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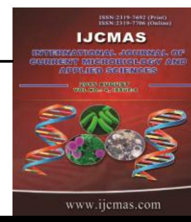

Original Research Article

Screening of Novel Actinomycetes from Near Lake Shore Sediment of the Chilika Lake, Odisha, India

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A B S T R A C T

The present study was carried to screen novel Actinomycetes that are potentially secret bioactive enzymes and secondary metabolites from near lake shore sediment of the Chilika Lake, Odisha, India. Five strains were isolated from the sediments. The isolate AC-I (*Streptomyces* sp.) showed a good antagonistic effect against Dermatophytes whereas AC-III is a good enzyme producer. The study reveals AC-III is a good producer of Lecithinase enzyme which is a haemolytic agent. The haemolysis titre reveals that the enzyme produced by AC-III (*Actinomycetes* sp.) is not lethal to the human & Sheep but is lethal to the fowl. The results of this investigation revealed that the actinomycetes of sediment of the Chilika Lake were potent source of novel antibiotics and bioactive compounds.

Keywords

Synthetic dyes, Textile industry, Decolourization, Bacteria

Introduction

Actinomycetes are the group of microbes that have been successfully utilized for social benefits but still remain a scope to explore and exploit. The name Actinomycetes was derived from a greek word "aktis" (a- ray) and "mykes" (fungus) and considered to be an intermediate group between bacteria and fungi, Now they are considered as a group of prokaryotic organisms, mostly free living, saprophytic bacteria found widely distributed in soil, water and colonizing in plant. Actinomycetes belong to the subdivision Actinomycetales of Prokaryotes. The Indian peninsula harbours its own diverse habitats which support the growth of various actinobacterial communities in specific microbial niches. Hence in India, actinobacterial diversity has been an important shorece for natural product discovery. Over the years, novel species of Actinomycetes have been discovered from diverse habitats of India. Mayilraj *et al.* (2006) reported Four novel species viz *Rhodococcus kroppenstedtii*, *Planococcus stackebrandtii*. *Agrococcus lahulensis* and *Kocuria himachalensis* from cold deserts of Himalayas by and Dhanjal *et al.* (2010) discovered two novel species of Actinomycetes viz. *Agrococcus carbonis* and *Yaniella fodinae* from coal mine. Actinomycetes perform significant

biogeochemical roles in terrestrial soils and are highly valued for their unparallel ability to produce biologically-active secondary metabolites. As on today a total of 22,500 bioactive secondary metabolites have been reported, out of which 16,500 compounds show antibiotic activities and 10,100 are reported to be produced by Actinomycetes in which 7630 from *Streptomycetes* and 2470 from rare Actinomycetes. A search of recent literature revealed that at least 4607 patents have been issued on Actinomycete related products and processes (Berdy, 2005). India the land of culture and heritage nurture a huge species of flora and fauna. According to Indian context diverse microflora are present in the scenario. Chilika Lake, the largest brackish water lagoon in India and second Largest in the world lies in the eastern coast from Longitude-19^{0} 43'' N & Latitude- 85^{0} 19'' E. Lake water is alkaline pH ranging from 6.1 to 9.6 with total alkalinity matching the salinity.

The southern part of the lake near Rambha has recorded the highest alkalinity. The ecological richness of the lake is of great value in preserving the genetic diversity because of the multiplicity of its habitat, flora and fauna. The Zoological Survey of India (ZSI) surveyed the lake between 1985 and 1988 and identified 800 species of fauna, including many rare, endangered, threatened and vulnerable species, but excluding terrestrial insects. Chilika Lake is the largest wintering ground for migratory birds, in the Indian sub-continent. It is one of the hotspots of biodiversity in the country. The abundance of microflora in the Chilika ecosystem is the major interest of the researchers for its diversity and uniqueness. Actinomycetes a dominant species in this brackish water is due to the alkaline pH of the lake (Patra *et al.,* 2009). *Steptomyces chilikensis*, a holophilic streptomycete is one of the major isolates from the Chilika Lake. Keeping in view of the importance the study was aimed to made to screen novel Actinomycetes that are potentially secret bioactive enzymes and secondary metabolites from near lake shore sediment of the Chilika Lake, Odisha, India which is a brackish water lake connected with the Bay of Bengal and have large diverse and largely unscreened ecosystem for the isolation of potent bioactive-compound producing Actinomycetes.

Material and Methods

Collection of sample from Chilka Lake

The samples were collected from the sediments of three different places of Chilika Lake (Rambha, Barkul, Balugaon), by inserting a spatula (previously sterilized) into the sediment of each sampling site. The samples were then transferred to a sterile polythene bag and transported immediately to the laboratory.

