Isolation and Screening of Phosphate Solubilizing Bacteria (PSB) from the Rhizosphere of Fenugreek Plants

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Abstract

This study focuses on the isolation and screening of phosphate solubilizing bacteria (PSB) in the rhizospheres of fenugreek plants from the agricultural lands of Saurashtra region of Gujarat. Plant Growth Promoting Rhizobacteria (PGPR) are the group of rhizobacteria which helps in maintaining soil fertility and plant development. The PGPR secretes acids, enzymes, and antibiotics, which helps in increasing population of the beneficial microorganism as well reducing the pathogenic group of population. This study involves screening of phosphate solubilizing bacteria qualitatively. A total of seven rhizospheric samples were collected from the agricultural land of Saurashtra region. A total forty-four bacterial colonies were isolated. Out of the forty-four isolates, eleven isolates were found to be phosphate solubilizing bacteria. The isolates were screened on Pikovskaya's agar media by observing the formed halo-zone. In addition, pH indicator dyes such as Congo red and Bromothymol Blue (BTB) have been incorporated into the Pikovskaya's media to observe more visually.

Keywords: Phosphate solubilizing bacteria (PSB), Rhizosphere, PGPR, Pikovskaya's agar

1. Introduction

India is one of the largest producer, consumer and exporter of spices, with their 44 percent share in output and 36 percent in global trade. The International Organization for Standardization has listed 109 spices of which nearly 75 spices are grown in India. Being known for their aroma and taste, spices are also a part of industries such as cosmetics, medical, and pharmaceutical. The estimated market size of spices by 2030 is $ 59.5 billion with a growth rate of 5.45% during 2022 to 2030 **(Size et al., 2022).** India the spices market value has reached $ 195.5 million and is estimated to reach around $ 364 million with a growth rate (CAGR) of 10.8% **(IMARC Group, 2023).** The most produced and exported spices are chilli, cardamom, turmeric, pepper, coriander, cumin, fenugreek, celery, fennel, garlic, nutmeg, and tamarind. According to the Spices Board India Ministry of Commerce & Industry, Government of India, total Indian spices to be produced in year 2021-2022 is estimated to be about 10875765 tonnes **(Spices Board India, 2021).**

All life forms directly or indirectly depend on soil. It is a resource category that requires continuous attention and upkeep in order to stay productive. Identifying issues like nutrient shortages is necessary for maintaining soil fertility. This promises the continuation of extremely beneficial fertile soils in the near future **(Sipai et al., 2022)**. Mineral fertilisers have been used in agricultural fields and have demonstrated a balance in the output of nutrients, boosting the yield. According to studies, fertiliser use has resulted in a 50% rise in crop yield over the course of the 20^{th} century the general soil heath and its ecosystem services may suffer from improper fertilisation practises. Errors made when applying chemical or mineral fertilisers to soils can change their pH, increase pest attacks, and reduce the community of beneficial microorganisms and carbon content. These kinds of issues result in stunted plant growth, which eventually reduces yield **(Krasilnikov & Taboada, 2022)**.

Plant growth-promoting rhizobacteria (PGPR) can colonize and multiply within the rhizosphere environment within the plant micro biome. Root exudates, which are of various types such as organic acids, amino acids, sugars, and other small molecules exuded by the plant roots and act as potent chemo-attractants of the soil microbiota **(Olanrewaju et al., 2019)**. Rhizosphere is the immediate area surrounding the root. These PGPRs have several roles like antioxidant activities, biofilm production, phosphate solubilisation, antibiotic compound production (like lytic enzymes and antibiotics), siderophore production, and iron solubilisation. Among these, the main purpose of our research is to isolate and screen Phosphate Solubilizing Bacteria (PSB) **(Santoyo, G.et al., 2021).**

