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Screening and Molecular Characterization of Actinomycetes from Mangrove Soil Producing Industrially Important Enzymes

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Abstract: **This study describes the isolation of actinomycetes, screening for their potential to produce industrially important enzymes and their molecular characterization. Twenty-five actinomycete strains were isolated from the soil samples of Pichavaram mangroves and were morphologically characterized. The isolates were screened for enzymes: L-asparaginase, lipase cellulase, amylase and protease. Five *Streptomyces* sp. showed a positive activity for all the enzymes studied. The isolates were further screened by quantitative method. The five *Streptomyces* spp. were further identified by conventional and molecular methods. Based on the molecular characterization, the novel isolates were identified as *Streptomyces* sp. LCJ10A (Accession no. KU 860466), *Streptomyces* sp. LCJ11A (Accession no. KU921107), *Streptomyces* sp. LCJ13A (Accession no. KU921109), *Streptomyces* sp. LCJ14A (Accession no. KU921110) and *Streptomyces* sp. LCJ16A (Accession no. KU 921112). These cultures showed good enzyme activity under submerged fermentation and therefore, they can be used in developing a low cost technology for industrial applications**

Index Terms: **Actinomycetes, Characterization, Pichavaram mangroves, *Streptomyces* sp and Submerged fermentation.**

I. Introduction

Actinomycetes are Gram positive bacteria with nearly 55% Guanine + Cytosine content in their DNA and are universally present in both terrestrial and aquatic environment (Chaudhary *et al.,* 2013). A substantial number of actinomycetes were isolated, described, and studied for the production of economically significant compounds. This group of microorganisms has additionally been identified to be a good source of numerous proteins, chemical inhibitors, immunomodifiers, vitamins besides antibiotics (Crawford *et al.,* 1993; Goodfellow *et al.,* 1988). *Streptomyces* belonging to the actinomycetes group are aerobic, filamentous and spore forming bacteria (Kim and Goodfellow, 2002; Kuster, 1968). Screening and isolation of microorganisms producing secondary metabolites and enzymes have been the main focus for several years (Ouhdouch *et al.,* 2001). *Streptomycetes* produce a wide range of bioactive metabolites along with different enzymes which have biotechnological applications (Saini *et al.,* 2015). Besides antibiotic production, actinomycetes have a diverse role in the mangrove habitat (Das *et al.,* 2006). In addition to other enzymes like aminotransferase, keratinase, chitinase, lipase, phosphoesterase, amylase, inulinase, collagenase, xylanase, hyalurinase and cellulase, actinomycetes have been studied for the production of a various types of heat tolerant, alkaline and neutral proteases (Ramesh *et al.,* 2009). Actinomycetes from mangrove habitats are an excellent source of enzymes with superior properties like heat tolerant and stability in alkaline environments (Shanmughapriya *et al.,* 2008). Recently, studies on enzyme production by mangrove actinomycetes have gained importance (Usha *et al.,* 2011). The mangrove habitat remains to be a promising source for novel actinomycetes, which are capable of producing unique, industrially vital enzymes. However, there are some reports available on enzyme production by mangrove actinomycetes. It is thus necessary to recognize novel microbes producing diverse extracellular enzymes that play a major role in different industrial applications. Hence the present study was mainly focused on isolation and screening of diverse mangrove actinomycetes for enzyme production.

II. Materials and Methods

A. Sample Collection

Soil samples were aseptically collected from various places of mangrove forest of Pichavaram, Chidambaram, Tamil

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Nadu, India (Latitude 11.43045 and Longitude 79.79294). Soil samples were taken in small plastic containers and transferred to research laboratory. At room temperature the soil samples were shade dried and pulverized well utilizing mortar and pestle. Then, the powdered samples were filtered using 30 µm pore size mesh to remove debris. The fine powdery soil was used for isolating actinomycetes.

B. Isolation and maintenance of actinomycetes

Serial dilution technique was used to isolate actinomycetes from the soil samples using three different sterilized ISP-2 medium, Actinomycetes isolation agar medium and Starch casein agar medium plates (Balachandran *et al.,* 2012). To reduce the microbial contamination, Nystatin (50 µg/ml), Actedione (50 µg/ml), and Nalidixic acid (25 µg/ml) were added. The plates were incubated at 30°C for 14 days and periodically observed for the growth of actinomycetes. The pure colonies were isolated and preserved on ISP-2 slants.

