JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1641-1649

# Isolation and Characterization of Anti Pathogenic Plant Growth Promoting Bacteria from Cotton Rhizosphere

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(Received: 09 January 2015; accepted: 01 March 2015)

**Sustainable farming systems strive to minimize the use of synthetic pesticides and to optimize the use of alternative management strategies to control soil borne pathogens. During the last decade numerous bio control experiments with bacteria have been reported. But when such bacterial formulation was tried on field, it was not able to give as nice response as its giving in lab. Therefore, it is necessary to develop efficient strains in field conditions. One possible approach is to explore soil microbial diversity for PGPR having combination of PGP activities and well adapted to particular soil environment. So keeping in view the above constrains, the present study was designed to screen certain rhizospheric bacterial isolates for their multiple plant growth promoting activities. In this research twelve bacterial isolates were successfully isolated from cotton rhizosphere which possess antagonistic activity against to main soil fungal pathogen of cotton as well as also shows plant growth promoting activities. All the potent isolates were characterized phenotypically and biochemically. One of them were also characterized molecularly by 16s r RNA sequencing and found to be novel. The selected isolates are antagonistic to *Rhizoctonia solani* and *Fusarium oxysporum* and could be developed into a valuable crop management tool to reduce the deleterious impact of plant pathogen**.

**Key words:** Plant Growth Promoting Rhizospheric Bacteria (PGPR), Bio control, Phenotypic characterization, Biochemical characterization, Molecular characterization.

Cotton is a soft, staple fibre that grows in a form known as a boll around the seeds of the cotton plant. India is the second largest producer of the cotton in the world after China. It is a cash crop and the economy of many farmers depends on this. The Textile industry of the India is one of the leading industries in the world. The Indian government earns a lot of foreign exchange through this and it has provided the jobs to millions of the people. As per Economic Survey of India, about 16% of total world production of cotton comes from India[1]

Cotton is also one of the most important crops in Gujarat and forms the major source for textile industry in India. Production is, however, low because of diseases caused by pathogenic fungi that reduce the quality and quantity of cotton production. Among these fungi, a number of soil borne pathogens seriously affects the cotton. Of these, *Fusarium oxysporum* and *Rhizoctonia solani* are now the most important fungal pathogen causing diseases generically called ‘‘cotton wilt”. The fungus can kill or damage the plant in a number of ways. Aerial infection in seedlings is usually lethal because the young plants are unable to photosynthesize. Aerial infection in mature plants reduces photosynthesis due to lesions on the leaf and reduction of photosynthetic potential by the fungus greatly reduces yield. Infections of root and vascular tissues have the potential to kill the plant by cutting off the supply of water and nutrients to the root[2]

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There for Cotton requires a great amount of chemical fertilizer and pesticides for good growth and yield and also to save itself from the diseases. PGPR inoculation is a promising agricultural approach that plays a vital role in crop protection, growth promotion or biological disease control and sustained soil fertility[1]

There are sound reasons for evaluating indigenous rhizosphere bacteria as possible pest control agents since naturally occurring strains can be found that are competitive and persistent under harsh environmental conditions, including moisture or temperature extremes, soil acidity, and salt tolerance. There are numerous reports of rhizobacteria that exert a beneficial effect on plant growth[3]

Bacterial diversity is of particular importance in human sustenance since these small creatures comprise the majority of earth's species diversity. Bacterial diversity is considered as one of the most useful resource with considerable significance in the global form of bioremediation and bio-prospecting. Interaction between bacteria and roots of plants has been reported to be beneficial, detrimental or neutral and this delicate balance is a consequence of both soil and plant type. Bacteria, beneficial to plants may be symbiotic or free living, and are abundant near the roots. Such beneficial free-living bacteria have been termed PGPR or plant growth promotory rhizobacteria[4]

The rhizosphere is a hot spot of microbial interactions as exudates released by plant roots are the main food source for microorganisms and a driving force for their population density and geochemical cycling of nutrients. Screening and selection of effective PGPR and their utilization in integrated practices is of great importance for enhancing the growth and yield of agricultural crops with maintaining sustainability of agro-ecosystems. Plant growth promoting rhizobacteria (PGPR) are group of bacteria that actively colonize plant roots region and increase plant growth and yield [1]. Various species of bacteria like *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance the plant growth. PGPR enhance plant growth either by direct or indirect mechanisms. The direct growth promoting mechanisms involve nitrogen fixation, solubilization of minerals, production of phytohormones and the indirect approach occurs when PGPR lessen or prevent the deleterious effects of plant pathogens on plants[5–12]

