



# ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF PGPR'S FROM PLANT ROOT SOIL.

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## Abstract:

Bacteria that colonize plant roots and promote plant growth and their yield are referred to as plant growth promoting rhizobacteria (PGPR). In this regard soil sample was collected from rhizospheric and non-rhizospheric region of a potted plant and preserved in polythene bag, later serial dilution was performed and incubated on LB media. For identification and characterization of bacterial species, combinations of DNA barcoding, biochemical tests- ammonia test, KOH test, organic acid test, catalase test, methyl red and voges-proskauer test, oxidase test and Sanger sequencing was performed. In this study, we have targeted universal gene 16srRNA for sequencing. The obtained species were identified as *Achromobacter xylosoxidans* 17SIN-B2, *Brevibacillus brevis* strain JZY2-32, *Bacillus subtilis* subsp. *Subtilis* strain WXZP9, *Serratia* sp. XT-1 and *Achromobacter xylosoxidans* strain MT3, which are PGPR bacteria help in growth of plant.

**keywords:** biochemical tests, DNA barcoding, Gram's staining, Sanger sequencing, 16s rRNA.

## Introduction:

The thin layer of soil surrounding plant roots is an important and active area for root activity and metabolism which is known as rhizosphere. Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as plant growth-promoting rhizobacteria (PGPR) (Verma *et al.*, 2010). Plant growth enhanced by PGPR is measured as an increase in seedling emergence, biomass, proliferation of root system and yield in various plant species (kloepper *et al.*, 1991; Vessey, 2003; Zahir *et al.*, 2004; Ping and Boland, 2004). PGPR has gained importance as agriculturally beneficial bacteria and can be used as an alternative way in place of chemical fertilizer, pesticides etc (Vejan *et al.*, 2016). ISR (induced systemic resistance) were found to be non-infected parts of previously pathogen-infected plants that become more resistant to further infection (Gupta *et al.*, 2015). PGPR increase availability of nutrients through solubilization of unavailable forms of nutrients and by the production of siderophores aiding in facilitating nutrient transport. And even phosphorous, organic acids and phosphates are made available showing symbiotic relationship between PGPR and plant (Xiao *et al.*, 2017).

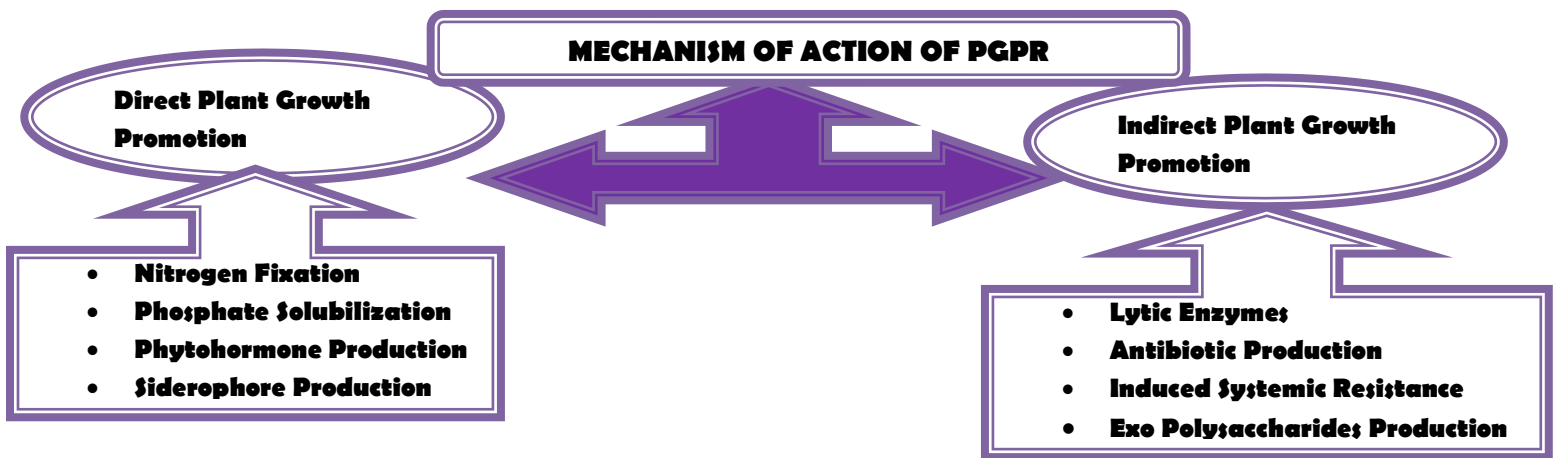


Fig 1: Showing mechanism of action of PGPR's.

