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Research Paper

ISOLATION AND PARTIAL CHARACTERIZATION OF ACTINOMYCETES FROM MANGROVE SEDIMENT SAMPLE

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Abstract

The study aimed at determining the *in vitro* antimicrobial activity of actinomycetes isolated from a mangrove ecosystem and their morphological, physiological and biochemical characterization. A total of seven isolates of actinomycetes were isolated from sediments of Koringa mangroves sample near Kakinada, East Godavari district (Andhra Pradesh) using cycloheximide (50 µg/mL) and rifampicin (25 µg/mL) as antifungal and antibacterial agents, respectively using perpendicular streak method and cup plate method. They were designated as M1 to M7. Of these seven isolates, one isolate exhibited significant antimicrobial activity against *Staphylococcus aureus* NCIM 2079 (19.5 mm), *Candida albicans* MTCC183 (22.6 mm). The isolates were further characterized based on the morphological, physiological and biochemical studies. The results revealed that the mangrove ecosystem, a rich consortium of many potent actinomycetes, which could synthesize novel bioactive compounds against pathogenic microorganisms.

Key words: Actinomycetes, Koringa mangrove, *Staphylococcus aureus*, *Candida albicans*, antimicrobial activity.

INTRODUCTION

Secondary metabolites are produced by some organisms such as bacteria, fungi, actinomycetes. Among them, actinomycetes occupy a prominent place[1-3]. Actinomycetes are Gram-positive bacteria but are distinguished from other bacteria by their morphology, DNA rich in guanine plus cytosine (G+C) and nucleic acid sequencing and pairing studies. They are characterized by having a high G+C content (>55%) in their DNA[4-6]. Actinomycetes are of universal occurrence in nature and are widely distributed in natural and man-made environments. They are found in large numbers in soils, fresh water, marine water and sediment, lake, river bottoms, manures, composts and dust as well as on plant residues and food products etc. However, the diversity and distribution of actinomycetes that produce secondary bioactive metabolites can be determined by different physical, chemical and geographical factors[5,6]. Actinomycetes provide many important bioactive substances that are of commercial value. Their ability to produce a variety of bioactive substances has been utilized in a comprehensive series of researches in numerous research and industrial laboratories. This has resulted in the isolation of metabolites from actinomycetes, which have found application in combating a variety of human infections[7]. Because of this reason 70% of naturally occurring

antibiotics have been isolated from different genus of actinomycetes[8]. Among them, *Streptomyces* is the largest genus known for the production of many secondary metabolites[9], which have different biological activities, such as antibacterial, antifungal, antiparasitic, anticancer and immunosuppressive actions[1,10,11]. Some antibiotics like penicillin, erythromycin, and methicillin which used to be one-time effective treatment against infectious diseases[12,13], are now less effective because bacteria have become more resistant to such antibiotics. Antibiotic resistant pathogens such as methicillin and vancomycin resistant strains of *Staphylococcus aureus* and others cause an enormous threat to the treatment of serious infections. To avoid this happening, immediate replacement of the existing antibiotic is necessary[13], and the development of novel drugs against drug resistant pathogens is important. Thus, finding and producing new antibiotics as well as using combined antibiotic therapy have been shown to delay the emergency of microbial resistance and can also produce desirable synergistic effects in the treatment of microbial infection. Antibiotic synergisms between known antibiotics and bioactive extracts are a novel concept and have an important activity against pathogens and host cells[14]. Research in finding newer antibiotics and increasing productivity of such agents has been a very important activity[3,7]. This is because some important drugs are expensive and/or have side effect to the host, some microbes have no successful antibiotics and others are developing multidrug resistance. This situation requires more attention to find solutions by searching and producing new and effective antibiotics from microbes like actinomycetes. However, there is no such scientific report on antibiotic producing actinomycetes from soil samples collected in waste disposal areas of Visakhapatnam, Vizianagaram and Srikakulam districts of Andhra Pradesh. Therefore, the objective of the present study was to isolate and screen antibiotic producing actinomycetes from soil samples. The outcome of this finding may be important to give direction for researchers and for future treatment of multidrug resistant human pathogens.

