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# ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM RHIZOSPHERE SOIL OF WEEDS OF KHEWRA SALT RANGE AND ATTOCK

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**Abstract**

During the present study rhizosphere soil of four halophytic weeds viz., *Chrysopogon aucheri*, *Lactuca dissecta*, *Solanum surattense* and *Sonchus arvensis* growing in saline soil of Khewra salt range and *Solanum surattense* growing in arid region of Attock were used for isolating phosphate solubilizing bacteria. The objective of this study was to explore the abilities of phosphate solubilization, production of stress hormone ABA (abscisic acid) and bacteriocin production of isolates. Only one isolate was selected randomly from culture of each plant rhizosphere soil. The isolates were preliminary identified on the basis of their morphology and biochemical characters**.** The isolates were able to solubilize tricalcium phosphate in broth cultures, the isolate 2P from rhizosphere soil of *Lactuca dissecta* and 1P from rhizosphere soil of *Chrysopogon aucheri* being more efficient. Least amount of phosphorous was solubilized by isolate 5P from rhizosphere soil of *Solanum surattense* growing in arid soil of Attock. Among all isolates maximum abscisic acid production was recorded in isolate 2P while least amount of ABA was produced by 1P. Bacterial isolates were also observed for bacteriocin production. Isolates 2P and 3P were able to produce bacteriocin, 2P produced bacteriocin of higher potency. The isolates used as inoculants, promoted growth of chickpea. The isolate 2P from rhizosphere soil of *Lactuca dissecta* growing in Khewra salt range having the ability of greater quantity of phosphorous solubilization, higher production of abscisic acid and bacteriocin, can be selected in the formulation of biofertilizer.

## Introduction

After nitrogen, phosphorous is an essential plant nutrient whose deficiency restricts crop yield severely. Tropical and subtropical soils are predominantly acidic, and often extremely phosphorous deficient (Gaume, 2000) with high phosphorous sorption (fixation) capacities. The low level of phosphorous is due to high reactivity of soluble phosphate with other elements.

The microbial system can derive out ample amount of nutrient from the natural reservoir and enrich the soil with important but scarce nutrients. The organisms with phosphate solubilizing potential increase the availability of soluble phosphates and can enhance plant growth by increasing the efficiency of biological nitrogen fixation or enhancing the availability of trace elements such as iron, zinc etc., and by production of plant growth promoting regulators (Ponmurugan & Gopi, 2006). Many rhizobacteria are able to solubilize sparingly soluble phosphates, usually by releasing chelating organic acids (Vessey *et al*., 2004). The solubilization effect is generally due to the production of organic acids by these microorganisms. Afzal *et al*., (2005) reported that inoculation of phosphate solubilizing *Pseudomonas and Bacillus* species on wheat (*Triticum aestivum* L.) resulted in increase in grain yield and phosphorous uptake. *Pseudomonas* inoculation had favourable effect on salt tolerance of *Zea mays* L., under NaCl stress (Bano & Fatima, 2009).

Field experiments revealed that P-solubilizing bacteria (PSB) not only improved the growth and quality of crops but also drastically reduced (1/3-1/2) the usage of chemical or organic fertilizers. Crop plants such as peanut, various horticultural plants, and vegetables were successfully inoculated with PSB to obtain higher yields (Young *et al*., 2003). The performance of PSB is severely influenced by environmental factors especially under stress conditions (Yahya *et al.,* 1989).

Bacteriocin producing rhizobacteria can enhance the growth of plant by increasing occupancy of nodules and may be useful as biocontrol agents. They can also act as anti-competitor compounds against rival bacteria, thus strengthening their rhizosphere competence. Possible applications of strains producing bacteriocin in agriculture include their use in biological control of soil borne or phyllosphere-inhabiting bacterial plant pathogens (Herlache & Triplett 2002). It has been proposed that bacteriocins may play a key role in bacterial population dynamics (Riley & Wertz, 2002).

Present investigation was aimed to isolate and characterize Phosphate solubilizing bacteria from rhizosphere soil of halophytes collected from Khewra salt range and compare them on the basis of their phosphate solubilizig abilities with the bacteria isolated from rhizosphere soil of *Solanum surattense* growing in arid soil of Attock.

