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Organic phosphate mineralization by *Bacillus sphaericus* and *Pseudomonas cepacia*

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Phosphorus (P) content of average soils is about 0.05% (w/w) but only about 0.1% of this P is available to plants. On the other hand an average utilization of added fertilizer P by the plants ranges from 15 to 25 % only, due to its fixation in soil. In order to become available to plants, organic P compounds must first be hydrolyzed by phosphatases. In soil, this process is predominantly mediated by the activity of soil microorganisms, and plant roots. In view of its wide spread deficiency in the soil, its low availability, high fixing tendency and high cost of industrial phosphate fertilizers the seed/soil inoculation with phosphate solubilizing microorganisms (PSMs) has a profound significance in the modern day of agriculture. Intensive screening of phosphate solubilizing bacteria with genetic potential for increased tolerance to high salt, high pH and high temperature could enhance production of food and forage in semi-arid regions. Emphasizing particularly on this hypothesis 165 phosphate solubilizing bacteria were isolated. Among these, two cultures *Bacillus sphaericus* and *Pseudomonas cepacia* were selected on the basis of salt tolerance property and PS activity with different forms of phosphates. In the present study both the bacterial culture were explored for their organic phosphate mineralization ability. Both the isolates were showed variable capability to mineralize various forms of organic phosphate. This organic P mineralizing ability can be profited to the village farmers by its successful use along with green manures, crop residues and recyclable waste.

Keywords: Phosphate mineralization, Microbial inoculants, Phosphate solubilizing bacteria, *Bacillus sphaericus, Pseudomonas cepacia.*

Introduction

Because of its complex nature, the organic fraction of soil P has received relatively little attention. This fraction may accounts 50 to 80% of the soil-P, depending upon the nature of soil and its composition (Dalal, 1978; McLoughlin *et al*., 1990). Vegetation and decaying plant residues entering the soil are the main sources of organic phosphorus compounds. The soil organic P fraction is composed chiefly of phytin, phospholipids, nucleic acids and their derivatives. Phytin and its derivatives are probably the main organic P – component (Pederson, 1953). The nucleic acid comprises not more than 5% of the organic P (Adams *et al*., 1954). The

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amount of lecithin in the soil is small, possibly about 1% of the total soil P. The remaining of the soil organic P occurs in unknown forms. Phytic acid, an organic phosphate form is of special interest to plants, because it is the principal storage form of phosphorus in mature seeds of both monocoat and dicoat plants. Phytin is a calcium magnesium salt of phytic acid (inositol phosphate). Inositol phosphate may have one to six phosphorus atoms per inositol unit. On germination, phosphate from phytin becomes available for the phopsphorylation reaction of the seedling. Phospholipids include lecithin and cephalin in which phosphate is esterified with a nitrogenous base. Lecithin is made up of glycerol, fatty acids, phosphate and choline.

In spite of relative abundance of P in nature, phosphate is sometimes a growth limiting factor for soil microorganisms also, because most of its natural supply occurs as insoluble salts. To cope up with this P limitations, most bacteria (Au *et al*., 1991), yeast and fungi (Furukawa *et al*., 1987; Goldman *et al*., 1987) produces extracellular phosphatases. Phosphatases are group of enzymes that catalyze the hydrolysis of both esters and anhydrates of H_3PO_4. These enzymes are classified as acid and alkaline phosphatases because their maximum activities occur at low (pH 6.5) and high (pH 11) pH ranges. Acid phosphatases are produced by both, microorganisms and plant, but alkaline phosphatases are produced mainly by microorganisms. Mineralization of organic compounds like nucleic acids and lecithin was carried out by nuclease and lecithinase respectively under favorable conditions. Phytase, breaks the phytic acid to myoinositol and phosphate via intermediate inositol polyphosphate. (Abul *et al*., 1990) Among microorganisms the phytase enzyme has been studied most extensively in *Aspergilli* species. Phytase activity is also known to be widespread in *Penicillium, Cunninghamella, Arthrobacter, Streptomyces. Proteus, Serratia, Pseudomonas,* and *Bacillus* are known to have ability.

