Antimicrobial potential of *Halophilic actinomycetes* against multi drug resistant (MDR) ventilator associated pneumonia causing bacterial pathogens

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Abstract: A collection of forty halophilic actinomycetes isolated from water and mud samples of the saline lake at Kalar Kahar, salt range, Pakistan, was screened to investigate their antimicrobial potential against multi drug resistant (MDR) ventilator associated pneumonia causing bacterial pathogens. The isolates exhibited significant tolerance to alkaline conditions and grew well at pH 9-11. The taxonomic status of the isolated strains was determined by morphological, biochemical and physiological characterization and by 16s rRNA gene sequencing. The results revealed that majority of the isolates (90%) belong to the genus *Streptomyces*. Most of the isolates exhibited remarkable antimicrobial activity up to 20mm zone of inhibition against MDR ventilator associated pneumonia causing bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* and *Acinetobacter* spp. Additionally the isolates showed moderate to high cytotoxicity in the range of 40 to 80% larval mortality against *Artemia salina* in a micro well cytotoxicity assay. The chemical screening or the so called metabolic fingerprinting of the methanolic extracts of each isolate, by thin layer chromatography (TLC) using various staining reagents and by high performance liquid chromatography (HPLC-UV), indicated an impressive diversity of the compounds produced by these strains. The study reveals that these halophilic actinomycetes are a promising source of bioactive compounds. The preparative scale fermentation, isolation, purification and structure elucidation of the compounds produced by them may yield novel antimicrobial or chemotherapeutic agents.

Keywords: Halophilic actinomycetes, biological screening, MDR ventilator associated pneumonia causing bacteria, chemical screening, 16s rRNA gene sequencing.

INTRODUCTION

Antibiotic resistance is increasing globally, and may make a long list of currently available antimicrobial agents that are now insufficient for the control of microbial infections (Baltz, 2007). Due to this reason, many scientists and pharmaceutical industries are actively involved in the extensive utilization of the most productive microbial groups *e.g.*, actinomycetes for the screening of antibiotics (Hube *et al*., 2009). In the past, actinomycetes and more specifically, streptomycetes, have remained the prolific sources of the large number of new antibiotics (Singh *et al*., 2012). As exploration of actinomycetes from terrestrial sources are nearly exhausted and there has been a subsequent redundancy in the screening of antibiotics from actinomycetes, the focus of industrial screening has therefore moved to various unexplored habitats with unusual environment including saline lakes, forests, deserts and marine sediments (Hozzein *et al*., 2011). Antibiotic discovery from microbial products relies on the proper combination of some key factors that need to be met at some point in the screening process including selection of appropriate producing strain, identification of adequate cultivation conditions and ensuring production of novel chemistry (Sajid *et al*., 2009). In order to expose the natural molecular diversity for drug development, different screening methods including target-directed biological, physical and chemical strategies have been developed (Taddei *et al*., 2006).

Since the time of their discovery, screening of actinomycetes from extreme environments has provided an attractive and challenging platform for scientists (Devi *et al*., 2006). In addition to unusual growth characteristics under extreme conditions, production of industrially invaluable compounds, such as antibiotics, enzymes *etc*. have captivated attention in present scenario. The reports on actinomycetes isolated from saline ecosystem are rather limited (Bredholt *et al*., 2008; Kumar *et al*., 2001) while those indicating their antimicrobial potential are rare.

The current study aimed at the isolation and thorough investigation of halophilic actinomycetes from Kalar Kahar Lake, (Punjab, Pakistan) for antimicrobial activity and compounds against multi drug resistant (MDR) ventilator associated pneumonia causing bacterial pathogens. The Kalar Kahar Lake is one of the renowned wetlands in Pakistan and due to high salinity it is home to a wide variety of species of plants, animals and microorganisms. To the best of our knowledge this rare ecological niche has never been investigated for actinomycetes diversity and their antimicrobial potential.

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A lot of global research work had explored antibiotic resistance among pathogens isolated from ventilator-associated pneumonia; a life threatening infection, which occurs in patients who are on mechanical ventilation through an endotracheal tube for more than 48 hrs (Foglia *et al*., 2007). This increased antibiotic resistance ultimately leads to increased morbidity, mortality and economic loss. It was anticipated that the investigation of halophilic actinomycetes may indicate some useful strains or may yield some potentially useful compounds to control the infections of ventilator associated pneumonia causing MDR bacteria.

