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PHOSPHATE SOLUBILIZING MICROORGANISMS AS BIOFERTILIZERS

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Abstract: In the present study, the soil samples were collected from Avicennia marina rhizosphere soil samples Muthukuda, Arasankarai and Mumbalai mangroves. Phosphate solubilizing fungi (PSF) were isolated from each sample by serial dilution and spread plate method. The isolated fungal species belongs to two division, Ascomycetes (2 genera) and Deuteromycetes (2 genera). The fungal strains were identified as Aspergillus flavus, A. humicola, A. niger, A. quercinus, A. ruber, A. rugulosus, A. sulphureus, A. ustus, A. wentii, Gliomastix cerealis, Paecilomyces elegans, Penicillium chrysogenum, Penicillium sp, P.oxalicum, P. nigricans and Sterile mycelium based upon their colony morphology, spore characteristics and microscopic studies. Phosphate solubilizing ability of fungal strains was tested in types of media. Fungi were isolated from mangrove soil samples were capable of solubilizing the phosphate. On 3rd day of observation, four isolates viz., Aspergillus flavus, A.niger, P.oxalicum and Penicillium sp has showed the clear zone measuring 1.7, 2.3, 1.5 and 1 cm on Modified Rose Bengal agar medium. Physico-Chemical characterization were analysed. Avicennia marina rhizosphere soil samples in three different sampling sites showed soil texture, pH, density, water holding capacity, electrical conductivity and micro and macro nutrients were analysed.

Keywords: *Avicennia marina,* Mangroves, Phosphate solubilizing fungi, Physico-Chemical characterization.

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INTRODUCTION

Fungi being ubiquitous organisms occur in all types of habitats and are the most adaptable organisms. Fungi play a major role in soil ecosystem and are the principal decomposers of forest litter or dung, fruits or other organic materials [1]. It has been found that more number of genera and species of fungi exist in soil than in any other environment [2] Contributing to the nutrient cycle and maintenance of ecosystem, fungi play an important role in soil formation, soil fertility, soil structure and soil improvement [3].

Phosphorus play a significant role in plant growth and metabolism by supplying energy needed for metabolic processes [4] and is considered obligatory for the synthesis of nucleic acids molecules. Moreover, in soil the main problem with phosphorus for plant uptake its availability in very minute quantity. Phosphorus containing chemical fertilizers after their application to agricultural soils either get fixed or precipitated in soils. A number of phosphate solubilizing bacteria and fungi act as plant growth promoters because of their ability to release IAA [5].

Phosphate solubilizing fungi that possess the capability to change the insoluble form of phosphorus into soluble one. Phosphorus is one the most essential element for plant growth second only to nitrogen in requirement for plant. Phosphorus plays a significant role in physiological and biochemical plant activities. But, due to different chemical reactions there is limited availability of this nutrient for plants especially in arid and semi arid soils. Most of the essential plant nutrients remain in insoluble form in soil.

Phosphate solubilizing activities were found to improve root length significantly [6]. Phosphorus is second only to nitrogen as essential macronutrients for plant growth and development [7]. The use of microbial inoculants (biofertilizers) possessing phosphate solubilizing activities in agricultural soils is considered as an environmental eco-friendly alternative to further applications of chemical based phosphate fertilizer.

MATERIALS AND METHODS

Collection of Samples

Soil samples were collected from *Avicennia marina* rhizosphere soil samples from Muthukuda mangroves. Samples were collected from 2 - 3 cm deep pits dug in the area to be sampled. The samples were collected with a surface sterilized trowel. Soil was scraped along the walls of the pits and collected in polythene bags. Soil from 3-5 pits was pooled together and mixed in the same polythene bag.

MAP SHOWING THE SAMPLING SITE

The study stations of the present investigation were on mangrove forest of three different unexplored areas at Pudukkottai (Dt), Tamil Nadu, India (Plate I).

