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Identification and Partial Characterization of Bacteria Exhibiting Antibacterial Activity Isolated From Soil Samples

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Abstract

Bacteria isolates from the soil having antibacterial activity against targeted bacteria were identified in present study. Six Soil samples were collected fromtwo region of India; Punjab (Jalandhar, Phagwara), and Jammu. Sampling sites includes dumping site areas, agricultural land, botanical garden and forest zone. Out of thirteen isolates isolated by crowded plate technique, screened only six (G1, G2, G3, G4, G5, G6) bacterial isolates from dumping site soil were identified exhibiting potent antibacterial activity against twogram negative and three gram positive bacteria *Bacillus subtilis*, *Staplylococcusaureus*, *Shigellaflexineri*, *Escherichia coli*, *Pseudomonas aeruginosa*. G5 has shown maximum antibacterial activity(2.3cm)against Gram positive *Staplylococcusaureus* while G6 exhibit minimum inhibition against *Shigellaflexineri* (0.6cm) All the isolates were selected for further studies concerning their morphological, physiological and biochemical and molecular identification which predict the isolates as *Pseudomonas spp., Klebsiellaspp., Micrococcus spp., Bacillus spp., Pseudomonas spp.* and *Klebsiella spp.*The identification of potent isolate G5 as *Pseudomonas aeruginosa*was further confirmedusingpartial sequencing, BLAST and phylogenetic analysis (16S rRNA). Present study on isolation of antibiotic producing bacteria gives an insight on identification of specific antibiotic showing resistivity against bacteria.

Keywords: Anti-bacterial activity, phylogenetic analysis, molecular characterization, dumping site soil, biochemical test

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Introduction

The demand of new class of antibiotics is being increased due to use and mismanagement of using the antibiotic which create the condition to screen the novel effective antimicrobial compound or new mode of action to deal with the infectious diseases [1].Widespread discovery of novel antibiotics and the strains which produces antibacterial agents contributes to the greater inhibition of MDR bacteria. Antibacterial substances are widely produced by a large number of microorganisms of genera *Bacillus, Streptomyces, Penicillium, Micromonospora* and *Cephalosporium*, as they are known to produce more than 5000 different antibiotics [2].Even though many antibiotics are already exist, increasing resistance ability or insensitivity for antibiotics among pathogen developed the platform to discover novel and effective antibiotics. The present report describes a simple rapid method for microbial isolation from soil, bacteria which produce antibacterial agents and their characteristics. Therefore, many bacteria; *Streptomyces*, *Bacillus* and *Penicillium* have been explored continuously for their potential to produce antibiotics [3].

The conventional way to identify the antibiotics from microorganisms or antimicrobial resistance is not the solely methods for discovering the new antibiotics now new ways and sources are being utilized. Soil is the one of the best source for the isolation of microorganism which may provide the novel compound to fight with pathogens and diversity of microorganism in a sample [4]. So the soil from different geographical area are preferred to isolate the bacteria and screen the antimicrobial potential and therefore hopefully used in research and development [5].

So the current study takes into consideration, bacterial strains isolated from soil and screened for their antibacterial activity against targeted bacteria.

Material and Methodology

Sampling procedure

Six samples of soil underlying solid waste andrhizospheric zone were taken from the dumping site, botanical garden soil,agriculture land and forest sites during spring seasons mentioned in table 1. These 4 soil sample sites were selected to get diverse isolates. All six samples were

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

collected at a depth of approximately 30 cm below the surface. The samples were transferred to sterile polythene bags, sealed and stored in a deep freezer (4^oC). The sample collection was done carefully to take care the some points as fully wet soil, and particle size, organic matter and color of the soil.

Table 1: Soil Sample's Site

Sample no	**Origin**	**Sample name**
Sample 1	Phagwara , India	Dumping site soil
Sample 2	Jammu, India	Forest soil
Sample 3	Jalandhar, India	Dumping site soil
Sample 4	Phagwara, India	Botanical soil
Sample 5	Phagwara , India	Agriculture soil
Sample 6	Jammu, India	Dumping site soil

Targeted Microorganisms:

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, *Shigellaflexneri* MTCC-1457 in lyophilized form were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India and revived by inoculating in nutrient broth.

