

Isolation and characterization of insoluble inorganic phosphate solubilizer rice rhizosphere strain *Enterobacter cloacae* BAU3

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Abstract

The objective of the present study was to isolate and characterize most efficient phosphate solubilizing bacteria (PSB) from rice rhizosphere. The study was carried out during the *Kharif* season 2018 at Department of Soil Science and Agricultural Chemistry, Bihar Agricultural University, Sabour, Bhagalpur, Bihar. The availability of phosphorous to plants for uptake and utilization is limited in soil due to fixation in the form of Fe-P, Al-P and Ca-P. The use of phosphate solubilizing bacteria can prove to be helpful measure to supply phosphorous to the crops to increase the productivity. In the present investigation, a total of 10 isolates were obtained from rice rhizosphere soil samples. All ten isolated isolates were shown phosphorus solubilization. Out of ten isolates BAU3 was found to be most potent phosphate solubilizers showing clear halo zone around its colony. The isolate BAU3 showed 20.00 mm phosphate solubilizing halo zone around its colony. The solubilization index (SI) of the isolate BAU3 was also calculated at the end of the incubation period and observed phosphate solubilization index (SI) of 3.22. The isolate BAU3 showed maximum insoluble phosphate solubilization of 450.24 $\mu\text{g ml}^{-1}$ and isolates BAU3 was selected for subsequent studies. The bacterial isolates BAU3 was gram negative, non-spore forming rods shaped. On the basis of the 16SrDNA sequencing, isolate BAU3 was identified as *Enterobacter cloacae* strain BAU3 (Genebank Accession No. MK033472). The isolated strain of bacterial has potential to solubilize insoluble phosphorus and it can be utilized for preparation of microbial inoculants or biofertilizers.

Keywords: *E. cloacae* BAU3, Phosphate solubilizing bacteria, Rice, Solubilization index

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INTRODUCTION

Phosphorus is one of the most important macro nutrient required by plants. It is a key nutrient for morphological, physiological and biochemical development of the plants. Also, it contributes to photosynthesis, energy and sugar production and nucleic acid synthesis (Saber *et al.*, 2005). Plants absorb inorganic form of phosphorus (P) which is essential element for plant growth and development making up to 0.2% of total plant dry weight. The level of available or inorganic phosphorus is very low in the soil and available P is insoluble form. Approximately 50 percent of the total phosphorus in legume seeds and 60 to 70 percent in cereal grains is stored as phytin (Braun, *et al.*, 1995). Movement of nutrients within the plant depends largely upon transport through cell membranes, which requires energy in the form of ATP and other P compounds to oppose the forces of osmosis (Leandro *et al.*, 2011). Phosphorus is taken up mostly as the primary orthophosphate ion (H_2PO_4^-), but some is also absorbed as secondary orthophosphate (HPO_4^{2-}). Phosphorus

may be stored in the root or transported to the upper portions of the plants. Plants obtain phosphorous from soil solution as phosphate anions which are extremely reactive and get immobilized through precipitation with cations viz., Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} , depending on the soil pH (Kundu *et al.*, 2009).

Plants acquire phosphorous from soil solution as phosphate anions which are extremely reactive and get immobilized through precipitation with cations such as Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} , depending on the particular properties of the soil. Several soil microorganisms known as phosphate solubilizing bacteria (PSB) have the ability to solubilizing insoluble mineral phosphate by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO_2 and H_2S . This results in acidification of the surrounding soil, releasing soluble orthophosphate ions (H_2PO_4^- , HPO_4^{2-} and PO_4^{3-}) which can be readily taken up by plants (Kundu *et al.*, 2009).

Almost 75 to 90% of added P fertilizer in agricultural soils is precipitated by iron, aluminum and calcium complexes present in soils. Furthermore,

phosphatic fertilizers are expensive, and excessive use of rock phosphate (RP) is potentially and environmentally undesirable (Panhwar et al., 2011). To overcome phosphorus deficiency problems in soils by safe ways, low costs and eco friendly environment strategies, Phosphorus Solubilizing Bacteria (PSBs) have been used to solubilize the precipitated phosphates through converting them into soluble forms, $H_2PO_4^-$ and HPO_4^{2-} that are available to plant (Coutinho et al., 2012). This occurs through principal mechanisms such as acidification of the medium, chelation, ion-exchange reactions and production of various low molecular weight organic acids (Chung et al., 2005 and Gulati et al., 2010). The majority of powerful PSBs that belong to bacteria as *Pseudomonas*, *Enterobacter* and *Bacillus* (Yadav et al., 2010 and Xiao et al., 2011). *Rhizobium leguminosarum* bv. *Viciae* have been demonstrated to solubilize inorganic phosphorus by Belal et al. (2013). The present study was designed to isolate, characterize and evaluate the phosphorous solubilization capacity of phosphate solubilizing bacteria.