Isolation and purification of Actinomycetes

The sediment sample was air-dried aseptically, and after one week, the sediment samples were incubated at 55° C for 5 min. Then one gm. of each soil sample was taken in three different test tube containing 10 ml of distilled water. To each test tube 0.1ml of sample solution was spread on three different sterile plates containing Actinomycetes isolation agar (AIA) media (Himedia). To minimize fungal and bacterial contamination, all agar plates were supplemented with 50 µg/ml of Nystatin and Ampicillin. All plates were incubated at 28° C for five days. Colonies were isolated and sub cultured. Pure culture of the isolates was maintained in the department.

Morphological and staining characteristics of the organisms

Morphological characters of the colonies and the isolates were studied following the standard microbiological methods.

Colony characters of the isolates

The isolates were streaked on Actinomycetes agar plates, incubated for five days at 30 ± 0.1 °C. The shape, size, colour, margin and opacity were recorded from isolated colonies.

Morphology of cells and motility

Morphology of the vegetative cells were observed under a microscope using a 100X objective from (for vegetative cells and motility) cultures grown on Actinomycetes agar plates at 30± 0.1^{0}C in an incubator.

Staining characters of vegetative cells

Staining characters of the organism were studied for vegetative cell character determination.

Direct visualization under microscope

The diluted suspensions of the Actinobacteria were smeared on clean slides and by putting a cover slip, they were observed under microscope using 40X objective.

Gram's stain

To study the Gram's reaction i.e. Gram (+ve) or Gram (-ve) characters of the isolates, diluted suspensions of the Actinobacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2–3 times. The slides were flooded with crystal violet solution for 1 min, washed with water and flooded with Gram's iodine for 1 min. The slides were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off alcohol. The slides were washed with distilled water and counter stained with safranin stain for about 30 sec and washed with water. The slides were air dried and examined under a microscope using 100X objective using a daylight filter.

Acid-fast stain

Acid fast staining was done to check whether the isolates were acid fast or non-acid fast. To study the acid fast characters of the isolates, diluted suspensions of the Actinobacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2-3 times. Then carbol fuschin stain was spread over the smear and steam was allowed from the opposite side of the smear for 10 min keeping in view that, the slide do not become dry. After treated with the primary stain, smear was washed with acid alcohol and counter stain (methylene blue) was given for 1 min and washed with distilled water. After drying the smear it was observed under oil immersion microscope under 100X objective.

Physiological characters

Physiological characters of the organisms were checked using the standard methods for identification of the isolates.

NaCl tolerance

Growth of the organisms in Actinomycetes isolation broth supplemented with 1 to 15% NaCl and incubated at 30 ± 0.1^{0}C for five days. After the incubation period, the cultures were streaked on the Actinomycetes isolation agar plates and growth was recorded.

Effect of pH on the growth of the isolates

The effect of pH were checked in Actinomycetes isolation broth in different pH (up to pH 14) and incubated at 30 ± 0.1°C for five days. After the incubation period, the cultures were streaked on the Actinomycetes isolation agar plates and growth was recorded.

Temperature tolerance

The isolates were grown in Actinomycetes isolation broth in different temperature (4° to 50°C) and incubated at 30 ± 0.1^{0}C for five days. After the incubation period, the cultures were streaked on the Actinomycetes isolation agar plates and growth was recorded.

Biochemical characters

Various biochemical tests were conducted to study activities of the isolates

Methyl red test

The test is used to detect acid production from glucose. Production of acid lowers the pH of the medium below 4.2 which is detected by the pH indicator methyl red. Actinomycetes were inoculated into tubes containing methyl red-Voges Proskauer (MRVP) broth and incubated at 30 ± 0.1^{0}C for 96 h. After incubation alcoholic methyl red indicator was added. Positive reaction was indicated by change of colour of medium to red.

Voges-Proskauer test

The isolates were tested on MR-VP broth medium to detect their ability to produce neutral products like acetoin (acetyl methyl carbinol) during metabolism of glucose present in the medium. After incubation, 0.6 ml of 5% naphthol was added followed by 0.2 ml of 40% KOH to about 1 ml of broth culture. The solution was allowed to stand for 30 minutes. A change in colour of the medium to wine red was as positive reaction while copper colour indicates a negative result.

Indole production test

The test is used to check ability of the organisms to form indole from tryptophan or to detect the presence of enzyme tryptophanase which converts tryptophan to indole. The test was performed by inoculating the bacterial cultures into tubes containing tryptone broth incubated at 30 ± 0.1^{0}C for 96 h. After inoculation, Kovac's reagent was added (1:1 by volume) and mixed to check for indole production which was indicated by a pink ring at the interface of the two solutions. Absence of pink ring indicated negative result. If required.

Citrate Utilization test

The capability of the isolates to utilize citrate as the sole shorece of carbon & energy was studied on Simmons citrate agar medium. Colour change of the slant from green to royal blue was considered as positive result while no change in colour was taken as negative.

