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Bacterial Strain Improvement via Random Physical Mutation to Improve Phosphate Solubilization Efficiency for Sustainable Crop Growth

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Abstract

Phosphorus deficiency in soil due to cation-mediated fixation reduces agricultural output from otherwise fertile lands. Phosphate solubilizing bacteria can solubilize this immobilized phosphate. The goal of this study was to use random UV mutagenesis to improve the phosphate solubilizing efficiency of the bacterial strains isolated from agriculture soils of Jaipur, Rajasthan. The phosphate solubilizing capacity was determined using the colorimetric chlorostannous reduced molybdo phosphoric acid blue method. When UV treated for 40, 50, and 60 min. Strain B5 depicted 58.54 %, 133.27 %, and 159.09 % enhanced phosphate solubilization, respectively, in the phylogenetic tree constructed using 16S rRNA gene sequencing, the isolate B5 clustered with *Pseudomonas putida* strains. Thus wild strains such as *Pseudomonas sp*. and *Bacillus sp.* can be mutagenically exploited to avail incapacitated phosphorus in soil. This can be an ecologically desired elucidation; however, more research is needed to investigate the underlying mechanisms involved and their repercussions.

Keywords: Mutagenesis; Phosphate solubilizing bacteria; Strain improvement; Sustainable agriculture.

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1. Introduction

There are many elemental nutritional factors which are inevitable for the soil fertility [1]. Nitrogen and phosphorus occupies the first and second position in limiting the agricultural yield. Phosphorus plays a vital role in nucleic acid and cell membrane structure, important for cell division, cell elongation, plant maturation, stress alleviation, and nitrogen fixation [2]. It is critical in terms of its role in advancing the stages of growth and differentiation of plants [3]. It has a comprehensive role in basal metabolic processes like energy production, respiration, and photosynthesis [4,5] and a conclusive role in anatomical development and modification of plant roots, hair density, and plant disease resistance [6]. But, multiple factors are associated with the concentration of available phosphorus in soil. Soil pH and concentration of divalent cations like calcium, iron, and aluminum are

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primely important [7]. In soils with high levels of these cations, naturally available or chemically applied phosphorus both gets precipitated and fixed, causing the decline in bioavailability of phosphate for root absorption [8]. There have been various observations where this phosphorus deficiency caused a significant cutback of crop output [9].

Moreover, meeting the requirement of using chemical fertilizers causes another cascade of environmentally distressing issues. Hence, the need for an ecologically competent solution brings phosphate-solubilizing bacteria into the limelight [10]. These bacteria have the potential to assist plants in preventing environmental stresses by solubilizing the stacked phosphate in soil and making it usable for plants [11,12]. Studies have shown that Bacillus megaterium increased phosphorus availability by nearly 30 % [13]. Likewise, several other microbial species belonging to the genera *Pseudomonas* [14], *Xanthomonas* [15], *Azotobacter* [16], *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Gordonia*, *Phyllobacterium*, and *Delftia* sp. [17]are also known to demonstrate elevated phosphorus. Various functional traits of microbes can be exploited to propose strategies that may enable enhanced phosphate utilization required for soils retaining a high amount of immobilized phosphate [9]. These traits can be improved by genetic alterations due to induced mutations, a common practice in the food industry [18]. However, few studies on bacterial mutation using UV treatment have been reported. The main objective of the current study was to conduct random mutagenesis of the isolated bacterial strains and evaluate the UV-induced mutation method's competence in the phosphate-solubilizing efficiency.

2. Material and Methods

2.1. *Soil sample collection*

The samples were collected from the agricultural fields of four different regions of Jaipur and Rajasthan (Chaksu, Chomu, Muhana, and Kanota). Soil samples were collected from various plant rhizospheres.

2.2. *Screening of phosphate solubilizing bacteria*

Soil samples were processed as soon as possible by plating them on nutrient agar and Pikovskaya's agar medium (Hi-media), which contains insoluble tricalcium phosphate as the source of phosphorus. Following incubation, bacterial colonies with a clearance zone on the media were picked and streaked on a fresh medium to test their phosphorus solubilizing ability. Phosphorus-solubilizing bacteria were streaked and grown on a nutrient agar slant [19].

