**Plant growth promoting properties of indigenous phosphate solubilizing bacteria from saline soils of the Urmia Lake basin**

**Abstract**

Plant growth promoting bacteria (PGPB) have been widely used recently to ameliorate adverse effects of salinity. Twenty five saline soil samples were collected from the Urmia Lake basin to isolate phosphate solubilizing bacteria (PSB). Of isolated bacteria, ten PSBs were characterized at the molecular level using 16S rRNA partial gene sequencing. Some selected growth promoting properties including zinc (Zn), phosphorus (P) and potassium (K) solubilizing potential, as well as making indole acetic acid (IAA), cyanide hydrogen (HCN), siderophore, and exopolysaccharide (EPS) were evaluated. Multiple sequence alignment of 16s rRNA of the bacteria with other sequences in GenBank indicated that RB93 and RB73 belong to *Pseudomonas aeruginosa* (100-99%), RB75 to *Pseudomonas fluorescens* (100-99%), RB80 and RB70 to *Stenotrophomonas* *maltophilia* (100-100%), and RB81 to *Paenibacillus* *sp* with (100-99%) similarity values. TCP solubilization activity of the strains varied between 90 to110 (mg l-1), which was more than the HA solubilization activity (72-88 mg L-1) in broth medium. Among the strains, three strains were positive for IAA, five for siderophore production, and seven were able to produce EPS. All screened *Pseudomonas* genus and *Paenibacillus sp.* produced HCN. Furthermore, strains, RB73 and RB88 were detected to be the superior strains in the field of Zn and P solubilization, which indicated the greater plant growth promoting activity compared to other strains. It is concluded that efficient PGPR strains may be used as bioinoculants, result in increasing of soil P and Zn availability, promote plant growth, reduce salt stress and agricultural fertilizer application, and promote sustainable agriculture finally.

**Keywords:** Plant growth promoting bacteria, Phosphate, Solubilization activity, Salinity, Zinc

**Introduction**

Salinity is one of the superlative serious environmental issues in the world, especially in arid and semi-arid lands. Salinity suppresses the phosphate availability and reduces the P activity. Soil sorption processes and low-solubility of Ca-P minerals firmly controlled phosphorus concentration in the soil solution (Grattan and Grieve, 1992). The importance of P nutrition for plants and harmful effects of its deficiency on plant development are well recognized, especially in saline soils hence, it is urgent to understand the adverse results of salinity in order to increase crop productivity in saline soil and overcome to increasing demand for food in the future (Vivekanandan et al. 2015). In Iran, about 55.6 million ha (i.e. 34%) of the country's total area is affected by salinity in different degrees (Banaei, 2000). It is predicted that by 2050, more than 50% of cultivated lands will be affected by salinity to different degrees (Jamil et al. 2011). Thus, the necessity of reclamation of salt-affected soils for potential cultivation is inevitable. Today, developing the efficacious, affordable, environment friendly strategies to manage abiotic environmental stresses is a major challenge.

Using of biological methods and the capability of plant growth promoting rhizobacteria (PGPR) in ameliorating the effects of biotic and abiotic stresses on plants has been recognized (Francisco et al. 2012; Srinivasan et al. 2012). PGP bacteria meliorate plant development and soil properties by different ways such as increased mobilization of insoluble nutrients (Lifshitz et al. 1987; Ahmad et al. 2008), biocontrol of phytopathogenic organisms (Weller et al. 2005) and/or by producing of phytohormones (Dubeikovsky et al. 1993; Spaepen et al. 2007). Direct effects of growth promotion by PGPR happens by increasing of soil nutrient bioavailability through biostabilization of nitrogen (N), potassium and P solubilization, inhibition of pathogenic agents, and producing plant growth regulating (Del Amor and Cuadra-Crespo, 2012). Using of PGPRs has beneficial effects on development of the plants growing in salt-affected soils and thereby improves the agricultural crop yields. Margesin and Schinner (2001) reported that some PGPRs act a serious duty in improving soil moisture by polymer production mechanisms (Exopolysaccharides), especially in salt stress conditions. Studies on the interaction of PGPR and their effect on the endurance and physiological reaction of plants under different soil salinity are still in the early steps. Utilization of the soil biota can answer as a possible method to lessen salinity impacts in salt-sensitive crops. Hence, vast research is required in this subject.

Salt-tolerant microorganisms can accommodate with the local conditions and compete with the indigenous heterotrophic bacterial population. In these conditions, the native microorganisms may perform better than introduced phosphate solubilizing bacteria (PSB) due to being better adapted to a particular environment. The isolation of superior strains of PSB is urgent for their usage as bioinoculants for improving the growth of plants in stress conditions. The main objectives of this study were to isolate and molecularly characterize the salt tolerant phosphate solubilizing bacteria, which are isolated from roots of the indigenous plants grown in the saline soils of the Lake Urmia basin. Meanwhile, the isolated PSBs were also investigated for their abilities to solubilize of Zn and K and their plant growth promoting potential properties.

