Int.J.Curr.Microbiol.App.Sci (2017) *6*(4): 1240-1260

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 4 (2017) pp. 1240-1260**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.604.152

Effect of P- Solubilizing Bacteria on Microbial Biomass P and Phosphatase Activity in Groundnut (*Arachis hypogaea* L) Rhizosphere

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Keywords

MBP, Phosphatase, PSB, Groundnut.

Article Info

Accepted:
12 March 2017
Available Online:
10 April 2017

A B S T R A C T

Phosphorus plays a major role in growth and development of crops and is often found limiting in almost all types of soils. Phosphorus (P) solubilizing bacteria do the vital processes of mobilization of P from its poorly available sources. In this study, the potency of five bacterial strains (2 strains of *Bacillus* and 3 strains of *Burkholderia*) have been evaluated to solubilize P from complexes of Ca-P, Al-P, Fe(II)-P and Fe(III)-P. *Bacillus amyloliquefaciens* CTC12 (KT633845) solubilized P more efficiently from complexes of Ca-P, Fe(II)-P and Fe(III)-P where as *Burkholderia cepacia* KHD08 (KT717633) solubilized highest P from Al-P in vitro. All the five strains significantly enhanced soil phosphatase activities, microbial biomass P and plant P concentrations when combinedly applied with SSP. *Bacillus amyloliquefaciens* CTC12 and *Burkholderia cepacia* KHD08 in combination with SSP solubilized 63 and 61 per cent more P compared to pots treated with SSP only. When these five isolates were inoculated to groundnut enhanced the soil available P and phosphatase activity.

Introduction

Phosphorus is the second most essential macro nutrient after nitrogen required for crop growth (Lal, 2002). Various metabolic processes *viz.*, energy transfer, signal transduction, macro-molecular biosynthesis, photosynthesis, respiration, etc. require P as the key ingredient (Shenoy and Kalagudi, 2005). Mostly soils contain only approximately 1 µmol l^{-1} soluble P, where as the P requirement by the crops is approximately 30 µmol l^{-1} soluble P for optimum productivity (Simpson *et al*., 2011). Plant roots usually absorb P as dihydrogen orthophosphate ($H_2PO_4^-$) and monohydrogen orthophosphate (HPO_4^{-2}) ion (Panda, 2009).

Declined soil reaction (pH) increases the concentration of Fe and Al in soil solution thereby making complexes with aluminum and iron-free oxide sand hydroxides (Fearnside, 1998; Richardson, 2001). Soil acidity thus triggers and increases rate of P fixation as well as immobilization (Fankem *et al*., 2006). Unlike N, P availability is highly dependent on the type of soil reaction (pH) and no big atmospheric source is there to supplement the P requirement of crops. Again, compared to N and K, total phosphorus level of soils is low and usually one tenth to one fourth of N and one twelfth of K (Jones and Eva, 2011). Thus, P is also

known as the limiting nutrient in almost all types of agricultural soils (Guiñazu *et al*., 2010).

P occurs in both organic and inorganic forms in soil. Foth (1990) reported that, approximately 70-80% of the total P in cultivated soils is inorganic. In the soil environment, P in the phosphate form (PO_4^{2-}), invariably forms compounds with calcium, aluminium and iron, making it unavailable for crop uptake. P fixation leading to deficiency is characteristics of weathered soils of tropics and subtropics (acid soil) (Hinsinger, 2001). On the other hand P in the organic forms occurs as phospholipids, nucleotides and inositol phosphate (Turner *et al*., 2002). All these forms of phosphates are unavailable for plant uptake.

Soil microorganisms play the major part in making P available. Soil microorganisms mostly secrete low molecular weight organic acids which perform as chelating agent to solubilize the inorganic fixed P (He *et al*., 2002). The organic P fraction can be mineralized by different soil enzyme processes (Sarapatka, 2003). Soil phosphatases (acid phosphatase and alkaline phosphatase) found in microorganisms, plant roots and also in extracellular forms in soil (Tabatabai, 1994). P is effectively mobilised by both plant and microbial phosphatases, but microbial phosphatases show greater effectiveness in releasing P (Tarafdar *et al*., 2001).

Hence, manipulation as well as efficient utilization of particular beneficial microorganisms for sustainable approach in agriculture and soil health is the need of the time. This is pertinent to the high-input production systems of the developed world, and also to developing countries where access to mineral fertilizers is restricted. The study revealed the potency of five PSB strains to solubilize P *in vitro* and in pot culture assay with test crop groundnut. The study also elaborated the dynamics of soil microbial biomass, microbial activity and phosphatase activity in relation to soil reaction, organic carbon, available P and plant P concentrations.

Materials and Methods

Collection of soil samples

One hundred six (106) nos. of soil samples were collected from acid soil areas of five districts *viz.*, Balasore, Cuttack, Khordha, Keonjhar and Mayurbhanj of Odisha. The samples were analyzed for population of heterotrophic bacteria and phosphorous solubilizing bacteria (PSB). From each district one efficient P solubilizer was selected for further characterization.

Identification of native phosphorus solubilizing bacteria

Five PSB isolates were identified by 16S rRNA gene sequencing. 16S region was PCR amplified with 16sF (5'AGAAAGGAGGT GATCCAGCC3') and 16sR (5'AGAGTTT GATCMTGGCTCAG3') primers after isolating DNA using Pure Link Genomic DNA kit (Invitrogen). Amplicon was electrophoresed in a 1% Agarose gel and visualized under UV-VIS gel doc system. The PCR product was then sequenced and aligned with other related sequences available in NCBI data base using multiple sequence alignment software ClustalW2 and MEGA4. Phylogenetic tree was constructed (Sambrook, 2001; Tamura *et al*., 2007).

Screening of native phosphorus solubilizing bacteria

NBRIP broth with inorganic phosphates of Calcium, Aluminium, Iron (II) and Iron (III)

were prepared and the five PSB strains were inoculated. The broth cultures were incubated at 30 ± 2^0 C till 192 h and then centrifuged at 10,000 rpm for 30 minutes. Phosphorus solubilizing efficiencies of the isolates were calculated by measuring the water-soluble phosphorus in the supernatant spectrophotometricaly at 660 nm by the chloromolybdic acid method as described by Jackson (1967).

Pot culture experiment

The five PSB strains were grown in nutrient broth at 28^0 C and 120 rpm for 72 h. The cultures were grown to achieve optical densities of 0.9 (10^8 to 10^9 CFU ml^{-1}) at 620 nm wavelength. Groundnut (*Arachis hypogaea* L cv. Tag 24) seeds were surface sterilized with 1 % NaOCl for 6 minutes and then repeatedly (6 times) rinsed with sterile distilled water for 15 – 20 minutes. Sterilized seeds were then placed in glass Petri dish and coated with bacterial suspension (OD_{620nm} 0.9). Three inoculated seeds were sown per 10 kg of unsterilized soil in earthen pots (Fernández *et al*., 2007).

The experiment comprised of twelve (12) treatments (T_1-Control, T_2-100% P as SSP, T_3-BLS18, T_4-CTC12, T_5-KHD08, T_6-KJR03, T_7-K1, T_8-BLS18 + 100% P as SSP, T_9-CTC12 + 100% P as SSP, T_{10}-KHD08 + 100% P as SSP, T_{11}-KJR03 + 100% P as SSP and T_{12}-K1 + 100% P as SSP) which were replicated thrice in a statistically randomized block design was conducted. The fertilizer sources N (20 kg ha^{-1}) as urea and K_2O (40 kg ha^{-1}) as Muriate of Potash (MOP) were given to all the treatments while P_2O_5 @ 40 kg ha^{-1} was applied as single super phosphate (SSP) following the treatment schedule. Due care and maintenance were followed till 110 days for growth of plants in the treated pots till maturity and then harvested.

