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ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA AND ANALYSING THEIR EFFECT IN *CAPSICUM ANNUM L.*

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ABSTRACT

The samples for the present study were collected from nearby firework industry respectively. After serial dilution and plating in Pikovskaya's agar medium, the phosphate solubilizing bacteria were identified by the formation of zone around the colony in Pikovskaya's agar plates. Five isolates of each sample were selected for further studies. The bacterial isolates were characterized by biochemical and functional tests. Phosphate solubilizing efficiency was estimated and the amount of phosphorous released was estimated using Pikovskaya's medium. The maximum amount of phosphatase was released in supernatant by the isolates A5 (82U/ml) and B3 (77U/ml) and the minimum amount of phosphatase was released by A1 (21U/ml) and A3 (22U/ml). The applications of bacterial isolates are used for *Capsicum Annum L.* plant growth studies. Bacterial isolates were tested for their efficiency in seed germination and analyzing the length of the shoots and leaves.

Key Words: Phosphate, Bacteria, Capsicum, shoot, leaves, seed germination.

INTRODUCTION

Phosphorus (P) is one of the key essential elements in modern agriculture. Fertilization of crops comprises the largest proportion of P used in agriculture. Phosphorus has many important functions in plants, the primary one being the storage and transfer of energy through the plant. The phosphate-solubilizing activity of the isolates was first qualitatively evaluated by the formation of halos (clear zones) around the colonies growing on solid medium containing tribasic calcium phosphate as a sole phosphorus source. (Vazquez *et al.*, 2000). Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields. Phosphate solubilizing microorganisms are routinely screened by a plate assay method using Pikovskaya (PVK) agar. The reliability of this halo-based technique is questioned as many isolates which did not produce any visible halo/zone on agar plates could solubilize various types of insoluble inorganic phosphates in liquid medium. (Shekhar Nautiyal, 1999). In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization. Currently, the main purpose in managing soil phosphorus is to optimize crop production and minimize P loss from soils. Recently, phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield. Plant growth promoting bacteria (PGPB) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms, and P-solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant P nutrition. Given the negative environmental impacts of chemical fertilizers and their increasing costs, the use of PGPB is

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advantageous in the sustainable agricultural practices. (Chen *et al.*, 2006). As P concentration in the nutrient solution increased, shoot and root growth increased by 19 and 15 %, respectively by day 35, with maximal growth at 4 mM P. A P concentration of 15 mM appeared to be toxic to plants. Phosphorus supply had no influence on nodule formation by day 12 but increased nodule number by day 35. (Tang *et al.*, 2001) . A critical P concentrations expected to cause noxious aquatic growth in downstream waters. (Wood *et al.*,2010). According to a global scenarios analysis, meeting future global phosphorus demand for food production will require a substantial reduction in the demand for phosphorus (through changing diets, reducing food waste and efficiency in agriculture) and ii) a high recovery rate of all sources of phosphorus – including human excreta – and also food waste, crop residues, manure, and other forms such as algae and ash. (Elisabeth Von Munch, 2009). The phosphate-solubilizing ability *in vitro* is not always correlated with phosphate mobilization to plant but it is very important to identify the target strain and to test its performance with the selected crops to avoid potential deleterious effect on plant growth. (Valverde *et al.*, 2003). In the present study, the phosphate solubilizing bacteria was isolated from the firework areas and it is used for improving the growth of *Capsicum annum* plants.

MATERIALS AND METHODS

Sample collection

Firework effluent was collected from the nearby area in Sivakasi.

Isolation of PSB

1ml of the firework effluent was dissolved in 9ml of water which is taken in test tube. Then that was serially diluted to 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6. Aliquots of 0.1ml of the sample from each of these dilutions were spread on to a Petri dish containing Pikovskaya's medium containing Tricalcium phosphate (10g Glucose, 5g $Ca_3(PO_4)_2$, 0.5g $(NH_4)_2 SO_4$, 0.2g KCl, 0.1g $MgSO_4.7H_2O$, 0.0001g $MnSO_4.H_2O$, 0.0001g $FeSO_4.7H_2O$,0.5g Yeast extract, 15g Agar in 1000mL distilled water). The pH of the media were adjusted initially to 7.0 with 1 mol L-1 NaOH or HCl and sterilized by an autoclave for 20 min at 115˚C. The plates were incubated aerobically at 28˚C±2 for 7 days in an incubator. Colonies showing clear zone of P-solubilization were counted. Different types of single well separated colonies, from each sample site, which grew on plates showing clear zones were picked and restreaked onto fresh Pikovskaya's solid medium. This procedure was repeated until pure culture with high P solubilization or mineralization was obtained. The strains which showed clear zones were incubated into nutrient broth and incubated at 28˚C±2 for 24 h at 200 r min-1.

