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EXPERIMENTAL ARTICLES

Studies on Mineral Phosphate Solubilization by Cyanobacteria *Westiellopsis* and *Anabaena*[1]

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Abstract—Two diazotrophic cyanobacteria, *Westiellopsis prolifica* and *Anabaena variabilis* were evaluated for elucidating the possible mechanism of mineral phosphate solubilization. Phosphate starved cyanobacteria evaluated for the presence of organic acids, extracellular compounds or enzymes that might have been produced and promoted the mineral phosphate solubilization with Mussorie Rock Phosphate and Tricalcium Phosphate as substrates. Both the cultures did not reveal production of organic acids throughout the incubation period when checked for decrease in media pH of the media and thin layer chromatography. Thin layer chromatography of culture filtrates showed the presence of hydrocarbon like compound. Further analysis of the culture filtrates with gas liquid chromatography, a single peak near to the retention time of 7.6 was observed in all extracts of culture filtrates irrespective of phosphate source. UV-visible spectra of culture filtrates revealed the absorption maxima of 276 nm. Gas chromatographic-mass spectrometric analysis of culture filtrates showed most intense peak in the electron impact (EI) ionization was at m/z 149 and molecular ion peaks at m/z 207 and 167, inferring the presence of phthalic acid. Among the mechanisms in mineral phosphate solubilization, it was evident that these cyanobacteria used phthalic acid as possible mode of P solubilization.

Keywords: *Anabaena variabilis*, gas liquid chromatography, GC-MS, *Westiellopsis prolifica*

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The importance of microorganisms in soil nutrient cycling and their role in plant nutrition has been realized for a long time. Diverse groups of microorganisms such as bacteria, fungi, actinomycetes and yeasts are known to solubilize insoluble forms of inorganic P [1] by bringing about favorable changes in soil reaction in the soil microenvironment leading to solubilization of inorganic phosphate sources. On an average the uptake of added phosphatic fertilizers to plants ranges from 15–25%, due to chemical fixation of P as either iron or aluminum phosphates in acid soils and calcium phosphates under alkaline soil conditions. Many microorganisms can solubilize inorganic phosphates, which are largely unavailable to plants.

Cyanobacteria, also called Blue-Green Algae (BGA) are primary colonizers in different habitats. The practical utility of these organisms as a source of organic nitrogen fertilizer for rice has been well recognized [2]; however, phosphorus is necessary for the growth and nitrogen fixation by these organisms. Blue green algae, like P solubilizing bacteria are known to have the ability to mobilize bound phosphates. They have been shown to solubilize insoluble $Ca_3(PO_4)_2$ [3], $FePO_4$ [4], $A1PO_4$ [5] and hydroxyapatite ($Ca_5(PO_4)_3 \cdot OH$) [6] in soils, sediments or in pure cultures. They are also known to solubilize organic sources of phosphorus [7]. Organic sources of phosphorus that are generated in plenty especially in deep water rice fields by breakdown of vegetative matters and animal excreta. In such situations, where cyanobacteria dominate over other microbes, they play a control role in P solubilization. Several theories have been advanced to explain the process of P solubilization by microorganisms. Among various mechanisms reported by which phosphate may be liberated from insoluble minerals by blue green algae include production of organic acids, synthesis of chelators, dissimilatory reduction of ferric ion and enzymatic solubilization or simultaneous operation of more than one of these methods [7]. However, no definite operative mechanism has been suggested for P solubilization and this makes it imperative to investigate and study phosphate solubilization in these microorganisms.

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Moreover, the ability of BGA to release soluble orthophosphate (Pi) from rock phosphate ore makes this phenotype of great potential importance for the development of eco-rational phosphate fertilizer technologies for agriculture.

MATERIAL AND METHODS

Cultures and growth conditions. Axenic cultures of heterocystous mineral phosphate solubilizing BGA *Anabaena variabilis* and *Westiellopsis prolifica* were obtained from the Centre for Conservation and Utilisation of Blue Green Algae of Indian Agricultural Research Institute, New Delhi, India [8]. The cultures were grown and maintained in N-free BG-11 medium [9] at 28 ± 2°C with 16/8 h L/D cycles at 2500–3000 Lux light intensity emanating from cool white fluorescent tubes. The mineral phosphate sources namely, Mussorie Rock Phosphate (MRP) was obtained from Pyrites and Phosphates Ltd., Dehradun, India and Tricalcium Phosphate (TCP) was procured from HiMedia Laboratories, Mumbai, India. The total phosphorus content in MRP and TCP was 18% and 20% respectively. The inoculum used for inoculating the experimental flasks was prepared by P starving the 10 day old actively growing cultures for 15 days on rotary shaker at 120 rpm. The P-starved cultures were grown in presence of alternate P sources MRP and TCP at varying concentrations (equivalent to 10, 20 and 30 mgP L^{-1} by replacing K_2HPO_4). Potassium chloride in equivalent amount (34.2 mg L^{-1}) was added in the medium to compensate potassium concentration. The experiments were conducted by taking 100 mL medium in 250 mL Erlenmeyer flasks in triplicate and inoculated with 2% P-starved inoculum.