## Materials and Methods

**Rhizospheric soil sampling:** Phosphate solubilizing bacteria were isolated from rhizosphere of plants growing at altitudes ranging from 313-350 m.a.s.l of Khewra salt range of Tehsil Pind Dadan Khan, District Jhelum and arid soil of Attock from altitude ranging from 305-1067 m.a.s.l of plain area of Tehsil Attock, District Attock. Naz *et al*., (2009) determined the EC; 2300 µS/cm; and pH; 8.6 of rhizosphere soil of four halophytes *Chrysopogon aucheri, Lactuca dissecta, Solanum surattense* and *Sonchus arvensis* from Khewra salt range and rhizosphere soil of *Solanum surattense* of arid region of Attock (EC 210 µS/cm; and pH;7.9).

The isolation of phosphate solubilizing bacteria were made from *Chrysopogonn aucheri* (Golden beared grass) family Poaceae, *Lactuca dissecta* (Wild lettuce) *Sonchus arvensis* (Noxious weed) family Asteraceae and *Solanum surattense* (Yellow berried night shade) family Solanaceae.

**Isolation of phosphate solubilizing bacteria:** For isolation of phosphate solubilizing bacteria 10g rhizosphere soil was suspended in 90ml of distilled water. An aliquot (100µl) from decimal dilutions was inoculated on Picovskaya's medium (pH 7.2) (Picovskaya, 1948), incubated at 30°C. Colonies (cfu) of PSB were counted after 24 hours. Single colonies appearing on Picovskaya's agar plates were transferred in liquid broth of Picovskaya's and on agar slants for further study.

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**Identification of bacterial isolates**

**Colony and cell morphology:** For identification of bacterial strains on the basis of colony and cell morphology, bacterial isolates from overnight grown cultures in Picovskaya's were spread on the agar plates of above-mentioned medium (Pikovskaya, 1948). The morphology of the colonies (colour and shape) was recorded after 24 hours. To study the cell motility and shape, single colony from the agar plates were transferred on glass slide with a drop of sterile water and observed under light microscope (Nikon, Japan). Following tests were perfomed to identify bacterial strains.

**Gram staining:** For observation under light microscope, slides of isolated bacterial cultures were prepared for Gram staining by the Vincent method (1970).

**Oxidase test:** Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates (Steel, 1961).

**Catalase test:** This test was performed to study the presence of catalase enzyme in phosphate solubilizing bacteria (McFadden, 1980).

**Miniaturized Identification System-QTS 24:** Physiological and biochemical tests of phosphate solubilizing bacteria were performed using QTS 24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan). The bacterial cultures (24 hours old) grown on media were used to inoculate QTS kits.

**Colony diameter, halozone and solubilization index of isolated *Pseudomonas* strain:** Sterilized Picovskaya's media was poured into sterilized Petri plates, after solidification of the media; a pin point inoculation of the Petri plates was made on plates under aseptic conditions (Fig. 1). The plates were incubated at 28 $^{o}$C for 7 days. Then the ability of PSM to solubilize the insoluble phosphate was studied by the determination of solubilization index: the ratio of the total diameter (colony + halozone) and the colony diameter (Edi-Premono, 1996).

$$SI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

**Quantification of available phosphorous solubilized by Phosphate solubilizing bacterial strain:** Phospho-molybdate blue colour method (Murphy & Riley, 1962) was used for determination of available phosphorous. Picovskaya's broth (100ml) (adjusted to PH 7) with sucrose and Tricalcium phosphate (0.3g /100ml) was poured in 250ml flasks. The flasks were autoclaved at 121$^{o}$C for 20 minutes. In each autoclaved flask, two loops full of phosphate solubilizing bacterial strain were inoculated and placed on rotary shaker at 12000 rpm for 12 days. The suspension was centrifuged (10000 rpm, for 15 min,) to remove bacterial cells and other insoluble materials. The available phosphorous (P) was determined using spectrophotometer at 882nm and calibrated with standard $KH_2PO_4$ curve. The available phosphorous (P) was a measure of extent of solublization of tri-calcium phosphate.

**Bacteriocin assays:** To assay bacteriocin production, 48h old culture of indicator strain of *Rhizobium leguminosarum* (VF39) grown in TY medium, was diluted ($10^{-2}$) and 1 ml was mixed with approximately 25 ml of soft TY agar (0.6% w/v) containing 5 mM $Ca^{+2}$. Single colonies of the strains to be tested for bacteriocin activity, were stab inoculated into the soft agar within 2h after the agar get solidified. Halos were visible as cleared zones surrounding the stab inoculated cultures. The plates were scored approximately after 48h of stab inoculation (Oresnil *et al*., 1999).

