NBU Journal of
Plant Sciences

Vol. 4, March 2010 p. 73-76
ISSN No. 0974 6927

Solubilization of rock phosphate by *Azotobacter spp*

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Abstract

To find out a potent phosphate solubilizing strain of diazotrophs, fifteen *Azotobacter* strains were isolated from local sources. They have been tested for the ability to solubilize rock phosphate *in vitro* and production of acid in the medium. Almost all the strains solubilized rock phosphate which ranged up to 45.5%. *Azotobacter* sp. R12 was found to be a potent one, which solubilized 30 to 46% phosphate from different rock phosphates. Almost maximum solubilization of P and acid production was achieved at four days ofgrowth of the organism. The strain produced ascorbic acid in the culture medium, the primary cause of rock phosphate solubilization. Addition of $(NH_4)_2SO_4$ in the culture medium reduced the solubilization of phosphate with increasing concentration beyond 0.25mg /ml for Jordon rock phosphate. Increasing concentration of $CaCl_2$ and $CaCO_3$ also reduced the P solubilization from Jordon rock phosphate by the strain. Kinetics of solubilization and acid production showed a linear relationship till the 5th day of incubation, then P level becomes static. Adding EDTA at 5mg/ml as chelating agent of calcium ions to 5 days old culture increased the P solubilization to 81%. Increasing EDTA concentration in 3 days old culture showed linear relationship between concentration of EDTA and P solubilization.

Keywords: *Azotobacter*, Rock Phosphate, Chelation, calcium activity

Phosphorus is one of the most essential plant nutrients. It plays a vital role in energy transfer and regulation. It is also an important constituent of macromolecules such as phosphor-lipids and nucleic acids. Most of the essential plant nutrients, including phosphorus, remain insoluble in soil. Crop plants get these nutrients from nature and from applied chemical fertilizer. A large portion of inorganic phosphate applied to soil as fertilizer is rapidly immobilized soon after application and become unavailable to the plants (Dey 1988). Applying phosphate solubilizing soil microbes to soil as biofertilizer may alleviate this problem by solubilizing these immobilized products. Several soil bacteria (Darmwal *et al.* 1989, Gaur *et al.* 1987, Loganathan *et al.* 2004, Rashid *et al.* 2004, Pandey *et al.* 2006), Cyanobacteria (Roychaudhury *et al.* 1989) and fungi (Agnihotri 1970, Chhonkar *et al.* 1967) have the property of solubilizing different inorganic and rock phosphates. So phosphatic rock may be used as a cheap source of phosphate fertilizer for crop production. In different states of India, there are about 40 million tons of phosphatic rocks deposited (Roychaudhury *et al.* 1989). We have screened 15 strains of *Azotobacter*, from natural sources of Burdwan district, which is an important part of cereal producing area in India. These bacteria fix atmospheric nitrogen and may be used as biofertilizers (Vincent 1970). Our primary object of this investigation was to assess the ability of isolated strains of *Azotobacter* to solubilize rock phosphate. We also studied the effects of several other factors on the extent of rock phosphate solubilization by these strains.

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Materials and Methods

Fifteen strains (Table 1) were isolated from roots of fully matured rice plants of alluvial soil from Burdwan district and screened to select potent phosphate solubilizers.The strains were maintained by routine transfer on Burk's N-free medium supplemented with 0.025% yeast extract.

The composition of the Burk's medium used to study rock phosphate solubilization by the strains of *Azotobacter* sp was as follows: D-glucose 20g;mgSO_4 $7H_2O$ 0.2g; $CaCl_2$ 0.09g; Fe-Mo Solution ($FeSO_4$ $7H_2O$ 50mg; $NaMoO_4$ $2H_2O$, 2.52mg); water to 1000ml; powdered phosphate rock (~240 mesh Size) at 2g per litre was added as a source of insoluble phosphate. The pH of the media was adjusted to 7.00 before autoclaving. The flasks were inoculated with cells of a stationary phase culture at 4% and incubated for 3 days on a rotary shaker at 28°C.

