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Diverse Actinomycetes from Indian Coastal Solar Salterns - A Resource for Antimicrobial Screening

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The prominence of naturally occurring antibiotics from actinomycetes has been deep-rooted upon their potential to produce numerous chemically diverse metabolites. However, microbial natural products research has declined due to increasing rediscovery of known compounds. Screening of actinomycetes inhabiting unexplored unusual environments is an important approach to avoid rediscovery of known compound and revitalize the antibiotic discovery from actinomycetes. In this study, a total of 69 actinomycete isolates derived from Indian coastal solar salterns were screened by two levels with four different production media to unravel their ability to produce antimicrobial compounds. Nine actinomycete strains demonstrated extracellular antimicrobial activity against at least one of the indicator bacteria (Gram positive or Gram Negative) or fungi in the preliminary agar plug method. In secondary screening, antimicrobial activities of the acinomycetes were confirmed in all the nine antagonistic actinomycetes. The actinomycetes, *Streptomyces* sp. JAJ06, *Streptomyces* sp. JAJ13 and *Nonomuraea* sp. JAJ18 were found to be the toppers among the antagonistic actinomycete population. The strain JAJ20 showed both antibacterial and antifungal activity. The antagonistic actinomycete population comprised both *Streptomyces* and non-streptomyces (rare actinomycetes). This study has delineated the antagonistic strains among the actinomycetes of solar saltern origin and recognized the solar salterns as resource of potent bioactive metabolite producing actinomycetes.

Key words : Actinomycetes, Rare-actinomycetes, Saltern, Agar-plug, Dual-culture, Antimicrobial.

Rapidly evolving resistance among the pathogenic microorganisms compromises the efficacy of available antibiotics and in turn intensifies the need of search for new antibiotics with novel structural and functional fractures. Traditionally, *Streptomyces* are considered as prolific sources of secondary metabolites, notably antibiotics[1] and being extensively used for commercial production of various medically important compounds. By the end of 20th century, microbial secondary metabolite based novel drug discovery vanished from large pharmaceutical companies, subsequently found shelter in research institutes and small biotechnology companies and continues to provide their fair share to clinical candidates and drugs[2-5]. Besides traditional antibiotic producing actinomycetes, rare actinomycetes (other than *Streptomyces*) are emerging as promising sources of bioactive lead compounds for the development of novel antibiotics[6]. The rare actinomycete species are often very difficult to isolate and cultivate might represent a unique source of novel biologically active compounds[7]. Rare actinomycetes are widely distributed in terrestrial and aquatic ecosystems while their distribution is affected by soil type, pH, humus content, and the characteristics of the humic acid content of the soil.

Screening of actinomycetes derived from conventional habitats have been led to rediscovery

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of known compounds[8-11], while those from poorly studied habitats promises a raise in the prospect of discovering new compounds that can be developed as resource for drug discovery[12-15]. In recent years, actinomycetes have largely been isolated from saline environments including sea water, saline soils, salt lakes, brines and alkaline saline habitats. These actinomycetes have been accepted as source of novel bioactive compounds, enzymes, biocompatible solutes and detoxifier compounds[16]. In this fashion, with an insight into acquiring diverse actinomycetes of industrial interest, previously, soil samples of largely unexplored solar salterns were processed and used for selective isolation of actinomycetes[17]. In the current communication we are describing antibacterial and antifungal activity of the saltern based actinomycetes assessed by using primary and secondary screening methods.

METHODS

A total of 69 actinomycete strains isolated previously from coastal[17] solar salterns were taken for this study. The strains were maintained either on ISP-2 or modified ISP4 (supplemented with 0.4% yeast extract) slants at 4° C for further evaluation of their antibacterial and antifungal activity. The following bacteria were used to screen the antibacterial activity of the actinomycetes: *Bacillus subtilis* MTCC 441, *Klebsiella pneumoniae* MTCC 109, *Salmonella typhi* MTCC 733, *Proteus vulgaris* MTCC 426, and Methicillin-resistant *S. aureus* (MRSA). Similarly, following fungal stains were used to screen antifungal activity of the actinomycetes: *Fusarium oxysporum*, *Aspergillus niger* and *Alternaria alternata*. The MTCC cultures were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungal strains were obtained from Tamilnadu agricultural college and research centre, Madurai, India. The bacterial and fungal strains were cultured and maintained either in Mueller Hinton broth or in nutrient broth and potato dextrose agar, respectively.

Primary screening for antimicrobials

All the actinomycete isolates were primarily screened for antibacterial and antifungal activity by using agar plug[18] and dual culture[19] methods, respectively with slight modifications.

