

Isolation and Partial Characterization of Bacteria Showing The Antibacterial Activity From Soil of Agricultural Land - Northern India, Against Bacteria

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A total of 21 different bacterial colonies were recovered from Agricultural land soil samples collected from different areas of Northern India (Punjab, Himachal Pradesh and Jammu). These were then assessed for their antibacterial activity against five different bacteria; *Staphylococcus aureus* MTCC-6908, *Pseudomonas aeruginosa* MTCC-424, *Bacillus subtilis* MTCC-121, *Escherichia coli* MTCC-1680, *Shigella flexneri* MTCC-1457. Results indicated that 29% of all isolates are active against, at least, one of the test organisms. Antibacterial activity shown by the soil isolates (P1, P2, P3, P4, P5, P6) lies in the range 0.6 cm to 1.8 cm against the targeted bacteria. According to their antibacterial activity, six of the selected soil isolates were characterized by conventional method; Morphological Characterization, Cultural Characterization and Biochemical Characterization. Out of six, three of the isolates are characterized as *Pseudomonas* sps., one for *Proteus*, one for *Salmonella* and one for *Klebsiella* sps. The soil isolate shown best antibacterial activity was also characterized at Molecular level (16S rRNA) and has shown 99% similarity and maximum homology with *Pseudomonas aeruginosa* CEBP2 strain, Accession No: JQ839146.1. The antibacterial activity of these isolates shows their potential as a source of novel antibiotics.

Key words: soil, characterization, antibacterial activity, screening, antibiotic.

Introduction

Microorganisms are playing the major role to the health and well-being of the people all over the world [1] and these microorganisms produce diverse group of chemical called antibiotics that have microstatic or microcidal activity against microorganism [2].

In the field of antibiotics, there are two parallel ways of discovery provided the success; isolation of natural product having antibiotic property and formation of new antibiotics synthetically during the 20th century [3]. Antibiotics are predominate in therapeutic and commercial importance [4].

The selection and sampling of natural environment is a random process [5]. Soil as sample collection provide the diversity of microorganism including bacteria and estimated to be one of the main determinants of microbial community structures [6]. Soil is also crucial to human health through their impact on human nutrition [7]. Soil residues formed mainly from the use of fertilizer on agricultural land and these residues increase the yield of antibiotics from soil microorganism [8]. One of the areas in soil where one can find abundance in microbial populations is the rhizosphere. It is a thin layer of soil adhering to a root system which is rich in microbial diversity [9].

Multi drug resistant microorganism are becoming problem in the field of medicine due to the failure of conventional antibiotic [10] with the use and overuse or misuse of antibiotics in human and animals [11]. This problem of antibiotic resistance has been recognized as a worldwide public health concern [12-13] because antibiotic-resistant strains becoming dominant in the bacterial communities in the 21st century [14-15] and has become one of the most powerful challenges in clinical therapy [16]. Multi drug resistance pathogen situation has been opened the window for the discovery of new antibiotic to treat microorganism [14].

The present study was undertaken to isolate and partially characterize (16S rRNA) the antibacterial activity showing bacteria from soil of Northern India, against multidrug resistant bacteria due to the wide range of increase in the antibiotic resistance, particularly multidrug resistance, among bacterial pathogens.

Materials and Methods

Collection of soil sample:

The Soil samples were collected from six different sites of Northern India: Jammu & Kashmir, Himachal Pradesh and Punjab, in table 1. Soil samples were taken in the sterilized polyethylene bags using sterilized spatula from rhizosphere (up to 20 cm deep) in the soil and stored at 4°C.

Table 1- Soil Sample for Bacterial Isolation and Screening

Sr. No.	Place of isolation
1	Jammu agriculture land (Deep Region)
2	Jammu agriculture land - Pakistan border area
3	Himachal Pradesh (kangra) Agriculture Land
4	Agriculture Soil, Phagwara
5	Agriculture Soil Near Well, Phagwara
6	Agriculture Land of LPU, Phagwara

Targeted Bacterial strains:

Two Gram positive bacterial strains; *Staphylococcus aureus* MTCC-6908, *Bacillus subtilis* MTCC-121 and three Gram negative bacterial strains; *Pseudomonas aeruginosa* MTCC-424, *Escherichia coli* MTCC-1680, *Shigella flexneri* MTCC-1457 in lyophilized culture form were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Isolation and culture condition for pure soil isolate:

1 gm of soil sample was weighed and added into the stock test tube with 9 ml of distilled water under sterile conditions for making the dilution 10^{-1} , vortexed for 1-2 mins, and allowed to stand for 2 min. 1 ml of sample from stock test tube was transferred appropriately to the next tube and shaken gently and the same is done for all the six tubes for each of the sample till the dilution 10^{-6} . Then to isolate bacteria by crowded plate method, nutrient agar medium was prepared and sterilized in an autoclave for 15 minutes at 121°C followed by proper pouring into Petri plates under aseptic conditions in a laminar air flow hood. 0.5 ml of sample was taken by the micropipette from the dilution test tubes 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} and spread plated over nutrient agar. The plates were inverted and incubated over night at 37°C . The colonies showing competitive inhibition were

picked for further sub-culturing, to isolate pure culture by Streak plate Method. Quadrant streaks were made from primary inoculums to make first streak on nutrient agar. The plates were incubated at 37°C for 24 hours and the same procedure was followed until the pure colony was not isolated. Single pure colonies of Antibiotic producing Microbes were picked from the plates and inoculated in nutrient broth, nutrient broth was prepared and sterilized in an autoclave for 15 minutes at 121°C by pouring in the test tube and placed in orbital shaker incubator at 37°C for 24 hours and maintained at 20°C for further use [17-18].

Anti-bacterial activity Screening:

Determination of antibacterial activities of soil isolates were tested *in vitro* against targeted bacteria by using well plate method. A cotton swab dipped in the inoculum suspension of targeted Bacteria in nutrient broth was swabbed over the entire surface of Muller Hinton agar plate to form a lawn culture and incubated for 20 min.

5 ml of broth culture of each soil isolate was centrifuged at 1000 rpm for 15 minutes. Supernatant was separate out and 10 µl pour down into the 2 mm diameter wells which was made by borer for antimicrobial screening and incubated at 37°C for 24-48 hours. Streptomycin (2mg/ml) was used as positive control and autoclaved nutrient broth was used as negative control.

Characterization of Antibacterial activity showing Bacteria:**Morphological Characterization:**

The air dried smear of culture was fixed with heat and then stained by Gram's stain. The smears were observed for its morphology under oil immersion microscope. Shape, size, colour, pigmentation and nature of colony were also observed under the microscope.

Cultural Characterization:**Biochemical Characterization**

The biochemical tests such as Indole, Methyl Red, Voges Proskauer, citrate, catalase, SIM, TSI, Gelatin Hydrolysis, Starch Hydrolysis, Motility test and Urease tests were performed with different isolates of antibiotic producing Microbes. The isolates were also subjected to sugar

fermentation test using Glucose, Sucrose. Morphological and biochemical characterization of isolated colony was done following the standard protocol of Aneja.[17]

Molecular Characterization of Soil Isolate P6

P-6 soil isolate, which has shown the best antibacterial activity, is further sent for DNA isolation, 16S rRNA sequencing; BLAST (Basic Local Alignment Search Tool) and phylogenetic tree analysis for molecular characterization to **MACROGEN** [19].

Results and Discussion:

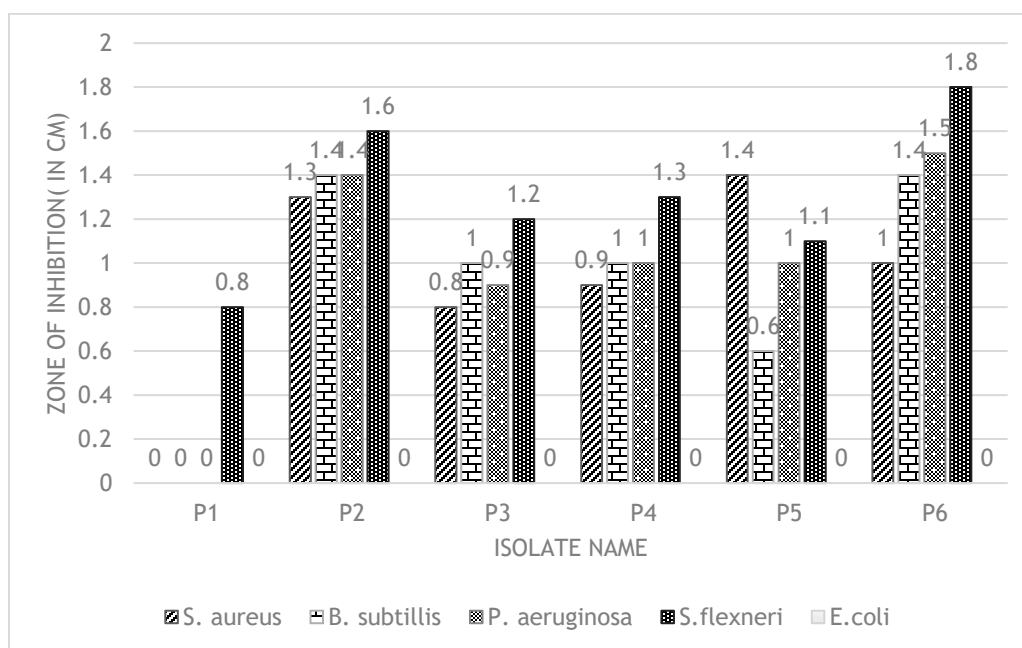
Isolation of bacteria by crowded plate technique and pure colony isolation