**Materials and Methods**

**Soil sampling and characterization**

A total of 25 soil samples were accumulated from the rhizospheric soil of plants that grown in saline soils of the Lake Urmia basin. The rhizospheric soils were manually separated from the roots and stored at 4°C until further processing. After drying and sieving non-rhizospheric soils, pH, electrical conductivity (EC) and the sodium absorption ratio (SAR) in saturated soil extract, available phosphorus (P) (Olsen and Sommers 1982), Soil texture (Gee and Bauder 1986) and total organic carbon (Walkley and Black, 1934) were measured. Significant presence of chloride (Cl) in the saline soils causes a overestimation error. To correct this error, the following correction coefficient was used as (Walkley, 1934):

%OC= (measured OC (%)) – (Cl (%)/12)

**Isolation and qualitative assay of phosphate solubilizing bacteria (PSB)**

Rhizospheric soil (1 g) was used for the isolation of PSBs by serial dilution. 10-3 to 10-5 dilutions were plated on NBRIP’s (National Botanical Research Institute’s Phosphate) agar medium plates containing 10 g glucose, 5 g Ca3(PO4)2, 5 g MgCl2·6H2O, 0.25 g MgSO4·7H2O, 0.2 g KCl, and 0.1 g (NH4)2SO4 in 1000 ml distilled water (Nautiyal, 1999). Also, hydroxyl apatite (Ca10(PO4)6(OH)2) was separately used as source of insoluble P at the concentration of 0.5%. The pH of the medium was adjusted initially to 7.0. After incubation at 30±2°C for 7 days, those colonies that showed a clean halo circa were purified, sub­­-cultured and stored as glycerol stocks at -20oC for further experiments.

All colonies indicating a clear halo around on the NBRIP solid medium were qualitatively tested. Petri dishes were dot-inoculated with 10 μl of nightly media on the NBRIP solid medium with three replicates then incubated at 30±2ºC for 7 days. The P solubilization ability was characterized by the solubilization index (SI) following formula (Edi-Premono et al. 1996):

(1) SI= Total diameter (Colony diameter + Halo zone diameter) / Colony diameter

The solubilization efficiency was assessed according to Silva Filho and Vidor,s scale (2000).

**Quantitative assay for P solubilization**

500 μl of bacterial inoculum was added into flasks containing 50 mL of the NBRIP broth medium, in triplicates, at 30± 2°C with shaking (120 rpm) on a rotary shaker with an equal number of un-inoculated controls. After seven days, the bacterial suspension was centrifuged at 10000 rpm for 10 min and filtered to analyze released P in the supernatants using molybdate-vanadat method using spectrophotometer at 470 nm (Cotteni, 1980). pH of the medium was recorded by pH meter. Hydroxyapatite (HA) at a concentration of 0.5% was used for quantitative analysis on NBRIP as discussed.

**Influence of NaCl on P-solubilizing ability of the PSB**

To distinguish the impact of the effect of salinity on ability of PSB to solubilize phosphate, 10 μl overnight culture of each isolate was spotted on NBRIP medium containing 0 (control), 2, 5, 7 and 10% NaCl in petri dishes. After seven days of incubation at 30 ±2°C, each isolate that formed a halo around the colony was transferred the NBRIP broth. The soluble P was determined using the standard method described previous and the pH of supernatant was recorded.

**Phenotypic characterization of bacteria**

Morphological (endospore, colony color, pigmentation, and motility) and biochemical characteristics (catalase, oxidase, citrate, urease, casein hydrolysis, starch hydrolysis, gelatin hydrolysis, H2S production, and glucose fermentation) of the PSBs studied by standard methods (Bergey’s Manual of Systematic Bacteriology, Holt et al. 1994).

**Molecular characterization of isolated bacteria**

The genomic DNA of selected bacterial isolates, based on the differences in phenotypic characteristics, was extracted from overnight grown cells according to the modified method of Sambrooket al. (1989).Briefly, 400 µL extraction buffer (Tris-HCl 50mM, pH: 8; EDTA 25mM; SDS 1%; Proteinase K 10 µgml-1) were added to 200 µL of bacterial suspension in sterile distilled water. Tubes were placed in Ban Mari with 55 °C for three hours. Then, 400 µL ammonium acetate (7.5 M) was added to tubes and DNA was recovered by isopropanol precipitation. Tubes were placed at -20 °C overnight, and then centrifuge and plates were washed with ethanol 70% twice. DNA re-dissolved in sterile distilled water and kept at -20 °C. To amplify the 16S rRNA gene, universal primers of fD1 (5′ AGAGTTTGATCCTGGCTCAG 3′) and rD1 (5’AAGGAGGTGATCCAGCC 3’) (Weisburget al.1991) were employed and PCR were carried out using Palm cycler model GP001 (Corbett Research Co., Australia). PCR amplification was performed in 25 μL of the reaction mixture containing 12 μL of PCR Master Mix Red (Ampliqon), 10 pM of each primer, 1 µL of 100 ng⁄ml purified DNA as template, and sterile ultra-pure water. Annealing conditions comprised 60 s at 53 °C. The quality and quantity of the final PCR product were resolved by electrophoresis in 1% w/v agarose gel in Tris-acetate EDTA buffer (TAE) and Gene Ruler TM 1 kb DNA ladder used as a marker. Staining of agarose gel was done by FluoroDye DNA Fluoresent Loading Dye 1 μL/mL (Smobio, Taiwan). The amplified fragments were sequenced and subjected to identification using BLAST and run against registered sequences in the GenBank database. The sequences of bacteria were deposited in GenBank databases under the accession numbers shown in Tables 3 (http://www.ncbi.nlm.nih. gov/BLAST).

