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CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA ISOLATED FROM RHIZOSPHERE OF PLANTS IN LAHORE DISTRICT OF PAKISTAN

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ABSTRACT

Different strains of Phosphate Solubilizing Bacteria (PSB) were isolated from the rhizosphere of plants from Lahore district of Pakistan. Characterization of purified isolates through amplification of 16S rRNA gene and its sequencing determined the identification of *Pseudomonas aeruginosa, Citrobacter freundii, Enterobacter aerogenes, Klebsiella pneumonia, Acinitobacter lwoffi, Enterobacter cloacae, Pseudomonas fluorescens, Proteous vulgaris, Burkholderia pseudomallei, Pasteurella hemolytical type A, Burkholderia capacia* and *Burkholderia mallei* in these isolates. These isolates were further categorized on the basis of their capabilities for phosphorus solubilization, IAA production, and antifungal activity. Isolates namely C-09, C-22 and C-29 were found to be capable of high IAA production without tryptophan. Similarly 73% phosphate solubilizing activity was detected in isolate namely C-18. Isolates C-06 and C-13 were found to have maximum antifungal activity.

Keywords: Phosphate Solubilizing Bacteria, IAA producing bacterial strains, antifungal activity, 16S rRNA

INTRODUCTION

The second most important nutritive component of plant is phosphorus after nitrogen (N). All major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration required phosphorus as major element [1]. It is present at levels of 400–1200 mg kg^{-1} of soil. In past, efforts have been made to improve soil fertility through microbial inoculants. Microorganisms play a central role in the natural phosphorus cycle. This cycle occurs by means of the cyclic oxidation and reduction of phosphorus compounds, where electron transfer reactions between oxidation stages range from phosphine (-3) to phosphate (+5) [2]. Mineral phosphate can also be found associated with the surface of hydrated oxides of Fe, Al and Mn, which are poorly soluble and assimilable. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants. Release of P by PSB from insoluble and fixed and adsorbed forms is an import aspect regarding P availability in soils [3].

Soil bacteria play important role in crop production and have been used for sustainable agricultural development [4]. The effect of phosphate solubilizing bacterial inoculants combined with phosphorit had stimulatory effects of bacterial species such *Pseudomonas, Bacillus, Arthrobacter and Rhizobium* on growth of wheat, maize and cotton growth, thus increased shoot and root length of plant. In addition, P contents significantly increased in cotton, inoculated with *Rhizobium meliloti* when combined with phosphorit [5,6]. Strains of *Bacillus liqueniformis* and *Bacillus amyloliquefaciens* were found to produce mixtures of lactic, iso-valeric, iso-butyric, and acetic acids. Phosphorus solubilization is carried out by a large number of saprophytic bacteria and fungi acting on sparingly soluble soil phosphates, mainly by chelation-mediated mechanisms [7,8]. Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, Ca) and decrease the pH in basic soils [9]. A study of ten isolates of bacteria was successfully characterized for their antagonistic activity against phyto-pathogenic fungi such as *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii.* The growth of PGPR isolates was optimized under different temperatures (10°C, 20°C, 28°C, 37°C and 45°C) for inducing Indole Acetic Acid (IAA), thus beneficial for rice cultivation [10-12]. Such inoculants with their beneficial traits would be considered as potential bio-fertilizer for the sustainable aerobic rice cultivation system [13].

Different strains of phosphate-solubilizing bacteria (PSB) isolated from the rhizosphere and rhizoplane of corn (*Zea mays L.*) and amplified ribosomal RNA restriction pattern analysis (ARDRA) exhibited different levels of tricalcium phosphate solubilizing activity and large genetic diversity The study mentioned above reported *A. incenata* strain as a PSB for the first time [14]. The present study was done to optimize the conditions for isolation and purification of the phosphate solubilizing bacteria from rhizosphere of plants (cotton and corn) and identifies the phosphate solubilizing bacteria using physiological, molecular and biochemical tests of bacterial isolates including antifungal activity, sensitivity and resistance, phosphorous and IAA quantification.