Isolation of bacterial strains from different soil samples:

One gram weight of soil samples weredissolved in 10 ml of distilled water in test tubes.Serial dilution has been done for all samples up to 10^{-7} dilution and shaken vigorously to make uniform suspension. Diluted samples were left at room temperature so that the bacterial populations present in the samples collected in the clear supernatant[2].The diluted sample were then plated on nutrient agar plate especially after vigorous shaking for isolation of bacteria showing inhibition zone around them from soil using crowded plate technique [6]. Incubation was given to cultured plates to maintain the aerobic condition at 37^o C for 24 hr. The developed colonies

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

showing inhibition zone around their vicinity were further subjected for purification by streak plate method on nutrient agar plate[7]. Isolated discrete pure colonies were inoculated in autoclaved nutrient brothfor further use[8].

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 nutrient agar plate to form a lawn culture and incubated for 20 min. The plates were then inoculated with 10 µl soil isolates by making wells of 2mm diameter with the well borer 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.

Identification

The developed colonies exhibiting antibacterial activity were submitted to Gram stain[9] observed for the morphology of the isolates under the microscope (100X), followed by biochemical tests for more authentic identification. These tests containIMViC test, nitrate reduction, oxidase test, catalase test, sugar fermentation, triple sugar iron (TSI), starch hydrolysis, motility test and urease test. All developed colonies with antibacterial activity in each plate were isolated for identification. According to Bergey's Manual of Systematic Bacteriology,biochemical test has been done and results were evaluated for the identification of bacteria [10].

Studies concerning the phylogenetic characteristic

The bacteria, isolate, showing maximum antibacterial activity against targeted microorganism was selected for molecular characterization. In Macrogen, DNA isolation has been done which was followed by PCR and then PCR product sequencing has been done. For PCR reaction, universal primers 518F and 800R was used for 16S ribosomal DNA amplification. PCR product was sequenced using by Sanger-Coulson method [11]. This sequenced was compared with the other deposited sequences at the National Center for Biotechnology Information (NCBI)

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

database by using BLAST. The Phylogenetic tree was constructed for the potent isolate and the analysis was performed using Geneious version 6.1.4 (Build 2013-04-09 14:51) created by Biomatters Ltd using Neighbor Joining algorithm, using distance matrix as input.

Result and Discussion

Antibacterial activity of isolates

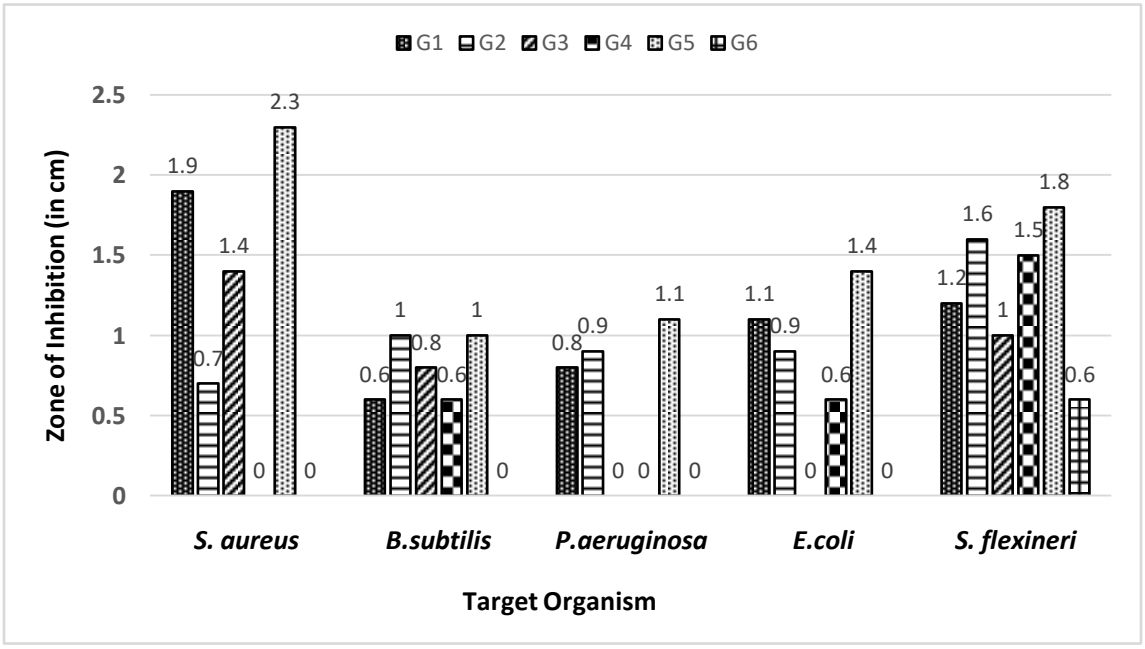
Thirteen isolates differ in their cultural and morphological characters were isolated in pure form from different soil sites of Punjab and Jammu region using nutrient agar mediumthroughcrowded plate technique. Among these isolates six isolates only exhibited antibacterial activity against tested organisms as illustrated in Table 2. The most potent isolate against all tested organisms was G5shown in Fig 1.

