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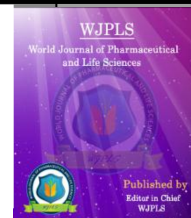

A COMPARISON OF NUTRIENT MOBILIZATION ACTIVITY OF RHIZOSPHERE MICROFLORA OF A LEGUMINOUS PLANT WITH THE RHIZOFLORA OF A NON-LEGUMINOUS PLANT AND NON-RHIZOSPHERIC SOIL

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ABSTRACT

The aim of the present study was to investigate and to compare the Nitrogen and Phosphorous mobilization activity by the microflora of rhizosphere of a leguminous plant with a non-leguminous plant and uncultivated soil. Sixty days old seedling of *Vigna radiata* and *Capsicum frutescens* was selected for this study. It was found that leguminous rhizospheric soil harbors high number of organisms (66 x10^{-5} CFU/g) than in non-leguminous rhizospheric soil (44 x 10^{-4} CFU/g) and non-rhizospheric soil sample (28x10^{-5} CFU/g). The activities studied also showed a similar trend. A total of ten bacterial colonies were isolated from leguminous, where nine colonies showed ammonification, six showed nitrite production and seven showed nitrate production. Among the seven colonies isolated from non-leguminous, two colonies showed ammonification activity, nitrate production and one colony showed nitrite production. Six colonies were isolated from soil and only one isolate showed ammonification. From leguminous rhizosphere two isolates (L8 and L10), showed all the activities analyzed. L8 showed more effective phosphate solubilization with a zone diameter of 4.5 cm, Solubilization Index of 3.25 and an efficiency of 2.25%. The study reestablishes the importance of rhizosphere microflora and the capacity to be used as good biofertilizer agents after proper optimization.

KEYWORDS: Rhizosphere, Plant Growth Promoting Rhizobacteria (PGPR), Phosphate Solubilising Bacteria (PSB), Nitrogen fixation, Ammonification, Nitrification.

INTRODUCTION

The soil is considered a storehouse of microbial activity, where microbial populations are immersed in a framework of interactions that can be beneficial or harmful (Parmar and Dufresne, 2011) depending upon the soil environment, plant defense mechanism or the type of microorganism in the rhizosphere zone (Cavigelli and Robertston, 2000). Such interactions are known to affect plant fitness and soil quality and thus influence the crop health and yield. It is well established that a minor volume of soil immediately surrounding a plant root is a richer source of such microbial populations (Nannipieri et al., 2003), than the bulk soil region. Such confined hotspots of microbial population and activities are termed as rhizosphere, where intense plant–microbe interactions can be seen, that involves colonization of different microorganisms in and around growing roots.

A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere, but bacteria are the most abundant among them; it is due to the high probability that bacterial populations can influence the plant's physiology to a greater extent, especially considering their competitiveness in root colonization (Kloepper and Antoun, 2001; Barriuso et al., 2008). These bacterial populations called as rhizobacteria are found to play a vital role in the transformation, mobilization and solubilization of soil nutrients compared to those from bulk soils (Hayat et al., 2010). Therefore, the rhizobacteria have an important role in recycling of soil nutrients and consequently, they are crucial for soil fertility (Glick, 2012) and improvement of soil fertility is one of the most common strategies to increase agricultural production. In accordance with Vessey (2003), rhizobacteria which grow in, on, or around plant tissues and stimulate plant growth through an array of diverse mechanisms can be collectively called as PGPR (plant growth promoting rhizobacteria).