Nitrate utilization test

Nitrate broth medium was used to determine the ability of the isolates to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. The ability of the isolates to denitrify nitrate to nitrite ultimately producing ammonia & molecular nitrogen was also tested simultaneously. After incubation, 1 ml each of sulphanilic acid (in 30% acetic acid) & napthylamine solution (in 30 % acetic acid) were added to the broth culture. Development of red colour indicated positive result for nitrate reduction. In case

of no change in colour, a pinch of powdered Zinc was added to the broth. Development of red colour in this step indicated a negative result while no change in colour after the addition of the Zinc powder, indicated a positive complete showing that denitrification took place & ammonia or molecular nitrogen were formed.

Mannitol motility test

The capacity of the isolates to utilize mannitol, reduce nitrate indicating the presence of the enzyme nitrate reductase & their motility was studied using this medium. The fermentation of mannitol was indicated by the acid production which changed the colour of the butt from red to yellow. Presence of air bubbles was taken as the positive indication of nitrate utilization. Diffused growth along the stabbed line was considered positive test for motility while only on the stabbed line indicated a negative test for motility.

Oxidase test

To detect presence of the enzyme oxidase in the bacteria which catalyses transport of electrons between bacteria and the redox dye e.g. N-tetramethyl-*p*-phenylene diamine dihydrochloride or dimethyl-*p*-phenylene diamine or methylene blue (oxidase reagents) and causes intense purple colouration was observed.

Catalase test

Presence of the enzyme catalase which catalyses breakdown of hydrogen peroxide into water and oxygen was studied on culture plates (NA) flooded with hydrogen peroxide solution. Positive reaction was indicated from effervescence of oxygen from the plate.

Urease test

To test for the presence of the enzyme urease in the isolates which split urea into ammonia and CO_2 was studied using Christensen's urea agar medium. Colour change of the slant from yellow to pink was considered as positive result while no change in colour was considered as negative.

Amino acid utilization test

The isolates were screened on Moeller decarboxycholate broth to test for their ability to utilize amino acid. Three amino acids were used under study viz. ornithine, arginine and lysine. The change of the colour of the medium to purple indicates positive test while yellow colour indicates negative test.

Oxidation-fermentation test

The test was performed on oxidation-fermentation medium to study the oxidative and fermentative mode of metabolic degradation of various sugars by the isolates. Various sugar used were Lactose, Dulcitol, Inocitol, Sucrose, Xylose, Ramnose, Inosine, Cellobiose, Mellibiose, Maltose, Serbitol, Arabinose respectively. Yellow coloration of the medium was considered positive while negative test was indicated by no colour change of the medium.

Esculin hydrolysis

The purpose is to see if the microbe can hydrolyse the compound esculin as a carbon shorece. When an organism hydrolyses the glycoside esculin to form esculetin and dextrose, the esculetin reacts with the ferric citrate to produce a dark brown or black phenolic iron complex. The bile-esculin agar

medium containing 0.1% esculin, & 0.05% ferric citrate was Prepared, pH was adjusted to 6.6. The medium was sterilized and poured filling the bottom half of the tube and creating a slant in the upper half. The isolates were and inoculate a bile-esculin tube by stabbing the medium then zigzag streaking on the surface of the slant. Reaction is considered positive if the slant turned blue-black and negative if no change from the original colour occurred.

Antagonistic effect of the isolates against some human pathogens

The microbial sensitivity of the isolates was analysed by spot inoculation method and disc diffusion method. Each of the isolate was spotted on the SDA (Sabouraud dextrose agar) and MHA (Muller Hinton agar) plates. It was incubated at 30^0C for 6 days after the sixth day different strains of microorganisms were swapped on the plates, and incubated at 37^0C for 24 h.

If the organism is susceptible to the antibiotics produced by Actinomycetes, then the growth is nil around the colony. And the clear zone formed was measured. The pathogens selected were *Candida albicans, Tricophyton mentagrophytes, Epidermophyton flocussum, Candida tropicalis, E. coli EPEC, E. coli ETEC, Salmonella* sp., *Aeromonas* sp., *Shigella* sp., *Staphylococcus epidermidis.*

Extracellular enzymatic activity of the isolates

The activities of various extracellular enzymes produced by the isolates were studied by the following tests.

Starch hydrolysis test

Capacity of the organisms to hydrolyze starch into simple substances like dextrin, glucose, maltose etc. by amylase enzymes was detected by spot inoculating the bacterial cultures on NA plates containing 1% soluble starch. After incubation for 96 hrs at 30 ± 0.1^0C, all the plates were then exposed to iodine vapour for 5 to 10 minutes. Starch hydrolysis was noted from a clear zone formed around the colonies. Reddish-brown area around the colonies indicated partial hydrolysis of starch.

Lipase test

Lipase production by the organisms was tested using different lipid shoreces on plates incubated at 30 ± 0.1^0C for 5 days.