Phosphorus solubilization and pH measurement. Total phosphorus and available phosphorus content in the algal cultures was estimated spectrophotometrically using method of Jackson [10] and Watanabe and Olsen [11] respectively. The pH of medium was recorded using undiluted temporal cell free supernatant using glass electrode at constant temperature of 28°C.

Thin Layer Chromatographic analysis. The organic acids produced by BGA strains were detected by thin layer chromatography as described by Gaur, [12] using the n-butanol : acetic acid : water solvent system (12 : 2 : 5) as described by Nordman and Nordmann [13]. The analysis of hydrocarbon like compounds in cell free extracts was carried out using hydrocarbon standards $C_{32,}$ C_{33}, C_{34} as reference prepared in hexane. The solvent system used was hexane : benzene (3 : 1).

Spectral determinations. The concentrated cell free extracts were subjected to scanning in UV-visible spectra of the compounds for analyzing absorbance maxima. Reactions were scanned in the range 230 nm to 700 nm. The absorption values obtained were plotted and an absorption maximum was calculated.

GLC and GC-MS analysis. The cell-free extracts containing the compound responsible for P-solubilization was separated by dissolving in ethyl acetate using column separator. The separated solvent (top layer) was filtered through anhydrous Na_2SO_4 in a funnel to absorb moisture content in the solvent. The filtered solvent was boiled in a water bath at 100°C. After solvent evaporated the residue in the vials left was dissolved in one mL of hexane and stored in screw cap vials. The samples (2 μL) were injected in Gas Liquid Chromatograph of Hewlett Packard 5890 A Series II Model with flame ionization detector and mega-bore column (HP-17, 10 m × 0.53 mm i.d.). Nitrogen was used as carrier gas with flow rate of 20 mL min^{-1} (operating conditions-column temperature: 100°C to 270°C with an increment of 10°C min^{-1}; injector temperature: 250°C; detector temperature: 250°C). The mass spectrometer was operated at electron ionization energy of 70 eV. Further same samples were also analyzed using GC-MS, HRGC Mega 2series of Fison Instruments (operating conditions-Initial temperature: 80°C; final temperature: 270°C; solvent delay: 3 min; ramp: 10°C min^{-1}; GC condition minutes: 2 min; GS standby minutes: 800 min). Instrumental library searches, comparison with authentic compounds and mass fragmentation patterns were used to identify the metabolites.

RESULTS

Phosphorus solubilization and pH of the medium. Periodic monitoring of total-P and available-P in culture supernatant showed that in case of P-starved cultures, total-P was negligible (Table 1) and in case of insoluble-P (MRP or TCP) it increases with concentration (20 mg and 30 mg P). In P-deficient cultures, most of the total-P was in available form and 70–80% of the total-P was as available-P, in presence of soluble source. In case of insoluble forms the available-P was less than total-P and available-P was more at lower concentration of insoluble form (10–20 mg P) than at higher concentration (30 mg P) in the medium (Table 2). However, available-P was marginally higher in case of MRP then TCP. In both the cyanobacterial case, amount of available-P was maximum during seventh day and gradually decreases with time and concentration. The rate of decrease was faster in case of soluble-P than in case of insoluble-P sources. The culture filtrates of both the cultures grown under soluble or insoluble source did not showed marked pH shift towards acidity and remains stable through the incubation period of 21 days.

