

*Chapter 2*  
*Isolation, Screening*  
*&*  
*Identification of*  
*Laccase-producing*  
*Marine Fungi*

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## **ABSTRACT**

Fungi were isolated from decaying wood pieces, leaves and other plant detritus from the mangrove swamps of Choraõ Island in Goa, India. Seventy-five fungi were isolated using techniques such as particle-plate, hyphal and single spore isolation methods out of which, 15 isolates were laccase-producers. From the laccase-producing fungi, the anamorphic fungi designated NIOCC #2a, NIOCC #7a and NIOCC #Xa were the most efficient dye decolourizers and laccase producers as well. NIOCC #2a was the laccase hyper-producing strain selected for this study. It was deposited at the Microbial Type Culture Collection (MTCC) under accession no. 5159. Since it was an anamorph, it was identified using rDNA homology (18S rDNA) to be a *Cerrena unicolor* strain. The fungus NIOCC #2a is therefore referred to as *Cerrena unicolor* MTCC 5159. These results were confirmed by ITS1-5.8S-ITS2 and D1/D2 of the 25-28S rDNA. Its identity as a marine-derived fungus was confirmed by obtaining maximum biomass production in full strength seawater of 34 ppt and maximum laccase production in seawater of 25 ppt.

## 2.1 INTRODUCTION

Marine fungi are an ecological rather than taxonomic group and comprise about roughly 1500 species, excluding those that form lichens. According to their biogeographical distribution, marine fungi can be grouped into temperate, subtropical, tropical and cosmopolitan species (Abdel-Wahab & El-Sharouny, 2002).

Fungi which grow and sporulate exclusively under marine conditions, have been defined as *obligate marine fungi*. In order to accommodate the possibility that terrestrial species might also be active in the sea, these authors offered a definition for fungi which originate from freshwater or terrestrial environment and are capable of growth and sporulation in the sea as *facultative marine fungi*. Marine obligate and facultative filamentous fungi are known to occur in algae, corals and detritus of marine macrophytes (Kohlmeyer & Kohlmeyer, 1979).

*Marine-derived fungi* mostly include facultative marine fungi, that are capable of growth in the marine environment but similar strains also occur on land. If the marine-derived strains have been actively growing and present for sufficient time in the marine environment, chances that specific adaptations occur, increase (Coumo *et al.*, 1995). These authors also suggest that five times as many new compounds from marine-derived strains could be isolated as compared to their terrestrial counterparts, suggesting that the rate of successful isolation of new compounds may be habitat dependant. Genetic comparison of the terrestrial and the marine-derived strains can determine whether these adaptations have occurred at the genetic level (Jensen & Fenical, 2002).

The vast majority of compounds reported from marine fungi are in fact from marine-derived strains, which based on morphological characteristics have been shown to be identical or at least closely related to terrestrial species. Given the inherent salt tolerance of many fungal species, transitioning the land / sea barrier may in fact be commonplace and most marine-derived strains may have been growing in the environments from which they were isolated. Since these strains are chemically prolific and compared to obligate marine fungi are fast growing as well as exhibit a high degree of salt tolerance, they have been the focus of a vast majority of research. Marine-derived

strains, which have been metabolically active in the marine environment for sufficient amount of time to allow secondary metabolite production to be influenced by specific marine parameters, are a good source for the isolation of novel marine metabolites (Jensen & Fenical, 2002).

The term ‘mangrove’, apart from indicating a tropical intertidal community, also refers to the constituent vegetation of this community (Tomlinson, 1986) that exists at the boundary between the terrestrial and marine environments. This covers approximately one-fourth of the entire tropical coastline and extend over 15.5 million ha worldwide (Bandaranayake, 1998). The mangroves have specialized features like pneumatophores (aerial roots), viviparous form of reproduction, prop roots and high salt tolerance all of which make them unique. The mangroves are home to mangrove fungi, which include some obligate marine fungi, marine-derived fungi and special *mangicolous fungi*, fungi specific for mangroves. The age of the mangrove stand, diversity of the mangrove and terrestrial tree flora as well as various microhabitats (e.g. salinity, temperature, humidity, pH) inside mangroves are the most important factors controlling the diversity of mangrove fungi (Kohlmeyer & Volkmann-Kohlmeyer, 1993).

These fungi play a mostly saprophytic role, in the microbial processes occurring in the mangroves especially the mangrove food web and the surrounding coastal areas, to release nutrients which can again be used by plants and animals as organic sources for metabolism (Raghukumar, 2004). This role involves the degradation and mineralization of lignocellulosic substrates and fungi that do so are termed as *lignicolous* or *lignin-degrading fungi* (this term applies for all fungi that have the capacity to degrade lignin, irrespective of their source). Mangrove plants, found in estuaries of the tropical and subtropical belts contain about 50 % lignocellulosic structural polymers and about 50 % soluble organics which include tanins and phenolics (Benner & Hodson, 1985).

Next to cellulose, lignin is the most abundant and widely distributed renewable aromatic polymer (Boominathan & Reddy, 1992) and is one of the major structural components of woody plants. About 70 % of the dry weight of woody plants consists of lignocellulose of which 20 - 30 % is contributed by lignin. The lignin-degrading fungi initiate the process of lignin degradation using a specialized set of

enzymes, the lignin-degrading enzymes (LDEs). These enzymes include the heme-containing peroxidases and the copper-containing glycoproteins, laccases. The peroxidases include the manganese dependant peroxidase (MnP), lignin peroxidase (LiP) and versitaile peroxidase (VP), which is a hybrid of MnP and LiP.

