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Comparative Analysis: Physiological and Biochemical Characterization of Phosphate Solubilization by *Pseudomonas* and *Bacillus* species from various agricultural areas of Dehradun, Uttarakhand, India

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ABSTRACT

The current research paper involve work on comparative physiological and biochemical characterization of phosphate solubilization *Bacillus* and *Pseudomonas*. *Bacillus* and *Pseudomonas* bacteria are ubiquitous in nature and most intensively studies microbial genera of soil. They are major phosphate solubilizer of soil that convert insoluble P into soluble P, thus improve the absorption and use of soil Phosphorus by plants. The Phosphorus (P) is an essential macro-nutrient present in the soil which are required by plant for their growth. It is a component of the complex nucleic acid structure of plants, which regulates protein synthesis thus play vital role in cell division and development of new tissue and important in plant metabolic process which such as respiration, photosynthesis, energy storage and nitrogen fixation. It is also important in seed germination, seedling establishment. Despite its importance it is unavailable to plant as it is present in the form of organic and inorganic compounds, which is mostly inactive to the plants. Thus, plant need an alternative method for utilization of phosphorus and in this phosphate solubilizing bacteria (PSB) play a vital role. Phosphate solubilizing bacteria are group of beneficial bacteria which solubilize inorganic phosphorus to plant-available forms. *Bacillus* and *Pseudomonas* are one of some PSB bacteria which are found in soil.

Keywords: Soil phosphorus, *Bacillus, Pseudomonas,* Phosphate solubilizing bacteria

INTRODUCTION

Phosphorus (P) is an important element in soil and second most crop nutrient after nitrogen, also an essential macronutrient required for the growth of plants (Abdalla *et al*., 2007). It is a crucial nutrient for increased sustained agricultural productivity which limits plant

growth in many soils section (Scervino *et al*., 2011, Ha and Tran, 2014). Phosphorus involves in many essential processes like cell division, development, photosynthesis, breakdown of sugar, nutrient transport within the plant, transfer of genetic characteristics from one generation to another and regulation of metabolicpathways (Schulze *et al*., 2006, Zhang *et al*., 2014; Balemi and Negisho, 2012). Therefore, Phosphorus (P) deficiency in plants result in the browning of leaves accompanied by small leaves, weak stem, and slow development by slowing the growth of stem (Fernández *et al*., 2014). The phosphorus constraint directly decrease photosynthesis in plant reducing the orthophosphate concentration in the chloroplast s to levels that inhibit ATP synthase activity (Mara *et al*., 2014). P also play important role in metabolism of nitrogen compounds, carbohydrate transportation, carbohydrate metabolism and fat metabolism (Vance, 2001, Vance *et al*., 2003).

Total phosphorus present in soil and its concentration in soil ranges from 0.01 to 0.2%, with an average approximately 0.05% (Lo´pez-Arredondo *et al*., 2014, Brady *et al*., 2008). But out of this only small portion is available to plants which makes about 0.2% of plant dry weight (Chen *et al*., 2008). Although concentration of P in soil is 2000-fold higher than the plant, its fixation in the form of aluminium/iron or calcium/ magnesium phosphates renders its unavailable for uptake by plants. Phosphorus in soil is found in two forms, namely organic and inorganic Approximately 30 to 65 percent of total soil phosphorus is in organic forms, which are not plant available, while the remaining 35 to 70 percent is in inorganic forms. Inorganic form includes Sorbed phosphorus, Mineral phosphorus while organic forms of phosphorus include dead plant/animal residues and soil micro-organisms (Weil & Brady, 2017). Soil micro-organisms play a key role in processing and transforming these organic forms of phosphorus into plant available forms, these microorganisms are referred as phosphorus solubilizing bacteria (PSB) (Prasad *et al*., 2019, Liu *et al*. 2015). Phosphate solubilizing bacteria (PSBs) convert unavailable P (both Pi and Po) into available P to satisfy the requirements of plants through dissolution and absorption. PSB are widely distributed in soil although their number varies based on climatic condition and properties of soil.

