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Screening and Isolation of antibiotic producing Actinomycetes from marine samples using rifampicin

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ABSTRACT

The total number of actinomycetes recovered from different samples varied between a maximum of 3.5 X 10^5 CFU/gm of samples of sea weeds and algae to a minimum of 6.2×10^2 CFU/ml of sea water. A total of 132 strains of actinomycetes were isolated, of these 76 (57.6%) exhibit antimicrobial activity to one and/ or more of bacterial, filamentous fungi or yeast strains. Most of the inhibitory activity was directed against bacteria than filamentous fungi and yeast. Among bacterial group *Bacillus subtilis* (39.4%) was the most susceptible while *Proteus vulgaris* (11.4%) was the least. Among fungal strains, *Penicillium chryseogenum* (34.1%) was the most susceptible while *Aspergillus oryzae* (10.6%) was the least. Thirty five (26.5%) of the total isolates exhibited broad spectrum activity. The result showed that, better recovery of actinomycetes was observed using rifampicin than using naldixic acid. Therefore, in the search for screening and isolation of bioactive producing actinomycetes, rifampicin might be used as a better drug of choice than naldixic acid for suppressing and inhibiting growth of non-filamentous bacterial contaminants in marine samples.

Key words: Actinomycetes, marine samples and Rifampicin.

INTRODUCTION

It is well established fact that microbial natural products remain one of the most important sources of lead compounds for the pharmaceutical industry. Among microorganisms, the actinomycetes are a fascinating group that they are sources of most of the antibiotics used in medicine today. They also produce metabolites that are used as anticancer drugs, antihelminthics and drugs that suppress the immune system in patients who have received organ transplants. This becomes apparent in 1940, following Selman Waksman's discovery of actinomycin from *S. gresous*[1] and was fully realized by the 1980s, when actinomycetes accounted for almost 70% of the world's naturally occurring antibiotics[2].

In addition to terrestrial sources actinomycetes have been isolated from marine water, sediments, plants and animals. Actinomycetes comprise about 10% of bacteria colonizing marine aggregates[3]. Despite their abundance, however, reports in marine actinomycetes are yet not explored. As a result in recent years marine environment be comes a major focus in the search for the next generation of pharmaceutical agents[4]. This is because; organisms in marine environment have developed unique adaptations that enable them to survive in dark, cold and highly pressurized environments. These novel adaptations can offer a wealth of opportunities for the discovery of new drugs for the treatment of infectious diseases [5, 6, 7].

The goal of this study were to screen and isolate antibiotic producing actinomycetes that are found in different samples of marine environment in the East Coast of Bay of Bengal using refampicin or naldxic acid as a selective inhibitor of bacterial contaminant**.**

MATERIAL AND METHODS

Sample collection and processing

A total of 37 different samples were taken from marine environment (10 sea water, 20 sediments, 1sample each from sponges, hard corals and snails, and 4 algae and sea weeds) at a various depth using sterile screw capped bottle and sterile plastic bags.

Samples were processed as soon as collected and some of the samples collected from distant site were processed in the following days of collection by preserving the samples with a sterile plastic bag in an ice box. Algae and sea weeds samples were dried overnight in a laminar flow hood and crushed with an alcohol sterilized mortar and pestle. Other samples like sponges, corals and shell of snail samples were processed as described by Jensen *et. al.* [8]**.** These samples were subjected overnight in a laminar flow hood and the samples were scrapped with a sterile spatula generating a powder.

About 1gm of the above samples (sediments, dried powdered samples) was suspended aseptically and were homogenized with sterile sea water and kept in 250 ml flask having 50 ml of sterile sea water and incubated in an orbital shaker at 26 ^{0}C with shaking at 140 rpm for 30 minutes. Mixtures were allowed to settle then ten fold serial dilutions were prepared. Depending on the condition water samples were either directly or serially diluted.

One millilitre of each of these dilutions was added to 50ml of starch casein agar (g/l: starch 10, casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01, agar 20) and oat meal agar (g/l: oat meal 20, agar 20, trace salt solution 1ml) when the temperature is about 40-45 ^{0}C. Samples were thoroughly mixed and poured in to a sterile Petri-plate (6 diam). One copy of the samples was plated following spread plating technique using sterile L-shaped glass rod. Inoculated samples were incubated at 28°C for 21 days. To inhibit growth of non-filamentous bacteria and fungi medium was supplemented with 5µg/ml rifampicin[8] or 20µg/ml naldixic acid and 50µg/ml cyclohexamide [9] respectively.

