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ENZYMATIC AND ANTIMICROBIAL ACTIVITIES OF ACTINOMYCETES SPECIES OBTAINED FROM FRESH WATER, MAYANUR DAM, TAMIL NADU, INDIA

R. Anbalagan and R. Sivakami*

PG & Research Department of Zoology,
Arignar Anna Govt. Arts College, Musiri -621211, Tamil Nadu, India.

*E-mail: drsiva17@gmail.com

Abstract: **Among bacteria, actinomycetes are well documented for production of a large number of antibodies belonging to a wide array of groups. Actinobacteria have been extensively reported to inhabit a variety of places including soil, fresh water and marine. As exploration of potential bacteria is an important approach to discovering novel antibiotics to meet the current needs, the present study was undertaken to estimate the enzymatic and antimicrobial properties of a few actinomycetes species obtained from Mayanur Dam in Tamil Nadu, India. All the species observed the presence of all enzymatic activities except *Actinomyces* and *Micromonospora* which did not show chitinases activity. All the actinomycetes species showed antimicrobial properties. Among these, *Streptomyces* recorded the maximum zone of inhibition to all bacteria except *Staphylococcus epidermidis*.**

Keywords: **Actinomycetes, fresh water, enzyme activities, antimicrobial property.**

1. INTRODUCTION

The continued and poorly controlled use of antimicrobial products has led to the emergence of resistant strains which have increased since the early 1960's (Davies and Davies, 2010). This has triggered the need for the discovery and development of new antimicrobial compounds with altered modes of action (Kapur *et al*., 2018). One of the primary goals in bioprospecting studies with microorganisms is to enhance the natural product discovery which can be used in pharmaceutical or other industry sectors (Brown *et al.*, 2014; Hotam *et al.* , 2013; Barka *et al.*, 2015). Among bacteria, actinomycetes are well documented for production of large number of antibiotics. Antibiotics produced by actinomycetes belong to different chemical groups like tetracyclines, β-lactams, macrolides, ansamycins, phephdes *etc.* possessing a range of biological activities (Mahajan and Balachandran, 2012). Actinobacteria have been extensively reported from different ecosystems such as soil, fresh water, marine and also as endophytes on plants. As exploration of potential bacteria is an important approach to discovering novel antibiotics to meet the current needs (Lazzarini *et al.*, 2012; Poulsen *et al.*, 2011), the present study was attempted to study antimicrobial activity of selected actinomycetes species obtained from the fresh water system.

2. MATERIALS AND METHODS

A total of 150 water samples were collected from three fresh water systems of Mayanur Dam, Tamil Nadu, India regularly every month during the year 2018. Water samples were collected in sterile one litre conical flask and brought to the laboratory by closing with a sterile cotton plug.

Actinomycetes strains were isolated from the air water samples by applying serial dilution and spread plate technique on three different media such as Glycerol-Arginine Agar (El-Nakeeb and Lechevalier, 1963), Starch-Casein Agar and Starch-Nitrate Agar (Cochrane, 1961). Different media were used in order to compare the recovery on different media as well as to obtain maximum diversity of actinomycetes strains. Plates were incubated at room temperature for 2-3 weeks. After incubation, typical actinomycetes colonies were selected on morphological basis (Shirling and Gottlieb, 1966) and were transferred to Glycerol-Arginine Agar and maintained at room temperature. The plates were incubated at 28 - 30 °C for 2 - 3 weeks. Isolated colonies were further subcultured on GA plates (Saadoun *et al*., 1999; Williams and Cross, 1971). Selected pure isolates were then used for antimicrobial assay.

Test organisms

Escherichia coli, *Klebsiella pneumoniae*, *Salmonella typhi*, *Bacillus cereus*, *Vibrio cholerae*, *Salmonella paratyphi* and *Staphylococcus epidermidis*. *Aspergillus niger*, *Fusarium oxyporum* and *Candida albicans* were used in the present study.

