Exploring the Phosphate Solubilization Potential of *Rhizobia* Isolated from *Sesbania Grandiflora*

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Abstract :

Rhizobia, are known to exhibit the ability to solubilize phosphate (P) in the soil, apart from their primary function of nitrogen fixation. Hence this study was conducted to evaluate and characterize *Rhizobia* isolates obtained from root nodules of *Sesbania grandiflora* for their potential in P solubilization. Two experiments were conducted under laboratory conditions arranged in a Complete Randomized Design (CRD) with three replicates. All data were analyzed using analysis of variance (ANOVA) and means were separated using the LSD test. Four bacterial strains (a,b,c,d) were isolated from root nodules using the trap plant method. They were initially identified as *Rhizobia* based on morphology and authenticated through gram staining, acid-alkaline tests, Congo Red Yeast Mannitol Agar (CRYMA), and Bromothymol blue Yeast Mannitol Agar (BRYMA) tests. The most effective *Rhizobia* for P solubilization was identified based on the Phosphorus Solubilization Index (PSI) and solubilized P in Pikovaskya (PVK) solid and liquid media. Subsequently, optimization of PVK liquid media was conducted for effective P solubilizer, varying with carbon (C), nitrogen (N), and P sources. All isolates were gram-negative and exhibited acid production, authenticated as *Rhizobia*. The significantly ($p<0.05$) highest PSI was recorded with isolate "c" and it also exhibited the highest ($p<0.05$) solubilized P. The PVK medium was optimized for isolate "c" and potassium dihydrogen phosphate (35.1±0.46 ppm), glucose (25.47±0.49 ppm), and ammonium sulphate (3.20±0.17 ppm) were identified as the optimum C, P, and N sources, for achieving significantly higher P solubilization by thriving *Rhizobia* "c" as an effective P solubilizer. However, further field studies are required to assess performance of *Rhizobia* "c" before introducing it as a P solubilizing inoculum.

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Keywords: Media optimization, Phosphorous solubilization, P solubilizing inoculum, *Rhizobium*

1. INTRODUCTION

Phosphorus is one of the most essential nutrients for plant growth. It is undoubtedly clear that P is one of the most essential macroelement required for growth and development of plants including photosynthesis, energy and sugar production. Moreover its promotes nitrogen fixation in legumes (Saber *et al.* 2005; Xiao *et al.*, 2011). Only 0.1% of the total P (0.5%) is available to plants in soils, while rest of the total P is present in the insoluble form and therefore, cannot be taken up by plants (Scheffer and Schachtschabel, 1988). In soils, Phosphoric acid (H_3PO_4), Dihydrogen phosphate ($H_2PO_4^-$) and Hydrogen phosphate (HPO_4^{2-}) are the primary forms of P taken up by plants. The solubilization of P is important in various biological and environmental processes.

Soil microorganisms, particularly phosphate-solubilizing bacteria, play a crucial role in solubilizing P. The principal mechanism for mineral P solubilization is the production of organic acids, and acid phosphatases play a significant role in the mineralization of organic P in soil. It is generally accepted that the major mechanism of mineral P solubilization is the action of organic acids synthesized by soil microorganisms (Rudresh *et al*., 2004). Apart from chemical fertilization, microbial P-solubilization and mineralization is the only possible way to increase plant-available P. Numerous microorganisms in the soil and rhizosphere are effective at releasing P from total soil P through solubilization and mineralization (Bhattacharyya and Jha, 2012). This group of microorganisms are referred to as Phosphorus Solubilizing Microorganisms (PSM).

Phosphate-solubilizing bacteria (PSB) are beneficial microorganisms capable of solubilizing inorganic P. Bacterial genera like *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, and *Pseudomonas* are reported as the most significant phosphate solubilizing bacteria (Bhattacharyya and Jha, 2012). Moreover, the ability to P-solubilization is found even among Rhizobiacae, comprising *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and other non-specified legume-nodulating bacteria (LNB) (Singh and Gera, 2018).