MATERIALS AND METHODS

Sample collection:

The rhizospheric soil samples from different crops were collected and transferred to laboratory. 1ml of each soil sample was taken from dilutions: 10^3 to 10^5 and was plated on NBRIP medium to isolate phosphate solubilizing bacteria, as described by Nautiyal [15]. Bacterial strains exhibiting halo zone were selected and glycerol stocks were preserved. Gram staining was performed to separate gram positive bacteria. Appearance of purple color indicated the presence of gram positive while pink color represents gram negative bacteria.

QTS-24 Miniaturized Identification System

QTS-24 miniaturized identification system (DESTO laboratories, Karachi) was employed to identify the gram negative bacteria. Physiological and biochemical tests of bacterial isolates were performed to identify the bacterial isolates as described by Yasmin and Bano [16].

DNA isolation

Total DNA of selected bacterial strains was extracted by using 24h old bacterial culture in L.B media. 3 ml from each culture was centrifuged at 13000 rpm for 5 minutes at 4°C and pellet was washed twice with distilled water. 400µl of CTAB buffer (2% CTAB, 2 µl/ml β-mercapto ethanol and 50 µl/ml proteinase K along with 150µl of lysis buffer (10% SDS and 5M NaOH) was added to each tube. Samples were mixed by inverting tubes 2-3 times. Lysis was done by incubation of tubes at 65°C for 2h followed by addition of 500µl of P:C:I (25: 24: 1) and centrifugation for 15 min. Supernatant was then transferred to a fresh 1.5ml tube and 300µl of chilled isopropanol was added and incubated at -20°C for 30 minutes to precipitate DNA. Pellet was washed with 70% ethanol, air dried pellet and re-suspended in TE buffer.

16S rRNA gene amplification

Amplification of 16S rRNA gene was done in a thermal cycler (PCR System 9700; Applied Bio-systems) in 20µl reaction mixture containing 0.1 mM of each primer, 2.5 mM $MgCl_2$, 1 mM of each dNTPs, 50 ng/µl template DNA and 2.5U Taq DNA polymerase (Thermoscientific). A 1.5kb product was amplified by using universal primers 5'-AACGCGAAGAACCTTAC-3' and 5'-CGGTGTGTACAAGACCC-3' under following conditions; initial denaturation was done at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. PCR products were analyzed on 1% agarose gel in 1X TAE buffer with ethidium bromide staining and visualized under UV. DNA gel elution kit (Thermoscientific; K0513) was used for elution and purification of the PCR products from gel. Amplified products were sequenced from Macrogen Company, Korea. Deduced DNA sequences were aligned with sequences present in Genbank database using BLASTN algorithm.

Qualitative analysis of Indole Acetic Acid (IAA)

Determination of IAA was done by employing modified qualitative method. Pure colonies of 39 bacterial isolates were grown in 500 ml conical flask containing 100 ml of LB broth without tryptophan and incubated at 28 ± 2°C for 72 hours on a shaker. The cultures were then centrifuged at 4000 rpm for 10 min. 1ml of bacterial culture was mixed with 2 ml of Salkowski reagent (1ml of 0.5M $FeCl_3$ in 50ml of 35% per chloric acid) in a test tube. After 20 - 25 min, the appearance of red color supernatant indicated the production of IAA while more red color emergence indicated more IAA production.

Phosphate solubilization activity

PSB isolates were characterized for their ability to solubilize tri-calcium phosphate $Ca_3(PO_4)_2$ by the formation of visible dissolution halos on NBRIP agar. Qualitative test for phosphate solubilization was performed.

Solubilization index (SI)

SI based on colony diameter and halo zone for each phosphate solubilizing bacterial isolate was calculated. The solubilization index was calculated after five days growth of pin point inoculation on NBRIP at 28°C as described originally by Freitas *et al.* [17].

The SI was calculated by following equation.

