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RESEARCH ARTICLE

Isolation, Screening and Evaluation of Multifunctional Strains of High Efficient Phosphate Solubilizing Microbes from Rhizosphere Soil

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ABSTRACT:
Phosphorus is one of the essential macronutrients which supports plant growth and is obtained from the soil. Phosphate solubilizing micro-organisms helps in solubilizing the insoluble form of phosphates into soluble forms. The present study is aimed to isolate phosphate solubilizing microorganisms from rhizosphere of four plants. P-solubilizing organisms were screened using the Pikovskaya's medium with Tricalcium phosphate as the sole source of phosphate. P-solubilization activity was measured in NBRIP medium and two fungal and bacterial strains obtained from the *Colocasia esculenta* were selected for their maximum solubilization indices. The selected strains were subjected for morphological, biochemical and genotypic identification and the strains were identified to be *Pestalotiopsis microspora, Aquabacterium commune, Bacillus* spp., and *Aspergillus* spp. All the identified strains were subjected to screening against various environmental stresses such as salinity, pH and temperatures. The strains were also tested for intrinsic antibiotic resistance. Finally, the strains were tested for the pesticide tolerance activity against 0.25% Cypermethrin. From these tests, it was observed that the strains were tolerant to salt concentration upto 4%, to temperature upto 37°C. and to pH of 7.8. The strains were tested for the indole acetic acid production activity and were found to be positive for the test. The strains were found to resistant against all the antibiotics except Gentamicin and Co-Trimoxazole. It was also found that the strains were tolerant towards the 0.25% Cypermethrin upto 1g/L. The identified strains were further analyzed by the molecular studies and constructed phylogenetic tree analysis.

KEYWORDS: Phosphate solubilizing microorganisms, Rhizosphere, NBRIP medium, Indole acetic acid, Pesticide tolerance.

INTRODUCTION:
Phosphorus is a second most needed major plant nutrients limiting the growth and yield[1,2]. It plays many structural and physiological functions in the plants[3] and its deficiency leads to the stunted growth in plants. Plants can utilize the soluble forms of phosphate from the soil which is present in low quantities. Hence chemical phosphatic fertilizers are used in the farm lands which are identified to be harmful to environment[4]. Moreover, a great proportion of the phosphorus in chemical fertilizer becomes unavailable in plants after its application in the soil[5,6].

Phosphate Solubilizing Microorganisms (PSM) play an important role in phosphorus cycle[7]. The organisms can convert insoluble phosphates into soluble forms[8]. The common phosphate solubilizing micro-organisms include the *Rhizobium, Pseudomonas* and *Enterobacter* bacterial species and *Penicillium, Aspergillus* of fungal species[9]. The phosphate solubilizing activity of these organisms is due to the secretion of low molecular weight acids such as citric acid, gluconic acid, 2-keto gluconic acid and oxalic acid, which via their hydroxyl and carboxyl group chelate the cations bound to the phosphate and convert it into soluble forms[10]. Other than phosphate solubilization, these organisms have also been identified to be stress tolerant and. These microbes can be used as inoculants to improve the soil growth and yield. Hence biofertilizers based on the phosphate solubilizing organisms are gaining an extensive interest of the agriculturists and the researchers. The aim of this work is to isolate and identify phosphate solubilizing

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micro-organisms from the rhizospheric soil of different plants. The phosphate solubilizing activity is to be calculated for the isolated strains. The selected strains are to be tested for tolerance against varying conditions of salt concentrations, pH and temperature. The antibiotic resistance activity and pesticide tolerance activity of the selected micro-organisms is also to be characterized.

MATERIALS AND METHODS:

Sample collection:

Soil samples were collected from rhizosphere soil of different economical crops. The crops include *Arachis hypogaea* (Groundnut), *Solanum lycopersicum* (Tomato), *Vicia faba* (Broad beans) and *Colocasia esculenta* (Taro root). The plants were selected on their commercial usability and the soil samples (Rhizophere) were collected at Mazhavanthangal, Villupuram District, TN, India.

Isolation of Phosphate Solubilizing Micro-organisms:

The collected soil samples were serially diluted into different concentrations and placed on Pikovskaya's (PVK) medium with Tri Calcium Phosphate (TCP) as the sole source of phosphate[11]. The medium is prepared with the ingredients manually and the pH of the medium was adjusted to 7.0 before autoclaving. The serially diluted soil sample solutions were spread on the Pikovskaya's agar plates and were incubated at room temperature. After 3 days of incubation the plates were observed for the clear zones that indicates the presence of P-solubilizing organisms.

