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Solubilization of tricalcium phosphate and rock phosphate by microbes isolated from chromite, iron and manganese mines

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Among 62 fungi and 253 bacteria obtained from heavy metal mines of Orissa (India) screened for phosphate solubilization properties, 12 fungi and 19 bacteria were found to solubilise tricalcium phosphate (TCP). *Penicillium* sp. 21 solubilised and released 81.48 μg P mL^{-1} whereas *Penicillium* sp. 2 showed better efficiency of rock phosphate solubilization and produced 4.87 μg P mL^{-1} into the liquid culture. Bacterial strains were comparatively poor solubilisers of TCP and rock phosphate in solid and liquid culture. The bacteria and fungi isolated from the mines of Orissa were endowed with phosphate solubilization properties. Phosphate solubilising fungi were acid producers and more efficient than bacterial isolates. *Penicillium* sp.21 and *Penicillium* sp. 2 were confirmed the best for TCP and rock phosphate solubilization.

Key words: Phosphate, solubilization, *Penicillium* fungi, bacteria

Introduction

Phosphorus availability to crops is subject to chemical fixation in soil with other metal cations, depending on soil pH. A large number of microorganisms including bacteria, fungi and actinomycetes are known to produce acidic metabolites which by change of soil pH or by direct chelation of metal cations, release fixed or insoluble phosphorus in available form (STORKANOVA et al. 1999, NARISEN and PATEL 2000, REYES et al. 2002). Although considerable work has been done on various aspects of phosphate solubilizing microorganisms from agriculture (ZAIDI and KHAN 2005, SON et al. 2006), phosphate solubilising microbes from mine environments have not been exploited for such properties. In the present study bacteria and fungi obtained from different mine sites of Orissa were investigated for their phosphate solubilization properties using TCP and rock phosphate in solid and liquid culture.

Materials and methods

A total of 263 bacteria and 62 fungi were obtained from microbial culture collections from RPRC, Bhubaneswar, which had been isolated previously from different mines: the

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Sukinda (Chromite), Talcher (Coal) and Joda (iron and manganese) mines of Orissa (India). The average temperatures in these mines range between 32 and 40 °C. Preliminary screening for phosphate solubilization was done by inoculating these organisms on Pikovskaya medium of 7.2 pH supplemented with tricalcium phosphate (HI media) (SRIVASTAV et al. 2004). The Pikovskaya medium had glucose (1%), TCP (0.5%) (with or without), ammonium sulphate (0.05%), sodium chloride (0.02%), magnesium sulphate (0.01%), potassium chloride (0.02%), manganese sulphate (trace amount), ferrous sulphate (trace amount), yeast extract (0.05%), agar (1.5%). The halo zone formation around the growing colony was considered as positive and selected for further studies. Standardization of culture conditions for selected organisms was done by inoculating them on Pikovskaya agar plates of three pH values (4.5, 7.2 and 9.0) to which Tricalcium phosphate (TCP) had been added. The halo zone formations around the growing colony of bacteria and fungi were measured after 8 and 5 days of incubation period at 30 °C and 37 °C. The selected organisms were grown in liquid Pikovsakaya medium of selected pH and temperature for the analysis of released phosphate content in the culture filtrate. Three experimental sets of completely random design (CRD) were prepared: media alone without phosphate (25 mL), media to which 0.5g TCP (Hi media) had been added and, media supplmented with 0.5 g Morocco rock phosphate (0.5 g), and inoculated with fresh culture of selected organisms in triplicate. The rock phosphate was characterized by 1.99 (% W/W) moisture, 32.76 P_2O_5, 51.0 CaO, 1.41 SO_3, 3.89 F, 2.64 SiO_2, 0.19 Fe_2O_3, and 0.36 MgO. No phosphate source was added to the control sets. These sets were incubated for 7 days (bacteria) and 12 days (fungi). The total phosphate content available in 25 mL of culture filtrate was measured by UV-vis spectrophotometer at 420 nm following the molybdate yellow colour method (BHARGAVA and RAGHUPATHI 1993). In each experimental set, the final pH of the culture filtrate was also measured. Finally, data were analyzed for variance (one way ANOVA) and critical difference (CD) among and between the bacteria and fungi used in this study (SOKAL and ROLF 1995).