Nitrogen (N) is the most vital nutrient for plant growth and productivity. Although, there is about 78% N_2 in the atmosphere, it is unavailable to the growing plants. The atmospheric N_2 is converted into a form that can be utilized by plants by a process called biological nitrogen fixation (BNF). Kim and Rees (1994) reported that during biological nitrogen fixation process, atmospheric nitrogen is changed to ammonia by nitrogen fixing microorganisms by using a complex enzyme system. These bacteria have the nitrogenase enzyme that mediates the mineralization of organic forms of N to ammonium (NH^{4+}) and its

subsequent nitrification to nitrate (NO^{3-}) by ammonia-oxidizing and nitrite oxidizing bacteria respectively. This process of BNF is of major significance to N availability and has influence on rhizosphere dynamics and proved to be advantageous that, it is economically beneficial and environmental friendly alternative to chemical fertilizers (Ladha et al., 1997).

Phosphorus (P), the second important plant growth-limiting nutrient after nitrogen, is abundantly available in soils in both organic and inorganic forms. Although there is a large reservoir of P in soil, plants are unable to utilize them as such because the majority of soil P is found in insoluble forms (Mckenzie and Roberts, 1990). Some microbes are capable of solubilizing the insoluble inorganic P of soil and make it available to the plants and are named as Phosphorus solubilizing bacteria (PSB). PSBs play role in phosphorus nutrition by enhancing its availability to plants through release from inorganic and organic soil P pools by solubilization and mineralization and is done by lowering of soil pH by microbial production of organic acids and mineralization of organic P by acid phosphatases. PSBs can be considered as important traits under PGPR because use of phosphorus solubilizing bacteria as inoculants have found to be very effective in increasing the plant available form P in soil and as well as influence the growth and yield of crops (Rodriguez, 2006 and Chen 2006). Chaiharn (2008) and Zaidi (2009) reported that rhizospheric phosphate utilizing bacteria could be a promising agent for promoting plant growth in agriculture.

Nitrogen fixation is done in both leguminous and non-leguminous plants and the microorganisms improve yield of nitrogen fixation (Swain, 2013). A wide diversity of nitrogen fixing bacteria, have the capacity to colonize the rhizosphere and to interact with the plant. Leguminous plants obtain their nitrogen by association with *Rhizobia* sp., forming nodules and some has been identified as colonizing root interior in a variety of cereals and grasses. Thus, both leguminous and non-leguminous plants were benefited differently (Claudine et al., 2008). Thus, it has been well documented that rhizosphere bacteria has a high potential to be used in the management of nutrient deficient soils. This project was done in order to study the mobilization of phosphorus and nitrogen by the microflora of rhizosphere of leguminous and to compare these activities by those of rhizosphere of non-leguminous and of uncultivated soil.

1. MATERIALS AND METHODS

1.1. Study Materials

The samples used for the study include rhizospheric soil of a leguminous plant, non-leguminous plant and soil away from the rhizospheric area. The leguminous and non-leguminous plants selected for the study are *Vigna radiata* (leguminous) and *Capsicum frutescens* (non-leguminous) respectively. Seeds of the plants were collected from College of Agriculture, Kerala Agricultural University, Vellayani and were grown in separate pots filled with soil. These plants were grown for sixty days and rhizosphere samples were collected from uprooted plants. Raw soil sample was collected using a sterile spatula from the soil away from the rhizospheric region and transferred to sterile fresh air tight bags. The samples were taken to the laboratory at the earliest and were subjected to microbial isolation procedures.

1.2. Isolation and Enumeration of Bacteria

The bacteria in the three soil samples (two rhizospheric soil and non-rhizospheric) were subjected to serial dilution and enumeration. Well isolated bacterial colonies were subjected to identification of the colony characteristics as per standard chart. Morphologically distinct colonies were selected for further studies after preparing pure cultures and doing gram staining.

1.3. Screening for Nitrogen fixing activity

Colonies isolated from all the samples were individually analysed for their ammonifying and nitrifying ability as per standard procedures, (Dubey and Maheshwari, 2012).

1.3.1. Identification of Ammonifying Bacterial colonies.

The screening of ammonifying bacteria was done by inoculating the isolated colonies in 4% peptone broth and incubated for 1 week at 28 ± 2°C and was tested for ammonia production using Nessler's reagent (Dubey and Maheswari, 2011).