Identification of phosphate solubilizing bacteria

The organisms were identified by the methods described in the Bergy's manual of Determinative Bacteriology.

Gram's staining

The isolated colonies from the soil sample were sub cultured and stained with crystal violet for two minutes and then fixed with gram's iodine. Followed by, de colorization with alcohol was carried out. Then counter stained with saffranin. The slides were then examined under the microscope to find out the gram's nature and shape of the organisms.

Biochemical characterization of PSMs

Indole production test

The indole test was performed by inoculating the bacterial isolates into the tryptone broth. The indole produced was detected by adding kovoc's reagent. Appearance of cherry red colour ring at the top indicated the positive reaction.

Methyl red test

MR-VP broth was inoculated with the isolated organisms and incubated at 37ºC for 24hours. Followed by incubation, methyl red indicator was added. Positive reaction was indicated by the developments of red colour.

Voges- Proskauer test

MR-VP broth was inoculated with the isolated cultures and incubated for 24 hours at 37ºC .after incubation about 40% KOH and 5%solution of alpha-napthal in absolute ethanol. Positive reaction was indicated by the development of pink colour in about 2.5 minutes which became crimson colored in about 30 minutes.

Citrate utilization test

The organisms were inoculant into simmon's citrate agar tube. Tubes were inoculated for 24 hours at 37ºC. Positive reaction was indicated by the change in colour of the medium from green to blue.

Catalase test

One loop full of the culture was placed on the slide to which one or two drops of 30% hydrogen peroxide was added and the slide was observed for the emergence of gas bubbles.

Oxidase test

Whatman No.1 filter paper disc was immersed freshly prepared solution of tetramethyl-phenylene-diamine dihydrochloride. The disc was placed the Petri disc moistened with distilled water. The colony to be tested was smeared over the moist area. Positive reaction was indicated by the formation of deep purple colour with in 5-10 seconds.

Starch hydrolysis test

One loop full of the culture was streaked on the sterile starch agar medium and the plates were incubated at 37ºC for 24 hours. Following incubation the plates were flooded with gram's iodine solution and observed for the formation of zone around the bacterial colony.

Estimation of phosphorous

The phosphate was estimated by the method of Boltz (1948)

Reagent A

1.25ml of acetone
0.88g Ascorbic acid
Add distilled water make up to 100ml.

Reagent B

0.5g of Ammonium hepta molybdate
3.1ml of conc. H_2SO_4
Add distilled water make up to 100ml.

Stock solution

100g/ml Potassium Dihydrogen Ortho phosphate was considered as the stock solution.

Procedure

Standard graph for phosphate

1. 1ml of different aliquots (10^{-1}g) of stock solution was taken in 10 test tubes.
2. 2ml of reagent A and 2ml of reagent B were added then the mixture were heated at 60ºC for 10 minutes in a water bath the reaction was stooped by placing the tubes on a ice bath for 10 minutes.
3. The blue color intensity of the solution was measured at 660nm.
4. Standard graph was prepared by plotting the concentration of potassium dihydrogen orthophosphate in X-axis and absorbance in –axis.

100ml of pikovskaya's broth was inoculated with 1ml of each isolate and incubated for days on a gyro rotary shaker at 37ºC. Culture broth was centrifuged at 10,000 rpm for 15 minutes at an interval of 24 hours. The amount of phosphorous released was estimated.

Estimation of phosphatase

The phosphatase was estimated by the method of De Freitas *et al.,* (1997).

Reagent A

0.1M Universal buffer
P H 6.5

Reagent B

0.05M p-nitro phenyl phosphate (pNPP) solution
Stock solution:

100g/ml p-nitro phenyl phosphate (pNPP) was considered as the stock solution.

Procedure

1.1ml of different aliquots (10^{-1}g) of stock solution was taken in 10 test tubes.

2. Aliquots of each sample were added to 0.48m L Reagent A.

3.0.12 m L of reagent B solution was added and followed by 1 h incubation at 37ºC.

4. Standard graph was prepared by plotting the concentration of p-nitro phenyl phosphate (pNPP) in X-axis and absorbance in –axis.

