Research Article

Evaluation of Antimicrobial Compounds from *Amycolatopsis decaplanina* JAR8 against Various Pathogens

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ABSTRACT

Amycolatopsis decaplanina JAR8 is rare actinomycetes which was isolated from agricultural field of Punjab, India. The 16S rRNA gene sequencing of strain JAR8 showed 98% similarity with *Amycolatopsis decaplanina*. The morphological, cultural and physiological characteristics were determined by International *Streptomyces* project by using various mediums. The bioactive metabolites produced by the strain were obtained from the optimized culture medium through solvent extraction method. The ethyl acetate extract obtained after extraction was characterized using UV, FTIR and GC-MS spectroscopic analysis. The minimum inhibitory concentration of active metabolite was found to be 60 µg/ml against *Staphylococcus aureus*. The present study deals with the exploration of rare actinomycetes which also produces various biological active compounds against infectious diseases.

Keywords: Antibiotics, Clinical pathogens, Gas chromatography, Minimum inhibitory concentration.

INTRODUCTION

The continual emergence of multi-drug-resistant bacteria is the major concern worldwide and antibiotic resistance continues to be a significant medical problem across the globe. Antimicrobials are natural, semi synthetic or synthetic substances that destroy and inhibit the growth of micro-organisms and antimicrobial resistance is the ability of micro-organisms to resist the effect of antimicrobials.[1] According to the recent reports released by Centers for Disease Control it has been stated that mortality rates between 40% and 50%, rose from 1.2% to 4.2% over the decade from 2001 to 2011 due to infections caused by carbapenem-resistant Enterobacteriaceae (CRE). Carbapenems are the last key antibiotics against Gram-negative bacteria and this steady erosion of their efficacy is especially concerning.[2] The cause is due to the spread of genes that encode enzymes that destroy these antibiotics, in particular KPC (*Klebsiella pneumoniae* carbapenemase) and NDM (New Delhi metallo-beta-lactamase).[3] The outbreaks of infectious diseases caused by multi-drug resistance bacteria has been reported in many health care centers across the world and the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be threat in the community as well as in hospitals. According to Wright, antibiotic resistance is the natural phenomenon which is the result of selection for genetic elements in bacteria that confer the ability to grow in the presence of toxic compounds. In various studies, it has been reported that resistance genes are prevalent in 30,000 year old permafrost samples, and in bacteria living in a cave, sealed from the surface 4 million years ago[4-5] the ability to exchange these genes through mobile genetic elements such as plasmids ensures that antibiotic resistance traits can spread efficiently through bacterial communities.

To overcome the ever increasing antimicrobial resistance among bacteria, natural products produced by fungi and bacteria serve as a starting point for antibiotic resistance. Over past 70 years, pharmaceutical industry has been responsible for bringing all the antibiotics in current clinical use to market and among microbes as actinomycetes is the largest producer of antibiotics. Actinomycetes are Gram-positive bacteria of the order actinomycetales; they are characterized by filamentous morphology, DNA with high G+C content and presence or absence of LL-Diaminopimelic acid (LL-DAP) with characteristic sugars in the cell wall.[6] Various antimicrobial agents have been isolated and characterized from actinomycetes including aminoglycosides, anthracyclines, glycopeptides, macrolides, beta-lactams, polyenes, phenazine, and tetracyclines.[6-7] The *Sterptomyces* genus exhibits a great numbers of diverse and versatile biological active compounds possessing antitumor[8], antibacterial[9], antiparasitic[10] antifungal and immunosuppressive.[11] About 70–80% of relevant secondary metabolites account under *Streptomyces*, with small contributions from other genera, such as *Saccharopolyspora*, *Amycolatopsis*, *Micromonospora* and *Actinoplanes*[12] *Amycolaptosis* falls under rare actinomycetes belonging to the family *Pseudonocardiaceae* which produces antibiotics against Gram positive bacteria such as *Streptococcus*, *Staphylococcus* and these organisms are not as easy to isolate as *Streptomyces* or other bacterial and fungal organisms.[13] *Amycolaptosis* is less explored genera for antibiotic production but recent studies suggest that it is a potent source of antibiotics and bioactive compounds.[14-19] The present investigation deals with isolation, characterization, optimization and biological activities of *Amycolatopsis decaplanina* strain JAR8.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from agricultural field near Batala, Punjab India in the month of January 2012 following sterilized conditions. Soil samples were pre-treated by heating at 100°C for 60 min to reduce the population of bacterial species. After which the samples were pre-treated with calcium carbonate for 7-10 d to isolate actinomycetes.