Besides existing as saprophytes, mangrove fungi also exist as '*endophytes*'. The term endophyte refers to all organisms inhabiting plant tissues, which at some time of their life cycle, colonize internal plant tissues without causing apparent harm to the host (Petrini, 1991). Successful colonization of the host tissues by endophytic fungi can be achieved only when they are able to breach the protective layers of the host. An endophyte occupies essentially the same ecological niche as most fungal pathogens and hence it is expected to adopt the same strategy as the pathogens to enter into the host tissues (Petrini *et al.*, 1992). Mangrove plants provide a hostile environment for endophytes, by the presence of phenolics like tannins, which are known to inhibit the growth of litter and soil fungi (Kumaresan *et al.*, 2002). Ability to grow in the presence of such phenolic compounds entails some adaptations on the part of the fungal endophytes. These include the production of extracellular cell wall degrading enzymes, ability to grow in the presence of phenolic compounds and halotolerance. Kumaresan *et al* (2002) have shown that most of endophytic mangrove fungi examined produced lipolytic and pectinolytic activities which degrade the cuticular waxes on the leaf surface and the middle lamella of the leaf cells. Most of them produced the non-specific enzyme, laccase which also degraded other phenolic compounds in addition to the lignin polymer, indicating their involvement in litter degradation as well.

Many mangrove plants either accumulate or exclude salt from their leaves; the salt concentration in their leaves was similar to that of seawater. In addition, salt excreting mangrove plants such as *Avicennia* and *Aegiceras*, mangrove plants often have crystals of salt on their leaf surface (Tomlinson, 1986). Thus, the foliar endophytes of mangrove plants have to encounter a saline milieu during and after leaf penetration. Halotolerance in endophytes, would be essential (Kumaresan *et al.*, 2002). Some latent pathogens, survive as endophytes on their host tissues and become active when the host is stressed (Carrol, 1988).

The ability of endophytic basidiomyceteous fungi to produce extracellular enzymes including laccase that lead to breakdown of the host cell walls, contributes to their overall success in the colonization of the host via entry through host cell walls. Endophytes can grow and produce fruiting bodies on dead and fallen mangrove leaves thus building up the endophyte inoculum (Kumaresan & Suryanarayanan, 2002). This suggests the ability of these endophytes to lead a saprobic lifestyle upon the senescence of the plant (Pointing, 2001).

Even with the comparatively lesser abundance of oligate and facultative marine fungi, their taxonomy is of importance and the presence and type of LDEs produced have also been used a criterion in fungal taxonomy, among the lignicolous fungi (Guarro *et al.*, 1999). Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. The baseline of traditional fungal taxonomy and nomenclature is morphological criteria or their phenotypes. Numerous alternative approaches have been developed including nutritional and physiological studies, serologic tests, secondary metabolites, ubiquinone system, and fatty acids. Although some of these are very useful for identifying poorly differentiated fungi such as yeasts and black yeasts, in most cases they are only complementary tools of morphological data. The fungus, as a whole, comprises a teleomorph (sexual state) and one or more anamorphs (asexual states). Traditional fungal classification is possible when the fungus is at its teleomorphic stage (asexual spore morphology also help); however the snag occurs in cases of fungi where only the anamorphic stage is available.

The dual modality of fungal propagation, i.e., sexual and asexual, has led to (Saccardo, 1880) a dual nomenclature. The anamorph and teleomorph generally develop at different times and on different substrates, (although in zygomycetes they often occur together). Each phase has been described in total ignorance of the existence of the other in many cases, although the International Code of Botanical Nomenclature (ICBN) maintains that it is legal to give them separate binomials, many mycologists regard it as unsatisfactory to have several names for a single fungus (Guarro *et al.*, 1999). For a long time, the anamorphs that occurred alone have been grouped into a separate major high-level taxon called Deuteromycotina or Deuteromycetes. The phenotypic approach has been largely criticized for its lack of standardized and stable terminology and for its high

subjectivity. Moreover, some phenotypic characteristics have been considered unstable and dependent on environmental conditions, as with growth in artificial culture. A clear limitation of phenotypic approaches is that they cannot be applied to fungi that do not grow or sporulate in culture. Consequently, many fungi will remain unclassified as long as taxonomists rely solely on phenotypic characteristics.

Another notable problem of classification based on morphological criteria is the above-mentioned dual system of classification, with no consistent correlation between the taxonomies of the Ascomycetes and Deuteromycetes (Hennebert & Sutton, 1994). This is an important difficulty in establishing the taxonomic concept of the fungus as a whole. In addition, traditional fungal systematics does not significantly contribute to the understanding of the evolutionary relationships among fungi. The simple morphology, the lack of a useful fossil record and fungal diversity have been major impediments to phylogenetic studies (Berbee & Taylor, 1992). Classically, studies on fungal evolution have been based on comparative morphology, cell wall composition, cytologic testing, ultrastructure, cellular metabolism and the fossil record, yet wide disparities remain (Guarro *et al.*, 1999). It is here where modern molecular techniques not only come to the rescue but also allow for the anamorphic and teleomorphic stages of a particular fungus to be connected and integrated into a more natural taxonomic scheme, which was previously possible only to a limited extent in traditional fungal taxonomy.

