3. Materials and Methods

Description of sampling sites

The study site is located in Point Calimere, East coast of India, Tamil Nadu (Lat. 10°18' N; Long. 79°51' E). Point Calimere wildlife sanctuary is located in Kodikharai, Vedaranyam taluk and Nagapattinam district of Tamil Nadu. The total area of the sanctuary is about 30 sq.km bordered by the Bay of Bengal on the east, Palk Strait on the south and salt pans of various chemical companies on the west and the north.

The present investigation was carried out in four different stations in Point Calimere namely Chola light house, Old light house, New light house (seashore) and Muniyappan lake (Mangrove) (Plate -1; Fig.1)

Sampling schedule

Randomly soil samples were collected in each sampling station once in every season for a period of two years (January 2004 to December 2005). For the sake of convenience year was divided as Post-monsoon (January – March), Summer (April – June), Pre-monsoon (July – September) and Monsoon (October – December).

Collection of samples

The soil samples were collected from 5 inches below the surface of the soil, using a metal spatula. The spatula was sterilized every time with 70 per cent alcohol. At each station 5 samples were collected randomly. The samples were kept in new polythene bags, sealed and transported to the laboratory. For the analysis of soil physico-chemical properties, one kg of soil was also collected separately from each station in polythene bags and brought to the laboratory for further investigation.
PLATE 1

Old Light house

New Light house

Chola Light house

Muniyappan lake

Panoramic view of sampling stations
LOCATION OF TAMULAMU

Old light house
New light house
Chola light house
Muniyappan lake

Fig. 1. The map showing the study area
Drift wood samples were collected from the above mentioned four stations in Point Calimere. At least 100 numbers of wood samples were collected from each station. The samples were kept in new polythene bags tied with string and transported to the laboratory for further investigation.

**Physico-chemical characteristics of soil**

**pH**

The collected soil samples were individually mixed with distilled water (1:2 w/v) and the supernatant was examined using pH meter (Elico, India) for the determination of pH.

**Electrical conductivity**

Soil samples were individually mixed in distilled water (1:2 w/v) and the electrical conductivity was determined by using electrical conductivity meter (Systronics, India, Model 631E).

**Nutrients**

Macro-nutrients such as available nitrogen, phosphorus, potassium and micronutrients Zn, Cu, Mn and Fe were analyzed by the methods described by Barnes (1959) and Muthuvel and Udayasoorian (1999).

**Isolation of fungi from soil**

Dilution plating technique described by Warcup (1950) was used for isolation of fungi from soil samples. 10 g of soil from the each sample was weighed separately and then dissolved in 100 ml distilled water. The flasks were shaken thoroughly in order to get uniform distribution of the soil. The soil suspensions were diluted in ten fold increment from $10^{-2}$ to $10^{-4}$. One ml of the diluted sample was plated on sterilized 50 per cent SWCMA medium (Sea Water Corn Meal Agar Medium - Corn meal powder 20 g, dextrose 20 g, peptone 20 g, agar 20 g) supplemented with 1% streptomycin (One gram of streptomycin was mixed thoroughly in 100 ml of sterilized distilled water).
The plates were incubated at the room temperature (28 ± 2°C) for 7 days. Three replicates were maintained for each sample.

After seven days of incubation, the colonies growing on 50% SWCMA plates, with different morphology were counted and purified in SWCMA medium separately. A portion of the growing edge of each colony was picked up with the help of a pair of needles and mounted in a clean slide with lactophenol cotton blue mountant (Hi-media). The slide was gently heated in a spirit lamp so as to remove air bubbles. The excess stain was wiped off with the help of tissue paper and then the cover slide was sealed with transparent nail polish / DPX mountant. The slide was observed under microscope and microphotographs of the individual fungal species were also taken using Nikon microphotograph microscope (Japan).

Identification

Colony colour and morphology were noted besides hyphal structure, spore size, shape and spore bearing structures. They were identified with the standard manuals such as A Manual of Penicillia (Raper and Thom, 1949); Manual of Soil fungi (Gilman, 1957); Manual of Aspergilli (Raper and Fennell, 1965); Hyphomycetes (Subramanian, 1971); Dematiaceous Hyphomycetes and More Dematiaceous Hyphomycetes (Ellis, 1971 and 1976).

Presentation of data

The percentage contribution was calculated as follows

\[
\text{% contribution} = \frac{\text{Total number of colonies of individual species}}{\text{Total number of colonies of all the species}} \times 100
\]

Simple correlation analysis was performed between the physico-chemical characteristics of the soil and fungal population density by computing the data in Microsoft Excel programme.
Fungi from litter samples (Direct Examination method)

The collected drift wood samples were packed in sterile polythene bags. Then sterilized 50% seawater was added to each bag in order to maintain moisture condition. The bag was tied with a string and incubated at room temperature.