In the present study, PGPR samples were isolated from plant root soil, serial dilution was performed to isolate pure colonies. Gram's staining was performed for microscopic characterization and biochemical tests such as ammonia test, KOH test, organic acid test, catalase test, methyl red, Voges-proskauer test and oxidase tests were conducted to check the properties and genus of obtained colonies. DNA was isolated and 16S rRNA sequencing was performed using Sanger method followed by data analysis.

## Materials and methods:

### Collection of soil sample:

Rhizosphere soil sample were collected by digging 13cm deep surrounding the roots of a potted plant in polythene bag and stored at 4°C.

### Serial dilution:

6 fold dilution was performed were 1gram of rhizospheric soil was taken into a vial containing 1000µl of sterile H<sub>2</sub>O and shaken this can be initial dilution 10<sup>-1</sup>. From this mixture 100µl was transferred to next dilution factor of 10<sup>-2</sup> same process is then repeated for the remaining tubes, taking 100µl from the previous tube and adding it to the subsequent 900µl diluents. The dilution was wrapped to 10<sup>-1</sup> to 10<sup>-6</sup>.

### Isolation of PGPR:

An aliquot (0.1ml) of 10<sup>-4</sup> and 10<sup>-2</sup> diluted suspension were spread on LB agar plates and also the obtained pure cultures were accustomed perform morphological, microscopic and molecular characterization.

## Morphological and microscopic identification of PGPR:

Cell morphology was observed thoroughly by understanding nature of colonies obtained. Gram staining method was performed to differentiate the bacteria into Gram Positive and Gram Negative, which further helps in selecting the strains for molecular characterization.

## Biochemical characterization of PGPR:

### Ammonia test:

The culture samples were inoculated in peptone water, incubated the tubes for 48hrs at 30°C. Nessler's reagent (0.5ml) was added to every tube and was observed for formation of brown to yellow color.

### KOH test:

3% KOH was added on slide with bacteria sample, mixed, left for 1min and observed for thread like mass appearance.

### Organic acid test:

Inoculate bacteria in minimal salt medium i.e., MM9 broth for two to three days at 30°C. When methyl red was added as an indicator, turning to pink color indicates positive for the test.

### Catalase test:

A drop of hydrogen peroxide was placed on the slide and therefore the bacteria colony was smeared on peroxide, production of bubbles or froth indicated the test as positive.

### Methyl red and Voges-proskauer test (MR-VP):

MR-VP broth was taken in tubes and bacteria was inoculated incubating the tubes for 48hrs. Followed by incubation, the tubes were vortexed and poured half of the broth into another clean tube to perform MR-VP tests separately. To one part of tubes a drop or two of pH indicator methyl red was added and swirled and if turns red it's positive. To another part of tubes Barritt's 1/A reagent and Barritt's 2/B reagent was added, shaken vigorously and waited for 1 or 2 hrs. The tubes turning pink or red indicates positive of VP test.

### Oxidase test:

Oxidase disc were taken and therefore the colony were gently rubbed on the disc, if there's appearance of purple or blue color within 30secs it's positive for the test.

### DNA isolation, purification and quantification:

Sample was enamored with CTAB extraction buffer, mixed and vortex. Centrifuged at 14,000 X g for 5min. cold isopropanol was added and incubated at -20°C(15mins). Transferred to silica based DNA column spin at 12,000 rpm(1min). 2 rounds of washes were performed using wash buffer and spinned at 12,000 rpm for 1min. Elution buffer was added and spun at 12,000 rpm for 1min to collect fresh DNA. RNAase was added and incubated at 37°C for 30mins. DNA thus obtained was mixed with bromophenol blue and loaded on gel to visualise the bands under UV light.