With the advent of universally applicable molecular approaches in fungal taxonomy, some mycologists have advocated abandoning the dual system of naming because unified classification of all fungi may be possible on the basis of the rDNA sequences of the anamorphs (Blackwell, 1993). Population studies and molecular data have shown that many widely used morphospecies actually comprise several biological or phylogenetic species. One of the problems for morphologists involves deciding how many base differences are required for strains to be considered different species. This has been partly solved by the phylogenetic-species concept, especially when based on cladistic analysis of molecular characteristics, which offers consistency in the delineation of species. Cladogram topology indicates the existence of monophyletic groups, which may represent species or supra- or subspecific taxa. Peterson and Kurtzman (1991) correlated

the biological-species concept with the phylogenetic-species concept by comparing the fertility of genetic crosses among heterothallic yeasts.

Two important technical advances have stimulated the use of molecular techniques. Firstly, the advent of PCR has allowed the analysis of small numbers of fungal cells or even single spores, dried herbarium material, or extinct organisms. Second, the selection of universal oligonucleotide primers specific to fungi, have provided easy access to nucleotide sequences (Guarro *et al.*, 1999).

One group of genes that is most frequently targeted for phylogenetic studies is the one that codes for ribosomal RNA (rRNA). Introns of several protein-encoding genes, such as the  $\beta$ -tubulin and actin, chitin synthase, acetyl coenzyme A synthase, glyceraldehyde-3-phosphate dehydrogenase, lignin peroxidase or orotidine 5'-monophosphate decarboxylase genes can also be applied and can provide important information (Guarro *et al.*, 1999). The main reasons for the popularity of rDNA are that it is a multiple-copy, non-protein-coding gene, whose repeated copies in tandem are homogenized by concerted evolution, and it is therefore almost always treated as a single-locus gene. Furthermore, ribosomes are present in all organisms, with a common evolutionary origin. Parts of the molecule are highly conserved (van de Peer *et al.*, 1997) and serve as reference points for evolutionary divergence studies. The conserved regions alternate with variable regions or divergent domains (Hassouna *et al.*, 1984). The 5.8S, 18S, and 25-28S rDNAs are transcribed as a 35S to 40S precursor, along with internal and external transcribed spacers (ITS and ETS). All spacers are spliced out of the transcript. Between each cluster is a non-transcribed spacer (NTS) or intergenic spacer (IGS) that serves to separate the repeats from one another on the chromosome. The 5S gene takes a variable position and is transcribed in the opposite direction. The total length of one DNA repeat is between 7.7 and 24 kb (Hibbett, 1992). Comparisons of the 18S (also called the small-subunit [SSU]) rRNA sequences have been performed to assess the relationships of the major groups of living organisms (Woese *et al.*, 1990). For phylogeny of filamentous fungi, the 18S sequence is mostly used completely or in subunits of over 600 bps (Wilmotte *et al.*, 1993). In the yeasts, the D1 and D2 variable regions of 25-28S rDNA regions are almost exclusively used (Demain *et al.*, 1998). This technique is currently being extended to Heterobasidiomycetes (Begerow *et al.*, 1997) and sometimes

