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# Assessment of two carrier materials for phosphate solubilizing biofertilizers and their effect on growth of wheat (*Triticum aestivum* L.)

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ABSTRACT

Biofertilizers are usually carrier-based inoculants containing beneficial microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers. Objective of the present study was to assess enriched biogas sludge and soil as biofertilizer carriers on growth and yield of wheat. Six phosphate solubilizing strains were used in this study. Three phosphate solubilizing strains, 77-NS2 (*Bacillus endophyticus*), 77-CS-S1 (*Bacillus sphaericus*) and 77-NS5 (*Enterobacter aerogenes*) were isolated from the rhizosphere of sugarcane, two strains, PSB5 (*Bacillus safensis*) and PSB12 (*Bacillus megaterium*) from the rhizosphere of wheat and one halophilic phosphate solubilizing strain AT2RP3 (*Virgibacillus* sp.) from the rhizosphere of *Atriplex amnicola,* were used as bioinoculants. Phosphate solubilization ability of these strains was checked *in vitro* in Pikovskaya medium, containing rock phosphate (RP) as insoluble P source, individually supplemented with three different carbon sources, i.e., glucose, sucrose and maltose. Maximum phosphate solubilization; 305.6 µg/ml, 217.2 µg/ml and 148.1 µg/ml was observed in *Bacillus* strain PSB12 in Pikovskaya medium containing sucrose, maltose and glucose respectively. A field experiment and pot experiments in climate control room were conducted to study the effects of biogas sludge and enriched soil based phosphorous biofertilizers on growth of wheat. *Bacillus* strain PSB12 significantly increased root and shoot dry weights and lengths using biogas sludge as carrier material in climate control room experiments. While in field conditions, significant increase in root and shoot dry weights, lengths and seed weights was seen by PSB12 and PSB5 *(Bacillus*) and *Enterobacter* strain 77-NS5 using biogas sludge as carrier. PSB12 also significantly increased both root and shoot dry weights and lengths in field conditions when used as enriched soil based inoculum. These results indicated that bacterial isolates having plant beneficial traits such as P solubilization are more promising candidates as biofertilizer when used with carrier materials.

## 1. Introduction

Plant growth promoting rhizobacteria (PGPR) reside in the rhizosphere of plants and directly or indirectly play an important role in plant health and soil fertility. They promote plant growth and enhance grain yield of various crops like rice, wheat, corn and sugarcane by production of compounds such as indole acetic acid (phytohormone), siderophores, HCN, solubilize minerals (P and Zn) and break organic materials for the easy uptake of plants and for their own use (Kumar et al., 2014; Goswami et al., 2016). From the rhizosphere of different members of family gramineae, large number of PGPR genera including *Bacillus, Burkholderia, Enterobacter, Serrratia Azospirillum* and *Pseudomonas* have been isolated and characterized (Berendsen et al., 2012; Gonzalez et al., 2015; Zaheer et al., 2016). PGPRs are used as biofertilizers and these are the best alternative to chemical fertilizers due to the fact that they reduce the cost of crop production and are environmental friendly as well (Mehnaz et al., 2001, 2010; Tahir et al., 2013).

Phosphorus is a macronutrient that plays a significant role in plant growth including photosynthesis, respiration, energy storage and transfer, and several other processes in the living plant cells (Yasmin and Bano, 2011; Solangi et al., 2016). It has been estimated that more than 90% of Pakistani soils contain insufficient amount of available phosphorus that is necessary to support plant growth (Rashid 1994; Malik et al., 2002; Jamil et al., 2016). Phosphate solubilizing activity has been reported in many PGPR genera i.e. *Bacillus, Enterobacter, Azosprillum* and *Pseudomonas* (Goes et al., 2012; Mehnaz et al., 2010; Tahir et al., 2013). These genera have ability to convert mineral phosphate into soluble phosphates (primary and secondary orthophosphates) by production of organic acids, i.e. malic acid, acetic acid, oxalic acid, citric acid and gluconic acid (Vessey et al., 2004; Tahir et al., 2013; Solangi et al., 2016).

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Use of economically cheap, low grade rock phosphate supplemented with P-solubilizing bacterial strains can replace the costly chemical and mineral phosphorus fertilizers. Biogas sludge based biofertilizers are used by the farmers to replenish essential nutrients into the soil (El-Gizawy, 2010). Enrichment of these biofertilizers with P-solubilizing bacteria result in conversion of insoluble indigenous rock phosphate (RP) into solubilized P, enhancing plant growth. Enrichment of biogas sludge and soil with less reactive rock phosphate has been used as an efficient and cost effective phosphate fertilizer for different crop production (Shrivastava et al., 2011). These biofertilizers are equally effective in alkaline as well as acidic soils. Species of *Bacillus, Enterobacter* and *Pseudomonas* have shown consistency in P-solubilizing ability by using rock phosphate from different sources (Afzal et al., 2005; Narayanan, 2012). Inoculation of P-solubilizing genera *Bacillus* and *Pseudomonas* on maize and wheat resulted in increase in grain yield and phosphorus uptake (Bonten et al., 2014).

Wheat, *Triticum aestivium*, is one of the major crops cultivated in South Asia and is the basis of food security in this part of the world. In order to make its cultivation cost effective and sustainable, it is essential to adopt measures to reduce the inputs of costly chemical fertilizers. The bacterial population associated with roots of wheat has been reported as an enhancer of yield and also acts as an antagonists to soil borne phytopathogens

One of the problems in the use of such biofertilizers (PGPR) is the selection of appropriate carrier material which should be inexpensive and should have ease of handling. The objective of present study was thus to compare the use of two inexpensive materials namely biogas sludge and soil enriched with PGPR as carrier materials. Another objective of this study was to characterize P-solubilizing bacteria from the rhizosphere of wheat, sugarcane and *Atriplex amnicola.*for use as microbial inoculants. The present study is the first report of its kind that deals with the comparative inoculation effect of both sludge and enriched soil based phosphorus biofertilizer on wheat plants by performing pot experiment in climate control room as well as in field experiment.

## 2. Materials and methods

### 2.1. Selection of phosphate solubilizing bacterial isolates

Twenty seven bacterial isolates from the rhizosphere of sugarcane, wheat and *Atriplex amnicola* were screened for phosphate solubilization on modified Pikovskaya agar plates (Pikovskaya, 1948) supplemented with rock phosphate (RP) instead of tri-calcium phosphate. Plates were incubated at 28 °C for 2–3 weeks and observed for clear zones around bacterial growth for P-solubilization.

### 2.2. Quantification of P-solubilization by bacterial isolates

Quantitative analysis of P-solubilization of bacterial isolates was done by molybdate blue color method (Watanabe and Olsen, 1965). Bacterial cultures were grown in modified Pikovskaya broth with RP, individually supplemented with three different carbon sources (sucrose, glucose and maltose) at 150 rpm for 21 days (Pikovskaya, 1948). Available P was calculated after 7, 14 and 21 days. Cell-free supernatants were used for the quantification of P-solubilization. After recording pH of cell-free supernatants, they were filtered with 0.2 µm sterile filters (Orange Scientific GyroDisc CA-PC, Belgium) to remove any residues. Solubilized phosphates (primary and secondary orthophosphate) were measured by spectrophotometer (Camspec M350-Double Beam UV–vis Spectrophotometer, UK) at 882 nm and values were calculated by using standard curve ($KH_2PO_4$ using 2, 4, 6, 8, 10, 12 ppm solutions). Based on high P-solubilization, six bacterial isolates were selected for detailed analysis (Fig. S1).

### 2.3. Quantification of indole-3-acetic acid (IAA) produced by bacterial isolates

Quantitative estimation of indole acetic acid produced by bacterial isolates was carried out by using colorimetric assay as described by Gordon and Weber (1950). For quantification of Indole-3-acetic acid (IAA), all bacterial isolates were grown in 10 ml LB broth supplemented with L-tryptophan (100 mg/l) for two weeks in shaking incubator (150 rpm) at 28 °C. The cell-free supernatant of the culture medium was obtained by centrifuging the cultures at 10,000 rpm for 15 min. Two ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ and 50 ml of 35% v/v $HClO_4$) were added to 1 ml of supernatant. After incubation of 30 min at room temperature, absorbance was noted at 535 nm using spectrophotometer. Pure Indole-3-acetic acid (Sigma) was used as standard. For standard curve, dilutions of IAA were prepared in triplicates, Salkowski's reagent was added and absorbance was checked at 535 nm. Standard curve was used to calculate the amounts of IAA produced (Fig. S2).