There were 21 different bacterial colonies observed from the soil samples collected from different sites and regions. The colonies forming zone of inhibition on the nutrient agar plate by spreading the 0.5ml of supernatant of serial dilution were selected. Colonies shown antimicrobial activity were streaked in the nutrient agar for primary, secondary and tertiary screening to isolate the pure culture.

Well plate method for Antibacterial Activity

Antibacterial activity of soil isolates were shown *in vitro* by making the zone of clearance or inhibition against targeted bacteria that included *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri* and *Staphylococcus aureus*, mentioned in figure no 1. Six colonies which has shown antibacterial activity against targeted microorganisms were named as P1, P2, P3, P4, P5 and P6. Out of six, no isolate had shown their antibacterial activity against *Escherichia coli*, whereas all the six isolates had shown their antibacterial activity against *Shigella flexneri*. Except P1, all the other five isolates (P2, P3, P4, P5, and P6) had shown their antibacterial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Zone of inhibition was measured by ruler scale in cm for all the soil isolates to compare their antibacterial activity against the targeted bacteria *S. aureus* MTCC-6908, *P. aeruginosa* MTCC-424, *B. subtilis* MTCC-121, *E. coli* MTCC-1680, *S. flexneri*. Effectiveness of antibiotics against targeted microorganism in the form of zone of inhibition, in cm, is mentioned in figure no 1.

Figure No 1: Antibacterial activity of isolates against target microorganism



Morphological and Biochemical Characterization of isolates

Six isolates (P1, P2, P3, P4, P5, P6) were morphologically characterized mentioned in table 2 and biochemically characterized mentioned in table 3. Triple sugar iron and sugar test also has been done for the identification of sugar fermentation capability and characterization, mentioned in table 4 and 5. Fluorescence has been shown by the soil isolate P6 on Nutrient Agar under the U.V. transilluminator.

Table 2- Morphological and Cultural Characterization of pure soil isolates

Sr No:	Sample site	Isolate code	Gram's staining	Morphology
1	Agriculture Land of LPU, Phagwara	P1	Gram negative	Rod Shaped
2	Agriculture Soil, Phagwara	P2	Gram negative	Rod Shaped
3	Himachal Pradesh (kangra) Agriculture Land	P3	Gram negative	Rod Shaped
4	Agriculture Soil, Phagwara	P4	Gram negative	Rod Shaped
5	Agriculture Soil Near Well, Phagwara	P5	Gram negative	Rod Shaped
6	Agriculture Soil Near Well, Phagwara	P6	Gram negative	Rod Shaped

Table 3-Biochemical Characterization of pure soil isolates

S.No	Biochemical Test	P-1	P-2	P-3	P-4	P-5	P-6
1.	MR	+	-	-	+	-	-
2.	VP	-	-	+	-	-	-
3.	Indole	-	-	-	-	-	-

4.	Citrate	+	+	+	+	+	+
5.	Catalase	+	+	+	+	+	+
6.	Urease	+	-	+	-	-	-
7.	Nitrate Reduction Test	+	+	+	+	+	+
8.	Gelatin	+	+	-	-	+	+
9.	Oxidase	-	+	-	-	+	+
10.	Motility	+	+	-	+	+	+
11.	Starch Hydrolysis	+	-	+	-	-	-

Table 4: Results of TSI Test

Sample Code	Slant	Butt	Gas Production	H ₂ S
P-1	K	A	+	+
P-2	K	K	-	-
P-3	A	A	+	-
P-4	K	A	+	+
P-5	K	K	-	-
P-6	K	K	-	-

Alkaline (K), Acidic (A), Positive (+), Negative (-)

Table 5: Results of Sugar Fermentation Test

Sugar fermentation test				
Sugar Isolates	Glucose	Sucrose	Lactose	Mannitol
P-1	+	+	-	-
P-2	-	-	-	+
P-3	+	+	+	+
P-4	+	-	-	+
P-5	-	-	-	+
P-6	-	-	-	+