5. The yellow color was measured at 405 nm (Tabatabai and Bremmer, 1969) and expressed in terms of units (U).

100ml of pikovskaya's broth was inoculated with 1ml of each isolate and incubated for days on a gyro rotary shaker at 37ºC. Culture broth was centrifuged at 10,000 rpm for 10 minutes at an interval of 24 hours. The amount of phosphatase released was estimated. One unit of phosphatase activity is the amount of enzyme required to release 1µg p NPP m $L^{-1}$of culture filtrate under assay conditions(1U=1 µg pNPP m L^{-1}).

Phosphate solubilization efficiency

Isolates were spot inoculated on Pikovskaya medium for detection of their phosphate solubilizing ability and incubated at 37^{o}C for 48 h. Halo surrounding the colonies was measured and the solubilizing efficiency (SE) was calculated by the following formula:

$$SE = \frac{\text{Solubilization diameter (S) X 100}}{\text{Growth diameter (G)}}$$

Detection of Organic Acid

The major mechanism for solubilization of insoluble inorganic phosphates by microorganisms is through production of organic acids. Hence, the organic acid production profile of the PSB was examined by paper chromatographic method. One milliliter of 24 h old culture of each isolate was inoculate to 50ml Pikovskaya's broth and incubated at 28±2ºC for 10 days. The broth was centrifuged at 10,000 rpm for 10 min. The supernatant so obtained was concentration to nearly $1/10^{th}$ of the original volume in a water bath maintained at 60ºC. The concentrated material was then used for determination of organic acid by paper chromatography in comparison with standard organic acid. Standards of organic acids were prepared at 20mg/ml stock. About 10µL of standards and 15µL of culture supernatants were spotted on Whatman No.1 chromatographic paper and dried with a hair dryer. A descending chromatography was run using a solvent mixture of n-butanol, acetic acid and water in 12:3:5 ratios in a chromatographic chamber pre saturated with solvent for 6h. The chromatogram was run for 16h and air dried for 3 days. The air dried paper was sprayed with 0.04% bromocresol green. The paper was dried at room temperature. The R_f values of the yellow spots of the organic acids developed on a blue background was measured and compared with the Rf values of the standard organic acid for identification. The R_f values can be calculated by using the following formula:

$$\mathbf{R_f = \frac{\text{Distance moved by the solute from the origin}}{\text{Distance moved by the solute from the origin}} \times 100}$$

Pot Experiment

Bacterial cells were grown overnight in 500mL Conical flasks containing 250mL of sterilized nutrient broth on a shaker at 100 rev/min at 37° C until late log phase. Healthy and viable seeds of Capsicum annum L. were surface sterilized with 0.1% mercuric chloride for one minute and washed with running tap water, followed by rinsing with distilled water. Various concentrations of paper mill effluent were prepared, such as 10%, 20% 30%, 40%, and 50% (v/v). The seeds were soaked in distilled water for two hours and allowed to grow in pots containing uniformly mixed red, black and sandy soil in 1:1:1 ratio. The experimental sets were kept in diffused light at room temperature. Seven days after sowing, the experimental plants were watered constantly every day with the respective concentration of the effluent. The control sets were treated with tap water. Both experimental sets and control sets were maintained in triplicates. On the twenty first day both the sets of plants were taken for analysis. Various concentrations (10%, 20%, 30% and 40%) of Bacillus were mixed separately with firework effluent. On the 21st day, the seedlings were carefully removed from the soil without any damage to the root system and analyzed the physical, biochemical and enzyme characteristics.

Chlorophyll analysis

The chlorophyll content of plant leaves was measured by the method of Arnon, (1949). 100 mg of leaf samples were ground with 80% acetone followed by centrifugation at 3000 g for 5 min. Absorbance of the supernatant was detected at 645 and 663 nm.

Total Chlorophyll mg L^{-1} = 20.2 x A645 + 8.02 x A663 x Vol/1000 x weight

Leaf and Shoot Growth analysis

Grown plants were removed carefully from the soil by mixing the total in sterilized distilled water and remove without any damage. The length of the leaves and shoots were estimated with commercially available scale.

