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Evaluation of Microbial Solubilisation of Nano Rock Phosphate

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A B S T R A C T

A laboratory study was conducted to assess the efficacy of the different microbes to solubilise nano rock phosphate. Nano rock phosphate was prepared using a 24 blade FRITSCH Rotar Mill (Model-Pulverizette-14) with a 80µm sieve, followed by high energy ball milling. The nano rock phosphate particles were characterized by Transmission electron microscopy (TEM), Field Emission Scanning Electron Microscopy (FESEM), Dynamic Light Scattering (DLS) and X-Ray Diffraction (XRD) analysis regarding their size, shape, morphology, structure and composition. The pure culture of fungi and bacteria were isolated form Bhanpur dumping site, Bhopal, India. The fungal and bacterial isolates were screened on the basis of their phosphate solubilisation potential. The dominant phosphate solubilising strains were Bacillus amyloliquefaciens, *Aspergillus flavus* and *Aspergillus terrus.* The quantitative study of phosphate solubilisation form nano rock phosphate was done with the positive strains. Results revealed that *Aspergillus flavus* and *Aspergillus terrus* were able to solubilize the nanorock phosphate particles more efficiently (2.87-18.16% more) than the tri -calcium phosphate particles as compared to bacteria. Solubilization of nano rock phosphate was the result of organic acid produced by microbes. The study reflected the potential of nano rock phosphate as a P supplying source along with P solubilising microbes. As to date, there is very meager report on microbial solubilisation of phosphorus from nano rock phosphate; the present investigation therefore has highlighted the scope for future research.

Introduction

Phosphorus (P) is one of the major essential plant nutrients limiting plant growth and yield on more than 40% of the world's arable land. The current depletion rate of global P reserves are alarming and could lead to no soil P reserve left by the year 2050 (8). Furthermore, India does not have adequate reserves of rock phosphate which is making crop production unsustainable. Phosphorus deficiency is one of the greatest limitations in agricultural production (15) (Lynch JP, Brown KM (2008), particularly in low-input agricultural systems occupying 5.7 billion hectare of global land. In India, almost 98% of cropland is deficient in available forms of soil P (25). After its application to soil, a large proportion

of fertilizer phosphorus is converted to insoluble form. A greater part of soil phosphorus, approximately 95%-99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants (29). P concentrations in soil solutions in arable lands seldom exceed 10^{-6} M which is below the critical level for the optimal performance of crops (8). Thus to maintain crop productivity we have to rely on large amount of mineral fertilizer application. However, the high production cost of fertilizer as well as dire need to import phosphatic fertilizer as evident by import of 6.01 million tonnes of DAP in 2015-16 by Govt. of India (5) have made this situation worse. This situation thus propelled us to find the alternative ways to release insoluble and fixed forms of phosphorus in soil to increase soil phosphorus availability. The main natural P reserve is composed by apatite ($Ca_5(PO_4)_3(F,Cl,OH)$) rock phosphates (RP), from which the conventional soluble fertilizers are obtained mainly by treatment with strong acids (19). It has also been recognized as a valuable alternative source for P fertilizer, especially for soils having pH < 6.0 (21), since substantial deposits of cheaper and low grade RP are locally available in many countries of the world. Different common efforts that have been made to get wide range application of this naturally available P sources, include very expensive and tedious processes like partial acidulation of RP with synthetic and natural organic acids and decreasing its particle size (27). Moreover, these processes also compromise the environmental health (10). Nevertheless, the latest effort is based on basic principles of nano-science and nano technology where nano rock phosphate (NRP) particles could be prepared by top down approach in laboratory for the direct application to crops in field. It is hypothesized that after application of NRP to soil, the fate of nano particles will be governed mainly by two phenomena viz. direct crop uptake of nano particle mediated by penetration through the root epidermal cell (33) or by conversion to soluble phosphate ion by soil microorganisms (1). Naturally occurring phosphate-solubilizing microbes (PSM) can solubilize insoluble P and release a soluble form of P into the soil solution to be absorbed by plant roots (7). The application of PSM to soils can replace or partially reduce use of inorganic P fertilizers and could be one of the most low-cost, highly efficient, and sustainable approaches to conserve P resources and prevent P pollution (4). Utilization of microbial mediated RP solubilization has several advantages over conventional chemical fertilizers for agricultural purposes. These advantages are as follows: (i) microbial products are considered safer than many of the chemical fertilizers now in use; (ii) neither toxic substances nor microbes themselves will be accumulated in the food chain; and (iii) self-replication of microbes circumvents the need for repeated application. PSM has traditionally been associated with chelation, ion exchange, and the production of organic acids, such as gluconic, keto-gluconic and lactic acid (7, 28, 30). The most efficient P solubilizers were reported to be bacterial strains from the genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi. Among the genera of *Bacillus* and Pseudomonas the *B. sub-tilis*, *B. polymyxa*, *B. sircalmous*, *Bacillus megaterium*, *B. circulans*, *Pseudomonas striata*, have been reported as the most significant strains of phosphate solubilizing bacteria (PSB) (3). However, fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria. Nevertheless, very meagre information is available on P solubilisation efficacy of microbes from NRP. Keeping the environmental concerns and sustainability issues in the view, research efforts are thus constantly being made on elaboration of

techniques that involve the use of less expensive, as well as less bio-available sources of plant nutrients such as RP and the application of phosphate solubilizing microbes for achieving sustainable productivity. The present work is in line with the above mentioned concept to explore the potential of less bioavailable P-source NRP to serve as a P supplying source. Against this backdrop, an attempt was made in the present investigation to examine the solubilisation potential of soil borne fungi and bacteria for phosphorus from NRP.