Contents of the culture flasks at the end of incubation period were centrifuged at 15000 rpm for 15 minutes to remove biomass and insoluble matter. The supernatant was taken as a test material. Quantitative estimation of soluble phosphate was done following the method of Chen et al.(1956). The reagent for phosphate estimation was prepared fresh before each experiment. One volume of 6(N) H_2SO_4, two volume of double distilled water and one volume of ammonium molybdate (1%) were taken together to which one volume of 10% ascorbic acid was added and mixed thoroughly 4ml of the reagent was added into each tube containing an aliquot of test material and the final volume of the reaction mixture was adjusted to 8ml and (incubated at 37° C for 1 hour). Absorbance was noted at 829 nm against a

Table 1: Strains of *Azotobacter* spp. isolated and used

Strains	Host	Locality*	Marker character
R3	*O sativa*	Kalna2,	Tet^r
R4	*O sativa*	Memari1	Amp^r, Chlo^r
R6	*O sativa*	Kalna 2	Amp^r
R7	*O sativa*	Kalna 1	Amp^r
R10	*O sativa*	Kalna 2	Chlo^r
R11	*O sativa*	Kalna 2	Amp^r,Chlo^r,Tet^r
R12	*O sativa*	Memari2	Tet^r
T1	*S indicum*	Kalna 2	Tet^r
T2	*S indicum*	Kalna 1	Tet^r
R20	*O sativa*	Memari 2	Amp^r
R21	*O sativa*	Monteshwar	Amp^r
R23	*O sativa*	Memari2	Chlo^r
R27	*O sativa*	Memari1	Amp^r,Chlo^r,Tet^r
R33	*O sativa*	Memari2	Amp^r,Chlo^r,Tet^r
R34	*O sativa*	Monteshwar	Amp^{r}Chlo^r,Tet^r

*Locations are in Burdwan District, West Bengal India

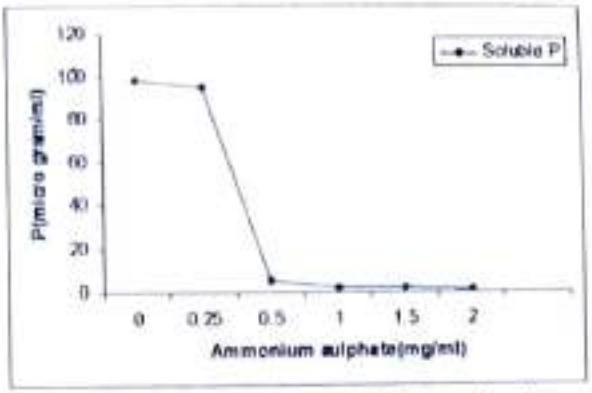

Fig 1: Effect of ammonium salt on solubilization of Jordon rock phosphate by the culture of *Azotobacter* sp. R 12. Ammonium sulfate in increasing level was added to Burk's medium. Release of phosphate, expressed as equivalent phosphorus, was measured after 4 days

corresponding blank in a spectrophotometer and at 619 nm in a visible spectrophotometer. The amount of solubilized phosphate was determined from a standard curve prepared by using K_2HPO_4 as standard and expressed as equivalent P.

The phosphate rocks are used as source of high grade and low grade phosphatic materials. Two phosphate rocks were collected from Phosphate Company, Rishra, Hooghly, P1 (off white Jordon Phosphate rock) and P2 (pale brown Udaypur Phosphate rock). P3 (yellowish gray Purulia Phosphate rock) was obtained from Sriniketan Agricuture farm, Sriniketan, Visva Bharati and P4 (black Mussoric phosphate rock) was collected from Das enterprise, Prof Lakshmi Narayan Das, Sure Kalna, Burdwan. The composition of those four phosphate rocks, as determined by the method of Shapiro and Brannock (1962), are given as Jordon phosphate rocks (JPR) P1 which contained 34.00% P_2O_5, 41.64% CaO, Udaypur phosphate rocks (UPR) P2 containing 31.00% P_2O_5, 46.82% CaO, Purulia phosphate rocks (PPR) P3 containing 32.7% P_2O_5, 51.20% CaO, were source of high grade phosphate. On the other hand Mussorie phosphate rocks (MPR) P4 contained 17.00% P_2O_5, 48.90% CaO and were source of low grade phosphate . In the media containing either P1, P2, P3 or P4 phosphate rock at 0.2%, the total phosphorus equivalents added were 362.5, 301, 310 or 189mg/ml respectively, if solubilized completely.

