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Original Research Article

Anti-MRSA potential and metabolic fingerprinting of actinobacteria from Cholistan desert, Pakistan

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Abstract

Purpose: *To investigate the actinomycetes from an extreme environment for their inhibitory potential against methicillin-resistant Staphylococcus aureus (MRSA), and the metabolic fingerprinting of the active strains.*
Methods: *A total of 80 actinomycetes strains were recovered from Cholistan desert, Pakistan. The isolated strains were identified by morphological, biochemical and physiological characterization and by 16S rRNA gene sequencing. The antimicrobial activity of the selected actinomycetes strains against MRSA was determined by agar well and disc diffusion assays. All the strains were screened against MRSA for the identification of potent antimicrobial producers. Further, validation of MRSA, strains was carried out using a portion of mec-A gene (533bp) of five strains including A1, A6, A7, A8 and A9, amplified and sequenced.*
Results: *The desert actinomycetes strains exhibited promising antimicrobial activity against MRSA with zone of inhibition of up to 25 mm recorded in agar diffusion and disc diffusion assays. The MRSA strains also showed maximum genetic similarity with methicillin-resistant Staphylococcus aureus in GenBank. Most of the actinobacterial strains exhibited 99 % genetic similarity with the genus Streptomyces, including strains AFD6, AFD12, AFD23, AFD25, and AFD26 while isolate AFD18 has 100 % similarity with a Pseudonocardia, named Saccharothrix xinjiangensis.*
Conclusion: *The results reveal that actinomycetes from the desert ecosystem studied are significant producers of useful antimicrobial agents, and should be explored further for novel drug candidates against MRSA.*

Keywords: *Anti-MRSA potential, Actinomycetes, Extreme environments, Metabolic fingerprinting, 16S rRNA gene sequencing, Mec-A gene characterization*

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INTRODUCTION

The multidrug resistant (MDR) pathogens are increasing significantly and have proved as a global economic and healthcare crisis. This phenomenon is listed to be a threat to global public health by the World Health Organization (WHO). *Staphylococcus aureus* is a component of the normal flora of the naso-pharynx of healthy humans. However, *S. aureus* has the capacity to strategically cause various diseases from simple skin infections to fatal necrotizing pneumonia and

infective endocarditis, resulting in substantial global human ailment and death [1].
There are reports of resistance against methicillin that is considered the drug of choice to treat serious infections. The first resistant isolate of *Staphylococcus aureus* appeared two years after the introduction of penicillin in 1944. The society for healthcare epidemiology of America suggested that it is the most common etiological agent of wound infections and respirator-associated pneumonia, causing 30 and 24 % of cases, respectively [2]. The data from Pakistan suggested that 4 - 51 % of healthcare-associated *S. aureus* cases were reportedly caused by MRSA. The use of glycopeptides (vancomycin and teicoplanin) is on the increase because of the increase in the incidence of MRSA infections. This rapid alteration in mechanism that confirms resistance is alarming [3].

Actinomycetes are biotechnologically invaluable class of prokaryotes producing bioactive secondary metabolites especially antibiotics, anticancer agents, immunosuppressive agents and enzymes [4]. These are the prime sources of clinically important antibiotics making three quarters of all known antibiotics, most of which are too complex to be synthesized by conjugative chemistry. Among actinomycetes, the *Streptomyces* are more competitive, producing approximately 80% of total antibiotic products. *Micromonospora* constitute the second best with less than one-tenth as many as *Streptomyces*. To avoid the redundancy in the discovery of antimicrobial compounds from normal habitats, scientists have been searching unexplored ecosystems like deserts, lakes, marines etc., for the discovery of novel bioactive compounds [5].

Desert is a rare ecological niche with respect to antimicrobial research. The present study specifically aims at the exploration of a unique and harsh ecosystem of desert in Pakistan, for the isolation and screening of actinomycetes for antimicrobial compounds against MRSA. Sand and soil samples were collected from Cholistan desert located in south-west of the Punjab province of Pakistan [6]. On the basis of parent material, topography and soil flora this desert is divided into two geomorphic regions Lesser Cholistan or northern region and Greater Cholistan or southern region. The climate of this desert is sub-tropical, rough, hot and tedious, and influenced by seasonal monsoons. A very notable feature of the Cholistan desert is the scarcity of rainfall for up to 4-6 years continually. In summer season the mean temperature varies from 35 to 50 ºC during May to June and in winter from 15 to 20 ºC during December to February [7].