Materials and Methods

Synthesis of rock phosphate nano particle

RP samples were collected from Udaipur, Rajasthan. At the initial stage, size of RP mineral was reduced to micron-level with a 24 blade FRITSCHRotar Mill (Model-Pulverizette-14) using an 80µm sieve. This process was followed for five times. After that, the same materials were ball milled at ambient temperature with high energy intensity (specific process or methodology is under patent application). The size of fine particles (< 100 nm) of RP was measured by dynamic light scattering technique (Fig. 1–4).

Culture source and isolation of bacterial and fungal strains

Soil samples were collected from Bhanpur dumping site (23°17'51.74"N, 77°26'9.10"E), Bhopal, India. Two sets of serial dilutions of soil samples were made and plated in nutrient agar medium and potato dextrose agar medium for isolation of bacteria and fungi respectively. The plates were incubated for 3 days at 30°C. Bacterial and fungal colonies were then isolated and sub cultured to obtain single strain. The purified cultures were maintained in sterile slants at 4^0C for further analysis.

Taxonomic characterization of isolated strains

For bacterial identification, the genomic DNA was extracted by the Ultra Clean Microbial DNA Isolation Kit (MO Bio, USA) and amplified using universal 16S rRNA primers (9) as mentioned in Table 1. The obtained 16S rDNA sequences of isolated bacteria were compared with those of other known species deposited in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN 2.2.24 program. The nucleotide sequences were deposited in GenBank with the accession number provided in Table 2. For fungal identification, the culture was submitted to ITCC, IARI for identification (Table 2).

Screening of P-solubilizing bacteria and fungi

The qualitative estimation of the P solubilizing activity of the bacterial and fungal isolates was carried out on Pikovskaya (1 L contain: glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4.H_2O$, 0.002 g; and $FeSO_4.7H_2O$, 0.002 g; agar, 18g) as well as National Botanical Research Institute's phosphate growth medium (NBRIP) medium (1 L contain: glucose, 10 g: $Ca_3(PO_4)_2$, 5 g; $MgCl_2.6H_2O$, 5 g; $MgSO_4.7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g; agar, 18 g) plating with bromophenol blue dye that shows decolorization of the media around the colonies. Based on positive response of the isolated strains they were again plated on modified Pikovskaya and modified NBRIP medium where $Ca_3(PO_4)_2$, the P source was replaced with 0.1% and 0.5% NRP and compared with the unmodified Pikovskaya and NBRIP medium. All the plates were incubated at 30°C for 72 hours. The plates which produced halo zone after 72 hours were considered as positive for P-solubilization

capacity (Fig. 5a and 5b). Morphological characteristics of these P-solubilizers were studied using standard phenotypic techniques (12).

Quantification of total organic acid production

Positive phosphate solubilizer strains were inoculated in the NBRIP broth and allowed to grow for 7 days and after incubation period the culture was centrifuged. Titrable acidity was estimated by titrating with 0.1 M NaOH (30). The titrable acidity was expressed as milliliter (ml) of 0.1 M NaOH consumed per 1.0 ml of culture filtrate.

Quantitative P-solubilization from nano-rock phosphate

Quantitative estimation of *in-vitro* P solubilisation was carried out as per the standard methodology (18) by inoculating 1 ml of bacterial suspension (3×10^7 cells ml^{-1}) in 50 ml of modified NBRIP (supplied with 0.1% and 0.5% NRP instead of $Ca_3(PO_4)_2$) broth in 150 ml conical flasks (Table 3). For quantitative study NBRIP broth was chosen over Pikovskaya broth due to higher efficiency of the former for distinguishing better solubilising ability of the phosphate solubilising strains (20). Two separate sets of incubation study were maintained. In one set cultures were inoculated to NBRIP medium containing bromophenol blue (NBRIP-BPB), a pH indicator dye, which changes its colour due to the decrease in pH of the medium (Fig. 6a and 6b). Hence, phosphate solubilising efficiency of microorganisms can be easily screened based on visual observation. In the other set, the NBRIP medium was devoid of BPB dye. Details of the incubation treatments have been presented in the table 3. Un-inoculated modified NBRIP broth served as the control. All the flasks were incubated for 3 days. At the end of the incubation period the cell suspension was centrifuged at 10,000 rpm for 10 min. The pH and the P content in the supernatant were estimated by pH meter and spectrophotometrically following the vanado-molybdophosphoric yellow color method, respectively (22).

Statistical analysis

All data obtained in the laboratory experiments were subjected to analysis of variance (ANOVA), assessed by Duncan's multiple range tests with a probability, P = 0.05 and analysis of Pearson correlation coefficient (r) by using SPSS version 16.0.

Results and Discussion

Identification of bacterial and fungal isolates

Isolated bacterial and fungal strains were identified as *Bacillus amyloliquefaciens*, *Aspergillus flavus and Aspergillus terrus*.