1.4. Identifying bacterial isolates involved in Nitrification

This process was demonstrated in 2 steps. In the first step, test for nitrite production and in second step test for nitrate production was done as per Dubey and Maheswari, (2011).

1.4.1. Identification of nitrite production

Isolated colonies obtained on nutrient agar plates were inoculated in ammonium sulphate broth and was incubated for a period of 1 week at 28 ± 2°C. After one week incubation, a drop of culture from the ammonium sulphate broth was transferred to a spot plate containing 3 drops of Trommsdorf's reagent mixed with 1 drop of dilute sulphuric acid, to check for nitrite production. The appearance of a blue-black colour indicated nitrite production.

1.4.2. Identification of nitrate production

Isolated colonies obtained on nutrient agar plates were inoculated in nitrite broth and was incubated for a period of 1 week at 28 ± 2°C. After one week incubation, one drop of culture from the nitrite broth was transferred to a spot plate containing 1 drop of Diphenylamine reagent mixed with 2 drops of dilute sulphuric acid and was checked for the appearance of a blue-black colour indicating nitrate production.

1.5. Screening for Phosphate Solubilizing Activity

The screening of phosphate solubilisation by bacteria was done by placing the isolated colonies on Pikovskaya's (PKV) agar medium (Hi Meida), (Pikovskaya 1948). These plates were then incubated at 28ºC for 5-7 days. The bacterial colonies showing clear zone around them were considered as phosphate solubilizing bacteria (PSB). The isolates thus obtained were sub cultured for 2-3 times on fresh PKV plates and were selected for further qualitative and quantitative assay (Tripti et al., 2012).

1.5.1. Qualitative Estimation of Phosphate Solubilisation

Isolates showing halo zones were selected for qualitative analysis by plate screening method, (Dubey and Maheswari, 2011). Bacterial isolates were spot inoculated on the center of PKV plate aseptically. All the plates were incubated at 28 ± 2°C for 5-7 days. A clear zone around a growing colony indicated phosphate solubilisation and the colony diameter and halozone diameter was measured. Using this, Phosphate solubilization index (SI) (Edi-Premono, 1996) and the solubilization efficiency (E) (Nguyen et al., 1992) were calculated as follows:

Solubilization Index (SI) = (Colony diameter + Halo zone diameter) /Colony diameter

Solubilization efficiency (SE) = (Solubilization diameter /Growth diameter) x100.

1.5.2. Quantitative Estimation of Phosphate Solubilisation

The bacterial colony found to be positive for Tri-Calcium Phosphate (TCP) solubilization were further analyzed to find their ability to solubilize phosphorus in liquid medium. For this, bacterial isolates showing the halo zone were inoculated in Pikovskaya's broth (Hi media).

Selected bacterial isolates were inoculated in 100 mL Pikovskaya's broth taken in 250 mL of Erlenmeyer flasks and incubated at 28 ± 2°C for 7 days. After incubation the bacterial cultures were filtered through Whatman No.1 filter paper and were clarified by centrifugation at 10,000 rpm for 10 minutes. From this 10 mL of clear filtrate was taken and 25mL of Baton's reagent was added. Final volume of this solution was made up to 50 mL using distilled water. After 10 minutes, the intensity of yellow colour was measured by UV-VIS spectrophotometer at 430 nm and the amount of phosphorous solubilise was extrapolated from the standard curve. Uninoculated broth served as control.

1.5.3. Standard Curve (Estimation of Phosphate Solubilisation)

Potassium dihydrogen orthophosphate (0.2195 g) was dissolved in distilled water and the final volume was made to 1 litre, so that 1ml of the solution contains 59 ppm of phosphate in it. Further dilution was made by taking 10 ml of this solution and it was made up to 250ml using distilled water. This was used as the stock solution, where 1ml of the solution contains 2ppm of phosphate in it. Aliquots of 2ml, 4ml, 6ml, 8ml, 10ml, 15ml and 20ml of stock solution were taken in 50ml volumetric flasks. To this 2.5ml of Barton's reagent was added. The final volume was made up to 50 ml using distilled water. After an interval of 10 minutes, the OD of each aliquot was measured at 430 nm and a graph was plotted between the OD and concentration of solution. From these graph, the amount of phosphorous solubilized for experimental samples were calculated. (Dubey and Maheshwari, 2012).