C. Physical and chemical pre-treatment methods

Various physical and chemical pre-treatment procedures were followed to eliminate redundant filamentous bacteria and to increase the count of unique and novel *Streptomyces* and actinomycetes genera. The pre-treatment methods followed were; Calcium Carbonate with soil treatment, centrifugation, dry heat, wet heat, SDS treatment and phenol treatment (Janaki *et al.,* 2014). All the pre-treatment methods were studied on Humic acid Vitamin Agar medium (HVA) to support the growth of rare actinomycetes.

D. Characterization and identification of the actinomycete isolates

After isolation of pure colonies, the actinomycetes were recognized based on their earthy smell and colour of their hyphae. The strains were further identified as actinomycetes depending on the colour of aerial and substrate mycelium, branching, nature of colony and texture. Actinomycete colonies were morphologically characterized using directions provided by Bergey's Systematic Bacteriology Manual and International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966; Locci 1989). Morphological characterizations were done by cover slip culture method (Buchanan and Gibbons, 1974; Gulve and Deshmukh, 2012). In ISP-2 medium, the isolates were grown for 7-14 days at room temperature for studying cultural characteristics. The colour of the mycelium, arrangement of spores and mycelium structure were observed through the oil immersion (100X). All the isolates were recognized to species level by relating spore bearing hyphae morphology and spore arrangement as elucidated in Bergey's Manual. Gram staining method was followed to observe the nature of the isolates (Prazeres *et al.*, 2006).

III. Qualitative and quantitative screening for different enzymes

A. Primary screening for lipase production

For the detection of lipase in actinomycete strains, Tributyrin agar clearing method was followed. On Tributyrin agar medium plates, the strains were grown for five days at 28°C. Lipolytic activity was observed as a zone showing a positive result. The zone was measured and the strains were further quantitatively screened by submerged fermentation (Veerapagu *et al.*, 2013).

B. Quantitative screening for lipase enzyme

The actinomycete strains were quantitatively screened for lipase production using the method of Nawani *et al.,* (2006), where p-nitrophenyl palmitate (p-NPP) was used as substrate. The yellow colored product was formed and absorbance read at 410 nm and lipase activity for all the isolates was studied.

C. Primary screening for amylase production

The actinomycete strains were studied for amylase production by starch hydrolysis test (Aneja, 2003). The strains were cultured on the starch agar plate for 48 hours at 37°C. Iodine reagent was inundated for 30 seconds on the plates after incubation. A strong hydrolysis zone surrounding the colonies shows that isolates produced amylase enzyme. The positive strains were further quantitatively screened.

D. Quantitative screening for amylase enzyme

The strains were quantitatively screened for amylase enzyme activity by Dinitrosalicylic acid (DNS) process (Miller, 1959). 0.2 ml of enzyme solution mixed with 1 ml starch substrate, was made up to 3 ml distilled water, and incubated for 20 minutes at 37°C. 1 ml of DNS reagent was added to the mixture to terminate the reaction and at 540 nm the absorbance was noted. The amount of amylase released from starch at 37°C by 1 µmol of maltose per minute at pH 7.0 corresponds to 1 unit (IU) amylase.

E. Qualitative screening for L-Asparaginase production

Actinomycete isolates were tested for L-asparaginase production using Gulati *et al.,*(1997) method. The isolates were grown on L-asparagine-glucose agar medium and incubated for 4 days. Pink color replaced yellow color media in isolates showing positive results and the colonies formed clear pink zones.

F. Quantitative screening for L-Asparaginase enzyme

For measuring L-Asparaginase activity, Imada *et al.* (1973) method was followed. 0.5 ml of 0.04 M L-asparagine solution was mixed with 0.5 ml of crude enzyme. To this mixture 0.5 ml of 0.05M Tris-HCl buffer (pH-7.2) was added followed by incubation for 10 minutes at 37°C. To terminate the reaction, 0.5 ml of 1.5 M Trichloro acetic acid (TCA) was added and subsequently 0.2 ml Nessler's reagent was added to know the

amount of ammonia released, by reading absorbance at 480 nm. The quantity of enzyme that catalyzed the development of 1µmol ammonia at 37°C per min gives one unit of asparaginase.