Effect of inoculation on pH dynamics and organic acid production

Initial pH of the modified NBRIP broth without microbial inoculation was approximately 7.0. In each set (Table 4) the un-inoculated control has the highest pH after three days (72 hours) of incubation period. Significant pH reduction has been noticed in all the sets with fungal and bacterial inoculation. In all the sets, i.e. NBRIP broth supplied with 0.5% $Ca_3(PO_4)_2$, NBRIP broth supplied with 0.1% and 0.5% NRP, the highest pH reduction was found with the inoculation of *Aspergillus flavus*, which was followed by inoculation of *Aspergillus terrus* and *Bacillus amyloliquefaciens* (Fig. 7). Total organic acid produced, estimated in terms of titrable acidity followed the opposite trend of pH. The highest amount of acid was produced with the inoculation of *Aspergillus flavus* in

all the sets, whereas the inoculation of *Bacillus amyloliquefaciens* resulted in the lowest organic acid production as compared to two fungal isolates during 72 hours of incubation period (Fig. 3).

Effect of inoculation on P solubilisation

The results of the quantitative estimation of *in-vitro* P solubilisation revealed that there was no significant difference between the microbial inoculation and un-inoculated treatment in percentage of P solubilisation from 0.5% $Ca_3(PO_4)_2$ after 72 hours of the incubation study (Table 4). Similar trend was also noticed with 0.5%NRP medium. However, the significantly higher percentage of P solubilisation was found with microbial inoculation from 0.1% NRP medium as compared to the un-inoculated control. The highest percentage of P solubilisation was recorded in the inoculation with *Aspergillus flavus* (31.88%) which was followed by inoculation of *Aspergillus terrus* (18.36%) and *Bacillus amyloliquefaciens* (13.72%). Without inoculation only 4.21 and 1.23% P solubilization occurred from 0.1% NRP and 0.5% NRP respectively, however there was no significant difference in percentage of P solubilization from 0.5% $Ca_3(PO_4)_2$ with microbial inoculation.

Table.1 PCR primers used in this study

Primer	Sequence (5'-3')	Target gene	Reference
pA-F	AGA GTT TGA TCC TGG CTC AG	16S rDNA	Edwards et al. (1989)
pH-R	AAG GAG GTG ATC CAG CCG CA		

Table.2 Identification of bacterial & fungal isolates

Code	Bacterial isolates	Accession number
B-16	*Bacillus amyloliquefaciens*	KF479460
Code	**Fungal isolates**	**ITCC No.**
SF-2	*Aspergillus terreus*	ITCC ID No.9943.15
SF-9	*Aspergillus flavus*	ITCC ID No.9938.15

Table.3 Treatment details of Quantitative P-solubilization from nano-rock phosphate

Treatments	1st set	2nd set	3rd set
Control	NBRIP medium with 0.5% $Ca_3(PO_4)_2$	NBRIP medium with 0.1% NRP	NBRIP medium with 0.5% NRP
With fungal inoculums	NBRIP medium with 0.5% $Ca_3(PO_4)_2$ + *Aspergillus flavus*	NBRIP medium with 0.1% NRP +*Aspergillus flavus*	NBRIP medium with 0.5% NRP+ *Aspergillus flavus*
With fungal inoculums	NBRIP medium with 0.5% $Ca_3(PO_4)_2$ + *Aspergillus terrus*	NBRIP medium with 0.1% NRP +*Aspergillusterrus*	NBRIP medium with 0.5% NRP +*Aspergillus terrus*
With bacterial inoculums	NBRIP medium with 0.5% $Ca_3(PO_4)_2$ + *Bacillus amyloliquefaciens*	NBRIP medium with 0.1% NRP + *Bacillus amyloliquefaciens*	NBRIP medium with 0.5% NRP + *Bacillus amyloliquefaciens*

#All the sets are made in triplicate.

Table.4 Effect of inoculations on solubilisation of P from nano-rock phosphate

Treatment	Amount of insoluble P added in medium (mg)	pH of the medium after incubation	Conc. of soluble P in the medium after incubation (ppm)	Amount of soluble P in the medium after incubation (mg)	% of the insoluble P solubilized due to microbial inoculation
NBRIP medium with 0.5% $Ca_3(PO_4)_2$	45.0	6.95 g	24.02 bc	1.20 cd	2.67 ab
NBRIP medium with 0.5% $Ca_3(PO_4)_2$+A. *flavus*	45.0	3.41 a	67.91 f	3.41g	7.57 b
NBRIP medium with 0.5% $Ca_3(PO_4)_2$+A. *terrus*	45.0	4.09 b	50.16 e	2.53 f	5.61 ab
NBRIP medium with 0.5% $Ca_3(PO_4)_2$+B. *amyloliquefaciens*	45.0	4.15 bc	30.93 cd	1.57 de	3.49 ab
NBRIP medium with 0.1% nano rock phosphate	6.11	6.84 g	5.09 a	0.26 a	4.21 ab
NBRIP medium with 0.1% nano rock phosphate+A. *flavus*	6.11	3.61 a	39.19 de	1.95 ef	31.88 e
NBRIP medium with 0.1% nano rock phosphate +A. *terrus*	6.11	4.39 cd	22.32 bc	1.12 cd	18.36 d
NBRIP medium with 0.1% nano rock phosphate + B. *amyloliquefaciens*	6.11	4.63 d	16.77 ab	0.84 abc	13.72 c
NBRIP medium with 0.5% nano rock phosphate	30.55	6.71 g	7.76 a	0.39 ab	1.23 a
NBRIP medium with 0.5% nano rock phosphate+A. *flavus*	30.55	4.99 e	35.11 cd	1.76 de	5.77 ab
NBRIP medium with 0.5% nano rock phosphate +A. *terrus*	30.55	5.50 f	23.75 bc	1.19 cd	3.90 ab
NBRIP medium with 0.5% nano rock phosphate + B. *amyloliquefaciens*	30.55	5.38 f	17.58 ab	0.89 bc	2.90 ab

The value in each column followed by different small case letters are significantly different as per DMRT test.