**Plant growth promoting (PGP) properties of the bacteria**

**Indole-3-acetic acid (IAA) production**

The production of IAA was quantitatively measured according to Gordon and Weber (1951). One mL inoculum of bacterial was added in 25 mL of Luria-Bertani (LB) broth containing 100 μg ml-1L-tryptophan. After 24 h incubation at 30±2°C, cultures were centrifuged (10000 rpm, 5 min) and two drips of phosphoric acid were stepped up to 2 mL of the culture supernatant in 4 mL of Salkowsky reagent and mixed, allowed to constant at room temperature for 30 min to extend color. The IAA concentration was determined using a spectrophotometer at 530 nm. Un-inoculated broth was considered as control. A standard curve was prepared with 5-100 μg ml-1 of IAA (Sigma Chemicals) for quantification.

**Siderophore production**

The ability of isolates to produce siderophores were tested on Blue CAS agar medium containing chrome azurol S and hexadecyltrimethylammonium bromide (HDTMA) as indicators (Schwyn and Neilands, 1987). All the strains were spot inoculated into the CAS agar medium and were incubated at 30 ±20 C for three days. The production of siderophores was determined by formation of an orange halo around the bacterial colony.

**HCN production**

To measure HCN production, isolates were first cultured on Agar Soy Tryptic medium (containing 5g enzymatically hydrolyzed casein, 5 g digested soy flour with pepsin enzyme, 5 g NaCl, 15 g agar in 1000 mL distilled water ) amended with glycine (4.4 g). Then a Whatman no. 1 filter paper soaked in 2% (w⁄v) sodium carbonate in 0.5% (w⁄v) picric acid solution was placed inside of a petri dish. The plate was then sealed with parafilm and incubated at 30° ±2C for three days. A change in filter paper color from yellow to reddish brown was considered to be an indication of HCN production (Donate et al, 2004).

**Exsopolysaccharide (EPS) production**

Exsopolysaccharide production was tested on MY culture medium containing 10 g Glucose, 5g Peptone, 3g malt extract, 3g yeast extract, 40 g NaCl, 4.85g MgSO47H2O, 3.5g MgCl24H2O, 1g KCl, 1.8g CaCl2,0.013g NaBr, 0.03g NaHCO3 in 1000 mL distilled water (Ventusa, 2004). Bacterial strains were grown in 100 mL of medium in 250 mL Erlenmeyer flasks at 30 ±20C for five days under shaking conditions (120 rpm). The supernatant was removed by centrifuging at 10000 rpm for 30 min. Ice cold ethanol (96%) was then added and the formation of a precipitate was considered positive for the production of EPS. After drying in an oven at 50 °C overnight, the dry weight of the precipitate was calculated as the amount of EPS produced per liter of culture medium.

**Solubilization of Zn**

Ten μL of a young culture of each isolate was transferred on basal medium containing (glucose-10.0 g, (NH4)2SO4-1.0 g, KCl-0.2 g, K2HPO4–0.1 g, MgSO4.7H2O–0.2 g, Agar-15.0 g, double distilled water-1000 mL and buffered to pH 7.0 and completed with 0.2% insoluble Zn, separately from three sources viz., ZnO, ZnCO3 and Zn3(PO4)2 (Saravanan et al. 2003). After seven days, SI was calculated (Edi-Premono et al., 1996). In order to quantify the amount of Zn solubilized, 500 μL of a young culture of each bacterial isolate was transferred to the flask containing 25 mL of liquid medium and kept in an incubator-shaker at 120 rpm with three replicates and uninoculated controls. After seven days, the medium was centrifuged at 10000 rpm for 5 min, and soluble Zn from the supernatant was estimated using AAS (Shimadzu AA-6300).

**Data Analysis**

Data were tested for statistical significance using the analysis of variance (ANOVA) by SAS package (SAS, 1999). Mean comparisons were conducted using a least significant difference (LSD) test (P = 0.05). Standard error and LSD results were calculated.