G. Primary screening for protease production

Pure isolates of actinomycetes were cultured on skim milk agar medium, for 4 days at 28°C. An opaque hydrolysis zone was observed in isolates which showed a positive result for protease enzyme (Hameş Kocabaş and Uzel, 2007).

H. Quantitative screening for protease enzyme

Depending on primary screening results, the potential isolates were selected for the production of protease. The activity of protease was measured according to Kaey and Wildi, (1970) method, using casein as substrate. To 50 ml of Protease Production Broth, 1 ml of the spore suspension of the isolate strains was supplemented and incubated at 30°C at 120 rpm, under shaking conditions. Harvesting of the culture was done at every two days interval. In order to remove medium debris and mycelia, the culture medium was centrifuged. The absorbance of the supernatant was noted at the wavelength of 660 nm after incubating the blend at room temperature for 30 minutes (Malathi and Chakraborty, 1991). The enzyme inactivated by TCA was used as the control. The amount of enzyme liberating 1µg of tyrosine per ml for 30 minutes at 37°C corresponds to one unit of protease activity (U) (Jayashree *et al.*, 2014). Lowry's method was used in estimating protein concentration where Bovine serum albumin (BSA) was the standard (1951). Protein content was measured in units/mg upon calculating specific activity of protease.

I. Primary screening for cellulase production

The isolates were quantitatively tested for cellulase production by means of 1% Carboxymethyl cellulose in basal salt medium using plate assay procedure, according to Hankin *et al.*, method (1977). Adding 0.1% Congo red reagent and counterstaining with 1 M NaCl for 20 minutes, resulted in cellulose hydrolysis that formed opaque zone surrounding the colony.

J. Quantitative screening for Cellulase enzyme

Standard method was used to determine total cellulase activity (Filter paper cellulase and Carboxymethyl cellulase). Cellulase assay was studied by measuring the quantity of reducing sugar from carboxymethyl cellulose (CMC). The activity of Carboxymethyl cellulase was calculated by adding 0.5 ml crude enzyme supernatant in 0.05 M sodium citrate buffer (pH 4.8) with 0.5 ml of 2% carboxymethyl cellulose. The blend was incubated at 55°C for 30 mins. Further to calculate filter cellulase activity, 1 ml of 0.05 M Sodium Citrate buffer (pH 4.8) containing Whatman no.1 filter paper (50 mg) was incubated with 0.5 ml of crude enzyme supernatant, for one hour at 55°C. For terminating the reaction, 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent was added to 1 ml of the mixture. Spectrophotometrically, using 3, 5-dinitrosalicylic acid with glucose as standard, reducing sugars were evaluated (Ghose, 1987). 1 µmol of reducing sugars (measured as glucose) released from the enzyme per ml per minute is considered as one unit of enzymatic activity.

IV. Molecular characterization using 16S rRNA sequencing

The cultures showing high enzyme activity were characterized using molecular techniques. Isolation of genomic DNA was carried out by Nucleospin tissue kit method (Machery-Nagel, Duren, Germany). The purity of the DNA was calculated at 260/280 nm and was later used for Polymerase chain reaction (PCR). Taq DNA Polymerase using 27F (5'AGAGTTTGAT CCTGGCTCAG3') and 14R (5'GGTTACCTTGTTACGACTT3') primers was used for amplifying 16S rRNA. PCR thermal cycler was used for PCR amplification. Sequencing was carried out at Rajiv Gandhi Centre for Biotechnology, Trivandrum. The sequence quality was checked using Sequence Scanner Software 1.0 (Applied Biosystems). Sequence alignment and necessary editing of the sequences obtained were performed using Geneious Pro v5.6 (Drummond *et al.*, 2012).

