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Isolation and Characterization of Phosphate-solubilizing Bacteria (PSB) from Bhitarkanika Mangrove Sediments

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Abstract: The phosphate solubilizing activity of twenty bacterial isolates obtained on Pikovskaya's medium from sediments of Bhitarkanika mangrove forest, India were studied. Out of the 20 isolates obtained, 16 were selected on the basis of halo zone on the above medium. These 16 isolates were identified using PIBW in software after Gram's staining, biochemical characterization, enzymatic assay, sugar utilization and antibiotic sensitivity tests. From the phosphate solubilization index, the most efficient phosphate solubilizers selected were $S_{7,}$ S_{12}, $S_{13,}$ S_{20} and identified as *Bacillus psychrophilus, Bacillus licheniformis, Pseudomonas aeruginosa* and *Pseudomonas cepacia* respectively, producing largest halos of approximately 19 - 23 mm within 8 days of incubation. Spectrophotometric quantification of phosphate solubilization showed that all bacterial species solubilized insoluble phosphate well in a liquid medium which was accompanied by a significant drop in pH from an initial pH 7.0 to 4.3- 5.0 of the medium after 72 hrs. The HPTLC analysis of cell-free spent culture medium from the four bacteria demonstrated that lactic acid was produced by all the bacterial isolates indicating production of organic acids by these mangrove microorganisms as a possible mechanism involved in the solubilization of insoluble calcium phosphate.

Key words: Mangrove, phosphate-solubilizing bacteria (PSB), P-solubilization index, organic acids, Pikovskaya's medium.

Introduction

Soil microbes play an important role in solubilizing the bound P into available forms. In addition, microorganisms such as phosphate solubilizers, free living and associated nitrogen fixers, can interact in the rhizospheric soil. The highly productive and diverse microbial community living in mangrove ecosystems continuously transforms nutrients from dead mangrove vegetation into sources of nitrogen, phosphorous and other nutrients that can be used by the plants and in turn the plant-root exudates serve as a food source for the microbes [22]. The saline and anaerobic condition makes survival of most microbes that are crucial in nutrient mineralization. Due to high productivity, turnover rates, and permanent exchange between terrestrial and marine ecosystems, mangroves play a crucial role in the biogeochemical cycling of phosphorus and other nutrients. Phosphorus being an essential and important element in transient zones such as estuaries and coastal environments is thought to control marine productivity over geological timescales [4, 11, 21]. The biogeochemical cycling of phosphorus in the sediment largely depends on its chemical speciation. It can occur in the sediment associated with calcium or iron or aluminum hydroxides or can be adsorbed on the surface of minerals or present in organic compounds. Fungi and bacteria have the ability to solubilize these

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compounds [12]. Although several mechanisms may be involved, the main one is through the production of organic acids [16]. It is assumed that these organic acids solubilize insoluble forms of phosphate to a usable form, such as orthophosphate, thus increasing the potential availability of phosphate for plants,[13] whereas the organic phosphorus compounds are decomposed and mineralized by many enzymatic complexes produced especially by heterotrophic bacteria. These enzymes occur either as free dissolved ones or are attached outside the cell. Analysis of microbial biodiversity from these ecosystems will help in isolating and identifying new and potential microorganisms having high specificity for various applications. The present work aims to study the occurrence and identification of PSB from the sediments of Bhitarkanika.

Materials and methods

Collection of samples

Sediment samples were collected aseptically in a sealed, sterile plastic bag from the Dangamala areas of Bhitarkanika mangrove forests. Two sediment samples were collected randomly from 30 cm below water surface in aseptic condition with the help of sterile spade. The collected sediment samples were mixed properly and transported to the laboratory where it was stored in refrigerator at 4°C for 24 hr before analysis.

Isolation of PSB

For the isolation of PSB, solubilization of precipitated tricalcium phosphate-TCP $Ca_3(PO_4)_2$ in unbuffered solid agar medium plates has been used widely as the initial criterion. Ten fold serial dilutions followed by spread plate technique were used to isolate various PSB from the sediment samples on Pikovskaya's agar and incubated at 30°C for 48 hours. All the experiments were conducted in triplicates. Observing the halo zone around the colony on Pikovskaya's agar medium, characteristics of PSB due to production of the enzyme phosphatase, preliminary isolation was done. Then colonies of different morphology were selected and sub-cultured on fresh nutrient agar plates to obtain pure culture of the isolates and maintained on nutrient agar slants at low temperature (4°C) for further characterization.

Identification of the bacterial isolates

Identification of bacterial isolates was done on the basis of their colony characteristics on the basal media, Gram's reaction, and biochemical tests[7]; keeping in view the tests required for bacterial identification using PIBWin software[5], sugar utilization tests and antibiotic tests. The isolates were also tested for various enzyme productions for amylase, gelatinase, urease, and caseinase on specific media[7].

Qualitative estimation of phosphate solubilization

A plate assay was done using Pikovskaya's agar medium for qualitative estimation of phosphate solubilization by all the 16 strains of PSB isolates. Total diameter (Diameter of colony + holo zone diameter) and diameter of colony was measured on different days i.e. 4, 6 and 8 to find out the solubilization index [9].

