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Research Article

ENZYMATIC ACTIVITY OF FRESH WATER ACTINOMYCETES

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ABSTRACT

Fresh water systems represent a largely untapped source for isolation of novel microorganisms. Gram-positive actinomycetes are of special interest, since they are known to produce chemically diverse compounds with wide range of biological activities. Twenty four actinomycetes with distinct characteristics were isolated from three freshwater systems of Karimnagar, Andhra Pradesh, India viz: Lower Manair Dam, Manakondur Pond and Kothapally Pond. These isolates were screened for their antagonistic activity. Among, eight showed antagonistic activity against test bacteria. Enzymatic activity of these antibacterial actinomycete isolates was performed which revealed that, out of eight actinomycetes, 8(100%), 7(87%), 6(75%), 6(75%), 8(100%), were possessing amylase, protease, urease, cellulase and lipase activity respectively. It was found that all tested active isolates were lacking chitinase activity. It was thus seen from above results that high numbers of actinomycetes were biochemically active in fresh water systems and play significant role in decomposition of complex organic matter.

Keywords: Fresh water, Actinomycetes, Cellulase, Protease, Amylase

INTRODUCTION

Actinomycetes are the group of Gram positive filamentous bacteria which are widely distributed in different terrestrial and aquatic habitats[1]. In aquatic habitats, actinomycetes play a great role in carbon cycle due to their ability to grow at low concentrations of carbonaceous substances and to degrade recalcitrant organic matter[2]. They makeup large part of microbial population of aquatic systems[3-6]. Diversity and bioprospecting studies on actinomycetes are mainly pertaining to the terrestrial and marine ecosystems and less importantly from fresh water systems[7]. There are many reports on actinomycetes from various fresh water systems at international level[8-10] but not in national level. In India, there are very few reports on fresh water actinomycetes[11-14]. Most of the studies reported the diversity of actinomycetes than studying their biological significance. Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, and enzymes.

The industrial enzyme sector in India and in the world is developing fast for meeting the needs of food processing, pharma, and textile industries. In addition to demand in Indian market, export opportunities are also there for bacterial enzymes. Research on enzyme producing actinobacteria is limited in our country. In view of the importance of bacterial enzymes and enzyme producing actinomycetes, it was interesting to carryout research in this direction[15]. Today more than 4000 enzymes are known, of which many are produced commercially. The majority of these industrial enzymes are microbial in origin. Besides antibacterial compounds, enzymes are the bioactive compounds that have focused attention by industrial research. The demand for bacterial enzymes in industrial fields is increasing day by day due to their application in clean, eco-friendly and cost effective biotechnological processes. Among microorganisms, actinomycetes are the most important because of their capacity to produce numerous bioactive molecules including antibiotics and enzymes[16,17]. Aquatic actinomycetes are emerged as rich source for the production of industrially important enzymes[18].

Amylase is one of the commonly used enzymes in different starch industries. It has many industrial applications in the production of syrup made from oligosaccharide and monosaccharide. In textile industries amylase enzyme is used for resizing of clothing materials[19,20]. Production of amylases was reported by some strains of actinomycetes[21,22].

Actinomycetes are good decomposers of organic materials through secretion of extracellular hydrolytic enzymes like amylases, chitinases and proteases. Proteases catalyse the hydrolysis of proteins to peptides and aminoacids. These proteases are important from industrial perspective and cater for the demand of 60 % world enzyme market[23]. Different microorganisms secrete urease enzyme. Urease enzyme plays an important role in degradation of organic nitrogen (N). The degradative process of the ureolytic microorganims like actinomycetes on animal and plant organic N wastes help to eliminate wastes and supply plant with available N.

Chitin is the most abundant biopolymer next to cellulose. It is the structural polymer of fungi and arthropods[24]. Both soil and aquatic systems acts as major sources of chitin degrading microorganims. The complex cellulolytic enzymes which convert cellulose to glucose, have shown to be extracellular product of several microorganisms[25]. Most work on cellulase has been focused on fungal systems which produce a complex of three distinct enzymes (exo-B-1,4 glucanase, endo-B1,4 glucanase and B-glucosidase) which act sequentially to hydrolyze cellulose to glucose[26]. A few reports only are found on cellulolytic activity of actinomycetes capable of degrading cellulose[27]. Actinobacteria are one of the potent cellulase synthesizing organisms has attracted considerable research interests[28,29].

