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Isolation and Characterization of Salt Tolerant Phosphate Solubilizing *Serratia marcescens* Isolated from Coastal Area

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The importance of rhizospheric microbial phosphate solubilization has now been well documented. However, the performance of these microbes is greatly affected by various environmental stresses. In this study strain of *Serratia marcescens* NAUKP5 was isolated from the rhizosphere of *Oryza sativa* from the coastal region of South Gujarat. The bacteria *Serratia marcescens* NAUKP5 was able to solubilize insoluble rock phosphate up to 625.16±0.01 μM with decrease in pH 4.1±0.01 after 10 days of incubation. The strain was also characterized for various morphological and biochemical tests. The growth of *Serratia marcescens* NAUKP5 was also tested for wide range of temperature, pH, carbon source, nitrogen source and buffering of the media. It was able to tolerate NaCl concentration up to 750mM and showed resistance against three antibiotics.

Keywords: Rock phosphate, Rhizosphere, Phosphate solubilization, Physiological studies.

Phosphorus, the macronutrient next only to nitrogen in its importance to life forms, plays a stellar role in the transfer of energy, cellular metabolism and preservation of genetic information. It plays an important role in virtually all major metabolic processes in plant including: photosynthesis, signal transduction, macromolecular biosynthesis and respiration[1,2]. Phosphorus availability in several soil is ~1 µmol per litre, but for optimum productivity, phosphorus requirement for plants is ~30 µmol per litre, It's deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development[3], which result in low yielding of crops[4]. The chemical fertilizers are widely used to provide the nutrient to boost the crop productivity. But almost 75 to 90% of added P fertilizer is precipitated by iron, aluminum and calcium complexes present in the soils[5] and is virtually unavailable to most plant species when applied to P deficient soil. In India, it is estimated that there are almost 40 million tons of phosphatic rock deposits and this material should provide a cheap source of phosphate fertilizer for crop production[6]. Many soil microorganisms have been reported to solubilize inorganic phosphate[7,8,9,10,11,12].

Moreover, salt stress causes adverse environment and hydrological impacts that restrict normal crop production throughout the year. The crop yields on these affected soils are considerably reduced compared to those on non- saline zonal soils[13]. Salinity also suppresses the phosphorus uptake by plant roots and reduces the available

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phosphorus by sorption processes and low solubility of Ca-P minerals[14]. Soil microorganisms play a key role in soil phosphorus dynamics and subsequent availability of phosphate to plants[9]. Microorganisms are an integral component of the soil phosphorus cycle and are important for the transfer of phosphorus between different pools of soil phosphorus. Phosphate Solubilzing Microorganisms (PSM) through various mechanisms of solubilization and mineralisation are able to convert inorganic and organic soil Phosphorus respectively[15] into the bioavailable form, facilitating uptake by plant roots. Microorganisms are involved in a range of processes that affect the transformation of soil phosphorus and are thus an integral part of the soil phosphorus cycle. In particular, soil microorganisms are effective in releasing phosphorus from inorganic and organic pools of total soil phosphorus through solubilization and mineralization[16]. There are considerable populations of phosphate-solubilizing bacteria in the soil and in the plant rhizosphere[17,18]. High proportion of PSB found in the plant rhizospheres are metabolically more active than other sources[19]. Larger populations of PSB are found in agricultural and range land soils[20]. Reports have been examined that the bacterial genera like Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium and Erwinia have the ability to solubilize insoluble inorganic phosphate compounds such as tri-calcium phosphate, di-calcium phosphate, hydroxyapatite and rock phosphate[21].

It is evident that soil microorganisms play an important role in the cycling of P in soil-plant systems and it is expected that better understanding of their contribution to the mobilization of soil P and plant P nutrition will provide opportunity for development of more P-efficient and sustainable agricultural systems. Moreover salinity is creating the major problem with phosphate solubilization and ultimately leading the less crop yield. Hence the present study has been carried out to isolate the salt tolerant phosphate solubilizing bacteria that can survive and solubilize the insoluble phosphate in the presence of salinity to increase the crop productivity.

