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ANTIMICROBIAL ACTIVITY OF *AMYCOLATOPSIS ORIENTALIS* JAR10 FROM AGRICULTURAL FIELD

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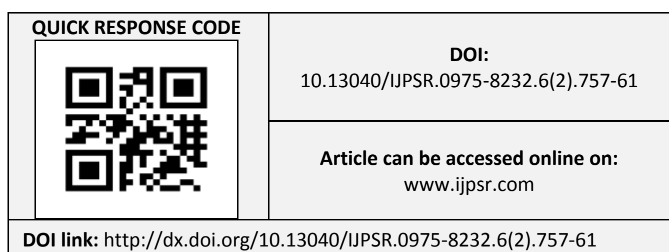
ABSTRACT: Actinomycetes are versatile and prolific source of microbial natural products with great advantage in medicine and agriculture. Recently there is growing interest in exploring biological active compounds from rare actinomycetes. Usually rare actinomycetes are regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of *Streptomycetes* strains. *Amycolatopsis orientalis* JAR10 rare genera of actinomycetes which have been isolated, characterized and antimicrobial activity against various pathogens have been determined in the present work. The morphological, cultural, genotypical and physiological characteristics of strain JAR10 has been examined by International *Streptomyces* Project. The bioactive metabolites produced by strain JAR10 were extracted by solvent extraction method and the crude extract obtained was further analyzed against various pathogens. The minimum inhibitory concentration of crude extract obtained from *Amycolatopsis orientalis* JAR10 was analyzed by broth dilution method and was found to inhibit *Staphylococcus aureus* at 60 µg/ml indicating the presence of biological active compounds.

INTRODUCTION: Antibiotics have always been considered as one of the greatest achievement of mankind against various infectious diseases however the rise of antibiotic resistance in hospitals, communities is alarming due to the indiscriminate use. The continuing problem of combating antibiotic resistance has obliged researchers to develop new antimicrobial agents. The antimicrobial research has been geared towards the discovery of novel chemical structures and therapeutic agents [1].



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Natural products are considered as low molecular weight compounds with therapeutic properties and have been the source of most of the active ingredients of medicines whereas, microbial natural products are the prolific source of therapeutic agents. Among microbes, actinomycetes are the largest producers of bioactive metabolites as they are Gram positive bacteria with high G and C content.

More than 70-80% of commercial antibiotics have been obtained from actinomycetes genera. With the discovery of streptomycin, a large number of antibiotic including major therapeutic agents including amino glycosides, chromaphenicol, tertacyclines, macrolides, β-lactam cephamycin group have been isolated from the group of streptomyces [2]. The discovery of new antibiotics

moved has to less exploited genera, the logic behind these approaches is to isolate strains with novel therapeutic values [3]. The less exploited rare genera of actinomycetes such as *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Microbiospora*, *Micromonospora* and *Streptosporangium* are difficult to isolate by conventional methods. This novel genera can be isolated by taking into account the several factors such as selection of appropriate habitat, chemical and physical treatment of sample collected, use of selective specific media and genus specific methodologies for screening isolates [4].

In the present work, isolation, characterization and antimicrobial activity of less exploited rare genera of actinomycetes, *Amycolatopsis orientalis* strain JAR10 have been determined.

MATERIALS AND METHODS:

Sample collection and isolation:

Soil samples were collected from agricultural field in Ludhiana, Punjab and isolation of actinomycetes was performed by standard microbiological serial dilution method followed by pretreatment of soil sample with dry heat at 100°C. The isolation of actinomycetes was carried out on Starch casein nitrate medium (soluble starch 10 g^{-1}, casein 0.3 g^{-1}, NaCl 2 g $^{-1}$, KNO_3 2 g^{-1}, K_2HPO_4 2 g^{-1}, $MgSO_4.7H_2O$ 0.5 g^{-1}, $CaCO_3$ 0.02 g^{-1}, $FeSO_4.7H_2O$ 0.01 g^{-1}) at pH 7.0 incubated at 28 °C for 10-14 d.

Morphological and physiological studies:

The morphological properties of isolated strain JAR10 was studied according to previously mentioned protocols by De Borr [5] and Good fellow [6]. The carbon and nitrogen source utilization was performed by following the method of Gordon by using basal medium. The spore morphology of isolated strain JAR10 was determined by Scanning electron microscope (FE-SEM). Strain JAR10 was studied for antibiogram study by performing Kirby-Bauer disc-diffusion assay described by Kavitha [8].

