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# Antimicrobial activity of the marine actinomycetes

**R.M. Gulve[1] and A.M. Deshmukh[2]**

[1]Department of microbiology, Kesherbai Sonajirao Kshirsager Alias Kaku Arts Science and Commerce College, Beed 431122 ( M.S.), India.
[2] Department of Microbiology, Dr. Babasaheb Ambedkar Marathwada University Sub – Center, Osmanabad 413 501 (M.S.), India.

**Abstract**
Antibacterial activity of 107 marine actinomycetes isolated form near sea shore sediment and seawater from Konkan coast of Maharashtra was studied. A total 107 actinomycetes were subjected to primary screening by perpendicular streak method against various test microorganisms. Among 107 actinomycetes 22,14,34,14,07,52,27 and 6 number of actinomycetal isolates were antagonistic against *Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris ,Escherichia coli, Klebsiella aerogenes , Pseudomonas aeruginosa.,Candida albicans and Aspergillus. niger* respectively. Out of 107 actinomycetal isolates 13 isolates showing maximum antagonistic activities that were subjected for the secondary screening by agar well method. Finally 0 5 isolates were selected for further study on the basis of maximum zone of inhibition and broad spectrum activity. Selected 05 isolates were inoculated in glucose soybean broth 7 days at $30^0$C and antibacterial substances were extracted with ethyl acetate.T L C of the ethyl acetate extract was carried out using N butanol : acetic acid : water ( 4:1:5 ) as a solvent system. Spots were observed under UV light and in iodine chamber. Bioautograhy of ethyl acetate extract of selected 05 isolates were carried using test organisms *B. subtilus* and *P. vulgaris.* Inhibition zones were observed and they were associated with the purple spots at the chromatograms as detected under UV light. This may indicate the same compound was responsible for the antibacterial activity of the actinomycetes.Finaly one potent actinomycetal isolate (GA-22) was selected and it's morphological, cultural, physiological and biochemical characters was studied. It was found that biochemically GA-22 was very active marine actinomycetes it was able to produce variety of enzymes and utilize number of sugars.
**Keywords:** Antimicrobial activity, Marine actinomycetes.

## INTRODUCTION

Marine microorganisms have become an important source of study in the search for novel microbial products. Today both academic and industrial interest in marine microorganisms is on the rise. Due to the physical and chemical conditions of the marine environment, almost every class of marine organism exhibits variety of molecules with unique structural features, which are not found in terrestrial natural products. Today researchers have isolated approximately 11,000 marine derived natural products compared with more than 155,000 natural, terrestrial products. Although , the oceans contain much greater biodiversity than is found on land , effort to exploit this biodiversity by identifying new chemical compounds have hardly begun [1]. Rare or novel actinomycete taxa has become a major focus in the search for pharmaceutical agents [2]. Actinomycetes have been looked upon as a potential source of antibiotic and the past experience proves that actinomycetes are the richest source of secondary metabolites. Physiologically active marine actinomycetes isolated from near shore marine sediments collected throughout Bahamas and Bye of Bengal sediment samples near Kakinada coast of Andhra Pradesh , India [3 and 4]. Actinomycetes isolated from mangrove sediments of Pichavaram southeast coast of India exhibiting prominant antibiotic activity against *C. albicans* [5] . Actinomycetes isolated from marine sediment samples of Bay of Bengal showing activity against multidrug resistant pathogens [6]. It is also reported that marine actinomycetes are useful and suitable source of new bioactive natural products. Marine actinomycetes *Salinospora* and *"Marinispora"* producing new macrolide antitumor-antibiotics, with specificities towards drug resistant bacteria and melaoma, of a novel class [7].

In the present investigation an effort was made to screen antagonistic marine actinomycetes from near sea shore sediments of the Konkan coast of Maharashtra, India which is large diverse and largely unscreened ecosystem for the isolation of potent antibiotic producing actinomycets.