Confirmation of Phosphate Solubilizing Micro-organisms:

The confirmation of P-solubilizing micro-organism was done by plating the isolated microbial colonies in NBRIP (National Botanical Research Institute's Phosphate) medium with Tri Calcium Phosphate acting as the sole source of phosphate. The plates were incubated at room temperature. After 3 days of incubation, the colonies were observed for clear halo zones which confirm the phosphate solubilization activity of the organisms. The zones were measured for calculating the phosphate solubilization. The activity of P-solubilizing microbes was measured by using phosphate solubilization index formula[12]. Higher the phosphate solubilization index, higher the phosphate solubilization activity.

Phosphate Solubilizing Index = Total Diameter/ Diameter of the colony

Morphological Identification:

After the confirmation of the microbes for the P-solubilization activity, the strains were primarily identified by morphological observation. The Phosphate solubilizing fungi (PSF) were observed by Lactophenol Cotton Blue staining and the bacteria (PSB) were identified by Gram's staining and biochemical tests.

Environmental Tolerance tests:

Salt Tolerance test:

The PSB was plated on Nutrient Agar (NA) prepared with different salt concentrations of 0.5, 1, 1.5 and 2gL^{-1} NaCl. Likewise PSF was also plated on Sabaouraud Dextrose Agar (SDA)[13] and incubated at 28ºC for a period of 24 hours – 4days.

pH Tolerance test:

The PSB was streaked on Nutrient Agar (NA) plates prepared with varying pH of 4.8, 5.8, 6.8 and 8.8. Likewise PSF was also plated on Sabaouraud Dextrose Agar (SDA). The pH was adjusted with NaOH and HCl. The plates were incubated for 24 hours – 24 days at 28ºC[14].

Temperature Tolerance test:

The PSB was streaked on Nutrient Agar (NA) plates and PSF was streaked on Sabaouraud Dextrose Agar (SDA) plates. Four plates were prepared for each selected strains and incubated at the temperatures of 10, 20, 28 and 38ºC respectively for 24 hours - 4 days.

Intrinsic Antibiotic Resistance test:

The bacterial culture was spread out on the MHA (Muller Hinton Agar) plates. The plates contained 9 different antibiotic discs and were incubated for 24 hours at 28ºC. The zone of inhibition or resistance was observed and recorded.

Pesticide Tolerance activity:

The PSB strains were streaked on Nutrient Agar (NA) plates and PSF was streaked on Sabaouraud Dextrose Agar (SDA) plates containing varying concentrations of 0.25% Cypermethrin such as 0.25, 0.5, 0.75 and 1 gL^{-1}. The plates were kept in incubation for 24 hours to 4 days at 28ºC[15] and growth was observed.

Gene Sequencing:

The identification of strains done by the gene sequencing. The gene sequencing was done by the 'Yaazh Xenomics', Coimbatore. The fungal strain was sequenced by 18s RNA sequencing and the bacterial strain by the 16s rRNA sequencing. Based on the results, phylogenetic trees were constructed [16].

RESULTS AND DISCUSSIONS:

In this present study, phosphate solubilizing microorganisms were isolated from rhizospheric soil based on the screening technique. Phosphate solubilization activity, Environmental tolerance, Indole Acetic Acid production activity and Pesticide tolerance activity were also determined.

Isolation of PSM:

For isolation of phosphate solubilizing microorganisms, rhizosphere soil collected from the plants of *Arachis hypogaea* (Groundnut), *Solanum lycopersicum* (Tomato), *Vicia faba* (Broad beans) and *Colocasia esculenta* (Taro root) the colonies were isolated in Pikovskaya's medium. The colonies with the zones are primarily identified to be of both fungal and bacterial organisms (Table 1). Among the plants, the soil samples obtained from *Colocasia esculenta* resulted in more number of colonies compared to the other crop plants.

Table 1: Number of colonies obtained after initial screening

S. No.	Samples	No. of bacterial colonies	No. of fungal colonies
1	*Arachis hypogaea*	3	2
2	*Solanum lycopersicum*	2	4
3	*Vicia faba*	4	3
4	*Colocasia esculenta*	5	6

Confirmation of PSM:

The colonies with the clear halo zones around the colonies were confirmed to be P-solubilizing organisms. From the 29 colonies obtained in the initial screening, 16 colonies were confirmed to be P-solubilizing organisms. Among those, four strains were observed for high solubilization indices. The strain CF1 obtained from the *Colocasia esculenta* (Taro root) (Fig. 1) showed highest phosphate solubilization index (Table 2).

