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Research Article
Identification of Potential Antifungal Metabolite Producing *Pseudomonas tolaasii* Strain GD76 Obtained from Contaminated Agar Plate

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Abstract

Background and Objective: Fungal pathogens develop resistance against antifungal agents available in market. Therefore, treatment of fungal infection is becoming problematic. On other hand, chemically synthesized antifungal agents are also causing adverse effect on human health. To overcome this issue, recently many drug industries are trying to isolate bioactive molecules from natural sources. The objective of the study was isolation and identification of bacterial isolate from contaminated agar plate with special reference to its antifungal property and explore bacterial contamination as new habitat for beneficial microbes. **Materials and Methods:** Antifungal metabolite producer *Pseudomonas tolaasii* (*P. tolaasii*) GD76 was isolated from contaminated yeast, peptone, dextrose (YPD) agar plate on to which it was streaked from casein agar plate. Morphological, biochemical and phylogenetic study was carried out for identification of the bacterial isolate by using 16S rRNA gene sequencing and evolutionary history was inferred using the maximum parsimony method. **Results:** Morphological and biochemical study of the bacterial isolate showed that the isolate was Gram negative, rod shape, capsule containing and ferment only glucose. On basis of position sequence of the isolate in phylogenetic tree, the isolate showed 99% similarity to *Pseudomonas tolaasii*, it is indicated that the isolate is the novel strain of *P. tolaasii* for which the name *Pseudomonas tolaasii* GD76 is affirm. Sequence of 16S rRNA gene was submitted to NCBI, Accession code; KU533778. **Conclusion:** On the basis of results obtained, present study indicates towards the potential of uncharacterized prokaryotic community associated with bacterial contamination.

Key words: Antifungal producer, *Pseudomonas tolaasii* GD76, phylogenetic analysis, bacterial isolate, gene sequencing

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The greatest part of the world's biodiversity still remains unexplored and the new high-speed approaches allow its successful exploitation[1]. Nowadays many industries are engaged in commercial production of bioactive compounds from microbial origin by using cloning and other strain improvement like approaches. Microbial metabolites are expected to govern future of drug discovery[2]. After Penicillin discovery microbial metabolites have had a great impression on scientific community as well as society[3]. Recently scientific community is attracted towards microbial origin novel metabolites due to rise in antibiotic resistance globally[4-6]. Among the under explored bacterial taxa, *Pseudomonadales* certainly deserve the spotlight[7]. Despite being long known as prolific producers of specialized metabolites, their systematic screening has been hampered by difficulties in their cultivation[8]. *Pseudomonas* species are well-known because of its bioactive compound producing ability, some Pyrrolnitrin like compounds leading commercialization of antifungal agents. Study of the biochemistry and mechanism of formation of these metabolites has proved useful in several ways. Pyrrolnitrin is very good example of metabolite produced by *Pseudomonas* sp. and due to its potent antifungal activity, it plays important role in production of many agriculturally important fungicides[9]. Present study was also carried out to analyze the taxonomic position and biochemical and morphological characteristics of antifungal metabolite producing strain of *Pseudomonas* isolated from contaminated agar plate. The objective of the study was isolation and identification of bacterial isolate from contaminated agar plate. With special reference to its antifungal property and explore bacterial contamination as new habitat for beneficial microbes.

MATERIALS AND METHODS

Isolation of bacterium: The bacterium was isolated from a contaminated YPD agar plate on to which it was streaked from casein agar plate where it was showing a pronounced proteolytic activity (Fig. 1).

Screening for antimicrobial activity: The selected colony was inoculated in sterile nutrient broth and broth was incubated at 37°C for 72 h. Incubated broth was then centrifuged at 20,000 rpm for 15 min at 4°C, to make it cell free[10]. For screening of antibacterial activity, two different methods were used.