Results

Screening of microbial isolates for phosphate solubilization revealed variations among different groups of organisms. In all 12 fungi and 19 bacteria were found to be TCP solubilisers in the solid state of the medium (Tab. 1). The selected bacterial and fungal strains were grown in different cultural conditions and evaluated for TCP solubilization on agar plates (Tabs. 2, 3). All fungi showed halo zones in 7.2 and 9.0 pH at 30 °C and 37 °C. Most fungi preferred pH 9 for good phosphate solubilization while four fungi performed better at pH 7.2. The value of pH 4.5 was suitable for very few fungi.. The maximum zone of solubilization was observed in *Aspergillus* sp MNF 6 i.e. 50 mm. Similarly, no bacteria performed better in 4.5 pH except MB93, MB99 and MB94, which exhibited 16.0–18.5 mm halo zones at 4.5 pH at 37 °C (Tab. 3). Most of the bacteria preferred pH 7.2 at 30 °C for phosphate solubilization. The highest halo zone measurement was recorded in MB93, i. e. 41mm in pH 7.2 and 30 °C.

The data recorded on solubilization of rock phosphate and TCP by fungi and bacteria are shown in tables 4 and 5. In control experimental sets (containing Pikovskaya medium without phosphate) pH decreased towards the acidic condition. Similarly, in test experimental sets pH also declined as compared to initial pH. However, the addition of rock phosphate did not have much effect on the pH of culture filtrate.

Tab. 1. Screening of fungi for tricalcium phosphate solubilization properties (Positive = halo zone formation around the colony)

S. NO.	Fungi	TCP solubilization	S. NO.	Fungi	TCP solubilization
1	*Aspergillus sp.1*	0	32	*Penicillium sp. 5*	0
2	*Aspegillus sp. 2*	0	33	*Pencillium sp. 6*	0
3	*Aspegillus sp. 3*	0	34	*Penicillium sp. 7*	positive
4	*Aspergillus sp. 4*	0	35	*Penicillium sp. 8*	0
5	*Aspergillus sp.5*	0	36	*Penicillum sp. 9*	0
6	*Aspergillus sp. 6*	0	37	*Penicillium sp. 10*	0
7	*Aspergillus sp. 7*	0	38	*Penicillium sp. 11*	0
8	*Aspergillus sp. 8*	0	39	*Penicillium sp. 12*	0
9	*Aspergillus sp.9*	0	40	*Penicillium sp. 13*	0
10	*Aspergillus sp. 10*	0	41	*Penicillium sp. 14*	0
11	*Aspergillus sp. 10*	0	42	*Penicillium sp. 15*	0
12	*Aspergillus sp. 11*	0	43	*Penicillium sp. 16*	positive
13	*Aspergillus sp. 12*	0	44	*Penicillium sp. 18*	0
14	*Aspergillus sp.13*	0	45	*Penicillium sp. 19*	0
15	*Aspergillus sp. MNF1*	positive	46	*Penicillium sp.20*	0
16	*Aspergillus sp. MNF2*	positive	47	*Penicillium sp. 21*	positive
17	*Aspergillus sp. MNF4*	0	48	*Penicillium sp. 21*	0
18	*Aspergillus sp. MNF5*	0	49	*Penicillium sp. 22*	positive
19	*Aspergillus sp. MNF6*	positive	50	*Penicillium camberti*	positive
20	*Aspergillus niger*	0	51	*Penicillium piscarium*	0
21	*Botrytis cenera*	0	52	*Penicillium restrictum*	0
22	*Cladochytrium tenue*	0	53	Undentified sp. 1	0
23	*Colleotrichum sp. MNF9*	positive	54	Undentified sp. 2	positive
24	*Cunnighmella elegans*	0	55	Undentified sp. 3	0
25	*Cunninghamella sp. MNF7*	0	56	Undentified sp. 4	positive
26	*Curvularia sp. 1*	0	57	Undentified sp. 5	0
27	*Paecilomyces sp. 1*	0	58	Undentified sp. 6	0
28	*Penicillium sp. 1*	0	59	Undentified sp. 7	0
29	*Penicillium sp. 2*	positive	60	Undentified sp. 8	0
30	*Penicillium sp. 3*	0	61	Undentified sp. 9	0
31	*Pencillium sp. 4*	0	62	Unidentified sp. 10	0

All fungi tested were found to be solubilsers of TCP in liquid medium. The P content released into the medium from TCP was 81.48 μg mL^{-1} by *Penicillium* sp. 21 (Tab. 4). It was followed by *Aspergillus* MNF1, which produced 37.07 μg P mL^{-1} into the medium and the remaining fungi showed similar responses and liberated P content in the range of 9.47 to 22.25 μg mL^{-1}.