The pH of the spent culture was measured by Systronic Digital pH meter 802.The organic acids were extracted after passing the spent culture broth through Dowex 50WX8-400 column (H^+ form). The eluent was concentrated by lyophilization. The acids were identified by Thin Layer Chromatography on silica gel plates and in comparison with authentic samples. The chromatograms were developed in a solvent system of butanol: acetic acid: water (2:1:1). Spots were identified by spraying with glucose-aniline and heating at 125°C for 5 min or by spraying with aniline phthalate.

Results and Discussion

The ability to solubilize rock phosphate by a number of Azotobacter strains (Table 1) isolated from local sources was evaluated. The strains were found to be Gram negative, small rods having cysts in their cells when stained with acrydine orange, produce smooth colonies, were indole and catalase positive and starch non utilizers.

After 6 days incubation, the end pH of the culture and the amount of phosphate solubilization from phosphatic rock by the strains in N_2 free Burks's medium are presented (Table 2). The pH of the culture media after autoclaving was near the pH 7.00 and level of soluble phosphate was 0.03mg/ml. After incubation the end pH was measured to find out whether solubilization of P was accompanied by the production of acid.

In Table 2 the data show that the solubilization of rock phosphate by strains of *Azotobacter spp.* ranged from 4.88% (R 21) to 29.89% (R 12) from P1, 6% (R 34) to 31.10% (R 12) from P 2, 3.75%(R 6) to 32.3% (R 12) from P 3 & 4.67%(R 21) to 45.5% (R 12) from P 4. The strain R 12 solubilized more than 32, 31, 32 and 45% of P from P 1, P 2, P 3 and P 4 respectively. The Strain (R 12) was found to be highest P solubilizer in all rock phosphates among the tested strains of *Azotobacter*. The pH of the media lowered by the production of acid, after incubation pH of the medium ranged from 6.00 (R 10) to 4.01 (R 12) in P1 , 5.88(R 10) to 3.92 (R 11) in P2 , 5.30 (R 4) to 4.32 (R 34) in P 3 , 6.1 (R 4) to 4.1 (R 12) in P4.

It is evident from the results (Table 2) that the strain R 12 of *Azotobacter sp.* was the highest P solubilizer of all the strains studied. Jordon phosphate rock (P1) proved to be a good source of P, so further investigation was carried out to characterize the process of P solubilization form only P1 with this strain. Bacteria belonging to the genera *Rhizobium*, *Bradyrhizoum*, *Bacillus*, *Enterobacter*, and *Pseudomonas* are known to bring about dissolution of insoluble phosphatic compound (Rashid *et al.* 2004, Roychaudhury *et al.* 1989, Halder *et al.* 1990, Rojas *et al.* 2001) along with production of organic acids. The degree of solubilization of P from the rock phosphates by the strains of *Azotobacter is* very comparable to that by other bacteria reported. All the tested organisms produced acid and lowered the medium

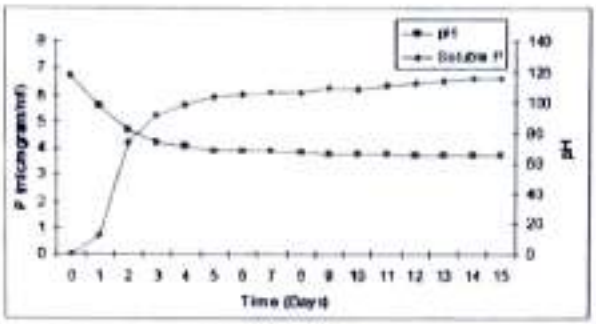

Fig 2: Kinetics of solubilization of phosphate and acid production by *Azotobacter* sp. R 12. The strain was cultured in Burk's medium containing 0.2% Jordon phosphate rock. Release of phosphate expressed as equivalent phosphorus, was measured at different time intervals.

pH and it showed that the acids produced by organisms were responsible for the P solubilization.