Thin Layer Chromatography and Spectral determinations. The presence of organic acid in culture filtrates in chromatogram failed to revel presence of organic acid in comparison to standard one (gluconic,

Table 1. Time course effect of different phosphate sources on total phosphorus content[e] in cell free supernatant of BGA

Treatments		Control (−P)	K_2HPO_4	MRP (mg P L^{-1})			TCP(mg P L^{-1})		
Strains	Days			10	20	30	10	20	30
Westiellopsis prolifica	7	0.31	4.54	7.60	15.50	24.25	7.43	15.64	24.68
	14	0.32	3.85	7.04	14.66	22.86	7.15	14.08	22.25
	21	0.40	2.41	5.61	12.13	20.63	5.64	13.15	20.12
	28	0.38	2.11	5.04	11.38	18.48	4.81	11.45	18.61
	35	0.30	1.44	4.83	10.12	18.32	4.24	10.03	17.45
Anabaena variabilis	7	0.33	4.27	7.71	15.71	24.88	7.56	15.23	24.81
	14	0.26	3.57	6.72	14.18	23.18	6.96	14.06	22.74
	21	0.27	2.73	5.78	13.02	21.63	5.37	12.73	21.05
	28	0.27	1.74	4.90	11.32	19.09	4.21	11.25	19.10
	35	0.18	1.54	3.92	10.20	18.77	4.04	10.02	17.56
	S			SXT			SXTXD		
SEM ±	0.0124			0.0350			0.0782		
CD ($P = 0.05$)	0.0344			0.0970			0.2168		

[e] Total phosphorus content in terms of μg mL^{-1}.

Table 2. Time course effect of different phosphate sources on available phosphorus[f] in cell free supernatant of BGA

Treatments		Control (−P)	K_2HPO_4	MRP (mg P L^{-1})			TCP(mg P L^{-1})		
Strains	Days			10	20	30	10	20	30
Westiellopsis prolifica	7	0.28	3.63	0.12	0.20	0.32	0.12	0.26	0.38
	14	0.29	2.85	0.11	0.16	0.27	0.12	0.22	0.32
	21	0.36	2.16	0.11	0.15	0.27	0.10	0.18	0.27
	28	0.33	1.43	0.05	0.13	0.22	0.07	0.13	0.25
	35	0.28	0.98	0.04	0.11	0.22	0.06	0.11	0.23
Anabaena variabilis	7	0.31	3.60	0.10	0.24	0.35	0.11	0.24	0.37
	14	0.24	3.03	0.10	0.23	0.28	0.10	0.21	0.34
	21	0.24	2.26	0.09	0.20	0.27	0.07	0.18	0.34
	28	0.25	1.41	0.05	0.18	0.25	0.06	0.18	0.30
	35	0.18	1.22	0.04	0.15	0.26	0.05	0.17	0.28
	S			SXT			SXTXD		
SEM ±	0.0052			0.014			0.0330		
CD ($P = 0.05$)	0.0144			0.040			0.0915		

[f] Total phosphorus content in terms of μg mL^{-1}.

