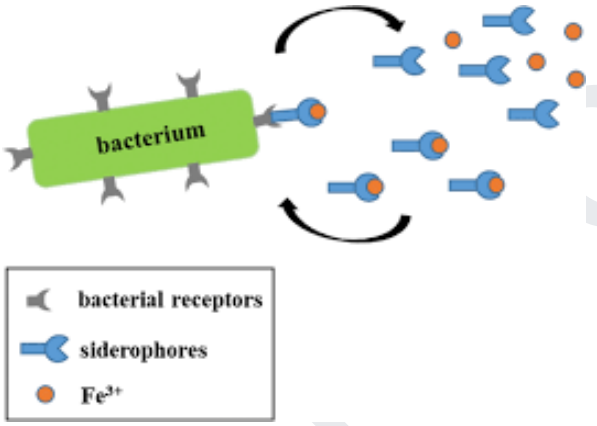

Fig.2. Application of Siderophore in Nitrogen fixation

Iron acquisition nevertheless poses a significant problem for many microbes despite the abundance of iron and the efficiency of Feo for uptake of Fe2+ because iron forms insoluble ferric hydroxides in the presence of oxygen, lowering the quantity of soluble Fe2+ below the threshold of Feo (**Niessen and Soppa, 2020).** To combat this, the siderophore-dependent iron uptake, a striking and popular tactic created to solubilize and collect iron from aerobic settings, has emerged. Prokaryotes and eukaryotes both manufacture siderophores, which have modest molecular weights (500–1,500 daltons) and a strong affinity for insoluble Fe3+**.** Catecholate, hydroxamate, phenolate, carboxylate, and mixed-type siderophores—which contain multiples of the aforementioned moieties—are the most prevalent categories used to categorise siderophores according to their iron-binding moieties (Figure 1). Non-ribosomal peptide synthetase (NRPS) modular multienzymes and NRPS-independent (NIS) enzymes are two different forms of enzymatic machinery that catalyse the biosynthesis of siderophores **(Barry and Challis, 2009).** The repetitive catalytic domain groups of NRPS enzymes, which are well known for their selectivity in assembling particular peptide products, are used to introduce one monomer into the developing peptide. The majority of the siderophores

produced by NIS enzymes, known as hydroxamates, are built by oligomerizing and macrocyclizing their -aminocarboxylic acid substrates through numerous cycles of amide bond formation **(Bloudoff and Schmeing, 2017)**.

Furthermore, numerous NIS synthetases might collaborate to take part in a special hybrid NIS-NRPS pathway for the production of siderophores. Iron, one of the most common metal elements on Earth, has a wide range of oxidation-reduction potential due to the transition between its two stable valences of ferrous (Fe2+) and ferric (Fe3+). Because iron participates in so many different biological processes, such as electron transport, metabolism, peroxide reduction, amino acid and nucleoside synthesis, DNA synthesis, photosynthesis, and gene expression, it is essential for almost all living things **(Liu Lulu *et al.*, 2020).**

Iron molecules may need to be put together form functional complexes, like iron-sulfur clusters and heme, or they may momentarily bind to apoproteins as mono- and bi-nuclear iron centres However, excessive iron may be extremely harmful to cells because it encourages the production of potentially lethal reactive oxygen species (ROS). Since the transcriptional and post-transcriptional levels of iron intake, storage, and consumption are tightly regulated, iron homeostasis in living cells must be carefully maintained through coordinated expression of these proteins (**Daniel, Trnka *et al*,. 2020).** Numerous iron-uptake mechanisms in bacteria have been discovered and characterised. The most important of them is Feo, a transporter that specifically takes in Fe2+ from its surroundings and supports proper bacterial metabolism and growth. Most bacterial Feo systems are made up of two subunits, FeoA and FeoB, but there are exceptions. For example, Escherichia coli and several other -proteobacterial species have three-subunit Feo systems (FeoA, FeoB, and FeoC), one fused FeoA/FeoB protein, and an isolated FeoB whose FeoA is still unknown. While FeoA is a small-molecule hydrophilic protein necessary for Feo activity by encouraging the formation of the Feo complex, FeoB operates as a Fe2+ permease with a cytosolic N-terminal G-protein domain and a C-terminal integral inner-membrane domain including two "Gate" motifs. Once formed, siderophores in Gram-negative bacteria are exported into the environment by certain transporters, where they bind Fe3+ to create ferrisiderophore complexes. Depending on the energy transduced by the TonB-ExbB-ExbD system located in the inner membrane (IM), the complexes are recognised and trafficked across the outer membrane (OM) by a TonB-dependent receptor (TBDR). Thereafter, the complexes are translocated across the inner membrane (IM) by the activity of ATP-binding cassette (ABC) transporters or permeases into the cytoplasm. Fe3+ in the ferrisiderophore complex is reduced to Fe2+ in the cytosol by ferrisiderophore reductase (FSR), which then releases Fe3+ from the complex. Iron can sometimes be released from ferrisiderophore complexes in the periplasm

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through reduction or alteration of the siderophore scaffold, and the resulting free siderophores can then be exported for further usage **(Caza, Mélissa *et al.*, 2016).**

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Review of Literature

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2.1 Siderophores and Siderophore Biosynthesis

More than 20 years ago, siderophores were initially discovered in Shewanella species. Shewanella putrefaciens quickly revealed that the siderophores were putrebactin, a cyclic homodimer of succinyl-(N-hydroxyputrescine) made from a single substrate, putrescine **(Ledyard and Butler., 1997)** Later discoveries made from Shewanella algae B516 included the identification of bisucaberin and avaroferrin in addition to putrebactin **(Böttcher and Clardy, 2014).** Avaroferrin is produced by combining the two substrates putrescine and cadaverine, whereas bisucaberin, also known as putrebactin, is a homodimeric result of two molecules of cadaverine. A NIS system catalyses the production of putrebactin, avaroferrin, and bisucaberin **(Codd *et al*., 2018)**. Because just a single siderophore could be found in the bacteria that were hosting any of these systems, it was first assumed that they functioned in a substrate-specific manner. But according to recent research, if the necessary substrates are added, AvbBCD can also create a variety of homodimeric and heterodimeric combinatorial products with 18 to 28 members, in addition to putrebactin, bisucaberin, and avaroferrin **(Böttcher and Clardy, 2014).** Therefore, it is plausible that any of these synthesising systems may be capable of producing a variety of cyclic hydroxamate molecules and that the ratios of the final products in the cell are determined by the substrate pool of precursor molecules, even though substrate preference may exist.

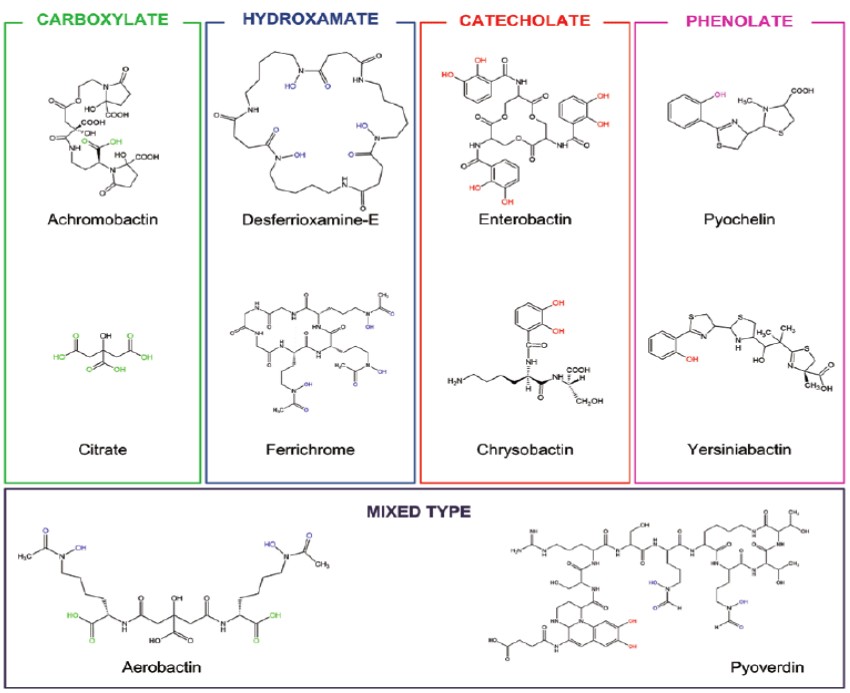

Fig. 3. The different types of siderophore structures and the functional groups involved in iron chelation.

