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Isolation and Characterization of Phosphate Sollubilizing *Bacillus* Consortia for Plant Growth Promotion

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ABSTRACT

Twenty five rhizospheric soil samples were collected from five different sampling sites and a total of 29 phosphate sollubilizing Bacillus isolates were obtained. From primary and secondary screening of these isolates, best 5 phosphate sollubilzers SST3, SST4, SLYV3, SDR1, SDR2 were found to produce 15 µg/ml, 11 µg/ml, 13 µg/ml, 18 µg/ml and 12 µg/ml solubilised phosphate respectively. These isolates were further selected for preparing compatible consortia for on field application. Along with phosphate sollubilization, isolates SST3, SST4, SLYV3, SDR1 and SDR2 showed considerable PGPR activities like 54 µg/ml IAA, 50 µg/ml, 40 µg/ml IAA, 36 µg/ml IAA and 34 µg/ml IAA production; 1.2 µg/ml, 0.79 µg/ml, 0.83 µg/ml, 1.01 µg/ml and 1.35µg/ml protease production. All the five isolates were found positive for siderophore production, N_2 fixation, cellulase production, HCN production while isolate SST3 showed antifungal activity against fungal pathogen Colletotrichum falcatum. From 16S rRNA sequencing SST3, SST4, SLYV3, SDR1 and SDR2 isolates were identified as Bacillus cereus, Bacillus pumilus, Bacillus cereus Bacillus subterraneus, Bacillus cereus respectively.

Key words: *Bacillus, Phosphate sollubilization, Plant Growth Promoting Rhizobactera, Bio fertilizer*

INTRODUCTION

Sugarcane is one of the principle crops of South Gujarat and Saurashtra region of Gujarat state, India. Sugarcane is an important cash crop for several countries and it is mainly used for sugar and ethanol production. Use of phosphate sollubilizing *Bacillus* consortia for plant growth promotion (PGPR) can minimize the cost of chemical phosphate fertilizer, environmental hazard, and suppress the diseases as well. Other very well known PGPR activities are nitrogen fixation, phytohormone production, cellulosic digestion, lipid digestion, protein digestion and acting as bio control agent. Natural association of these bacteria with plants, especially with grasses is very well studied.

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Along with phosphate sollubilization, this consortium can be applied for improvement of physical, chemical and biological properties of soils, improvement of soil fertility and nutrient efficiency thereby necessitating less fertilizer, systematic soil suppression to diseases by improving microbial populations of beneficial strains. *Bacillus* is spore forming and so can survive in harsh environmental condition, reported for all plant growth promoting activity and having minimum compatibility issues with consortia. These consortia includes thermophiles, mesophilic and psychrophilic microbes which work across a wide range of temperatures from 4°C to 55°C , microbes which can work over a range of pH from 4 to 12. Bio fertilizers are inputs containing microorganisms which are capable of mobilizing nutritive elements from non usable form to usable form through biological processes[1]. It is well-recognized that microbial inoculants constitute an important component of integrated nutrient management that leads to sustainable agriculture. In addition, microbial inoculants can be used as an economic input to increase crop productivity; fertilizer doses can be lowered and more nutrients can be harvested from the soil.

MATERIALS AND METHODS

1. Collection and preparation of soil sample

Total twenty five soil samples of sugarcane rhizosphere were collected from the Main Sugarcane Research Centre, Navsari Agricultural University, Gujarat, India. Samples were collected in aseptic begs and immediately transported to lab under cold condition (4^o C) for further process[2]. Under sterile conditions, 1 g of each soil samples was added to 5 mL of nutrient broth and incubated at 35^oC for 24 hours.

2. Isolation of *Bacillus* spp.

After the incubation period, 0.1 mL of the supernatant of each tube containing suspension of soil and culture media were inoculated in nutrient agar plates by streaking at 30°C for 24 hours. After that, the plates were examined and the suspected colonies were stained by Gram staining method. The Gram-positive, rod-shaped, spore forming bacilli were selected for additional identification tests. Subsequent identification tests including susceptibility test to penicillin, citrate hydrolysis, motility, Voges-Proskauer, indole production, catalase, nitrate reduction, and production of H_2S were performed.

3. Primary screening for phosphate sollubilizing *Bacillus* spp.

The plates were prepared with Pikovskya's medium. The culture of bacterial isolates were streaked on the plates and incubated in an incubator at 30°C for 7 days. The plates were then examined for formation of halo zone around the colony[3].

4. Secondary screening for phosphate sollubilizing *Bacillus* spp.

The positive *Bacillus* isolates from primary screening were further quantitatively measured using Reyes basal medium. Inoculated flasks and un-inoculated controls were incubated at 28° C on a rotary shaker at 150 rpm in the dark. After 4 days 2 ml sub-sample of the culture supernatant was aseptically withdrawn from each flask which was centrifuged and used for colorimetric determination of dissolved phosphate.

