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Rhizobacteria of sugarcane: *In vitro* screening for their plant Growth Promoting potentials

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Abstract

Plant growth promoting Rhizobacteria (PGPR) are group of naturally occurring soil microorganisms that aggressively colonize plant root and enhance growth of plant and yield by direct and indirect mechanism. In search for efficient PGPR strains associated to Saccharum officinarum, a total of 50 isolates were obtained from the rhizospheric soil, root, stem and leaf of sugarcane plant that were grown in agricultural field of Kamrej, Surat district, India. Among 50, eleven isolates exhibited multiple Plant growth promoting traits viz., direct mechanisms: Solubilization of Phosphate, Biological Nitrogen fixation, Zinc solubilization, Potassium solubilization, Indole-3-acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase production, 46 isolates exhibited positively for Tri-calcium phosphate solubilization on pikovskaya agar. 39 isolates were able to fix atmospheric nitrogen, 25 isolates were able to solubilizing zinc, 24 isolates were able to solubilizing Potassium, 40 isolates were able to cleave 1-aminocyclopropane-1-carboxylate, and all isolates produced IAA in the presence of L-Tryptophan. Such multiple positive PGP traits isolates can be further explored as effective bio-inoculants to improving plant growth and yield.

Keyword: Rhizobacteria, PGPR, nitrogen fixation, Indole-3-acetic acid.

Introduction

Plant Growth Promoting Rhizobacteria (PGPR) are free living soil born microorganisms isolated from rhizosphere, which have ability to either enhance the plant growth or reduce the damage through the soil borne plant pathogen when applied to seed and crops[1]. PGPR are potentially capable for stimulating growth of plant and increasing yields of crop has evolved over the past several years to till today researchers are able to successfully use them in field experiments[1-4]. A number of bacterial species associated with the plant rhizosphere belonging to genera *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Pseudomonas, Rhizobium, Bradyrhizobium and Serratia* are able to exert a beneficial effect on growth of plant[5, 6]. PGPR have the potential to contribute in sustainable plant growth promotion in agricultural system[7]. The growth promotion channel by these bacteria that enhances the plant growth was not fully known while in few ways it is understood[8]. Mainlly, PGPR works by three different ways[6,9]: synthesizing particular compound for the plants[10, 11], facilitating the uptake of certain nutrients from the soil[12,13] and preventing the plant from diseases[14,15,16]. The well known mechanism for the plant growth promotion is through producing hormones including Indole-3-acetic acid (IAA)[17], Solubilization of insoluble phosphate[18], Fixation of atmospheric nitrogen[19], Hydrogen cyanide (HCN) production[20] and by producing vital enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase[21,22]. Researchers have also inoculated phosphate solubilizing bacteria and nitrogen fixing bacteria in the field level condition which have capable of enhancing the plant growth and better crop yield[23].For microorganisms and plants zinc is an essential micronutrient which is present in the enzyme system as co-factor or metal activator of many organisms[24] Growth-promoting capability of many bacteria may be highly specific to certain plant species, cultivar and genotype[25]. However the effect of *in vitro* condition to the field level condition is not the same when the isolates were inoculated in the field which has to be continuously monitored for improvement of crop inoculants[26]. So it is necessary to develop rhizobacterial population which has significant PGP capacity for the improvement of agricultural practices and crop yield, the present study was mainly focused to the isolates having multiple PGP attributes from the rhizosphere of sugarcane plant.

Material and methods

Sample collection: The root adhering samples of five sugarcane plants were collected from the agricultural field of Kamrej, Surat, India. The sample were collected in aseptic bags and immediately transported to lab for further process[27]. Sugarcane leaves and stems were collected from a 4 month old plantation and processed thereby[28].