EXPERIMENTAL

Sample collection

Fifteen soil and sand samples were collected from various sites of Cholistan desert in district Rahim Yar Khan, Pakistan, in clean polythene bags. The samples were processed by physical and chemical treatments (Heat treatment at 50 - 55°C for 3-7 days) following the methods described by Hayakawa and Nonomura [8] for the enrichment of actinomycetes.

Isolation of actinomycetes

One gram of each soil sample was suspended in 10 mL of sterile water and vortexing was done for 45 sec. Glycerol-casein-KNO_3 agar (glycerol 10 g, KNO_3 2 g, casein 0.3 g, NaCl 2 g, K_2HPO_4 2 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.01 g, agar 18 g in one liter) was prepared and sterilized at 121 ºC in 15 lbs pressure for 15 min. The media was supplemented with 50 µg/mL of nystatin to prevent the growth of fungal contaminants. The sand and soil samples were serially diluted and 0.1 mL of the dilution 10^{-3} was spread on the surface of the glycerol-casein-KNO_3 agar. The plates were then incubated at 28ºC for 7 - 15 days. The presumptive actinomycetes colonies were selected and were transferred to the cultivation medium for actinomycetes, *i.e.*, GYM agar (10 g malt extract, 5 g yeast extract, 5 g glucose, 15 g agar in one liter of distilled water) [9]. The selected colonies were purified by continuous sub culturing on GYM agar.

Identification of the actinomycetes

The selected actinomycetes strains were identified through morphological, biochemical and physiological characterization and by 16S rRNA gene sequencing. The morphological characteristics including colony morphology, aerial and substrate mycelia and pigmentation were studied after cultivating the individual strains on GYM agar by the methods described by Bensultana *et al*. [10]. The selected strains were investigated for biochemical characteristics including melanin production, sugar utilization as carbon source, formation of organic acids, organic acid and oxalate utilization, hydrolysis of esculin and urease. For 16S rRNA gene sequencing, genomic DNA of selected desert actinomycete strains was isolated from mycelia, by using tissue genomic DNA kit (FavorPrep™, Cat# FATGK001-1). PCR amplification of 16S rRNA gene was done by using primers (27f:

AGAGTTTGATCCTGGCTCAG) and (1522r: AAGGAGGTGATCCARCCGCA). The PCR products were purified by gel purification kit (FavorPrep^{TM}, Cat# FAGPK001-1) and the purified product was sequenced. In order to determine the genetic similarity of the strains with already reported data in gene bank, the sequenced data was analyzed through the BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST.

Collection and identification of MRSA

The clinical strains of methicillin resistant *S. aureus* (MRSA) were collected from Citi Lab, Lahore, Pakistan. Molecular characterization of MRSA was performed by *mecA* gene amplification [11] and by disc diffusion antibiotics sensitivity assay using methicillin (10 µg), oxacillin (1 µg) and cefoxitin (30 µg) discs according to CLSI standards 2017 [12].

Small scale cultivation of desert actinomycetes and preparation of crude extracts

The selected desert actinomycetes were cultivated on small scale as shaking cultures in GYM broth (500 mL to 1L culture broth) and the crude extracts were obtained which were screened biologically and chemically. The crude extracts were obtained by solvent-solvent extraction using ethyl acetate.

Determination of antimicrobial activity of desert actinomycetes against MRSA

Antimicrobial activity of the strains was determined by agar plug and well diffusion methods against MRSA strains by using the method described by Sajid *et al*. [13]. The results of the assays were recorded after overnight incubation by measuring the zones of inhibition around the wells in mm.

Metabolic fingerprinting of the methanol extracts

The extracts were screened chemically by TLC using two staining reagents and HPLC-UV as described below.