After incubation, the wood samples were taken out from the bag and allowed to air dry. This facilitates the development of fungal structures on the wood substratum. Then, the wood samples were examined under dissection microscope for the presence of ascocarps, basidiocarps, pycnidia or conidia. Such fruit bodies were transferred with a needle to a microscopic slide, torn apart in a drop of water to expose the spores and carefully squeezed under a cover glass.

Morphology and septation of ascospores, basidiospores or conidia were used for their identification. In some cases, asci and sterile elements of the ascocarps such as paraphyses and pseudoparaphyses of the fruit bodies were used as characters for identification.

Microphotographs were made and the fungi were identified using the Manual of Marine Mycology – The Higher Fungi (Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer and Kohlmeyer, 1992), Synoptic key to higher marine fungi – Sarma and Hyde (2000) and Pictorial key to higher marine fungi – Hyde and Sarma (2000).

Percentage frequency was calculated and recorded.

\[
\text{\% frequency} = \frac{\text{No. of wood samples in which a particular fungus occurred}}{\text{Total No. of wood samples examined}} \times 100
\]
Based on the frequency of occurrence, the fungi were grouped into four frequency classes viz., rare (1-10%), occasional (11-20%), frequent (21-30%) and common (31-50%).

Screening of fungi for extracellular enzyme production

The method described by Hankin and Anagnostakis (1975) and Rohrman and Molitoris (1992) were used to detect the production of extracellular enzymes.

Amylolytic activity

Glucose yeast extract peptone agar medium (GYPA) was prepared (g/l glucose, 1; yeast extract, 0.1; peptone, 0.5; agar 18; soluble starch, 0.2). pH of the medium was adjusted to 6.0. The medium was sterilized in an autoclave and poured into the Petri plates. After solidification, the fungi were inoculated into the agar medium separately and the plates were incubated at 30 ± 2°C for 3-5 days. After the period of incubation, the plates were flooded with iodine solution. The formation of clear zone was observed, which indicated the amylolytic activity.

Proteolytic activity

GYPA medium was prepared (pH 6.0). A solution of gelatin in water (8%) was prepared separately and added to GYPA medium at the rate of 5 ml per 100 ml of medium. The medium was poured into the Petri plates. After solidification, the test fungi were inoculated separately and incubated at 30 ± 2°C for 3-5 days. After incubation, degradation of gelatin was seen as clearing the somewhat opaque agar around the colonies. The plates were flooded with a saturated aqueous solution of ammonium sulphate, which resulted in the formation of a precipitate. This made the agar opaque and enhanced the clear zone around the fungal colony, which indicated proteolytic activity.
Cellulolytic activity

Yeast-extract-peptone-agar (YPA) medium (g/l Yeast extract 1, Peptone 0.5, agar 18) containing carboxy methyl cellulose was prepared (0.5%) and the pH was adjusted to 6.0. The medium was sterilized and poured into the Petri plates. After solidification, the fungal isolates were inoculated into the medium separately and the plates were incubated at 30 ± 2°C for three days. After the colony growth, the plates were flooded with 0.2% congo red solution and destained with 1 M NaCl (15 min each). Appearance of yellow zone around the fungal colony or red medium indicated as cellulase activity.

Lipolytic activity

Tween 20 was sterilized in an autoclave. One ml of Tween 20 was added to 100 ml of sterilized and cooled agar medium (g/l Peptone 10, NaCl 5, CaCl₂.2H₂O 0.1, agar 20, pH 6.0) and poured into the Petri plates. After solidification, the fungal isolates were inoculated separately and incubated. Clearing or precipitation around the fungal colony indicated the lipolytic activity.

Xylanase activity

Akiba and Horikoshi medium (g/l Xylan 5, Peptone 5, Yeast extract 1, NaCl 5, K₂HPO₄ 1, MgSO₄ 0.2, CaCl₂ 0.1, Na₂CO₃ 10) was prepared and pH was adjusted to 7. The medium was sterilized and poured into Petri plates. After solidification, the fungi were inoculated individually and the plates were incubated at 37°C. Colonies were evaluated on the basis of the formation of clearing zones due to the activity of xylanase.

Laccase activity

GYPA medium (g/l glucose, 1, yeast extract 0.1; peptone, 0.5; agar 18) was prepared with 0.05 g 1-naphthol L⁻¹ and pH was adjusted to 6. The medium was sterilized in an autoclave and poured into Petri plates. After solidification, the fungi were inoculated separately into the medium and incubated. The fungus
grew over the colourless medium and the medium turned blue colour due to the oxidation of 1-naphthol by the production of laccase.