Phosphate Solubilization Index = A/B

A= total diameter (colony + halo zone), B= diameter of colony

Quantitative assay for phosphate solubilization

The PSB isolates found to be positive for TCP solubilization were further analyzed for their ability to solubilizing the inorganic tri-phosphates in the liquid medium. Hundred milliliter of Pikovskaya's broth in a 250 ml Erlenmeyer flask were inoculated with the four best bacterial isolates and incubation was done at 30°C for 3 days in a rotary shaker incubator at 200 rpm. Each treatment was done in triplicate. The pH of the medium was recorded by pH meter (pH system 361, Systronics, India). After incubation the bacterial cultures were centrifuged for 10 minutes at 10,000 rpm. Uninoculated broth was taken as control. The amount of soluble phosphate was measured spectrophotometrically at OD 600nm[18].

Extraction of Organic Acids

Ten ml of bacterial samples were taken from each of 4 samples, and centrifuged at 2000 rpm/min (Remi centrifuge) for 20 minutes followed by filtration of the upper phase using Whatman No.1 filter paper. The centrifugation was repeated 3 times & the filtrates were collected.

High performance thin layer chromatography

The stock solutions of the standards (0.1M of lactic, oxalic and citric acids) were prepared [15]. From this stock solution, suitable concentration of working standard was prepared. The samples and standards were spotted on 10 cm × 10 cm pre-coated silica gel 60 F TLC plate (Merck) of uniform thickness (0.2mm) by using HPTLC-linomat 5 system. After this, the plate was developed in saturated developing tank containing ethyl acetate and chloroform in the ratio 3:2. After development of the plate, the plate was removed from the tank, dried well, viewed in a UV cabinet HPTLC Reprostar-3 at visible UV (210 nm). The plate was scanned at 210 nm using HPTLC-TLC scanner and the spectrum was taken which indicate the presence of the organic acids.

Results and discussion

In the present study, 20 isolates of PSB obtained from the sediment of Bhitarkanika mangrove forest were preliminary screened for phosphate solubilization on Pikovskaya's medium [1]. From these, 16 isolates were found to be positive for phosphate solubilization based on the halo zone on Pikovskaya's medium. The isolates were identified on the basis of morphological, Gram's staining and biochemical tests using PIBWin software (Table 1). Out of the 16 isolates 5 were gram positive rod, 10 were gram negative rod and one was gram positive cocci. Again the occurrence of *Pseudomonas* sp. (n=5), *Bacillus* sp. (n=4) is similar to the previous report of [25], who had isolated 6 phosphate solubilizing bacteria such as *Pseudomonas* sp. (n=2), *P. cepacia*, *P. stutzeri*, *B. lichiniformis*, *B. schlegelii* and *Bacillus* sp. from Bhitarkanika mangrove soils. But in addition to the above organisms, the other isolates were i.e., *Brevibacillus laterosporus*, *Cellobiosococcus* sp., *Ralstonia pickettii*, *Burkholderia pseudomallei*, *Burkholderia gladioli*. The study corroborates with the present findings where they had isolated PSB like *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus atrophaeus*, *Paenibacillus macerans*, *Vibrio proteolyticus*, *Xanthobacter agilis*, *Enterobacter aerogenes*, *Enterobacter taylorae*, *Enterobacter asburiae*, *Kluyvera cryocrescens*, *Pseudomonas stutzeri*, and *Chryseomonas luteola* from the rhizosphere of mangroves [28].

All the 16 isolates were tested for the production of different enzymes i.e. amylase, gelatinase,

Table 1. Identification of the PSB isolates

Isolates code	Name of the isolates	Id score
S_1	*Bacillus cereus*	0.995
S_2	*Cellobiosococcus sp.*	0.973
$\mathbf{S_3}$	*Brevibacillus laterosporus*	0.985
S_4	*Bacilluscarotarum*	0.974
S_5	*Burkholderiagladioli*	0.979
S_6	*Burkholderiapseudomallei*	0.965
S_7	*Bacilluspsychrophilus*	0.983
S_8	*Pseudomonasluteola*	0.956
S_9	*Ralstonia pickettii*	0.963
S_{12}	*Pseudomonasaeruginosa*	0.992
S_{13}	*Bacilluslicheniformis*	0.953
S_{14}	*Pseudomonas luteola* (formerly classified as CDC group Ve-1)	0.968
S_{15}	*Flavobacterium meningosepticum*	0.973
S_{18}	*Pseudomonasalcaliphila*	0.967
S_{19}	*Stenotrophomonas maltophila*	0.985
S_{20}	*Pseudomonascepacia*	0.993

urease and caseinase (Table 2). All the isolates were positive for urease production. Except S_3 & S_5, all other isolates were caseinase positive. Only S_6, S_8, S_{13}, S_{14}, S_{15} &S_{19} were amylase positive. Gelatinase were produced by all the isolates except S_2.

