

INCLUSION OF BROMOTHYMOL BLUE DYE IN CONVENTIONAL PIKOVSKAYA'S MEDIUM FOR RAPID SCREENING OF PHOSPHATE SOLUBILIZING BACTERIA

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(Received 10 April, 2025; Accepted 30 May, 2025)

Key words: *Rhizobium*, *Bromothymol blue (BTB)*, *Pikovskaya's medium*, *Halo zone formation*, *Phosphate solubilizers*.

Abstract– The present study developed a modified plate assay and compared with previously reported techniques for the isolation and screening techniques of P-solubilizing bacteria. The newly developed plate assay is based on the inclusion of bromothymol blue (BTB), an acid-base indicator dye, which improves visualisation of halo zone formation surrounding the colonies on agar plates. Phosphate released in liquid medium showed a significant correlation ($R = 0.91248$) with the halo zone. This approach enhanced the visualisation of possible phosphate solubilizers and may be used to identify weak acid producers. The modified medium with BTB showed increased sensitivity and promising results, with all 9 isolates exhibiting clear halo zone formation, positively correlated with quantified phosphate. As a result, it is anticipated that using this qualitative analysis-based protocol will be salutary for the rapid screening of bacteria that solubilize phosphate.

INTRODUCTION

Phosphorus (P) is one of the essential major macronutrients necessary for plant growth but cannot be found in the atmosphere like nitrogen (Kumar and Ram, 2014). Plants can't manage to use the majority of the P (95-99%) present in the soil, as it is present in unavailable form to the plants (insoluble organic and inorganic phosphates) (Corona *et al.*, 1996). Significant amount of phosphorous applied to the soil as a fertilizer rapidly becomes immobile by chemical fixation when combined with metal ions like Al^{3+} and Fe^{3+} in acidic soils and Ca^{2+} in alkaline or normal soils (Khan *et al.*, 2009). It's interesting to note that a set of heterotrophic microbes solubilize these inorganic forms by producing organic acids and synthesising acid phosphatase (Arcand and Schneider, 2006).

Compared to fungus, bacteria are better at solubilizing phosphate (Alikhani *et al.*, 2007). Symbiotic nitrogen fixing bacteria are being more effective due to their shielded environment, as they are protected inside the nodule and face little competition with indigenous rhizospheric microflora (Alikhani *et al.*, 2007). Since early 1900s,

numerous media including Pikovskaya's and National Botanical Research Institute Phosphate (NBRIP) medium have been developed to screen PSMs based on the visual detection of clear zones in media containing insoluble mineral phosphates (Hii *et al.*, 2020).

Pikovskaya (1948) was the first person who developed calcium phosphate-based PSB detection medium. A halo or clear zone is produced into the surrounding medium by any microorganism is chosen as a possible phosphate solubilizer. The solubilization of insoluble phosphates, which is mostly caused by the release of organic acids into the surrounding medium, is what leads to this halo to form. However, there are consistently doubts about this halo-based technique's dependability. Numerous investigators have observed that a large number of isolates were able to solubilize different kinds of insoluble inorganic phosphates in liquid medium, even in the absence of any discernible halo zone on agar plates. When the halo is modest or non-existent, the current plate test is therefore ineffective (Gupta *et al.*, 1994).

Gupta *et al.* (1994) developed an enhanced Pikovskaya's solid medium containing bromophenol

blue, which formed yellow-coloured halos. Nevertheless, there was no discernible relationship between the liquid-culture based phosphate solubilization and plate halo detection seen (Joe *et al.*, 2018).

Hence, the focus of this study was to improve visualization of halo zone formation around the colonies on agar plates by modifying the existing medium, through inclusion of an acid-base indicator dyes and correlation studies between plate-based and broth-based assay.

METHODOLOGY

Sample collection and isolation of *Rhizobium*

In order to isolate *Rhizobium*, instead of soil, soybean root nodules were selected from various locations in Amravati region, Maharashtra. Actively growing plants with elite characters were selected. Pinkish, healthy and unbroken root nodules were detached and washed with running tap water. Further those were surface disinfected by 0.1 % (w/v) $\text{HgCl}_{2(\text{aq})}$ for 4-5 min and subsequent treatment with 70 % (v/v) ethanol and then, thoroughly washed thrice with sterile distilled water to remove the traces of disinfectants to avoid its deleterious effect on inner rhizobia. Nodules were crushed into sterile saline (0.85 % (w/v) $\text{NaCl}_{(\text{aq})}$) to release bacteroids into suspension (Vincent, 1970; Singh *et al.*, 2008; Sawale *et al.*, 2024).