MATERIALS AND METHODS

Selective isolation and identification of bioactive halophilic actinomycetes strains

The water and mud samples were collected from the Kalar Kahar Lake and were treated physically and chemically for the enrichment of actinomycetes. Serial dilutions of each sample were prepared and the dilutions 10^{-2}, 10^{-3} were spreaded on the selective media, *i.e.* glycerol casein KNO_3 agar (glycerol 10g, KNO_3 2g, casein 0.3g, NaCl 2g, K_2HPO_4 2g, $MgSO_4.7H_2O$ 0.05g, $CaCO_3$ 0.02g, $FeSO_4.7H_2O$ 0.01g, agar 18g in one liter distilled water) using nystatin as an antifungal agent. The pH of the medium was adjusted up to 9.5 by using 2N NaOH for the proper growth of halophilic actinomycetes. After incubation at 28^{o}C for 7-21 days, the sample plates were examined for the crowding of hard and embedded actinomycetes colonies. The presumptive actinomycetes were transferred to the Glucose, Yeast extract and Malt extract (GYM) agar (glucose 10g, yeast extract 5g, malt extract 5g, agar 18g in one liter distilled water, pH adjusted to 9.5) and the pure cultures were characterized morphologically, biochemically and physiologically (Williams *et al*., 1983). A preliminary antimicrobial activity was determined using agar plug method (Sajid *et al*., 2009) against *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas* spp. and *Proteus* spp. The active strains were preserved as glycerol stocks for further studies.

Preparation of methanolic crude extracts

In order to get the methanolic crude extracts of the bioactive compounds produced by the selected strains, the isolates were grown as 100ml shaking cultures in glass flasks for 5-7 days at 28^{o}C. The resulting culture broth was lyophilized, mixed with ethyl acetate and the cells were disrupted by sonication for 15min in a sonication bath (Ultrasons Med I-II). The mixture was taken in a separating funnel and was shaken physically for 10 minutes then the content was allowed to settle making two distinct layers and the upper layer was separated carefully. The solvent was recycled through rotary evaporator (D-91126 Schwa bach, Germany); dried extract was dissolved in 3-5 ml of methanol and was used for biological screening and for chemical analysis.

Determination of antimicrobial activity and cytotoxicity

The antimicrobial activity was determined by agar diffusion assay against a set of multi drug resistant (MDR) bacteria causing ventilator associated pneumonia which includes; *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas* spp. and *Proteus* spp. These MDR bacteria were isolated from endotracheal secretions of mechanically ventilated patients admitted in intensive care unit of Mayo Hospital, Lahore. The sensitivity or resistance pattern of the test organisms against commonly used antibiotics was determined by the standard Kirby-Bauer disc diffusion assay (Bauer *et al*., 1966). For determination of antimicrobial activity, the test organisms were cultured in Lauria Bertani broth (Gerhardt *et al*., 1994) at 37^{o}C for 24 hrs. The test plates were prepared by pouring 14 ml of LB-agar as base layer, after solidifying; it was overlaid with 5 ml of the molten seed agar. Later with the help of a sterile cork borer agar wells were made (inside diameter 5mm) and about 60µl of the methanolic crude extract were loaded in each well. After incubation, the diameter of zone of inhibition was measured in mm. A micro well cytotoxicity assay was used for determining the cytotoxicity of crude extracts against the larvae of brine shrimps (*Artemia salina*). Dried eggs of *Artemia salina* were added to a separating funnel filled with artificial sea water; this suspension was aerated and kept at room temperature for 24-48 hrs. With the help of a pipette active larvae were transferred to the wells of a micro titer plate filled with artificial sea water. The dead larvae were counted (value N), then a solution of 15 µg of crude extract in 5µl of DMSO was added and the plate was kept at room temperature in the dark. After 24 hrs, the value A of the dead larvae was counted under the microscope. Then surviving larvae were killed by the addition of 0.5 ml methanol so that the total number G of the larvae could be determined. The mortality rate was calculated using the formula, M= [A-B-N/G-N]×100 (Solis *et al*., 1993).