Phosphate solubilizing bacteria (PSB) are an integral part in phosphorus cycle and are also responsible for the cycling of insoluble phosphorus to soluble phosphorus orthophosphate , which is the only form that are taken up by plant roots (Balemi and Negisho, 2012, Ahemad & Kibret, 2014, Datta & Basu,2000). PSB produce indole acetic acids (IAA) which evoke plants cell to grow, protein synthesis which stimulates the production of longer roots and roots laterals (Sadaf *et al*., 2009, Kim *et al*.,1997; Santana *et al.,* 2016) and it is documented that IAA improve growth parameters , photosynthesis and NPK concentration in soil which effect the overall growth of plant (Mittal *et al*., 2008; Yousefi *et al*., 2011). PSB also promotes plant growth by producing phytohormones such as auxin, gibberellins, cytokinin's, other than that it produces organic acid such as carboxylic, glycolic, malonic, fumaric and alpha ketoglutaric acid (Vikram and Hamzehzarghani, 2008). They help in production antibiotics, hydrogen cyanate a type of phytopathogens which help in defence mechanism of plants (Afzal and Bano, 2000; Whitelaw, 2000). Numerous research has been done on screening of PSB and some of the most common examples are the species of *Pseudomonas, Bacillus, Micrococcus, Flavobacterium, Aspergillus, Penicillium, Fusarium, Sclerotium, etc. Strains from bacterial genera Pseudomonas, Bacillus, Rhizobium and Enterobacter* along with *Penicillium and Aspergillus* fungi are the most powerful P solubilizer (Subbarao, 1998). *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata,* and *Enterobacter* are referred as the most important strains (Kucey *et al.,* 1989, Rodríguez& Fraga, 1999), butmost of PSB are Gram- negative bacteria in nature which belong to *Pseudomonas, Acinetobacter Pantoea*, and *Enterobacter* and some PSB are Gram-positive bacteria belonging to *Bacillus*and among them Bacillus and pseudomonas are mostly worked bacteria (Osborne *et al*., 2002).

MATERIAL AND METHODS

Sample collection About 10 soil samples were collected from different locations of Dehradun, Uttarakhand by digging1-1.5cm depth from surface and kept in sterile zip-lock bag and transported to laboratory for further studies. (Table 1)

Isolation, selection, and maintenance of phosphorus solubilising bacteria (PSB)

Serial dilutions were made of the different soil samples. The same was done by dissolving 1g of

TABLE 1: Collection of Samples from various agricultural areas

Sample No.	Location
1.	Anarwala
2.	Mohkampur
3.	Karanpur
4.	Naya Gaon
5.	Badripur
6.	Prince chowk
7.	Robber's cave
8.	Jogiwala
9.	Rajpur
10.	Survey chowk
11.	Dilaram
12.	Miyanwala
13.	Premnagar
14.	Doiwala
15.	Garhi Cantt

sample in 10ml of sterile normal saline (N.S). Then 25µl of above diluted samples were lawn cultured was plated on National Botanical Research Institute's phosphate growth medium (NBRIP) agar plates (Nautiyal, 1999) [including per litre: 10 g glucose, 5.0 g MgCl2.6H2O, 0.25 g MgSO4.7H2O, 0.1 g (NH4)2SO4, 0.2 g KCl, 5.0 g Ca3(PO4)2, and 15 g agar, with pH adjusted to 7.5–8.0 media plates, which were then incubated at 27-30°C for 48 hours. After incubation, the selected colonies with zone were streaked on freshly made NBRIP plates for pure colony isolation. Different samples were taken from which 20 strains were isolated through streaking. The colonies were further streaked in Nutrient agar plates followed by blood agar and cetrimide agar for pure culture and identification.

ASSESSMENT OF PHOSPHATE SOLUBILIZATION

Primary Screening on the basis of zone pattern

The NBRIP plates having zone forming colonies were re-streaked on NBRIP plates in triplicates or quadruplicate form. Then the phosphate solubilization index and efficacy were calculated using the formula below:

$$\textit{Phosphate solubilizing Index}\ (\%) = \frac{\textit{zone diameter} + \textit{Colony diameter}}{\textit{colony diamter}} X100$$

Qualitative Estimation of Phosphate Solubilization

The bacterial cultures were screened for their phosphate-solubilizing ability by inoculatingthe phosphate solubilizing strains in 30 ml capacity test tube containing 5ml NBRIP-BPB broth.Autoclaved uninoculated broth serves as control. Test tubes were incubated for 10 days at 30°c inshaker and observed for 10 days visually.