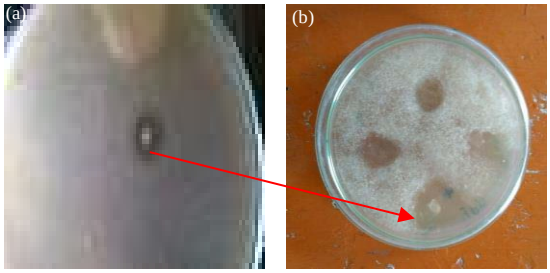

Fig. 1(a-b): (a) Contaminated casein agar plate with zone of proteolysis and (b) Serendipitously observed antifungal activity

Agar cup diffusion method: The cell free broth was tested for antimicrobial activity against *A. niger*, *A. flavus*, *F. oxysporum*, *Alternaria* sp. bacteria *S. typhi, B. subtilis* by using agar cup diffusion technique. Plates were incubated at 37°C for 24 h. and after incubation they were observed for zone of inhibition.

Dual culture technique (for fungus)[11]: In first step sterile potato dextrose agar (PDA) plate was spot inoculated with bioactive metabolite producing bacterium. Then fungal spores of *A. niger*, *A. flavus* spread on inoculated PDA plate and incubated at 37°C for 48 h.

In second step sterile PDA plate was inoculated with bioactive metabolite producing bacterium[12]. Plate was incubated at 37°C for 72 h. After incubation spores of *A. niger* and *A. flavus* spread on inoculated plate and again incubated at 37°C for 48 h. Both plates are observed for antifungal potential of bacterial metabolite.

Methods for identification and characterization of the isolate

Morphological characterization: The isolate was grown on sterile NA plate and colony characteristics of the isolate on nutrient agar medium after incubation on 37°C for 24 h was observed. Gram staining and motility (hanging drop technique) was also performed.

Biochemical characterization: Biochemical tests for characterization of isolate were carried out according to the Bergey's Manual of Determinative Bacteriology 9th edition. Catalase Test, Oxidase Test, Indole Test, Methyl red Test, Voges Proskauer Test and Citrate utilization tests were performed for the biochemical characteristic of the isolated bacteria[13].

Sugar fermentation test: Sugar fermentation tests were performed by inoculating the organism in sterile peptone water base supplemented with 1% of required sugars

Table 1: Primers used for 16S rRNA region amplification

Primers	Primer sequence (5'-3')
519F (Forward)	CAGCAGCCGCGGTAATAC
1385R (Reverse)	CGGTGTGTACAAGGCCC

Table 2: Concentration of antibiotics on disc (for antibiogram)

Antibiotics	Symbols	Concentrations (mcg)
Amikacin	AK	30
Lomefloxacin	LOM	10
Cefadroxil	CFR	30
Sparfloxacin	SPX	5
Ampicillin/SUL	AmP/SUL	30
Ceftazidime	CAZ	30
Ceftriaxone	CTR	30
Ciprofloxacin	CIP	5
Cefotaxime	CTX	30
Gentamicin	GEN	10

(Glucose, Lactose, Maltose, Arabinose, Xylose, Galactose, Raffinose, Mannitol) containing phenol red as a indicator and an inverted Durham's tube. After incubation for 24 h at 37°C, change in colour of the medium from red to yellow was recorded as positive for acid production and formation of bubble in Durham's tube indicated the formation of gas.

Lysine decarboxylase test: The isolate was inoculated in Bromocresol purple Falkow medium. Incubated at 37°C for 24 h. The change in colour of medium from purple to yellow was recorded as positive test.

Starch hydrolysis test: The isolate was inoculated on starch agar plate and incubated at 37°C for 24 h. After Incubation Gram's iodine was poured onto a plate and observed for zone of hydrolysis.

Cellulose degradation test: The isolate was spot inoculated on sterile carboxymethyl cellulose agar medium. Incubated at 37°C for 24 h, after incubation flood the plate with 1% congo red solution, this was kept for 15 min then decolorized it with 1M NaCl solution and observed for zone of hydrolysis.

Phylogenetic analysis of the isolate: Partial sequencing of 16S rRNA gene of Isolate was done from Codon Bioscience, Panjim Goa (Table 1). Similarities of obtained sequence can be studied using BLAST and FASTA programs. From the resulting multiple sequence alignment phylogenetic analysis can be conducted to assessed sequence shared evolutionary relationship[14,15].

