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Article

Evaluation of Phosphate Solubilizing Potential of Three *Burkholderia* Species Isolated from Green House Soils

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***Burkholderia anthina* R-4183, *Burkholderia diffusa* R-15930 and *Burkholderia stabilis* LMG 14294 isolated from green house soils (Gongju-Gun area, South Korea) were characterized and their phosphate solubilizing ability was assessed. Under *in vitro* culture conditions, all three species were proved to be effective in solubilizing phosphates in varying degrees. Strain *Burkholderia anthina* exhibited the highest phosphate solubilization in NBRIP medium (665 μg ml^{-1}) followed by *Burkholderia diffusa* (630 μg ml^{-1}) and *Burkholderia stabilis* (578 μg ml^{-1}). However, solubilization of $FePO_4$ and $AlPO_4$ was found to be poor in all the strains. Acidification by means of gluconic and oxalic acids accumulation in the culture medium could be the possible mechanism responsible for phosphate solubilization. Glucose at the rate of 3% was found be the best carbon source for *Burkholderia anthina* while other two *Burkholderia* species showed maximum phosphate solubilization at 2% of glucose. In the case of nitrogen sources, ammonium and nitrate were equally effective in solubilizing phosphates by *Burkholderia* species. Despite a slight decrease in phosphate solubilization observed at increasing temperature, all three *Burkholderia* species could withstand a temperature of 30-35℃, pH at the range of 7-9 and the presence of NaCl (up to 2.5%) without much compromising the phosphate solubilization. As shown with potted mung bean seedlings, all the three isolates could enhance soil fertility and plant growth indicating their great potential to be used as bio-inoculants.**

Key words: Phosphate Solubilization, *Burkholderia* species, Acidification

Introduction

Phosphorus is one of the major essential macronutrients for plant growth and it is applied as chemical fertilizer. However, a large portion of the applied soluble forms of phosphorus fertilizers are easily immobilized in to insoluble forms particularly, $CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$ and then become unavailable to plants (Mundra et al., 2011). Therefore, most soils are deficient in soluble forms of phosphorus. This leads to an excess application to crop plants and continuous application of phosphate fertilizers cause economical and environmental problem due to soil and water pollution (Park et al., 2010).

There are some microorganisms effectively involved in the solubilization of insoluble phosphate (Vassilev et al., 2006) called as phosphate solubilizing microorganisms (PSMs). Recently, PSMs have attracted the attention of agriculturists to apply them to soil as bio-inoculants to mitigate the phosphate problems (Fasim et al., 2002).

Solubilization of phosphate by PSMs depends on various physico-chemical factors viz. nature and amount of phosphate sources, pH, temperature, salt and acid concentration. Apart from this, carbon and nitrogen sources and their concentrations also have strong influence on phosphate solubilization (Dave and Patel, 2003). Therefore effective phosphate solubilization mainly depends on optimum combination of various physico-chemical factors along with the energy sources.

Previous studies have demonstrated that the ability of some *Burkholderia* species to efficiently solubilize inorganic phosphates, which subsequently results in increased availability of phosphorus for plants (Lin et al., 2006; Song et al., 2008). In present study three *Burkholderia* species having potential to solubilize insoluble phosphates were isolated from green house soils in South Korea and their characteristics of phosphate solubilization were investigated. In addition, the capacity of these isolates as bio-inoculants was assessed in pot experiments.

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Materials and Methods

Isolation of strain Bacterial strains were isolated from the soils collected from Chungchugnam-do province, Gongju-Gun area in South Korea. Serial dilution of soil solution were spread on NBRIP (National Botanical Research Institute Phosphorus) agar plates containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 2000). The plates were incubated for 5 days at 30℃. Formation of clear halo zone around the colonies after 5 days of incubation indicates phosphate solubilizing ability. It was further purified by re-culturing on the fresh NBRIP agar plates.