RESULT AND DISCUSSION

Isolation and identification of Phosphate solubilizing microorganisms

On Pikovskaya's agar, many bacteria and fungi (Plate.1) shown very large, clear and transparent solubilization zone and highest percentage of phosphate solubilization in Pikovskaya's broth. All isolates released inorganic phosphate from TCP indicating the potential of these strains to release inorganic soluble phosphate from fixed phosphates sources for plant uptake. A number of ten (10) isolated PSB strains were tested for phosphate solubilizing activity in Pikovskaya's agar plate, a clear halo zone indicating phosphate solubilization . The highest phosphate solubilizing activity was found in A5 (68%) strain while the lowest solubilizing activity (47%) was found in A1 strain. Because of their solubilization efficiency all strains solubilize the TCP in the medium. The efficiency may be different for each strain (Table 1).

Biochemical analysis was carried out for all the strains and the results were shown in the Table-2. In phenol red media, strain A5 showed yellow and pink color. All biochemical characteristics and result in phenol red media showed positive result for the presence of *Bacillus subtilis* in A5 strain. Starch hydrolysis test for A5 strain showed the presence of *Bacillus subtilis* which is shown in Plate.2. *Bacillus subtilis* has the capacity to solubilize the starch in the medium. The PSB strains forming clear zones in Pikovskaya's medium were able to release P from tricalcium phosphate. P release was observed from 3rd day onwards and gradually increased until 7th day. Statistical analyses were performed between all the strains from 3rd to 7th day of incubation. PSB strains A and B solubilized higher amount of P. Bacterial strain A5 released high amount of P on 3rd day (114 µg mL^{-1}) and A4 and B5 (82 µg mL^{-1}) released low amount of P. Statistical analyses on 5th day revealed comparatively higher amount of P release by A5 strain (161 µg mL^{-1}) compared to other strains A3 (143 µg mL^{-1}), B3 (135 µg mL^{-1}) and A2 and B2 (134 µg mL^{-1}). On 7th day, high P release was observed in all strains isolated from A and B samples and maximum P release was seen in A5 strain (461 µg mL^{-1}) compared to all other strains. From the statistical analysis it is clear that P released by A5 strain was significantly higher when compared with other PSB strains. All the PSB strains isolated were efficient in solubilizing tricalcium phosphate. Significant difference was observed in between the days in each strain and between the strains. The efficiency of the PSB strain to release P can be correlated with the amount of P released into the media (Figure 1).

Phosphatase activity by PSB strains

The acid phosphatase released into the media on 3rd, 5th and 7th day . The enzyme activity between the days were compared and found to be significantly different. It was seen that the production of phosphatase decreased from 3rd to 7th day. The amount of phosphatase released into the media was high on 3rd day. The phosphatase released by A5 (82U) was high compared to other strains on 3rd day. All the strains released acid phosphatase into the liquid medium ranging between 21 to 82 units (Figure 2).

The PSB strains B3 (77 U), B2 (73 U), B4 (62 U), A4 (42 U) also significantly released higher amount of phosphatase compared to other strains. On 5th day, B4 (59 U) strain released high amount of phosphatase compared to other strains which showed significant reduction of phosphatase, indicating the slow growth of the strain. B4 strain was at growth phase on 5th day, when other strains entered death phase, except A5 and B2 which produced high amount of phosphatase on 7th day indicating slow growth of the strains. Because of the slow growth the

amount of phosphatase released was low by the other strains (Figure 3).

Organic acid detection

The PSB strains isolated from firework effluent were able to produce organic acids in the broth culture containing different P sources during 48 h of incubation period. The PSB strain A5 (*Bacillus subtilis*) was selected for the organic acids determination. Different organic acids like citric acid, boric acid, lactic acid, oxalic acid, succinic acid, etc., were produced by PSB strains. Lactic acid, boric acid, citric acid and oxalic acid were used as standard organic acids for the determination. The sample A5 (*Bacillus subtilis*) produced two different organic acids such as citric acid and boric acid. The R_f values showed citric acid was produced in high amount and boric acid was produced in small amount. These organic acids are produced to dissolve the phosphate molecules (Figure 4 and plate 3).

Plant study

Leaf and Shoot length analysis

Different concentrated effluent with different concentration of *Bacillus subtilis* were mixed with each other and given to plants. Low concentration of effluent with high concentration of *Bacillus subtilis* showed high plant growth. The shoot length of the plant which was treated with 10% effluent and 40% *Bacillus subtilis* culture gives 6.8cm when compared with the other plants. Similarly the shoot length of the plant which was treated with 50% effluent and 10% *Bacillus subtilis* culture gives 6.05cm.

