

## Isolation and Characterization of Plant Growth Promoting Rhizobacteria Associated with *Saccharum officinarum* L.

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### Abstract

Plant growth promoting rhizobacteria is free living soil microorganisms that directly or indirectly exert beneficial effects on plants. Hundred strains were characterized morphologically and biochemically. Primary screening was carried out for all hundred isolates for IAA, phosphate solubilization, siderophore and nitrogen fixation. Eight isolates F181, F271, F323, F372, F373, F531, ELB1 and ESB4 from different sites and sugarcane cultivars have showed potential for all PGPR activities and were further investigated quantitatively. The isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. The results shown, F372 achieved the highest phosphate solubilization of 15 mg L<sup>-1</sup>, and F271 was the most promising IAA producer with 63 mg L<sup>-1</sup>. Furthermore, most of the PGPR isolates shown antifungal properties like HCN chitinase and protease production. The present study, therefore, suggests that the use of selected PGPR isolates as inoculants might be beneficial for sugarcane cultivation.

**Keywords:** Bio-control; Rhizobacteria; Phosphate solubilization; Siderophore; Sugarcane

### Introduction

Sugarcane (*Saccharum officinarum* L.) is a monocotyledon and belongs to the tribe Andropogoneae of the family Poaceae. It grows best under warm conditions with high light intensity and fertile soil. Sugarcane differs from many common grasses in its long period of vegetative growth, resulting in the large size and they have stout, jointed, fibrous stalks that are rich in sugar. It is common in tropical and subtropical countries throughout the world. It is one of the principle crops of South Gujarat and Saurashtra region of Gujarat state. Importance of sugar in human diet needs no introduction; it has become a part and parcel of daily life. As sugarcane is a long duration crop and faces many biotic and abiotic stresses during growth phase, the microorganism associated with sugarcane roots may be potent and useful for application to promote plant growth [1]. Soil microorganisms play a significant role in regulating the dynamics of organic matter, decomposition and the availability of plant nutrients such as Nitrogen (N), Phosphorous (P) and Sulfur (S). 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 [2]. Biofertilizer are the formulation of living microorganisms, which are able to fix atmospheric nitrogen in the available form for plants either by living freely in the soil or being associated symbiotically with plants. Biofertilizer are inputs containing microorganisms which are capable of mobilizing nutritive elements from nonusable form to usable form through biological processes [3]. A number of bacterial species associated with the plant rhizosphere belonging to genera *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are able to exert a beneficial effect on plant growth [4]. Plants play an important role in selecting and enriching the types of bacteria by the constituents of their root exudates. Rhizospheric bacterial communities have efficient systems for uptake and catabolism of organic compounds present in root exudates [5]. We are trying here to isolate the PGPR from sugarcane having potential to act as biofertilizer with the objectives such as screening of IAA production, Phosphate

solubilization, Nitrogen fixation, Siderophore production, anti-fungal activity, protease activity, HCN production, cellulase activity, chitinase activity.

### Material and Methods

#### Sampling sites, isolation and characterization

Soil samples were collected from Sugarcane crop rhizosphere from different villages of Bardoli region includes Sevani, Vansdarundhi, Vihan and Dhamdod. Sugarcane leaves and stems were collected from a 4 month old plantation. Isolation was carried out by following two ways. (a) Soil sample was diluted and plated on nutrient agar plates. Colonies were randomly selected on the basis of morphology and further purified by streaking [6]. (b) The leaves and stem were washed macerated separately and dilutions were placed on nutrient agar. Morphology and texture of each colony was recorded. Colonies were randomly selected and further purified and maintained on nutrient agar slant at 4°C for further studies [7].

#### Phosphate solubilization

Phosphorus is an essential element next to nitrogen influencing the plant growth and production. It is absorbed as various organic and inorganic forms in the plant. The Pikovskya's medium plates were inoculated with isolates then examined for formation of halo zone around the colony [8]. The quantitative phosphate solubilization by bacterial isolates was measured using Reyes basal medium [9]. The blue color intensity of the solution was measured at 600 nm.

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## IAA production

The production of Indole acetic acid (IAA) was assayed by using Salkowski method [10]. Productions of pink colour indicate presence of IAA in the medium. And then light absorbance was measured immediately at 535 nm spectrometrically. The amount of IAA produced was calculated using the standard curve prepared with known concentration of IAA.