Table 2: Isolation of Bacterial Colonies

Sample No.	Place	Sample Site	Name of isolates (Agar well diffusion method)
Sample 1	Phagwara , India	Dumping site soil	G1 G2
Sample 3	Jalandhar, India	Dumping site soil	G3 G4
Sample 6	Jammu, India	Dumping site soil	G5 G6

Fig. 1: Anti-bacterial activities of isolate showing zone of inhibition (in cm)

ISSN: 0971-1260
Vol-22-Issue-17-September-2019



Morphological and Physiological Analysis of the Isolates

Six bacterial isolates obtained has shown different growth with yellow and white colony appearance in nutrient agar plate and different shades of yellow and green pigmentation in supernatant of inoculated Nutrient broth [12] as in Table 3.There were three gram positive and three gram negative bacteria identified. Gram negative bacteria appear pink due to presence of outer membrane it prevent the penetration of the stain and retain[9] out of which only G3 isolate show spherical shaped while other are rod shaped morphology at 100 x microscope.

Table 3: Phenotypic characteristics for some of the bacterial colonies isolated from soil sample

Isolates	**Surface Texture**	**Elevation**	**Margin**	**Chromogenesis**	**Gram Staining**	**Shape**
G1	Circular, Mucoid	Flat	Entire	Bluish Pigmentation	G-ve	Rod
G2	Mucoid	Raised	Entire	colourless	G-ve	Rod
G3	Circular	Convex	Entire	Cream Yellow	G+ve	Spherical
G4	Pinpointed	Flat	Entire	Yellow	G+ve	Rod

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

G5	Punctiform, Mucoid	Flat	Entire	Bluish Pigmentation	G-ve	Rod
G6	Mucoid	Raised	Entire	sticky	G-ve	Rod

Biochemical Identification of Isolates

The biochemical characterization and sugar fermentation properties of bacterial isolates identified from the soil samples helped to characterize them under particular family (genus and species) different biochemical test make it possible[13]. Catalase test result showed all the isolates to be positive ensuring the presence of catalase enzyme whereas, the increase activity of catalase and urease enzymeshave profound effect on the breakdown ofhydrogen sulfide bond present in cell wall of the cells[13].All the isolates showed strictly negative result in Indole test as illustrated in Table 4. Unable to produce indole due to the absence of the enzyme typtophanase [10].G3 show positive Methyl red testhave the ability to produce and maintain stable acid end products from glucose fermentation.Oxidase test are positive for G1, G3, G6 and G5show positive and G2, G4 show negative resultsthis can be due topresence of strong hydrogen bonds [13].Starch hydrolyzing for G2, G4 and G6 is positive showing capability of hydrolyzing starch to maltose [9]. Citrate utilization test showed 100% positive results as all isolates were capable of utilizing citrate as the sole source of carbon and energy.TSI test was done to check the ability to reduce sulfur and ferment carbohydrates in Table 5[14]. Characterization according to sugar fermentation includes the use of Glucose, Lactose, Sucrose and Mannitolas illustrated in Table 6[13]. These results Predict G1 isolate as *Pseudomonas spp.*, G2as*Klebsiellaspp.*, G3 as *Micrococcus spp.*, G4 as *Bacillus spp.*, G5 as *Pseudomonas spp.* and G6 as *Klebsiella spp.*,which was further confirmed by molecular characterization of potent strain G5.