The goals of this study were to enumerate and characterize the major heterotrophic bacterial genera that inhabit the cotton rhizosphere over one growing season and to examine a collection of potential PGPR isolates for fungal repression activity[3]

## MATERIALS AND METHODS

### Soil sampling and analysis

Different soil samples were collected from districts (Rajkot, Jamnagar, Junagadh, Bhavnagar, Porbandar, Surendranagar, Amreli) of Saurashtra region of Gujarat at latitude 21.2 and longitude 72.8. Soils sample were analyzed for moisture holding capacity, pH and macro as well as micro minerals content as describe in Table-1[12]

### Isolation of Rhizobacteria from Various Sources

Bacterial strains were isolated from cotton rhizospheric soil samples collected from different district of Saurashtra Region. A composite sample of 10 g rhizosphere soil (root adhering soil) was carried to the laboratory in an ice-box. Isolation of rhizobacteria was completed within 48 h of sample collection. Loosely adhering soil was removed from the roots by washing with sterile distilled water. Serial dilutions of soil samples were spread on Nutrient agar. Following growth, bacterial colonies were purified and preserved on nutrient agar slants for further study[14-15]

### Screening of bacteria for plant growth enhancement

Cotton (*Gossypium hirustum*) seeds were surface sterilized with 0.1% mercuric chloride for 5 min, rinsed with sterilized distilled water and soaked in bacterial suspension ($3\times10^8$ cfu $ml^{-1}$) using 1% carboxymethyl cellulose (CMC). Air dried seeds were placed on a paper towel (ten seeds per paper) and incubated at $28\pm2^0$C for 4 days in a growth chamber. Percentage germination was recorded along with root and shoot length. Non-bacterized seeds served as control. Three replicates were used for this experiment[15]

**Preliminary screening of PGPR isolates for anti-pathogenic activity**

Fifty two plant growth promoting isolates out of two hundred eleven bacterial isolates were tested for their ability to produce antifungal substances against *Fusarium oxysporum* and *Rhizoctonia solan*i using a dual-culture in vitro assay on PDA plates. Five µl of each bacterial suspension ($10^8$ cfu/ml) was placed on the plate. After 48 h incubation at 28°C, a single 6 mm diameter mycelial disc was placed at the centre of plates. Then plates were incubated at 27-29 °C in darkness and after 7 days the zone of inhibition was measured. This experiment was conducted twice. Bacteria with inhibitory potential were selected for further experiments[16,17]

**Phenotypic and biochemical characterization of potent isolates**

Morphological features and biochemical characteristics of the potent isolates were studied. The colonies (shape, size, colour, contour, etc.) were studied on N-agar plates after 5 days of incubation at 28 $^0$C. Cells were examined for morphology using Gram-stained smears and phase-contrast microscopy. Biochemical characterization including motility, sugar fermentation (cellobiose, raffinose, mannose, galactose, fructose, arabinose, melibiose, adonitol, glucose, xylose), sodium citrate utilization, urease production, nitrate reduction starch hydrolysis were performed. Anaerobic growth was determined in tubes of oxidative and fermentative test medium (Difco) under mineral oil. Casein hydrolysis was detected after incubation of strains for 3 days on nutrient agar supplemented with 2% skimmed milk. Determinations of catalase and oxidase activities were performed using disc of Highmedia kit. Growth at different temperatures was determined by inoculating on nutrient agar plate for 3 days in water baths set at different temperature[18]

**Effect of environmental conditions on bacterial growth**

There are many parameters which affect growth of bacterial isolates viz. temperature, pH, aeration, salt concentration, nutrient availability, radiation, presence of heavy metals, carbon source, etc. Out of these, some important parameters viz. pH, temperature, salt concentration affecting bacterial growth was considered[19]