Antibiogram of the bacterial isolate: Antibiotic susceptibility pattern of isolate was studied by disc diffusion method. From 24 h old culture of isolate 0.1 mL bacterial suspension with 0.5 McFarland standards was spread on Muller Hinton agar plate. The antibiotic disc of HiMedia, Dodeca G-I-Minus of known potency was then placed on agar surface and plate was incubated at 37°C for 24 h and the zone of inhibition around the antibiotic disc was observed for the antibiotics (Table 2).

RESULTS AND DISCUSSION

The bacterial strain producing antifungal compound was isolated from contaminated YPD agar plate by using routine isolation techniques (Fig. 1a and b).

Screening for antimicrobial activity of cell free broth: By performing antimicrobial activity it was found that cell free broth shows maximum zone of inhibition against test fungi *A. niger*, *A. flavus*. But it fails to inhibit growth of test bacteria viz. *S. typhi*, *B. subtilis*.

Screening for antifungal activity by dual culture technique: In dual culture technique bacterium inhibit growth of fungus *A. niger* and *A. flavus*. No inhibition was observed when both the isolate and test fungi were inoculated together i.e., on the same day. While, in other set, where bacterial isolate was grown on PDA for 3 days and then followed by inoculation of test fungi, Clear zone of inhibition were observed.

Morphological and biochemical characterization: Colony characteristics of the isolate on nutrient agar plate were observed after 24 h incubation at 37°C. For Gram staining *B. subtilis* was used as Gram positive and *E. coli* as Gram negative bacterium for comparison and found that isolate was Gram negative, rod shaped, nonmotile bacterium. It produces capsule which was detected by Maneval's method. Where cells appeared red background is blue (Table 3). Biochemical characterization of isolate shows that isolate is Catalase positive and Oxidase negative (Table 4 and 5) (Fig. 2a,b).

Phylogenetic analysis of the isolate: The molecular phylogeny of the isolate was determined by analyzing 16S rRNA gene (Fig. 3) sequences. On the basis of the position of sequence of the bacterial isolate in the phylogenetic tree, isolate shows (B1) showed closest similarity to *Pseudomonas tolaasii*.

Maximum parsimony analysis of taxa: The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

Table 3: Colony characteristics of isolate on nutrient agar after 24 h incubation at 37°C

Size	Shape	Colour	Margin	Opacity	Consistency	Elevation
Pinpoint (<1mm)	Circular	Off white	Erose	Opaque	Sticky	Flat

Table 4: Biochemical characterization of Isolate

Catalase	Fine bubbles arising from the colony after addition of H_2O_2	+ve
Oxidase	No colour change on contact with reagent on filter paper	-ve
Lysine decarboxylase test	Bromocresol purple falkow medium changes colour from purple to yellow	+ve
Cellulose degradation test	Zone of inhibition was observed on CMC agar plate followed by addition of 1% congo red solution and decolorizing it by NaCl solution	+ve
Starch hydrolysis	Zone of decolorization was observed on starch agar plate on addition of Gram's iodine	+ve
Indole test	No development of pink coloured ring on the surface of tryptone broth after addition of Kovac's reagent	-ve
Methyl red test	No development of red colour of GP broth after addition of Methyl red indicator	-ve
Voges proskauer test	Development of red colour of GP broth after addition of VP I and VP II reagent	+ve
Citrate utilization test	No colour change of Simmon's citrate slant from green to blue	-ve

Table 5: Sugar fermentation test of the isolate

Test	Glucose	Lactose	Maltose	Mannitol	Arabinose	Xylose	Galactose	Raffinose
Acid	++	-	-	-	-	-	-	-
Gas	++	-	-	-	-	-	-	-

Table 6: Antibiotic susceptibility of test observations

Antibiotics	Zone of inhibition (mm)	Interpretation
Amikacin (30 mcg)	22	Intermediate
Lomefloxacin (10 mcg)	33	Sensitive
Cefadroxil (30 mcg)	25	Sensitive
Sparfloxacin (30 mcg)	30	Sensitive
Ampicillin/sulbactam (10/10 mcg)	30	Sensitive
Ceftazidime (30 mcg)	30	Sensitive
Ceftriaxone (30 mcg)	25	Sensitive
Ciprofloxacin (5 mcg)	35	Sensitive
Cefotaxime (30 mcg)	25	Sensitive
Gentamycin (10 mcg)	25	Sensitive

