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Isolation and characterization of phosphate solubilising bacteria undernath *Excoecaria agallocha* L. of muthupet mangrove reserve

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ABSTRACT

Mangrove forests provide numerous benefits, serving as essential ecosystems for both the environment and human populations. Mangrove forests benefit from microbial development in an array of ways, including nutrient cycling, decomposition, and carbon capture. Microbes perform an important role in breaking down organic matter, releasing critical nutrients for plant growth, and boosting ecosystem productivity. They also contribute to carbon storage and can even yield useful molecules for biotechnological purposes. The current study focuses on the isolation and characterization of Phosphate solubilizing bacteria (PSB) that has the capability to convert the inorganic form of phosphate into its bioavailable form using Pikovaskaya's (PKA) agar medium containing insoluble tricalcium phosphate. Among the 32 isolates obtained only 8 isolates showed increased Phosphate Solubilizing Index (PSI) and further only one isolate that showed the highest value of 2.3. The obtained genomic DNA was PCR amplified and subjected to 16S rRNA sequencing and was identified as *Bacillus pumilus* ST3-23-10. This can further be quantitatively estimated with several other parameters and can be utilized as a bioinoculant for plant growth and development.

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INTRODUCTION

Mangroves have always been a phenomenon to the fascinating world and scientists (Mondal *et al.*, 2016) Mangroves are the woody halophyte species of vital ecological significance. In the coastal food web, tidal flats is an energy to source and these mangrove sediments are the first line of defense against many of the natural calamities like cyclone, tsunami etc. These mangroves are a diverse group of plants and trees found along the world's tropical and subtropical coasts. They are also known as the natural nutrient recyclers and strainers as they safeguard the coastal zone from seawater intrusion and also assists flood water management (Arumugam *et al.*, 2012) *Excoecaria agallocha* L. commonly known as 'Thillai' in "Tamil", 'Komatti' in 'malayalam', 'Gangtiva' in 'Hindi', 'Agaru' in 'Sanskrit' and 'Milky Mangrove' in 'English' is widely distributed in Australia, temperate and tropic zones throughout Asia and the southern Pacific region of the world (Mondal *et al.*, 2016). The Taxonomic classification of *E. agallocha* L. is tabulated. This mangrove is known for its significance in terms of ecology, economy and medicine (Patil and Manohar *et al.*, 2012).

Kingdom	Plantae
Phylum	Charophyta
Class	Equisetopsida
Subclass	Magnoliidae
Order	Malpighiales
Family	Euphorbiaceae
Genus	*Excoecaria*
Species	*agallocha* L.

It is applied to sores and ulcers and used internally in the treatment of bites from poisonous marine creatures and as a purgative. The bark oil derived from *E. agallocha* L. has therapeutic uses in the treatment of Leprosy, Paralysis and Rheumatism (Arumugam *et al.*, 2012).

The pharmacological activities of *E. agallocha* L. gained the attention of researchers as it possesses antioxidant, anti-inflammatory and antimicrobial activities. Some activities like antiulcer, anticancer, anti-reverse transcriptase, antidiabetic, DNA damage protective activity, anti-filarial, antitumor protecting activity and antihistamine release, seeked the attention of medical enthusiasts (Patil *et al.*, 2012) This study is indended to identify the microbial diversity of Phosphate Solubilizing Bacteria (PSB) unterneath the roots of *Excoecaria agallocha* L.

Phosphorus (P) is a promising limiting growth factor. Like nitrogen (N_2) there is no greater atmospheric pool of P that can be readily opened up to the plants (Ezawa *et al.*, 2012). The attributes associated with the phosphorus includes improving the quality, maturity and yield of crops, root development, strength of stalk and stem, formation of flower and seed , Biological N-fixation in legumes and associated with resistance to plant diseases (Gyaneshwar *et al.*, 2002). The stability of phosphate compounds in soils is primarily attributed to its strong reactivity with other compounds (such as Al_{3}^{+} , Fe_{3}^{+} and Ca_{2}^{+}), which will progressively become insoluble in the soil. Furthermore, the and mobilization release of fixed and insoluble forms of phosphorus is critical to improving phosphorus availability in soil. In an effort to escape the inconvenience of P deficiency, majority of the farmers switched to the chemical phosphatic fertilizers that is adsorbed into the soil and encourages plant development. This applied phosphorus readily converts the insoluble form of P into its stable form with minimal availability for plants (Gyaneshwar *et al.*, 2002).

