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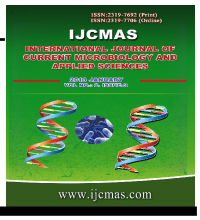

Original Research Article

Evaluation of Indole-3-Acetic Acid in phosphate solubilizing Microbes isolated from Rhizosphere soil

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Phosphate Solubilizing; Microbes; Tri Calcium Phosphate; Salkowski reagent; TLC.

A B S T R A C T

Phosphorous is one of the major nutrients needed to dry plants and micro organisms for performing in crop fields. Among micro organisms several bacterial species posses the ability to solubilize tri calcium phosphate. From the Rhizosphere soil many species of phosphate solubilizing bacteria like *Bacillus, Pseudomonous* sp , *Xanthomonas* sp. were isolated and fungi like *Aspergillus niger, Aspergillus flavus , Aspergillus fumigatus and Penicillium* spp. were isolated. Then the PSM solubilizing effects were studied in pikovsyakas medium. Then the PSM isolates were subjected to production of IAA in pikovskayas broth *in-vitro* condition by adding L- tryptophan as a substrate. Then pH range of *Pseudomonous* 3sp .was initially at 6.8 and finally decreased to 4.63 then produce more Indole acetic acid. The pH range of *Aspergillus niger* was initially at 6.8and finally reduced to 4.29 and produce more Indole acetic acid in Fungi. Then finally the IAA was observed in thin layer Chromatography. There is increase evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing phosphorous.

Introduction

Phosphorus is one of the essential mineral macronutrients, which are required for maximum yield of agriculturally important crops. Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated as a consequence of regular applications of phosphate fertilizers (Richardson, 1994).

However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dey, 1988; Yadav and Dadarwal, 1997). Farmers are thus asked to apply phosphorus fertilizers in

several-fold excess in order to overcome this problem. Therefore, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability.

Plant root-associated phosphate solubilizing bacteria (PSB) have been considered as one of the possible alternatives for inorganic phosphate fertilizers for promoting plant growth and yield (de- Freitas *et al.,* 1997; Rodriguez and Fraga, 1999; Richardson, 2001; Vessey, 2003; Thakuria *et al.,* 2004). Seed or soil inoculation with PSB is known to improve the solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yield (Yahya and Al-Azawi, 1989; Abd-Alla, 1994; Mehta and Nautiyal, 2001). In fact, PSB render more phosphates into the soluble form than required for their growth and metabolism by secreting organic acids and/or enzymes (*e. g.* phosphatases), the surplus get the plants (Vessey, 2003). The interest in PSB has increased due to the prospective use of efficient strains as bio-inoculant (biofertilizer) components in organic agriculture, which is emerging as an alternative to chemical inputs in intensive agriculture (Ryder *et al.,* 1994; Bashan and Holguin, 1998). However, their root colonization, persistence and performance in the rhizosphere are severely affected by environmental factors, especially under stressful soil conditions.

Micro organisms plays an important role for transformation of phosphorous in water and sediments and the phosphate ions are reported strongly absorbed by sediments with a high content of slit and clay (Seshadri *et al*., 2002). Phosphate solubilizers were isolated based on the halozones produced around the colonies (Seshadri, 1995). Unfortunately, phosphorous is one of the least available and the least mobile mineral nutrient for plants in the soil (Takahashi and Anwar, 2007). Among PSM various species of soil bacteria, fungi and mycorrhizae have been reported to be involved in this bio-conversion. The organic acids secreted by PSM seem to be involved in phosphate solubilization (Subba Rao, 1982). The decomposing root also release phosphorous as a result of autolysis, directly in to the soil solution mainly as inorganic orthophosphates. The ultimate results of this also in phosphorous fixation (Martin and Cunnighamun, 1973).

Materials and Methods

Collection of Sample

The Rhizospheric soil samples from the plants were collected in and around Kalavai, Vellore district, Tamil Nadu in different agricultural lands like sugarcane, groundnut and paddy fields. The collected samples were transferred in a polythene bag aseptically and it was transported to the laboratory.

Isolation of phosphate solubilizing microorganism

The collected rhizosphere soil samples were serially diluted in the sterile distilled water and plated on Pikovskaya's medium. The plates were incubated at 28^{o}C up to 7 days. The clear zone around the microbial colonies in the plates shows the phosphate solubilization. Then it was isolated and maintained in a slant was used for further study.

