Int. J. Adv. Res. Sci. Technol. Volume 7, Issue 1, 2018, pp. 728-733.

IJARST

International Journal of Advanced Research in Science and Technology

journal homepage: www.ijarst.com

ISSN 2319 – 1783 (Print)

ISSN 2320 – 1126 (Online)

Isolation and Characterization of Actinomycetes from Visakhapatnam Coast and Screening for Antimicrobial Activities of Actinomycetes against Pathogens.

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A R T I C L E I N F O

Article history:

Received : 23 Apr. 2018

Accepted : 10 July. 2018

Available online : 15 July. 2018

Keywords:

Actinomycetes,
Antimicrobial activity,
Characterization and
Submerged fermentation.

A B S T R A C T

Actinomycetes are one of the most attractive sources of antibiotics. In the present study, 32 isolates were isolated from marine soil sediments from the Bay of Bengal, Visakhapatnam. Isolation of Actinomycetes strain was obtained by serial dilution method and grown on actinomycetes isolation agar. Isolated strains were identified for their antibacterial activity of which only five isolates showed good result. They were evaluated for their inhibitory activity on 10 strains of microorganisms (Staphylococcus aureus (MTCC (Microbial Type Culture Collection) 3160), Bacillus subtilis (MTCC 441), Pseudomonas aeruginosa (MTCC 424), Bacillus cereus (MTCC 430), Proteus vulgaris (MTCC 426), Escherichia coli (MTCC 443), Saccharomyces cerevisiae (MTCC 170), Aspergillus niger (MTCC 961), Aspergillus flavus (MTCC 3396), Candida albicans (MTCC 227)). Antibacterial compounds were produced by submerged fermentation and their activity was checked against bacterial culture by antibiogram analysis where extracellular compounds showed positive result. Whereas KRBT-205, KRBT-401 and KRBT-411 showed antibiotic activity against all tested bacterial organisms, the zone of inhibition ranged from 10 mm to 24 mm and KRBT-103 and KRBT-401 showed antibiotic activity against all tested fungal organisms, the zone of inhibition ranged from 10 mm to 24 mm 10mm to 26 mm.

PAPER-QR CODE

IJARST

Volume 7, Issue 1

Citation: *Narsingarao.et.al, Isolation and Characterization of Actinomycetes from Visakhapatnam Coast and Screening for Antimicrobial Activities of Actinomycetes against Pathogens. Int. J. Adv. Res. Sci. Technol. Volume 7, Issue 1, 2018, pp.757-762.*

Introduction:

Actinomycetes are the most widely distributed groups of micro-organisms in Soil. They provide many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. Almost 80% of the world's antibiotics are known to come from *actinomycetes*, mostly from the genera *Streptomyces* and *Micromonospora* (Panday, *et al.,* 2004; Oskay et al 2004). According to the World Health Organization over prescription and the improper use of antibiotics has led to the generation of antibiotic resistance in many bacterial pathogens (Oskay, *et al*., 2004).They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. 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 *et al*., 1988). In these days most of the disaeases caused by bacteria have become resistance to most of the antibiotic (Alanis, 2005). *Staphylococcus aureus* is a commonly known pathogen that is responsible for infections like pneumonia, diabetes, cancer, vascular disease and lung disease have developed resistance to most classes of antibiotics (Enright 2003). Physicians acquired methicillin-resistant *S. aureus* (MRSA) which also bears resistance to many antibiotics. During this time, vancomycin has been the therapeutic answer to MRSA and Vancomycin resistant strains have emerged clinically (Hiramatsu

1998, Bozdogan *et al*. 2003, Chang *et al*. 2003, Anonymous 2004).