The solubilization index was calculated at different day's interval like 4, 6 & 8 days (Table 3). Most strains were able to solubilize tri- calcium phosphate $Ca_3(PO_4)_2$ with maximum indexes of solubilization on 8th day of incubation. From the phosphate solubilization index, the most efficient P solubilizers were S_7, S_{12}, S_{13}, S_{20}, i.e. *Bacillus psychrophilus, Bacillus licheniformis, Pseudomonas aeruginosa* and *Pseudomonas cepacia* respectively. These 4 isolates were selected among all the other PSB isolates; which show maximum phosphate solubilizing halozone (≥15 mm) (Table 3). The solubilization index of the above 4 isolates is well comparable with the findings of [8]. According to them good phosphate solubilizers produce halos around their colonies having diameter higher than 15 mm. P solubili-zation efficiency of one *Bacillus* sp. NPSBS isolate after 96 hours of incubation [17]. However some other authors have reported 3 days, more than 10 days and even up to 15 days to be the optimum incubation period of P solubilization by various bacterial isolates [23,24,3].

The result showed that *Pseudomonas aeruginosa* was the most efficient phosphate solubilizer on Pikovskaya's agar plates with solubilization index of 1.72 at 8th day of incubation. Measurement of solubilization index ranged from 1.0 to 1.72. The solubilization of tri- calcium phosphate in the liquid medium by the different phosphate solubilizing bacterial isolates was accompanied by a significant drop in pH from an initial pH of 7.0 to 4.3 of the medium after 72 hrs. The drop in pH was correlated with elevated levels of P solubilization by the isolates. In case of *Bacillus* sp. the maximum pH drop was recorded as 3.5 [26]. The decline in pH clearly indicated the pro-duction of organic acids which suggested that acidification of the culture supernatants can be the main mechanism for phosphate solubilization [27,19,14].

Our findings are very well supported to the occurrence of *Pseudomonas* sp. as a potent phosphorus solubilizer [10]. The occurrences of PSB indicate that the bacteria play a crucial role in phosphorus cycling in the soil sediments of mangrove forest[2] and also a good indicator of recycling of organic and inorganic matter in mangrove environment. IPSB *Vibrio* sp. and

Table 2. Enzymatic activity of the PSB isolates on different media

Isolates code	Amylase	Gelatinase	Urease	Caseinase
S_1	-	+	+	+
S_2	-	-	+	+
S_3	-	+	+	-
S_4	-	+	+	+
S_5	-	+	+	-
S_6	+	+	+	+
S_7	-	+	+	+
S_8	+	+	+	+
S_9	-	+	+	+
S_{12}	-	+	+	+
S_{13}	+	+	+	+
S_{14}	+	+	+	+
S_{15}	+	+	+	+
S_{18}	-	+	+	+
S_{19}	+	+	+	+
S_{20}	-	+	+	+

Table 3. Halo zone at different days (mm) and Solubilization index at different days

Isolates code	Halo zone at different days(mm) and Solubilizationindex at differentdays					
	4 days		6 days		8 days	
	Halo zone	Solubilization index	Halo zone	Solubilization index	Halo zone	Solubilization index
S_1	11	1.25	12	1.42	12	1.28
S_2	7	1.1	8	1.22	9	1.12
S_3	10	1.04	11	1.06	13	1.00
S_4	6	1.05	7	1.08	8	1.07
S_5	9	1.07	10	1.09	10	1.08
S_6	6	1.00	6	1.00	6	1.00
S_7	15	1.43	16	1.48	19	1.39
S_8	4	1.00	4	1.00	4	1.00
S_9	9	1.1	10	1.22	11	1.12
S_{12}	17	1.62	20	1.67	23	1.72
S_{13}	14	1.43	17	1.48	19	1.39
S_{14}	12	1.34	15	1.37	19	1.35
S_{15}	5	1.04	6	1.06	7	1.00
S_{18}	14	1.09	15	1.15	15	1.12
S_{19}	8	1.03	11	1.05	11	1.07
S_{20}	15	1.53	18	1.57	20	1.54

Significant PSB > 15mm halo zone

Table 4. Quantitative assay for phosphate solubilization

Isolates code	pH	Soluble phosphorus (mg L^{-1})
S_7	4.83	1.3392
S_{12}	4.39	1.8723
S_{13}	4.91	1.1982
S_{20}	4.68	1.6605

Pseudomonas sp. strain solubilize phosphorus 0.5 to 0.55 mg/L isolated from the marine sediment. The phosphate solubilizing activity of the present isolates are comparable to their study [20].

These four efficient PSB were also used for the determination of organic acid production by HPTLC analysis. Referring the literature, lactic, oxalic and citric acid were taken as standards (Fig. 1-3). The HPTLC analysis showed that lactic acid was produced by all the bacterial isolates but oxalic and citric acids were not found in any of them (Fig. 4-7). There are previous reports of PSB in favour of *Bacillus* sp. i.e. *Bacillus amyloliquefaciens*, *Bacillus licheni-formis*, *Bacillus atrophaeus* isolated from mangrove rhizosphere producing lactic acids in addition to succinic, isovaleric, isobutyric and acetic acids [28]. Other workers had detected the production of citric, gluconic, lactic, succinic and propionic acids by PSB [6], which corroborates with the present findings.