**Identification and quantification of ABA produced by bacterial isolates:** Isolated PSB strains were analyzed for abscisic acid production in pure culture. The Picovskaya's growth media (100ml) were inoculated with one-day-old bacterial cultures and placed on a shaker (100rpm). These bacterial cultures were harvested after one week by centrifugation at 10,000rpm for 15 minutes and supernatant was used for extraction of growth hormones excreted in the growth medium. pH of supernatant was adjusted to 2.8 with 1N HCl.

ABA was extracted with equal volumes of ethyl acetate, following the method described by Tien *et al*., (1979). The ethyl acetate extract was evaporated to dryness at 35 ◦C and the residue was dissolved in 1.5 ml of methanol. The samples were analyzed on HPLC (Agilent 1100) using UV detector and $C_{18}$ column (39 x 300mm). For identification of hormones, 100µl sample filtered through 0.45µ syringe filter (Millipore) was injected into column. ABA was identified on the basis of retention time and peak area of the standard. Methanol, acetic acid and water (30:1:70) were used as mobile phase. Flow rate was adjusted at 1.0 ml/min. with an average run time of 20 min/sample. The wavelength used for detection of ABA was adjusted at 254 nm (Li *et al*., 1994).

**Inoculation of chickpea (*Cicer arietinum* L.) plant by isolated strain:** Growth promoting effects of isolates were studied on chickpea (Accession no. C44). Surface sterilized seeds were soaked in 48 hr old cultures of PSB isolates for two hours and were sown in pots ($18\times19cm^2$) containing sterilized soil. Pots were placed in green house with temperature range (max/min 20.32/5.88 35$^{o}$C) and humidity 93% (8am) to 59% (2pm). Hoagland nutrient solution and urea (0.32g/200ml) was applied to each pot after 3-4 days of sowing for better growth. Three replicates were used for each treatment. Harvest was made 30 days after inoculation and root and shoot length and weight were measured.

**Results and Discussions**

**Isolation identification and characterization of phosphate solubilizing bacteria:** Bacterial colonies were obtained on Picovskaya's media specific for the phosphate solubilizing bacteria. One isolate was selected randomly from rhizosphere soil of each plant species. These Isolates were designated as 1P (from rhizospheric soil of *Chrysopogon aucheri*), 2P (from rhizospheric soil of *Lactuca dissecta)*, 3P (from rhizospheric soil of *Solanum surattense* growing in Khewra salt range), 4P (from rhizospheric soil of *Sonchus arvensis*) and 5P (from rhizospheric soil of *Solanum surattense*) growing in Attock.

Colonies of all the isolates were round and creamy in colour. All the isolates were gram negative and catalase positive. Oxidase test was positive with isolates 3P, 4P and 5P while isolates 1P and 2P were not positive to oxidase test. Furthermore, 24h old bacterial cultures of the isolates were tested by using microbial identification kits QTS-24, which was based on carbon/nitrogen source utilization. Among these five isolates 2P and 5P exhibit tryptophan deaminase activity. While only 5P showed activity in the utilization of_lysine decarboxylase, arginine dehydrolase and ornithine decarboxylase, which differed from 1P in utilization of Ortho nitro phenyl β-D-galactopyranoside and malonate (Table 1).

Among PSB isolates from rhizospheric soil of Khewra salt range isolate 4P from rhizospheric soil of *Sonchus arvensis* showed better utilization of carbohydrates as compared to isolates 1P, 2P, 3P.