Vancomycin-resistant *S. aureus* (VRSA) challenges clinicians, not only because of vancomycin and methicillin resistance, but also because of resistance to many other antibiotics, including aminoglycosides, macrolides, and fluoroquinolones. Certain undesirable side effects and the spread of pathogens with this new antimicrobial drug resistance emphasize the need for the development of other newer antimicrobial agents with activity against such gram positive bacteria (Jevitt *et al*. 2003, Meka and Gold 2004, Nathwani 2005). The other reason for this is the gram negative antibiotic-resistant opportunistic pathogens. Gram negative environmental and enteric organisms currently threaten patients in hospitals and communities with multi-drug resistance, including broad resistance to first, second, and third generations of penicillins and cephalosporins (Urban *et al*. 2003, Obritsch *et al*. 2004, Paterson *et al*. 2004). These bacteria such as *Pseudomonas aeruginosa* are common organisms which were found in environment and act as opportunistic pathogens in clinical cases where the defense system of the patient is compromised (Lyczak et al. 2000). In addition, other intrinsically antibiotic-resistant organisms such as *Stenotrophomonas maltophilia* are emerging as opportunistic pathogens. The end result of this phenomenon is that many strains of bacteria have become resistant, and in many cases multi-resistant to these therapeutic agents. To overcome this problem, a new antibiotic is needed which will show positive result (Alanis 2005).

Actinomycetes are capable of degrading many complex organic substances and consequently play an important role in building soil fertility. They are also noted for their ability to synthesize and excrete antibiotics. The isolation and characterization of actinomycetes were performed in different biochemical methods (Dhanasekaran *et al.,* 2009). The mycelium structure, color and arrangement of conidiospores and arthrospores on the mycelium were originally considered as an intermediate group between bacteria and fungi. Most of actinomycetes grow slowly as branching filaments while many actinomycetes will grow on the common bacteriological media used in the laboratory such as Nutrient agar and, isolation media. Antibiotics are the best known product of actinomycetes.

The present study is carried out by isolation of *Actinomycetes* culture and to check antibacterial activity of extracellular compounds against different types of pathogens.

Materials and methods:

Sampling and isolation:

A total of 8 marine sediment samples were collected from 4 different locations of the Visakhapatnam coast, Bay of Bengal, India. Actinobacteria were isolated from marine sediment samples by plating them on Chitin agar media (Hsu and Lockwood, 1975), Starch casein agar media (Küster and Williams, 1964) and Glycerol asparagine agar media (Shirling and Gottlieb, 1966) with different dilutions. Actinobacteria colonies can easily be distinguished on the plate from those of fungi and non-filamentous bacteria.

One gram each of the above marine sediment samples were serially diluted up to 10^{-7} level. 0.1 ml of each of these dilutions were added to the media in Petri plates (6 inch diameter) and the dilutions were dispersed throughout the media with an L-shaped glass rod. The Petri plates were incubated at 28°C and are observed from one week to three weeks for colonies of actinobacteria. The isolation media plates were also supplemented with Rifampicin 5µg/ml (Pisano *et al.,* 1989) and Nystatin 25µg/ml to inhibit bacterial and fungal contamination respectively. The pH of the media was maintained at 7.9.

Actinomycetes colonies were scored out and the selected colonies were sub cultured on Starch casein agar, Glucose yeast extract, malt extract agar slants and incubated at 28°C for one week to three weeks.

Screening of Actinomycetes for Antimicrobial Activity:

Primary Screening for Antimicrobial Activity:

The antimicrobial activities of the isolates were tested by Cross-Streak method employing Nutrient agar for bacteria and Potato dextrose agar medium for fungi. After 7 days, test organisms were streaked perpendicular to the growth of the isolate; 4 day cultures of bacteria and fungi were used as the test organisms. All the test organisms employed in the present investigation were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The test organisms used for the determination of antifungal activities are (Staphylococcus aureus (MTCC (Microbial Type Culture Collection) 3160), *Bacillus subtilis (MTCC 441), Pseudomonas aeruginosa (MTCC 424),* ***Bacillus cereus*** *(MTCC 430),* ***Proteus vulgaris*** *(MTCC 426),* ***Escherichia coli*** *(MTCC 443),* ***Saccharomyces cerevisiae*** *(MTCC 170),* ***Aspergillus niger*** *(MTCC 961),* ***Aspergillus*** flavus *(MTCC 3396),* ***Candida albicans*** *(MTCC 227)*).