16S rDNA gene sequencing and Phylogenetic analysis of the isolated bacteria The partial sequencing of 16S rRNA of the strains was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F and 1492R. The online program BLAST was used to find out the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Three *Burkholderia* species were selected for further studies and a Phylogenetic tree was constructed using CLUSTAL X program, which involved sequence alignment by neighbor joining method and maximum parsimony using the MEGA4 program. Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Assay of Inorganic phosphate solubilizing abilities Bacterial strains were grown in sterilized liquid NBRIP medium (20 ml) at 30℃ for 2 days with continuous shaking at 150 r min^{-1}. Aliquots of culture (1 ml) were then transferred to a 500 ml flask (n=3 per strain) containing sterilized liquid NBRIP medium (200 ml) and incubated for 7 days with continuous shaking at 30℃. Sterilized uninoculated medium served as a control. A sample (10 ml) of each cultured and control were taken daily and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the amount of phosphorous released into the medium. The pH of the culture medium was also recorded with the pH meter equipped with glass electrode. The phosphorus availability was determined using phospho-molybdate blue color method (Murphy and Riley, 1962).

For the analysis of organic acids, 10 μl of filtrate was injected to high-performance liquid chromatography. The used column was Inertsil ODS 3V and a UV detector set to 210 nm at 40℃. Mobile phase consisted of 0.008 M H_2SO_4 run at a flow rate of 0.2 ml min^{-1}. HPLC profiles of the culture filtrates were analyzed by comparison with the elution profiles of pure organic acids (gluconic acid, oxalic acid and citric acid) injected separately. Peaks were identified by retention times against a set of standards from known three organic acids.

The $Ca_3(PO_4)_2$ solubilization assay performed as described above and $AlPO_4$ and $FePO_4$ solubilization was assayed by adding 4 g l^{-1} $AlPO_4$ or 6 g l^{-1} $FePO_4.2H_2O$ instead of $Ca_3(PO_4)_2$ in NBRIP medium. Rock phosphate solubilization ability was assayed using rock phosphate having P_2O_5 content 28% instead of $Ca_3(PO_4)_2$ in NBRIP medium. These amounts are equal to phosphorus as in the standard NBRIP medium.

Phosphate solubilizing ability of bacterial strains was tested under different carbon and nitrogen sources. Effect of carbon source on phosphate solubilization was tested by adding fructose, galactose, sorbitol, mannitol, xylose, sucrose, maltose and lactose instead of glucose in the NBRIP medium. To test the effect of the nitrogen source, $(NH_4)_2SO_4$ in the NBRIP medium was replaced byNH_4Cl, NH_4NO_3, KNO_3, $NaNO_3$ and $Ca(NO_3)_2$.

The effect of salt on phosphate solubilization was tested by growing the strain on NBRIP containing various amounts of NaCl (0%, 2.5%, 5% and 10%). Further, the effect of pH on phosphate solubilization was tested by adjusting the pH of NBRIP medium using HCl or NaOH to different pH levels (7-10). For estimation of high temperature induced phosphate solubilization NBRIP medium inoculated strain was incubated at different temperature conditions (30 - 40℃). In all cases, phosphate solubilization and pH of the culture medium were measured described earlier. The absolute value of the control refers to the amount of phosphorus solubilized (μg ml^{-1}) by each strain (PSB-1, PSB-2 and PSB-3) when individually grown for 3 days in NBRIP medium under the 30℃ temperature, pH 7 and in the absence of salt (NaCl).

Plant growth promotion bioassay on mungbean (*Vigna radiata*) Pot culture assay for plant growth

promotion ability of the *Burkholderia* species was determined in sterilized soil for 4 weeks. Seeds of mungbean were soaked in bacterial suspensions separately at the concentration of 10^8 cells ml^{-1} about 30 min prior to plantation. At the end of 4 weeks, seedlings were uprooted, washed under running water and root and shoot length were measured.

Values are given as means ± SD for triplicate samples. All the data were analyzed by analysis of variance or by regression analysis. Differences were considered to be significant at the $P \leq 0.05$ level.