### 2.4. Molecular characterization and phylogenetic analysis

Genomic DNA was isolated by CTAB method (Winnepenninckx et al., 1993). PCR amplifications of 16S rRNA were performed by using universal forward and reverse primers P1 (5′-GggatccAGAGTTTGATC-CTGGTCAGAACGAAC-3′) P6 (5′CGggatccTACGGCTACCTT

GTTACGACTTCACC-3′) for prokaryotes (Tan et al., 1997). A PCR reaction of 50 µl was prepared by using Taq polymerase (5U) 0.5 µl, Taq buffer (10X) 2 µl, $MgCl_2$ (25 mM) 2.5 µl, dNTPs (2.5 mM) 2 µl, 2 µl each of forward and reverse primer (10 pmol), 36 µl of dd·$H_2O$ and 3 µl of template DNA. First denaturation step was performed at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and a final extension step was at 72 °C for 10 min as described by Tan et al. (1997). PCR products were analyzed by using 1% agarose gel and purified by using GeneJET PCR Purification Kit (K0702 − Thermo Fisher Scientific). Purified PCR products were sequenced by using forward and reverse primers (Eurofins, Germany).

Acquired sequences were assembled and analyzed with the help of Chromus Lite 2.01 sequence analysis software (Technelysium Pty Ltd. Australia). The gene sequences were compared to those deposited in the GenBank nucleotide database using the BLAST program. Sequences were aligned using Clustal X 2.1 program and phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei, 1987). Bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011). There were a total of 1457 positions in the final dataset. The sequences were submitted to NCBI GenBank data base under the accession numbers LT703511-LT703516.

### 2.5. Preparation of biogas sludge (BGS) and soil based phosphorus biofertilizers for pot and field experiments

Three experiment sets were designed to study the effects of phosphate solubilizing bacteria on the growth of wheat plants in climate control room and field conditions.

Seeds of local variety (FSD, 2008) were surface sterilized using 0.1% NaClO (Chlorex) for 8–10 min and washed three times with sterile d.$H_2O$. Seeds were coated with $10^7$ CFU/mL of individual bacterial strains and incubated with 3% PVP (Polyvinyl pyrrolidone) for 3 h. Mixture of bacterial strains, $10^7$ CFU/mL, was also applied on the seeds to observe the effect of bacterial consortium. Mixture was prepared by adjusting the OD of each bacterial isolate at $10^7$ CFU/mL and taking

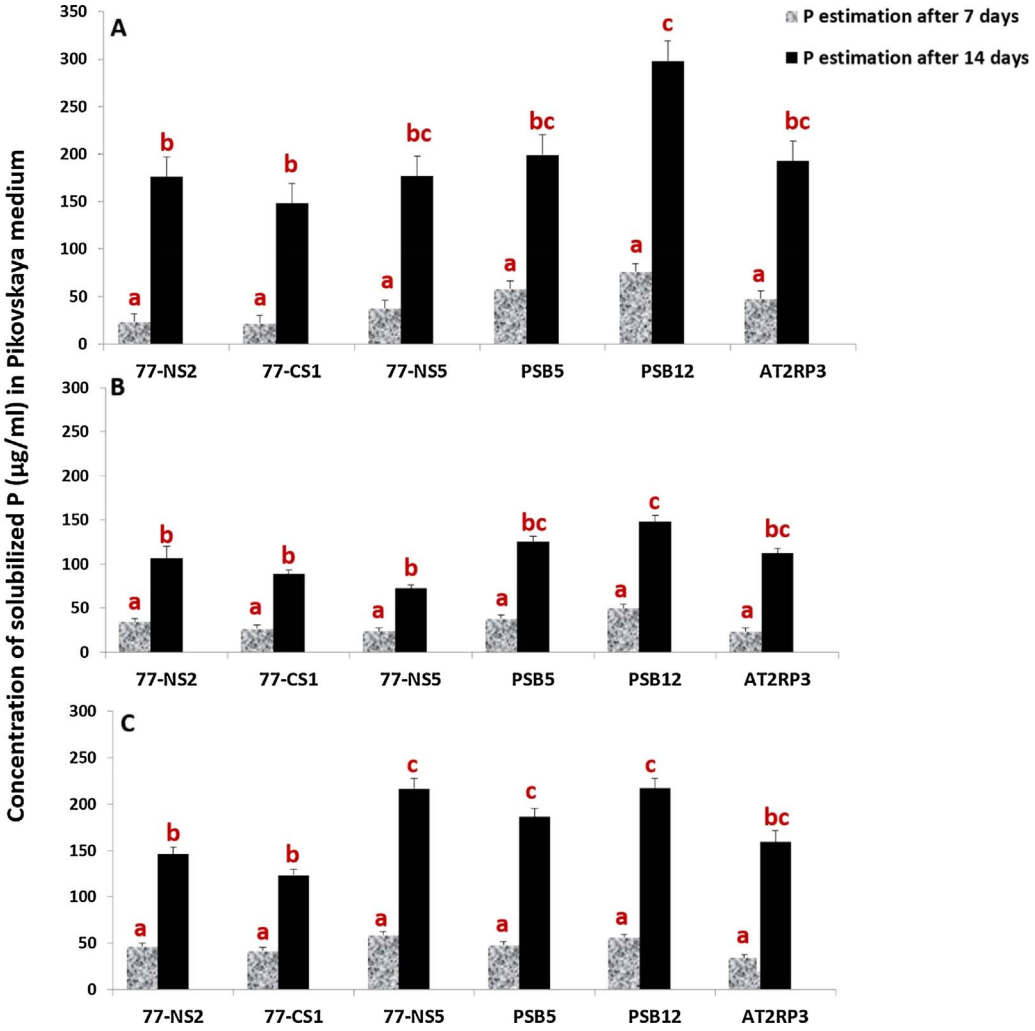


**Fig. 1.** Phosphate solubilization (µg/ml) by bacterial isolates in Pikovskaya broth medium supplemented with carbon sources **(A)** sucrose **(B)** glucose **(C)** maltose after incubation of 7 days and 14 days. The values are an average of three determinations. Alphabets on graph bars represent statistically significant values at 5% level.

1 ml from each of them. Un-inoculated seeds served as control for this first set of experiment (Experiment Set A).

Phosphorous based biofertilizers were applied in two sets of experiments. For one experiment set (Experiment Set B), biofertilizer was prepared by enriching biogas sludge. Biogas sludge sample was collected in marked cans from Ittehad Chemicals Limited that is located near Kala Shah Kaku, Sheikhupura, Punjab, Pakistan. These samples were oven dried at 70 °C for 48 h. After drying and grinding, sludge sample was used as a carrier material to prepare Phosphorous based biofertilizer. It contained 500 g of un-sterilized biogas sludge (BGS) + 1% RP (rock phosphate) with 50 ml ($10^7$ CFU/ml) of individual phosphate solubilizing bacterial isolates (PSBs). Separately, to monitor the effect of bacterial consortium, one BGS bag was inoculated with mixture of six strains, 50 ml ($10^7$ CFU/ml). Un-inoculated sack (500 g BGS + 1% RP) was used as control.

For Experiment Set C, biofertilizer was prepared by doing soil enrichment with PSBs (phosphate solubilizing bacteria). About 500 g of un-sterilized soil (S) was supplemented with 1% glucose + 1% RP. Six bags were inoculated with 50 ml of individual PSBs ($10^7$ CFU/ml) and one soil bag was enriched with 50 ml mixture of PSBs ($10^7$ CFU/ml). Soil bags without bacterial inoculation were used as control. Biogas sludge and soil based phosphorous biofertilizers were also analyzed for change in electrical conductivity (dS/m), pH and availability of soluble phosphorous after 7 and 14 days of incubation.

### 2.6. Pot experiments in climate control room with microbial enriched BGS, soil and coated seeds

Wheat plants of variety Faisalabad-2008 were grown in plastic pots containing 500 g of sandy loam unsterilized soil (EC 2.5 ds/m, pH 8.2, organic matter 0.4%, available phosphorous 2.5 mg/kg and total nitrogen 0.06%). First set of pot experiment was done with coated seeds (Experiment Set A) and every pot was supplemented with 1% RP before sowing. Coated seeds, one seed/pot, were transferred in pots. Six replicates for each treatment; wheat seeds coated with individual bacterial strains and mixture of bacterial strains, were used in experiment. Un-inoculated/sterilized seeds were sowed to the pots as control.