Collection of soil sample from pots

Soil samples were collected from each pot at 40, 75 days after sowing (DAS) and at harvest for soil microbiological analysis.

Soil microbial analysis

Enumeration of total heterotrophic bacteria and P- solubilizing bacteria

The soil microbial population was determined by dilution plate technique. 1 g of the soil samples were added to each of ten tubes containing 9 ml of sterile distilled water, serially diluted and spread over Nutrient Agar and NBRIP (National Botanical Research Institute's Phosphate growth) media for enumeration of total bacteria and phosphorus solubilizing bacteria (PSB) respectively (Nautiyal, 1999). The plates were incubated at 30°C for 24 hours for bacterial isolation and at 30°C for 48 hrs for PSB. The total no. of bacterial colonies was expressed in terms of log CFU per 1 g dry wt. soil.

Microbial Biomass Carbon and Phosphorus

Microbial biomass carbon (MBC) was measured by fumigation-extraction [soil: extractant (0.5 M K2SO4) ratio 1:4] method using a conversion factor (Kc) of 0.38 (Vance *et al*., 1987; Hu and Cao, 2007). Microbial biomass phosphorus (MBP) was also measured by fumigation extraction [soil extractant (0.5 M $NaHCO_3$) ratio 1:20] method using a Kp factor of 0.4 (Brookes *et al*., 1982).

Phosphatase Activity

Soil phosphatase activity was calculated by colorimetric estimation of the p-nitrophenol released by phosphatase activity when the soil was incubated with buffered disodium p-

nitrophenyl phosphate tetrahydrate of pH 6.5 and 11 respectively for acid and alkaline phosphatase at 37^0 C for 1 h (Tabatabai and Bremner, 1969).

Soil chemical analysis

Soil samples were analyzed for soil chemical parameters *viz.*, soil pH (Jackson, 1967), organic carbon (Page *et al*., 1982) and available phosphorus by Bray's 1 method (Bray and Kurtz, 1945) as out lined by Page *et al.*, (1982).

P concentration of shoot and kernel

Shoot and kernel samples of groundnut were digested in diacid mixture [HNO_3:$HClO_4$ (3:2)]. Phosphorus concentrations were estimated spectrophotometricaly at a wavelength of 470 nm (Page *et al*., 1982).

Statistical analysis

Data were statistically analyzed by the software R (version 3.2.2) and tested with Duncan's new multiple range test at 5% critical range using the package "agricolae".

Results and Discussion

Two universal primers were used for determination and identification of the 16S rRNA gene of the isolates. The primer amplified the gene for the five isolates successfully. The size of the 16S rRNA gene product of four (BLS18, CTC12, KHD08 and KJR03) of the PSB isolates was about 3 kbp while that of for K1 isolate was 1.5 kbp (Fig. 3). The 16S rRNA gene sequences were compared with the Genbank database and accession number was received. Phylogenetic trees for the five isolates (BLS18, CTC12, KHD08, KJR03 and K1) revealed them as *Bacillus cereus* (KT582541), *Bacillus amyloliquefaciens* (KT633845), *Burkholderia cepacia* (KT717633), *Burkholderia cepacia* (KT717634), *Burkholderia cepacia* (KM030037) respectively (Fig IV-V). The result showed that two (BLS18 and CTC12) of the PSB strains belong to class Firmicutes and the rest three (KHD08, KJR03 and K1) belong to Beta subdivision of Proteobacteria (Table I).

Five PSB strains BLS18, CTC12, KHD08, KJR03 and K1 were analysed for their P solubilization efficiency (PE) in liquid NBRIP medium supplemented with $Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$ respectively. Study revealed that isolate CTC12 showed significantly higher PE in the mediums with $Ca_3(PO_4)_2$, $FePO_4$ and $Fe_3(PO_4)_2$ whereas KHD08 recorded significantly higher PE with $AlPO_4$ at 48 h of incubation (Fig. 1). Continuing the incubation till 192 h, all five isolates showed an increasing trend of P solubilization efficiency (Fig. 2). CTC12 showed maximum PE in $Ca_3(PO_4)_2$, $FePO_4$ and $Fe_3(PO_4)_2$ compared to other four isolates, however, was found at par with KHD08 at 192 h. But in case of $AlPO_4$, isolate KHD08 recorded maximum PE (4.87%). Results further revealed that, after 8^{th} day of incubation, isolate K1 recorded least P solubilization efficiency in Ca-P (36.78%), KJR03 in Fe(III)-P (8.55%) and BLS18 in Al-P (2.50%) and Fe (II)-P (5.20%). Among the inorganic P sources, $AlPO_4$ was least preferred substrate for all the isolates.

A pot culture experiment was conducted with Groundnut (*Arachis hypogaea* L cv. Tag 24). The soil was sandy loam in texture with sand - 73.5%, silt- 16.75% and clay- 9.00% with acidic (pH – 5.40) reaction. Initial soil organic carbon and available P were 0.46 % and 5.24 mg ha^{-1} respectively. Population of total heterotrophic and P solubilizing bacteria were 6.58 and 6.54 log CFU g^{-1} dry wt. soil respectively. Initial values for soil enzymes *i.e.* acid and alkaline phosphatases were 0.056

and 0.006 µM PNP g^{-1} soil h^{-1} and microbial biomass carbon, biomass phosphorus and C/P ratio were 102.23 µg C g^{-1} soil, 8.25 µg P g^{-1} soil and 12.39 respectively.

Treatment of groundnut plants with five [*Bacillus cereus* BLS18 (KT582541), *Bacillus amyloliquefaciens* CTC12 (KT633845), *Burkholderia cepacia* KHD08 (KT717633), *Burkholderia cepacia* KJR03 (KT717634), *Burkholderia cepacia* K1 (KM030037)] of the PSB strains resulted in a significant increase of 80 % or more of the parameters measured as compared to uninoculated control plants. Soil samples collected at 40 days after sowing (DAS), 75 DAS and harvest were analyzed for population of heterotrophic bacteria and P-solubilizing bacteria (Table II). The treatment T_{12} (K1 + 100% P as SSP) recorded highest population of culturable heterotrophic bacteria (7.863 log CFU g^{-1} dry wt. soil) at 40 DAS followed by T_{11} (KJR03 + 100% P as SSP). At 75 DAS, the pots imposed with CTC12 + 100% P as SSP (T_9) recorded highest bacterial population (7.934 log CFU g^{-1} dry wt. soil) followed by T_{10} (KHD08 + 100% P as SSP). In the post harvest soil, T_{11} (KJR03 + 100% P as SSP) maintained highest culturable bacterial population (7.914 log CFU g^{-1} dry wt. soil). Study revealed that heterotrophic bacterial population was significantly increased due to application of PSB strains in combination with P fertilizer (single super phosphate) over sole application of inorganics. Data further revealed that treatment T_{10} (KHD08 + 100% P as SSP) maintained highest colonies of PSB (7.982 and 7.991 log CFU g^{-1} dry wt. soil respectively) at 40 and 75 DAS whereas T_9 (CTC12 + 100% P as SSP) recorded highest PSB population (7.968 log CFU g^{-1} dry wt. soil respectively) at harvest. PSB population significantly increased due to application of PSB compared to control.