Results and Discussion

Isolation and Identification of Burkholderia species

All three *Burkholderia* species had a marked insoluble phosphate solubilizing ability as visualized by the clear zone developed around the colonies after 5 day incubation at 30℃. It was seen that halo zone increased with increase in colony diameter. Solubilization Index (colony diameter + halo zone diameter/ colony diameter) was found to be reached to the peak at 5 days after inoculation (2.75, 3.25 and 2.25 respectively for PSB-1, PSB-2 and PSB-3) followed by gradual reduction.

According to 16S rRNA sequence analysis, the strains were identified as *Burkholderia anthina* R-4183 (PSB-1), *Burkholderia diffusa* R-15930 (PSB-2) and *Burkholderia stabilis* LMG 14294 (PSB-3). A phylogenetic tree was constructed with 16S rRNA sequences of strains with other *Burkholderia* species using neighbor-joining method (Fig. 1).

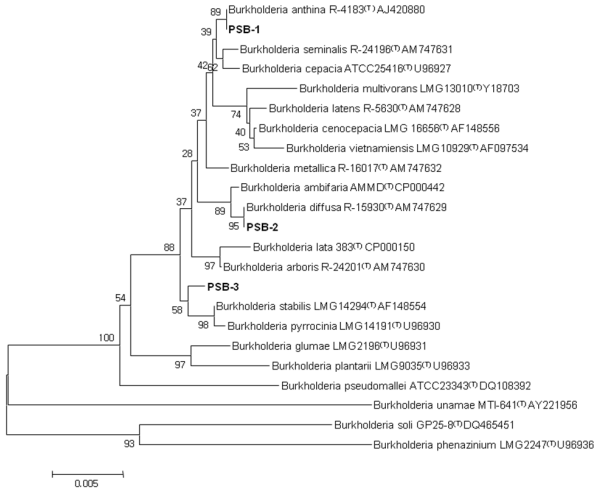

Fig. 1. Phylogenetic tree based on 16S rDNA gene sequences, showing the position of isolated efficient phosphate solubilizing bacterial strains with respect to related species. The scale bar indicates 0.005 substitutions per nucleotide position and accession numbers are given in parenthesis.

Assay of inorganic phosphate solubilizing abilities

Periodic changes of the soluble phosphorus content which has been released from the inorganic phosphorus in the NBRIP medium, due to addition of three Burkholderia inoculants during 7 days of incubation are presented in Fig. 2. Results showed that the isolates had different capabilities to release soluble phosphorus from inorganic phosphate. Among the three isolates, PSB-1was shown to release the highest content of soluble phosphorus (665 μg ml^{-1}) into the medium, followed by PSB-2 and PSB-3 with 630 and 578 μg ml^{-1} of soluble phosphorus respectively (Fig. 2). Results also showed that the content of soluble phosphorus released by the isolates in culture medium increased significantly during the first 2 days of the incubation remained high for several days. However subsequently a significant drop in soluble phosphorus level was observed on later days when incubation period progressed. This may be due to the availability of soluble phosphorus in the culture medium, which has an inhibitory effect on further phosphate solubilization (Xiao et al., 2009). Some researchers suggested that it is due to the depletion of nutrients in the culture medium especially carbon source for the production of organic acids and microbial activity (Kang et al., 2002; Kim et al., 2005; Chaiharn and Lumyong, 2009). There was no significant change in the content of soluble phosphorus under the control, which only resulted in a negligible slight increment throughout the incubation period.

All three strains produced acid and lowered the pH of culture medium from 7 to 3.63-3.93 after one day. Thereafter, the pH of the medium remained constant for

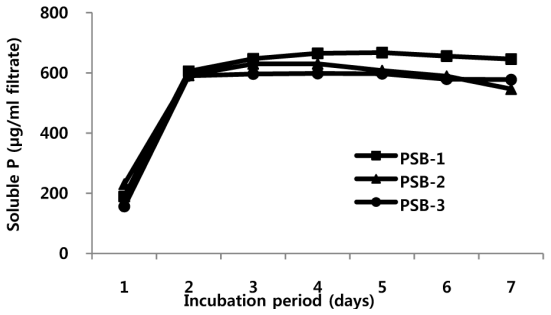

Fig. 2. Soluble phosphorus content in the NBRIP culture medium due to phosphate solubilization by three *Burkholderia* species. Results represent the mean of three replicates ± SD.

