BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, August 2014. Vol. **11**(2), 401-406

In vitro Phosphate Solubilization by *Bacillus* sp. NPSBS 3.2.2 Obtained from the Cotton Plant Rhizosphere

Narveer, Asish Vyas, Harsh Kumar and Chayanika Putatunda*

School of Biotechnology and Biosciences, Lovely Professional University, Punjab, India.

doi: http://dx.doi.org/10.13005/bbra/1288

(Received: 10 April 2014; accepted: 15 May 2014)

Cotton is one of the important cash crops of India. Apart from being an important source of fibres, cotton seeds also form an important source of edible oil. A lot of Phosphatic fertilizers are used to during cotton cultivation but a major portion of this Phosphate gets converted into insoluble form which is not available to the plants. However, there are certain Phosphate Solubilizing Bacteria which can solubilize inorganic insoluble phosphorous to inorganic soluble form and this form of phosphate can be easily used by plants. In present study isolation of Phosphate Solubilizing Bacteria was carried out from rhizosphere soil sample of cotton plants growing in various villages of District Mahendergarh, Haryana. A total of 15 bacterial isolates were obtained which were subjected to primary and secondary screening. On the basis of secondary screening NPSBS3.2.2 was found to solubilize the maximum amount of Phosphorous (0.129¼g/ml). The isolate was grown in different conditions and it was found that sucrose was the most suitable Carbon source at pH 7 at the incubation time period of 96 hours. The isolate was characterized morphologically, biochemically and on the basis of 16s rDNA sequencing (Genebank Accession Number- KF974682) and was found to be a member of the genus *Bacillus*.

Key words: Phosphate solubilization, bacteria, rhizosphere, cotton.

Phosphorus (P) is one of the most important macronutrient and plays a central role in conservation and transferring energy in cell metabolism. A reduction in seedling, plant establishment and root development may be resulted from deficiency of phosphorous. Deficient plants appear stunted dark green in color and exhibit delayed flowering, boll setting and crop maturity (Khan *et al*, 2009). Phosphorus exists in nature in variety of organic and inorganic form but mainly in either insoluble or in poorly soluble inorganic form. It is applied in the form of phosphate fertilizers. But a large portion of this inorganic phosphate applied to soil through these fertilizers is easily immobilized and are not in the usable form through precipitation reaction with cations like Al^{2+} ,Fe^{2+} and Ca^{2+} present in soil (Glodstein,1986; Gyaneshwar *et al.,* 2002; Hao *et al.*, 2002) Over application of phosphate fertilizer results into the phenomenon of overphosphatisation resulting in still lesser phosphate availability to plants(Landweert *et al.,* 2001) and also adds to the overall crop production cost. The excess of fertilizers may also be washed away and reach nearby water bodies resulting in eutrophication Many soil microorganisms like mycorrhizal fungi and the bacteria like *Rhizobium, Enterobacter, Bacillus, Pseudomanas* have ability to convert insoluble forms of phosphorus into bioavailable soluble form phosphorus *(*Igual *et al.*, 2001; Whitelaw, 2000). These microbes secrete organic acids like citric acid, glutamic acid which dissolve the mineral phosphates by anion exchange or

* To whom all correspondence should be addressed.
Mob.: +91-9779028811;
E-mail: putatunda7@gmail.com

chelation of both Fe^{3+} and Al^{3+} ions associated with phosphate (Gaur *et al.,* 1973). This leads to an increase in the availability of phosphorous to plants which in turn may result in better plant growth (Guiñazú *et al.,* 2010; Mohamed and Ibrahim, 2011).

Isolation of phosphate solubilising bacteria has been reported from a wide variety of habitats like alpine and sub-alpine regions (Selvakumar *et al*., 2009) and cold deserts (Gulati *et al*., 2009), arid and semi-arid regions (Srividya *et al*., 2009) saline-alkaline soils by some workers(Sharan *et al.,* 2008), phosphate mine (Farhat *et al.* 2009) brine well (Xiang *et al.* 2011).

Cotton (*Gossypium* sp.*)* is a very important cash crop grown across the globe. India is the second largest producer of cotton in the world with about 12.2 million hectares land. The yield per hectare is however, the lowest against the world average (http://cotcorp.gov.in/shares.aspx). The problems of many conventional farmers have increased because of decrease soil fertility, decrease in cotton price and enhancement in production cost. The cotton crop requires many of the nutrients for its growth out of which Nitrogen and Phosphorus are the major ones. The phosphorus requirement for cotton crop varies from land to land. But in a hectare of land farmers apply 10 to 20 kg of phosphorus in the form of fertilizers. The deficiency of this element cause the reduced quality of the crop (Qureshi *et al*,2012). So the main purpose of present research effort was to isolate those strains of bacteria which are able to convert this insoluble form of Phosphate to soluble form. This work may help to maintain the fertility of the soil and the use of fertilizers may be reduced.