Rhizospheric soil samples were analyzed for soil microbial biomass carbon (MBC) and biomass phosphorus (MBC) at 40, 75 DAS and harvest (Table III). Soil MBC significantly increased due to sole inoculation of four PSB strains (CTC12, KHD08, KJR03 and K1) as compared to the uninoculated pots at 40 and 75 DAS and harvest, while all five strains (BLS18, CTC12, KHD08, KJR03 and K1) in combination with SSP resulted in a significant increase in MBC values over the uninoculated pots. Soil MBP values also increased significantly owing to inoculation of the five strains as sole or in combination over the uninoculated ones at 40, 75 DAS and harvest. In addition, C/P ratio was also calculated for soil samples collected at 40, 75 DAS and harvest (Table IV). The results showed a decrease in C/P ratio in all the inoculated pots compared to the uninoculated ones. The pots treated with two (CTC12 and KHD08) of the PSB strains combined with P fertilizer (SSP) resulted in the lowest C/P ratio at 40, 75 DAS and harvest.

Acid phosphatase activity significantly increased as a result of sole inoculation of four (CTC12, KHD08, KJR03 and K1) of the PSB strains over the uninoculated pots at 40 and 75 DAS (Table V) except the strain KHD08 which showed a significant increase even at harvest.

When combined with P fertilizer all the five strains significantly promoted the acid phosphatase activity over the uninoculated treatments at 40, 75 DAS and harvest. However, no significant increase was observed in the alkaline phosphatase enzymes in the pots treated with PSBs only compared to the uninoculated treatment (T_2 – 100% P as SSP). Four (CTC12, KHD08, KJR03 and K1) of the five strains showed a significantly higher alkaline phosphatase activity when applied in combination with P fertilizer (SSP) at 40, 75 DAS and harvest.

Soil reaction (pH), organic carbon and available P values were recorded at harvest of the plants (Table VI). Soil pH and organic carbon ranged from 5.36 to 5.48 and 0.40 to 0.48 per cent respectively. No great variations were observed with respect to soil pH and organic carbon among the inoculated and uninoculated pots. However, three (CTC12, KHD08 and K1) of the strains resulted in significantly higher soil available P either sole or in combination with P fertilizer compared to the uninoculated treatments (T_1 and T_2). The pots inoculated with strain CTC12 combined with P fertilizer recorded highest soil available P.

Kernel P and shoot P concentrations determined after harvest of the plants revealed that all the five strains when combined with P fertilizer significantly enhanced the kernel P concentrations over sole application of SSP (Table VII). The two PSB strains CTC12 and KHD08 when applied without P fertilizer recorded higher kernel P concentration which was found at par with the pots treated with only inorganic P fertilizer (T_2). Except the strain BLS18, rest of the four (CTC12, KHD08, KJR03 and K1) strains enhanced shoot P concentration and the values were at par with the treatment T_2 (P fertilizer). However all the five strains showed an enhanced shoot P concentration when applied combined with P fertilizer (single super phosphate). Shoot and kernel P contents were significantly correlated with soil acid phosphatase activity (Fig VI and Fig VII).

Soil microbiota is the vital constituent of P cycle. These microbes represent the inevitable part in mobilizing P among various pools of soil P. Generally P in soils exist in three (3) pools i.e. solution P, active P, fixed P. The solution P pool is very small and found mostly in the orthophosphate form with small amounts of organic P. Orthophosphate form of P is the only form, the plants can take up for nutrition (Richardson and Simpson, 2011). Growing crops quickly deplete the P in the soluble P pool. Therefore, the active P pool is the main source of available P for crops. The active P pool contains inorganic phosphate which is attached to small soil particles. The fixed P pool contains the insoluble inorganic and organic P compounds. This P pool has negligible impact on soil fertility and crop nutrition. Soil microorganisms scavenge the P from fixed pool and provide the P to crop for uptake. Consequently, long established interest is there for manipulation of rhizosphere microflora to improve soil fertility and crop uptake. In the present context five P- solubilizing bacteria were isolated from acid soil areas of Odisha and screened for its P solubilization potency in groundnut rhizosphere. Out of five PSB strains two of them *viz.,* BLS18, CTC12 were identified as *Bacillus cereus* and *Bacillus amyloliquefaciens* while three (KHD08, KJR03 and K1) were found to be strains of *Burkholderia cepacia*.

Crop rhizosphere harbours so many microbial groups that play the vital roles in performing various soil biochemical processes i.e. mineralization and immobilization of nutrients (N, P, K and C) (Lundberg *et al*., 2012). There occur specific microorganisms which are the essential part of soil P transformations. Inorganic phosphate in acidic soil occur mostly in the forms of variscite [$Al(OH)_2H_2PO_4$ or $AlPO_4.2H_2O$] and strengite [$Fe(OH)_2H_2PO_4$ or $FePO_4.2H_2O$] (Fearnside, 1998; Richardson, 2001; Bashan *et al*., 2013) and vivianite [$Fe_3(PO_4)_2.8H_2O$] (Mohsin *et al*., 1995). In alkaline soils tricalcium phosphates are abundant. These minerals are highly stable and poorly soluble, which makes them unavailable for plants, although soils contain high concentration of total P (Merbach *et al*., 2010). Consequently, we have taken four representative types of P minerals [$Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$] commonly found in acid or alkaline soil.

The study demonstrated the effectiveness of five bacterial strains (BLS18, CTC12, KHD08, KJR03 and K1) as P solubilizers in vitro. In this experiment, the ability of five strains was assessed in the liquid NBRIP medium each containing four types insoluble phosphates [$Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$] as the sole source of inorganic P. Among them, $Ca_3(PO_4)_2$ was the best phosphate source for all PSB strains followed by $FePO_4$. $Fe_3(PO_4)_2$ and $AlPO_4$. As revealed in the results all the strains especially *Bacillus amyloliquefaciens* CTC12 released highest amounts of P in the mediums where $Ca_3(PO_4)_2$, $FePO_4$ and $Fe_3(PO_4)_2$ have been given as the insoluble P sources. However, the strain *Burkholderia cepacia* KHD08 released highest concentration of P in the medium containing $AlPO_4$ as the sole P source. Balamurugan *et al.*, (2010) also reported PSB strains solubilizing $AlPO_4$. After 8th day, the P solubilization efficiency increased compared to the data at the 2nd day of incubation. At 48 h, the strain *Burkholderia cepacia* K1 solubilized least P in the medium with tricalcium phosphate and aluminium phosphate. However, after 8th day the strain *Bacillus cereus* BLS18 recorded with lowest P solubilization efficiency in medium containing $AlPO_4$. Moreover the strains KJR03 and K1 showed least solubilization in mediums with $FePO_4$ and $Fe_3(PO_4)_2$ respectively till the 8th day of incubation. It can be stated here that, tricalcium phosphate was the most preferred P source for all the strains whereas aluminium phosphate the least preferred one (Balamurugan *et al*., 2010; Dave and Patel, 2003). Pérez *et al.*, (2007) reported strains of *Burkholderia*, *Serratia*, *Ralstonia* and *Pantoea* to effectively solubilize $Ca_3(PO_4)_2$ in liquid cultures while found less effective in solubilizing $FePO_4$.

The bacteria and PSB population showed an increasing trend at 75 DAS but decreased towards harvest. This may be attributed to poor root activity in the harvest soil. Root exudates supply several carbon compounds that attract the heterotrophic soil microbiota (Naher *et al*., 2008). Markedly, the uninoculated pots showed lower P solubilization compared to inoculated pots. Further, the application of PSB along with the water soluble P fertilizer (single super phosphate) appeared to have increased the population (log CFU per g soil) of PSB as well as heterotrophic bacteria. Panhwar *et al.*, (2012) reported that, addition of inorganic P sources positively affected the bacterial population which in turn enhances its association with the plant roots.

