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Phosphate Solubilization and Organic Acid Production of Efficient Phosphate Solubilizing Bacteria Isolated from the Rhizosphere Soil of *Tephroshiapurpurea* Linn.

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A B S T R A C T

Phosphate solubilizing bacteria had the ability to convert insoluble form of phosphate into soluble form by lowering the pH of the medium. Acidification was due to the solubilization of calcium and aluminum phosphates by the organic acids produced by the phosphate solubilizing bacteria. Production of organic acids was considered as one of the important mechanism of phosphate solubilization. Organic acids chelate the calcium cations that bound to phosphate by its hydroxyl and carboxyl groups which lead to the increased solubilization. In the present study among 86 strains were isolated from the rhizosphere soil of *Tephroshia purpurea.* Out of these four efficient strains SS2, SS5, SS7 and SS11 were found to be very effective in phosphate solubilization. The efficient strains SS2, SS5, SS7 and SS11 were subjected to 16S RNA sequencing and identified as *Stenotrophomonas rhizophila*_SS2, *Stenotrophomonas rhizophila*_SS5, *Stenotrophomonas rhizophila*_SS7, *Stenotrophomonas rhizophila*_SS11 (GenBank Accession no OM131765, OM131766, OM131767, OM13178). The strains were further characterized for organic acid production by HPLC analysis. The peak values in retention time were observed for SS2 (2.463), SS5 (2.258), SS7 (2.251) and SS11 (2.724) respectively. The organic acids as involved in the reduction of pH of the medium and aids effective phosphate solubilization.

***Key words*:** Phosphate solubilizing bacteria, *Tephroshia purpurea,* Organic acids, Acidification, Retention time

Phosphorus is one of the key elements necessary for the plant growth and development. The availability of phosphorus in soil is in limited amount and cannot utilize by the plants directly. The limitation of phosphorus is overcome by the addition of phosphate fertilizers to the soil. The added phosphorus and the phosphorus in the environment mostly present as insoluble form. This has been accumulated in the soil leads to eutrophication. Phosphate solubilizing bacteria plays an important role in converting the insoluble form of phosphate into soluble form of which the plants can utilize directly. Phosphate solubilizing bacteria were effective in releasing phosphorus from total phosphorus in soil by the process of solubilization (inorganic phosphorus) and mineralization (organic phosphorus) [1]. The mechanism of inorganic phosphate solubilization by the organisms in the medium is by the mineral dissolving compounds production such as organic acids, exopolysaccharides, siderophores, liberation of protons, Chelation, bound to phosphate through their hydroxyl ions and CO_2 [2]. Phosphate solubilization efficiency has been observed with the reduction in pH of the medium [3]. The acidity of the medium is mainly due to the production of organic acids by lowering the pH of the medium, chelate the cations (especially calcium) that bound to phosphate by its hydroxyl and carboxyl groups, compete with phosphate for adsorption sites which leads to the increased solubilization of phosphorus and availability of mineral phosphates to plants. Organic acids may also increase P availability by blocking P absorption sites on soil particles or by forming complexes. The present study aimed for the identification of efficient strains from the rhizosphere soil of *Tephroshia purpurea* and further analyzed for the production of organic acids.

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MATERIALS AND METHODS

Rhizosphere soil samples of *Tephrosia purpurea* L, plants were collected from thirty sites of different districts in Tamil Nadu. The rhizosphere soil samples that adhere to the root hairs and 0-15 cm below the surface soil of *Tephrosia purpurea* (Linn.) were collected randomly and air dried, sieved and stored in sterile plastic containers [4].

Isolation of phosphate solubilizing bacteria

One gram (1g) of each soil sample was measured and transferred to 9 ml of sterile distilled water to form 10^{-1} dilution

and from this 1 ml of sample was serially diluted up to 10^{-9}.0.1 ml from the dilutions 10^{-4} to 10^{-9} was spreaded over the sterile Pikovskaya medium and incubated at 37°C for 2-7 days. Triplicates were done and the uninoculated plate served as control [5].