**16S r RNA gene amplification and sequencing.**

Total DNA from isolated strains was extracted using the method of Cullen and Hirsch (1998). DNA quality was estimated by electrophoresis in 1% agarose gels in 1× TAE buffer (40 mM Tris, pH 8.3; 20 mM acetic acid; 1 mM EDTA) and stained with 0.5 gm L"[1] ethidium bromide solution (ETB). DNA concentrations were determined spectrophotometrically and a A260/ A280 ratio of 1.8 was considered to be acceptable[14]

Isolated microorganisms were identified by the similarity and phylogenetic analysis of the 16S rRNA gene partial sequence. The amplification of 16S rRNA genes was performed with a PCR System 9700 (Applied Biosystems). Reaction mixtures contained 5 ng of template DNA, 1X reaction buffer, 25 mM $MgCl_2$, 0.25 mM of each dNTP, 10 pM of each primer, and 1 U of Taq Polymerase, adjusted to 25 µL. Ribosomal 16S rRNA genes were amplified using the universal bacterial primer 8 (5'-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG-3') forward and 1492 (5'-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3') reverse. The following PCR conditions were used: an initial denaturation step at 94$^0$C for 5 min; 35 cycles of 45 s at 94$^0$C, 45 s at 55$^0$C, and 90 s at 72$^0$C; and a final extension step at 72$^0$C for 10 min. The PCR products were electrophoresed and stained as mentioned above. PCR products corresponding to the 16S rRNA genes were purified using the PCR purification kit according to the manufacturer's instructions (Qiagen Inc)[14]

DNA sequencing was performed with an ABI 3130 XL DNA sequencer (Applied Bio systems). Sequences were aligned, and a consensus sequence was computed with fragment assembly tools in the Genetics Computer Group software package (MEGA 5). Nucleotide sequence similarities were determined by using BLAST (National centre for Biotechnology Information databases)[20]

## RESULTS AND DISCUSSION

**Soil Analysis**

All the soil sample collected from different location of Saurashtra region were analysed for below describe parameters. Result of one of the soil sample collected from Junagadh district (J-1) were describe in the table-2.

**Isolation of rhizospheric bacteria**

211 bacterial colonies with different growth characteristics were isolated and purified by further streaking on nutrient agar media and pure cultures were used for further experiments. The criteria taken in to the consideration were Pigmentation, Elevation, Texture and size. Such criteria for all the selected bacterial colonies were note down.

**Table 1.** Parameters and Method used for soil analysis

| Parameter | Method |
|---|---|
| PH | $P^H$ Meter |
| E C | EC Meter |
| Available C | Walkey and Black Method |
| Available N | Kjeldhal Method |
| Available P2O5 | Olsen's extracted Mehod |
| Available K2O | Flame Photometry Method |
| Sand | International Pippet method (Piper 1950) |
| Slit | |
| Clay | |
| Soil type | By observation |
| Water holding capacity (Moisture) | Balance |
| Zn | DTPA extractor (Instrument-AAS) |
| Fe | |
| Cu | |
| Mn | |
| B | |

**Screening of isolates for plant growth promotion (PGP)**

52 isolates out of 211 primary isolates improve plant health and promote growth by increasing of seedling emergence and the vigour when tested in vitro for cotton germination.

All 52 PGPR isolates were selected on the basis of their performance in seed germination assay, significantly enhanced the seedling length. Some of the isolates also showed a significant increase in % germination as compared to untreated control. Seeds coated with all bacterial isolates showed 100% germination. Seeds coated with bacterial isolates V-3 showed highest seedling length and seedling vigour after 4 days of germination as shown in table-3. In this study, seed treatment with the bacterial isolates significantly improved seed emergence together with plant root and shoot length as shown in figure-1.

The overall improvement in seedling vigour through a significant increase in various physiological parameters suggests that these strains have a plant-growth promoting ability on cotton seedlings and hence could be used for seed inoculation for better establishment of seedlings. The plants with enhanced seedling vigour can help in better establishment of plantations.

**Screening for antagonism**

Isolated rhizobia showing plant growth promotion in germination assay were studied for antagonistic activity against the fungal pathogen.