Isolation

Amycolatopsis decaplanina JAR8 was isolated from $CaCO_3$ pre-treated soil samples on starch casein nitrate agar 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 d. The isolated strain was further maintained on yeast extract-malt extract-dextrose (YMD) agar medium at 4°C.[20]

Phenotypic and taxonomic studies

The cultural characteristics of mature sporulating aerial and substrate mycelium of *Amycolatopsis decaplanina* JAR8 were observed after 14 d of incubation on International *Streptomyces* project (ISP) and non-ISP medium described by Shirling and Gottlieb.[21] The utilization of different carbon sources by the isolated strain was examined using Shirling and Gottlieb[21] protocol. The melanin production was observed on peptone-yeast extract iron agar (ISP medium 6) and tyrosine (ISP medium 7) agar. The sensitivity of isolated strain was determined against various antibiotics using Kirby-Bauer method. The morphology of strain JAR8 was analyzed on 14 d of grown culture and the arrangement of the spores were determined by field-emission scanning electron microscope (FE-SEM). The strain JAR8 was allowed to grow on oatmeal agar for 14 d and healthy spores were scrapped from the petridish. The collected spores were then washed with ethanol and stored in refrigerator at 4°C. The spores were completely dried on the stubs and then coated with the thin film of gold in vacuum evaporator. The spore morphology of the strain JAR8 was examined by scanning electron microscopy (HITACH, Model S-3400N) coated with gold to avoid charging.

Genotypic characterization

The genomic DNA extraction of *Amycolatopsis decaplanina* JAR8 was performed by using Rainey[22] 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 *Amycolatopsis decaplanina* JAR8 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 is KJ396079.

Optimization of culture medium

250 ml of Erlenmeyer flask containing 50 ml of basal medium $(NH_4)_2SO_4$ 2.64 g^{-1}; KH_2PO_4 2.38 g^{-1}; $MgSO_4.7H_2O$ 1.00 g^{-1}; $CuSO_4.5H_2O$ 0.0064 g^{-1}; $FeSO_4.7H_2O$ 0.0011 g^{-1}; $MnCl_2.4H_2O$ 0.0079 g^{-1}; $ZnSO_4.7H_2O$ 0.0015 g^{-1} at pH 8 was sterilized using autoclave.[23] Various carbon and nitrogen sources including glucose, lactose, starch, sucrose, maltose, mannitol and nitrogen sources including peptone, sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), yeast extract, casein and soybean meal were added to basal medium at 1% of concentration. Spore suspension was prepared from 10 d well grown culture of *Amycolatopsis decaplanina* JAR8 in 0.05% of Tween 20 solution. 5% of spore suspension was added to 50 ml basal medium supplemented with various carbon and nitrogen sources and were incubated on rotary shaker at 28°C for 15 d. The inorganic salt medium without carbon and nitrogen sources served as control. 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 *Amycolatopsis decaplanina* JAR8 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 soluble starch 10 g^{-1}, casein 0.3 g^{-1}, soybean meal 10 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 7.2. The fermentation was carried out for 10 d at 28°C with continuous agitation at 260 rpm. The fermented *Amycolatopsis decaplanina* JAR8 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. 1.19 g of the brownish gummy metabolic product was recovered from separating funnel.

Fatty acid methyl ester analysis

The strain JAR8 was cultivated on trypticase soy broth for 10 d and biomass was harvested and completely dried before analysis. 40 mg of the harvested biomass was extracted, methylated and characterized through gas-chromatography mass spectroscopy using standard microbial identification system (MIDI).