Phosphate solubilization

Phosphate solubilizing ability of isolates SS2, SS5, SS7 and SS11 were checked in Pikovskya's agar medium [6]. The isolates were streaked on to the Pikovskya's agar plate and incubated at room temperature for 5 to 6 days. Phosphate solubilization was detected by clear halo zones around the colony.

Effect of pH on efficiency of Phosphate solubilization

Phosphate solubilizing efficiency was analyzed by the change in the pH of broth media. For this the identified efficient phosphate solubilizing bacterial culture was inoculated in Pikovskaya broth and incubated for 14 days. The initial pH and the change in pH were checked and recorded from day 1 to day 14 of incubation [7].

Bacterial Identification using 16S rRNA gene sequence

The selected strains were identified by partial sequencing of the 16S rRNA gene. Genomic DNA were isolated from the bacterial culture by using EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd.16S rRNA gene was amplified using universal primers forward (5´-'AGAGTTTGATCTGGCTCAG-3') and reverse (5´-TACGGTACCTTGTTACGACTT-3'). 50µL reaction mixture were prepared containing 5 µL of DNA template, 5µL 10X PCR Reaction Buffer,0.75µL10µ, M of each primers, 1µL10µ MdNTPs mix, 0.5µL5µ/µL Taqpolymerase, 3µL25µM $MgCl_2$ and 34µL ultra-pure water. PCR reactions were carried out in a thermal cycler. The PCR product was sequenced using the primers. Sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with Ampli Taq® DNA polymerase (FS enzyme). (Applied Biosystems) using cycles as follows: 5 min at 95°C,30 sec at 95°C, 30 sec at 50°C, 1 min at 72° C and final extension for 5 min at 72°C. The amplified 16SrRNA gene was purified with a Gel/PCR DNA Fragments Extraction Kit. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors by using BLAST (NCBI, USA).

Analysis of organic acids

HPLC was used for the analysis of organic acids produced by the straining broth medium. On 8th day of incubation, the supernatant was collected from the bacterial culture and centrifuged at 13,000 rpm for 15 min. Sample were filtered through 0.45 mM Millipore filter and from this 20µl of filtrates was injected into an HPLC column using a glass syringe. 20 mMKH_2PO_4 and methanol (60:40v/v) were used as mobile phase at a constant flow rate of 1 ml/min and the column was operated at 30^0C. Retention time (RT) of each signal was recorded at a wavelength of 256 nm. HPLC profiles of the culture filtrates were compared with the elution profiles of pure organic acids and the peak areas of their standards [7].

RESULTS AND DISCUSSION

A total of 86 efficient phosphates solubilizing bacterial strains isolated from the rhizosphere soil samples of *Tephrosia purpurea* (Linn.) plants. Out of the 86 strains the isolates SS2, SS5, SS7 and SS11showed maximum zone formation in Pikovskaya medium. From the four strains SS11 showed the highest solubilization index of 60.1. The isolates SS2 and SS7 was with the solubilization index 58.8, 57.5 respectively followed by SS5 showed 52.4 of solubilization index at the incubation period of 7 days (Table 1, Fig 1). The data of (Table 2) shows the quantitative estimation of isolates on PKV broth with initial pH 7.0. The maximum reduction in pH was noted in 7^{th} day of incubation which falls down to 2.5 and 2.3 respectively. The strain SS2 and SS11 showed maximum reduction in pH 7.0 to 2.3 whereas SS5 and SS7 reduce pH 7.0 to 2.5after seven days of incubation no reduction changes in pH of the medium (Table 3, Fig 1). PSM were grown in vitro for seven days on Pikovskaya's medium and performed the analysis such as solubilization index, pH change, phosphorus (P) solubilized, P immobilized and organic acids produced under invitro conditions [8]. P solubilization index of the isolates ranged from 1.63-3.29. Drop in pH of the medium were ranged from 7 to 3.2 with the continuous growth of the isolates for seven days and confirmed that more P was immobilized (0.2-0.46%) than solubilized (0.088-0.22%). They concluded that bacteria found to be more active than fungi in conversion of insoluble P to soluble P and Citric and oxalic acids was the two common organic acids produced by PSM.