**Table 2.** Result of soil analysis for soil sample J-1

| Parameter | Value | Method |
|---|---|---|
| PH | 7.02 | PH Meter |
| E C | 0.60 dSm-1 | EC Meter |
| Available C | 0.093 % | Walkey and Black |
| Available N | 270 Kg/Hector | Kjeldhal |
| Available P2O5 | 51 Kg/Hector | Olsen's extracted |
| Available K2O | 290 Kg/Hector | Flame Photometry |
| Sand | 22 % | International Pippetmethod (Piper 1950) |
| Slit | 23 % | |
| Clay | 55 % | |
| Soil type | Medium black calcareous | By observation |
| Water holding capacity | 45 % | Balance |
| Zn | 1.47 ppm | DTPA extractor(Instrument-AAS) |
| Fe | 13.7 ppm | |
| Cu | 3.24 ppm | |
| Mn | 10.01 ppm | |
| B | 0.86 ppm | |

**Table 3.** Showing result for different parameters of Germination Assay

| Isolates | Seed Germination | Total length | | | Average length | Seedling vigour index |
|---|---|---|---|---|---|---|
| Control | 80% | 3.8 | 4.2 | 4.5 | 4.1 | 410 |
| A-1 | 100% | 11 | 10.8 | 11.5 | 11.1 | 1110 |
| A-2 | 100% | 13 | 12.5 | 13 | 12.8 | 1280 |
| A-3 | 100% | 9.1 | 7.2 | 8.8 | 8.3 | 830 |
| A-4 | 100% | 9 | 7.7 | 9 | 8.5 | 850 |
| A-5 | 100% | 8 | 11 | 8 | 9.0 | 900 |
| A-6 | 100% | 14.2 | 11.5 | 11 | 12.2 | 1220 |
| A-7 | 100% | 10.2 | 10.4 | 11 | 10.5 | 1050 |
| K-1 | 100% | 12 | 11.5 | 12.4 | 11.9 | 1190 |
| K-2 | 100% | 8.2 | 8.5 | 11 | 9.2 | 920 |
| K-3 | 100% | 11 | 10.5 | 10.9 | 10.8 | 1080 |
| K-4 | 100% | 11.8 | 8.2 | 12.4 | 10.8 | 1080 |
| K-5 | 100% | 12 | 11.5 | 12.1 | 11.8 | 1180 |
| K-6 | 100% | 8.2 | 10 | 8.6 | 8.9 | 890 |
| S-1 | 100% | 12 | 13 | 12.1 | 12.3 | 1230 |
| S-2 | 100% | 9.1 | 10 | 9.4 | 9.5 | 950 |
| S-3 | 100% | 7.5 | 8 | 9.1 | 8.2 | 820 |
| S-4 | 100% | 9.9 | 7.9 | 8.5 | 8.7 | 870 |
| S-5 | 100% | 10 | 10.4 | 12 | 10.8 | 1080 |
| S-6 | 100% | 11.2 | 13 | 11.5 | 11.9 | 1190 |
| P-1 | 100% | 9.1 | 9.7 | 13 | 10.6 | 1060 |
| P-2 | 100% | 8.9 | 10.6 | 10.2 | 9.9 | 990 |
| P-3 | 100% | 10.5 | 8.5 | 8.5 | 9.1 | 910 |
| P-4 | 100% | 8.2 | 9.5 | 9.4 | 9.0 | 900 |
| P-5 | 100% | 13 | 12.8 | 16 | 13.9 | 1390 |
| P-6 | 100% | 14 | 12 | 11.5 | 12.5 | 1250 |
| P-7 | 100% | 8.7 | 9.2 | 12.3 | 10.0 | 1000 |
| J-1 | 100% | 16 | 10.8 | 14 | 13.6 | 1360 |
| J-2 | 100% | 12.3 | 12.5 | 13.3 | 12.7 | 1270 |
| J-3 | 100% | 15.1 | 16.2 | 14.9 | 15.4 | 1540 |
| J-4 | 100% | 14 | 15.8 | 15.9 | 15.2 | 1520 |
| J-5 | 100% | 10.2 | 10.7 | 9.3 | 10.0 | 1000 |
| J-6 | 100% | 12 | 11.8 | 9.6 | 11.1 | 1110 |
| U-1 | 100% | 10 | 14.4 | 15 | 13.1 | 1310 |
| U-2 | 100% | 15.1 | 13.4 | 12.2 | 13.5 | 1350 |
| U-3 | 100% | 10.1 | 12.2 | 12 | 11.4 | 1140 |
| U-4 | 100% | 8.2 | 9.1 | 10.1 | 9.1 | 910 |
| U-5 | 100% | 14.3 | 14.9 | 16 | 15.0 | 1500 |
| U-6 | 100% | 15.8 | 11.8 | 14.3 | 13.9 | 1390 |
| U-7 | 100% | 7.8 | 9.2 | 9.8 | 8.9 | 890 |
| .V-1 | 100% | 10.5 | 9 | 9.5 | 9.6 | 960 |
| V-2 | 100% | 10.2 | 14.9 | 16.1 | 13.7 | 1370 |
| V-3 | 100% | 17.1 | 15.6 | 16.1 | 16.2 | 1620 |
| V-4 | 100% | 12.6 | 13 | 14.2 | 13.2 | 1320 |
| V-5 | 100% | 8.2 | 10.9 | 9.1 | 9.4 | 940 |
| V-6 | 100% | 13.1 | 10.2 | 10.4 | 11.2 | 1120 |
| B-1 | 100% | 10.2 | 10.6 | 13 | 11.2 | 1120 |
| B-2 | 100% | 12 | 13.3 | 14 | 13.1 | 1310 |
| B-3 | 100% | 6.4 | 7.8 | 10.2 | 8.1 | 810 |
| B-4 | 100% | 9.1 | 12 | 8.8 | 9.9 | 990 |
| B-5 | 100% | 7.2 | 6.8 | 6 | 6.6 | 660 |
| B-6 | 100% | 9.1 | 8.9 | 9 | 9 | 900 |
| B-7 | 100% | 6.2 | 6 | 7.2 | 6.4 | 640 |