MATERIALS AND METHODS

The present study was undertaken to isolate and characterize most efficient phosphate solubilizing bacteria from rice rhizosphere at Bihar Agricultural University, Sabour, Bhagalpur, India.

Soil sample collection: Soil samples were collected during *khari* season of 2018 from rhizosphere of healthy rice plants from Research Farm of Bihar Agricultural University, Sabour (longitude 87°2'42" East and latitude 25°15'40" North at altitude of 46 meters above mean sea level in the heart of the vast Indo-Gangetic plains of North India.) Bhagalpur, India, stored in polyethylene bags and brought to the laboratory for further studies. The collected soil samples were silty clay loam having pH 7.1, Electrical conductivity 0.24, dSm^{-1} , organic carbon 0.53 per cent, available phosphorus 11.98 $kg\ ha^{-1}$, available potassium 195.00 $kg\ ha^{-1}$ and nitrogen 185.00 $kg\ ha^{-1}$.

Isolation of phosphate solubilizing bacteria (PSB): Phosphate solubilizing bacterial (PSB) isolates were obtained by dilution plating method, the air-dried ten grams soil from each soil sample was suspended in a 90 ml of sterilized water blank and successively diluted up to seven time dilution, than one ml suspension were transferred to the sterilized petri plate. Transfer 15 ml of sterilized Pikovskaya's agar medium (PKV) to the plate (Pikovskaya 1948) (The composition is as follows ($g\ l^{-1}$): Glucose, 10 g; tricalcium phosphate (TCP), 5 g; ammonium sulphate, 0.5 g; sodium chloride, 0.2 g; potassium chloride, 0.2 g; magnesium sulphate, 0.1 g; yeast extract, 0.5 g; manganese sulphate, trace; ferrous sulphate, trace; agar, 15 g; the pH was adjusted to 7.0 ± 0.2). Pure culture of

the isolates were made by repeated sub culturing on fresh PKV plates and maintained on PKV slants at the refrigerated condition. The one ml suspensions of desired dilutions were plated on Pikovskaya's agar medium and plates were incubated at $28 \pm 2^\circ C$ for 3-5 days. The bacterial colonies surrounded with a halo zone were purified by streaking method then maintained on Pikovskaya's Agar slants at $4^\circ C$.

Screening of isolates for phosphate solubilization Primary screening: All the halo zone bearing colonies were screened for phosphate solubilization on PKV medium in petriplates. The isolates showing phosphate-solubilizing ability were inoculated on the PKV plate and incubated at $28 \pm 2^\circ C$ for 3-7 days. The diameter of colony and solubilization zone was measured. Phosphate solubilization index (SI) was calculated by measuring the colony diameter and the halo zone diameter and the colony diameter, using the following formula of Premono et al., 1996.

Phosphate Solubilization Index (SI) = (Colony diameter + Halo zone diameter) \times / Colony diameter
.....Eq.1

50 ml of Pikovskaya's broth medium with tricalcium phosphate was prepared and sterilized then inoculated with the specific amount (5-10% of the broth) of the isolate. Then the inoculated sample was incubated for 48 hours on rotatory shaker at 150 rpm at $28 \pm 2^\circ C$ for 3-7 days, after incubation culture broth was centrifuged at 10,000 rpm for 5min and the supernatant was used to estimate the P release. The P was measured spectrophotometrically (Systronic UV-VIS Spectrophotometer 117) at 430 nm according to Subba (1993).

Characterization of bacterial isolates: All bacterial isolates were screened on the basis of formation of clear halo zone around the colonies. The highest phosphorus solubilizing activity of isolate (wider halo zone having more diameter and thickness) were selected for morphological, biochemical tests Bergey's Manual of Systemic Bacteriology (Buchanan, 1974).

16S rRNA gene sequencing and analysis: Molecular characterization (included DNA extraction and polymerase chain reaction (PCR) and 16S rDNA gene sequencing technique was conducted using ABI 3730xl DNA sequencer at Xcelris Labs Ltd. Gujrat, India). To determine the phylogenetic relationship, the 16S rRNA gene-sequence of isolated and screened bacterial strain was obtained using the 16S rRNA gene-specific universal primers: 8F and 1492R. The 16S rRNA gene sequence of the isolated strain was analyzed at NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) using BLAST (N) program (Zhang et al, 2000). Phylogenetic tree was constructed by neighbor joining method for this alignment using the MEGA 6 (Molecular Evolutionary Genetics Analysis) software (Tamura et al, 2013)

a,b). The final sequence was submitted at Gen-Bank (Thompson, et al, 1997).