MATERIALS AND METHODS

Isolation of Phosphate solubilizing Bacteria

Soil samples were collected from rhizosphere of cotton plants growing in various villages viz. Dongra Ahir, Bhalkhi, Mundia Khera of District Mahendergarh, Haryana. The soil samples were diluted up to 10^{-6} and plated on Pikovskaya's medium (Pikovskaya, 1948) and incubated at 37 ^{O}C for 48 hours. The composition of Pikovskaya's medium (gm/l) was: Glucose-10, Ca*f* ($PO_{,,}$), -5, ($NH_{,,}$), $SO_{,,}$ -0.5, NaCl-0.2, $MgSO_{,,}$ .7H, O-0.1,KCl-0.2, Yeast extract-0.5, $MnSO_{,,}$ .H, O- 0.001, $FeSO_{,,}$ .7H, O- 0.001;pH-7.0). The bacterial isolates were purified by streaking on fresh Pikovskaya's media & purified isolates were transferred on to slants and stored in refrigerated conditions at 4^{o}C.

Primary screening of bacterial isolates

The purified phosphate solubilising bacterial strains were spotted over Pikovskaya's media plates and incubated for 72 hours at 37 ^{0}C. The phosphate solubilization efficiency (PSE) was as per the given formula: PSE (in %) = (Z-C)/C X 100, where, Z= Solubilization zone diameter and C = Diameter of bacterial colony (Kundu *et al.,* 2009)

Secondary screening of bacterial isolates

One loop-full bacterial culture of isolates was transferred from 24 hrs old slants to 10 ml Pikovskaya's broth and incubated at 37 ^{0}C for 48 hrs. One ml of bacterial culture (O.D = 0.5 $\lambda_{600\ nm}$) was transferred to 100 ml Erlenmeyer flask containing 25 ml of Pikovskaya's broth. The flasks were incubated at 37 ^{0}C under shaking condition for 72 hrs. Subsequently, the bacterial culture was subjected to centrifugation at 10,000 rpm for 10 min. The pellet was discarded and the quantity of solubilized phosphorus in the supernatant was assessed by John's method (1970). The isolate showing highest phosphate solubilising activity was used for subsequent experiments.

Standardization of conditions

The conditions for maximum phosphate solubilization by selected bacterial isolates were optimized by varying the cultural conditions like pH (3 to 9), carbon source (Fructose, Sucrose, Mannitol, Lactose, Starch), nitrogen source (beef extract, peptone, tryptone, ammonium sulphate, ammonium chloride, potassium nitrate,) and agitation conditions etc. Temperature taken as 37ºC and incubation period for optimization was 48 hrs. During optimization process one of the conditions was varied in each experiment keeping the other variables constant.

Characterization of selected bacterial isolate

Selected strains were characterized for some morphological and biochemical characteristics according to Bergey's manual of determinative bacteriology (Kreig and Holt,1994). The 16s rDNA sequencing was done by Samved Biotech, Ahmedabad, India with the help of

27F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence sequence obtained was used to carry out BLAST with the nrdatabase of NCBI genbank database.

RESULTS AND DISCUSSION

A total of three soil samples were collected from cotton rhizosphere grown in different villages i.e. Dongra Ahir, Bhalkhi, Mundia Khera of District Mahendergarh, Haryana, India. A total of 15 bacterial isolates were obtained. Many scientists have isolated PSB from the rhizosphere soil of different plants like tomato, groundnut, maize, soyabean, mungbean, potato, Cotton, Wheat, Jowar, Sugarcane, Gram, Onion, Sunflower, Cabbage etc. (Chabot *et al.,* 1996; Ponmurugan and Gopi , 2006; Rajankar *et al*., 2007)

All purified bacterial isolates were spotted over solid Pikovskaya's media plates to check their insoluble phosphorus solubilizing efficiency. The Phosphate Solubilization Efficiency of all the isolates ranged between 25 to 33%. All the isolates were subjected to secondary screening under liquid culture. The isolate NPSBS3.6 showed the least activity of 0.02 µg/ml while the isolate NPSBS3.2.2 which was having less phosphorous solubilizing efficiency from primary screening(25%) solubilized maximum amount of Phosphorous in Pikovskaya broth(0.129¼g/ml). So this shows that there is no significant relation between primary and secondary screening. Similar observations were also made by some other workers (Balamurgan *et al*., 2010 and Banerjee *et al.,* 2010).