V. PHYLOGENETIC ANALYSIS

The nearest actinomycetes with similar sequence were evaluated with GenBank database using BLAST with our sequence as the query sequence. The similarity of the isolates was determined through the nucleotide BLAST search. MUSCLE software (Multiple Sequence Comparison by Log-Expectation) was used to align the sequences and phylogenetic analysis performed by MEGA (Molecular Evolutionary Genetics Analysis) software 6.0. The phylogeny was structured with MEGA 6.0 software, by neighbor tree joining method (Tamura *et al*., 2013).

VI. RESULTS AND DISCUSSION

A. Isolation

Twenty five different novel actinomycetes were isolated from the soil samples of Pichavaram mangrove forest. Among the 3 different media studied for isolation, ISP-2 medium yielded maximum number of actinomycete colonies followed by Starch casein agar medium and Actinomycetes isolation agar medium. Pre-treatment methods studied on Humic acid Vitamin agar medium (HVA) facilitated in obtaining rare *Streptomyces* sp.

B. Characterization and identification of the isolates

1) Morphological characterization

Morphological characterization of the isolates is important to determine the nature of the isolates. Primary identification

of selected isolates LCJ10A, LCJ11A, LCJ13A, LCJ14A and LCJ16A was performed based on morphological characterization using spore chain arrangements and colour of aerial and substrate mycelia (Table I). The colony morphology of the isolates was distinct when observed on ISP-2 medium where the colonies showed powdery consistency with flat edges. The spores were organized in chains and the spore morphology explained that aerial coiled mycelia were noticed in all the isolates. Spore chain arrangements were observed using microscope at 100X showing that the isolates formed a knob like structures. Gram staining of the strains indicated they were gram positive in nature. The results of qualitative screening for the isolates for the selected enzymes were recorded and shown in table II. All the isolates showed positive for the enzymes tested.

Table I. Morphological characteristics and Gram staining results of the isolates

Strain Number	**Colour of aerial mycelium**	**Colour of substrate mycelium**	**Spore chain morphology**	**Gram staining**
LCJ10A	Grey	Light brown	Spiral	+
LCJ11A	White	Pale yellow	Flexibilis	+
LCJ13A	White	brown	Spiral	+
LCJ14A	Grey	Pale yellow	spiral	+
LCJ16A	Dark grey	grey	spiral	**+**

Table II. Qualitative Screening results of isolates

Strain No	**Protease**	**Amylase**	**L-Asparaginase**	**Lipase**	**Cellulase**
LCJ10A	+++	+++	++	+++	++
LCJ11A	+++	+++	+++	+++	+++
LCJ13A	+++	++	++	+++	++
LCJ14A	+++	+++	+++	++	++
LCJ16A	++	+++	+++	++	++
+++ ; high activity, ++ ; moderate activity					

2) *Qualitative and quantitative screening for enzyme production*

Among 25 isolates tested, five isolates showed highest protease, amylase, L-asparaginase, cellulase and lipase activity in the primary screening using suitable agar medium. These five isolates were further identified for quantitative test, as they showed optimal hydrolyzed zones (Fig.1) and quantitatively screened. Extracellular enzyme production of selected isolates was monitored and measured during alternative days for 12 days. The isolates showed maximum enzyme production on 8th day which was considered as the peak day as represented in Table. III. Based on the qualitative and quantitative results, isolates were selected for molecular characterization.

(a)

(b)

Fig 1. Qualitative screening for protease, (a) & (b) Shows the hydrolytic zone due to proteolysis

C. Molecular characterization

Among the 25 isolates, LCJ10A, LCJ11A, LCJ13A, LCJ14A and LCJ16A exhibited good enzyme activity for various enzymes studied. Hence they were subjected to molecular identification. Genomic DNA was isolated and amplification of the genomic DNA in PCR was performed by the universal primers. DNA was sequenced using Sanger's dideoxy method using big dye terminator v3.1. The isolates sequences were matched using Basic Local Alignment Search Tool (BLAST) with those in the National Centre for Biotechnology Information (NCBI) nucleotide Sequence database. Subsequently the sequences were submitted in NCBI

using 16S rRNA submission tool through BankIt and accession numbers for all the isolates had been obtained.