## Siderophore production

Siderophore is an important for the survival and growth of bacteria in the soil and aqueous environment [11]. Siderophore production was tested qualitatively using chrome azurole S (CAS). Orange halos around the colonies after 48 hours incubation indicated siderophore production. Quantitative estimation of siderophores was done by CAS-shuttle assay. All positive cultures were compared for siderophore production ability as described by Payne [12] where percent decolorization was calculated by using the following formula:

$$\% \text{ Siderophore Units} = (A_r - A_s / A_r) \times 100$$

Where  $A_r$ =Absorbance of reference at 630 nm (CAS reagent) and  $A_s$ =Absorbance of sample at 630 nm.

## Nitrogen fixation

During the biological nitrogen fixation, molecular nitrogen is reduced in multiple electron transfer reactions, resulting in the synthesis of ammonia and the release of hydrogen [13]. Ammonium is then used for the subsequent synthesis of biomolecules. Screening of nitrogen fixing organisms was carried out by using semisolid malate medium (NFB). Growth of bacterial isolates indicates nitrogen fixation [14].

## Protease production

Protease is a single class of enzyme used in detergents, pharmaceuticals, leather, and the food agriculture industries [15]. It constitutes over 40% to 60% of the enzyme industries. Protease production was assayed using skim milk agar. Proteolytic activities were identified by clear zone formation around the cell. The enzyme activity was determined by using McDonald and Chen method [16]. After the completion of the time the absorbance was read out at 700 nm spectrophotometrically.

## Cyanide production

Production of HCN by certain strains of *Pseudomonas* fluorescence

has been involved in suppression of soil borne pathogens [17]. HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens. Hydrogen cyanide production was assayed by the method suggested by Lorck [18] and Castric [19]. Discoloration of the filter paper from yellow to brown indicates production of cyanide.

## Chitinase production

Chitin is the second most abundant natural polymer and widely distributed as a structural component of crustaceans, insects, and other arthropods, as well as a component of the cell walls of most fungi and some algae. For chitinolytic activity bacterial isolates were streaked on chitin agar medium. Isolates were detected by the appearance of clear zones around colonies grown on chitin agar plates. Chitinase activity was measured with colloidal chitin as a substrate. The amount of reducing sugar produced was measured by the dinitrosalicylic acid (DNSA) method.

## Results and Discussion

### Isolation and characterization of rhizobacteria

In this study total 100 isolates were isolated from 45 samples collected from different villages of Bardoli region (Table 1).

Among all sites, F4 which contained Co 86032 sugarcane variety was found good for colonizing higher number of rhizospheric bacteria, while F2 site where sugarcane variety Co-0411 was found poor for colonization of rhizospheric bacteria. In addition leaves and stem of Co 86249 were used for isolation of bacteria. Among the total isolates, 59 isolates were found as IAA producer, 61 isolates were found as phosphate solubilizes, 39 isolates were found as siderophore producer, 59 isolates were found as nitrogen fixer and 65 isolates were found as protease producer. The results of primary screening are summarized in Table 2.

After initial screening for all four PGPR activities, F181, F271, F323, F372, F373, F531, ELB1 and ESB4 were selected for further studies as they have shown positive result. Microscopic observations were performed to investigate the Gram reaction of PGPR isolates. Effort was made to characterize all 8 isolate morphologically (Table 3). Two isolates F181 and ESB4 were Gram positive while rests of the isolates were Gram negative bacteria. All the isolates were found to having a rod shape, small in size. Various biochemical tests were performed to

Sample site	Source	No. of sample collected	Sugarcane variety	Sampling site	No. of isolates obtained
F1	Rhizosphere	08	Co 86032	Sevani	16
F2	Rhizosphere	08	Co 0411	Vansdarundhi	14
F3	Rhizosphere	08	Co 86249	Vihan	21
F4	Rhizosphere	08	Co 86032	Dhamdod	23
F5	Rhizosphere	08	Co 0411	Bardoli	18
F6	Leaves & Stem	05	Co 86249	Vihan	08

Table 1: Details of isolates collected from sugarcane.

Sample site	Total Isolates			
	IAA production	Phosphate solubilization	Siderophore production	Nitrogen fixation
F1	07	12	04	07
F2	09	11	05	04
F3	12	16	06	14
F4	10	05	07	10
F5	17	12	10	15
F6	04	05	07	07

Table 2: PGPR activities among the total isolates.

Characteristics	Selected Isolates							
	F181	F271	F323	F372	F373	F531	ELB1	ESB4
Grams nature	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Positive
Size	Small	Large	Small	Small	Small	Small	Small	Small
Shape	Round	Round	Round	Round	Round	Round	Round	Round
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Opacity	Translucent	Opaque	Opaque	Opaque	Opaque	Transparent	Transparent	Opaque
Elevation	Raised	Convex	Flat	Flat	Flat	Raised	Convex	Convex
Consistency	Moist	Dry	Moist	Moist	Moist	Moist	Moist	Moist
Colour	Yellow	Green	Colorless	Orange	Green	Orange	Colorless	Colorless

Table 3: Morphological characterization of selected isolates.