The leaf length of plants which was treated with different concentration of effluent and *Bacillus subtilis* culture gives increase in the length. Plants which were treated with 10% effluent and 40% *Bacillus subtilis* culture gives 3.80cm of leaf length and plants with 50% effluent and 10% *Bacillus subtilis* culture gives 2.93cm of leaf length. This gives the result that PSB can stimulate the plant growth with increase in shoot length and leaf length (Table 4 and Table 5).

Chlorophyll Estimation

The plants treated with the 10% effluent and 40% PSB showed significantly higher level of leaf chlorophyll content (92mg) in the leaf of *Capsicum annum L* when compared with the other plants and control. Because of the PSB which was given to the plants that solubilized the large molecules of phosphate into small molecules of phosphorous and make it plant usable form (Table 6).

Table 1. Estimation of Phosphate solubilizing efficiency

Colony	Z.F	C.F	%
A1	2.1	1.0	47
A2	1.7	0.9	52
A3	1.9	1.1	57
A4	1.9	1.1	57
A5	1.6	1.1	68
B1	2.0	1.2	60
B2	1.9	1.1	57
B3	1.9	1.0	55
B4	2.1	1.2	57
B5	2.0	1.2	60

Table 2. Biochemical analysis for PSB

S.No	Indole	MR	VP	Citrate	Starch hydrolysis
A1	-	-	+	-	+
A2	-	-	+	-	-
A3	+	+	-	+	+
A4	-	+	+	+	+
A5	-	-	+	+	+
B1	-	+	-	+	+
B2	-	+	+	+	+
B3	-	-	+	-	+
B4	+	+	-	+	+
B5	-	+	-	+	+

Table 3. Organic acid detection

Organic acids	R_f values (mg/L)
Citric acid- std	0.81
Lactic acid- std	0.51
Boric acid- std	0.31
Oxalic acid- std	0.72
Citric acid- Bacillus	0.87
Boric acid- Bacillus	0.34

Table 4. Differences in Shoot length of *Capsicum annum L*.

Control with water	Control with effluent	Different concentration of effluent and PSB			
5.91cm	5.01cm	10%E+10%PSB 6.54cm	10%E+20%PSB 6.58cm	10%E+30%PSB 6.7cm	10%E+40%PSB 6.8cm
		20%E+10%PSB 6.40cm	20%E+20%PSB 6.45cm	20%E+30%PSB 6.48cm	20%E+40%PSB 6.50cm
		30%E+10%PSB 6.30cm	30%E+20%PSB 6.31cm	30%E+30%PSB 6.35cm	30%E+40%PSB 6.37cm
		40%E+10%PSB 6.22cm	40%E+20%PSB 6.24cm	40%E+30%PSB 6.25cm	40%E+40%PSB 6.28cm
		50%E+10%PSB 6.05cm	50%E+20%PSB 6.11cm	50%E+30%PSB 6.15cm	50%E+40%PSB 6.20cm

Table 5. Differences in Leaf length of *Capsicum annum L.*

Control with water	Control with effluent	Different concentration of effluent and PSB			
2.8cm	2.1cm	10%E+10%PSB 3.55cm	10%E+20%PSB 3.67cm	10%E+30%PSB 3.73cm	10%E+40%PSB 3.80cm
		20%E+10%PSB 3.27cm	20%E+20%PSB 3.35cm	20%E+30%PSB 3.40cm	20%E+40%PSB 3.48cm
		30%E+10%PSB 3.93cm	30%E+20%PSB 4.0cm	30%E+30%PSB 4.06cm	30%E+40%PSB 4.15cm
		40%E+10%PSB 3.60cm	40%E+20%PSB 3.77cm	40%E+30%PSB 3.80cm	40%E+40%PSB 3.84cm
		50%E+10%PSB 2.93cm	50%E+20%PSB 3.09cm	50%E+30%PSB 3.15cm	50%E+40%PSB 3.41cm

Table 6. Chlorophyll estimation in *Capsicum annum L.*

Control with water	Control with effluent	Different concentration of effluent and PSB			
70mg	**56mg**	10%E+10%PSB 83mg	10%E+20%PSB 87mg	10%E+30%PSB 90mg	10%E+40%PSB 92mg
		20%E+10%PSB 75mg	20%E+20%PSB 76mg	20%E+30%PSB 79mg	20%E+40%PSB 80mg
		30%E+10%PSB 69mg	30%E+20%PSB 70mg	30%E+30%PSB 72mg	30%E+40%PSB 74mg
		40%E+10%PSB 63mg	40%E+20%PSB 65mg	40%E+30%PSB 66mg	40%E+40%PSB 68mg
		50%E+10%PSB 58mg	50%E+20%PSB 59mg	50%E+30%PSB 61mg	50%E+40%PSB 62mg