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Rhizobia, belonging to the family *Rhizobiaceae*, are a group of soil bacteria known for their remarkable ability to form symbiotic relationships with certain leguminous plants, particularly legumes (Abeysingha and Weerarathne, 2010). This unique association leads to the development of specialized structures called root nodules (Wang *et al*., 2012). While their primary function is nitrogen fixation, some strains of *Rhizobia* also exhibit the ability to

solubilize phosphate in the soil. The phosphate-solubilizing ability of *Rhizobia* is crucial for plant nutrition and contributes significantly to plant growth when used as a component of biofertilizers for crops. This mutualistic interaction between *Rhizobia* and leguminous plants has significant agricultural and ecological importance.

Sesbania grandiflora, a small, erect, fast-growing, and sparsely branched perennial tree belonging to the Leguminosae family, creates a favorable rhizosphere environment that supports the growth of *Rhizobium.* This study was conducted to assess and characterize *Rhizobia* isolates from *Sesbania grandiflora* root nodules for their potential in P Solubilization and optimization of the media for effective P solubilization.

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2. METHODOLOGY

2.1 Seed Collection

Seeds of *Sesbania grandiflora* were collected from three locations of *Anuradhapura*, *Puliyankulama* (DL1b) area of Sri Lanka.

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2.2 Sample Preparation and Isolation of *Rhizobia* Using Trap Plant Method

The collected seeds were planted in pots containing soil samples known to have Reddish Brown Earth (RBE) (Panabokke, 1959) collected from the field (at the faculty of Agriculture, Rajarata University of Sri Lanka).

After approximately 45 days of planting, healthy pink root nodules were separated from the plants. Then, the separated nodules were placed on Potato Dextrose Agar (PDA) medium for isolation of ~~ed~~ *Rhizobacterial* ~~*bia*~~ strains. The plates were incubated at 28±2°C for 2-7 days. After incubation, four colors of single gummy colonies were successfully identified: (a) colorless, (b) whitish, (c) yellowish, and (d) pinkish. To obtain pure cultures, the isolates were further purified by sub-culturing multiple times on the same medium.

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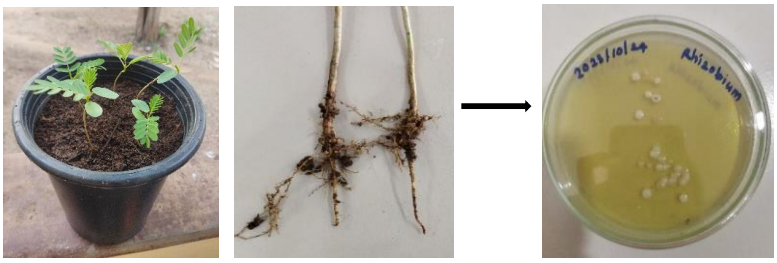

Figure .1. Isolation of *Rhizob*acteria from ~~*ia nodulating*~~ *Sesbania grandiflora* plant using trap plants method from RBE soils at the faculty of Agriculture, Rajarata University of Sri Lanka field.

2.3 Authentication of *Rhizobia* Isolates

All four isolated *Rhizobia* cultures were subjected to check their authenticity using the following methods.

Acid alkaline production test

The production of acid and alkali was detected in this test by following the four treatments to grow on Yeast-extract-mannitol (YEM) broth supplemented with Bromothymol Blue (BTB) . The change in color and the pH of the YEM broth was recorded after incubation at 28±2 ^{0}C for 24-48 hours (Somasegaran and Hoben, 1994).

CRYMA test

YMA media was prepared, the 2.5 ml Congo red was added to the solution, and it was put in Autoclave. The media was sterilized and it was poured into sterilized Petri plates and cultured isolated four treatments separately (Wijesundara *et al*., 2000).

BRYMA test

YMA media was prepared and the 1.25 ml BTB was added to the solution and it was put in Autoclave. The media was sterilized and it was poured into sterilized petri plates and cultured isolated four treatments separately (Wijesundara *et al*., 2000).

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Microscopic observation

Four treatments were observed using a light microscope to identify morphological characteristics to trap the *Rhizobia* colonies.

Gram's staining test

Gram staining technique was used to differentiate gram-negative and gram-positive bacteria colonies using a microscope (Tripathi and Sapra, 2020).