### 16S rRNA PCR:

PCR methodology was used to amplify partial gene region of 16S rRNA gene. PCR reaction and program was set using 27F and 1492R primers. Amplifications was dispensed in 25µl reaction mixture by adding 10µM forward and reverse primer, 10µM dNTPs, 0.5µl Taq DNA polymerase, 2.5µl of 2X buffer and approximately 40ng of plant genomic DNA. Initial denaturation (95°C) for 5mins, final denaturation for 30secs, annealing (55°C) for 30secs, elongation (72°C) for 1min, repeated for 30 cycles, final elongation (10mins), held at 4°C. The obtained PCR products were run on gel and eluted to urge pure variety of template for sequencing.

### Gel purification:

Gel DNA bands were cut and solubilized in buffer by heating at 55°C. 200µl of isopropanol was added and transferred to DNA column spinning at 12,000 rpm for 1min. Wash buffer was added and spinned at 12,000 rpm for 1min, followed by dry spin for 2mins. 2µl of elution buffer was added and spinned at 12,000 rpm for 1min, using this DNA as template for Sanger sequencing.

### Sanger sequencing:

It had been performed to spot the nucleotide sequence of the DNA using automated sequencing machine. It is also called as chain termination and automatic method having all ddNTPs, where in each dNTP incorporates a unique fluorescent label ddNTPs lacking 3'-OH group required for phosphodiester bond formation and hence extension ceases on binding. The results of chain-termination PCR is millions to billions of oligonucleotides copies of the DNA sequence of interest, terminated at a random lengths by 5'-ddNTPs. All nucleotide fragments are run in capillary gel electrophoresis within the sequencing machine. The output data obtained is electropherogram which shows a florescent peak of every nucleotide along the length of the template. Further it is converted to other formats for data analysis. The results were compared at NCBI site using BLAST server.

### Data analysis:

Sequencing files are obtained in .ABI format which can be viewed using software FinchTV. Quality of sequence was observed through electropherogram peaks and obtained FASTA file was analyzed for the presence of nucleotide pattern and performed blast for further analysis. From the blast, the unknown sequence was predicted supported the share similarity and e-value. The obtained DNA sequences were aligned with top hits from BLAST in Clustal Omega to construct Phylogenetic tree.

### Results and discussion:



Fig 2: Soil sample taken for study



Fig 3: Showing performed  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  dilutions.

Soil sample was collected from the rhizosphere region surrounding the roots of the plant (fig2) by digging 14cm deep and serial dilution was performed to get  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  dilutions (fig 3).



Fig 4: Growth of bacterial colonies.



Fig 5: Isolation of pure colonies-PR1, PR2, PR3, PR4, PR5, PR6.

More bacterial colonies were observed on  $10^{-2}$  dilution plate when compared to  $10^{-4}$  dilution plate (fig4). Six unique colonies were selected from 2 different concentration plates (fig5) and inoculated them on fresh LB media to obtain their pure colonies by streak plate method.

Table 1: Table showing the morphological characterization of bacteria colonies

Sl No.	Code	Size	Shape	Texture	Color	Gram staining	shape
1	PR1	Small	circular	smooth	white	-ve	Bacillus
2	PR2	Large	rhizoid	smooth	white	+ve	clostridium
3	PR3	Pinhead	circular	shiny	white	+ve	bacillus
4	PR4	Small	irregular	rough	white	-ve	bacillus
5	PR5	Small	punctiform	shiny	white	+ve	Bacillus
6	PR6	Medium	Irregular with hypae	rough	white	-ve	Bacillus
7	PR7	Medium	irregular	rough	white	+ve	clostridium
8	PR8	Medium	Filamentous	rough	white	+ve	clostridium