Although the employment of microbial inoculants that favors the promotion of soil fertility has started in the last century, the existing work on Phosphate solubilization is relatively sparse compared to the nitrogen fixation. The dynamics of soil P is characterized by physico-chemical (desorption-sorption) and biological (mineralization-immobilization) processes.

Addition of high amount of Phosphatic fertilizer precipitates and enters in to the immobile pools and reacts with highly reactive Al_3^+ and Fe_3^+ in acidic soils and Ca_2^+ in calcareous or normal soils (Gyaneshwar *et al.*, 2002; Zaidi *et al.*, 2009). The main thrust of the investigation is to identify the microbial diversity and to isolate the microorganisms that has the ability to solubilize Phosphate underneath the widespread mangrove of Muthupet mangrove reserve *Excoecaria agallocha* L.

MATERIALS AND METHODS

Collection of soil sample

The soil samples [(10.35797, 79.53291 (±4m) NE) (10.35797, 79.53294 (±4m) SE) (10.35797, 79.53293 (±4m) E)] were collected using pit drilling method underneath the roots of the mangrove *Excoecaria agallocha* L. (Fig. 1) of the Muthupet Mangrove Reserve situated at Tiruvarur district of Tamil Nadu. Approximately 250 g of soil samples were collected at 3 different locations at 3 different points notably surface soil, 1 foot and 2 feet in depth at a distance of 1m from each location along with several sub samples using a sterile zip-lock cover and the samples were immediately transferred to the laboratory (Annizah *et al.*, 2021).

Fig. 1. *Excoecaria agallocha* L. of Muthupet mangrove

Isolation of PSB

100 ml of sterile distilled water was suspended with 1000 mg of soil sample and labeled as 10^{-1}. Serial dilution was carried out for the dilutions from $10^{-1}$to 10^{-7} and 100 micro litres of the suspension was uniformly spread on the petri dish containing autoclaved Pikovskaya's agar medium (PKA). The medium consists of $(NH_4)2SO_4$ - 0.50 (g/l), KCl 0.20 (g/l), $FeSO_4$ 0.0001 (g/l), yeast extract 0.50 (g/l), $MnSO_4$ 0.0001 (g/l), $MgSO_4$ 0.10 (g/l), dextrose 10.00 (g/l), agar 15 (g/l) and $Ca_3(PO_4)_2$ 5.0 (g/l). The pH of the media was set to 7.0 prior to autoclave at 15 lbs pressure (121 °C) for 15 min.

Plates were incubated in an inverted form for a period of 15 days at 28–35 °C and colonies with distinct clear halo zone exhibiting positivity towards phosphates solubilization and were considered as PSB.

These chosen colonies were then sub cultured a number of times by streaking technique until pure cultures were achieved and preserved on nutrient agar plates at 4 °C for later research (Pande *et al.*, 2017).

Morpho-biochemical characterization

The bacterial species showed different culture characters on the Pikovskaya's Agar Media. The Gram's staining method was used in the identification of the morphology of the isolates by using the standard procedure. The cells were stained and examined under a compound microscope. The Gram's reaction and the cellular structures for potent PSB strains were recorded (Isenberg, 1998) The Biochemical tests such as Glucose and Sucrose fermentation along with the IMVIC tests were carried out for the isolate (Haswania *et al.*, 2021).

Identification of the isolated strain

Total genomic DNA was isoalted using the CTAB method. Subsequent to DNA extraction, PCR reaction was carried out in Prima- 96 thermal cycler (Himedia). Universal primers (27F Forward primer 50-AGAGTTTGATCCTGGCTCAG-30 and 939r reverse primer 50-CTTGTGCGGGCCCCCGTCAATTC-30) were employed for 16S rRNA genome amplification prior to sequencing. The bacterial isolates were classified on the basis of their 16S rRNA gene partial sequences by using BLAST analysis (Hamid *et al.*, 2010).