16S rRNA gene sequencing:

The extraction of genomic DNA of strain JAR10 was performed by using Rainey [9] protocol. The amplification of 16S-rRNA gene was carried out by using forward primer of 400 ng 5′-AGAGTRTGATCMTYGCTWAC-3′ and reverse primer of 400 ng 5′- CGYTAMCTTWTTACGRCT-3′, 2.5 mM each of dNTPs, 10X Taq polymerase assay buffer and Taq DNA polymerase enzyme keeping the reaction volume upto 100 µl. The PCR amplification reaction was further followed by initial denaturation at 94°C for 5 min to improve the denaturation of the DNA 5% (v/v) DMSO was added to the reaction mixture. After denaturation, annealing at 55°C for 30 s was carried out leading to final extension at 72°C using $MgCl_2$ with 1.5 mM final concentration.

The amplified product was sequenced with the primer using ABI 3730xl genetic analyzer (Amnion Biosciences Pvt. Ltd.). The phylogenetic position of strain JAR10 was determined by performing a nucleotide sequence database search using the BLAST program from National Centre for Biotechnology Information (NCBI) GenBank. The nucleotide sequencing result was submitted to the GenBank NCBI and accession number obtained was KJ3960801.

Optimization of culture medium:

250 ml of Erlenmeyer flask containing 50 ml of different medium yeast extract-malt extract medium (ISP2), Starch casein nitrate broth (SCNB), Inorganic salt medium (ISP4), Maltose-tryptone broth (MTB) and Bennett medium (BM) was sterilized using autoclave. Spore suspension was prepared from 10 d well grown culture of strain JAR10 in 0.05% of Tween 20 solution. 5% of spore suspension was added to 50 ml different medium and were incubated on rotary shaker at 28 °C for 15 d. The biomass with the bioactive metabolite was recorded at 600 nm (optical density) and antimicrobial activity was determined against clinical pathogens after 3, 6, 9, 12 and 15 d.

Fermentation, isolation and extraction of bioactive metabolites:

Spores of strain JAR10 was scrapped from 10 d old slant and was cultivated in YMD broth as seed medium and incubated in rotary shaker at 220 rpm at 28°C for 48 h. 10% of the seed medium was inoculated into the optimized fermentation medium for the production of bioactive metabolites consisting of Maltose tryptone medium. The fermentation was carried out for 7 d at 28°C with continuous agitation at 260 rpm. The fermented

Amycolatopsis orientalis JAR10 culture of 2.5 L was obtained after 10 d when the fermentation was completed. The culture filtrate was centrifuged at 2000 x g at 4°C for 10 min. The organic solvent (ethyl acetate) and culture filtrate was vigorously shaken for an hour in separating funnel and kept stationary for another 30 min to separate the aqueous layer. The dark brown color filtrate was obtained after extraction.

Antimicrobial activity:
The crude extract obtained was analyzed against Gram negative bacteria such as *E. coli, Shigella* sp., *Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella* sp. and Gram positive bacteria *Staphylococcus aureus, Enterococcus* sp.

The pathogenic microorganisms were procured from Microbial Biotechnology Laboratory, SBST, VIT University, Vellore, India. The antibiosis studies of clinical bacterial pathogens were determined against standard antibiotics vancomycin (30 µg/disc), tigecycline (15 µg/disc), erythromycin (15 µg/disc), ciprofloxacin (30 µg/disc), penicillin (10 µg/disc), ofloxacin (5 µg/disc) and fungal clinical plant pathogen were screened against flucanazole (25 µg/disc) and voriconazole (5 µg/disc) by disc-diffusion method [10].

Minimum inhibitory concentration:
The MIC of bioactive metabolite produced by strain JAR10 was determined by broth dilution method in the culture tube containing nutrient broth and the final volume was adjusted to 5 ml according to Boruwa protocol [11].

The bacterial pathogens were adjusted to a final inoculum size of 3×10^5 colony forming units (cfu/mL) and nutrient broth without active compound served as control. After inoculation the culture tubes were shaken well and then incubated at 37°C for 24 h and were observed for turbidity. Turbidity was observed in all the tubes including control tubes and to determine the MIC of bacterial pathogens 10 µl content from each tube was spread onto nutrient agar plates at different intervals for 24 h. MIC of bioactive compound was defined as the lowest concentration at which the pathogens were inhibited 100% as against control.