Antimicrobial activity

Velvety actinomycetes isolates were examined microscopically to determine if they are gram-positive, filamentous or had long cells. Mycellium structure, arrangement of conidospore and arthrospore on the mycelium was observed microscopically (Olympus, 201, Japan) using oil immersion (100X) objective. The observed structure was compared with Berger's Manual of Determinative Bacteriology and the organism identified. Morphologically distinct actinomycetes isolates were selected for antimicrobial activity screening against the pathogenic test organisms. This was done by using Kirby-Bauer method (Bauer *et al*., 1966). Test group isolates were grown on antibiotic producing medium (GS medium) in an orbital shaker (150 rpm ambient temperature 28 °C). The fermentation broths were centrifuged after third, fifth and seventh days of incubation and the supernatant used as crude antibiotic extract of the isolates. Test pathogens were spread on the test plates - Nutrient Agar (NA) for bacteria and Sebouround agar (SA) for yeast and fungi. Sterile antibiotic discs (Himedia, 5 mm diameter) impregnated with the crude antibiotic extract of the test isolates were transferred to the test plates and incubated for 24-48 h. The zone of inhibition (in mm diameter) were read and taken as the activity against the test pathogen.

Enzymatic screening of actinomycetes

The active isolates obtained from fresh water systems of Karimnagar were subjected to screening to test their enzymatic activity. Two stages of enzymatic screening were done. All active isolates were subjected to primary screening, while secondary screening was done for those isolates which showed enzymatic activity in primary screening.

Primary enzymatic screening

In primary enzymatic screening, all active isolates were inoculated on suitable medium by spot inoculation method in order to check different enzymatic activity (amylase, protease, urease, cellulase, lipase and chitinase). The plates were incubated at 30 °C for 7 days and the results were recorded. The details are given in Table 1.

Secondary enzymatic screening

A further study for enzymatic activity of the actinomycetes was done by using shake flask method. The glycerol aspargine broth were incubated with enzymatically active isolates of actinomycetes at 30 °C for 7, 14 and 21 days. After incubation, broth cultures were filtered and partially purified enzyme was used for enzyme assay.

Enzyme assay

Cell free supernatant of the fermentation broth obtained by the centrifugation of the whole fermentation broth at 12,000 rpm for 15 min was used for enzyme assay.

Amylase

Assay of amylase was done by mixing 0.2 ml of enzyme extract, 0.25 ml of starch and 0.5 ml of phosphate buffer. The mixture was incubated at room temperature for ten minutes. The reaction was stopped by adding 0.25 ml of 0.1 N HCl

and the color developed by adding 0.25 ml of I/KI solution. The optical density was determined using a colorimeter at 690 nm. One unit of the enzyme activity is defined as the quantity of enzyme that caused 0.01% reduction of blue color intensity of starch iodine solution at 50 °C in min per ml.

Protease

Two ml of 0.5% casein in Tris HCl buffer with 0.5 ml of crude enzyme was incubated for 20 min at 37 °C in orbital shaker. The reaction was stopped by adding 1 ml of 30% TCA with shaking. After 15 minutes of incubation, the mixture was centrifuged at 2500 rpm for 30 min. 1 ml of supernatant was mixed with 1 ml of 1 M NaOH and the blue color absorbance was read at 440 nm by using colorimeter. The enzyme activity was calculated from standard curve of L-tyrosine. Quantitative estimation of Protein of the enzyme preparation was done by following the method of Lowry *et al*. (1951).

Urease

One ml of enzyme extract, 2 ml phosphate buffer (pH 6.5), 2 ml urea solution , 2 ml ZnSO4 and 0.25 ml 6N NaOH were taken in a test tube and the contents were kept for 30 min incubation and filtered through Whatmann N0.1 filter paper. The filterate was added with 10 ml DW, 1 drop of EDTA, 1 ml of Nessler's reagent and 20 ml DW. The developed orange color was read at 440 nm using colorimeter. The urease activity was expressed in enzyme units.

Cellulase

Cellulase assay was done by DNS method. The amount of reducing sugars liberated from carboxy methyl cellulose (0.5% CMC) was solubilized in 50 µl Tris-HCl buffer (pH 7.0). This mixture was added with 1 ml of enzyme kept for 20 min at 70 °C. The reaction was terminated by the addition of DNS solution. The mixture was boiled for ten minutes, cooled in water for stabilization of color, and the OD recorded at 550 nm using colorimeter. The Cellulase activity was determined by using standard graph of glucose.