Lipases (triacyl glycerol acilyl hydrolase) are widely used in the food, pharmaceutical, diagnostic and detergent industries[30]. Furthermore that have been used in biotechnological industries for synthesis of biopolymers and biodiesel[31,32]. Lipase enzyme production in fungi, bacteria and actinomycetes has been reported by many workers[33].

The present study deals with screening of industrially important enzymes cellulase, protease, amylase, urease, lipase and chitinase producing actinomycetes.

MATERIAL AND METHODS

A total of 144 Water and sediment samples were collected from three freshwater systems of Karimnagar, Andhra Pradesh, India viz: Lower Manair Dam, Manakondur Pond and Kothapally Pond regularly every month during July 2006 to June 2008. Water samples were collected in a sterile one liter conical flask and brought to the laboratory by closing with sterile cotton plug. Sediment samples were collected in a sterile Petri dish by using sterile spatula. Actinomycetes from these collected samples were isolated by Double Agar Layer (DAL) method on actinomycetes isolation agar containing cycloheximide (50µg/ml) to minimize fungal contamination[34]. All plates were incubated at 28°C for 1-2 weeks. The actinomycetes colonies that appeared on petri plates were counted from 5th day onwards up to 14th day.

Screening of actinomycetes isolates for antibacterial activity

A total of 24 different actinomycetes were collected from these samples viz: eleven from Lower Manair Dam (LAM1 to LAM11) seven from Manakondur Pond (MAM1 to MAM7) and six from Kothapally Pond (KAM1 to KAM6). All isolates were, sub cultured and maintained in agar slants. The isolated actinomycetes strains were tested for their antibacterial activity against ten test bacteria namely *Bacillus subtils* (MTCC 431), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424), *Enterobacter aerogenes* (MTCC 111), *Salmonella typhi* (MTCC 733), *Escherichia coli* (MTCC 40), *Sarcina lutea* (MTCC 1541), *Shigella flexneri* (MTCC 1457) *and Klebsiella pneumonia* (MTCC 7162). Among, eight isolates (LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5) showed good antagonistic activity against test bacteria which were selected for enzymatic screening.

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 was 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 7days 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 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 is done by mixing 0.2 ml of enzyme extract, 0.25 ml of starch and 0.5 ml of phosphate buffer. The mixture is incubated at room temperature for ten minutes. The reaction was stopped by adding 0.25 ml of 0.1N HCl and the color was 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 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 r.p.m. for 30 min. 1 ml of supernatant was mixed with 1 ml of 1M 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*[35].

Urease One ml of enzyme extract, 2 ml phosphate buffer (pH 6.5), 2 ml urea solution , 2 ml $ZnSO_4$ 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) solubilized in 50 µl Tris-HCl buffer (pH 7.0). This mixture was added with 1ml of enzyme kept for 20 min at 70°C. The reaction was terminated by the addition of DNS solution. The mixture is boiled for ten minutes, cooled in water for stabilization of color, and the O.D was 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 0.1M 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. Liberated fatty acid was titrated against 0.1 M NaOH using phenolphthalein as an indicator. End point is an appearance of pink color. A unit 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[36]. 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 is 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 is measured at 540 nm using colorimeter. A standard curve for N-acetyl glucosamine was carried out parallel to measure the concentration of reducing sugar released.

Table 1 Primary enzymatic screening of bioactive actinomycetes

Enzyme	Medium	Incubation	Criteria of positive enzyme activity
Amylase	Starch agar	7 days	Clearing around the growth after flooding with 2% Iodine
Protease	Skim milk agar	7 days	Clearing around the growth
Urease	Christenson's agar	7 days	Development of pink color
Cellulase	Czepeck mineral salt agar	7 days	Clearing around the growth
Lipase	Rhodamine B agar plate assay	7 days	Colonies showing fluorescence under UV irradiation indicates lipase activity.
Chitinase	Colloidal chitin agar	7 days	Clearing around the growth

Table 2 Enzyme activities of selected antagonistic actinomycete strains (Primary enzymatic screening)

Enzymes	Antagonistic actinomycetes strains							
	LAM1	LAM2	LAM7	LAM11	MAM3	MAM6	KAM2	KAM5
Amylase	+	+	+	+	+	+	+	+
Protease	+	+	+	+	-	+	+	+
Urease	+	+	+	+	+	-	-	+
Cellulase	+	+	+	-	-	+	+	+
Lipase	+	+	+	+	+	+	+	+
Chitinase	-	-	-	-	-	-	-	-

