RESEARCH ARTICLE

ISOLATION AND MOLECULAR CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA FROM THE HIGH ALTITUDE HIMALAYAN REGION OF UTTARAKHAND

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Abstract: The objective of this study was to isolate and characterize a rhizospheric bacterium from Munsyari, (2200 feet, 30.06°N/80.23° E) Uttarakhand, western Himalayas, (India). Plant growth promoting rhizobacteria (PGPR) are known to influence plant growth by various direct or indirect mechanisms. Isolated strain was tested for various PGP traits like 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, phosphate solubilisation, indole acetic acid production, production of siderophore, carbohydrate utilization test. Bio-control ability of isolate was also screened. Further identification of isolate was performed by PCR based 16S rRNA gene sequencing. The isolate PS03 was found to be most effective.

Keywords: Rhizobacteria, Isolation, Molecular, Himalaya region

INTRODUCTION

World population is expected to be 9.7 billion by the year 2050 and to feed this population we need to grow 60% more food (United nation Population Division 2015).This demand can met by increasing the input of chemical fertilizers and increasing the land area under cultivation. But the area under agriculture is already under the urbanization pressure. However, the prices and availability of these chemical fertilizers become the limiting factor for crop production especially in developing countries around the world. Continuous application of Chemical fertilizers may result in negative impacts on agro-ecosystem such as leaching, pollution. Chemical fertilizers pose a detrimental effect on plant, animals and soil health by interference with their natural structure, function and mechanism. In the past few years, researchers all around the world proved the worth and role of plant growth promoting rhizobacteria (PGPR) and mycorrhiza in sustainable, cost effective and nature friendly importance in agriculture. PGPBR may promote plant growth directly usually by either resource facilitation and modulating plant hormone level or indirectly by decreasing the inhibitory effect of various pathogenic agent on plant growth and development, that is, by acting as a biocontrol agent (Glick et al; 1995) Root-colonizing plantbeneficial bacteria, commonly referred to as plant growth-promoting rhizobacteria (PGPR), are capable of stimulating plant growth when cultivated in association with a host plant (Vessey 2003; Hayat et al. 2010). These bacteria are associated with the rhizosphere, the narrow zone of soil surrounding the root that is under the immediate influence of the root system (Dobbelaere et al. 2003) and that provides an important soil ecological environment allowing plant-microbe interactions (Hayat et al. 2010). The rhizosphere provides a rich source of energy and nutrients to the bacteria resulting in higher bacterial diversity and larger populations when compared with bulk soil (Gray and Smith 2005). Likewise, rhizobacteria also secrete a wide variety of metabolites into the rhizosphere that are utilized by plants (Van Loon 2007). Hence it becomes imperative to isolate and characterize new plant growth promoting bacteria and mycorrhiza from soil. Hence this study was aimed to isolate and characterize PGPRs.

MATERIAL AND METHOD

Soil Sample Collection

Rhizospheric soil sample was collected from agricultural soil of Munsyari, (2200 feet, 30.06°N/80.23° E) Uttarakhand, western Himalayas.

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Isolation of Bacteria

Serial dilutions were prepared from the soil samples, and 100 µL aliquots from each dilution of 1 × 10-6, 1 × 10-7, and 1 × 10-8 CFU mL-1 were spread on agar plates and incubated for 24 hours at 25 ± 2°C. Morphologically distinct bacterial colonies were selected for further purifications. The purified isolates were preserved temporarily in 20% glycerol solution at -20°C. Initially 40 strains were selected as potential plant growth promontory bacteria but after different experimentation only five strains were found suitable for further study.

P solubilization Assay

Phosphate solubilization by bacterial isolates was done by the method of Pikovskaya (Pikovskaya et al. 1948) Plates were made in triplicate for each bacterial isolate using Pikovskaya agar medium. Bacterial culture was point inoculated at the center of Pikovskaya agar plate and incubated in incubator at 28°C for 7 day. The plates were then examined for halo zone around bacterial culture and solubilization index (S.I.) was calculated as: S.I. = (colony diameter + halo zone diameter)/colony diameter (Edi-PremonoMoawad et al 1996).