RESULTS

Isolation of phosphate solubilising bacteria

In the current investigation, soil samples were tested in vitro to determine the efficiency of P solubilizing bacteria on Pikovskaya's (PKV) agar medium plates. As a result of halo zone formation 32 isolates were obtained in the PKV medium containing Tricalcium Phosphate as a sole source of Phosphate.

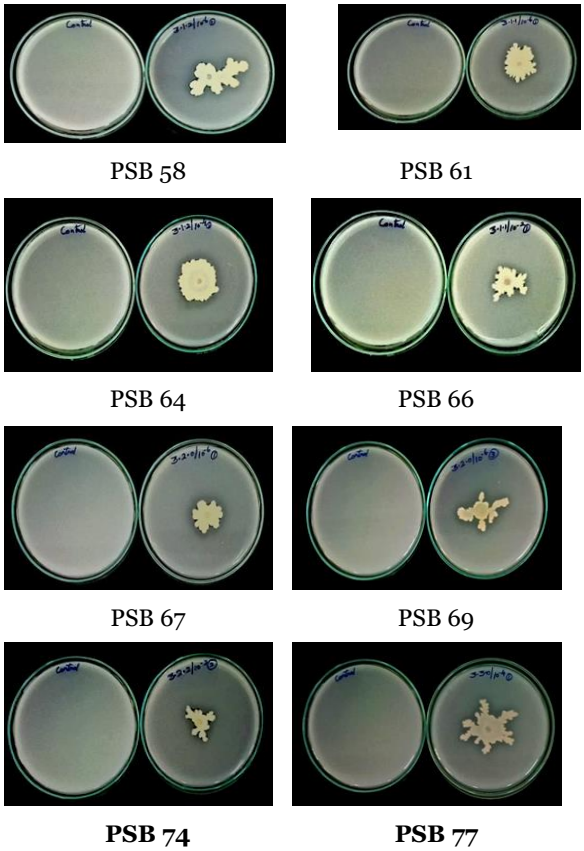

Fig. 2. Plates representing the zone of solubilization of phosphate on PKA plates (15th day)

After repeated subculture the pure colonies were obtained for 24 isolates and it was further stored in nutrient agar medium. 0.5cm of inoculum was placed in the centre of the plate to qualitatively measure the ability of the isolates to solubilize the phosphate available in the medium. The plates were maintained till 15 days and photographed on regular intervals (3,6,9,12,15 days) to check the increase in P utilization. Out of which 8 bacterial isolates (Fig. 2) were found to be highest solubilizers showing the clear zone of utilization around the colony. According to the Phosphate Solubilization Index (PSI) PSB 67 qualitatively showed the highest solubilizing ability (Table 1) and it is further characterized.

Morpho-biochemical characterization

The aerobic spore forming rod shaped bacterium trapped the crystal violet stain as it has thick peptidoglycan layer resulting the Gram-positive bacterium. The obtained microbial isolate responded positive to indole, urease, catalase, VP and citrate and negative to oxidase and MR (Table 2).

Table 1. Qualitative measurement of the isolates - (PSI)

Bacterial isolates	Colony diameter (cm)	Zone of Solubilization (cm)	PSI
PSB 58	2.1	2.4	2.1
PSB 61	2.8	3.4	2.2
PSB 64	3.0	2.6	2.2
PSB 66	3.2	3.8	2.1
PSB 67	2.1	2.9	2.3
PSB 69	2.5	3.1	2.2
PSB 74	2	2.4	2.2
PSB 77	2.2	2.7	2.2

PSI – Phosphate Solubilization Index

Table 2. Biochemical characterization of the microbial isolate

Bacterial isolate	Cell shape	Gram's staining	Oxidase	Indole	Urease	Catalase	VP	MR	Citrate
	Rod shaped, whitish cream coloured	+	+	-	-	+	+	-	+

Molecular identification

The PCR amplified DNA (Fig. 3) was sequenced for 16S rRNA, and the results were visually compared. The Clustal W program was used to align the sequences, and the distances between the phylogenetic trees were calculated. Using sequence analysis, the isolates were identified as *Bacillus pumilus* ST3_23_10 and the sequence was deposited to Genbank with the Accession No: PV936924 and the distance between the tree was identified by constructing the phylogenetic tree of closely related species using MEGA 12 software and one out group is

used from the partial genome sequencing of *E. coli* obtained from NCBI (Fig. 4).