2.3. *Isolation and identification of phosphate solubilizing bacteria*

These soils were blended and serially diluted using the stock soil suspension. The Nutrient agar plates were examined after two days of incubation for bacterial colony enumeration,

whereas the Pikovskaya (PVK) agar medium plates were examined after 3-4 days of incubation. Various cultural, morphological, and biochemical assays were conducted on the bacterial cultures to narrow their identification to the genus level. Bergey's manual of determinative bacteriology was used as a reference to identify bacterial culture based on the observed results.

2.4. *Quantitative analysis of phosphate solubilization by phosphate solubilizing bacteria*

The ability of PSB isolates to solubilize inorganic phosphorus from tri-calcium phosphate (TCP) was tested using PVK broth. On days 3, 6, and 9, the soluble phosphorus was determined using a colorimetric chlorostannous reduced molybdo phosphoric acid blue method [20]. A standard graph was prepared using KH_2PO_4 (2-10 ppm/mL) as a standard reagent.

2.5. *Random UV mutation of bacterial isolates*

The nutrient agar plate cultures containing the isolates were exposed to UV light, keeping at a distance of 15 cm and intervals for 40, 50, 60, 70, and 80 min and one plate was kept as a growth control without UV light exposure. Following the above procedure, the plates were covered with black paper to avoid light-induced repair mechanisms and incubated at 37 ºC for two days. Following the above procedure, the plates were covered with black paper to avoid light-induced repair mechanisms and incubated at 37 °C for two days [21]. The exposed cultures were again evaluated on days 3, 6, and 9 for phosphate solubilization using the process described above. The quantitative analysis of soluble phosphate for both wild isolated and mutant strains was carried out for comparison.

2.6. T*axonomical assignment*

The 16s rRNA sequencing was performed using the NCBI blast similarity search tool. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis, and HKY85 as a Substitution model. The program Tree Dyn 198.3 was used for tree rendering.

3. Results and Discussion

3.1. *Screening and isolation of phosphate-solubilizing microorganisms*

Phosphate solubilizing bacteria were isolated on nutrient agar plates and pikovskaya agar plates. On nutrient agar plates inoculated with 10^{-5} serially diluted suspension, the maximum and easily countable colonies were observed, producing 258, 211, 198, and 201 colony-forming units, respectively, from the soil of regions A, B, C, and D. Similarly, Pikovskaya agar plates inoculated with a 10^{-5} diluted bacterial suspension revealed 18, 17, 10, and 8 colony forming units from soil from regions A, B, C and D, respectively (Table 1).

Table 1. Rhizosphere microbial density as found on nutrient agar and Pikovskaya agar plates.

Soil Sample Regions	Microbial Density(Cfu/mL)*							
	Nutrient Agar Plate				Pikovskaya Agar Plates			
	10-4	10-5	10-6	10-7	10-4	10-5	10-6	10-7
Region A	TNTC	258	31	5	TNTC	18	4	-
Region B	TNTC	211	29	5	TNTC	17	2	-
Region C	TNTC	198	26	2	TNTC	10	2	1
Region D	TNTC	201	28	4	TNTC	8	-	-

*mean value of duplicates, Cfu= Colony forming Units; TNTC= Too numerous to count

3.2. *Morphological and biochemical identification of the isolated phosphorus solubilizing bacteria*

Further research was carried out with the best eight isolates labeled as B1, B2, B3, B5, B6, B8, B11, and B16 (Fig. 1), which were chosen based on their zone of clearance on Pikovskaya agar media (Table 2). All of the phosphate-solubilizing bacteria isolates were Gram-negative, small/medium-sized rods with a diverse array of colony morphological features (Table 3). By assorted characteristics displayed in various biochemical tests (Table 4), the bacterial isolates were identified as *Pseudomonas spp.* and *Bacillus spp.* Several findings showed that these organisms were prominently as phospho-solubilizers [22-24].