RESULTS AND DISCUSSION: The strain JAR10 was isolated from agricultural field of Ludhiana, Punjab and on the basis of morphological, physiological and cultural characteristics it was confirmed that strain JAR10 belongs to rare actinomycetes genera. The soil sample was subjected to dry heat at 100°C following standard sterilization procedure. The 16S r-RNA gene sequencing of strain JAR10 resulted in 99% of similarity with *Amycolatopsis orientalis*, therefore the strain JAR10 was designated as *Amycolatopsis orientalis* strain JAR10 which was submitted in the NCBI genbank and accession number KJ396080.1 was obtained. The phylogenetic tree of strain JAR10 was constructed by calculating bootstrap values by neighbor tree joining method which has been presented in **Figure 1.**

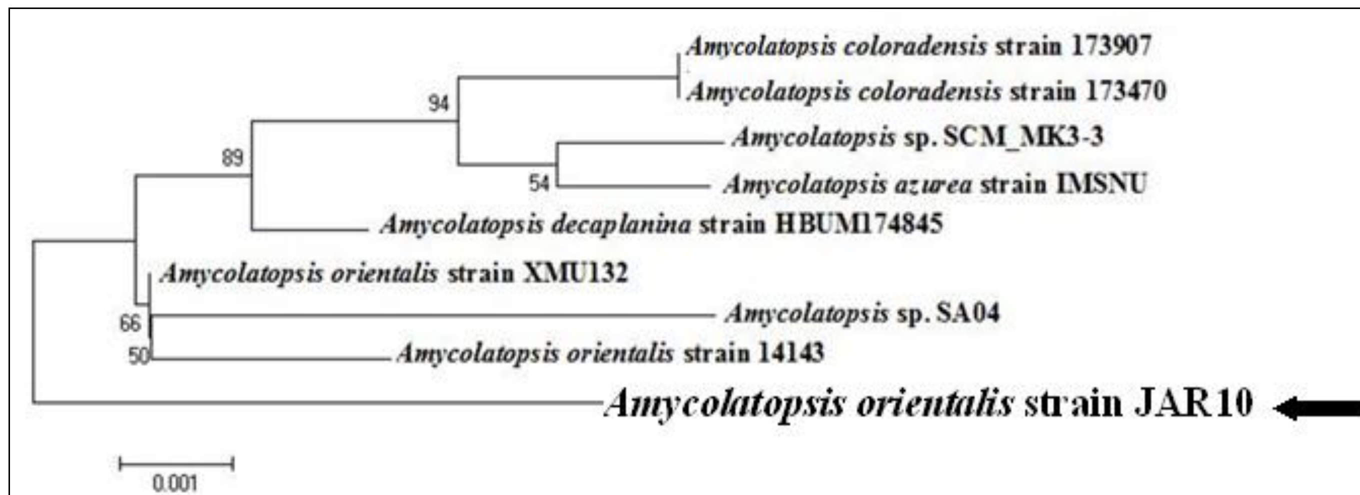

FIGURE 1: PHYLOGENETIC TREE REPRESENTING POSITION OF STRAIN JAR10 WITH NEIGHBOR JOINING METHOD.

The color of aerial mycelium was found to be pale white color whereas, color of substrate mycelium was found to be peach and strain JAR10 exhibited no reverse and melanoid pigment production after 7 d of incubation on ISP2 medium. Pronounced growth of strain JAR10 was noticed on ISP5 and Maltose tryptone agar medium whereas poor growth was monitored on ISP1 and ISP4.

The strain JAR10 utilized almost all the carbon sources such as D-Glucose, D-sucrose, D-mannitol, D-lactose, D-fructose, arabinose, xylose, maltose, inositol and rhamanose but among all sugars glucose and maltose. The antibiotic resistance of strain JAR10 against commercial antibiotics resulted in resistance to most of the antibiotics such as tigecycline (15 µg/disc), penicillin (10 µg/disc), chloramphenicol (30 µg/disc), vancomycin (30 µg/disc), erythromycin (15 µg/disc), fluconazole (25 µg/disc) and methicillin (10 µg/disc) and was found to be sensitive against streptomycin (10 µg/disc), gentamicin (10 µg/disc), ampicillin (10 µg/disc), kanamycin (10 µg/disc), ciprofloxacin (30 µg/disc) and tetracycline (30 µg/disc) which states that the biological active compounds produced by strain JAR10 may be may be responsible for the resistance of the strain to these antibiotics [8]. The morphological, cultural and physiological parameters of strain JAR10 has been presented in **Table 1** and **Table 2**.