Quantitative Estimation of Phosphate Solubilization

The bacterial strains were inoculated in 150 ml conical flask containing 50 ml NBRIP broth. Conical flask was incubated at 30°c in rotary shaker for 10 days and phosphate solubilization was quantified for 1,3,5,7, &10respectively by using Fiske (Fiske and Subbarow, 1925) method. After incubation period,1ml of culture was dispensed in Eppendorf tubes and centrifuged at 10,000rpm for 10 mins.100µl of aliquot was transferred to fresh test tube and 4.2 ml of distilled water was added. After this 500µl of ammonium molybdate solution was added followed by addition of 200µl of amino-naphthol solution in each test -tube to make up the volume 5.0 ml. The mixture was mixed properly and kept at room temperature for 30 min. Absorbance was measured at 660 nm against blank.

Utilization of Nitrogen (N) sources

The strains were stabbed on M9-Minimal salt media plates in which ammonium chloride was replaced by various 'N' sources as ammonium sulphate and ammonium acetate. The plates were incubated at 30°and growth was observed for 10 days.

ENZYMATIC ACTIVITY OF ISOLATED STRAINS

Cellulolytic activity

The test tubes containing 5ml of Luria-Bertani broth were inoculated with isolated strains and incubated at 30°c for 24 hours. 10µl of bacterial suspension was spotted onto the cellulolytic media plate containing substrate of enzyme to be tested and incubated at 28°c for 48 hrs. For Visualization of β-D glucan hydrolysis, the agar medium was flooded with an aqueous solution of Congo red (1mg/ml) for 15min and then with NaCl (58.4g/l). Clear Yellow halo formed around colonies exhibiting the Carboxymethyl Cellulose (CMCase) activity.

Proteolytic activity

The test tubes containing 5ml of Luria-Bertani broth were inoculated with isolated strains and incubated at 30°c in rotary shaker for 24 hours. 10µl of bacterial suspension was spotted onto the Nutrient agar plates containing 1% skimmed milk and incubated at 28°c for

48 hrs. The clear zone formed around colonies showed the presence of proteolytic activity.

Amylolytic activity

The bacterial strains were stabbed on starch agar media plates and incubated at 280C in BOD incubator for 48 hrs. The plates were flooded with iodine solution for 10 min. The clear halo around bacterial colonies exhibits amylolytic activity.

Quantitative Estimation of Auxin Production

The test tubes containing 5ml of NBT were inoculated with *Pseudomonas* and *Bacillus* strains and incubated at 300C in rotary shaker for 48 hours. After incubation, the 5ml culture was centrifuged at 10,000 rpm for 10 mins and supernatant was collected. An aliquot of 2ml supernatant was pipetted out to a fresh tube to which 100µl of 10mM orthophosphoric acid and 4ml Salkowski's reagent was added and mixed thoroughly. The reaction mixture was incubated at room temperature for 25 min. and the absorbance of pink color developed was measured at 530 nm against a reagent blank.

PQQ (Pyrrole Quinoline Quinone Activity)

The ability of PQQ independent/dependent activity of GDH (Glucose dehydrogenase) of 20 bacterial strains were checked in MacConkey +1% glucose plate at 30°C. The plates were stabbed with 20 bacterial strains. These plates were kept for overnight at 30°C in incubator.