Isolation of Rhizobacteria: For the isolation of rhizospheric bacteria, 1 gm of sample was suspended in 9 ml of sterial distilled water, after sedimentation of solid particles dilution was made up to 10^{-8}. For endorhizospheric sample the roots

were surface sterilized with 70% ethanol and washed 2 to 3 times to remove excess disinfectant. Root material was suspended in phosphate buffer and crushed with sterial scalpel. The leaves and stem were washed with sterile distilled water and their surface disinfected by washing with 70% ethanol. 10 g of stems and leaves were macerated separately in sterile 10 mM Tris-HCL, pH 8. All these suspension were serially diluted up to 10^{-8}. One hundred micro liters of appropriate dilutions was taken and performed spread plate on different media *viz.* king's B medium (KBM), ashby's mannitol agar (AMA), nitrogen free medium(NFM), yeast extract mannitol agar (YMA), azospirillum medium (AZ), pikovskaya medium (PM), bacillus medium(BM). The plates prepared were incubated in room temperature for 48 h. Bacterial cultures were maintained on the respective slants and stored at 4°C for further use.

In vitro screening of Plant Growth Promoting (PGP) Attributes: Phosphate solubilization assay: The solubilization of phosphate was tested using Pikovskaya agar medium (Glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.1g; yeast extract 0.5 g; $MnSO_4$, and $FeSO_4$ trace; D/W, 1 l; agar, 15 g; pH 7. Overnight test culture grown in nutrient broth (beef extract, 1 g; yeast extract, 2 g; peptone, 5 g; NaCl, 5; pH 7). All the plates were incubated for the 4 days at 28±2°C. The halo zone around the colony was measured and considered as phosphate solubilizing bacteria.

Nitrogen Fixation: All the isolates were subjected for N-fixation on semisolid nitrogen free medium DL-malic acid, 5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; NaCl, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; : $NaMoO_4 \cdot 2H_2O$, 2 mg; $MnSO_4 \cdot H2O$, 10 mg; 0.5 % bromothymol blue in 0.2 N KOH- 2 ml; 1.64% Fe-EDTA solution 4 ml; agar 2 g, 1000 ml distilled water, pH 6.8). Bacterial strains were incubated for five days at 30°C, pellicle growth was considered to be positive for N-fixation. The culture was further inoculated in fresh N free semisolid medium for the final confirmation of N-fixation activity[29].

Zinc solubilization assay: All the isolates were inoculated on to the modified Pikovskaya medium[30] (Glucose, 10 g; $Ca_3(PO_4)_2$, 5.0 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; Yeast extract, 0.5 g; $MnSO_4$, Trace; $FeSO_4.7H_2O$, Trace; Agar, 15 g; Water, 1000 ml; pH, 7.0±0.2) containing 1% insoluble zinc compound (ZNO). All the plates were incubated for 48 h at 28°C. The halo zone around the colony was measured and considered as zinc solubilizing bacteria.

Potassium solubilization assay: All the isolates were spot inoculated on to the modified Aleksandrov medium (composition: Glucose, 5g ; $MgSO_4.7H_2O$, 0.5 g; $CaCO_3$, 0.1 g; $FeCl_3$, 0.006g; $Ca_3(PO_4)_2$, 2.0 g; Insoluble Mica powder, 3 g, agar,20 g; D/W, 1000ml[31] . All the plates were incubated for 3 days at 30°C. The halo zone around the colony was measured and considered as potassium solubilizing bacteria.

ACC deaminase activity: ACC deaminase activity was determined by the method[6]. 1 µl of LB pure bacterial culture was inoculated into agar plates having NFb (composition: Malic acid, 5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; NaCl, 0.1 g; 0.5 % bromothymol blue in 0.2 N KOH- 2 ml; Vitamin solution, 1 ml; Micronutrient solution, 2 ml; 1.64% Fe-EDTA solution, 4 ml; KOH,4.5 g) or NFb-ACC modified by including unique nitrogen source 1-aminocyclopropane-1-carboxylate. Plates were incubated for 4 days at 28°C and observed daily for formation of colony. Colonies were re-inoculated and incubated in the same experimental conditions. Newly formed colonies in NFb with addition of ACC were considered positive for ACC deaminase activity.

Indole acetic acid (IAA) assay: 500 µl of 24 h old bacterial culture was inoculated in fifty milliliter of Nutrient broth containing 0.1% DL-tryptophan and incubated in refrigerated incubator Shaker for 48 h at 30±0.1 °C and 180 rpm in dark than centrifuged at 10,000 rpm for 10 min at 4 °C. From the supernatants estimation of indole-3-acetic acid (IAA) was done using colorimetric assay[32]. Colorimetric estimation: One millilitre of supernatant was mixed with 4 ml Salkowski reagent and absorbance of the pink color was read after 30 min at 535 nm in UV/Visible Spectrophotometer. Presence of pink color in test tubes indicated IAA production[33].