Tween 20 hydrolysis test

Tween AIA plates were prepared using AIA/5, 1% (V/V) tween-20 & 1.5% agar. All the isolates were spot inoculated on the plates & incubated at 37^0C for 96 hours. All the plates were then stored at 4^0C for 2-3 hours to precipitate the lipid in presence of $CaCl_2$.

A clear zone around the colony indicated positive lipolytic activity of the isolate caused by the production of lipase.

Lecithin hydrolysis (Lecithinase) test

Some bacteria produce the enzyme lecithinase which splits lipoprotein complexes. Egg yolk when added to the medium, the activity of the enzyme causes turbidity around the colony. An opaque zone around the colony indicated lecithinase activity and a halo zone around the opaque zone indicates lipolytic activity. Egg yolk suspension: Fresh hen egg was washed with disinfectant (90% ethanol), the egg white (albumin) was withdrawn, and diluted yolk with equal volume of 0.85% NaCl and add required amount to the presterilized medium.

Cellulase test

Carboxy methyl cellulose (CMC) - AIA plates were prepared using AIA/5, 1% (W/V) CMC and 1.5% agar. All the isolates were spot inoculated on the plates & incubated at 37^0C for 24 to 48 hours. All the plates were then exposed to 1% Congo red for 5 to 10 minutes, 1N HCL for 5 minutes and finally treated with 1N NaOH for 5 minutes respectively.

A clear zone around a Colony on a red background indicated positive cellulolytic activity of the isolate caused by the production of cellulase.

Gelatin hydrolysis test

Gelatin liquefaction was tested by spot inoculating the Actinobacteria on AIA plates containing 1% gelatin followed by incubation at 30 ± 0.1^0 C for 4-5 days. The plates were flooded with acidic mercuric chloride solution (15%), waited for 5-10 min, the excess solution was decanted off and appearance of a clear zone around the colonies was indicative of hydrolysis of gelatin by the enzyme gelatinase.

The clear zones depicted the activity levels of the organisms. Otherwise, unhydrolyzed and continuous opaque zone around the Actinobacterial growth i.e. white opaque precipitate was taken as no gelatinase enzyme production.

Casein hydrolysis test

Casein hydrolyzing activity of the bacteria was recorded from liquefaction of casein by the bacteria on AIA plates containing 1% casein, spot inoculated and incubated at 30 ± 0.1^0C for 96 h. The plates were flooded with acidic $HgCl_2$ (15%) or 1% tannic acid, excess solution was decanted off and clear zone formation was observed.

Pectin hydrolysis test

The ability of the microorganisms to hydrolyze pectin into pectic acid was assessed by the test. AIA plates containing 1% pectin was spotted with the bacteria, incubated at 30 ± 0.1^0C for 96 h. The plates were flooded with 1% hexadecyltrimethyl ammonium bromide i.e. cetyl trimethyl ammonium bromide (CTAB) which precipitates pectin and a clear zone around the colony will be formed. The ratio of clear zone to the growth of bacteria gave the activity levels of the organism.

Chitin hydrolysis test

Chitin hydrolysis ability of the microorganisms to glucosamine was assessed by the test. Chitin at 1% level was added to AIA medium; organisms were spotted and incubated at 30 ± 0.1^0C for 96 h. A clear zone formation was observed after the incubation period.

DNase test

DNase agar plates were prepared on which all the isolates were spot inoculated and incubated at 30°C for 96 hours. A faint color with clear zone around a CFU on an opaque background indicates production of DNase.

Extraction of crude lecithinase

Standardization of production media

For extraction of extracellular Lecithinase, strain Actinomycetes Ш were taken. For subculture brain heart infusion broth was used. For production of the lecithinase, Actinomycetes were grown on trypticase glucose yeast (TGY) broth prepared as follows.

This broth is supplemented with different

micronutrients to check the effect of these nutrients on the production media. The different micronutrients taken were 0.1mM $ZnSO_4$, 0.1mM $CuSO_4$, 0.1mM $NiSO_4$ and 0.1mM $FeCl_3$ were taken. The pH was adjusted to 7.5 and culture broth was autoclaved at 115^0C for 20 minutes.

Sterile glucose was then added to a final concentration of 1% before inoculation. With these production media egg yolk broth and nutrient broth were also taken to check the better production in these media. In all these media the Actinomycetes culture were added and incubated for 7 days at $30^0 \pm 1^0$C.

Optimization of pH and days of incubation for the extraction of

Lecithinase

The production media was prepared by taking TGY broth and it was supplemented with 0.1 mM $ZnSO_4$ and 20 ml of each media was transferred to six 50 ml conical flask respectively and the pH of these six conical flash was set as 5 to 11 respectively and then the six conical flask were sterilized and Actinomycetes were inoculated and incubated at 30^0 C in a shaker incubator.