Thin-layer chromatography (TLC)

The crude extracts were analyzed on TLC plate (TLC Silica gel 60 F_{254}, Merck, Germany) to determine the presence of various compounds adopting the procedure described by Sajid *et al* [13]. Each of the samples was spotted repeatedly on the TLC plate by means of a capillary which was then developed with 5 % MeOH/CH_2Cl_2 solvent system and visualized under U.V. (254 and 366 nm) by a UV lamp (CAMAG). One of the plates was sprayed with anisaldehyde/sulfuric acid (methanol, acetic acid, H_2SO_4, anisaldehyde) and the other with Ehrlich's reagent (methanol, HCl 37 %, 4-dimethylamino benzaldehyde) for the detection of different compounds. The colored bands that appeared were marked and recorded [13].

High performance liquid chromatography (HPLC-UV) analysis

HPLC-UV analysis was performed on clarity chromatography data system (single channel serial port) Sykum S1122 delivery system. For the chromatographic separation, the column used was RPC18 from ThermoHypersil-Keystone (dimensions 250 x 4.6 mm and 5 µm particle size). Methanol and water (9:1) was used as mobile phase and flow rate was adjusted to 1 mL/min. The crude extract was dissolved in HPLC grade methanol and 20 µL of sample was injected into the system with the help of a micro-syringe. All of the samples were run for 20 min and UV absorbance of the crude extract of actinomycetes strains was determined at 254 nm. The peaks observed at different retention times (t_R) were later compared with the UV absorbance data of secondary metabolites in order to get an idea about the nature of compounds produced by the selected desert actinomycetes.

Bioautography

The crude extracts of desert actinomycetes obtained by solvent extraction were spotted on TLC plates (TLC Silica gel 60 F_{254}, Merck, Germany), which were developed with 5 % CH_2Cl_2/MeOH solvent system. For each of the sample, TLC plate was cut into two halves, one was used as a reference and sprayed with staining reagent (anisaldehyde/H_2SO_4) and the other half was placed inverted on the LB agar seeded with MRSA strain. This plate was then incubated for 24 h at 37°C. The zone of inhibition was measured after incubation and the active components were compared with the reference plate, marked and were scanned [13].

RESULTS

Taxonomic characteristics of the desert actinomycetes

A total of 30 desert actinomycetes strains were isolated, all of the strains showed hard, deep-rooted and rough colonies with regular or irregular margins and different colony sizes

ranges from pinpoint to large (*e.g.*, 3 mm). The color of the spores produced by these strains and their consistency was different. The strain AFD2, AFD3, AFD5, AFD6, AFD7, and AFD8 had greenish colored spores. The strains AFD1, AFD4, AFD9, AFD11, AFD23 and AFD29 produced yellow colored spores, while AFD10, AFD12 and AFD13 produced pink colored spores. The strains AFD15 and AFD20 produced orange colored spores, while all other strains produced white colored spores.

In biochemical and physiological characterization, the strains displayed the production of melanin pigment and exhibited their growth on different sugars as carbon source. Sixteen out of thirty strains tested were able to produce melanin including the strains AFD1, AFD2, AFD3, AFD6, AFD6, AFD7, AFD9, AFD12, AFD13, AFD15, AFD18, AFD20, AFD21, AFD21, AFD22, AFD23 and AFD29. All of the strains utilized glucose and mannose as carbon sources except only one strain AFD23 which could not grow on mannose. Fructose was utilized, except four strains namely AFD6, AFD14, AFD20 and AFD26. L-arabinose was used as a carbon source by nineteen of the strains, galactose and mannose by seventeen strains as carbon. The least utilized sugar was inositol as half of the strains were able to grow on it and the sucrose, only eight strains were able to use it namely AFD1, AFD2, AFD4, AFD9, AFD10, AFD19, AFD22 and AFD24 (Table 1). The comparison of taxonomic characteristics of these desert actinomycete strains with already reported data in Bergey's Manual of Systematic Bacteriology [14] provided a hint about their genus.