Table 1 Efficiency of phosphate solubilization of isolated bacterial strains

Isolates	Colony diameter (mm)	Halozone diameter (mm)	Solubilization index (mm)
*Stenotrophomonas rhizophila*_SS2	1.2	69.1	58.8
*Stenotrophomonas rhizophila*_SS5	1.4	71.4	52.4
*Stenotrophomonas rhizophila*_SS7	1.2	67.6	57.5
*Stenotrophomonas rhizophila*_SS11	1.1	64.9	60.1

Table 2 Effect of pH on efficiency of phosphate solubilization

Day	SS2	SS5	SS7	SS11
1	7.0	7.0	7.0	7.0
2	5.6	5.8	5.9	5.5
3	5.5	5.5	4.6	4.2
4	4.6	4.7	4.0	3.8
5	4.0	4.1	3.5	3.4
6	3.4	3.6	3.1	2.8
7	2.3	2.5	2.5	2.3
8	2.3	2.5	2.5	2.3
9	2.3	2.5	2.5	2.3
10	2.3	2.5	2.5	2.3
11	2.3	2.5	2.5	2.3
12	2.3	2.5	2.5	2.3

Table 3 HPLC analysis of Organic acids produced by the bacterial isolates

Bacterial isolates	Retention times (minutes)
SS2	2.463
SS5	2.258
SS7	2.251
SS11	2.724

Their correlation studies suggested that besides organic acids there might be other factors responsible for P solubilization. In previous studies Pérez *et al*. [9] studied the characterization and molecular identification of *Stenotrophomonas rhizophila* strains isolated from the rhizosphere soil of maize and reported a common bacterial

genus *Stenotrophomonas* in many rhizosphere soil of different crops as a plant growth promotors. In the present study the efficient bacterial isolates SS2, SS5, SS7 and SS11 isolated from the rhizosphere soil samples of *Tephrosia purpurea* (Linn.) were identified as *Stenotrophomonas rhizophila*_SS2, *Stenotrophomonas rhizophila*_SS5, *Stenotrophomonas rhizophila*_SS7 and *Stenotrophomonas rhizophila*_SS11 using 16S rDNA gene amplification.