2 RESULTS AND DISCUSSION

2.1. Isolation and Enumeration

Enumeration of microorganisms showed that there were 66 $x10^{-5}$ CFU/g of the *Vigna radiata* rhizosphere, 44 x 10^{-4} CFU/g *Capsicum frutescens* rhizosphere and $28x10^{-5}$ CFU/g from bulk soil. The exudates released by roots always occur in their highest concentrations adjacent to the root surface. Therefore, more soil organisms are attracted to this region and is the reason for abundance of soil organisms adjacent to plant roots, *i.e.,* the rhizospheric region (Newman and Watson 1977).

2.2. Gram Staining and Morphological Analysis

Of the isolated microbes, 23 bacterial colonies were selected on the basis of colony morphology for further studies. It included ten colonies from leguminous rhizosphere, seven colonies from non-leguminous rhizosphere and six colonies from soil sample. The morphological characteristics of the isolated microbial colonies from leguminous rhizosphere are given in **Table 1**, non-leguminous rhizosphere are given in **Table 2** and those from raw soil in **Table 3**. The samples were subjected to gram staining and the results for leguminous, non-leguminous and raw soil are represented as **Table 4**, **Table 5**, and **Table 6** respectively.

2.3. Screening for Ammonification

Ammonification was detected by the formation of an orange to brown precipitate in peptone broth upon adding few drops of Nessler's reagent into it. Some of the organisms inoculated into the broth are capable of degrading the peptone (protein) and produce ammonia in the medium. If ammonia is formed, it reacts with Nessler's reagent added and forms an orange to brown precipitate, indicating the presence of ammonifying bacteria (**Figure 1**). It was seen that nine isolates from leguminous rhizosphere (L1, L2, L3, L4, L6, L7, L8, L9 and L10), two isolates (NL1 and NL4) from non-leguminous rhizosphere and one isolate (S5) from soil sample showed ammonia production. This finding is in accordance with the results done by earlier workers (Ewel, 1986), that the leguminous plants have a greater tendency to colonize nitrogen fixing and ammonifying microbes when compared to non-leguminous rhizoflora and microbes from non-rhizospheric soil.

2.4. Identification of Nitrifying Bacterial colonies

Nitrite producers were detected by the formation of a blue-black coloration in ammonium sulphate broth upon adding 3 drops of Trommsdorf's reagent mixed with 1 drop of dilute sulphuric acid. Ammonium sulfate broth is a nitrite forming broth. When nitrite producing bacteria are inoculated into this broth, they have the potential of converting ammonia in the medium to nitrites producing a blue black coloration to the broth upon the addition of Trommsdorf's reagent (**Figure 2**). A positive result was shown by six isolates from leguminous rhizosphere (L1, L2, L4, L7, L8, and L10) and one isolate from non-leguminous rhizosphere (NL6). None of the colonies isolated from non-rhizospheric soil gave a positive nitrite production. This is also in accordance with the earlier studies, (Ewel, 1986; Starkey, 1929). Lyon and Wilson (1921) explained this stimulation is due to the organic matter given off by the roots.

Nitrate producers were detected by the formation of a blue-black coloration in nitrite broth upon adding 1 drop of Diphenylamine reagent mixed with 1 drop of dilute sulphuric acid into the media inoculated with the isolates. Nitrite broth is a nitrate forming broth. The presence of bacterial colonies that are capable of converting nitrites to nitrates produces a blue black coloration in the medium after adding diphenylamine reagent (**Figure 3**). The test was given positive by seven isolates from leguminous rhizosphere (L1, L2, L4, L5, L8, L9, and L10) and two isolates from non-leguminous rhizosphere (NL3 and NL5).