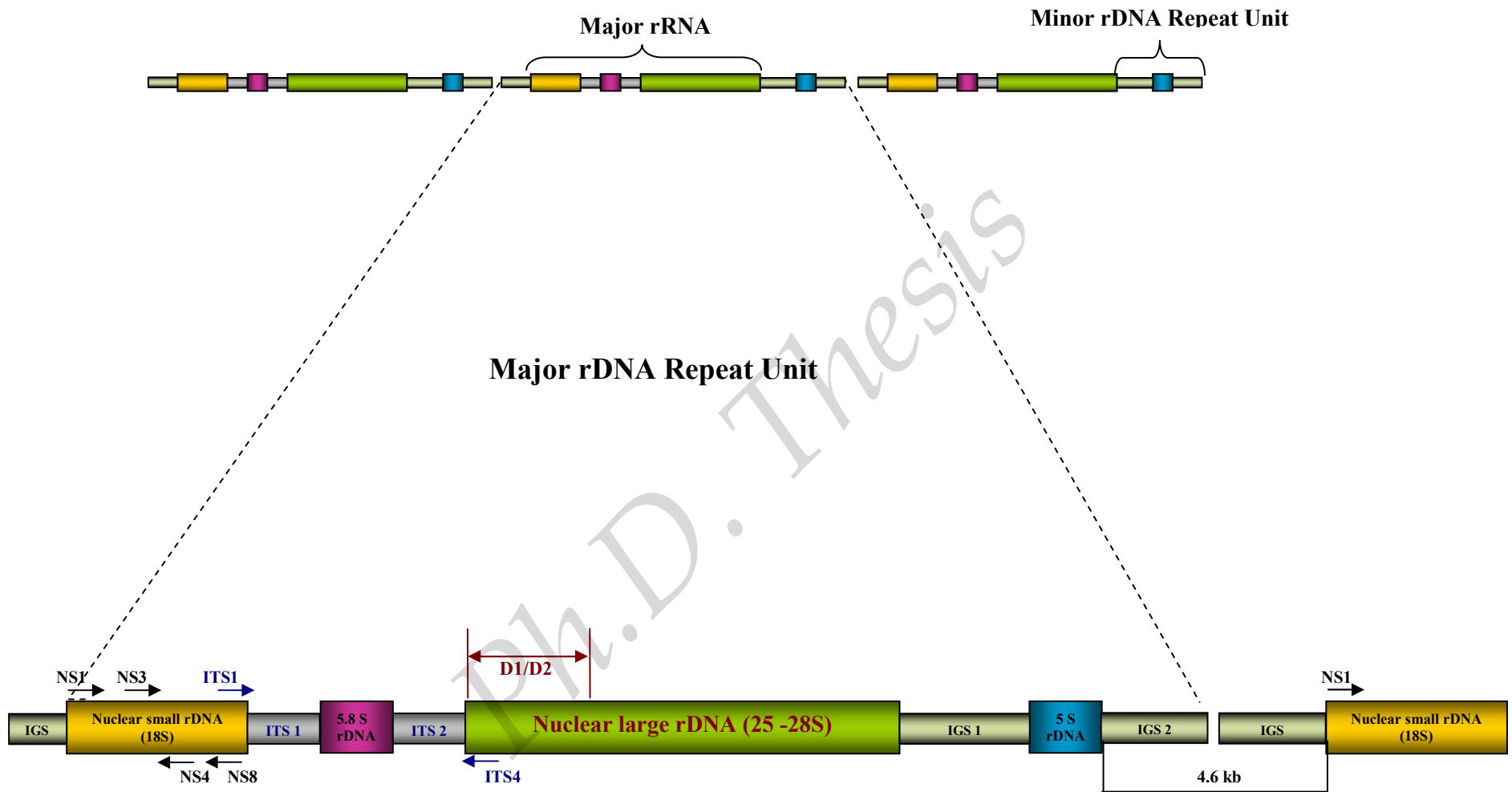


also to filamentous Ascomycetes (Masclaux *et al.*, 1995). Peterson and Kurtzman (1991) demonstrated that the D2 region, a variable region of the 25-28S rDNA gene, is sufficiently variable to recognize biological species of yeasts and that con-specific species generally show less than 1 % nucleotide substitution. A schematic diagram representing various rDNA regions is illustrated in Fig. 1.

In only a limited number of fungi have both regions been sequenced. Due to this different choice of target regions, comparison of fungi to all possible relatives is hampered. The 25-28S variable domains are very informative and allow comparisons from high taxonomic levels down to the species level, although only a limited number of variable positions remain (Guého *et al.*, 1993). In the 18S gene, the variable domains mostly provide insufficient information for diagnostic purposes (de Hoog *et al.*, 1997), and thus large parts of the molecule must be sequenced to obtain the resolution required (Haase *et al.*, 1995). The ITS regions are much more variable but sequences can be aligned with confidence only between closely related taxa. These regions are generally used for species differentiation but may also demonstrate patterns of microevolution (Gräser *et al.*, 1999). In contrast, 5.8S rDNA is too small and has the least variability compared to the ITS regions. The 5S rDNA has been used mainly to infer relationships, based on the secondary structure of the ribosomal molecule (Blanz & Unseld, 1987) and not merely the DNA sequence.

The evolutionary distance is generally displayed in the form of trees, and a wide diversity of algorithms is available to construct them. Two basic methods are available: distance matrix methods, resulting in phenograms, and maximum-parsimony methods, resulting in cladograms. The statistical significance of the tree found is tested by using resampling algorithms such as the bootstrap method (Hillis *et al.*, 1994). The tree is usually rooted with an organism at a moderate distance (outgroup) that still can be aligned with confidence. For groups which have unexpected heterogeneity such as the fission yeasts and the black yeasts, the correct choice of out-group is quite important (Stasz *et al.*, 1989).

Among the classical DNA-based methods is the determination of the nuclear DNA (nDNA) guanine-plus-cytosine (G+C) content. The G+C content of nDNA has been established for many fungi, primarily yeasts.



**Fig. 1: Schematic representation of the fungal rRNA gene.**

The primers used to amplify the various rDNA regions used in this study are represented as one-direction (5' to 3') arrows. The D1/D2 region of the 25-28 S rDNA is represented by a double sided arrow (<http://plantbio.berkeley.edu/~bruns/picts/results/map.pdf> ; <http://www.biology.duke.edu/fungi/mycolab/primers.htm> ; Sugita & Nishikawa, 2003).

A difference of 2 % in the G+C content has been considered to indicate that two strains should be assigned to different species (Kurtzman, 1994). In some insufficiently resolved fungal groups, a difference of 8 % has been allowed within species (Boekhout, 1991). In more precise recent studies with ecologically defined taxa, this difference was reduced to 1 % (Guého *et al.*, 1992). The G+C content is determined by using the  $T_m$  from the S-shaped melting curve of the DNA. Occasionally the shape of the curve is deviant; by determination of the first derivative, this could be traced back to the presence of DNAs with different melting velocities. Guého *et al.* (1997) found that these differences may be characteristic of sub-specific entities.

The identity or nonidentity of closely related strains can be determined by DNA-DNA hybridization, estimating the velocity of heteroduplex formation compared to the standard kinetics of the individual strains. A relative hybridization value of over 80 % is generally regarded as indicating membership in the same species, whereas values of less than 20 % are proof of nonidentity (Vilgalys, 1988). Intermediate values have increasingly been found recently, and these probably indicate subspecific entities (Smith *et al.*, 1995).

In recent years, the methods most widely used for taxonomy at the species level have been sequencing and electrophoretic methods. Many authors have sequenced closely related species to investigate the relationship of the taxa. Such studies have been carried out with larger genera such as *Penicillium*, *Fusarium* and *Trichoderma*. Teleomorph and anamorph variation is not always congruent. The speed of evolution seems heterogeneous and characterized by different rates of variation between groups. In general, about 2 % intraspecific variability is maintained within species, when ITS homology is used as the basis for distinction (Guarro *et al.*, 1999).