Table 4: Results of Biochemical Tests

S.No	**Name of the Test**	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**
1.	Methyl Red	-	-	+	-	-	-
2.	Voges-	-	+	-	+	-	-

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

	Proskaur						
3.	Indole production	-	-	-	-	-	-
4.	Simmons' citrate	+	+	+	+	+	+
5.	Catalase	+	+	+	+	+	+
6.	Urease test	-	+	-	-	+	-
7.	Nitrate reduction	+	+	+	+	-	
8.	Gelatin	+	-	+	+	+	+
9.	Oxidase	+	-	+	-	+	+
10.	Motility	+	-	-	+	+	+
11.	Starch Hydrolysis Test	-	+	-	+	-	+

Table 5: Results of TSI Test

Sample Code	**Slant**	**Butt**	**Gas Production**	**H_2S**
G1	K	K	-	-
G2	A	A	+	-
G3	K	A	-	-
G4	K	A	-	-
G5	K	K	-	-
G6	A	A	+	-

Table 6: Results of Sugar Fermentation Test

Sugar fermentation test

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Sugar / Isolates	Glucose	Sucrose	Lactose	Mannitol
G1	-	-	-	+
G2	+	+	+	+
G3	+	-	-	+
G4	+	+	-	+
G5	-	-	-	+
G6	-	-	-	+

Molecular Characterization and Phylogenetic Analysis

According to colonial growth and morphological and biochemical analysis, G5 was predicted under *Pseudomonadaceae*family, as it showed fluorescence under UV exposure. Isolate was further conformed using 16s rDNA sequencing.

16srDNA gene sequencing:It was defined to the isolate G5, the blast result of 16S rDNA gene sequence showed the isolate G5 (Coded as G5_11115020) has 99% identity withdifferent *Pseudomonasaeruginosa*strains; CMG 586 and CMG581as shown in Table 8 and with various other strains.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Table 8: BlastN Report for G5 Isolate

Query name : G5_11115020_g-5_contig_1

Query length : 1501

Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct.(%)	Strand
5	1475	Pseudomonas aeruginosa strain CMG586 16S ribosomal RNA gene, partial sequence	EU194236.1	1476	5	1473	2682	1452	0.0	1467	1473	99	Plus/Plus
5	1475	Pseudomonas aeruginosa strain CMG581 16S ribosomal RNA gene, partial sequence	EU126608.1	1474	5	1473	2682	1452	0.0	1467	1473	99	Plus/Plus
5	1471	Pseudomonas aeruginosa strain 67 16S ribosomal RNA gene, partial sequence	JN995661.1	1472	4	1468	2680	1451	0.0	1463	1468	99	Plus/Plus
5	1475	Pseudomonas aeruginosa strain CMG860 16S ribosomal RNA gene, partial sequence	EU037096.1	1488	21	1487	2680	1451	0.0	1466	1472	99	Plus/Plus
5	1471	Pseudomonas aeruginosa strain RI-1 16S ribosomal RNA gene, partial sequence	JQ773431.1	1529	18	1481	2678	1450	0.0	1463	1468	99	Plus/Plus
5	1471	Pseudomonas aeruginosa strain CEBP2 16S ribosomal RNA gene, partial sequence	JQ839146.1	1476	3	1466	2678	1450	0.0	1463	1468	99	Plus/Plus
5	1471	Pseudomonas sp. BT 302 16S ribosomal RNA gene, partial sequence	JQ782891.1	1495	13	1476	2678	1450	0.0	1463	1468	99	Plus/Plus
5	1471	Pseudomonas aeruginosa strain D1 16S ribosomal RNA gene, partial sequence	JN995662.1	1485	17	1480	2678	1450	0.0	1463	1468	99	Plus/Plus
5	1471	Pseudomonas sp. YGJ2 gene for 16S ribosomal RNA, partial sequence	AB691548.1	1485	7	1470	2678	1450	0.0	1463	1468	99	Plus/Plus
5	1471	Pseudomonas aeruginosa strain CGR 16S ribosomal RNA gene, partial sequence	JN128893.1	1498	17	1480	2678	1450	0.0	1463	1468	99	Plus/Plus