**Results**

Soil samples of organic carbon content, calcium carbonate equivalent (CCE) pH, EC, and SAR were characterized (Table 1). The pH of the soils ranged from 7.3 to 8.5, EC from 1.0 to 53 dS m−1, SAR from 1.7 to 10.5 meq L-1 . According to these attributes, the soil samples were classified as saline and non-saline soils, respectively (Brady, 1990).

**Table 1** Some physicochemical Properties of the soil samples used for isolation of phosphate-solubilizing bacteria

|  |  |  |  |
| --- | --- | --- | --- |
|  | Minimum | Maximum | Mean ± SD |
| pH | 7.3 | 8.5 | 7.9 ± 0.39 |
| EC (dS/m) | 1.0 | 53 | 10.4 ± 8.7 |
| CCE% | 0.8 | 17.5 | 7.15±5.2 |
| SAR (meq/L) | 1.7 | 10.5 | 6.6 ±2.69 |
| Na+ | 32.4 | 138.7 | 48.8 ± 31.4 |
| Ca 2+  Mg2+ | 36.8  20.4 | 298.8  135.2 | 87.9 ± 54.2  44.4±34.4 |
| OC % | 0.06 | 1.93 | 1.04 ±0.46 |
| Clay % | 15 | 55 | 26.6 ±10.94 |
| Silt % | 12.5 | 47.5 | 28.5±10.33 |
| Sand % | 7.5 | 70 | 44.9±18.8 |
| Olsen-P (mg/kg) | 5.8 | 80 | 21.2±15.2 |

**Biochemical and molecular characterization**

In this study, a total of 10 PSB were isolated from 25 saline soil samples. All strains were identified based on their morphological and biochemical attributes. Among the 10 isolates, one isolate was a gram positive endospore former, and the remains were gram negative and non-spore forming (Table 2). All strains were motile and positive for pigmentation, catalase and gelatinase. Six strains were positive and four were negative in terms of citrate utilization. The other biochemical parameters are presented in Table 2.

To accurately identify the PSB strains, the 16S rDNA gene sequence analysis was carried out. Based on morphological and biochemical resemblances, the strains were grouped into five groups, and from each group one or two strains were selected for molecular identification. Multiple sequence alignment of 16s rDNA of the bacteria with other sequences in GenBank indicated that RB93 and RB73 belong to *Pseudomonas aeruginosa* with (100-99%) similarity values, RB75 to *Pseudomonas fluorescens* with (100-99%) similarity values, RB80 and RB70 *Stenotrophomonas maltophilia* (100-100%)*,* RB81 to *Paenibacillus sp* (100-99%) similarity values (Table 3).

**Table 2** Morphological and biochemical characteristics of the isolated bacterial strains

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Characteristics | RB70 | RB71 | RB73 | | RB75 | RB80 | RB81 | RB82 | RB88 | RB91 | RB93 |
|  | Morphology | | | | | | | | |  |  |
| Gram stain | - | - | - | - | | - | + | - | - | - | - |
| Endospore | - | - | - | - | | - | + | - | - | - | - |
| Colony color | white | yellow | green | yellow | | white | yellow | green | green | green | green |
| Pigmentation | - | + | + | + | | + | + | + | + | + | + |
| Motility | + | + | + | + | | + | + | + | + | + | + |
| Biochemical parameters | | | | | | | | | | | |
| Catalase | + | + | + | + | | - | + | + | + | + | + |
| Oxidase | - | - | + | - | | - | + | - | + | + | + |
| Citrate | + | + | + | + | | + | - | + | + | + | + |
| Lactose | - | - | - | - | | - | - | - | - | - | - |
| Sucrose | - | - | - | - | | - | - | - | + | + | + |
| Dextrose | + | + | + | + | | + | + | + | + | + | + |
| Urease | + | + | - | + | | + | - | + | + | + | - |
| Starch hydrolysis | - | - | - | - | | - | - | - | - | - | - |
| Gelatine hydrolysis | + | + | + | + | | + | + | + | + | + | + |
| H2S production | + | - | - | + | | + | - | + | - | - | + |
| Fluorescence | - | - | + | + | | - | - | - | + | + | + |
| Lipase Activity | + | + | + | - | | - | - | + | + | - | + |
| M-R test | + | - | - | - | | + | - | - | - | - | - |
| V-P test | - | - | - | - | | - | - | + | + | + | - |

+ indicates presence or positive; - indicates absence or negative

**Qualitative trial for P solubilization**

All ten strains showed a clear halo around the colony on NBRIP supplemented with TCP and HA. Based on the Silva Filho and Vidorʾ scale, RB88, RB93 and RB73 were classified as high solubilizers, RB82, RB75 and RB91 as medium solubilizers and others were classified as low or very low solubilizers. All isolates were classified as low or very low solubilizer when HA was used as a P source (Table 3).

**Quantitative assay for P solubilization**

The statistical results indicated significant differences between the isolates in solubilizing insoluble P compounds and reducing the medium pH (Table 4). The P solubilizing abilities of the strains in TCP broth were higher than in HA broth. RB73 and RB91 showed significantly higher P solubilization than other strains in the two broth media (Figure 1A). All strains were more effectual in solubilizing TCP than HA.