Table.5 Correlation among phosphate solubilisation, pH and titrable acidity in the solution

	pH	Conc. of soluble P in the medium after incubation (ppm)	Amount of soluble P in the medium after incubation (mg)	% of the insoluble P solubilized due to microbial inoculation	Titrable Acidity
pH	1				
Conc. of soluble P in the medium after incubation (ppm)	**-0.76****	1			
Amount of soluble P in the medium after incubation (mg)	**-0.76****	**1.00****	1		
% of the insoluble P solubilized due to microbial inoculation	-0.56	0.205	0.20	1	
Titrable Acidity	**-0.72****	**0.78****	**0.77****	0.49	1

Fig.1 TEM micrograph of Udaipur NRP particle

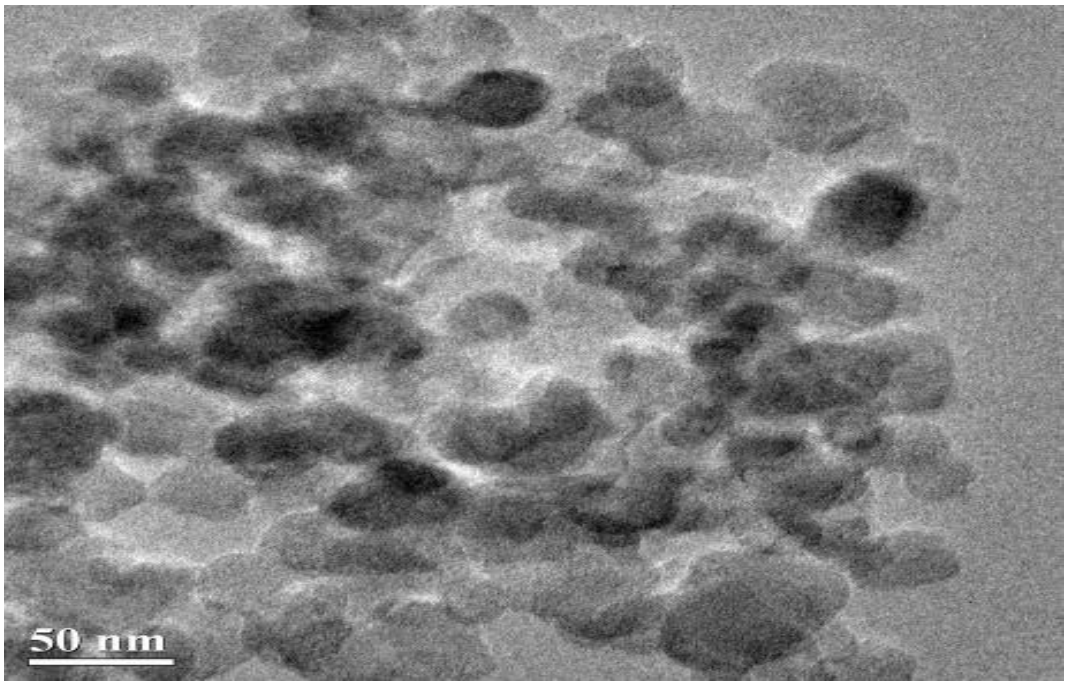


Fig.2 SEM micrograph of Udaipur NRP particle

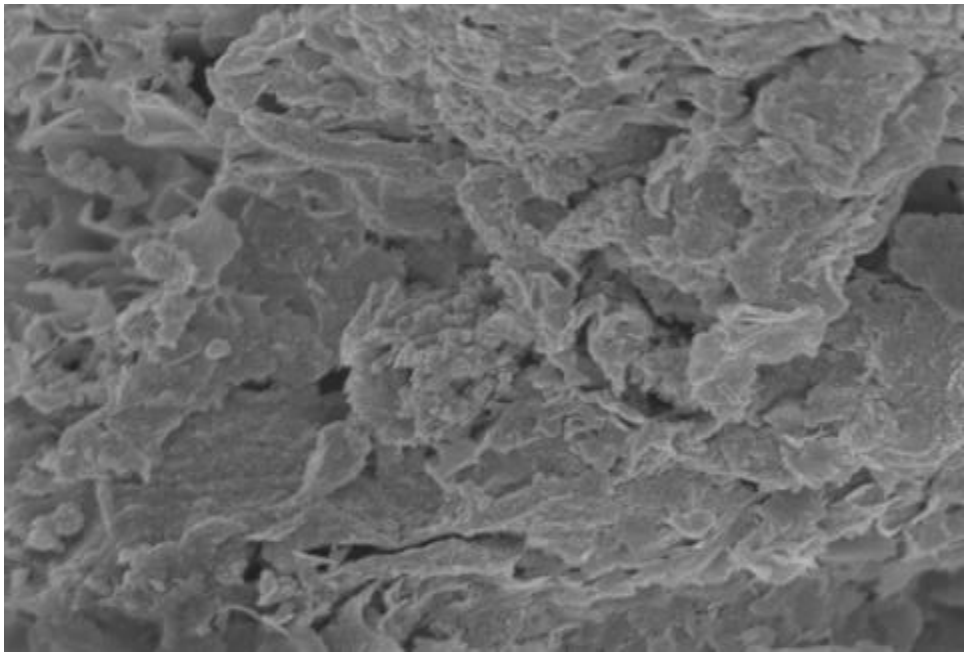


Fig.3 Size of Udaipur NRP particles as measured by DLS technique

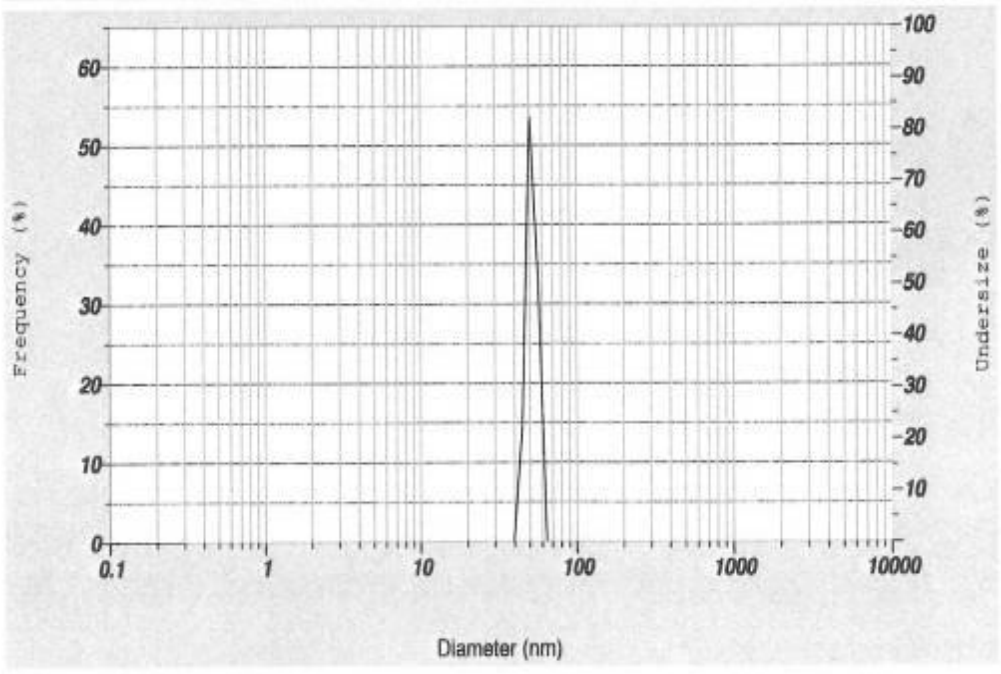


Fig.4 XRD spectra of Udaipur NRP particle

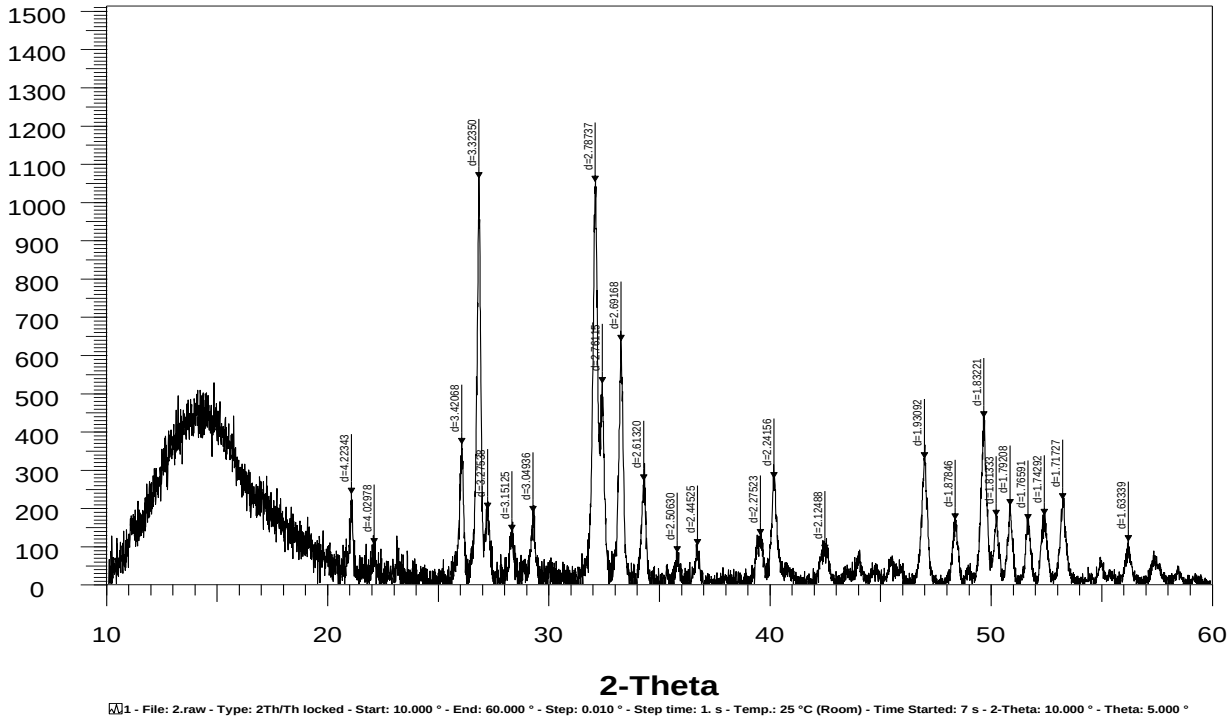


Fig.5a Growth and zone of phosphate solubilization by *Aspergillus terrus* at 72 h on 0.1% and 0.5% nano rock phosphate-Pikoskavya agar plate-containing bromophenol blue (pH