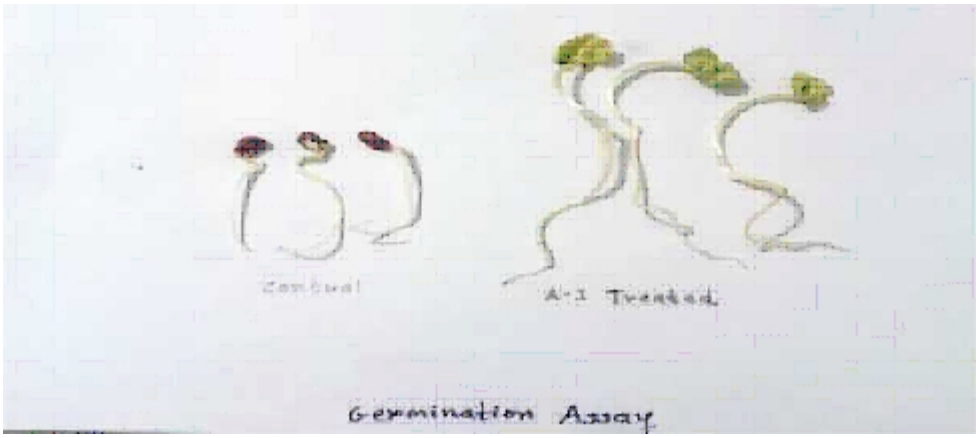


**Fig. 1.** Photo of seed germination assay after 4 days of incubation

**Table 4.** Results for antagonism against both fungal plant pathogens

| Isolates | Diameter of inhibition zones($mm^2$) Against F.oxysporum | Against R.solani |
|---|---|---|
| A-2 | 10.0 | 11.2 |
| A-3 | 14.0 | 18.2 |
| A-4 | 16.0 | 15.2 |
| K-1 | 12.5 | 11.1 |
| K-2 | 8.2 | 8.9 |
| K-4 | 14.1 | 12.0 |
| S-1 | 6.6 | 9.1 |
| P-3 | 8.8 | 20.1 |
| J-1 | 6.1 | 9.2 |
| U-1 | 5.2 | 4.2 |
| V-1 | 9.0 | 16.1 |
| B-1 | 11.0 | 16.0 |

Twelve out of 52 bacterial isolates had shown high antifungal activity against both pathogens, were selected and used in the subsequent in vitro inhibition tests and field experiments.