## MATERIALS AND METHODS

The actnomycetes used in this study were isolated from near shore sediment samples from different sites of Konkan coast of Maharashtra.viz Mumbai,Ghuhagar, Ganpatipule, Vengurle and Ratnagiri. Twenty near sea shore sediment samples were collected 10 cm in depth in sterile Petri plates. The collected samples were labeled as (MU) for Mumbai, (RA) for Ratanagiri, (GA) for Ganpatipule, (GH) for Guhagar and (VE) for Vengurle. Dilution of sample in sterile sea water was made and temperature shock 70 $^0$C for 5 min was given to each diluted sample to depress associated gram negative bacteria. Then inoculated in enrichment medium of composition (Starch 2g, yeast extract 0.8 g, peptone 0.4 g, sea water – 1000 ml with antifungal tablet rifampicin (5 µg/ml.) and incubated at $30^0$ C for 10 days [9].

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*Corresponding Author
R.M. Gulve
Department of microbiology, Kesherbai Sonajirao Kshirsager Alias Kaku Arts Science and Commerce College, Beed 431122 ( M.S.), India.

Tel:; Fax:
Email: rekhagulve@gmail.com

Actinomycetes from enrichment medium was isolated by streak plate method, using starch nitrate agar, glycerol asparagine agar, actionomycetes agar.Plates were incubated at $30^0$ C for 7 days. After incubation dry leathery colonies of actinomycetes were isolated and preserved on actionomycetes agar slants.

**Screening of actinomycetes for antibacterial activity**

In primary screening, the antibacterial activity of actinomycetal isolates was determined by using perpendicular streak method. Single streak of the actinomycetes was made on the surface of the nutrient agar.

The test organisms used were *B. subtilis* NCIM 2195 *S. aureus* NCIM 2602,*P. vulgaris* NCIM 2027,*K. aerogenes* NCIM 2283 *P. aeruginosa* NCIM 2945, *C. albicans* NCIM 3466 and *A. niger* NCIM 50l. After observing good ribbon- like growth of the actinomycetes on the Petri plates, the test organisms were streaked at right angles to the original streak of actinomycetes and plates were incubated at $30^0$C for 24 hours. After incubation length of streak growth was measured. Decrease in length of growth than the inoculate streak indicate inhibition of growth. Those actinomycetal isolates showed maximum inhibition were selected for secondary screening.

**Secondary screening**

In the primary screening13 actinomycetes showing positive antibacterial activities were tested by using secondary screening. Actinomycetes were inoculated in sterile10 ml of glucose soybean medium of composition (glucose- 20g, soyabean meal-20 g, Nacl-4g, $K_2HPO_4$-0.05g, $MgSO_4$-0.5g, $CaCO_3$ – 5 g, seawater 1000 ml) in 50 ml conical flask with 2.5% inoculums of actinomycetal isolate and incubated on rotary shaker at 220 rpm at $30^0$C for 8 days. Then the culture was collected and centrifuged at 4000 rpm for 20 minute and filtrate used to test antibacterial activity. Antibacterial activities were assayed by using well diffusion method against the standard test organisms as listed above [5] .

The test organisms were grown in sterile nutrient broth at $37^0$C for 12 hours and swabbed on Muller –Hinton agar surface. Agar wells were prepared using sterile cork borer (diameter 4 mm). Subsequently, 100 $\mu$l of the filtrate broth was added in the well and kept in freeze for 30 min. for diffusion and incubated at $37^0$C for overnight and the zone of inhibition (in mm) were measured using a ruler. Out of these 13 actinomycete isolates only five isolates MU-15, GA-22, VE-25, VE-35 and GH-58 showed maximum zone of inhibition against test organisms. They were selected for further studies.

**Extraction of antibacterial metabolites**

Five actinomycetal isolates MU-15, GA-22, VE-25, VE-35, and GH-58 were selected for further studies. Antibiotic fermentation was carried in 500ml Erlenmeyer flask. [9]. Five selected actinomycetes isolates viz. MU-15, GA-22, VE-25, VE-35, and GH-58 were inoculated in 100ml of glucose soybean medium separately in aseptic condition and flasks were incubated at $30^0$C for 8 days on rotary shaker. After fermentation filtrate was separated by centrifugation and antibiotic was extracted from filtrate by solvent extraction method. Ethyl acetate was added to the filtrate in the ratio 1:1 (V/V) and shaken vigorously for an hour for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase using separating funnel. Ethyl acetate layer was concentrated by evaporating to dryness at $40^0$C and residue obtained was purified using methanol [10]. This obtained compound was used to determine antibacterial activity against test organisms by disc diffusion method and bioautography [11].