The correlation analysis between SI and P solubilization in liquid media indicated a significant relationship (r = 0.57\*\*). Isolates that were more efficient solubilizers on solid medium also solubilized more P on liquid medium.

**Table 3** Mean solubilization index (SI) values corresponding to 10 bacteria isolates studied

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Isolate Code | Most frequent genus/species | Accession number | | SI (TCP) | Classification | | SI (HA) | Classification |
| RB88 | *Pseudomonas* |  | 3.3a | | HS | 1.43b | | LS |
| RB93 | *Pseudomonas aeruginosa* | [MT454630](https://www.ncbi.nlm.nih.gov/nuccore/MT454630) | 3.1a | | HS | 1.5ab | | LS |
| RB73 | *Pseudomonas aeruginosa* | [MT449509](https://www.ncbi.nlm.nih.gov/nuccore/MT449509) | 3.0a | | HS | 1.6a | | LS |
| RB82 | *Stenotrophomonas* |  | 2.76b | | MS | 1.13c | | LS |
| RB75 | *Pseudomonas fluorescens* | MT454668 | 2.4c | | MS | 1.03c | | LS |
| RB91 | *Pseudomonas* |  | 2.3c | | MS | 1.0c | | VLS |
| RB81 | *Paenibacillus sp.* | MT449014 | 1.33d | | LS | 0.58d | | VLS |
| RB70 | *Stenotrophomonas maltophilia* | MT454071 | 1.23d | | LS | 0.52d | | VLS |
| RB80 | *Stenotrophomonas maltophilia* | MT449476 | 1.2d | | VLS | 0.5d | | VLS |
| RB71 | *Stenotrophomonas* |  | 1.0d | | VLS | 0.45d | | VLS |
| LSD |  |  | 0.22 | |  | 0.14 | |  |
| CV |  |  | 7.1 | |  | 9.1 | |  |

The means followed by the same letters indicate no significance difference (P < 0.05), HS: high solubilization, MS: medium solubilization, LS: low solubilization, VLS: very low solubilization, Classification formulated by Silva Filho and Vidor (2000).

In NBRIP medium with TCP, all ten strains decreased pH of the medium (Figure 1 B). The maximum decline in pH was recorded with RB88 (*Pseudomonas*) from 7.0 to 3.5 and RB70 (*Stenotrophomonas* *maltophilia*) from 6.7 to 3.7. We observed a significant negative correlation (r = -0.87) between the quantity of P liberated and pH of the medium amended with TCP (Table 4)

**Table 4** Variance analysis (mean squares) of solubilized P and of pH values in TCP and HA media

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | Df | MS | | | | r  pH(TCP)-SP1 | r  pH(AH)-SP2 |
| Solubilized TCP (μg/ml) | Solubilized AH (μg/ml) | pH (TCP) | pH (HA) |
| Isolate | 9 | 90.6\*\* | 64.8\*\* | 1.43\*\* | 0.58\*\* | -0.87\*\* | -0.14ns |
| Insoluble-P | 1 | 52.93\* | 16.97\* | 0.63\* | 0.02\* |  |  |
| P × Isolates | 9 | 21.7\* | 8.7\* | 0.04\* | 0.01\* |  |  |
| Error | 38 | 1.68 | 10.3 | 1.8 | 0.057 |  |  |
| CV |  | 2.6 | 4.5 | 10.5 | 8.3 |  |  |

\*\* and \* Significant at probability level of 1% and 5% (P< 0.01, P< 0.05) ; 1 and 2: R2 between pH and soluble P in TCP and AH medium respectively.

**Influence of NaCl on P-solubilizing ability of the PSB**

In order to investigate the intrinsic resistance of PSB to salt stress, the strains were streaked on NBRIP agar supplemented with various concentrations of NaCl (2, 5, 7, 10 g L-1). The results of variance analysis indicated that the effect of the salinity levels and strains were significant at 1% probability level (Table 5). The results showed that all strains lost their ability to grow and solubilize P in the presence of 5, 7, and 10% NaCl (Table 6). Among the ten strains, six strains showed clear haloes around the colonies in increased NaCl concentration up to 5% NaCl and did not show growth thereafter. Mean comparison of data showed that of the six isolates, RB88 had the highest insoluble phosphate solubilization at the salinity levels of 2 and 5% (Table 6).

