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Research Article

ISOLATION AND SCREENING OF *STREPTOMYCES* SP. FROM CORINGA MANGROVE SOILS FOR ENZYME PRODUCTION AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

This study was carried out to isolate actinomycetes which have the potential to produce antibiotics as well as enzymes from under-explored mangrove soils. The soil samples were collected from Coringa mangrove forest, Andhra Pradesh and the isolations were carried out using dilution-plate technique. 27 isolates were obtained and were characterized morphologically, biochemically, physiologically. Screening tests were performed to determine enzymatic activity and antimicrobial activity. Results indicated that *Streptomyces* sp. strain A exhibited amylase (0.59 µmol/ml/min), cellulase (1.388 µmol/ml/min), L-asparaginase (3.67 µol/ml/min), chitinase (1.356 µmol/ml/min) and protease (0.248 µmol/ml/min) activities; specific activities of cellulase, L-asparaginase, chitinase and protease were found to be 10.475 µmol/ml/mg protein, 10.194 µmol/ml/mg protein, 6.027 µmol/ml/mg protein and 0.4332 µmol/ml/mg protein respectively. Strain A exhibited antimicrobial activity against test micro organisms like *Staphylococcus aureus, Pseudomonas aureginosa, Pseudomonas fluroscence, Lactobacillus acidophilus, Lactobacillus casei, Candida albicans, Streptococcus mutans, Bacillus subtilis, Bacillus megaterium, Xanthomonas.* Maximum activity was exhibited against *Xanthomonas* where as minimum against *Candida albicans.* 96 hr incubation resulted in optimum antimicrobial activity.

Keywords: *Actinomycetes, Mangroves, Streptomyces sp., antimicrobial activity.*

INTRODUCTION

Penetration of biotechnology into marine environment has opened up unexpected new horizons for finding novel organisms for trapping their potential resources. However, culturally independent methods have demonstrated that marine sediments contain wide range of unique microorganisms. Mangroves are a unique woody plant community of intertidal coasts in the tropical and subtropical zones, which are regarded as highly productive ecosystems and abode to unexplored microbial diversity including actinomycetes[1]. Exploitation of less/ unexplored ecosystems for actinomycetes is highly necessary for the discovery of novel bioactive metabolites[2].

Actinomycetes are the dominant group of soil population together with bacteria and fungi. They are Gram-positive bacteria having high G+C (>55%) content in their DNA and they are originally considered as an intermediate group between bacteria and

fungi. They are free living, saprophytic bacteria, and a major source for production of antibiotics. They play a major role in recycling of organic matter, production of novel pharmaceuticals, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. *Streptomyces* are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites[3].

MATERIALS AND METHODS

1) Sample collection

The soil samples were collected from mangrove forests of Coringa (17° 0' 0" N and 82° 19' 60" E) in the east coast region of southern India. The samples were collected by inserting a polyvinyl corer (previously sterilized with alcohol) into the sediments. The central portion of the top 2 cm sediment sample was taken out with the help of a sterile spatula and this sample was transferred to a sterile polythene bag and transported to the laboratory. The samples were air dried at room temperature.

2) Isolation of actinomycetes from the soil sediments

The air dried samples were incubated at 55^{0}C in an Incubator for 5 min. 1g of soil was dissolved in 100ml of distilled water (10^{-2} dilution) and 1ml of 10^{-3}, 10^{-4}, 10^{-5} serial-dilutions were spread plated on ISP-2 media (International Streptomyces Project type-2 media) using Dilution-plate technique.

Antibiotics like nalidixic acid and nystatin were added to minimize microbial contamination. All the plates were incubated at 30^{0}C in an Incubator for 2-3 weeks. After incubation, actinomycete colonies were selected and maintained by sub-culturing on ISP-2 agar slants and stored at 4^{0}C for further use.

3) Identification of actinomycete isolates

Morphological characterization which includes colony and spore morphology was performed. Physiological parameters like pH, temperature, NaCl concentration, carbon source utilization in the growth media were analyzed. Biochemical tests such as IMViC test, nitrate reduction test, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, hydrogen sulfide production test, Urease test, Catalase test were performed.

