Eco. Env. & Cons. 30 (Nov. Suppl. Issue) : 2024; pp. (S95-S100)
Copyright@ EM International
ISSN 0971–765X

DOI No. http://doi.org/10.53550/EEC.2024.v30i07s.018

Effect of pH and salinity on mineral phosphate solubilization by bacterial strains isolated from the rhizosphere of *Lablab purpureus* (L.) Sweet

***R. Saikia[1,2] and L.R. Saikia[2]**

[1]Department of Botany, Tinsukia College, Tinsukia, Assam, India
[2] Department of Life Sciences, Dibrugarh University, Dibrugarh, Assam, India

(Received 23 April, 2024; Accepted 15 June, 2024)

ABSTRACT

Phosphate Solubilizing Bacteria (PSB) solubilizes insoluble phosphates. Two strains of PSB, 2b3 and 2b5 were isolated from *Lablab purpureus* (L.) Sweet rhizosphere soil based on the formation of halozone in Pikovskaya agar plates. Phosphate solubilization in Pikovskaya broth containing either aluminium, ferric or tricalcium phosphates in different pH conditions was estimated. Both the strains were subjected to salinity stress. Morphology and biochemical characters of the bacterial isolates were studied and identified by 16s rRNA gene sequencing. The two bacterial strains 2b3 and 2b5 were identified as *Burkholderia* sp. and *Burkholderia cepacia* respectively. The Phosphate Solubilization Index of *Burkholderia* sp. and *Burkholderia cepacia* were 2.43±0.057 and 2.36 ± 0.200 respectively. Both the strains showed higher phosphate solubilization in broth containing tricalcium phosphates in comparison to aluminium and ferric phosphates. *Burkholderia cepacia* (2b5) showed higher phosphate solubilization than 2b3 (*Burkholderia* sp.) after 144 hours in all the three media. Phosphate solubilized in broth containing aluminium phosphate, ferric phosphate and tricalcium phosphate by 2b5 after 144 hours were 117.53±9.13, 109.15±5.43 and 260.17±11.81 respectively. Both the isolates showed ammonia production and *Burkholderia cepacia* (2b5) showed better growth in agar plates with NaCl than *Burkholderia* sp. (2b3). The results indicate that the isolated bacterial strains are potent mineral phosphate solubilizers and further studies will help in the use of these two strains as biofertilizers.

Key words: *Phosphate Solubilization Index, Halozone, Biochemical, Molecular*

Introduction

Phosphorus (P) is a major plant growth limiting nutrient after nitrogen (Anasuya and Jayaranjan, 1998; Gyaneswar *et al.,* 2002). The amount of P in plants ranges from 0.05% to 0.5% of total dry weight of plant and in soil it is 2000-fold higher (Malhotra *et al.,* 2018). P is less available to plants due to its fixation in the form of aluminium/iron or calcium/magnesium phosphates (Malhotra *et al.,* 2018). P is taken up by the plants in the form of inorganic orthophosphate ions $H_2PO_4^-$ and HPO_4^{2-}. Deficiency of P reduces plant growth and affects crop yield and quality (Malhotra *et al.,* 2018). To overcome the deficiency of P in soil, chemical fertilizers are applied and large amount of these chemical substances are converted to insoluble forms (Halder and Chakrabartty, 1993). Chemical fertilizers are expensive and have negative impact on the environment (Rodríguez *et al.,* 2006). Overuse of chemical fertilizers in agriculture sector now becomes a critical environmental issue. Recently, the use of Phosphate Solubilizing Bacteria (PSB) as alternative to chemical fertilizers is being encouraged. There are several

PSB such as *Pseudomonas, Burkholderia, Bacillus, Enterobacter, Rhizobium, Serratia, Arthrobacter and Acinetobacter* that can solubilize phosphates (Rodrìguez and Fraga, 1999). PSB has the ability to solubilize insoluble mineral phosphates by secreting organic acids (Rodrìguez and Fraga, 1999). Plate screening methods which show clearing or halo zones formed due to the solubilization of insoluble mineral phosphates present in the plates when inoculated with PSB are used for visual detection (Rodrìguez and Fraga, 1999). The plant growth promoting rhizobacteria provide fixed nitrogen, phosphorus, iron, phytohormones synthesized by the bacteria and can lessen the negative impacts of some pathogens also (Glick and Bashan, 1997). *Bacillus megaterium* is one of the commonly used PSB for preparation of commercial biofertilizer. Application of PSB led to increase in wheat and cotton yield, increase in potato biomass (Hameeda *et al.,* 2008; Qureshi *et al.,* 2012; Malboobi *et al.,* 2009). The aim of our study was to isolate and characterize such PSB from the rhizosphere of *Lablab purpureus* (L.) Sweet.