After the third day onwards from the culture media, 2ml media from each conical flask were taken and centrifuged and the supernatant which contains the crude enzyme were taken and 100µl of supernatant was poured to the wells of already prepared egg yolk media plates and the plates were incubated at 30^0C for 24 hours and the zone formation by the crude enzyme was measured. The same process was repeated for 4th day to 9th day

Optimization of temperature for production of lecithinase

The production media was prepared in six conical flasks by taking TGY broth supplemented with 0.1mM $ZnSO_4$ and sterilized. Isolate AC-III was inoculated and incubated at different temperature from 20^0C to 45^0C.

Haemolysis

Haemolysis of the RBC by the isolates was done by streaking the isolates viz. (AC I-V) on sheep blood agar plates. Sheep blood agar plates were prepared and isolates were streaked. The plates were incubated for seven days. Clear and opaque zone indicates positive for haemolysis. When alpha haemolysis is present, the agar under the colony is dark and greenish.

This is otherwise called as incomplete haemolysis. Beta haemolysis sometimes called complete haemolysis causes a complete haemolysis of red cell in the colonies. The area appears lighten (yellow) and transparent. If a microorganism does not induce haemolysis the agar under and around the colony is unchanged, and the microorganism is called non-haemolytic or said to display gamma haemolysis.

Haemolytic assay of crude extract

Collection of blood sample

The blood samples were collected from fowl, sheep and human. The blood were drawn from fowl's wing vein, sheep's juggler vein, human's hepatic vein and transferred into test tubes containing 2ml of PBS containing 1% of EDTA.

Preparation of RBC solution for haemolytic assay

The different blood samples were centrifuged at 3000 rpm in 4^0C. After centrifugation the supernatant were discarded and washed with PBS. The

process was continued for three times with intermittent centrifuge then the supernatant was discarded. The pellets contained washed RBC. 100 ml of 0.15M NaCl and 100 ml of 1M $CaCl_2$ were prepared and both were mixed in equal proportion to make the buffer. 2% of the washed RBC was poured to 10 ml of buffer and mixed well to prepare the RBC solution.

Clean micro titre plate was taken, and in 12 wells of the micro titre plate 100 µl of buffer was taken, in first well containing the buffer crude enzyme was poured and serial two fold dilution was done up to last well containing buffer then from last well 100µl of the solution was discarded.

On to each well 100 µl of prepared RBC solution was added into the wells containing buffer and different dilution of crude enzyme solution. The micro titre plates were incubated for 18 hours at 37^0C after incubation the result was observed. Appropriate controls were taken along with test samples.

Results and Discussion

Actinomycetes constitute a major group in soil micro flora and the isolates from the sediment of Chilika Lake were characterized for its various proportion. Observations were also statistically analysed.

Colony characters of the isolates on Actinomycetes isolation agar

The colony characteristics of the cultures *viz*. AC-*I* to AC-*IV* were white and off white in colour.AC-II and AC-III colonies were spherical and bulging whereas AC-IV were small whitish colour with irregularity in their structure. AC-*V* was creamy, oval in shape. All were gummy, attached to the agar plate after an incubation period of 5 days (Table 1).

Staining characters of the isolates

Gram staining and acid fast staining characters were studied. All of the Actinomycetes were gram positive, non-acid fast. Among the isolates, AC-*I*, AC-*II*, AC-*III*, AC-*IV* are filamentous and AC-*V* is rod shaped (Table 2).

Tolerance capacity of isolates to different physiological conditions (NaCl, pH, Temperature)

All the isolates were found to grow well at pH range between 7.0to11.0.and from pH 12 onwards they showed declined growth, after pH 12 no growth was observed (Table 3). Growths of the organisms were reported between the temperature ranges of 10^0 C to 40^0 C. Some are inhibiting in 45^0 C and all are inhibiting in 50^0 C (Table 4). The isolates tolerated up to 5-10% 0f salt concentration and they showed inhibition at 15% salt concentration (Table 5).

Biochemical properties of the isolates

The isolates were further selected for biochemical characterizations. The main objective of any microbial classification system is to identify at Genus level, which is the basic unit of the taxonomic grouping. The biochemical properties of the cultures are presented in tables 6. And a few representative test results are given in all of the organisms were urease positive, indole negative, VP negative. The isolates shown negative for MR except AC-*I*, and positive for nitrate except AC-*II*. All the isolates are positive for aesculin hydrolysis. Other results were variable.

Fermentation of Organic Carbon Shoreces

Fermentation reactions of different isolates were checked by taking 12 different sugars.

Among all the isolates AC-*I* have fermented all the 12 sugars. Other results were variable (Table 7).

Identification of the isolates

Comprehensive results of biochemical characters of the isolates (AC-I to AC-V) for identification up to species levels are presented (Table 8). The isolates were identified as *Streptomyces* spp, *Actinomycetes* spp, *Nocardia* spp, and *Rhodococcus group*.