Station - I	Station - II	Station - III
Arasankarai	Muthukuda	Mumbalai
Latitude -9˚ 50'15"N	Latitude -9˚ 51'43"N	Latitude -10˚ 4'25"N
Longitute-79˚ 6'19"E	Longitute-79˚ 7'23"E	Longitute-79˚ 14'23"E

Isolation and Identification of Phosphate Solubilizing Fungi

Phosphate solubilizing fungi (PSF) were isolated from each sample by serial dilution and spread plate method [8]. One gram (1g) of soil sample was dispersed in 9ml sterile sea water and was thoroughly shaken serial dilution of soil samples were prepared in the range of 10^{-2} to 10^{-8} dilution. 0.1 ml of each dilution was spread on Rose Bengal agar medium and incubated at 28^0±30^0C for 3 days. Colonies showing halo zones were picked and purified for further identification. The colonies that show clearing of phosphate were purified separately on Rose Bengal agar and were maintained on slants, respectively for further studies.

Fungal strains were isolated from the mangroves soil samples and predominant, morphologically distinct colonies were selected, purified by repeated culturing and maintained on agar slants at 4^0C. Isolates were identified by their colony characteristics, spore morphology and microscopic observation.

The individual colonies were picked out and allowed the phosphate solubilization index test on Rose Bengal agar medium. A clear halo zone was formed around the colonies after 5 days of incubation on solidified agar medium supplemented with indicating phosphate solubilizing ability of the fungal isolates selected for further studies.

Optimization of Media and Growth Conditions for Phosphate Solubilization

Phosphate solubilizing ability of fungal strains was tested in types of media. Flask were inoculated with 8 % spore suspension and incubated on shaker at 28^0C for 6 days.

Effect of Temperature on Efficiency of Phosphate Solubilization

Fungal isolates were able to solubilize phosphate (P_2O_5) in the pH ranges of 5 to 8. Maximum phosphate solubilization and growth was monitored at pH 7.

For all isolates 35˚C is the optimum temperature for the growth and phosphate solubilization. Phosphate solubilization was also observed at 30˚ C and it was found that after 35˚ C there was growth retardation and fall in phosphate solubilization. Different temperature has been reported by earlier workers for solubilization, most of them have found 25˚C to 28˚C to be optimum temperature.

Effect of pH on the Efficiency of Phosphate Solubilization

pH ranging from 5 to 8 at an interval of 1 pH was altered in Rose Bengal agar medium by adding 1N NaOH (to increase the pH) and 1 N HCl (to reduce the pH). The proper sterilization of the medium poured in pertiplates and inoculated the isolated fungi. The plates were incubated at 30^0C for 48-72 hrs. After incubation period the plates were observed the clear halo zone.

Composition of Modified Rose Bengal Agar Medium

Ingredients mg/ml

Glucose - 10.0g

Peptone - 2g

KH_2PO_4 - 0.5g

$MgSO_4$ - 0.5g

Rose Bengal dye - 100 µg

Agar agar - 16g

Distilled water - 1000ml

pH - 5 – 6.5

Rose Bengal agar medium was prepared and sterilized.

10% $CaCl_2$ solution was prepared and sterilized separately. 10% KH_2PO_4 solution also prepared and sterilized in a separate conical flask. To this, sterilized 60 ml of 10% $CaCl_2$ and 40 ml 10% KH_2 PO_4 solutions were mixed. Then 1 N NaOH solution was added as drops till the precipitation is formed. After the formation of white precipitate the medium was poured into petriplates.

Analysis of Physico – Chemical Characterization of the Soil Samples

pH

After removing the debris, the soil samples were suspended in distilled water (1; 2 w/v) and allowed to settle down the sand particles. The pH of the suspension was determined using pH meter (systronics, India).

Electrical conductivity

Electrical Contuctivity Bridge as described by [9], cation exchange capacity (CEC) of the soil was determined by using 1N ammonium acetate solution.

Nutrient analysis

Organic carbon (OC) content was determined by adopting chromic acid wet digestion method as standard procedure of [10], available nitrogen was estimated by alkaline permanganate method [11], and available phosphorus by Bray method [12]. Available potassium was extracted from soil with neutral 1N ammonium acetate (1:5) and the potassium content in the extract was determined by using flame photometer [13]. Calcium (Neutral 1N NH_4 OAC extractable 1:5) was extracted with neutral 1N ammonium acetate and the available calcium in the extract was determined by versenate method [9]. Available micronutrients such as Zn,Cu and Mn were determined in the diethyl triamine penta acetic acid extract of soil using [14]. Atomic Absorption Spectrophotometer Pekin-Elmer Model 2280. Other nutrients such as magnesium, sodium and available iron contents were also analyzed [15].