RESULTS

All the 20 isolates were screened for Phosphate solubilization on NBRIP medium and showed positive result in primary screening. All the isolates produce zone forming colonies in NBRIP medium

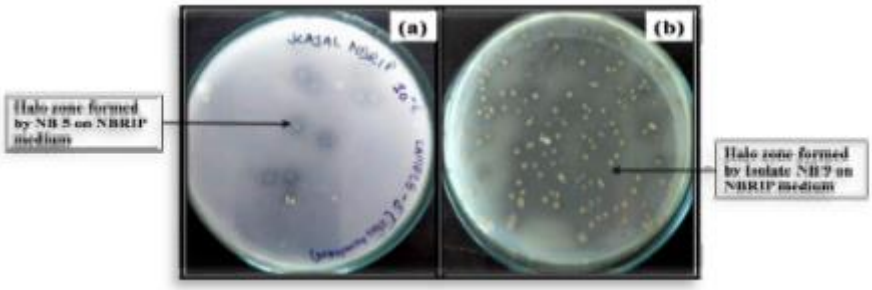

FIGURE 1:- Culture in NBRIP Medium

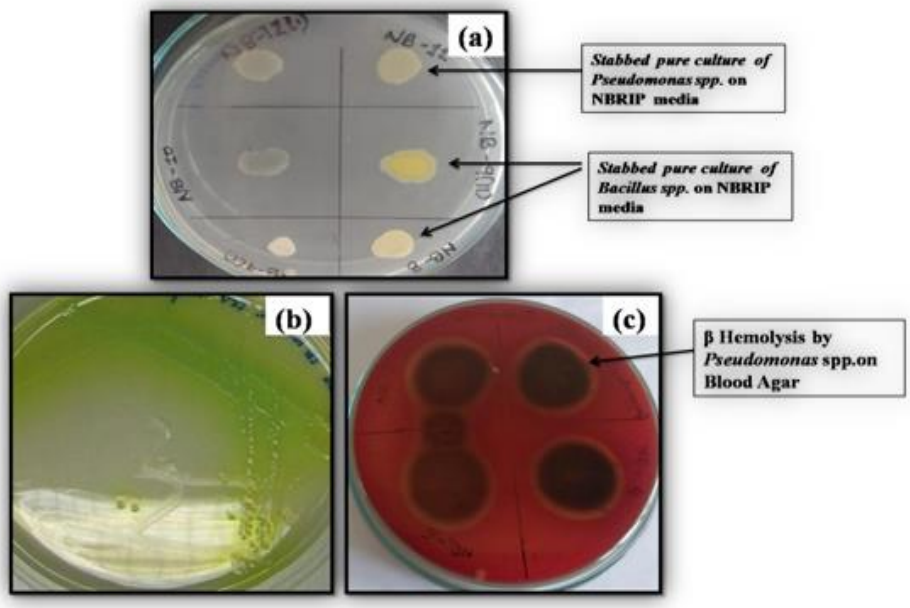

FIGURE 2:- Cultural Characterisation (a) Pure culture of *Bacillus spp*. (NB 8) and *Pseudomonasspp*.(NB11)onNBRIPmedium(b)*Pseudomonasspp*(NB3)onCetrimideagar. *Pseudomonas spp*. (NB3, NB4, NB5,and NB6) on Bloodagar

Qualitative estimation of solubilized Phosphate
Around 20 Bacterial isolates were tested for their phosphate solubilization ability using NBRIPBPB medium. Solubilization of mineral phosphate in NBRIP-BPB media resulted in decolorization of blue color of the media after overnight incubation.

Quantitative estimation of solubilized Phosphate
Inoculation of Bacterial strains in NBRIP liquid medium resulted in gradual increase in the amount of soluble phosphate in the medium up to 10 days. The result indicates 'P' solubilization ability by *Bacillus* and *Pseudomonas* strains. 'P' solubilization by efficient 'P' solubilizer ranges from 40-100µg/ml pi whereas in case of moderate phosphate solubilizer the range was 10-40µg/ml pi. Among all the test cultures NB5 & NB13(i) was selected as the best with 146.75 µg/ml pi followed by NB6 with 141.75µg/ml pi ,NB12(i) with 137.89µg/ml, NB12(ii) with 107.46µg/mlpi, NB14(ii) with 102.57µg/ml pi,NB13(ii) with 101.20µg/ml,NB2 with 94.88µg/ml pi, NB8 with 94.85µg/ml,NB12(iii) with 94.13µg/ml pi on 5th day of incubation.