Enzymatic activity of different isolates

Enzymes have been isolated and purified from microorganisms, animals and plants; among them microorganisms represent the most common shorece of enzymes because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. The isolates were tested for their ability to produce extracellular enzymes and those capable of producing the enzymes are assigned a "+ve" and those do not possessing enzymatic activity are assigned a "-ve".

Starch hydrolysis

Amylase activities of the isolates were checked by inoculating the isolates in NA plates containing 1% starch. None of the isolates showed positive result (Table 9).

Cellulase activity

Among the five isolates screened for the cellulose activity all the isolates have showed a good cellulase activity (Table 9).

Caseinase activity

Caseinase activity of the isolates was checked and it was found that all the isolates except AC-V showed positive result (Table 9).

Gelatinase activity

All the five isolates were screened for the Gelatinase activity. Among the five isolates AC-I, AC-II and AC-III showed clear zone on the tween-20 plates. It reveals that these three isolates are good gelatinase producer (Table 9).

Pectinase activity

Pectinase activity of the isolates was checked. None of the isolates were pectinase positive (Table 9).

Lipase activity

Primary screening of lipase activity of the isolates were also checked in the lipase plates and it was found AC-III and AC-V showed lipase activity (Table 9).

Chitinase activity

The chitin hydrolysis of the isolates was checked on chitin containing media plates and it was found that no isolates were positive (Table 9).

Lecithinase activity

Lecithinase activity of the isolates was checked by streaking the isolates on egg yolk media plates. Among five isolates three isolates (AC-I, AC-II, AC-III) have showed lecithinase activity. As compared to all other

Enzyme at different pH	Zone diameter
5	10mm
5.5	10mm
6	12mm
6.5	13mm
7	15mm
7.5	17mm
8	14mm
9	13mm

enzymes the lecithinase activity was maximum in case of AC-III (Table 9).

Catalase activity

Catalase activities of the isolates were checked by checking the effervescence on the plates by applying H_2O_2. It was found that all the isolates except AC-V are positive (Table 9).

Antagonism against some human pathogens

The isolates were showing antagonism against some human pathogen. AC-I showed antagonism against seven pathogens which was highest as compared to all other isolates. Antagonistic activity of AC-II against four, AC-III against three, AC-IV against two and AC-V against one pathogen was observed respectively (Table 10).

Enzyme production

The production of enzyme was checked in TGY media supplemented with 0.1mM $ZnSO_4$/ 0.1mM $CuSO_4$/ 0.1mM $NiSO_4$ or 0.1mM $FeCl_3$ respectively and also checked in simple NB and egg yolk broth. It was found that the enzyme production was more in case of TGY medium supplemented with 0.1mM $ZnSO_4$ with showing a zone size of 17mm on the seventh day. The zone size of other crude extract in egg yolk media plates were considerably low on different days (Table 11).

Optimization of pH for lecithinase enzyme production

The lecithinase activity was measured using different pH such as 5, 5.5, 6, 6.5, 7, 7.5, 8, 9.here different pH of the fermentation medium was adjusted. The results are shown in the graph below.

Optimization of temperature for lecithinase enzyme

The lecithinase activity was measured using different temperature such as 20^0, 25^0, 30^0, 35^0, 40^0C respectively. The results are shown in the graph.

Enzyme at diff. temperature	Zone diameter
20^0C	10mm
25^0C	13mm
30^0C	18mm
35^0C	16mm
40^0C	13mm

The haemolytic capacity of the isolates were checked by streaking all the isolates in blood agar plates and all the isolates shown clear and opaque zone indicating all the isolates are gamma haemolytic. They have shown haemolysis against sheep blood.

Haemolytic assay

The haemolytic assay of the crude enzyme from isolate-III- were done and it was found that the crude enzyme was not lethal against human and sheep RBCs but it was lethal against fowl RBCs. The haemolysis titre was found to be 1:64. Microbes, though invisible constitute a major part of living form in the Earth. Potentialities of microbes have been utilised for social benefits since time immemorial and till date there is exploration & application of these diverse group of organism for benefit of human civilization. Actinomycetes, which constitute one of the dominant groups of microbes, have been of immense benefit to human civilization, mostly through production of antibiotics, various enzymes active metabolites for antagonistic activity against plant & human pathogens, etc.