4) Screening of actinomycetes for enzyme production

The isolated actinomycetes strains were screened for the presence of different enzymes like amylases, cellulases, L-asparaginase, proteases, and chitinase.

i. Amylases

a. Primary screening for amylase production

The strains were grown on starch-agar media (starch-20g, peptone-5g, beef extract-3g, agar-20g, NaCl- 3 or 4% depending on the strain, distilled water-1000ml) for 3 to 4 days and then were flooded with Gram's Iodine. It was allowed to stand for few minutes and then it is poured off. Clear distinct zones have appeared around the colonies which produce amylase. The clear zone area was measured.

b. Amylase assay

50ml of starch broth was prepared for each positive strain and inoculated with the strain. It was incubated for 48-72 hrs and then centrifuged at 5,000 rpm for 20 min in a refrigerated centrifuge. The cell-free supernatant (crude enzyme extract) was collected and enzymatic assay was performed. 1ml of starch substrate was added to 0.2 ml of enzyme solution and made up to 3ml distilled water, and incubated for 20 min at 37°C. The reaction was stopped by addition of 1ml of DNS reagent (1g of 3, 5- Dinitrosalicylic acid, 20ml of NaOH and 30g of sodium potassium tartarate in 100ml distilled water) and the absorbance was read at 540 nm.

1 unit (IU) is defined as the amount of enzyme that released 1µmol of maltose from starch per minute at pH 7.0 at 37°C.

ii. Cellulases

a. Primary screening for cellulase production

The actinomycetes strains were grown on ISP-4 media (carboxymethyl cellulose-10g, K_2HPO_4- 10g, $MgSO_4$-10g, Nacl-3 or 4%,

$CaCO_3$- a pinch, $(NH_4)_2SO_4$-2g, $MnCl_2$-1mg, $ZnSO_4$-1mg, $FeSO_4$-1mg, agar-20g) for 3-4 days and then flooded with 1% 5ml of iodine solution along with 1ml mercuric iodide. Clear yellow zones were observed and the area was measured.

b. Cellulase assay

50 ml of ISP-4 broth was taken for each positive strain and inoculated with the strain. The strains were incubated for 48-72hrs and then centrifuged in refrigerated centrifuge for 10min at 2,000 rpm. The cell-free supernatant was collected and enzymatic assay was performed. 0.1ml of crude enzyme extract was added to 1 ml of carboxymethyl cellulose substrate and made up to 3ml with distilled water. The mixture was incubated at 60˚C for 20min and then reaction was stopped by addition of DNS reagent. The absorbance was measured at 540nm[4].

1 unit (IU) is defined as the amount of enzyme that released 1µmole of glucose from carboxymethyl cellulose per minute at pH 7.0 at 60˚C.

iii. L-Asparaginases

a. Primary screening for L-asparaginase production

The actinomycetes strains were grown on L-asparagine-glucose agar media (glucose-1g, yeast extract-2g, L-asparagine-10g, K_2HPO_4-0.5g, agar-20g, Nacl-3 or 4%, phenol red-3 drops, pH-7.3) for 3-4 days. The yellow color media turns pink and clear pink zones were formed around the colonies.

b. L-Asparaginase assay

50ml of glucose asparagine broth was taken and the positive strains were inoculated and incubated at 30˚C in rotary shaker for 7 days at 125 rpm. Then the cultures were centrifuged at 10,000 rpm for 15 min and the cell free supernatant was collected which is considered to be the crude enzyme extract. The L-asparaginase activity was assayed using the method of Imanda *et al*., 1973. Reaction was started by addition of 0.5 ml of crude enzyme to 0.5 ml 0.04M L-asparagine and 0.5 ml 0.05M tris (hydroxymethyl) amino methane (Tris-HCl) buffer, pH 7.2 and incubated at 37˚C for 10 min. The reaction was stopped by the addition of 0.5 ml 1.5 M Trichloro acetic acid (TCA) and the absorbance of ammonia released by addition of 0.2 ml Nessler's reagent was measured at 480 nm[5].