The significant increase in the microbial biomass carbon and phosphorus with addition of inorganic P fertilizer suggest that the applied water soluble P enhanced immediate P availability for crop uptake, which in turn might have increased the activities of root and rhizosphere microorganisms (Attar *et al*., 2012). Increased microbial activities obviously make the bacteria to transform available P into microbial biomass P (MBP) (Wu *et al*., 2007). Microbial biomass P is one of the most labile forms of P in soil and plays a vital role in biogeochemical cycling of P in soil. By taking consideration of microbial biomass C and P, we have calculated the C/P ratio, which showed a decreasing trend in the inoculated pots. In addition, the pots treated combinedly with PSB and SSP (P fertilizer) C/P ratio declined. Zhang *et al.*, (2014) reported that addition of inorganic P could decrease the C/P ratio in a low P soil. It is evident from the study that, microorganisms compete with plant roots for the orthophosphate and assimilate the P making it temporarily unavailable for the crop. Thus microbial biomass P protects the orthophosphates from binding with cations of Ca, Al and Fe in the soil solution (Olander and Vitousek, 2004).

Table.1 Identification and relationship of five PSB isolates based on 16S rDNA

Isolate	Kingdom	Phylum	Class	Order	Family	Genus	Species	NCBI Accession number
BLS18	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*cereus*	KT582541
CTC12	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*amyloliquefaciens*	KT633845
KHD08	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	*cepacia*	KT717633
KJR03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	*cepacia*	KT717634
K1	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	*cepacia*	KM030037

Table.2 Effect of PSB on microbial population in groundnut rhizosphere

Treatments	Total heterotrophic Bacteria (log CFU g^{-1} dry wt. soil)			P- Solubilizing Bacteria (log CFU g^{-1} dry wt. soil)		
	40 DAS	75 DAS	Harvest	40 DAS	75 DAS	Harvest
Control	7.519 ± 0.035^{e}	7.580 ± 0.003^{d}	7.568 ± 0.018^{e}	7.580 ± 0.007^{c}	7.591 ± 0.003^{c}	7.505 ± 0.006^{d}
100% P as SSP	7.681 ± 0.033^{bc}	7.756 ± 0.268^{c}	7.748 ± 0.014^{d}	7.591 ± 0.014^{c}	7.663 ± 0.005^{c}	7.623 ± 0.003^{c}
BLS18	7.59 ± 0.060^{de}	7.771 ± 0.006^{bc}	7.740 ± 0.007^{d}	7.633 ± 0.009^{bc}	7.833 ± 0.010^{b}	7.799 ± 0.004^{b}
CTC12	7.633 ± 0.008^{cd}	7.792 ± 0.006^{abc}	7.771 ± 0.004^{cd}	7.716 ± 0.539^{abc}	7.813 ± 0.004^{b}	7.778 ± 0.006^{b}
KHD08	7.663 ± 0.060^{bcd}	7.771 ± 0.012^{bc}	7.724 ± 0.002^{d}	7.748 ± 0.006^{abc}	7.799 ± 0.004^{b}	7.792 ± 0.007^{b}
KJR03	7.681 ± 0.025^{bc}	7.763 ± 0.005^{bc}	7.748 ± 0.006^{d}	7.756 ± 0.005^{abc}	7.813 ± 0.003^{b}	7.785 ± 0.005^{b}
K1	7.716 ± 0.007^{b}	7.806 ± 0.003^{abc}	7.799 ± 0.004^{bcd}	7.799 ± 0.004^{abc}	7.806 ± 0.004^{b}	7.806 ± 0.005^{b}
BLS18 + 100% P as SSP	7.806 ± 0.006^{a}	7.857 ± 0.002^{abc}	7.839 ± 0.003^{abc}	7.940 ± 0.004^{a}	7.949 ± 0.004^{a}	7.934 ± 0.006^{a}
CTC12 + 100% P as SSP	7.813 ± 0.008^{a}	7.934 ± 0.007^{a}	7.857 ± 0.004^{ab}	7.954 ± 0.007^{a}	7.982 ± 0.004^{a}	7.968 ± 0.004^{a}
KHD08 + 100% P as SSP	7.833 ± 0.005^{a}	7.929 ± 0.004^{a}	7.851 ± 0.004^{ab}	7.982 ± 0.004^{a}	7.991 ± 0.002^{a}	7.964 ± 0.004^{a}
KJR03 + 100% P as SSP	7.857 ± 0.016^{a}	7.919 ± 0.004^{ab}	7.914 ± 0.007^{a}	7.875 ± 0.003^{ab}	7.934 ± 0.004^{a}	7.954 ± 0.003^{a}
K1 + 100% P as SSP	7.863 ± 0.009^{a}	7.881 ± 0.007^{abc}	7.869 ± 0.006^{ab}	7.919 ± 0.003^{a}	7.949 ± 0.003^{a}	7.929 ± 0.002^{a}
CV (%)	0.571	1.043	0.550	1.814	0.745	0.818

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different.
Data given in above are average values of three replicates ± standard error of mean (SEM)

Table.3 Effect of PSB on microbial biomass carbon and phosphorous in groundnut

Treatments	Microbial biomass carbon (µg C g^{-1} soil)			Microbial biomass phosphorous (µg P g^{-1} soil)		
	40 DAS	**75 DAS**	**Harvest**	**40 DAS**	**75 DAS**	**Harvest**
Control	105.356±2.603^{f}	112.560±2.709^{e}	105.652±2.784^{e}	8.522±0.166^{c}	8.962±0.021^{e}	8.562±0.006^{e}
100% P as SSP	118.650±4.305^{e}	121.360±2.952^{d}	120.362±3.466^{d}	9.360±0.131^{c}	10.368±0.109^{d}	10.253±0.158^{d}
BLS18	119.522±5.258^{e}	125.328±2.616^{cd}	125.325±2.710^{cd}	11.524±0.102^{b}	12.320±0.036^{c}	12.210±0.613^{c}
CTC12	125.366±4.207^{de}	132.562±5.335^{bc}	133.520±2.902^{bc}	12.360±0.220^{b}	12.352±0.014^{c}	12.208±0.161^{c}
KHD08	135.650±3.482^{c}	138.520±2.885^{b}	137.256±3.155^{bc}	11.256±0.526^{b}	12.382±0.335^{c}	12.310±0.196^{c}
KJR03	133.560±4.435^{cd}	138.500±5.388^{b}	138.250±3.124^{b}	12.365±0.268^{b}	13.524±0.414^{bc}	13.216±0.114^{bc}
K1	138.646±0.984^{bc}	139.860±4.521^{b}	135.622±2.490^{bc}	12.220±0.104^{b}	12.480±0.386^{c}	12.255±0.498^{c}
BLS18 + 100% P as SSP	145.682±1.841^{ab}	158.253±5.278^{a}	162.350±3.619^{a}	13.850±0.192^{a}	14.450±0.247^{ab}	14.252±0.267^{ab}
CTC12 + 100% P as SSP	152.653±2.804^{a}	159.365±3.685^{a}	166.422±2.724^{a}	14.256±0.229^{a}	15.655±0.116^{a}	15.286±0.240^{a}
KHD08 + 100% P as SSP	153.365±3.900^{a}	162.422±1.722^{a}	168.352±3.736^{a}	15.360±0.282^{a}	15.685±0.294^{a}	15.322±0.171^{a}
KJR03 + 100% P as SSP	155.360±1.822^{a}	163.520±2.775^{a}	166.522±1.784^{a}	14.235±0.436^{a}	15.852±0.894^{a}	15.226±0.042^{a}
K1 + 100% P as SSP	150.380±2.703^{a}	163.560±2.482^{a}	168.354±2.847^{a}	14.250±0.195^{a}	15.689±0.316^{a}	15.302±0.212^{a}
CV (%)	3.916	3.591	4.698	6.834	5.877	6.173

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different.
Data given in above are average values of three replicates ± standard error of mean (SEM).