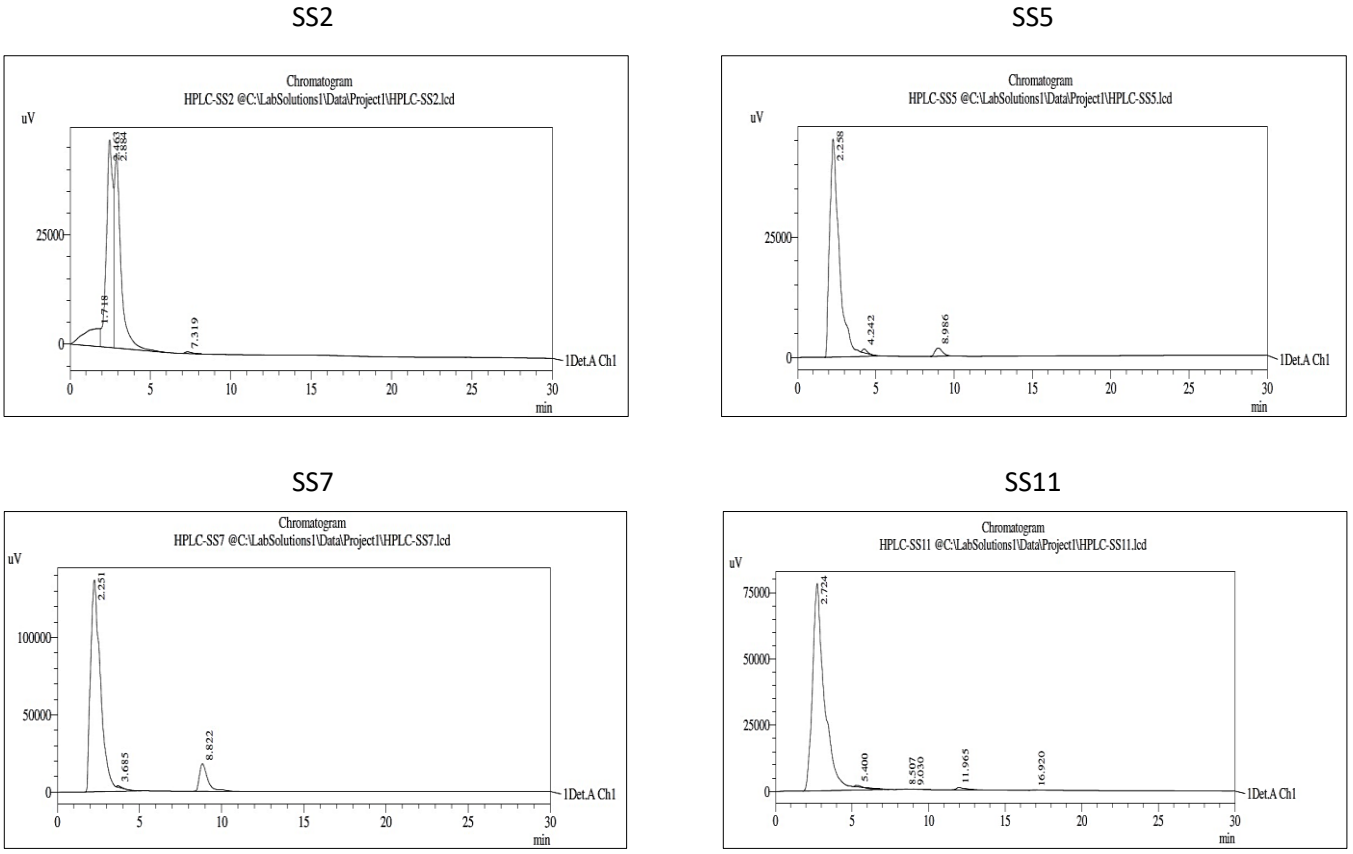

Fig 1 HPLC chromatogram of bacterial isolates

In the previous reports Rodríguez and Fraga [10] reported phosphate solubilizing bacteria as inoculants for the increased uptake of P by the plant and crop yield. The production of organic acids and acid phosphatases plays a major role in organic phosphorous mineralization in soil which was the principal mechanism in phosphate solubilization. Behera *et al.* [11] studied on the phosphate solubilizing bacterium PSB-37 isolated from mangrove soil of Mahanadi River delta in NBRIP agar medium and NBRIP-BPB broth that contains tricalcium phosphate as the phosphate source and the strain based on the phenotypic and molecular studies was identified as species of *Serratia*. The strain exhibits maximum phosphate solubilizing activity of 44.84l g/m with reduction in pH from 7.0 to 3.15. Many organic acids were detected in phosphate solubilization such as malic acid (237 mg/l), lactic acid (599.5 mg/l) whereas low levels of 5.0 mg/l acetic acid were detected. Satyaprakash *et al*. [12] suggested the principal mechanism for solubilization of phosphorus in soil by lowering the pH due to the microbial production of organic acids or the release of protons., Phosphate had the ability to precipitate in alkaline soils which leads to formation of calcium phosphates, including rock phosphate that are insoluble in soil and its solubility level raises with decrease pH in soil. Therefore, the P availability was increased with the production of organic acids which reduces the soil pH. Khalil *et al*. [13] reported organic acids secreted by solubilizing bacteria or by plants include oxalic, tartaric, acetic, citric, butyric, propionic, malonic, lactic, succinic, malic, acetic, fumaric and adipic acids, gluconic acid and keto-gluconic acid plays an important role in phosphate-solubilizing process. The type and amount of organic acids secreted by different PSB and plant are variable and different organic acids play various roles in the phosphate-solubilizing process.