Plate 1. Phosphate solubilizing microorganisms

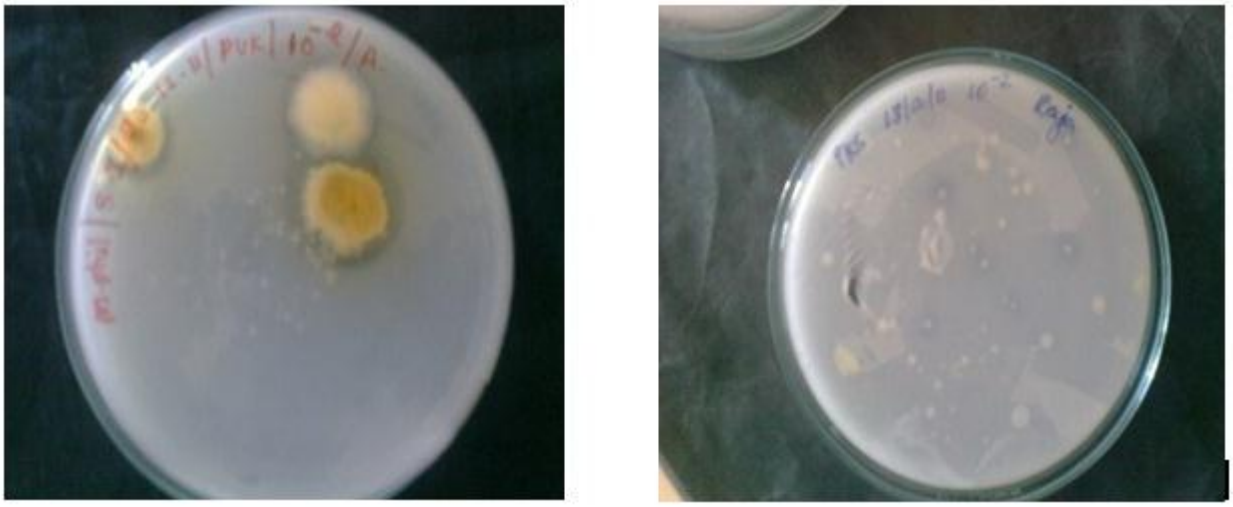


Plate 2. Starch hydrolysis by *Bacillus subtilis*

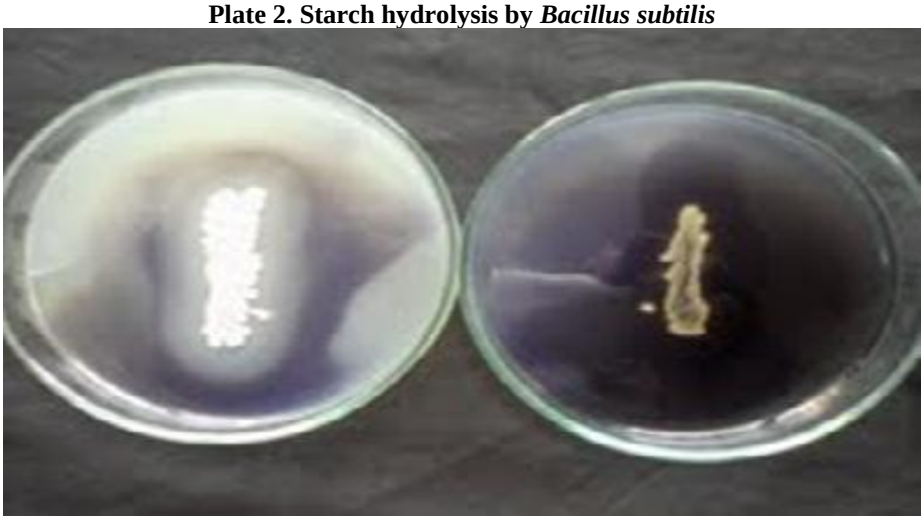


Plate 3. Organic acid detection

