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ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING DIAZOTROPHIC BACTERIA FROM RHIZOSPHERE OF RICE PLANT OF INDO-NEPAL BORDER

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Abstract

Free-living nitrogen fixing bacteria were isolated from the rhizosphere of rice plant of different regions of Indo-Nepal border. 33.78% isolates among them showed mineral phosphate solubilization. On the basis of bacterial characterization and identification with Bergey's manual of Determinative Bacteriology (Holt et al, 1994), 38% isolates showed maximum similarity with Pseudomonas and Azotobacter, 30% with members of Enterobacteriaceae, 4% with Gram's positive Microbacterium sp. and 28% was categorized unknown. ECI 12A isolate showed maximum solubilization index (S.I.), 0.809 on solid medium whereas 188.65 mg P/mg dry weight phosphate solubilization in liquid medium. The highest phosphate solubilizing isolate ECI 12A was sequenced after amplification of partial 16S rRNA gene, blast with BLASTn 2.2.17 program of NCBI showed 99 % identity having 99 % query coverage with Microbacterium sp. This isolate has been named as Microbacterium sp. Strain ECI-12A.The sequence of this strain has been deposited in NCBI Gene Bank under accession number EU155122 and this strain has promising potential for developing as a plant growth promoting rhizobacteria (PGPR) as well as biofertilizer.

Key words

Rhizobacteria; PGPR; phosphate solubilization; biofertilizer; phosphate solubilizing bacteria (PSB)

Introduction

The narrow zone of soil surrounding the root is known as rhizosphere, which is under the immediate influence of the root system. This zone is rich in nutrients in comparison to the bulk soil, due to the accumulation of a variety of organic compounds released from roots by

exudation, secretion, and deposition (Curl and Truelove, 1986). Because these organic compounds can be used as carbon and energy sources by microorganisms, microbial growth and activity is particularly intense in the rhizosphere. This is reflected by the number of bacteria that are found around the roots of plants and that is generally 10 to 100 times higher than in the bulk soil (Weller & Thomashow, 1994). Plant-associated bacteria that are able to colonize roots are called rhizobacteria and can be classified into beneficial, deleterious, and neutral groups on the basis of their effects on plant growth. Beneficial rhizobacteria that stimulate plant growth are usually referred to as Plant- Growth-Promoting Rhizobacteria or PGPR (Davison, 1988; Kloepper *et al.*, 1989). Diazotrophic bacteria, by their ability to convert N_2 into ammonia, which can be used by the plant, also belong to the PGPR (Dobbelaere *et al.* 2003). Because of their competitive advantages in a C-rich, N-poor environment, diazotrophs may selectively enriched in the rhizosphere (Döbereiner and Pedrosa, 1987), putting themselves in a good position to promote plant growth. PGPR, that solubilize the insoluble phosphate present in the soil, are called mineral phosphate solubilizing rhizobacteria. By the activities of mineral phosphate solubilizing rhizobacteria plants are benefited to use the plant available phosphorus. Soil microorganisms are able to solubilize insoluble mineral phosphate by producing various organic acids (Taha *et al.*, 1969; Banik and Dey, 1982; Halder *et al.*, 1990; Illmer *et al.*, 1995; Jones, 1998). This results in acidification of the surrounding soil, releasing soluble orthophosphate ions ($H_2PO_4^{-1}$ and HPO_4^{-2}) that can be readily taken up by plants. Furthermore, they are able to solubilize organic P compounds by means of phosphatase enzymes (Greaves and Webley, 1965; Tarafdar and Junk, 1987; Garcia *et al.*, 1992). A large number of P-solubilizing bacteria (PSB) have been isolated from the rhizosphere of several crops. It was estimated that P-solubilizing microorganisms may constitute 20 to 40% of the culturable population of soil microorganisms and that a significant proportion of them can be isolated from rhizosphere soil (Kucey, 1983; Chabot *et al.*, 1993). Phosphorus is one of the major plant nutrients limiting plant growth. Most agricultural soils contain large reserves of P, a considerable part of which has accumulated as a consequence of regular applications of chemical fertilizers. However, a large proportion of soluble inorganic phosphate added to soil is rapidly fixed as insoluble forms soon after application and becomes unavailable to plants (Rodriguez *et al.*, 1999). Phosphorus fixation and precipitation in soil is generally highly dependent on pH and soil type. In acid soils, free oxides and hydroxides of Al and Fe fix P, while in alkaline soils Ca, causing a low efficiency of soluble P fertilizers, fixes it. The calcium super phosphate fertilizer, which contains about 15% of phosphorus pentoxide, normally loses its available P proportion when it comes in contact with soil minerals containing calcium carbonates (Lindsay, 1979). The physical chemical properties of soil of different fields are variable hence it seems that the bacterial populations may vary from place to place.