indicator dye). Phosphate solubilizing fungus shows decolourization in BPB containing Pikoskavya agar plate

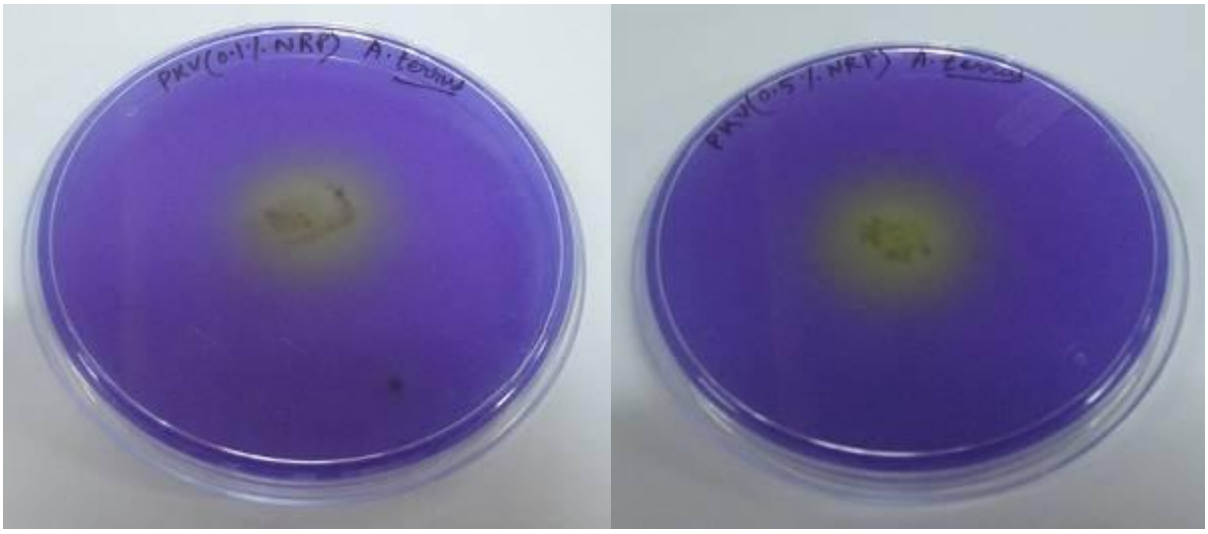


Fig.5b Growth and zone of phosphate solubilization by *Aspergillus flavus* at 72 h on 0.1% and 0.5% nano rock phosphate-Pikoskavya agar plate-containing bromophenol blue (pH indicator dye). Phosphate solubilizing fungus shows decolourization in BPB containing Pikoskavya agar plate.

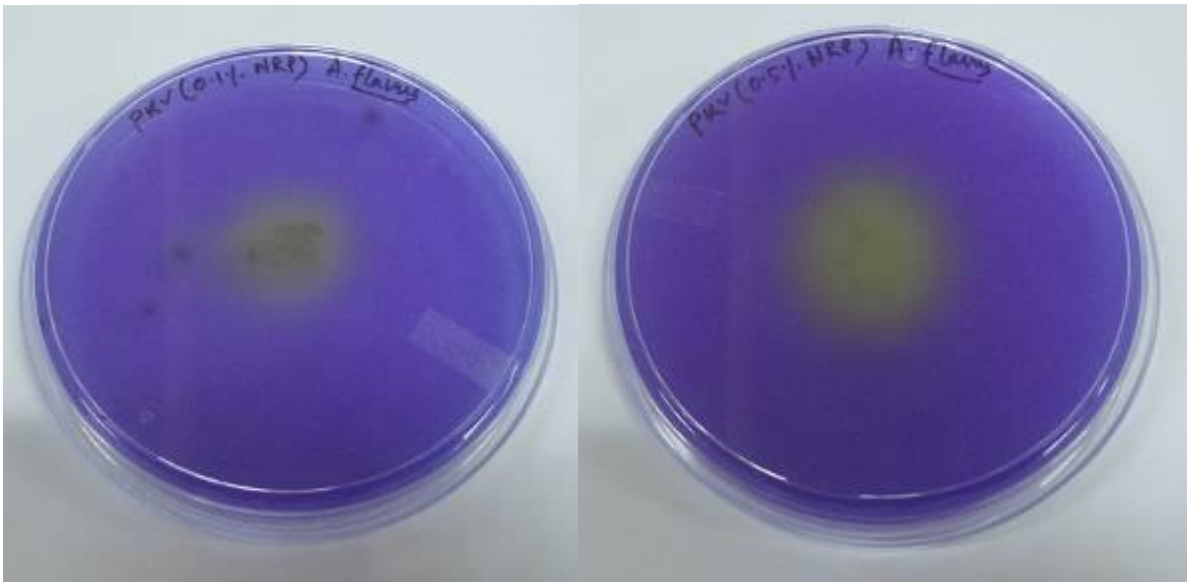


Fig.6a Phosphorus solubilization in NBRIP broth and corresponding pH change in the solution