Materials and Methods

Top soil rhizosphere samples of *Lablab purpureus* (L.) Sweet were collected with the help of trowel from the agricultural field of Garudharia Charaihibi Gaon, Dibrugarh, Assam, India (Latitude-27.34206°, Longitude-94.809513°). The samples were placed in sterile plastic bags. The soil samples were stored at 4°C in the laboratory after collection and PSB were isolated within 2 weeks.

For isolation of PSB, Pikovskaya media containing tricalcium phosphates was used. Soil suspension consisting of 10 g soil in 90 ml distilled water was made and then serially diluted. The samples were then spread on Pikovskaya plates and the colonies that formed halo zone were considered as PSB.

Phosphate Solubilizing Index (PSI) was determined by spot inoculation of isolates in Pikovskaya agar plates containing tricalcium phosphates and incubated for 48 hours at 31°C. The diameter of colony and halo zone was measured after 48 hours. Phosphate Solubilizing Index was found out using formula given by Edi Premono *et al.,* 1996. PSI= (Colony diameter + Halozone diameter/Colony diameter).

Phosphate solubilized in Pikovskaya broth was estimated containing any of the three phosphate sources, aluminium phosphate ($AlPO_4$), ferric phosphate ($FePO_4$) and tricalcium phosphate ($Ca_3(PO_4)_2$). The pH of the medium with tricalcium phosphate was 7 before inoculation, whereas for aluminium phosphate and ferric phosphate the pH of the medium was acidic i.e. 5. Pikovskaya broth was inoculated with the isolated bacterial strains. Centrifugation of the broth was done at 10000 rpm for 10 min. Amount of phosphate solubilized was determined in the supernatant after 48,96 and 144 hours by the method given by Murphy and Riley, 1962.

The pH of the three different broths (aluminium, ferric and tricalcium) were noted after 48,96 and 144 hours.

Salinity test was performed using LB medium with 2%, 5% and 8% NaCl concentration. The plates were inoculated with the isolated bacterial strains and were observed after 72 hours at 28 °C (Gupta *et al.,* 2019; Barra *et al.*, 2016).

Ammonia production test was performed in peptone water 10 ml peptone water was taken and inoculated with 2b3 and incubated for 48 hrs at 28 °C. Similarly in another 10 ml, 2b5 was inoculated. After 48 hrs, 0.5 ml of Nessler's reagent was added to each tube. Production of brown to yellow colour indicated production of ammonia (Ahmad *et al.,* 2008).

Shape and colour of the isolated colonies were studied. Gram staining was done. Catalase test, sucrose fermentation test, citrate test, oxidase test, nitrate reduction test and gelatin hydrolysis test were performed for biochemical characterization of the isolates as listed in Bergey's manual of determinative bacteriology by following standard protocols.

Sequencing of 16S rRNA gene was done for analyzing the phylogenetic relationships. For the isolate 2b3,DNA was isolated from the bacterial culture and evaluated on 1.0% Agarose Gel. Amplification of DNA isolated was done with 16SF and 16SR primers. BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer was used for Sanger sequencing. For molecular characterization of 2b5, DNA isolated was evaluated on 1.8% Agarose Gel and amplified with 16S rRNA Specific Primer (27F and 1492R) where Veriti® 96 well Thermal Cycler was used. Sanger Sequencing using BDT v3.1 Cycle sequencing kit on ABI 3500Dx Genetic Analyzer was used. NCBI Nucleotide BLAST was used to find the sequences producing significant alignments. For both the isolates, phylogenetic analysis was done with MEGA11. Neighbour joining was the statistical method used for construction of phylogenetic tree. Number of bootstrap replications for bootstrap con-

sensus tree was 1000. Maximum composite likelihood was the substitution model.

The experiments were conducted in triplicates. Means and standard deviation (SD) were computed using Microsoft Excel.

Results and Discussion

Isolation of PSB

PSB were isolated on production of halo zone in Pikovskaya plates. Two isolates 2b3 and 2b5 which showed good amount of solubilization in Pikovskaya agar plates, i.e. the Phosphate Solubilization Index was higher than the rest of the bacterial colonies were selected for further study (Fig. 1).

