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ISOLATION AND CHARACTERIZATION OF EFFECTIVE AND EFFICIENT PLANT GROWTH-PROMOTING RHIZOBACTERIA FROM RICE RHIZOSPHERE OF DIVERSE PADDY FIELDS OF INDIAN SOIL

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ABSTRACT

Plant growth-promoting rhizobacteria, which are generally known as PGPR, are such kind of bacteria, which can support the growth of host plants by living in the rhizospheric region by variety of mechanisms. In present study, we had collected rice rhizospheric soil samples from different field of rice from Surat region of Gujarat, India. Total 22 PGPR bacterial isolates were isolated and screened for their various PGPR activities like siderophore production, phosphorus solubilisation, IAA production, and HCN production as well as for protease and chitinase activity. 10 isolates in case of siderophore production, 6 isolates in case of phosphorus solubilisation, 13 isolates in case of IAA production were found to be positive whereas 4 isolates shown positive HCN production. However, 10 and 9 isolates shown protease enzyme production and chitinase enzyme production. However, out of 22 isolates, 2 isolates were found to be most potent and gives majority of the PGPR activities. So, present study suggested that, isolated PGPR will be used to attain significant productivity and soil fertility in rice fields and can be used as biofertilizers.

Keywords: PGPR, rhizobacteria, siderophore, phosphorus solubilization, IAA, HCN

INTRODUCTION

Rice (*Oryza sativa L.)* is the most important staple food throughout the world and mainly in the East Asia, South Asia, Southeast Asia, Middle East, and the West Indies [1]. Majority of peoples consumes rice as their daily food. However, according to the data of FAOSTAT - 2012, after the maize and wheat, rice is the highest producing grain [2]. Rice and its different products provide higher nutrition and calories to the humans [3].

However, now a days due to the use of chemical fertilizers for getting higher production in the field of rice, which are not only expensive for farmers but also inhibit the natural flora of soil which support the growth of plant as well as also decrease the fertility of soil. So, it is necessary and important to find out another way, which not only enhance the fertility of soil, but can also increase the rice production without the uses of chemical fertilizers. So, the best alternative is the use of PGPR. "Plant growth promoting rhizobacteria are those bacteria which are living in to the rhizospheric regions and support plant growth by different kinds of biological processes" [4].

Various PGPRs have been reported which can enhance the growth of plants, increase yield of crop and production by their various biological processes [5-7]. PGPR enhances plant growth by variety of processes such as, by producing siderophore, by solubilizing phosphorus, by uptake of nitrogen, by producing certain kinds of compound, which inhibit the growth of pathogenic organisms or help plants to fight against adverse environmental conditions [8-10].

Gujarat is one of the major rice producing state of India. Average rice production of the state is about 0.9-1.1 million tons in a year [11]. In Gujarat, rice is grown in 14 districts. Surat is one of the rice producing district of Gujarat. In Surat, rice is grown in both summer (in few area) as well as monsoon (in larger area). Out of this, average rice production of Surat is low (1500 – 2000 kg/ha) of the state [12]. So, we had started our work mainly for the isolation and characterization of novel PGPR bacteria from the rice field, which can enhance average production of rice in Surat and will be used as an alternative of chemical fertilizers.

MATERIAL AND METHODS

Collection of rhizospheric soil sample from rice field

Rice plants from different fields of Surat districts of Gujarat, India were selected for this work. Soil samples were collected from 0–15 cm depth from the rhizosphere of 2 months old rice plants. Whole plant after chopping off the shoots, was carefully uprooted (along with the adhering soil; without breaking the secondary and tertiary roots), labelled and collected the soil from roots for the isolation of PGPRs.

Isolation of PGPR from rice rhizosphere soil

Rice rhizospheric soil was used for isolation of PGPR. 10 g of rhizosphere soil were taken into a 250 ml of conical flask, and 90 ml of sterile distilled water was added to it. The flask was shaken for 30 min on a rotary shaker. 1 ml of suspension was added to 10 ml vial and shaken for 2 min. Serial dilution technique was performed up to 10^{-7} dilution. An aliquot (0.1 ml) of this suspension was spread on the plates of Nutrient agar (NA) medium.