Since a few phosphate-solubilizing bacteria were reported earlier but most efficient strain is lacking. The mail objective of this present investigation to find out the bacterial population and most efficient diazotrophic phosphate solubilizing rhizobacteria present in the rice fields of India as well as Nepal and to investigate so as to use as biofertilizer.

Materials and methods

Soil sample collection and isolation of bacteria

Rice (*Oryza sativa L.*) plant of Parsa district, Bara district of Nepal and East Champaran district of Bihar and Varanasi district of Uttar Pradesh of India were selected for study. Rhizosphere soil sample were collected carefully by uprooting the root system and placed in a sterile polythene bag

for transport and stored at 4°C. 1.0 gram of rhizosphere soil was suspended in 1.0 ml. of sterile DDW in sterile test tube and shaken by hands vigorously for proper mixing. After 1h of sedimentation process, 1.0 ml. of water was taken form the tube and it was mixed in another 9.0 ml. of sterile DDW for dilution. 1.0 ml of bacterial suspension was further diluted in another sterile test tube containing 9.0 ml. DDW. In this way it was diluted 10^{-7} dilution. 100µl aqueous phase was kept in solid $JNFb^-$ agar plate and spreading was done. It was incubated for 3 days at 37°C in BOD incubator and morphologically different colonies appeared on the plates were isolated, sub cultured and enrichment was done in N-free $JNFb^-$ medium (Döbereiner, 1995) devoid of combined nitrogen.

Search and quantification of isolates for their mineral phosphate solubilizing activities

Search of phosphate solubilizing isolates was done as per the method of Goldstein (1986) as well as Mehta and Nautial (2001) solid medium after the incubation of 3 days at 37°C for the detection of phosphate solubilizing isolates. Solubilization index (S.I.) was calculatedusing solid plates of MPVK medium with a few modifications. The MPVK medium comprises 1.0% (w/v) glucose, 0.05% (w/v) $(NH_4)_2SO_4$, 0.02% (w/v) NaCl, 0.02% (w/v) KCl,0.01% (w/v) $CaCl_2$-$2H_2O$, 0.01% (w/v) $MgSO_4$ -$7H_2O$, 0.05% (w/v) $MnSO_4$ -$7H_2O$, 0.05% (w/v) FeSO4-$7H_2O$, 0.05% (w/v) yeast extract and 0.5% (w/v) $Ca_3(PO_4)_2$ in distilled water (pH 7.5). $Ca_3(PO_4)_2$ was autoclaved first. Then, the other sterile ingredients were aseptically mixed after autoclaving. 1.0 ml bromophenol blue was added from 0.4% (w/v) stock solution of the dye prepared in ethanol (pH 6.7 adjusted with 0.1N NaOH) in 100 ml MPVK medium. For S.I. study, 1.0 ml of overnight NB grown bacterial culture was taken; it was washed with normal saline (0.85% w/v NaCl) after adjusting equal O.D. (0.3) for all isolates. Now, it was resuspended in 1.0 ml normal saline. 10µl suspended bacterial isolates were inoculated on solid MPVK medium in triplicate for estimation of S.I. of phosphate solubilization. Colony diameter (CD) and colony diameter + halozone (TCD) were recorded after 3 days incubation at 37°C. The S.I. was calculated using formula (S.I. = (TCD – CD)/ TCD). The quantification of phosphate solubilization was done in liquid Nautial medium after 3 days of incubation at 37°C. Calcium tri phosphate was used as standard for quantification by spectrophotometer. Estimation of free phosphate released in the medium as a result of phosphate solubilization by the bacterial isolates was estimated as per the method of Marinetti (1962). Reagent A: 10% Ascorbic acid (stored at 4°C) Reagent B: 0. 42% Ammonium molybdate in 1 N H_2SO_4 (stored at room temperature) Reagent C: 1 part of reagent A + 6 part of reagent B. 1.5 ml of 3 days old grown cultures was harvested by centrifugation at 8000 rpm for 2 min. 3.5 ml of reagent C was added to the culture supernatant of each bacterial sample and after mixing, incubated at room temperature for 1h. Optical density was measured at 660 nm against blank. Simultaneously, standard with various concentrations of K_2HPO_4 was prepared separately.