The ribonucleases and deoxyribonucleases are synthesized by certain species of *Aspergillus, Penicillium, Strepromyces, Achromobacter* and *Clsotridium*. Lecithinase produced by bacteria including actinomycetes and fungi. Somani and Saxena (1971, 1975, 1976) also reported the reduction in the content of organic phosphorus in soil was associated with simultaneous increase in the numbers of microorganisms capable of dephosphorylating these substances, which show their involvement in the mineralization process.

In the present investigation *Bacillus sphaericus* and *Burkholderia cepacia* were evaluated for their organic phosphate mineralizing ability.

Materials and methods

Organisms: *B. sphaericus* and *B. cepacia* maintained on Pikovskaya's agar slants were used.

Culture Media: Modified Pikovskaya's broth: TCP in Pikovskaya's broth was replaced with 0.5% each of deoxyribonucleic acid(DNA), ribonucleic acid(RNA), sodium-β-glycerophosphate (SGP), calcium phytate(CP), sodium phytate(SP) and adenosine triphosphate (ATP).

Nutrient broth: The medium was used to prepare bacterial inoculum.
The compositions of Pikovskaya's broth and N. broth are mentioned in chapter 2. All media were sterilized by autoclaving for 15 minutes at 15 lb p.s.i (121° C).
Preparation of an inoculum: An inoculum of each culture was prepared as described in chapter 2.

Inoculation and growth condition

Two sets, each of six flasks containing 100ml modified Pikovskaya's broth, were inoculated aseptically with 1ml inoculum in each flask. The inoculated flask were incubated at 28° ± 0.2° C for 21 d for phosphate mineralization under static condition and shaken at 12 h intervals. Respective uninoculated flasks were incubated as control. The experiment was carried out in triplicate.

Phosphorus estimation and pH measurement

At periodic intervals, 10 ml medium was withdrawn from each flask aseptically and centrifuged at 10,000 r.p.m. for 20 minutes. Supernatant was analysed for its water soluble -P content by chlorostannous reduced molybdophosphoric acid blue method. (Jackson, 1973) The pH of the medium was determined by 'Elico' pH meter.

Results and discussions

Table 1 and 2 show soluble P released from the organic phosphorus compound and drift in the pH of the medium. *B. sphaericus* and *B. cepacia* were found to mineralize different organic phospatic compounds in variable amounts. The breakdown of organophosphates indicates the presence of phosphatases such as nuclease or phytase.

Maximum P released in presence of calcium phytate by both the organisms. The hydrolysis of different organic phosphates with subsequent release of soluble P by *B. sphaericus* and *B. cepacia* can be arranged in decreasing order as follows;

For *B. sphaericus*;

CP	>	SGP	>	ATP	>	RNA	>	DNA	>	SP
(118.96)		(110.38)		(101.59)		(74.53)		(35.90)		(26.70)

For *B. cepacia*;

CP >	SGP	> ATP	> RNA	> SP	>	DNA
(111.11)	(106.98)	(85.81)	(50.81)	(23.01)		(20.18)

Above results clearly indicate that calcium phytate stand distinctly above among all organophosphates as the best substrate for minerlization. Dave (1999) also reported calcium phytate as the most preferred compound among different organic phosphates tested, by *Psudomonas fluorescens.* This observation is in affirmation with our results. However, Samaha (1998) found that SGP was mineralzed maximally by *Aspergillus niger* among different organic phosphates tested.

B. sphaericus solubilized SGP, RNA, DNA and SP maximally on 21st d and CP and ATP on 15th d and 9th, respectively. In case of B. *cepacia*, SGP, ATP, DNA and SP hydrolyzed maximally on 18th d while CP and RNA on 9th d and 21st d, respectively.

Table 3 shows acid phosphatases production by *B. sphaericus which* is distinctly higher in ATP and DNA containing medium in initial phase and later phase of the experiment, respectively. Among the two phytates, the enzyme production was more in calcium salt than in sodium salt of the phytic acid and among nucleic acids, double in DNA than in RNA. Among the six substrates, RNA supported acid phosphatase production least.