SI = (Total Zone diameter - bacterial colony diameter)

Quantitative method

The quantitative method was used for the estimation of inorganic phosphate. For this, pure colonies of all isolates were grown in 50 ml of NBRIP broth with 0.5% TCP (pH 7.0) and incubated at 28 ± 2°C for 5 days on a shaking incubator at 180 rpm. Each bacterial strain was inoculated in triplicate. An un-inoculated control was also kept under same conditions. Subsequently, the cultures were centrifuged at 10,000 rpm for 10 min and phosphorus in supernatant was estimated by vanado-molybdate-yellow color method [18]. To 1ml aliquot of the supernatant, 5ml nitrovanado-molybidic reagent (deionized water, 5% ammonium molybdate solution, 0.25% ammonium vanadate solution, dilute nitric acid) was added and volume was kept at 100ml. The absorbance was measured at 410 nm in UV/visible spectrophotometer. 4.3937g potassium dihydrogen phosphate in 1000 ml distilled water was used for preparation of the standards of 1, 2, 4 and 8 ppm. The total soluble phosphorus was calculated from the regression equation of standard curve. The values of soluble phosphate liberated were expressed in percentages. The pH was also recorded.

Fungus growth inhibition

Antagonistic activity of fungi was assayed for antifungal activities of all isolates by using Potato Dextrose Agar (PDA) medium. *Fusarium oxysporum* was taken from the peripheral edge of five days old cultures and was placed at the center of the plates. The bacterial strains were individually streaked 2.5 to 3 cm from the fungi at 4 opposite locations around the periphery of the plate and the dual culture plates were incubated at room temperature. The barrier between isolates and fungi indicated antagonistic interaction between them.

RESULTS

Characterizations of Bacterial Isolates

Total 28 isolates were characterized to be phosphate solubilizer. PSB zone results showed that out of these isolates, 16 were considered to be best phosphate solubilizer whereas others were categorized as intermediary or low phosphate solubilizers. Bacterial strains that solubilize 40% or above phosphorous are incorporated in this study.

Identification of PSB by 16S rRNA

Four isolates shown high Phosphorous solubilization ability and IAA production (C-09, C-28, C38 and C-39) were identified by 16S rRNA. Sharp amplification of 1.5kb fragment amplification of 16S rRNA gene from bacterial isolates was obtained (figure 1). The bacterial isolates were further characterized through sequencing of amplified product. The deduced sequence was compared with NCBI available sequences. The nucleotide sequences of four 16S rRNA gene segments obtained in this study were deposited in Genbank isolates C-09 *Enterobacter* sp., C-28 *Bacillus* sp., C-38 *Burkholderia* sp. and C-39 *Pseudomonas* sp., under accession numbers KF487556, KF487557, KF487546 and KF176374, respectively.

QTS-24 Miniaturized Identification

Total 12 PSB isolates showed high quantitative phosphate solubilization efficiency as detected through biochemical identification. Identified bacterial isolates include two *Pseudomonas* strains, 1 *Klebsiella* strain, 5 *Burkholderia* strains, 1 species of *Citrobacter*, *Enterobacter* and *Acinitobacter* each and these were assigned code numbers as depicted in table 1.

Qualitative analysis of IAA

All isolated bacterial strains were subjected to qualitative analyses of IAA, on the basis of color intensity 7 were graded best, 4 medium, 23 were characterized as low Indole producer while 5 with no IAA producer. Data is presented as table 2.

Solubilizing Index (SI)

Solubilizing Index of different bacterial isolates given in figure 2. These bacterial isolates produced the maximum halos of 11 mm and minimum of 5 mm within 7 days of incubation. C-38 *Burkholderia* sp. showed maximum SI index. 1 2 3 4

Phosphorous quantification

Mean values of pH and soluble Phosphorous percentage are shown in Table 2. Soluble Phosphorous percentage ranged between 40 to 61%and pH value 3.4 to 4.9. C-38 isolate was found to have maximum phosphate solubilization .i.e. 61% with pH decrease of 3.4 while isolates C-12, C-21, C22 and C-34 were found to be showing minimum phosphate solubilization which was estimated 35% to 40%.

Fungus growth inhibition

The application of bacterial strains against fungi (*Fusarium oxysporum*) showed inhibitory effect. Bacterial isolates C-24 and C-33 showed maximum inhibition as compared to C-14 and C-20 which resulted in minimum inhibition as shown in figure 3.