**Table 5** Effect of different concentrations of NaCl on phosphate solubilization in NBRIP medium

|  |  |  |  |
| --- | --- | --- | --- |
| Source of variation | Df | P2O5 (μg /ml) | pH |
| Salinity | 2 | 7811.9\*\* | 3.1\*\* |
| Isolate | 5 | 172.4\*\* | 2.71\*\* |
| S\*I | 10 | 89.9\*\* | 0.69\*\* |
| Error | 36 | 1.36 | 0.072 |
| CV | 4.5 | 6.3 |  |

\*\* and \* Significant at probability level of 1% and 5% (P< 0.01, P< 0.05)

**Table 6** Amounts of solubilized phosphate by isolates under different concentrations of NaCl

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| NaCl% | RB70 | RB73 | RB75 | RB80 | RB88 | RB93 |
| 0 | 90.86e | 110a | 102.3bc | 94.3d | 99.4c | 102.6b |
| 2 | 73.7a | 68.7b | 63c | 61.6c | 75a | 62.9c |
| 5 | 54.3c | 62.4b | 58.6c | 55.7d | 71.4a | 62.4b |

The means followed by the same letters indicate no significance difference (P < 0.05)

|  |
| --- |
| **A** |
| **B** |

**Figure 1** P solubilized (mg L-1) by isolated bacteria and pH in NBRIP medium supplemented with TCP and HA.Vertical bars represent the standard deviation. Means with the same letters are not significantly different according to LSD (P < 0.01).

**Assay of PGP characteristics of the bacteria**

In the present study, all strains were screened for PGP activities (Table 7). Out of ten strains, only three strains (*Pseudomonas fluorescens, Paenibacillus sp and Stenotrophomonas maltophilia*) indicated the production of IAA.

Out of the 10 PSB strains, RB75 (*Pseudomonas fluorescens*) RB73 (*Pseudomonas aeruginosa*) RB93 (*Pseudomonas aeruginosa*), RB88 (*Pseudomonas*) and RB81 (*Paenibacillus* *sp*) were positive for the production of siderophores.

As for the other PGP characteristics, HCN production potential of the strains was measured qualitatively (Table 7). HCN production was found by observation of filter paper color changing from yellow to reddish brown after two days incubation. Among all PSBs, only four strains, RB73, RB75, RB81 and RB93 were positive for HCN production.

Among all PSBs, seven strains were able to produce EPS. In this study, the highest amount of EPS production was found in isolates RB71 (5.3 g/ L), RB70 (4.7 g/ L) and RB80 (4.3 g L-1) the lowest was found in isolates RB81 (0.5 g/ L) and RB93 (0.4 g /L) which was significantly lower than for other strains.

**Table 7** Growth promotion Capability of the isolated bacterial isolates

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolate No. | IAA | Siderophores | HCN | EPS (g L-1) |
| RB70 | - | - | - | 4.7b |
| RB71 | - | - | - | 5.3a |
| RB73 | - | + | + | 2.3c |
| RB75 | + | + | + | 1.32d |
| RB80 | + | - | - | 1.2cd |
| RB81 | + | + | + | 0.5e |
| RB82 | - | - | - | - |
| RB88 | - | + | + | - |
| RB91 | - | - | + | - |
| RB93 | - | + | + | 0.4e |

**Solubilization of Zn and K**

Zinc and K solubilization potential is another PGP trait that was evaluated in this study. The strains isolated from this study were not able to release K from the silicate sources. After 15 days of incubation, the clear haloes around the colonies didn’t found (Table 8). RB93 and RB88 produced the largest clear haloes in the media with different insoluble Zn compounds. The diameter of the clear halo produced by RB93 was not significantly altered by changing the insoluble Zn compounds (Figure 2). RB70, RB75 and RB88 showed the highest SI with ZnO, RB81with Zn3(PO4)2 and RB80 with ZnCO3. Strain RB71 only produced a clear halo with Zn3(PO4)2.

All of the eight strains were further evaluated for their capability to solubilize inorganic Zn in liquid medium. The solubilized Zn amount ranged from 21 mg/L to 42 mg/L in ZnO, from 12 mg/L to 65 mg/L in Zn3(PO4)2 and from 5 mg/L to 50.1 mg/L in ZnCO3 (Table 8). In the presence of all three sources of insoluble Zn, the Zn solubilizing capacities of RB93, RB88 and RB80 were higher than for other strains. Strains RB75 and RB80 showed significantly higher solubilization of ZnCO3, while they showed almost no solubilization of the other insoluble Zn compounds (Table 8).

**Table 8** Zn solubilized (mg/L) by bacterial isolates in liquid medium with several insoluble Zn minerals

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Isolates | Solubilized Zn (mg L-1) | | |  |  |
| Zn3(PO4)2 | ZnCO3 | ZnO |  | Illite |
| RB70 | 12f | 48b | 36c |  | - |
| RB75 | 32d | 32c | 28d |  | - |
| RB80 | 49b | 27d | 21e |  | - |
| RB88 | 63a | 50.1a | 42a |  | - |
| RB93 | 65a | 21 | 39b |  |  |
| RB73 | 24 | 15f | - |  | - |
| RB81 | 38c | 5g | - |  | - |
| RB71 | 26e | - | - |  | - |
| LSD | 2.1 | 1.1 | 1.8 |  | - |
| CV | 5.4 | 3.1 | 2.8 |  | - |

Means with the same letters aren't significantly differencing according to LSD (P < 0.01)

**Figure 2** Zinc solubilisation zone (mm) of bacterial strains in solid medium with different Zn sources. Vertical bars indicate standard deviation; LSD (P < 0.01) for Zn3 (PO4)±2.4; ZnCO3±2.5; ZnO±2.