Statistical analysis: All experiments were performed in triplicate, and the results were expressed as the mean.

RESULTS AND DISCUSSION

Isolation and screening of phosphate solubilizing bacteria: Rice rhizosphere soil samples were collected from five rice crops of Bihar Agricultural University Research farm, Sabour, Bhagalpur, Bihar and a total of 10 morphologically distinct colonies isolates were isolated (Table 1). The colony of isolate BAU3 was observed gram negative, round in shaped, white in colour and having entire edge. Similar results were obtained by Borham et al., 2017, who isolated the most efficient phosphate solubilizing bacterial strain

isolated from rhizosphere of healthy rice plants and identified as *Enterobacter cloacae* (B1) based on morphological and biochemical characteristics and 16S r DNA. Till today numerous works have been done by different researchers, like Vazquez et al. (2000) who isolated many isolate of PSBs from mangrove soil and reported that out of all isolates *V. proteolyticus* was found to be most active. Similarly, Kannapiran et al. (2011), isolated *Pseudomonas, Bacillus, Vibrio, Micrococcus, Flavobacterium, Corynebacterium, Alcaligenes* and *Enterobacter* from samples collected from different stations of the Thondi coast.

Inorganic phosphorus solubilization efficiency: In the present investigation, the phosphate solubilizing efficiency (Qualitative) of isolates was determined by the plate assay where 10 isolates were spotted on Pikovskaya's agar medium plate as per

Table 1. Colony characteristics of phosphate solubilizing bacterial isolates isolated from rice rhizospheric soil.

Isolate	Shape	Edge	Opacity	Colour	Elevation	Texture	Gram staining
BAU1	Irregular	Undulate	Opaque	White	Raised	Mucoid	Negative
BAU2	Round	Undulate	Opaque	White	Raised	Mucoid	Negative
BAU3	Round	Entire	Opaque	White	Raised	Mucoid	Negative
BAU4	Round	Undulate	Opaque	White	Flat	Mucoid	Negative
BAU5	Irregular	Undulate	Translucent	White	Raised	Mucoid	Negative
BAU6	Irregular	Undulate	Opaque	White	Raised	Mucoid	Negative
BAU7	Round	Undulate	Opaque	White	Raised	Mucoid	Negative
BAU8	Irregular	Undulate	Opaque	White	Flat	Mucoid	Negative
BAU9	Round	Entire	Opaque	White	Raised	Mucoid	Negative
BAU10	Round	Undulate	Opaque	White	Raised	Mucoid	Negative

Table 2. Phosphate solubilization by PSB isolates isolated from rice rhizosphere.

Isolate	Colony diameter (mm) at 7 th day		P solubilization zone (mm) at 7 th day		Solubilization index (SI)
	day	day	day	day	
BAU1	8	15	2.88		
BAU2	9	12	2.33		
BAU3	9	20	3.22		
BAU4	10	18	2.80		
BAU5	6	10	2.67		
BAU6	7	11	2.57		
BAU7	6	12	3.00		
BAU8	8	14	2.75		
BAU9	9	8	1.89		
BAU10	6	12	3.00		

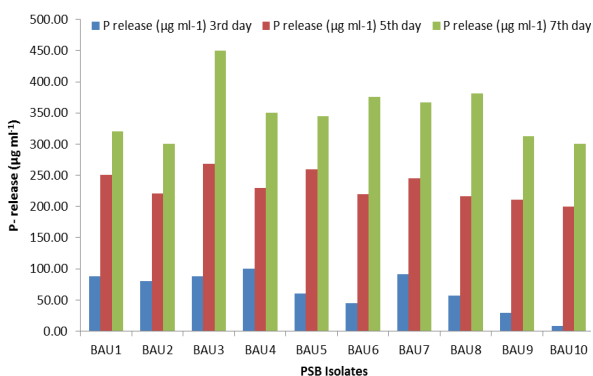


Fig. 1. P -release (µg ml⁻¹) by PSB isolates isolated from rice rhizosphere.