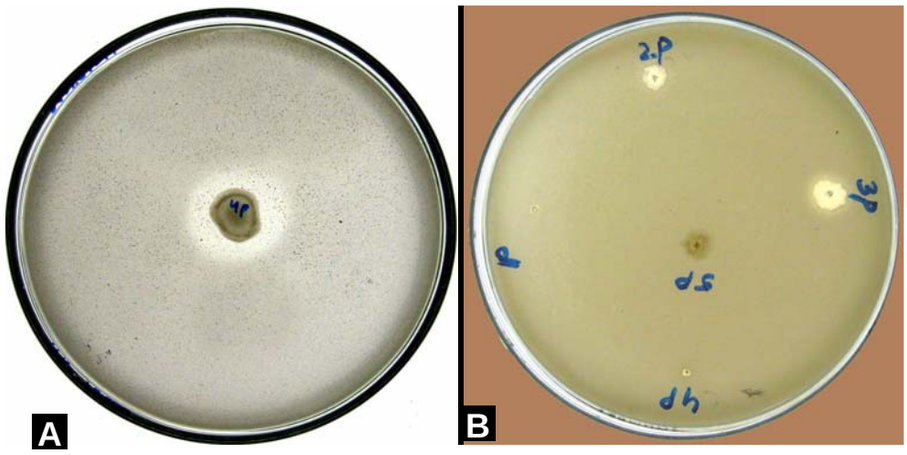


Fig. 1. (A). Photograph showing halozone formation due to solubilization of phosphorous by the isolate 4P. (B). Halozone formation indicating bacteriocin production.

**Table 1. Morphological and biochemical characteristics of phosphate solubilizing bacterial isolates from rhizosphere soil of weeds collected from Khewra and Attock.**

| Tests | 1P | 2P | 3P | 4P | 5P |
|---|---|---|---|---|---|
| Colony morphology | Spherical Creamy, Round | Spherical Creamy, Round | Spherical Creamy, Round | Spherical Creamy, Round | Spherical Creamy, Round |
| P-solubilization | + | + | + | + | + |
| Catalase test | + | + | + | + | + |
| Oxidase test | _ | _ | + | + | + |
| Gram test | Gram negative | Gram negative | Gram negative | Gram negative | Gram negative |
| QTS tests | | | | | |
| ONPG | - | + | + | + | + |
| CIT | - | - | - | - | + |
| MALO | - | + | + | - | + |
| LDC | - | - | - | - | + |
| ADH | - | - | - | - | + |
| ODC | - | - | - | - | + |
| $H_2S$ | - | - | - | - | - |
| URE | - | - | - | - | - |
| TDA | - | + | - | - | + |
| IND | - | - | - | - | - |
| VP | - | - | - | - | - |
| GEL | - | - | - | - | + |
| GLU | - | + | - | + | + |
| MAL | + | - | + | + | + |
| SUC | + | - | - | + | + |
| MAN | + | - | - | - | - |
| ARA | - | - | - | + | - |
| RHA | + | - | - | + | - |
| SOR | + | - | - | + | - |
| INO | + | - | - | + | - |
| ADON | - | - | - | + | - |
| MEL | + | - | - | + | - |
| RAF | + | - | - | - | - |

Microbial identification kits (Qts-24, DESTO Labs., and Karachi) were used for these biochemical tests.

For these tests 24-hour-old bacterial cultures were used and results were noted after 18 hours of incubation at 30 $^0$C.ONPG = ortho nitro phenyl β-D-galactopyranoside; CIT=sodium citratrate; MALO=sodium malonate; LDC=Lysine decarboxylase; ADH=Arginine dihydrolase; ODC=Ornithine decarboxylase; H2S= H2S production; URE=Urea hydrolysis; TDA=Tryptophan deaminase;IND=Indole; VP=(Voges proskauer)=Acetion; GEL=Gelatin hydrolysis; GLU=Acid from glucose; MAL=Acid from maltose; SuC=Acid from sucrose;MAN=Acid from mannitol;ARA=acid from arabinose; RHA=Acid from Rhamnose; SOR=Acid from sorbitol; INO=Acid from inositol; ADO=Acid from adonitol; MEL=Acid from Melibiose; RAE=Acid from Raffinose

1P: bacterial isolates from rhizosphere soil of *Chrysopogon aucheri* of Khewra salt range

2P: bacterial isolates from rhizosphere soil of *Lactuca dissecta* of Khewra salt range

3P: bacterial isolates from rhizosphere soil of *Solanum surattense* of Khewra salt range

4P: bacterial isolates from rhizosphere soil of *Sonchus arvensis* of Khewra salt range

5P: bacterial isolates from rhizosphere soil of *Solanum surattense* of arid field of Attock