The selected isolate NPSBS3.2.2 was then followed up for the optimization process. In this conditions were varied to check the maximum solubilization of the selected strain with respect to, pH, carbon sources, nitrogen sources and incubation period

The medium pH is also one of the very crucial factors, influencing the phosphate solubilization efficiency. Out of all different values, i.e. pH 3, 4, 5, 6, 7, 8 and 9 it was found that

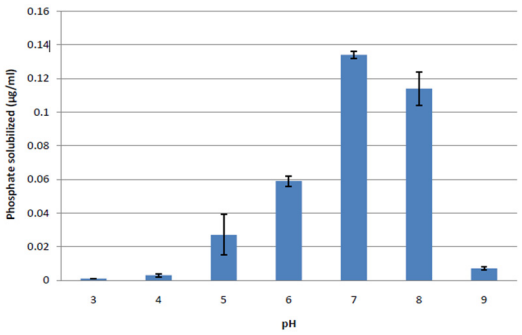

Fig. 1. Effect of pH on phosphate solubilizing efficiency (µg/ml) of isolate NPSBS3.2.2.

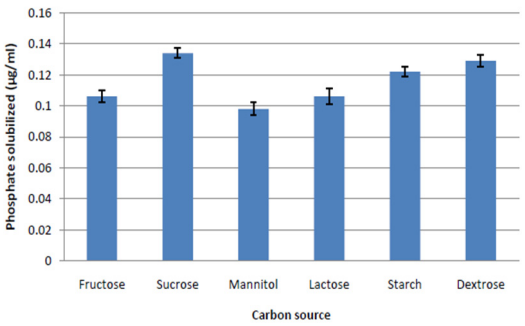

Fig. 2. Effect of various carbon sources on phosphate solubilizing efficiency (µg/ml) of isolate NPSBS3.2.2

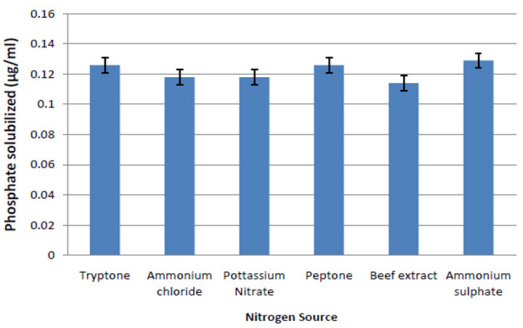

Fig. 3. Effect of various nitrogen sources on phosphate solubilizing efficiency (µg/ml) of isolate NPSBS3.2.2

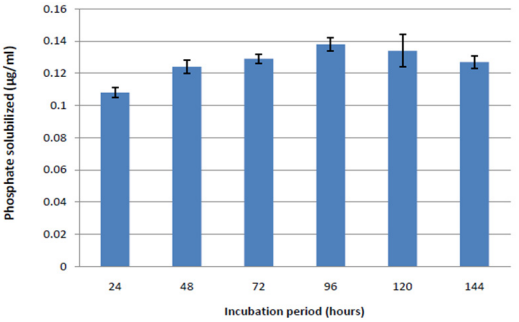

Fig. 4. Effect of incubation period on phosphate solubilizing efficiency (µg/ml) of isolate NPSBS3.2.2

pH 7 was optimum for maximum solubilization (0.134 ±0.002) ¼g/ml of calcium phosphate. The Phosphate solubilizing efficiency of the isolate was almost negligible pH 3(0.001¼g/ml) and it gradually increase up to pH 7 then it started to decrease on further increase in pH (Fig-1). Chen *et al*. (2005) also reported that in case of *Acinetobacter* CR 1.8, the maximum phosphate solubilization was recorded at pH 7 (3,820.6 lg ml^{-1}) followed by pH 5 (1,042.2 lg ml^{-1}). Zhu *et al*.(2011)and Sahu *et al*. (2007) also reported that pH7 was best suitable for Phosphate solubilization activity by isolates *Kushneria sp.* and *Streptomyces gallus* PS3 respectively. Several other workers have reported a range of pH 6 to 8 for phosphate solubilization (Naik *et al.,* 1982; Promod and Dhevendaran, 1987; Seshadri *et al.,* 2002).