On the basis of Biochemical Characterization; Out of six, three (P2, P5, P6) of the isolates could be characterized as *Pseudomonas* sps., one (P1) for *Proteus*, one for *Salmonella* (P4) and one for *Klebsiella* sps. (P3)

Molecular Characterization of Soil Isolate P6

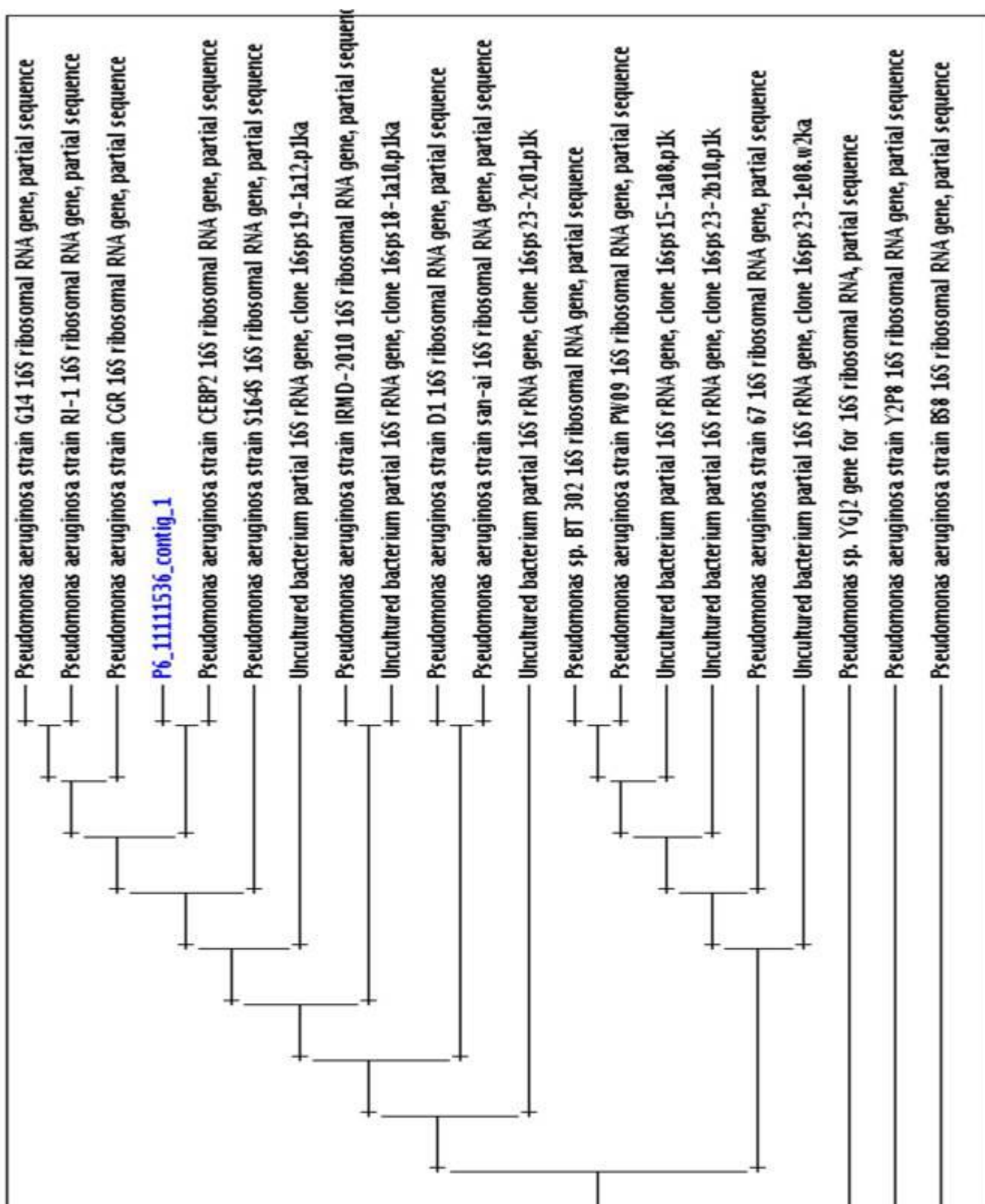
16S rRNA supported with phylogenetic tree analysis sequence report obtained from MACROGEN Company. Universal primers 518F (Forward primer) and 800R (reverse primer) was used to amplify the 16S rDNA. Sequencing report shown the Read length (normal) of 1462 bp, Forward Sequence (normal) of 930 bp and Reverse Sequence (normal) of 716 bp. BlastN (Nucleotide Basic Local Alignment Search Tool) report shown the 99% similarity and maximum homology of soil isolate with CEBP2 strain of *Pseudomonas aeruginosa* (Accession No: JQ839146.1) in figure no 2. It is evident through phylogenetic tree, in fig. no. 3, that 99% similarity and maximum Homology of soil isolate P6 with CEBP2 strain of *Pseudomonas aeruginosa* showed that P6 belong to the species *Pseudomonas aeruginosa*, genus *Pseudomonas* in the family Pseudomonadaceae.