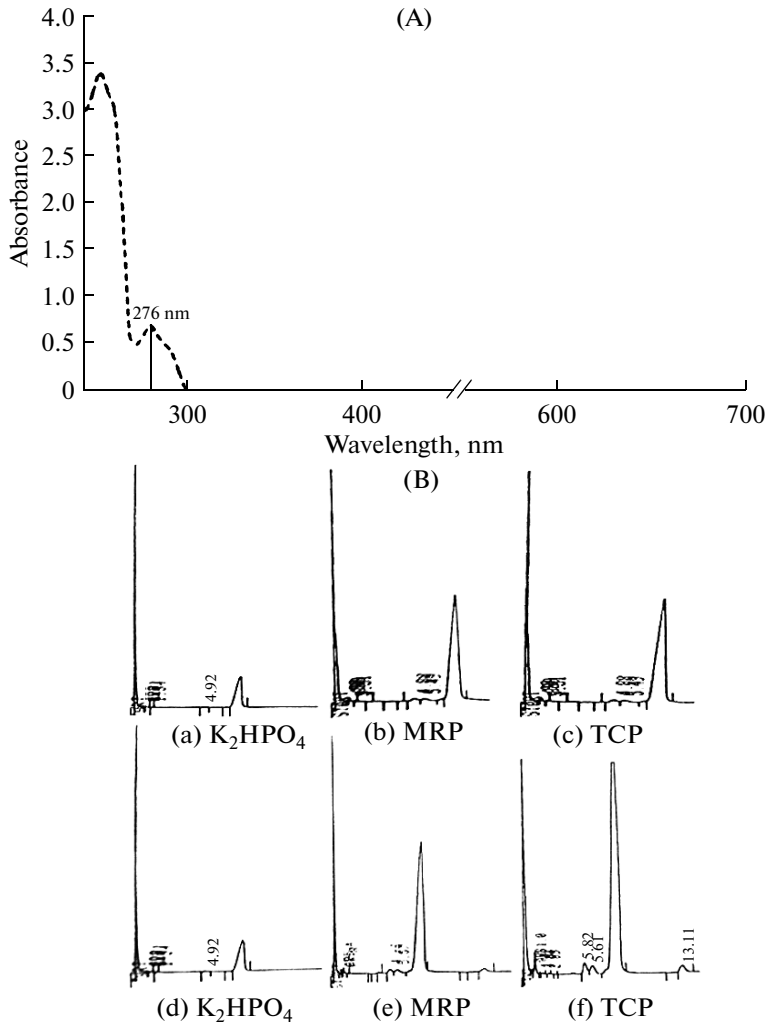

Fig. 1. (A) – The absorbance spectra of culture filtrate in the UV/VIS region. (B) – Gas chromatographic analysis of culture filtrate extracts of *Westiellopsis prolifica* (a, b, c) and *Anabaena variabilis* (d, e, f). No peak was detected in controls.

citric, malic, tartaric, α-ketoglutaric acids) leading to inference of absence of detectable level of organic acid production by studied BGA *Anabaena* and *Westiellopsis*.

Preliminary screening for hydrocarbon like compounds in culture filtrate showed presence of broad spots at variable distance on chromatogram (Fig. 2b). The concentrated cell free extracts has a typical UV-visible spectra of the compounds and is shown in Fig. 1a.

GC and GC-MS. The GC analysis of filtrate showed prominent peak near to the retention time of 7.6 in all the extract (Fig. 1b) irrespective of phosphate source. However the peak in all insoluble phosphate source showed higher amount of compound production than the soluble phosphate source. GC-MS of selected extract were performed to establish empirically the molecular weight and identity of these compounds that might be involved in solubilization process of inorganic phosphate. The GC-MS chromatogram (Fig. 2a) reveled the prominent peak at m/z 207 and 167. The base peak appeared at m/z 149 (100%). As compared to standard indices phthalate esters with saturated alkyl side chains (without oxygen) has a most intense peak in electron impact (EI) ionization mass spectrum at 70 eV was always at m/z 149 due to rapid formation and stability of the ions, leading to identification of produced compounds as phthelic acid and esters with saturated alkyl chains which are produced by *Westiellopsis prolifica* and *Anabaena variabilis* under different treatments.

DISCUSSION

Blue green algae are considered as the ideal biological fertilizers for the improvement of soil fertility and long-term sustainability under wetland rice ecosystems. Their practical utility as a source of organic