Chemical screening

In chemical screening or the so called metabolic fingerprinting, the methanolic extracts were analyzed by thin layer chromatography (TLC) using various staining reagents (anisaldehyde/sulfuric acid and Ehrlich's reagent) and by high performance liquid chromatography (HPLC-UV).

Thin layer chromatography (TLC)

In thin layer chromatography each sample extract was loaded onto the TLC plate drop wise, with allowing the former spot to dry before superimposing it. The plates were developed with a 10% methanol/CH_2Cl_2 solvent system. After developing the TLC plates were air dried and were visualized under UV light at 254 nm and 366 nm. The components showing UV absorbance under short UV or florescence under long UV were marked and were documented. Then the plates were stained by spraying

with anisaldehyde/H_2SO_4 and Ehrlich's reagent separately for further localization and documentation of the colored components.

High performance liquid chromatography (HPLC-UV) analysis

The crude extracts were analyzed on the HPLC system (Sykum S3210) using a software clarity. The column used was C18 from Phenomenex with 30cm length. Mobile phase used was a mixture of 95% methanol and 5% water and the flow rate was adjusted to 1ml/min. The samples were prepared by dissolving the crude extracts in HPLC grade methanol and 20µl of the sample was injected through a micro syringe. The sample was run for 15min and UV absorbance was determined at 254nm. The peaks of each sample were analyzed and were compared at different retention times with standard UV absorption data of secondary metabolites.

16s rRNA gene sequencing

The genomic DNA of the selected halophilic actinomycetes was prepared from GYM grown mycelia following the methods of Kieser *et al*. (2000). The PCR amplification of the 16s rRNA gene was carried out using the universal primers 27f: 5≠ AGAGTTTGATCCTGGCTCAG 3≠ and 1522r: 5≠ AAGGAGGTGATCCAACCGCA 3≠. The reaction mixture contained 25 µl master mix, 2.0 µl each forward and reverse primers, 3 µl template DNA, deionized water 18 µl. Amplification was performed in primus 96 advanced^R thermocycler (peQLab Biotechnologie GmbH_V.1106E) according to the following amplification profile: an initial denaturation at 94^{o}C for 5 min; 30 cycles at 94^{o}C for 1 min, 55^{o}C for 1 min, and 72^{o}C for 2 min, and a final extension at 72^{o}C for 5 min. The PCR product was analyzed by agarose gel electrophoresis and the DNA fragments were purified (FavorPrep^{TM} gel purification mini kit, Taiwan) from the gel. The gene sequencing was done on automated sequencer (3730 DNA analyzer ABI) and the obtained 16s rRNA gene sequence data was analyzed with BLAST program (http://www.ncbi.nlm.nih.gov/). The nucleotide sequence data was submitted to the GenBank and the accession numbers were obtained.