Table III. Quantitative Screening of different enzyme activity by *Streptomyces* sp. on 8th day (U/mL)

Isolates	**Protease**	**Amylase**	**L-Asparaginase**	**Lipase**	**Cellulase**
LCJ10A	56.91±1.3	87.24±2.1	78.91±1.9	56.11±1.5	67.22±1.8
LCJ11A	73.32±1.5	83.44±2.1	62.36±1.9	63.77±1.9	69.45±1.4
LCJ13A	72.41±1.5	58.29±1.8	67.19±2.1	56.55±1.9	64.94±1.7
LCJ14A	56.91±2.3	87.24±2.6	48.91±1.6	29.62±2.3	54.38±1.4
LCJ16A	81.27±2.1	63.48±1.9	55.85±1.7	69.42±1.8	52.27±1.7
Values are mean ± standard deviation					

D. Phylogenetic analysis

MEGA 6.0. software was used in making the phylogenetic tree. LCJ10A phylogeny shows a 99% sequence similarity with *Streptomyces ginglanensis*, when matched with the genetically similar sequences in NCBI database. LCJ11A phylogeny shows a similarity with *Streptomyces flocculus* with 98% bootstrap support. LCJ13A phylogeny showed a similarity with *Streptomyces ginglanensis* with 99% bootstrap support. LCJ14A phylogeny showed a similarity with *Streptomyces smyrnaeus* 99% bootstrap support. LCJ16A phylogeny showed a similarity with *Streptomyces smyrnaeus* with 99% bootstrap support. (Fig 2-6).

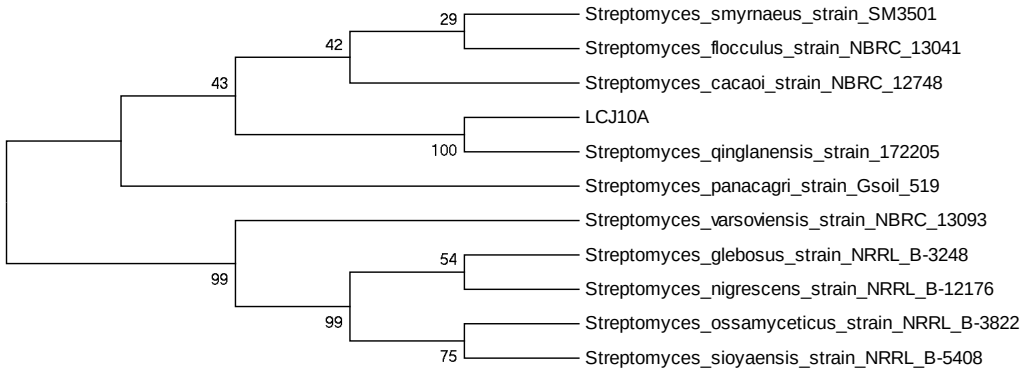

Fig 2**.** Neighbor-Joining tree of the ITS region of the strain LCJ10A constructed with higher similarity sequences from the Genbank selected from the results of a BLAST search using MEGA 6.0 software.

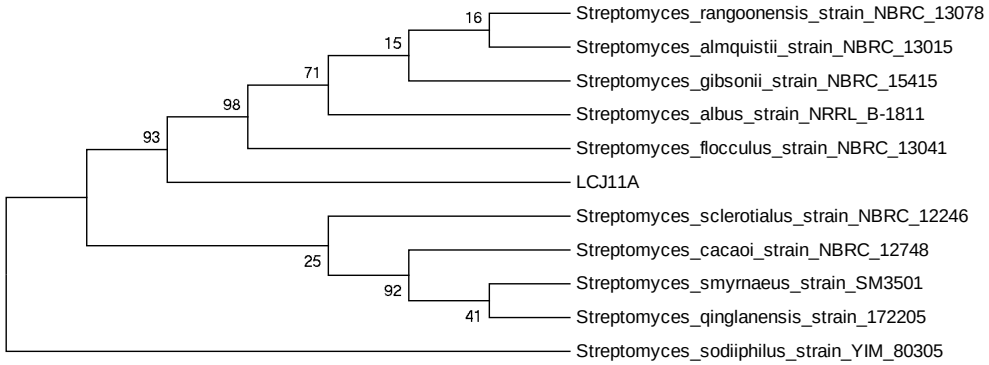

Fig 3**.** Neighbor-Joining tree of the ITS region of the strain LCJ11A constructed with higher similarity sequences from the Genbank selected from the results of a BLAST search using MEGA 6.0 software.