Characteristics	Selected Isolates							
	F181	F271	F323	F372	F373	F531	ELB1	ESB4
Glucose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	-	-	-	-
Lactose	-	+	+	+	+	-	-	+
Mannitol	+	-	+	+	-	-	+	+
Xylose	-	+	-	-	+	-	-	-
Sucrose	-	-	+	+	-	-	+	+
Methyl Red Test	-	+	-	-	-	-	-	-
Voges-Proskauer	+	-	+	+	+	+	+	+
Oxidase test	-	-	+	-	+	-	-	-
Citrate Utilization	+	+	+	+	+	+	-	+
Urea Hydrolysis	+	-	-	-	-	-	-	-
H <sub>2</sub> S Production	-	-	-	+	-	-	+	-
Phenyl alanine	-	-	+	-	-	-	-	-
Nitrate Reduction	+	+	+	+	+	+	+	+
Ammonia Production	+	-	+	-	-	-	-	-
Gelatin Hydrolysis	+	+	+	-	-	-	+	+
Catalase	+	+	+	+	+	+	+	+
T S I	Gas	+	-	-	-	-	-	-
	H <sub>2</sub> S	-	+	+	-	-	+	-
	Lac fermentor	-	+	+	-	-	-	-

Table 4: Biochemical characterization of selected isolates.

Isolates	Phosphate (mg L <sup>-1</sup> )	IAA (mg L <sup>-1</sup> )	Siderophore	Protease (IU)	Chitinase (IU)
F181	10	56.23	92	19.5	0.35
F271	6	63.69	72	5.41	0.2
F323	8	38.48	91	7.58	-
F372	15	12.43	94	12.78	-
F373	12	31.91	95	15.81	-
F531	9	36.18	95	11.7	-
ELB1	7	49.66	96	17.3	-
ESB4	14	29.49	88	13.43	-

Table 5: Quantitative estimation of PGPR properties.

identify the isolate including sugar fermentation, oxidase test, urea, TSI etc. (Table 4).

Phosphorus is second highest required mineral nutrient after nitrogen in the growth of plants. Phosphate solubilising rhizobacteria have been considered as one of the best alternatives for inorganic phosphate fertilizers for promoting plant growth and yield [20-22]. Out of 61 bacterial isolates, eight isolates shown prominent phosphate solubilization were further evaluated quantitatively. Among all eight isolates F372 (15 mg L<sup>-1</sup>) was found best isolates as phosphate solubilizers followed by ESB4 (14 mg L<sup>-1</sup>) (Table 5). Similar consistent results of phosphate solubilization in both agar and broth assay were observed earlier [1,23]. Quantification of IAA was carried out for eight different isolates. The phytohormone regulates a whole repertoire of plant

development processes. As a critical plant hormone, auxin modulates such diverse processes as tropic responses to light and gravity, general root and shoots architecture, organ patterning, vascular development and growth in tissue culture [24]. IAA production was found 93.69 mg L<sup>-1</sup> by F271 isolate which was highest in all isolates being analyzed. F372 isolate was found poor in IAA production. F181, F271 and ELB1 found to produce more than 40 mg L<sup>-1</sup>. All eight organisms were grown well on N free media which indicates that isolates are able to fix maximum nitrogen. A plant is better able to achieve its optimized physical growth when it receives enough nutrients such as fixed nitrogen and this can be influenced by the presence of such bacteria in association with the host plants [25]. These biological processes can help reduce overreliance on chemical fertilizer. Siderophore by PGPR is one of the biocontrol mechanisms under iron limiting condition. PGPR produces a range

of siderophore which have a very high affinity for iron and reduced availability of iron in the soil would suppress the growth of pathogenic organisms [26].

## Conclusion

In present study, among all eight isolates ELB1 was found to produce maximum of 96% units of siderophores. In addition to siderophore, there are other mechanisms of biocontrol including enzyme secretion like protease production was also studied. Production of protease is one of the anti-pathogenic mechanisms present in PGPR as it degrades the cell wall of other pathogenic organism.

HCN production was only found in F323 isolate which was obtained from sample F3. Siderophore activity was generally found against pathogenic organism. Two isolate were obtained positive in primary screening were analyzed quantitatively for chitinase production. F271 and F323 were recorded to produce 0.35 and 0.2 IU of chitinase respectively. Thus, this study yielded some of the promising isolates which need to be tested for their *in vivo* plant growth promotion potential.

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