RESULTS

Taxonomic characteristics of the selected halophilic actinomycetes

A total of 40 actinomycete strains were isolated from water and mud samples collected from the saline lake (Kalar Kahar). The strains were assigned symbols on the basis of source of isolation, number of sample and number of the sequence on which they were isolated. For instance, symbols used were $KL_2$3 and $KL_3$2 where KL representing Kalar Kahar Lake, number in subscript representing the number of water or mud sample (2 & 3) while 3 and 2 was the sequence at which these isolates were recovered. The isolates showed embedded, dry, rough and colored colonies with regular or irregular margins, colony size ranges from pinpoint to large (*e.g.* 3 mm), hard and convex surfaces. However, the spore color and consistency was different in nearly all isolates. In some strains the spores were grey in color, as in case of $KL_2$9, $KL_3$2, $KL_3$6, $KL_3$8, $KL_4$1, $KL_5$9, $KL_7$3, $KL_8$2, $KL_9$8 while in others it was white as observed in $KL_1$4, $KL_2$3, $KL_3$7, $KL_5$2, $KL_6$8, $KL_7$1, $KL_9$14 isolates and pink in $KL_2$12 and $KL_6$6. In physiological characterization, the strains showed the production of melanin pigment and exhibited the ability to grow on different carbon sources. Only five out of 40 strains (*i.e*., $KL_2$9, $KL_2$12, $KL_3$6, $KL_9$1, $KL_9$14) did not produce melanin. All the strains were able to grow on glucose, mannose and fructose but sorbitol, inositol, sucrose, raffinose, lactose, ribose and galactose were not as efficiently utilized by the isolates. The strains $KL_3$2, $KL_3$6, $KL_3$7, $KL_3$8, $KL_6$8, $KL_7$1, $KL_8$2, $KL_8$4, $KL_9$4, $KL_9$6 and $KL_9$8 were found incapable to utilize arabinose sugar (table 1). Comparison of the taxonomic characteristics of these strains with those of known actinomycetes as described in the International *Streptomyces* Project (Williams *et al*., 1983), suggested that these strains belong to the genus *Streptomyces*. In addition, genomic DNA of most promising actinomycetes strains *i.e.*, $KL_2$1, $KL_7$2, $KL_7$3, $KL_8$1, KL_{11}2, $KL_6$8, $KL_9$3, $KL_2$3, $KL_3$7 and $KL_9$2 was also isolated. Out of ten isolates, only seven gave amplified PCR product. The BLAST analysis of 16s rRNA gene sequence data of the selected strains showed alignments of these sequences with already reported 16s rRNA gene sequences in GenBank. Strain $KL_2$1 showed 99% similarity with *Streptomyces albogriseolus* strain SCSAAB0028, $KL_2$3 showed 99% similarity with *Streptomyces lilaceus* strain NBRC 13676, $KL_7$2 showed 99% similarity with *Streptomyces ambofaciens* strain HBUM174927, $KL_7$3 showed 99% similarity with *Streptomyces griseorubens* strain 2418, KL_{11}2 showed 99% similarity with *Streptomyces cinereoruber subsp. cinereoruber*, strain: NBRC 12756 and $KL_9$3 showed 98% similarity with *Streptomyces rochei* strain HBUM174697 (table 2).

Antimicrobial activity and cytotoxicity

All the strains were selected for detailed analysis on the basis of their preliminary antimicrobial activity. The strains were found to be active against a variety of test microorganisms in biological screening (table 3, fig. 1). Among the selected strains, $KL_1$4, $KL_2$1, $KL_2$9, $KL_3$2, $KL_3$7, $KL_4$1, $KL_6$3, $KL_7$2, $KL_7$3, $KL_8$1, $KL_9$3, $KL_9$6, $KL_9$8 and KL_{11}6 were found to be most biologically active. In case of MRSA as test organism the isolates $KL_3$7, $KL_2$9, $KL_8$4 and KL_{11}6 exhibited significant antibacterial activity showing zones of inhibition of 16mm, 17mm, 19mm, 24mm respectively. While $KL_4$3, $KL_6$4, KL_{11}7 gave zones of inhibition of 10mm, 11mm and 13mm respectively. In case of *E. coli*, maximum

antibacterial activity was exhibited by $KL_8$1, $KL_7$3, $KL_2$9, $KL_5$2, $KL_5$9, $KL_4$1 and $KL_9$2. While $KL_8$1, KL_{10}3, KL_{11}7, $KL_7$2, $KL_3$7 and $KL_9$2 isolates were observed to exhibit maximum activity against *Klebsiella pneumoniae*. In case of *Proteus vulgaris*, maximum zones of inhibition were shown by $KL_9$4 (13mm), $KL_6$8 (16mm), KL_{10}3 (17mm) and $KL_8$4 (18mm). The maximum cytotoxicity against *Artemia salina* in terms of %age larval mortality was observed in case of isolates $KL_2$1 (89.7%), $KL_3$2 (75.6%), $KL_3$7 (64.1%), $KL_6$3 (87.8%), $KL_6$7 (76.1%), $KL_6$8 and $KL_7$1 (85.0%), $KL_8$1 (71.7%), $KL_9$1 (70.02%), $KL_9$3 (75.6%), $KL_9$2 (69.2%) and KL_{11}2 (77.7%) strains. The strains $KL_6$4, $KL_5$2, $KL_4$1, $KL_2$9, $KL_2$12, $KL_8$4, $KL_9$6, $KL_9$14, KL_{11}5, and $KL_3$8 were found to exhibit less cytotoxicity against larvae of *Artemia salina*. While only two strains, $KL_2$4 and $KL_4$3 exhibited no lethal effect against *Artemia salina* larvae.

