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ORIGINAL ARTICLE **OPEN ACCESS**

"Molecular Characterization of Potential Phosphate Solubilizing bacteria *Enterobacter hormaechei*from Soil"

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ABSTRACT:
A total of 10 phosphate solubilizing bacteria were isolated from 5 different soil samples collected from the Sarud region. Out of 10 phosphate solubilizing isolates, 5 capable to solubilize phosphate would be tested using primary and secondary screening methods. Out of 5 phosphate solubilizing bacterial isolates, one isolate was sorted out based on the zone of phosphate solubilizing on Kartznelson and Bose *and Pikovskaya's medium. The phosphate-solubilizing bacterium was identified using morphological, physiological, and biochemical characteristics. Genetic identification of potential isolate was carried out by 16S rRNA gene sequencing. The phosphate-solubilizing bacterium (SW3) was identified as an Enterobacter hormaechei.The purified potential phosphate-solubilizing bacterium was tested under different environmental conditions. Enterobacter hormaechei was grown at optimum physicochemical parameters such as temperature 40^{0}C, pH- 7.5, and agitation speed- 150 rpm. Enterobacter hormaechei showed growth on a 4% salt concentration.*
Keywords: *Screening, Phosphate solubilizing, Isolation, Identification, Physicochemical.*

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INTRODUCTION

A nation's development is directly proportional to the amount of food or nutrient available to the population. The human population growth creates a demand for more food grains. The productivity of the soil is increased by the incorporation of any substance known as fertilizer. Fertilizers can promote soil fertility by incorporating nutrients into the soil, which helps in plant growth. Chemical fertilizers are composed of raw chemicals in the solid or liquid form manufactured in factories targeting the nutritional requirement of the plants. The food demand of the increasing population is completed by the use of fertilizers.Nitrogen, Phosphorous, and Potassium together called NPK are normally present in these chemical fertilizers along with other nutrients (13).The substances are normally present in a form that is easily absorbed by the plant. The use of chemical fertilizers has some harmful side effects on the environment (11). The most important issue includes groundwater contamination, especially nitrogen compounds, which break down into nitrate and accumulates in the groundwater.

Biofertilizers can be used as supplements to chemical fertilizers; they are comparatively inexpensive and renewable sources of plant nutrients. Biofertilizers are selected strains of microorganisms that are beneficial to the growth of plants. These microorganisms are cultured in the laboratory, mixed with suitable carrier materials, and then applied to the fields. They maintain soil health and minimize pollution of the environment by lowering the use of chemicals (10). Biofertilizers are used to treat seeds, plantlets, and grown plants. The popularity of biofertilizers is due to their eco-friendly, non-hazardous, and non-toxic nature. The living microorganism colonizes the rhizosphere or colonizes the interior of the plant, they promote growth by increasing the availability of the nutrients and help in the breakdown of inorganic substances in an organic form, increasing the supply of growth stimulus to the seeds of crops, plant surfaces and even in the soil can help in greater productivity. Examples of bio fertilizers are numerous and varied like *Rhizobium*, *Azospirillium*, and *Azotobacter* – Nitrogen fixing biofertilizers. *Pseudomonas*, *Bacillus*, and *Aspergillus*, are examples of PSB or Phosphate solubilizing biofertilizers. Mycorrhiza is an example of a Phosphate mobilizing biofertilizer. *Pseudomonas* species are also commercialized as Plant growth-promoting biofertilizers. Soil microorganisms play an important role in

maintaining the ecological balance, they participate actively in carbon, nitrogen, phosphorus, potassium, and sulfur recycling in nature and thus facilitate uptake in the plants (1).
The role of microorganisms in converting insoluble forms of nutrients into soluble forms is well known. After nitrogen, phosphorus is second in terms of importance for growth in plants. Phosphorous is 0.2% of the dry weight in plants. Phosphorus is obtained by the plant as phosphate anions. Phosphate solubilizing bacteria possess the capability to convert phosphorus from insoluble to soluble form (7). Phosphatic fertilizer when applied to the soil it has been seen that only a small amount is actually utilized by the plants. In India, it has been estimated that about 98% of the soil has some amount of deficit in phosphorus. Chemical fertilizers having phosphorus have a disadvantage, inorganic phosphates when applied to the soil are immobilized and thus not totally available to the plant (6). PSBs or Phosphate solubilizing bacteria helps in converting phosphorus into soluble forms by acidification by organic acids, and chelating oxo acids from sugars. They also produce enzymes like phosphatase enzymes that help in further degradation. Inoculation of PSBs in soil or near the rhizosphere of the plants has been shown to promote the growth of plants as a stimulatory effect. Plant roots can take up different forms of phosphorus like $H2PO^{4}$, and HPO^{2-}, this take-up normally depends upon the soil pH, temperature, moisture content, and other nutrients or minerals present in the soil(9).

MATERIAL AND METHOD

Collection of soil samples

The soil samples were collected from agricultural field areas of the Sarud region, Maharashtra. Near about five different areas were selected and the soil sample was collected from a depth of 15-20cm. The soil samples were collected separately in sterile polythene bags and brought to the laboratory.

