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PHOSPHATE SOLUBILIZING BACTERIA AND ITS APPLICATION

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Abstract:
Phosphate solubilizing bacteria are PGPRs which are usually present in very less concentration in the soil (in ppm) but they have a very crucial role to be played in making the inorganic phosphate available in the organic form so that the plants can absorb it improve their growth and yield. This project includes comparison between qualitative and quantitative analysis of the phosphate solubilization of 4 strains- *Bacillus subtilis*, *Pseudomonas putida*, *Pseudomonas fluorescens andRhizobia* and checking which will produce the maximum improvement in the growth of plants as biofertilizers if applied. Qualitative and quantitative assays are performed in order to check which strain has best phosphate solubilizing ability among the above-mentioned bacteria. HPLC analysis for organic acid produced and the plant hormone synthesized by the bacteria were also performed. This gives a much clearer idea of the entire process of how these biofertilisers work. Further extension of the study is identifying the phosphate solubilizing bacterial strain that has the capacity to resist the heavy metal accumulation which was carried out by plate assay and microtitre plate.

Keywords: Phosphate Solubilization Bacteria, High Pressure Liquid Chromatography, Biofertilizer, Heavy metal resistance, Plant growth promoting bacteria.

Introduction:

Phosphate is the second most abundant element after nitrogen. The macro elements are very important for the growth of plants. Especially phosphate has vital role in metabolism and physiology of plants. It is vital for meristem growth, hormone production like IAA production [*Lakshmi priya D., Ramya Anandan, Rajendran P.,*] and entire plant's growth overall. It is usually present in tri-calcium phosphate form but this form of phosphate is not soluble and is inorganic. Thus, it is not available for the plant's uptake. Phosphate is very important element and plays a very crucial role in the plant's growth and development hence its presence in soil is important but it should be in the organic form for the plant's absorption. Iron chelation, organic acid production also plays important role in the phosphate solubilization process. More the organic acid production, lesser the pH and more the phosphate solubilization. These PGPRs play various other roles like nitrogen fixation, prevention from pathogens, other growth promoting substances like siderophores. This is where the microbes play a role. They have a very critical role in phosphate cycle. They convert the inorganic phosphate

to organic form which is in water soluble form thus can be absorbed by plant's roots. Some of the microbes which have this unique property are *Azatobacter, Rhizobia, Pseudomonas, Azospirillum* [*Pacôme A. Noumavo, EméricKochoni, 2013*]. Usually, phosphate is present in hydrogen phosphate, hydrogen diphosphate. Anions get precipitated along with cations like Fe2+, Ca2+, etc. Hence, are immobilized. The anion forms of phosphate are insoluble in water. Phosphate can be released from organic compounds in soil by three groups of enzymes:

a) Non-specific phosphates
b) Phytases
c) Phosphatases and lyases

There are certain other chemicals that are synthesized by plants that support Plant Growth Promoters Ribosomes (PGPR) that have a role to be played in the solubilization of these insoluble phosphates. Some of the strains are responsible for heavy metal removal from soil. For eg.*Pseudomonas putida* and *Bacillus safensis* Strain. It has been implemented for removal of nickel in dhapa industrial area which is a wasteland in Kolkata, India. The PGPRs are the microbes which thrive in the rhizosphere region and make the phosphate present in the soil available to the plants for their survival. These PGPRs are the ones that are responsible for converting the insoluble form of phosphates to soluble forms present in the rhizospere (near the root areas where high density of microbes thrives). One of the major hormones produced by plants that is responsible for the growth of PGPRs is IAA (Indole Acetic Acid). Unfortunately, due to the pollution caused by manmade activities these are either completely absent or present in negligible number. Since the soil is no longer able to support the PGPR the conversion of phosphate from inorganic form to organic form does not take place. The survival of microbes in rhizosphere also depends on root morphology, root exudates and physical and chemical characteristics of the soil.

Few decades back, since the level of urbanization and modernization was taking place at a very nominal rate as compared to the present scenario, the soil was at a much better and healthier state than today. The PGPRs were present in the soil thus, the phosphate availability to the plant was sufficient for their growth and development. Unlike today, when one of the major deficiencies observed in plants is phosphate deficiency which needs to be taken care of at the earliest. A good number of heavy metals are observed as pollutant in soil [*Tanoy Mukherjee, Avijit Ghosh and Santanu Maitra, 2014*]. This is the need of the hour. Since, a lot of cereals and other important plants require phosphate for their survival eg.Maize [*A.Gholami, S. Shahsavani, and S. Nezarat 2009*], soyabean, sesame, wheat, lettuce, rice, pepper, etc. Hence, this is the thought process that made me take up this as the project. This is the unique point and the significance of this entire effort.