Secondary Screening for Antimicrobial Activity:

The active isolates resulting from the primary screening were tested for their extra cellular anti-bacterial and anti-fungal compound production capabilities under submerged fermentation conditions. The production medium containing glucose 1%, soyabean meal 1%, NaCl 1% and $CaCO_3$ 0.1% with pH 7.6 was used for anti-fungal compound production employing isolates obtained from marine sediments samples. Well-sporulated, 7-day old slants of the selected isolates were used for antibiotic production studies.

Cup-plate Method:

Cups of 6 mm diameter were made using sterile cork borer. 50 ml of clear supernatant from fermentation broth was added to each cup. The plates were kept in incubator at 28°C for fungal growth and at 37°C for bacterial growth. The inhibition zones were measured using an antibiotic zone reader after 48 h of incubation.

Selection of Media for Taxonomic Studies:

The biochemical reactions that were determined by employing the prescribed media in International *Streptomyces* Project (ISP) reports, Bergey's Manual of Determinative Bacteriology (1957), Bergey's Manual of Systematic Bacteriology (1989) include Melanin formation, H_2S production, tyrosine reaction, gelatin hydrolysis, coagulation and peptonization of milk; casein hydrolysis, starch hydrolysis, nitrate reduction, utilization of carbon source, sodium chloride tolerance, effect of various nitrogen sources on growth, resistance to various antibiotics, effect of temperature on growth, pH tolerance and cell wall composition.

Characterization of Actinomycetes:

Morphological, macromorphology, micromorphology and biochemical characteristics were observed for all isolates and the results were tabulated.

Results and discussion

Isolation of Actinomycetes

Soil sediment samples were obtained from five locations at different depths of the Bay of Bengal area. 32 isolates of actinomycetes were obtained from marine soil sediments from the Bay of Bengal in our investigation. 11 isolates were showed activity against the bacterial test organisms. 5 isolates were showed activity against the bacterial and fungal test organisms. The 5 organisms which showed potent antimicrobial activity against the test organisms were selected for further studies.

The antibiotics produced by these organisms have a wide spectrum, although some are highly active against yeast like fungi and others against filamentous fungi (Waksman et al., 1952; Mustafa Oskay et al., 2009). Alexopoulus et al., (1941) were the first to show that as many as 56% of all cultures of actinomycetes isolated from the soil possessed some antifungal properties. Thirty seven actinomycete strains were isolated from soil sample collected from Zagazig districted, El-Sharkia governorate, Egypt. All these strains were screened for their antifungal activity against pathogenic fungi (Kathiresan et al., 2005). Primary Screening for Antimicrobial Activity**.**

During the course of survey, a total of 32 actinobacteria were isolated from the 6 marine sediment samples, by using Chitin agar, Starch Casein Agar, Glycerol Asparagine Agar and Glucose Yeast extract Malt extract Agar media. The culture of the isolation plates are shown in Figure 1 and the pure cultures of some of the isolates were shown in Figure 2. The isolates were analyzed for antimicrobial activity preliminarily by cross streak method. Among these isolates, 11 isolates exhibited antibacterial activity, out of which 6 isolates showed potent antifungal activity. All the isolates which exhibited antifungal activity also showed effective antibacterial activity. These active isolates were later subjected to submerged fermentation studies.

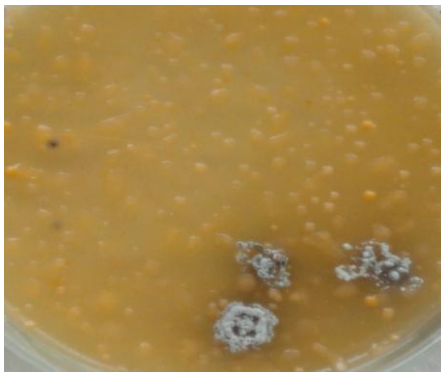
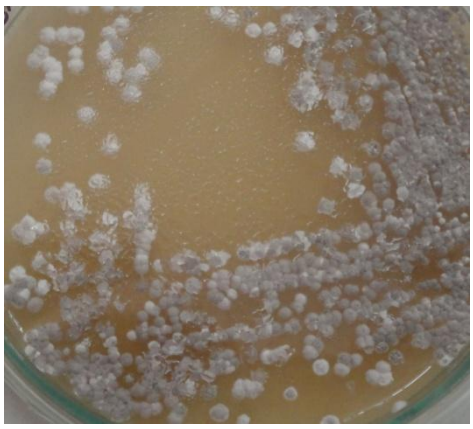


Figure: 1. Isolation of Actinobacteria on GYM agar plate showing grey colony of Actinomycetes Isolate.