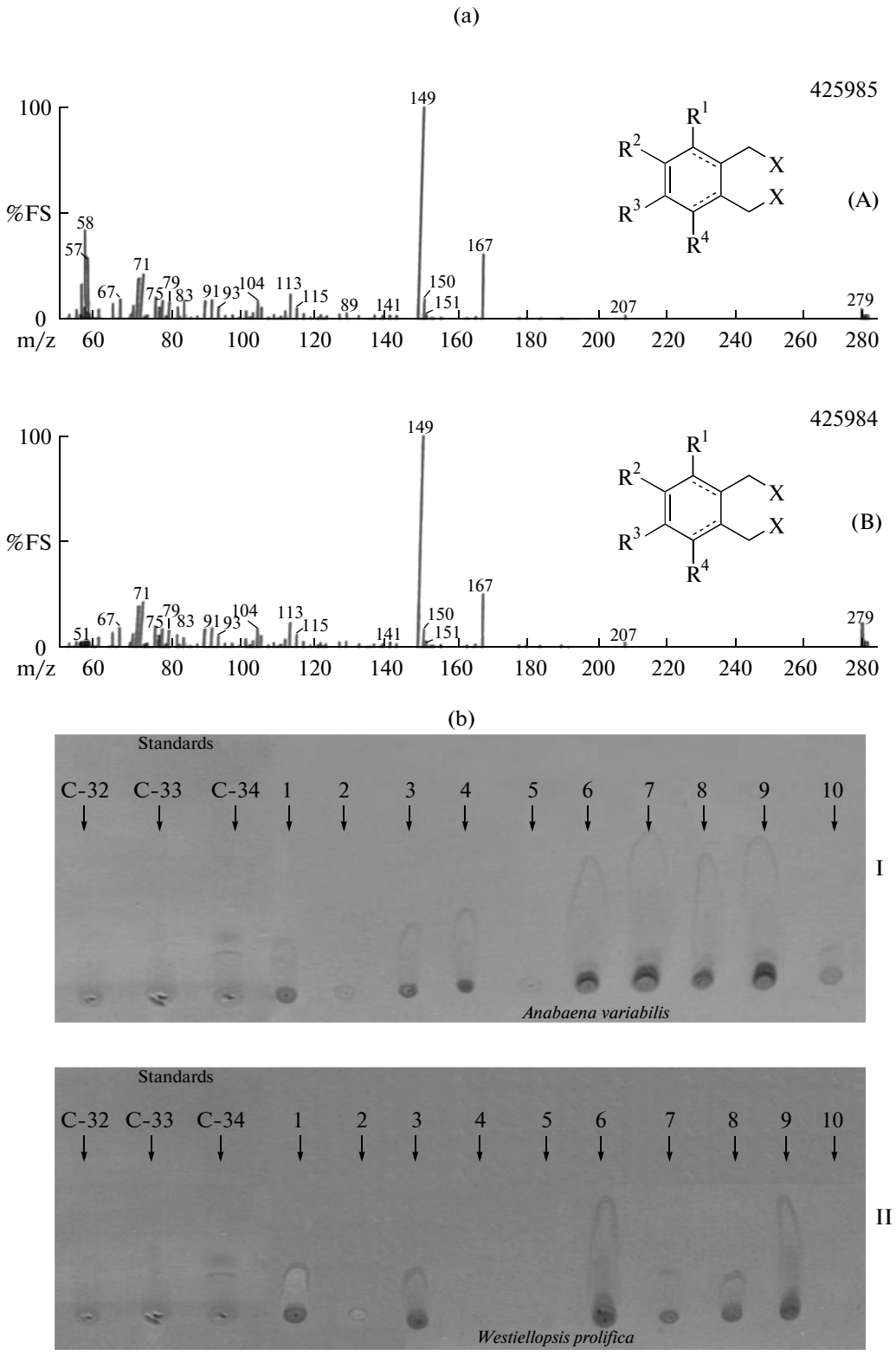

Fig. 2. a – GC Mass Spectra of the extracts of (A) *Anabaena variabilis* and (B) *Westiellopsis prolifica*. (I) Thin layer chromatography analysis of cell free extracts of *Anabaena variabilis* grown under different phosphate sources [C-32, C-33, C-34: Standard hydrocarbon compounds; 1– P deficient; 2 – K_2HPO_4; 3 and 4 – MRP (10 mg); 5 and 6 – MRP (20 mg); 7 and 8 – TCP (10 mg); 9 and 10 – TCP (20 mg)]; (II) thin layer chromatography analysis of cell free extracts of *Westiellopsis prolifica* grown under different phosphate sources [C-32, C-33, C-34; Standard hydrocarbon compounds; 1 – P deficient; 2 – K_2HPO_4; 3 and 4 – MRP (10 mg); 5 and 6 – MRP (20 mg); 7 and 8 – TCP (10 mg); 9 and 10 – TCP (20 mg)].

nitrogen fertilizer for rice has been well recognized. They have also been shown to possess the property of solubilization of phosphatic compounds, which is of great economic importance in plant nutrition. Since phosphorus is another essential input and often a major constraint, the successful crop production in India, therefore, its solubilization and bioavailability is of utmost importance. Blue green algae have also been shown to effectively solubilize TCP [3] and Mussorie rock phosphate [13]. In the present study, observations on the growth and P solubilization by BGA in insoluble phosphate medium shows that there exists lot of variability among different strains to solubilize and make use of insoluble phosphates. Arora and Gaur [12] have also reported that among different strains tested, *Aspergillus awamori* was the best followed by *Pseudomonas striata* and *Bacillus polymyxa*. The selection of two strains i.e. *Westiellopsis prolifica* and *Anabaena variabilis* was based on enhanced growth and P solubilization in comparison to other strains of BGA. The mineral phosphates used in the study namely MRP and TCP supported the growth of BGA. Roychoudhury and Kaushik [13] have reported that MRP supported luxuriant growth of BGA and biomass of some BGA was more in media containing MRP, compared to medium containing K_2HPO_4. But on the other hand the biomass of *Tolypothrix* in presence of K_2HPO_4 was more than in MRP. This suggests that the organisms vary in their capacity to grow in MRP. This may be attributed to the fact that, although the organisms are obtaining its P requirement from insoluble source, all may not be equally efficient and may also have different ways to make it available. Because the most efficient BGA cultures were selected, thus they showed very good growth in presence of insoluble phosphate source. The results have shown that both *Westiellopsis prolifica* and *Anabaena variabilis* were able to solubilize insoluble phosphates. Most of the soluble P (K_2HPO_4) was present as available P and the available P in case of insoluble phosphate sources showed gradual increase with increase in incubation. It shows that the organisms were continually solubilizing the insoluble phosphates and the part of it was used by them for their own growth and metabolism and remaining released in the medium. There was in fact only a marginal increase in available phosphorus in cell free supernatants of cultures supplied with insoluble phosphates. The higher concentrations of insoluble phosphate in medium did not seem to have much affect on the amount of available P and there seems to be an optimum concentration after which the amount of available P. During the field experiment, Saha and Mandal [15] showed that the Olsen-P content of submerged soils decreased during first 90 days of growth of BGA and then began to increase which was attributed to the assimilation of phosphorus by the living cells initially and later released during death and decay. The results also showed that not all insoluble phosphate could be brought into soluble form and a major portion remained insoluble. This is because certain portions of insoluble phosphates remain bound and are not solubilized by microorganisms.