Conclusion

The above study demonstrated that many of the

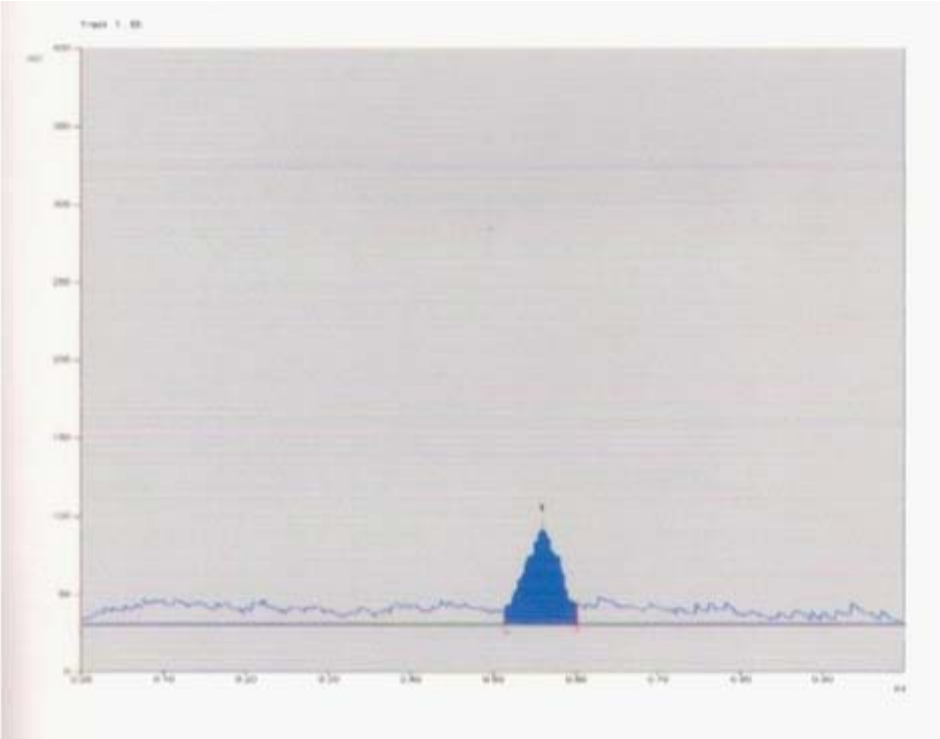

Figure 1. HPTLC Charomatogram of standard citric acid

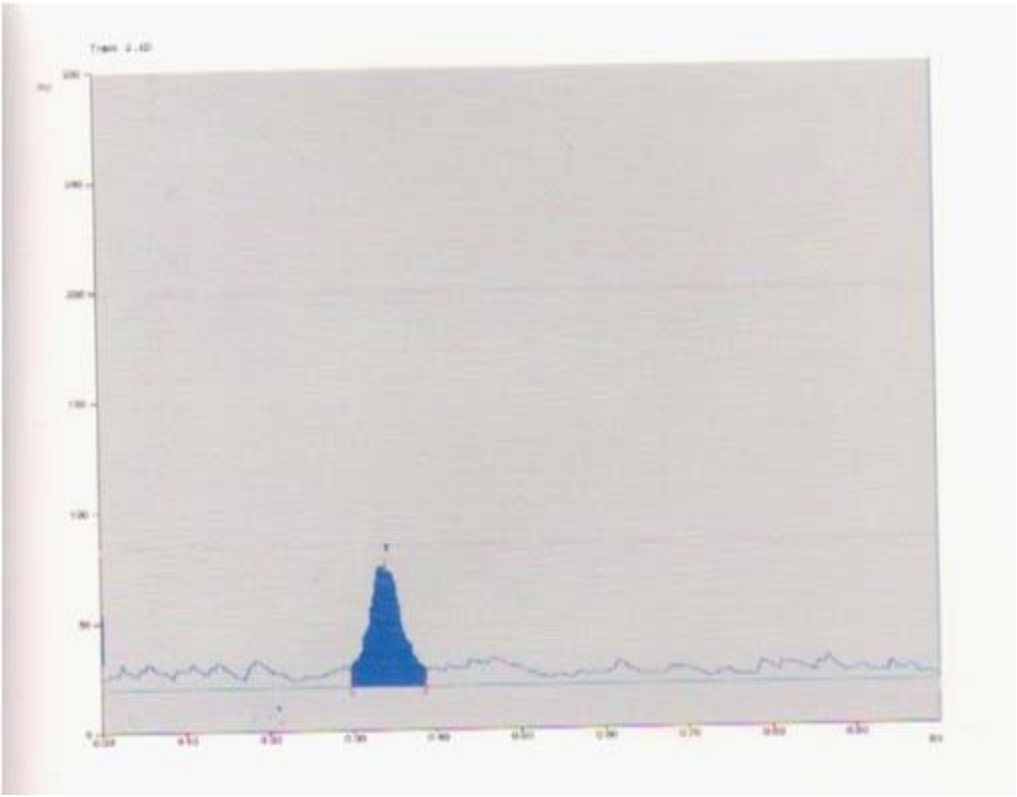

Figure 2. HPTLC Charomatogram of standard oxalic acid

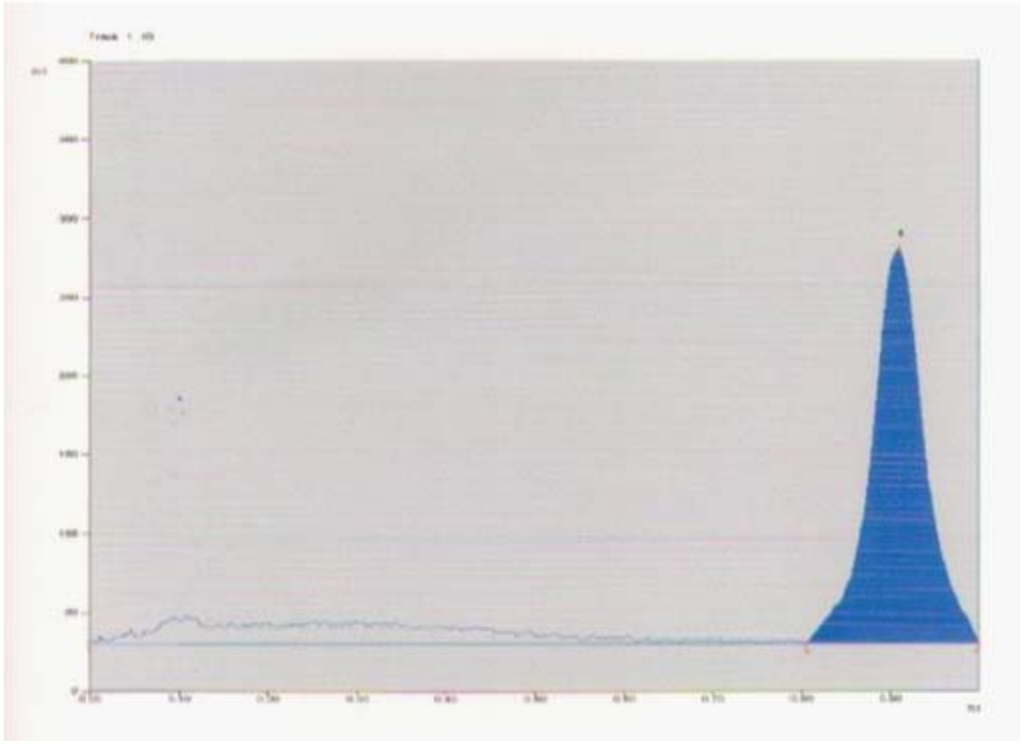

Figure 3. HPTLC Charomatogram of standard lactic acid