**Discussion**

Phosphate solubilizing microorganisms dissolve insoluble P and make it available to the plant. All strains indicated different levels of P solubilizing activity during quality and quantity screening (Table 3). In this study, PVK and NBRIP media were used to evaluate P solubilizing ability of the bacterial strains based on the development of the halo zone around the colonies. None of the isolates could show clear halo on the PVK solid medium after incubating. All strains were more efficient in solubilizing TCP than HA. These results are in similar to the results of most of the studies that reported a clean halo around the bacterial colonies in the NBRIP medium with Ca3 (PO4)2 to other insoluble P sources (Ca10 (PO4)6(OH) 2, FePO4 and AlPO4) (Kumar *et al.* 2010; Parasanna et al. 2011; Chang and Yang, 2009).

In this study all strains were identified to genus level based on the standard morphological, physiological and biochemical tests (Table 3). Among the 10 strains, the genera *Pseudomonas* and *Stenotrophomonas* were found to be the most common in the saline soils of Lake Urmia. In several studies, *Bacillus* was reported as the most predominant genus in stressed environments, though genera such as *Pseudomonas, Azospirillum, Enterobacter, Arthrobacter, Flavobacterium, Azotobacter, Micrococcus, Clostridium,* and *Achromobacter* are also prevalent (Dinesh et al. 2018; Dinesh et al. 2015; Felici et al.2008; Forchetti et al.2007; Swain and Ray, 2009; Wani et al, 2007).

The ability of PSB strains in reducing pH of the surroundings, either in releasing organic acids or proton represents their potential to solubilize the P (Hariprasad and Niranjana, 2009). In NBRIP medium with TCP, all 10 strains lowered pH of the medium (Figure 1 B). The maximum decline in pH was recorded with RB88 (*Pseudomonas*) from 7.0 to 3.5 and RB70 (*Stenotrophomonas* *maltophilia*) from 6.7 to 3.7. We observed a significant negative correlation between the quantity of P liberated and pH of the medium amended with TCP (Table 3) which is in accordance with previous works (Srinivasan et al, 2012; Cherif et al, 2013). These results suggest that reducing the pH has a prominent role in P solubilization (Son et al. 2006; Keneni et al. 2010). In NBRIP medium with HA, the pH of medium was less to be changed by growth of strains, and RB70 (*Stenotrophomonas maltophilia*), RB73 (*Pseudomonas aeruginosa*) and RB91 (*Pseudomonas*) actually increased pH of this growth medium up to 7.3, 7.26 and 7.2 respectively, after seven days of incubation (Figure 1B). The alkalinization and absence of organic acids in the growth medium of *Actinobacteria* isolated from a Togolese phosphate mine during the solubilization of rock phosphate was reported previously (Hamdali et al,2012). This explains that other mechanisms, such as siderophore production, are involved in phosphate solubilization (Hamdali *et al.* 2012). Lack of a significant correlation between pH and the amount of solubilized P in NBRIP medium with HA (r = -0.14) proposed that microbial P solubilization is an intricate phenomenon that depends on many factors such as nutritional, physiological, and growth conditions of the culture (Pradhan and Sukla, 2005; Son et al. 2006; Sagervanshi et al.2012).

The effect of salinity stress was studied using different concentrations of NaCl (2, 5, 7 and 10 %) on growth and the ability of the strains to solubilize TCP in NBRIP broth medium. In the present study, all strains loss their ability to grow and solubilize P in the presence of 5, 7 and 10% NaCl (Table 6). Among the 10 strains, six strains showed clear halo around the colonies to increase in NaCl concentration up to 5% NaCl and did not show thereafter. Kumar et al. (2010) found increased P solubilization with an increase in NaCl concentration (0.5-2%). A similar study on PSB from salt affected soils observed that the strain (*pseudomonas.* *striata*) indicated a significant lessen in P solubilization with increasing of NaCl concentrations. Their results indicated that PSB strains of salt-affected soils had better performance in releasing P from TCP even under salt stress conditions (Srinivasan et al.2012). Rehman and Nautiyal (2002) reported that resistance to salinity, drought and high temperature of *Bacillus* *subtilis* was higher than *Pseudomonas reactans* which can be due to the shape of the *Bacillus* cell wall making them more salt-tolerant, which can reduce the effects of salinity stress in the rhizhosphere and improve soil fertility (Barazani and Fridman, 2000). Barin et al (2015) found that increasing salinity on the eastern coastal plain of the Urmia Lake, didn’t affect the microbial biomass, but the microbial communities were adapted to salinity in the studied area. They suggested that organic carbon can mitigate salinity stress in microorganisms.