Tab. 2. Effect of pH and temperature on tricalcium phosphate solubilization by fungi

Fungi	Halo zone (mm) at different pH and temperature					
	4.5		7.20		9.00	
	30 °C	37 °C	30 °C	37 °C	30 °C	37 °C
Penicillium camberti	20	17	0.00	18.00	0.00	0
Penicillium sp. 22	23	0	23.00	22.50	28.00	33.5
Penicillium sp. 2	0	28.5	25.50	31.50	35.50	31.5
Penicillium sp. 7	0	0	0.00	28.00	30.00	23.5
Unidentified sp. 4	0	0	35.00	0.00	0.00	35.5
Penicillium sp. 16	0	21.5	31.50	25.50	26.00	23.5
Penicilliuim sp. 21	0	0	20.00	0.00	22.00	21
undentified ap. 2	0	0	26.00	24.00	32.50	28.5
Aspergillus sp. mnf 1	0	0	0.00	28.50	18.50	17.5
Aspergillus sp. mnf6	0	0	0.00	12.50	0.00	50
Aspergillus sp. mnf2	0	0	38.50	20.00	0.00	0
Colletotrichum sp.	0	0	26.00	19.50	27.00	18.5

Tab. 3. Effect of pH and temperature on tricalcium phosphate solubilization by bacteria

Bacteria	Halo zone (mm) at different pH and temperature					
	4.5		7.20		9.00	
	30 °C	37 °C	30v	37 °C	30 °C	37 °C
MB 3	0	0	0	0	0	18.5
MB 103	0	0	17.5	0	18	16
MB 12	0	0	16	17	14.5	15.5
MB 37	0	0	16.5	19	16	17
MB 31	0	0	0	10	9	9.5
MB 98	0	0	26.5	24.5	0	21.5
MB1	0	0	11	11	14.5	10
MI 2	0	0	0	16.5	23	25
MI1	0	0	29.5	16	21.5	18
MI 21	0	0	21	18	32	21
MI 20	0	0	33	18.5	15.5	15
MB93	0	18.5	41	30	21.5	19.5
MB99	0	16	22	18	0	16.5
MB 94	0	18.5	24.5	21.5	12.5	13.5
MB 95	0	0	23	14.5	11	11.5
MB 89	0	0	26	0	19.5	28.5
MB11	0	0	23.5	22	17.5	18.5
S1	0	0	14.5	17	17.5	13.5

Tab. 4. Solubilization of rock phosphate and tricalcium phosphate in liquid culture by fungi. Mean – denotes mean value of 6 replications. SD – Standard deviation. The level of significant difference (CD) at p<0.001 (**) and p<0.05 (*).

Fungi	Phosphate source								
	culure condition		pH /culutre	Tricalcium phosphate			Rock phosphate		
	usedin experiment		Filtrate	Final pH	P content (µg/25 mL)		Final pH	P content (µg/25mL)	
	pH	Temp. (C)	(–P)		MEAN	SD		MEAN*	SD**
Penicillium camberti	7.2	37.00	2.56	5.86	348.25	31.25	7.77	0.00	0.00
Penicillium sp. 22	9	37.00	3.96	6.63	363.75	15.00	6.91	40.00**	22.00
Penicillium sp. 2	9	30.00	2.83	6.28	490.25	77.25	7.22	121.75**	9.75
Penicillium sp. 7	9	30.00	7.10	6.05	422.00	5.50	7.08	0.00	0.00
Unidentified sp. 4	7.2	30.00	3.06	5.73	417.25	17.50	5.68	0.00	0.00
Penicillium sp. 16	7.2	30.00	4.22	5.59	448.25	17.25	7.52	0.00	0.00
Penicillium sp. 21	9	30.00	3.46	5.09	2037.00**	46.50	5.99	16.75*	5.25
Undentified ap. 2	9	30.00	4.27	5.49	563.75*	21.50	7.09	8.50	5.50
Aspergillus sp. mnf 1	7.2	37.00	3.02	5.52	926.75**	56.50	7.03	0.00	0.00
Aspergillus sp. mnf6	9	37.00	5.17	5.71	313.75	66.25	6.87	3.50	3.75
Aspergillus sp. mnf2	7.2	30.00	4.42	5.90	236.75	8.25	7.24	0.00	0.00
Colletotrichum sp.	7.2	30.00	6.08	5.40	419.75	11.25	5.74	0.00	0.00