The genetic characterization of the strains was carried out by isolating the genomic DNA of eleven most anti-MRSA strains including AFD1, AFD2, AFD5, AFD6, AFD12, AFD15, AFD18, AFD20, AFD23, AFD25 and AFD26. The BLAST analysis of 16S rRNA gene sequences of the selected desert actinomycetes strains showed similarity with already reported 16S rRNA sequences in GenBank. The strain AFD6 showed 99 % similarity with *Streptomyces thermolilacinus* strain NBRC 14274, AFD12 and AFD25 showed 99 % similarity with *Streptomyces werraensis* strain NBRC 13404, AFD18 showed 100 % similarity with *Saccharothrix xinjiangensis* strain AS 4.1731, AFD23 and AFD26 showed 99 % similarity with *Streptomyces albaduncus* strain NBRC 13397, (Table 2).

Table 1: Melanin production and utilization of different sugars as carbon source by desert actinomycetes strains

Strains	Melanin production	D-glucose	D-fructose	L-arabinose	D-mannitol	Sucrose	Inositol	D-galactose	Mannose
AFD1	+	+	+	+	+	+	+	+	+
AFD2	+	+	+	+	+	+	+	+	+
AFD3	+	+	+	+	-	-	+	+	+
AFD4	-	+	+	-	-	+	-	-	+
AFD5	-	+	+	+	+	-	+	-	+
AFD6	+	+	-	+	+	-	-	+	+
AFD7	+	+	+	-	+	-	-	-	+
AFD8	-	+	+	+	-	-	+	-	+
AFD9	+	+	+	+	+	+	+	+	+
AFD10	-	+	+	+	+	+	+	+	+
AFD11	-	+	+	-	+	-	+	+	+
AFD12	+	+	+	-	+	-	+	-	+
AFD13	+	+	+	+	-	-	-	-	+
AFD14	-	+	-	+	-	-	-	-	+
AFD15	+	+	+	+	-	-	-	+	+
AFD16	-	+	+	-	-	-	-	-	+
AFD17	-	+	+	-	-	-	-	-	+
AFD18	+	+	+	+	-	-	-	+	+
AFD19	-	+	+	+	-	+	-	-	+
AFD20	+	+	-	+	-	-	-	-	+
AFD21	+	+	+	-	+	-	+	+	+
AFD22	+	+	+	+	+	+	+	+	+
AFD23	+	+	+	-	+	-	+	-	-
AFD24	-	+	+	+	+	+	+	+	+
AFD25	-	+	+	+	+	-	+	+	+
AFD26	-	+	-	+	+	-	+	+	+
AFD27	+	+	+	-	+	-	-	+	+
AFD28	-	+	+	-	+	-	-	+	+
AFD29	+	+	+	-	-	-	-	-	+
AFD30	-	+	+	+	-	-	-	+	+

Key: (+) = melanin production and growth on sugar, **(-)** = no melanin is produced and no growth on sugar

Table 2: GenBank accession numbers of the selected desert actinomycetes and MRSA strains along with their percentage similarities with different strains reported in GenBank

Desert actinomycete strains	No. of nucleotides sequenced (bp)	% Similarity with		Gene bank accession numbers
AFD6	1270	*Streptomyces thermolilacinus* strain NBRC 14274	99 %	KX131166
AFD12	950	*Streptomyces werraensis* strain NBRC 13404	99 %	KX131167
AFD18	1030	*Saccharothrix xinjiangensis* strain AS 4.1731	100 %	KX094938
AFD23	1430	*Streptomyces albaduncus* strain NBRC 13397	99 %	KX131165
AFD25	1000	*Streptomyces werraensis* NBRC 13404	99 %	KX131168
AFD26	1250	*Streptomyces albaduncus* strain NBRC 13397	99 %	KX131169
MRSA strains				
A1	1219	*Staphylococcus aureus* strain ATCC 12600	100 %	KU662352
A2	1190	*Staphylococcus aureus* strain NBRC 100910	100 %	KU662353
A5	1000	*Staphylococcus aureus* subsp. *aureus* N315 strain	100 %	KR862284
A6	1250	*Staphylococcus aureus* subsp. *aureus* N315 strain	100 %	KR862285
A7	1530	*Staphylococcus aureus* strain S33 R	99 %	KR862291
A8	1050	*Staphylococcus aureus* subsp. *anaerobius* strain MVF-7	100 %	KU662354
A9	1070	*Staphylococcus aureus* strain NBRC 100910	99 %	KR862287
A11	1290	*Staphylococcus aureus* strain ATCC 12600	100 %	KR862288
A12	1410	*Staphylococcus aureus* subsp. *aureus* JH1	97 %	KR862289
A14	1120	*Staphylococcus aureus* subsp. *anaerobius* strain MVF-7	100 %	KU662355