Agar plug method

The actinomycetes were initially streaked over four different production media (PM) solidified with 2% agar: PM1, 10 g of starch, 4 g of yeast extract, 5 g of NaCl, 2 g of NH_4SO_4, 1 g of $MgSO_4.7H_2O$, 1 g of K_2HPO_4 and 1 liter of distilled water; PM2, 10 g of Starch, 0.3 g of casein, 2 g of KNO_3, 4.6 g of NaCl, 2 g of K_2HPO_4, 1 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$, 1 mg of $ZnSO_4.7H_2O$, 18 g of agar and 1 litre of distilled water PM3, 10 g of Starch, 4 g of Yeast extract, 5 g of NaCl, 2 g of NH_4SO_4, 2 g of $MgSO_4.7H_2O$, 1 g of K_2HPO_4, 1 gm of $CaCO_3$, 0.010 g of $FeSO_4.7H_2O$, 0.001 g of $ZnSO_4.7H_2O$, 0.001 g of $MnCl_2.4H_2O$, 0.001 g of $CuSO_4.5H_2O$ and 1 litre of distilled water; PM4, 10 g of starch, 4 g of yeast extract, 2 g of peptone, 0.010 g of $FeSO_4.7H_2O$, 0.1g of KBr and 1 liter of natural seawater. The plates were incubated at 29°C for 7 to 10 days to attain enough growth over the production media. For the initial antibacterial screening, agar plugs of 6 mm in diameter were cut from the 10 d old agar plates and plugged into the wells bored using sterile cock borer (diameter of 6 mm) in Mueller Hinton agar plates seeded with different bacteria. The agar plugged plates were incubated at 37°C for 24 h and observed for zone of inhibition around the inserted agar plugs.

Dual culture method

The actinomycetes were point inoculated on production media at 30 mm distance from the center of plate. Fungal mycelial-disks (6 mm in diameter) prepared from growing margin of cultures of test fungal strains and placed in the center of plate. Antifungal activity was indicated as mycelial growth of fungal isolates was inhibited in the direction of active actinomycete isolates.

Secondary Screening for antimicrobials

Those actinomycests showed positive antimicrobial activities in the primary screening were subjected to antimicrobial compound production in submerged culture and their antimicrobial proficiency was confirmed by disc diffusion method[18,20].

Production and extraction of bioactive compounds using submerge culture

Spore suspensions of active actinomycetes were prepared in distilled water from cultures grown on ISP-4 medium either supplemented with 4% of NaCl (w/v) or prepared

with 90% sterile sea water. The suspensions were added either to ISP-2 broth or modified ISP4 broth (supplemented with 0.4% yeast extract) in 250 ml Erlenmeyer flasks at a rate of 10^8 spores in 50 ml liquid medium incubated on a shaker at 120 rpm at 30°C for 3 to 7 d. From the seed cultures, 25 ml aliquots were transferred to 250 ml production media (PM1, PM2, PM3 and PM4) and the flasks were incubated for 7 to 10 d at 30°C while shaking at 120 rpm. The culture broth was centrifuged at 10,000 rpm for 10 min to separate the mycelial biomass. Ethyl acetate was added to the

Table 1. Antibacterial activity of actinomycetes isolated from Indian coastal solar salterns