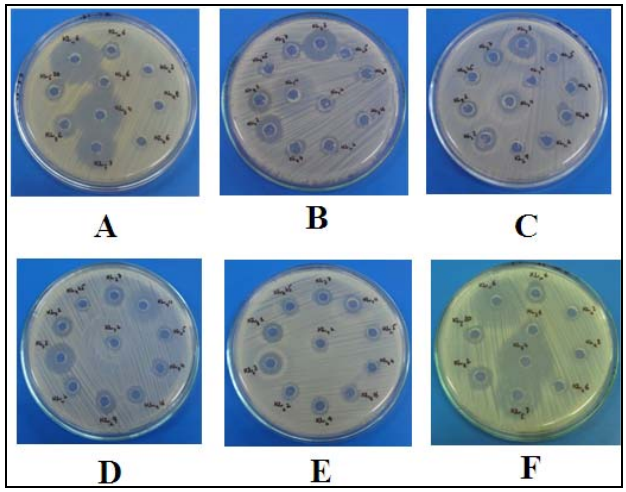

Fig. 1: Antimicrobial activity of the selected halophilic actinomycete isolates against various multi drug resistant ventilator associated pneumonia causing bacterial pathogens. A Activity of various *Streptomyces* extracts against *methicillin resistant Staphylococcus aureus.* B Activity against *Klebsiella pneumoniae.* C Activity against *Acinetobacter spp.* D Activity against *E. coli*. E Activity against *Pseudomonas spp*. F Activity against *Proteus vulgaris*.

Metabolite diversity and chemical characteristics of the active extracts

In thin layer chromatography a highest variety of the UV absorbing spots was visualized under UV at 254 nm and 366 nm (fig. 2a, 2b). The pattern of colored bands on TLC plates after treatment with staining reagents (anisaldehyde/H_2SO_4 and Ehrlich's reagent) is visible in fig. 2c, 2d. The separated fractions of each of the crude extracts produced different colors including pink, blue, purple, dark brown and yellow depending on the staining reagent. A unique pattern of colored bands was observed in crude extracts of strains $KL_9$8, $KL_6$3, $KL_2$9, $KL_7$3, $KL_8$1, KL_{11}6, $KL_8$3 and $KL_7$11. The isolates KL_{10}3, $KL_6$3, $KL_9$8, $KL_8$1, $KL_6$7 and $KL_9$3 gave numerous green, violet, brown, orange, pink and blue bands with anisaldehyde/H_2SO_4 reagent. After staining with Ehrlich's reagent, isolates $KL_6$3, KL_{10}3, $KL_8$3, $KL_9$8, $KL_2$12 gave purple bands which were probably of indole like compounds, while yellow spots produced by $KL_6$7, $KL_7$1, $KL_8$1 and $KL_2$9 isolates indicating the presence of N-heterocyclic compounds. In HPLC-UV analysis, each of the crude extracts exhibited a varying number of peaks at different retention times. The crude extract of $KL_7$1 showed six peaks and major peak appeared at retention time (t_R) of 4.260 min with a peak area of 8349.769 [mV.s]. In chromatograms of $KL_7$2, $KL_6$3 and $KL_6$4 two major peaks appeared per extract while in case of $KL_9$14 and $KL_5$9, three distinct peaks appeared at different retention times (fig. 3).