+ = positive - = Negative

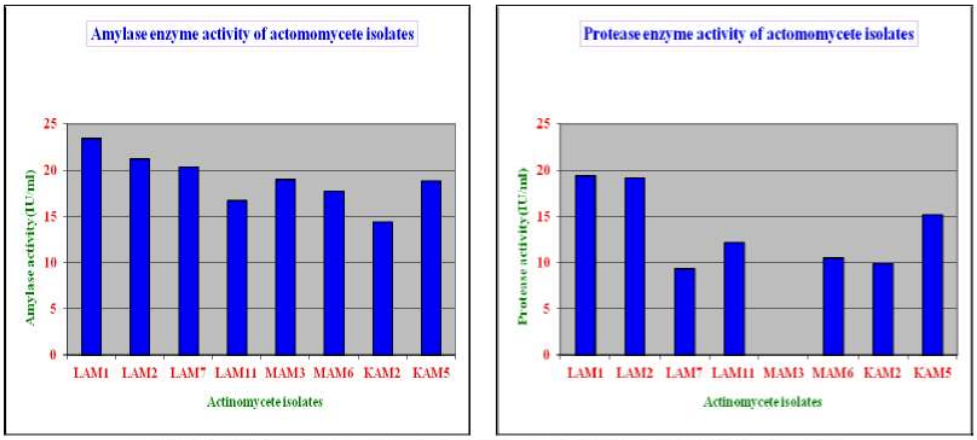


Figure 1 Amylase and protease enzyme activity of bioactive actinomycete isolates

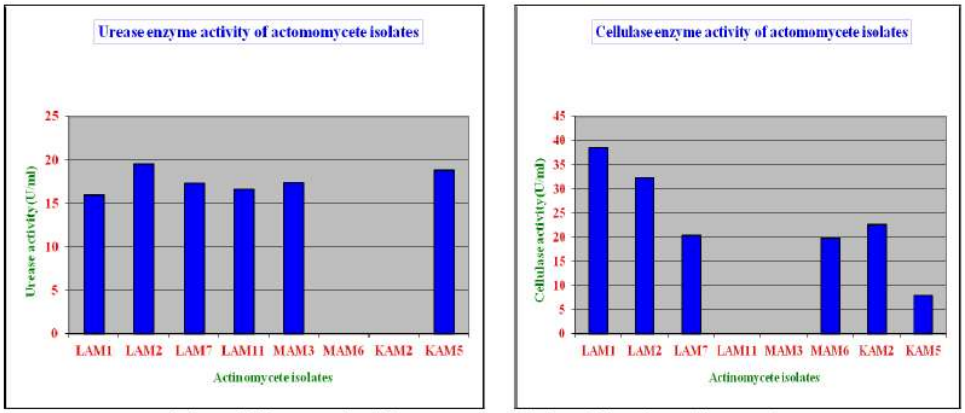


Figure 2 Urease and cellulase enzyme activity of bioactive actinomycete

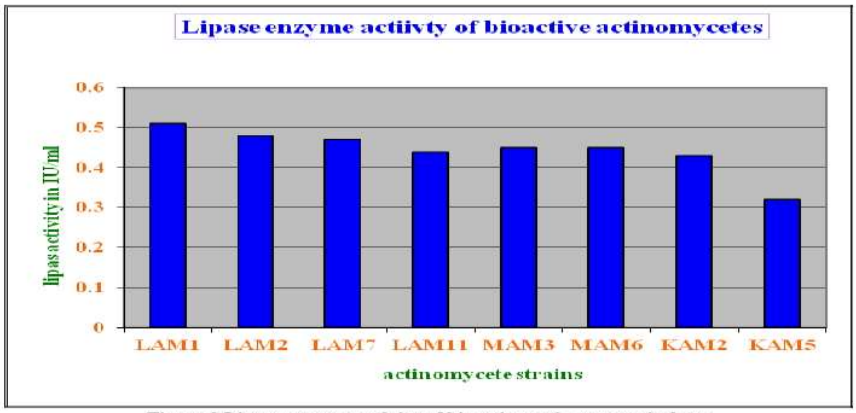


Figure 3 Lipase enzyme activity of bioactive actinomycete isolates

RESULTS

In the present study, 24 different actinomycetes were gained from the fresh water systems of Karimnagar, Andhra Pradesh, India. Among, eight showed antibacterial activity against test bacteria. These potent antibacterial actinomycetes were subjected to enzyme activity screening. Amylase and protease screening were carried out on starch and skim milk agar plate assay method respectively. Amylase producing strains were identified based on the formation of clear area against dark blue plates. Protease producing strains were identified by formation of clear area in skim milk agar plates.