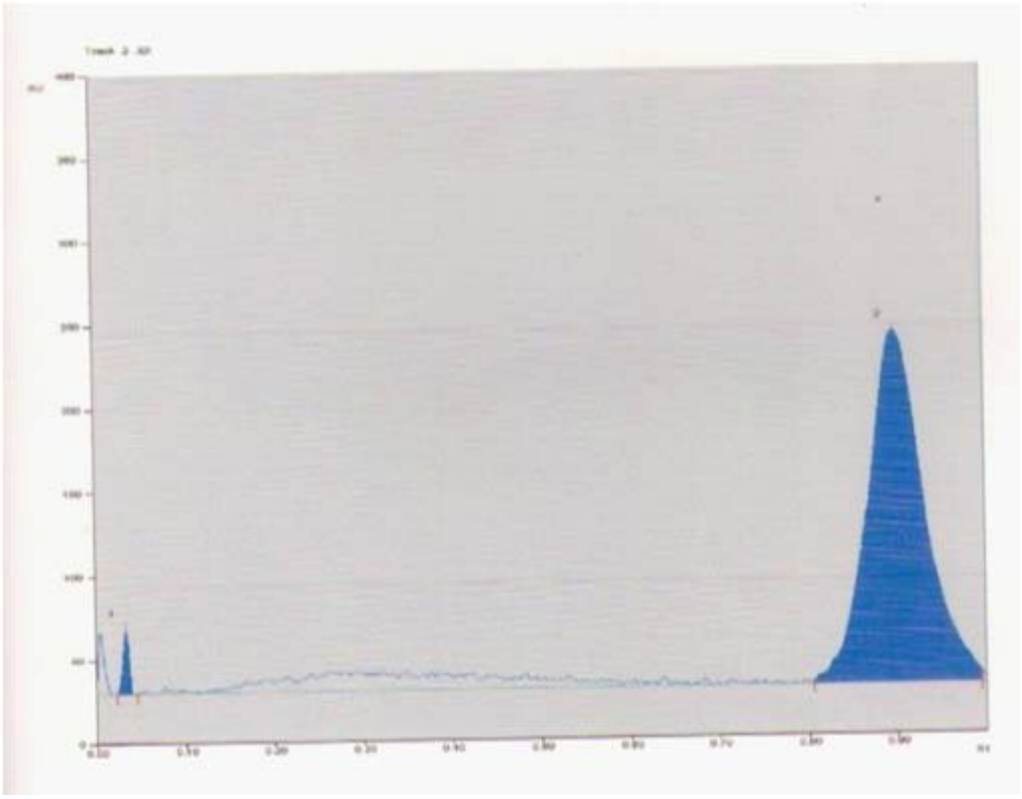

Figure 4. HPTLC Charomatogram of isolate S_7

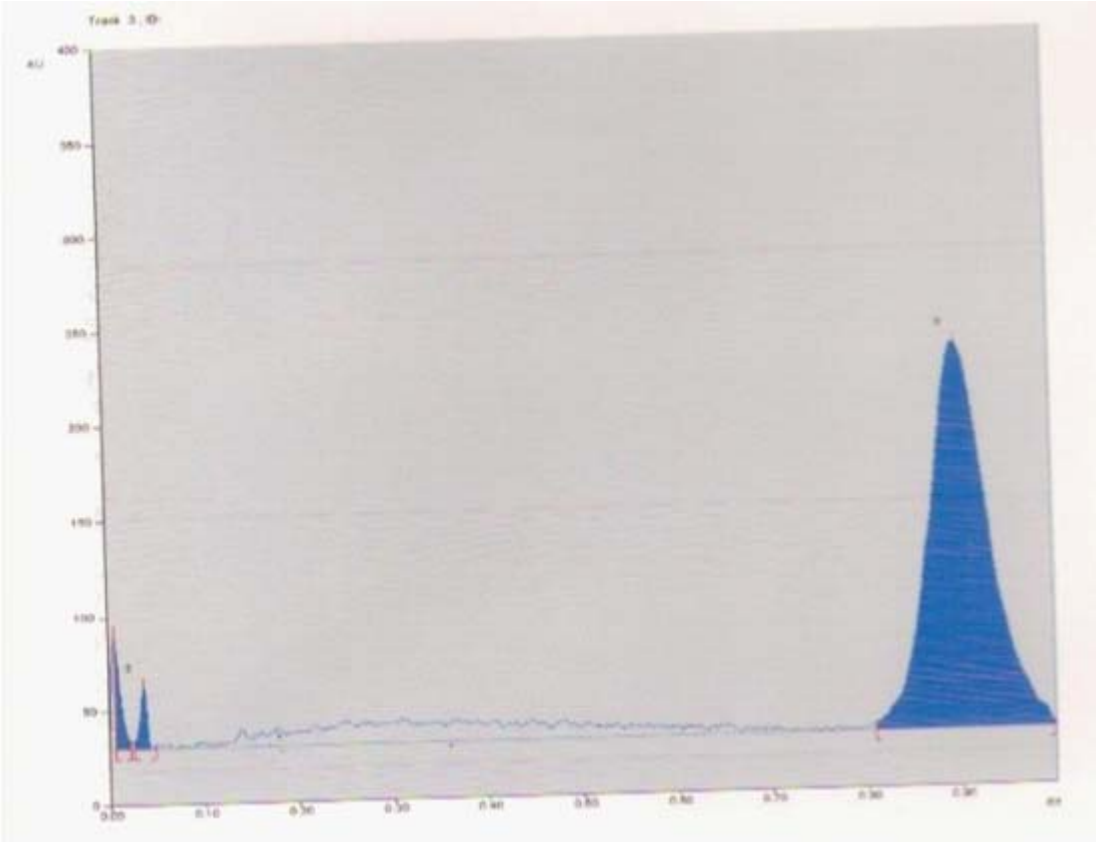

Figure 5. HPTLC Charomatogram of isolate S_{12}

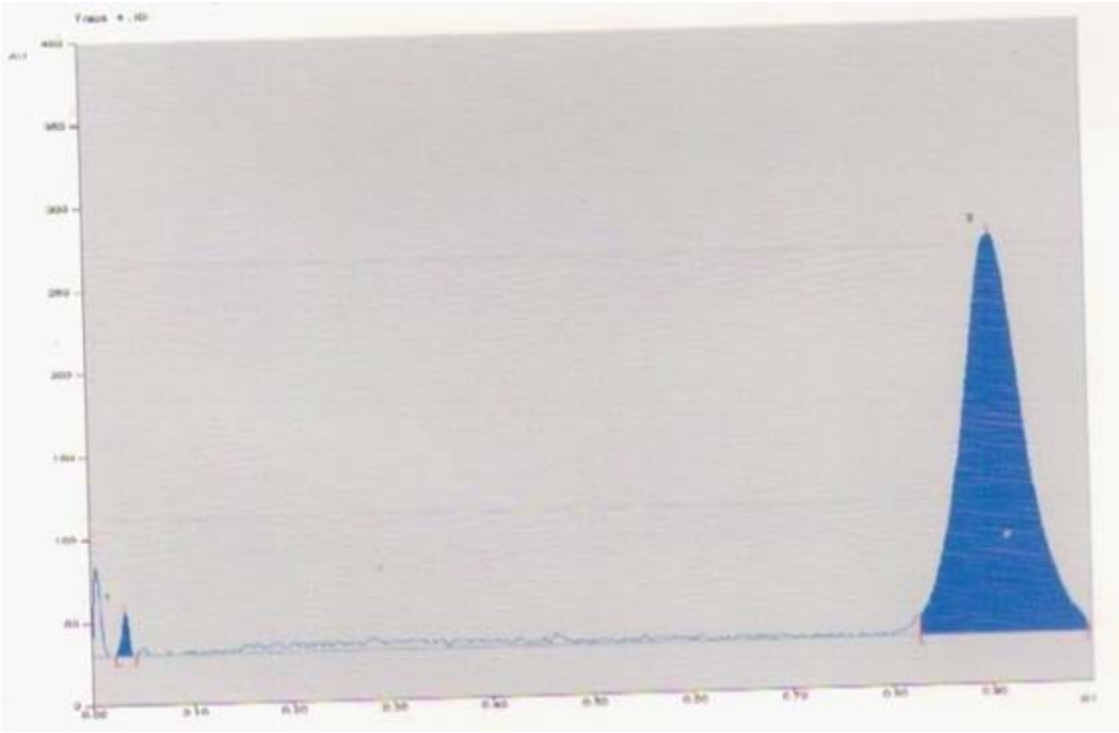

Figure 6. HPTLC Charomatogram of isolate S_{13}

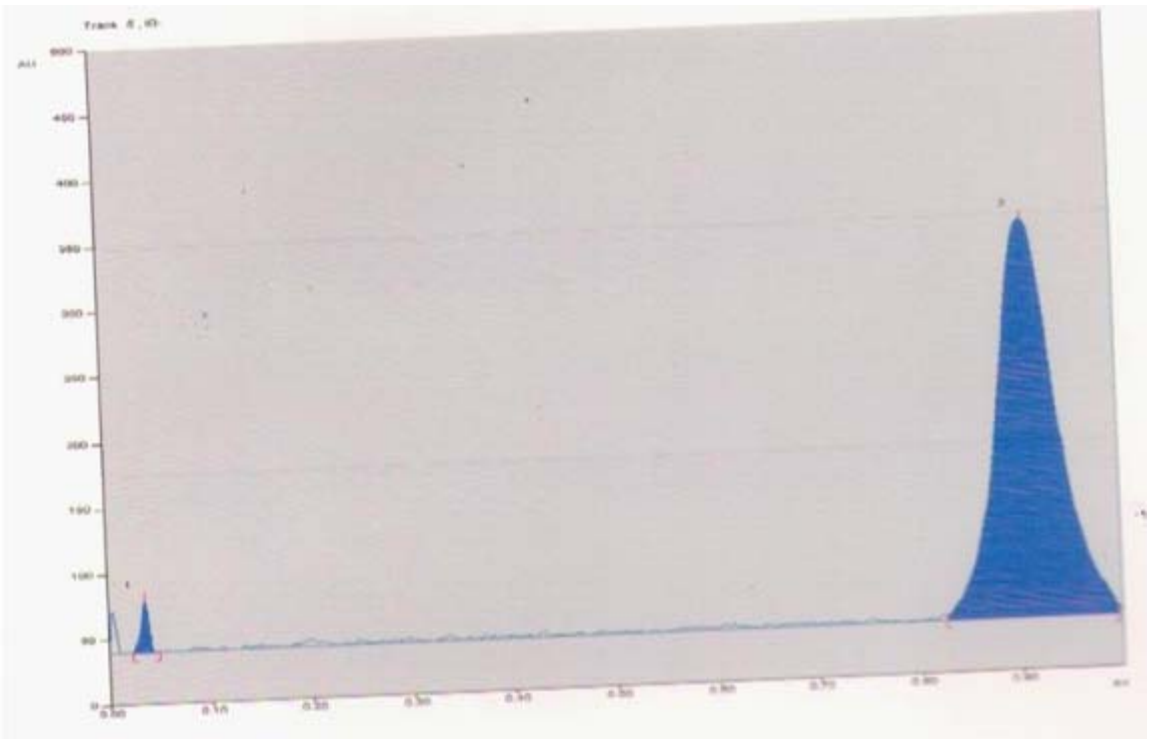

Figure 7. HPTLC Charomatogram of isolate S_{20}

bacteria had phosphate solubilizing or mineralizing properties and the ability was found in different organisms like *Bacillus* sp., *Pseudomonas* sp., *Brevibacillus laterosporus, Cellobiosococcus* sp., *Ralstonia pickettii, Burkholderia pseudomallei* and *Burkholderia gladioli*. The microbial diversity of Bhitarkanika mangrove ecosystem is not fully explored so this is probably a pioneer study to reveal that this mangrove ecosystem is a good source of diverse genera of phosphate solubilizers, which are responsible for the maintenance of the mangrove ecosystem and can be exploited in field condition for mineralization of insoluble phosphates to the available form for plant uptake.