The concentration of insoluble phosphate does affect the P-solubilization. Gaur and Sachar [16] found that lower the quantity of phosphate added with the medium, the greater was the conversion of insoluble form by the organism. It was also observed that the media receiving lowest quantity of rock phosphate remained more acidic than media receiving higher amounts. Similarly, here a concentration of insoluble phosphates equivalent to 10 mg P or 20 mg P rather than 30 mg P has been found optimum for the growth as well as phosphorus availability by *Westiellopsis prolifica* and *Anabaena variabilis* used in this study. Several investigations have shown that, under cultural conditions phosphorus solubilization starts after a lag phase and reaches to a maximum as the growth progresses. There has been no information about the effect of incubation time on the availability of insoluble phosphates using BGA under laboratory conditions and most of the authors who investigated microbial P solubilization have detected the available P content at specific times and not throughout the whole incubation period. However, in the present study, a periodical observation of P-availability and growth of BGA has revealed that insoluble phosphates are solubilized gradually and their availability increases with incubation time. The maximum availability has been observed after 21 or more days of incubation.

It has been established that BGA can solubilize bound phosphates and they have also been shown to solubilize different inorganic and organic phosphate sources in soils, sediments or in pure cultures. Till now, a number of hypotheses are known about the microbial solubilization of insoluble phosphates. Although most of the workers [17, 18] have attributed P solubilization to the release of organic acid, none of them was able to find a connection between the amount of organic acids in culture solutions on the one hand and the solubilized P on the other hand [19]. Therefore, Kucey [20], Asea et al. [21] and Cunningham and Kuiack [22] also believed that other factors exist which appear to be involved but, they do not offer alternative theories for solubilization. In the literature, citrate [22], α-ketogluconate, lactate and succinate [20] and few other acids are thought to be responsible for P solubilization. Compared to biotic leaching these investigations always lead to low yields of solubilized P. Therefore, reasonable doubts arouse whether organic acids are really and exclusively involved in the solubilization mechanism [19, 21, 23, 24]. Illemer and Schinner [19] who had earlier suggested that solubilization of inorganic P is solely caused by the release of organic acids have shown that complex formation with gluconic acid has no part in solubilizing apatite and brushite, although the acid is produced by phosphate solubilizing microorganisms. In the present study, the analysis of culture solutions for organic acids did not

reveal organic acids. This is in confirmation to the previous investigation [6, 14, 25] and stands in contrast to the popular opinion that P solubilization is always caused by the release of organic acids. No change in pH of the medium containing soluble or insoluble source of phosphate further strengthen the fact that no acid like compounds were produced during growth of these organisms and resultant P-solubilization. Roychoudhury and Kaushik [14] and Natesan and Shanmugasundram [25] observed a rather slight increase in pH by BGA in medium containing MRP and categorically ruled out the production of organic acids.

The synthesis of chelators without changing the pH of growth medium has also been suggested as a mechanism to aid P solubilization [6, 14, 26]. The calcium chelation by chelators synthesized by microorganisms has been reported to be responsible for the release of P from insoluble 'Ca' compounds. Many other extracellular compounds having chelating properties might form chelates with Al and Fe, leading to the release of P originally bound by these metals [27]. Considering this, the cell free extracts analyzed for the presence of compounds showed enhanced amount of an unidentified small molecular weight compound. The increased concentration of this compound in presence of insoluble phosphates can be attributed to its role in P solubilization. However, further investigation and critical examination is required to support this view. Illemer and Schinner [19] have, however, ruled out the role of chelating agents as no calcium accumulation at or in the microbial biomass could be observed and considering that indirect processes too are ineffective for solubilizing P.