Table 1. HPLC analysis of organic acids production by phosphate solubilization by PSB-1, PSB-2 and PSB-3. Values given here are the means of three replicates (n = 3).

	Gluconic acid mM				Oxalic acid mM			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
PSB -1	1.12	3.19	5.59	5.29	0.007	0.009	0.012	0.009
PSB -2	0.02	4.97	7.98	7.71	0.075	0.099	0.117	0.101
PSB -3	1.16	4.87	5.78	4.51	0.003	0.004	0.006	0.004

several days (data not presented). The decrease in pH clearly indicated the production of organic acids, which is considered to be responsible for phosphate solubilization. Phosphate solubilization was concomitant with a significant pH decrease and microorganisms which decrease the medium pH during growth are considered as efficient phosphate solubilizers (Halder et al., 1991).

According to HPLC analysis, gluconic acid was the major acids produced by all the tested bacteria strains followed by oxalic acid (Table 1). No strain was capable for production of citric acids. Among *Burkholderia* species PSB-2 produced the highest amount of gluconic acid (7.98 mM) and oxalic acid (0.117 mM) than other two species. In agreement with this work, gluconic acid was the major organic acid produced by *Burkholderia cepacia* DA23 (Song et al., 2008) *Burkholderia cepacia* CC-A174 (Lin et al., 2006) and it was detected maximum 9.4 mM and 16.3 mM respectively in the culture medium. Results revealed that organic acid production in the culture medium increased with the incubation period, reaching a maximum at 2-3 days. This finding was also evident from the pH decreased during the period of the increase of organic acids. Therefore, the production of organic acids played a significant role in the acidification of culture medium and following by the decrease of pH in culture medium and thus facilitated the inorganic phosphate solubilization.

Strains were tested for their ability to solubilize hardly soluble phosphate sources ($FePO_4$ and $AlPO_4$) and results are shown in Fig. 3. The ability to solubilize $FePO_4$ or $AlPO_4$ by isolates was lower when compared with the $Ca_3(PO_4)_2$ solubilizing ability. Therefore, isolated *Burkholderia* species are not effective in solubilizing hardly soluble Fe and Al phosphate. This observation in consistent with earlier reports, which have shown that the very low Al phosphate solubilization by *Burkholderia cepacia* DA23 (Song et al., 2008). This may be probably due to the adaptive nature of the enzyme that is responsible

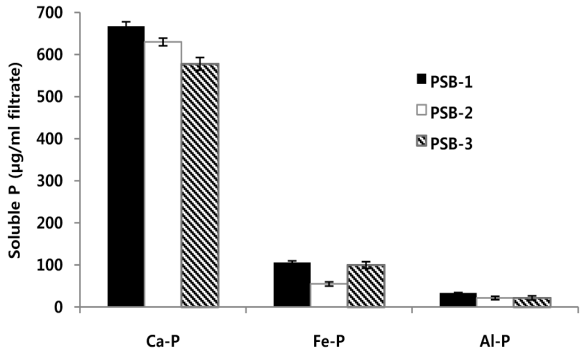

Fig. 3. Soluble phosphorus content under different phosphate sources (Ca-P : $Ca_3(PO_4)_2$, Fe-P : $FePO_4$ and Al-P : $AlPO_4$). Results represent the mean of three replicates ± SD.

for solubilizing $Ca_3(PO_4)_2$ (Banik and Dey, 1982). However, a few reports have indicated the other soil microorganisms to solubilize hardly soluble Fe or Al phosphates (Barroso et al., 2006; Antoun, 2002). *Penicillum rugulosum* was more efficient for solubilizing $AlPO_4$ and $FePO_4$ than hydroxyapatite (Reyes et al., 1999).