2.4 Determination of P Solubilizing Index (PSI) on PVK Agar Medium

Four isolated *Rhizobia* strains produced a clear holo appearance in PVK agar solid media, which were incubated at 28±2°C for 3-7 days, and the highest PSI was selected as an effective

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P solubilizer. After 7th day of incubation, a clear zone was observed around the colony of four isolates. Then zone diameter and colony diameter were measured in each four isolates. Finally, PSI was calculated using the following formula.

$$\text{Phosphate Solubilization Index (PSI)} = \frac{Colony\ diameter\ (mm) + Zone\ diameter(mm)}{Colony\ diameter\ (mm)}$$

(Saiyad *et al.*, 2015)

2.5 Evaluation of the Efficiency of Phosphate Solubilization in PVK Broth Medium

Quantification of solubilized P was assessed by measuring the available P content in PVK culture broths (Sati, 2019). All samples were shaken at 1 rpm for 3-7 days at room temperature. After 3-7 days of culture, the media was filtered through the Whatman No. 42 filter paper (Kumari *et al.*, 2010). Finally, supernatants were collected to estimate the available P concentration using the Murphy and Relay method (2002), and the remaining suspension was used to measure the pH values.

2.6. Identification of Effective P Solubilizer

The most effective P solubilizer among four isolates ~~treatments~~ was determined based on the PSI and solubilized P.

2.7 Optimization of Media for Effective P Solubilizer

Different sources of N, P, C, were tested to optimize the media for higher solubilization of P with selected P solubilizer.

P sources are potassium dihydrogen phosphate [KH_2PO_4], sodium phosphate [Na_2PO_4], tri-calcium phosphate [$Ca_3 (PO_4)_2$], N sources are ammonium sulphate [$NH_4)_2SO_4$], urea, and sodium nitrate [$NaNO_3$], C sources are glucose, fructose and sucrose were tested in PVK Broth. Three replicates were arranged for each nutrient sources as treatments separately. Culture broths were shaken at 1 rpm in room temperature 3-7 days (Fasim *et al.*, 2002). After 7 days, pH was measured and media was filtered through Whatman No. 42 filter paper and 1 ml of each culture was taken out to estimate the amount of solubilized P concentration using the Murphy and Relay method (2002).

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2.8 Statistical Analysis

The experiment data were analysed using R software, followed by ANOVA, CRD. Mean separations were done using LSD mean comparison test.

3. RESULTS AND DISCUSSION

3.1 Microscopic Observation

Isolates were acquired from the root nodules of *Sesbania grandiflora* plants, demonstrated rapid growth within a temperature range of 25 to 30°C. The colonies of the isolates are individually cultured for purification, as illustrated in figure 2 During the initial stages, colony appearances exhibited variation, encompassing four treatments colorless (a), whitish (b), yellowish (c), and pinkish (d) morphologies.

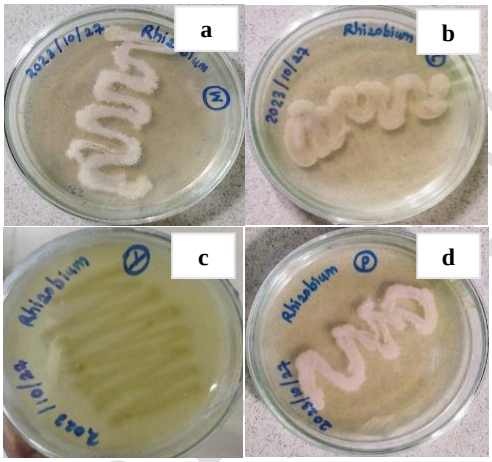

Figure 2. Colony morphology of isolates from *Sesbania grandiflora* plant using trap plants method: (a) colorless, (b) whitish, (c) yellowish and (d) pinkish colonies

3.1 Authentication of *Rhizobia* Isolates

Acid alkaline production test

Inoculation of these isolates in YEM broth supplemented with BTB changed the color of the broth to yellow after five days of growth, showing the production of acid, which is characteristic of *Rhizobia* (Singh and Gera, 2018). The pH of the culture broth was also decreased to 5.4-6.6 from an initial pH of 7.0 (Table .1).

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Table .1. pH of the isolated *Rhizobia* strains at the end of the acid alkaline production test.

Rhizobia ioslates	Acid alkaline production test (pH)
a	6.71[a]
b	6.61[a]
c	6.61[a]
d	5.91[b]

The YEMA medium incorporated with bromothymol blue was streaked with active culture of bacterial isolates, incubated 3-4 days and was observed either for yellow colour due to production of acids (fast growers) or blue colour due to production of alkali (slow growers) as per method described by Somasegaran and Hoben (Mir *et al*., 2020).