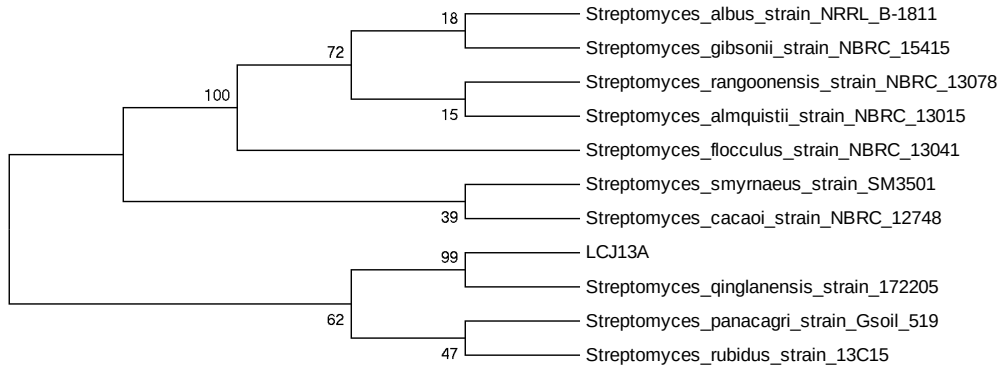

Fig 4. Neighbor-Joining tree of the ITS region of the strain LCJ13A constructed with higher similarity sequences from the Genbank selected from the results of a BLAST search using MEGA 6.0 software

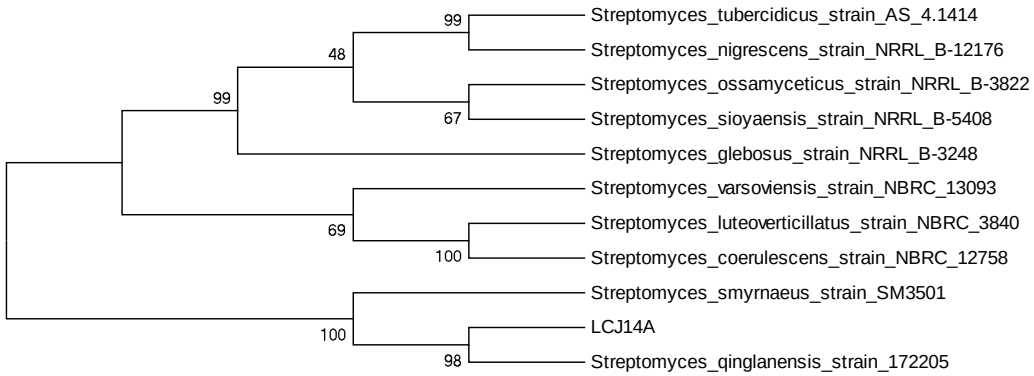

Fig 5. Neighbor-Joining tree of the ITS region of the strain LCJ14A constructed with higher similarity sequences from the Genbank selected from the results of a BLAST search using MEGA 6.0 software