Different carbon sources were used in broth to see the optimum result of the strain. Fructose, Sucrose, Mannitol, Lactose, Starch were used as carbon sources. The isolate was having the maximum activity in the Sucrose(0.134¼g/ml) followed by dextrose and starch. Least activity of 0.098 ¼g/ml was observed in the presence of mannitol. Similarly (Srividya *et al,* 2009) found that the isolate DASA 68056 was having the highest Phosphate Solubilization Efficiency when Sucrose was used as carbon source (Fig-2). However, Patel *et al*. (2008) observed higher Phosphate solubilization by *Citrobacter* sp. DHRSS in the presence of glucose and maltose as compared to sucrose and fructose.

Different nitrogen sources were used in broth to see the optimum result of the strain. Although the isolate showed comparable activity with all the Nitrogen sources, Ammonium sulphate was the best suitable nitrogen source, closely followed by peptone and tryptone (Fig-3). Similar results were shown by (Balamurgan *et al*, 2010) while optimizing the conditions the strain PSB22 and PSB37 showed best results in Ammonium sulphate. Many other workers have also reported ammonium sulphate to result in better Phosphate solubilization activity (Thakker *et al*.,1993; Kumari and Gupta, 2013). Several workers (Illmer and Schinner, 1992; Lapeyrie *et al*., 1991) have reported that a significant number of bacteria have been found of being capable to solubilizing phosphate only in the presence of ammonium as the nitrogen source.

The activity of the isolate was checked at different incubation time periods i.e. 24hrs, 48hrs, 72hrs, 96hrs, 120hrs, and 144hrs at 37^0C. The activity of the isolate after 24 hours was quite good (0.108µg/ml) and was maximum after 96 hours(0.138¼g/ml). Phosphorous Solubilizing Efficiency however decreased with further increase in time (Fig.-4). Kumar and Rath (2013) found that maximum activity of the strain of *Bacillus* sp. and *Pseudomonas* sp. were after 72hours of incubation. Banerjee *et al*. (2010) also reported maximum phosphate solubilization by the bacterial isolate TRSB10 after incubation of 3 days. However some other workers have reported more than 10 days (Sridevi and Mallaiah 2009)and even upto 15 days (Pandey *et al*., 2006; Sahu *et al*., 2007) to be the optimum time for P solubilization by various bacterial isolates. When the isolate was grown under the optimized conditions (pH-7, Carbon source-sucrose, Nitrogen source-ammonium sulphate and Incubation time-96 hrs under shaking conditions) and the amount of phosphorous solubilized was enhanced from 0.129µg/ml to 0.432µg/ml.

Selected isolate was characterized for various morphological biochemical characteristics and was found to be Gram positive, with spore formation, rod shaped, motile, indole negative, Methyl Red positive , Voges-Proskauer negative, Citrate positive, Catalase positive. The isolate was also characterized on the basis of 16s rDNA sequence(Genebank Accession Number-KF974682) and on the basis of it's biochemical and molecular characteristics, the isolate seemed to be similar to *Bacillus* sp.

So, in the current study, a total of 15 isolates were obtained from rhizosphere of cotton plant, out of which the isolate NPSBS3.2.2 showing highest phosphate solubilization was selected and conditions for highest phosphate solubilization in vitro were assessed. The pH-7, sucrose (Carbon source) , -ammonium sulphate (Nitrogen source) and Incubation time of 96 hrs under shaking conditions were found to be suitable for maximum phosphate solubilization by the isolate. The morphological, biochemical and molecular characteristics the isolate was found to be the member of genus *Bacillus.* Since, the isolate *Bacillus* NPSBS3.2.2 has shown good phosphate solubilization activity under *in vitro* conditions,

it may prove to be a promising isolate for *in* vivo phosphate solubilization also.