Among the electrophoretic methods, restriction fragment length polymorphism (RFLP) is particularly significant for taxonomy. This technique involves digesting DNA samples with a panel of restriction enzymes. The patterns can be tabulated and compared or phenetic trees can be constructed. The RFLP technique is widely used in taxonomy to compare patterns of mitochondrial DNA (mtDNA). This DNA is generally indicative of differences somewhat below the species level but in groups where microspecies are currently distinguished, such as in the dermatophytes, the differences

seem to correspond to teleomorph species (Mochizuki *et al.*, 1990). RFLP-based typing methods have been used to reveal anamorph-teleomorph connections (Gené *et al.*, 1996). Most commonly, the RFLP of PCR-amplified rDNA is used. This technique is also known as amplified rDNA restriction analysis (Vaneechoutte *et al.*, 1992) and provides a quick insight into relationships between moderately distant fungi (de Hoog *et al.*, 1997). Therefore, homogeneity of ITS profiles corresponds well to final ITS sequencing diversity (Uijthof *et al.*, 1998). The method is primarily confirmatory; i.e. new strains are quickly assigned to sequenced strains with the use of restriction maps (Vilgalys & Hester, 1990). This strategy was used, for example, by Yan *et al.* (1995) to study *Phialophora*. Amplified rDNA restriction analysis is particularly useful as an inexpensive and simple alternative to SSU rDNA sequencing when broad relationships have already been determined. However, the frequent occurrence of introns in SSU rDNA (Gargas & DePriest, 1996) may hamper quick comparison of strains.

Random primed methods (Hadrys *et al.*, 1992) are particularly useful to determine relationships below the level of species but depending on the length of the primers and the recognized taxonomic diversity of the group under study, the method may help to discriminate species (Uijthof *et al.*, 1994). A popular technique is random amplified polymorphic DNA (RAPD) with 10-mer primers. However, this method is gradually being abandoned due of poor reproducibility. Microsatellites are a special class of tandem repeats, which have a base motif of up to 10 bps that is frequently repeated (up to about 100 times); they are found in many genomic loci with an almost ubiquitous distribution (Tautz, 1993). A general profile comparison of microsatellites enables species recognition (Niesters *et al.*, 1993). In addition, due to the high level of polymorphism, individual bands can be informative for the characterization of strains (Longato & Bonfante, 1997).

Karyotyping, the migration of chromosomes under the influence of an electric field, has been commonly used for a long time in plant, mammal and bacterial taxonomy. However, some problems have complicated the use of this technique in fungal taxonomy (e.g. the small size of the fungal chromosomes and the difficulty of observing condensed chromosomes during meiosis) (Kohn, 1992). Techniques such as electron microscopy, pulsed-field gel electrophoresis (Mills & McCluskey, 1990) and its recent

modification, contour-clamped homogeneous electric field electrophoresis (Tateishi *et al.*, 1996) contribute to the precise determination of karyotypes. The high intraspecific variability of the electrophoretic karyotypes of fungi makes this technique especially applicable for studies of populations in pathogenic yeasts (Doebbeling *et al.*, 1993) and filamentous fungi (Tateishi *et al.*, 1996).

Before DNA-sequencing methods became available, it was practically impossible to infer a reliable evolutionary tree containing all forms of life. Molecular biology techniques especially the analysis of rRNA sequences are currently used for reliable phylogenetic studies. These enable a more natural classification system to be established. However, the reliability of the new system depends on the skill of the investigators and the quality of data submitted to the database, which cannot be always verified. This is the Achilles heel of classification based solely on molecular techniques.

Traditionally, marine-derived lignicolous fungi have been classified into Ascomycetes and Basidiomycetes based on their distinct spore structures that set them apart from their terrestrial counterparts. The anamorphic fungi are placed under 'Deuteromycetes' and it is the taxonomy of this group of fungi that benefit the most from the use of molecular techniques. The integration of traditional fungal taxonomy and molecular taxonomy would lead to a more natural taxonomic scheme.

## **2.2 OBJECTIVES**

The objectives were to isolate lignin-degrading marine fungi, make a comparison between their respective lignin-degrading capabilities in order to select the fungal strain with the maximum lignin-degrading capacity and identification of this strain for further study.

## **2.3 MATERIALS AND METHODS**

### **2.3.1 SAMPLING**

The sampling site for the isolation of lignin-degrading fungi was the mangrove swamps of Choraõ island in Goa, India (73° 55' E and 15° 30' N). The physico-chemical conditions prevalent at the site during the time of sampling were recorded. These included dissolved oxygen content, chlorinity, ([Appendix 8.2.1.1 & 8.2.1.2](#)) pH, temperature and salinity of the immediate surrounding surface water. Salinity was determined by measurement of the conductivity of the water sample with Autosal, Laboratory Salinometer (Guildline instruments Ltd., Canada). For pH determination, a pH meter (LabIndia, India) was used. Temperature was measured with a Celsius thermometer.

The decaying wood and leaf matter were rinsed with the surrounding estuarine water to remove attached soil particles and other extraneous matter. They were then placed in sterile (pre-rinsed with 70 % ethanol) transparent plastic 'zip-lock' bags to seal the moisture in. They were examined within 3 h after collection under a stereomicroscope to detect fruiting bodies (ascocarps, basidiocarps, pycnidia) and spores of higher marine fungi. Fungal structures are usually confined to the outer 1 cm unless the wood is at an advanced stage of decay, although such wood usually supports fewer fungal species, most of them are exclusively involved in lignin degradation ([Vrijmoed, 2000](#)).

Whether the fruiting bodies were present or not, the samples were incubated at ambient temperature in sterile bags lined with moist filter paper for a fortnight or until, fungal mycelia were observed to colonize the detritus pieces and/or produce fruiting bodies.