2.2. Regulation of Siderophore Biosynthesis

The majority of microorganisms cannot survive without iron, yet cells can become toxic from intracellular iron excess because it breaks DNA, proteins, and lipids biomolecularly via the Fenton reaction. Therefore, it is crucial to carefully manage iron homeostasis, which calls for coordination between iron acquisition and consumption, including iron intake, consumption, storage, and outflow **(Andrews et al., 2013).** Transcription factor Fur coordinates the coordination in many bacteria by detecting internal iron levels and regulating the transcription of genes involved in iron acquisition and utilisation. According to recent research, the global regulator Fur controls the transcription of genes involved in a variety of biological processes via intricate and unique mechanisms that change depending on the microorganisms. In order to sense intracellular iron balance, Fur can additionally interact with a [2Fe-2S] cluster. The traditional Fur regulation still exists despite them. According to this model, Fur binds to Fe2+, and the dimeric Fe2+-Fur complex (holo-Fur) recognises the target sequences (Fur-box) upstream of iron-regulated genes and inhibits transcription of those genes under iron-replete conditions while de-repressing transcription under iron-limiting conditions **(Fillat, 2014).** One of these genes produces the short RNA RyhB, which functions posttranscriptionally to promote an iron-sparing response by suppressing the translation of proteins that require iron cofactors and preserving iron for critical proteins. An N-terminal DNA-binding domain connected to a C-terminal dimerization domain by a hinge region makes up fur proteins, which function as a

homodimer of polypeptides. Fur-box variations with lengths ranging from 15 to 23 bp (19 bp in E. coli) have been observed, but they are all AT-rich, mostly constructed from the base sequence GATAAT, and share a great deal of sequence similarity. Since the advent of omics technology, numerous bacterial genes involved in siderophore biology have been found to be directly regulated by Fur and RyhB.

2.3. Role of siderophores in Sustainable Agriculture

a. Soil mineral weathering

The microbial communities found in soils atop mineral surfaces are distinct from those found in the surrounding soil. A microenvironment that shields the microorganisms from external challenges is created as a result of microbial adhesion to mineral surfaces. Mineral nutrients in these microenvironments can either be directly chelated from the soil minerals or distributed among the local microorganisms **(Roberts Rogers and Bennett, 2004).** The mineral dissolution of insoluble phases can be aided by siderophores generated by soil microorganisms. For siderophore-promoted Fe dissolution, various methods have been proposed. The Fe(III)-siderophore complex is generally thought to develop at the mineral surface, move into the surrounding soil solution, and then become available for uptake by microbes or plants. Because siderophores form more stable complexes with Fe, their influence on soil mineral weathering may be greater than that of low molecular mass organic acids (LMMOAs). The constants of siderophores with Fe(III) range from K = 1030 to K = 1052 **(Matzanke, 1991),** whereas oxalic and citric acids have constants with Fe(III) of K = 108 and 1012, respectively. The synergistic effects of LMMOAs and siderophores, as opposed to siderophore alone, may cause a greater rate of mineral dissolution when both are present **(Reichard et al., 2007).** The role of siderophores in the dissolution of Fe minerals has been well researched due to the significance of microbial siderophores in weathering and soil formation. For instance, Suillus granulatus produces hydroxamate siderophores that are highly effective at causing goethite to dissolve, according to Watteau and Berthelin (1994). Because Suillus sp. continuously produces siderophores, significant amounts (109 mol m2 h1) of Fe were mobilised in its presence. Additionally, the ferrichrome family of fungal siderophores like ferrichrome and ferricrocin helped alter the surface structure of biotite and accelerate its breakdown in podzolic forest soil. In comparison to the dissolution of Fe by a synthetic siderophore, it has also been shown that the dissolution of Fe from hornblende is higher in the presence of actinobacteria that produce siderophores (Streptomyces and Arthrobacter) (desferrioxamine B). By increasing the dissolution of some Fe-containing minerals like ferrihydrate and goethite, some studies have also demonstrated that

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phytosiderophores can play a substantial role in mineral weathering processes. According to Reichard and associates (2005), the greatest rate of goethite dissolution (1.7 nmol m2 h1) in the presence of phytosiderophores (2′-deoxymugineic acid) was discovered at pH 6. The expression of Fe-phytosiderophore transporter genes in barley increases its capacity to dissolve Fe from soil minerals, as recently observed by genetic engineering applications **(Gómez-Galera et al., 2012).**

b. Biogeochemical cycling of Fe in the ocean

There is growing concern over the biogeochemical cycling of trace metals in the oceans. Fe has drawn the most attention out of all the trace metals found in marine waters because it is a crucial micronutrient for marine life and because its low concentration in the ocean regulates phytoplankton productivity and community structure. The majority of the organic Fe chelators found in seawater are produced by marine bacteria, which contributes significantly to the biogeochemical cycling of Fe in the ocean. These bacteria produce various types of siderophores that have a significant impact on the quantity and solubility of Fe in the marine environment and compete with phytoplankton for the element (**Cordero et al., 2012).** Snychobactins, petrobactins, aerobactins, and marinobactins are examples of marine siderophores that have a hydroxyl-carboxylate functional group that is either given by citrate or by b-hydroxyaspartate (i.e. aquachelins, loihichelins and alterobactin). By producing Fe(III)-siderophore complexes that increase Fe availability for phytoplankton in the surface water, siderophores take part in the photochemical cycling of Fe. Ferrioxamine E demonstrated a more variable distribution among depths, but ferrioxamine G was found to be extensively dispersed in surface waters throughout the Atlantic Ocean. These results imply that marine siderophores have a significant role in increasing the abundance and availability of Fe in the Atlantic Ocean's water column and, consequently, in the biogeochemical cycle of Fe **(Amin et al., 2012).**

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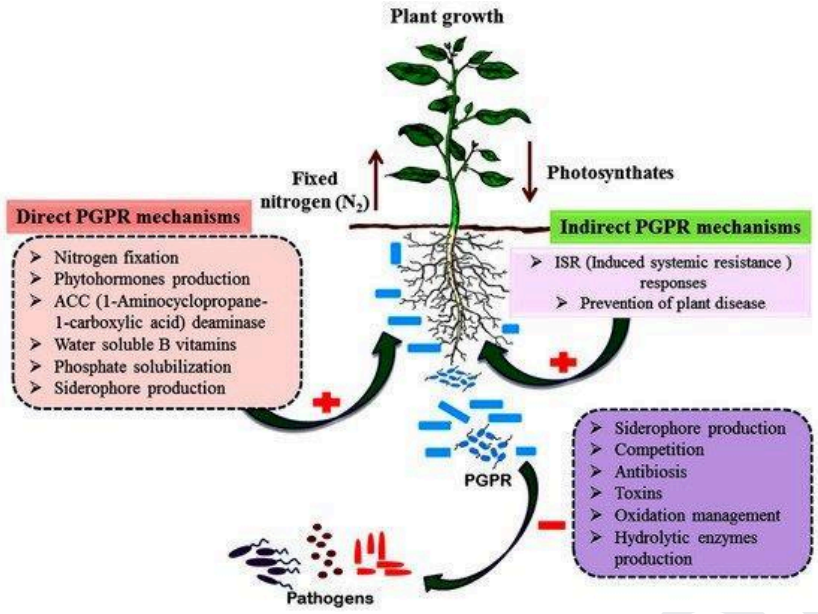

Fig.4. Plant Growth-Promoting Rhizobacteria

2.3 Applications of Siderophores

a. Enhancing growth and pathogen biocontrol of plants

Although the precise mechanism is not well understood, it is known that microbial siderophores provide plants with Fe nutrition to promote their growth when the bioavailability of Fe is low. Plants may receive Fe from microbial siderophores through one of two potential mechanisms: I High redox potential microbial siderophores can be reduced to donate Fe(II) to the plant's transport system. According to this method, siderophore reduction may take place in the plant root's apoplast, where the microbial Fe(III)-siderophores are delivered.