Effect of utilization of different Nitrogen sources (M9 Media)
Utilization of Nitrogen (N) sources
The strains were stabbed on M9 media plates with the substitution of different Nitrogen source and growth was observed for 10 days at 30°c. The Nitrogen sources used were Ammonium chloride, Ammonium sulphate, Ammonium acetate, Magnesium nitrate. All the strains show good utilization of Ammonium chloride, Ammonium acetate, Ammonium sulfate. Magnesium nitrate was utilized by Bacillus spp NB2, NB7, NB8, NB9(i), NB9(ii), NB12(ii), NB13(iii), NB14(i), NB15.

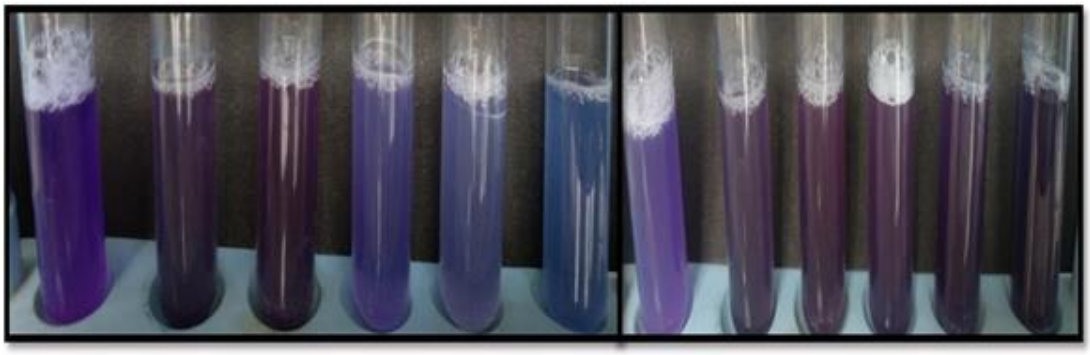

FIGURE 3: Qualitative estimation of solubilized Phosphate

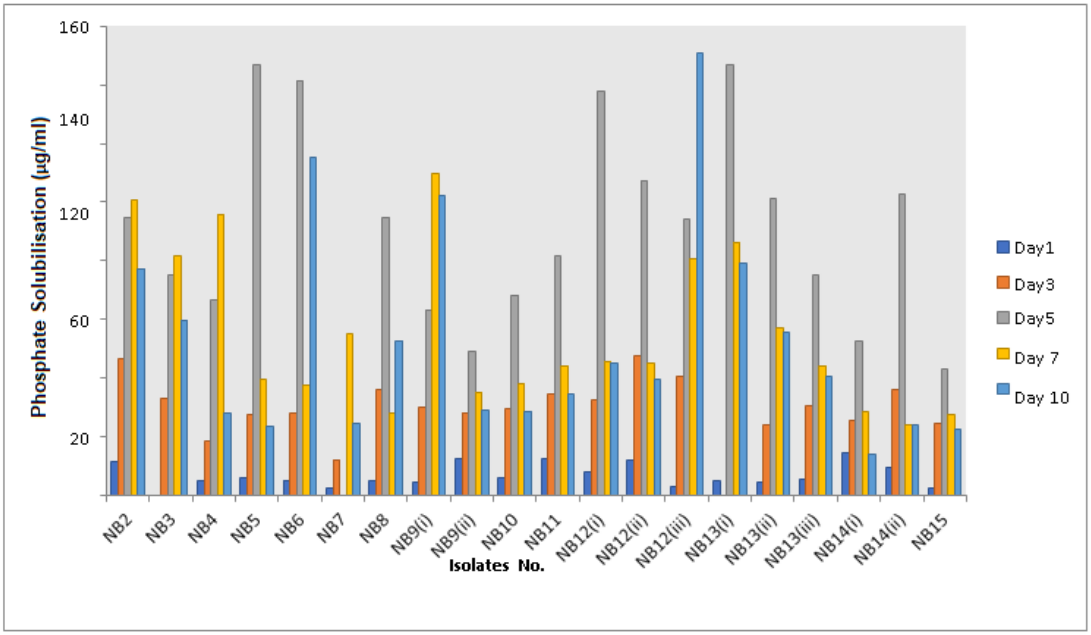

FIGURE 4: Phosphate solubilization ability of isolated *Pseudomonas* and *Bacillus* strain