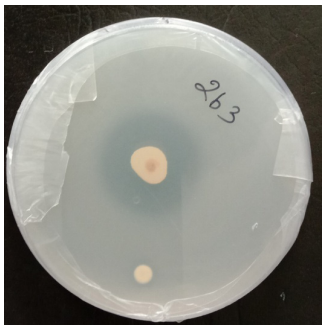


Fig. 1. Halo zone produced by 2b3 in Pikovskaya agar plate

Phosphate Solubilizing Index (PSI)

In the present study, both the isolates 2b3 and 2b5 produced halo zone in Pikovskaya agar plates. The PSI of 2b3 and 2b5 were 2.43±0.057 and 2.36 ± 0.200 respectively after 48 hours incubation in 31 °C. Organic acids production by PSB leads to the formation of halo zone (Halder and Chakrabartty, 1993; Rodrìguez and Fraga, 1999).

Phosphate Solubilized in liquid broth

Solubilization of phosphates was higher in Pikovskaya broth containing tricalcium phosphates than aluminium and ferric phosphates (Table 1). 2b5 showed higher phosphate solubilization than 2b3 in all the three types of media. Amount of phosphate solubilized in medium containing aluminium phosphates, ferric phosphates and tricalcium phosphates after 144 hours by 2b5 are 117.53 ±9.13, 109.15 ± 5.43 and 260.17±11.81 respectively.

Change in pH

The decline in pH correlated with increase in solubilization of insoluble phosphates (Table 1). Production of organic acids which help in the solubilization of insoluble mineral phosphates declines the pH of the broth (Halder and Chakrabartty, 1993).

Table 1. Amount of Phosphate solubilized in Pikovskaya liquid broth (aluminium, ferric, tricalcium) and the change in pH of the broth

	Aluminium Phosphate ($AlPO_4$)					
	48 hours		96 hours		144 hours	
	Phosphate Solubilized (µg/ml)	pH	Phosphate Solubilized (µg/ml)	pH	Phosphate Solubilized (µg/ml)	pH
2b3	87.05±5.01	3.92±0.02	94.84±5.62	3.91±0.02	104.27±4.06	3.73±0.03
2b5	89.08±10.16	3.90±0.01	109.6±5.69	3.83±0.02	117.53±9.13	3.60±0.02
	Ferric Phosphate ($FePO_4$)					
	48 hours		96 hours		144 hours	
	Phosphate Solubilized (µg/ml)	pH	Phosphate Solubilized (µg/ml)	pH	Phosphate Solubilized (µg/ml)	pH
2b3	58.96±3.90	4.21±0.01	95.15±5.52	4.11±0.01	103.05±14.02	3.89±0.01
2b5	66.90±4.67	4.08±0.005	107.6±6.95	3.94±0.02	109.15±5.43	3.75±0.01
	Tricalcium Phosphate ($Ca_3(PO_4)_2$)					
	48 hours		96 hours		144 hours	
	Phosphate Solubilized (µg/ml)	pH	Phosphate Solubilized (µg/ml)	pH	Phosphate Solubilized (µg/ml)	pH
2b3	143.11±4.23	5.32±0.02	174.57±6.91	4.51±0.02	207.14±27.74	4.34±0.04
2b5	148.24±6.53	5.24±0.02	226.66±5.73	3.44±0.05	260.17±11.81	3.20±0.02

Salinity tolerance test

2b5 showed better salinity tolerance in comparison to 2b3. Both the isolates showed growth in 2% NaCl. Growth of 2b5 was seen in 5% NaCl concentration but 2b3 showed no growth in 5% NaCl concentration. Both the isolates were not able to grow at 8% NaCl concentration (Fig. 2).