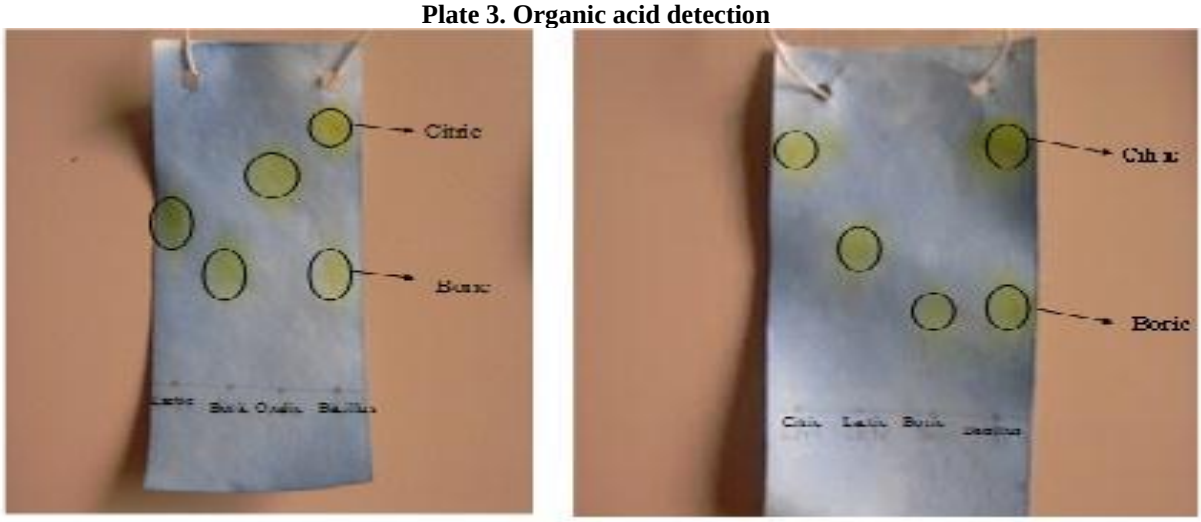


Figure 1. Determination of phosphate solubilising efficiency after 5 days

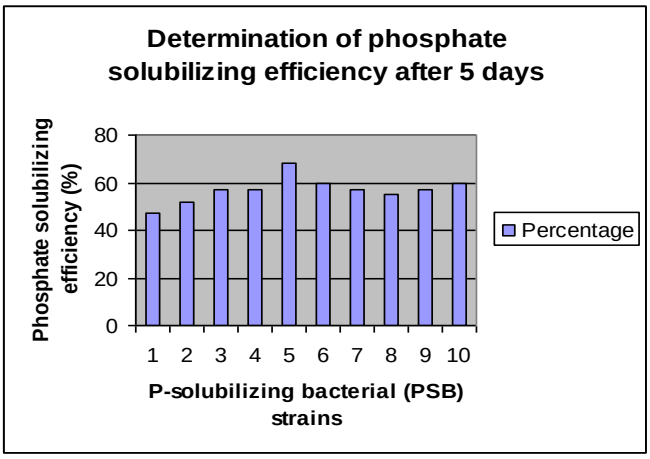


Figure 2. Phosphorous released by PSB strains on 3rd, 5th and 7th day for sample A1-B5