16 rRNA gene amplification and sequencing

Amplification of 16S rRNA gene of the best phosphate solubilizing isolate ECI 12-A was performed. The PCR reaction mixture contains 36.0 µl H_2O, 5.0 µl of 10X buffer, 0.125 µl each primer (Forward: 5'- ACT GGC GGA CGG GTC AGT AA- 3'and Reverse: 5'- CGT ATT ACC GCG GCT GCT GG-3'), 0.5 µl of each dNTPs (10 mM), 0.5 µl of 3.0 U Taq polymerase and 4.0 µl template DNA. Amplification was performed with thermal profile having initial denaturaton at 95°C for 3 minute, 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extention at 72°C for 1 minute 10 second with final extention at 72°C for 5 minutes.

The amplified *16S rRNA* gene was sequenced

with ABI PRISM model 3730 version 3.0 DNA sequencer.

Sequence analysis and multiple alignments with known P-solubilizing bacteria

The generated *16S rRNA* gene sequence of isolate ECI 12-A was analyzed by blast with BLASTn program of NCBI online on website: http://www.ncbi.nlm.nih.gov/BLAST/. 16S rRNA gene sequences of few well known p-solubilizing bacteria were taken from NCBI Gene Bank and aligned with our generated sequence of *Microbacterium* sp. strain ECI 12-A with ClustalW program online on website: http://align.genome.jp/.

Nucleotide sequence accession number

Sequence obtained in this study has been submitted in NCBI Gene Bank which can be viewed under accession number EU155121. *16S rRNA* genes of known phosphate solubilizing strains were taken from data of Gene Bank for alignments with gene sequence of this study.

Statistical analysis

Statistical calculation of the S.I was calculated for the t-test and significance study using online tool with the website http://home.calara.net/sisa/t-test.html. The confidence interval was taken 95% the P value (<0.05) was the level of significance.

Results

Mineral Phosphate solubilization Characterization

All the phosphate-solubilizing isolates were analyzed by measuring the CD and TCD of the isolates for the determination of S.I. on the MRVK agar plate containing bromophenol pH indicator. The yellow or orange halozones were formed around phosphate solubilizing bacteria (Fig. 1). There were three categories of bacteria were found depending on the formation of halozone. First category of isolates did not grow, the second, grew on the plate but did not produce halozone and the third, produced yellow or orange halozones (Fig. 1) due to acid production presence of bromophenol pH indicator in the medium. The change in colour was found due to acidification of the medium by the p-solubilization. It was found that's.I. ranges from 0.118 to 0.809 as shown in Table1. The highest S.I. was recorded in isolate ECI 12A. For the confirmation and most accurate determination, quantification was also done. According to quantification data ECI 12A again showed maximum phosphate solubilizing potential (188.65 µg P/mg dry weight). The phosphate solubilizing potential of ECI 12A was found promising sufficient to be an efficient PGPR. The pH of the medium was measured at the time of quantification of P; it was found 3.0 to 5.4 (data not shown) due to production of acids in the medium.

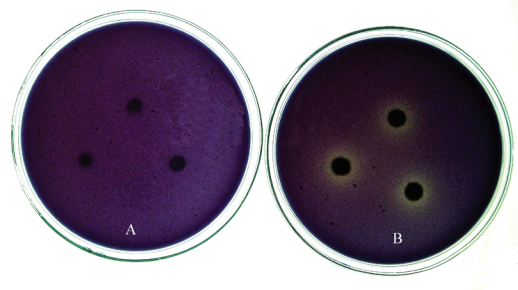

Fig. 1: Phosphate solubilization plate assay showing halozones of phosphate solubilization. A. No solubilization, B. Halozones of solubilization. Inoculation was done in triplicate of same isolate in a plate.

Statistical calculation for t-test was done online with ewbsite http:// home.clara.net/ sisa/t-test.html CD = Colony diameter, TCD = Colony diameter + halozone diameter, S.I = solubilization index. All records are mean of three independent readings.

Table 1: Phosphate solubilization records showing CD, TCD, S.I. P- value and quantification of phosphate solubilizing isolates