Production of plant growth promoting hormones as a secondary metabolite is one of the direct effects of PGPR on plant growth (Mangang et al. 2015). Out of 10 strains, only 3 strains (*Pseudomonas fluorescens, Paenibacillus sp and Stenotrophomonas maltophilia*) indicated the production of IAA. Production of IAA is a pivotal PGP trait displayed by the majority of the bacteria in the rhizosphere, which develops the plant root system and improves the nutrient uptake by plants in stress conditions (El-Azeem, 2007). Siderophore production is another important PGP trait that was detected by the formation an orange halo around the colonies due to the chelation of iron (Fe3+). Out of ten PSB, four strains formed the orange halo around the colonies due to removing the Fe3+ from the complex that alters the medium colour from blue to orange (Table 7). Under the present study, six strains were positive for HCN production (Table 7). Mohamed and Gomaa (2012) reported that HCN is a potential and environmentally compatible mechanism for the biological control of pathogens. Several studies reported that the activity of some PGPR in biocontrol of fungal phytopathogens might be due to the HCN and siderophores production and have reported a close relationship between HCN and siderophore production and antifungal activity. (Scher and Baker 1982; Ahmad et al. 2008; Komal et al.2016). Some PGPR that produce EPS are highly important in binding cations such as Na+, suggesting a role in mitigation of adverse salinity effects by reducing the content of Na+ available for plant uptake (Arora et al.2013). The use of EPS-producing bacteria, in addition to enhancing the solubility of insoluble phosphorus to provide the plant with this essential nutrient for plant growth and development, also provides protection in plants against abiotic stresses (Gupta et al.2015). A previous study also discussed that plants treated with EPS producing bacteria showed increased resistance to abiotic stresses and improved soil structure. EPS can also make Na+ less available to plants in saline conditions (Sandhya et al.2009).

Characterize the bacteria for their Zn and K solubilization potential is another PGP trait that was evaluated in this study. Eight out of the ten bacterial strains showed solubilization haloes in solid medium with Zn3(PO4)2 followed by seven with ZnCO3 and four with ZnO (Figure 2). The present results are in agreement with previous reports that bacterial strains had different abilities to solubilize inorganic Zn compounds (Sunithakumari et al. 2016; Sharma et al.2012). Some strains like RB75 and RB81 exhibited multiple PGP traits, which can improve plant growth. Since plant nutritional requirement increase in salt stress conditions and physiological processes require more energy under stress, these strains may act as sources of nutrients to the plants subject to salinity conditions (Gupta et al. 2015). Numerous studies indicated that native salt tolerant bacteria usually have one or more different PGP traits (Gouda et al. 2018; Komal et al. 2016). Among the strains, only RB88 solubilized high amount of Zn in all three insoluble Zn sources, but the rest of the strains did not have a constant trend in the solubilization of Zn. Reports in this context are contradictory, with some studies demonstrated that the largest solubilization was found with ZnO (Dinesha et al. 2018; Kumar et al. 2016), or Zn3(PO4)2 (Sharma et al. 2012) or ZnCO3 (Vidyashree et al. 2016). Other researchers also reported variation among the insoluble Zn minerals with ZnO also being more impressively solubilized than ZnCO3 or Zn3(PO4)2 (Saravanan et al. 2007; Madhaiyan et al. 2004). Some bacterial strains such as RB70 in ZnCO3 medium had a smaller SI than RB93 and RB80 but released a high amount of soluble Zn in liquid medium (Table 8). Therefore, it is necessary to use both quantitative and qualitative screening methods to determine an efficient strain for bioinoculant production.

**Conclusions**

Salinity stress is an important factor in reducing plant growth. Therefore, it is necessary to study different strategies and carry out new research to increase the yield of agricultural products under salt stress conditions. The present study has provided useful information regarding P-solubilizing bacteria from the saline soils of the Urmia Lake basin and their PGP characteristics, which can be very useful information regarding the expansion of bioinoculants for sustainable agriculture under salt stress conditions. In this study, ten salt-tolerant P-solubilizing bacteria were isolated from saline soils. Based on the biochemical tests and molecular characterization the strains were categorized into four groups including *Pseudomonas aeruginosa, Pseudomonas fluorescens, Paenibacillus sp,* and *Stenotrophomonas maltophilia.* The results showed diverse levels of P solubilization activity during quantitative and qualitative screening, and strains RB73 and RB93, which indicated the highest activity during quantitative screening, were selected as efficient strains. Assessment of the PGP characteristics of the ten strains suggested that eight of all had the ability to solubilize inorganic Zn compounds, but none were able to solubilize insoluble K compounds. Based on the results of Zn solubilization, RB73 and RB88 are proposed to be efficient isolates. Further studies, however, are required to scrutinize the efficiency of these strains in PGP under glasshouse and field conditions and to evaluate the effectiveness of RB73 and RB88 that exhibited multiple PGP traits to improve the growth, physiology and quality of plants in the soil-plant system under saline conditions.

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