Table.4 Effect of PSB on C/P ratio in groundnut rhizosphere

Treatments	C/P ratio		
	40 DAS	**75 DAS**	**Harvest**
Control	12.363	12.560	12.340
100% P as SSP	12.676	11.705	11.739
BLS18	10.372	10.173	10.264
CTC12	10.143	10.732	10.937
KHD08	12.051	11.187	11.150
KJR03	10.801	10.241	10.461
K1	11.346	11.207	11.067
BLS18 + 100% P as SSP	10.519	10.952	11.391
CTC12 + 100% P as SSP	10.708	10.180	10.887
KHD08 + 100% P as SSP	9.985	10.355	10.988
KJR03 + 100% P as SSP	10.914	10.315	10.937
K1 + 100% P as SSP	10.553	10.425	11.002

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different. Data given in above are average values of three replicates ± standard error of mean (SEM)

Table.5 Effect of PSB on soil enzymes in groundnut rhizosphere

Treatments	Acid phosphatase (µM PNP g^{-1} soil h^{-1})			Alkaline phosphatase (µM PNP g^{-1} soil h^{-1})		
	40 DAS	75 DAS	Harvest	40 DAS	75 DAS	Harvest
Control	0.086±0.002^{f}	0.097±0.002^{f}	0.102±0.005^{e}	0.006±0.002^{e}	0.013±0.002^{e}	0.016±0.002^{e}
100% P as SSP	0.106±0.009^{e}	0.115±0.003^{e}	0.121±0.003^{d}	0.016±0.004^{d}	0.026±0.004^{cd}	0.026±0.003^{d}
BLS18	0.116±0.003^{de}	0.120±0.003^{de}	0.122±0.004^{d}	0.017±0.002^{cd}	0.028±0.002^{bcd}	0.029±0.003^{cd}
CTC12	0.128±0.003^{bcd}	0.133±0.003^{bcd}	0.136±0.003^{bcd}	0.018±0.002^{bcd}	0.028±0.001^{bcd}	0.030±0.002^{cd}
KHD08	0.126±0.004^{cd}	0.135±0.003^{abc}	0.138±0.004^{bc}	0.021±0.002^{abcd}	0.026±0.002^{cd}	0.032±0.003^{bcd}
KJR03	0.128±0.005^{bcd}	0.130±0.003^{cd}	0.130±0.002^{cd}	0.018±0.002^{bcd}	0.025±0.003^{cd}	0.031±0.003^{bcd}
K1	0.126±0.001^{cd}	0.133±0.003^{bcd}	0.135±0.002^{cd}	0.017±0.002^{cd}	0.024±0.003^{d}	0.029±0.001^{cd}
BLS18 + 100% P as SSP	0.135±0.002^{abc}	0.138±0.003^{abc}	0.140±0.002^{bc}	0.022±0.002^{abcd}	0.026±0.002^{cd}	0.030±0.003^{cd}
CTC12 + 100% P as SSP	0.148±0.003^{a}	0.148±0.003^{a}	0.158±0.004^{a}	0.026±0.002^{a}	0.036±0.003^{ab}	0.038±0.005^{ab}
KHD08 + 100% P as SSP	0.144±0.002^{ab}	0.146±0.002^{ab}	0.152±0.003^{ab}	0.024±0.002^{ab}	0.038±0.002^{a}	0.042±0.003^{a}
KJR03 + 100% P as SSP	0.138±0.003^{abc}	0.139±.003^{abc}	0.141±0.001^{bc}	0.023±0.002^{abc}	0.033±0.003^{abc}	0.036±0.003^{abc}
K1 + 100% P as SSP	0.142±0.002^{abc}	0.145±0.003^{ab}	0.145±0.002^{abc}	0.026±0.002^{a}	0.035±0.001^{ab}	0.037±0.002^{abc}
CV (%)	6.883	5.718	6.399	16.994	16.339	13.519

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different. Data given in above are average values of three replicates ± standard error of mean (SEM)

Table.6 Effect of PSB on changes in soil pH and organic carbon in groundnut rhizosphere

Treatments	**pH**	**Organic carbon (%)**	**Available P (mg kg^{-1})**
Control	5.43±0.030^{abc}	0.40±0.017^{a}	4.16±0.100^{f}
100% P as SSP	5.37±0.056^{bc}	0.41±0.017^{a}	4.97±0.043^{ef}
BLS18	5.45±0.030^{abc}	0.40±0.053^{a}	5.89±0.030^{de}
CTC12	5.46±0.052^{ab}	0.45±0.026^{a}	6.88±0.046^{bcd}
KHD08	5.36±0.053^{c}	0.42±0.020^{a}	6.57±0.026^{cd}
KJR03	5.38±0.053^{bc}	0.41±0.046^{a}	6.13±0.020^{cde}
K1	5.41±0.036^{abc}	0.40±0.060^{a}	6.44±0.021^{cd}
BLS18 + 100% P as SSP	5.40±0.030^{abc}	0.45±0.046^{a}	6.86±0.030^{bcd}
CTC12 + 100% P as SSP	5.39±0.056^{abc}	0.46±0.052^{a}	8.11±0.026^{a}
KHD08 + 100% P as SSP	5.48±0.017^{a}	0.47±0.046^{a}	8.02±0.026^{ab}
KJR03 + 100% P as SSP	5.40±0.036^{abc}	0.46±0.010^{a}	6.77±0.036^{cd}
K1 + 100% P as SSP	5.38±0.020^{bc}	0.48±0.020^{a}	7.20±0.026^{abc}
CV (%)	0.942	8.447	10.266

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different. Data given in above are average values of three replicates ± standard error of mean (SEM).

Table.7 Effect of PSB on N and P concentrations in shoot and kernel of groundnut

Treatments	**P concentration in kernel (%)**	**P concentration in shoot (%)**
Control	0.206±0.003^{d}	0.126±0.003^{c}
100% P as SSP	0.389±0.003^{c}	0.240±0.003^{ab}
BLS18	0.386±0.003^{c}	0.200±0.003^{b}
CTC12	0.414±0.006^{bc}	0.242±0.004^{ab}
KHD08	0.416±0.004^{bc}	0.245±0.003^{ab}
KJR03	0.402±0.005^{c}	0.232±0.003^{ab}
K1	0.394±0.006^{c}	0.238±0.003^{ab}
BLS18 + 100% P as SSP	0.506±0.003^{ab}	0.253±0.004^{a}
CTC12 + 100% P as SSP	0.573±0.005^{a}	0.285±0.003^{a}
KHD08 + 100% P as SSP	0.557±0.002^{a}	0.280±0.003^{a}
KJR03 + 100% P as SSP	0.512±0.005^{ab}	0.268±0.002^{a}
K1 + 100% P as SSP	0.508±0.003^{ab}	0.256±0.004^{a}
CV (%)	12.239	11.654

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different. Data given in above are average values of three replicates ± standard error of mean (SEM).