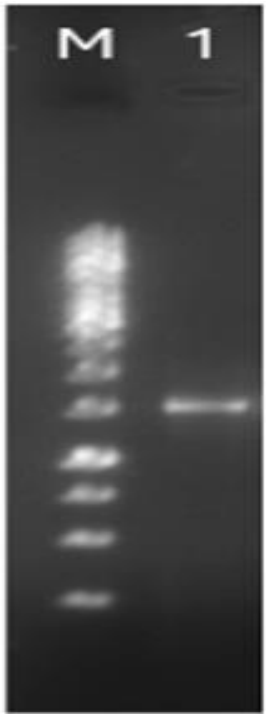

Fig. 3. PCR amplified genomic DNA of the microbial isolate (M – Ladder, 1-PSB 67)

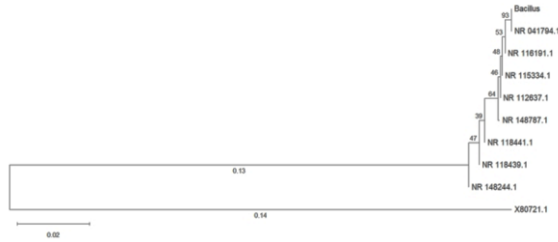

Fig. 4. Phylogenetic tree based on the 16S rRNA sequences of *Bacillus pumilus* was generated by the pairwise alignemnt using the neighbour-joining method included in MEGA12 software. The scale bar indicats 0.02 substitutions per nucleotide

DISCUSSION

Pikovskaya's agar is a selective medium that can be used routinely to screen the effeciency of phosphate solubilization of the microorganisms resulting the formation of clear halo zone around the colonies by converting the insoluble form of calcium phosphate in the medium into its soluble form (Nilsson *et al.*, 2003). Previous report suggests that the *Bacillus pumilus* can effectively be used as a phosphate solubilizing organism to enhance the plant growth and development by releasing the organic acids such as oxalic acid, formic acid, maleic acid, citric acid, etc., which in turn lowers the pH and aids in the conversion of the phosphate present in its insoluble form (Dipta *et al.*, 2017).

Since Mangroves are the boon to the scientific community because of its astonishing microbial community, the existing study deals with the isolation of Phosphate solubilizing bacteria from the Muthupet mangrove sediments with the geographical coverage of 120 square kilometers commonly known as Muthupet lagoon as many rivers gets connected to it.

This forest is primarily found in Tiruvarur district extending to Thanjavur and Nagapatinam is yet an unexplored area of research in PSB.

The soil samples collected underneath *E. agallocha* L contains the phospahte solubilizing bacteria evidenced by the development of distinct halo zones around the microbial colonies as a result of utilizing the tri calcium phosphate $(Ca_3Po_4)_2$ present in the medium. These isolates were then screened and the pure cultres were obtained. These isolates were then measured quantitatively and the PSI was calculated to obtain the isolate with highest solubilization. Then the isolate was PCR amplified and subjected to genome identification by Sanger sequencing. The phylogenetic tree based on the16S rRNA of the isolate PSB 67 was constructed with the closely related species of *Bacillus* and *E. coli* as an outgroup obtained from NCBI. The sequences were submitted to genbank. Due to their highest solubilization efficiency, these cultures might be checked for various parameters like quantitative analysis, pH, temperature and several other parameters that help to determine the usage of these isolates as a bio inoculum for the plant growth promotion. Additional researches need to be carried out in the soil to ensure the functional competency of those PSB for crop growth before entering actual field conditions.

CONCLUSION

In conclusion, the bacterial strain from the Muthupet mangrove soil was isolated and purified and characterized phenotypically and identified through 16S rRNA gene sequencing. The bacterial strain was recognized as belonging to the Bacillus genus. From this study , the effective Phosphate solubilizing bacteria isolated was measured qualitatively. Further studies should be carried out in quantifying the amount of phosphate solubized by the bacteria and several other parameters. It is also necessary to evaluate the plant growth promoting properties of the obtained isolate.