### **2.3.2 FUNGAL ISOLATION METHODS**

**2.3.2.1 Particle plating method:** The leaves were cut into pieces of 1 cm<sup>2</sup> under sterile conditions and surface sterilized for 5 min with a mixture of 0.5 % sodium hypochlorite and 0.1 % detergent prepared in sterile distilled water ([Zak & Bryan, 1963](#)). The solution

was then drained and the leaf pieces washed using sterile seawater. These pieces were homogenized using sterile mortar and pestle and passed through sterile stainless steel sieves of 200  $\mu\text{m}$  and 100  $\mu\text{m}$  in succession, followed by washing with sterile seawater. The particles retained on the 100  $\mu\text{m}$  sieve were then plated on malt extract agar prepared with 50 % seawater and fortified with 10 % antibiotic solution, to prevent bacterial growth. The antibiotic solution contained 400,000 U of procaine penicillin and 1 g of streptomycin sulphate in 100 mL of sterile distilled water. In addition, 1 % (w/v) of benomyl, a benzimidazole fungicide, was added in order to select for the slower growing wood decay fungi (Maloy, 1974). The plates were then incubated at room temperature.

**2.3.2.2 Single-spore isolation method:** Decaying wood pieces, which were incubated in a moist chamber for over a fortnight, were thoroughly examined for the presence of fruiting bodies of sporulating fungi using a stereo-microscope. A sterile glass needle was used to transfer a single fruiting body onto the slide, containing a drop of sterile seawater. This was then placed under the low power of a stereo microscope and crushed to release the spores. By capillary action, the drop was sucked into a sterile Pasteur pipette, which was then released into a Petriplate containing malt extract agar, which was gently tapped so that the drop flows downwards in a straight line. The malt extract agar was prepared in 50 % seawater fortified with 10 % antibiotic solution and 1 % (w/v) benomyl. The plates were examined under the microscope for the presence of isolated spores, the area on the plates on which isolated spores were present were marked and incubated at room temperature and examined daily for germination under the light microscope. The germinated spores were excised and transferred onto fresh malt extract agar prepared with half-strength seawater and again incubated at room temperature and observed for growth.

For non-sporulating fungi as well as fungi which produce spores that tightly adhere to each other, the hyphae or the cluster of spores were directly spotted / transferred onto plates containing malt extract agar in 50 % seawater fortified with streptomycin and penicillin.

### **2.3.3 SCREENING FOR LIGNIN-DEGRADING FUNGI**

The fungi obtained from the above methods were qualitatively screened for laccase.

#### **2.3.3.1 Qualitative assay for laccase.**

The fungal isolates were screened for laccase production by growing them on plates of Boyd & Kohlmeyer (B&K) medium ([Appendix 8.1.2](#)) ([Kohlmeyer & Kohlmeyer, 1979](#)) containing 4 mM guaiacol / 2 mM ABTS / 0.02 % Poly-R 478. The production of an intense brown colour under and around the fungal colony in guaiacol-supplemented agar and a deep green colour in ABTS-supplemented plates was considered as a positive reaction for the presence of laccase activity. The transformation of the Poly-R 478 plate from an initial pink colour to yellow indicated the presence of peroxidase activity.

#### **2.3.3.2 Quantitative assay for lignin-degrading enzymes.**

The fungal isolates, which were laccase-positive as well as showed dye decolourization ability, were grown in various liquid media like Malt extract broth (MEB) ([Appendix 8.1.3](#)), B&K and Low nitrogen (LN) media ([Appendix 8.1.1](#)) ([Tien & Kirk, 1988](#)), prepared with half strength sea-water. The week-old cultures were filtered through Whatman GF/C filter paper. Laccase and the peroxidases; lignin peroxidase (LiP) and manganese peroxidase (MnP) were estimated in the mycelia-free culture filtrate ([Niku-Paavola et al., 1988](#); [Tien & Kirk, 1988](#)). Their assay procedure and the definition of the enzyme unit are given in [Appendix 8.2.2.1.1](#), [8.2.2.2](#) and [8.2.2.3](#) respectively.

#### **2.3.3.3 Qualitative assay for dye decolourization ability.**

The laccase-positive fungal isolates were grown in malt extract agar (MEA) and low nitrogen (LN) agar prepared with half strength sea-water containing various dyes. Trypan blue, aniline blue and remazol brilliant blue R (RBBR) at 0.04 %, methylene blue, crystal violet, brilliant green, congo red and Poly-R 478 at 0.02 % and reactive orange 16 (RO 16) at 0.015 % concentration were used. The decolourization of the dye under and around the fungal colony indicated the dye decolourization potential of the isolate.



## **2.3.4 FUNGAL IDENTIFICATION METHODS**

### **2.3.4.1 Classical method.**

The classical method of identifying fungi is by using light microscopy. The fungi with their telomorphic stages were identified based on the morphology of their spores and fruiting bodies. This was carried out by mounting them in sterilized seawater and identification were made with the help of the keys of [Kohlmeyer & Volkmann-Kohlmeyer \(1991\)](#).

### **2.3.4.2 Molecular identification.**

The most efficient laccase producing strain was an anamorph. Hence, molecular techniques like rDNA sequencing were used for its identification.

#### **A) Isolation of genomic DNA:**

The biomass of the selected fungal strain in its log phase of growth (2 - 3 day-old culture) was lyophilized and the DNA was extracted by the method described by [van Burik \*et al.\* \(1998\)](#) ([Appendix 8.3.1](#)).