Nutrition condition of the culture medium affects microbial growth as well as phosphate solubilization (Jain et al., 2012). Inorganic phosphate solubilizing capacity of PSB-1, PSB-2 and PSB-3 was assessed in the presence of eight carbon sources and five nitrogen sources by replacing glucose and $(NH_4)_2SO_4$ respectively in NBRIP medium (Table 2). All *Burkholderia* species showed diverse levels of phosphate solubilizing activity in the presence of various carbon and nitrogen sources.

The effect of various carbon sources on phosphate solubilization by *Burkholderia* species (Table 1) revealed that glucose was the preferred carbon source for all three *Burkholderia* species followed by galactose (PSB-1 : 88.9%, PSB-2 : 93.8% and PSB-3 : 76.4% when compared to the control). Xylose was found to be a poor source of carbon for all the species in solubilization of phosphate. The highest pH reduction was recorded by glucose (3.63, 3.82 and 3.93 respectively for PSB-1, PSB-2 and PSB-3) followed by other carbon sources. The nature of carbon sources affects the type and concentration of organic acid

Table 2. Effect of various carbon and nitrogen sources on phosphate solubilization by PSB-1, PSB-2 and PSB-3.

	Phosphate solubilization compared to control (%)		
	PSB-1	PSB-2	PSB-3
Control	100 (3.63)	100 (3.82)	100 (3.93)
Absolute value	665 ± 12.5	630 ± 10.8	578 ± 14.5
Carbon source			
Fructose	28.2 (4.88)	15.7 (5.18)	3.8 (6.01)
Galactose	88.9 (4.12)	93.8 (4.13)	76.4 (4.01)
Sorbitol	12.3 (5.67)	9.6 (5.95)	6.3 (5.69)
Mannitol	32.7 (5.01)	33.5 (5.81)	19.2 (5.59)
Xylose	3.1 (6.28)	4.4 (5.62)	2.5 (6.26)
Sucrose	15.3 (6.42)	89.6 (4.31)	7.3 (6.33)
Maltose	72.9 (4.18)	69.9 (4.42)	67.5 (4.16)
Lactose	72.6 (4.16)	84.4 (4.25)	61.5 (4.18)
Nitrogen source			
NH_4Cl	99.2 (3.65)	102.5 (3.93)	100 (3.75)
NH_4NO_3	84.9 (4.19)	93.5 (4.05)	88.9 (4.33)
KNO_3	100 (3.75)	94.6 (3.96)	97.2 (3.76)
$NaNO_3$	94.1 (3.82)	100 (3.83)	82.7 (3.82)
$Ca_3(NO_3)_2$	99.8 (3.78)	99.8 (3.78)	92.5 (3.85)

Control strains were grown for 3 days in NBRIP medium
Autoclaved, un-inoculated medium served as control. Final pH of the growth medium is given within the parentheses.
Absolute value of phosphate solubilization (μg ml^{-1}) of control corresponding to 100%. Values are means ±SD for triplicates.

produced by the phosphate solubilizing microorganisms which in turn controls the amount of phosphate solubilization by lowering the pH and ability to chelate different metal ions that are associated with phosphate. Our results showed a significant negative correlation for soluble phosphorus and pH with all carbon sources. Corresponding to this result, it has been reported that glucose is the best carbon source for phosphate solubilization in *Burkholderia vietnamiensis* M6 (Park et al., 2010) and *Burkholderia cepacia* DA23 (Song et al., 2008).

To determine the effect of glucose concentration on the insoluble phosphate solubilization, various glucose concentrations ranging from 0.5% to 5% were added to the medium. As depicted in Fig. 4, phosphate solubilization was enhanced with increasing amounts of glucose up to 2% in PSB-1 and PSB-2 and up to 3% in PSB-3, but beyond this limit, there was reduction in the level of phosphate solubilization. It has been reported that insoluble phosphate solubilization by was enhanced with increasing amounts of glucose up to 2.5% in *Burkholderia vietnamiensis* M6 (Park et al., 2010) and up to 3% in *Burkholderia cepacia* DA23 (Song et al., 2008).