One unit of asparaginase is the amount of enzyme which catalyzed the formation of 1µmol ammonia per min at 37˚C.

iv. Proteases

a. Primary screening for protease production

Skim milk agar medium contained: skim milk powder, 10g; peptone, 5g; NaCl, 3%; and agar 20g per 1000ml of distilled water; pH 7.0. The actinomycetes strains were streaked on the medium and incubated at 28˚C for 4 days such that colonies were grown. A clear zone appears around the actinomycete colonies if the strain is positive for protease activity[6].

b. Protease assay

Proteolytic activity was assayed using casein as the substrate. A 0.5 ml aliquot of the enzyme extract was incubated with 1 ml of 2.0% casein solution in 0.1 M Tris HCl buffer, pH 7.0 at 37˚C for 10 min. The reaction was stopped by the addition of 5.0ml 5% trichloroacetic acid and incubated for 30 min. The mixture was filtered and 2.0ml of filtrate was added to 4.0ml of 0.1N NaOH and 0.5ml diluted Folin-Cocalteau reagent and incubated for 30 min and then the amount of tyrosine released into the filtrate was measured from its absorbance at 670 nm. Protein was estimated using BSA as the standard[6].

One unit of protease activity is expressed as the amount of enzyme which converts 1µg of tyrosine per 1min at 37˚C[7].

v. Chitinases

a. Primary screening for chitinase production

Screening for chitinase production of all the isolates was done by plate agar assay. The colloidal chitin medium contained: colloidal chitin-15g; yeast extract-0.5g; $(NH_4)_2SO_4$-1g; $MgSO_4 \cdot 6H_2O$-0.3g; KH_2PO_4-1.36g; agar-15g, and distilled water- 1000ml[8]. The plates were incubated for 5 days at 30˚C. These isolates produced large clear zones on (colloidal chitin agar) CCA[9].

b. Chitinase assay

50ml of colloidal chitin broth was incubated with inoculum at 30˚C in the incubator shaker at 150 rpm for 7 days. The cell free supernatant was collected and the assay was performed. Colloidal chitin was used as a substrate to assay chitinase activity: 0.1 g in 1 ml of phosphate buffer (pH 7.0) was incubated with 0.5 ml of enzyme at 37˚C for 60 min. The reducing sugars in the reaction mixture were measured by colorimetric method.

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1µmol of N-acetylglucosamine per ml in 60 min[9].

5) Screening for antimicrobial activity

The test organisms include *Staphylococcus aureus, Pseudomonas aureginosa, Pseudomonas fluroscence, Lactobacillus acidophilus, Lactobacillus casei, Candida albicans, Streptococcus mutans, Bacillus subtilis, Bacillus megaterium, Xanthomonas* etc., were used to test the antimicrobial activity of potent strains.

The actinomycete isolates were grown in ISP-2 broth for specific time period. After incubation, the culture was filtered using Whatman filter paper no.1. The filtrate was partially purified using separating funnel and the partially purified filtrate was evaporated at 80^{0}C in a water bath. Then 50µl metabolite obtained after evaporation was loaded on to nutrient agar plates containing 20µl of 24hr old test organisms. The plates were incubated at 37^{0}C for 24 hrs and analyzed for zone formation.

RESULTS

1. Isolation of mangrove actinomycetes

27 actinomycetes were isolated from the Coringa mangrove soils by dilution-plate technique on ISP-2 media. The isolates were sub-cultured to obtain pure cultures and were stored at 4^{0}C for further use. Among the isolates few strains released characteristic camphor like smell including strain A.

2. Identification

Strain A was identified as *Streptomyces sp.* based on morphological, physiological, biochemical and molecular characteristics. When the strain was grown on ISP-2 agar plate, its aerial mycelium appeared ash in color whereas substrate mycelium was dark brown in color with pigmentation.