In second and third set of experiments, BGS (Experiment Set B) and enriched soil (Experiment Set C) based phosphorus biofertilizers were applied as inoculum. Pots with 500 g soil were prepared and inoculated with 2 g of enriched BGS (Experiment Set B) and soil (Experiment Set C), respectively. Six replicates for each treatment were also maintained for these two sets (six pots with BGS and six with soil enriched individual strain). Mixtures of strains incubated in BGS and soil were also applied in same number of replicates for combined effect study. Parallel controls with un-inoculated BGS and soil were also run with same number of replicates. All pots were watered with half strength Hoagland's solution (Hoagland and Arnon, 1950). Plants were kept in climate control room at relative humidity of 60% with 12 h photoperiod (200 µM $m^{-2}$ $s^{-1}$at pot heights with fluorescent lights, 15°/20° C). The experiment was set up in completely randomized design (CRD). Plants were watered daily and harvested at vegetative stage (after 5 weeks). Data for root and shoot weight (fresh and dry) and length was recorded.

### 2.7. Field experiment with BGS and soil based P biofertilizer

Field experiment was also performed with the same two sets of

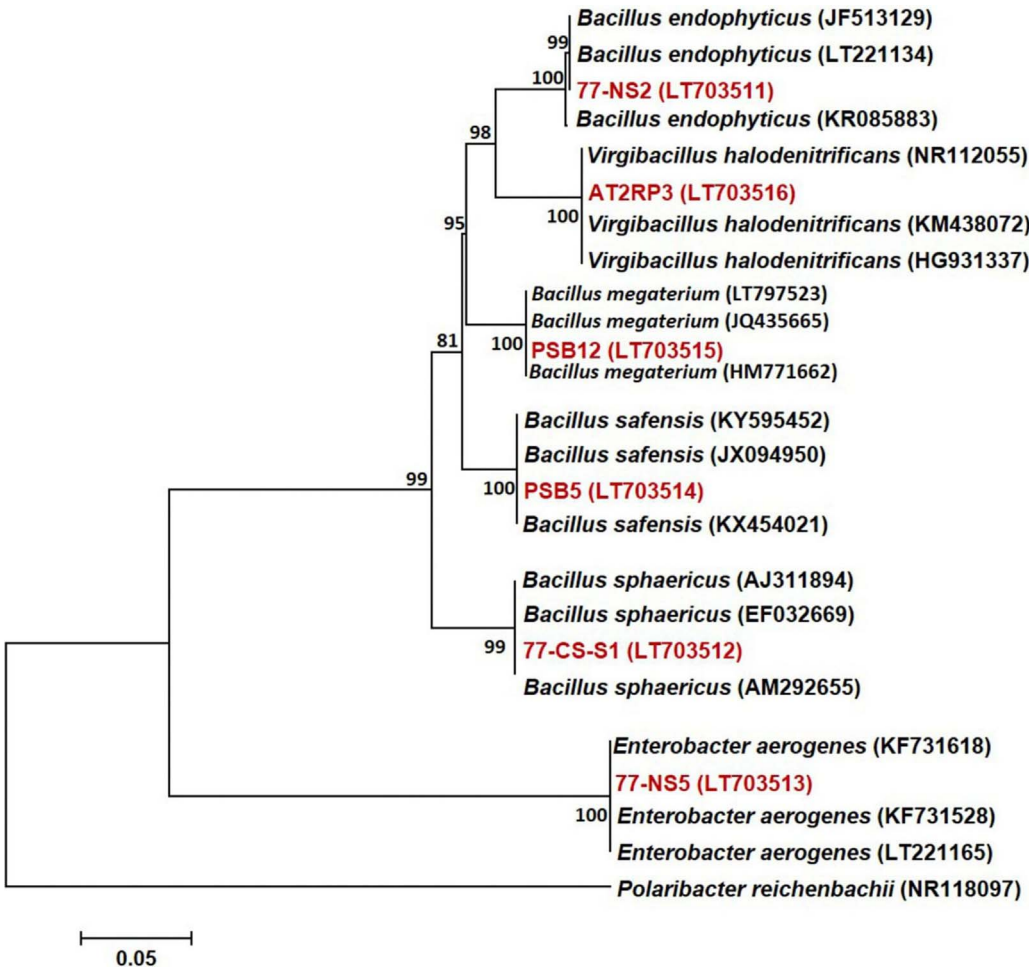


**Fig. 2.** Neighbor-joining tree of the 16S rRNA gene sequences of phosphate solubilizing bacterial isolates. There were 1436 nucleotides in final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of number of base substitutions per site.

biofertilizers (B, C) and inoculum coated seeds (Experiment Set A) as done in climate control room. Field area of 80 × 60 sq. feet was selected for experiment. Field area was subdivided in 24 plots with 9 × 11 feet dimensions. Each plot was further divided into three sections, each section contained three rows. Each plot was designed to cater three different treatments at a time and had thirty plants of same treatment in one sub-section of the plot, in three rows (Fig. S3).

Field experiment was conducted with twenty-four treatments. Experiment Set A (coated seeds), Experiment Sets B and C (BGS and soil based P biofertilizers) had total eight treatments each; six bacterial strains individually applied, mixture of strains and un-inoculated control. Ninety plants (replicates) for each treatment in RCBD were grown in field area. Plots were irrigated four times during the plant growth. Data of shoots and roots weights (fresh and dry) and length was recorded after one month while data of number of spikes and grain yield was recorded at plant maturity (after 4 months).

### 2.8. Physico-chemical analysis of field soil before sowing and after harvesting of wheat

Three soil samples from the selected plots before sowing of wheat and nine soil samples after harvesting of wheat were selected for physico-chemical analysis. Each soil sample (300 g) was thoroughly mixed and sieved through a pore size of 2 mm. Physical properties (moisture content, pH, salinity and temperature) of soil samples from different pots were determined. Electrical conductivity (dS/m) was measured by 1:1 (w/v) soil to water mixtures at 25 °C (Adviento-Borbe et al., 2006), pH was measured by 1:2.5 (w/v) soil to water suspension, moisture (%); temperature ( °C) and texture class were determined as described by Anderson and Ingram (1993). Organic matter (Corg) was calculated by the Walkley-Black method (1934), phosphorous was estimated by extraction with sodium bicarbonate (Olsen et al., 1954) and calcium and magnesium were detected by atomic absorption spectrometry. Nitrate ions were measured by Raney-Kjeldahl (1883) method and potential acidity (H + Al) was determined by an equation based on the pH in SMP (Shoemaker, McLean and Pratt) buffer solution (pH SMP). Cation exchange capacity (CEC) and sodium adsorption ratio (SAR) were calculated as the ratio of the sodium to the magnesium and calcium (Hendershot and Duquette, 1986).

### 2.9. Root colonization assay

Root colonization by inoculated bacteria was determined after 4 weeks at tillering stage in pot and field experiments using serial dilution plating method on Pikovskaya agar (Pikovskaya, 1948) and total P-solubilizing bacterial population was determined. Bacterial population was biochemically and morphologically characterized and compared with previous biochemical results to confirm the survival of inoculated strains. CFU of inoculated P-solubilizing strains were also calculated.

### 2.10. Statistical analysis

Experiment was laid out in Randomized Complete Block Design (RCBD) with six replicates of all individual and mixture strains for each type of biofertilizer applied. Results were subjected to analysis of variance (ANOVA) and significance at the 5% level was tested by Least Significance Difference Test (LSD) by using a computer software program IBM SPSS Statistics (version 23). Mean values and the standard error were calculated.