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References

1. **Ahuja, A., Ghosh, S.B., and D'Souza, S.F. (2006).** Isolation of a starch utilizing, phosphate solubilizing funguson buffered medium and its characterization. Nucl. Agri. and Bio. Division. 46: 120-129.
2. **Alongi, D.M. (1988).** Bacterial productivity and microbial biomass in tropical mangrove sediments. Microb. Ecol. 15: 59-79
3. **Banerjee, S., Palit, R., Sengupta, C. and Standing, D. (2010).** Stress induced phosphate solubilization by Arthrobacter sp. and Bacillus sp. isolated from tomato rhizosphere. Australian Journal of Crop Science. 4: 378-383
4. **Bauerfeind, E., Hickel, W., Niermann, U., Westernhagen, H.V. (1990).** Phytoplankton biomass and potential nutrient limitation of phytoplankton development in the southeastern North Sea inspring1985 and 1986. Neth J. Sea. Res. 25: 131-142
5. **Bryant, T.N. (2004).** PIBWin-software for probabilistic identification. J. Appl. Microbiol. 97:

1326-1327.

6. **Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., Young, C.C. (2006).** Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil Ecol. 34: 33-41.
7. **Collins, C.H., Lyne, P.M., Grange, J.M. (1989).** Collins and Lyne's Microbiological Methods. 6th Edition, Butterworth and Co. Ltd. London.
8. **DeFreitas, J.R., Banerjee, N.R. and Germida, J.J. (1997).** Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol. Fertil. Soils. 24: 358-364.
9. **Edi-Premono, Moawad, M.A., Vleck, P.L.G. (1996).** Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Ind. J. Crop Sci. 11: 13-23.
10. **Gupta, A., Meyer,J.M. and Goel, R. (2002).** Development of Heavy metal- resistant Mutants of phosphate Solubilizing Pseudomonas sp. NBRI 4014 and their characterization. Current Microbiology. 45: 323-327.
11. **Harrison, P.J., Hu, M.J., Yang, Y.P., Lu, X (1990).** Phosphate limitation inestuarine and coastal waters of China. J. Exp. Mar. Biol. Ecol. 140: 79-87
12. **Illmer, P. (1995).** Solubilization of hardly-soluble AIPO4 with Phosphate solubilizing microorganism. Soil Biol. Biochem. 27: 265-270.
13. **Kucey, R.M.N. (1983).** Phosphate-solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. Can. J. Soil Sci. 63: 671-678.
14. **Ma, Y., Rajkumar, M. and Freitas, H. (2009).** Inoculation of plant growth promoting bacterium Achromobacter xylosoxidans strain Ax10 for the improvement of copper phytoextraction by Brassica juncea. Journal of Environmental Management. 90: 831-837.
15. **Malboobi, M. A., Parviz, O., Mandana, B., Elaheh, S., Sara, M., Bagher, Y, Ali, D., Kambiz, M.H. (2009).** "Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates". World Journal of Microbiology and Biotechnology 25(8): 1471-1477.
16. **Nahas, E. (1996).** Factors determining rock phosphate solubilization by microorganism isolated from soil. W J. Microbiol. Biotechnol. 12: 567-572.
17. **Narveer, Vyas, A., Kumar, H. and Putatunda, C. (2014).** In vitro phosphate solubilization by Bacillus sp. NPSBS 3.2.2 obtained from the cotton plant rhizosphere. Biosciences Biotechnology Research Asia. 11: 401-406.
18. **Patel, D. and Parmar, P. (2013).** Isolation and screening of phosphate solubilizing bacteria from sunflower rhizosphere. Global. Journal of Bioscience and Biotechnology. 2(3): 438-441
19. **Pérez, E., Sulbarán, M., Ball, M.M. and Yarzábal, L.A. (2007).** Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biology and Biochemistry. 39: 2905-2914.
20. **Promod, C. K. and, Dhevendaran K. (1987).** Studies on phosphobacteria in Cochin backwater. J. Mar. Biol. Assoc. India 29: 297-305
21. **Ruttenberg, K.C. (2004)** The global phosphorus cycle. In: SchlesingerWH (ed) Treatise on geochemistry (Volume 8) biogeochemistry.Elsevier Pergamon, Amsterdam, pp. 585-43
22. **Sahoo, K. and Dhal, N.K. (2009).** Potential microbial diversity in mangrove ecosystems: A review. Indian Journal of Marine Sciences. 38(2): 249-256.
23. **Sahu, M.K., Sivakumar, K. and Kannan, L. (2007).** Phosphate solubilizing actinomycetes in the estuarine environment: An inventory. Journal of Environmental Biology. 28: 795-798.
24. **Sridevi, M. and Mallaiah, K.V. (2009).** Phosphate solubilization by Rhizobium strains. Indian Journal of Microbiology. 49: 98-102.
25. **Thatoi, H.N., Behera, B.C. and Mishra, R.R. (2013).** Ecological role and biotechnological

potential of mangrove fungi: a review, Mycology: An International Journal on Fungal Biology, 4(1): 54-71

26. **Tripti, V.K. and Anshumali. (2012).** Phosphate Solubilizing Activity of Some Bacterial Strains Isolated from Chemical Pesticide Exposed Agriculture Soil. International Journal of Engineering Research and Development. 39: 01-06.
27. **Vassilev, N., Medina, A., Azcon, R. and Vassileva, M. (2006).** Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake. Plant and Soil. 287: 77-84.
28. **Vazquez, P., Holguin, G., Puente, M.E., Lopez-Cortes, A., and Bashan, Y. (2000).** Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biol. and Fert. of Sols. 30: 460-468.