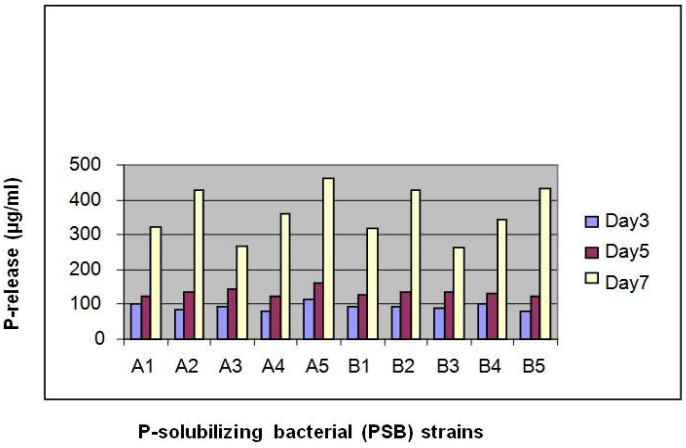
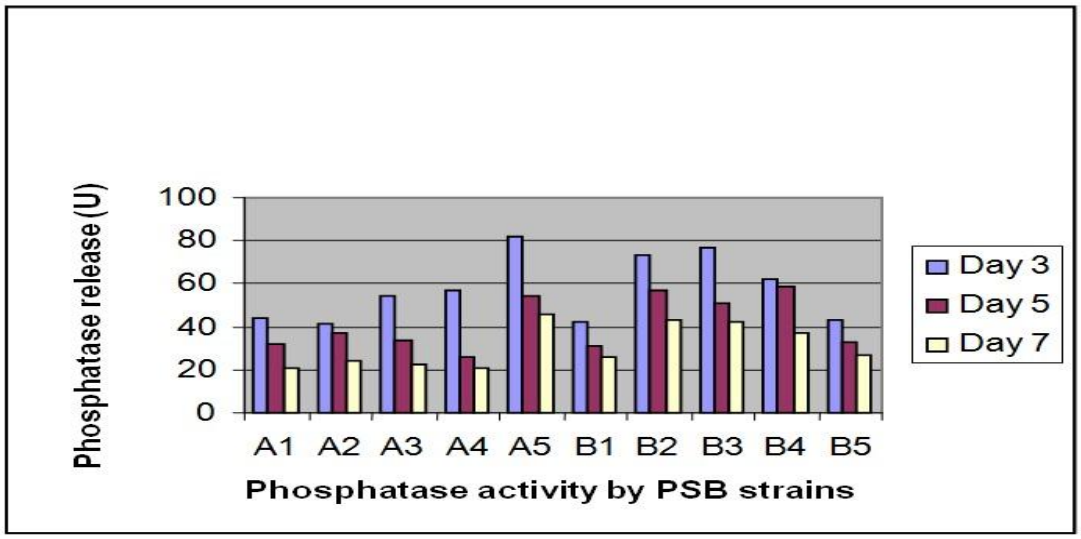


Figure 3. Phosphatase released by PSB strains on 3rd, 5th and 7th day for sample A1-B5



CONCLUSION

The bacterial isolates were characterized by biochemical and functional tests. Phosphate solubilizing efficiency was estimated and the amount of phosphorous released was estimated using Pikovskaya's medium. The maximum amount of phosphatase was released in supernatant by the isolates A5 (82U/ml) and B3 (77U/ml) and the minimum amount of phosphatase was released by A1 (21U/ml) and A3 (22U/ml). The applications of bacterial isolates are used for *Capsicum Annum L.* plant growth studies. Bacterial isolates were tested for their efficiency in seed germination and analyzing the length of the shoots and leaves.

REFERENCES

Boltz FD and Mellon GM. Spectrophotometric determination of phosphorus as molybdophosphoric acid. *Anal. Chem.* 1948; 20: 749-751.

Chen YP, Rekha PD, Arun AB, Shen FT, Lai W A, Young CC. Phosphate solubilizing bacteria from subtrophical soil and their tricalcium phosphate solubilizing abilities. *Applied soil Ecology*. 2006; 34: 34-41.

DeFreitas JR, Banerjee MR and Germida JJ. Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of Canola(*Brassicanapus* L.). *Biol. Fertil. Soils*. 1997; 24: 358Ð364.

Elisabeth von Munch. Quarterly newsletter on ecological and sustainable sanitation worldwide. A service by the GTZ ecosan program, issue 33.for multi-functional biofertilizer preparation. *Bioresource Technology*. 2009; 100: 1648–1658.

Selvarathi P, Ramasubramanian V, and Jeyaprakash R. Bioremedial effect of Azotobacer and Phosphobacterium on the growth and biochemical characteristics of paper mill effluent treated Lycopersicum esculentum Mill. *J. BioSci. Res*. 2010; 1(1): 58-64.

Shekhar Nautiyal C. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*. 1999; 170: 265-270.

Tang C, Hinsinger P, Drevon JJ and Jaillard B. Phosphorus Deficiency Impairs Early Nodule Functioning and Enhances Proton Release in Roots of Medicago truncatula L. *Annals of Botany*. 2001; 88: 131-138.

Valverde A, Igual JM, and Cervantes E. Polyphasic characterization of phosphate-solubilizing bacteria isolated from rhizospheric soil of the north-eastern region of Portugal. *First International Meeting on Microbial Phosphate Solubilization*. 2007; 273-276.

Vazquez P, Holguin G, Puente M E, Lopez-Cortes A, Bashan Y. Phosphate- solubilizing microorganisms associated with the rhizosphere of Mangroves in a semiarid Coastal lagoon. Biol Fertil Soils. 2000; 30: 460-468.

Wood SM, Lee M K,Hong IS, Poonguzhali S and Sa TM. Isolation and characterization of phosphate solubilizing bacteria from Chinese cabbage. *World congress of soil science*, soil solutions for a changing world, 2010.