Overall, *Penicillium* sp. 21 was found to be best among the 12 fungi evaluated in this experiment. A critical analysis of variance for P content in different fungi was found significantly variable ($p < 0.001$). The mean P contents solubilised in different fungi are significantly different ($p < 0.001$). The mean P contents solubilised by *Penicillium* sp. 21 and *Aspergillus* sp. mnf 1 were significantly higher than other fungi ($p < 0.001$). Similarly, MF 15 (unidentified fungal isolate) showed a critical difference (at 0.001 level) from all other fungi except *Aspergillus* MNF1 and MNF6.

Among them, 5 fungi, namely *Penicillium* sp. 22, *Penicillium* sp. 2, *Penicillium* sp. 21, unidentified fungus 2 and *Aspergillus* sp. MNF 2 were able to solubilise rock phosphate in liquid culture and produced 0.14 to 4.87 25 µg PO_4 mL^{-1} into the medium. Critical difference (at 0.001 level) was also observed among the rock phosphate solubilisers.

Tab. 5. Solubilization of rock phosphate and tricalcium phosphate in liquid culture by bacteria. Mean – mean value of 6 replications, SD – standard deviation. Level of significant difference (CF) at at p<0.001 (**) and p<0.01 (*). Ns -non significant

Bacteria		Phosphate source								
		Culure condition used		pH / culutre filtrate	Tricalcium phosphate			Rock phosphate		
		In experiment		Without phosphate	Final pH	P content (μg mL^{-1})		Final Ph	P content (μg mL^{-1})	
		pH	T (°C)		CF	MEAN	SD	CF	Mean	SD
MB96	Gram + cocci	7.20	30.00	5.78	5.60	18.33*	0.78	7.49	0	0
MB 3	Gram + branched	9.00	37.00	7.14	5.77	17.52*	1.41	7.66	0	0
MB 103	Gram – Rods	9.00	30.00	6.97	6.90	13.71^{ns}	2.13	7.55	0	0
MB 12	Gram + branched	7.20	37.00	6.76	5.64	13.85^{ns}	2.17	7.54	0	0
MB 37	Gram + branched	7.20	37.00	6.75	5.17	26.87**	0.78	7.61	0	0
MB 31	Gram + branched	7.20	37.00	6.58	5.70	15.85	0.94	6.79	0	0
MB 98	Gram + cocci	7.20	30.00	5.89	5.43	28.47**	4.98	7.20	0	0
MB1	Gram + branched	9.00	30.00	7.28	6.01	10.48^{ns}	0.49	7.68	0	0
MI 2	Gram + branched	9.00	37.00	6.55	6.05	7.21^{ns}	0.21	7.32	0.6^{ns}	0.22
MI1	Gram + branched	7.20	37.00	7.48	6.01	8.05^{ns}	0.33	7.71	0.00	0.00
MI 21	Gram + branched	9.00	30.00	6.82	5.83	8.77^{ns}	0.41	7.67	0.00	0.00
MI 20	Gram + branched	7.20	30.00	4.32	5.82	10.67^{ns}	0.65	7.47	0.00	0.00
MB93	Gram + cocci	7.20	30.00	3.38	5.62	25.33**	1.75	7.09	0.00	0.00
MB99	Gram + cocci	7.20	30.00	3.38	5.62	22.07**	3.40	7.09	0.00	0.00
MB 94	Gram neg cooci	7.20	30.00	5.48	5.67	8^{ns}	2.52	7.47	0.00	0.00
MB 95	Gram + cocci	7.20	37.00	4.17	5.56	17.53*	0.85	7.56	0.00	0.00
MB 89	Gram + cocci	9.00	37.00	5.14	6.04	7.88^{ns}	0.44	7.26	0.27^{ns}	0.10
MB11	Gram + cocci	7.20	30.00	5.63	5.82	8.93^{ns}	0.41	7.38	0.00	0.00
S1	Gram + cocci	9.00	30.00	3.86	6.21	3.21^{ns}	0.79	7.32	0.00	0.00

