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ISOLATION AND CHARACTERIZATION OF SUGARCANE PHYLLOSPHERIC BACTERIA AND EVALUATION OF THEIR *in vitro* EFFICACY FOR PLANT GROWTH PARAMETERS

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Abstract: Leaf associated phyllospheric bacteria exert beneficial effects on host plant through various direct and indirect mechanisms. For the isolation of native candidate phyllospheric bacteria, leaf samples were collected from five popular varieties of sugarcane from the sugarcane farms of Navsari Agricultural University, Navsari, Gujarat, India. A total of 18 morphologically diverse phyllospheric isolates were obtained in pure culture form and further studied for their *in vitro* plant growth promoting traits. Among all the isolates, 38.89% were solubilizing mineral phosphate, and 44.44% of the isolates possessed potash and zinc solubilization potential. Surprisingly, none of the isolates showed nitrogen fixation potential. Further, 44.44% of isolates showed antagonistic activity against *Fusarium oxysporum*, and 33.33% were positive for siderophore production. In terms of extracellular enzyme secretion, 66.66%, 38.89%, and 55.55% were found positive for extracellular proteases, lipases, and amylases, respectively. Additionally, 33.33% and 22.22% of the isolates demonstrated IAA production and ACC deaminase activities, respectively. Based on *in vitro* screening, the two most potent isolates with multiple plant-growth promoting (PGP) traits were identified by 16S r-RNA sequencing method, as *Bacillus amyloliquefaciens* and *Enterobacter cloacae*. These native phyllospheric isolates can be further explored for their plant growth promotion potential as an alternative of synthetic chemicals in sustainable agricultural practices.
Keywords: Phyllosphere, Plant growth promotion, ACC deaminase, Phytohormone, Antagonism, phosphate solubilization

INTRODUCTION

The interactions of plants and their associated microbes play a crucial role in the overall growth and performance of the host plant. Historical plant microbiome research was mainly focused on the interaction between rhizospheric microbes, but there has been good progress in phyllospheric microbiome studies during the last decade [1]. Similar to rhizospheric microbes, phyllosphere inhabiting microbes are considerable in size with bewildering diversity and could have tremendous potential with applications in agriculture, biotechnology, medicines, etc. [2].

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The terrestrial leaf surface that could be colonized by microbes is approximately 6.4×10^8 km^2 area, encompassing large bacterial population, may be as high as 10^{26} cells [3]. Phyllospheric microbiomes have been swiftly adapted to the ever-changing environmental conditions such as UV radiation, atmospheric temperature, light, less availability of nutrients and water, and so on. Changes in these external conditions largely affect the composition and biodiversity of phyllospheric microbes [4]. Exploring the mode of action through which the phyllosphere develops and maintains its unique microbiome is crucial for in depth analysis of plant microbe interactions.

Phyllospheric microbes have the capacity to increase leaf development, apical growth, flowering, seed mass, fruit development, plant growth and development through direct and indirect mechanisms [5]. Such microbes are important to fix atmospheric nitrogen, increase micro- and macronutrient uptake through solubilization/mobilization, phytohormone production, siderophore production, plant pathogen inhibition, etc. [6]. Sugarcane (*Saccharum* spp. hybrid complex), a C4 plant is most efficient in converting physical and chemical energy (solar energy, Carbon dioxide and water) into biological energy (sucrose) on the earth fulfilling 80% per cent of world's sugar demand [7]. Franco *et al*., [8] by using the radioactive nitrogen confirmed that nitrogen derived from the chemical fertilizers (root) contributed only up to 40% of the total nitrogen in the plant cane at initial stages of development which decreases during stages of maturity to approximately 10% of total nitrogen at harvest. The study signifies the role of phyllospheric microbes in sugarcane ecosystem. These bacteria can also be used in agriculture for the augmentation of plant growth and reduce chemical fertilizers. However, systematic studies on the presence and their efficacy of phyllospheric microbes have not performed so far. Therefore, the present investigation has been carried out to isolate and characterization of sugarcane phyllospheric bacteria and evaluation of their *in vitro* efficacy for plant growth parameters.

MATERIALS AND METHODS

Collection of sugarcane leaf samples

Sugarcane leaf samples were randomly collected from different sugarcane farms at Navsari Agricultural University, Navsari, Gujarat, India (20.9248° N, 72.9079° E). Attempts were made to select leaf samples from five popular sugarcane varieties (CoN 05071, CoN 13072, CoN 13073, CoN 15071, and CoN 05072).