Characterization

The characterization of metabolic product recovered after fermentation was analyzed by thin layer chromatography using various solvent systems such as hexane:formic acid:acetic acid (2:1:1) and $CHCl_3$: MeOH (90:10). The bands were observed by keeping the TLC plate in iodine vapor chamber. The active elutes were characterized by UV-Vis absorption spectra, fourier-transform infrared spectroscopy (FT-IR), gas chromatography mass spectrometry (GC-MS).

Biological assays

Antimicrobial effect

Test organisms

The Gram negative bacteria including *E. coli, Shigella* sp., *Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella* sp. and Gram positive bacteria *Staphylococcus aureus, Enterococcus* sp. and fungal strains including *Candida tropicalis, Fusarium* sp. and plant pathogens *Aspergillus terreus* strain JAS1, *Scedosporium* sp. JAS1, *Ganoderma* sp. JAS4 were procured from Microbial Biotechnology Laboratory, SBST, VIT University, Vellore, India. Bacterial and fungal clinical and plant pathogenic isolates were maintained on Nutrient agar and Potato Dextrose Agar respectively. The antibiosis studies of clinical bacterial isolates 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.[20]

Primary and secondary screening

The isolated strain *Amycolatopsis decaplanina* JAR8 was streaked horizontally onto modified nutrient agar medium consisting glucose 5 g^{-1}, peptone 5 g^{-1}, beef extract 3 g^{-1}, NaCl 3 g^{-1}, agar 15 g^{-1} and incubated for 5 d at 28°C. The pathogenic Gram negative and Gram positive bacteria were streaked perpendicular to the isolated strain on the modified nutrient agar plates and then incubated at 37°C for 24 h. The active metabolite inhibition was determined by measuring the zone of inhibition against test organism.

Secondary screening of the isolated strain was examined by Kirby-Bauer method on Muller-Hinton Agar. The Muller-Hinton agar plates were seeded with 100 µl of test organism, 6 mm diameter of four wells was punctured onto agar plates. The active elute with different concentration of 25 µl, 50 µl, 75 µl and 100 µl was added into four wells. The agar plates were further incubated at 37°C for 24 h and zone of inhibition was measured.

Minimum Inhibitory concentration

The MIC of bioactive metabolite produced by strain JAR8 was determined in the culture tube containing nutrient broth and the final volume was adjusted to 5 ml according to Boruwa[24] protocol. The nutrient broth without active compound served as control. The bacterial pathogens were adjusted to a final inoculum size of 3×10^5 colony forming units (cfu/mL). 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.

In-silico modeling studies

The crystal structure of Topoisomerase II chain A (PDB ID: 3ILWA) was obtained from protein data bank (PDB). The 3D structure of isolated bioactive metabolites were prepared in chem draw 3D and were saved as MDL mol file. All the docking studies were performed by iGemdock software tool. Crystallographic water molecules and non polar hydrogen atoms were removed and Topoisomerase A chain was used as receptor and bioactive metabolites produced by strain JAR8 were used as ligand for docking studies. The best docked files were chosen for the analysis of binding free energy and interaction between the ligand and receptor.

RESULTS AND DISCUSSION

The strain JAR8 was isolated from agricultural field and was identified as *Amycolatopsis decaplanina* by 16S rRNA gene sequencing. The particular sequence was subjected to similarity searches against public databases to infer possible phylogenetic relationships of strain JAR8 and it showed 98% similarity with *Amycolatopsis decaplanina*. Figure 1 represents the phylogenetic relation of strain JAR8 with other neighboring species. The morphological observations were recorded on the bases of International Streptomyces Project (7) which revealed the abundant growth of aerial and vegetative mycelium on ISP-4, ISP-2, ISP-5, ISP-6, ISP-7 and Kuster agar whereas poor growth was recorded on ISP-1, ISP-3 and Maltose-Tryptone agar. The aerial mycelium was recorded as pale white and strain JAR8 did not produce any diffusible pigment. The morphological characteristics of strain JAR8 has been presented in Table 1. The spore chain morphology has been presented in Figure 2.