Plates were incubated for 3 days at 28 ± 2°C to observe the colonies of bacteria [13]. Bacterial colonies were streaked to other NA plates and were incubated at 28 ±2°C for 3 days. Well-isolated single colony was picked up and re-streaked to fresh NA plates and incubated similarly. Isolated colonies were streaked to obtain single isolation and transferred to NA slants to make pure culture.

Characterization of isolates

Total number of 22 rhizobacterial isolates were obtained by pure culture and designated as CG1, CG2, CG3, CG4, CG5, CG6, CG7, CG8, CG9, CG10, CG11, CG12, CG13, CG14, CG15, CG16, CG17, CG18, CG19, CG20, CG21, and CG22. Morphological characteristics of each isolated colony were examined on NA plates. After 3 days of incubation, different characteristics of colonies such as size, shape, elevation, margin, opacity, pigmentation, etc. were recorded. All the rhizobacterial isolates were biochemically characterized for Gram's reaction [14].

PGPR activity of isolated bacteria

Siderophore production

Siderophore production ability of the all PGPR isolates was carried out on to the CAS agar medium (Chrome Azurol S medium) [15]. All the isolates were streaked on to the CAS agar plate and incubated at room temperature for 24 to 72 hours. Siderophore productions were detected with orange halos around the colonies.

Phosphate solubilization

Phosphate solubilizing ability of all PGPR isolates was checked on to the Pikovskya's agar medium [16]. All the isolates were streaked on to the Pikovskya's agar plate and incubated at room temperature for 5 to 6 days. Phosphate solubilization was detected by clear halo zones around the colony.

IAA (Indole-3-acetic acid) production

IAA production of PGPR isolates was carried out by using Salkowaski's method. All the isolates were enriched in to peptone broth containing tryptophan for 24 hours. Then, the culture medium was centrifuged at 9000x rpm for 10-15 minutes to separate bacterial cells from the culture medium. Further, Salkowaski's reagent (4.5 grams of $Fecl_3$ per liter in 10.8 M H_2SO_4) were mixed with the supernatant and incubated in dark at room temperature for 30-40 minutes. Absorbance was taken at 530 nm.

HCN production

HCN production was evaluated by streaking the bacterial isolates on King's B agar medium supplemented with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plate. The plates were then sealed airtight with parafilm and incubated at 30°C for 48 h. A colour change of the filter paper from deep yellow to reddish-brown colour was considered as an indication of HCN production [17].

Extra cellular enzyme activities

Protease activity

Protease production assay was carried on sterile skim milk agar plate (Pancreatic digest of casein 5.0 g, Yeast extract 2.5 g, Glucose 1.0 g, Agar 15.0 g, distilled water 1000 ml, and skim milk 7% was added as inducer). All the PGPR isolates were streaked on to the sterile skim milk agar plates and incubated at room temperature for 72 hours. Protease production was detected by a clear zone around the colony [18].

Chitinase production

Suspension was prepared using chitin powder to 10 M HCl and kept overnight at 4°C with vigorous stirring. It was then added to chilled ethanol and kept overnight at 25°C with vigorous stirring. The precipitate was collected by centrifugation at 10000 rpm for 20 minutes and washed with sterile distilled water until colloidal chitin became neutral (pH 7.0) [19]. Chitinase production was determined on NA plates containing colloidal chitin. Bacterial cultures were streaked on NA plates and incubated for 7 days at 30°C. The ability of chitinase production was shown by a clear halo around bacterial colonies [20].

Experimental replication

Data from all experiment represent the averages of two or more independent experiments.

RESULTS AND DISCUSSIONS

Isolation of PGPR

22 bacterial isolates were successfully isolated from the rhizosphere soils of rice field from different areas of Surat District in Gujarat, India (Table 1). They were designated as CG1, CG2, CG3, CG4, CG5, CG6, CG7, CG8, CG9, CG10, CG11, CG12, CG13, CG14, CG15, CG16, CG17, CG18, CG19, CG20, CG21 and CG22.

Table-1. List of isolates from diverse paddy fields.