**Influence of environmental parameters on enzymatic activity**

Among the 62 species tested, the promising enzymatic activities of fungi such as *Thielavia terricola, Aspergillus fumigatus, A. niger, A. terreus, Curvularia lunata, Fusarium oxysporum, Penicillium chrysogenum, P. citrinum, Trichoderma harzianum* and *Verticillium tenerum* were screened and the environmental parameters were optimized, which favoured the enzyme production.

**Production media employed for the enzymes**

The above mentioned 10 fungi were inoculated into enzyme production media such as starch broth (peptone 5 g, beef extract 3 g, soluble starch 3 g) for amylase (alpha amylase) and modified Reese’s medium (glucose 0.25 g, casein 0.5 g, yeast extract 0.05 g, K2HPO4 1.0 g, MgSO4 – 0.20 g) for protease (alkaline protease). Modified Czapek’s cellulose medium (cellulose – 10.0 g, KNO3 – 3 g, K2HPO4 – 1.0 g, MgSO4 – 0.5 g, FeSO4 – 0.01 g) for cellulase, production medium with coconut oil (trypitone – 1%, yeast extract – 0.5%, NaCl – 0.5% supplemented with CaCl2·2H2O – 0.01%, Tween 80-1%) for lipase, Akiba and Horikoshi medium (xylan – 5 g, peptone – 5 g, yeast extract – 1 g, NaCl – 5 g, K2HPO4 – 1 g, MgSO4 – 0.2 g, CaCl2 – 0.1 g, Na2CO3 – 10 g) for xylanase and liquefied basal medium (1.0 mM ammonium tartarate, 1.0 g of KH2PO4, 1.0 g of K2HPO4, 1.4 g of (NH4)2SO4, 0.5 g of MgSO4·7H2O, 0.1 g CaCl2·2H2O, 0.5 ml of vitamin B complex, and 7.0 ml of trace element solution. It also contained 0.2 g of Tween 80) for laccase activity.

**Effect of pH**

The production media were prepared individually and the pH was adjusted to 5, 6, 7, 8, 9 using 0.1 N HCl and 0.1 N NaOH and the fungi were inoculated into the medium individually and enzyme assay was performed.
Effect of salinity

The production media with different salinity levels such as 10, 20, 30, 40 and 50 ppt were prepared with seawater diluted in distilled water and also by the addition of Salt (NaCl). The medium was sterilized and the fungi were individually inoculated and incubated at room temperature.

Effect of temperature

Media for enzyme production were prepared and sterilized. The test fungi were inoculated individually and incubated at different temperatures viz., 10, 20, 30, 40 and 50°C, and enzyme assay was performed.

Enzyme Assay

Alpha amylase (Peter Bernfield, 1955)

One ml of starch solution was added with 1 ml of diluted enzyme solution and incubated at 27°C for 15 minutes. 2 ml of dinitrosalicylic (DNS) reagent was added and kept in boiling water bath for 5 minutes. 1 ml of potassium sodium tartrate was added and allowed to cool down in running water. This solution was made upto 10 ml with sterile distilled water and absorbance was read at 560 nm.

Alkaline protease (Keay et al., 1970)

0.2 ml of enzyme sample was taken and made upto 1 ml with distilled H₂O. 3 ml of 0.4 M carbonate – bicarbonate buffer (pH 9) and 1 ml of casein was added. The mixture was incubated for 30 minutes. 2 ml of TCA was added and again incubated at 37°C for 30 minutes. The mixture was filtered through Whatman No.1 filter paper and the clean filtrate was collected. To 0.1 ml of the filtrate 0.5 ml of 0.4 N sodium carbonate and 1 ml of Folin-Phenol were added. The solution was mixed well and incubated at 37°C for 20 minutes. Then, absorbance was read at 660 nm.
Cellulase (Denison and Koehn, 1977)

0.5 ml of the enzyme extract was added to the test tube containing 0.45 ml of 1% carboxy methyl cellulose and incubated at 55°C for 15 minutes. 0.5 ml of dinitrosalicylic acid reagent (DNS) was added and kept in a water bath for 5 minutes. 0.1 ml of potassium sodium tartrate was added and made up to 5 ml with distilled water. The optical density was measured at 540 nm.

Lipase (Safarik, 1999)

250 mg of olive oil was taken into a test tube and added with 2 ml of phosphate buffer (pH 6.3). 1 ml of enzyme sample was added and vortexed for 15 seconds. The mixture was incubated at 37°C in a water bath under static conditions for 30 minutes. 1 ml of concentrated HCl was added and vortexed for 10 minutes. Then, 3 ml of benzene was added and vortexed for 90 seconds. From this 2 ml of benzene layer was taken and added to 1 ml aqueous solution of 5 per cent cupric acetate (pH 6.2). Then it was vortexed for 90 seconds and centrifuged at 5000 rpm for 10 minutes. Clear organic phase of benzene layer was removed and used for the estimation of liberated fatty acids by measuring the optical density at 715 nm.