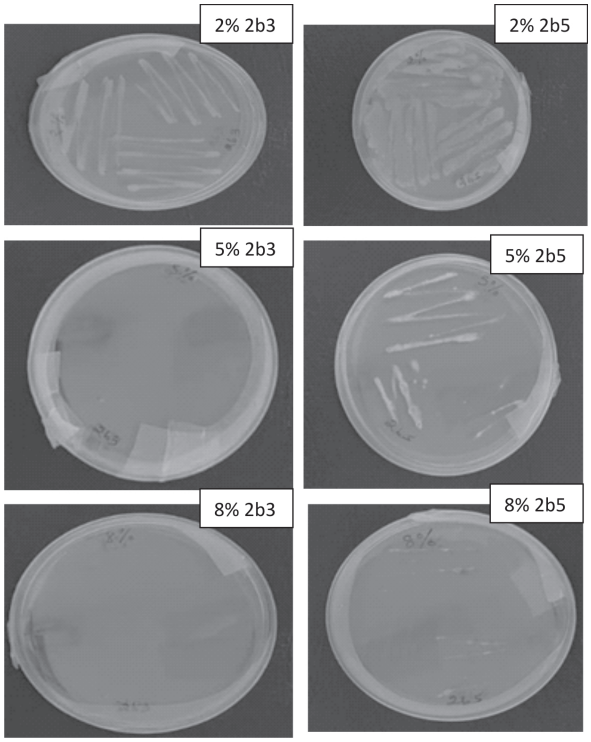


Fig. 2. Growth of the isolates in LB Agar supplemented with 2%, 5% and 8% NaCl concentrations

Ammonia production test

2b3 and 2b5 showed positive results for ammonia production which was based on the production of yellow colour in peptone water. This shows that the bacteria can help in supply of nitrogen to the plant.

Morphological and biochemical characteristics

Both the colonies were white and round. The isolates 2b3 and 2b5 were gram negative. 2b3 and 2b5 showed positive catalase and citrate utilization test. 2b3 showed negative sucrose fermentation test while the sucrose fermentation test was positive for 2b5.2b3 showed negative gelatin hydrolysis test and oxidase test. Both gelatin hydrolysis and oxidase test were positive for 2b5. Both was positive for nitrate reduction test.

Molecular characterization

For molecular identification, 16S rRNA gene sequencing was done. According to the NCBI BLAST results, 2b3 showed 98.97 percent identity with MH698887.1 and 2b5 showed 99.80 percent identity with MN398146.1. Both 2b3 and 2b5 FASTA sequences were submitted to NCBI GenBank. The accession number of 2b3 and 2b5 are OR985028 and OR947461 respectively (Table 2). According to the phylogenetic analysis in MEGA11, 2b3 exhibited close relation with *Burkholderia* sp. and so 2b3 was identified as *Burkholderia* sp. The other isolate 2b5 was identified as *Burkholderia cepacia*.

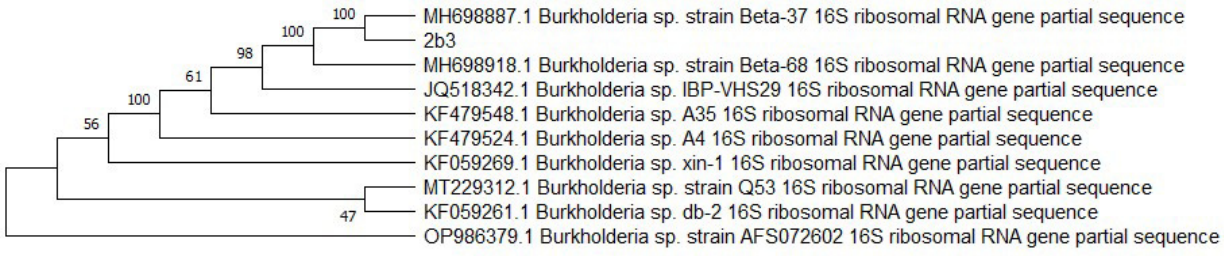
Burkholderia sp. from the rhizosphere of sweet corn showed phosphate solubilizing potential in both liquid and solid media (Pande *et al.*, 2020). *Burkholderia* sp. isolated from maize was the highest phosphate solubilizer among all the bacterial strains (Zhao *et al*., 2014). According to the study done by Megu *et al*., 2024, *Burkholderia* could tolerate salinity stress upto 2.5 %. *Burkholderia* sp. could release phosphates from tricalcium, ferric and aluminium phosphates (Ghosh *et al*., 2016). Phosphate Solubilizing Bacteria are promising natural fertilizers. In the present study, two bacterial strains *Burkholderia* and *Burkholderia cepacia* exhibited phosphate solubilizing ability in Pikovskaya medium containing either tricalcium phosphates, aluminium phosphates or ferric phosphates. Both the strains could grow at 2% salinity stress. Further studies will help in the development of these strains as potent biofertilizers that can be used in agricultural fields.

Acknowledgement

The authors are thankful to CSIR-UGC for providing JRF fellowship and Dibrugarh University for providing the facilities to carry out the research work.

Table 2. Molecular characterization of the isolates

Isolates	Percent identity	Identification	Accession number alloted
2b3	98.97% to MH698887.1	*Burkolderia* sp.	OR985028
2b5	99.80% to MN398146.1	*Burkholderia cepacia*	OR947461



(a)

(b)

Fig. 3. Phylogenetic tree showing the position of (a) 2b3 and (b) 2b5

Conflict of interest

There is no conflict of interest.