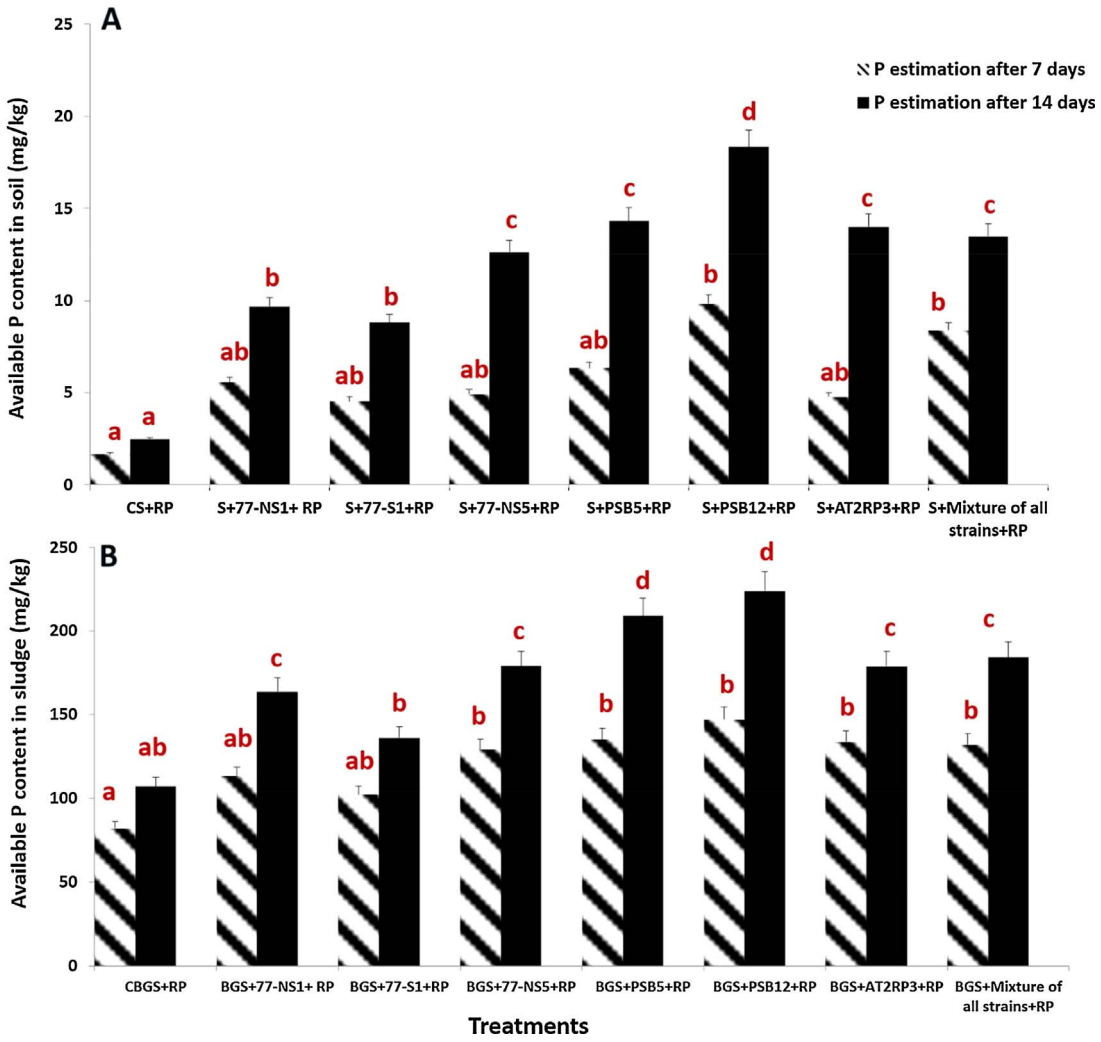


**Fig. 3.** Quantification of available phosphorous in sludge and soil based phosphorous biofertilizers. (A) Phosphate solubilization (mg/kg) by enriched soil after incubation of 7 and 14 days. (B) Phosphate solubilization (mg/kg) by enriched biogas sludge after incubation of 7 days and 14 days. The values are an average of three determinations. Alphabets on graph bars represent statistically different values at 5% level. CS = Control soil sample; CBGS = Control biogas sludge sample; S = Soil; BGS = Biogas sludge; RP = Rock phosphate.

## 3. Results

### 3.1. Phosphate solubilizing activity of bacterial isolates

Six bacterial strains that showed the ability to solubilize P on Pikovskaya agar were selected for quantification. When grown in Pikovskaya broth individually supplemented with sucrose, maltose and glucose, all six isolates (77-NS2, 77-CS-S1, PSB5, PSB12, AT2RP3 and 77-NS5) showed increase in P-solubilization and decrease in pH with the passage of time, up to 14 days. Non-significant increase in solubilized P was observed after 21 days of cultivation by all isolates.

With sucrose, all strains solubilized phosphate in the range of 23.2 µg/ml to 75.9 µg/ml after 7 days. Highest P solubilization was shown by PSB12, however, the difference was non-significant among all strains. After 14 days significant P solubilization was shown by PSB12 (305.6 µg/ml). No significant change in solubilized P was noted after 21 days for all strains (Fig. 1A).

Highest P solubilization using glucose as C-source in Pikovskaya broth was also shown by PSB12 that solubilized 49.92 µg/ml after 7 days and 198.66 µg/ml after 14 days. No significant increase in solubilized P after 21 days was noted for all strains. Minimum P solubilization was shown by 77-CS-S1 (89.15) µg/ml after 14 days (Fig. 1B).

PSB12 showed maximum P solubilization using maltose as well as compared to all other strains. After 7 days, all strains solubilized P in the range of 26.22 µg/ml to 55.92 µg/ml and non-significant difference was observed among all strains. But after 14 days, PSB12 showed highest amount of solubilized P (217.16 µg/ml). Minimum solubilized P was shown by 77-NS2 (99.96 µg/ml) using maltose as C-source (Fig. 1C).

### 3.2. Quantification of indole-3-acetic acid produced by bacterial strains

Bacterial isolates were also screened for their ability to produce plant growth hormone indole-3-acetic acid (IAA) in medium containing L-tryptophan as precursor for IAA synthesis. 77-NS5 produced higher amount of IAA (87.20 µg/ml) with respect to all other isolates. While 77-NS2 produced 6.11 µg/ml, 77-CS-S1; 13.11 µg/ml, PSB5; 12.54 µg/ml, PSB12; 5.42 µg/ml and AT2RP3 9.57 µg/ml of IAA.

### 3.3. Identification of bacterial isolates based on molecular method

All isolates were motile, rod shaped and five of them were Gram-positive while one was Gram-negative. Bacterial isolates, 77-NS2, 77-CS-S1, PSB5, PSB12 and AT2RP3 were identified as strains of *Bacillus* and 77-NS5 as *Enterobacter*. Phylogenetic analysis of isolate 77-NS2 showed 99% similarity (1452/1500) with *Bacillus endophyticus* (Accession No. JF513129), 77-CS-S1 showed 99% similarity (1398/1425) with *Bacillus sphaericus* (Accession No. HM771671), PSB5

**Table 1**
Electrical conductivity (dS/m) and pH of enriched soil and sludge based phosphorus biofertilizers after 14 days of incubation.

| Treatments | $EC_{1:1}$ (soil: water) (dS/m) | | $pH_{1:2.5}$ (soil: water) | |
|---|---|---|---|---|
| | Before Microbial Enrichment | After 14 days of Microbial Enrichment | Before Microbial Enrichment | After 14 days of Microbial Enrichment |
| CS + RP | 2.41[a] | 2.79[a] | 7.44[b] | 7.21[c] |
| S + 77-NS1 + RP | 2.36[a] | 2.81[a] | 7.61[b] | 6.72[b] |
| S + 77-S1 + RP | 2.37[a] | 2.85[a] | 7.54[b] | 6.77[b] |
| S + 77-NS5 + RP | 2.32[a] | 2.89[a] | 7.78[b] | 6.54[b] |
| S + PSB5 + RP | 2.35[a] | 3.35[ab] | 7.64[b] | 6.64[b] |
| S + PSB12 + RP | 2.41[a] | 3.55[b] | 7.79[b] | 6.34[a] |
| S + AT2RP3 + RP | 2.26[a] | 2.95[a] | 7.45[b] | 6.74[b] |
| S + Mixture of all strains + RP | 2.29[a] | 3.19 [a] | 8.01[bc] | 6.64[b] |
| CBGS + RP | 2.75[b] | 3.11[a] | 6.91[a] | 6.65[b] |
| BGS + 77-NS1 + RP | 2.71[b] | 3.23[ab] | 6.69[a] | 6.39[a] |
| BGS + 77-S1 + RP | 2.69[b] | 3.24[ab] | 6.62[a] | 6.43[a] |
| BGS + 77-NS5 + RP | 2.71[b] | 3.39[b] | 6.89[a] | 6.52[a] |
| BGS + PSB5 + RP | 2.71[b] | 3.44[b] | 6.88[a] | 6.22[a] |
| BGS + PSB12 + RP | 2.71[b] | 3.61[b] | 6.78[a] | 6.35[a] |
| BGS + AT2RP3 + RP | 2.75[b] | 3.24[ab] | 6.76[a] | 6.29[a] |
| BGS + Mixture of all strains + RP | 2.79[b] | 3.41[b] | 6.69[a] | 6.21[a] |

Note: Values are the mean of three replicates (mean ± SE). Comparisons between mean of treatments were made by the least significance difference (LSD) test ($P < 0.05$).
CS = Control soil sample; CBGS = Control biogas sludge sample; S = Soil; BGS = Biogas sludge; RP = Rock phosphate. Significance value P = 0.05.

showed 99% similarity (1478/1505) with *Bacillus safensis* (Accession No. KX454021) and PSB12 showed 99% similarity with *Bacillus megaterium* (Accession No. HM771662). AT2RP3 showed 98% similarity (1462/1489) with halophilic *Virgibacillus* sp. (Accession No. HG931337). Bacterial isolate 77-NS5 showed 99% similarity with *Enterobacter aerogenes* (Accession No. KF731618) (Fig. 2).