Fig.1 Phosphorous solubilizing efficiency of the bacterial isolates with different inorganic P sources after 48 h of incubation

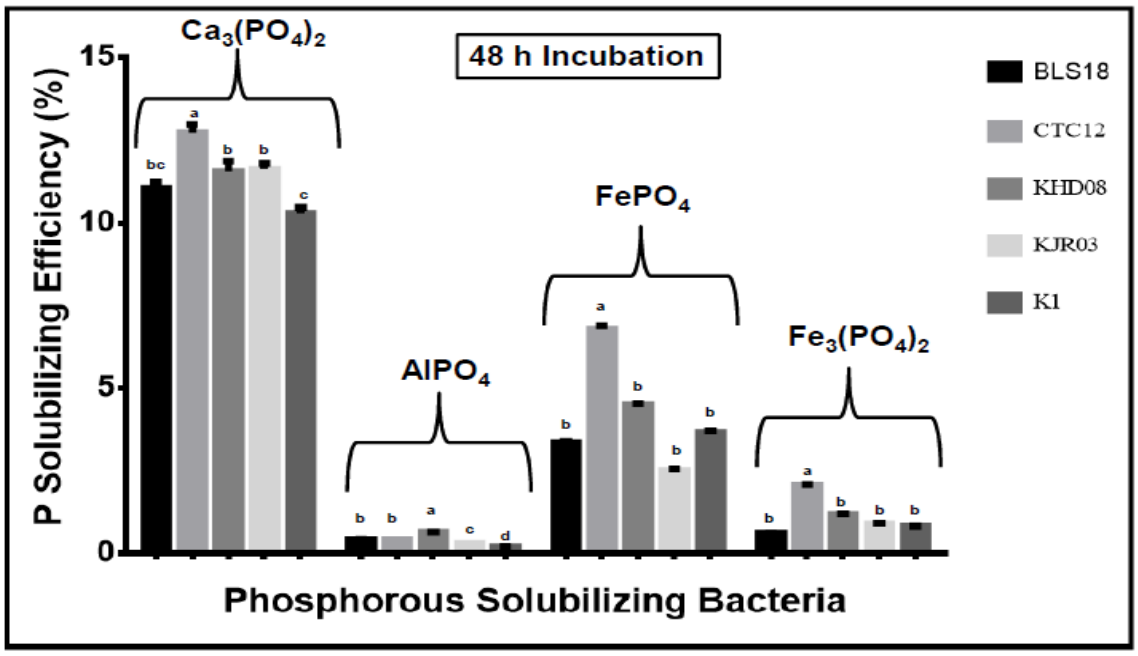


Fig.2 Phosphorous solubilizing efficiency of the bacterial isolates with different inorganic P sources after 192 h of incubation

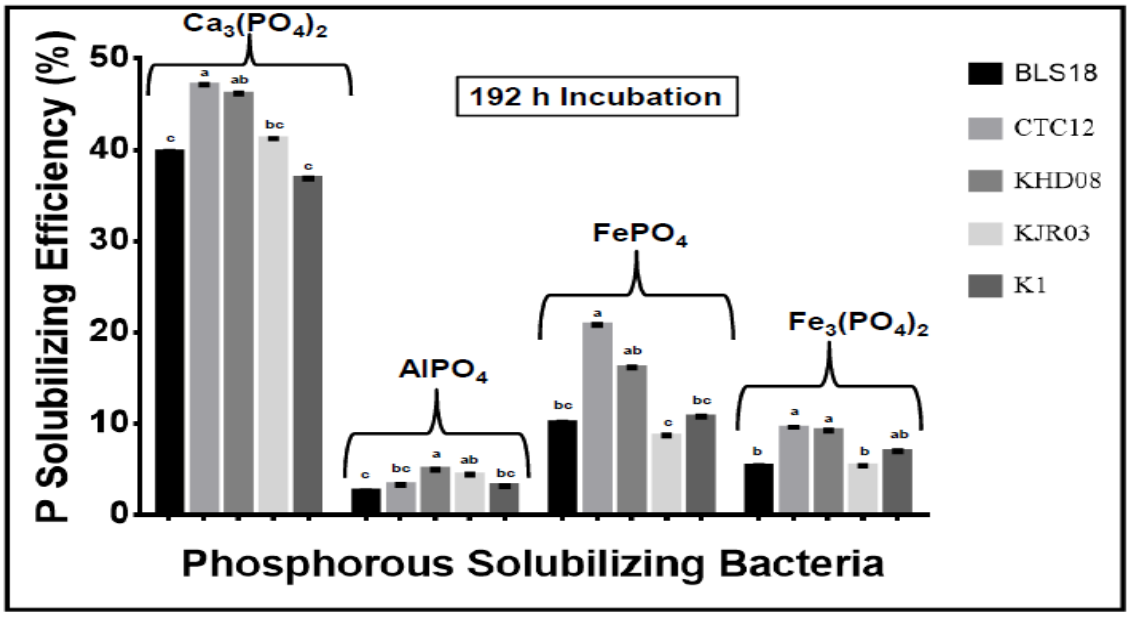


Fig.3 PCR products of 16S rRNA genes of the five PSB isolates (Lane 1 – BLS18, Lane 2 – KHD08, Lane 3 – CTC12, Lane 4 – KJR03 and Lane 5 – K1). The size of the 16S rDNA gene of four of the isolates (BLS18, KHD08, CTC12 and KJR03) was 3 kbp and that of K1 isolate was 1.5 kbp