5. Other PGPR activities

Best phosphate sollubilizing *Bacillus* isolates were checked for other plant growth promoting activities

5.1 IAA production

The production of indole acetic acid (IAA) was assayed by using Salkowski method[4].

5.2 Protease production

The protease enzyme activity was determined by using McDonald and Chen method[5].

5.3 Siderophore production

Siderophore production was tested qualitatively using Chrome Azurole S agar as described by Alexander and Zuberer[6].

5.4 Nitrogen fixation

Screening of nitrogen fixing organisms was carried out by using Jensen's Medium[7].

5.5 Antifungal production

In-vitro antagonistic ability of *Bacillus* isolates were investigated against sugarcane pathogen *Colletotrichum falcatum* by dual culture technique[8]. Observations on width of inhibition zone and mycelia growth of test pathogens were recorded.

5.6 Cyanide production

Hydrogen cyanide production was assayed by the method suggested by Lorck and Castric[9,10].

5.7 Cellulase Production

Bacterial isolates were screened for cellulase activity by inoculating CMC agar according to Cattelan *et al.*[11].

6. DNA extraction and 16S rRNA Gene sequencing

Extraction and amplification of genomic DNA for 16S rRNA gene sequence analysis was carried out as described by Cui *et al.*[12].

RESULTS AND DISCUSSION

1. Isolation and purification of PGPR

In this study twenty five rhizospheric soil samples were collected from 5 different sites of Main Sugarcane Research Station, Navsari Agricultural University, Gujarat, India. Total twenty nine different *Bacillus* isolates were obtained from different sites as shown in Table 1.

Table 1: Total numbers of *Bacillus* isolated and purified from different sampling site

Sampling site	No of samples collected	Sugarcane variety	No. of *Bacillus* isolates obtained
Plot no 16, MSRC, NAU, Gujarat	5	*S. Spontaneum* 86002	4
Block 1, MSRC, NAU, Gujarat	5	Co 86032	7
Plot no 15, MSRC, NAU, Gujarat	5	CoC 90063	5
Plot no 5, MSRC, NAU, Gujarat	5	*S. officinarum* 4131	8
Plot no 12, MSRC, NAU, Gujarat	5	CoC 1148	5

2. Primary and secondary screening for phosphate sollubilization

Phosphorus is a primary essential nutrient element. The bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species, nutritional status of soil, presence of effective microorganisms and soil conditions. To enhance phosphorus uptake efficiency, PSB play an important role in supplying phosphate to plants, which is environment friendly and sustainable approach.

All twenty nine rhizospheric *Bacillus* isolates were studied for its Phosphate sollubilization potential. Out of total twenty nine isolates, total twelve isolates were found positive for phosphate sollubilization on pikovaskya medium. As shown in Figure 1, clear zone of sollubilized phosphate was observed. The *Bacillus* consortia can survive in wide range of temperature by spore formation. The spore forming ability of *Bacillus* isolates to survive under adverse environmental condition can be utilized as potential advantage as biofertilizer. The same *Bacillus* isolates also showed growth on 4-7 pH range.

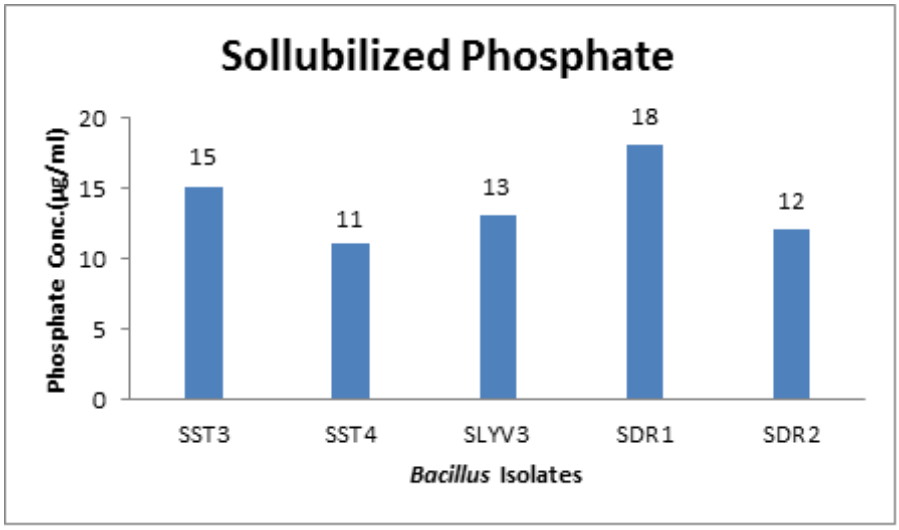

Fig. 1: Graphical representation of Phosphate sollubilization

As shown in Figure 1 and Figure 2, out of twelve isolates that are positive for phosphate sollubilization, isolates SST3, SST4, SLYV3, SDR1, SDR2 sollubilized 15 µg/ml, 11 µg/ml, 13 µg/ml, 18 µg/ml, 12 µg/ml phosphate respectively.