Isolation of phyllospheric bacteria

The collected leaves were washed with sterilized distilled water separately to remove the dirt present on the leaf surface under aseptic conditions. Isolation of phyllospheric bacteria was performed by the leaf imprinting method [9]. After getting well isolated colonies on agar medium, colonies showing distinct morphology were carefully transferred on fresh N-agar plate by the four sector streaking method. Pure cultures of the isolates were preserved on N-agar slants at refrigerated temperatures for further studies.

Colonial, morphological and biochemical characterization

All the isolates were characterized in terms of their colonial and morphological parameters as well as the potent isolates were characterized for different biochemical characters [10] to judge their diversity.

In vitro screening of phyllospheric bacteria

After judging distinctness among bacterial isolates, each of them was screened for *in vitro* efficacy of plant growth promotion based on different parameters, as below:

Nitrogen fixation:

The nitrogen fixation ability of isolates was checked on a nitrogen-deficient Jensen's medium [11]. After incubation at 28±2ºC for 3 days, the presence of a yellow coloured zone around the colonies was considered evidence of positive BNF.

Phosphate solubilization:

Phosphate solubilization efficacy of isolates was checked by spot inoculation of bacteria on Pikovskaya's medium [12] amended with 0.5% (w/v) tri-calcium phosphate. Inoculated plates were further incubated at 28±2ºC for 3 days. A clear zone of tri-calcium phosphate solubilization around the colonies indicated positive P solubilization. The zone ratio was calculated by following formula:

Zone ratio=Zone diameter (mm)/Colony diameter (mm)

Potash mobilization:

The rate of K mobilization was checked by inoculating bacterial isolates on glucose, yeast extract, calcium carbonate (GYC) medium [13]. The development of a halo zone surrounding the bacterial colony after incubation at 28±2ºC for 3 days indicated a positive test for K mobilization. The zone ratio was worked out as discussed earlier.

Zinc solubilization:

The Zn solubilization potential of the isolate was investigated in Bunt and Rovira's medium containing 0.1% insoluble ZnO [14]. Isolates were spot inoculated on the medium followed

by incubation at 28±2ºC for 3 days. A clear zone around the bacterial colony indicated zinc solubilization potential. The zone ratio was worked out to find the efficiency of isolates.

Biological control (antagonistic potential):

The biocontrol efficiency of isolates was checked against *Fusarium oxysporum* by the dual culture method. The efficiency of isolates was judged by percent growth inhibition (PGI) method [15].

Siderophore test:

Siderophore production of isolates was determined using chrome azurol S (CAS) agar medium [16]. Isolates were spot inoculated on CAS agar and incubated at 28±2ºC for 7 days in the dark. The yellow or orange halo zone around the colony against a blue background on CAS agar indicated positive siderophore production.

Extracellular enzyme secretion:

Various extracellular enzymes, such as Proteases [17], amylases [18], and lipases [19] were evaluated on a medium amended with specific substrates. The development of a halo zone on the medium was considered evidence of a positive test.

IAA production:

IAA estimation was done using the Salkowski method [20]. Bacterial cultures were inoculated in a minimal medium amended with tryptophan. After incubation period, 2-3 drops of orthophosphoric acid+salkowski reagents were added to the broth. The development of the pink colour was considered evidence of IAA production.

ACC deaminase activity:

ACC deaminase producer microbes were screened on Dworkin and Foster minimal medium containing ACC (3.0 mM) as the sole source of nitrogen [21].

Identification of isolates

For the identification of the two most potent isolates, DNA was extracted from cultures followed by PCR amplification of 16s r-DNA gene with primer 27F using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on the maximum identity score, the first ten sequences were selected and aligned using multiple alignment software programs. Gene sequences were also deposited on NCBI with successful accession number generation.

RESULTS AND DISCUSSION

Isolation and characterization of phyllospheric bacteria

Sugarcane leaf samples were randomly collected from the farms at Navsari Agricultural University, Navsari, Gujarat, India from five popular sugarcane varieties of the South Gujarat. Total 18 isolates were obtained in pure form and characterized (Table 1). The data clearly indicated that all the isolates were diverse in terms of different characters studies.