Purified bacterial isolates were obtained by streaking 0.1 mL aliquot of suspension on yeast extract mannitol agar (YEMA) with Congo red (Congo red_(aq) 0.0025% (w/v)) and incubated at 28 ± 2 °C for 2-7 days (Gachande and Khansole, 2011; Sawale and Phirke, 2022). Growth was sub-cultured and preserved for further study.

Determination of cultural, morphological and biochemical traits of isolates

After incubation, single well isolated colonies were selected and further characterized for confirmation of those as *Rhizobium* as per the Bergey's manual of determinative bacteriology (Halt *et al.*, 1994).

Prescreening of phosphate solubilizing potential of *Rhizobium*

Qualitative analysis of Phosphate solubilization

Total 9 isolates were obtained. These isolates were prescreened on their phosphate solubilizing ability with Pikovskaya's agar, calcium phosphate as the

insoluble phosphate source. *Rhizobium* were spotted and incubated at 28 ± 2 °C and observed until formation of transparent 'halos' around each colony (Wang *et al.*, 2017).

Quantitative analysis of Phosphate solubilization

In Pikovskaya's broth quantitative estimations were carried. Rhizobial isolates were inoculated, separately, in Pikovskaya's broth, following 72 h at 28 ± 2 °C. Removing 1 ml of broth and centrifuged at 10,000 rpm for 20 min. A test tube was filled with 0.1 ml of supernatant plus 1.9 ml of double distilled water and 2 ml colour reagent (comprising sulfuric acid, ammonium molybdate, ascorbic acid solution and potassium antimonyl tartrate solution). The optical density was recorded at 882 nm after allowing the reaction happened for 20 min. Blank, containing only water and colour reagent, were used to set zero of spectrophotometer (Adhikari and Pandey, 2019).

Optimization of assay using Bromothymol blue (BTB) dyes

Modification in Pikovskaya's agar medium was done by amending the medium with different concentrations of bromothymol blue acid-base indicator dye. Bromothymol blue from stock solutions (5 mgL^{-1}) made with 70% (weight/volume) ethanol. Dye solutions (from stock) ranging from 1.0 to 5.0 mL, mixed in 100 mL of Pikovskaya's agar medium to get the final concentrations of 10.0, 20.0, 30.0, 40.0, and 50.0 mgL^{-1} . After adding the measured amounts of dye solution, the medium was autoclaved and poured into Petri plates. Plates containing Pikovskaya's agar medium devoid of any dye solution was kept as a control.

RESULTS AND DISCUSSIONS

It is well known that microbes significantly contribute to increased P availability in soil through solubilizing these inorganic forms by producing organic acids and synthesizing acid phosphatase. There were total of 9 isolates obtained on CR-YEMA medium, these isolates were further screened for phosphate solubilization using qualitative assay involving spot inoculation on Pikovskaya's agar plates. Based upon the halo zones observed in the plate assay, the positive isolates were grown in Pikovskaya's broth.

Several authors suggest that the synthesis of organic acids and chelating oxo acids from sugars is

what causes microorganisms to solubilize inorganic insoluble phosphate. Therefore, most of the quantitative tests to assay the relative efficiency of the phosphate-solubilizing bacteria are based on the lowering of pH, owing to production of organic acids into the surrounding medium (Mehta and Nautiyal, 2001).

The plate assay using Pikovskaya's medium, which is currently utilized for screening of P-solubilizing bacteria, is not rapid, reproducible and there is no discernible correlation between it and the quantitative estimation (Joe *et al.*, 2018). To quickly evaluate phosphate solubilization by isolates, the medium was modified with BTB dye, which turn yellow with a drop in pH.

Optimization of dye concentrations

Among the 3 different acid-base indicator dyes phenol red, bromocresol purple, and bromothymol blue, BTB was found to be superior in producing clear, well defined and distinct halo zones around the colonies (Rajawat *et al.*, 2016). Therefore, BTB dye with different concentrations was added into the Pikovskaya's medium for further analysis. As the organic acids were produced by the isolates while solubilizing phosphate, the colour of medium around colonies was turned yellow. The conventional Pikovskaya's agar takes at least 48 h to start the forming halo zone of solubilization. By using BTB dye yellow-coloured halo zones were appeared within 48 h and also the visibility and clarity of solubilization zone was improved.

Among the 9 Rhizobial isolates analysed qualitatively for P solubilization, all the 9 isolates have showed small halo zones on Pikovskaya's agar plates (Fig. 1. A). However, on the modified medium developed using BTB, all the 9 isolates showed large sized yellow coloured halo zone formation with better visibility and clarity (Fig. 1. B-C).