Serial dilution of the soil sample

1gm of weighed soil sample was added in 10 ml sterile distilled water as a diluent in a test tube. This makes the first dilution labelled as 10^{-1}. Aseptically dilute soil sample up to 10^{-9}. The process was performed under Laminar Air Flow to maintain sterile conditions.

Isolation of Phosphate Solubilizing Bacteria

The selective media prepared for isolation and growth of Phosphate Solubilizing Bacteria had the following constituents: Yeast extract, Dextrose, Tricalcium Phosphate, Ammonium Sulphate, Potassium Chloride, Magnesium Sulphate, Manganese Sulphate and Ferrous Sulphate, Agar and Distilled water. The media was sterilized by autoclaving at 121^{0}C for 15 minutes. The media was poured into Petri plates and allowed to solidify. The soil solution of about 0.1 ml was spread on to the plate by Spread-plate technique. The plates were incubated for 24 hours at 37^{0} C (12).
After incubation for 24 hours, the plates were taken out and the growth of microorganisms was seen on the plates. Plates of dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}, and 10^{-7} were chosen for further screening and formation of halo zone around the selected colonies.

Halo zone test

Isolated colonies from the isolation plates were chosen and utilized for screening and halo zone test. The isolates were screened for their capability of TCP solubilizing activity. These were performed in PKV plates. Isolated colonies were streaked on PKV plates. They were kept in an incubator for 5-7 days at 37^{0} C. Clear zone around the growth of the microorganism was regarded as a positive test. All the observations and experiments were carried out in triplicates for better results. The strains that developed clear zones around the colonies of microorganism growing on the plate were easily identified as PSBs (8).

Characterization of the isolated microorganism

Morphological characterization:

The isolated strains of microorganisms were grown on EMB plates. Eosin Methylene Blue helps to identify whether the strain is a Gram positive or Gram negative (4). Only Gram negative strains grow on EMB plates. It is thus selective in nature. Identification and characterization of the isolated strain was done following standard bacteriological techniques (2,5).

Gram Staining

Isolate was tested for the fundamental test like Gram staining. Isolate was smeared on clean and grease free slides. It was then heat fixed. The smears were stained using Gram staining procedure. The slide was examined under the 100 X oil immersion microscope.

pH test

The isolate was quantitatively tested in liquid media such as PKV broth. The isolated strains were grown in PKV broth. The broth was autoclaved at 121^{0}C for 15 minutes, then allowed to cool down to a suitable temperature. Then the broths were incubated for 5-7 days at 37^{0}C. Phosphate solubilization was measured by recording final pH of the media.

Biochemical characterization of the organism Isolates were tested by biochemical testes such as MR-VP test, Hydrogen sulphide test, Starch hydrolysis test, Catalase test. The above tests were performed as per Bergey's Manual of Systematics Bacteriology (3).

Effect of temperature on isolates:

Isolate was tested for growth at various temperature in broth culture method. Temperature values selected were 30^0C, 35^0C, 40^0C, 45^0C and 50^0C. Optimum temperature value for maximum growth of isolate was determined.

Effect of pH on isolates:

Isolate was tested for growth at various pH values in broth culture method. pH values selected were pH-7, pH-7.5, pH-8.0, pH-8.5, and pH-9.0. Optimum pH value for maximum growth of isolate was determined.

Effect of agitation speed on growth of isolate:

Isolate was tested for growth at various agitation speed in broth culture method. Agitation speed values selected were in (rpm) 50, 100, 150, 200 and 250. Optimum agitation speed value for maximum growth of isolate was determined.

Effect of salt on growth of isolate:

Isolate was tested for growth at various salt concentration in broth culture method. Salt concentrations selected were in (%) 1, 2, 3, 4 and 5. Optimum salt concentration for maximum growth of isolate was determined.

Molecular characterization of isolate:

Out of 10 isolates, only isolates showing potential ability for maximum phosphate solubilization was selected and identified at molecular level by 16S rRNA technique. 16S rRNA sequencing of isolates were carried out by using different steps.

Preparation & Production of PSB biofertilizer

Selected strain was grown in 1000 ml PKV broth as a production medium in optimum environmental conditions. The growth rate of the PSB culture was determined at regular time interval. The culture was mixed with cow dung as the carrier material. The ratio of PSB and carrier material was kept at 1:1. The mixture was packaged and kept for storage at 4 ^{0}Covernight.

Field trial of the biofertilizer on maize seeds.

Maize seeds were treated with mixture of bacterial isolates and carrier. Treated seeds were then sown in a field, at Sarud and Shirala district. The fields were divided into various segments which were denoted as Control field (C), Fertilizer field (N) and the PSB biofertilizer field (P). The seeds were regularly watered and growth was measured in terms of plant height, number of tassels, flowers and fruit that grew on the maize plant.