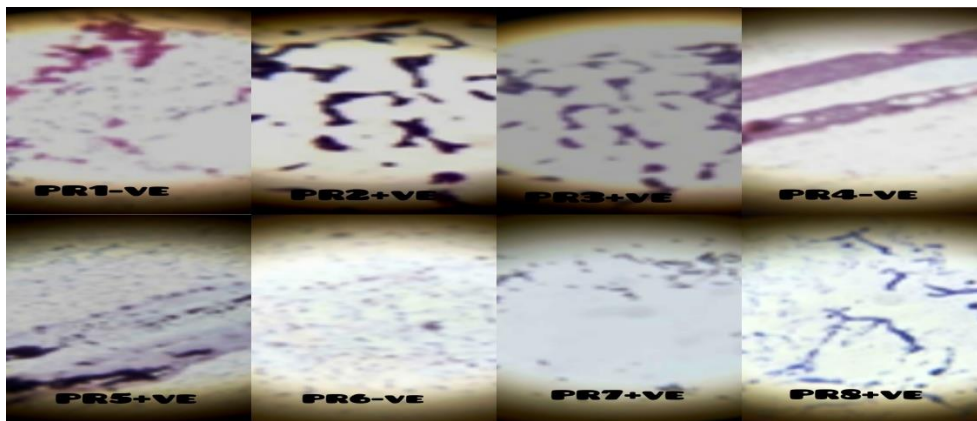


Fig 6: Gram's staining results of all the 8 bacterial colonies.

5 Gram positive (purple) and 3 were Gram negative (pink). They were classified based on shape that is bacillus or rod shaped bacteria, clostridium are rod-shaped in chain bacteria.

PR1	PR2	PR3	PR4	PR5	PR6
-ve	-ve	-ve	+ve	-ve	-ve



Fig 7: Showing results of Ammonia test

Table 2: Result table of catalase test.

Formation of brown to yellow color indicated positivity of results. Here, PR4 showed positive result, whereas PR1, PR2, PR3, PR5 and PR6 were found negative. Hence 1 positive and 5 negative results were obtained for the ammonia test.

PR1	PR2	PR3	PR4	PR5	PR6
+ve	+ve	+ve	+ve	+ve	+ve

Table 3: Result table of KOH test.

Formation of thread like mass indicated of positivity of results. All samples PR1,PR2,PR3,PR4,PR5,PR6 were found to be positive for the KOH test by forming thread like masses.

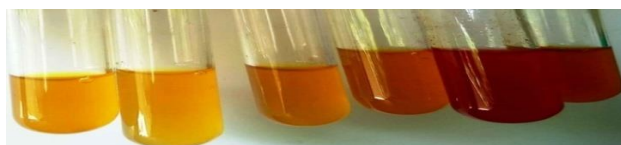
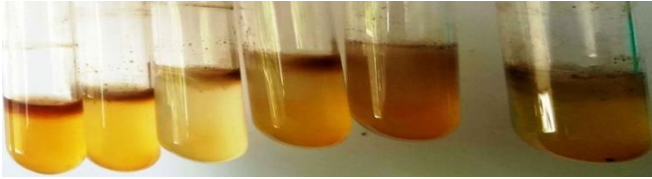


Fig 9: Showing color indication of strains in MR test.

PR1	PR2	PR3	PR4	PR5	PR6
-ve	+ve	-ve	-ve	-ve	-ve

**Table 6: Result table of MR test.**

Appearance of red color indicated positivity of results. 1 positive (PR2) and 4 negative (PR1,PR3,PR4,PR5,PR6) results were obtained for MR test by the pink color formation.

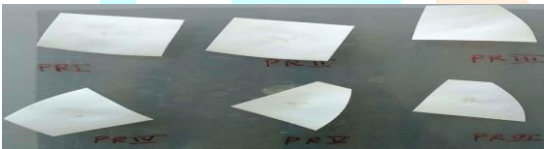


**Fig 10: Showing color indication of strains in VP Test.**

PR1	PR2	PR3	PR4	PR5	PR6
-ve	-ve	+ve	+ve	-ve	+ve

**Table 7: Result table of VP Test.**

Appearance of brown color ring junction indicated positivity of results. 3 negative (PR1,PR2,PR5) and 3 positive (PR3,PR4,PR6) results were obtained for the VP test as shown in above fig 10.