Phosphorus is considered the second most important macronutrient by plants next to nitrogen. It is known to be a crucial factor for many crop production systems, because of its unavailability in soluble forms in the soil **(Charana Walpola, 2012)**. Phosphorus/Phosphate (P) is a vital component of living, serving as the structural support for molecules like DNA (deoxyribonucleic acid), RNA (ribonucleic acid), and phospholipids in the cells of both plants and animals **(Kishore et al., 2015)**. The nucleic acid pool usually contains 40–60% of the P found in the combined organic P pool, making it the largest organic P pool in a plant. At least 85% of the nucleic acid mass is typically made up of RNA, and the remaining 15% is made up of DNA **(Veneklaas et al., 2012)**. Soils have phosphorus in both organic and inorganic form. The organic phosphorus makes up more than 50% of total phosphorus in soil and they include phytic acid, phospholipids nucleic acids etc. The remaining portion, i.e., inorganic form, include rock phosphates, orthophosphate ions, phosphoric acid and phosphate minerals that contain P, such as Iron, Calcium and Aluminium phosphates. Even if phosphorus is present in soils in high amount, these aren't available for plant uptake due to the mechanism of adsorption and precipitation. The phosphates which solubilised readily in water or 25% citric acid solution can be readily utilized by plants such as $(HPO_4)^{2-}$, $(H_2PO_4)^{2-}$ **(Kishore et al., 2015).**

Phosphate Solubilizing Bacteria (PSB) present in the soil make these unavailable phosphates available for utilization by plants via phosphate solubilisation and mineralisation **(Raj et al., 2014).** The mechanism for phosphate solubilisation mainly includes production of acids and acid phosphatases **(Charana Walpola, 2012)**. Acids like gluconic acid and 2-ketogluconic acid seems to be most common acids produced by these microorganisms for phosphate solubilization, but apart from those, citric acid, malonic acid, lactic acid fumaric acid are also some of the known solubilizers of phosphates **(Ahmed & Shahab, 2012; Charana Walpola, 2012; Song et al., 2008)**. To screen these PSBs, Pikovskaya's agar plating seems to be an

efficient method. In the Pikovskaya's agar (PVK) it can be an clearly observe microorganisms producing a halo zone which in turn depicts the production of acid for phosphate solubilization in the medium **(Katznelson et al., 1962; Mehta & Nautiyal, 2001)**. But the PVK agar plating method is said to be unreliable since not all PSBs produce acid that give a clear halo zone **(LOUW & WEBLEY, 1959; Mehta & Nautiyal, 2001; Singal et al., 1994; Sipai et al., 2022)**. Some of the studies reported that Pikovskaya agar (PVK) incorporated with colour changing indicator dyes such as Congo red and Bromothymol blue (BTB) are an effective method for screening of PSB **(Joe et al., 2018)**.

2. Materials and Methods

2.1 Sample collection and Isolation

Soil samples were collected aseptically from the rhizospheric zones of *Trigonella foenum-graecum* (fenugreek) from seven different locations of agricultural lands of Saurashtra region; Jetpur, Metoda, Haripar, Bhichri, Jamnagar, Khodapipar, and Junagadh.

2.2 Isolation of rhizobacteria

The samples were serially diluted with 0.85% NaCl solution and 0.1 millilitre of the solution with dilution of 10^{-2} to 10^{-7} pipetted out and spreaded on nutrient agar plates and incubated for 24 hours at 37°C. The Isolated bacterial colonies were purified by streaking them on a sterile nutrient agar plate. The isolates were identified by performing Gram staining and Potassium Hydroxide (KOH) test.

2.3 Screening of rhizobacteria for their phosphate solubilizing activity

The isolates were screened for their phosphate solubilizing activity by using Pikovskaya's (PVK) medium. The isolates were spot inoculated on PVK agar media which were incorporated with indicator dyes such as Congo red and Bromothymol blue (BTB) of 0.4% and 0.5% respectively and incubated at 28±2°C for 7-14 days **(Joe et al., 2018)**.

3. Result and Discussion

3.1 Isolation of Rhizobacteria

A total of forty-four isolates were with different morphology were isolated from the rhizospheric soil of fenugreek plants. The isolates were named according to the location they were collected from such as SRJ1-8 (Jetpur), SRM9-15 (Metoda), SRH16-22 (Haripar), SRB23-28 (Bhichri), SRJG29-32 (Jamnagar), SRKH33-38 (Khodapipar), SRJU39-44 (Junagadh). Further, these isolates were selected for screening for Phosphate Solubilizing Bacteria.