References

Ahmad, F., Ahmad, I. and Khan, M.S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research*. 163(2):173-181.

Anusuya, D. and Jayarajan, R. 1998. Solubilization of phosphorus by *Trichoderma viride*. *Current Science*. 74(5): 464-466.

Barra, P.J., Inostroza, N.G., Acuña, J.J., Mora, M.L., Crowley, D.E. and Jorquera, M.A. 2016. Formulation of bacterial consortia from avocado (*Persea* americana Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. *Applied Soil Ecology*. 102: 80-91.

de Bang, T.C., Husted, S., Laursen, K.H., Persson, D.P. and Schjoerring, J.K. 2021. The molecular-physiological functions of mineral macronutrients and their consequences for deficiency symptoms in plants. *New Phytologist*. 229(5): 2446-2469.

Edi-Premono, M., Moawad, A.M. and Vlek, P.L.G. 1996. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Sciences*. 11:13-23.

Ghosh, R., Barman, S., Mukherjee, R. and Mandal, N. C. 2016. Role of phosphate solubilizing *Burkholderia* spp. for successful colonization and growth promotion of *Lycopodium cernuum* L. (Lycopodiaceae) in lateritic belt of Birbhum district of West Bengal, India. *Microbiological Research*. 183: 80-91.

Glick, B.R. and Bashan, Y. 1997. Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. *Biotechnology Advances*. 15(2): 353-378.

Gupta, S. and Pandey, S. 2019. ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in French bean (*Phaseolus vulgaris*) plants. *Frontiers in Microbiology*. 10: 1506.

Gyaneshwar, P., Naresh Kumar, G., Parekh, L.J. and Poole, P.S. 2002. Role of soil microorganisms in improving P nutrition of plants. *Plant and Soil*. 245: 83-93.

Halder, A.K. and Chakrabartty, P.K. 1993. Solubilization of inorganic phosphate by Rhizobium. *Folia Microbiologica*. 38: 325-330.

Hameeda, B., Harini, G., Rupela, O., Wani, S. and Reddy G. 2008. Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. *Microbiological Research* 163: 234-242.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staloj, J.T. and Williams, S.T. 1994. *Bergey's Manual of Determinative Bacteriology*- 9th Ed. Williams & Wilkins. Baltimore.

Malboobi, M., Behbahani, M., Madani, H., Owlia, P., Deljou, A., Yakhchali, B., Moradi, M. and Hassanabadi, H. 2009. Performance evaluation of

potent phosphate solubilizing bacteria in potato rhizosphere. *World Journal of Microbiology and Biotechnology*. 25: 1479-1484.

Malhotra, H., Vandana, Sharma, S. and Pandey, R. 2018. Phosphorus nutrition: plant growth in response to deficiency and excess, p.171-190. In: *Plant Nutrients and Abiotic Stress Tolerance*. Springer: Singapore.

Megu, M., Paul, A. and Deb, C. R. 2024. Isolation and screening of stress tolerant and plant growth promoting root nodulating rhizobial bacteria from some wild legumes of Nagaland, India. *South African Journal of Botany*. 168:260-269.

Murphy, J. and Riley, J.P. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta.* 27: 31-36.

Pande, A., Kaushik, S., Pandey, P. and Negi, A. 2020. Isolation, characterization, and identification of phosphate-solubilizing *Burkholderia cepacia* from the sweet corn cv. Golden Bantam rhizosphere soil and effect on growth-promoting activities. *International Journal of Vegetable Science*. 26(6): 591-607.

Qureshi, M.A., Ahmad, Z.A., Akhtar, N., Iqbal, A., Mujeeb, F. and Shakir, M. A. 2012. Role of phosphate solubilizing bacteria (PSB) in enhancing P availability and promoting cotton growth. *The Journal of Animal and Plant Sciences.* 22: 204-210.

Rodrìguez, H. and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*. 17: 319-339.

Rodríguez, H., Fraga, R., Gonzalez, T. and Bashan, Y. 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant and Soil*. 287: 15-21.

Zhao, K., Penttinen, P., Zhang, X., Ao, X., Liu, M., Yu, X. and Chen, Q. 2014. Maize rhizosphere in Sichuan, China, hosts plant growth promoting *Burkholderia cepacia* with phosphate solubilizing and antifungal abilities. *Microbiological Research.* 169 (1):76-82.