Results and Discussion

The presence of bacteria in rhizosphere is based on the concentration of nutrient available. Due to constant delivery of nutrients from plant roots, soil microbes are found to dominate the niche and their by helps in plant growth promotion under various mechanisms[34, 6]. PGPR are mainly used as inoculants for enhancing the growth and yield of agriculture crops, however screening for the efficient PGPR strains selection needs to be very critical. This study mainly focuses on the screening for a potential PGPR strains on the basis of direct plant growth promoting traits *viz.,* Solubilization of Phosphate, Biological Nitrogen fixation, Zinc solubilization, Potassium solubilization, ACC Deaminase activity, Indole acetic acid production. The solubilization is based on the production of low molecular weight organic acid[35]. The production of gluconic acid, acetic acid, formic acids during the solubilization of insoluble tri-calcium were also reported[36] by using the *pseudomonas* spp. higher concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere soil as compared to nonrhizospheric soil[37]. Phytohormone IAA works as a signal molecule in the regulation of plant development. Plant system uses auxin like hormones for their optimal growth[38]. In our study majority of all the isolates were produce IAA in the presence of L-Tryptophane. IAA synthesis by PGPR can vary among different species and strains which are also influenced by culture conditions, growth stage and substrate availability[39]. IAA by *Pseudomonas aeruginosa* isolate produced 80 $\mu g ml^{-1}$ of IAA on tryptophan supplementation[40] ($0.1 g l^{-1}$). *Pseudomonas* strains producing high amounts of IAA have a great potential

for use as PGPR for large scale production of inoculums for sugarcane[39]. Nitrogen, phosphorus and potassium are major essential macronutrients for plant growth and development. Inoculation with phosphate solubilizing bacteria (PSB) increased sugarcane yield by 12.6 percent. Among All isolates were screened for phosphate solubilization on modified PVK medium, 46 isolates were positive for phosphate solubilization, from which rhizospheric isolates shown maximum phosphate solubilization activity. The organisms were isolated using N free media and 39 isolates able to fix atmospheric nitrogen, among which Rhizospheric, Rhizoplane and stem endophytes isolates were able to fix maximum nitrogen. 25 isolates were able to solubilizing insoluble zinc compound, from which stem endophytes isolates were able to solubilizing zinc on modified Aleksandrov medium.40 isolates were able to cleave 1-aminocyclopropane-1-carboxylate. In our study, 11 isolates exhibited multiple plant growth promoting activity under *in vitro* condition. However, natural variations make it difficult to predict how PGPR may respond when applied to field level conditions.