There are a number of PGPRS that have been isolated, characterized and identified. Some of the most common strains of microbes are bacillus (*B.subtilis), pseudomonas (P.putida, P.flluorescens), azospirillium, azatobacter, rhizobia*, etc. There have been reports in the past where the application of phosphate solubilizing bacteria have brought about great results in the growth of plants .Eg. Wheat yield has increased due to inoculation of Azatobacter strain in the rhizosphere region and also rice.

The basic mechanism by which the PGPR increases the growth of plant is through number of ways and not understood thoroughly. The major roles of PGPRs that have been reported are as follows: [*BS Saharan, V Nehra, 2011*]

1. PGPRs have the ability to produce phytohormones
2. They also play role in AsymbioticNitrogn fixation against phytopathogenic microbes. Thus, in turn preventing the plants from diseases and increasing the life span of the crops.
3. They produce siderophores [*Ste´phaneCompant, Brion Duffy, et. al, 2005*]
4. They also synthesize certain antibiotics, thus improving the immunity of the plant.
5. In certain cases, they also produce certain vital enzymes
6. There are examples of certain PGPRs which produce fungicidal compounds
7. One of their major functionalities is to solubilize mineral phosphates and also other nutrients.
8. The phosphate solubilizing bacteria also increases the plant and crop yield.
9. PGPR are also responsible for the production of HCN.

10. Some of the strains of rhizobia are known to produce plant hormones [*Tarun Sharma, Nishant Rai, 2014-2015*].
11. PGPR also have been reported to reduce salinity and osmotic stress by altering phytohormones and antioxidants as shown in *Cucumis sativus* [*Sang-Mo Kang, Abdul Latif Khan, et. al, 2014*]

Mechanism:

Since this process of solubilization is not a simple one step production method, it requires a good amount of literature review and in-depth knowledge about plant metabolism. This process is a complex one and includes a great number of steps and sub-steps. This is not an isolated process that happens in an isolated manner on the contrary it takes place with a number of other vital hormone production processes. Phosphates solubilization and phosphate mobilization are two different terms and processes. Phosphate solubilization is making the inorganic phosphate present in the soil available for the plants to absorb. Phosphate mobilization is related to it assimilation. As mentioned earlier the anionic forms of phosphate are deposited along with certain cationic elements thus making the phosphate immobile and static. Thus, concentrated in one area of soil. These terms are inter-related but not interchangeable! Former is related to transformation and later is related to availability. But still for the simplicity of understanding I have concentrated only on the solubilization process alone and considered only those cycles which are closely linked to it [*BS Saharan, V Nehra, 2011*].

So, the general scheme of the solubilization is:

The plants produce tryptophan which are released from the rhizosphere

Bacteria that are present in the soil produce tryptophase (An enzyme that digests tryptophan).

This reaction produces IAA as one of the products

These IAA are important for the microbes to thrive

These microbes (PGPRs) convert the inorganic, anionic form of phosphates to organic forms (soluble form).

These organic are now available for the plants to absorb and assimilate.
Thus, the plant growth and development are enhanced [*MuneesAhemad , Mulugeta Kibret , 2013*] along with the phosphate solubilization there are 2 other important factors pH and the organic acid production.
The general flowchart goes like this PGPR increase the seed emergence hence causes increase in the synthesis of hormones like gibberellins (enzymes responsible for promoting early germination). It enhances the seedling vigor due to increased production of auxin. Hence PGPR plays has 3 important functions – one, nitrogen fixation ability, two, phosphate solubilizing capacity and third, phytohormone production.

Since one of the PGPRs major role includes production of plant hormones, HPLC analysis is included in order to quantify and characterize them. Through the phosphate solubilization qualitative assay it has been observed that the halo-zone formation is due to the production of organic acid. Thus, characterization of these acids is also significant and will help in the further studies.

Materials and Methods:

The pure culture of *Rhizobium*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *bacillus subtilis* were collected from NCIM (National Collection of Industrial Microorganism, Pune) and MTCC (Microbial Type Culture Collection – Gene Bank). *Pseudomonas* species and *Bacillus subtilis* were sub-cultured in incubator for 24 hours in Nutrient agar and broth whereas *Rhizobium* was cultured in rhizobium media at 30'C for 48 hours.