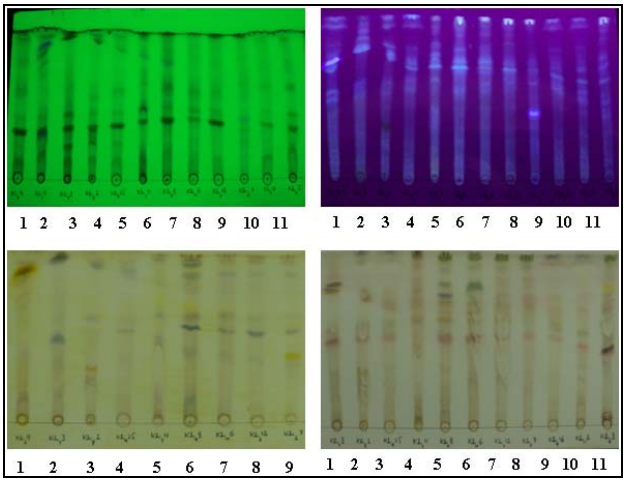

Fig. 2: Chemical screening using TLC with Anisaldehyde/H_2SO_4 and Ehrlich's spraying reagents. TLC plates A under UV at 254 nm, B under UV at 366 nm, C after treatment with Ehrlich's reagent, D after treatment with anisaldehyde/H_2SO_4 solution. Numbers 1-11: Crude extracts of *Streptomyces* strains 1=$KL_8$4, 2=$KL_6$7, 3=$KL_8$3, 4=$KL_8$1, 5=$KL_9$2, 6=$KL_7$1, 7=KL_{10}3, 8=$KL_2$12, 9=KL_{11}2, 10=$KL_6$3, 11=$KL_6$4

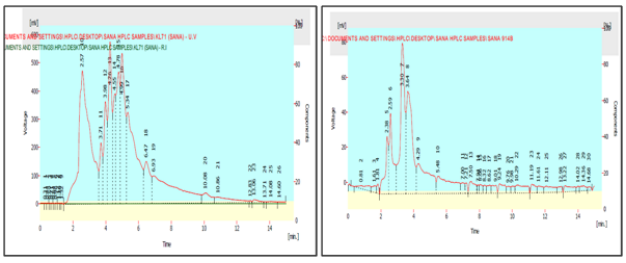

Fig. 3: HPLC-UV chromatograms of strains $KL_7$1 and $KL_9$4 showing distinct peaks at different retention times (t_R)

DISCUSSION

The appearance and dissemination of antibacterial resistance is well recognized as a serious problem worldwide which threatens to return us to the time before

Table 1: Melanin production and sugar utilization profile of the selected *Halophilic actinomycetes*

Lake actinomycete isolates	Glu	Man	Fru	Ara	Sor	Ino	Suc	Raf	Lac	Rib	Gal	Melanin
$KL_1$4	++	++	+	+	+	+	-	+	-	+	+	++
$KL_2$1	++	++	++	++	++	+	+	-	+	++	-	+
$KL_2$9	++	+	++	+	+	++	++	-	-	+	+	-
$KL_2$12	+	+	+	-	-	-	+	+	+	+	+	-
$KL_3$2	++	+	+	-	+	+	-	-	+	-	-	+
$KL_3$6	++	++	++	-	+	+	-	-	-	+	+	-
$KL_3$7	+	+	+	-	++	+	+	+	-	-	+	++
$KL_3$8	++	+	++	-	+	+	-	-	+	+	+	+
$KL_4$1	++	+	++	+	+	++	+	-	-	+	++	+
$KL_4$3	++	++	++	++	+	+	+	+	-	++	+	+
$KL_5$2	+	+	+	+	-	+	-	+	-	-	-	+
$KL_5$9	++	++	++	+	+	+	-	+	-	-	-	++
$KL_6$3	++	++	++	++	++	++	-	-	-	+	++	++
$KL_6$4	+	++	+	+	-	+	+	-	-	-	-	+
$KL_6$8	+	+	+	-	+	+	+	-	-	+	+	++
$KL_7$1	++	++	+	-	+	-	+	+	-	+	+	+
$KL_7$2	++	+	+	+	+	+	-	+	-	+	++	++
$KL_7$3	++	++	+	++	++	+	-	-	+	+	++	+
$KL_8$1	++	+	+	+	+	-	+	-	-	-	-	++
$KL_8$2	++	++	+	-	+	+	-	+	-	++	+	+
$KL_8$3	++	++	+	+	+	+	-	-	+	-	+	++
$KL_8$4	++	++	+	-	+	-	+	+	-	-	+	++
$KL_9$1	+	+	+	+	-	+	+	-	+	-	+	-
$KL_9$2	++	+	++	+	++	+	-	-	+	-	++	++
$KL_9$3	++	+	+	+	+	++	+	-	-	-	+	+
$KL_9$4	++	+	+	-	+	++	-	+	+	+	+	++
$KL_9$6	+	+	+	-	-	+	+	-	+	+	++	+
$KL_9$8	++	+	+	-	-	-	+	-	+	-	+	+
$KL_9$14	++	+	++	+	-	+	+	+	-	-	-	-
KL_{10}3	++	+	+	+	+	++	-	+	-	-	+	++
KL_{11}7	++	+	+	+	-	+	-	-	-	-	+	++
KL_{11}6	++	++	+	++	+	++	+	+	+	+	++	++