CRYMA test

Rhizobial isolates colony on YEMA medium did not absorb the supplemented Congo red dye and by this ditinguished *Rhizobium* from other bacteria (Singh and Gera, 2018). The isolates demonstrated the ability to grow and absorb the Congo red dye in the YEMA media plates, serving as an authentication test for *Rhizobium* in Figure 3

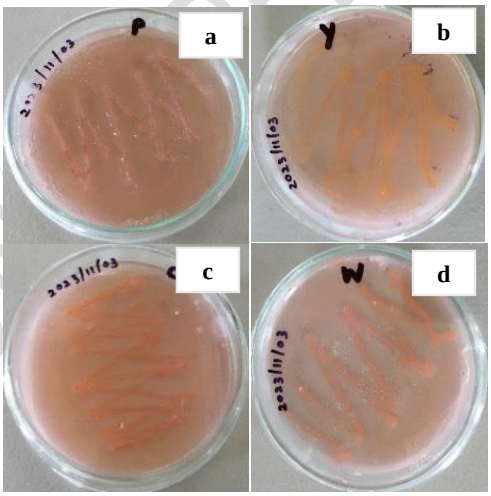

Figure 3.. Congo Red YEMA Plates on CRYMA test authentication of *Rhizobium* from isolated four *Rhizobia strains* as (a) colorless, (b) whitish, (c) yellowish and (d) pinkish.

The purity of the isolates was detected by addition of congo red in YEMA medium. Most *Rhizobia* produce white colonies, whereas many other bacteria take up the dye strongly. In previous study all isolates did not absorb the congo red color when streaked on YEMA medium except (IHRG), and such nature differentiates Rhizobium from Agrobacterium and other bacterial contaminants (Mir *et al.*, 2020).

BRYMA test

Isolates were able to produce yellow colour change in BTB YEM media plates which is an indicator of fast growth an authentication test of *Rhizobium*. Yellow colour changed because acid producers as they react with pH indicator BTB dye in an acidic reaction (Dhiman *et al.*, 2022). In this study, all four treatments formed colonies on YEMA medium containing BTB. The isolates exhibited the ability to generate a yellow color in BTB with YEM media plates, signifying rapid growth and serving as an authentication test for *Rhizobia.*

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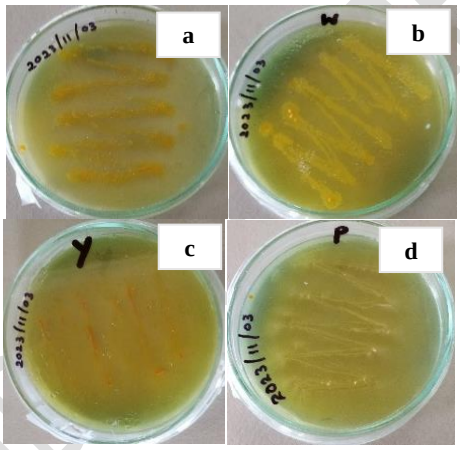

Figure 4. Bromothymol Blue Test (Fast growing) is an authentication test of *Rhizobium* from isolated four *Rhizobia strains*: (a) colorless, (b) whitish, (c) yellowish and (d) pinkish

Microscopic Observation

Four treatments were evaluated for their morphology under the microscope to authenticate *Rhizobia.* The first treatment showed cocci-shaped cells, while treatments b, c, and d exhibited rod-shaped structures.

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Rhizobia typically exhibit a rod-shaped morphology, are aerobic, and possess motility *(Mir et al*., 2020). The genus encompasses various species including *Rhizobium, Mesorhizobium,*

Bradyrhizobium, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium* (Berrada, 2014). Through a chemotactic response to flavonoid molecules released by the legume host, these bacteria form symbiotic relationships with legumes (Poonia, 2011).