Lipase

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis. One ml of the culture supernatant was added to the reaction mixture containing 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), 2.5 ml of deionised water and 3 ml of olive oil. The solution was mixed well and kept at 37 °C for 30 minutes. Both test and blank were prepared. After 30 minutes the test solution was transferred to a 50 ml Erlenmeyer flask. The reaction is terminated by adding 3 ml of 95% ethanol. The liberated fatty acid was titrated against 0.1 M NaOH using phenolphthalein as an indicator. End point was appearance of pink color. A unit of lipase is defined as the amount of enzyme which releases one micromole fatty acid per minute under specified assay condition.

Chitinase

Chitinase activity of bioactive isolates in the cell free broth was determined by using swollen chitin as a substrate and by measuring the release of reducing sugars with dinitrosalicylic acid method (Omura, 1986). 1 ml of reaction mixture contained 0.3 ml of substrate (1% w/v aqueous solution of the swollen chitin), 0.6 ml of 0.05 M citrate phosphate buffer (pH 6.0) and 0.1 ml of the enzyme sample. The mixture was incubated in a shaker water bath of 100 rpm at 35 °C for 60 min. Reaction was terminated by adding 1 ml of dinitrosalicylic acid followed by boiling in water bath for ten minutes. The solution was then filtered by using Whatman filter paper. After incubation, the optical density was measured at 540 nm using colorimeter. A standard curve for N-acetyl glucosamine was carried out parallelly to measure the concentration of reducing sugar released.

3. RESULTS AND DISCUSSION

The potent antibacterial actinomycetes chosen for the present study were subjected to enzyme activity screening and is presented in Table-1. Amylase and protease screening were carried out on starch and skimmed milk agar plate. While amylase producing strains were identified based on the formation of clear area against dark blue plates, the protease producing strains were identified by the formation of a clear area in the skimmed milk plates. Cellulose screening was carried out by plate assay method and the organism were identified by the formation of clear zones against the dark brown plates. Urease producing strains on the other hand were identified by the formation of pink colour in Christenson's agriculture. Lipase producing strains were identified by the formation of orange fluorescent halo in the Rhodomine. B agar plates and chitinase producing strains by the formation of clear areas on colloidal chitin agar.

Tables 2-4 records the enzymatic activities recorded by the various actinomycetes species. As evident from the table, all the species under study showed the presence of all enzymatic activities except *Actinomyces* and *Micromonospora* which did not record chitinases activity. Among the various actinomycetes species, *Streptomyces* recorded maximum enzymatic activity for all the enzymes except uease.

The maximum urease activity was recorded by *Actinopolyspora*. Among the various species, *Micromonospora* recorded minimal enzymatic activities with regard to protease, urease, cellulose, and chitinases. Mohan and Charya (2012) while analyzing the enzymatic activities of actinomycetes also recorded similar results. However, they reported the absence of chitinases activity in all the samples analysed. In the present study, only *Actinomyces* and *Micromonospora* did not record any chitinases activity while the remaining species recorded this activity. Ningthoujam and Shovarani (2018) while studying the actinomycetes species of Nambul river in Manipur also reported the presence of chitinases activity as reported in the present study. Nevertheless, the present study clearly reveals the fact that most of the actinomycetes species are active and can play a significant role in the decomposition of organic matter as already suggested by Mohan and Charya (2012).

The results of the antimicrobial activity of the various actinomycetes species to various bacteria are presented in Table-3. The results clearly indicate that all the actinomycetes species recorded antimicrobial activity but at different levels.

Among the various actinomycetes species, *Streptomyces* record the maximum zone of inhibition against all the bacteria except *Staphylococcus epidermidis.* in addition, *Streptomyces* was the most efficient actinomycetes species against all the bacteria analysed except for *S. epidermis* where *Actinopolyspora* recorded the maximum zone of inhibition. Further, the present study also suggested that *Streptomyces* and *Actinomyces* are equally efficient against *Vibrio cholerae* as both the species recorded the same amount of the zone of inhibition. Among the various *Actinomyces* species, *Micromospora* appeared to have the least inhibitory effect for most of the bacteria analysed. The study also suggests that the antimicrobial effect of the above actinomycetes species drastically decreased towards *Aspergillus*, *Fusarium* and *Candida* as evident by the decreased zones of inhibition in all actinomycetes species.