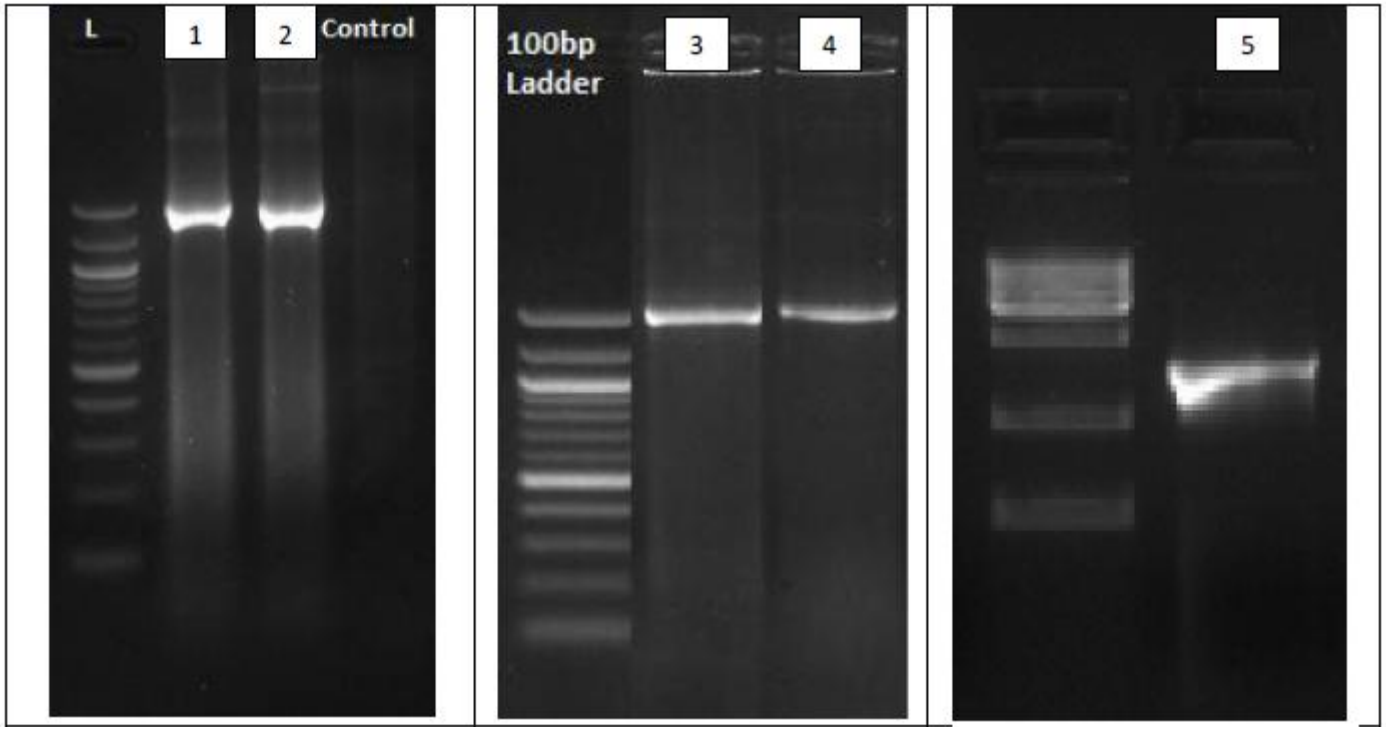
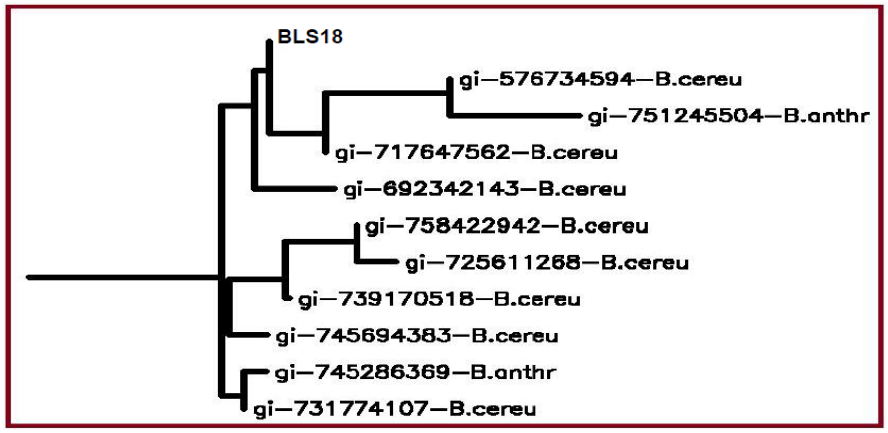
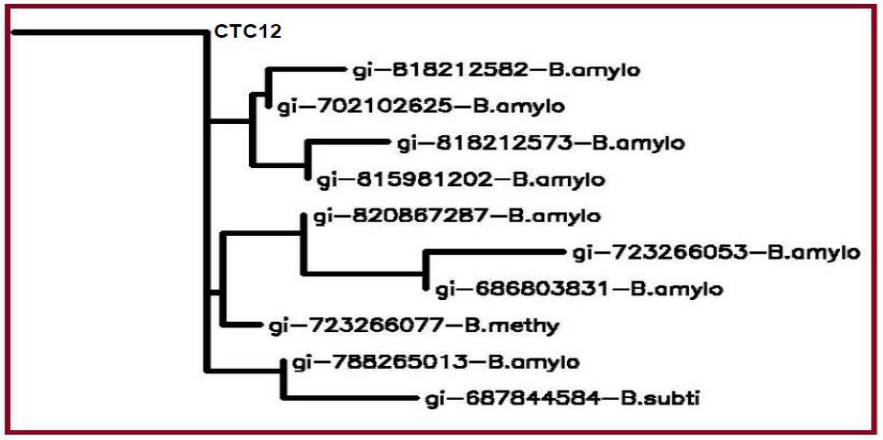
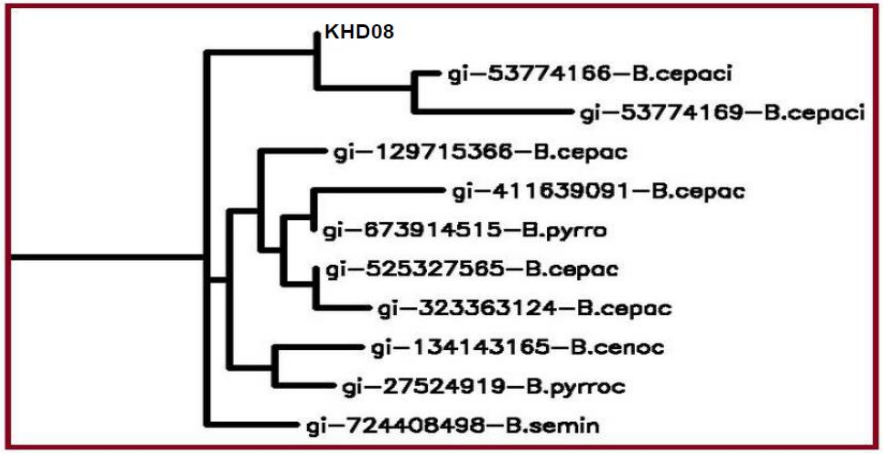


Fig.4 Phylogenetic tree of four strains *viz.*, BLS18, CTC12, KHD08 and KJR03 (clockwise), constructed using ClustalW2







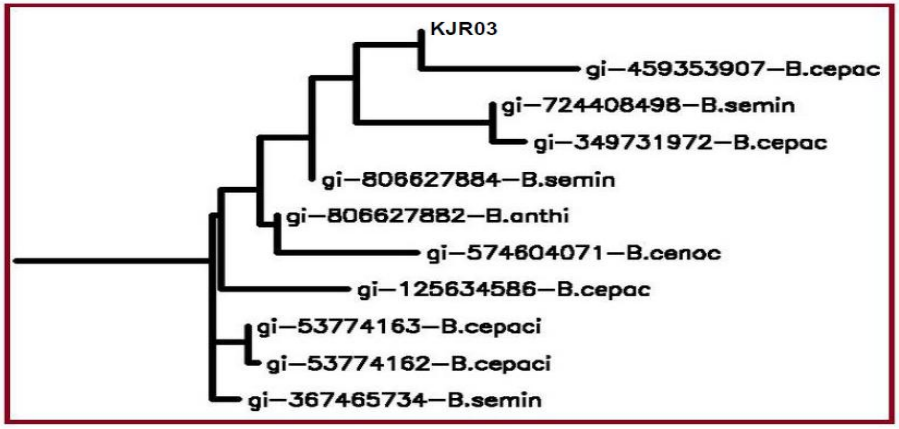


Fig.5 Phylogenetic tree of strain K1, constructed using MEGA4

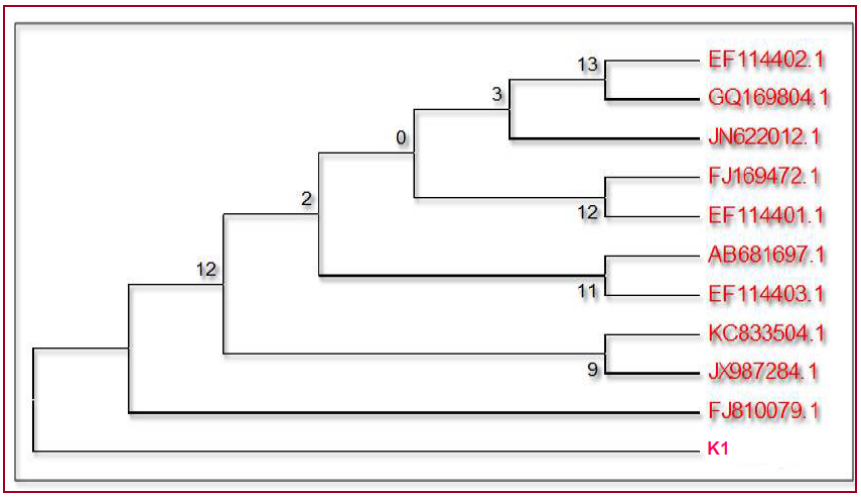


Fig.6 Correlation between acid phosphatase activity and shoot P content. Each dot inside the figure represents the mean data of each treatment in the experiment