TABLE 1: MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF JAR10 STRAIN

S. No.	Culture Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusable pigment	Melanoid Pigment
1	Tryptone-yeast agar medium (ISP-1)	Poor	-	-	-	-
2	Yeast extract malt-extract agar (ISP-2)	Very poor	Pale white	-	-	-
3	Oatmeal agar (ISP-3)	Good	White	-	-	+
4	Inorganic salt-starch agar (ISP-4)	Poor	White	Cream	-	-
5	Glycerol asparagine agar (ISP-5)	Very Good	White	Cream	Red	+
6	Peptone yeast iron agar (ISP-6)	Good	White	White	-	-
7	Tyrosine agar (ISP-7)	Good	White	Cream	-	-
8	Starch-casein nitrate agar	Good	White	Cream	Peach	-
9	Sabourad agar	Good	White White	White	Peach	-
10	Maltose-tryptone agar	Poor		White	-	-

*Note: Present- +, Not Present –

TABLE 2: PHENOTYPICAL CHARACTERISTICS OF JAR10 STRAIN

S. No.	Utilization of carbon sources	Strain JAR9	Antibiotic	Zone of inhibition in mm
1	D-glucose	++ +	Tigecycline (15 µg/disc)	S (22)
2	D-sucrose	+	Penicillin (10 µg/disc)	R
3	D-mannitol	+	Streptomycin (10 µg/disc)	S (18)
4	D-lactose	+	Chloramphenicol (30 µg/disc)	R
5	D-Fructose	+	Vancomycin (30 µg/disc)	R
6	Arabinose	+	Gentamicin (10 µg/disc)	S (9)
7	D-xylose	++	Ampicillin (10 µg/disc)	S (25)
8	Maltose	+ ++	Kanamycin (30 µg/disc)	S (28)
9	Inositol	++	Ciprofloxacin (30 µg/disc)	S (42)
10	Rhamanose	++	Erythromycin (15mcg/disc)	R
11	H_2S production	-	Methicillin (10 µg/disc)	R
12	Citrate utilization	-	Tetracycline (30 µg/disc)	S (30)
13	Gelatin	-	Fluconazole (25 µg/disc)	R
14	Urease	-	Voriconazole (5 µg/disc)	S (18)

*Note: P-positive, W-weak, N-negative result, R-Resistant, S-Sensitive, mm-millimeters, µg-micrograms.

The optimized culture medium, Maltose tryptone medium was used for fermentation and for the production of bioactive metabolite. After 7 d of fermentation the clear supernatant was extracted with liquid-liquid extraction using ethyl acetate in separating funnel. The obtained crude extract was dried under reduced atmospheric pressure and it was found to be active against *Staphylococcus aureus* with 24 mm of zone of inhibition. The minimum inhibitory concentration was found to be 60 µg/ml against *Staphylococcus aureus* broth dilution method. The rare genera of actinomycetes

exhibited good antimicrobial effect against multi drug resistant *Staphylococcus aureus* which indicates the potential biological aspects of rare actinomycetes. *Micromonospora*, *Nocardia*, *Actinoplanes* and *Amycolaptosis* belong to the rare genera of actinomycetes which contribute to bioactive metabolites at lesser account. But *Amycolaptosis* with good antimicrobial effect against Gram positive bacteria have been previously reported by Zhang [12]. As *Streptomycetes* is the largest and efficient producers of bioactive metabolites, the rare genus was never focused on antibiotic production. With the discovery of *Nocardia* sp. researchers started focusing on rare genera of actinomycetes in order to produce bioactive metabolites in terms of pharmaceutical applications.

CONCLUSION: The present study concludes that the rare actinomycetes are prolific source of biological active compounds and *Amycolatopsis orientalis* JAR10 is a potent inhibitor of *Staphylococcus aureus.* Pretreatment of soil sample by dry heat, addition of phenol and calcium carbonate enhances the production of rare actinomycetes than *Streptomyces* strain. Furthermore, studies are to be carried out regarding pharmaceutical applications of *Amycolatopsis orientalis* JAR10.

DECLARATION OF INTEREST: The authors report no conflicts of interest.

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