Where,

- stands for negative in test

+ stands for positive in test

**Survival efficiency of PSB isolates:** Bacterial population determined on gowth media revealed that colony counts of isolates 2P and 3P from rhizosphere soil of weeds of Khewra salt range were higher than that of 5P isolate of Attock; 4P isolate has cfu similar to that of 5P (Table 2). The higher colony count of PSB isolates from rhizosphere soil of weeds of Khewra salt range as compared to that of isolate from Attock indicate the survival efficiency of these isolates related to phosphate content of soil. The higher cfu of 1P, 2P and 3P isolates of Khewra salt range suggest their better salt tolerance. Tolerance to high salt, high pH and high temperature may be important in the survival, multiplication and spread of bacterial strain in saline soils (Nautiyal, 1999). PSB spp. S1 alleviated greatly salt stress and promoted significantly the seedling growth of annual ryegrass under either 5 or 10 g/kg NaCl stress in genotobiotic growth pouch assay (Ji & Huang, 2008).

**Phosphate solubilization:** When grown in culture media supplemented with Tricalcium phosphate, all the isolates produced halozone around the colonies, indicating the solubilization of phosphate source used. Phosphate solubilizing microbes are detected by the formation of clear halos around their colonies. The halo is produced due to solubilization of insoluble phosphates, which in turn is mediated via the production of organic acid in the surrounding medium (Gaur, 1990). The isolate 1P showed maximum solubilization index, which differed non-significantly from 2P, 3P, 4P and 5P (Table 2). These isolates were confirmed for their P solubilization ability by phospho- molybdate test. Phospho-molybdate test for quantitative determination of available phosphorous indicated that the isolates 1P and 2P solubilized significantly higher phosphate than all other bacterial strains. The isolates ranked for P-solubilization as1P=3P>2P>4P=5P. An inverse relationship was observed between pH value of culture medium and concentration of phosphate solubilized, indicating the organic acid secretion (Table 2). Difference in the P solubilization was reflected by the change in the pH of the culture medium. The most efficient isolates 1P and 2P have significantly decreased pH of the culture media.

The PSB isolates from rhizosphere soil of weeds of Khewra salt range solubilized higher quantity of phosphorous as compared to that of the Attock. Nautiyala *et al*., (1999) reported that the strains isolated from alkaline soil have the potential to solubilize phosphates at high salt, high pH and high temperature concentration. It may be due to the production of organic acids by these strains that lowers the pH of medium. The inverse relationship between pH and soluble phosphate concentration was observed among all the isolates from Khewra salt range and Attock. The inverse relationship between pH and soluble phosphate was reported earlier (Rashid *et al*., 2004).

**Bacteriocin production:** Bacteriocin production was detected by 2P and 3P isolates. The diameter of inhibition zone by 2P was greater than that of 3P (Table 3). Bacteriocins are the proteinaceous compounds that are produced by bacteria able to kill bacterial competitors while producing little and no harm to the producing cells because of a specific immunity mechanism and post translational machanisms. Among all the isolates from rhizospheric soil of weeds of Khewra salt range and Attock two isolates designated as 2P and 3P have shown clear zone of inhibition for bacteriocin production. The isolate 2P produced bacteriocin with maximum potency, while 3P showed lower potency of bacteriocin production. Antagonism amongst mixtures of inoculant strains of *Rhizobiaceae* on the basis of bacteriocin production was assessed (Hafeez *et al*., 2004).

**Production of ABA:** Among the isolates of PSB tested, 2P produced maximum amount of ABA (1.64µg/ml), while least amount of ABA was produced by the isolate 1P (0.15µg/ ml). The ranking order of ABA production by the strains from rhizospheric soil of plants of Khewra salt range and Attock was 2P>5P≥4P>3P>1P (Table 3). Salinity causes increased biosynthesis and accumulation of abscisic acid (ABA), which can modulate physiological reactions in plant responses to salinity (Chang *et al*., 2006). Cohen *et al*., (2007) reported ABA production in plant growth promoting rhizobacteria. Abscisic acid production by the phosphate solubilizing bacteria was reported first time by Mateen (2008).

**Inoculation of chickpea (*Cicer arietinum* L.) plant by isolated strain of PSB:** The inoculation with the isolates from rhizosphere of weed of Attock and that 1P and 2P from the rhizosphere of weeds of Khewra salt range did not result in any significant increase in root length of chickpea as compared to uninoculated control, however, isolates 3P and 4P significantly increased the root length as compared to control. All the isolates used as inoculant exhibited significant increase in shoot length of chickpea over control, the minimum increase was shown by isolate 1P (Table 4).