(A)



(B)

Figure: 2. Pure Culture of Actinobacteria. A) White Coloured Actinobacterial Colonies with Chalky Appearance over the Surface; B) Grey Coloured, Folded Colonies.

Secondary Screening for Antimicrobial Activity:

Secondary screening is useful in sorting out microorganisms that have real commercial value from many isolates obtained during fermentation process. 5 isolates showed potent antimicrobial activity (Table 1 & 2; Fig 3 & 4). Among them, the isolate number. KRBT – 401 exhibited highest antimicrobial activity when compared to the other isolates. The isolate KRBT – 411 showed good antibacterial activity against *E. coli*, *B. subtilis* and other test organisms. But it showed less antifungal activity on the fungal test organisms viz., *Aspergillus niger flavus*, *Saccharomyces cerevisiae*, and *Candida albicans*, and no activity against *A. niger*. Similarly, the isolate KRBT-103 showed good antifungal activity against *A. niger and A. flavus*. Some of the isolates such as KRBT-205, KRBT-411 and KRBT-601 inhibited good antimicrobial activity against a specific organism and showed mild activity against the other test organisms. Lim et al., (2000) reported that among 32 thermophilic actinomycetes which showed antifungal activity against 6 plant pathogenic fungi on V8 agar, 12 actinomycetes were selected for further *in vitro* bioassay for antifungal activity of culture filtrates.

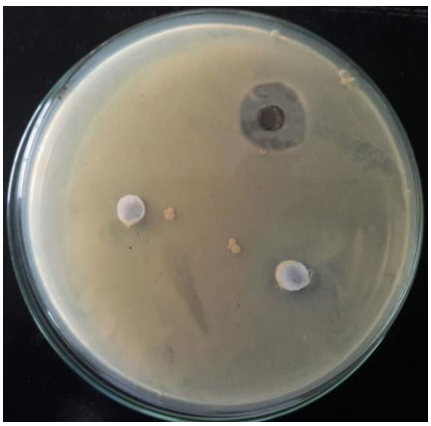


Figure 3: Figure showing antibacterial activity

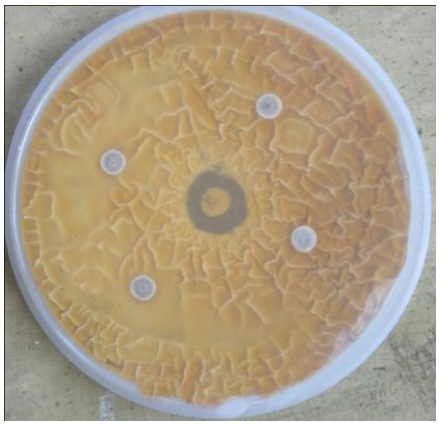


Figure: 4. Figure showing antifungal activity

Research on the biodiversity of marine actinobacteria is not only important for the basic studies but also necessary for their exploitation. There are only few comprehensive reports on the marine antagonistic actinobacteria from India. The existence of these bacteria in the marine sediments collected at different depths, along the south east coast of Bay of Bengal, India specifies that it is an underexploited and highly diversified ecosystem suitable for screening actinobacteria that are capable of producing bioactive compounds.

Morphological Characteristics

All the five isolates which showed promising antimicrobial activities were identified as belonging to the genus *Streptomyces and* family Streptomycetaceae (spore chain with coiling and branching). The predominance of *Streptomyces* in any actinomycete population is a well-known fact. Jensen *et al.*, (1991) reported the number of marine *Streptomyces* in the actinomycete population in relation to the depth, where the distribution accounted for by a rapid decrease in *Streptomycetes* and an increase in actinoplanetes and *Micromonospora* with increasing depth.