Phosphate solubilisation assay was carried both quantitative and qualitatively. For the qualitative assay Pikovskaya media was chosen which has TCA (tri- calcium) and 0.1gm/lt Bromocresol purple was added. It is a pH indicator which turns yellow in acidic medium and purple in basic medium. It also makes the Halo-Zone formation very evident. The pH of the media was checked and was found to be alkaline. After incubation for a week at room temperature the plates were inoculated with 10µL of culture at the centre of the petri-plate.

Quantitative phosphate analysis was carried out using the same Pikovskaya media followed by addition of sucrose (5gm/lt) as carbon and energy source. The broth was kept for incubation for 2 weeks in Arbitary shaker at 150rpm at room temperature. Then 1mL of the media was taken and centrifuged at 10,000 rpm for 10min. The pellet was discarded and the supernatant was subjected to pass through 0.2µm filter for filter sterilization. 0.5mL of supernatant was used for HPLC analysis to determine the organic acid produced by the microbe and the remaining 0.5mL was stored at -40'C for further analysis.

Results and Discussion:

There are a lot of significant conclusions which can be drawn from the results obtained. The pure cultures of *Rhizobium* and *Pseudomonas putida* were collected from NCIM (National Collection of Industrial Microorganism, Pune) and *Bacillus subtilis* and *Pseudomonas fluorescens* were collected from MTCC (Microbial Type Culture Collection – Gene Bank). The varied morphology can be observed of the four microbes that we have chosen for our study. Phosphate solubilization can be analysed qualitatively which is observed in fig1. Pikovskaya media is used with Bromocresol Purple for clear observation of the halo-zone formation. Since, bromocresol purple is pH indicator, in the acidic pH it turns yellow/orange while in alkaline pH it turns purple. Hence, orange color formation depicts the acidic pH near the phosphate solubilization zone. According to the qualitative analysis, maximum halo-zone formation is observed in *Bacillus subtilis* followed by *Rhizobium*. Quantitative analysis was also performed in order to check if any correlation is obtained between the qualitative and quantitative assays. According to the O.D. value obtained from the Microtiter plates analysis (Table1), - Standard graph of Phosphate solubilization was plotted. Using the equation obtained, phosphate solubilization for each of the microbe was calculated. Hence, we can conclude that Rhizobium is the best phosphate solubiliser (Table2: Quantitative assay result), followed by *Bacillus subtilis, Pseudomonas fluorescens* and *Pseudomonas putida*. This discrepancy between the qualitative and quantitative has been reported by many authors. Relatively, quantitative results are more authentic and accurate.

Different organisms produce varied organic acid during the phosphate solubilization process. These organic acids have a vital role to be played in the growth and development of plants. The organic acids that we have chosen for our study includes Aconitic acid, Ascorbic acid, Citric acid, Mallic acid, Fumaric acid, Oxalic acid and Tartaric acid. HPLC analysis was done in order to characterize and quantify the amount of the mentioned acids produced by these organisms. Media was taken as control and the peaks obtained in it were not considered for measurement of the samples. From Table: 1.3 we observe that *Bacillus subtilis* produces maximum of Aconitic acid i.e 0.42ppm and minimum

by *Pseudomonas fluorescens* , $7.2*10^{-3}$ ppm. Citric acid is produced maximum by *Rhizobium* and is not produced at all by *Pseudomonas fluorescens* and *Bacillus subtilis.* Bacillus produces highest amount of Ascorbic acid (5.6ppm) and Malic acid (54). *P.putida* produced maximum amount of oxalic acid (3.3ppm) and Tartaric acid is generated in highest amount by *Rhizobium*. Hence, we can conclude from the above results that the microbes showed maximum resistance to lead and nickel and are highly sensitive to mercury and Cadmium. Maximum phosphate solubilization is carried out by *Rhizobium* and least by *Pseudomonas putida.* Maximum heavy metal resistance was shown by *Pseudomonas fluorescens*.

Study should also be done on measuring the number of Organic acids produced by the microbes in the presence of Heavy metals. These microbes can be co-inoculated after checking the antagonist effects. It is of prime importance to check the two microbes that we are applying has any disastrous effect on the other if so then the entire effort will be futile. Hence, a good detailed study is required in the microbe's inoculation and its effect. Co-inoculation [*Badawi, F. Sh F., A. M. M. Biomy,*et. al, 2011] will produce ideal result if the chosen microbes produce symbiotic effect i.e. one involved in phosphate solubilization and the other displaying the heavy metal resistance property. Hence further study has to be done in order to check if *Rhizobium* and *Pseudomonas fluorescens* can be co-inoculated.