Table.1 Colony characters of the isolates

Isolate No.	Colour	Form
AC-*I*	White	Oval Sticky colonies, and are filamentous
AC-*II*	Off white	Bulgy sticky colonies and filamentous
AC-*III*	Off white	Spherical and bulging colonies, filamentous
AC-*IV*	white	Small white colonies with irregular shape
AC-*V*	Creamy colour	Small oval colonies

Table.2 Gram's staining and acid fast staining

Isolate No.	Staining	
	Gram's stain	Acid fast stain
AC I	+	-
AC II	+	-
AC III	+	-
AC IV	+	-
AC V	+	-

Table.3 Effect of pH on growth of the isolates

Isolate No.	pH 3.0	pH 5.0	pH 7.0	pH 9.0	pH 12.0
AC I	-	-	+	+	-
AC II	-	-	+	+	-
AC III	-	-	+	+	-
AC IV	-	-	+	+	-
AC V	-	-	+	+	-

Table.4 Effect of temperature on growth of the isolates

		Growth at different temperature					
	4°C	10°C	15°C	25°C	35°C	45°C	50°C
AC I	-	+	+	+	+	+	-
AC II	-	+	+	+	+	-	-
AC III	-	+	+	+	+	+	-
AC IV	-	+	+	+	+	-	-
AC V	-	+	+	+	+	+	-

Table.5 Tolerance to NaCl Concentration by the isolates

	Growth at different salt concentration				
	5%	7%	9%	12%	15%
AC I	+	+	+	+	-
AC II	+	+	+	-	-
AC III	+	+	+	-	-
AC IV	+	+	+	+	-
AC V	+	+	_	_	_

Table.6 Biochemical properties of the isolates

Test	Isolate No.				
	AC-I	AC-II	AC-III	AC-IV	AC-V
Indole	-	-	-	-	-
M.R Test	+	-	-	-	-
V.P Test	-	-	-	-	-
Nitrate Reduction	+	-	+	+	+
Urease Production	+	+	+	+	+
Citrate Utilisation	+	-	+	-	-
Oxidase	-	+	-	-	-
Arginine Dehydrlase	-	+	+	-	+
Ornithin	+	-	+	-	-
Dehydrolase					
Aesculin Hydrolysis	+	+	+	+	+
Catalase	+	+	+	+	-

Table.7 Fermentation of organic carbon shoreces by the isolates

Isolate No.	Lactose		Dulcitol		Inocitol		Sucrose		Xylose		Ramnose		Inosine		Cellobiose		Mellibiose		Maltose		Sorbitol		Arabinose	
	F	O	F	O	F	O	F	O	F	O	F	O	F	O	F	O	F	O	F	O	F	O	F	O
AC-I	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	+	+	+
AC-II	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+	+	-	+	-	-	+	-	+
AC-III	-	+	+	-	-	-	+	-	+	-	+	-	-	-	+	-	-	-	+	-	-		-	-
AC-IV	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-		-		-	+
AC-V	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-		-		-	-

Table.8 Identification of the isolates based on biochemical characterization

Isolate Name	Identified organism
AC-I	***Streptomyces* sp.**
AC-II	***Actinomycetes* sp.**
AC-III	***Actinomycetes* sp.**
AC-IV	***Nocardia* sp.**
AC-V	***Rhodococcus* sp.**

Table.9 Enzymatic activity of the isolates

	Isolates showing enzymatic activity				
	AC-I	AC-II	AC-III	AC-IV	AC-V
Amylase	-	-	-	-	
Cellulase	+	+	+	+	-
Caseinase	+	+	+	+	+
Gelatinase	+	+	+	-	-
Pectinase	-	-	-	-	-
Lipase	-	-	+	-	-
Chitinase	-	-	-	-	+
Lecithinase	+	+	+	-	-
DNase	-	-	+	+	-
Catalase	+	+	+	+	-
Oxidase	-	+	-	-	-

Table.10 Antagonism against some human pathogens (Fungal & Bacterial)

Isolate No.	Dermatophytes			Bacterial Pathogen							
	Candida albicans	*Tricophyton mwntagrophytes*	*Epidermophyton flocussum*	*Candida tropicalis*	*E.coli EPEC*	*E.coli ETEC*	*Salmonella*	*Aeromonas*	*Shigella*	*Streptococcus epidermidis*	*E.coli*
AC-I	+	+	-	-	+	+	+	-	-	+	+
AC-II	-	+	-	-	+	+	-	+	-	-	+
AC-III	-	-	-	-	+	-	-	-	-	+	+
AC-IV	-	-	-	-	+	-	-	-	-	-	-
AC-V	-	+	-	-	-	-	-	-	-	-	-

Table.11 Enzyme production in different media on different days

		Zone of inhibition by enzyme of different media				
incubation	TGY with $ZnSO_4$	TGY with $CuSO_4$	TGY With` $NiSO_4$	TGY with $FeCl_3$	Egg yolk	NB
D3	6	5	5	6	5	5
D_4	7	8	6	7	6	5
D5	13	12	10	11	8	8
D_6	17	14	12	16	11	10
D_7	17	15	14	16	12	10
D_8	16	13	12	15	11	9
D_9	12	12	11	11	9	9