IAA, Siderophore, Carbohydrate utilization and other PGPR activities:

Indole acetic acid (IAA) production by bacterial isolates was determined in LB broth supplemented with L-Tryptophan (500 µg/mL) at 24, 48, and 72 h as described by Patten and Glick (Pattern and Glick et al 2002). For this, bacterial cells were removed by centrifugation at 10,000 rpm for 5 min at 4°C. One mLof the supernatant was mixed with 4 mL of Salkowski's reagent in the ratio of 1: 4 and incubated at room temperature for 20 min. Development of a pink colour indicated indoles. Siderophore production was determined by using blue indicator dye and chrome azurol S agar (Schwyn B, Neilands JB et al 1987). Bacterial isolates exhibiting orange halo zone on chrome azurol S agar after 5 d of incubation at 28°C were considered positive for the production of siderophores.

Antibiotic Sensitivity

Antibiotic sensitivity profile of the strainswas checked by using OD043- 1PK Octadiscs (HIMEDIA, INDIA)

Carbohydrate Utilization Test

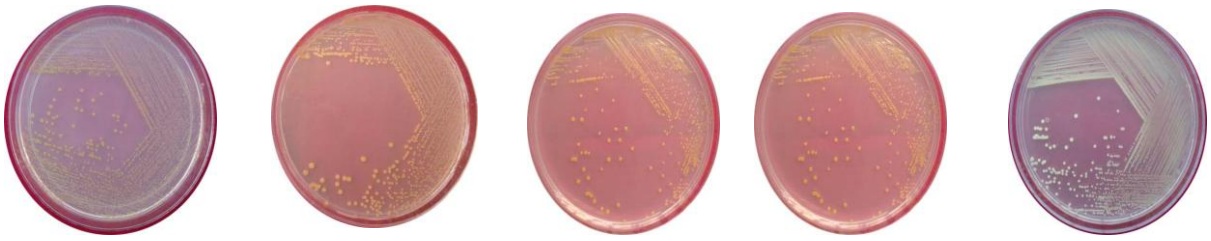
Biochemical characterization was performed using Biochemical test kit i.e. KB009A and KB009B1 (Hi-media, India) according to the manufacturer guidelines. 24h old culture was used for these biochemical tests. 1 mLaliquot of culture was added in the wells and the results were observed after 24h.

Identification of Potent PGPR based on 16S rRNASequencing Genetic characterization based on 16s rRNA gene sequence was analyesd. Genetic DNA was extracted as described by Neumann et al; 1992 and PCR amplification of 16s rDNA was carried out by using primers RDNA 1A 5'AGAGTTTGATCCTGGCTCAG 3' and RDNA 1B 5'AAGGAGGTGATCCAGCCGCA 3' PCR was done as a hot start of 940C for 3minutes followed by 35 cycles of 940C for 1minute, 540C for 1minute, 720c for 1.5 minutes. Amplified PCR products were purified QIAquick gel extraction kit (QIAGEN, GERMANY) and sequenced at automated DNA sequencer (Applied Biosystems 3730). Obtained sequences were compared with Genebank database of NCBI with blastnprogramme and then deposited in Genebank under accession no. KU925853, KU925855 KU925854 KU925856 and KU925857 for PS-01, PS-02, PS-03, PS-04 and PS-05 respectively.

RESULT AND DISCUSSION

Characterization of Bacterial Isolates

All studied bacterial cultures were gram negative except PS02 strain.(Fig.1) Out of five bacterial isolates PS-01 were found to light yellow, PS-03 were found to form yellow brown and rest were showing off-white colour under microscope(Table3.1).Based on 16S rDNA gene sequences, PS-01 and PS-03 isolates were identified as Bacillus species (GenBank accession number KU925853, KU925855 for PS-01 and PS-03 respectively) while the PS-02, PS-04 and PS-05 isolates wereidentified as Microbacterium sp., Pseudomonas sp., and Arthobacter sp. (GenBank accession number KU925854, KU925856 and KU925857 for PS-02, PS-04 and PS-05 respectively).



PS01 PS02 PS03

Fig. 1. Colony characteristics of the bacterial isolates

Phosphate Solubilisation

In the fields, phosphorous is the second most essential macronutrients for the plant growth and development.In the soil, 20–80% of phosphate is in organic form (Richardson et al; 2000) and plant may poorly/not possess an innate ability to acquire phosphorus directly from soil phytate (Greiner et al; 2001). Therefore the availability of P_i is highly dependent on the chemical composition and biological processes occurring in the soil, especially in rhizosphere. Hence these isolated PSB's were analyzed for their P solubilisation activity which degrades the soil phytate to lower phosphate esters which are available to plants. All of the five bacterial cultures showed translucent region around colonies on phytase screening medium described by (Kerovuo et al 1998). The bacterial isolates solubilized tricalcium phosphate in Pikovskaya media. Microbacterium sp. PS-02 showed highest solubilisation index of 4.9, while Bacillus sp. PB-01 showed least solubilisation index 1.39. Bacillus sp. PS-03 and Pseudomonas PS-04 showed the intermediate solubilisation index of 4 (Fig. 3.2, Table 3.2).The results indicate that indigenous bacterial strains could serve as efficient biofertilizer candidates P nutrition of crop plants.