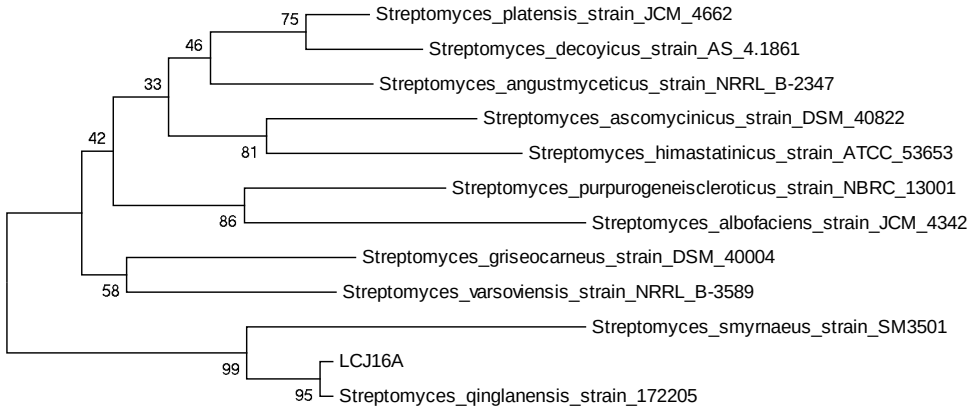

Fig 6. Neighbor-Joining tree of the ITS region of the strain LCJ16A constructed with higher similarity sequences from the Genbank selected from the results of a BLAST search using MEGA 6.0 software

The best and most abundant source of economically valuable microbes is the nature and this microbial variety can be utilized for acquiring the organism of interest (Sahoo and Dhal, 2009). Actinomycetes are the most treasured prokaryote, biotechnologically and economically (Lam, 2006). Considerably, a great deal of metabolic variety and biotechnological uses have been identified in mangrove microbes (Hamedi *et al.*, 2013). In the present situation, the interest for industrial enzymes for specific procedures like paper, food industry, textile, fuel ethyl alcohol production and in processing the feed, and also for certain purposes like, using enzymes in the preparation of detergents, is significantly a lot extra than the production (Sarethy *et al.*, 2011). Extracellular enzymes have gained increased consideration due to their large

scale applications in various industries (Sharma 2014). There is a remarkable diversity and uniqueness among the mangrove actinomycetes. There are few studies in the literature which aimed at isolating new actinomycetes from the samples of various mangrove habitats and environments, as they are the rich source of rare actinomycetes (Chavan Dilip *et al.*, 2013). Currently, there have been many research findings associated with mangrove microbes, as they are proved to be stable and active than enzymes from animals or plant source. Earlier studies by Deepthi *et al.*, (2012) explained that a *Streptomyces* sp., isolated from Coringa mangroves was capable of producing industrially important extracellular enzymes. This study also showed that there are many rich extracellular enzyme producing actinomycetes available in less explored environments. Naikpatil *et al.*, (2011) explained about the effect of pre-treatment of soil from mangrove sediments of Karwar which helped in isolating rare actinomycetes. It also revealed that uncommon *Streptomyces* species are efficient enzyme producers. Therefore various unexplored habitats and less explored environments are the great source of actinobacteria that produce industrially useful enzymes which have a great commercial significance.

CONCLUSION

This study mainly focused on the isolation of actinomycetes from soil samples of Pichavaram mangroves and screening them for different enzymes. Out of the 25 isolates, 5 were positive for all the enzymes studied which explains that besides antibiotic purposes actinomycetes can also be extensively studied for obtaining industrially vital enzymes like protease, amylase, lipase etc. The present study highlights the importance of *Streptomyces* sp., the constantly available actinobacteria which showed a favorably high enzyme production. The present study also showed the molecular identification of the five isolates which have not been reported earlier for enzyme production. The molecular techniques of identification are highly significant than morphological methods. All the five isolates exhibited a higher enzyme activity for different enzymes studied. Subsequently all the isolates were novel and they were deposited in NCBI and a unique accession number had been obtained for each strain. From our study it can be concluded that *Streptomyces* sp. LCJ10A, *Streptomyces* sp. LCJ11A, *Streptomyces* sp. LCJ13A, *Streptomyces* sp. LCJ14A and *Streptomyces* sp. LCJ16A isolated from Pichavaram mangroves are highly efficient in producing commercially essential enzymes lipase, cellulase, asparaginase, amylase, and protease. Such enzymes can serve as outstanding resources for innovative biotechnological processes and can play an important role in the development of new biological insights. In this view, there is ample scope for further research on optimization studies of the best isolate for an extensive range of applications. In summary, these isolates can be further used for optimizing the culture conditions using a suitable production medium besides the characterization of more enzymes would help to establish the biochemical and structural basis for the molecular stability of the organisms isolated from mangrove environment.

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