Identification of actinomycetes

Actinomycetes colonies were recognized by their characteristics tough leathery colonies that adhered to the agar surface, branched vegetative mycelia, and when present, aerial mycelia and spore formation [8,10]**.** Actinomycetes colonies were counted on different plates after 10-21 days incubation at 28 ^{0}C. Data are expressed as colony forming unit (CFU/ml) for water or CFU/gm for dry weight samples. Actinomycetes colonies were characterized morphologically and physiologically following the methods given in the International Streptomyces Project (ISP) [11]**.** Species were identified by the morphological characteristics of colonies, substrate and aerial mycelium, structure of spore chains and pigment production on different ISP media. Detailed physiological and biochemical characterization of promising isolates were performed following the standard procedure[12].

Determination of antimicrobial activity

Primary screening of antimicrobial activities of actinomycetes isolated from different samples were screened following the streak plating technique**.** The test organisms employed in this study were supplied by the National Chemical Laboratory (NCIM), Pune and Microbial Type Culture Collection and Gene bank (MTCC), Chandigarh. The following test organisms were used for antimicrobial activities. *Bacillus pimilus* (NCIM-2327), *Bacillus subtilis* (NCIM-2063), *Staphylococcus aureus* (NCIM-2079), *Escherichia coli* (NCIM-2065), *Shigella flexinari* (MTCC-1457), *Salmonella typhimerium* (MTCC-98), *Aspergillus niger* (NCIM-548), *Aspergillus oryzae* (NCIM-643), *Penicillium cryseogenum* (NCIM-738)*, Candidia albicans*, *and Saccharomyces cervisae.*

Further screening of activities were made using an agar overlay methods following methods of Willims *et.al*. 1983a [13]. That is, spot inoculated 5 days old plated colonies were killed by inverting them

over 1.5ml chloroform for 40 minutes. The dead colonies were overlaid with 5ml of nutrient agar (bacteria) and 5 ml of potato dextrose agar (yeast and fungi) that has been inoculated with the test organism. Zone of inhibition around the colonies was recorded after 24 hours at 37 °C for bacteria and 48 hours at 28°C for yeast and filamentous fungi.

RESULT AND DISCUSSION

The total number of actinomycetes recovered from different samples showed that the maximum population density was recorded in samples of sea weeds and algae, 3.5×10^5 CFU/gm followed by samples of sponges, corals and snails, 4.7×10^4 CFU/ml; sediments 2.6×10^3 CFU/ml and water with least population counts, 6.2×10^2CFU/ml. (Table 1). Sahu *et.al*[14] observed similar patterns of population density of marine actinomycetes from sediment and water samples collected in the East coast of India. The maximum count of actinomycetes in algae and sea weeds samples may be because of nutrient availability. That is, nutrient availability in plant and animal residues is higher and this may create a fertile ground for spores of actinomycetes to germinate. However, the population of actinomycetes decrease in water samples than other samples, this is also because dormancy of spore imposed by lack of nutrients in water and a spore population will decline[15] and results low number of actinomycetes in water sample than other samples.

Table 1: Total microbial count obtained in different samples using Rifampcin

Type of sample	Total number of samples	Average total count of actinomycetes	Medium used	Antifungal and antibacterial agents
Water	10	6.2×10^2 CFU/ml	Starch casein agar	Cyclohexamide 50µg/ml and rifampicin5µg/ml
Sediment	20	2.6×10^3 CFU/gm	Starch casein agar	Cyclohexamide 50µg/ml and rifampicin5µg/ml
Sponges, corals and snails	3	4.7×10^4CFU/gm	Starch casein agar	Cyclohexamide 50µg/ml and rifampicin5µg/ml
Algae and sea weeds	4	3.5×10^5 CFU/gm	Starch casein agar	Cyclohexamide 50µg/ml and rifampicin5µg/ml

Table 2: Comparison of actinomycetes recovery from same samples using rifampicin and naldixic acid as inhibitors of bacterial contaminants. (Only 21 samples were compared)

Type of sample	Number of samples assessed	Antibiotics used	Recovery of actinomycetes		
			Yes	No	Average count
Water	4	5µg/ml Rifampicin	1	3	1.5×10^1CFU/ml
		20µg/ml Naldixic acid	-	4	-
Sediment	10	5µg/ml Rifampicin	5	5	1.1×10^3 CFU/gm
		20µg/ml Naldixic acid	3	7	1.3×10^2 CFU/gm
Corals, sponges, shells	3	5µg/ml Rifampicin	2	1	4.7×10^4 CFU/gm
		20µg/ml Naldixic acid	2	1	2.8×10^3 CFU/gm
Algae and sea weeds	4	5µg/ml Rifampicin	3	1	3.6×10^5 CFU/gm
		20µg/ml Naldixic acid	2	2	3.0×10^4 CFU/gm