It has been considered that effective P solubilizing microorganisms decreased the pH during their growth (Agnihotri 1970, Halder *et al.* 1990, Seshadri *et al.* 2000, Arora *et al.* 1979). Characteristic of P solubilization is not dependent on the taxonomic grouping.

To study the effect of ammonium salt on solubilization of rock phosphate by the strain of *Azotobacter sp.*, ammonium sulphate as ammonium salt was added in increasing concentrations to the composition of Burk's medium with powdered P1 phosphate rock at 2gm / litre. The results of phosphate solubilization from the phosphatic rock and end pH of the culture after 4 days incubation are presented (Figure 1). The strain released comparable amount P from P 1 in the culture adding 0.25mg /ml with that of zero $(NH_4)_2SO_4$. The results show that (Fig. 1) in *Azotobacter* the presence or absence of NH_4SO_4 in medium has no effect on P solubilization in lower dose. But higher dose of $(NH_4)_2SO_4$ beyond 0.25mg/ml in culture inhibits the phosphate solubilization.

Kinetics of solubilization of phosphate and acid production by the strain R 12, are presented (Figure 2). The figure shows the kinetics of solubilization of P and corresponding values of culture pH in their respective medium at different time intervals for 15 days. It appeared from the results that as the mean pH decreased, the level of solubilized P increased linearly till the 4th day of incubation and the pH was near 4, after reaching in highest level of P solubilization , it became static and further incubation did not improved the extent of solubilization drastically. Complete solubilization of the rock phosphate was never achieved. In term of percentage, about 32% of solubilization of P was achieved after 15 days of incubation. Further lowering of pH beyond 4th day was probably due to the accumulation of soluble P in the form of phosphoric acid (H_3PO_4).The organic acid produced by R12 turned out to be ascorbic acid as compared with the authentic sample.

Adding increasing amounts of calcium as $CaCO_3$ to the culture of strain R 12, in medium change of phosphate was recorded. The data (Figure 3) indicated that after 4 days incubation the pH of the cultures became higher as compared to control. The extent of P solubilization was reduced gradually with an increasing concentration of $CaCO_3$. The dissolution of P was reduced to zero at the concentration of 2mg/ml of $CaCO_3$. It also indicated that increasing amounts of calcium as $CaCl_2$ to the culture of strain R 12 in medium made little difference in the culture pH after 4 days of growth from that of the control culture with no $CaCl_2$ added. the extent of P solubilization reduced significantly with addition of

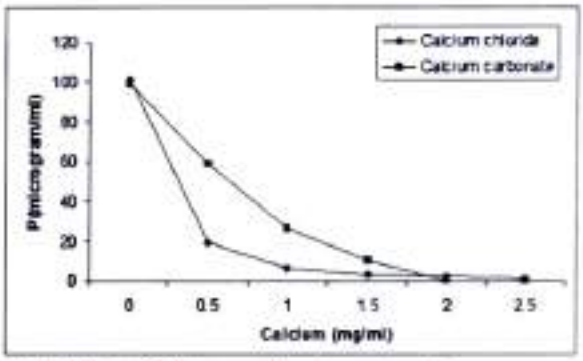

Fig 3: Effect of calcium supplement on phosphate solubilization from Jordon phosphate rock by culture of *Azotobacter* sp. R 12. Calcium in increasing levels was added to the Burks medium. Release of phosphate expressed as equivalent phosphorus, was measured after 4 days

Table 2: Solubilization of rock phosphate by the strains of *Azotobacter spp* on N-Free Burks medium after 6 days and end pH of cultures