MATERIALS AND METHODS

Experimental site

The experiment was carried out at Department of Plant Molecular Biology and Biotechnology, N. M. College of Agriculture, Navsari Agricultural University, Navsari, Gujarat, India during year 2012-2016.

Collection of rhizospheric samples

The rhizospheric soil samples were collected from different undisturbed, uncultivated and wild ecosystem around the sea bank of the South Gujarat to isolate different native isolates of the phosphate solubilizing bacteria. Around 100 g soil samples from both the vigorously grown plant as well as poorly grown plants were collected in sterile plastic bags and brought in laboratory for further investigation. 1 g of the soil strongly adhered to roots was dissolved in 10 mL of Tris HCl (pH 8.0) buffered solution in flask for enrichment.

Isolation of bacteria

The flasks containing Tris minimal rock phosphate (TRP) medium (pH 8.0) [22] amended with xylose as carbon sources and 500 mM NaCl were added with one percent of the enriched sample. The decrease in the pH was observed in media. The isolate was further transferred on the petri-plate containing TRP agar with 0.03 % methyl red. Colonies showing clear halo pink zone formation was observed and solubilization index was calculated using following formula[23].

SI = Halo zone + Colony diameter ? Colony diameter
Further isolate was inoculated in to TRP broth and decrease in the pH and P solubilization was again monitored [24].

Characterization of bacteria

Morphological characterization

Selected bacterial isolates were grown on TRP agar plates at 37 °C. That culture was used for morphological characterization and Gram's staining.

Physiological characterization

To optimize the physiological parameters of phosphate solubilization, the selected bacteria was inoculated in media with different pH, temperature, carbon source, nitrogen source and salt concentration. P solubilization for all the treatments was analysed.

Salt tolerance

The flask containing TRP broth with selected isolate was supplemented with the different concentration of NaCl (0mM, 250mM, 500mM and 750mM) and incubated at optimum temperature to check the salt tolerance with optimum P solubilization of the isolates.

Effect of carbon source

The flask containing carbon sources like Glucose, Xylose and Sucrose were studied in TRP Broth.

Effect of nitrogen source

To check effective source of nitrogen for phosphate solubilization TRP broth was added with NH_4Cl, $(NH_4)_2SO_4$ and NH_4NO_3.

Effect of Temperature

The flask containing TRP broth for temperature profiling were inoculated and placed at different temperatures (20°C, 28°C, 37°C, 40°C and 60°C).

Effect of pH

The flask containing TRP broth with different initial pH (6, 7, 8, 9, 10 and 12) were inoculated with the isolate to check optimum pH range for phosphate solubilization.

Effect of buffering

The isolate was point inoculated on 0mM, 50mM and 100mM Tris-Cl buffered tris minimal medium plates (pH 8.0, containing 0.03% methyl red dye). The size of the halo/clear zones around the colonies was measured. Solubilization index at different buffering was calculated [23].

Biochemical characterization

Biochemical characterization was carried out according to Bergey's Manual of Systematic Bacteriology [9] to study the biochemical characters.

Antibiotic study

The bacterial strain was inoculated in the Luria broth and incubated at 37 °C for overnight. The fresh culture was spreaded on Luria Agar plate. The inoculum spreaded was allowed to dry, and after drying the discs containing antibiotics (Hi-Media Laboratories, ICOSA MINUS GI and PLUS GI) were placed on the plates. The discs should be placed on the Luria agar with sterile forceps and tapped gently to ensure the adherence to the agar. The plates containing the disks were incubated at 37°C for 16-18 h. After incubation, the diameter of each zone of inhibition was measured.