Microscopic Examination

The slides were examined under microscope and the morphological characters were observed.

Growth in Different Temperature

The fungi were inoculated individually in the plates containing Modified Rose Bengal agar medium and grown under 15, 28, 35°C, in an incubator, for a period of a week. The growth and phosphate solubilizing efficiency were estimated.

Growth in Different pH

pH ranging from 4 to 8 at an interval of 1 pH was altered in the Rose Bengal agar medium by adding 1 N NaOH (to increase the pH) and 1 N HCl (to reduce the pH). The composition of the medium in the pH range from 4 to 8 were inoculated with the fungi, *Aspergillus fumigatus, A.niger, A.terreus, Penicillium* sp. The plates were incubated at room temperature (28± 2^0 C) for a period of 7 days. Their growth and solubilization zone was measured using mm scale. From this, solubilization efficiency was worked out as described earlier.

Growth in Different Salinity

Modified Rose Bengal agar medium with different salinity ranging from 0 to 1%, 2%, 3%, 4% and 5% intervals was prepared using addition of salt (NaCl). The fungi were inoculated individually in the plates containing different salinity medium incubated, their growth and phosphate solubilizing efficiency were estimated after 7 days of incubation as described earlier.

RESULTS

Physico-Chemical characterization of soil

In the present study, the soil were collected from *Avicennia marina* rhizosphere soil samples in three different sampling sites showed soil texture in clay, pH ranges from 7.5 - 7.8, bulk density 1.340 – 1.410 g/cm^3, water holding capacity 26 -29%, Electrical conductivity 0.615 – 0.670 dsm^{-1}, organic carbon 0.54 – 0.61%, Available nitrogen 139.5 -152.6 mg/g, Available phosphorus 6.5 – 7.6 mg/g, Available Potassium 210 – 251 mg/ g and salinity 4.8 – 5.0.

The macro nutrients are nitrogen, phosphorous and potassium in the ranges from 0.165- 0.185, 0.170 – 0.190 and 1.40- 1.60 %. Available micronutrients viz.; calcium, magnesium, sodium and potassium, zinc, copper, iron, manganese and boron the ranges from 1.95 – 2.10, 1.10 – 1.35, 4.65 – 5.20, 2.10 – 2.65 and 0.460 – 0.510 % were tabulated (Table:1).

Table 1 Physico-chemical properties of soil samples

S.No	Sampling places	Texture	pH	Bulk density (g/cm^3)	Water holding capacity (%)	Electrical conductivity (dsm^{-1})	Organic carbon (%)	Available Nutrients(mg/gm)		
								N	P	K
1	Muthukuda	Clay	7.60	1.390	29	0.625	0.58	141.0	6.5	**229**
2	Arasankarai	Clay	7.80	1.340	26	0.615	0.54	152.6	7.6	**251**
3	Mumbalai	Clay	7.50	1.410	28	0.670	0.61	139.5	7.1	**210**

S.No	Sampling sites	Salinity	Macro nutrients (%)		Available Micro nutrients (%)					
			N	P	K	Zn	Cu	Fe	Mn	B
1	Muthukuda	4.8	1.70	0.170	1.55	2.00	1.20	4.65	2.40	0.510
2	Arasankarai	5.0	1.85	0.185	1.60	2.10	1.35	5.20	2.65	0.480
3	Mumbalai	4.9	1.65	0.190	1.40	1.95	1.10	4.85	2.10	0.460

Density of fungal growth in mangrove soil

The dilution plating of the soils collected from the mangrove showed the fungal population in the range from 14 to 17 of CFU/ml on isolated from Rose Bengal agar plates. In plates they expressed identical colony based on morphology and therefore randomly four isolates were selected and raised in pure culture and subjected to lactophenol cotton blue study (Table 2; Plate I & II).