S. No.	Isolates	Sample	Location	No. of Isolates
1	CG1, CG2, CG3, CG4	Sample-1	Olpad, Field-1	4
2	CG5, CG6	Sample-2	Olpad, Field-2	2
3	CG7, CG8, CG9	Sample-3	Tarsadi, Field-3	3
4	CG10, CG11, CG12	Sample-4	Mahuva, Field-4	3
5	CG13, CG14	Sample-5	Olpad, Field-5	2
6	CG15, CG16, CG17	Sample-6	Mahuva, Field-6	3
7	CG18, CG19, CG20	Sample-7	Bamroli, Field-7	3
8	CG21, CG22	Sample-8	Kim, Field-8	2

Morphological characteristics of PGPR isolates

The morphological characteristics mainly colony characteristics and Gram's nature of all 22 PGPR isolates varied widely and are described in Table-2.

Table-2. Description of the PGPR isolates on the basis of morphology.

Isolate name	Size	Shape	Elevation	Margin	Opacity	Pigmentation	Gram's reaction
CG1	Medium	Round	No	Entire	Opaque	Nil	Positive
CG2	Small	Round	No	Entire	Transparent	Nil	Positive
CG3	Medium	Round	Slightly	Rough	Opaque	Yellow	Negative
CG4	Small	Round	No	Entire	Opaque	Nil	Positive
CG5	Small	Round	Slightly	Entire	Opaque	Nil	Negative
CG6	Medium	Round	No	Entire	Transparent	Nil	Positive
CG7	Large	Irregular	No	Rough	Opaque	Yellow	Positive
CG8	Small	Round	No	Entire	Transparent	Nil	Positive
CG9	Small	Round	No	Entire	Opaque	Nil	Positive
CG10	Small	Round	Slightly	Rough	Transparent	Nil	Negative
CG11	Large	Irregular	No	Rough	Opaque	Nil	Positive
CG12	Small	Round	Slightly	Entire	Transparent	Nil	Positive
CG13	Small	Round	No	Entire	Opaque	Nil	Positive
CG14	Medium	Round	No	Rough	Opaque	Nil	Negative
CG15	Small	Round	No	Entire	Opaque	Yellow	Positive
CG16	Small	Round	No	Entire	Transparent	Nil	Negative
CG17	Medium	Round	No	Entire	Opaque	Nil	Positive
CG18	Small	Irregular	Slightly	Rough	Transparent	Nil	Positive
CG19	Small	Round	No	Entire	Opaque	Nil	Positive
CG20	Large	Round	No	Entire	Transparent	Nil	Positive
CG21	Small	Round	No	Rough	Opaque	Nil	Negative
CG22	Large	Irregular	Slightly	Rough	Transparent	Yellow	Positive

Plant growth promoting activities of PGPR isolates

Direct as well as indirect PGPR activities like Siderophore production, Phosphorus solubilization, IAA production, HCN Production, Protease production and Chitinase production of all 22 PGPR traits is described in Table-3.

Table-3. List of plant growth promoting activities (Production of Siderophore, IAA, HCN, Protease, Chitinase and Phosphorus solubilization) of all 22 PGPR isolates.

Isolates	Siderophore production	Phosphorus solubilization	IAA Production	HCN Production	Protease activity	Chitinase activity
CG1	Negative	Not Solubilize	+	-	+	+
CG2	Negative	Not Solubilize	-	-	-	-
CG3	Positive	Solubilize	++	-	+	+
CG4	Positive	Not Solubilize	-	-	+	-
CG5	Negative	Not Solubilize	++	+	-	+
CG6	Positive	Not Solubilize	++	-	+	-
CG7	Positive	Solubilize	+	-	-	-
CG8	Negative	Not Solubilize	-	-	-	-
CG9	Positive	Not Solubilize	++	+	+	+
CG10	Negative	Not Solubilize	-	-	+	-
CG11	Positive	Solubilize	+++	+	+	+
CG12	Negative	Not Solubilize	-	-	-	-
CG13	Negative	Not Solubilize	+	-	-	+
CG14	Positive	Not Solubilize	++	-	+	-
CG15	Positive	Solubilize	+	-	-	+
CG16	Negative	Not Solubilize	-	-	-	-
CG17	Positive	Solubilize	+	-	-	-
CG18	Negative	Not Solubilize	+++	+	+	+
CG19	Negative	Not Solubilize	-	-	-	-
CG20	Negative	Not Solubilize	-	-	-	-
CG21	Positive	Solubilize	+	-	+	+
CG22	Negative	Not Solubilize	-	-	-	-