Root weight was significantly increased in seedlings inoculated with PSB isolates 1P to 4P from rhizosphere soil of weeds grown at Khewra salt range. The PSB isolates from rhizosphere of weeds of Attock did not show any significant increase in root weight over uninoculated control. The PSB isolate 3P, 4P and 5P showed significant increase in shoot weight as compared to uninoculated control while 1P and 2P showed no significant difference in shoot weight as compared to uninoculated control (Table 4). Positive growth promotion by inoculation with P-solubilizing bacteria has been is attributed to the ability of these bacteria to solubilize P and produce siderophores and hormones (Khan *et al*., 2009).

**Table 2. Colony count, solubilization index and quantification of Phosphorous solubilized by isolates of PSB from the rhizosphere soil of weeds obtained from Khewra salt range and Attock. Only one isolate similar in colony morphology and cell shape was selected from the pure culture (on Pikovskaya's media) of rhizosphere soil of each weed. The isolates were grown in culture medium supplemented with Tricalcium phosphate.**

| Bacterial isolates | Cfu (log cfu $g^{-1}$ soil) | Colony diameter (cm) | Halozone diameter (cm) | Solubilization index | pH of culture media | P conc. (µg/ml) |
|---|---|---|---|---|---|---|
| 1P | 12.16A | 1.30 | 1.6 | 2.23A | 5.8 | 16.7 A |
| 2P | 11.32B | 1.93 | 2.15 | 2.11 B | 5.26 | 17.4 A |
| 3P | 12.17A | 0.58 | 0.64 | 2.10B | 6.2 | 9.7 B |
| 4P | 6.536C | 1.2 | 1.3 | 2.08 B | 6.5 | 6.0 C |
| 5P | 6.503C | 1.93 | 2.23 | 2.15 AB | 6.9 | 1.52 D |

**Table 3. Abscisic acid (ABA) productions (µg/ml) and bacteriocin production by the isolates of PSB from rhizosphere soil of weeds obtained from Khewra salt range and Attock against VF39 strain of *Rhizobium leguminosarum*. For ABA production the isolates were grown in culture medium supplemented with Tricalcium phosphate for 7 days. The inhibition zone for bacteriocin was measured after 48 h.**

| Isolates | ABA (µg/ml) | Bacteriocin inhibition zone (mm) |
|---|---|---|
| 1P | 0.15 D | Not detected |
| 2P | 1.64 A | 6.000 A |
| 3P | 0.80 C | 4.333 B |
| 4P | 1.087 B | Not detected |
| 5P | 1.31 B | Not detected |

**Table 4. Effect of inoculation with PSB isolates on length and fresh weight of root and shoot of chickpea plants.**

| Treatments | Root length (cm) | Shoot length (cm) | Root weight (g) | Shoot weight (g) |
|---|---|---|---|---|
| T1 | 6.887 C | 6.730 B | 1.443 B | 1.289 BC |
| T2 | 6.013 C | 8.40 A | 1.583 B | 1.293 BC |
| T3 | 9.000 B | 8.480 A | 1.820 AB | 1.663 A |
| T4 | 10.75 A | 9.047 A | 2.160 A | 1.453 AB |
| T5 | 6.667 C | 9.150 A | 1.310 BC | 1.430 AB |
| T6 | 5.687 C | 5.767 C | 0.8767 C | 1.007C |

T1, T2, T3, T4 represents the inoculation treatments with isolates 1P, 2P, 3P, 4P from rhizospheric soil of *Chrysopogon aucheri, Lactuca dissecta, Solanum surattense* and *Sonchus arvensis* collected from Khewra salt range, respectively. While T5 treatment represents inoculation treatment with 5P isolated from rhizospheric soil of *Solanum surattense* collected from Attock. T6 representing uninocuated plant. All means which share same letter are insignificantly different.

## Conclusion

It is inferred from the results that PSB isolate 2P was more efficient in terms of phosphorous solubilization, produced maximum amount of ABA and exhibited bacteriocin production. The isolate 2P may be used in the production of biofertilizer in areas where P availability is limited or P is fixed and is unavailable. However, identification of isolated PSB strains is required on the basis of molecular techniques. Complete spectrum of phytohormones and secondary metabolites produced needs further investigation.

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