**Fig 11: Results of Oxidase test.**

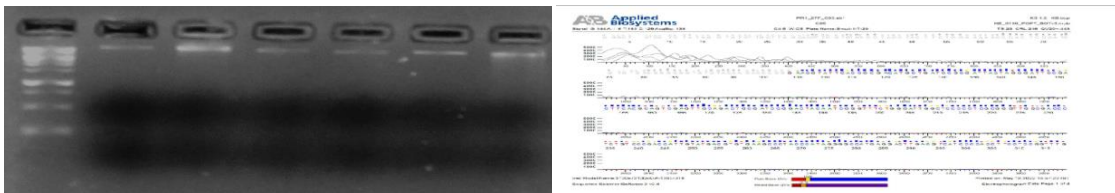
PR1	PR2	PR3	PR4	PR5	PR6
+ve	-ve	+ve	-ve	+ve	+ve

**Table 8: Result table of oxidase test.**

Oxidase disc should turn red to confirm the oxidation process of bacterial strains. PR2, PR4 were negative as there was no color change after few minutes, where as PR1, PR3, PR5, PR6 were positive as the disc had turned into red spot after the application on inoculums on disc and leaving it for few minutes.

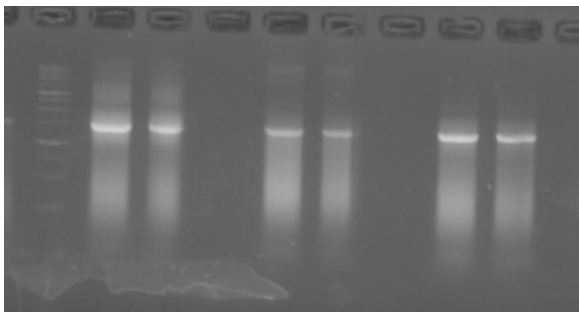
Biochemical tests	PR1	PR2	PR3	PR4	PR5	PR6
Ammonia test	-ve	-ve	-ve	+ve	-ve	-ve
KOH test	+ve	+ve	+ve	+ve	+ve	+ve
MR test	-ve	+ve	-ve	-ve	-ve	-ve
VP test	-ve	-ve	+ve	+ve	-ve	+ve
Oxidase test	+ve	-ve	+ve	-ve	+ve	+ve

**Table 9: Representing overall Biochemical test profile of samples.**

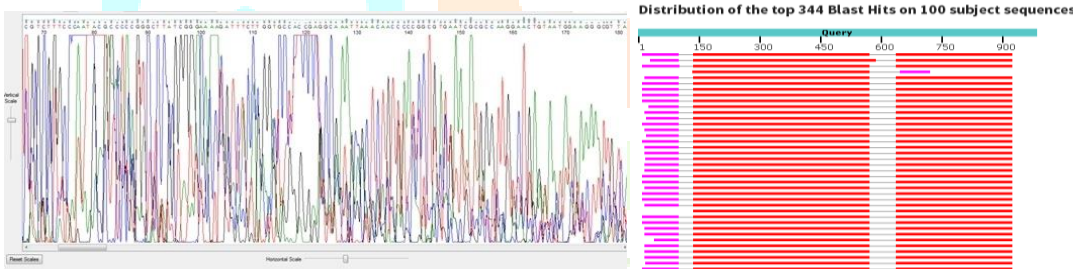


**Fig 12: Genomic DNA Bands appeared on gel under UV light. Fig 13: Samples obtained in AB1 format.**

The dye make the bands visible under UV light (fig 12) and this makes easy to cut the PCR products as gel bands (fig 14). The results are obtained in the form of AB1 format, which was then converted using software (fig 15).