Table 2 Isolation of fungi from mangrove soil samples

S.No	Place	Dilution	No. of colonies
1	Arasankarai	10^{-2}	15
		10^{-3}	11
		10^{-4}	8
2	Muthukuda	10^{-2}	14
		10^{-3}	9
		10^{-4}	5
3	Mumbalai	10^{-2}	17
		10^{-3}	12
		10^{-4}	10

Isolation of fungi from the rhizosphere soils of Arasankarai site

Control

10^{-2}

10^{-3}

10^{-4}

Plate II Isolation of fungi from the rhizosphere soils of Muthukuda site

Control

10^{-2}

10^{-3}

10^{-4}

III Isolation of fungi from the rhizosphere soils of Mumbalai site

Control

10^{-2}

10^{-3}

10^{-4}

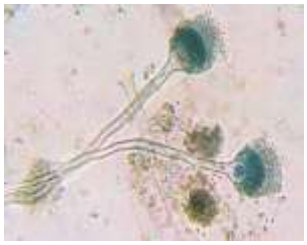
The fungal strains were identified as *Aspergillus flavus, A. humicola, A. niger, A. quercinus, A. ruber, A. rugulosus, A. sulphureus, A. ustus, A. wentii, Gliomastix cerealis, Paecilomyces elegans, Penicillium chrysogenum, Penicillium* sp, *P.oxalicum, P. nigricans* and Sterile mycelium based upon their colony morphology, spore characteristics and microscopic studies. The isolated fungal species belongs to two division, Ascomycetes (2 genera) and Deuteromycetes (2 genera) (Table 3 and Plate III).

Table 3 List of fungi recorded in three different sites of mangrove soil

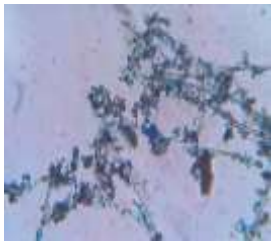
S. No	Division	Fungal species
1	Ascomycetes	*Oidiodendron echinulatum* Barron *Paecilomyces elegans* (Corda) Mason et Hughes
2	Deuteromycetes	*Aspergillus flavus* (Link) *A.humicola* (Chaudhuri) *A.niger* (Van Tieghem) *A.quercinus* (Bain.) Thom & Church *A.ruber* (Spieckermann and Brenner) *A.rugulosus* (Thom and Rafer) *A. sulphureus*(Fresenius) – Thom and Church *A.ustus* (Bainier) – Thom and Church *A.wentii* Wehmer *Gliomastix cerealis* (Kart.) Dickinson *Penicillium chrysogenum* Thom

		P.nigricans (Bainier) *P. oxalicum* (Thom) Sterile mycelium

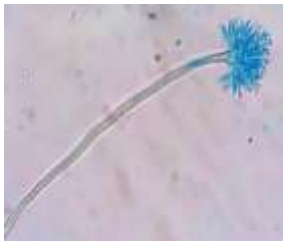
Plate III Microphotography (400 X) of isolated fungal species