Taxonomic characteristics of MRSA

All of the MRSA strains showed yellowish, round, smooth, small colonies (pinpoint to 2 mm) with regular margins. Under the compound microscope (OLYMPUS CX21), all of these appeared as clusters of cocci which were gram positive. In biochemical characterization, all the strains showed the production of catalase enzyme by converting hydrogen peroxide to water and oxygen. All of these were capable to produce DNase enzyme. These were confirmed as MRSA based on their resistance to antibiotics methicillin (10 µg), oxacillin (1 µg) and cefoxitin (30 µg). According to the CLSI standards of 2017 [12] all of the strains exhibited less than 19 mm zone of inhibition against cefoxitin disc which was used as their confirmation test.

The genomic DNA of all the MRSA strains was extracted, amplified and characterized by using PCR amplification. The 16S rRNA gene sequence data of all the MRSA strains was analyzed by BLAST analysis which showed similarity with already reported 16S rRNA sequences in GenBank. The strain A1 showed 100 % similarity with *Staphylococcus aureus* strain ATCC 12600, A2 showed 100 % similarity with *Staphylococcus aureus* strain NBRC 100910, strain A5 showed 100 % similarity with *Staphylococcus aureus* subsp. *aureus* N315 strain, A6 showed 99 % with *Staphylococcus aureus* subsp. *aureus* N315 strain, A7 showed 99 % similarity with *Staphylococcus aureus* strain S33 R. The strain A8 showed 100 % similarity with *Staphylococcus aureus* subsp. *anaerobius* strain MVF-7, strain A9 showed 99 % similarity with *Staphylococcus aureus* strain NBRC 100910, the strain A11 showed 100 % similarity with *Staphylococcus aureus* strain ATCC 12600, A12 showed 97 % similarity with *Staphylococcus aureus* subsp. *aureus* JH1, strain A14 showed 100 % similarity with *Staphylococcus aureus* subsp. *anaerobius* strain MVF-7 (Table 2).

For further confirmation of the strains as MRSA, a portion of *mec-A* gene (533bp) of five strains including A1, A6, A7, A8 and A9 was amplified and sequenced. The BLAST analysis of this gene sequence data showed alignments with already reported *mec-A* gene sequences in GenBank. The *mec-A* gene sequence of MRSA isolate A1 showed 100 % similarity with *Staphylococcus aureus* JCSC6945 *mec-A* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase *mec-A*. The *mec-A* gene sequence of isolate A6 showed 100 % similarity with *Staphylococcus aureus* TN/CN/1/12 *mec-A* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase *mec-A*. The *mec-A* gene sequence of MRSA isolate A7 showed 100 % similarity with *Staphylococcus aureus* JCSC6943 *mec-A* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase *mec-A*. The *mec-A* gene sequence of isolate A8 showed 100 % similarity with *Staphylococcus aureus* TN/CN/1/12 *mec-A* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase *mec-A*. The *mec-A* gene sequence of MRSA isolate A9 showed 100 % similarity with *Staphylococcus aureus* subsp. *aureus* N315 *mec-A* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase *mec-A* (Table 3).