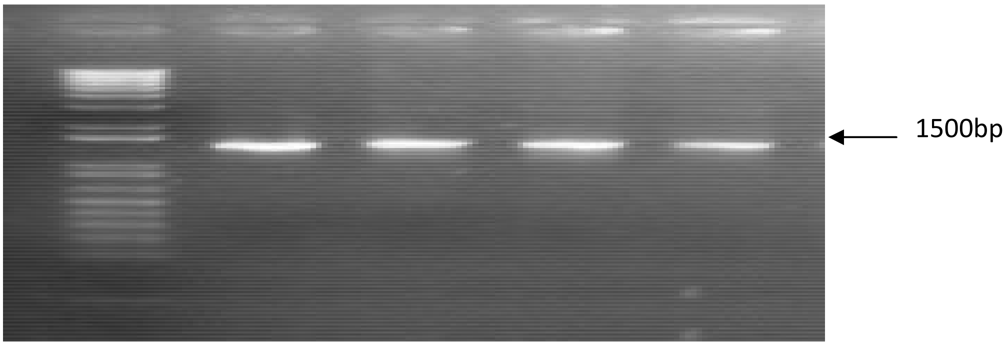

Figure 1: PCR amplification of16S rRNA gene using universal primers
Lane 1: 1kb plus DNA Ladder, Lanes 2-5: bacterial samples

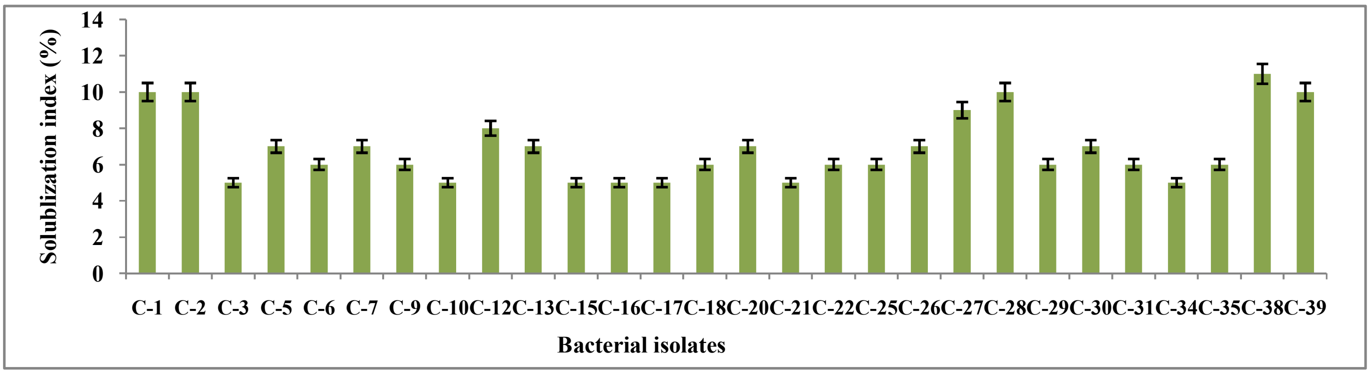

Figure 2: Solubilization Index of different bacterial isolates

Figure 3: Bacterial isolates showing antagonistic activity against fungus (*Fusarium oxysporum*)

Table 1: Screening of Phosphate solubilizing isolates through QTS-24 miniaturized identification kit

Isolate No	Results
C-01	*Pseudomonas aeruginosa*
C-02	*Citrobacterfreundii*
C-18	*Klebsiella pneumonia*
C-05	*Enterobacterspp*
C-15	*Acinitobacterlwoffi*
C-20	*Enterobacter cloacae*
C-13	*Pseudomonas fluorescens*
C-27	*Burkholderiapseudomallei*
C-10	*Burkholderiapseudomallei*
C-06	*Burkholderiacapacia*
C-25	*Burkholderia mallei*
C-26	*Burkholderiacapacia*

Table 2: Characterized bacterial isolates showing pH drop, qualitative and quantitative analysis of phosphate solubilization and production of indole acetic acid