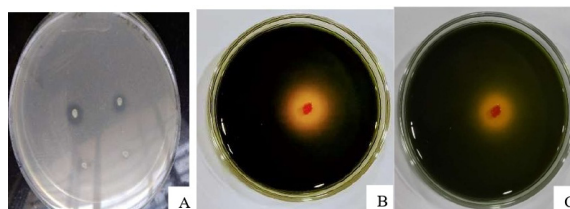


Fig. 1. Clarity of zone of Phosphate solubilization against incubation period: (A) Serving as a control, Rhizobial isolate inoculated on normal Pikovskaya's agar, (B) Same isolate inoculated on Pikovskaya's agar modified with BTB, incubated at 48h, (C) Same isolate inoculated on Pikovskaya's agar modified with BTB, incubated at 72 h.

In the modified plate assay, halo zone formation based on organic acid production showed a significant correlation with P released in liquid medium (Table 1). Similar inverse relationships between the concentration of solubilized minerals and the pH value of the culture medium were noted by Rajawat *et al.* (2016).

A concentration of 25 mgL⁻¹ of BTB was found to be optimum for the detection of P solubilizing

Table 1. Qualitative and quantitative comparison of P solubilizing potential of 9 Rhizobial isolates after 72 h incubation.

| Isolates | Pikovskaya's agar Halo zone (mm) After 2 days of incubation | Modified with BTB Pikovskaya's agar Halo zone (mm) After 2 days of incubation ^a | Solubilized available P ^a in liquid medium ($\mu\text{g ml}^{-1}$) | pH of the culture filtrate |
|----------|--|--|---|-------------------------------|
| BJ1 | 3.13 | 16.66 | 29.0 \pm 0.81 ^b | 4.8 |
| BJ2 | 2.53 | 11.33 | 24.3 \pm 1.24 | 5.1 |
| BJ3 | 3.03 | 18.33 | 31.0 \pm 0.81 | 4.9 |
| BJ4 | 3.00 | 15.00 | 26.3 \pm 0.47 | 5.1 |
| BJ5 | 3.26 | 18.33 | 34.3 \pm 1.24 | 4.8 |
| BJ6 | 2.66 | 11.66 | 24.0 \pm 1.63 | 5.2 |
| BJ7 | 3.23 | 18.66 | 34.6 \pm 1.69 | 4.5 |
| BJ8 | 3.03 | 16.00 | 26.6 \pm 2.05 | 5 |
| BJ9 | 2.86 | 12.33 | 21.6 \pm 0.94 | 5.2 |

a The correlation coefficient between the halo zone size with the modified plate assay for P-solubilization and the solubilized available P in liquid medium is 0.91248, showing significant correlations among the values obtained from the modified plate assay and phosphate quantification in broth.

b Means \pm standard deviations.

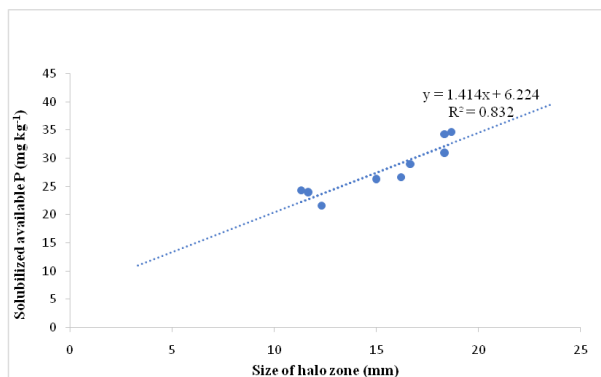


Fig. 2. Scatter plot illustrating the correlation between the size of halo zone with the modified plate assay and the solubilized available phosphate in culture broth.

bacteria. The incubation period for screening of P solubilizers was greatly shortened in this investigation. Using the modified plate assay, the minimum incubation period for screening P solubilizers was 36 hours; in the case of the traditional Pikovskaya's media, this duration typically exceeded 3 days. A direct significant correlation between the size of halo zone and quantitative solubilization of P in liquid medium was observed using this method. When it came to the isolation of P-solubilizing bacteria, it was more quick, reliable, and sensitive.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge the University Grant's Commission, New Delhi for providing instrumental facilities through major research project 41-1134/2012 (SR) awarded to NVP and Senior research fellowship 952/2017 to SKS and Rashtriya Uchchatar Shiksha Abhiyan for providing infrastructural facilities.