Antimicrobial activity of desert actinomycetes against MRSA

The selected desert actinomycete strains exhibited very promising antimicrobial activity against MRSA. In case of the actinomycete strain AFD2, maximum zone of inhibition recorded was 36 mm against MRSA isolate A11 and 16 mm zone against MRSA isolate A5, 24 mm against MRSA strains A6 and A7; 28 mm zone of inhibition against MRSA isolate A9 and 25 mm zone of inhibition against MRSA isolate A12. The other highly active actinomycete strain was AFD9, which exhibited maximum zone of inhibition against MRSA isolate A12 up to 30 mm, 26 mm zone of inhibition against MRSA isolate A5, 24 mm zone against MRSA strains A6, A7 and A11 while 23 mm zone of inhibition against MRSA isolate A9. Among other desert actinomycete, the strain AFD10 also showed very good antibacterial activity as it expressed 28 mm zone of inhibition against MRSA strains A5 and A12, and 22 mm zone of inhibition against MRSA isolate A9. The strain AFD22 was another very active strain and it exhibited 24 mm zone of inhibition against MRSA isolate A12, 22 mm zone of inhibition against MRSA isolate A11 while 20 mm zone of inhibition against MRSA strains A6, A7 and A9 and 19 mm zone of inhibition against MRSA isolate A5. The desert actinomycete strain AFD24 also exhibited good antimicrobial activity against different MRSA strains, it showed 22 mm zone of inhibition against MRSA isolate A12, 21 mm zone of inhibition against MRSA isolate A7, 20 mm zone of inhibition against MRSA strains A6 and A11, 18 mm zone of inhibition against MRSA isolate A5 and 17 mm zone of inhibition against MRSA isolate A9 (Figure 1 A, B and D, Table 4).

Chemical profile of methanol extracts of desert actinomycetes

In chemical profiling, the crude extracts of desert actinomycetes were analyzed by TLC and HPLC-UV. In case of TLC the UV visible spots were analyzed under UV at short and long wavelength (254 and 366 nm).

Table 3: Similarity of *mec-A* gene of MRSA strains with genes already repoted in GenBank

MRSA strains	No. of nucleotides sequenced	Similarity with	Score (%)
A1	510	*Staphylococcus aureus* JCSC6945 *mecA* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100
A6	510	*Staphylococcus aureus* TN/CN/1/12 *mecA* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100
A7	510	*Staphylococcus aureus* JCSC6943 *mecA* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100
A8	500	*Staphylococcus aureus* TN/CN/1/12 *mecA* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100
A9	500	*Staphylococcus aureus* subsp. *aureus* N315 *mecA* gene for PBP2a family beta-lactam-resistant peptidoglycan transpeptidase MecA	100

Table 4: Antimicrobial activity of the selected desert actinomycetes against various MRSA (methicillin resistant *Staphylococcus aureus*) strains

Actinomycete Strains	Zone of inhibition (mm) against various MRSA strains					
	A5	**A6**	**A7**	**A9**	**A11**	**A12**
AFD1	18	11	11	10	14	18
AFD2	16	24	24	28	36	25
AFD3	18	12	13	12	15	12
AFD4	16	13	12	10	10	14
AFD5	16	12	14	-	18	12
AFD6	8	-	-	10	-	-
AFD7	-	-	-	8	-	8
AFD8	10	-	-	10	10	-
AFD9	26	24	24	23	24	30
AFD10	28	22	21	22	20	28
AFD11	14	12	-	11	-	12
AFD12	16	12	12	14	14	12
AFD13	14	11	11	9	11	12
AFD14	-	17	23	18	11	-
AFD15	11	10	-	-	-	-
AFD16	14	14	12	11	11	10
AFD17	11	10	-	10	-	10
AFD18	12	-	-	-	-	-
AFD19	-	-	-	-	-	10
AFD20	15	16	14	18	14	18
AFD21	12	17	16	16	10	14
AFD22	19	20	20	20	22	24
AFD23	-	16	18	20	16	16
AFD24	18	20	21	17	20	22
AFD25	12	16	16	12	14	10
AFD26	12	16	16	14	12	12
AFD27	11	16	18	17	10	16
AFD28	11	13	11	14	10	11
AFD29	10	10	10	11	10	10
AFD30	14	24	13	-	12	10

Zone of inhibition in millimeters (mm)

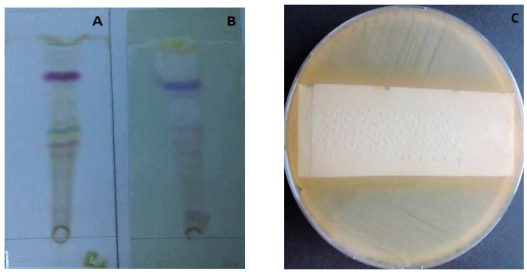

Plate 1: (A) Metabolic fingerprints of actinomycete strain AFD2 on TLC after treatment with anisaldehyde/H_2SO_4 and **(B)** Ehrlich's reagent; **(C)** Bioautography of actinomycete strain AFD9