Xylanase (Nanmori et al., 1990)

Enzyme solution (0.5 ml) was added to 2 per cent xylan suspension (0.5 ml) in 0.1 M acetate buffer, pH 6.0, and the mixtures were incubated at 55°C for 30 minutes. After incubation the mixtures were cooled rapidly in ice water and the insoluble xylan was removed by centrifugation. To the resulting supernatant (0.5 ml), 1 ml of 3, 5-dinitrosalicylaldehyde (0.5%) solution was added, and the mixture was kept in boiling water. Colour development was measured using the spectrophotometer at 535 nm. The enzyme activity was expressed as μmol of xylose released per ml/min.

Laccase (Ruttimann et al., 1992)

Laccase activity was assessed in 1.0 ml of reaction mixture containing catechol as substrate in 50 mM sodium phosphate buffer (pH 5.0). 0.2 ml of
enzyme extract was added to it. The progress of the reaction was monitored at 440 nm for 10 minutes.

All the enzyme activities were calculated as follows

\[
\text{Enzyme activity (IU)} = \frac{\text{Test OD}}{\text{Std. OD}} \times \frac{\text{Conc. of Std.}}{\text{Vol. of Sample taken}} \times \frac{1}{\text{Time of incubation}} \times \frac{1}{\text{mg of protein}} \times \frac{1}{\text{Mol. wt. of Std.}}
\]

**Molecular characterization**

**DNA Extraction**

Total genomic DNA was extracted by a modified method used for the isolation and purification of DNA from fungi (Belen Florez et al., 2007).

The fungal strains were grown in 50% SWCM broth at 25°C for 72 h with shaking. The mycelial mat of the cultures were then centrifuged at 14,000 xg for 5 min and the pellet was washed with TE buffer (10 mmolL⁻¹ Tris-hydrochloride, 1 mmolL⁻¹ EDTA, pH 8.0) and frozen overnight at -20°C. The pellets were then suspended in 500 μl of a lysis buffer (200 mmol L⁻¹ Tris-hydrochloride, 15 mmol L⁻¹ EDTA, 250 mmol L⁻¹ NaCl, and 0.5% SDS pH 7.5). Chloride acetate (300 μL) was then added, along with 10 μl of 150-212 μm glass beads. Lysis was accomplished by vortexing for 10 min, after which proteinase K was added at a final concentration of 2 mg mL⁻¹ and the mixture was incubated for 30 min at 50°C. One hundred microliters of 1.5 mol L⁻¹ NaCl was added to this solution, and incubation proceeded for 5 min at room temperature. Cell debris was eliminated by centrifugation and the clear lysate was transferred to a new tube. The DNA was purified by phenol: chloroform: isoamyl alcohol (24:24:1) extraction and precipitated with equal volume of isopropyl alcohol. The pellet was washed twice with ethanol at 70%, vacuum dried, and suspended in 100 μL of TE buffer. The purity and concentration of the DNA was assessed by electrophoresis in agarose gels; visualization was achieved with ethidium bromide. DNA was quantified by recording the absorbance at 260 nm using TE buffer as the blank.
Oligonucleotide primers and PCR amplification

All the ten fungal extracts were amplified with 10-base pair primer OPN-03 GGTACTCCCC (Doherty et al., 2003). PCR amplification was performed using RAPD analysis beads. Amplification was conducted in a 50 μl volume containing 50ng of genomic DNA (1 μl), 100pmols of a single RAPD primer (2 μl), 10 X PCR buffer (5 μl), dNTP mix (1 μl), Taq DNA polymerase (0.5 μl) and Sterile Double distilled water (40.5 μl). The DNA amplification was performed in eppendorf Thermal cycler-gradient (Germany) with the following program: 1 cycle at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, with a final extension of 10 min at 72°C.

The PCR products were separated electrophoretically on 1.5% agarose gels with TBE buffer (45.0mM Tris-borate, 1.0mM EDTA). DNA bands were visualized by staining with ethidium bromide (0.5 μg/ml), observed under UV light and photographed.

Calculation of genetic distance with RAPD markers

Each band visualized on a gel was considered a RAPD marker and part of the total RAPD fingerprint generated for a species of fungi. The presence or absence of a band at any position on the gel was used to construct a binary matrix for fungal RAPD markers from the described reactions. Genetic distances between species were calculated by using the algorithm of Nei and Li (1979), as provided in the RAPDistance software package developed by Armstrong et al. (1994). A pair wise comparison of genetic distance for all fungal patterns was used to create a phenogram based on the Neighbor Joining method and the program NJ Tree (Saitou and Nei, 1987).