The strain JAR8 utilized almost all the carbon sources including glucose, starch, inositol, lactose, dextrose, mannitol, maltose and fructose. The strain JAR8 tolerated

up to 3% of sodium chloride for its abundant growth, it showed coagulation of milk and lipolytic activity. Resistant to tigecycline, clindamycin, ampicillin, oxacilin, methicillin, penicillin, chloramphenicol, fluconazole and was only sensitive to vancomycin. The antibiotic sensitivity test suggests that biological active compounds produced by strain JAR8 may be responsible for the resistance of the strain to these antibiotics[25]. The physiological characteristics of strain JAR8 is represented in Table 2.

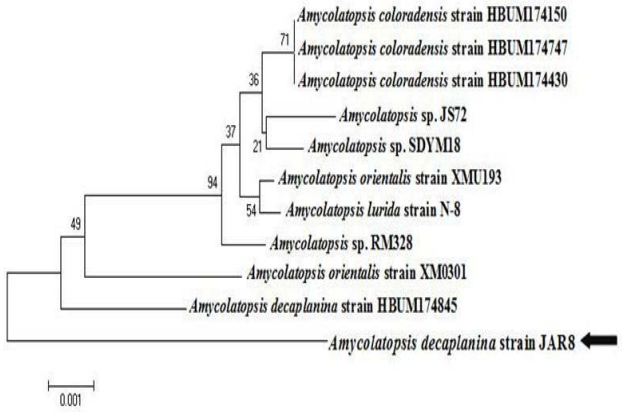

Figure 1: Phylogenetic tree representing position of strain JAR8 with neighbor joining method.

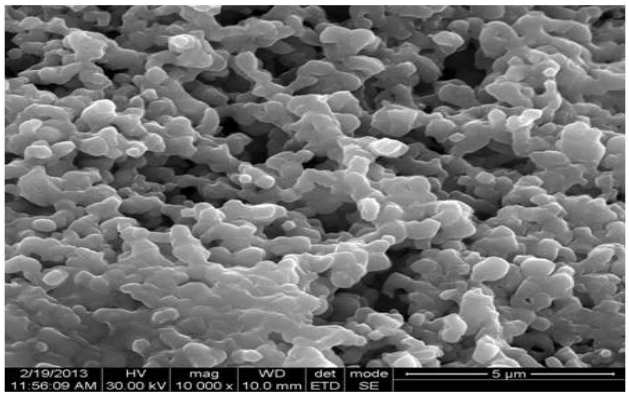

Figure 2: Spore morphology of *Amycolaptosis decaplanina* JAR8 on oatmeal agar medium

To extract bioactive metabolites strain JAR8 was grown in optimized medium for 10 d. After the fermentation was over bioactive metabolites were extracted using ethyl acetate as the organic solvent. The gas-chromatography of bioactive metabolite produced by strain JAR8 revealed various compounds: 10-Bromodecanoic acid ethyl ester with molecular weight of 278 at retention time 18.540, Ethyl 9-Hexadecenoate with molecular weight of 282 at retention time 18.34, Diethyl phthalate with molecular weight 222 at retention time 14.313 and Hentriacontane with molecular weight of 161.24 at retention time 16.4.

Table 1: Morphological and Cultural characteristics of strain JAR8

Culture Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment	Melanoid Pigment
Tryptone-yeast agar medium (ISP-1)	Poor	Creamish White	-	-	-
Yeast extract malt-extract agar (ISP-2)	Very Good	White	Red	Pale red	-
Oatmeal agar (ISP-3)	Good	White	-	-	-
Inorganic salt-starch agar (ISP-4)	Very Good	White	-	-	-
Glycerol asparagine agar (ISP-5)	Good	White	-	-	-
Peptone yeast iron agar (ISP-6)	Poor	-	Light red	-	-
Tyrosine agar (ISP-7)	Good	White	Pink	-	-
Starch-casein nitrate agar	Good	White	Light red	-	-
Sabourad agar	Poor	White	-	-	-
Maltose-tryptone agar	Good	White	-	Red	+