High Fe concentrations in the root are the result of Fe(II) being trapped in the apoplast. (ii) Microbial siderophores have the ability to exchange ligands with phytosiderophores after chelating Fe from soils. The stability constants and concentrations of both microbial and phytosiderophores, as well as the pH and redox conditions of the root environment, all affect this mechanism **(Crowley, 2006).**

According to Schenk et al. (2012), siderophores have been proposed as a safe substitute for harmful pesticides. Since it has been known for more than 30 years that certain Pseudomonas species can enhance plant growth by generating siderophores (pyoverdines) and/or shielding plants from pathogens, this bacterial genus has been referred to as "plant growth-promoting bacteria." In addition to pseudomonads, other bacteria, like Azadirachta indica, that make ferrioxamines may help plants

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get the iron they need and encourage the growth of their roots and shoots. In order to improve plant growth, mycorrhizal fungus can also be utilised as biofertilizers. It has been demonstrated that plants with mycorrhizal sorghum uptake greater amounts of Fe than nonmycorrhizal plants. It has been proposed that the role of fungal siderophores in ectomycorrhizal fungi relationships in plant nutrition. Aspergillus niger, Penicillium citrinum, and Trichoderma harzianum siderophores were discovered to lengthen the shoot and roots of chickpeas during a recent investigation on the plant growth-promoting effects of fungus..

b. Biocontrol of fish pathogens

By restricting Fe, which is crucial for virulence and bacterial interactions, siderophores play a significant part in the management of fish disease **(Li and Chi, 2004).** The pathogenic bacterium infects the fish host in two different ways: I by producing damaging enzymes like proteases and cholesterol acyl transferases to thwart the host's defensive mechanisms; and (ii) by producing transferrin to compete with the host for Fe and stunt its growth. The essential component of the biocontrol method is the competition between the siderophore produced by the biocontrol agents and the transferrin produced by pathogens to form complexes with iron. The siderophore always prevails because of its significantly higher Fe stability constants.

Fish farming uses siderophore-producing bacteria (Pseudomonas fluorescens) as probiotics because it can stop the growth of a number of fish pathogens, including Vibrio anguillarum, Vibrio ordalii, Aeromonas salmonicida, Lactococcus garvieae, Streptococcus iniae, Flavobacterium psychrophilum, and C. ruckeri. For instance, the Bacillus sp. strain NM 12 produced siderophores with a broad antibacterial spectrum that prevented the growth of 62.5 percent of the 363 intestinal bacteria found in the fishes' intestines. Additionally, it has been discovered that siderophores made by Bacillus cereus prevent the growth of the fish disease Aeromonas hydrophila. According to recent research **(Sugita et al., 2012),** a number of siderophore-producing bacteria isolated from fish intestinal tracts inhibit fish infections like Aliivibrio logei, Vibrio ichthyoenteri, V. anguillarum, Vibrio splendidus, and A. salmonicida.

c. Microbial ecology and taxonomy

Siderotyping is the process of classifying microbial strains based on the kinds of siderophores they produce. The analytical method and the biological method are the two different approaches of siderotyping. The physicochemical characteristics of siderophores are the foundation for the

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analytical techniques utilising high-performance liquid chromatography (HPLC) and mass spectrometry. The biological approaches, on the other hand, rely on the detection of particular DNA sequences linked to siderophores or on the direct measurement of siderophore-mediated Fe in the microbial cells **(Bach et al., 2000).** Along with a number of other siderophores, Pseudomonas produces more than 50 different forms of pyoverdine siderophores **(Cornelis, 2010)**. The fluorescent pyoverdine's peptide chain differs between species, and this variation makes it simple to ascertain how closely related different species are to one another. In a prior study, siderotyping was applied to 400 fluorescent and non-fluorescent Pseudomonas spp. strains, and these strains were divided into 28 taxa, including 15 clearly characterised species, based on the various siderophore types. Siderotyping was suggested as a useful technique for researching microbiological diversity and taxonomy since it allowed for the identification of 68 fluorescent Pseudomonas strains utilising mass spectrometry analysis of their pyoverdines. Based on the variation in the chemical structures of ornibactins and mycobactin, further research have discovered that siderophore synthesis can be utilised as a chemotaxonomic marker for the identification of other species of bacteria, such as Burkholderia spp. and Mycobacterium spp. Due to its ability to quickly and accurately identify microorganisms at the species level, siderotyping may prove to be an effective technique in environmental research **(Meyer et al., 2002).**

d. Bioremediation of environmental pollutants

Metals Metals are essential for the growth of human civilizations, however they are polluted due to the manufacturing sector, sludge applications, nuclear power plants, and mining **(Wasi et al., 2013).** A wide variety of metals, including Cd, Cu, Ni, Pb, Zn, and the actinides Th(IV), U(IV), and Pu, can be made more soluble and mobile using siderophores (IV). Siderophores can have strong affinities or selectivities for certain metals other than Fe depending on the stability constants of this metal-siderophore complex. This capacity of siderophores is mostly dependent on the functionalities of their ligands. As a result, siderophores can be used in bioremediation, a technology that is both affordable and environmentally beneficial **(Rajkumar et al., 2010).**

e. Nuclear fuel reprocessing

Because siderophores have anionic hydroxamate or catecholate functional groups, they can attach to Lewis acids to produce hard oxodonors with exceptionally high stability constants. It has been proposed that siderophores could bind actinides with a complexation constant estimated to be K = 1016 because actinides form potent complexes with hard oxygen anions. U and Pu are separated for reuse from fission products like Ti and Np using solvent extraction techniques in the Purex process,

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which has been used commercially to reprocess radioactive nuclear fuel. U and Pu flow into the solvent during this process and pick up Np contamination. Siderophores could be utilised in the Purex process to make the actinides removal process simpler because it has been demonstrated that they enable the selective removal of Np from the solvent phase. Desferrioxamine B, a ligand that has been suggested for actinide complexation, forms a stable complex with U(VI) where its hydroxamate functional group is comparable to acetohydroxamic acid **(Mullen et al., 2007).** According to a recent study by Marshall and colleagues (2010), employing synthetic desferrioxamine B or pyoverdine made by P. fluorescens did not appear to make a substantial difference in the possible influence siderophores could have on the disintegration of spent nuclear material. Siderophores have been suggested for the cleanup of radioactive waste and reprocessing of nuclear waste based on those findings..

f. Optical biosensor

In order to improve the signal to noise ratio and enable the detection of many sorts of responses through specially developed systems, a biosensor is a biomolecule coupled to an electrical device such as a transducer, amplifier, or noise filter **(Gupta et al., 2008)**. The properties of pyroverdines, which are yellow-green water-soluble fluorescent siderophores, are as follows: I They form a strong complex with Fe(III) and have a weak or negligible affinity for Fe(II); and (ii) The stability constants of the Fe(III) complexes are extremely high (roughly K = 1032). (Kurtz and Crouch, 1991). Pyoverdine is a promising substance for the development of optical biosensors due to these properties.