A review of literature reveals that Mohan and Chanya (2012) also reported antimicrobial activity against some of the bacteria used in the present study. Kumar *et al.* (2013) suggested the actinomycetes show maximum inhibitory effect on *S. ureane* and *E. coli* while Saravanan *et al.* (2015) also suggested maximum antimicrobial activity against *K. pneumoniae* and *E. coli* while Kapur *et al.* (2018) suggested actinomycetes to show maximum inhibitory effect towards *B. cereus* and *F. oxysporum*. Thus actinomycetes appear to show differential inhibitory effect towards each pathogen. Nevertheless, the study clearly indicates that these organisms can be used to control a large number of bacteria.

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APPENDIX - A

Table-1: Antimicrobial Activity of Actinomyctes Strphomyces against Microbial pathogen (Zone of inhibition in mm)

S. No.	Bacteria & Fungi	*Streptomyces*	*Saccharopolyspora*	*Actinopolyspora*	*Actinomyces*	*Micromonospora*
1.	*Escherrichia coli*	20.6	18.9	18.9	17.8	16.4
2.	*Bacillus substilis*	18.4	12.6	17.6	16.8	17.8
3.	*Salmonella typhi*	17.9	17.8	16.2	13.6	11.8
4.	*Vibrio cholerae*	16.4	15.6	13.2	16.4	7.8
5.	*Staphylococcus aureus*	17.6	8.9	8.9	7.8	9.8
6.	*Staphylococcus epidermidis*	16.4	17.2	18.2	14.6	10.8
7.	*Klebsiella pneumoniae*	9.2	8.2	8.3	7.2	8.4
8.	*Aspergillus niger*	8.4	7.2	3.2	1.8	1.2
9.	*Fusarium oxysporum*	7.4	4.0	5.6	4.2	3.1
10.	*Candida albicans*	6.4	3.2	2.2	1.4	1.8

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Table-2: Primary Enzymatic Screening of Bioactive Actinomycetes Isolated from Mayanur Dam

S. No.	Enzyme	Medium	Incubation	Criteria of Positive Enzyme Activity
1.	Amylase	Starch agar	7-8 days	Clearing around the growth treating with 2% iodine
2.	Protease	Skim milk agar	7-8 days	Clearing around the growth treating with 2% iodine
3.	Urease	Chritenson's agar	7-8 days	Development of pink colour
4.	Cellulase	Cepek mineral salt agar	7-8 days	Clearing around to growth
5.	Lipase	Rhodamine B agar plant assay	7-8 days	Colonius showing fluorescent under gradation indicates lipase activity
6.	Chitnase	Colloidal Chitin agar	7-8 days	Clearing around to growth

Table-3: Enzymatic activities of selected antagonistic Actinomyctes strains

S. No.	Enzymes	*Streptomyces*	*Saccharopolyspora*	*Actinopolyspora*	*Actinomyces*	*Micromonospora*
1.	Amylase	+	+	+	+	+
2.	Protease	+	+	+	+	+
3.	Urease	+	+	+	+	-
4.	Cellulase	+	+	+	+	+
5.	Lipase	+	+	+	+	+
6.	Chitnase	+	+	+	-	-

Table-4: Enzymatic activities of selected antagonistic Actinomyctes strains

S. No.	Enzymes	***Streptomyces***	***Saccharopolyspora***	***Actinopolyspora***	***Actinomyces***	***Micromonospora***
1.	Amylase	21.4	18.4	16.4	18.6	17.2
2.	Protease	19.4	16.4	18.4	15.4	12.8
3.	Urease	16.7	12.6	17.8	6.3	4.4
4.	Cellulase	14.6	14.2	4.6	3.3	1.6
5.	Lipase	15.6	7.8	9.6	14.8	9.2
6.	Chitnase	6.8	3.9	5.8	-	-