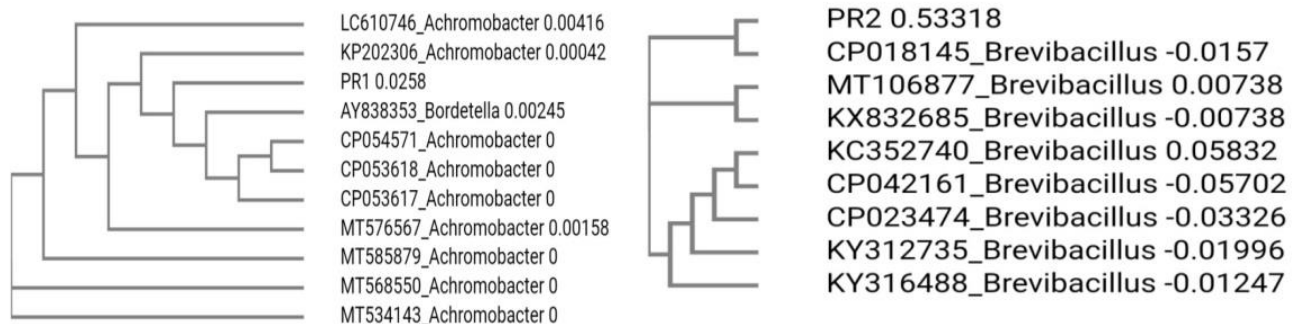
**Fig 14: 16s gene amplification using PCR process.**



**Fig 15 : Electropherogram peaks. Fig 16: Graphical summary of BLAST**

Quality of the obtained sequence were observed through Electropherogram peaks (fig 15). BLAST was performed to produces a graphical summary of the sequence with their similar sequence (fig 16).

**Phylogenetic tree representing the evolutionary of bacteria.**



**Fig 17: PR1 Fig 18: PR2.**



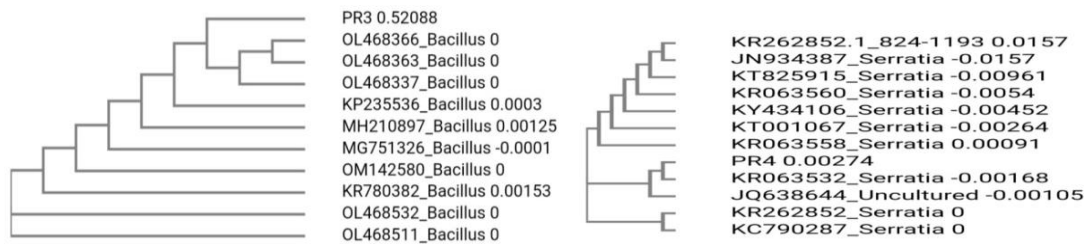


Fig 19: PR3 Fig 20: PR4

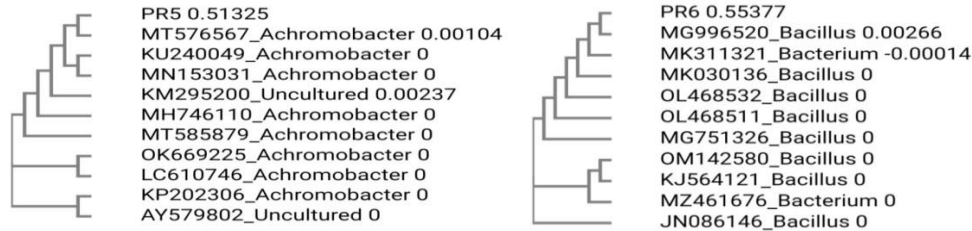


Fig 21: PR5. Fig 22: PR6.