MATERIALS AND METHODS

Collection of sample

The sediments sample were collected from Koringa mangrove environment (16°-30' to 17°-00' N latitudes and 82°-14' to 82°-23'E longitudes), near Kakinada, East Godavari district (Andhra Pradesh, India). The sediment sample was collected from 5-10 cm depth during the month of July – August 2014 by inserting sterile corer into the soil. The sample was transferred to a sterile polythene bag and transported immediately to the laboratory for further processing.

Sampling and isolation of actinomycetes

From the sediment sample collected above, 1 g of sample was then added in different test tubes containing 10 mL sterile distilled water and shaken well using vortex mixer. These test tubes were considered as stock cultures for different soil sample sites. From the stock cultures, a volume of 1 mL was transferred aseptically and added to a test tube containing 9 mL of sterile distilled water and mixed well. From this test tube, 1 mL of aliquot was again transferred and mixed with another 9 mL of sterile distilled water to make 10^{-2} dilution factor. Similarly, dilutions up to 10^{-8} were made using serial dilution technique for all soil samples. A volume of 1 mL of suspension from 10^{-7} and 10^{-8} serially diluted tubes were taken and spread evenly with sterile glass rod over the surface of sterile starch casein agar (the medium was prepared by using 50% v/v sea water) plates aseptically using spread plating technique. Cyclohexamide (50 µg/ mL) and Rifampicin (25 µg/ mL) were added in medium to inhibit fungal and bacterial contamination, respectively. The plates were incubated aerobically at 27 °C up to 7 d and observed intermittently during incubation[15]. After incubation, morphologically different actinomycetes colonies on the plates were picked and further subcultured onto their respective isolation media. The actinomycete colonies were purified by streak plate method[16,17]. Once the pure colonies were obtained, each colony was further identified on the basis of its earthy like smell, colony morphology, colour of hyphae and the presence or absence of aerial and substrate mycelium. Then, selected and identified colonies of actinomycetes were transferred from the plate to starch casein agar slant and incubated at 27 °C for their growth. After

incubation, the slants containing pure isolated actinomycetes were stored at 4 °C and preserved as glycerol stocks at -20 °C for further studies.

Morphological and cultural characteristics

Morphological studies of the actinomycete isolates were determined by following International Streptomyces project (ISP) procedures[18]. The Yeast extract malt extract agar (ISP-2), Oat meal agar (ISP-3), Inorganic salts- starch agar (ISP-4), Glycerol-asparagine agar (ISP-5) media were recommended by ISP were used for morphological studies and colour determinations.

Physiological and biochemical characterization

Effect of temperature on growth

Ability of the actinomycetes to grow at different temperatures was studied at 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. The isolates were streaked on starch casein agar slants and incubated at different temperatures[19] and after 7-8 d observed their growth.

Melanin production

The production of melanin is an important characteristic for the identification and classification of actinomycetes especially *Streptomyces*. Melanin is a dark pigment produced by the microorganisms on tyrosine containing medium. The tryptone yeast extract broth (ISP-1), yeast extract iron agar (ISP-6), tyrosine agar (ISP-7) were used for testing melanin production by the actinomycete isolates[18]. Seven day old culture of isolates was inoculated on each media mentioned above and incubated at 28°C for 4 d and the uninoculated media serves as a control. The color change was observed after 2 d and 4 d. Greenish brown and brown to black diffusible pigments were considered as positive for melanin production.

Gelatin hydrolysis

Gelatin is a protein produced by the hydrolysis of collagen. Microorganisms can produce gelatinase that can hydrolyze gelatin to amino-acids. For this the actinomycete isolates were streaked on gelatin agar and incubation at 28°C for 5 d. Following incubation, the plates were flooded with 10 mL of mercuric chloride solution and observed zone of hydrolysis[20].

Peptonization and coagulation of milk

Milk coagulation and peptonization test was carried out with skim milk. The skim milk test tubes were inoculated with actinomycetes and incubated at 28°C for 7-8 days. The extent of coagulation and peptonization was recorded on 3rd and 8th d[21].

Casein hydrolysis

The proteolytic activity of isolates was studied using milk casein agar (g/L: Peptone 1.0, sterile skimmed milk (10%), Agar 20.0). The isolates were streaked on milk casein agar and incubated at 28°C for 5-7 d. Following incubation, isolates secreting protease enzyme will exhibit a zone of proteolysis, which is demonstrated by clear zone surrounding their growth. This loss of opacity is the result of a hydrolytic reaction yielding soluble, non-colloidal amino acids, and it represents a positive for casein hydrolysis[21].