Table 2. Four efficient strains of phosphate solubilizing organisms with higher solubilization indices

S. No.	Source	Strains	Phosphate Solubilization Index
1	*Colocosia esculenta*	CF1 (Fungal)	3.84
2	*Colocosia esculenta*	CF2 (Fungal)	2.85
3	*Colocosia esculenta*	CB3 (Bacterial)	2.00
4	*Colocosia esculenta*	CB4 (Bacterial)	1.25

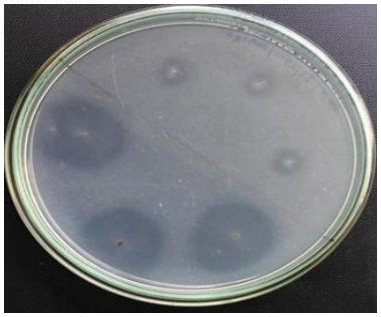

Fig. 1: Clear zones formed around the fungal strains in NBRIP medium showing the maximum solubilization index

Morphological Identification:

The selected strains based on their ability was morphologically identified. The strain CF2 was identified as *Aspergillus* spp. The selected bacterial strain CB3 was observed as blue coloured rod shaped Gram negative colonies and the strain CB4 was observed as Gram positive pink coloured rods arranged in chains (bamboo stick appearance) and identified as *Bacillus* spp.

Environmental Tolerance tests:

Salt Tolerance test:

All the strains showed tolerance against different concentrations of NaCl. Among the strains, only the fungal strain CF2 showed moderate tolerance towards the different salt concentrations. This shows that the strains were capable of surviving in soils with different amount of salts

pH Tolerance test:

All the strains showed tolerance against pH. Among the strains, only the fungal strain CF1 showed no tolerance towards the pH values of 4.8 and 5.8. This shows that the strains were capable of surviving in soils with different pH values.

Temperature Tolerance test:

The plates incubated for different temperatures were observed after 24 hours for tolerance against thermal stress. All the strains showed tolerance against the different temperatures. Among the strains, only the fungal strain CF1 showed no tolerance at temperatures of 10 and 20ºC.

Intrinsic Antibiotic Resistance test:

The plates that were incubated with nine different types of antibiotics for 24 hours were observed for their antibiotics resistance. All the bacterial strains were resistant for the antibiotics other than Gentamicin and Co-Trimoxazole (Fig. 2).

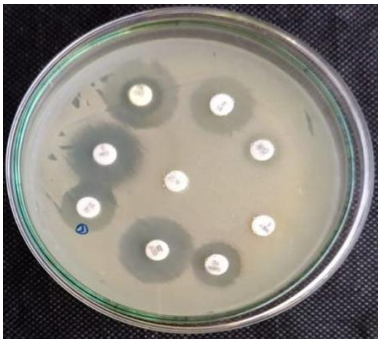

Fig 2: Bacteria spread on HMA medium with 9 different antibiotic discs showing different range of resistances.

Pesticide Tolerance activity:

All the strains showed tolerance against different concentrations of 0.25% Cypermethrin. Among the strains, only the fungal strain CF2 showed low tolerance towards the higher concentrations of 0.75 and 1.0gL^{-1} (Table 3). This strain showed variation in tolerance according to the pesticide concentrations. The bacterial

strains CB3 and CF4 showed growth in all concentrations and have higher tolerance towards the pesticide. This shows that the strains were capable of surviving in soils with different amount of pesticide concentrations.

Table 3. Pesticidal resistance activity different strains

S. No	Pesticide concentration (gL^{-1})	Fungal strains		Bacterial strains	
		Strain CF1	Strain CF2	Strain CB3	Strain CB4
1	0.22	+	+	+	+
2	0.5	+	±	+	+
3	0.75	±	±	+	+
4	1.0	±	-	+	+

* Note: (+: growth, ±: low growth, -: no growth)

Gene Sequencing:

The sequence obtained from the fungal strain was compared with the data available in NCBI using BLAST algorithm and the strain was identified to be *Pestalotiopsis microspora*. Likewise the sequence of the bacterial strain was identified to be *Aquabacterium commune*.

CONCLUSION:

Among the soil samples collected, *Colocasia esculenta* showed phosphate solubilizing strains (two fungal and two bacterial strains) with higher efficiency. From the morphological and genotypic identification study, the CF1 and CB3 strains were concluded that *Pestalotiopsis microspora* and *Aquabacterium commune* respectively. The CF2 and CB4 strains were identified form morphologically identified as *Aspergillus* spp. and *Bacillus* spp. From the environmental tests, it was observed that the strains were tolerant to salt concentration upto 4%, to temperature upto 37°C. and to pH of 7.8. The strains were found to resistant against all the antibiotics except Gentamicin and Co-Trimoxazole. It was also found that the strains were tolerant towards the 0.25% Cypermethrin upto 1g/L. Therefore, it can be concluded that the strains obtained from the study are efficient as well as multi-functional. The conventionally used biofertilizers are mostly derived from the strains of Phosphobacteria. From this study, it can be also concluded that the obtained strains are efficient than the already existing biofertilizers. Hence, the identified strains can be further used in mass producing the carrier based inoculums for biofertilizers in future.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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