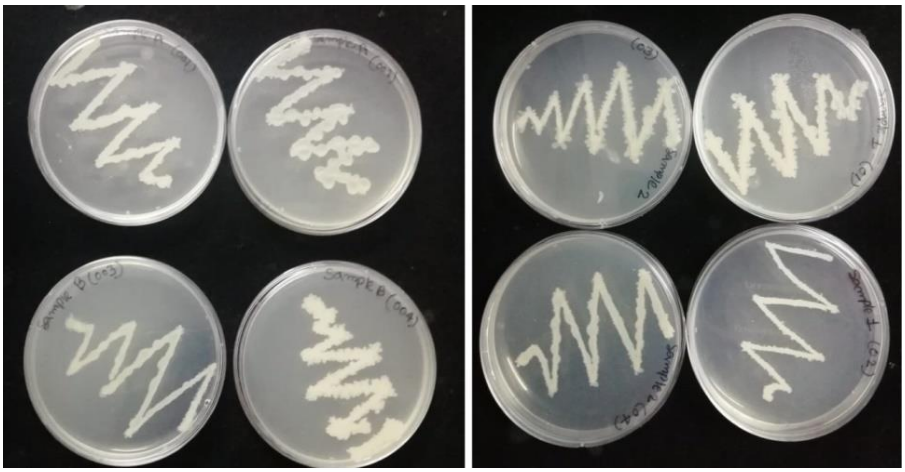

Fig. 1. Phosphate solubilizing Microbes on nutrient agar plates.

Table 2. Zone of clearance formed by eight best-selected isolates from different regions on pikovskaya agar medium.

S. No.	Isolate No.	Place of soil sample collection and serial dilution	Zone of clearance* (mm)
1	B1	Jaipur Region A	15.6 ± 1.1
2	B2		11.3 ± 1.1
3	B3		7.0 ± 1.0
4	B5	Jaipur Region B	13.3 ± 1.5
5	B6	Jaipur Region C	8.6 ± 1.5
6	B8		8.3 ± 1.5
7	B11		5.0 ± 1.0
8	B16	Jaipur Region D	11.1 ± 1.5

**Values are the mean of three replicates, ± indicates the standard deviation*

Table 3. Colony morphology of the isolates.

Sl. No.	Isolate	Characteristics
1	Isolate B1	Opaque, rounded, slightly bulged, and small colonies
2	Isolate B2	Creamish, rounded, shiny, opaque
3	Isolate B3	Creamish, rounded, slightly bulged, small colonies, opaque.
4	Isolate B5	Whitish, rounded, bulged, opaque, small colonies.
5	Isolate B6	Creamish, shiny, rounded, bulged, opaque.
6	Isolate B8	Whitish, rounded, opaque, flat colonies
7	Isolate B11	Creamish, rounded, opaque, wrinkled, slightly bulged.
8	Isolate B16	Creamish, shiny, rounded, slightly bulged, medium-sized colonies.

Table 4. Morphological and biochemical characteristics of the selected eight best isolates.

Strain/ Test	Isolate B1	Isolate B2	Isolate B3	Isolate B5	Isolate B6	Isolate B8	Isolate B11	Isolate B16
Gram Reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Catalase	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve
Methy red	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
VogesProskauer	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Indole	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve
Citrate utilization	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve
Oxidase	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve
Lactose fermentation	LF	LF	LF	NLF	NLF	NLF	NLF	LF

LF: Lactose fermenting, NLF: Non-lactose fermenting

3.3. *Quantitative assay for tricalcium phosphate (TCP) solubilization in liquid medium*

The phosphorus solubilizing activity of all eight isolates varied with the soluble phosphate in the range of 69.1-167 µg/mL on day 9 using tricalcium phosphate as a source of insoluble phosphate (Fig. 2). The phosphate solubilization was accompanied by a decrease in medium pH, indicating the production of organic acids. This was consistent with the results of other experiments [25,26].

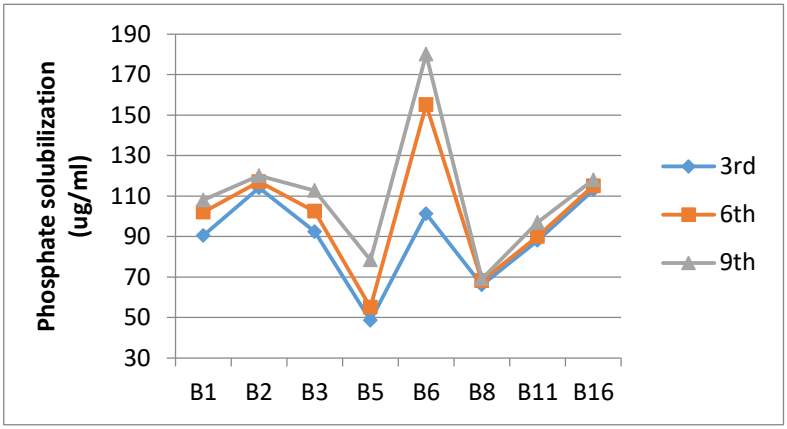

Fig. 2. Quantitative assay for tricalcium phosphate (TCP) solubilization in liquid medium by wild isolates.