40^{0}C was found to be optimum for the growth of strain A on both starch casein agar media and Yeast extract malt extract agar media. The growth was moderate even at 30^{0}C on ISP-2 agar media. Strain A has shown abundant growth when maltose and glucose were used as carbon sources individually with 3-4% of NaCl at pH 7.0.

Various biochemical tests were performed and strain A was found to be positive for VP test, starch hydrolysis, casein hydrolysis, catalase tests.

3. Enzymatic activity of Strain A

i. Amylase activity

For amylase screening, the isolates were grown on starch agar plates for 4-5 days and then were tested for amylase production by flooding the plates with Iodine solution. A clear white zone is formed surrounded by violet background. Strain 13, 15, A and D were found to be positive for amylase production.

Amylases hydrolyze starch into simple sugars like maltose. 0.1mg/ml maltose was taken as standard. Here amylase activity was determined using DNS method. Strain A exhibited optimum activity of 0.590µmol/ml/min on day 3 at 37^{0}C and pH 7.0.

ii. Cellulase activity

For cellulase screening, the isolates were grown on ISP-4 modified agar media where cellulose or CMC was used instead of starch. After 4 to 5 days incubation, the plates were flooded with 5ml of iodine solution along with 1ml mercuric iodide. Here pale yellow color zones were formed indicating cellulase production. Strain A and B were found to be positive.

For cellulase assay, glucose (0.1mg/ml) was used as standard. DNS method was used to measure absorbance at 540nm. Strain A has exhibited an optimum activity of 0.611µmol/ml/min on day 5 at 37^{0}C and pH 7.0.

iii. L-asparaginase activity

Strain 10 and A were found to be positive for L-asparaginase screening. L-asparagine-glycerol agar media and phenol red indicator were used. The yellow color background turns pink in color which implies the test was positive.

L-asparaginase assay was performed using the method of Imanda *et al.,* 1973. Ammonium sulphate was used as standard. In this method, the amount of ammonia liberated was measured at 480nm. Strain A has exhibited optimum activity of 3.67µmol/ml/min at 30^{0}C and pH 8.0 when maltose was used as carbon source and L-asparagine as nitrogen source.

iv. Chitinase activity

Strains A, B and D were found to be positive for chitinase production. Here colloidal chitin agar media was used. Different concentrations of colloidal chitin were tested among which 1% colloidal chitin was found to be optimum.

In chitinase assay, NAG (N-acetyl glucosamine) was used as standard. Strain A has exhibited optimum activity of 5.83µmol/ml/hr when ISP-2 media was used and incubated till day 7 at 37^{0}C. Similarly when colloidal chitin media was used the optimum activity was found to be 1.356µmol/ml/hr on day 7.

v. Protease activity

Strain 10 and A were found to be positive for protease production. Clear zone appears around the actinomycete colonies when grown on skim milk agar media.

In protease assay tyrosine was used as standard. Strain 'A' showed enzymatic activity of 0.248µmol/ml/min on day 4 at pH 8.0 and temperature 37^{0}C.

4. Protein Estimation and Specific activity

Lowry *et al*., 1951 method was used and 0.2mg/ml BSA (Bovine serum albumin) was used as standard. Specific activity was measured as the ratio between enzyme activity and the protein concentration.

Table 1 depicts the protein concentrations and specific activities of enzymes cellulase, L-asparaginase, protease and chitinase.

Antimicrobial activity of strain A

Antimicrobial activity of different isolates was tested and among them strain A was found to have potential activity against most of the test microorganisms. Day 4 was found to be the optimum incubation period for antimicrobial activity. Figures 1 and 2 depict the zone of inhibition exhibited by strain A against *Xanthomonas* and *Pseudomonas aeruginosa*.

