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Antimicrobial Activity of Actinomycetes Strain Isolated From Alkaline Water of Lonar Lake.

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Abstract: The main focus of this study was to isolate some antibiotic producing actinomycetes strains from Halo alkaline water of Lonar Lake. Isolation of soil actinomycetes was done by culture-dependent methods and Furthermore, microscopic examination.

Total of 16 actinomycetes strains were isolated from the alkaline water of Lonar Lake and screened for their anti-bacterial activity. They were evaluated for their inhibitory activities on four test microorganisms. Eleven actinomycetes isolate which exhibited antimicrobial activity against at least two of the test organisms and were characterized by conventional methods. The cultural characteristics of isolates were also studies in different culture media. The results indicated that two isolates were highly active against *Staphylococcus aureus* strains. Most of the isolates inhibited growth of the Gram negative bacteria tested. These microorganisms may have capability to produce some of the most important medicines ever developed.

Key words: Actinomycetes, Antimicrobial activity, Antibiotic Producer, soil sample, *Staphylococcus aureus, E. coli, K. pneumoniae, E. faecalis*

INTRODUCTION:

Actinomycetes, the filamentous bacteria, are primarily, saprophytic microorganisms of the soil (Ramasamy Vijaykumar *et al.,* 2007). According to Baltz only a fraction of the World's biodiversity has been explored with less than one part of the Earth's soil surface screened for potential Actinomycetes. The terrestrial soil has been widely exploited for the isolation of Actinomycetes wherein they perform significant biogeochemical role contributing to the turnover of complex biopolymers (Sonashia and Kamat, 2013)

Actinomycetes are Gram-positive bacteria with high G+C content. Actinomycetes play an important role in recycling wastes in the environment and they are also the producers of thousands of metabolic products which exhibit biological activity. After the discovery of the broad spectrum antibiotic Streptomycin by Waksman and Schatz, more attention was paid towards the actinomycetes for isolation of many more antibiotics. Actinomycetes have been exploited successfully for their biologically potential secondary metabolites. They produce diverse group of antimicrobial metabolites notably glycopeptides, beta-lactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycins and tetracyclins (Gunasekaran and Sekar, 2013).

Many researchers have isolated novel antibiotics from the marine environment (Sujatha *et al*., 2005; Biabani *et al*., 1997; Maskey *et al*., 2003; Charan *et al*., 2004; and Li *et al*., 2005). The marine actinomycetes produce variety of enzyme inhibitors, antibiotics and anticancer compounds. The marine actinomycetes are the good source of enzyme inhibitors (Imade, 2005). Some of the novel secondary metabolites from marine actinomycetes have been isolated recently include Abyssomicin C, from *Verrucosispora* sp., a secondary metabolite with potent inhibitory action on para aminobenzoic acid synthesis (Riedlinger *et al*., 2004). Salinosporamide A, an anticancer compound from *Salinispora* species (Fehling *et al*., 2003) and a novel marinopyrroles from *Streptomyces* species (Hughes *et al*., 2008).

Soda lakes represent a specific type of salt lake, which contain an alkaline sodium carbonate or bicarbonate fraction among the dominant salts. They are mostly confined to dry areas with high evaporation rates that facilitate salt accumulation in local depressions. The presence of sodium carbonate in variable combinations with sodium chloride and sodium sulfate creates a unique, buffered haloalkaline habitat appropriate for a stable development of obligately (halo) alkaliphilic microorganisms growing optimally at pH around 10 (Sorokin and Kuenen, 2005). *Bacillus* sp. is one of the dominant genus among the gram-positive isolates from soda lakes and their soil (Nielsen *et al*., 1995). Industrial applications of these microorganisms have been investigated extensively and some of their enzymes such as alkaline amylases have been put to use on an industrial scale (Horikoshi, 1999; Aygan *et al*., 2008).

Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (Okami and Hotta, 1988). Actinomycetes are abundant in terrestrial soils, a source of the majority of isolates shown to produce a number of bioactive compounds. The result of intensive screening program carried out over the past several decades is that there is a growing problem of rediscovery of already known bioactive compounds (Nolan and Cross, 1988). An approach to address this problem is to expand the source of actinomycetes by carrying out ecological assessment of environments other than terrestrial soils. There is growing interest in the Streptomycete actinomycetes from Vellar Estuary soil and it was found that majority of isolates were *Streptomycetes*, indicating that the Vellar Estuary soil is a suitable source of actinomycetes to screen for production of novel bioactive compounds (Dhanasekaran *et al*., 2009).