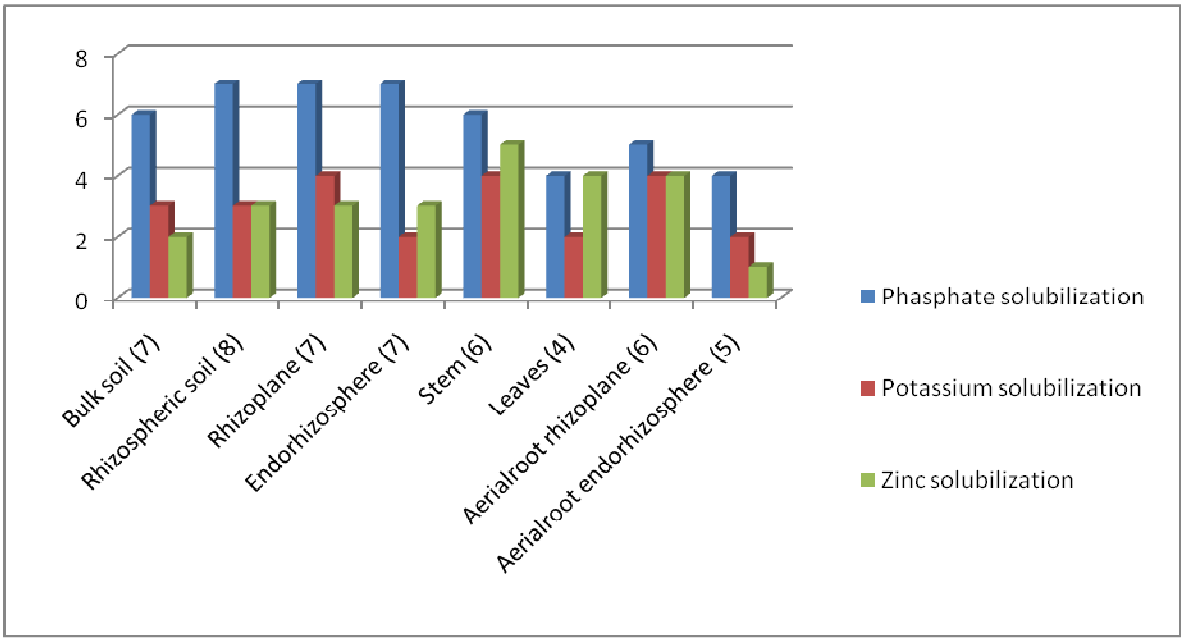


Figure-1
Plant growth promoting traits of isolates