### 3.4. Quantification of available phosphorus in prepared BGS and soil based biofertilizers

Available P in sludge and enriched soil based phosphorus biofertilizers was quantified after 7 and 14 days of incubation (Fig. 3). All soil based biofertilizer samples showed significant P solubilization after incubation of 14 days. Soil biofertilizers inoculated with *Bacillus* strain PSB12 showed maximum amount of available P (8.45 mg/kg) after 7 and 14 days (18.3 mg/kg) as compared to other soil biofertilizer samples (Fig. 3A). In case of sludge based biofertilizers, there was also gradual increase in available P with the passage of time. Maximum P was solubilized by the sludge samples inoculated with PSB12 (179. 34 mg/kg and 207 mg/kg) after 14 days (Fig. 3B). Biogas sludge biofertilizer samples showed more amount of available P as compare to enriched soil based biofertilizer. Available P was also quantified after 21 days but no significant difference was found when values were compared with 14 days data of P quantification.

It was also observed that microbial enriched biogas and soil based phosphorus biofertilizers had a significant difference in the pH and electrical conductivity after 14 days of incubation. Biofertilizer samples showed a gradual decrease in pH value and increase in EC (Table 1). Gradual decrease in pH can be due to production of organic acids like acetic acid, oxalic acid, citric acid during the phosphate solubilization by *Bacillus* and *Enterobacter* strains. The change in electrical conductivity is attributed to the increase of ions due the solubilization of rock phosphate. Dissolution of this mineral result in higher solution concentration of $Ca^{++}$ and $H_2PO_4$ and other cations and anions present in the rock phosphate.P = 0.05

### 3.5. Effect of enriched BGS and soil based P biofertilizers on wheat growth

#### 3.5.1. Pot experiment

Phosphate solubilizing bacterial isolates exerted a significant influence on growth characteristics of wheat, shoot and root dry weight and shoot and root lengths.Plants with coated seeds (bacterial strains with 3% PVP) showed 28–51% increase in dry weight of root (Fig. 4A) and 31–105% increase in dry weight of shoots (Fig. 4B) as compared to un-inoculated control (3% PVP, no inoculum). Wheat plants inoculated with sludge based phosphorus biofertilizers showed 6–49% increase in root dry weight (Fig. 4A) and 18–86% increase in shoot dry weight as compared to control (BGS + RP, no inoculum). Similarly, plants treated with enriched soil based phosphorus biofertilizers showed significant increase in dry weights of root (28–64%) and shoot (19–154%) as compared to control (S + RP, no inoculum) (Fig. 4A,B).

The relative increase in length varied between 10 and 82% for shoots (Fig. 4C) and 37–111% for roots (Fig. 4D) and in plants with coated seeds (bacterial strains with 3% PVP) over the control (3% PVP, no inoculum). Wheat plants treated with sludge based phosphorus biofertilizers showed 38–107% and 28–72% increase in root and shoot lengths, respectively (Fig. 4C,D) while plants inoculated with enriched soil based P biofertilizers showed significant increase in root length 33–116% and shoot length 36–87% as compared to control (S + RP, no inoculum) (Fig. 4C,D). Data indicated that plants treated with enriched BGS P biofertilizers showed better results as compared to other two experiment sets.

#### 3.5.2. Field experiment

In field conditions, all bacterial strains positively affected the plant growth and grain yield as compared to non-inoculated control plants. Plants treated with mixture of bacterial strains and 3% PVP showed significant increase in dry weights of root (11–68%) and shoot (14–48%) as compared to control plants (3% PVP, no inoculum). Plants treated with enriched BGS showed 7–39% increase in root weight and 19–61% increase for shoot weights when compared with control plants for this set of experiment while enriched soil based P biofertilizer indicated 20–41% increase for root and 19–62% increase for dry weight of shoot (Fig. 5A, B). *Bacillus* strain PSB12 showed the most significant increase in shoot and root weights among all strains.

Plants with coated seeds (bacterial strains with 3% PVP) showed relative increase in root (15–46%) and shoot (10–35%) length over the non-inoculated controls (3% PVP, no inoculum). The overall increase in root and shoot length due to BGS P biofertilizers ranged 9–38% and 8–36% respectively when compared with control plants. While plants inoculated with enriched soil based P biofertilizers showed significant increase in root length 11–41% and shoot length 8–43% as compared to un-inoculated controls (Fig. 5C, D).

Increase in grain yield in plants inoculated with coated seeds (bacterial strains with 3% PVP), biogas sludge and soil based phosphorus biofertilizers was 40–86%, 30–100% and 42–78% respectively (Fig. 5E, F). PSB12 showed most significant increase in seed weight and number of seeds/plant when used as inoculum with BGS carrier. The efficacy of different isolates for growth characteristics was variable. *Bacillus* strains PBS5 and PSB12 and mixture of all strains showed better results as compared to un-inoculated and other treated plants. Data indicated that BGS based P biofertilizer showed the maximum increase in plant growth and grain yield in field conditions.