Most of the components of the crude extracts of these strains had the absorptive capacity for UV and some noticeable bands were observed in the extracts of strains AFD2, AFD9, AFD10, AFD22 and AFD24 (Figure 2 A and B). The pattern of colored bands on TLC plates after treatment with different staining reagents (anisaldehyde/H_2SO_4 and Ehrlich's reagent) is visible in Figure 1 (E and F). The components of the crude extracts of strains AFD9, AFD10, AFD20, AFD22 and AFD24 produced different colors when treated with anisaldehyde/H_2SO_4 blue, green or violet spots appeared in 10 min (Figure 1 E) representing the presence of sugar molecules [15]. The treatment with Ehrlich's reagent resulted in blue colored bands representing the presence of indole or indole like compounds in the crude extract of AFD2 (Figure 1 F).

In HPLC-UV analysis of the crude extracts of the selected desert actinomycetes each of the strains displayed variety of peaks at different retention times (t_R). For instance, crude extract of the strain AFD23 exhibited 8 peaks and the major peak was at t_R 2.37 min, with the peak area of 5104.438 mV.s. (Figure 3).While the crude extracts of AFD6, AFD9, AFD18 and AFD27 showed number of peaks at different retention times (t_R) with different peak areas.

The bioautographic assay (Figure 1C) performed with crude extracts of actinomycete strains AFD13, AFD9 and AFD23 revealed that different fractions in the extracts exhibited antimicrobial activity against MRSA strains. The strain AFD9 produced 5 fractions, two of them exhibited pronounced antimicrobial activity against MRSA

strain A5 and one fraction exhibited antimicrobial activity against MRSA strain A6 (Figure 2 C, D).

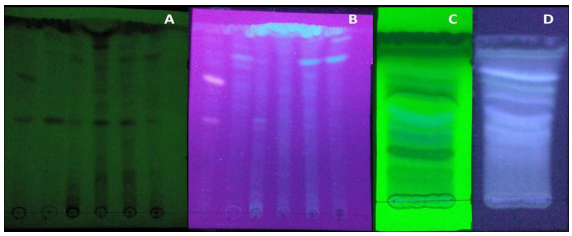

Plate 2: Metabolic fingerprints of various desert actinomycete strains on TLC under UV **(A)** 254 nm **(B)** 366 nm **(C)** bioautography of strain AFD9 under UV 254 nm **(D)** under 366 nm wavelength

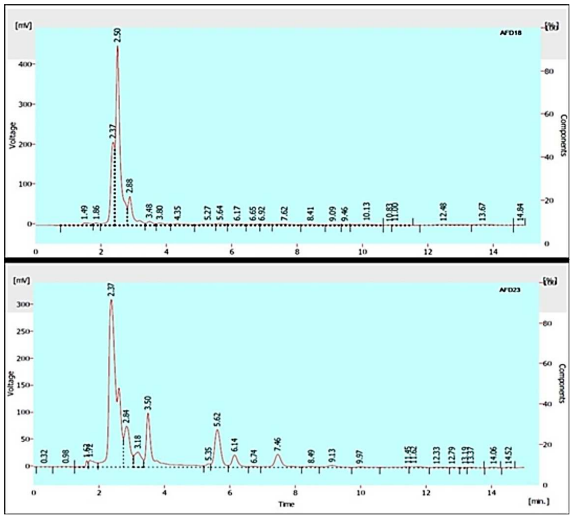

Figure 3: HPLC-UV chromatogram of methanol extract of actinomycete strains AFD18 (1st line) and AFD23 (2nd line) with peaks at different retention times (t_R)

DISCUSSION

Antibiotic resistance is emerging at a rate that exceeds the discovery of new drugs. Most antibiotics are produced by actinomycetes which are prolific producers of specialized metabolites or natural products. Due to increase in the advancements of technology, the need to explore the unexplored areas is also increasing. In the present study 30 strains of actinomycetes were isolated from the soil and sand collected from Cholistan desert which is an unexplored ecosystem located in the southern Punjab, Pakistan. These actinomycetes were characterized and identified on the basis of physiological, biochemical and genetic protocols. All of the strains produced hard and embedded colonies on glycerol-casein-KNO_3 agar medium which is a characteristic growth pattern of actinomycetes [16].