Identification of fungi and bacteria

For the identification of fungi lactophenol cotton blue and slide culture technique was performed and structures were observed under microscope .The Characters of each isolates were differentiated based upon the colony morphology and spores presentation. From the microscope observation the fungi like *Aspergillus niger, Aspergillus fumigatus, Penicillium* spp. were isolated Gram straining and Biochemical methods were done for the identification of bacteria. From the above methods *Pseudomonous* spp, *Xanthomonas* sp., *Bacillus* sp. were isolated.

Zone formation in PKA medium in addition of tricalcium phosphate

100ml of Pikovskaya's agar medium with 250mg of tri calcium phosphate was prepared. 1ml of sample is incubated in the plates separately. The plates were incubated for upto 7days at 28^{o} to 37^{o}C. The colonies were show clear zone was measured.

Phosphate estimation method

The amount of phosphorous present in the isolates were determined by Subba Rao and Fiske method. 1ml of sample was taken in two test tubes and its volume was made upto 8.6ml with distilled water.1ml of ammonium molybdate was added to the tubes and vortexed. The color intensity was read out after 10mts in samples at 660nm. Concentration of phosphorous in sample was calculated (Subba Rao *et al* ., 1966).

Phosphate solubilization by Qualitative method

All the suspected colonies from PKA medium were subjected to spot inoculative individually at the centre of PKA medium. The plates were incubated at 28^{o}C-37^{o}C upto 7 days. The diameter of clear zone. Was measured as follows, the phosphate solubilization efficiency (PSE) is the ratio of total diameter (i.e) clearance zone including bacterial growth (Z) and the colony diameter (C) multiply by 100. PSE=(Z-C)/CX100.

Phosphate solubilization by Quantitative method

100ml of Pikovskaya's broth medium with 250mg of Tricalcium phosphate was prepared and stored 1ml of each isolates were inoculated into the broth medium separately. Then the inoculated samples were incubated for 14 days, on the rotary shaker at 37^{o}C. After incubation culture broth was centrifuged at 10000 rpm for 30mts, pH of all isolates were measured.

Production of indole acetic acid *in-vitro* conditions

Five days old culture of phosphate solubilizing organisms both bacteria and fungi were transferred separately into Pikovskaya's broth containing L-tryptophan as a substrate for the production of Indole acetic acid, Inoculated cultures were kept in a rotary shaker for about 5 days under room temperature. The samples were centrifuged at 1000rpm for 20mts. Then the supernatant were subjected to spectrophotometric analysis.

Estimation of indole acetic acid by Spectrophotometer analysis

After centrifugation (1000rpm 20mts) the liquid portion of aliquots of each broth is mixed with salkowski reagent (2:1) and the color developed within 30mts was measured at 530nm.

Analysis of indole acetic acid by Thin layer chromatography

In a successful approach the concentrated aliquots 100ml (4:1) the liquid portion of centrifuged sample of each broth were brought to pH 3.0 and extracted 3 times ethyl acetate. The organic phase was concentrated to dryness and then diluted with 0.5ml of ethanol application of these solution on silica gel (20cm-50cm) was diluted with mixture of chloroform. Ethylacetate formic acid (5:3:2) and salkowski reagent giving the correct RF value (0.57).

Results and Discussion

Phosphorous is one of the important macro nutrients in crop nutrition. Plants need constant supply of phosphorous of either growth and development phosphatic fertilizers are often applied to enrich phosphorous content in soil. A portion of the applied phosphorous often gets immobilized in soil due to chemical fixation is reordered available by phosphate solubilizing bacteria and fungi.

The present study concentrated on phosphate solubilizing organisms in rhizosphere soil of various agricultural yields. From the rhizospheric soil bacteria like *Pseudomonas* spp., *Bacillus* sp. *Xanthomonas* sp. are isolated. The fungi like *Aspergillus niger, Aspergillus fumigatus and Penicillium* sp. were isolated. The isolates were separately inoculated in PKA medium and tricalcium phosphate is added. The zone was observed upto 7days in the 1st, 3rd and upto 7th days. (Table.1). The pH range of *Pseudomonas* 3 sp. was initially at 6.8 and finally it decreased to 4.63 then produced more Indole acetic acid (Table.2). The pH range of *Aspergillus niger* also initially at 6.8 and finally it reaches 4.29 then produce more IAA (Table.3.). The thin layer chromatography shows the presence of Indole acetic acid with the help of salkowski reagent (Table 4).

Phosphate activity of a soil is due to the combined functioning of the soil microflora and any free enzymes present. Phosphate activity in soil increased by the organic content. But also it is affected by pH moisture temperature and other factor.

The variation in the population of phosphobacteria might be attributed to many soil factors organic matter and soil enzymes activities. The result thus throws light on existence of microbial solubilizing of phosphate in rhizosphere soils of different agricultural fields.