Strains	P1		P2		P3		P4	
	%P*	pH	%P	pH	%P	pH	%P	pH
R3	27.7	4.8	26.66	4.82	20.47	5.12	23.2	5.7
R4	28.04	4.6	19.62	5.77	17.81	5.3	21.05	6.1
R6	5	5.7	8.88	5.6	3.75	4.62	7.1	5.8
R7	23.31	5	28.51	5.07	16.32	4.96	25	5.1
R10	7.43	6	17.66	5.88	11.89	5.07	16.42	6.24
R11	20.6	4.2	18.51	3.92	17.44	4.1	19.07	5.7
R12	29.89	4.0	31.1	4.8	32.3	4.77	45.5	4.1
T1	20.71	4.8	28.51	5	23.05	4.85	23.68	5.36
T2	28.37	4.68	27.77	4.72	20.44	4.75	32.89	5.77
R21	4.88	5.1	7.32	4.77	9.32	5.22	4.67	5.46
R23	14.74	4.8	12.8	4.91	10.52	4.76	15.21	4.86
R27	12.26	4.8	8.31	5.02	7.28	5.16	11.53	4.91
R33	6.5	5.0	9.11	4.98	8.02	5.06	6.32	5.12
R34	5.86	5.0	6.45	4.79	9.9	4.32	7.02	5.04
R20	13.12	4.9	10.66	4.84	5.36	4.86	9.72	5

*%age of soluble P

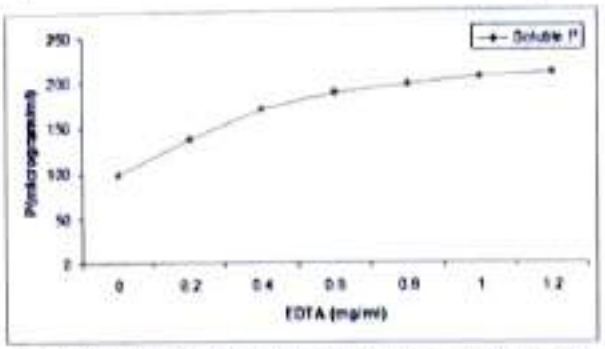

Fig.4:Effect of adding EDTA on phosphate solubilization from Jordon phosphate rock in culture of *Azotobacter* sp. R12. The strain was allowed to grow in the presence of rock phosphate for 4 days in Burk's medium when neutralized EDTA was added to the culture in increasing concentration. Soluble phosphate, expressed as equivalent phosphorus, was measured in the culture filtrate after incubation of 24 h

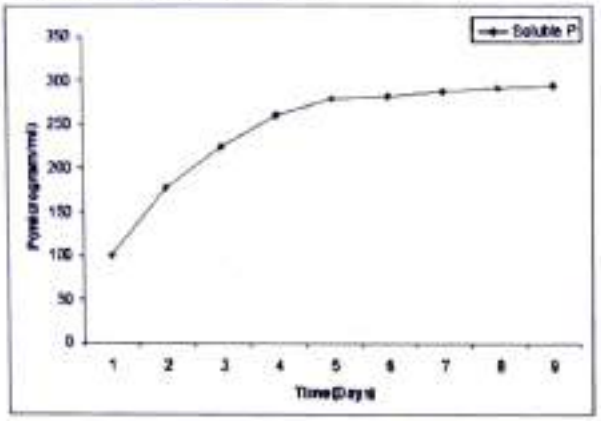

Fig.5: Kinetics of solubilization of phosphate by *Azotobacter* sp. R12 upon addition of EDTA to a 5 days old culture. The strain was allowed to grow in the Burk's medium containing 0.2% Jordon phosphate rock for 5 days when nutralised EDTA was added to the cultures to a final concentration of 5mg perml. Release of soluble phosphate, expressed as equivalent phosphorus, was then measured at time intervals

$CaCl_2$ to their medium. The role of calcium activity in the dissolution of P from phosphate rocks is important. Studies of Wilson & Ellis (1984) involving soil solution suggested the calcium activity as an important factor controlling the rate and extent of dissolution of rock phosphate.

Adding di-sodium salt of ethylenediamine tetra acetic acid (EDTA) neutralized to pH 7.0 to 4 days old cultures of R 12 and further incubation of the culture for another 24 h showed that increasing concentration of EDTA increased the solubilization of P from rock phosphate (Figure 4). The extent of P solubilization increased up to 55%. It is apparent that probably calcium which inhibits P solubilization as observed in Figure 3 are chelated by EDTA when applied to the medium[13]. Adding neutralized EDTA to a final concentration of 5mg/ml to a 5 day old culture gradually increased the solubilized P upon incubation for the next 5 days. The P reached a plateau when more than 81% of the P from P I was released (Figure 5).

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