PS01 PS02 PS03 PS04 PS05

Fig. 2. Phosphate solubilization potential on Pikovskaya agar plates of isolates.

Table 1. Growth promotory properties of the bacterial strains

S.No.	Culture ID	Species Name	Phosphate Solubilization (SI)	IAA Production	Siderophore production
1.	PS01	*Bacillus sp strain* 1	+ (1.39)	+ve	-ve
2.	PS02	*Microbacterium sp* strain 2	+ (4.9)	+ve	+ve
3	PS03	*Bacillus sp.* strain 3	+ (4.3)	+ve	-ve
4	PS04	*Pseudomonas sp.* strain 4	+(4.1)	+ve	+ve
5	PS05	*Arthrobacter sp.* strain 5	_	+ve	+ve

IAA, and Siderophore Production

Indole acetic acid (IAA) is one of the most physiologically active auxins. PGPRs enhance the growth of plants by proliferation of lateral roots and root hairs. Rhizospheric bacteria also produce the siderophores which helps the strategy II plants like cereals to uptake the iron and zinc from the soil. All five bacterial cultures were found to be IAA producing, which may enhance plant growth and may help plant to gain good vigour index.PS02, PS04 and PS05 were positive in siderophore production with pink halo zones around colonies (Table 3.2, Fig. 3.3). The bacterial strain PS02 was found to be positive for IAA and siderophore production which is good for the plant productivity.

PS01PS04 PS02 PS03PS05 PS04 PS05

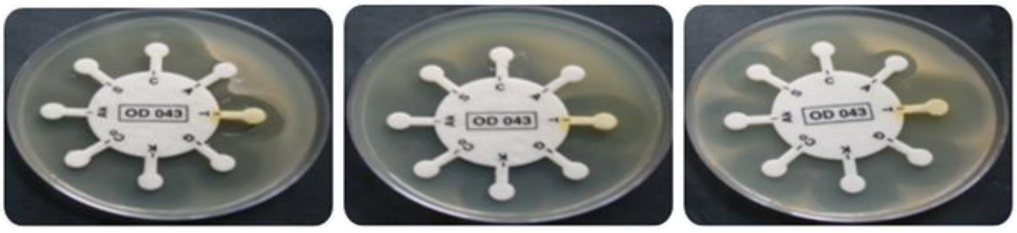
Fig. 3. Siderophore production by potential bacterial strains at 28^{o}C on CAS-blue agar plates

Antibiotic Sensitivity

Plant roots are the main part of the plant for the uptake of minerals and water from the soil. The rhizosphere is the narrow zone of soil specifically influenced by the root system. This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Gray and Smith, 2005). Root associated bacteria can be deleterious to the plant. The PGRs indirectly helps the plant by inhibiting the growth of pathogenic bacteria in the rhizosphere(Glick, 1995). Culture PS01, PS02 and PS04 were resistant to Ampicillin but PS03 and PS05 were sensitive to Ampicillin. All remaining bacterial cultures were sensitive to Tetracycline, Gentamycin, kanamycin Co-Trimoxazole, Amicamycin, Streptomycin and Chloramphenicol. (Fig 3.4 & Table 3.4)

Table 2. Antibiotic sensitivity profile of the strains using OD043- 1PK Octadiscs (HIMEDIA, INDIA)

S.N.	Antibiotics	PS01	PS02	PS03	PS04	PS05
1.	**Ampicillin**	-	-	+	-	+
2.	**Tetracycline**	+	+	+	+	+
3.	**Gentamycin**	+	+	+	+	+
4.	**Kanamycin**	+	+	+	+	+
5.	**Co-Trimoxazole**	+	+	+	+	+
6.	**Amikamycin**	+	+	+	+	+
7.	**Streptomycin**	+	+	+	+	+
8.	**Chloremphenicol**	-	+	+	+	+