Table 4 shows alkaline phosphatase production by *B. sphaericus* using different organic phosphorus compounds. The order of the compounds on the basis of phophatase production was as follows;

RNA >SGP>CP>SP > DNA = ATP

On comparing two enzymes, the organism produced more alkaline phosphatase with RNA and SGP and more acid phosphatase with DNA, CP and ATP while, production of both enzymes was equal with SP.

Table 5 reveals acid phosphatase production with different substrates by *B. cepacia*. The enzyme activities was doubled with DNA than with RNA. CP showed more phosphatase production than SP. Acid phosphatase production was highest in case of ATP followed by DNA and CP.

Table 6 shows alkaline phosphatase production by *B. cepacia* in presence of different organic phosphorus compounds. A phosphatase activity was maximum on 21st d with RNA, SGP and CP and on 18th and 15th d with SP, ATP and DNA, respectively. Maximum alkaline phosphatase activity was noted with SGP followed by SP, RNA, CP, ATP and DNA. Phosphatase activity was nearly equal with DNA and ATP. The organism showed more alkaline phosphatase activity with RNA, SGP, CP, SP and ATP and more acid phosphatase activities with DNA and ATP. On comparing two enzyme activity, maximum acid phosphatase activities was

more (with ATP) than that of maximum alkaline phosphatase activity (with SGP).

The reduction in enzyme activity with some organic compounds tested once attaining its maxima may be due to the increase in soluble phosphorus concentration in the culture medium which repress the native phosphatase activity. Utilization of phytate by both the organisms suggests the presence of phosphatases and its beneficial roll in phosphate uptake by plants, as phytate is present in large quantity in the plants.

The pH drift was observed with different organic phosphates mineralized by *B. sphaericus* and *B. cepacia*. The pH drift was more towards acidic side, ranged between pH 3.8 to 2.9 and 3.9 to 3.2 with DNA and ATP, respectively, compared to rest of the compounds. While, with all other organic phosphorus sources the pH drifted towards neutral to alkaline side. This is may be due to the presence of a very sensitive control mechanism for regulation of gene involved in phosphate utilization by microorganisms, which not allowing organic acid production but permit the release of extracellular acid or alkaline phosphatases in presence of different organophosphate compounds.

Taking together, all above results suggest that both the isolates are also able to scavenge phosphate from organic P sources. This organic P mineralizing ability can be profited to the village farmers by its successful use along with green manures, crop residues and recyclable waste.

Table 3. Acid phosphatase activity of B. sphaericus with different organic P sources

Organic P sources	**Acid Phosphatase (EU)**						
	Days of incubation						
	3	6	9	12	15	18	21
DNA	0.40	0.45	0.50	0.50	0.52	0.80	0.82
RNA	0.22	0.20	0.18	0.24	0.29	0.30	0.30
Sodium β glycerophosphate	0.12	0.18	0.22	0.19	0.22	0.30	0.40
Calcium phytate	0.32	0.33	0.29	0.30	0.60	0.28	0.26
Sodium phytate	0.19	0.19	0.20	0.25	0.32	0.38	0.40
ATP	0.60	0.70	0.69	0.65	0.42	0.61	0.58

Table 4. Alkaline phosphatase activity of B. sphaericus with different organic P sources

Organic P sources	Alkaline Phosphatase (EU)						
	Days of incubation						
	3	6	9	12	15	18	21
DNA	0.10	0.15	0.10	0.20	0.15	0.10	0.16
RNA	0.80	1.10	1.09	1.12	1.20	1.22	1.30
Sodium β glycerophosphate	0.20	0.40	0.30	0.28	0.40	0.60	0.80
Calcium phytate	0.30	0.28	0.22	0.30	0.42	0.32	0.30
Sodium phytate	0.18	0.20	0.32	0.36	0.38	0.40	0.21
ATP	0.12	0.12	0.12	0.12	0.15	0.18	0.20