Almost all bacteria isolates used in this study were producers of acid into the medium as a decline in the final pH of culture filtrate was observed (Tab. 5). However, addition of rock phosphate did not much affect the pH of the medium, and rather a minute increase in pH could be observed. The three bacterial strains i. e. MB 98 (28.47 25 $\mu g\ mL^{-1}$, MB 37 (26.87 $\mu g\ mL^{-1}$) and MB 93 (25.33 $\mu g\ mL^{-1}$) produced higher amounts of P solubilised phosphate content in the medium. The amount of P content solubilised by MB 98 was significantly higher than by MB93 ($p<0.001$) and MB 37 ($p<0.05$). Only two bacterial isolates MI 2 and MB 9 could be observed as rock phosphate solubilisers and they released 0.6 μg and 0.27 $\mu g\ P\ mL^{-1}$ of medium, respectively.

Discussion

In the present study, the occurrence of phosphate solubilising organisms useful for both tricalcium phosphate and rock phosphate has been confirmed. It is well known that phosphate solubilising microorganisms in soil solubilize insoluble phosphates mainly by secreting acids into the medium (DAVE and PATEL 2003, CHUNG et al. 2005). Our isolates might have used the same mechanism as a decline in the culture filtrate was observed in inoculated experimental sets. A large number of microorganisms are known to produce acidic metabolites. This is also confirmed by the fall of pH in culture filtrate, which was at a maximum with TCP and not rock phosphate and also higher in fungi than in bacteria. Instead, in rock phosphate pH drifted to the alkaline side. The effect of pH on phosphate solubilization by fungi and bacteria showed that pH 9 and 7.2 was suitable for solubilization of TCP and rock phosphate in their presence.

Not all the fungi and bacteria screened were able to solubilise TCP in solid culture state. However, the degree of phosphate solubilization varied with the type of organisms involved. It was reported that dicalcium phosphate could be solubilised more readily than tricalcium phosphate by some bacteria (SUJATHA et al. 2004). *Penicillium* sp. 21 was found to be superior to other fungi in phosphate solubilization. Both tricalcium phosphate and rock phosphate were solubilised by these fungi but not in equal efficiency.

Overall, fungi showed better solubilization of both the phosphates than the bacteria. Though bacteria have been reported well for their mineral solubilization properties, in present study they performed poorly as far as the phosphate solubilization is concerned.

The findings of 10 organisms as rock phosphate solubilisers, including *Aspergillus* and *Penicillum*, are important as rock phosphate can be directly applied to soil and can be solubilised by such types of phosphate solubilising microbes. This study is corroborated by YU et al. (2005), SILVA and VIDOR (2001) who investigated the solubilization of rock phosphate in liquid culture by *Aspergillus niger* and *Penicillium oxalicum*. Several reports have mentioned the effects of carbon and nitrogen sources on phosphate solubilization capacity and its enhancements. Similarly, nutritional modification and standardization of the C: N ratio can also enhance the phosphate solubilization activity of our microbial strain. SRIVASTAV et al. (2004) reported the phosphate solubilization and antifungal activity of *Asepergillus niger, Curvularia lunata, Rhizoctonia solani* and *Fusarium oxysporium* and suggested the beneficial prospects of these organisms for better crop productivity. Further analysis of these microbial strains for their antimicrobial properties is required. However, the present study retains its importance due to the habitat from which the microbial strains

were isolated and collected. These fungal and bacterial strains were isolated from the chromite and iron ores mines of Orissa, India. Therefore, the special characteristics of metal tolerance may also be assumed in these organisms.

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