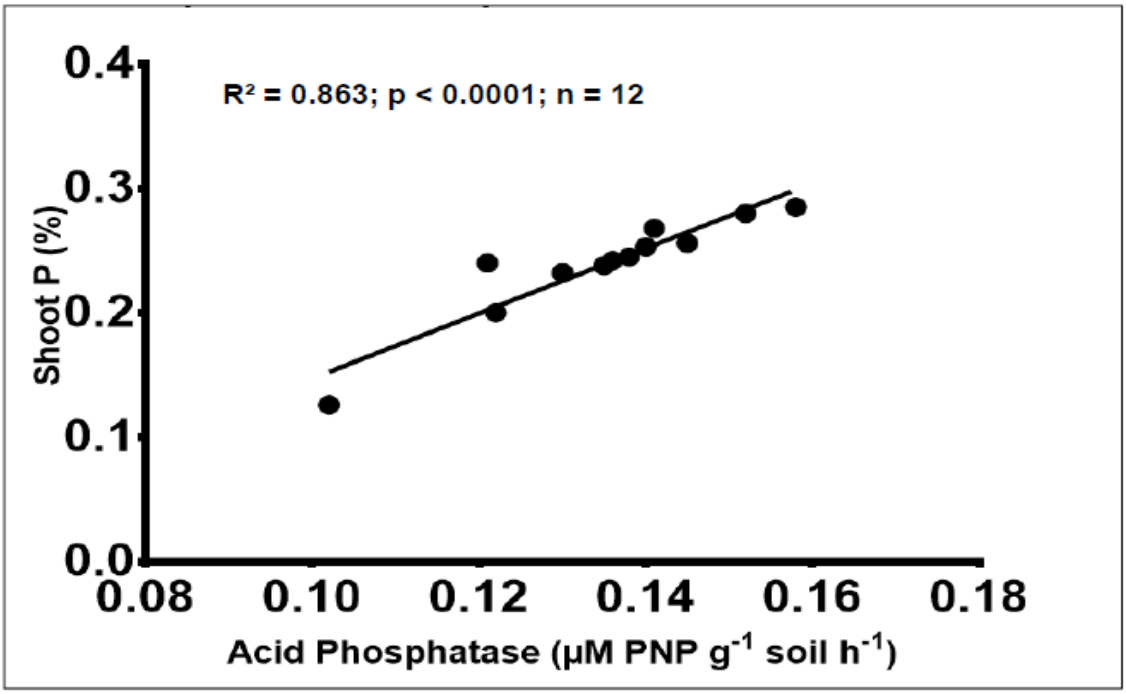
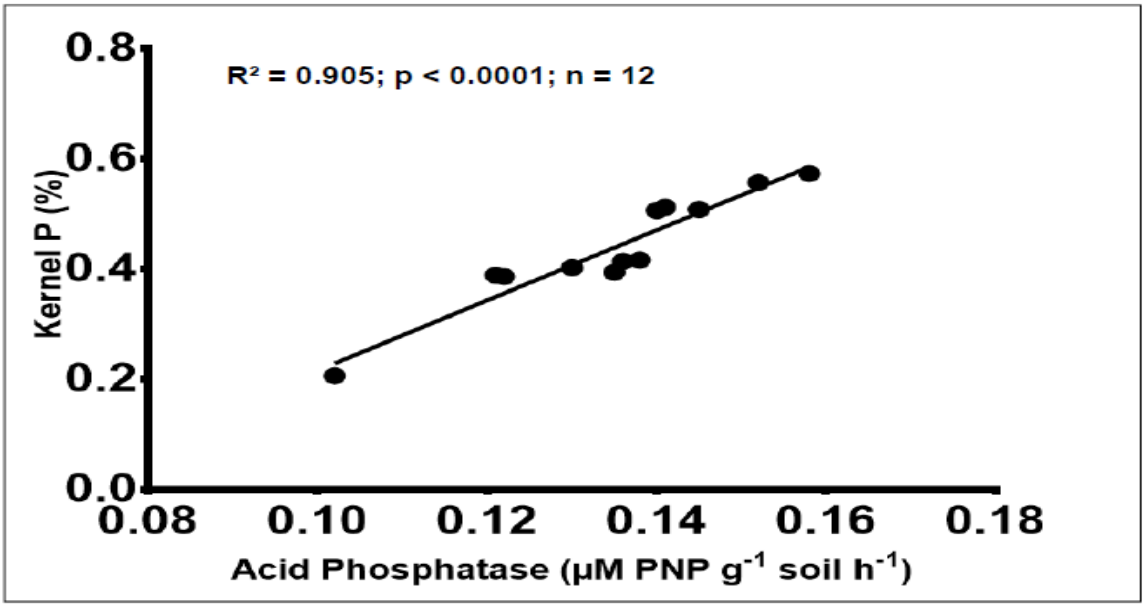


Fig.7 Correlation between acid phosphatase activity and kernel P content. Each dot inside the figure represents the mean data of each treatment in the experiment



All the five PSB strains (BLS18, CTC12, KHD08, KJR03 and K1) effectively increased microbial biomass P in presence of inorganic P fertilizer and no significant differences were observed among them. Further PSB application enhanced soil phosphatase activity and pots treated with combined application of PSB and SSP favoured phosphatase activity. This may be attributed to higher root and microbial activity (Zhang *et al*., 2014). The two strains (CTC12 and KHD08) showed more phosphatase activity either in combination or sole compared to other three. However, more acid phosphatase activities were recorded compared to alkaline phosphatase. Prior to sowing of groundnut seeds the soil pH was acidic and it seemed to be not affected by application of PSB. Similarly organic carbon didn't show any response to PSB application. Soil available P values were increased in the PSB treated soil. Strains CTC12 and KHD08 either sole or in combination with inorganic P (SSP) recorded more soluble P. Soil bacteria solubilize soil inorganic P by secreting low molecular weight organic acids and organic P by producing phosphatases (Rodríguez *et al*., 2006). More than 40% of culturable bacteria possess the ability to mobilize insoluble and organic P (Jorquera *et al*., 2008) and release orthophosphates ($H_2PO_4^-$ and HPO_4^{2-}) that can be absorbed by plant roots (Rodríguez and Fraga, 1999). Mobilization of insoluble organic P by soil bacteria can be regulated by addition of inorganic P fertilizer, which can directly affect the activity of soil microbiota and indirectly enhance the plants ability to take orthophosphates from organically fixed P (Zhang *et al*., 2014). Anzuay *et al* (2015) reported that strains of *Serratia* sp. J260, *Enterobacter* sp. J33, *Acinetobacter* sp. L176, *Enterococcus* sp. L185, *Enterococcus* sp. L191 and *Bacillus* sp. L55 enhanced soil available P and plant P uptake in groundnut. Similar studies were also previously reported by Dey *et al.*, (2004). This piece of work also reported a significant increase in P content of shoot and kernel in PSB treated pots compared to the uninoculated ones. The significant correlation between acid

phosphatase and P content of plant proved that, PSB inoculation appeared to be a major factor in the mineralization of organic P which might have improved plant P nutrition.

In conclusion, the five PSB strains when combined with SSP (inorganic P fertilizer) increased microbial biomass P and phosphatase activity while simultaneously reduced C/P ratio. All the strains (BLS18, CTC12, KHD08, KJR03 and K1) proved to solubilize P in the pot culture assay and improved nutrition of groundnut. However, the two strains *Bacillus amyloliquefaciens* CTC12 (KT633845), *Burkholderia cepacia* KHD08 (KT717633) performed better in mobilizing soil P and producing phosphatases.

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How to cite this article:

Madhusmita Pradhan, Chinmay Pradhan and Santanu Mohanty. 2017. Effect of P- Solubilizing Bacteria on Microbial Biomass P and Phosphatase Activity in Groundnut (*Arachis hypogaea* L) Rhizosphere. *Int.J.Curr.Microbiol.App.Sci.* 6(4): 1240-1260.
doi: https://doi.org/10.20546/ijcmas.2017.604.152