TABLE 2: Utilization of nitrogen source by various Samples

S. No.	Isolate No.	Nitrogen utilization			
		Ammonium chloride	Ammonium sulphate	Ammonium acetate	Magnesium Nitrate
1	NB2	+(1)	+(1)	+(1)	+(1)
2	NB3	+(1)	+(1)	+(1)	-
3	NB4	+(1)	+(1)	+(1)	-
4	NB5	+(1)	+(1)	+(1)	-
5	NB6	+(1)	+(1)	+(1)	-
6	NB7	+(1)	+(1)	+(1)	+(1)
7	NB8	+(1)	+(1)	+(1)	+(1)
8	NB9(i)	+(1)	+(1)	+(1)	+(1)
9	NB9(ii)	+(1)	+(1)	+(1)	+(1)
10	NB10	+(1)	+(1)	+(1)	-
11	NB11	+(1)	+(1)	+(1)	-
12	NB12(i)	+(1)	+(1)	+(1)	-
13	NB12(ii)	+(1)	+(1)	+(1)	+(1)
14	NB12(iii)	+(1)	+(1)	+(1)	-
15	NB13(i)	+(1)	+(1)	+(1)	-
16	NB13(ii)	+(1)	+(1)	+(1)	-
17	NB13(iii)	+(1)	+(1)	+(1)	+(1)
18	NB14(i)	+(1)	+(1)	+(1)	+(1)
19	NB14(ii)	+(1)	+(1)	+(1)	-
20	NB15	+(1)	+(1)	+(1)	+(1)

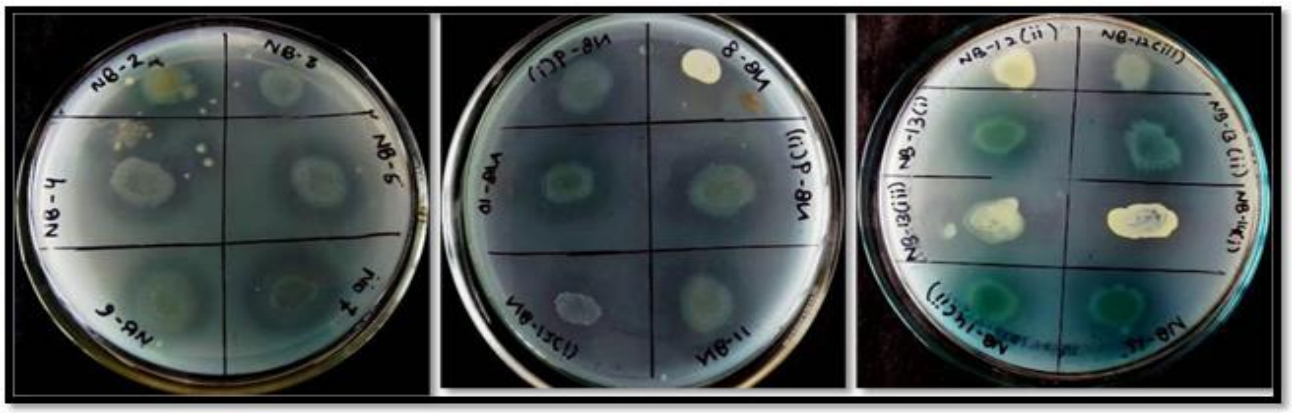

FIGURE 5: Detection of protease production by bacterial strains (Halo zones indicate positive results)

ENZYMATIC ACTIVITIES

Cellulase activity of isolated bacterial strains All the strains were tested for their cellulolytic properties. None of the P-solubilizer strains having cellulolytic activity.

Proteolytic activity of bacterial strains Proteolytic property of bacterial strains is attributed to bacterial strains for making the complex nutrient into a simple form to make it available for the plants. Among all isolates except NB12(ii) formed a clear zone around the colonies exhibiting proteolyticactivity.

Amylolytic activity of isolated strains Amylolytic activity is a character of solubilizing Amylase. All the 20 isolates were tested for amylolytic activity. Among the 20 isolates, the clear zone was observed around colonies of strains NB7, NB8 & NB14(i), depicting its amylase producing activity.