S.N.	Isolates	Phosphate solubilization				
		CD (mm.)	TCD (mm.)	S.I.	P Value	µg P mg $^{-1}$ dry weight
		Mean ± S.D.	Mean ± S.D			
1	ECI 2A	4.67 ± 0.577	11 ± 1	0.575	0.0027	92.13
2	ECI 2B	4.67 ± 0.577	7 ±1	0.333	0.0099	16.04
3	ECI 5B	6.33 ± 0.577	18.67 ± 1.155	0.661	0.0005	140.06
4	ECI 7A	4.67 ± 0.577	25.67 ± 0.577	0.818	0	52.88
5	ECI 7D	4.33 ± 0.577	6.67 ± 0.577	0.351	0.0077	16.04
6	ECI 9A	5.33 ± 0.577	7.667 ± 0.577	0.305	0.0077	12.9
7	ECI 9B	5.33 ± 0.577	9.333 ± 0.577	0.429	0.0011	40.37
8	ECI 10A	4.33 ± 0.577	6.333 ± 0.577	0.316	0.0132	13.25
9	ECI 11C	5.67 ± 0.577	9.333 ± 1.155	0.392	0.0015	40
10	ECI 12A	4.33 ± 0.577	22.67 ± 1.155	0.809	0.0001	188.65
11	AF 1C	4.67 ± 0.577	5.067 ± 0.577	0.176	0.101	12.47
12	AF 1D	5.33 ± 0.577	6.667 ± 1.155	0.201	0.1677	12.74
13	AF 4B	5 ± 1	7 ± 1	0.286	0.0705	18.05
14	AF 4C	5.67 ± 0.577	7 ±0	0.19	0	14.08
15	AF 5A	5.67 ± 0.577	7.333 ± 0.577	0.227	0.0242	17.47
16	AF 5D	4.33 ± 0.577	7.33 ± 0.577	0.41	0.0031	34.45
17	PN 4D	5.33 ± 0.577	7.667 ± 0.577	0.305	0.0077	17.47
18	PN 7C	5.67 ± 0.577	6.667 ± 0.577	0.15	0.1018	12.47
19	BN 1A	5.33 ± 0.577	7.667 ±1.155	0.305	0.0506	15.66
20	BN 1C	5 ± 0	6.333 ± 0.577	0.21	0.775	14.27
21	BN 2A	4.33 ± 0.577	6.667 ±1.155	0.351	0.0506	20.78
22	BN 4A	5.33 ± 1.155	8.667 ± 0.577	0.385	0.0202	33.17
23	BN 5C	5.33 ± 0.577	7.333 ± 0.577	0.273	0.0131	12.23
24	BN 7C	7.33 ± 0.577	8.667 ±1.155	0.154	0.1677	12.17
25	BN 9C	5 ± 1	5.667 ± 0.577	0.118	0.404	11.52

16 rRNA gene amplification and sequence analysis

The sequence of *16S rRNA* gene of ECI 12-A showed 99% maximum identity with 99% query coverage with most of the *Microbacterium sp* and Microbacteriaceae bacterium such as *Microbacterium sp* 022s1-13 (accession no- AB266330), *Microbacteriaceae bacterium* KVD-unk-67 (accession no- DQ490447.1), *Microbacterium laevaniformans* LA (accession no- AF535159). (See Fig 2)

Discussion

Plant growth promotion has been reported by the solubilization of insoluble P in the soluble form by the activities of PGPR (Rodríguez and Fraga, 1999; Richardson, 2001). It is well known that P is an essential nutritional element for plant. But, it is one of the least soluble nutrient ions in the environment; therefore, less than 5% of total soil phosphate is available to plants (Epstein, 1972; Brown, 1974). Phosphorus exists in nature in a variety of organic (derived from microorganisms and plants) and inorganic (originating from