Gram staining test

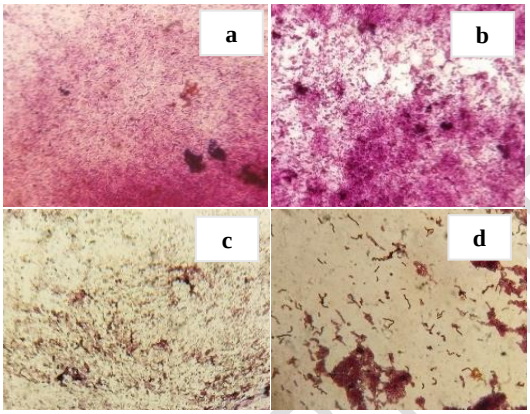

Figure.5. Gram negative colonies of *Rhizobia* isolates authenticated by gram staining test; (a) colorless, (b) whitish, (c) yellowish and (d) pinkish.

All isolates exhibited pink-colored cells in the Gram staining test under microscopic observation. Gram-negative organisms typically appear pink or red in color (Tripathi and Sapra, 2020). *Rhizobia* are Gram-negative bacteria capable of inducing the formation of specialized organs called root nodules on leguminous host plants (Fauvart and Michiels, 2008). Therefore, all treatments were confirmed as *Rhizobia*.

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3.2 Efficiency of Phosphate Solubilization

Four isolated *Rhizobia* strains evaluated for their phosphate solubilization efficiency using PSI (Table .2)

Table .2 PSI for isolated *Rhizobia* strains

Rhizobia isolates	PSI ± SE
a	2.23 ± 0.03^{b}
b	1.8 ± 0.003^{d}

c	2.53±0.04[a]
d	2.09±0.04[c]

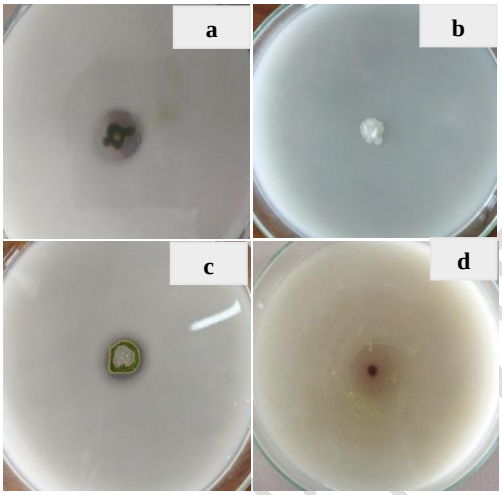

Figure.6 P solubilization zones exhibited by four isolated *Rhizobia strains* from *Sesbania grandiflora* on PVK agar medium plates ~~for *Rhizoba;*~~ (a) colorless, (b) whitish, (c) yellowish and (d) pinkish.

Among the isolated *Rhizobia* strains, *Rhizobia* "c" displayed the highest average PSI (2.53±0.04), indicating excellent P solubilization compared to the other *Rhizobia* strains. These isolates hold tremendous potential in the near future for utilization as biofertilizers, not only improving P solubilization but also enhancing the overall plant growth of *Sesbania grandiflora*.

3.3 Efficiency of Phosphorus Solubilization in PVK Broth Medium

The significantly highest solubilization (36.90±4.75 ppm), was observed in treatment “c” compared to the other isolates. In this study, the reduction of pH in the culture media was synchronized with the process of P solubilization, as evidenced by the lowest pH value (4.97) recorded in the medium containing *Rhizobia* “c”, which also exhibited the highest solubilized P.

Table .3 Changes pH levels on the P solubilization efficiency

Rhizobia isolates	pH value
a	5.17
b	5.12
c	4.97
d	5.44

The pH is the vital factor in solubilization, P solubilization is the result of the organic acid production. The pH of the media was set at pH 7 using NaOH or HCl and growth recorded as described above. The result showed that lowest pH value recorded *Rhizobia* "c" also exhibited the highest solubilized P concentration.

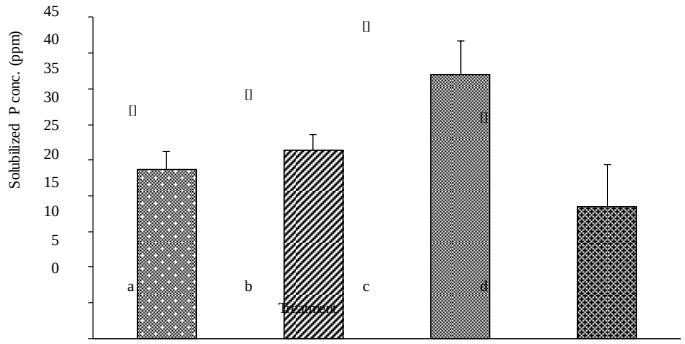

Figure 7 Average solubilized P in PVK broth for four treatments; (a) colorless, (b) whitish, (c) yellowish and (d) pinkish. Vertical bars with different letters indicate statistically significant differences at $p \leq 0.05$ probability level according to the Least mean comparison test.