The UV-visible spectrum revealed the absorption maxima of the culture filtrate compounds as 276 nm. Analysis of the filtrate using GLC did confirm the presence of the compound at retention time of 7.6. GC-MS is useful method for separation and identification of complex mixtures of tropane alkaloids [28]. GC-MS analysis revealed molecular ion peak at m/z 149. Most intense peak in the electron impact (EI) ionization mass spectrum at 70 eV was at m/z 149 due to the rapid formation and stability of the ion and thus, the identity of the compound was found as phthalic acid. The GC-MS spectrum was compared with standard online NIST database library for confirming the phthalic acid. The role of organic acids such as phthalic acid has been implicated in P solubilization and release of phosphates from tricalcium phosphate up to 34.58 μg mL^{-1} and rock phosphate up to 26 μg mL^{-1} in 24 hours incubation period [29]. The absorption maxima of 276 nm of the compound in the culture filtrates compared with Spectra Data Base (http://www.science-softcon.de/spectra/orgacid/ orgacid_l.htm) confirmed the presence of phthalic acid. The phthalic acid might have been synthesized from the TCA cycle through the intermediate protocatechuic acid.

The results have demonstrated that different blue green algae vary in their capacity to solubilize insoluble phosphates like MRP and TCP. Although many BGA strain tested could solubilize insoluble phosphates but *Westiellopsis prolifica* and *Anabaena variabilis* showed much higher solubilizing capacity than other strains. Both these strains were also capable of nitrogen fixation and showed luxuriant growth and metabolic activity in laboratory medium containing MRP or TCP using organic acids like phthalic acid as one of the possible mechanism of P solubilization. These can, therefore, be exploited for the efficient utilization of low cost, low grade rock phosphate fertilizers where phosphate rock can be applied directly. This would be especially beneficial in developing countries like India with limited P resources. These are the results of a laboratory study which needs to be further extended and investigated in the field to predict P availability regarding P-solubilizing BGA and to decide the amount of external P addition for optimum plant growth based on sufficiency approach [30] applying just the amount needed for a crop for optimum yields.

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REFERENCES

1. Kucey, R.M.N., Jenzen, H.H., and Leggett, M.E., Microbially Mediated Increases in Plant Available Phosphorus, *Adv.Agron.*, 1989, vol. 42, pp. l99–228.
2. Singh, A.L. and Singh, P.K., Phosphorus Fertilization and the Growth and Nitrogen Fixation of *Azolla* and Blue-Green Algae in Rice Field, *Indian J. Plant . Physiol.*, 1989, vol. 33, pp. 21–26.
3. Bose, P., Nagpal, U.S., Venkataraman, G.S., and Goyal, S.K., Solubilization of Tricalcium Phosphate by Blue Green Algae, *Curr. Sci.,* 1971, vol. 40, pp. 165–166.
4. Wolf, A.M., Baker, D.E., Pionke, H.B and Kunichi, H.M., Soil Test for Estimating Labile, Soluble, and Algae Available Phosphorus in Agricultural Soils. J. *Environ. Qual.*, 1985, vol. l4. pp. 341–348.
5. Dorich, R.A., Nelson, D.W., and Sommers, L.E., Estimating Algal Available Phosphorus in Suspended Sediments by Chemical Extraction., *J. Environ. Qual.,* 1985, vol. l4, pp. 400–405.
6. Cameron, H.J. and Julian, G.R., Utlization of Hydroxyapatite by Cyanobacteria as Their Sole Source of Phosphate and Calcium, *Plant Soil*, 1988, vol. 181, pp. 123–124.
7. Whitton, B.A., Grainger, S.L.J., Hawley, G.R.W., and Simon, J.W., Cell-Bound and Extracellular Phosphatase Activities of Cyanobacterial Isolates, *Microb. Ecol.*, 1991, vol. 21, pp. 85–98.

8. Yandigeri, M.S., Yadav, A.K., Meena, K.K. and Pabbi, S., Effect of Mineral Phosphates on Growth and Nitrogen Fixation of Diazotrophic Cyanobacteria *Anabaena variabilis* and *Westiellopsis prolifica, Antonie van Leeuwenhoek*, 2010, vol. 97, pp. 297–306.

9. Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazier, G., Purification and Properties of Unicellular Blue Green Algae (Order *Chroococcales), Bact. Rev.*, 1971,vol. 5, pp. 171–205.

10. Jackson, M.L., *Chemical analysis*, New Delhi: Prentice Hall of India, 1973.

11. Watanabe, F.S. and Olsen, S.R., Test of Ascorbic Acid Method for Determining Phosphorus in Water and Sodium Bicarbonate Extracts of Soil, *Proc. Soil Sci. Soc. Am.*, 1965, vol. 29, pp. 677–678.