3.1 Screening for Phosphate Solubilizing Activity

The isolated rhizobacteria as mentioned in the Table 1, were screened for phosphate solubilizing activity by using Congo red and Bromothymol blue incorporated plates as mentioned in the methodology. Out of forty-four isolates eleven (11) isolates showed zone of solubilization on Pikovskaya's plate with indicator dyes. The 8 isolates named SRB25, SRH22,

SRJN31, SRJ1, SRKH36, SRJG42, SRJ4, SRB28 showed zone in PVK media along with BTB dye. In which SRB25 and SRH22 showed clearer zone of solubilization. Where other 6 isolates showed moderate zone. In Figure.1, the Pikovskaya's plates incorporated with bromothymol blue have changed their colour from light blue to yellow, which infers to the fact that acid was produced in order to solubilize the insoluble phosphate. Similarly, in Figure.2 the Pikovskaya's plates incorporated Congo red also showed zone of hydrolysis but there was no significant change in the colour of the plates. The colour change in the Congo red plates were less than moderate.

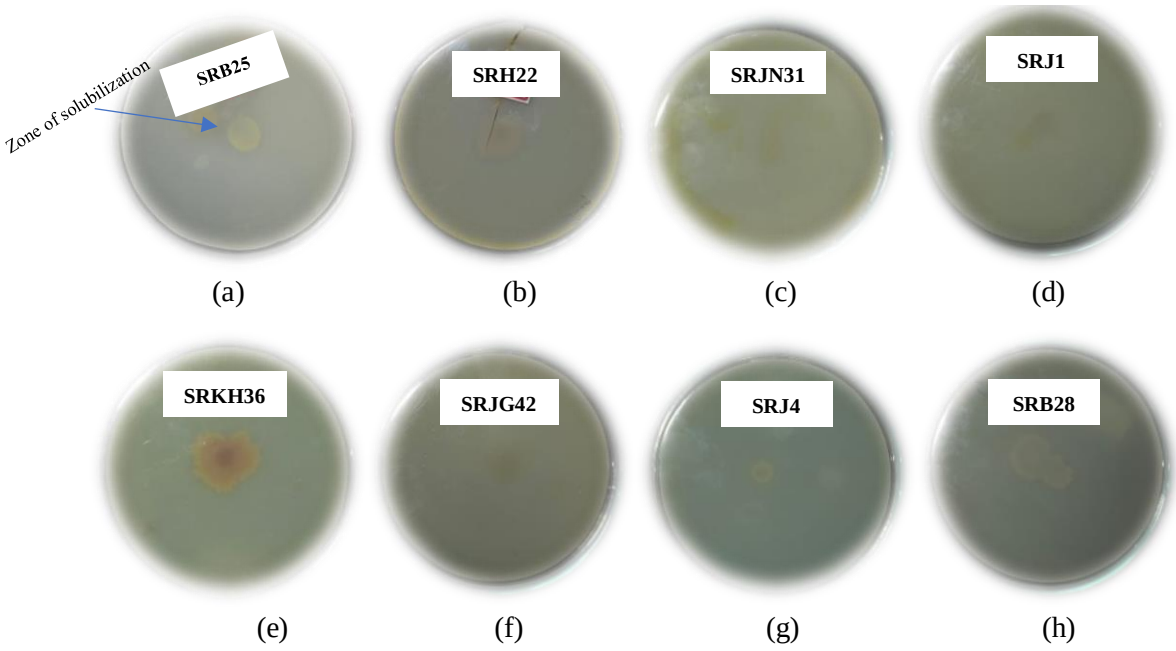

Figure.1: Zone of solubilization of Tri Calcium Phosphate by Phosphate solubilizing bacteria on PVK plate along with Bromothymol blue dye: (a) SRB25, (b) SRH22, (c) SRJN31, (d) SRJ1, (e) SRKH36, (f) SRJG42, (g) SRJ4, (h) SRB28.