REFERENCES

1. Khan A. A., Jilani G., Akhtar M. S., Naqvi S. M. S., Rasheed M. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *J. Agric. Bio. Sci.*, 2009; **1**: 48-58
2. Goldstein, A.H.. Bacterial solubilization of mineral phosphates: historical perspectives and future prospects. *Am. J. Altern. Agricult*., 1986; **1**: 57–65.
3. Gyaneshwar P., Kumar G. N., Parekh L. J., Poole P. S. Role of soil microorganisms in improving P nutrition of plants. *Plant Soil.,* 2002; **245**: 83–93
4. Hao X., Cho C. M., Racz G. J., Chang C. Chemical retardation of phosphate diffusion in an acid soil as affected by liming. *Nutr. Cycl. Agroecosys*., 2002; **64**: 213-224
5. Landweert R., Hooffland E., Finlay R.D., Kuyper T.W. , van Breemen N. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends Ecol. Evol.,*2001; **16**: 248–254
6. Igual, J. M., A. Valverde, E. Cervantes, E. Velázquez. Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. *Agronomie*., 2001; **21**: 561-568.
7. Whitelaw, M. A. Growth promotion of plants inoculated with phosphate solubilizing fungi. *Adv. Agron*., 2000; **69**: 99-151
8. Gaur, A.C., Madan M. and Ostwal K. P. Solubilization of phosphatic compounds by native microflora of rock phosphates, *Indian J. Exptl. Biol.,*1973; **11**: 427–429,
9. Guiñazú , L.B., Andrés, J.A., Papa, M.F.D., Pistorio, M., Rosas, S.B. Response of alfalfa (*Medicago sativa* L.) to single and mixed inoculation with phosphate-solubilizing bacteria and *Sinorhizobium meliloti. Biol Fertil Soils*., 2010; **46**: 185-190.
10. Mohamed, H. M., Ibrahim, E. M. A. Effect of inoculation with *Bacillus polymyxa* mutants on growth, phosphorus and iron uptake by tomato (*Lycopersicon esculentum* L.) in calcareous soils. *Inter. J. Soil Sci.,* 2011; **6**:176-187
11. Selvakumar, G., Joshi, P., Mishra, P. K., Bisht J., Gupta, H. S. Mountain aspect influences the genetic clustering of psychrotolerant phosphate solubilizing *Pseudomonads* in the Uttarakhand Himalayas. *Curr. Microbiol*.,2009; **59**: 432–438
12. Gulati, A., Rahi, P., Vyas P. Characterization of phosphate-solubilizing fluorescent Pseudomonads from the rhizosphere of Seabuckthorn growing in the cold deserts of Himalayas, *Curr Microbiol.,*2008; **56**: 73–79
13. Srividya, S., Soumya, S., Pooja, K. Influence of environmental factors and salinity on phosphate solubilization by a newly isolated *Aspergillus niger* F7 from agricultural soil. *Afri. J. Biotechnol*., 2009; **9**: 1864-1870
14. Sharan, A., Shikha, Darmwal, N. S., Gaur, R., *Xanthomonas campestris*, a novel stress tolerant, phosphate-solubilizing bacterial strain from saline–alkali soils, *World J. Microbiol. Biotechnol.,*2008; **24**: 753–759
15. Farhat, M. B., Farhat, A., Bejar, W., Kammoun, R., Bouchaala, K., Fourati, A., Antoun, H., Bejar, S., Chouayekh H. Characterization of the mineral phosphate solubilizing activity of *Serratia marcescens* CTM 50650 isolated from the phosphate mine of Gafsa, *Arch Microbiol*., 2009; **191**: 815–824
16. Xiang, W., Liang, H., Liu, S., Luo, F., Tang, J., Li, M., Che, Z. Isolation and performance evaluation of halotolerant phosphate solubilizing bacteria from the rhizospheric soils of historic Dagong Brine Well in China, *World J. Microbiol. Biotechnol.,* 2011; **27**: 2629–2637
17. Qureshi, M.A., Ahmed, Z.A., Akhtar, N., Iqbal, A., Mujeeb, F., Shakir, M.A., Role of Phosphate Solubilizing Bacteria in Enhancing P Available Promoting Cotton Growth. *J. Animal Plant Sci.* 2012; **22**: 204-210.
18. Pikovskaya, R.I., Mobilization of phosphorus in soil in connection with vital capacity of source microbial species, *Microbiologiya,* 1948;**17**: 362–370.
19. Kundu, B. S., Nehra, K., Yadav, R., Tomar M., Biodiversity of phosphate solubilizing bacteria in rhizosphere of chickpea, mustard and wheat grown in different regions of Haryana, *Indian J. Microbiol*.,2009; **49**:120–127.
20. John, M.K. Colorimetric determination of phosphorus in soil and plant materials with ascorbic acid. *Soil Sci.*1970;**109**: 214–220.
21. Krieg N. R. and Holt G. Bergey's Manual of Determinative Bacteriology, 9th Edn, Baltimore: Williams and Wilkins,1994
22. Chabot, R., Antoun H., Cesas, M. P., Growth promotion of maize and lettuce by phosphate solubilizing *Rhizobium leguminosarum* biovar *phaseoli, Plant Soil*. 1996; **184**: 311–321
23. Ponmurugan, P., Gopi C., Distribution pattern and screening of phosphate solubilizing bacteria isolated from different food and forage crops. *J. Agron*. 2006; **5**: 600-604.
24. Rajankar, P.N., Tambekar, D.H., Wate, S.R. Study of phosphate solubilization efficiencies of fungi and bacteria isolated from saline belt of

Purna river basin. *Res. J. Agric. Biol. Sci*. 2007; **3**: 701-703.