12. Arora, D. and Gaur, A.C., Periodic Microbial Solubilization of P32 Labelled Hydroxy Apatite, *Indian J. Microbiol.*, 1978, vol. 10, p. 315.

13. Nordmann, J. and Nordmann, R., *Organic Acids in Chromatographic and Electrophoretic Techniques*, Vorsmith, Ed., New York: Intersciences, 1960.

14. Roychoudhury, P. and Kaushik, B.D., Solubilization of Mussorie Rock Phosphate by Cyanobacteria, *Curr. Sci.*, 1989, vol. 58, pp. 569–570.

15. Saha, K.C. and Mandal, L.N., Effect of Algal Growth on the Availability of P, Fe and Mn in Rice Soils., *Plant and Soil*, 1979, vol. 52, pp.139–149.

16. Gaur, A.C. and Sachar, S., Effect of Rock Phosphate and Glucose Concentration on Phosphate Solubilization by *Aspergillus awamori, Cur. Sci.*, 1980, vol. 49, pp. 553–554.

17. Sperber, J.I., Solution of Mineral Posphate by Soil Bacteria, *Nature*, 1957, vol. 180, pp. 994–995.

18. Illemer, P. and Schinner, F., Solubilization of Inorganic Phosphates by Microorganisms Isolated from Forest soils, *Soil Biol. Biochem.*, 1992, vol. 24, pp. 389–395.

19. Illemer, P. and Schinner, F., Solubilization of Inorganic Calcium Phosphate Solubilization Mechanisms, *Soil Biol. Biochem.,* 1995, vol. 27, pp. 257–263.

20. Kucey, R.M.N., Phosphate Solubilizing Bacteria and Fungi in Various Cultivated and Virgin Alberta Soils, *Can. J. Soil Sci.,* 1983, vol. 63, pp. 671–678.

21. Asea, P.E.A., Kucey, R.M.N., and Stewart, J.W.B., Inorganic Phosphate Solubilization by Two *Penicillium* spp. in Solution Culture and Soil, *Soil Biol. Biochem.*, 1988, vol. 20, pp. 459–464.

22. Cunningham, J.E. and Kuiack, C, Production of Citric and Oxalic Acids and Solubilization of Calcium Phosphate by *Penicillium bilaji, Appl. Environ. Microbiol.*, 1992, vol. 58, pp. 451–1458.

23. Jurinak, J.J., Dudley, L.M., Allen, M.F. and Knight, W.G., The Role of Calcium Oxalate in the Availability of Phosphorus in Soils of Semiarid Regions: a Thermodynamic Study, *Soil Sci.*, 1986, vol. 142, pp. 255–261.

24. Parks, E.J., Olson, G.J., Brinckman, F.E and Baldi, F., Characterization by High Performance Liquid Chromatography (HPLC) of the Solubilization of Phosphorus in Iron ore by a Fungus, *J. Industrial Microbiol.,* 1990, vol. 5, pp. 183–190.

25. Natesan, R. and Shanmugasundaram, S., Extracellular Phosphate Solubilization by the Cyanobacterium *Anabaena* ARM 310, *J. Biosci.*, 1982, vol. 14, pp. 203–208.

26. Luo, A.C., Sun, X. and Zhang, U.S., Species of Insoluble Phosphate Solubilizing Bacteria in Rhizosphere and Their Effect on Yield and Nutrient Uptake of Wheat Crop, *Plant Soil*, 1993, vol. 57, pp. 223–230.

27. Mandal, B., Das, S.C. and Mandal, L.N., Effect of Growth and Subsequent Decomposition of Blue-Green Algae in the Transformation of Phosphorus in Submerged Soils, *Plant Soil*, 1992, vol. 143, pp. 289–297.

28. Witte, L., Muller, K., and Alfermann, H.A., Investigation of Alkaloid Pattern of *Datura innoxia* Plants by Capillary Gas-Liquid-Chromatography/Mass Spectrometry, *Planta Medica*, 1987, vol. 52, pp. 192–197.

29. Pareek, R.P. and Gaur, A.C., Release of Phosphate from Tri Calcium and Phosphate by Organic acids, *Curr. Sci.*, 1973, vol. 42, pp. 278–279.

30. Fixen, P.E. and Grove, J.H., Testing Soils for Phosphorus, in *Soil Testing and Plant Analisis*, Westerman R.L., Ed., SSSa, Madison, UI, 1990, pp. 141–180.