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>MK033472.1 Enterobacter sp. strain BAU3 16S ribosomal RNA gene, partial sequence
GTTCCGGGGAACCGGTAGCACAGAGAGCTTGCCTCGGGTGACGAGTGGCCGACGGGTGAGTAATGTCTG
GGAACTGCCGTATGAGGGGGATAACTACTGGAAACGGTAGCTAATACCCGATAACGTCCGAAGACCAA
AGAGGGGGACCTTCGGGCTCTGCCATCAGATGTGCCAGATGGGATAGCTAGTAGGTTGGGTAACCG
CTCACCTAGCCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACTGGAACCTGAGACACGGTCCA
GACTCCTACGGGAGCAGCAGTGGGGAATTTGCACAATGGCGCAAGCCTGATGCAGCCATGCCCGGTG
TATGAAGAAGCCCTTCGGGTTGTAAGTACTTTCAGCGGGGAGGAAGGTTGTTGGTTAATAACCCGACGC
AATTGACGTTACCCGCGAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA
AGCGTTAATCGGAATTACTGGGCTAAAGCCACGACGAGCGGCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCAACCTGGGAACCTGCATTCGAAACTGGCAGGCTAGAGTCTTGAGAGGGGGTGAATTCAGGTTG
AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGCCGCCCTGGACAAGACTGACGC
TACGTTGCGAAAGCGTGGGAGCAACAGGATTAGATACCCCTGGTGTCCACCGCTAAACGATGTCGAT
TTGAGGTTGTGCCCTTGAGCGGTGGCTTCGGGAGCTAACCGCTTAAATCGACCCGCTGGGGAGTACGGC
CGAAGGTTAAAACTCAAATGAATGACGGGGCCCGCACAGCGGTGGAGCATGGTTAATTCGATG
CAACCGAAGAACCCTACCTGGTCTTGACATCCACAGAACCTTCCAGAGATGGATGGTGCCTTCGGGAA
CTGTGAGACAGGTGCTGCATGGCTGTGCTCAGCTCGTGTGTAATGTTGGTTAAGTCCCGCAACGAG
CGAACCCCTTATCCTTTGTTCCAGCGGTAGGCCGGGAACCTCAAAGGAGACTGCCAGTGAATAACTGGA
GGAAGTGGGGATGACGTCAAGTCAATCATGCGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCA
TACAAGAGAGAACGACCTCCGAGAGCAAGCGGACCTCATAAAGTGGCTCGTAGTCCGGATGGAGCTG
CAACTCGACTCATGAAGTCGAATCCGATGTAATCGTAGATCAGAAATGCTACGGTGAATAGCTTCCCGG
GCCTTGTACACACCCCGCTCACACCATGGGAGTGGGTTGCTAAAAGAAGTAGGTAGCTTAACCAAGTGGT
AGC
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Fig. 2. FASTA sequences of the PSB (*E. cloacae* strain BAU3).

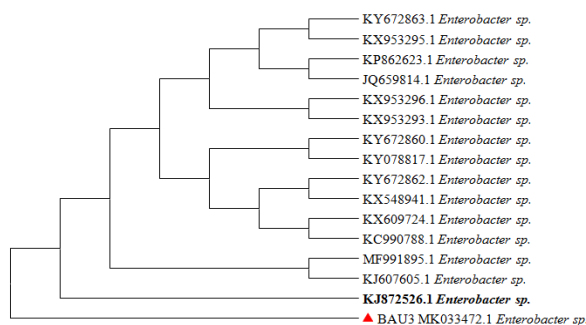


Fig.3. Phylogenetic tree: Amplified 16S rRNA gene fragment from the isolated strain *Enterobacter cloacae* BAU3 was sequenced and blast searched through NCBI database. Closely related sequences were downloaded and aligned using CLUSTAL W. These sequences were analyzed using maximum likelihood (ML) method. The bootstrap replicates (BS) values of 70% or greater represent well supported nodes and thus only those were retained. *Enterobacter cloacae* AJ1 with accession number **KJ872526.1** taken as out group. *Enterobacter* strain BAU3 present in the group containing *Enterobacter cloacae* strains.

the primary screening technique described by Kundu *et al.* (2009). On the basis of the halo zone and isolate diameter Solubilization Index (SI) was calculated (Kannapiran *et al.*, 2011). The PSB isolate BAU3 was found to be most potent phosphate solubilizer showing clear halo zone around its colony. The PSB isolate BAU3 showed 20.00 mm phosphate solubilizing halo zone around its colony (Table 2). The solubilization index (SI) of the isolated strain BAU3 was also calculated at the after ten days of incubation period and observed phosphate solubilization index (SI) of 3.22. The halo zone formation around the bacterial colonies could be due to the production of low molecular weight organic acids viz., succinic acid, palmitic acid, glutamic acid, oxalic acid or due to the production of polysaccharides or due to the activity of phosphatase enzymes of phosphate solubilizing bacterial strains (Paul and Sinha, 2013). The obtained results were conformity with the Kumar *et al.* (2012) who observed the halo zone of solubilization up to 20 mm on Pikovskaya agar plates by *Enterobacter sp. Pseudomonas sp.* was the most efficient phosphate solubilizers on Pikovskaya's agar plates it gaven solubilization index 228 ± 6.12 at 7th day incubation which were shown by Kannapiran *et al.* (2011).