In the present study on the analysis of HPLC the *Stenotrophomonas rhizophila*_SS2 shows the peak value with the retention time (RT) of 2.463, *Stenotrophomonas rhizophila*_SS5 and *Stenotrophomonas rhizophila*_SS7 with 2.258 RT and 2.251 RT whereas *Stenotrophomonas rhizophila*_SS11 with 2.724 retention time respectively.

In the previous studies Kaewkod *et al*. [14] reported that gluconic acids were detected by HPLC in kombucha tea with the retention time of 2.433. In the present study the bacterial isolate *Stenotrophomonas rhizophila*_SS2 also shows the with the retention time of 2.463. Also, glucuronic acid was detected by HPLC in kombucha tea with the retention time of 254. In the present study bacterial isolates *Stenotrophomonas rhizophila*_SS5 and *Stenotrophomonas rhizophila*_SS7 showed the peak value with the retention time in HPLC as 2.258 and 2.251 respectively. The plant extracts of *Berkheya coddii* showed the presence of Phthalic acid at the retention time of 2.79 in HPLC [15]. In the present study a bacterial isolate *Stenotrophomonas rhizophila*_SS11 shows the retention time peak of 2.724. The production of by microbes organic acids such as gluconic, citric, malic, malonic, oxalic, succinic, lactic and tartaric [16]. These acids provide both organic anions and protons which serve as chelating agents. The anions with the negative charge form complexes to the ions of positively charged (Ca^{+2}, Al^{+3}, Fe^{+3}) particles present in the soil thereby releasing the phosphorus that is precipitated or occluded.

Inorganic phosphate solubilization by bacteria was associated with the release of low molecular weight organic

acids, such as citric, oxalic, malic and gluconic acids that chelate phosphate-bound cations through their hydroxyl and carboxyl groups and converts them into bioavailable forms [17-18]. In the previous report Song *et al.* [19] reported that the acidification of the medium was due to the production of organic acids which was one of the mechanism of phosphate solubilization and the type of organic acids produced by the organisms differ with other microbes. Among the organic acids glucuronic acid and 2-Ketogluconic acid were most frequent acids in mineral phosphate solubilization and in the previous study Munir *et al.* [20] reported that phthalic acid (1,2 benzene dicarboxylic acid) belongs to the member of benzoic acid had the capacity of phosphate solubilization produced by a fungus. The other only report of Yandigeri *et al*. [21] the role of phthalic acid in solubilization of phosphate was from the study of diazotrophic cyanobacteria of *Westiellopsis prolific* and *Anabaena variabilis* secreted phthalic acid in culture filtrates as mineral phosphate solubilizing agent. The organic acids in addition to phosphate solubilization also used as a carbon source for microorganisms which consume and reduce the phosphate [22]. Hence the effect of solubilization had allowed determining the lifespan between 0.5 and 12 h of these acids and they were produced and secreted continuously. *Stenotrophomonas rhizophila* strains were able to utilize organic acids as carbon sources such as acetic acid, citric acid, butyric acid, ketobutyric acid, keto-glutaric acid, lactic acid, malonic acid, propionic acid, succinic acid and succinamic acid [23].

CONCLUSION

Our study concluded that strains *Stenotrophomonas rhizophila*_SS2, *Stenotrophomonas rhizophila*_SS5, *Stenotrophomonas rhizophila*_SS7, *Stenotrophomonas rhizophila*_SS11 isolated from the from the rhizosphere soil of *Tephroshia purpurea* can able to produce the effective agents of solubilization of phosphate such as organic acids thereby reducing the pH of the medium. The use of these strains as bioinoculants can able to solubilizes greater amount of phosphate even in stress conditions thereby increasing the plant growth and yield besides improvement in the soil health. The strains of *Stenotrophomonas rhizophila* might secrete the organic acids of gluconic acid, glucuronic acid and Phythalic acid as correlate with other report retention times.

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