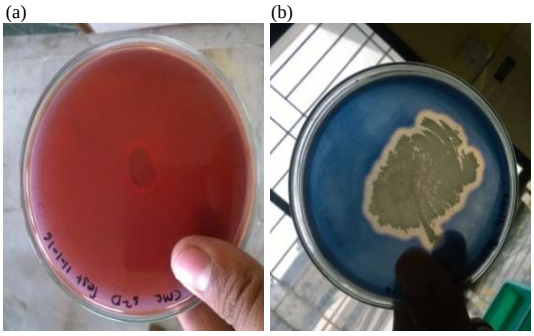

Fig. 2(a-b): (a) Cellulose degradation of bacterial isolate and (b) Starch hydrolysis

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the close-neighbor-interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale; with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 21 nucleotide sequences. There were a total of 1542 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Fig. 4).

Antibiogram of the bacterial isolate: After incubation of inoculated MH agar plate at 37°C for 24 h zone of inhibition was observed around all antibiotics (Table 6) (Fig. 5).

DISCUSSION

Immune-compromised individuals are the target of invasive fungal infections. The number of therapeutic options for the treatment of invasive fungal infections is quite limited when compared with those available to treat bacterial infections. Indeed, only three classes of molecules are currently used in clinical practice and only one new class of antifungal drugs has been developed in the last 30 years[15]. Microorganisms from environmental sources are the good candidates for production of bioactive metabolite is common thing but some insect gut microorganisms are also well-known for their ability to produce bioactive compounds[16]. Microbes have made a phenomenal contribution to the health and well-being of people throughout the world. In addition to producing many primary metabolites, such as amino acids, vitamins and nucleotides, they are capable of making secondary metabolites, which constitute half of the pharmaceuticals in the market today and provide agriculture with many essential products. American author Demain, A.L. and Mexican author Sanchez, S. jointly published review article which strongly support the term 'contribution of microorganisms in field of agriculture and healthcare'. Authors reviewed last 80 years progress in development of drugs from the bacterial origin[17]. Authors also