Table 1: Colonial and morphological characters of different sugarcane phyllosphere isolates

Colony characters	Sugarcane variety						
	CoN 05071						
	S 1.1	S 1.2	S 1.3	S 1.4	S 1.5	S 1.6	S 1.7
Size	Medium	Small	Small	Pin point	Small	Medium	Large
Shape	Round	Round	Round	Round	Round	Irregular	Undulate
Elevation	Flat	Flat	Flat	Flat	Flat	Convex	Flat
Margin	Entire	Convex	Entire	Entire	Entire	Irregular	Curled
Texture	Smooth	Moist	Smooth	Smooth	Powdery	Butyrous	Smooth
Opacity	Translucent	Transparent	Transparent	Transparent	Translucent	Transparent	Translucent
Pigment	White	White	White	Yellow	White	Yellow	Greyish
Morphological characters							
Cell shape	Cocci	Short rod	Short rod	Short rod	Long rods	Short rods	Cocci
Cell arrangement	Bunch	Singly	Singly	Singly	Singly and chain	Singly	Chain

Colony characters	Sugarcane variety					
	CoN 13072				CoN 13073	
	S 2.1	S 2.2	S 2.3	S 2.4	S 3.1	S 3.2
Size	Small	Small	Medium	Medium	Large	Small
Shape	Round	Round	Round	Irregular	Round	Round
Elevation	Flat	Flat	Flat	Raised	Flat	Flat
Margin	Entire	Entire	Entire	Undulate	Entire	Undulate
Texture	Smooth	Moist	Dry	Smooth	Moist	Smooth
Opacity	Transparent	Transparent	Translucent	Translucent	Translucent	Translucent
Pigment	White	Yellow	White	White	Yellow	White
Morphological characters						
Cell shape	Short rod	Short rod	Cocci	Long rods	Long rod	Short rods
Cell arrangement	Singly	Singly	Singly	Singly and chain	Chain	Singly
Gram's reaction	Negative	Negative	Positive	Positive	Positive	Negative
Gram's reaction	Positive	Negative	Negative	Negative	Positive	Negative	Positive

Colony characters	Sugarcane variety	
	CoN 15071	CoN 05072

	S 4.1	S 5.1	S 5.2	S 5.3	S 5.4
Size	Small	Pinpoint	Small	Large	Medium
Shape	Round	Round	Round	Round	Filamentous
Elevation	Flat	Flat	Flat	Flat	Flat
Margin	Entire	Entire	Convex	Curled	Filiform
Texture	Moist	Moist	Dry	Smooth	Powdery
Opacity	Transparent	Transparent	Transparent	Transparent	Translucent
Pigment	Colourless	White	White	Colourless	White
Morphological characters					
Cell shape	Short rod	Short rod	Short rod	Long rods	Cocci
Cell arrangement	Singly	Singly	Singly	Singly and chain	Bunch
Gram's reaction	Negative	Negative	Negative	Positive	Positive

In vitro screening of isolated bacteria

In vitro screening data (Table 2) suggested that out of all the 18 isolates, none of the isolate was found positive for nitrogen fixation. In terms of nutrient solubilization, 07 (38.89%), 08 (44.44%), and 08 (44.44%) isolates were found positive for phosphate, potash, and zinc solubilization, respectively. Microbes possess tremendous biological weathering capabilities that may enhance mineral dissolution, results into release of macro- and micronutrients in the soil solution [22], and thus make them available for plants growth.

Another direct mechanism for plant growth promotion exerted by phyllospheric bacteria is biocontrol (antagonistic) potential against plant pathogens and siderophore productions [23]. Among different phyllospheric bacterial isolates, 8 (44.44%) showed antagonistic effects against *Fusarium oxysporum* with PGI in the tune of 29.65-51.22 percent. Siderophore production was noted in the case of 6 (33.33%) isolates. Bacterial mediated hydrolytic enzymes (proteases, lipases, amylases and cellulases) secretion was found to be associated with bacterial competitiveness, which in turn beneficial for plant protection and boosting plant development [24]. Experimental data revealed that 12 (66.66%), 7 (38.89%), and 10 (55.55%) isolates were found positive for extracellular proteases, lipases, and amylases, respectively.