Fig.6b Phosphorus solubilization in modified NBRIP broth (where Ca_3PO_4 was replaced with 0.1% Nano rock phosphate) and corresponding pH change in the solution

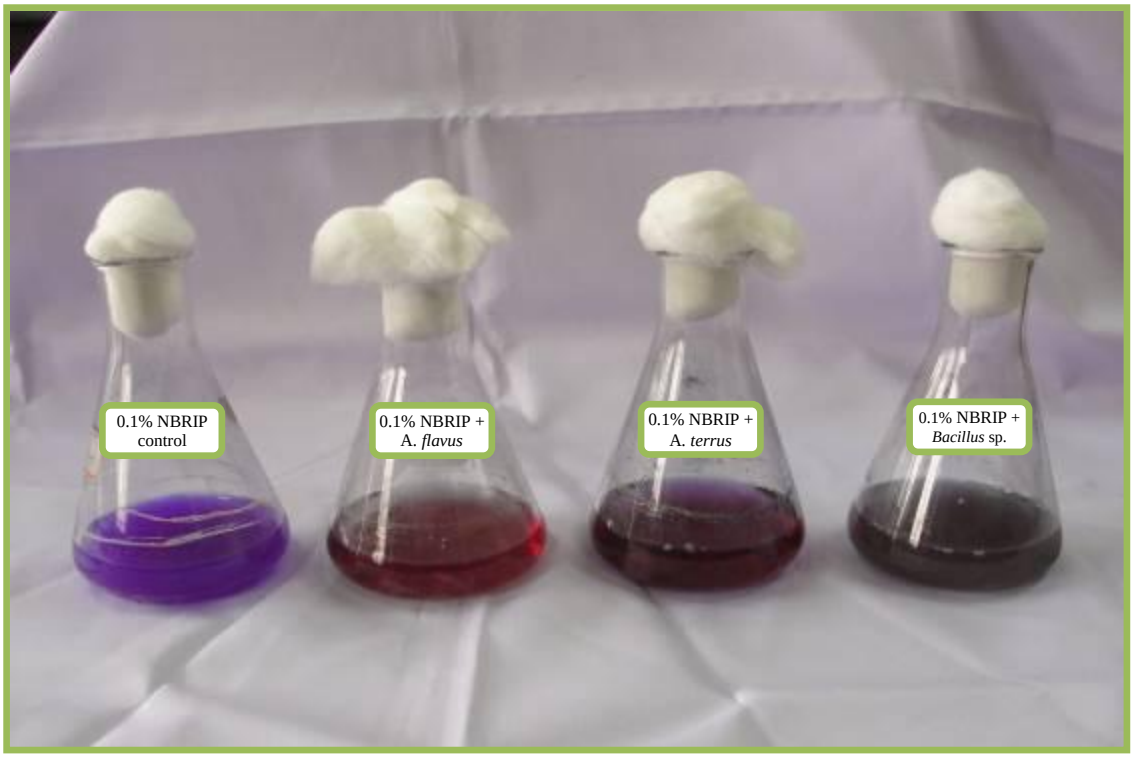
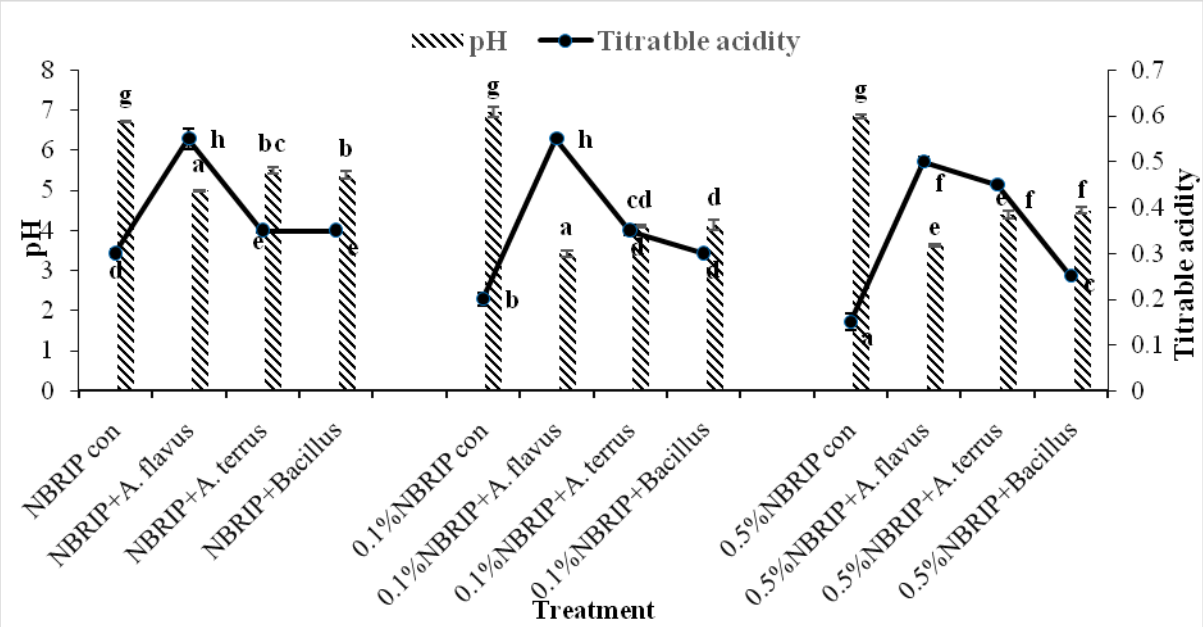