The field study of the control plant (non-inoculated) should be done against the sample plant (inoculated) and their yield and growth can be compared to check the effect of these Plant Growth Promoting Rhizobacteria (PGPR) on the growth and biomass. Form the HPLC results it is very evident that *Bacillus subtilis* produces the greatest number of organic acids i.e Aconitic acid, Ascorbic acid and Malic acid in the highest quantity compared to the rest of the organisms. Followed by *Rhizobium*, which produced two different organic acids and the rest 2 species produced one of each acid in the maximum amount. Citric acid was not produced by *Pseudomonas fluorescens* and *Bacillus*. *Pseudomonas putida* produced Oxalic acid in maximum quantity and Tartaric acid was produced by *Pseudomonas fluorescens* in the highest amount. Further detailed study is required so as to discover the reason behind the same. Hence the focus of the future venture should be to theoretically and experimentally observe the feasibility of co-inoculation of these species of PGPRs and check the effect it has on the growth and yield of the plants.

Thus, on a large scale this can act as bio-fertilizer and enhance the cultivation of crops and its yield.

Fig.1.Phosphate Solubilisation – Qualitative Assay

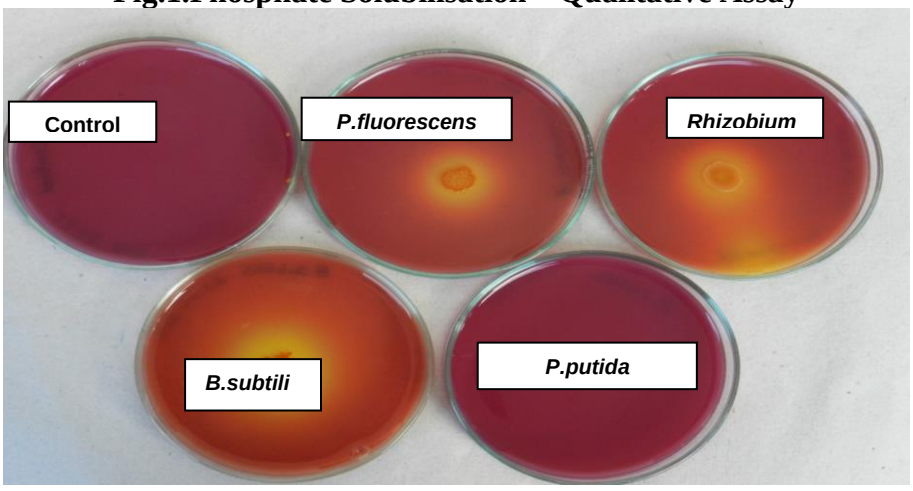

Table1.1: Qualitative Assay – Halo-Zone Readings

Microbes	**Colony diameter (cm)**	**Entire Halo-Zone diameter (cm)**	**Solubilization Index (SI)**
Pseudomonas fluorescens	1.00	1.30	0.70
Bacillus subtilis	1.20	1.50	0.80
Pseudomonas putida	1.00	No zone	-
Rhizobium	1.00	1.40	0.71

Table1.2: Quantitative assay result

Microbes	**O.D.**	**Phosphate Solubilized (µM)**
Rhizobium	2.122	10.771
Bacillus subtilis	1.699	8.606
Pseudomonas fluorescens	1.582	8.008
Pseudomonas putida	0.938	4.712

Table 1.3: HPLC analysis of Organic acid produced by microbes

S.No.	**Organic Acid**	***Rhizobium***	***Pseudomonas putida***	***Pseudomonas fluorescens***	***Bacillus subtilis***
1.	Aconitic Acid (ppm)	0.01	0.02	$7.2*10^{-3}$	**0.42**
2.	Citric Acid (ppm)	**433.80**	50.55	-	-
3.	Ascorbic Acid (ppm)	2.05	4.30	1.30	**5.60**
4.	Malic Acid (ppm)	21.00	41.00	42.00	**54.00**
5.	Fumaric Acid (ppm)	**1.45**	0.60	0.90	0.10
6.	Oxalic Acid (ppm)	1.50	**3.30**	1.50	1.30
7.	Tartaric Acid (ppm)	**18.90**	4.00	5.00	2.00

Table 1.4: Organic acid with respective Retention time in minutes

S.No.	**Organic Acid**	**Retention Time (min)**
1.	Aconitic Acid	25.947
2.	Citric Acid	14.162
3.	Ascorbic Acid	9.111
4.	Malic Acid	8.544
5.	Fumaric Acid	17.714
6.	Oxalic Acid	8.900
7.	Tartaric Acid	7.572

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