Isolate No.	pH	Soluble P (%)	Qualitative IAA	SI(mm)	Isolate No.	pH	Soluble P (%)	Qualitative IAA	SI(mm)
C-01	4.1	57%	+	10	C-21	4.7	36%	++	5
C-02	3.9	57%	+	10	C-22	4.6	36%	+++	6
C-03	4.6	46%	+	5	C-23	-	---	+	
C-04	-	---	+		C-24	-	---	-	
C-05	4.3	54%	+	7	C-25	4.4	47%	+	6
C-06	4.2	48%	+	6	C-26	4.6	47%	+	7
C-07	4.3	46%	+	7	C-27	4.8	50%	++	9
C-08	-	---	+		C-28	4.3	58%	+++	10
C-09	4.1	59%	+++	6	C-29	4.0	40%	+++	6
C-10	4.2	49%	+++	5	C-30	4.4	44%	+	7
C-11	-	---	+		C-31	4.6	43%	+	6
C-12	4.8	35%*	+	5	C-32	-	---	-	
C-13	4.3	50%	+	7	C-33	-	---	-	
C-14	-	---	+		C-34	4.9	40%	++	5*
C-15	4.6	54%	+	5	C-35	4.5	45%	+++	6
C-16	4.5	40%	+	5	C-36	-	---	-	
C-17	4.5	46%	+	5	C-37	-	---	-	
C-18	4.9**	53%	+	6	C-38	3.4*	**61%	+++	11*
C-19	-	---	+		C-39	3.9	60%	++	9
C-20	4.5	54%	+	7					

***Show minimum value; **Show maximum value; + Represent low IAA producer; ++ Represent medium IAA; producer; +++ Represent high IAA producer**

DISCUSSION

In the present study, bacterial strains exhibiting growth promoting properties like IAA production and solubilization of phosphate were selected and used in different treatments for characterization. Bacterial isolate C-18, C-02, and C-01 were characterized to possess maximum 51 to 73% phosphate solubilization in NBRIP medium as compared to *Burkholderia* and other bacterial species that were found to have 40-50% phosphate solubilization. These results are similar to results of Khosro, [19] who declared microorganisms such as *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* as powerful phosphate solubilizers on the basis of observation through testing *in-vivo* for their efficiency. It has also been demonstrated by Walpola *et al*., [20] that the ability of phosphate solubilization decreases as the buffering capacity of the medium increases and it is maximum at acidic pH. Similar results were obtained in the present study. Similarly variable concentration of IAA production was obtained on evaluation of different bacterial strains for their ability of IAA production. Maximum IAA production i.e. 27 µg/ml was obtained from bacterial isolate C-09, C-22 and C-29 even in the absence of precursor, determined the high efficacy of IAA production by these bacterial strains in pure culture. Similar type of results were obtained by Leinho and Vacek [21] in a study conducted on *Pseudomonas* and *Acinotobacter* species isolated from wheat and rye rhizosphere for IAA production which ranges from 0.01 - 3.98 mg L^{-1}.While11.40 mg L^{-1}IAA production was obtained in the absence of precursor by Asghar *et al.* [22] from one bacterial strain out of hundred which he used in his study conducted on bacterial strain isolated from rapeseed plant. According to Frankenberger and Arshad [23] the production of IAA could also be influenced by other factors, such as culture and medium conditions, strains of rhizobacteria, growth stage and availability of substrates.

In present study different rhizobacterial strains were also evaluated for their ability against soil born fungal pathogen. The results clearly demonstrated that out of the total 37 rhizobacterial isolates used only 16 were found to be antagonistic at varying degree to the soil borne fungal pathogens. These results are similar to finding of Safiyazov *et al*. [24] who reported that *Enterobacter, Klebsiella* and *Pseudomonas* have the ability to inhibit the growth of *Fusarium* sp and these bacterial isolate, inhibited the growth of *Rhizoctonia* sp. around cotton giving an inhibition zone on application. *Rhizoctonia* and *Pythium* have been known to be notorious plant disease causing soil microorganisms responsible for root rot disease of sweet potato [25-27]. Park *et al*. [28] have also shown the beneficial effect of plant growth promoting rhizobacteria through inhibiting the growth of *Rhizoctonia, Pythium* and *Phytopthora* sp. i*n-vitro*.

CONCLUSION

In the perspective of the present study, it is concluded that PSB isolates have the potential to be used as inoculant for plant growth improvement through availability of nutrients and inhibition of growth of various soil born fungal pathogens.

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