The purity of the pure DNA was calculated from 260 / 280 nm ratios. This was used as the template for further polymerase chain reactions (PCR) using the DNA Engine Thermal Cycler (BioRad, NSW, Australia).

#### **B) PCR amplification:**

- a)** The NS<sub>1</sub>- NS<sub>4</sub> (~1030 bps) region of the 18S region of the rDNA of this strain was amplified using the forward and reverse primers (NS<sub>1</sub>F and NS<sub>4</sub>R) ([White \*et al.\*, 1990](#)). The PCR was carried out using the high fidelity proof reading enzyme Vent DNA Polymerase (0.3 U) in 25 µL reaction mixture containing 1X PCR buffer, 1.5 mM MgSO<sub>4</sub>, 0.2 mM dNTP mix, 0.5 µM each of NS<sub>1</sub>F and NS<sub>4</sub>R and 50 ng of fungal genomic DNA as template. The PCR was standardized to get maximum amplification with least non-specificity. A negative control reaction (without DNA template) was also set up. PCR conditions are given in [Table 1](#). The reaction was stopped by chilling the mixture at 4°C.

b) The NS<sub>3</sub>- NS<sub>8</sub> (~1230 bps) region of the 18S region (Table 1) was amplified using identical concentrations of chemicals as well as PCR conditions mentioned above with the exception of the primers, which, in this case were NS<sub>3</sub>F and NS<sub>8</sub>R (forward and reverse primers respectively) used at a final concentration of 0.5 μM.

**Table 1: PCR parameters & primers used for rDNA amplification**

PRIMER PAIRS	SEQUENCE (5' → 3')	PCR PROFILE		PRODUCT SIZE	PRODUCT
		Parameters	Cycles		
NS <sub>1</sub> F NS <sub>4</sub> R	GTAGTCATATGCTTGTCTC CTTCCGTCAATTCCTTTAAG	94°C; 4 min	1	~ 1033 bps	NS <sub>1</sub> - NS <sub>4</sub> region of 18S rDNA
		94°C; 1 min	35		
		62.7°C; 30 sec			
		72°C; 1 min			
		72°C; 10 min	1		
NS <sub>3</sub> F NS <sub>8</sub> R	GCAAGTCTGGTGCCAGCAGCC TCCGCAGGTTACCTACGGA	94°C; 4 min	1	~ 1230 bps	NS <sub>3</sub> - NS <sub>8</sub> region of 18S rDNA
		94°C; 1 min	35		
		62.7°C; 30 sec			
		72°C; 1 min			
		72°C; 10 min	1		
ITS 1 ITS 4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	94°C; 5 min	1	~ 650 bps	ITS1-5.8S- ITS2 rDNA
		94°C; 1 min	35		
		55°C; 30 sec			
		72°C; 1 min			
		72°C; 10 min	1		

2. A ~650 bps region of the ITS1-5.8S-ITS2 region of the rDNA of the fungus was amplified using ITS1 and ITS4 primers (Table 1). polymerase chain reaction was carried out using the high fidelity proof reading enzyme Vent DNA Polymerase (0.5 U) in 25 μL reaction mixture containing 1X PCR buffer, 1 mM MgSO<sub>4</sub>, 0.2 mM dNTP mix, 0.5 μM each of the primers (mentioned above) and 25 ng of the fungal genomic DNA as template. The PCR was standardized to get maximum amplification with least non-specificity. A negative control reaction (without DNA

template) was also set up. PCR conditions are given in [Table 1](#). The reaction was stopped by chilling the mixture at 4°C.

The PCR products along with the appropriate DNA ladder were electrophoresed on a 1 % agarose gel.

### **C) Purification of PCR products from gel:**

The gel slice containing the amplified PCR products was excised out with a sterile blade and the DNA was eluted using the Sigma Gel Extraction Kit as per the manufacturer's protocol. The eluted DNA was then checked for purity by agarose gel electrophoresis. This pure DNA was lyophilized and used for sequencing.

For the large subunit (LSU) rDNA gene sequence data acquisition, the genomic DNA of the selected fungal strain was lyophilized and sent to MIDI Labs, Newark, USA. The MicroSeq™ D2 rDNA Fungal Kit was used for this purpose. Here the primers used correspond to positions 3334 and 3630 in the *Schizosaccharomyces japonicus* LSU rRNA gene. These primers amplify a portion of the D2 region of the LSU rDNA.

### **D) Analysis of sequence data:**

All the sequences obtained were edited using the algorithm 'ChromasPro'. Two sets of sequences were obtained per PCR amplicon, one sequence obtained using the forward primer and the other using the reverse primer. For the reverse primer sequence, the reverse complement of this was derived using the algorithm 'Nucleic Acid Sequence Massager' available at the web site: <http://www.attotron.com/cybertory/analysis/seqMassager.htm>. The sequence for each rDNA was processed to remove the overlap area obtained from sequencing using forward and reverse primers. A comparative study of other rDNA sequences with the rDNA from the selected fungus was accomplished using the BLAST ([Altschul et al., 1997](#)) algorithm, at the website: <http://www.ncbi.nlm.nih.gov>. Phylogenetic and molecular evolutionary analyses in relation to NIOCC #2a were conducted using MEGA v. 4 ([Tamura et al., 2007](#)).

## 2.4 RESULTS

The sampling site for the isolation of lignin-degrading fungi was the mangrove swamps of Choraõ Island in Goa, India. The physico-chemical conditions prevalent at the site at the time of sampling are shown in Table 2. The physico-chemical parameters of the mangrove sites at the sampling times varied greatly between the pre-monsoon (sampling I) and monsoon periods (sampling II & III).