A siderophore was used as a biosensor to measure the concentration of Fe in the water **(Chung Chun Lam et al., 2006).** The parabactin generated by Paracoccus denitrificans was used as a biosensor in that work by being encapsulated in a sol-gel thin film on a quartz substrate. A flow cell installed in the sample partition of the fluorescence spectrometer was used to analyse the seawater samples. Additionally, siderophores offer the potential for an effective, sensitive, and focused detection method that would resemble the biological uptake procedure. A modified design that relies on the encapsulation of the azotobactin in sol-gel matrices without noticeably losing its fluorescence signal, for instance, has been used to use A. vinelandii's azotobactin as an optical biosensor for Fe(III). Additionally, the Fe biosensor pyoverdine's Fe(II and III) selectivity was improved by immobilising it in three different porous sol-gel glass formulations (A, B, and C), each of which included different levels of water **(Yoder and Kisaalita, 2011).** Pyoverdine immobilised in sol-gel C,

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which included more water than A and B, showed the most linear and specific response for binding to Fe(II and III) in that investigation.

g. Bio-bleaching of pulps

According to **Singh et al. (2008)**, the pulp and paper industry is a major contributor to a number of environmental issues, including acidification, nitrification, photochemical oxidation, global warming, human toxicity, ecotoxicity, and solid wastes. The bleaching procedures are the primary cause of issues in the pulp and paper producing industry. While some contaminants are released into the air, others are flushed into wastewater. Siderophores are considered to be efficient pulp treatment agents since they can cut the amount of chemicals required to bleach Kraft pulp by 70%, making them an environmentally friendly alternative. more prone to degradation and so aid in the bio-bleaching of pulps.

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Methodology

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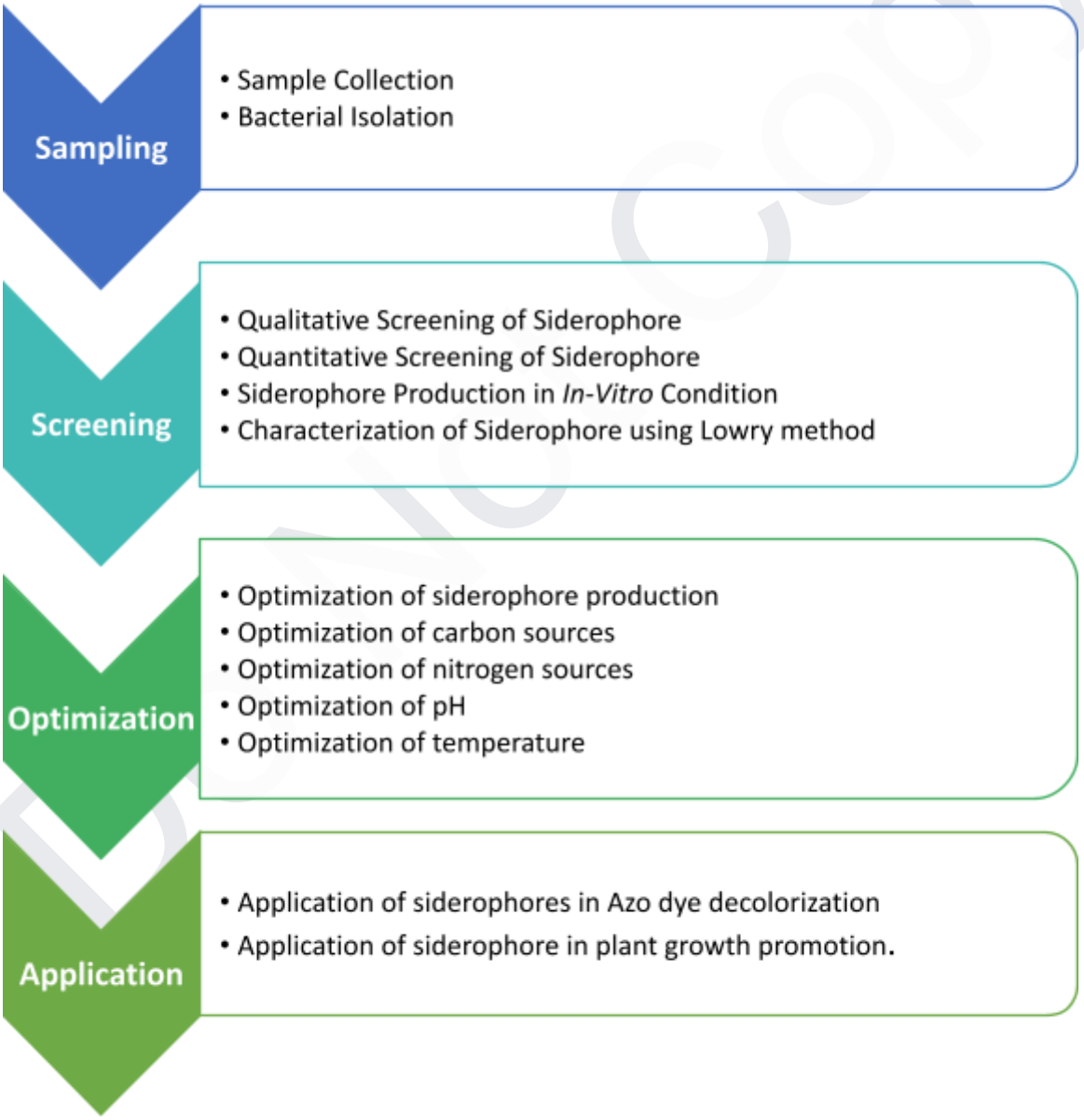
In the present study, Bacterial culture were collected and isolated. Qualitative and quantitative estimation of siderophore production was then examined. Optimization of Carbon source, Nitrogen source, Temperature, pH was also conducted. Application of siderophores in Azo dye decolorization was examined to investigate their application in plant growth promotion.

Methodology of Research Work



1. **Sample collection and bacterial isolation**

In the present study, bacterial samples collected from soil were isolated using microbiology techniques and cultured in the laboratory.

- **Bacterial strains**

The 23 siderophore-producing bacterial strains taken in the study belong to species amongst diverse genera including Pseudomonas, Rhizobium, Enterobacter, Chronobacter, Kosakonia, Beijerinckia, and Pantoea. All of these bacterial genera and species are well-known PGPR and common inhabitants of rhizo- sphere.

- **Bacterial culture**

Isolated bacterial culture were collected from Rhizosphere Microbiology Laboratory, Department of Environmental Science, Gautam Buddha University, Greater Noida, India. Total 23 Siderophore-producing bacterial strains were isolated to investigate the plant growth promoting rhizobacteria (PGPR). Bacterial strains were cultivated for 48 hours on Luria–Bertani (LB) agar media (Himedia). All strains were kept at 4 ° C in LB slants and at - 80 ° C in a 25% glycerol stock solution.

2. **Qualitative Screening of Siderophore**

The ability of bacterial strains to produce siderophores was tested using a universal CAS assay (**M. Kannahi *et al.*, 2014)**. Glassware was cleaned with 3 mol/l hydrochloric acid (HCl) to eliminate iron before the experiment and then washed in deionized water. The CAS reagent was made according to Schwyn and Neilands' instructions (1987). In a nutshell, 121 mg of CAS was dissolved in 100 ml pure water and 20 ml of a 1 mM ferric chloride (FeCl3-6H2O) solution produced in 10 mM HCl. Under stirring, this solution was added to a 20 mL hexadecyl trimethyl ammonium bromide (HDTMA) solution. 729 mg HDTMA was mixed with 400 mL distilled water to make the HDTMA solution. Before being used again, the CASHDTMA solution was sterilised.The modified Hu and Xu methodology was used to do qualitative siderophore screening (2011). 100 mL CAS reagent was mixed with 900 mL sterilised LB agar medium to make CAS agar plates. Each plate was injected with four different bacterial strains. As a control, an uninoculated plate was used. Plates were inoculated and incubated for 5– 7 days at 28 ° C, looking for the formation of an orange zone surrounding the bacterial colonies (Louden et al. 2011).