To avoid bacterial contaminant from different samples, 5µg/ml rifampicin or 20µg/ml naldixic acid have been used for comparison. Comparing to naldixic acid, the rate of recovery of antibiotic producing actinoomycetes using rifampicin is relatively high (Table 2). Similar report by Pisano *et al.*[16] showed that, in pre-treated samples 16 % of the actinomycetes produced bioactivity while in rifampicin treated marine samples 46% of actinomycetes showed bioactivity[17].

Table 3: Distribution of actinomycets exhibiting antimicrobial activities

Type of test organisms		Number of actinomycetes	%
	Gram positive	27	20.5
Bacteria	Gram negative	5	3.8
	Gram positive and negative	32	24.2
	Total number of isolates	64	48.9
	Filamentous fungi	19	14.4
Fungi	Yeast	2	1.5
	Filamentous fungi & yeast	29	22.0
	Total number of isolates	50	37.9
Number of isolates with broad spectrum activity (bacteria, filamentous fungi and yeast)		35	26.5
Total actinomycetes showing activities		76	57.6
Total actinomycetes with weak and/or no activities		56	42.4

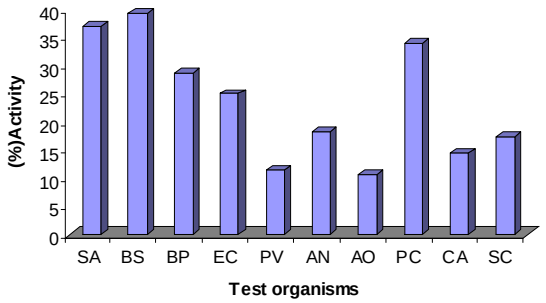
Table 4: Activity of promising isolates on different test organisms using an agar overlay method

Code of isolate	Inhibition zone (mm)												
	Filamentous fungi and yeast					Gram-positive and gram-negative bacteria							
	AN	AO	PC	SC	CA	SA	BM	BS	EC	PV	SF	ST	MRSA
WS1/31	25	20	28	25	15	15	25	20	-	-	-	-	-
SS4/2	-	-	-	-	-	50	20	55	-	-	-	-	-
SS4/4	-	_	_	_	_	-	20	-	-	-	-	-	-
SS4/61	_	_	15	_	_	-	-	20	-	-	-	-	-
SS16/2	15	-	35	12	23	32	15	57	45	25	45	47	32
SS18/2	-	-	-	-	-	10	25	15	-	-	-	-	-
SS18/3	-	-	10	30	23	60	25	65	25	-	30	-	-
CS19/10	-	-	-	-	-	55	-	-	23	-	55	-	-
CS19/11	-	-	-	-	-	40	50	-	30	-	28	-	-
CS19/13	-	-	-	-	-	55	-	45	23	-	28	-	30
SS23/2	18	20	40	18	-	60	60	70	-	-	23	-	-
SS31/1	-	-	-	-	-	30	45	25	10	-	-	-	-
SS31/2	-	-	-	-	-	48	50	41	-	-	-	-	-
SS31/32	-	-	-	-	-	47	45	42	-	40	-	-	-
SS31/T2	-	-	-	-	-	42	45	38	-	-	55	-	-

AN= A. niger (NCIM-548); *AO=A. oryzae* (NCIM 643); *PC= P. cryseogenum; SC= S. cervisae; CA= C. albicans; SA= S. aureus* (NCIM 2079); *BP=B. pimilus* (NCIM2327); *BS=B. subtilis* (NCIM 2063); *EC= E. coli* (NCIM 2065); *PV= P. vulgaris; SF= S. flexinari* (MTCC1457); *ST=S. typhimerium* (MTCC 98); *MRSA*= Methicillin Resistant *Staphylococcus aureus.*

As indicated in table-2, four water samples were taken for comparison of rifampicin and naldixic acid. It was found that actinomycetes colonies were recovered only in one sample using rifampicin (1.5×10^{1}CFU/ml). Out of 10 sediment samples taken for comparison, actinomycetes were recovered in 5 samples for rifampicin (1.1×10^{3} CFU/gm) and 3 samples for naldixic acid (1.3×10^{2} CFU/gm). One sample each from corals, sponges and shells were also compared, both rifampicin (4.7×10^{4} CFU/gm) and nalixic acid (2.8×10^{3} CFU/gm) were showing similar recovery with high population density in rifampicin. Four samples from algae and sea weeds were also compared, 3 of the samples were showing high density count using rifampicin (3.6×10^{5} CFU/gm) and 2 of the samples showing also good count using naldixic acid (3.0×10^{4} CFU/gm). As naldixic acid, rifampicin is also effective against some gram-positive and gram-negative bacteria. However, rifampicin is mostly effective when there is combination of other drugs (Examples, treatment of tuberculosis, leprosy); this might be the reason that using rifampicin alone may favour recovery of more actinomycetes populations than using the broad spectrum antibiotic, naldixic acid.