Starch hydrolysis

The actinomycete isolates were streaked on starch agar plates and incubated at 28°C for 5-7 d. Then, the plates were flooded with iodine solution. Starch in the presence of iodine will impart a blue-black color to the medium indicating the absence of starch splitting enzymes and representing a negative result. However, the presence of clear colorless zone surrounding the growth of the organism represents a positive result for starch hydrolysis[21].

Nitrate reduction test

The isolates were inoculated into 5 mL of nitrate broth and incubated at 28°C for 7 d and uninoculated nitrate broth serves as a control. After incubation, the ability of isolates to reduce nitrates to nitrites was determined by the addition of two reagents: Solution A (sulfanilic acid), followed by Solution B (α-naphthylamine). The addition of solution A and B produce an immediate cherry red color which is recorded as positive for nitrate reduction[22].

Use of carbon sources **(1% w/v)**

The ability of the isolates utilizing various carbon sources for energy was studied by following the method recommended by ISP[18]. The carbon sources (1% w/v) such as D-glucose, D-xylose, L-arabinose, D-fructose, D-galactose, raffinose, D-mannitol, sucrose, maltose, lactose, cellulose were used in this study. and then the observations were noted after 7th, 14th and 21st d

by comparing with that of non-supplemented basal medium and on positive control containing D-glucose.

Screening for antimicrobial activity

The actinomycetes isolates were screened for their antimicrobial spectrum against bacteria and fungi. The test bacteria used for primary screening were Methicillin resistant *Staphylococcus aureus* (clinical isolate, King George Hospital, Visakhapatnam), *Psedomonas aeruginosa* (clinical isolate from KGH), *Escherichia coli* NCIM2065, *Klebsiella pneumonia*, *Bacillus subtilis* NCIM2063, and *Bacillus pumilis* NCIM2327. Antifungal activity was determined using *Saccharomyces cerevisiae* MTCC170, *Candida albicans* MTCC183, *Aspergillus niger* NCIM 548, *Penicillium chrysogenum* NCIM 738 and *Aspergillus oryzae* NCIM 643 as test organisms. Activities were assessed using nutrient agar for bacteria and potato dextrose agar for fungi by cross streak method[23] and agar overlay method[24-26]. Each plate was streaked and stabbed with each isolate at the centre of a plate and incubated at 27 °C for approximately 6-7 d. Then, 24 h subcultured bacteria and 48 h old culture of fungi were streaked perpendicular to the actinomycete isolate and also by agar overlay method. Then, the plates were incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. After incubation, the zone of inhibition was measured and recorded.

RESULTS AND DISCUSSION

Actinomycetes are diverse groups of prokaryotic microorganisms, which are known to produce a wide range of novel bioactive metabolites such as enzymes, antibiotics, anticancer and immuno-suppressive agents[27]. They are specially recognized for their ability to produce various bioactive metabolites with antibacterial, antifungal and antiprotozoal activities[28]. The actinomycetes mainly occur along the coastline at the confluence of rivers and sea. This ecosystem produces large amounts of organic matter due to microbial decomposition of fallen leaves, twigs, flowers and fruits. Abundance of these organic matters, salinity and high degree of moisture content favour the predominance of antimicrobial population and other life forms in the mangrove ecosystem[29].

Sampling and isolation of actinomycetes

In the present study, a total of 7 actinomycetes were isolated from mangroves sediment sample, morphologically distinct colonies were isolated and they were designated as M-1, M-2, M-3, M-4, M-5, M-6, and M-7. These isolates were used for morphological, biochemical, cultural characterization and antimicrobial activity.

Morphological and cultural characteristics

The isolates were developed as round, powdery, convex colonies with spreading edges. The morphological and characteristics of the actinomycete isolates on starch casein agar were shown in Table 1.