By biochemical characterization 16 of the strains were capable of producing melanoid pigments which is a characteristic feature of the genus *Streptomyces* [17]. The strains were able to utilize glucose, fructose and mannose as carbon sources as reported earlier [18]. *Streptomyces* was considered the most dominant genera isolated from desert soil worldwide in different studies. The identification of any organism up to species level is not only possible through biochemical and physiological testing, but it also requires extensive genetic level identification by comparing 16S rRNA gene sequences with type strains [19]. In the present study, 16S rRNA gene sequencing proved that all of the strains isolated from desert belong to different species of the genus *Streptomyces* (Table 2) which is in accordance with other reports [20].

An important focus of this study was to identify and maintain a set of test pathogens, *i.e* methicillin resistant *Staphylococcus aureus* (MRSA).These strains were confirmed and identified as different strains of MRSA by using the methods described by Abdalrahman and Fakhr [11]. The genetic characterization of 16S rRNA gene of these strains also proved that they belong to the genus *Staphylococcus aureus* and the findings of other researchers also supported this result. Different researchers also characterized MRSA through 16S rRNA gene amplification by PCR [21]. For the identification of MRSA, the genotypic detection of *mec-A* gene is used as a reference standard worldwide; it is used as an authentic test for the confirmation of MRSA [22]. So, we also used this as a confirmation test for our strains of MRSA along with antibiotics sensitivity against oxacillin, cefoxitin etc.

The main focus of this study was to isolate and identify potent actinomycete strains active against the most resistant pathogens, the MRSA, so for this purpose all of the strains of desert actinomycetes were screened biologically and chemically. A good number of desert actinomycetes exhibited very promising antimicrobial activity (Table 4). The strain AFD2 was very active strain with 36 mm zone of inhibition, another significant anti-MRSA strain was AFD9 with 30 mm zone of inhibition, strain AFD10 exhibited 28 mm zone of inhibition. Altogether 71 % of the total strains of desert actinomycetes exhibited very promising activity against all MRSA strains and this percentage is more than described in various studies [23] conducted for desert actinomycetes. But this percentage is less than that described previously by Tiwari *et al* [24] who studied the antimicrobial activity of actinomycetes of Thar Desert India. Some actinomycete strains like AFD6, AFD7 did not exhibit any significant antimicrobial activity

which shows these strains are different in their antimicrobial behavior even then they are obtained from the same source.

In metabolic fingerprinting on TLC plates the crude extracts of these actinomycetes strains showed significant results when treated with anisaldehyde/H_2SO_4 different colored spots including blue, green or violet spots representing the presence of sugar molecules [15]. The treatment with Ehrlich's reagent exhibited blue and yellow colored bands representing the presence of N-heterocyclic compounds as described by Aslam *et al* [25].

HPLC-UV chromatograms of the crude extracts of these desert actinomycetes provided an insight into the chemical fingerprints of the compounds they contain. Some strains like AFD23 exhibited total 8 peaks at different retention times (t_R) with highest at 2.37 min. Strain AFD18 showed 4 peaks with highest at 2.50 retention time and these results were comparable with the results of Anwar *et al* [26]. These results showed that all of these strains produce varied antimicrobial compounds that may have the capability to inhibit the growth of resistant pathogens like MRSA. The capacity of the potentially active compounds to resist the pathogenic organisms can be confirmed further by following the extraction and purification methods and by using different column chromatographic techniques. Thin layer chromatography coupled with bioautography is a very useful alternative to detect the antimicrobial compounds in crude extracts. Bioautographic assay of the crude extracts of desert actinomycetes strain specially AFD23 exhibited different fractions of bioactive compounds which showed good antimicrobial activity against MRSA strains. These results are also supported by the findings of Sajid *et al* [13].

CONCLUSION

Cholistan desert is an important and rare source of diverse actinomycetes in Pakistan with very potent antimicrobial compounds. The flora of actinomycetes in this unique ecological environment should be explored further by purification and structure elucidation of the active compounds and by affirming their antimicrobial activity for novel drugs against MRSA and other MDRs.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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