PS-01 PS-02 PS-03

PS-04 PS-05

Fig .4. Antibiotic Sensitivity profile of strains 1,2,3,4,5

Table 4. Carbohydrate Utilization Test - All the strains were found to be different for utilization of different carbohydrates

Bacteria/ Glucose	PS01	PS02	PS03	PS04	PS05
Lactose	_	_	+	_	_
Xylose	_	+	+	+	+

Maltose	_	+	+	_	+
Fructose	_	+	+	+	+
Dextrose	_	_	+	+	+
Galactose	+	_	+	+	_
Raffinose	_	_	+	_	+
Trehalose	_	_	+	_	_
Mellibiose	_	_	+	_	+
Sucrose	_	+	+	+	+
Arabinose	+	+	+	+	+
Mannose	_	+	+	+	_
Inulin	-	-	+		+
Sodium Gluconate	-	-	-	-	-
Glycerol	-	+	-	+	+
Salicin	-	+	+	+	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Mannitol	-	+	-	+	+
Adonitol	-	-	-	-	-
Arabitol	-	-	-	+	+
Erythrytol	-	-	-	-	-
Alpha Methyl D Glucoside	-	+	-	-	-

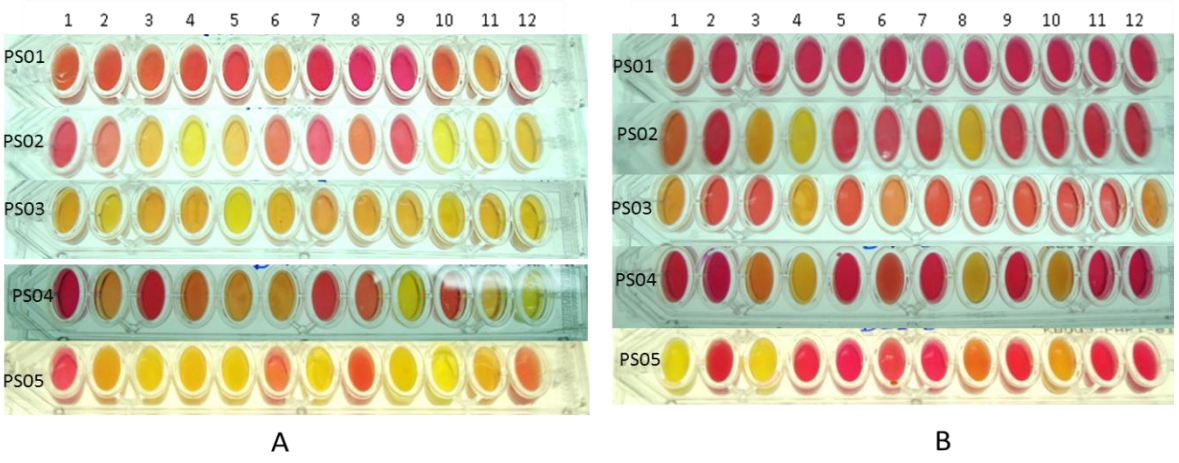


Fig. 4. Carbohydrate utilization tests (Positive-Yellow; Negative-Pink)(A) (1-Lactose,2-Xylose,3-Maltose,4-Fructose, 5-Dextrose, 6-Galactose, 7-Raffinose, 8-Trehalose,9- Melibiose,10- Sucrose,11- L-Arabinose, 12-Mannose), (B) (1-Inulin, 2-Sodium gluconate,3- Glycerol,4- Salicin, 5-Dulcitol,6-Inositol, 7-Sorbitol,8-Mannitol,9- Adonitol,10-Arabitol,11- Erythritol,12- alpha-Methyl-D-glucoside)

CONCLUSION

Biofertilizers is very important for the agriculture as they are cheap, ecofriendly and efficient, hence important for the sustainable agriculture. Hence, plant growth promoting rhizobacteria (PGPR) isolation and characterization is very important for utilizing them as Biofertilizers. If a PGPR is isolated from the harsh environment like high altitude it has the added advantage of to withstand the abiotic stress. In our study we have isolated the different bacterial strains from high altitude. All the isolates gram negative except the one. Two isolates were identified as Bacillus species (PS-01 and PS-03) rest three (PS-02, PS-04 and PS-05) were identified as Microbacterium sp., Pseudomonas sp., and Arthobacter sp. Isolate PS03 was found to be most efficient for Phosphate utilization, siderophore production and carbohydrate utilization and

antibiotic profiling. Hence this isolate PS-03 should prove a better PGPR.

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