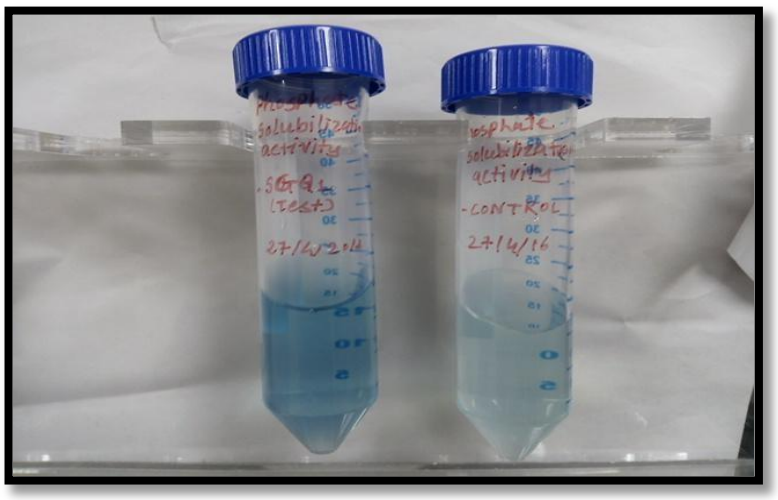

Fig. 2: Secondary screening for phosphate sollubilization

3. Characterization of other PGPR activities

Table 2: Best five *Bacillus* phosphate sollubilizers showing different PGPR activities

Isolate number	Various PGPR activities						
	N2 fixation	IAA production	Siderophore production	Protease production	HCN production	Cellulase activity	Antifungal activity
SST3	+	+	+	+	+	+	+
SST4	+	+	+	+	-	-	-
SLYV3	+	+	+	+	+	-	-
SDR1	+	+	+	+	-	-	-
SDR2	+	+	+	+	+	+	-

The best five *Bacillus* isolates positive for phosphate sollubilization were checked for other important PGPR activities such as nitrogen fixation, IAA production, Siderophore production, Protease production, HCN production, Cellulase activity and antifungal activity against sugarcane pathogen *Colletotrichum falcatum* as shown in Table 2. All the five *Bacillus* phosphate sollubilizers were also found positive for nitrogen fixation. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops [13]. Same five isolates were also efficient IAA and siderophore producers. Isolates SST3 and SST4 were found to produce 54 µg/ml IAA and 50 µg/ml IAA. While isolates SLYV3, SDR1 and SDR2 were found to produce 40 µg/ml IAA, 36 µg/ml IAA, 34 µg/ml IAA correspondingly. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake [14]. IAA stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis and inducing protein synthesis. It promotes embial activity, inhibit It promotes embial activity, inhibit or delay abscission of leaves, induce flowering and fruiting [15]. Orange halo zone observed around each inoculated bacterial spot indicates siderophore production which is responsible for the suppression of soil

borne plant pathogens for competitions for iron. Isolates were spot inoculated and followed by incubation at 30° C and zone of clearance around the colony indicating the enzymatic degradation by protease As shown in Graph3, isolate SST3, SST4, SLYV3, SDR1, SDR2 produced 1.2 µg/ml, 0.79 µg/ml, 0.83 µg/ml, 1.01 µg/ml and 1.35µg/ml 17 U protease in sequence. Isolate SST3, SLYV3, SDR2 were found positive for HCN production. Isolate SST3 and SDR2 were positive for cellulose activity and isolate SST3 showed considerably antifungal activity against fungal pathogen *Colletotrichum falcatum.* The ability to synthesize fungal cell wall-lysing enzymes such as protease, cellulase or hydrogen cyanide (HCN), which suppress the growth of fungal pathogens; promotes a successful compete with pathogens for nutrients or specific niches on the root surface[16,17].

4. Molecular characterization of isolates by 16 S r RNA

From 16S rRNA sequencing SST3, SST4, SLYV3, SDR1, SDR2 isolates were identified as *Bacillus cereus, Bacillus pumilus, Bacillus cereus Bacillus subterraneus, Bacillus cereus* respectively.

CONCLUSION

Sugarcane Rhizospheric soil sample were collected and checked for *Bacillus* Spp. capable of sollubilizing phosphate which can work in wide range of temperature and pH. The same isolates also showed other significant plant growth promoting activities such as nitrogen fixation, IAA production, siderophorte production, protease, cellulose, and antifungal activity against sugarcane pathogen *Colletotrichum falcatum*. This *Bacillus* consortia can be utilized as efficient biofertilizer minimizing the cost of chemical fertilizer and can rejuvenite soil health.

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