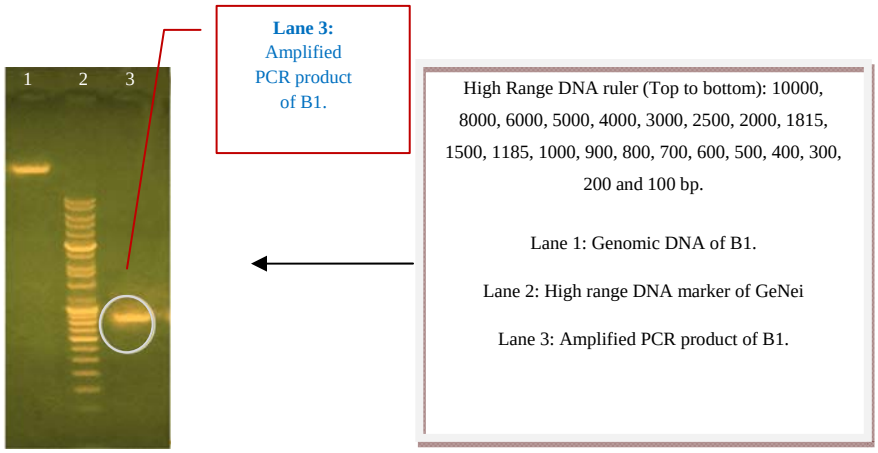

Fig. 3: Amplified partial 16S rRNA gene

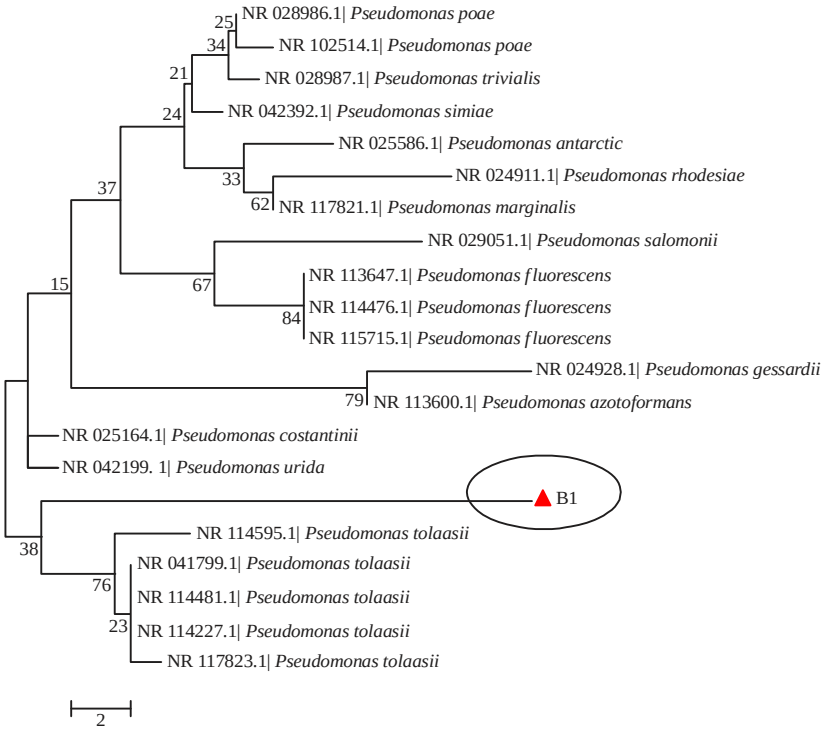

Fig. 4: Position of bacteria in phylogenetic tree

focused on economic importance of the progress in microbial metabolites in accordance with pharmaceutical industries. This article also suggested a novel phenomenon which is useful for the formulation of new drug molecules from old reported antibiotics. In short "Reborn of old antibiotics"[17,6]. *Streptomycetes* species are also in spotlight of scientific community, Charousova *et al.*[18] screened antimicrobial metabolite producing *Actinomycetes* species from costal area and mentioned that these species are the potential candidate for industrially important enzymes and bioactive compounds. Medicinal plants associated entophytic fungi are also considered as a potent source for bioactive microbial metabolites; study carried out by Gangwar *et al.*[19] strongly suggested that the endophytes associated with medicinal plant have very good antimicrobial activity and could be new sources of agricultural and medicinally important bioactive compounds. Scientific community is engaged in isolation and characterization of bioactive metabolite producing

Fig. 5: Antibiotic susceptibility test

microbes by using routine enrichment and screening techniques[20-21] but this article reported slightly different way to screen out bioactive compound producing bacteria. The present study is very good example of identification of antifungal compound producing bacterial species isolated from contaminated nutrient media plate. A contaminated YPD agar plate with a bacterial species isolated from soil and fungal growth around showed a clear inhibition of fungal growth in vicinity of bacterial colony, while the fungus grew luxuriantly in entire plate otherwise; continued incubation for up to 10-12 days showed strong inhibition of fungi by bacterial species. This spurred interest for the detection, extraction, purification and characterization of antifungal metabolite from the bacterial isolate which could a potential candidate for biotechnological or medicinal use. Primary screening confirmed that bacterial metabolite was active only against fungal species while no or very less activity was observed against the tested bacterial species. This indicates towards the notion that the compound is active against eukaryotic cell system rather than prokaryotes. The antifungal activity too, was observed only if the bacterial isolate was pre- incubated for about 3-4 days and then fungi inoculated in same plate (dual culture), while no activity was observed if the isolate and test compound inoculated on same day. This indicates that the metabolite may be secondary metabolites which is produced only after sufficient growth of the producer organism.

CONCLUSION

The morphological, biochemical and molecular identification revealed the organism to be a Gram negative bacterium *Pseudomonas tolaasii.* Phylogenetic relationship analyses of the bacterial isolate indicating to be a novel strain. The sequence of 16S rRNA gene was submitted to NCBI, Accession code; KU533778. On the basis of result obtained from the present study was proved that bacterial contamination is may be a very important habitat for diverse type of microbes.

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