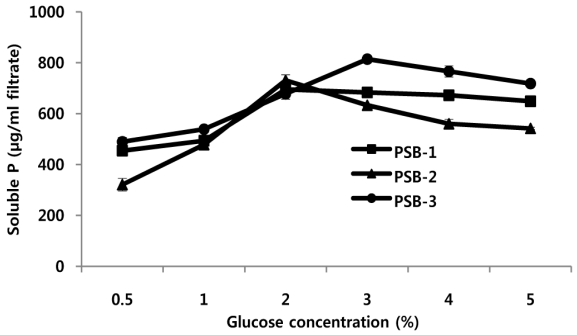

Fig. 4. Effect of glucose concentration on phosphate solubilization by PSB-1, PSB-2 and PSB-3. Values given here are the means of three replicates (n = 3).

Various ammonium and nitrate sources were added separately to the medium to assess their effects on phosphate solubilization and results are shown in Table 2. According to the results it is clear that ammonium and nitrate sources to be equally effective for phosphate solubilization PSB-1, PSB-2 and PSB-3. In agreement with this, Nautiyal et al. (2000) also observed all ammonium and nitrate sources were utilized for phosphate solubilization by NBRI0603, NBRI12601, NBRI13246 and NBRI14003 strains. However, there are some earlier

Table 3. Effect of various pH, temperature and salt concentration (NaCl %) on phosphate solubilization by PSB-1, PSB-2 and PSB-3.

	Phosphate solubilization compared to control (%)		
	PSB-1	PSB-2	PSB-3
Control	100 (3.63)	100 (3.82)	100 (3.93)
Absolute value	665 ± 12.5	630 ± 10.8	578 ± 14.5
Initial pH			
8	94.7 (3.89)	104.2 (3.69)	140.9 (3.69)
9	88.6 (4.06)	106.3 (3.83)	129.9 (3.74)
10	23.6 (4.92)	1.0 (7.95)	2.2 (6.31)
11	0.7 (8.91)	0.9 (8.63)	1.2 (6.96)
12	0.4 (9.83)	0.6 (9.79)	0.2 (9.54)
Temperature (℃)			
35	91.1 (3.78)	95.8 (3.91)	105.6 (3.85)
40	22.3 (4.58)	27.7 (4.53)	20.3 (6.41)
NaCl (%)			
2.5	87.3 (4.13)	76.9 (4.34)	89.2 (4.05)
5	1.8 (6.51)	2.0 (6.65)	2.4 (6.79)
7.5	1.8 (6.55)	1.9 (6.71)	2.1 (6.76)
10	1.8 (6.53)	1.7 (6.76)	1.9 (6.75)

Control strains were grown for 3 days in NBRIP medium

Autoclaved, un-inoculated medium served as control. Final pH of the growth medium is given within the parentheses.

Absolute value of phosphate solubilization (μg ml^{-1}) of control corresponding to 100%. Values are means ±SD for triplicates.

reports contrary to these findings showing differences in phosphate solubilization and also different mechanisms involving in acidity generation when ammonium and nitrate was used (Kpomblekou and Tabatabai, 1994; Halder et al., 1991). They have been reported that phosphate solubilization accelerates due to inorganic acids production by proton exchange mechanism in the presence of ammonium ion (Ahuja et al., 2007). However, Production of organic acids was greatly affected by carbon source not by nitrogen source. Therefore, organic acid production is not the sole factor responsible for phosphate solubilization.

The *Burkholderia* species were grown under high pH (8, 9, 10, 11 and 12), high temperature (35 and 40℃) and high salt (2.5, 5, 7.5 and 10% NaCl) conditions to study the effect of such conditions on the phosphate solubilizing ability of *Burkholderia* species (Table 3). The phosphate solubilizing abilities of the two strains (PSB-2 and PSB-3) were higher than the controls at the pH 8 and 9. The amount of soluble phosphorus produced seemed to be lower with the increasing pH beyond 9, but PSB-1 showed 24% phosphate solubilizing ability at pH 10 when compared to the control. This suggests that these bacteria favor alkaline conditions.