**Determination of antibacterial activity**

The ethyl acetate extract were evaporated to dryness then recuperated in 1 ml of methanol and tested for their antimicrobial activities using disk of 6 mm diameter against *B subtilis, S. aureus* and .*P vulgaris* [12].

**Thin layer chromatography**

Ethyl acetate extract were used for primary analysis of the antibacterial substances. It was performed by thin layered chromatography (TLC) on silica gel slides by using n- butenol ,acetic acid and water (4:1:5) as a solvent system. Chromatograms were observed under UV light and exposed to iodine vapours [13]. Bioautography of TLC slides were carried with test microorganisms B *subtilis* and *P. vulgaris*.

**Bioautography**

Antibiotic compound was separated by using TLC slide. TLC slide was dried and was put in empty sterile Petri plate, in which 15 ml of sterile, molten nutrient agar seeded with 2% test organism was poured. Nutrient agar plate was incubated at$37^0$C for 24 hrs. After incubation zone of inhibition around the spot of antibiotic was observed. . The sterile zone on the media proved the presence of active antibacterial components in the studied samples [14]. The Rf value of antibacterial compounds were determined.

Observing bioautography results of five selected actinomycetal isolates one actinomycetal isolate GA-22 was selected for further study.

**Characterization of antinomycetes**

The potent actinomycetal isolate selected from primary, secondary and final screening processes was characterized by morphological methods. Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method. The cover-slip cultures of actinomycetal isolates were prepared and morphological characteristics were studied. The isolates were grown on glycerol aspargine agar as simple cover –slip cultures.A sterilized cover slip was carefully inserted at an angle of about $45^0$ in glycerol aspargine agar plate until about an half of the cover slip was in the medium. An actinomycetes isolate was then inoculated along the line where the upper surface of the cover slip meets agar. The plates were incubated at $30^0$C for 7 days. After incubation, the cover slip was carefully removed with respect to its orientation and placed in upward on a slide and used for microscopic observations.

Mycelium structure and arrangement of conidiospores on the mycelium was observed through microscope.The observed structure was compared with Bergeys manual of determinative bacteriology ninth edition and the organisms were identified [15]. Actinomycetes growth observed on different medium Isp2, Isp3, Isp4 Isp6,

actinomycetcs agar, tyrosine agar. Its physiological and biochemical characters were studied.

## RESULTS

Antimicrobial activity of marine actinomycetal isolates and distribution of antagonistic marine actinomycetes in sea sediments of Konkan coast inhibiting growth of various microorganisms is given in table 1 and plate1.

22,14,34,14,07,52,27 and 6 number of actinomycetal isolates were antagonistic against *B. subtilis, S.aureus, P. vulgaris ,E. coli, K. aerogenes , P. aeruginosa.,C. albicans* and *A. niger* respectively.

From secondary screening five potent antibiotic producing strains were selected according to maximum zone of inhibition and broad spectrum of activity they are MU- 15, GA- 22, VE- 25, VE- 35, and GH- 58 ( plate2).

Antimicrobial efficacy of ethyl acetate extract of fermentation broth of MU 15, GA 22, VE25, VE35, and GH 58 actinomycetal isolates was determined by disk diffusion method against microorganisms and results are given in table 2.

It was observed that crude ethyl acetate extracts obtained from actinomycetal isolates MU 15, GA 22, VE25, VE35, and GH 58 were showing prominent antagonistic activity against Gram positive and Gram negative test organisms but not against fungi.

From this observation it was concluded that antibacterial compound produced by actinomycetal isolates was extracted in ethyl acetate but antifungal compound was not extracted in ethyl acetate.