During screening of 37 different marine samples, 420 suspected actinomycetes colonies were recovered. Among these, on the basis of their colony morphology, colour of their aerial mycelium 150 colonies suspected to be different were selected



SA= *S. aureus* (NCIM 2079); BS=*B. subtilis* (NCIM 2063); BP=*B. pimilus* (NCIM2327); EC= *E. coli* (NCIM 2065); PV= *P. vulgaris*; AN= *A. niger* (NCIM-548); AO=*A. oryzae* (NCIM 643); PC= *P. cryseogenum*(NCIM-738); CA= *C. albicans*; SC= *S. cervisae*

Fig. 1: Actinomycetes isolated from different marine samples showing bioactivity against bacteria and fungi

and looping out. Although rifampicin can suppress the growth of some of the strains of actinomycetes, 5µg/ml concentration added to the sample during primary screening found to be effective to protect rapidly growing members of non-filamentous bacteria.

After detail study of their morphology and characteristics of aerial and substrate mycelium, a total of 132 isolates were confirmed to be actinomycetes (Table 3). Among these, 76 (57.6%) exhibit antimicrobial activity to one and/ or more test organisms of bacterial, and filamentous fungi or yeast strains while the other 56 (42.4%) isolates don't show and/or poor activities. This result was lower than reported by Pisano et.al.[18], 73% of chitinolytic actinomyctes were showing bioactivity. The reason may be due to correlation between chitinolysis and bioactivity of actinomycetes[18].

Most of the inhibitory activity was directed against bacteria than filamentous fungi and yeast. As shown in table-3, 64 (48.9%) of the total number isolated, proved to be active to one or more bacteria, while 50 (37.9%) were active to filamentous fungi and yeast.

Among bacterial group *B. subtilis* (39.4%) was the most susceptible organism followed by *S. aureus* (37.1%), *B. pimilus* (28.8%), *E. coli* (25%); *P. vulgaris* (11.4%) was the least susceptible. Among fungal group *Penicillium cryseogenum* (34.1%) was the most susceptible followed by *Aspergillus niger* (18.2%), *Saccharomyces cervisae* (17.4%), *Candidia albicans (*14.4%); *Aspergillus oryzae (10.6%)* was the least susceptible (Figure-1). Total number of actinomycetes exhibiting broad spectrum activity (bacteria and fungi) were 35(26.5%).

Fifteen promising isolate were selected and their detailed biochemical and physiological study showed that 10 of them were under the *Sterptomyces* species and 5 of them were under the group *Micromonospora* species. This result was in agreement with reports of Das *et.al*., [19] and Peela *et.al*., [20] as the major genera isolated in the bay of Bengal were the *Streptomyces* and *Micromonospora* species. However, this result is different from reports by Bredholt *et.al*.[21] that *Micromonospora* species are dominant. The reason

may be because of difference in depth of the sample collected. The deep water sediments contained a higher relative amount of *Micromonospora* compared to the shallow water sediments[22]. Although, the other isolate showed strong antagonistic activities against different test organisms two of the *Streptomyces* species were also strongly active against methicillin resistant *S. aureus* (MRSA (Table 4). The antimicrobial spectrums of these species were also evaluated by submerged fermentation and their activities are still very promising against the test organism of bacterial and fungal strains. The diversity of these promising isolates and their content of biological activities are understudy.

CONCLUSION

As it is well documented drug resistance pathogens such as methicillin resistant *S. aureus* become the most problematic gram positive bacterium in public health not only because it is highly prevalent but also it has reported that, the organism shows reduced susceptibility to the last resort antibiotics of vancomycin and teicoplanin. Therefore, despite the challenges of identifying new natural product antibiotics; searching and screening of antibiotic producing actinomycetes from new unexplored area using different pre-treatment of marine samples such as rifampicin as selective inhibitors for non filamentous bacterial contaminants during primary screening may help to identify new novel bioactive molecules.

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