*Note: Present- +, Not Present –

The bioactive metabolite was analyzed for antimicrobial effect and it was found that strain JAR8 only inhibited *Staphlococcus aureus* with 23 mm zone of inhibition and minimum inhibitory concentration was found to be 60 µg/ml to inhibit the growth of *S. aureus* and did not show any inhibition against Gram negative bacteria. However effective fungal inhibition with 40 mm of zone of inhibition against *Candida tropicalis* was recorded. Among four antimicrobial metabolites diethyl phthalate showed docking with Topoisomerase II. Furthermore, *in-silico* docking studies of diethyl phthalate represent the binding of 'O4' atom with 'N6' of receptor with the measurement of 3.3 Å distance, the binding of 'N5' of with 'N1' of receptor with the measurement of 2.9 Å distance and the binding of 'OP1' of with 'NZ' with the measurement of 2.8 Å (Figure 3). *In-silico* results explain the binding resulting in the interaction of ligand with receptor with nucleotides at specific site which halts the further replication of particular receptor. *Amycolaptosis* is a rare actinomycetes genera and in the previous studies it has been mentioned that *Amycolatopsis mediterranei* is a rifamycin producer which is active against Gram positive bacteria. Among

actinomycetes, *Streptomyces* genera is the largest producer of antimicrobial agents and antibiotics whereas rare actinomycetes such as *Micromonospora*, *Nocardia*, *Actinoplanes* and *Amycolaptosis* contributes very less number of bioactive compounds but recent findings have shown that rare genera of actinomycetes are also valuable sources of pharmaceutical active compounds.

Table 2: Phenotypical characteristics of strain JAR8

Utilization of carbon sources	Strain BS-1	Antibiotic	Zone of inhibition in mm
D-glucose	P	Tigecycline (15 µg/disc)	R
D-sucrose	W	Penicillin (10 µg/disc)	R
D-mannitol	W	Streptomycin (10 µg/disc)	R
D-lactose	P	Chloramphenicol (30 µg/disc)	R
D-Fructose	P	Vancomycin (30 µg/disc)	R
Arabinose	W	Gentamicin (10 µg/disc)	R
D-xylose	P	Ampicillin (10 µg/disc)	R
Maltose	P	Kanamycin (30 µg/disc)	S (29)
Inositol	P	Ciprofloxacin (30 µg/disc)	R
Rhamanose	W	Erythromycin (15mcg/disc)	R
H_2S production	N	Methicillin (10 µg/disc)	R
Citrate utilization	N	Tetracycline (30 µg/disc)	R
Gelatin	N	Fluconazole (25 µg/disc)	R
Urease	N	Voriconazole (5 µg/disc)	R

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

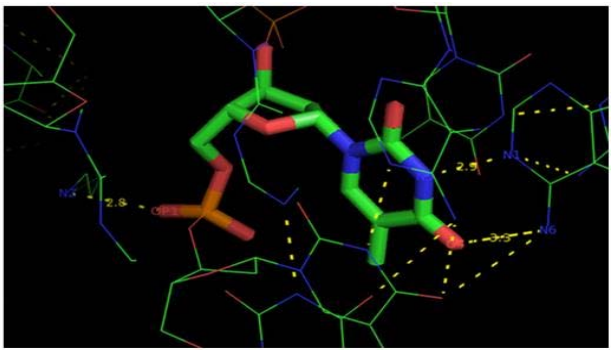

Figure 3: Docking of diethyl phthalate with Topoisomerase II representing interactions and surface docked pose

CONCLUSION

The present investigation deals with the exploration of biological activities of rare actinomycetes as *Amycolatopsis* is the less explored genus. Rare actinomycetes are the fastidious organisms with diverse, unique and excellent bioactive potential which have been presented in our study through *in-vitro* as well as *in-silico* studies.

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