But even today, microbial diversity in general and Actinomycete diversity in particular with their potential effect remains a scope for scientists to explore. Chilika, the largest lake of India harbours diverse group of flora, fauna and microbes including Actinomycetes. Actinomycetes of Chilika origin and their potential have neither been explored nor have been exploited. In an attempt to study, five Actinomycetes were isolated from sediments of Chilika Lake grown in Actinomycetes isolation agar. Reichardt *et al.* (2001) opined that for viable count (CFU) method is most acceptable for determination of Actinomycetes population in the soil. CFU method reveals only1-5% of Actinomycetes in soil. Thus viable count method was adopted for assessment of Actinomycetal population in the sediment of Chilika. Five different types of Actinomycetes (AC-I to AC-V) were found in the sediment which were characterized and studied for their potential applications. By using morphological characteristics and simple staining reaction (Eikelboom, 1975) distinguished 27 bacterial samples. This identification key has some general limitations. Morphology of the isolates varies depending upon the collection site. All the isolates were subjected to Gram's stain and acid fast stain distinguished accordingly. All the isolates were positive to Gram's reaction and were non Acid fast.The isolates were found to be AC-I as *Streptomyces* sp, AC-II as *Actinomyces* sp., similarly AC-III *Actinomyces* sp., AC-IV *Nocardia* sp. and AC-V *Rhodococcus* group. The antagonistic activities of the isolated Actinomycetes from the soil sediments of Chilika Lake against some human pathogens were studied. Actinobacteria from marine ecology are showing antagonistic effect against several human, plant and fish pathogens (Sivakumar *et al*., 2006). Isolates from marine ecosystem exhibited prominent activity against human pathogens, the most potential being a *Streptomyces* sp.

According to Saurav *et al.* (2010) the most potential Actinomycetes isolated was inhibitory to *Aspergillus niger*, *Aspergillus fumigatus* & *Candida albicans* and was identified as a *Streptomyces* sp. as reported in the present investigation it is observed that four isolates (except AC-V) are showing a good antagonistic effect against *Candida albicans, Tricophyton mentagrophytes, E. coli, Aeromonas Sp.* and *Staphylococcus epidermidis* showing a good inhibition zone to the dermatophytes. Hence it can be opined that AC-I, AC-II, AC-III & AC-IV can be explored as potential shorece of good inhibitors of skin disease causing dermatophytes.

Enzymes are one of the major secondary metabolites produced by different microorganisms. Enzymes act as a catalyst in different metabolic reactions in the microbes and simultaneously help in degrading complex, toxic and harmful substances in nature. Gulve *et al.* (2011) reported various enzymes such as pectinase, gelatinases, amylases, lecithinases, cellulases and ureases from the Actinomycetes strains. The study depicts four the isolates (AC-I, AC-II, AC-III & AC-IV) showed a positive result for the enzymes Cellulase and Caseinase. Whereas isolate AC-III showed the best result among all with a potential to produce Gelatinase, Lipase, Caseinase, Cellulose, Urease & Lecithinase.

Geoffroy *et al.* (1991) investigated the biological properties of the 29-kDa PLC. The exoenzyme was active at a pH range (6.0 to 7.0). It was weakly haemolytic for erythrocytes from various animal species, including guinea pig, horse, and human, but was not hemolytic for sheep erythrocytes. Interestingly, sheep erythrocytes are practically devoid of phosphatidylcholine,

whereas guinea pig, horse, and human erythrocytes contain 29 to 42% of this compound. In the present investigation, the crude lecithinase was extracted following the protocol of Geoffroy *et al.* (1991). The haemolytic assay of the crude extract was undertaken to check the lethal action of the enzymes on different blood (Sheep, Fowl & Human). The experimental observation show lecithinase is maximum lethal to Fowl and less lethal to sheep and human.

The enzymatic activity was strongly enhanced in culture supernatants when bacteria were grown in TGY broth supplemented with $ZnSO_4$ but was not stimulated in the presence of Fe^{3+}, Cu^{2+}, Co^{2+}, or Ni^{2+}. It is observed that by amendment of $ZnSO_4$ to the medium increased enzymatic activity of the isolates. This suggests that lecithinase produced by the Actinomycetes, isolated from the Chilika Lake is also Zinc dependent. It is evident from the above finding that the Actinomycetes isolated from the sediments of Chilika lake have the potential to produce antibacterial compound and can be used as shorece of various enzyme such as Lecithinase for industrial application. The present investigation was undertaken to explore the potential Actinomycetes from the sediments of Chilika Lake followed by their bioactive potential. Five strains were isolated from the sediments. The isolate AC-I (*Streptomyces* sp.) showed a good antagonistic effect against Dermatophytes whereas AC-III is a good enzyme producer. The study reveals AC-III is a good producer of Lecithinase enzyme which is a haemolytic agent. The haemolysis titre reveals that the enzyme produced by AC-III (*Actinomycetes* sp.) is not lethal to the human & Sheep but is lethal to the fowl. So it can conclude that isolate AC-III can be used as a shorece of antibiotics and can be used as a potential enzyme producer even for industrial use.

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