The marine actinomycetes, although easy to isolate, their ecological role in the marine ecosystem was largely neglected, and various assumptions meant that there was little incentive to isolate strains for search and discovery of new bioactive molecules. However, current research suggests that the marine actinomycetes are a prime resource in search and discovery for novel natural products and biological diversity. Striking advances made in marine microbial ecology using molecular techniques and metagenomics have projected actinobacteria as an often significant, sometimes even dominant clade in the marine environment. Approaches, culture-dependent methods and culture-independent techniques are leading to new insights into marine actinobacterial biodiversity and biogeography. Very different views of actinobacterial diversity emerge from these, however, and the true extent and biogeography of this are still not clear (Ward and Bora 2006). A review by Lam (2006) also describes marine actinomycetes as a prolific but underexploited source for the discovery of novel secondary metabolites. There is a tremendous diversity and novelty among the marine actinomycetes present in marine environments. Progress has been made to isolate novel actinomycetes from samples collected at different marine environments and habitats (Jensen and Mafnas 2006; Bredholdt *et al.*, 2007).

Characterization of indigenous actinomycetes and the extent of their adaptations in their habitat that affect secondary metabolite synthesis will provide a basis on which to develop a new source of pharmaceutical compounds (Jensen and Fenical 1994). Much of the success of biotechnology relies upon investigating, characterising and maintaining the biodiversity of microorganisms and to study the correlation between different factors affecting the occurrence of species (Fenical *et al*., 2002).

Antimicrobial activity influences the structure and the function of the microbial community, hence influencing the soil property of that habitat and, overall, the nature and transport of biogeochemical substances. Ghanem *et al.*, (2000) reported the distribution of actinomycetes populations from different types of marine sediment collected from four different sites in Alexandria, Egypt. Jensen *et al.*, (1991) reported a bimodal distribution of actinomycetes in near-shore tropical marine environments of 15 island locations throughout the Bahamas. Although marine actinomycetes diversity has been well studied in recent times, the relationship between the distribution of actinomycetes and their antagonistic behavior with the physicochemical characteristics of the habitat has not yet been explored (Mitra *et al.*, 2008).

MATERIAL AND METHODS:

Isolation of actinomycetes:

1. Collection of water and sediment samples of Lonar Crater.

Water samples will be collected in sterilized bottles and sterile polyethene bags from different locations of Lonar Lake.

2. Isolation of diversity of actinomycetes from water and sediment samples of Lonar Crater.

Actinomycetes will be isolate from Lonar water and sediment samples by spread plate on selective Actinomycetes Isolation Agar medium using serial dilution method. Isolated colonies will use for further work.

3. Screening of actinomycetes in pure form.

Actinomycetes isolate will purified by spread plate on Actinomycetes Isolation Agar medium using serial dilution method. Pure isolates of actinomycetes will use for further work and identification.

4. Screening of antibiotic producing actinomycetes.

The antibiotic producing actinomycetes will be screened from isolated actinomycetes species.

5. Screening of soil samples by crowded plate technique:

A series of culture tubes containing 9 mL of sterile water was taken. From the stock culture, 1 mL suspension was transferred aseptically to the 1st tube (10^{-1}) and mixed well. Further serial dilutions were made to produce 10^{-5} suspensions were made. Suspension (0.1 mL) from each culture tube was spread on sterile Nutrient agar medium plates and Actinomycetes Isolation agar medium plates aseptically in a laminar-air flow cabinet. The plates were incubated at 27 ± 2^0C for 72 hrs. The plates were observed intermittently during incubation. After 72 h, whitish pin-point colonies, characteristic of actinomycetes and with a clear zone of inhibition around them were seen. The pinpoint colonies with inhibitory or clear zone of inhibition were selected and purified into actinomycetes agar slants. The selected strains were further purified by multiple streaking method. The stock cultures of each selected strain was prepared and maintained in nutrient agar slants at 4oC. The actinomycetes colonies isolated from the crowded plate were selected for the further studies and labeled ACM 1, ACM 3, ACM 4, ACM 7, ACM 8, ACM 9, ACM 10, ACM 12, ACM 13, ACM 15, and ACM 16.

Test microorganisms

1. *E. coli* (MTCC 118)
2. *K. pneumoniae* (MTCC 109)
3. *S. aureus* (MTCC 1430)
4. *E. faecalis* (MTCC 2729)

Primary screening of the antimicrobial activity:

The primary antimicrobial activity was done by perpendicular streak method. In this method bacterial colonies were streaked on center of nutrient agar plates as a linear culture and incubated at 28ºC for 7 days. After 7 days, the test microorganisms were inoculated perpendicularly to the linear cultures and incubated at 37ºC for 48 h. Antagonism was measured by determination of size of inhibition zone (Table 1). The antimicrobial producer isolates inhibited the growth of test microorganisms and were selected for further experiments.

Preparation of sample:

1. The mycelium of respective sample was taken and was grinded in a sterile mortar & pestle.
2. The grinded mycelium was then squeezed through sterile muslin cloth in order to extract the liquid.
3. This liquid (extract) was further used for disc diffusion test.