RESULTS AND DISCUSSION

Isolation

Xylose being predominant monosaccharide found in plant root exudates secreted in the rhizospheric soils where it is a nutrient source for microbial growth[25]. As the xylose was the most preferable carbon source for the bacteria, the number of isolates were obtained from the rhizospheres of different plant species by screening it in presence of RP as the sole P source and xylose as a sole carbon source under 100mM buffering conditions. From which the Serratia marcescens NAUKP5 was selected for further study, which was isolated from the plant Oryza sativa. It can able to solubilize insoluble phosphate in tris minimal rock phosphate medium containing the 500mM NaCl. The amount of P released by the strain *S. marcescens* NAUKP5 was 625.16±0.01µM with decrease in pH up to 4.1±0.01 after 10 days of incubation (Fig. 1 and 2). An inverse relationship was observed between dropped pH value of culture medium and concentration of phosphate solubilized. This inverse relationship between pH and soluble phosphate was reported earlier [26]. The solubilization index on TRP agar was 2.20±0.01 in the presence of 500mM NaCl. The solubilization index also decreased with increase in buffering of the media. The similar results were observed by[22].

Characterization

Morphological and biochemical characterization

The microscopic observation revealed that the strain of the isolated bacteria was Gram negative rods (Fig. 3 and Table 1). The biochemical parameters were performed according to the

Table 1. Morphological parameters of *S. marcescens* NAUKP5

Colony characteristics	Observation
Gram's staining	-ve
Shape	Rod
Motility	+
Size	Small
Shape	Round
Surface	Smooth
Elevation	Raised
Opacity	Opaque
Pigmentation	Red
Consistency	Viscous

method described by Bergey's Manual of Systematic Bacteriology which revealed that *S. marcescens* NAUKP5 was from the class gamma proteobacteriaceae. The *S. marcescens* NAUKP5 showed various types of biochemical characters. (Table 2). It was able to utilize different types of sugars and amino acids. However there was absence of gas production. The bacteria showed catalase production and it was motile in nature. It was negative for MR and VP test. All these results indicated that different fermentation pathways were involved in it. On the basis of biochemical and morphological parameters tentative identification of isolate was done. The conformation of its identity was further done by molecular characterization i.e., by sequencing of 16s rRNA using a pair of primers and it was identified as Serratia marcescens NAUKP5 (data not shown).

Table 2. Biochemical characteristics of *S. marcescens* NAUKP5

Biochemical Characteristics	*S. marcescens* NAUKP5	Biochemical Characteristics	*S. marcescens* NAUKP5
Arginine	+	Mannitol	++
Arabinose	+	Melibiose	++
Adonitol	+	Ornithine	+
Cellobiose	+	Proline	+
Dextrose	++	Rhamnose	+
Xylose	++	Raffinose	++
Sucrose	++	Sorbitol	+
Ducitol	++	TSI slant	+
Fructose	++	Citrate Slant	+
Galactose	++	MR test	-
Inulin	+	VP test	-
Lactose	++	Urea hydrolysis	-
Lysine	++	Nitrate reduction test	+
Maltose	++	Gelatine hydrolysis	-
Mannose	++	Catalase	++

+++ High acid production, ++ Medium acid production,
+ low acid production, - No acid production, G-Gas production

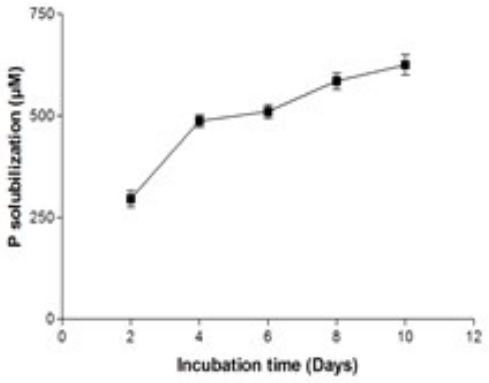

Fig. 1. Phosphate solubilization of *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean.

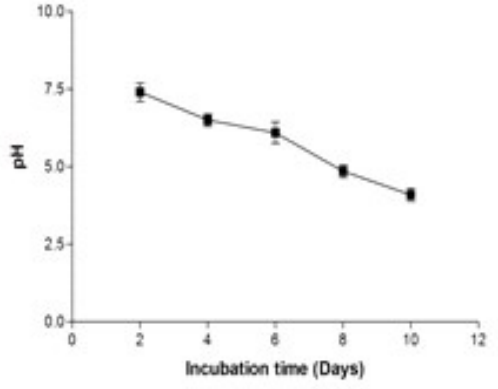

Fig. 2. pH profile of *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean.