The phosphate solubilizing ability of the strain PSB-3 was higher than the control at the 35℃ temperature. Even though the phosphate solubilizing activity was highest at 30℃, other two strains also able to produce relatively high amount of soluble phosphorus at the 35℃ temperature (91% and 96% for PSB-1 and PSB-2 respectively when compared to the control). Generally bacteria growth was reduced at high temperature (Malboobi et al., 2009). This was more pronounced for our *Burkholderia* species such that phosphate solubilization dramatically decreased when incubated at 40℃.

Even though a slight decrease observed in phosphate solubilization in all *Burkholeria* species, they could tolerate to added NaCl concentrations up to 2.5% suggests that isolates would be functionally active where salinity is below 2.5%. In all cases except 2.5%, there was a dramatical decrease in the production of soluble phosphorus with increasing concentrations of NaCl.

In a similar study, Malbodi et al (2009) isolated *Pantoea agglomerans* P5, *Microbacterium laevaniformans* P7 and *Pseudomonas putida* P13 that could withstand 42℃ temperature, wide range of pH (5-11) and high concentration of NaCl (up to 5%) without much compromising the phosphate solubilization.

Plant growth assay Plant growth promotion assay of strains showed that the all three *Burkholderia* species had significantly different effect on shoot and root growth when compared with uninoculated seeds. This may be due to enhanced phosphorus nutrition which affects overall plant growth and root development (Jones and Darrah, 1994). As shown in Fig. 5, it was observed that inoculated seedlings (PSB-1, PSB-2 and PSB-3 respectively) recorded 24.41%, 22.31%, 27.73% and 19.11%, 17.43%, 30.11% higher shoot and root lengths respectively compared to uninoculated control. PSB-3 inoculated seedlings showed significant shoot and root growth when compared with PSB-1 and PSB-2 (Fig. 6).

It is clear from the results that the strain PSB-3 which demonstrated lower phosphorus release in the culture filtrate showed higher plant and root growth in the soil. Therefore, in addition to providing available phosphorus to plants, phosphate solubilizing microorganisms can enhance plant growth through several different mechanisms when inoculated in soil (Mundra et al., 2011). However, plant growth period was short and further works are required for their suitability as bio-inoculants.

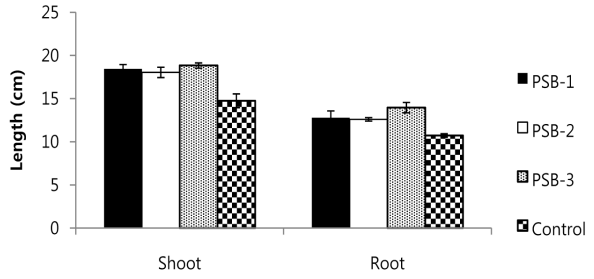

Fig. 5. Soot length and root length of green gram seedlings after inoculation of PSB-1, PSB-2 and PSB-3. Values given here are the means of three replicates (n = 3).

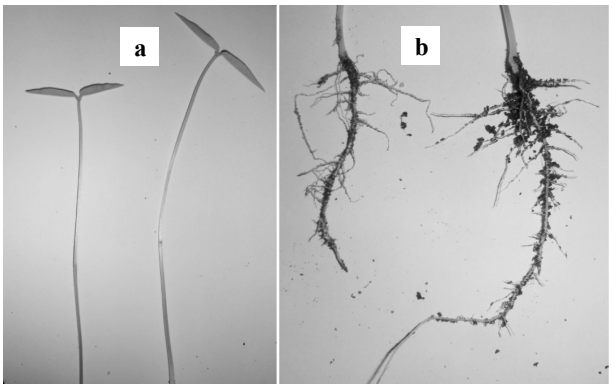

Fig. 6. Soot length (a) and root length (b) of control (left) and PSB-3 (right) inoculated green gram seedlings.

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