Antagonistic activity of the bacterial isolates was evaluated in terms of inhibition zone diameter as an indicator of the reduction in growth

**Fig. 2.** Isolates A-4 and P-3 Showing Antagonistic activity against cotton plant pathogen *R. solani*, *F. oxysporum* respectively

**Table 5.** Colony characteristics of bacterial isolates on nutrient agar plate

| Isolates | Size | Shape | Character Elevation | Colour | Consistency | Opacity |
|---|---|---|---|---|---|---|
| A-2 | 2mm | Circular | Flat | Whitish | Moist | Translucent |
| A-3 | 3mm | Circular | Convex | Krimish | Moist | Opaque |
| A-4 | 1mm | Circular | Flat | Yellowish | Moist | Opaque |
| K-1 | 5mm | Irregular | Convex | Greenish | Mucoid | Translucent |
| K-2 | 8mm | Irregular | Convex | Greenish | Mucoid | Translucent |
| K-4 | 2mm | Circular | Flat | Yellowish | Moist | Opaque |
| S-1 | 7mm | Circular | Convex | Whitish | Moist | Translucent |
| P-3 | 5mm | Irregular | Convex | Whitish | Mucoid | Translucent |
| J-1 | 5mm | Circular | Convex | Whitish | Dry | Opaque |
| U-1 | 6mm | Circular | Convex | Whitish | Dry | Opaque |
| V-1 | 8mm | Circular | Convex | Whitish | Viscous | Opaque |
| B-1 | 3mm | Irregular | Convex | Whitish | Moist | Opaque |

of pathogenic fungi. The maximum zone of inhibition was observed in isolate P-3 against *Fusarium oxysporum* and isolate A-4 against *Rhizoctonia solani* as shown in table-4 and figure-2.

Overall some bacterial isolates were found to be potent inhibitors of the test fungi whereas others showed only mild activity or no activity. This suggests that the mode of action exerted and/or the type of antifungal metabolite produced by the isolates may vary.

The antagonistic activity of all these isolates is may be due to inhibitory compound, siderophores production, cell-wall degrading

**Table 6.** Result of biochemical characterization of all the isolate

| Characteristics | K-1 | K-2 | K-4 | A-2 | A-3 | A-4 | P-3 | J-1 | U-1 | S-1 | V-1 | B-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Anaerobic growth | - | - | - | - | - | - | - | - | - | - | - | - |
| Oxidase | + | + | + | + | + | + | + | + | + | + | + | + |
| Maximum growth temp | 45 | 40 | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 |
| Minimum growth temp | 20 | 20 | 15 | 18 | 20 | 18 | 18 | 20 | 20 | 15 | 15 | 20 |
| Nitrate reduction | + | + | + | + | + | + | + | + | + | - | + | + |
| Catalase | + | + | + | + | + | + | + | + | + | + | + | + |
| Arabinose | - | - | + | + | + | + | - | + | + | + | + | + |
| Adenittol | - | + | + | - | - | - | - | - | + | - | + | - |
| Cellobiose | - | - | + | + | + | + | - | + | + | + | + | + |
| Galactose | + | - | + | + | + | + | + | + | + | + | - | + |
| Fructose | + | + | + | + | + | + | + | + | + | + | + | + |
| Mannose | + | - | + | + | + | + | - | + | + | + | + | + |
| Melibiose | - | - | - | - | + | + | + | + | + | + | + | + |
| Raffinose | - | - | - | + | - | + | + | + | + | + | + | + |
| Xylose | - | - | + | - | + | + | - | + | + | + | + | + |
| Sucrose | + | + | + | + | + | + | + | + | + | + | + | + |
| Citrate Utilization | + | + | - | - | - | - | + | + | - | - | - | - |
| Urease | - | - | - | - | - | - | - | - | - | - | - | - |
| H2S Production | - | - | - | + | - | + | - | - | - | + | - | - |
| Acid Production | + | + | + | + | + | + | + | + | + | + | + | + |
| Gas Production | - | - | - | - | - | - | - | - | - | - | - | - |
| Starch Hydrolysis | - | - | - | - | - | - | - | + | + | + | + | + |
| Motility | - | - | - | - | + | - | - | + | + | - | - | - |

**Table 7.** Results for effect of environmental condition on growth of isolates

| | Experimental condition | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature | | | | pH | | | | | | | Salt | | | | | |
| | $15^0$C | $25^0$C | $35^0$C | $45^0$C | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 1% | 2% | 3% | 4% | 5% | 6% |
| K-1 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - |
| K-2 | - | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | - |
| K-4 | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| A-2 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| A-3 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| A-4 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| P-3 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | - |
| J-1 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| U-1 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| S-1 | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| V-1 | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |
| B-1 | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + |

enzymes, hydrogen cyanine production, ammonia and antibiotics production.