Physiological characterization

The pH and temperature is the major factor responsible for controlling the metabolic activity of the bacteria. It has optimum range of pH and temperature while increase or decrease in any parameter may affect the P solubilization. The isolate was able to solubilize the insoluble P ranging from 28 to 60°C (Fig. 4). However, the optimum P solubilization was observed at 28 to 37°C. The higher and lower temperatures were not suitable for the bacteria. It was also able to solubilize the P at different range of initial pH which ranges from 6 to 12 (Fig. 5). However, the optimum P solubilization was observed at pH 7 to 8. As soon as the initial pH increased from 8.0 to more, there was decreased in the P solubilization. The isolate was also grown on media containing different concentration of NaCl. The optimum P solubilization was observed at 0mM of NaCl. However, it was able to tolerate the salt concentration of the medium up to 750mM however the amount of P solubilization was decreased. The amount of solubilized P found to be decreased with increase in salt concentration (Fig. 6). The P solubilization capacity also depends on the types of carbon and nitrogen source used. The optimum P solubilization observed for carbon sources were in order of Glucose > Xylose >Sucrose (Fig. 7). Similar study was also performed [27]. The better nitrogen source for P solubilization were

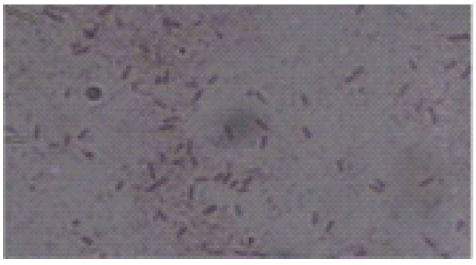

Fig. 3. Gram staining of *S. marcescens* NAUKP5

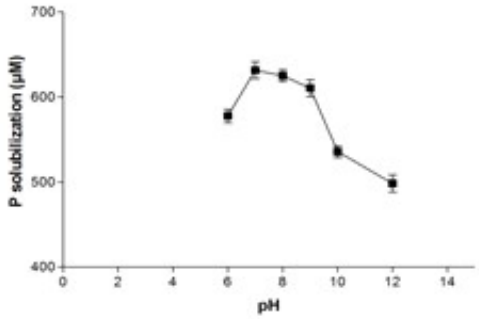

Fig. 5. Effect of pH on P solubilization by *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean.

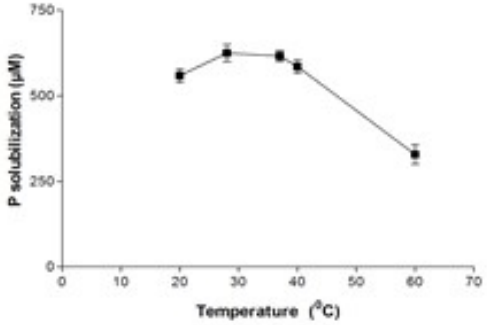

Fig. 4. Effect of temperature on P solubilization by *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean.

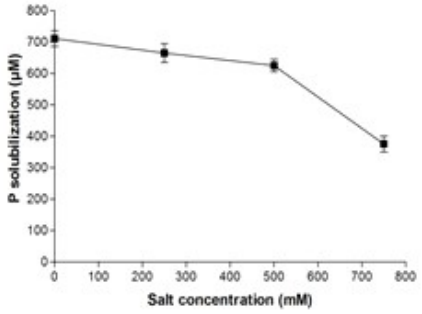

Fig. 6. Effect of NaCl concentration on P solubilization by *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean.