3.4 Effective P solubilizer

The *Rhizobia* isolated from *Sesbania grandiflora* root nodules, particularly *Rhizobia* "c" was identified as the most effective P solubilizer. This determination was made based on the highest PSI and its capacity to solubilize the highest P in PVK broth medium.

3.5 Media Optimization for Effective P Solubilizer

Different sources of P, C, N were tested to optimize the PVK broth media for highest solubilization of P with *Rhizobia* “c”.

P sources

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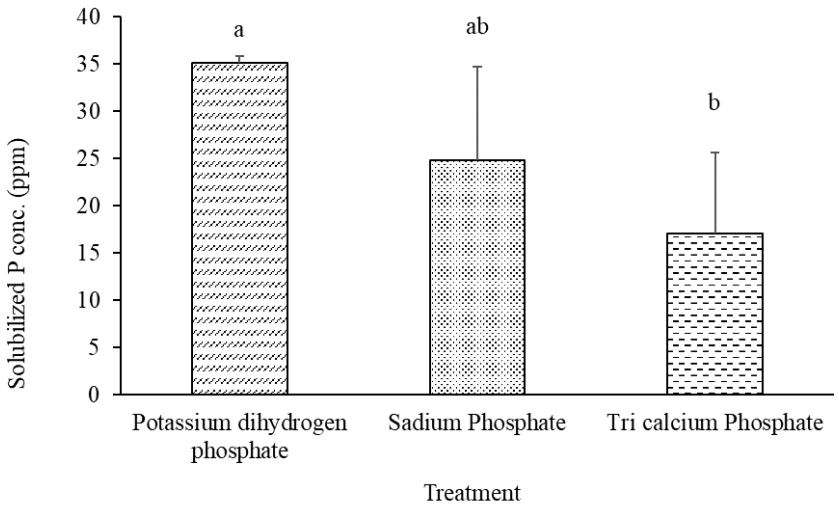

Figure 8 Effect of various P sources on the efficiency of P solubilization with *Rhizobia* "c". Different letters indicate statistically significant differences at $p \leq 0.05$ probability level to the Least mean comparison test

The culture was grown in different P sources in PVK liquid media the effects of various P sources, such as potassium dihydrogen phosphate, sodium phosphate and tri-calcium phosphate showed that potassium dihydrogen phosphate was the best phosphate source, with a solubilized rate 35.1±0.46 ppm. Then the sodium phosphate which exhibited moderate P solubilization and while the minimal solubilized P was recorded with tri-calcium phosphate. *Rhizobia* “c” showed a higher P solublization with potassium dihydrogen phosphate than other different P sources. potassium dihydrogen phosphate is an acidic source, directly provides $H_2PO_4^-$ that are readily available for uptake by plants or microorganisms. This immediate availability of phosphate can promote plant growth or support microbial activity, including phosphate solubilization by phosphate-solubilizing microorganisms (PSMs). potassium dihydrogen phosphate dissolves P more efficiency. The reason for that can be pointed to the high solubility of its water (Nguye *et al*,. 1992).