Aspergillus sulphureus

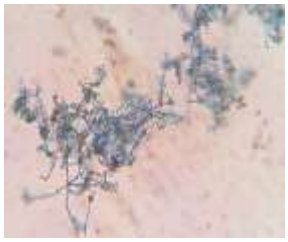


Gliomastix cerealis



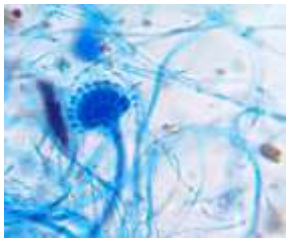
Aspergillus ustus

Penicillium oxalicum

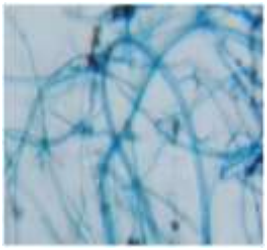


Paecilomyces elegans

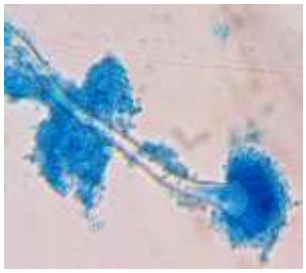
Aspergillus quercinus



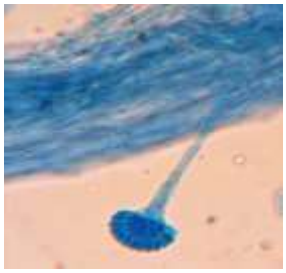
Aspergillus ruber



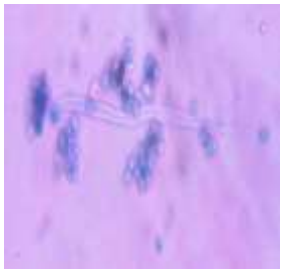
Sterile mycelium



Aspergillus wentii

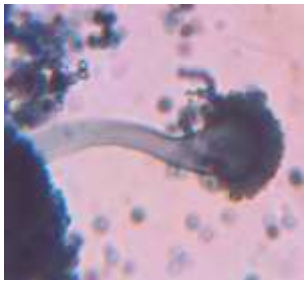


Aspergillus humicola

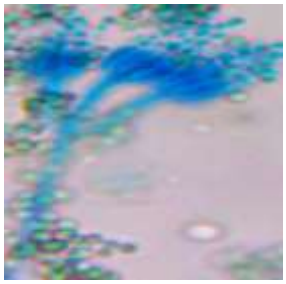


Oidiodendron echinulatum

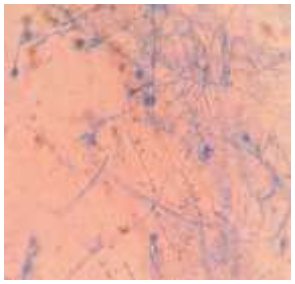
Aspergillus niger



Aspergillus rugulosus



Penicillium nigricans



Penicillium chrysogenum

Aspergillus flavus

Phosphate solubilization by different isolates

Fungi were isolated from mangrove soil samples were capable of solubilizing the phosphate. On 3rd day of observation, four isolates viz., *Aspergillus flavus, A.niger, P.oxalicum* and *Penicillium* sp has showed the clear zone measuring 1.7, 2.3, 1.5 and 1 cm on Modified Rose Bengal agar medium (Table 4; Plate IV).

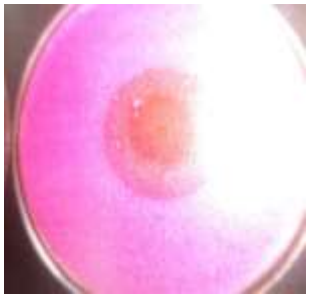
Table 4 Phosphate solubilization index of isolated fungi

S.No	Microorganisms	Halo Zone of clear of zone (mm)
1	*Aspergillus flavus*	1.7
2	*A. niger*	2.3
3	*Penicillium* sp.	1
4	*P. oxalicum*	1.5

Plate IV Phosphate solubilization assay of isolated of fungi

Penicillium oxalicum


A.niger

A.terreus

Effect of pH on growth of phosphate solubilizing fungi

Optimum pH for better phosphorus solubilizatioin was 7 in *Aspergillus niger.*

Table 5 Effect of pH on the growth of Phosphate solubilizing fungi

S.No	Microorganisms	Ph				
		5	**6**	**7**	**8**	**9**
1	*Aspergillus flavus*	-	++	++	+	+
2	*A. niger*	+	++	+++	++	++
3	*Penicillium* sp.	-	++	++	+	-
4	*P. oxalicum*	-	+	++	+++	+++

(-) - No growth, (+) - Slight growth, (++) - Moderate growth, (+++) - High growth

Salinity tolerance of fungal isolates

Aspergillus niger and *Penicillium oxalicum* are tolerate to grow at all the concentration of Nacl. *Aspergillus flavus* can grow only in 0.5% concentration of Nacl.

Table 6 Effect of NaCl on the growth of Phosphate solubilizing fungi

S.No	Microorganisms	NaCl (%)				
		0.5	**1**	**2**	**3**	**4**
1	*Aspergillus flavus*	+	-	-	-	-
2	*A. niger*	+	++	+++	+++	+++
3	*Penicillium* sp.	+	+	+	-	-
4	*P. oxalicum*	++	++	+++	+++	+++

(-) - No growth, (+) - Slight growth, (++) - Moderate growth, (+++) - High growth

Optimization of temperature on the growth of phosphate solubilizing fungi

All the fungi grow in 28°C but *Penicillium* sp grow at 15°C.