It was further observed that GA-22 actinomycetal isolate was most potent antibacterial compound producing actinomycetes. Bioautography was performed to confirm the position of the active component on the TLC slides. Spots on TLC slides were analyzed by using bioautography against *P. vulgaris* and *B. subtilis*. TLC spots of ethyl acetate extract of all the selected actinomycetes viz. MU -15, GA -22, VE- 25, VE- 35 and GH- 58 were showing zone of inhibitions against test organisms. Zone of inhibition against *P.vulgaris* by actinomycetal isolates MU -15, GA -22, VE- 25, VE- 35 and GH- 58 were 18,31,17,16 and 17mm respectively.

Zone of inhibition against *B.subtilis* by actinomycetal isolates MU- 15, GA- 22, VE -25, VE- 35 and GH -58 were 17,29,177,18 and 19mm respectively.

Thus it was found that the actinomycetal isolate GA-22 was giving maximum zone of inhibition. The zone of inhibition on the nutrient agar medium where antibiotic spot diffuses proved the presence of antibiotic in the separated TLC spot (plate 3).

TLC and bioautography were carried by many researchers during the study of antibiotics. Considinet et al.(1965) [16] used this technique for determination of position of the active spot on TLC slide.

Awais et al. (2007) [17] used this technique for checking the potency of antibiotic spots on TLC slides. Peneka Moncheva et al. (2002) [14]; Muhammad Awais et al. ( 2007) [11] ; Xue-Chang Wu., (2007) [10] have reported importance of TLC during antibiotic study.

From the results of primary, secondary screening, TLC and bioautography it was observed that the marine actinomycetal isolates GA- 22 was giving maximum zone of inhibition against test bacteria. The antibiotic potential of this actinomycetes retain in ethyl acetate extract.

Thus it was concluded that in future actinomycetal isolate GA - 22 has better potential of antibiotic production on large scale. By considering this view in mind actinomycetal isolate GA- 22 was used for further study.

The potent marine actinomycetal isolate GA- 22 form dry, leathery colonies with solid colony texture (plate 4). On ISP2 and ISP3 nutrient media good growth was observed. On ISP4, ISP7 and actinomycetes agar media moderate growth was observed but poor growth was present on ISP6 medium after seven days of incubation. Aerial mycelium color was white on ISP2, ISP4, ISP6, ISP7 and actinomycetes agar but faint rose violet color was observed on ISP3 medium. Color of vegetative mycelium was bright yellow on ISP2 and ISP3 medium; yellow on ISP4, ISP6 and actinomycetes agar media and pale yellow on tyrosine agar. Diffusible pigments were not observed on any nutrient media tested (Table 3).

Table1.Distribution of antagonistic marine actinomycetes in sea sediments of Konkan coast

| Sr No. | Test organism | Total Active Actinomycetes | | Zone of Inhibition (mm) | | | | | | No Inhibition | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | > 20 mm | | 10-20 mm | | < 10 mm | | | |
| | | No | % | No | % | No | % | No | % | No | % |
| 1 | *B.subtilis* | 22 | 20.56 | 3 | 13.63 | 7 | 31.81 | 12 | 54.54 | 85 | 79.43 |
| 2 | *S.aureus* | 14 | 13.08 | 3 | 21.42 | 4 | 28.57 | 7 | 50 | 93 | 86.91 |
| 3 | *P.vulgaris* | 34 | 31.77 | 5 | 14.76 | 12 | 35.29 | 17 | 50 | 73 | 68.22 |
| 4 | *E.coli* | 14 | 13.8 | 2 | 14.28 | 5 | 35.21 | 7 | 50 | 93 | 86.91 |
| 5 | *K.aerogenes* | 7 | 7.47 | 3 | 37.5 | 01 | 14.28 | 3 | 42.85 | 100 | 93.45 |
| 6 | *P.aeruginosa* | 52 | 48.59 | 5 | 9.61 | 18 | 34.61 | 29 | 55.76 | 55 | 51.40 |
| 7 | *C.albicans* | 27 | 25.23 | 4 | 14.81 | 8 | 29.62 | 15 | 55.55 | 80 | 74.76 |
| 8 | *A.niger* | 6 | 5.60 | 3 | 50 | 2 | 33.33 | 1 | 16.66 | 101 | 94.39 |