Table 2: *In vitro* characterization of isolates

Sr. No.	Isolate No.	Nitrogen fixation	Phosphate solubilization (mm)	Potash mobilization (mm)	Zinc solubilization (mm)	Antagonistic potential against *Fusarium oxysporum*	Siderophore production	Protease production	Lipase production	Amylase production	IAA production	ACC deaminase
1	S 1.1	-	-	-	2.2	34.12%	-	+	-	+	-	-
2	S 1.2	-	-	-	-	43.22%	-	-	+	-	-	-
3	S 1.3	-	1.9	3.1	1.3	-	-	+	+	-	+	-
4	S 1.4	-	2.7	2.1	-	-	-	-	-	+	-	-
5	S 1.5	-	-	-	3.2	44.21%	+	+	-	+	-	-
6	S 1.6	-	-	-	-	-	-	+	+	-	+	+
7	S 1.7	-	2.5	3.4	3.1	-	-	+	-	+	-	-
8	S 2.1	-	3.3	1.9	2.2	-	-	+	-	+	-	-
9	S 2.2	-	-	-	-	33.89%	+	-	+	-	+	-
10	S 2.3	-	-	-	-	51.22%	-	-	-	-	+	-
11	**S 2.4**	**-**	**4.8**	**3.8**	**3.4**	**48.35%**	**+**	**+**	**-**	**+**	**+**	**+**
12	S 3.1	-	-	-	-	-	-	+	+	+	-	-
13	S 3.2	-	-	-	-	-	-	+	+	+	-	-
14	**S 4.1**	**-**	**3.9**	**4.4**	**2.1**	**44.52%**	**+**	**+**	**+**	**-**	**+**	**+**
15	S 5.1	-	-	-	-	-	-	+	-	-	-	-
16	S 5.2	-	-	-	-	29.65%	+	-	-	-	-	+
17	S 5.3	-	3.7	2.7	-	-	-	-	-	+	-	-
18	S 5.4	-	-	3.2	1.6	-	+	+	-	+	-	-

'+' = Positive and '-' = negative reaction

Plant associated bacteria also exerted a beneficial effect on plant growth by producing several useful metabolites like the IAA along with ACC deaminase activity for the conferring of biotic and abiotic stress tolerance in the host plant [25,26]. Data indicated that 6 (33.33%) showed IAA production trait and 4 (22.22%) were positive for ACC deaminase activity.

Biochemical characterization of potent bacteria

Out of all the 18 isolates screened under *in vitro* conditions, isolates S 2.4 and isolate S 4.1 were found to be potent for individual as well as multiple plant growth parameters. Therefore, both the isolates were further characterized biochemically (Table 3). Data indicated that methyl red, starch utilization, nitrate reduction, urease, acid and gas production from galactose, and xylose tests were dissimilar among both the isolates, suggesting wide biochemical diversity among both the potent isolates.

Table-3: Biochemical characterization of isolates

Sr. No.	Name of the Test	S 2.4	S 4.1
1	Methyl Red test	Negative	Positive
2	Voges-Proskauer test	Positive	Positive
3	Starch hydrolysis	Positive	Negative
4	Indole test	Positive	Positive
5	Citrate utilization test	Positive	Positive
6	Nitrate Reduction test	Positive	Negative
7	Urease test	Negative	Positive
8	Phenylalanine deamination test	Negative	Negative
9	Gelatin hydrolysis	Positive	Positive
10	Casein hydrolysis	Positive	Positive
11	Motility	Positive	Positive
12	Catalase	Positive	Positive
13	H_2S production test	Negative	Negative
14	Acid and gas production		
	Glucose	Positive	Positive
	Fructose	Positive	Positive
	Sucrose	Positive	Positive
	Mannitol	Positive	Positive
	Galactose	Negative	Positive
	Xylose	Positive	Negative

Identification of bacteria

Both the isolates were identified on the basis of 16s r-RNA sequence analysis. Isolate S 2.4 was *Bacillus amyloliquefaciens* (Fig. 1) and S 4.1 was belonging to *Enterobacter cloacae* (Fig. 2). The bacterium *B. amyloliquefaciens* is a well known phyllospheric bacteria important in plant pathogen inhibition and growth promotion [27, 28]. The positive impact on plant growth promoting by *Enterobacter cloacae* was also reported by other researchers [29, 30].