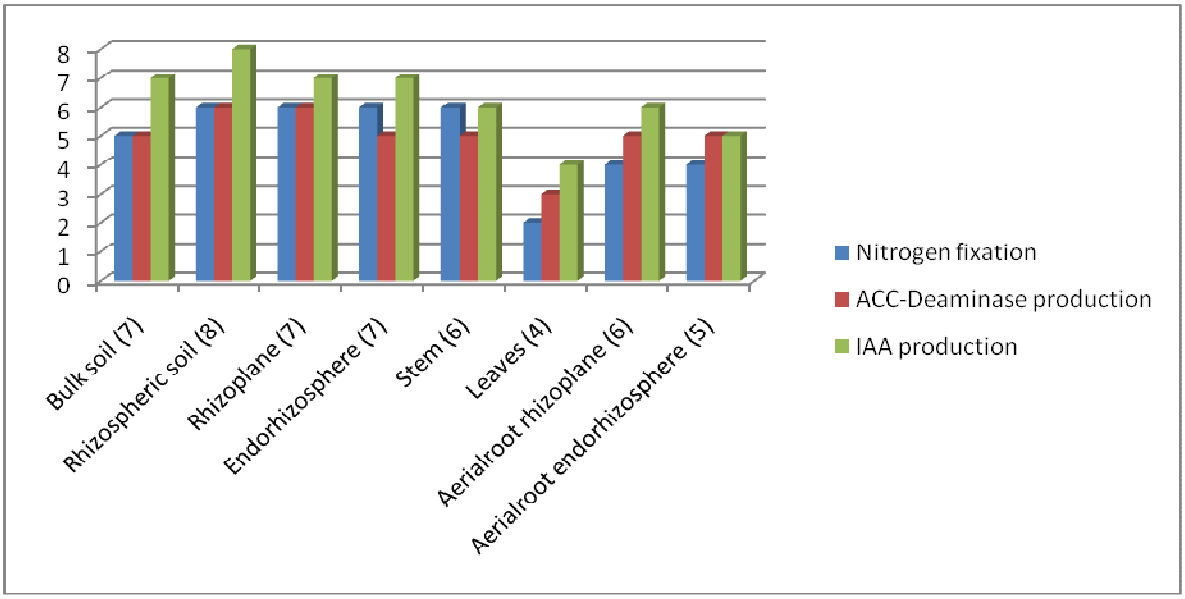
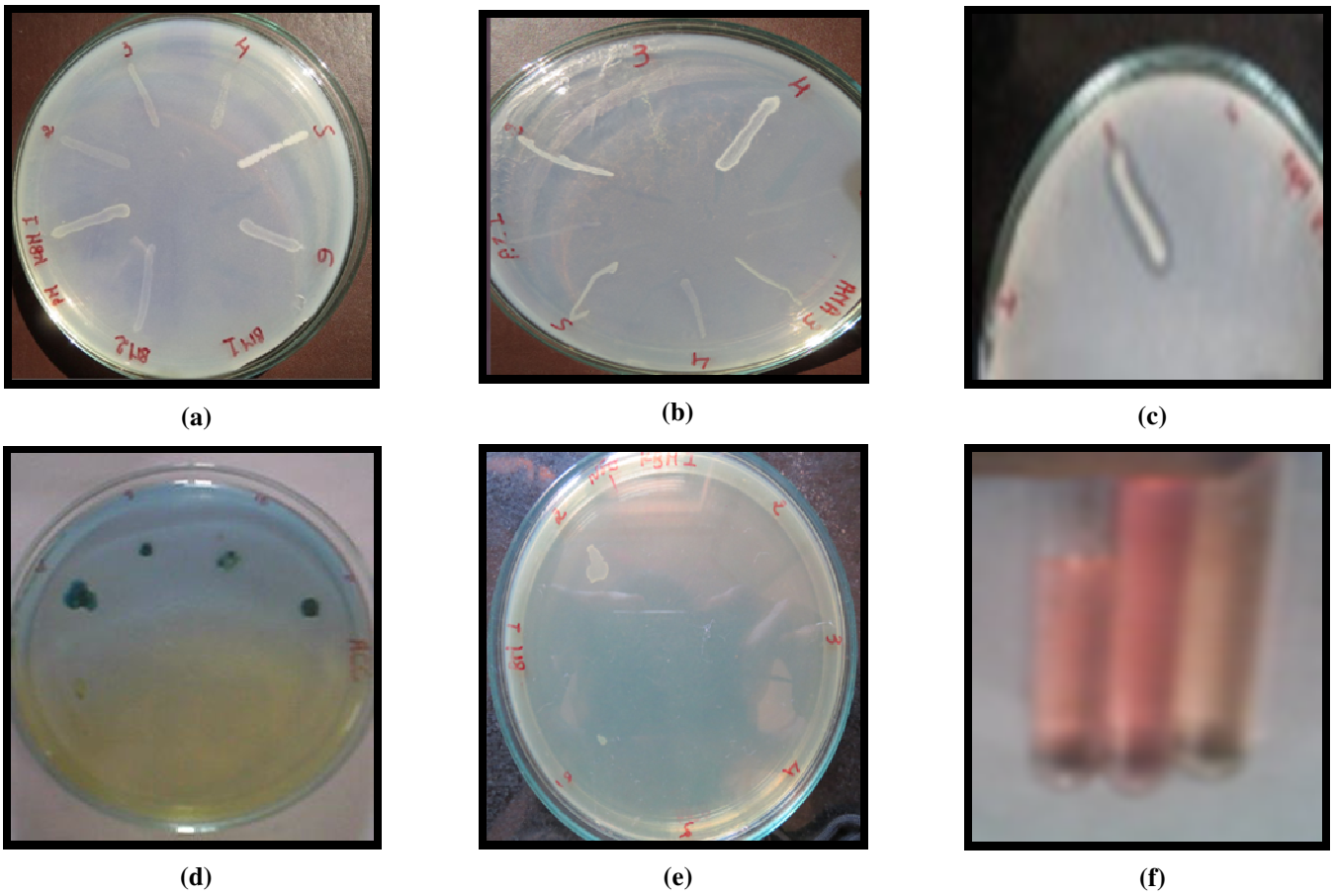