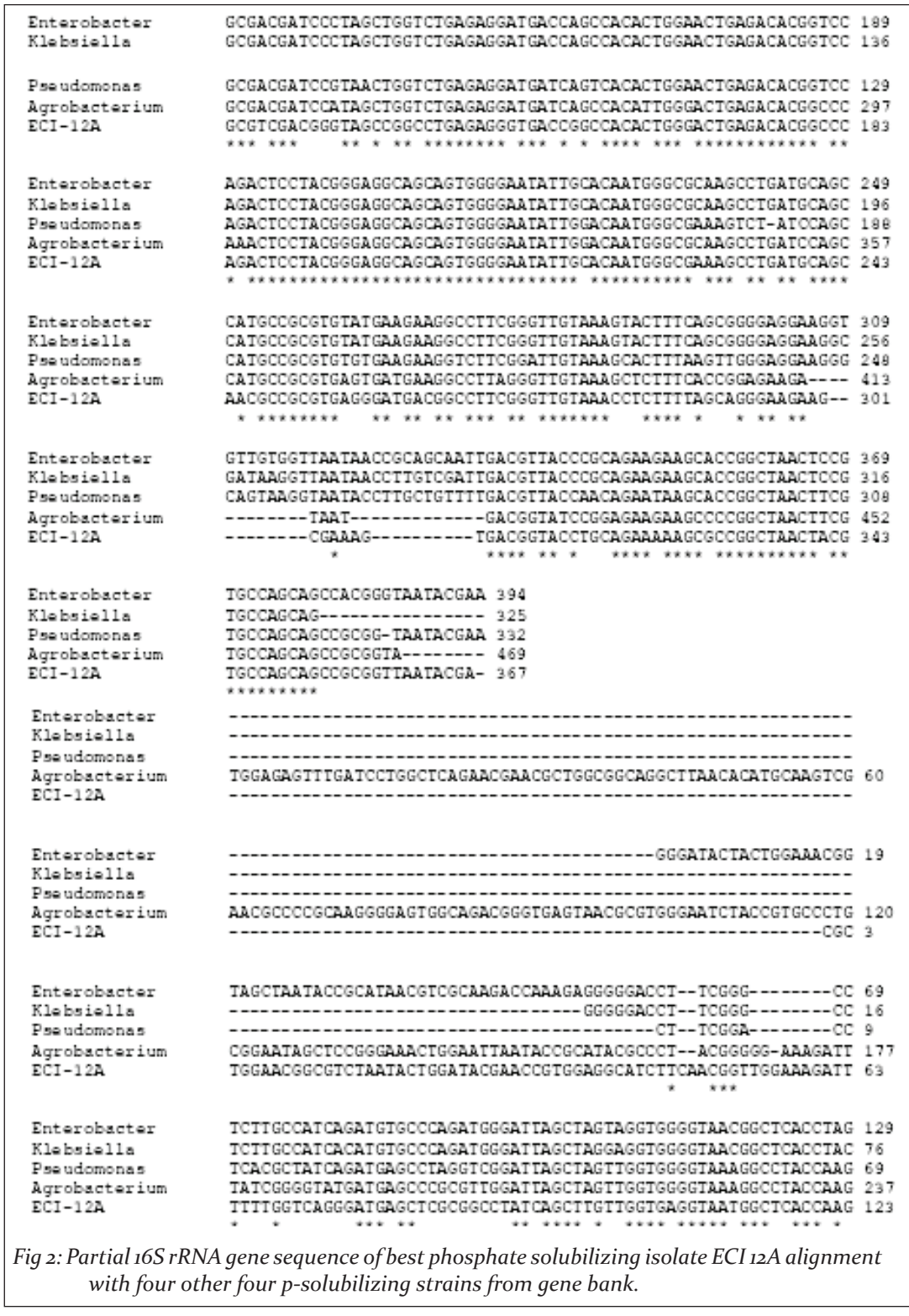

Fig 2: Partial 16S rRNA gene sequence of best phosphate solubilizing isolate ECI 12A alignment with four other four p-solubilizing strains from gene bank.

applied P fertilizer) forms that are insoluble to very poorly soluble (Paul and Clark, 1989). Therefore, the addition of phosphate fertilizers has become a common practice in modern agriculture. However, a large portion of the soluble inorganic phosphate applied to soil as fertilizer is rapidly immobilized by the iron and aluminium in acid soils and by calcium in calcareous soils soon after application, thus becoming unavailable to plants (Chang and Chu, 1961; Lindsay, 1979; Sanyal and De Datta, 1991; Holford, 1997). Soil microorganisms are able to solubilize insoluble mineral phosphate by producing various organic acids (Taha *et al*., 1969; Banik and Dey, 1982; Halder *et al*., 1990; Illmer *et al*., 1995; Jones, 1998). This results in acidification of the surrounding soil, releasing soluble orthophosphate ions ($H_2PO_4^{-1}$ and HPO_4^{-2}) that can be readily taken up by plants. In our investigation 33.78% phosphate solubilizing isolates were found among the total diazotrophic isolates, which shows agreement with the estimation of Kucey, 1983 and Chabot *et al*., 1983. According to them 20 to 40% of the culturable bacterial population of soil solubilize P. Although there is good evidence for P-solubilization by these microorganisms in pure culture (Taha *et al*., 1969; Bajpai and Sundara Rao, 1972; Banik and Dey, 1981; Chabot *et al*., 1993), it is not easy to demonstrate P solubilization in plant-microorganism systems. Experiments performed with P-solubilizing diazotrophs are few, and the results obtained quite diverse, varying according to plant or bacterial species. *Bacillus megaterium* and *P. polymyxa* are able to enhance growth and yield but not the P uptake of canola, indicating that P-solubilization

is not the main mechanism responsible for positive growth response (de Freitas *et al*., 1997). Kumar and Narula (1999) used chemically induced mutants of *Azotobacter chroococcum*, isolated from the wheat rhizosphere, with higher phosphate-solubilization activity to inoculate wheat and found significantly positive effects of inoculation on percent germination and growth emergence, with the mutant strains performing better than the parent strain. We are now investigating the response of the highest p-solubilizing isolate on rice plant and uptake of P in this plant.

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