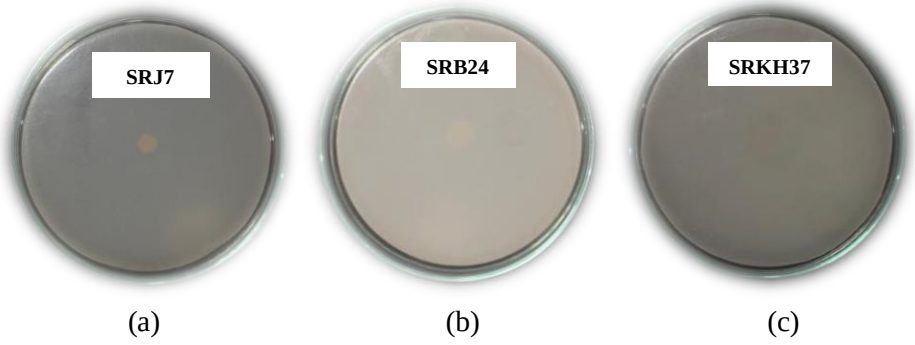

Figure.2: Solubilization of Tri Calcium Phosphate by Phosphate solubilizing bacteria on PVK plate along with Congo red dye: (a) SRJ7, (b) SRB24, (c) SRKH37.

It can be observed from Figure.2 that there is very light decolouration in the plates. Phosphate solubilizing activity by the rhizobacteria enables the plants to avail the insoluble part of phosphate in soil. Phosphate solubilization could be due to production of either acid phosphates or acids like gluconic acid, 2-ketogluconic acid, citric acid, malonic acid, lactic acid, fumaric acid etc. The zone of hydrolysis formed due to phosphate solubilization.

Table 1: Various Rhizospheric isolates along with their phosphate solubilizing activity

Isolate	Gram's Staining	KOH test	Zone of Clearance
SRJ1	-ve	-ve	++
SRJ2	+ve	+ve	-
SRJ3	+ve	+ve	-
SRJ4	+ve	+ve	+
SRJ5	+ve	+ve	-
SRJ6	+ve	+ve	-
SRJ7	+ve	+ve	++
SRJ8	+ve	+ve	-
SRM9	+ve	+ve	-
SRM10	+ve	+ve	-
SRM11	-ve	-ve	-
SRM12	+ve	+ve	-
SRM13	+ve	+ve	-
SRM14	+ve	+ve	-
SRM15	+ve	+ve	-
SRH16	+ve	+ve	-
SRH17	-ve	-ve	-
SRH18	-ve	-ve	-
SRH19	+ve	+ve	-
SRH20	-ve	-ve	-
SRH21	+ve	+ve	-
SRH22	+ve	+ve	+++
SRB23	+ve	+ve	-
SRB24	-ve	-ve	+
SRB25	+ve	+ve	+++
SRB26	-ve	-ve	-
SRB27	+ve	+ve	-
SRB28	+ve	+ve	+
SRJN29	+ve	+ve	-
SRJN30	+ve	+ve	-
SRJN31	+ve	+ve	++
SRJN32	+ve	+ve	-
SRKH33	+ve	+ve	-
SRKH34	-ve	-ve	-
SRKH35	+ve	+ve	-
SRKH36	+ve	+ve	++
SRKH37	-ve	-ve	+++
SRKH38	+ve	+ve	-
SRJG39	+ve	+ve	-
SRJG40	+ve	+ve	-
SRJG41	+ve	+ve	-
SRJG42	+ve	+ve	++
SRJG43	+ve	+ve	-
SRJG44	+ve	+ve	-

+: Mild zone, ++: Moderate zone, +++: Significant zone

4. Conclusion

Plants require phosphates for various metabolic pathways and ATP generation, they are also a vital component of their genetic material. Phosphate Solubilizing Bacteria (PSB), make the unavailable phosphates available to plants. Based on the findings, it can be concluded that bromothymol blue in Pikovskaya's agar media is an effective method for qualitative screening of phosphate-solubilizing bacteria. This study involves isolation and screening of phosphate

solubilizing bacteria (PSB) from fenugreek crop. Out of forty-four isolates 11 isolates showed positive results for PSBs. Since there was no colour shift with Congo red, it seems that this method of identifying phosphate-solubilizing bacteria may not be very successful.

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