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3. Quantitative Screening of Siderophore

The supernatant of bacterial cultures grown in LB broth medium was used to estimate siderophore quantitatively (**Arora *et al.*, 2017).** For this, 10 ll of freshly produced bacterial culture (108 colony forming units (cfu) per ml) were inoculated with 1 ml broth in 1.5 ml centrifuge tubes (Thomas Scientific, US) (one for each bacterial culture). For each strain, four replicates (tubes) were taken. Apart from that, a control tube (without infected broth) was kept. Bacterial cultures were centrifuged at 10,000 rpm for 10 minutes after being incubated at 28 ° C for 48 hours, the cell pellets were discarded, and the supernatant was utilised to calculate siderophore. Each bacterial culture's supernatant (0.5 ml) was mixed with 0.5 ml CAS reagent, and optical density was measured at 630 nm (Spectrophotometer) after 20 minutes. The amount of siderophore produced by strains was quantified in percent siderophore unit (psu), which was derived using the formula: (Ar-As) 100/Ar = siderophore production (psu)

{where Ar represents the absorbance of the reference (CAS solution and uninoculated broth) and As represents the absorbance of the sample (CAS solution and cell-free supernatant of sample}.

4. Optimization of Siderophore production

In order to achieve maximum siderophores production, the bacteria isolates were allowed to grow in a diversity of fermentation parameters, including pH, temperature, nitrogen supply, carbon source, iron concentration, and organic acid. The isolates were cultivated for 48 hours in succinate medium under various fermentation conditions. The supernatant was centrifuged at 5000 rpm for 10 minutes for siderophores analysis, and the cell-free supernatant was tested using the CAS assay test. At 630 nm, siderophore production was measured and computed (**A.E. Fazary *et al.*, 2016).**

5. Total protein were also estimated by Lowry Method.

Total cell protein was isolated and estimated by Lowry method. Blank, Standard and Test samples were Pipette out a series of test tubes. The volume to 1 ml in all the test tubes was made. A tube with 1 ml of water serves as the blank. 5 ml of reagent C to each tube including the blank is added and mixed well and allow to stand for 10 min. OD was taken at 570nm (**Eric Larson *et al.*, 1986**).

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6. Siderophore Production under submerged culture conditions.

For 48 hours, siderophores were cultured at room temperature. A bacterial culture was obtained from garden soil and used for the experiment. After that, the culture was inoculated for 48 hours on agar medium (Fig).

7. Azo dye decolorization Assay

Azo dye experiment was performed to examine the efficacy of siderophores in treating pollutants and improving the fertility of soil. 3mg Solid Azo Dye was dissolved in 100ml of distilled water. 30ppm conc and 50ppm conc. was maintained in 100 ml nutrient broth. 0.1ml of isolated bacterial culture was inoculated and kept at room temperature 24h. After 24 h colour intensity of reference and sample was checked at 663nm **(Khan *et al.*, 2010).**

8. Application of siderophore in plant growth promotion.

To examine the efficacy of PGPR in plant growth and soil fertility, 0.25kg of compost was weighed and placed in two beaker. Both the beakers were autoclaves and checked for decontamination. Then 100ml distilled water was added in the control beaker and 100ml + Bacterial inoculates were added and mixed. The mixture was then placed in two sterilized pots with corn seeds for 15 days.

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Result & Discussion

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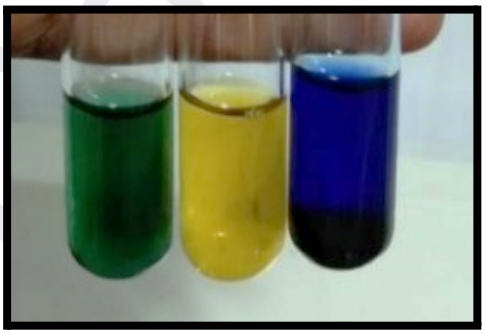
A diverse group of bacteria known as plant growth-promoting rhizobacteria (PGPR) is associated to many plant tissues. Through a variety of processes, such as the generation of siderophores, they can promote plant development, improving the plant's nutrition and protecting it from phytopathogens. Many bacteria have siderophores, which are a high-affinity method for absorbing iron from the environment. This system involves iron mobilisation through competitive complexation or dissolution of iron-bearing molecules, biosynthesis, exudation in the extracellular space, recognition and uptake of ferric siderophore complexes by highly efficient transport systems, or iron liberation from the siderophore complex and uptake of iron. In the present study the use of Siderophore for PGPR as biofertilizers or biocontrol agents constitutes a good alternative to support an eco-friendly and sustainable agricultural crop production.

Fig. 5.Experimental setup to conduct the Study

A. Qualitative Estimation of Siderophore production

From the soil sample, a total of 29 samples were extracted and pure cultivated. By using CAS assay, the different siderophore-producing bacterial isolates were eliminated (both qualitatively and quantitatively). The cultures were cultivated for 48 hours in broth medium, and then the supernatant was removed and subjected to a spectrophotometric analysis for the CAS assay test. Two of the five bacterial isolates showed turbidity in the medium and had positive CAS results. By plating the bacterial isolates using the CAS agar plate method, the presence of siderophores was further established. It was discovered that the bacterial isolates had distinct orange zones that appeared to be producing siderophores (Fig. 1). Siderophore formation is plainly seen from the orange zone appearance. Siderophore formation is plainly seen from the orange zone appearance. Similar outcomes were reported by Ghosh et al., who produced siderophores using the bacterial strains Bacillus subtilis-1, B. megatericus 1, and Pseudomonas aeroginosa1 and the fungal strains Trichoderma viride-1, T. harzianum-1, and Candida famata-1 [23]. When iron chelators like siderophores are added to the medium, they remove the iron from the dye complex, causing the colour to gradually change from blue to orange. The iron chelators, such CAS or HDTMA, form a tight complex with the ferric ion to create a blue colour in the medium.

It was seen that an orange-colored zone formed surrounding the bacterial colonies, indicating that certain bacterial strains were producing siderophores. All of the bacterial strains included in the study tested positive for siderophore, it was found.



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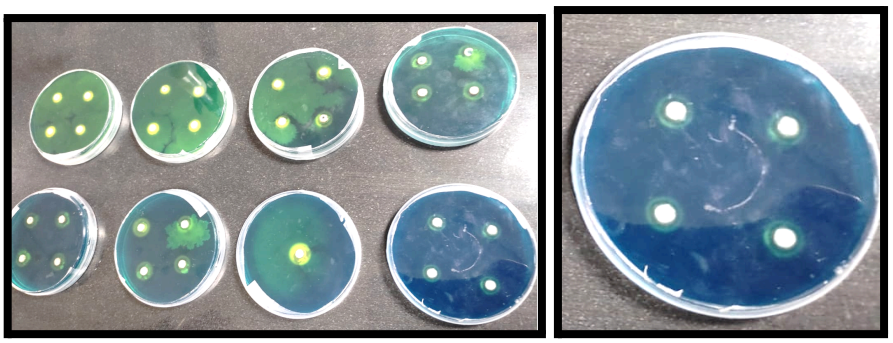

Figure 6. a Sample showing the formation of siderophores as compared to the reference.
b. Formation of orange colorurged zone around the bacterial colonies was observed which indicated siderophore production by bacterial strains.