Sample	Hit	Query coverage	E value	Percentage identity	Accession number
PR1	<i>Achromobacter xylosoxidans</i> 17SIN-B2 gene for 16S rRNA, partial sequence.	82%	0.0	95.19%	LC610746.1
PR2	<i>Brevibacillus brevis</i> strain JZY2-32 16S ribosomal RNA gene, partial sequence	96%	0.0	97.68%	MT106877.1
PR3	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> strain WXZP9 16S ribosomal RNA gene, partial sequence.	54%	0.0	96.66%	OL468532.1
PR4	<i>Serratia</i> sp. XT1 16S ribosomal RNA gene, partial sequence.	99%	0.0	99.89%	KR063532.1
PR5	<i>Achromobacter xylosoxidans</i> strain MT3 16S ribosomal RNA gene, partial sequence	84%	5E-164	95.20%	OK669225.1
PR6	<i>Bacillus subtilis</i> subsp. <i>Subtilis</i> strain WXZP9 16S ribosomal RNA gene, partial sequence	77%	0.0	96.66%	OL468532.1

Table 9: predicted Hits from individual gene sequences using NCBI BLAST.

For all the 6 samples their top similar hits, query coverage, E Value, percentage identity, accession number which was obtained through performing BLAST. Direct and indirect mechanisms showed that PGPR's could even be a stronger alternative for the chemical fertilizers utilized in agriculture accustomed kill pathogens, pests and weeds which cause harmful impact on the ecosystem (Gupta *et al.*, 2015). Soybean and alfalfa plants growing in several soil types using deep Illumina 16S rRNA sequencing. The rhizomicrobiome encompasses a pronounced influence on plant growth, so the rhizomicrobiome assemblage and plant. Few scientists have collected soil from single source and studied about differing types of PGPR bacteria present in them (Xiao *et al.*, 2017). The influence of 4 PGPR strains on the degradation of 5 soil applied pesticides and their effects on bacterial growth were studied. The author here introduced the known strains of PGPR to review their effects (Myresiotis *et al.*, 2012). Bacterial treatments significantly affected all parameters tested. The sole result was obtained from 637Ca treatment, which significantly increased fruit yield, number and weight, compared to manage (Ipek *et al.*, 2014). Tryptophan addition is additionally a competent source for increasing potential of PGPR, thereby improving wheat growth and physiology. Same methods were performed explaining inoculation of bacteria on LB media after serial dilution, DNA extraction, PCR amplification, sequencing for 16S rRNA and analysis by Hassan *et al.*, 2015). *Achromobacter xylosoxidans* bacterium has the potential to boost plant growth under a high-salinity conditions as a PGPR. *Brevibacillus brevis* exerts beneficial effects through combined modes of actions, including PGP traits like phosphate solubilization, IAA production, Acetylene reduction and anti-fungal activity. It also accelerates the expansion and development of the plant, enhancing root function and also has capacity to survive higher temperatures. *Bacillus subtilis* strain assist plants to endure from stress. They also secrete exopolysaccharides and siderophores that inhibit the movement of toxic ions and help maintain ionic balance, additionally because of the uptake of water by roots. These compounds also inhibit pathogenic microbial populations. *Serratia* restores membrane integrity by minimizing oxidative damages and promotes plant growth under various levels of salt stress. It's also effective to suppress the fungal pathogens and address biotic stress responses.

## Conclusion:

PGPR are free living soil bacteria, that can be isolated from the rhizosphere, when it applied as seed or crop inoculation, to push growth and yield by several mechanism e.g., phytohormones production, provide available nitrogen, available phosphorous etc. In our study the soil sample was collected, serial diluted and plated  $10^{-2}$ ,  $10^{-4}$  concentrations on LB agar plates. Unique bacterial colonies were identified and characterized them supported their morphology and microscopic (Gram staining). Biochemical tests such as ammonia test, KOH test, organic appraisal, catalase test, methyl red, voges-proskauer test and oxidase test were performed. DNA was isolated, PCR amplification, 16S rRNA sequencing and Sanger sequencing was performed. The results were obtained in .AB1 format analysis of results was performed by BLAST and a phylogenetic tree was constructed. 6 rhizobacteria strains were predicted as *Achromobacter xylosoxidans* 17SIN-B2, *Brevibacillus brevis* strain JZY2-32, *Bacillus subtilis* subsp. Subtilis strain WXZP9, *Serratia* sp. XT-1 and *Achromobacter xylosoxidans* strain MT3 that are proved to be having background in improving the growth and yield of the plant being PGPR- plant growth promoting Rhizosphores.

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