Table 1: Morphological and cultural characteristics of the actinomycete isolates

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Starch casein agar	Abundant	M1 Grey	M1 Grey	-
		M2 White	M2 Dark brown	
		M3 White	M3 Black	
		M4 Grey	M4 Pink	
		M5 White	M5 Light blue	
		M6 Dark grey	M6 Dark grey	
		M7 White	M7 Dark red	
Yeast extract malt extract agar (ISP-2)	Abundant	M1 White	M1 Dark grey	-
		M2 Light brown	M2 Dark brown	
		M3 White	M3 Black	
		M4 Dark Grey	M4 Pink	

		M5 White	M5 Blue	
		M6 Grey	M6 Dark grey	
		M7 White	M7 Dark red	
Oat meal agar (ISP-3)	Abundant	M1 Grey	M1 Grey	-
		M2 White	M2 Brown to red	
		M3 White	M3 Black to grey	
		M4 Grey	M4 Pink	
		M5 White	M5 Light blue	
		M6 Dark grey	M6 Grey	
		M7 White	M7 Light red	
Inorganic salt agar (ISP-4)	Moderate	M1 Grey to black	M1 Grey	-
		M2 White to grey	M2 Brown	
		M3 White	M3 Dark Black	
		M4 Grey	M4 Pinkish white	
		M5 White	M5 Bluish white	
		M6 Dark grey	M6 Dark grey	
		M7 White	M7 Dark red	
Glycerol aspergine agar (ISP-5)	Abundant	M1 White	M1 Grey	-
		M2 White	M2 Dark brown	
		M3 White	M3 Black	
		M4 Dark Grey	M4 Pink	
		M5 White	M5 Light blue	
		M6 Grey	M6 Dark grey	
		M7 White	M7 Dark red	
Nutrient agar	Moderate	M1 Greyish to black	M1 Black	-
		M2 Whitish pink	M2 Brown	
		M3 White	M3 Black to grey	
		M4 Grey	M4 Pinkish white	
		M5 White	M5 Light blue	
		M6 Grey	M6 Dark grey	
		M7 White	M7 Light red	

Physiological and biochemical characteristics

The selected actinomycete isolates exhibited optimum growth within the mesophilic range of 24-35 °C. Interestingly, all the selected isolates grew well at pH ranging from 6.0 to 9.0 but optimum growth was observed between pH 7.0- 8.0. Furthermore, they were also tolerant to 0.05- 3% NaCl but failed to grow at above 6% salt concentration. The may be due to the hypertonic medium where the isolates would have suffered from severe osmotic shock. In comparison to the present findings, a marine *S. rochei*, isolated from Visakhapatnam coast in India, Reddy et al.[17] reported that the actinomycete was tolerant to pH 10.5 and 6% NaCl. From these observations, it was clearly evident that mangrove actinomycetes were generally alkaliphilic and halotolerant. However, all the isolates demonstrated negative reaction for melanin production. The reduction of nitrate to nitrite is used mainly for species differentiation. $NaNO_3/KNO_3$ is used as an electron acceptor by some microorganisms. NO_3^- and NO_2^- serve as sources of nitrogen for the synthesis of organic nitrogenous compounds. Except isolate M6, all isolates had shown positive result for starch hydrolysis (maximum hydrolyzed zone observed with isolate M3 as shown in Figure 1). Starch is a polymer of glucose, which is a source of carbon for microorganisms. In order to utilize, the microorganisms has to hydrolyze the insoluble starch into soluble form with the help of enzymes. Starch degrading microorganisms transport the soluble form across the cytoplasmic membrane of the cell. Actinomycetes possess the ability to produce amylase that breaks starch into maltose. The amylase is an extracellular enzyme, which is released from the cell of microorganisms. The starch hydrolysis positive isolates produced amylase and hence, they failed to produce blue colour (zone of hydrolysis)

upon treatment with iodine solution (Figure 1). Only the isolate M4 was hydrolyzed casein and M1, M2, M5 were shown positive result for gelatin hydrolysis whereas the M5 isolate was positive for coagulation of milk (Table 2). Many microorganisms produce extracellular enzymes that are capable of hydrolyzing gelatin. Some microorganisms hydrolyze casein, and others simultaneously may cause fermentation and hydrolysis. The fermentation of lactose produces an acid, which forms an insoluble complex of calcium and casein which results in coagulation of milk. The digestion of coagulated mass by the action of proteolytic enzymes is termed as peptonization. Some microorganisms may peptonize milk without coagulation and if enzyme caseinase is not present in the microorganisms, peptonization however, does not follow precipitation. Utilization of carbon sources by the microorganisms can be used as an index for species determination[30]. Good fellow and Orchard[31] reported that the antibiotic sensitivity of some nocardioform bacteria as one of the valuable information for the taxonomic differentiation. The carbon source utilization by these isolates were shown in Table 3.