Urease producing strains were identified by the formation of pink color in Christenson's agar. Cellulase screening was carried out by plate assay method. Cellulase producing strains were identified by formation of clear zones against dark brown plates.

Screening of lipase was carried out by Rhodamine B agar plate assay method. Lipase producing strains were identified by formation of orange fluorescent halo around the colonies. Chitinase producing strains were identified by formation of clearing areas on colloidal chitin agar. Among the eight strains, no one showed chitinase activity.

The results of primary enzymatic screening were mentioned in the table 2. It was found that out of eight actinomycetes 8(100%), 7(87%), 6(75%), 6(75%), 8(100%), were possessing Amylase, protease, urease, cellulase and lipase activity respectively. It was found that all tested active isolates were lacking Chitinase activity. It was thus seen from above results that high numbers of actinomycetes were biochemically active in fresh water systems and play significant role in decomposition of complex organic matter.

Secondary enzymatic screening

All the positive isolates of primary screening were confirmed for their enzymatic activities by submerged fermentation conditions and the results of promising isolates are presented in Fig 1 –3.

Spectrophotometric assay of amylase production was carried out for the strains LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5 and their activity was found to be 23.4, 21.2, 20.3, 16.7, 19, 17.7, 14.4 and 18.8 U/ml. From the above results, strain LAM1 and LAM2 showed maximum amylase activity.

Colorimetric assay of protease production was carried out for the strains LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5 and their activity was found to be 19.4, 19.1, 9.3, 12.2, 0, 10.5, 9.9 and 15.2 U/ml. From the above results, strain LAM1 and LAM2 showed maximum protease activity.

Assay of urease production was carried out for the strains LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5 and their activity was found to be 16, 19.5, 17.3, 16.6, 17.4, 0, 0 and 18.8 U/ml. From the above results, strain LAM2 and KAM5 showed maximum urease activity.

Spectrophotometric assay of cellulase production was carried out for the strains LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5 and their activity was found to be 38.6, 32.3, 20.3, 0, 0, 19.8, 22.6 and 8.0 U/ml. From the above results, strain LAM1 and LAM2 showed maximum cellulase activity.

Assay of lipase production was carried out for the strains LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5 and their activity was found to be 0.51, 0.48, 0.47, 0.44, 0.45, 0.45, 0.43 and 0.32 U/ml. From the above results, strain LAM1 and LAM2 showed maximum lipase activity.

DISCUSSION

Actinomycetes are unparalleled sources of bioactive metabolites including antibiotics, plant growth factors and enzymes[37,38]. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds[39]. These searches have been remarkably successful and many naturally occurring antibiotics and enzymes of medical importance have been isolated from actinomycetes[40]. Fresh water systems represent a largely untapped source for isolation of novel bacteria. Gram-positive actinobacteria are of special interest, since they are able to synthesize chemically diverse compounds with wide range of biological activities.

Totally, 24 actinomycetes with distinct characteristics were isolated from sediment and water collected from three major fresh water systems of Karimnagar. These isolates were screened for their antibacterial activity. Among, eight isolates (LAM1, LAM2, LAM7, LAM11, MAM3, MAM6, KAM2 and KAM5) exhibited potent antibacterial activity against test bacteria. Enzymatic activity of these antibacterial actinomycete strains were performed which revealed that, out of eight actinomycetes, 6(75%), 7(87%), 8(100%), 6(75%) were possessing cellulase, protease, amylase and urease activity respectively. The results of secondary enzyme screening revealed that among eight isolates, two isolates namely LAM1 and LAM2 showed superior enzymatic activity compared to other isolates. Studies on enzymatic activity of the active isolates shows that approximately 90% of isolates produced one or more enzymatic activity[41-43]. This indicates that actinomycetes posses the potential to create broad range of enzymes, which may be the result from natural selection of microorganism in order to survive in a competing environment[44].

It was found that all tested active isolates were lacking chitinase activity. It was thus seen from above results that high numbers of actinomycetes were biochemically active in fresh water systems and play significant role in decomposition of complex organic matter. It also appears that fresh water actinobacteria are one of the important resources for screening useful enzymes and bioactive metabolites.

CONCLUSION

From this study, it is clearly indicated that fresh water systems can provide rich source of enzyme producing actinomycetes. These enzymatically active actinomycetes may be effectively used in large scale production for commercial and pharmaceutical applications in the coming future.

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