Table 2. Antimicrobial activity of crude ethyl acetate extract by disk diffusion method**.**

| | | Zone of inhibition (mm) against | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sr. No. | Actinomycete isolate | *Bs* | *Sa* | *Pv* | *Ec* | *Ka* | *Pa* | *Ca* | *An* |
| 1 | MU-15 | 17 | 15 | 12 | 12 | 15 | 16 | - | - |
| 2 | GA-22 | 25 | 20 | 22 | 20 | 21 | 22 | - | - |
| 3 | VE-25 | 22 | 15 | 12 | 12 | 15 | 16 | - | - |
| 4 | VE-35 | 20 | 18 | 12 | 15 | 16 | 18 | - | - |
| 5 | GH-58 | 18 | - | 12 | 15 | 16 | 19 | - | - |

Table 3. Cultural Characteristics of actinomycetes isolate GA- 22

| Sr. No | Medium | Growth | Aerial mycelium color | Vegetative mycelium color | Diffusible pigment |
|---|---|---|---|---|---|
| 1 | Yeast extract malt extract agarI SP2 | Well | White | Bright yellow | None |
| 2 | Oat meal agar .I SP3 | Well | Faint rose violet | Bright yellow | None |
| 3 | Inorganic salt starch agar. ISP4 | Moderate | White | Yellow | None |
| 4 | Peptone yeast extract agar. ISP6 | Poor | White | Yellow | None |
| 5 | Actinomycetes agar | Moderate | White | Yellow | None |
| 6 | Tyrosine Agar . ISP7 | Moderate | White | Pale yellow | None |

Table 4. Physiological and biochemical character of actinomycetal isolate GA- 22

| Sr No. | Test | Result |
|---|---|---|
| | **Utilization of sugars** | |
| 1 | | |
| 2 | D- Glucose | +++ |
| 3 | Sucrose | +++ |
| 4 | Lactose | + |
| 5 | D-Mannitol | ++ |
| 6 | D- xylose | -- |
| 7 | RAffinose | -- |
| 8 | L-Rhamnose | ++ |
| 9 | L-Arabinose | + |
| | Maltose | +++ |
| | **Biochemical Tests** | |
| 10 | | |
| 11 | Indol | -- ve |
| 12 | Methyl red | + ve |
| 13 | Vogus proskauer | -- ve |
| 14 | Citrate utilization | -- ve |
| 15 | Nitrate reduction | -- ve |
| 16 | $H_2S$ production | + ve |
| 17 | Catalase | + ve |
| 18 | Oxidase | + ve |
| 19 | Gelatinase | + ve |
| 20 | Caseinase | + ve |
| 21 | Cellulase | + ve |
| 22 | Amylase | + ve |
| 23 | Lecithinase | + ve |
| | Urease | -- ve |

+++ =Very good growth, ++ =Moderate growth , + = Poor growth ,
-- =No growth + ve = Positive test, --- ve = Negative test .

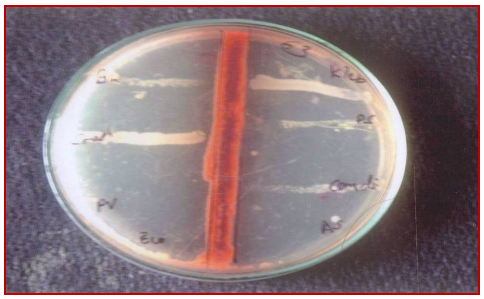

Plate 1. Actinomycetal isolates showing growth inhibition of test organisms (quadrant streak method)

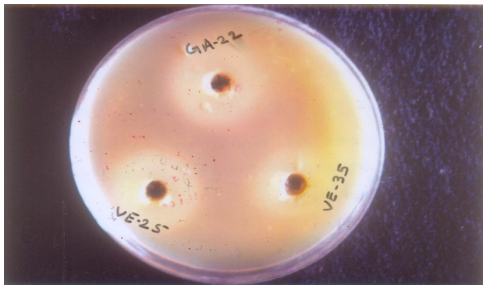


Plate 2. Actinomycetal isolate showing zone of inhibition against *E. coli (*agar well method antibiotic