Siderophore assay

Siderophores are small compounds produced by microorganisms, which are strongest Fe^{3+} binding agents and higher capacity to chelate iron. In respiration, synthesis of DNA and in other processes iron plays an important role. However, organisms cannot utilize iron directly because, in soil iron is present in the form of oxides and hydroxides therefore its bioavailability is very low. However, PGPR release siderophores, which can chelate iron from these phases by formation of soluble Fe^{3+}. On the other hand, growth of pathogenic organisms will be suppressed due to the low iron availability because siderophore have higher affinity towards the iron [21]. CAS medium is most commonly and widely used for siderophore assay. This assay is colour-based assay. Blue colour of the medium is due to the Chrome Azurol S dye. When the iron removes from the medium, dye changes the colour to orange from blue. In the absence of siderophore, the plates remain blue in colour. This assay is used for both qualitative as well as quantitative estimation of siderophore present in to the samples. Out of 22 isolates, ten isolates have shown the ability for siderophore production on CAS medium with different efficacy. Siderophore production of all isolates is described in Table - 3.

Phosphate solubilization

Phosphorus plays an important role in the growth of plant and is one of the major nutrients, second only to nitrogen in requisite for plants. However, in soil large amount of phosphorus is present in the form of insoluble phosphates, therefore, cannot be utilized by plants [22, 23]. Phosphate solubilizing bacteria convert this insoluble phosphate in to soluble form by producing organic acid. The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists. Availability of phosphorus greatly enhanced the production of rice in comparison to non-rhizospheric soil [23]. Out of 22 isolates, 6 isolates exerted ability for phosphate solubilization on Pikovskya's medium with different efficacy. Out of 6, 3 strains showed maximum degree of phosphate solubilization. The maximum phosphate solubilization was identified in CG11 strain. The phosphate solubilizing activity characterizes the microorganisms with ability to produce and release metabolites such as organic acids that chelate the cations

bound to phosphate, converting them into soluble forms [16].

IAA Production

Indole-3-acetic acid is a naturally occurring plant hormone of the auxin class, which is the most investigated hormone among plant growth regulators. However, IAA carried out growth and development of plant by inducing cell division and elongation. In the processes of organogenesis, IAA also serves as a signalling molecule and is by far the most common as well as the most studied auxin [24, 25]. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability [26]. Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil [27, 28]. Out of 22 isolates, 13 isolates were able to produce indole-3-acetic acid growing in medium (Table 3). In our study, maximum IAA production was recorded in CG11 and CG18 strain as compared to other isolates.

HCN Production

HCN is an organic compound and identified as a major component of antibiotics produced by the Gram negative bacteria which is suppressor of weeds [28] and insects [29]. *Meloydogyne incognita* [30], Carabid beetles such as *Harpalus pensylvanicus* are common in many crop fields, which destroys large numbers of weed seeds. However, certain rhizobacteria are noted as active against weeds [31]. In this study, out of 22 isolates, only 4 (CG5, CG9, CG11 and CG18) were able to produce HCN in the rhizosphere soil (Table 3).

Extra cellular enzyme (Protease and Chitinase) activities

Extracellular protease and chitinase producing bacteria inhibit the growth of phyto-pathogenic organisms and protect plants from various kinds of diseases [32, 33]. In the present study 10 out of 22 (CG1, CG3, CG4, CG6, CG9, CG10, CG11, CG14, CG18, and CG21) and 9 out of 22 (CG1, CG3, CG5, CG9, CG11, CG13, CG15, CG18 and CG21) isolates were shown positive for protease and chitinase production (Table 3).

CONCLUSIONS

PGPR helps in plant growth by variety of mechanisms but exact mechanism by which they stimulate growth is not clearly known. Although, there are several mechanisms such as, production of siderophore, phosphate solubilization, phyto-hormones production, suppression of pathogenic organisms etc. It can be concluded from the above discussion that PGPR enhance the plant growth. Collectively, our results indicated that the use of PGPR isolates CG11 and CG21, which are most potent strains in our finding which carry out production of IAA, Phosphate solubilization, Siderophore production, HCN production, Protease and Chitinase production. These two potent strains may be used in the rice fields as a biofertilizers. Simultaneous screening of PGPR from various paddy fields is a good tool to select effective PGPR for biofertilizers development technology.

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