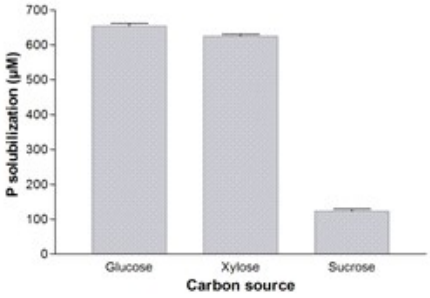

Fig. 7. Effect of carbon source on P solubilization by *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean

NH_4NO_3 > $(NH_4)_2SO_4$ > NH_4Cl (Fig. 8). The ammonium salt as a nitrogen source produces acid by proton exchange mechanism which solubilize the insoluble P [7]. The bacteria was also tested for its P solubilization capacity at different level of buffering of the media. The P solubilization efficiency decreased with increase in the buffering of the media (Fig. 9). The similar results were found by other scientist [22, 28]. They found that the incorporation of buffer into the medium drastically reduced the P solubilization properties of the PSMs tested.

Antibiotic study

The *S. marcescens* NAUKP5 was tested for its ability to resist different types of antibiotics by using antibiotic discs (HiMedia laboratories). Total 27 antibiotics were tested from which it showed the resistance against three antibiotics i.e., Clarithromycin, Co-Trimoxazole and Erythromycin antibiotics where as *S. marcescens* NAUKP5 was susceptible to rest of the antibiotics (Table 3).

Table 3. Antibiotic sensitivity test of *S. marcescens* NAUKP5

Antibiotics	*Serratia marcescens* NAUKP5	Antibiotics	*Serratia marcescens* NAUKP5
Norfloxacin	S	Oxacillin	S
Gentamicin	S	Erythromycin	R
Chloramphenicol	S	Nalidixic acid	S
Ciproflaxin	S	Tobramycin	S
Clarithromycin	R	Vancomycin	S
Co-Trimoxazole	R	Impenem	S
Azithromycin	S	Moxifloxacin	S
Penicillin	S	Ofloxacin	S
Amikacin	S	Sparfloxacin	S
Ampicillin	S	Levofloxacin	S
Amoxyclav	S	Colistin	S
Ceftriaxone	S	Augmentin	S
Kanamycin	S	Gatifloxacin	S
Streptomycin	S		

R- Resistance; S-Susceptible

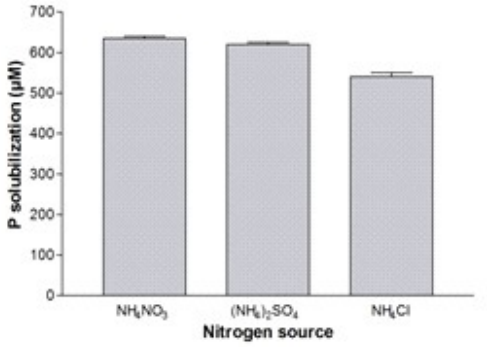

Fig. 8. Effect of nitrogen source on P solubilization by *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean

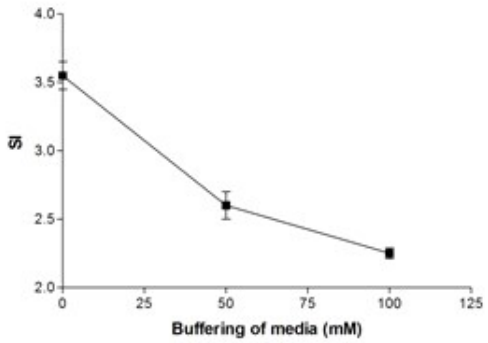

Fig. 9. Effect of buffering of media on P solubilization by *S. marcescens* NAUKP5. Each value is the mean of three replicates. Error bars show standard error of mean.

CONCLUSION

From the present investigation it can be concluded that *S. marcescens* NAUKP5 has ability to solubilize RP in presence of salt. Physiological parameters for optimum RP solubilization was analysed. *S. marcescens* NAUKP5 was the efficient salt tolerant PSB which can be used in saline area to overcome P deficiency in soil. However further study like HPLC profiling of organic acids and metabolic enzyme activity need to be performed to find out metabolic pathway for P solubilization.

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