Table 7 Effect of Temperature on the growth of Phosphate solubilizing fungi

S.No	Microorganism	Temperature (°C)		
		15	**28**	**35**
1	*Aspergillus flavus*	-	++	+++
2	*A. niger*	+	+++	+++
3	*Penicillium* sp.	-	+	-
4	*P. oxalicum*	++	+++	+++

(-) - No growth, (+) - Slight growth, (++) - Moderate growth, (+++) - High growth

Discussion

Physico- chemical parameters

[16], reported that the physico-chemical analysis of the soil indicated a low concentration of phosphates in the soil samples tested. Specifically, 2 soil samples beerur sample I and the

Bangalore sample, showed low concentrations of phosphates at pH 7.1 and the presence of high levels of organic carbon. The second soil samples from Beerur, with a pH of 7.3, had higher organic carbon and higher phosphorus, which was shown to limit other nutrients such as potash, zinc, copper and manganese.

Similarly in the present study, the soil were collected from *Avicennia marina* rhizosphere soil samples in three different sampling sites showed soil texture in clay, pH ranges from 7.5 - 7.8, bulk density 1.340 – 1.410 g/cm^3, water holding capacity 26 -29%, Electrical conductivity 0.615 – 0.670 dsm^{-1}, organic carbon 0.54 – 0.61%, Available nitrogen 139.5 -152.6 mg/g, Available phosphorus 6.5 – 7.6 mg/g, Available Potassium 210 – 251 mg/g and salinity 4.8 – 5.0. The macro nutrients are nitrogen, phosphorous and potassium in the ranges from 0.165- 0.185, 0.170 – 0.190 and 1.40- 1.60 %. Available micronutrients viz.; calcium, magnesium, sodium and potassium, zinc, copper, iron, manganese and boron the ranges from 1.95 – 2.10, 1.10 – 1.35, 4.65 – 5.20, 2.10 – 2.65 and 0.460 – 0.510 % were tabulated

Isolation of fungi

In the present study the potential phosphate solubilizing fungi *Aspergillus* and *Penicillium* species was reported. In agreement with several published reports, the present study confirms high phosphate solubilizing potential of common soil fungal strains *Aspergillus* and *Penicillium* (Fenice *et al*.,2000, Khan and Khan 2002, Reyes *et al.,* 2002).

Effect of pH **on growth of Phosphate solubilizing fungi**

[17], reported that the estimation of solubilized phosphate was done by the method reported by [8] *in vitro.* The four isolates were subjected to estimate their phosphate solubilization activity in Pikoviskaya`s medium in *Bacillus subtilis*, and *Pseudomonas aeuroginosa*, recorded maximum reduction at pH at 6.0 on second day on the incubation with 1.0 % phosphate concentration.on the other hand, on third day of incubation the reduction in 1.0% phosphate concentration was observed with *Aspergillus niger* and *Penicillium* at 5.0. Similarly in the present study four strains, *Aspergillus flavus, A. niger and Penicillium, P.oxalicum* were used for phosphate solubilization. Optimum pH for better phosphorus solubilizatioin was 7 in *Aspergillus niger.*

Salinity tolerance of fungal isolates

[18], pointed out that the total of 12 soil yeasts were isolated on PYG medium from collected soil samples. After purifying isolated soil yeasts on PYG media, salt tolerance of soil yeasts were screened on PYG media supplemented with various NaCl concentrations (ranging 1-14%). Among 12 isolates, four isolates (I1, I2, I3 and I4) tolerated NaCl to 14% and selected after plate screening. Similarly in the present study *Aspergillus niger* and *Penicillium oxalicum* are tolerate

to grow at all the concentration of Nacl. *Aspergillus flavus* can grow only in 0.5% concentration of Nacl.

The maximum P solubilization activity was produced at 28 ºC by *Pseudomonas* isolates. However, decline in yield of these activities was observed above and below 28 ºC. Similarly in the present study, *Aspergillus flavus, A. niger, Penicillium and P. oxalicum* grow in 28°C.

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