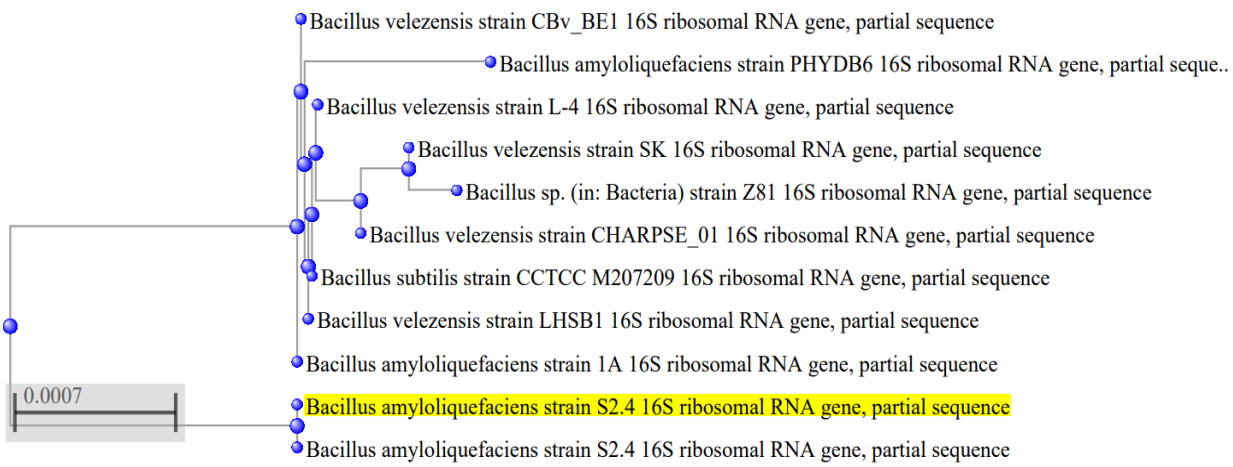

Fig-1 Phylogenetic tree of isolate *Bacillus amyloliquefaciens* S 2.4 [Accession no. ON054025]

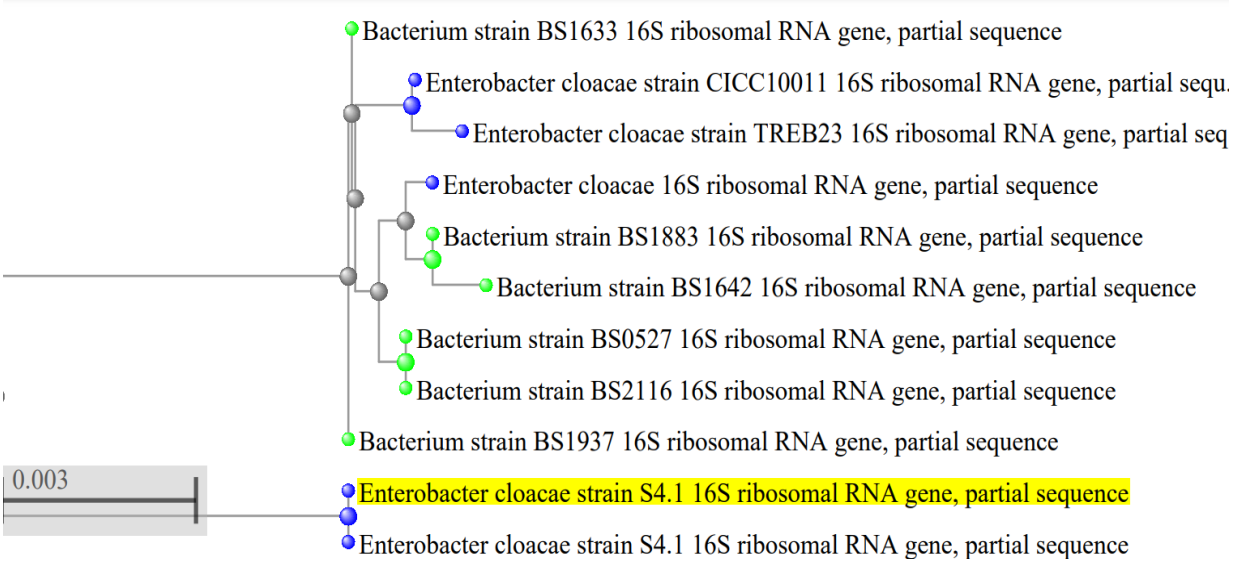

Fig-2 Phylogenetic tree of isolate *Enterobacter cloacae* S 4.1 [Accession no. ON054026]

CONCLUSION

Phyllospheric bacteria form symbiotic or commensal relationships with their host and promote plant growth by direct and indirect mechanisms. The isolated potent phyllospheric bacteria *Bacillus amyloliquefaciens* S 4.1 and *Enterobacter cloacae* S 4.1 possessed highest plant growth promoting traits under *in vitro* conditions. The use of these beneficial bacteria in agriculture is a cost effective and eco-friendly way to mitigate various biotic and abiotic stresses. Application of efficient and native bacteria in agricultural might be a step ahead in augmentation of plant growth and minimization of the synthetic chemicals to without compromising plant, soil and human health.

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