Table 5. Acid phosphatase activity of B. cepacia with different organic P sources

Organic P sources	Acid Phosphatase (EU)						
	Days of incubation						
	3	6	9	12	15	18	21
DNA	0.39	0.48	0.50	0.60	0.68	0.79	0.65
RNA	0.20	0.28	0.21	0.20	0.19	0.30	0.32
Sodium β glycerophosphate	0.12	0.18	0.20	0.15	0.18	0.16	0.12
Calcium phytate	0.22	0.28	0.35	0.32	0.29	0.28	0.29
Sodium phytate	0.18	0.19	0.23	0.19	0.26	0.30	0.28
ATP	0.60	0.80	0.82	0.81	0.60	1.20	0.70

Table 6. Alkaline phosphatase activity of B. cepacia. with different organic P sources

Organic P sources	Alkaline Phosphatase (EU)						
	Days of incubation						
	3	6	9	12	15	18	21
DNA	0.18	0.10	0.12	0.18	0.20	0.19	0.16
RNA	0.30	0.35	0.36	0.35	0.60	0.65	0.70
Sodium β glycerophosphate	0.20	0.28	0.37	0.39	0.46	0.68	0.79
Calcium phytate	0.12	0.26	0.30	0.38	0.40	0.42	0.59
Sodium phytate	0.40	0.42	0.48	0.59	0.60	0.78	0.69
ATP	0.10	0.12	0.15	0.16	0.20	0.22	0.19

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Table 1. Mineralization of organic phosphates by *B. sphaericus*

Organic phosphorus sources	Days of Incubation													
	3		6		9		12		15		18		21	
	P_2O_5*	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH
DNA	22.44	3.8	24.52	3.6	27.89	3.6	25.80	3.6	28.53	3.0	29.97	2.9	35.90	2.9
RNA	12.98	5.5	17.95	5.6	11.70	7.8	61.39	7.8	68.60	8.2	73.25	8.6	74.53	7.8
Sodium β glecerophosphate	12.21	5.2	16.28	5.3	15.90	5.8	37.54	5.8	46.03	6.0	71.20	6.0	110.38	6.2
Calcium phytate	46.23	5.6	59.53	5.8	62.67	5.9	78.54	5.9	118.96	6.0	34.30	6.0	24.20	6.3
Sodium phytate	19.81	6.4	19.01	6.4	20.93	6.5	19.01	6.5	19.65	6.5	20.13	6.5	26.70	6.8
ATP	70.88	3.9	101.59	3.5	69.21	3.4	53.04	3.4	25.29	3.4	61.68	3.4	53.10	3.2

*P solubilized as net mg % P_2O_5 after deducting respective control.

Table 2. Mineralization of organic phosphates by *B. cepacia*

Organic phosphorus sources	Days of Incubation													
	3		6		9		12		15		18		21	
	P_2O_5*	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	pH	P_2O_5	p H	P_2O_5	pH
DNA	24.84	3.8	27.41	3.6	16.39	3.5	31.25	3.7	25.16	3.5	31.41	3.6	20.18	3.6
RNA	15.86	5.6	16.19	5.6	16.63	5.6	15.38	5.6	10.74	5.7	16.03	5.7	50.81	5.8
Sodium β glecerophosphate	69.12	4.6	88.35	4.9	31.37	4.9	89.15	6.2	59.82	6.4	106.98	6.6	99.03	6.4
Calcium phytate	33.18	4.6	100.25	4.6	111.11	4.7	79.73	5.5	65.94	5.5	60.49	5.6	39.97	5.9
Sodium phytate	17.24	4.6	15.00	5.0	20.93	5.7	21.89	6.2	15.00	6.1	23.01	6.2	19.81	6.1
ATP	41.16	3.9	85.56	3.4	36.83	3.5	56.39	3.5	45.81	3.5	85.81	3.4	42.28	3.4

* P solubilized as net mg % P_2O_5 after deducting respective control.

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