2.5. Isolation of Phosphate Solubilizing bacteria (PSB)

Phosphate solubilizing activity was detected by the formation of a halo zone around the bacterial colonies inoculated on Pikovskaya's (PKV) agar medium. The halo zone results from the production of organic acids into the surrounding medium by the bacterial isolates (**Figure 4**). Only two colonies from leguminous rhizosphere (L8 and L10), showed phosphate solubilization activity. Rodríguez and Fraga (1999) found that strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. This could be the reason why only isolates from leguminous rhizosphere gave phosphate solubilizing activity.

2.5.1. *Qualitative Estimation of Phosphate Solubilization*

Isolates showing halo zones (L8 and L10) were spot inoculated on the center of PKV plate and incubated for 5-7 days. The colony diameter and halo zone diameter was measured for both the colonies showing a halo zone. Using these values, the phosphate solubilization index and solubilization efficiency were calculated. The results are as shown in **Table 7**. Of the two isolates the isolate L8 showed a zone with greater diameter of 4.5 cm whereas the diameter of zone by L10 was 3 cm.

2.5.2. *Quantitative Estimation of Phosphate Solubilization*

The positive isolates for P solubilization were analyzed for their ability to solubilize P in liquid medium using Pikovskaya's broth. Standard Curve (**Figure 5**) was prepared for Qualitative Estimation of Phosphate Solubilization. The values for the Standard Curve are as given in **Table 8**. From this graph, the quantity of phosphorus solubilized was estimated for both the colonies based upon their OD values as 4mg/mL and 2mg/mL respectively in both the samples.

Both L8 and L10 isolates were found to be potent phosphate solubilizers showing clear halo zone around their colonies. Out of these 2 bacterial isolates, L8 isolates showed highest phosphate solubilization activity, qualitatively and quantitatively. L8 isolates showed a solubilization index of 3.25 along with high soluble phosphate production of 4mg/mL in broth culture.

Table 1. The colony morphology of bacterial isolates from leguminous rhizosphere.

Culture	**Form**	**Size**	**Surface**	**Texture**	**Colour**	**Elevation**	**Margin**
L1	Irregular	Medium	Dull	Moist	Opaque	Raised	Undulate
L2	Rhizoid	Large	Dull	Dry	Transparent	Flat	Rhizoid
L3	Irregular	Medium	Glistening	Viscous	Opaque	Umbonate	Lobate
L4	Irregular	Large	Dull	Dry	Opaque	Flat	Lobate
L5	Circular	Small	Glistening	Moist	Opaque	Convex	Entire
L6	Circular	Small	Glistening	Moist	Opaque	Convex	Entire
L7	Circular	Small	Dull	Dry	Opaque	Flat	Entire
L8	Irregular	Medium	Glistening	Viscous	Opaque	Umbonate	Lobate
L9	Circular	Punctifom	Dull	Dry	Transparent	Flat	Entire
L10	Circular	Small	Glistening	Moist	Translucent	Convex	Entire

Table 2. The colony morphology of bacterial isolates from non-leguminous rhizosphere.

Culture	**Form**	**Size**	**Surface**	**Texture**	**Color**	**Elevation**	**Margin**
NL1	Filamentous	Large	Glistening	Viscous	Opaque	Umbonate	Lobate
NL2	Rhizoid	Medium	Wrinkled	Dry	Opaque	Flat	Rhizoid
NL3	Irregular	Large	Dull	Moist	Translucent	Flat	Undulate
NL4	Irregular	Small	Dull	Dry	Opaque	Flat	Undulate
NL5	Circular	Small	Dull	Dry	Opaque	Flat	Entire
NL6	Irregular	Medium	Glistening	Moist	Opaque	Pulvinate	Curled
NL7	Circular	Small	Dull	Dry	Opaque	Flat	Entire

Table 3. The colony morphology of bacterial isolates from raw soil.