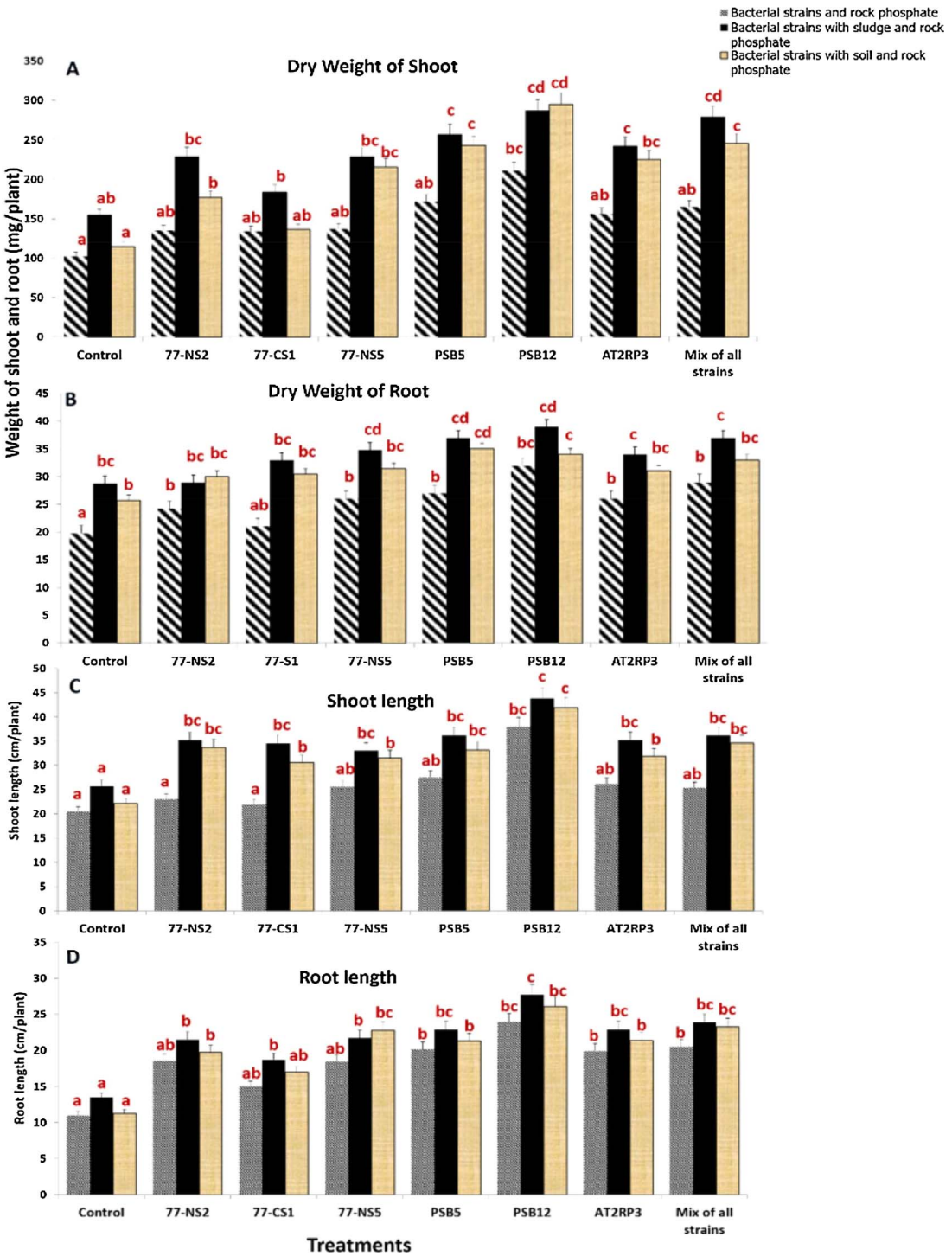


**Fig. 4.** Effect of coated bacterial inoculum, enriched BGS and soil on dry weights and length of roots and shoots of wheat plants. Alphabets on graph bars represent statistically different values at 5% level. Fig. 4A (Dry weights of roots), Fig. 4B (Dry weights of shoots), Fig. 4C (Shoot lengths), Fig. 4D (Root lengths).

### 3.5.3. Root colonization potential

Total P-solubilizing bacterial population (log CFU/g soil) in the rhizosphere of wheat was determined at tillering stage in pot experiment as well as under field conditions. Significant variation in bacterial population was observed in pot and field experiment at tillering stage. Population of P-solubilizing bacteria in the rhizosphere of wheat plants with coated seeds (bacterial strains with 3% PVP) varied from $10^6$-$10^8$ (CFU/g soil) in pot experiment and $10^6$-$10^7$ in field experiment (Fig. 6A). Plants inoculated with sludge based phosphorus biofertilizers showed bacterial population $10^7$-$10^8$ in pot experiment and $10^6$-$10^7$ (CFU/g soil) in field experiment (Fig. 6B). Plants treated with enriched soil based phosphorus biofertilizers showed bacterial population $10^7$-$10^8$ in pot experiment and $10^6$-$10^7$ (CFU/g soil) in field experiment (Fig. 6C). Overall bacterial population was higher in pot experiment than in field experiment at tillering stage.

### 3.5.4. Physico-chemical properties of field soil before and after sowing

The detail data on the physico-chemical properties was shown in Table 2. There was very little difference in the soil physical properties (pH, salinity, moisture, temperature and texture) before sowing and

after harvesting of wheat. The amount of organic matter, available P and K increased in the soil samples collected after harvesting. No noticeable change in V (base saturation index), CEC (Cation exchange capacity) and SAR (Sodium adsorption ratio) in the soil samples collected before sowing and after harvesting was observed.

## 4. Discussion

The plant growth promoting potential of rhizobacteria isolated from rhizosphere of sugarcane, wheat and *Atriplex amnicola* was analyzed by using biogas sludge and enriched soil based phosphorus biofertilizers. Effect of inoculation of bacterial strains, biogas sludge and enriched soil based P biofertilizers was investigated by pot experiments in climate control room as well as field experiment. Twenty seven bacterial isolates from the rhizosphere of sugarcane, wheat and *Atriplex amnicola* were screened for phosphate solubilization on Pikovskaya agar plates (Pikovskaya, 1948). Out of twenty seven, six bacterial strains were selected on the basis of P solubilizing activity for further assessment of sludge and enriched soil based phosphate biofertilizers. These strains were identified on the basis of morphological, biochemical and molecular characterization. On the basis of 16S rRNA gene sequence analysis, bacterial isolates 77-NS2, 77-CS-S1, PSB5 and PSB12 were identified as *Bacillus* spp., isolate AT2RP3 was identified as *Virgibacillus* sp. and the isolate 77-NS5 was identified as *Enterobacter aerogenes*. Isolation of different PGPR genera including *Enterobacter, Azospirillum, Bacillus* and *Pseudomonas* from the rhizosphere of wheat, rice and sugarcane has also been reported previously (Mehnaz et al., 2001, 2010; Tahir et al., 2013; Kruasuwan and Thamchaipenet, 2016).

When grown in Pikovskaya broth containing rock phosphate as insoluble P-source, individually supplemented with three carbon sources; sucrose, glucose and maltose, all isolates of genus *Bacillus* (77-NS2, 77-CS-S1, PSB5, PSB12 and AT2RP3) showed maximum P-solubilization after 14 days. *Bacillus* strains PSB5 and PSB12 isolated from the rhizosphere of wheat showed more P-solubilization and decrease in pH of the medium as compared to other *Bacillus* strains (77-NS2, 77-CS-S1 and AT2RP3) and *Enterobacter* strain 77-NS5 isolated from the rhizosphere of sugarcane and *A. amnicola*. P-solubilizing activity of these bacteria have been previously detected by Pikovskaya broth containing calcium triphosphate (Nautiyala et al., 1999; Yasmin and Bano, 2011; Karpagam and Nagalakshmi, 2014) but in this study, rock phosphate is used as main P-source in Pikovskaya medium. All bacterial strains showed ability to utilize three different carbon sources used in this study. It has been reported that various sugars such as glucose, maltose, sucrose and galactose are present in exudates of the wheat rhizosphere (McRae and Monreal, 2011). These bacterial isolates were also investigated for the production of plant growth promoting hormone indole-3-acetic acid (IAA). All bacterial isolates produced IAA in culture medium containing L- tryptophan as a precursor of IAA. *Enterobacter* strain 77-NS5 produced highest amount of IAA as compared to *Bacillus* strains (77-NS2, 77-CS-S1, PSB5, PSB12 and AT2RP3). Production of IAA in pure cultures of *Enterobacter, Bacillus*, *Pseudomonas* and *Azospirillum* strains has been reported previously (Vessey et al., 2003; Mehnaz et al., 2010; Kumar et al., 2014).