Table 1. Specific activities of different enzymes

Isolate type	Enzyme	Enzyme activity (µmol/ml/min)	Protein conc. (mg)	Specific activity (µmol/ml/mg ptn)
Strain A	Cellulase	1.388	0.1325	10.475
Strain A	L-asparaginase	3.67	0.360	10.194
Strain A	Protease	0.248	0.5725	0.4332
Strain A	Chitinase	1.356	0.225	6.027

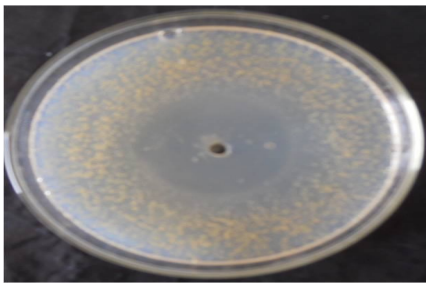

Fig. 1: Antimicrobial activity of strain A against *Xanthomonas*
(*The zone of inhibition of* Streptomyces *sp. strain A against* Xanthomanas *after 4 days of incubation was found be 32 mm (diameter)***)**

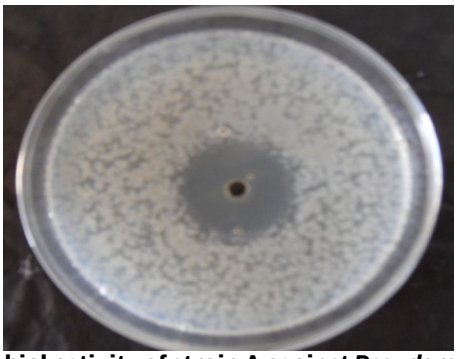

Fig. 2: Antimicrobial activity of strain A against *Pseudomonas aeruginosa*
(Streptomyces *sp. strain A exhibited zone of inhibition of 20 mm (diameter) against* Pseudomonas aeruginosa *on day 4**)**.

DISCUSSION

Among the 27 different actinomycete strains isolated from Coringa mangrove forests, 5 isolates exhibited amylase activity, 2 isolates exhibited cellulase activity, 2 isolates exhibited L-asparaginase activity, 3 isolates exhibited chitinase activity and 2 isolates exhibited protease activity. Cultural, morphological, biochemical and physiological characterization of the potent actinomycetes has revealed that the isolate belongs to *Streptomyces sp*.

Strain A (*Streptomyces sp.*) has a unique characteristic i.e., it releases camphor like smell. The growth was optimum at 40^{0}C and pH 7.0 when maltose or glucose was used as substrate at 3-4% NaCl concentration. Strain A was found to be positive for amylase, cellulase, chitinase, protease, L-asparaginase production. It has exhibited cellulase, L-asparaginase, protease and chitinase enzymatic activities of 1.388µmol/ml/min (day 4), 3.67µmol/ml/min (day4), 0.248µmol/ml/min (day 4) and 1.356µmol/ml/min (day 7) respectively. The specific activities of cellulase, L-asparaginase, protease and chitinase were 10.475µmol/ml/mg protein, 10.194µmol/ml/mg protein, 0.4332µmol/ml/mg protein and 6.027 mol/ml/mg protein respectively. Among the five enzymes produced by strain A L-asparaginase, cellulase and chitinase are found to be in higher concentrations compared to amylase and protease. Here these three enzymes are of high commercial value as L-asparaginase is used as anti-tumor agent, cellulase in paper and pulp industry and chitinase acts against fungal pathogens.

Strain A has also exhibited antimicrobial activity against various test microorganisms. Strain A exhibited zone of inhibition of 32 mm (diameter) against *Xanthomonas* on day4 where as it exhibited zone of inhibition of 10 mm (diameter) on day4 against *Candida albicans* which were maximum and minimum activities respectively. There was a steep increase in the antimicrobial activity of strain A against test micro organisms from day1 to day4 after which there is a decrease. Day7 recorded the minimal antimicrobial activity against all test micro organisms.

CONCLUSION

From the above study, it can be concluded that strain A (*Streptomyces sp.*) has both enzyme producing and antimicrobial activity characteristics. As the isolate is salt tolerant there would be minimal chances of contamination and also tolerant to various physiological conditions.

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