3.4. *Effect of UV on phosphate solubilization capacity of bacterial isolates and comparative analysis*

There was no growth observed in the plates inoculated with culture exposed to UV for 70 and 80 min.. The bacterial cultures were selected from the plates 40, 50, and 60 min. UV exposure time depicted a gradual decrease in colony count with increasing exposure time. Various studies using random mutagenesis *via* UV have been conducted on bacterial and fungal strains for improved production of various enzymes [27,28], but the literature on bacterial studies is very limited. All eight isolates responded positively by increasing phosphate solubilization as compared to the wild for all three treatments, *i.e.*, UV treatment for 40, 50, and 60 min. They also followed a general trend where they showed increased phosphate solubilization on day 3^{rd,} which again increased more on the 6th day and then decreased on the 9th day. The best responses were recorded by isolate B5uv40, isolate B5uv50, and isolate B5uv60 on day 6, which was 58.54 %, 133.27 %, and 159.09 % increased phosphate solubilization as compared to wild isolates (Figs. 3-5). B5uv60 was the most efficient phosphate-solubilizing mutant isolate that responded positively to the treatment. The soluble phosphate level was 142.5 µgP/mL, more than 159.09 % more than the wild strain. Various researchers have also used this type of random mutagenic treatment to improve the production of various metabolites [28,29]. Also, the mutant strains were categorized as high phosphate solubilizers, moderate phosphate solubilizers, and low phosphate solubilizers on the basis of best treatment for 50 min. on day 6 (Table 5).

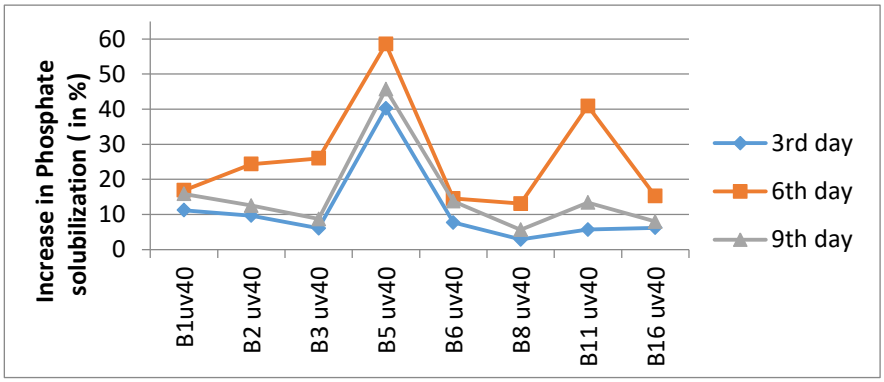

Fig. 3. Phosphate solubilization increase (in %) by eight mutant isolates treated with UV for 40 min. on day 3rd, 6th and 9th.

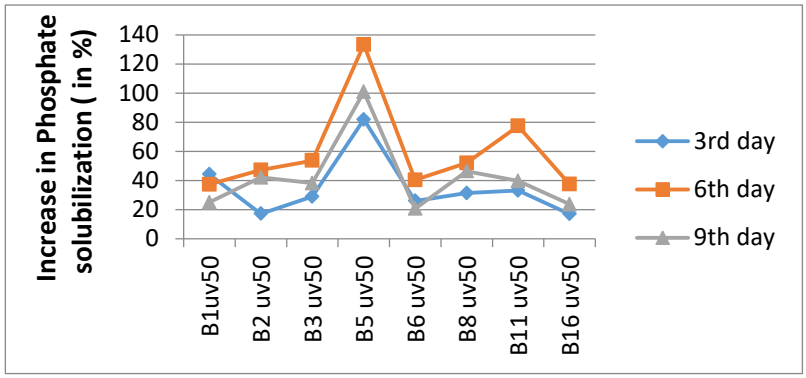

Fig. 4. Phosphate solubilization increase (in %) by eight mutant isolates treated with UV for 50 min. on days 3rd, 6th and 9th.