**Phenotypic and bio chemical characterization**

The distinguishing phenotypic and biochenmical characters of all the isolates were shown in table-5 and table-6. Morphological and phenotypic characterization revealed that out to twelve microbial isolates eleven were found to be bacterial and one were actinomycete. From eleven bacterial isolates, five were gram positive, and six were gram negative. Biochemical characterization shown that out of twelve isolates three isolates belongs to *Pseudomonas* spp., four isolates belongs to *Bacillus* spp., remaining five belongs to each one of *Azotobacter* spp., *Azospirillum spp.*, *Agromyces spp.*, *Burkholderia* spp. *Guconobacter* spp., which was need to further characterize by 16s r RNA sequencing.

**Effect of environmental condition on bacterial growth**

All the isolates showed ability to grow at wide range of temperature, pH and NaCl concentration as shown in table-7. The effect of temperature on bacterial growth was checked with temperature range of $15^0$C to $45^0$C. Maximum growth was observed at $35\pm2^0$C temperature. The effect of pH on bacterial growth was observed in the pH range of 4 to 10 from which the maximum growth was seen at pH 7. All isolates were unable to grow at pH 4. The optimum NaCl concentration for all six isolates was found to be 1 %. All isolates except K-1, K-2 and P-3 were grown up to 6 % NaCl concentration. This results show ability of this isolates to grown up in saline soil.

**Molecular characterization**

The nucleotide sequence of nearly full length (1,300 bp approximately) 16S r RNA gene from isolates K-1 was determined and compared to sequences available in NCBI data bank remaining isolates are under sequencing process so their data of molecular characterization were not shown here. Result of BLAST was shown close similarity with *Pseudomonas putida* (Accession No. KF640237). And the similarity found were 95% which confers finding of novel strain, needs to be further characterize. Data of sequenced isolates strains have been deposited under the following accession number KM204146 to the NCBI.

## CONCLUSION

This study illustrates the significance of screening of antagonistic rhizobacteria containing multiple PGPR traits. This can lead to the selection of effective PGPR isolates which can be used as a bio control agent and as a result of their multiple PGPR traits can prove to be effective in improving the productivity of cotton crop and maintenance of soil fertility. As this isolates were isolated from rhizosphere assures their long survival of it in to field level, which were not found in case of other biofertilizers which do not associates with root.

## ACKNOWLEDGMENTS

Authors are thankful to the Head, Department of Biotechnology, Junagadh Agricultural University, Junagadh, India for providing laboratory facilities and valuable guidance. Authors are also thankful to Solar Agrotech Pvt. Ltd., Rajkot, India for providing seeds of cotton required in this research.

## REFERENCES

1. B. S. Saharan and V. Nehra, "Assessment Of Plant Growth Promoting Attributes Of Cotton ( Gossypium Hirsutum ) Rhizosphere Isolates And Their Potential As Bio-Inoculants," *Journal of Environmental Research And Development*, 2011; **5**(3), pp. 575–583.
2. M. Chaiharn and Æ. S. Chunhaleuchanon, "Screening siderophore producing bacteria as potential biological control agent for fungal rice pathogens in Thailand," *World J Microbiol Biotechnol*, 2009; pp. 1919–1928.
3. C. Hagedorn, W. D. Gould, and T. R. Bardinelli, "Rhizobacteria of Cotton and Their Repression of Seedling Disease Pathogens," *Applied And Environmental Microbiology*, 1989; **55**(11), pp. 2793–2797.
4. A. P. Kumar, A. Shruti, S. K. Singh, S. Kumar, and S. K. Prashad, "Characterization Of Plant Growth Promoting Bacteria From Soil Of Central And Upper Himalayan Region", *International Journal of Applied Biology and Pharmaceutical Technology*, 2011; **1**: pp. 363–369.
5. A. Kumar, A. Kumar, S. Devi, S. Patil, C. Payal, and S. Negi, "Isolation , screening and characterization of bacteria from Rhizospheric

soils for different plant growth promotion ( PGP ) activities: an in vitro study," *Recent Research in Science and Technology*, 2012; **4**(1), pp. 1–5.