Fig.6c Phosphorus solubilization in modified NBRIP broth (where Ca_3PO_4 was replaced with 0.5% Nano rock phosphate) and corresponding pH change in the solution

Fig.7 Effect of inoculations on pH and the titrable acidity of the P-solubilizing liquid medium



The error bars followed by different small case letters are significantly different as per DMRT test

Correlation among phosphate solubilization, pH and titrable acidity

Pearson correlation coefficient analysis revealed significant negative correlation between pH of the medium and the amount of soluble P in medium after incubation and the titrable acidity (Table 5). Similarly, titratable acidity also had significant positive correlation with the amount of soluble P in medium after incubation and negative correlation with pH of the medium. The major identified processes of soil P cycle that affect soil solution P concentrations are (i) dissolution–precipitation, (ii) sorption–desorption due to interactions between P in solution and soil solid surfaces, and (iii) mineralization–immobilization mediated through soil microorganisms (26). The RP is an alternative and natural source of P. Dissolution of RP in soil is prerequisite for the P from RP to become available to plants. Acidification of the medium either by microbially secreted organic acids or abiotically released acid may favour P solubilization from RP. Here our concern is the first mechanism involving microbe. The main P solubilization mechanisms employed by soil microorganisms include: (i) release of complexing or mineral dissolving compounds e.g. organic acid anions, siderophores, protons, hydroxyl ions, CO_2, (ii) liberation of extracellular enzymes (biochemical P mineralization) and (iii) the release of P during substrate degradation (biological P mineralization) (17). P-solubilisers mainly secret organic acid in the periplasmic space by the direct oxidation pathway (32), which in turn leads to a drop in pH due to the acidification of the microbial cells and the surroundings. The P ions are thus released by substitution of H^+ for Ca_2^+. In this regard the assimilation theory of acidification by H^+ proposed by Illmer and Schinner (13) holds importance where they explain that H^+ released is associated with cation assimilation. Another reported mechanisms involved in P solubilisation are (i) enhanced chelation of the cations bound to P release phosphate in soil solution, (ii) competes with P for adsorption sites on the soil (iii) forms soluble complexes with metal ions associated

with insoluble P (Ca, Al, Fe)and thus P is released (16, 31). Organic acids that solubilise phosphate are mainly citric, lactic, gluconic, 2-ketogluconic, oxalic, tartaric and acetic, etc (14).

$2\ Ca_5F(PO_4)_3 + 20\ (HX) = 10\ CaX_2 + 6\ H_3PO_4 + 2\ HF$ (i)

$2\ Ca_5F(PO_4)_3 + 14\ (HX) = 7\ CaX_2 + 3\ Ca(H_2PO_4)_2 + 2\ HF$ (ii)

where HX is citric acid ($C_6H_8O_7$), oxalic acid ($C_2H_2O_4$) and gluconic acid ($C_6H_{12}O_7$).

However gluconic acid is reported to be the most promising one (4). These organic acids are sources of biologically generated H^+ ion that are able to dissolve the mineral phosphate and make it available for the plants. Similarly, different studies indicated that the rock phosphate solubilization is a consequence of the decrease in pH of the medium due to production of organic acids evident from a significant negative correlation between the final pH of the culture medium and the concentration of soluble phosphate (23, 11). Furthermore, fungi have been reported to possess greater ability to solubilize RP than bacteria as found in the present study. They are considered as one of the key groups of soil micro flora involved in P cycling. Among them, *Penicillium* and *Aspergillus* are two genera frequently used for phosphate solubilisation (24). However, till now report on direct microbial solubilisation from NRP is almost nil where only one paper reported direct solubilisation of nano rock phosphate particle by phosphate solubilizing fungi and bacteria due to reduction of pH of the medium (2) and another paper reported about reduction of soil pH due to the combined effect of NRP and PSB in an incubation study (6). Similar reduction of pH has been noticed in the present experiment which would have led to P solubilisation from NRP.

In conclusion, the overall results showed that both *Aspergillus flavus* and *Aspergillus terrus*, were able to solubilize the NRP particles more efficiently than the micron sized tri calcium phosphate particles as compared to bacteria. The present investigation also revealed that the fungi have more potential (2.87-18.16%) to solubilize the NRP than bacteria. However, it is also clear that increasing concentration of NRP from 0.1 to 0.5% has reduced the phosphate solubilisation efficiency of the microbes. Nevertheless, the present study highlighted the potential of NRP as a P supplying source in combination with P solubilizers for sustainable agriculture. Thus, further studies are also warranted to harness the true potential of rock phosphate in nano form as sustainable P source as well as to characterize the organic acids involved in microbial phosphate solubilization activity.

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