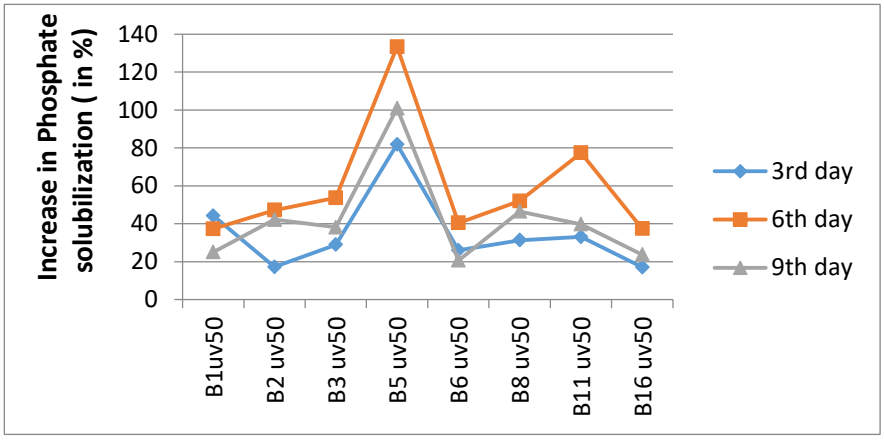

Fig. 5. Phosphate solubilization increase (in %) by eight mutant isolates treated with UV for 60 min. on days 3rd, 6th and 9th.

Table 5. Isolates are categorized according to %increase in phosphate solubilization post Mutagenic Treatment.

Sl. No.	Category	
1.	≥100 % increased phosphate solubilization; High Phosphate solubilizers	$B5_{UV60}$; $B5_{UV50}$
2.	50-100 % increased phosphate solubilization; Moderate phosphate solubilizers	$B11_{UV50}$; $B11_{UV60}$; $B5_{UV40}$; $B16_{UV60}$; $B8_{UV60}$; $B3_{UV50}$; $B8_{UV50}$
3.	≤ 50 % increased phosphate solubilization; Low phosphate solubilizers	$B2_{UV60}$; $B2_{UV50}$; $B6_{UV60}$; $B11_{UV40}$; $B6_{UV50}$; $B3_{UV60}$; $B16_{UV50}$; $B1_{UV50}$; $B1_{UV60}$; $B3_{UV40}$; $B2_{UV40}$; $B1_{UV40}$; $B16_{UV40}$; $B6_{UV40}$; $B8_{UV40}$

4. Nucleotide sequence accession numbers and taxonomical assignment

B5uv60 displayed high sequence similarity with *Pseudomonas putida* (91. 89%) using BLAST at the National Centre for Biotechnology Information website (www.ncbi.nlm.nhi.gov). In the phylogenetic tree constructed using 16S rRNA gene sequences of the genus Pseudomonas, the isolate B5uv60 clustered with *Pseudomonas putida* strains (Fig. 6). The 1,323 bp genomic sequences were submitted to GenBank with accession number ON358430.1 and version ON358430.1.

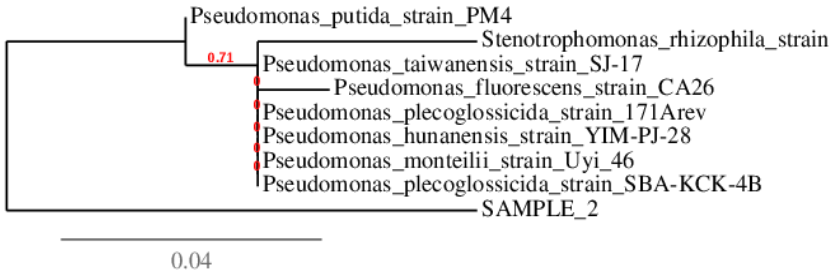

Fig. 6. Phylogenetic tree of bacterial isolates based on 16S rRNA gene sequences..

5. Conclusion

The current study's findings led us to believe that induced random mutagenesis can improve the efficacy of bacterial strains for phosphate solubilization. Inoculation with wild strains has already become a standard practice among researchers. Using these mutant strains as bio-inoculum could be a significant step toward achieving a sustainable goal of meeting food demand. The stability of these mutations and their performance with different plant species can be studied in the future.

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