Culture	**Form**	**Size**	**Surface**	**Texture**	**Color**	**Elevation**	**Margin**
S1	Irregular	Medium	Dry	Moist	Opaque	Raised	Undulate
S2	Irregular	Medium	Dry	Dry	Transparent	Flat	Undulate
S3	Irregular	Medium	Dry	Moist	Opaque	Flat	Undulate
S4	Irregular	Small	Dry	Moist	Transparent	Pulvinate	Curled
S5	Circular	Small	Glistening	Viscous	Opaque	Convex	Entire
S6	Circular	Large	Glistening	Moist	Translucent	Convex	Undulate

Table 4. Gram staining results for bacterial isolates from non-leguminous rhizosphere.

Culture	Gram (+/-)	Shape
L1	'+'ve	Cocci
L2	'+'ve	Rod
L3	'-'ve	Cocci
L4	'+'ve	Cocci
L5	'-'ve	Rod
L6	'+'ve	Rod
L7	'+'ve	Cocci
L8	'-'ve	Cocci
L9	'-'ve	Cocci
L10	'-'ve	Rod

Table 5. Gram staining results for bacterial isolates from non-leguminous rhizosphere.

Culture	Gram (+/-)	Shape
NL1	'-'ve	Cocci
NL2	'-'ve	Cocci
NL3	'+'ve	Rod
NL4	'-'ve	Cocci
NL5	'-'ve	Rod
NL6	'+'ve	Cocci
NL7	'+'ve	Cocci

Table 6. Gram staining results for bacterial isolates from non-rhizospheric soil.

Culture	Gram (+/-)	Shape
S1	'+'ve	Cocci
S2	'+'ve	Cocci
S3	'+'ve	Cocci
S4	'+'ve	Rod
S5	'-'ve	Cocci
S6	'-'ve	Cocci

Table 7. Qualitative Estimation of Phosphate Solubilization.

Colony	Colony Diameter (cm)	Halo Zone Diameter (cm)	SI (cm)	SE (%)
L8	2	4.5	3.25	2.25
L10	1.6	3	2.875	1.875

Table 8. Standard values for quantitative estimation of Phosphate Solubilization.

P (mg/ml)	Absorbance
1	0.01
2	0.02
3	0.03
4	0.04
5	0.06
6	0.07
8	0.08
10	0.11
15	0.12

Figure 1. Brown precipitate formed by ammonifying bacteria in peptone broth.

Figure 2. Blue black colour in ammonium sulphate broth showing nitrite production.

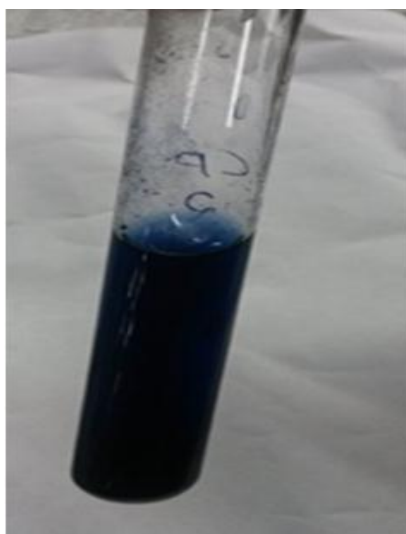
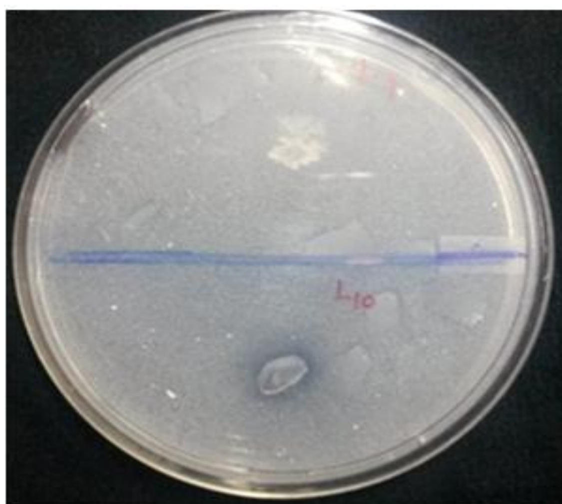


Figure 3. Blue black colour in nitrite broth showing nitrate production.