Isolates[17]	Medium	Antibacterial activity / Zone of Inhibition (mm ± SEM*)				
		B. subtilis	*P. vulgaris*	*K. pneumoniae*	MRSA	*S. typhi*
Streptomyces sp. JAJ07	PM1	NA	NA	NA	NA	NA
	PM2	18 ± 0.66	21 ± 2.66	12 ± 0.33	23 ± 0.88	10 ± 0.57
	PM3	22 ± 0.33	24 ± 0.88	15 ± 0.57	26 ± 0.66	13 ± 1.20
	PM4	18 ± 0.57	20 ± 0.57	11 ± 0.33	17 ± 0.88	11 ± 0.57
Streptomyces sp. JAJ13	PM1	18 ± 0.33	15 ± 0.57	09 ± 1.20	13 ± 0.57	10 ± 0.33
	PM2	11 ± 0.88	16 ± 0.33	12 ± 0.88	17 ± 0.66	14 ± 0.57
	PM3	25 ± 1.15	21 ± 1.30	19 ± 1.30	26 ± 0.57	19 ± 1.00
	PM4	14 ± 1.45	16 ± 0.66	12 ± 0.57	14 ± 0.66	14 ± 0.66
Nonomuraea sp. JAJ18	PM1	15 ± 1.33	18 ± 0.33	11 ± 0.88	13 ± 0.57	14 ± 1.73
	PM2	20 ± 0.57	20 ± 0.57	13 ± 0.57	18 ± 0.33	18 ± 0.66
	PM3	30 ± 1.15	26 ± 0.66	17 ± 0.33	27 ± 0.57	23 ± 1.45
	PM4	23 ± 0.88	18 ± 0.57	10 ± 0.33	13 ± 0.88	14 ± 1.15
Streptomyces sp. JAJ19	PM1	ND	ND	ND	ND	ND
	PM2	12 ± 0.33	12 ± 0.33	11 ± 1.30	13 ± 1.15	11 ± 1.30
	PM3	17 ± 0.66	17 ± 0.57	15 ± 0.57	19 ± 0.33	14 ± 0.88
	PM4	12 ± 1.00	15 ± 1.15	14 ± 0.66	19 ± 1.15	14 ± 1.70
Micromonospora sp. JAJ20	PM1	19 ± 0.33	22 ± 0.66	14 ± 0.57	17 ± 0.57	12 ± 1.85
	PM2	16 ± 0.66	18 ± 0.57	15 ± 1.20	21 ± 1.33	13 ± 0.88
	PM3	21 ± 1.00	25 ± 0.57	16 ± 0.33	22 ± 2.00	15 ± 1.70
	PM4	NA	NA	NA	NA	NA
Streptomyces sp. JAJ41	PM1	10 ± 0.57	NA	NA	14 ± 0.33	ND
	PM2	12 ± 0.66	NA	NA	16 ± 1.20	ND
	PM3	16 ± 1.15	NA	NA	17 ± 0.66	ND
	PM4	17 ± 0.57	NA	NA	15 ± 0.33	ND
Streptomyces sp. JAJ59	PM1	11 ± 0.57	11 ± 1.2	11 ± 0.88	11 ± 0.88	14 ± 0.66
	PM2	13 ± 1.20	10 ± 0.88	13 ± 0.66	16 ± 1.15	15 ± 0.33
	PM3	16 ± 1.00	13 ± 1.15	13 ± 1.45	10 ± 1.20	16 ± 0.88
	PM4	NA	NA	NA	NA	NA

ND - Not Detected; NA - No Activity

Table 2. Antifungal activity of actinomycetes isolated from Indian coastal solar salterns

Isolates[17]	Medium	Antifungal activity / Zone of Inhibition (mm)		
		A. niger	*F. oxysporum*	*A. alternata*
Micromonospora sp. JAJ20	PM2	10 ± 0.33	10 ± 0.88	12 ± 0.88
	PM3	14 ± 1.15	15 ± 0.33	17 ± 0.57
	PM4	09 ± 1.40	12 ± 1.15	13 ± 1.15
Streptomyces sp. JAJ28	PM2	11 ± 1.76	10 ± 1.20	15 ± 1.15
	PM3	16 ± 0.88	17 ± 1.15	22 ± 1.45
	PM4	15 ± 1.45	12 ± 0.88	19 ± 0.57

supernatants in 1:1 proportion and the mixtures were agitated for 45 min. The solvent layers were separated from broths and centrifuged at 5000 rpm for 15 min to remove traces of fermentation broth. The ethyl acetate fractions were evaporated and the resultant crude compounds were resuspended in 50 µl of methanol which were then assayed for antimicrobial activity.

For the evaluation of antimicrobial activity, Mueller Hinton agar plates were inoculated with test microorganisms by spreading the microbial inoculums on the surface of the media. The extracts were loaded on 6 mm sterile discs, dried and placed on the surface of the medium inoculated with pathogens and the plates were incubated at 37 °C for 24 h. At the end of incubation period, inhibition zones formed around the disc were measured with transparent ruler in millimeter. The same procedure was followed for the yeast also. In the case of antifungal assay the same procedure was practiced on potato dextrose agar instead of Mueller Hinton agar.

RESULTS

Primary screening

All the actinomycete isolates were primarily screened for antibacterial and antifungal activity by using agar plug and dual culture methods respectively. In the primary screening, nine (13%) isolates were found to be active against at least one of the tested bacteria and fungi. Among the nine antagonistic actinomycetes, seven isolates showed antibacterial activity, two isolates showed antifungal activity. Of these, one isolate showed both the antifungal and antibacterial activity. The traditional antibiotic producing genus *Streptomyces* contributed 78% antagonistic actinomycete population while the remaining 22% constituted by actinomycetes other than *Streptomyces* (non-*Streptomyces*).