6. D. Egamberdieva, "Plant Growth Promoting Properties of Rhizobacteria Isolated from Wheat and Pea Grown in Loamy Sand Soil," *Turk J Biol*, 2008; : pp. 9–15.
7. A. T. Wahyudi and R. I. Astuti, "Screening of Pseudomonas sp . Isolated from Rhizosphere of Soybean Plant as Plant Growth Promoter and Biocontrol Agent," *American Journal of Agricultural and Biological Sciences*, 2011; **6**(1), pp. 134–141.
8. S. Z. Ali, V. Sandhya, and L. V. Rao, "Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescentPseudomonas sp .," *Annals of Microbiology*, 2013; pp. 1–22.
9. B. Mohite, "Isolation and characterization of indole acetic acid ( IAA ) producing bacteria from rhizospheric soil and its effect on plant growth," *Journal of Soil Science and Plant Nutrition*, 2013; **13**(3), pp. 638–649.
10. P. K. Agrawal and S. Agrawal, "Original Research Article Characterization of Bacillus sp . strains isolated from rhizosphere of tomato plants ( Lycopersicon esculentum ) for their use as potential plant growth promoting rhizobacteria," *Int.J.Curr.Microbiol.App.Sci*, 2013; **2**(10), pp. 406–417.
11. S. R. Naphade, A. A. Durve, M. Bhot, J. Varghese, N. Chandra, and D. Thane, "Isolation , characterization and identification of pesticide tolerating bacteria from garden soil," *European Journal of Experimental Biology*, 2012; **2**(5), pp. 1943–1951.
12. M. Madhaiyan, S. Poonguzhali, J. Lee, M. Senthilkumar, K. C. Lee, and S. Sundaram, "plant-growth-promoting bacteria isolated from cotton rhizosphere soils," *International Journal of Systematic and Evolutionary Microbiology*, 2010; pp. 2451–2457.
13. J. I. Qazi, N. Qaiser, and A. S. Bano, "Isolation of antifungal bacteria from soil samples,"*Mycopathology*, 2009; **7**: pp. 5–10.
14. Y. E. Navarro-Noya, E. Hernández-Mendoza, J. Morales-Jiménez, J. Jan-Roblero, E. Martínez-Romero, and C. Hernández-Rodríguez, "Isolation and characterization of nitrogen fixing heterotrophic bacteria from the rhizosphere of pioneer plants growing on mine tailings," *Applied Soil Ecology*, 2012; **62**: pp. 52–60.
15. B. Joseph, R. R. Patra, and R. Lawrence, "Characterization of plant growth promoting rhizobacteria associated with chickpea ( Cicer arietinum L .)," *International Journal of Plant Production*, 2007; **1**.
16. K. Karimil, J. Aminil, and B. Harighil, "*Australian Journal of Crop Science*," 2012; **6**(4), pp. 695–703.
17. A. R. Fathilah, "Determination of the percentage inhibition of diameter growth ( PIDG ) of Piper betle crude aqueous extract against oral Candida species," *African Journal of Microbiology Research*, 2011; **5**(6), pp. 878–884.
18. O. N. Reva, V. V Smirnov, B. Pettersson, and F. G. Priest, "Bacillus endophyticus sp . nov ., isolated from the inner tissues of cotton plants ( Gossypium sp .)," *International Journal of Systematic and Evolutionary Microbiology*, 2002; pp. 101–107.
19. D. Arroyo and J. Scellekens, "Characterization of blue pigmented bacteria isolated from Puerto Rico,"*Current Research*, 2010; pp. 117–123.
20. R. M. Dhingani, M. V Parakhia, R. S. Tomar, and B. J. Malviya, "Functional characterization of PGPR and its identification through 16 S rRNA sequencing," *Indian Journal Of Applied Research*, 2013; **3**: pp. 47–50.