FIGURE 6: Detection of amylase production by bacterial strains (Yellow zones indicate positive results)

Auxin assay

Auxin is a plant growth promoting enzymes produced by the bacterial strains having plant growth promoting properties. So, all the isolates were screened for their IAA production properties and their concentration is quantitatively estimated. Out of all the 20 strains NB11 shows the best result with 64.40µg/ml, followed by NB12(i) with 60.31µg/ml, NB10, with 55.42µg/ml, NB2 with 52.98µg/ml, NB9(i) with 49.73µg/ml, NB6 with 47.28µg/ml.

PQQ Activity

GDH holoenzyme in bacteria converts glucose into gluconic acid can be observed by the appearance of red colonies on MacConkey indicator plates containing glucose. The conversion of glucose to gluconic acid is attributed to P-solubilization. Strains having PQQ positive are PQQ independent and can be an efficient P-solubilizer. Among the 20 strains except for NB13(i) all isolates form pink colour zone which indicates PQQ independent activity of GDH. Isolate NB8 &NB14(ii) did not show any growth in the medium.

DISCUSSION

Phosphorusis key nutrient for plant as it helps higher agricultural productivity. It is important part of adenosine triphosphate (ATP) which is an energy unit that regulate several biochemical cycles in plants (Brady and Weil, 2008).Phosphorus line up in many such as photosynthesis, nitrogen fixation , respiration, seed germination, maturation (Plaxton and Lambers, 2015). Despite its importance, soil have common phosphorus deficiency which make 42% of land barren in world (Liu *et al*., 1995). Mehta and Nautiyal, 2001) reported the formulation to quickly screen the phosphate solubilizing bacteria on a qualitative basis using an efficient media NBRIP containing Bromophenol Blue as dye. So accordingly, the isolated bacterial strains were selected as low and high phosphate solubilization and qualitatively based on decolorization of the dye colour.

In this study, 20 strains were characterized, and all the strains seem to be well adapted in the environment. On quantitative basis strains NB5, NB6, NB12(i), NB12(ii), NB13(i), NB13(ii), NB14(ii) solubilize more than 100µg/ml insoluble phosphate and consider as the most efficient P solubilizer. NB2, NB3, NB4, NB7, NB8, NB9(i), NB10, NB11, NB12(iii), NB13(iii), NB14(i) solubilize P in the range between 50µg/ml to 100µg/ml and considered as high p solubilizers. NB9(ii), NB15 solubilize P below 50µg/ml, hence considered as low P solubilizer. While based on nitrogen source utilization, Ammonium chloride, Ammonium acetate and Ammonium sulphate were highly utilized by all bacterial isolates. Magnesium nitrate was utilized by only Gram +ve bacilli, which are NB2, NB7, NB8, NB9(i), NB9(ii), NB12(i), NB12(ii), NB13(iii), NB14(i), NB15 as their Nitrogen source, shown that only Bacillus spp have the capability to utilize Magnesium Nitrate as their Nitrogen source.

All the 20 isolates except NB13(i), NB13(ii) & NB14(ii) do not require PQQ as a cofactor for their Phosphate solubilization ability, which governed by Glucose dehydrogenase gene (GDH).Out of all the 20 strains, Auxin was best produced by NB11 with 64.40µg/ml concentration, followed by NB12(i) with 60.31µg/ml, NB10, with 55.42µg/ml, NB2 with 52.98µg/ml, NB9(ii) with 49.73µg/ml, NB6 with 47.28µg/ml. All the 20

strains have proteolytic and lipolytic activity except NB12(ii). Only NB8 and NB14 (i) isolates showed Amylolytic activity.

CONCLUSION

All the 20 isolates that are used have phosphate-solubilizing ability. Out of 20 isolates, 10 were Gram +ve which are identified as Bacillus spp and other 10 were Gram-ve bacteria, identified as Pseudomonas spp. Among the 20 strains of the present study NB5, NB6, NB8 and NB12(i) can be good strains to be used as a bioinoculant. These strains showed positive results in most of the tests. So, we can conclude that these strains have multiple characters suitable to prepare bioinoculants that can enhance plant growth and hence can be used for commercialization at field level.

Conflict of interest: The authors declare that they have no conflict of interest.

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