C sources

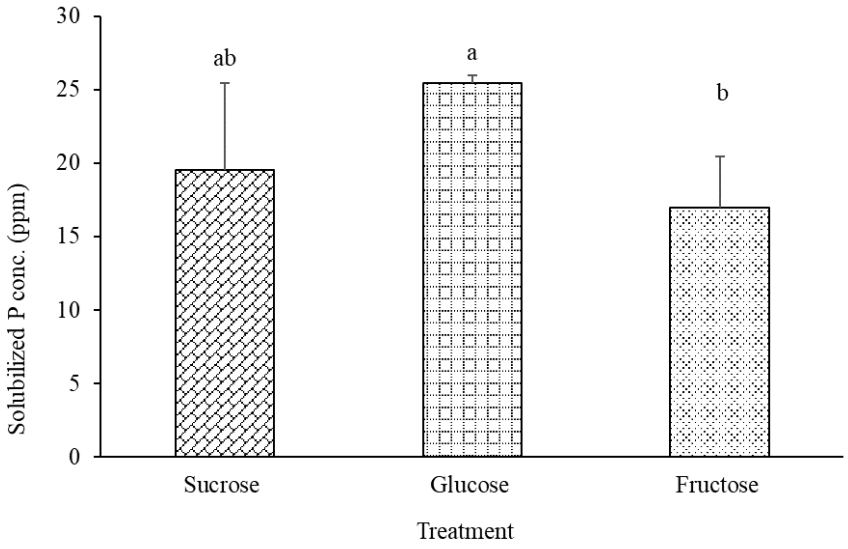

Figure 9 Effect of various Carbon sources on the efficiency of P solubilization with *Rhizobia* "c". Different letters indicate statistically significant differences at p≤ 0.05 probability level to the Least mean comparison test .

The amount of glucose as a carbon source played an important role in the phsophate solubilization. In this study, the effect of various C sources was investigated. Glucose displayed the highest P solubilization at 25.47±0.49 ppm, while fructose showed the lowest P solubilization. PSB isolated from different C sources revealed that glucose resulted in the highest P solubilization accompanied by a decrease in pH (Sagervanshi *et al*., 2012).

N sources

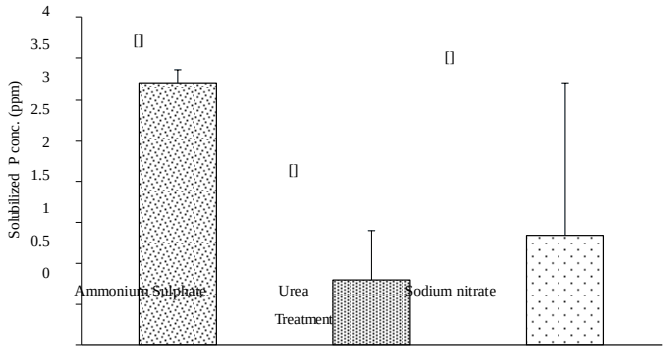

Figure.10 Effect of various N sources on the efficiency of P solubilization with *Rhizobia* "c". Different letters indicate statistically significant differences at $p \leq 0.05$ probability level to the Least mean comparison test .

While studying the effect of various N sources on the P solubilization it was found that ammonium sulphate recorded the significantly highest solubilized P at a rate of 3.2 ± 0.17 ppm, while the lowest solubilized P was observed with urea at 0.79 ± 0.61 ppm. In a previous study, the impact of different N sources solubilization was investigated by substituting five sources in the PVK medium: ammonium sulphate, sodium nitrate, potassium nitrate, calcium nitrate, and urea. It was found that ammonium sulphate resulted in the highest P solubilization (Sridevi and Mallaiah, 2009). Ammonium as the N source of several bacteria and fungi only have been reported to be able to solubilize phosphate (Illmer and Schinner, 1992; Lapeyrie, 1991). This finding aligns with previous reports indicating that many fungi and bacteria can solubilize phosphate effectively only in the presence of ammonium as the nitrogen source (Illmer *et al*., 1995).

In another literature investigating the effect of various N sources on P solubilization, ammonium sulphate exhibited the highest P solubilization, followed by casein. Urea and sodium nitrate showed very low P solubilization. In the control group with no N source, substantial growth and a decrease in pH were observed, along with slight P solubilization, likely due to yeast extract and glucose in the medium, which were utilized by bacteria as nitrogen sources (Sagervanshi *et al*., 2012).

4. Conclusion

Four bacterial strains (a,b,c,d) were isolated from *Sesbania grandiflora* root nodules and they were initially identified as *Rhizobia* based on morphology and authenticated through gram-staining, acid-alkaline tests, CRYMA and BRYMA tests. Out of the four isolates tested, isolated *Rhizobia* "c" was identified as effective P solubilizers based on PSI in PVK solid media and efficacy on PVK liquid media. Results of media optimization tests revealed that potassium dihydrogen phosphate as P source, glucose as C source and as N source performed better in the highest solubilization of P with modified PVK media with different nutrient sources. Moreover, this effective solubilizer *Rhizobia* "c" showed good potential for developing an inoculum for soil with its optimized media.

However, further improvements would be essential prior introducing *Rhizobia* "c" as a P solubilizing inoculum.

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