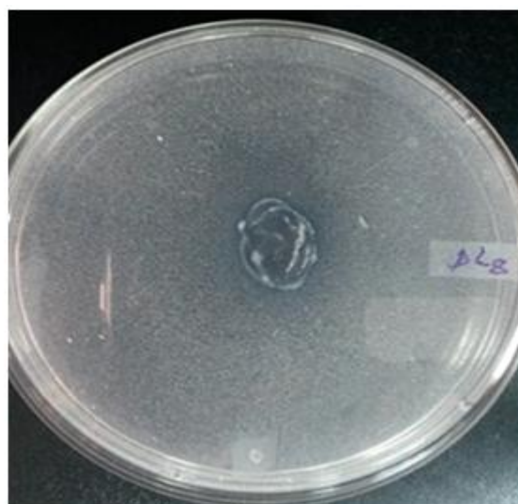


Figure 4. Halo zone produced by phosphate solubilizing bacteria

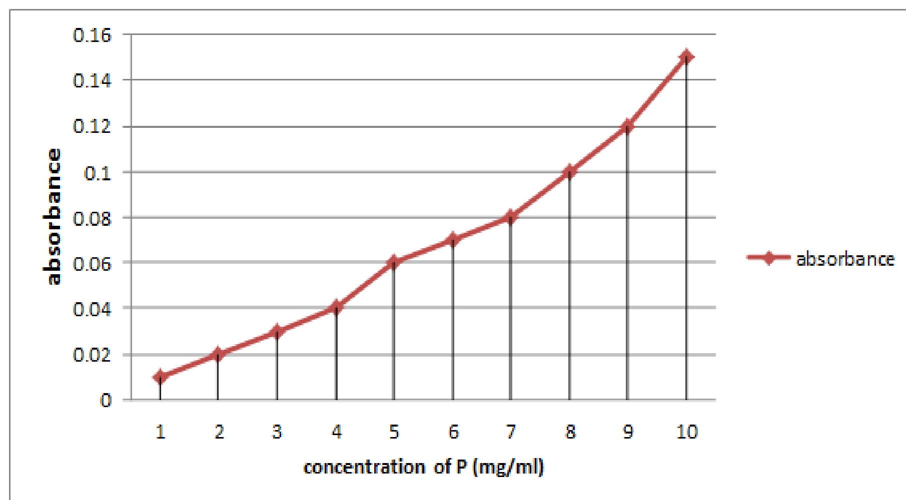


Figure 5. Standard Curve for qualitative estimation of Phosphate Solubilization.

3 CONCLUSION

Out of the 23 isolates analyzed for nutrient mobilization activities, two isolates showed all the activities analyzed, *viz.* ammonification, nitrification and phosphate solubilization. It was found to have roles in both nitrogen and phosphorus mobilization. The use of these strains as bio-fertilizers helps in reducing the use of chemical fertilizers and also effective in reducing the cost of cultivation and maintaining the natural fertility of soil and its self-rejuvenation capacity.

The activities of these PGPRs show that they can be proposed as candidates for use as biofertilizer in sustainable agriculture practices solely or in combination after proper optimization and characterization. In this context, the exploitation of soil root interface (rhizosphere) is emphasized in agriculture to provide an environment friendly alternative to application of chemical fertilizers. Further research and understanding of mechanisms of PGPR would pave the way to find out more competent rhizobacterial strains which may work to stimulate soil fertility and thus plant growth and yield.

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