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REFERENCES

Annizah IN, Alawiyah DD, Susetyo RD, Surtiningsih T, Nurhariyati T. 2021. Phosphate solubilizing bacteria isolated from Tuban mangrove soil, Indonesia. IOP Conference Series: Earth and Environmental Science **762**(1), 012007. https://doi.org/10.1088/1755-1315/762/1/012007

Arumugam M, Panneerselvam R. 2012. Micropropagation and phenolic exudation protocol for *Excoecaria agallocha* – an important mangrove. Asian Pacific Journal of Tropical Biomedicine **2**(2), S1096-S1101. https://doi.org/10.1016/S2221-1691(12)60368-2

Arumugam M, Pawar UR, Gomathinayagam M, Lakshmanan GMA, Panneerselvam R. 2012. Antibacterial and antioxidant activity between micropropagated and field grown plants of *Excoecaria agallocha* L. International Journal of Plant, Animal and Environmental Sciences **3**(3), 235.

Dipta B, Kirti S, Bhardwaj S, Gupta S, Kaushal R. 2017. Phosphate solubilizing potential of *Bacillus pumilus* for the enhancement of cauliflower (*Brassica oleracea* var. *botrytis* L.). Ecology, Environment and Conservation **23**, 1541-1548.

Ezawa T, Smith SE, Smith FA. 2002. P metabolism and transport in AM fungi. Plant and Soil **244**, 221-230. https://doi.org/10.1023/A:1020258325010

Gyaneshwar P, Naresh Kumar G, Parekh LJ, Poole PS. 2002. Role of soil microorganisms in improving P nutrition of plants. Plant and Soil **245**, 83-93. https://doi.org/10.1023/A:1020663916259

Hamid AAA, Hamdan S, Ariffin SHZ, Huyop F. 2010. Molecular prediction of dehalogenase producing microorganism using 16S rDNA analysis of 2,2-dichloropropionate (Dalapon) degrading bacterium isolated from volcanic soil.

Haswania H, Karim H, Azis AA, Iriany N, Jumadi O. 2021. Isolation and characterization of phosphate solubilizing bacteria from corn rhizosphere.

Isenberg HD. 1998. Essential procedures for clinical microbiology. ASM Press, Washington, DC, pp. 216-221.

Mondal S, Ghosh D, Ramakrishna K. 2016. A complete profile on blind-your-eye mangrove *Excoecaria agallocha* L. (Euphorbiaceae): Ethnobotany, phytochemistry, and pharmacological aspects. Pharmacognosy Reviews **10**(20), 123. https://doi.org/10.4103/0973-7847.194049

Nilsson WB, Paranjype RN, DePaola A, Strom MS. 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. Journal of Clinical Microbiology **41**(1), 442-446. https://doi.org/10.1128/jcm.41.1.442-446.2003

Pande A, Pandey P, Mehra S, Singh M, Kaushik S. 2017. Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. Journal of Genetic Engineering and Biotechnology **15**(2), 379-391. https://doi.org/10.1016/j.jgeb.2017.06.005

Patil RC, Manohar SM, Katchi VI, Rao AJ, Moghe A. 2012. Ethanolic stem extract of *Excoecaria agallocha* induces G1 arrest or apoptosis in human lung cancer cells depending on their P53 status. Taiwania **57**(2), 89-98.

Patil RC, Manohar SM, Upadhye MV, Katchi VI, Rao AJ, Mule A, Moghe AS. 2012. Anti reverse transcriptase and anticancer activity of stem ethanol extracts of *Excoecaria agallocha* (Euphorbiaceae). Ceylon Journal of Science (Biological Sciences) **40**(2). http://dx.doi.org/10.4038/cjsbs.v40i2.3931

Zaidi A, Khan MS, Ahemad M, Oves M, Wani PA. 2009. Recent advances in plant growth promotion by phosphate-solubilizing microbes. In: Microbial strategies for crop improvement, pp. 23-50. https://doi.org/10.1007/978-3-642-01979-1_2