(-) = no growth, (+) = growth, (++) = good growth, (+++) = best growth Key: Glu, Glucose; Man, Mannose; Fru, Fructose; Ara, Arabinose; Sor, Sorbitol; Raf, Raffinose; Lac, Lactose; Rib, Ribose; Gal, Galactose.

the development of antibiotics (Gold and Moellering, 1996). Over the last decade, bacterial resistance particularly among ventilated patients in ICUs has been a constant challenge for the clinicians. This situation recommends the need for the exploration of new effective antimicrobials for replacement of invalidated antimicrobials (Smith *et al*., 1999). In this perspective, intensive studies on alkaliphilic and halophilic actinomycetes in less exploited ecosystems for the discovery of new antibiotics would be a helpful count in recent times. Keeping in view the above facts, the present study was undertaken to isolate actinomycetes from less explored ecosystem such as saline lake (Kalar Kahar) and to screen for antimicrobial potential against various multi drug resistant bacteria. The morphological and physiological characteristics of the isolated strains strongly suggested that most of them belong to the genus *Streptomyces* (table 1). The 16s rRNA gene sequencing also proved that all the isolates were different species of the genus *Streptomyces* (table 2). Various researchers reported that the *Streptomyces* spp. have found to be the most frequent isolates among the actinobacterial flora of saline lakes and wetlands (Sanasam *et al*., 2011; Singh *et al*., 2012). As our major concern was to explore the potential of the isolated strains for the production of unique secondary metabolites, so representative strains were selected on the basis of biological and chemical screening. In biological screening, multi drug resistant isolates causing ventilator associated pneumonia were used as test organisms. Approximately 97% of the isolates showed antimicrobial activity against test strains while 3% did not show any antimicrobial activity. This was clear indication that almost all isolates in this study are having a substantial amount of bioactive compounds.

Table 2: Gene bank accession numbers and %age similarity of the selected isolates with known *Streptomyces* species in gene bank

Lake actinomycete isolates (common name)	BLAST analysis		Gene bank Accession Number
	Similarity with other microbes	%age of similarity	
$KL_2$1	*Streptomyces albogriseolus* strain SCSAAB0028	99	JX573192
$KL_2$3	*Streptomyces lilaceus* strain NBRC 13676	99	JX573193
$KL_7$2	*Streptomyces ambofaciens* strain HBUM174927	99	JX573194
$KL_7$3	*Streptomyces griseorubens* strain 2418	98	JX573195
$KL_8$1	Uncultured bacterium clone BFM134X3RFb07	100	JX573196
$KL_9$3	*Streptomyces rochei* strain HBUM174697	98	JX573197
KL_{11}2	*Streptomyces cinereoruber subsp. cinereoruber*, strain: NBRC 12756	99	JX573198

Table 3: Antimicrobial activity and cytotoxicity of the selected halophilic actinomycetes strains

Actinomycetes strains	Antimicrobial activity against multi drug resistant bacteria (zones of inhibition in mm)								Cytotoxicity (%age mortality)
	S. aureus	MRSA	*Acinetobacter spp.*	*Pseudomonas spp.*	*P. vulgaris*	*E. coli*	*Klebsiella pneumoniae*	*Enterobacter spp.*	
$KL_1$4	12	9	10	11	10	9	-	9	50.0
$KL_2$1	13	13	12	13	7	13	12	11	89.7
$KL_2$9	10	15	12	14	9	11	13	12	38.8
$KL_3$2	23	11	13	15	9	12	10	-	37.5
$KL_3$6	11	10	-	-	9	9	11	10	75.6
$KL_3$7	10	10	13	14	9	13	10	-	75.6
$KL_4$1	14	12	15	17	10	12	13	13	64.1
$KL_5$2	11	10	-	-	14	13	10	9	63.8
$KL_5$9	10	11	-	-	10	12	11	10	81.0
$KL_6$3	12	14	14	18	16	15	13	14	21.6
$KL_7$2	16	11	13	13	-	10	13	11	87.8
$KL_7$3	10	18	20	19	10	13	12	11	55.0
$KL_8$1	15	13	18	19	10	14	13	-	65.8
$KL_9$2	13	11	20	14	10	15	15	-	69.2
$KL_9$3	12	12	12	-	9	15	16	12	71.7
$KL_9$6	11	10	13	15	7	17	13	17	75.6
$KL_9$8	15	15	12	-	11	-	11	-	48.7
KL_{10}3	19	21	13	-	17	13	11	12	71.7
KL_{11}6	18	28	13	13	10	16	13	15	75.0
KL_{11}7	11	14	-	10	-	10	-	10	68.2