Therefore as per need of industry a various type of micro-organisms, which are capable of producing high levels of this enzyme have been isolated. Taking into study the extracellular production, its efficient recovery, pH tolerance and a good purified enzyme activity, CHO produced by *B. lichiniformis* should prove to be an industrially important enzyme. The COP was also determined to be 20α-Hydroxycholesterol. Our preliminary work led to the conclusion that *B. lichiniformis* might be considered as potentially sources of extracellular cholesterol oxidase for clinical and commercial purposes.

Table. 1 Change in zone appearance during tri calcium phosphate solubilization by bacterial and fungal isolates at various incubation periods

S.No	Bacterial and fungal isolates	Diameter of Zone (nm) Day			
		1st	3rd	5th	7th
1	*Bacillus sp*	1.0	1.4	3.5	-
2	*Pseudomonas sp.1*	1.2	2.1	3.3	-
3	*Pseudomonas sp.2*	2.3	2.8	3.1	-
4	*Pseudomonas sp.3*	3.1	3.3	3.5	-
5	*Pseudomonas sp.4*	2.5	2.9	3.2	-
6	*Xanthomonas sp.*	1.5	1.9	2.2	-
7	*Aspergillus niger*	-	3.4	4.5	4.5
8	*Aspergillus fumigatus*	-	3.5	4.0	4.0
9	*Aspergillus flavus*	-	3.7	4.1	4.3
10	*Penicillium sp.*	-	2.7	3.0	3.5

Table. 2 Screening of selected phosphate solubilizing isolates

S.No	Phosphate solubilizing Bacteria and fungi	Initial pH	Final pH	Solubilization Zone Diameter mm (Z)	Spot Diameter mm (C)	PSE= (Z-C)/ CX100
1	*Bacillus sp.*	6.8	5.0	3.5	1.5	150.00
2	*Pseudomonas sp.1*	6.8	4.68	3.3	1.0	230.00
3	*Pseudomonas sp.2*	6.8	4.63	3.1	1.0	210.00
4	*Pseudomonas sp.3*	6.8	4.63	3.5	1.0	250.00
5	*Pseudomonas sp. 4*	6.8	4.63	3.2	1.0	220.00
6	*Xanthomonas sp.*	5.7	4.25	2.2	2.0	10.0
7	*Aspergillus niger*	6.8	4.29	4.5	1.5	200.00
8	*Aspergillus fumigatus*	6.8	4.64	4.0	1.5	166.66
9	*Aspergillus flavus*	6.8	4.59	4.3	1.6	168.75
10	*Penicillium sp*	6.8	4.76	3.5	2.0	75.00

Table. 3 *In - vitro* phosphate solubilization capacity

S.No	Name of Organism	Available P
1	*Bacillus* sp.	29.41
2	*Pseudomonas* sp.1	42.38
3	*Pseudomonas* sp.2	35.56
4	*Pseudomonas* sp.3	44.08
5	*Pseudomonas* sp.4	40.69
6	*Xanthomonas* sp.	24.88
7	*Aspergillus niger*	34.47
8	*Aspergillus fumigatus*	30.44
9	*Aspergillus flavus*	28.08
10	*Penicillium* sp.	29.41

Table. 4 *In- vitro* production of indole acetic acid

S.No	Phosphate Solubilization Organisms	Indole Acetic Acid (IAA)
1	*Bacillus* sp.	35.18
2	*Pseudomonas* sp.1	40.25
3	*Pseudomonas* sp.2	39.15
4	*Pseudomonas* sp 3	44.11
5	*Pseudomonas* sp.4	39.22
6	*Xanthomonas* sp.	31.01
7	*Aspergillus niger*	34.25
8	*Aspergillus fumigatus*	33.25
9	*Aspergillus flavus*	31.25
10	*Penicillium* sp.	33.14

Phosphorus (P) is one of the major essential macronutrients for plants and is applied to soil in the form of phosphatic fertilizers. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants (Goldstein, 1986). Microorganisms are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization (Hilda and Fraga, 1999). Currently, the main purpose in managing soil phosphorus is to optimize crop production and minimize P loss from soils. Recently, phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield (Young, 1994; Young *et al*., 1998; Goldstein *et al*., 1999; Fasim *et al*., 2002).

Plant growth promoting bacteria (PGPB) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms (Glick, 1995), and P-solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant P nutrition. Given the negative environmental impacts of chemical fertilizers and their increasing costs, the use of PGPB is advantageous in the sustainable agricultural practices. It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Kim *et al*., 1997), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). However, P-solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al*., 1999). There is experimental evidence to support the role of organic acids in mineral phosphate solubilization (Halder *et al*., 1990).

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