Table 2: Biochemical characteristics of the isolates

Actinomycete isolate	Starch hydrolysis	Casein hydrolysis	Gelatin hydrolysis	Coagulation of milk	Nitrate reduction
M1	+	-	+	-	-
M2	+	-	+	-	-
M3	+	-	+	-	-
M4	+	+	-	-	-
M5	+	-	-	+	-
M6	-	-	+	-	-
M7	+	-	+	-	-

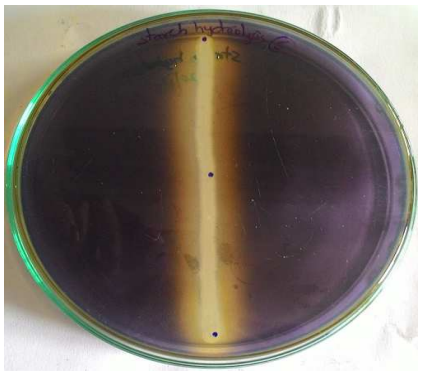

Figure 1: Starch hydrolysis of isolate M3

Table 3: carbon source utilization

Isolate	D-Glucose	Galactose	L-Arabinose	D-Fructose	Raffinose	Meso-Inositol	D-Mannitol	Sucrose	Salicin	Rhamnose
M1	+	-	+	+	-	-	-	+	-	+
M2	+	+	-	+	-	-	-	+	+	+
M3	+	+	-	+	-	-	-	-	-	-
M4	+	+	-	-	-	-	+	-	-	-
M5	+	+	+	-	-	-	+	-	+	-
M6	+	-	-	+	-	-	-	+	-	-
M7	+	-	-	+	-	-	-	+	-	-

Antimicrobial activity

As a result of primary screening by perpendicular streak method and cup plate method, among all actinomycete isolates, only one isolate (M3) had shown promising antimicrobial activity

against one or more test bacteria and fungus as shown in Table 2. The actinomycete isolate M3 was active against *E. coli, S. aureus, Bacillus pumilis* and C*andida albicans*. The isolate had shown maximum zone of inhibition against *S. aureus* (19.5 mm) and *C. albicans* (22.6 mm). A novel *Streptomyces* having antifungal activity was isolated from the soil sample of Cheonan, Korea[32]. The present study of primary screening methods indicated that one actinomycete isolate showed potential antimicrobial activity against bacteria (*S.aureus*) and fungus (*Candida albicans*). Sharma et al.[33] isolated actinomycetes from Pulicat, Muttukadu, and Ennore estuaries and they had shown antibacterial and antifungal activity. Thakur et al.[34] reported previously that a total of 110 actinomycetes strains were isolated from the soil samples collected from the protected forest soil from two states in Northeast India. The actinomycete isolate in the present study had shown dissimilar activities between Gram positive and Gram negative bacterial strains. The results clearly demonstrate that a Gram positive bacterium was highly susceptible to the tested actinomycete isolate compared to Gram negative bacteria.

Table 2: Zone of inhibition (mm) of the Isolate M3 against the tested bacteria and fungi.

Isolate	*E. coli*	*B.subtilis*	*S.aureus*	*B.pumilis*	*MRSA*	*P.aureginosa*	*S. cerevicia e*	*A. niger*	*C. albicans*
M3	6	10.5	19.5	9.4	16.3	5.9	13.6	11.7	22.6

Figure 2: Agar overlay method of isolate M3 showing zone of inhibition against *S.aureus.*

CONCLUSION

The present study had shown that starch-casein agar supplemented with cycloheximide and rifampicin was found to be suitable for isolation of actinomycetes from Koringa Mangrove sediments near Kakinada (Andhra Pradesh). Out of seven isolates, only one isolate M3 exhibited significant antimicrobial activity against *S. aureus* and *C. albicans*. Further the biochemical test and cultural characterization showed the positive for the actinomycetes. Further studies on optimization, purification and structure elucidation of bioactive compound are currently in progress. It is more expected that the attempt for the isolation and characterization of actinobacteria from Koringa mangrove ecosystem will be useful for the production of novel antibiotics for the treatment of various pathogenic diseases.

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