Secondary Screening

Results of secondary antimicrobial screening of active actinomycetes were given in Table 1 and 2. Those actinomycetes showed antimicrobial activity in primary screening were found to produce bioactive compound in the batch of submerged fermentation process. Among the four production media, PM2 and PM3 facilitated production of bioactive compound in all the actinomycetes except JAJ06, which produced antimicrobial compound in PM4 in the presence of sea water. An extented study carried out in JAJ06 has already been reported elsewhere[21]. Some actinomycetes, JAJ20 and JAJ59 failed to produce antimicrobial compound in the presence of sea water while others able to produce antimicrobial compound in the presence of seawater. PM1 facilitated antimicrobial compound in almost all the tested antagonistic actinomycetes except JAJ07.

The actinomycetes inhibited the growth of bacteria and fungi with inhibition zone ranging from 9 to 44 mm. The strains, JAJ06, JAJ21, JAJ07, JAJ13 and JAJ18 showed significant antimicrobial activity with inhibition zone ranging from 30 mm to 17 mm against tested bacteria and fungi. Maximum antimicrobial activity was recorded against MRSA and *F. oxysporum*.

DISCUSSION

Since the discovery of streptomycin in 1943, the antimicrobial products from actinomycetes, especially those from *Streptomyces* have made phenomenal success in drug discovery for the past seven decades[22]. Recently, not only *Streptomyces*, other actinomycetes derived from unexplored environments also deserve the attention of researchers as a novel source for antibiotic research[23-25,6]. It is widely accepted that actinomycetes inhabiting in saline environments will provide a valuable resource for novel products of antimicrobial importance[16,21,26]. The diverse actinomycetes including non-*Streptomyces* (rare actinomycetes) engaged in the current antimicrobial screening program have previously been isolated from the largely unexplored Indian coastal and inland solar salterns[17].

Once a microbial library of diverse actinomycetes has been generated, a systematic screening should be employed to disclose antimicrobial proficiency of actinomycetes of our interest. Primary screening step is most critical since it eliminates the bulk of unwanted non producer actinomycetes. Actinomycetes have typically been screened by either cross streak method[27, 28] or agar plug method[18,29,30]. In agar plug method, separate media can be employed to facilitate antibiotic production and highly sensitive antimicrobial screening, while it is not applicable

in the case of cross streak method which employs a single media which may not be suitable either for antibiotic production or antimicrobial screening. For example, *Streptomyces* sp. JAJ06 produces antimicrobial compound in the presence of seawater, its antibacterial activity has been detected by growing on specific sea water based production medium and subsequent antimicrobial assay on MH agar plate seeded with indicator bacteria[21], while cannot be detected by growing on MH agar plates and cross streaking the indicator bacteria. In this study, the actinomycetes were grown on four different production media to facilitate the production and diffusion of antimicrobial substance into the agar; subsequently agar plugs were cut and tested against the indicator bacteria spread over MH agar (ideal media for antibiotic sensitivity assay). This method successfully discriminated the antimicrobial compound producing strains from non-producers.

Antimicrobial secondary metabolite production highly depends on the nutrients and the cultural conditions[31, 32]. In the current study four different production media were employed to facilitate the successful screening of antimicrobial compound producing actinomycetes. An actinomycete fails to produce antimicrobial substance in one media can produce it in another media. Use of multiple media for screening of antimicrobial compound production offers wide choice of nutrients to explore the antagonistic ability of the actinomycetes and also declines the probability of losing actinomycetes with antimicrobial compound producing capability.

In the current study antimicrobial activity was observed in both *Streptomyces* and rare actinomycetes. Members of genus *Streptomyces* was still the highest contributor in the antagonistic acinomycete population derived from solar saltern and it is accordance with the well known fact that *Streptomyces* are predominant source of natural antibiotics[33]. However, the rare actinomycetes also provided a fair share among the antagonistic population. The rare actinomycetes are currently emerging as potential natural bioactive compound producers[34]. In the resent study, antimicrobial activity was recognized in a rare genus *Nonomurae*. Broad spectrum activity exhibited by most of the antagonistic actinomycetes intensifies the importance of further study on these actimycetes.

In conclusion, the present study provides comprehensive report on in-vitro screening of coastal saltern based actinomycetes for their extracellular antimicrobial characteristic against a range of microorganisms. The results indicate the occurrence of antimicrobial compounds producing *Streptomyces* and non-streptomyces and evidence that coastal solar salterns are reservoir of antimicrobial compound producing actinomycetes. The observed antimicrobial activity against a range of bacteria and fungi may worth to further characterization of persuasive actinomycetes and their potential antimicrobial secondary metabolites.

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