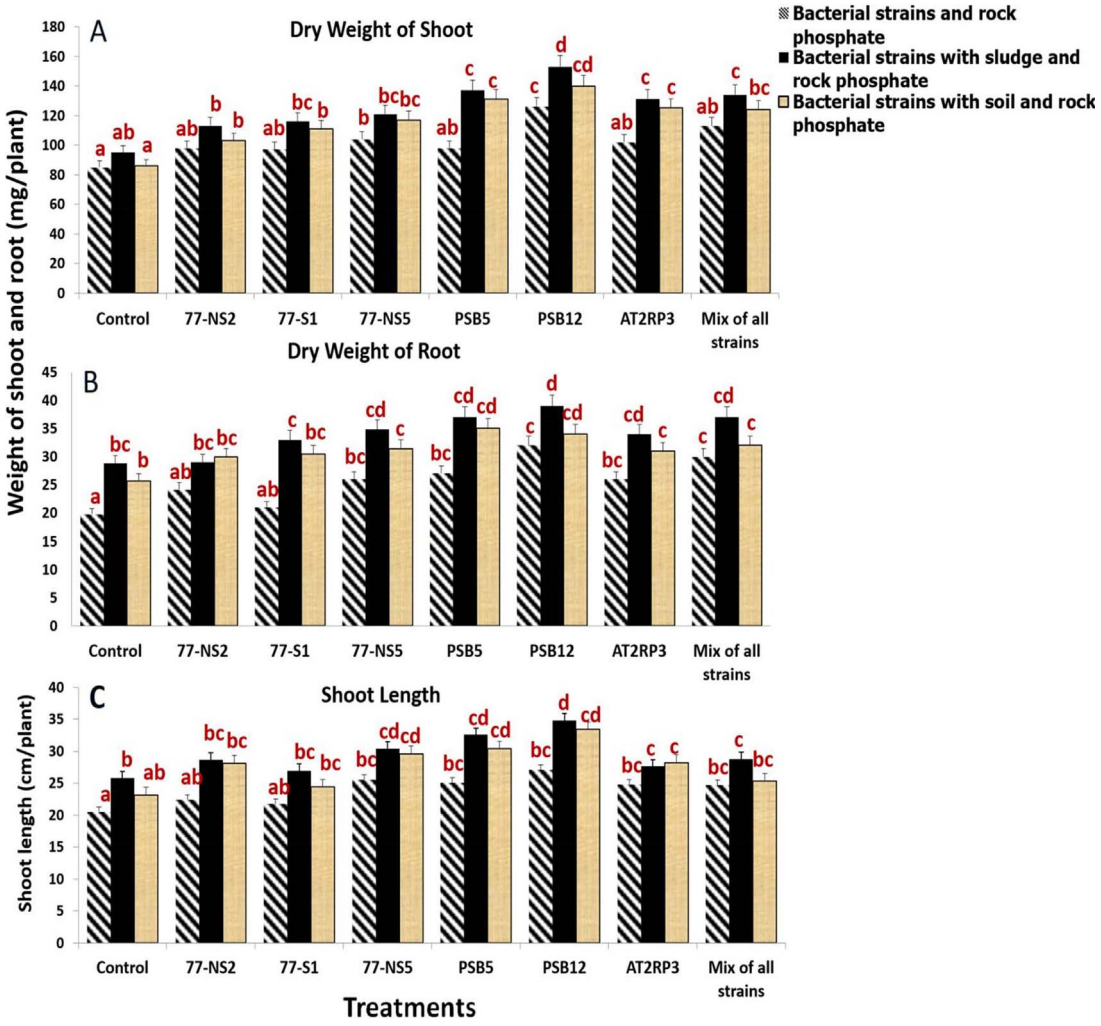


**Fig. 5.** Effect of bacterial inoculation on dry weight, length of roots and shoots and grain yield of wheat grown in fields. Alphabets on graph bars represent statistically different values at 5% level. Fig. 5A (Dry weights of shoot), Fig. 5B (dry weights of root), Fig. 5C (Shoot lengths), Fig. 5D (Root lengths), Fig. 5E (Seed weights (g/plant), Fig. 5F (Number of seeds/plant).

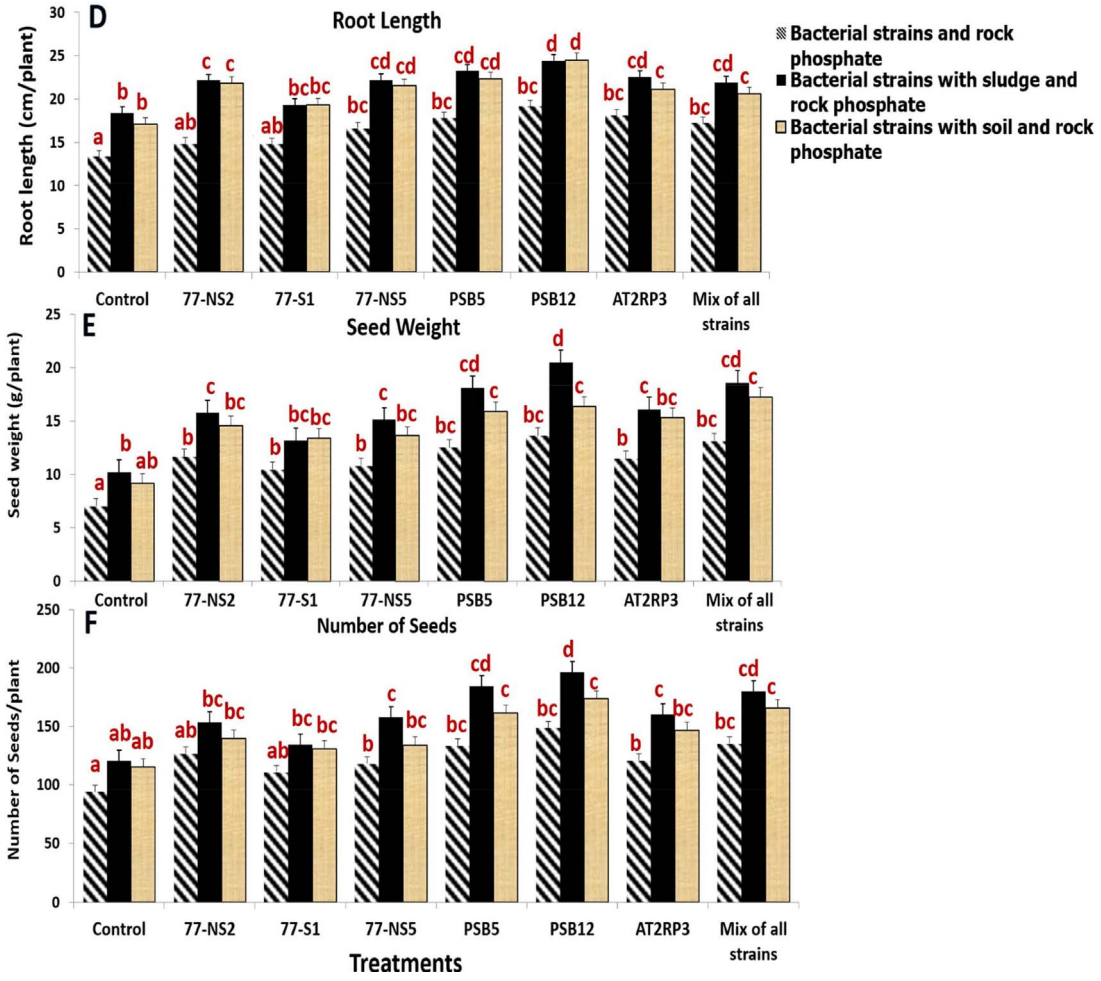


**Fig. 5.** (*continued*)

In this study, effect of phosphate biofertilizers on wheat growth have been assessed by using two carrier materials; biogas sludge and enriched soil. Initially there was slight difference among the various treatments with respect to the amount of available P in biogas sludge and enriched soil based phosphate biofertilizer but with the passage of time, pH of medium was decreased and available P-content was increased in all treatments (Fig. 3). Integrated use of organic wastes, biogas sludge, rock phosphate and microbial inoculants have been reported by a number of workers to increase the phosphate efficiency and stability of crop yields (Mahmoud and Mohamed, 2008; Shrivastava et al., 2011). This study showed the comparative effect of biogas sludge and enriched soil based phosphorus biofertilizers on P-solubilization and plant growth promotion. Biogas sludge and enriched soil based phosphorus biofertilizers inoculated with *Bacillus* strains PSB5 and PSB12 showed maximum P-solubilization as compared to other bacterial isolates.

A number of *Bacillus* species have been involved in P-solubilization by converting insoluble phosphate into available forms (Kumar et al., 2011). The presence of phosphate solubilizing bacteria in rhizosphere of plants may be considered a positive indicator of utilizing the microbes as biofertilizers for crop production and beneficial for sustainable agriculture. The observed increase in available P-content could be due to production of organic acids like acetic acid, oxalic acid and citric acid by the P-solubilizing bacteria which could affect the release of P from the rock phosphate (Fernandez et al., 2009; McRae and Monreal, 2011).