Figure-2
Plant growth promoting traits of isolates

Table-1
Plant Growth Promoting Traits of isolates

NO	Isolate code	Direct plant growth promoting traits						
		PO_4	N_2	K	ZN	ACC	IAA	Total
1	KBM/F3/BS/1	+	+	+	-	+	+	5
2	NFM/F3/BS/2	+	+	-	-	+	+	4
3	AMA/F3/BS/1	+	+	-	+	+	+	5
4	PM/F3/BS/2	+	+	+	-	+	+	5
5	AZ/F3/BS/2	+	+	-	-	+	+	4
6	BM/F3/BS/1	-	-	-	-	-	+	1
7	YMA/F3/BS/2	+	-	+	+	-	+	4
8	KBM/F3/RH/3	+	-	+	+	-	+	4
9	NFM/F3/RH/2	+	+	-	-	+	+	4
10	AMA/F3/RH/1	+	+	-	+	+	+	5
11	AMA/F3/RH/5	+	+	+	+	+	+	6
12	PM/F3/RH/2	+	+	-	-	+	+	4
13	AZ/F3/RH/2	+	+	-	-	+	+	4
14	BM/F3/RH/1	-	-	-	-	-	+	1
15	YMA/F3/RH/4	+	+	+	-	+	+	5
16	KBM/F3/RP/1	+	+	+	-	+	+	5
17	NFM/F3/RP/3	+	+	+	+	+	+	6
18	AMA/F3/RP/2	+	+	-	+	+	+	5
19	PM/F3/RP/2	+	+	+	-	+	+	5
20	AZ/F3/RP/2	+	+	-	-	+	+	4
21	BM/F3/RP/4	+	-	+	-	+	+	4
22	YMA/F3/RP/1	+	+	-	+	-	+	4
23	KBM/F3/ER/5	+	-	-	+	+	+	4
24	NFM/F3/ER/2	+	+	-	-	+	+	4
25	AMA/F3/ER/2	+	+	-	+	+	+	5
26	PM/F3/ER/2	+	+	+	-	-	+	4
27	AZ/F3/ER/3	+	+	-	-	+	+	4
28	BM/F3/ER/3	+	+	+	-	+	+	5
29	YMA/F3/ER/1	+	+	-	+	-	+	4
30	NFM/F3/S/3	+	+	+	+	+	+	6
31	NFM/F3/S/4	+	+	+	+	+	+	6
32	AMA/F3/S/2	+	+	-	+	+	+	5
33	PM/F3/S/2	+	+	+	-	+	+	5
34	AZ/F3/S/4	+	+	+	+	+	+	6
35	YMA/F3/S/1	+	+	-	+	-	+	4
36	KBM/F3/L/5	+	-	-	+	+	+	4
37	NFM/F3/L/3	+	+	+	+	+	+	6
38	PM/F3/L/6	+	+	+	+	+	+	6
39	YMA/F3/L/2	+	-	-	+	-	+	3
40	KBM/F3/ArRP/4	-	-	-	-	+	+	2
41	NFM/F3/ArRP/3	+	+	+	+	+	+	6
42	PM/F3/ArRP/2	+	+	+	-	+	+	5
43	PM/F3/ArRP/7	+	-	-	+	-	+	3
44	AZ/F3/ArRP/4	+	+	+	+	+	+	6
45	BM/F3/ArRP/5	+	+	+	+	+	+	6
46	KBM/F3/ArER/4	-	-	-	-	+	+	2
47	NFM/F3/ArER/3	+	+	+	+	+	+	6
48	PM/F3/ArER/2	+	+	+	-	+	+	5
49	AZ/F3/ArER/1	+	+	-	-	+	+	4
50	BM/F3/ArER/3	+	+	-	-	+	+	4



(a) **(b)** **(c)**

(d) **(e)** **(f)**

Figure-3
(a) Phosphate solubilizing bacteria (b) Potassium solubilizing bacteria (c) Zinc solubilizing bacteria (d) ACC deaminase activity (e) Nitrogen fixing bacteria (f) Indole acetic acid production

Conclusion

The experiment performed under in vitro condition has three major conclusions, i. Out of 50 isolates, 6 isolates were weak promoter for plant growth were as rest of all isolates may promote plant growth extensively, from which 11 isolates were found positive for all the plant growth promoting potentials. Such multiple positive PGP Traits isolates can be further explored as potential biofertilizer for the sustainable agriculture. ii. 2 isolates which were reported in bulk soil and in rhizospheric soil also reported in the whole part of sugarcane plant. These characteristic is useful for whole plant. iii. The direct promotions of plant growth by PGPR include facilitating the uptake of certain nutrient like nitrogen, phosphorus, potassium and zinc in accemiable form for nutrient avability from the soil and root environment by producing plant hormone indole-3-acetic acid and by producing vital enzyme 1-aminocyclopropane-1-carboxylate(ACC) deaminase.

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