Conflict of Interest – None

REFERENCES

Adhikari, P. and Pandey, A. 2019. Phosphate solubilization potential of endophytic fungi isolated from *Taxus wallichiana* Zucc. roots. *Rhizosphere*. 9: 2-9.

Arcand, M.M. and Schneider, K.D. 2006. Plant-and microbial-based mechanisms to improve the agronomic effectiveness of phosphate rock: a review. *Anais da Academia Brasileira de Ciências*. 78: 791-807.

Bashan, Y., Kamnev, A.A. and de-Bashan, L.E. 2013. Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a

proposal for an alternative procedure. *Biology and Fertility of Soils*. 49(4): 465-479.

Gachande, B.D. and Khansole G.S. 2011. Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn. and *Bradyrhizobium japonicum* of Soybean. *Bioscience Discovery*. 2(1):1-4.

Gupta, R., Singal, R., Shankar, A., Kuhad, R.C. and Saxena, R.K. 1994. A modified plate assay for screening phosphate solubilizing microorganisms. *The Journal of General and Applied Microbiology*. 40(3): 255-260.

Halt, J.G., Krieg, N.R., Sneath, P.H. and Williams, S.T. 1994. *Bergey's Manual of Determinative Bacteriology*. 9th ed. Lippincott Williams and Wilkins. 95.

Hii, Y.S., San, C.Y., Lau, S.W. and Danquah, M.K. 2020. Isolation and characterisation of phosphate solubilizing microorganisms from peat. *Biocatalysis and Agricultural Biotechnology*. 26: 101643.

Joe, M.M., Deivaraj, S., Benson, A., Henry, A.J. and Narendrakumar, G. 2018. Soil extracts calcium phosphate media for screening of phosphate-solubilizing bacteria. *Agriculture and Natural Resources*. 52(3): 305-308.

Khan, A.A., Jilani, G., Akhtar, M.S., Naqvi, S.S. and Rasheed, M. 2009. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *Journal of Agricultural and Biological Science*. 1(1): 48-58.

Kumar, G.K. and Ram, M.R. 2014. Phosphate solubilizing rhizobia isolated from *Vigna trilobata*. *American journal of Microbiological research*. 2(3): 105-109.

Mehta, S. and Nautiyal, C.S. 2001. An efficient method for qualitative screening of phosphate-solubilizing bacteria. *Current microbiology*. 43: 51-56.

Perez Corona, M.E., Van der Klundert, I. and Verhoeven, J.T.A. 1996. Availability of organic and inorganic phosphorus compounds as phosphorus sources for *Carex* species. *New Phytologist*. 133(2): 225-231.

Pikovskaya, R. I. 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Microbiologiya*. 17: 362-370.

Qureshi, M.A., Shakir, M. A., Iqbal, A., Akhtar, N. and Khan, A. 2011. Co-inoculation of phosphate solubilizing bacteria and rhizobia for improving growth and yield of mungbean (*Vigna radiata* L.). *Journal of Animal and Plant Sciences*. 21(3): 491-497.

Rajawat, M.V.S., Singh, S., Tyagi, S.P. and Saxena, A.K. 2016. A modified plate assay for rapid screening of potassium-solubilizing bacteria. *Pedosphere*. 26(5): 768-773.

Sawale, S. K. and Phirke, N. V. 2022. Exploring the possibilities of using brady *Rhizobium japonicum* as a nitrogen fixing bio resource in soybean cultivation in Purna-river basin. *Sci Temper*. 13(1): 8-14.

Sawale, S. K., Pawar, A. S. and Phirke, N. V. 2024. Evaluation of plant growth promoting traits of Soybean (*Glycine max* L.) root nodulating *Rhizobium* and its characterization. *Ecology, Environment & Conservation*. 30: 383-390.

Singh, B., Kaur, R. and Singh, K. 2008. Characterization

- of *Rhizobium* strain isolated from the roots of *Trigonella foenumgraecum* (fenugreek). *African Journal of Biotechnology*. 7(20): 3671-3676.
- Vincent, J.M. 1970. *A Manual for the Practical Study of Root Nodule Bacteria*. IBP Handbook No. 15. Oxford, USA: Blackwell publication.
- Wang, Z., Xu, G., Ma, P., Lin, Y., Yang, X. and Cao, C. 2017. Isolation and characterization of a phosphorus-solubilizing bacterium from rhizosphere soils and its colonization of chinese cabbage (*Brassica campestris* ssp. *chinensis*). *Frontiers in Microbiology*. 8: 1270.
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