RESULTS AND DISCUSSION

All ten PSB isolates were tested for Gram nature and pH test. Their results were represented in **table no.1**. Isolates SW1, SW6,SW7 and SW8 were showed Gram positive nature while isolates SW2, SW3, SW4, SW5, SW9 and SW10 were showed Gram negative nature after Gram staining. Gram staining showed that 60% isolates were Gram negative while 40% isolates were Gram positive in nature. The isolate SW2 (pH-4.5) was produced minimum amount of an organic acid while isolate SW3 was produced maximum amount of an organic acid (pH-3.42) in liquid production medium. All other isolates were being producer of organic acid but their value ranges in between pH-3.42 to pH-4.5. SW3 was potential producer of organic acid so it was selected for production of biofertilizer.

Table no.1: Gram nature and pH test

Sr.No.	Isolate	Gram nature	pH
1	SW1	Gram positive	4.2
2	SW2	Gram negative	4.5
3	SW3	Gram negative	3.42
4	SW4	Gram negative	3.82
5	SW5	Gram negative	3.68
6	SW6	Gram positive	3.96
7	SW7	Gram positive	3.78
8	SW8	Gram positive	3.79
9	SW9	Gram negative	4.12
10	SW10	Gram negative	4.35

Biochemical test of selected isolates was done by various biochemical test such as MR test, VP test, Catalase test, Oxidase test, starch hydrolysis and H_2S production test. Results of biochemical test for

selected isolate was represented in **table no.2.** MR test, VP test, Catalase test, Oxidase test, starch hydrolysis was positive and H_2S production test was negative for selected isolate.

Table no.2: Biochemical test of isolates

Iso. / B.T.	Methyl red test	Voges Proskauer	Catalase test	Oxidase test	Starch hydrolysis	H_2S
SW3	Positive	Positive	Positive	Positive	Positive	Negative

Where: *Iso. : - Isolat B.T.: - Biochemical test*

Effect of temperature on selected isolate was done at various temperatures such as 30^0C, 35^0C, 40^0C, 45^0C and 50^0C and results were recorded in **table no.3.**Minimum temperature value for growth of isolate was 30^0C and maximum temperature for growth of isolate was 45^0C. The optimum temperature value for maximum growth of isolate was 35^0C. This temperature was used for production of PSB biofertilizer.

Table no.3: effect of temperature

Iso. / Temp.^{0}C	30	35	40	45	50
SW3	+	+++	++	+	-

Where: "+" = Growth
"-"= No growth

Effect of pH on selected isolate was done at various pH such as pH-7, pH-7.5, pH-8.0, pH-8.5, and pH-9.0. and results were recorded in **table no.4.** Minimum pH value for growth of isolate was 7 and maximum pH for growth of isolate was 9. The optimum pH value for maximum growth of isolate was 7.5. This pH value was used for production of PSB biofertilizer.

Table no.4: effect of pH on growth

Iso. / pH	7	7.5	8	8.5	9
SW3	+	+++	++	+	-

Where: "+" = Growth
"-"= No growth

Effect of agitation speed on selected isolate was done at various agitation speed in rpm such as 50, 100, 150, 200 and 250 and results were recorded in **table no.5.** Minimum agitation speed for growth of isolate was 50 rpm and maximum agitation speed for growth of isolate was 250 rpm. The optimum agitation speed for maximum growth of isolate was 150 rpm. This agitation speed was used for production of PSB biofertilizer.

Table no.5: effect of agitation speed on growth

Iso. / Agitation speed	50	100	150	200	250
SW3	+	++	+++	+	+

Where: "+" = Growth
"-"= No growth

Effect of salt concentration on selected isolate was done at various salt concentrations in % such as 1, 2, 3, 4 and 5 and results were recorded in **table no.6.** Minimum salt concentration for growth of isolate was 1% and maximum salt concentration for growth of isolate was 5%. The optimum salt concentration for maximum growth of isolate was 4%. This agitation speed was used for production of PSB biofertilizer.

Table no.6: effect of salt concentrations on growth

Iso. / Salt %	1	2	3	4	5
SW3	+	++	++	+++	+

Where: "+" = Growth
"-"= No growth

16s rRNA identification of isolates was carried out in molecular characterization and isolates SW3 was identified as *Enterobacter hormaechei.*

Field trial of the PSB biofertilizer made from isolate SW3 on maize seedswas completed. The growth of maize seeds was measured in terms of plant height, number of tassels, flowers and fruit that grew on the maize plant. Significant difference in parameters studied on the maize plant such as plant height, number of tassels, flowers and fruit that grew when it compared with control growth of maize seeds.

CONCLUSIONS:

Out of 10 isolates 5 isolates were potential to solubilize phosphate. The biofertilizers are environmental friendly and alternative for chemical fertilizers. The drastic shift in pH of medium at acidic side shows the production of organic acid, enzymes by the microorganisms. This can be helpful for solubilization of the phosphate provided in the medium.

Field trial output of the PSB biofertilizer shows marked difference in soil fertility, increased plant height, number of tassels observed in the maize plant. The maize seeds grown under the influence of PSB biofertilizer of SW3 isolate had better quantity and quality of fruits and flowers, than those that grew on the control field which was supplemented with available chemical fertilizer.

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