Table: 1. Isolates showing antibacterial activity

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm)					
	E. coli	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Bacillus cereus*	*Staphylococcus aureus*
KRBT – 103	12	--	--	10	--	--
KRBT – 205	14	12	11	12	12	10
KRBT – 401	18	12	14	16	12	10
KRBT – 411	16	14	14	14	18	24
KRBT – 601	10	12	--	12	--	--

'—' Inhibition zone not observed.

Table: 2. Isolates showing antifungal activity

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm)			
	A.niger	*A.flavus*	*C.albicans*	*S.cerevisiae*
KRBT – 103	16	22	12	18
KRBT – 205	10	10	--	10
KRBT – 401	22	24	20	26
KRBT – 411	--	10	10	12
KRBT – 601	--	16	20	18

'—' Inhibition zone not observed.

The morphological and cultural properties of promising isolates are shown in Table 3. The results indicated that among 6 isolates, 1 isolate was characterized as spiral type, one as flexous type and two were retinaculum apertum type of spore bearing hyphae. Morphological observation of 14-day old culture of isolates grown on different ISP media revealed that the vegetative mycelia and the aerial hyphae were abundant, well developed and varied from grey – white, creamy – yellow and yellow – light brown colour on different test media. Most of the isolates grew well on the ISP -2, ISP-4 and ISP -5 media whereas the vegetative mycelia and aerial hyphae were poorly developed on ISP -3 medium.

Table: 3. Morphological and Cultural Properties of Potent Isolates

Morphological and Cultural characteristics of the active isolates						
Isolate No.	Morphological characteristics			Cultural characteristics		
	Spore bearing hyphae	Spore mass colour	Growth	Vegetative mycelia colour	Aerial mycelia colour	Soluble pigment
KRBT – 103	Flexous	Light yellow	Good	Yellow	Brown	Brown
KRBT – 205	Retinaculum apertum	Dark brown	Abundant	Dark yellow	Brown	Reddish brown
KRBT – 401	Retinaculum apertum	Brown	Abundant	Light brown	Brown	Reddish Brown
KRBT – 411	spirals	Grey	Good	Light brown	Grey	Brown
KRBT – 601	Monoverticillus	Black	Abundant	Light brown	Brown	Nil

Physiological and Biochemical Characteristics:

The physiological and biochemical properties of 6 promising isolates are shown in Table 3.4. Melanin pigmentation was shown by KRBT-205, KRBT-401 and KRBT-411 on all the three test media. The isolate, KRBT-103 produced melanin in only two test media while the isolate, KRBT-601 did not show melanin pigmentation in any of the media used in the study. All the isolates were H_2S-positive except KRBT-601. Tyrosine reaction was positive for all the isolates. Starch hydrolysis was positive for all isolates expect KRBT-205. Casein hydrolysis was positive for isolates (KRBT-103, KRBT-401 and KRBT-601) and negative for the remaining isolates. Gelatin hydrolysis was positive for all the isolates. Milk coagulation and peptonization test was negative for 2 isolates (KRBT-401 and KRBT-411) while the remaining isolates showed positive test. Nitrate reduction test was negative for 1 isolates (KRBT-601) while the other isolates exhibited positive test. All the promising isolates showed negative reaction to Voges-Proskauer and oxidase tests. The isolates KRBT-205, KRBT-411 and KRBT-601 showed positive test for methyl red. The citrate and urease tests were positive for all the isolates. Out of 6 isolates, only 2 isolates (KRBT-205 and KRBT-601) were positive for catalase test. The isolates grew well at all the temperatures ranging from 20 to 37˚C.

The cell wall peptidoglycan of all the 6 isolates contained LL-diaminopimelic acid and glycine.The presence of LL-diaminopimelic acid was indicated by a characteristic olive green ninhydrin reaction fading to yellow during paper chromatography. It is a rapid method of differentiation between the *Streptomycetes* and most other actinomycetes by paper chromatography of whole cell hydrolysates (Becker *et al.,* 1964).

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