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2. Quantitative Estimation of Siderophore production

a. Liquid culture media and CAS reagent are used to quantitatively estimate siderophore. The cell-free supernatant (0.5 ml) is taken for spectrophotometric measurement in a cuvette following growth in the conventional way. However, in the suggested procedure, only 100 ul of the supernatant was placed onto the microtiter plate wells. In contrast to the traditional method, which took 0.5 ml of CAS reagent per tube, the microplate method only required 100 ul. As a result, the quantity of reagents and broth utilised was drastically reduced. In fact, the amount of CAS reagent needed has been reduced by 80%, and the amount of broth utilised has decreased by 50%. After incubation and centrifugation to get cell-free supernatant, our calculations reveal a 88% of siderophore generated (Fig.2). To assess the effectiveness of the suggested and conventional approaches, the siderophore production rates of all 23 strains were tested and compared. Both for the conventional and modified microplate methods, the absorbance of the reference (Ar) or control (uninoculated broth and CAS reagent) was noticeably similar. When measured using the conventional approach, the concentration of siderophore generated by bacterial strains 88 psu, while when quantified using the suggested microplate method. These results indicates the Siderophore production efficacy of bacterial spices selected in the experiments.

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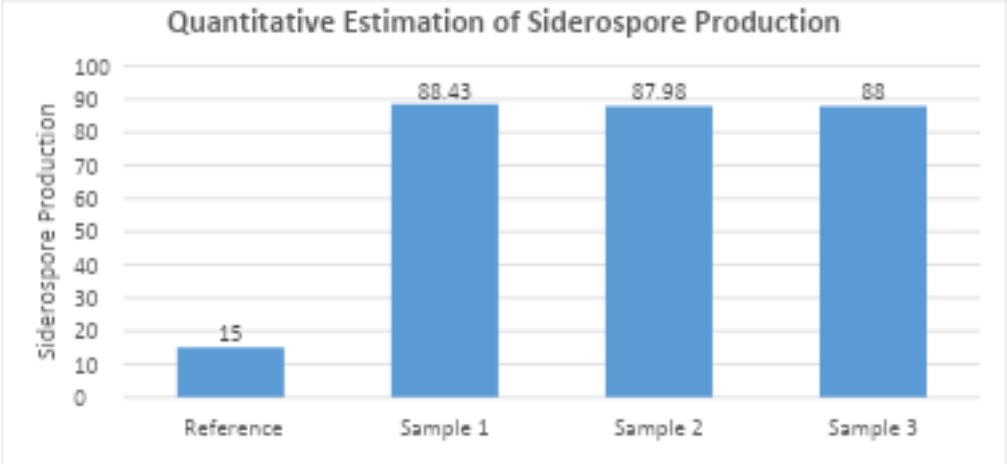

Reference	**15**	**1.764**
Sample 1	88.43	0.204
Sample 2	87.98	0.212
Sample 3	88	0.206

Figure 7. Quantitative Estimation of Siderophore production, Bar graph represents the PSU of total Siderophore production, results calculated from the absorbance of reference and samples.

b. Total protein were also estimated by Lowey Method.

Total cell protein estimation assay indicated the presence high conc. of protein when compared with standard solution. As the results indicated in Table.

	O.D	**Protein Conc.**
Blank	00.00	
Standard	0.494	
Sample	0.295	29.85g/dl

3. Siderophore Production under submerged culture conditions.

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Siderophores were cultured at room temperature for 48h. To perform the experiment bacterial culture was isolated from garden soil. The culture was then inoculated on agar medium for 48h (Fig). Bacterial siderophores were find to grow with 48h of inoculation.

Figure 8. Siderophore Production under submerged culture conditions for 48 h.

4. Optimization of Siderophore Production

Optimization was carried out in order to comprehend the important relationship between different culture conditions and bacterial growth and siderophore production. Although iron is the primary component in the synthesis of siderophores, other aspects of culture conditions also have a substantial impact on siderophore production. circumstances such varying pH, temperature, carbon source, nitrogen source, etc. are taken into account.

a. Optimization of Carbon Source

The ideal carbon source circumstances under which the bacterial isolate produces the siderophore were examined. The ability of bacteria to grow and produce siderophores is increased by adding carbon sources to the growth media. We have chosen glucose, fructose, sucrose, and maltose as the main four carbon sources as a result. When affected by a carbon source like sucrose, as seen in Fig. 3, all of the bacterial isolates produced siderophores in increased concentrations. In the presence of sucrose, a significantly greater siderophore synthesis of 80 % was observed. The majority of siderophore-producing bacteria are classified as Bacilli sp. according to results of earlier research of bacillus species [28], [29].

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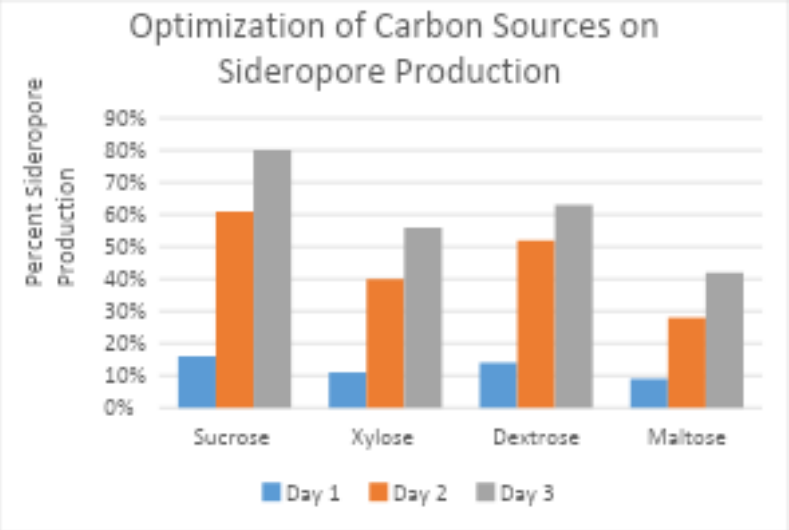
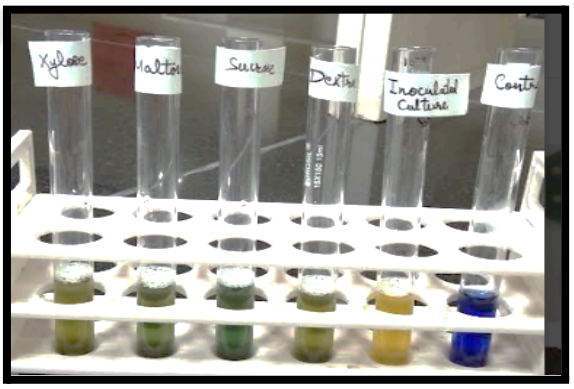


Carbon Source	Day 1	Day 2	Day 3
Sucrose	16%	61%	80%
Xylose	11%	40%	56%
Dextrose	14%	52%	63%
Maltose	9%	28%	42%