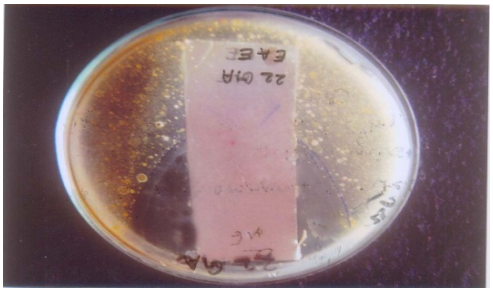

Plate 3. Bioautography of ethyl acetate extract of antibiotic obtained from actinomycetal isolateGA-22

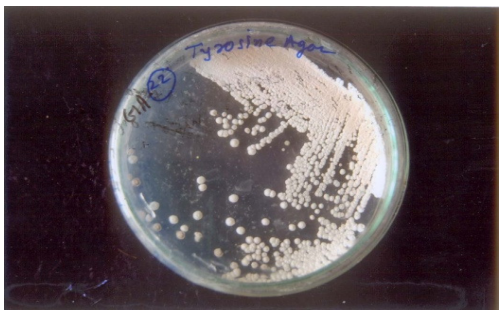


Plate 4 Colony morphology of actinomycetal isolate GA-22 on tyrosine agar

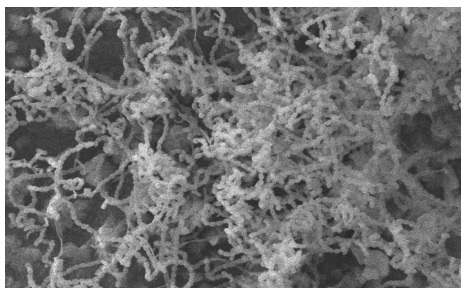

Plate 5. Spore chain morphology of *Streptomyces* (X1000)

The aerial mycelium at maturity forms spiral chains of spores. It belongs to genera *Streptomyces* according to Bergeys Manual of determinative bacteriology [15] (Plate 5). The ability of *Streptomyces* GA- 22 to utilize different sugars was tested. The biochemical characters of *Streptomyces* GA-22 are given in table 4. *Streptomyces* GA- 22 was able to utilize D-glucose, sucrose and maltose , D- lactose, D- mannitol , L- rhamnose, L-arabinose but unable to utilize in D- xylose and raffinose *Streptomyces* GA -22 was giving Indol test negative ;methyl red test positive; vogus proskauer and citrate test negative and $H_2S$ production test positive.

*Streptomyces* GA- 22 was able to produce catalase, oxidase, gelatinsase, caseinase, cellulase, amylase and lecithinase enzymes but unable to produce urease. Thus it was found that the potent antagonistic actinomycetal isolate *Streptomyces* GA- 22 was biochemically versatile and was able to produce number of enzymes. It has good potential in biodegradation of variety of organic compounds in marine environment.

## DISCUSSION

Many research workers have reported that antibiotic production from different species of actinomycetes by using similar work.

Fiedler et al. (2005) [18] isolated 600 actinomycetes strains from various sites of Pacific and Atlantic Oceans were screened for the production of bioactive secondary metabolites. Abyssomicin producing new rare genus *Verrucosisspora* were isolated during screening process.

Hala Rifat (2006) [19] Isolated actinomycetes from bottom sediments of Nile River and screened for antimicrobial activity.

Harald Bredholt et al. (2008) [20] studied antimicrobial potential of actinomycetes from sediments in Trondheim Fjord.

Glen P. et al. (2008) [21] used different screening process to isolate antibiotic producing microorganisms from Sunshine Coast in Australia and isolated gentamycin producing *Micromonospora* from sediment samples

## CONCLUSION

Based on the screening results, it has been show that sediments of Konkan sea coast Maharashtra possess antibiotic producing actinomycetes and may be tapped as one of the India's potential source of novel antibiotics.

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