The phosphate solubilizing efficiency of all ten isolates Pikovskaya's broth indicated that the all isolates efficiently solubilized inorganic phosphate in the medium containing 0.5% tri-calcium phosphate (Fig 1). PSB isolate BAU3 was produced $450.24 \mu\text{g P ml}^{-1}$ in soluble phosphate in the PKV broth after the 7th day of the incubation period. It might be due to the more secretion of low molecular weight organic acids, secrete phosphatases

enzymes which increase the solubilization of insoluble phosphate. The similar results were obtained by Banerjee *et al.*, 2010, who reported that maximum phosphate solubilization efficiency of the isolated *Bacillus sp.* was after incubation of 96 h. The results are also in agreement with the results of Thakur and Putatunda (2017), who isolated the isolate IH37 (*Enterobacter aerogenes*) and the isolate IKas4 (*Enterobacter sp.*) which were showed maximum phosphate solubilization of $0.070 \mu\text{g}$ and $0.99 \mu\text{g P ml}^{-1}$ respectively. Both bacterial isolates were gram negative, non-spore forming rods. On the basis of the 16SrDNA sequencing isolates IKas4 and IH37 were identified as *Enterobacter aerogenes* and *Enterobacter sp.* Respectively.

The results are also conformity with the findings of Kundu *et al.* (2009) who demonstrated that the P-solubilization in PVK broth, the maximum number of bacteria showed P-solubilization $< 50 \mu\text{g P/ml}$. As we compare the results of solubilization index and quantitative P solubilization we find that all the 10 isolates showed phosphorus solubilization. Out of the 10 isolates, highest phosphate solubilizing efficiency was shown by BAU3 and on its basis isolates showing maximum solubilization index and phosphate solubilization were selected for subsequent studies.

Characterization of selected *Enterobacter cloacae* strain BAU3: To identify the selected isolated bacteria isolate BAU3, 16S rRNA sequencing was performed. BLAST search analysis was carried out for the 16S rRNA sequences thus obtained using NCBI-Gene Bank database that showed a sequence identity of 99.0% with *Enterobacter cloacae* strain AJ1 (GeneBank accession number KJ872526.1). The FASTA sequence was submitted to GenBank with the accession number MK034472 (Fig. 2). Since the BLAST analysis revealed the alignment of the 16S rRNA sequence with a number of species of the genus *Enterobacter*, we used the most similar sequences to construct a phylogenetic tree using neighbour joining method (Fig. 3). Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program CLUSTAL W (Thompson *et al.*, 1997). These sequences were analyzed using maximum likelihood (ML) method as outlined by Mirza *et al.*, (2009). It was evident from the phylogenetic tree that the isolated organism *E. cloacae* was assigned as *E. cloacae* MK033472. Similarly, Ahemad and Khan (2010) was isolated *Enterobacter asburiae* strain PS2 from the mustard rhizosphere and was assessed for the fungicide-tolerance and production of plant growth promoting traits (phosphate solubilization, siderophores, indole acetic acid, exopolysaccharides, hydrogen cyanide and ammonia production) both in the presence and absence of fungicides.

Nucleotide sequence accession numbers: The 16S rDNA sequences were carry out by BLAST alignment search tool at the National Centre for Biotechnology Information web site (www.ncbi.nlm.nih.gov), and submitted to GenBank. Based on maximum identity score first Fifteen sequences were selected and aligned using multiple alignment software program ClustalW with accession number; stored in the NCBI database E. cloacae strain AJ1 (GeneBank accession number KJ872526). The NCBI has issued the nucleotide accession number MK033472 to *Enterobacter cloacae* strain BAU3. The similar organism E. cloacae strain BAB-6019 (GeneBank Accession Number KY672863) was isolated (Nagar et al., 2017).

Conclusion

A total of 10 isolates were obtained from five rice rhizosphere soil samples. Out of ten isolates, BAU3 was found most potent insoluble phosphate solubilizer. BAU3 was identified as *Enterobacter cloacae* (Genebank Accession Number MK033472) by 16s rDNA sequencing. The isolated bacterial strain seemed to be highly potent to solubilize insoluble phosphate. The biological nitrogen fixation efficiency of this strain may be explored and can be utilized for preparation of biofertilizers.

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