Disc Diffusion

1. In the disc diffusion test sterile Whatman filter paper (number 1) discs were impregnated with 20µl of different samples.
2. The broth culture's CFU was 10^8 used for the study.
3. A broth culture of each of the respective species mentioned above was spread on the surface of sterile Mueller Hinton Agar plates.
4. The impregnated discs with respective samples were then placed on the inoculated surface of the agar plates (maximum of 8 discs per plate including negative control).
5. The agar plates were incubated at 37ºC for 24hr. Antimicrobial activity of each sample against the test species was measured by growth free "zone of inhibition" near the respective discs.
6. The assay was performed in triplicate.

RESULTS AND DISCUSSION

Actinomycetes strains are characterized by the production of important extracellular bioactive compounds and majority of those strains belong to species within the genus *Streptomyces* which produce two–thirds of

the clinically important antibiotics. This genus was confirmed to be promising bacteria against several pathogens and is well known for their potential to produce a large number of inhibitory metabolites. (Dhanasekaran, 2009). Total 70 Water and sediments samples were collected from different areas of Lonar Lake. To kill spores of fungus heat treatment was applied on all samples. After heat treatment serial dilution was done on the soil samples to reduce the colony count on agar plate and to reduce other bacterial colony to final countable range. Rough, chalky, powdery and single white, yellow, pink colonies were observed on Nutrient agar plates. Some colonies were very hard to pick from agar surface, which is also a characteristic of actinomycetes. These kinds of colonies were picked with help of hot Nicrhome loop. A total of 16 actinomycetes isolates were obtained in all. Further, actinomycetes colonies that were showing point of zone of inhibition on nutrient agar media were selected for antibacterial screening. Total eleven isolates showed zone of inhibition on Mullet Hinton Agar and Nutrient agar plates. After sub-culturing, slants of isolates were stored at 4^0C in refrigerator and labeled ACM 1, ACM 2, ACM 3, ACM 4, ACM 7, ACM 8, ACM 9, ACM 10, ACM 12, ACM 13, ACM 15, AND ACM 16. Eleven isolates that were showing zone of inhibition were further tested for antibacterial activity against Pathogenic Gram positive and Gram negative bacteria. After 7^{th} day of streaking of active actinomycetes isolates, related test organisms were streaked on nutrient agar plates. Observation was done on different times and reference pathogens and observations were recorded.

Table 1: Sensitivity of various bacteria to the Actinomycetes isolates

S.N	Samples	Zone of Inhibition (In mm)			
		E. coli	***K. pneumoniae***	***S. aureus***	***E. faecalis***
1	**ACM 1**	---	11 mm	10 mm	---
2	**ACM 3**	13 mm	13 mm	12 mm	11 mm
3	**ACM 4**	---	11 mm	---	---
4	**ACM 7**	---	---	---	11 mm
5	**ACM 8**	12 mm	---	11 mm	---
6	**ACM 9**	---	---	---	10 mm
7	**ACM 10**	11 mm	---	---	---
8	**ACM 12**	---	10 mm	10 mm	11 mm
9	**ACM 13**	---	---	---	---
10	**ACM 15**	15 mm	14 mm	16 mm	14 mm
11	**ACM 16**	---	11 mm	08 mm	---

Isolate ACM 1 showed activity against both Gram positive and Gram negative bacteria. Isolate ACM 3 showed broad spectrum of activity against both Gram positive and Gram negative bacteria. ACM 4 isolate showed activity against Gram negative bacteria only i.e. *K. pneumoniae*. ACM 7 showed activity against *E. faecalis.* ACM 8 showed activity against *E. coli* and *S. aureus.* ACM 9 showed antibacterial activity against *E. faecalis.* ACM 10 showed antibacterial activity only against *E. coli.* ACM 12 showed antibacterial activity against *K. pneumoniae, S. aureus* and *E. faecalis.* ACM 15 showed broad spectrum of activity against both Gram positive and Gram negative bacteria. ACM 16 *K. pneumoniae* and *S. aureus.* ACM 13 does not show antibacterial activity. (Charan et.al, 2004).

On the basis of macroscopic and microscopic characteristics, Gram reaction and biochemical characterization, all selected actinomycetes isolates were found to belong to *Streptomyces* genus. Isolate ACM 5, ACM 6, ACM 7, ACM 12, and ACM 16 showed broad spectrum against both Gram positive and Gram negative bacteria and was therefore selected for further analyses (Table 1). The morphology of isolate ACM 5 showed a well-defined colony on Nutrient Agar plate; colony color was white; aerial mycelium was observed with long chain of spore containing more than 50 spores in rectiflexibiles chains in macroscopic characterization and Gram positive reaction was observed in Gram staining (George et. al, 2010).

In biochemical tests, it showed positive reaction in Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, and urease test. For utilization of sugar on Triple sugar iron agar it showed fermentation of lactose, sucrose with H_2S production. No growth has been observed on MacConkey agar. (Gunasekaran et. al, 2013).

Conclusion: Actinomycetes are well known bioactive substance producers. The actinomycetes present in Lonar Lake are rich source of antimicrobial agent.

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