Figure 9. Optimization of Carbon Sources on Siderophore Production



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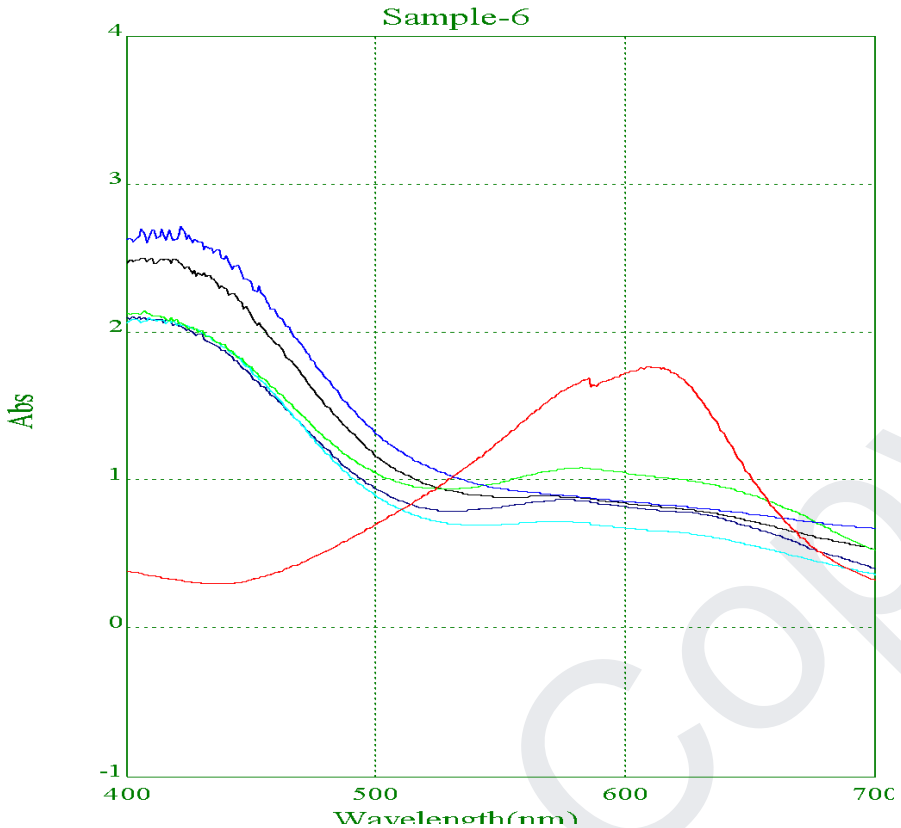
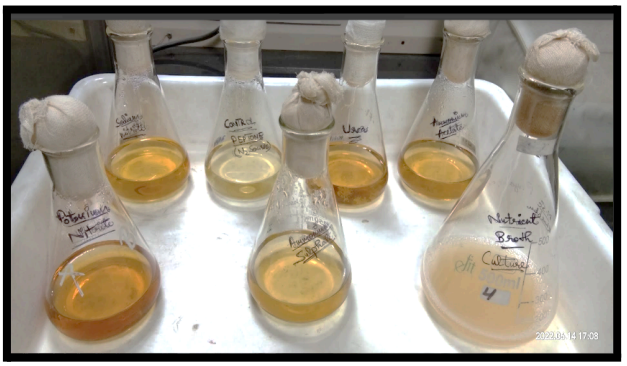


Figure 10. UV-Visible spectrophotometer reading graph of carbon sources indicating Sucrose as the highest carbon producing source.

b. Optimization of Nitrogen Source

Ammonium sulphate, Ammonium acetate, Potassium Nitrate, sodium nitrate, and urea were used as different sources of nitrogen during the optimization process. All bacterial isolates produced siderophore at a considerable rate in all nitrogen sources. The outcomes were comparable to the siderophore synthesis by isolates of Rhodotorula sp. under various nitrogen sources [10]. In the presence of Sodium nitrate, bacterial culture displayed their highest levels of (73%) siderophore generation on third day [16], [24]. Sodium nitrate was considered as the highest siderophores producing nitrogen source for further experiment (Fig11.)



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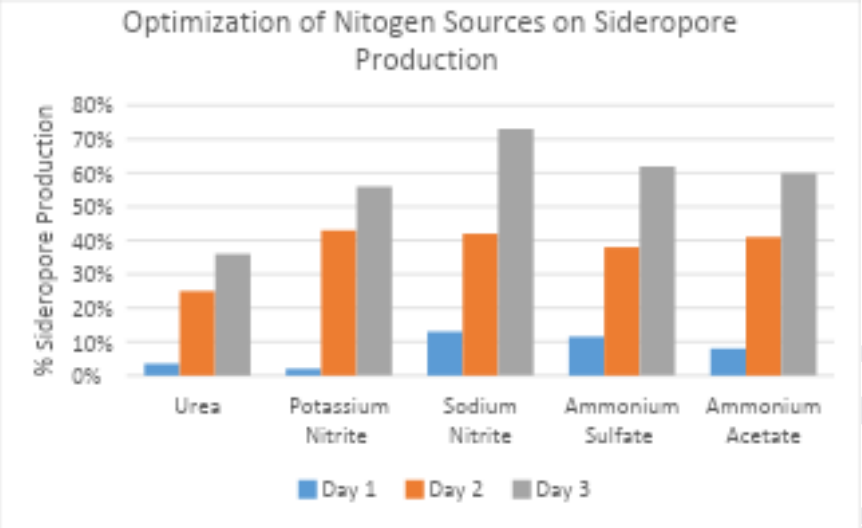

Nitrogen Source	Day 1	Day 2	Day 3
Urea	4%	25%	36%
Potassium Nitrite	2%	43%	56%
Sodium Nitrite	13%	42%	73%
Ammonium Sulfate	12%	38%	62%
Ammonium Acetate	8%	41%	60%

Figure 11. Optimization of Nitrogen Sources on Siderophore Production

c. Optimization of pH

The impact of pH on microbial growth is important. The pH of the medium affects both the solubility of iron and its accessibility to growing bacteria. Given that iron is insoluble at neutral pH and that bacteria can grow best in a physiological environment, it was observed that the highest generation of siderophore occurs at pH 8 **Fig**. According to our research, a pH of 8 triggers the highest generation of iron chelators and bacterial isolates exhibit higher siderophore production concentrations at pH 8 than the siderophore-producing bacteria Rhizobacteria and Pseudomonas, which are active in promoting plant development and stress tolerance [24, 26]. Similar findings were also reported by Calvente et al. [10].

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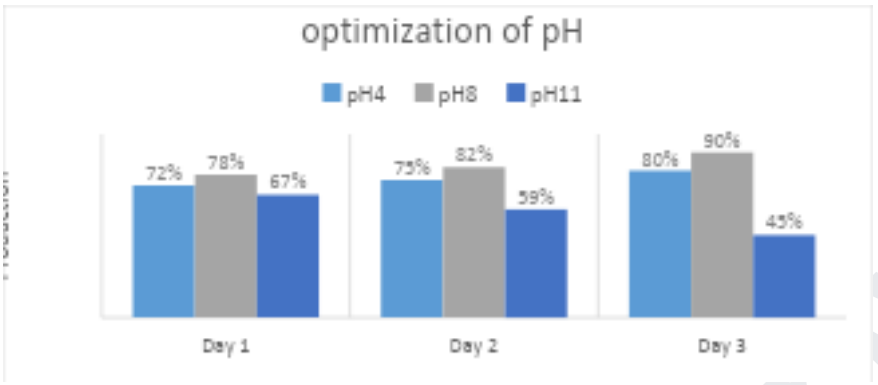

pH	Day 1	Day 2	Day 3
pH4	72%	75%	80%
pH8	78%	82%	90%
pH11	67%	59%	45%

Figure 12. Optimization of pH on Percent Siderospore Production

a. Optimization of Temperature

Additionally, a steady pH and variable temperatures were added to the variation in culture conditions. The bacterial isolate displayed greater siderophore concentration at 37°C, according to other investigations on siderophore production and optimization using the Plackett–Burman method **[27].** When compared to the findings of the prior investigation, bacterial isolates produced siderophores in high concentrations across the board at all temperatures (90 percent of siderophores) **(Fig. 4).** 37°C demonstrates optimal siderophore synthesis for both strains, even though room temperature may be the best temperature for microbial development.