Phylogenetic Analysis

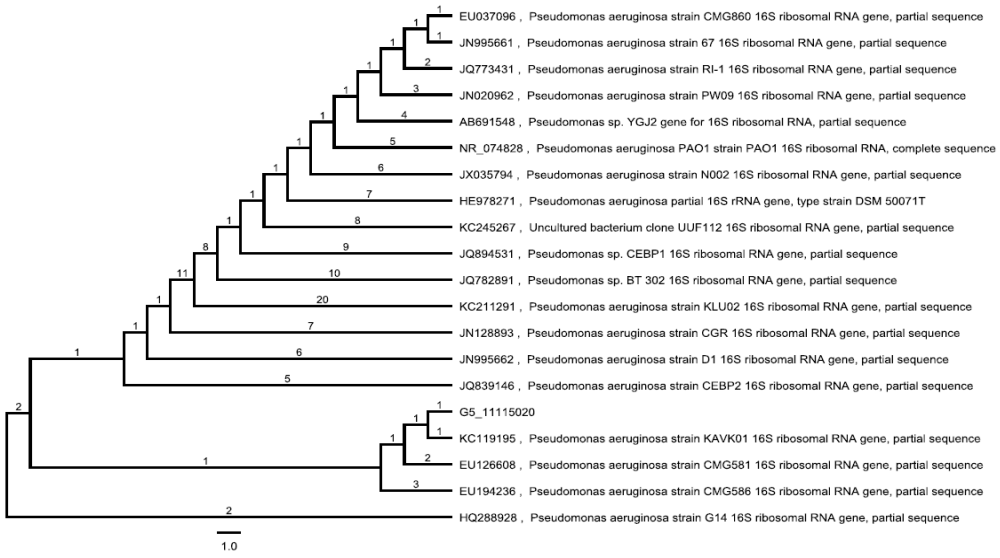
Phylogenetic analysis is biologically important it shows the evolutionary relationship between different biological species [15]. The phylogenetic analysis was performed for G5 Isolate (G5_11115020) and nineteen other similar strains. The constructed phylogenetic tree diagram can also be denoted in the Newick'sformat as,

((((((((((((((EU037096:1.0,JN995661:1.0):1.0,JQ773431:2.0):1.0,JN020962:3.0):1.0,AB691548:4.0):1.0,NR_074828:5.0):1.0,JX035794:6.0):1.0,HE978271:7.0):1.0,KC245267:8.0):1.0,JQ894531:9.0):1.0,JQ782891:10.0):8.0,KC211291:20.0):11.0,JN128893:7.0):1.0,JN995662:6.0):1.0,JQ839146:5.0):1.0,(((G5_11115020:1.0,KC119195:1.0):1.0,EU126608:2.0):1.0,EU194236:3.0):1.0):2.0,HQ288928:2.0); G5 Isolate (G5_11112050), KC119195, EU126608 and EU194236 form a clade group with sixteen other strains including EU037096, JN995661, JQ773431, JN020962, AB691548, NR_074828, JX035794, HE978271, KC245267, JQ894531, JQ782891, KC211291, JN128893, JN995662 and JQ839146 as shown in Fig 2. The points at which branches separate

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

are clades (being a group of taxa) of that node.These all form the sister taxa which share a recent common ancestor at the node that joins them together. Though G5_11112050 isolate showed identity with various Pseudomonas strains still there are at least four base difference variations with its closest group genome sequences.

Figure 2: Phylogenetic tree diagram for G5 Isolate along with various other similar strains



Conclusion

It has been concluded from this study that most of the isolates from soil are active against Multidrug resistant bacteria showing inhibition against variety of antibiotics. The inhibition ability of soil isolates may be due to production of antibiotics or some chemical agents active against them. The selection of dumping site soil serve as a reservoir for variety of bacterial isolates which were partially characterized as*Pseudomonas spp., Klebsiella spp., Micrococcus spp., Bacillus spp., Pseudomonas spp.* and *Klebsiella spp*. The range of zone of inhibition was 2.3-0.6 cm against target strain by different soil isolates. Maximum zone of inhibition (2.3 cm) showing G5 isolate was chosen for further molecular characterization, 16s rRNA sequencing.

Furthermore, the BLAST result of most potent isolate with other sequences submitted in NCBI database confirms it showing 99% similarity with the various *Pseudomonas* strains also showing variation in number of base and this 1% variations of isolate G5 which is producing some

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

secondary metabolites that are effective against common pathogenic bacteria. Biochemical, morphological and partial molecular characterization confirmed that G5 belong to the species *Pseudomonas aeruginosa*, genus Pseudomonas in the family Pseudomonadaceae.So the G5 isolate may be consideredas a good potential source for the production of new antibiotics in future.

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ISSN: 0971-1260
Vol-22-Issue-17-September-2019

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