25. Balamurgan, A., Princy, T., Vidhyapallavi, R., Nepolean, P., Jayanthi, R. and Premkumar, R. 2010. Isolation and Characterization of Phosphate Solubilizing Bacteria in Tea. *J. Biosci.,* 2010; **1**: 285-293.
26. Banerjee, S., Palit, R., Sengupta, C., Standing, D., Stress induced phosphate solubilization by *Arthrobacter* sp. and *Bacillus* sp. isolated from tomato rhizosphere, *Aus. J. Crop Sci*., 2010; **4**: 378-383.
27. Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., Young C.C., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, *Appl. Soil Ecol.,* 2005; **34**: 33-41.
28. Zhu F, Qu L, Hong X, Sun X, Isolation and characterization of a phosphate-solubilizing halophilic bacterium *Kushneria* sp. YCWA18 from Daqiao Saltern on the coast of Yellow Sea of China. *Evid. Compl. Alternat. Med*, 2011; article ID-615032:6
29. Sahu, M. K., Sivakumar, K., Kannan, L., Phosphate solubilizing actinomycetes in the estuarine environment: An inventory, *J. Environ. Biol.,* 2007; **28**: 795-798.
30. Naik, V.M., D'Souza J., Araujo A. Phosphorus solubilizing yeasts in estuarine environment. *Indian J. Mar. Sci*., 1982; **11**: 197-198.
31. Promod, K., Dhevendaran K. Studies on phosphobacteria in Cochin backwater. *J. Mar. Biol. Ass. India*, 1987; **29**: 297-305.
32. Seshadri, S., Ignacimuthu S., Lakshminarsimhan C. Variation in heterotrophic and phosphate solubilizing bacteria from Chennai, southeast coast of India. *Indian J. Mar. Sci*., 2002; **31**: 69-72.
33. Srividya, S., Soumya, S., Pooja, K. Influence of environmental factors and salinity on phosphate solubilization by a newly isolated *Aspergillus niger* F7 from Agricultural Soil. *Afr. J. Biotechnol,* 2009; **8**: 1864-1870.
34. Patel, D. K., Archana, G., Kumar G. N. Variation in the Nature of Organic Acid Secretion and Mineral Phosphate Solubilization by *Citrobacter* sp. DHRSS in the Presence of Different Sugars, *Curr Microbiol*., 2008; **56**: 168-174.
35. Thakker, J., Narsian, V., and Patel, H. H. Inorganic phosphate solubilization by certain soil bacteria. *Indian J Exp Biol*., 1993; **31**: 743-746.
36. Kumari, P. P., Gupta, P. C. Effect of different carbon and nitrogen sources on solubilization of insoluble inorganic phosphate by psychrotolerant bacterial strains. *Bioscan*, 2013; **8**: 1299-1302
37. Illmer, P., Schineer, F. Solubilization of hardly-soluble $AlPO_4^-$ with solubilizing microorganisms. *Soil Bio. Biochem.,* 1992; **24**: 389-395.
38. Lapeyrie, F., Ranger J., Vairelles, D. Phosphate solubilizing activity of ectomycorrhizal fungi *in vitro. Canadian J. Bot.* 1991; **69**: 342-346.
39. Kumar, S., Rath, C. Optimization of culture conditions of phosphate solubilizing activity of bacterial sp. isolated from Similipal Biosphere Reserve in solid-state cultivation by response surface methodology. *Int. J. Curr. Microbiol. App. Sci.* 2013; **2**: 47-59.
40. Sridevi M., Mallaiah K.V., Phosphate solubilization by *Rhizobium* strains, *J. Microbiol. Biotechnol.,* 2009; **49**: 98-102.
41. Pandey, A., Trivedi, P., Kumar, B., Palni, L. M. S. Characterization of a phosphate solubilizing & antagnostic strain of *Pseudomonas putida* (BO) isolated from a sub-Alpine location in the Indian Central Himalaya, *Curr. Microbiol.,*2006; **53**:102-107.