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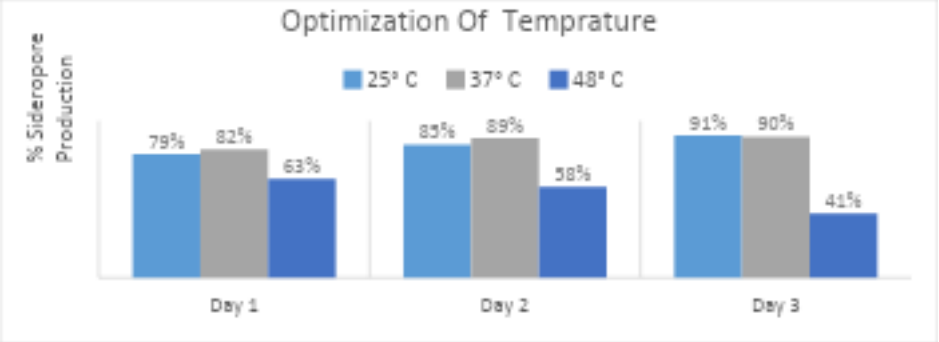

Temprature	Day 1	Day 2	Day 3
25° C	79%	85%	91%
37° C	82%	89%	90%
48° C	63%	58%	41%

Figure 13. Optimization of Temprature on Percent Siderospore Production

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4. Azo Dye Decolourization assay

Environmental application of siderophore producing microbes was examines by azo dye decolourization assay. Results suggested that bacterial siderophores significantly reduce the pollutant and improve the biodegradability, soil fertility and nutrient cycle. Azo Dye decolourization assay shows that, 26% to 37% and 18% to 22% dye was decolourized in 30ppm and 50ppm conc. respectively within 24h. These results indicate the efficacy and efficiency of siderophores in the application of plant growth.

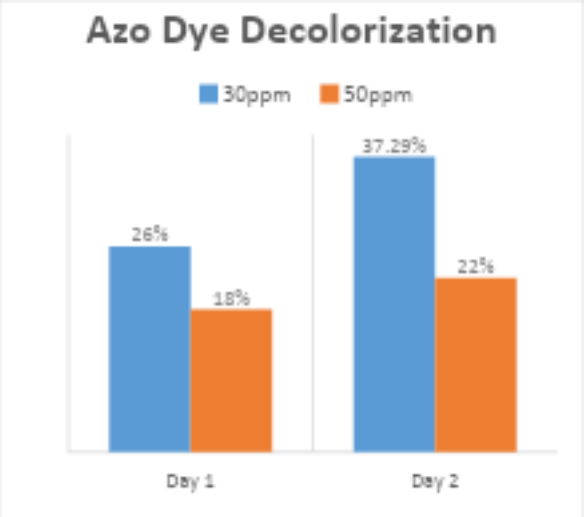
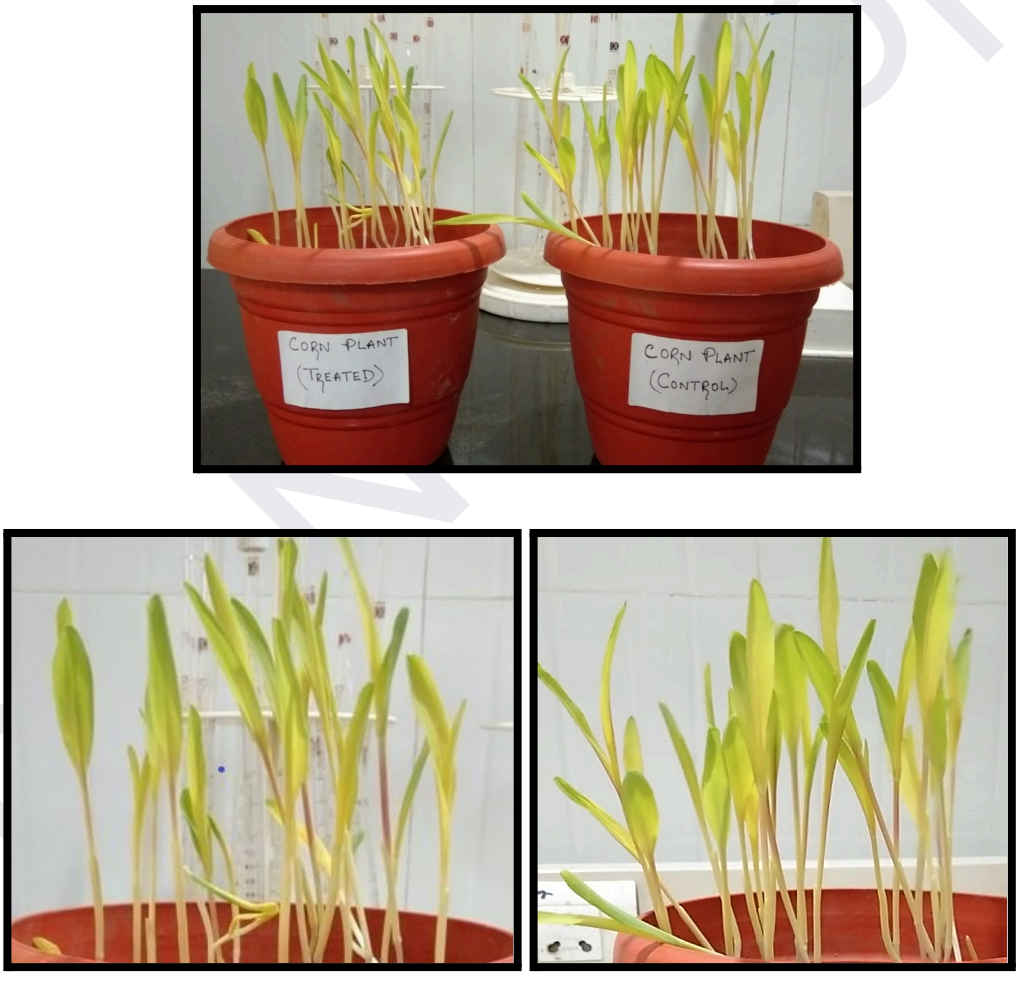


Figure 14. Azo Dye Decolourization Assay indicated decolourizing efficacy of bacterial species.

5. Application of Siderophore in Plant Growth and Bio fertilization of the soil.

Zea mays or corn plant were seeded in controlled and treated condition. Treated soil was inoculated with siderophore producing bacteria. After 15 days the plants were growth and examined. It was observed that plant treated with bacterial isolates showed higher plant as compared to the control plants. However the treated plant shown higher productivity and fertility (Figure. 14). These results confirms the application of Siderophore producing bacteria in sustainable crop production. However, further research is required to validate these results on molecular level.



Treated **Controlled**

Figure 15. Plant Growth under treated (Bacterial inoculates) and controlled condition.

Summary

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Conclusions

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High output yields, increased crop production, and soil fertility are required in today's world in order to produce in an environmentally sustainable way. Therefore, research must focus the novel idea of "rhizoengineering,". As a vital element, iron is needed by all the living organisms from unicellular to multicellular for their numerous cellular processes. Microorganisms under iron-deficient conditions produce siderophores, low molecular weight chelators that trap iron molecules from the atmosphere, host etc., for their survival. The characterization of siderophore production by CAS assay test showed positive which was confirmed by qualitative and quantitaively CAS agar plate test. The appearance orange color and halo zone formation confirmed that the bacterial isolate had the ability to producing siderophores. Further, these preliminary results paved an idea on proceeding with optimization parameter analysis where the bacterial isolates were allowed to grow in different culture conditions such as pH, temperature, carbon source, nitrogen source, organic acid and iron concentration. Results of the influence of different culture conditions showed that these bacterial isolate had the efficiency of producing siderophore in higher concentration at pH 8, at 37°C, with glucose and sucrose as carbon source, azo dye assay depicted the application of siderophore in bioremediation and plant growth. After a certain concentration, an increase in iron concentration decreased siderophore production, which may be related to the negative transcriptional control of genes involved in siderophore production. The increase in iron concentration increased the production of siderophore. This study supports the significance of siderophores in crop yield and bioremediation, the manufacture and purification of the siderophore from the bacterial isolate as well as its use in diverse domains, however, require more investigation.

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