To study the plant growth promoting effect of phosphate solubilizing bacteria, six bacterial isolates (five isolates of genus *Bacillus* and one *Enterobacter aerogenes* isolate) were used as inoculants in the form of biogas sludge and enriched soil based phosphorous biofertilizers. All bacterial strains positively affected the plant growth as compared to non-inoculated control plants both in pot experiments as well as under field conditions. Maximum increase in weight and length of root and shoot was observed in plants inoculated with sludge and enriched soil based biofertilizers as compared to plants inoculated with coated seeds (bacterial strains with 3% PVP) and non-inoculated control plants. Under natural field conditions, plants inoculated with *Bacillus* strains PSB5 and PSB12 based sludge biofertilizers showed maximum increase in grain yield 18.13% and 20.13% respectively (Fig. 5E). The significant increase in weight and length of roots and shoots upon inoculation of *Bacillus* and *Enterobacter* strains indicated that PGPR strains have ability to provide better nutrient flux to the plant host which resulted in the increase of plant biomass. Several studies have previously reported that application of phosphate solubilizing bacterial strains like *Bacillus*, *Pseudomonas* and *Enterobacter* as bio-inoculants promote plant growth and enhance grain yield in various crops including wheat, rice and sugarcane (Mehnaz et al., 2001; Saikia et al., 2012; Tahir et al., 2013; Karpagam and Nagalakshmi, 2014). However no such study has used biogas sludge and enriched soil as carier materials. Comparative effect of individual bacterial strains, biogas sludge and enriched soil based P biofertilizers on plant growth has been discussed for the first time in this study. Previously, a number of studies have been reported that sludge based phosphate biofertilizers have improved the soil health, increase nutrient availability and more microbial activity that enhanced plant growth and yield (Medina et al., 2006; Criquet et al., 2007; Narayanan, 2012). It has also been documented that combined use of

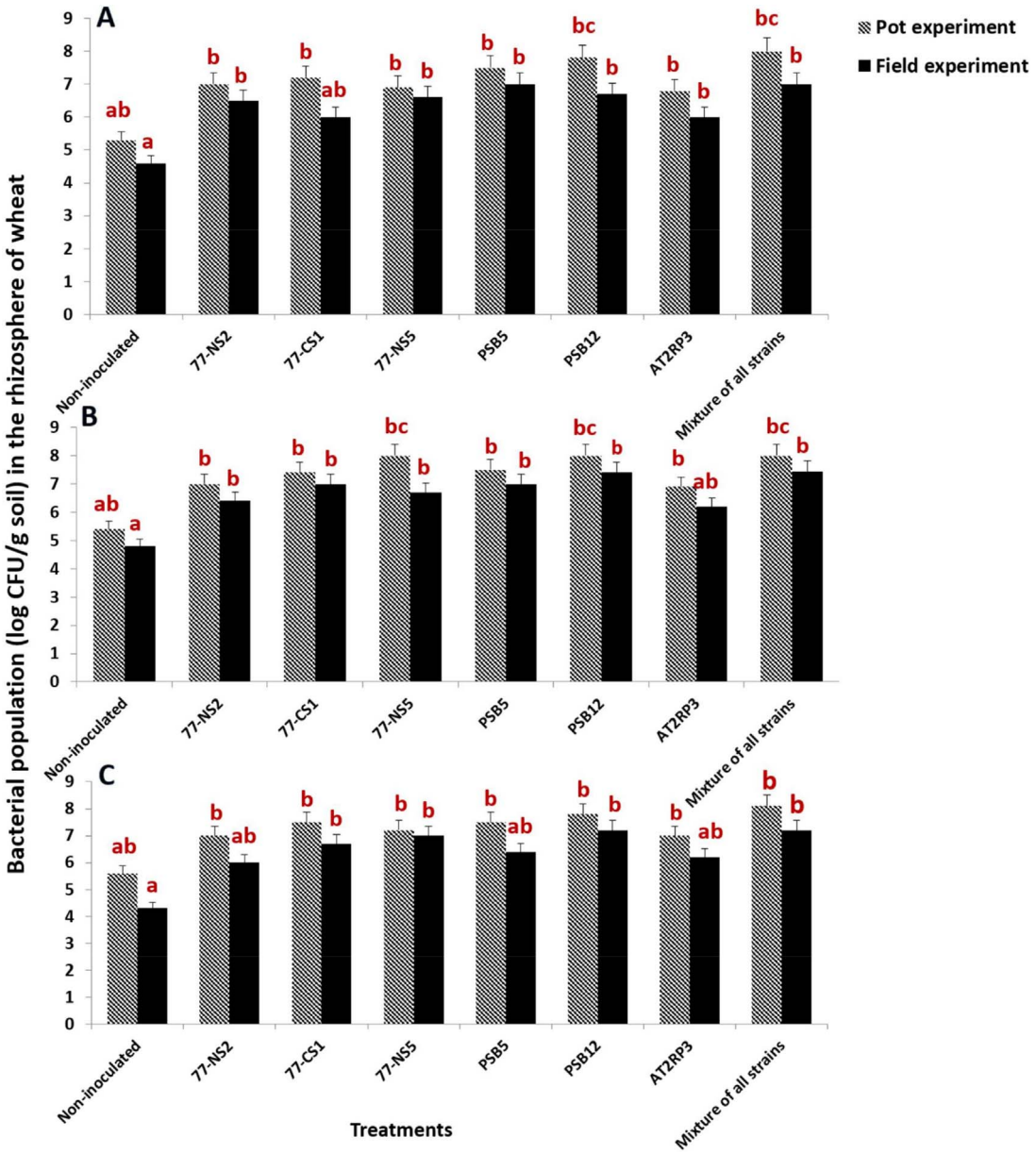


**Fig. 6.** Population density of P solubilizing bacteria (log cfu/g soil) in the rhizosphere of wheat grown in pots and under field conditions at tillering stage. Fig. 6A (Inoculum coated seeds + 3% PVP), Fig. 6B (BGS based P biofertilizer), Fig. 6C (Soil based P biofertilizer).

inorganic phosphate, bacterial strains and some carrier material like sludge and manure is beneficial in improving soil pH, surface area of roots through enhanced root proliferation (El Husseini et al., 2012). The data on the bacterial population (CFU/g of soil) showed that there was significant difference in pot and field experiment at tillering stage of wheat. It has been reported that microbial populations are usually maximum during early growth stages of plants as compared to maturity stage (Roesti et al., 2006; Moutia et al., 2010).

## 5. Conclusion

This study has investigated the comparative effect of biogas sludge and enriched soil based P biofertilizers on wheat growth. Strains used in this work utilized different sugars (sucrose, maltose and glucose), produce organic acids and showed plant growth promoting traits like P-solubilization and IAA production. Highest phosphorus solubilization activity was observed by *Bacillus* strain PSB12 grown on sucrose supplemented Pikovskaya medium. These isolates increased the wheat growth by 20.13% and 15.51% as compared to non-inoculated controls using biogas sludge and enriched soil based P biofertilizers, respectively. Therefore, these strains can be the potential candidates for field applications as microbial enriched biogas sludge and soil based biofertilizers.

## Acknowledgments

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**Table 2**
Physico-chemical properties of field soil before and after sowing.

| Parameters | Physico-chemical properties of soil after sowing | Physico-chemical properties of soil after harvesting | | |
|---|---|---|---|---|
| | | Samples from Treatment 1* | Samples from treatment 2* | Samples from treatment 3* |
| $EC_{1:1}$ (dS/m) | 1.15[a] | 1.18[a] | 1.13[a] | 1.14[a] |
| pH | 7.65[a] | 7.45[a] | 7.56[a] | 7.68[a] |
| Temperature (°C) | 22.5[a] | 34.51[b] | 33.24[b] | 32.84[b] |
| Moisture (%) | 25 [a] | 20[b] | 21[b] | 20[b] |
| Texture class | Silty loam | Silty loam | Silty loam | Silty loam |
| OM (g $kg^{-1}$) | 29.7[a] | 34.34[b] | 36.11[b] | 34.55[b] |
| P (mg $kg^{-1}$) | 3.31[a] | 3.95[b] | 4.16 [b] | 4.21[b] |
| K (mg $kg^{-1}$) | 0.48[a] | 0.51[a] | 0.52 [a] | 0.49[a] |
| Ca (mg $kg^{-1}$) | 1.37[a] | 1.45[a] | 1.42[a] | 1.39[a] |
| Mg (mg $kg^{-1}$) | 1.15[a] | 1.16 [a] | 1.31[ab] | 1.18[a] |
| $NO^{-3}$ (mg $kg^{-1}$) | 10.94[a] | 12.54[ab] | 10.28[a] | 12.13[ab] |
| H + Al (mg $kg^{-1}$) | 47.3[a] | 49.2[a] | 50.1[a] | 47.1[a] |
| V (mg $kg^{-1}$) | 4.02[a] | 4.18[a] | 4.51[ab] | 4.15[a] |
| CEC (mg $dm^{-3}$) | 52.93[a] | 54.25[a] | 55.46[a] | 53.33[a] |
| SAR | 6.46[a] | 6.61[a] | 6.55[a] | 6.39[a] |

Note: EC (Electrical conductivity); OM (Organic matter); P (Phosphorous); K (Potassium); Ca (Calcium); Mg (Magnesium); $NO^{-3}$ (Nitrate ion); H + Al (potential acidity); V (base saturation index); CEC (Cation exchange capacity) and SAR (Sodium adsorption ratio). *Values are mean of three samples (mean ± SE). Significance value P = 0.05.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2017.08.011.

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