Report

Gene : P6_11111536_contig_1

Length : 1462

Query		Subject				Score			Identities		
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Strand
1	1460	Pseudomonas aeruginosa strain Y2P8 16S ribosomal RNA gene, partial sequence	EU221384.1	1478	20	1478	2678	1450	0.0	1458	Plus/Plus
1	1462	Pseudomonas aeruginosa strain RI-1 16S ribosomal RNA gene, partial sequence	JQ773431.1	1529	18	1477	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas aeruginosa strain CEBP2 16S ribosomal RNA gene, partial sequence	JQ839146.1	1476	3	1462	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas sp. BT 302 16S ribosomal RNA gene, partial sequence	JQ782891.1	1495	13	1472	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas aeruginosa strain san-ai 16S ribosomal RNA gene, partial sequence	JQ012798.1	1461	2	1461	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas aeruginosa strain D1 16S ribosomal RNA gene, partial sequence	JN995662.1	1485	17	1476	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas aeruginosa strain 67 16S ribosomal RNA gene, partial sequence	JN995661.1	1472	4	1464	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas sp. YGJ2 gene for 16S ribosomal RNA, partial sequence	AB691548.1	1485	7	1466	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas aeruginosa strain CGR 16S ribosomal RNA gene, partial sequence	JN128893.1	1498	17	1476	2676	1449	0.0	1459	Plus/Plus
1	1462	Pseudomonas aeruginosa strain PW09 16S ribosomal RNA gene, partial sequence	JN020962.1	1498	1482	23	2676	1449	0.0	1459	Plus/Minus



Soil is the one of the best source as sample for the isolation of bacteria. There are 26 different colonies of bacteria were observed through six sample and among them 6 potent bacterial colonies were screened against the targeted microorganism. In the changing environment scenario, development of drug resistant condition in bacteria is creating condition for the identification of new antibiotics. This experiment has shown the diversity of microorganism in different site of soil sample and potent microorganism which can be utilized in future for the novel antibiotic production.

Conclusion

From six agricultural land soil samples collected from different areas of Northern India- Punjab, Himachal Pradesh, Jammu. 21 isolates of antibacterial activity showing bacteria were obtained by the crowded plate of serial diluted soil samples on nutrient agar. Approximately 29% (6 isolates: P1, P2, P3, P4, P5, P6) of the isolates shown their antibacterial activity against bacteria. Antibacterial activity shown by the soil isolates (P1, P2, P3, P4, P5, P6) lies in the range 0.6 cm to 1.8 cm against the bacteria (Gram positive - *Staphylococcus aureus* MTCC-6908, *Bacillus subtilis* MTCC-121, Gram negative- *Pseudomonas aeruginosa* MTCC-424, *Escherichia coli* MTCC-1680, *Shigella flexneri* MTCC-1457). Soil isolates partially characterized in Enterobacteriaceae (*Proteus*, *Salmonella*, *Klebsiella*) and Pseudomonadaceae (*Pseudomonas aeruginosa*) on the basis of their morphological, cultural, biochemical characterization. 16S rRNA sequence analysis (1462bp) and phylogenetic tree analysis of the isolate P6, which has shown best antibacterial activity, was carried out at Macrogen shown 99% similarity with CEBP2 strain of *Pseudomonas aeruginosa*, Accession No: JQ839146.1 and the isolate P6 belong to the species *Pseudomonas aeruginosa*, genus *Pseudomonas* in the family Pseudomonadaceae. Interesting observation in this experiment, P6 isolate belong to the species and showing activity against multidrug resistant - *Pseudomonas aeruginosa* MTCC-424.

The increasing number of MDR Bacteria by the excessive use or misuse of antibiotics and the continuous demand for new leading antibiotics in the medical science for the treatment of drug resistant infectious pathogens has emerged the search for secondary metabolites or antibiotics in the ecosystem. Hence, the rhizosphere of agricultural land soil of Northern India is a goldmine of microbial diversity. Present studies will establish the rich bacterial diversity of the region and also help conserve and utilize the antibacterial activity showing bacteria against MDR bacteria in bioindustry to synthesize the new and leading antibiotics.

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