(-) = No antimicrobial activity against the test organism was observed

Various other studies also indicated that alkaliphilic and halophilic actinomycetes from saline habitats are wealthy in bioactive compounds (Manam *et al*., 2005; Sarkar *et al*., 2008).

The results of biological screening reveal that majority of the strains were found to be more active against gram positive bacteria than gram negative bacteria. Earlier studies also revealed that many species of *Streptomyces* exhibit a strong antibacterial activity against gram-positive bacteria *e.g.*, *Staphylococcus aureus* (Fuat *et al*., 2010; Reddy *et al*., 2011). The reason for different sensitivity between gram positive and gram-negative bacteria could be attributed to the morphological differences between these microorganisms as gram negative bacteria have an outer membrane carrying the lipopolysaccharide components which make their cell walls impermeable to lipophilic solutes. The gram positive should more incline having only an outer peptidoglycan layer, which is not an effective permeability barrier. Maha *et al*. (2010) also reported antagonistic activity of actinomycetes against multidrug resistant gram-positive isolates. Among selected strains, $KL_1$4, $KL_2$1, $KL_2$9, $KL_3$2, $KL_3$7, $KL_4$1, $KL_6$3, $KL_7$2, $KL_7$3, $KL_8$1, $KL_9$3, $KL_9$6, $KL_9$8 and KL_{11}6 were found to be most biologically active as they exhibited promising activity against all the test strains of multi drug resistant pathogens (table 3, fig. 1). This might be due to the reason that the targeted ecosystem was mineral rich and also inhabited by a large diversity of microbial flora, so the secretory products of residing actinomycetes are intended to antagonize microbial community. Brine shrimp micro

well cytotoxicity assay is a valuable technique to assess the toxicity of the microbial secondary metabolites. In the study reported here, 95% of the selected strains showed lethal effects against *Artemia salina* among which 35% isolates were found to be highly cytotoxic and 25% were less cytotoxic while two isolates were found to be nonresponsive (table 3). In the previous studies, this assay has been used by various researchers to detect the cytotoxicity of bacterial metabolites and plant extracts (McLauglin *et al*., 1991).

Chemical screening by TLC using selected spraying reagents showed a unique pattern of secondary metabolites specific for each strain. In case of anisaldehyde/H_2SO_4 spraying reagent the best results were obtained due to its capacity to stain the various spots with different colors. The isolates $KL_6$3, $KL_7$11, $KL_8$1, $KL_8$3, $KL_9$8 and $KL_9$6 gave purple, yellow, orange, dark pink, blue and green bands with anisaldehyde/H_2SO_4 reagent respectively; while with Ehrlich's reagent they gave mostly yellowish bands which are a clear hint of the presence of indole like compounds and N-heterocycles in the extract. The colored bands produced on TLC plates were marked (fig. 2). For separation, identification and quantification of compounds present in crude extracts of isolates HPLC was performed where chromatogram revealed various peaks at different retention times indicating the presence of various concentrations of bioactive compounds in an extract. Remya and Vijayakumar (2008) have also demonstrated the metabolic diversity of crude extracts from *Streptomyces* spp by using HPLC-UV analysis for the detection of "talented" strains, as an essential step of proficiently applied biological high-throughput assays.

Overall the study reveals that these halophilic actinomycetes harbor an immense antimicrobial potential against multidrug resistant (MDR) bacterial pathogens. The actinomycetes flora of this rare ecological niche (Kalar Kahar Lake) should be exploited further by purification and structure elucidation of the active compounds produced by them and by validating their antimicrobial, antiviral, antiparasitic, antitumor and anticancer activities.

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