**Table 2: Physico-chemical conditions of the estuarine water prevalent at the sampling site (mangrove swamps) of Choraõ island in Goa, India.**

S. No	DATE	TIME	TEMP	CHEMICAL CHARACTERISTICS			
				pH	DISSOLVED OXYGEN CONTENT (mg L <sup>-1</sup> )	CHLORIDE CONTENT (g L <sup>-1</sup> )	SALINITY (g L <sup>-1</sup> )
I	29.04.2003	4.00 pm	34°C	7.8	9.6	19.99	36.68
II	03.07.2003	7.45 am	27°C	7.2	13.44	0.175	0.321
III	13.08.2003	6.15 am	28°C	7.1	8.24	0.154	0.238

Fungi were isolated from mangrove detritus by the single-spore-isolation and particle-plating methods (Table 3). Leaves were chosen due to their high lignocellulosic content (Kumaresan *et al.*, 2002). Although the particle-plating method using mangrove leaves yielded maximum number of fungal isolates, none of them were found to be laccase-positive. Mangrove wood, however with their greater lignin content yielded laccase positive fungal strains. The single spore isolation yielded several laccase-positive isolates. Wherever possible, morphology of fruiting bodies and spores was used in the identification of some of fungal isolates using classical light microscopy. The spores of

the obligate marine fungus, *Halocyphina villosa* had been isolated only during the first sampling but could not be maintained *in-vitro*.

**Table 3: Fungi obtained by screening for laccase production, their source and method of isolation.**

S. No	SOURCE	ISOLATION METHOD	LACCASE PRODUCTION	ISOLATES & IDENTIFICATION
1	Decaying wood	Hyphal isolation	Positive	NIOCC #1, NIOCC #2a, NIOCC #7a & NIOCC #Xa (non-sporulating fungi)
2	Decaying wood	Single spore isolation	Positive	NIOCC #2, NIOCC #6, NIOCC #11, NIOCC #13 & NIOCC #15 are <i>Cirrenalia pygmea</i> (Kohlm); NIOCC #X ( <i>Cirrenalia pseudomacrocephala</i> ), NIOCC #4 ( <i>Thielavia terricola</i> [Gilman & Abbot Emmons]) & NIOCC #8, NIOCC #5, NIOCC #9 & NIOCC #10
3	Decaying leaves	Particle plating	Negative	57 isolates

The identification of isolates was carried out by the classical method using their teleomorphic state.

**Table 4: Qualitative tests for dye decolourization by the laccase-positive fungi.**

ISOLATE	DYE	LOW NITROGEN MEDIUM		MALT EXTRACT MEDIUM	
		GROWTH	DECOLOURIZATION	GROWTH	DECOLOURIZATION
NIOCC #2a & NIOCC #Xa	Brilliant Green	++++	++++	++++	++++
	Crystal Violet	++++	++++	++++	++++
	Congo Red	++++	++++	++++	++++
	Poly-R 478	++++	++++	++++	++++
	Remazol Brilliant Blue R	++++	++++	++++	++++
	Methylene Blue	++++	++++	++++	++++
	Aniline Blue	++++	++++	++++	++++
	Trypan Blue	++++	++++	++++	++++
NIOCC #7a	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	++	++	++	+
	Poly-R 478	++	++	++	+
	Remazol Brilliant Blue R	++	++	++	++
	Methylene Blue	++	++	++	+
	Aniline Blue	++	++	++	++
	Trypan Blue	++	++	++	++
NIOCC #1	Brilliant Green	-	-	-	-
	Crystal Violet	+	-	+	-
	Congo Red	+	+	+	+
	Poly-R 478	+	+	+	++
	Remazol Brilliant Blue R	+++	+++	+++	+++
	Methylene Blue	++	+	++	++
	Aniline Blue	+	+	++	++
	Trypan Blue	++	++	++	++
NIOCC #4	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	++	++	++	++
	Methylene Blue	++	++	++	+
	Aniline Blue	++	++	++	+
	Trypan Blue	++	++	++	++
NIOCC #9	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	+	-	-
	Poly-R 478	+	+	+	-
	Remazol Brilliant Blue R	+	+	+	+
	Methylene Blue	+	-	+	-
	Aniline Blue	+	+	+	+
	Trypan Blue	+	+	+	+

Ph.D. Thesis

**KEY:**

SYMBOL	GROWTH	DECOLOURIZATION
++++	Excellent	Complete
+++	Good	Good
++	Average	Average
+	Poor	Poor
-	None	None

**Table 4 (contd): Qualitative tests for dye decolourization by the laccase-positive fungi.**

ISOLATE	DYE	LOW NITROGEN MEDIUM		MALT EXTRACT MEDIUM	
		GROWTH	DECOLOURIZATION	GROWTH	DECOLOURIZATION
NIOCC #5 & NIOCC #10	Brilliant Green	+	-	-	-
	Crystal Violet	+	-	+	+
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	+	+	+	+
	Aniline Blue	+	-	+	+
	Trypan Blue	+	-	+	-
NIOCC #3	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	-	++	-
	Poly-R 478	+	-	++	+
	Remazol Brilliant Blue R	+	-	++	-
	Methylene Blue	+	-	++	-
	Aniline Blue	+	-	++++	++++
	Trypan Blue	+	-	++	-
NIOCC #8	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	-	-	+	+
	Aniline Blue	+	-	+	-
	Trypan Blue	+	-	+	+
NIOCC #2, NIOCC #6, NIOCC #11, NIOCC #13 & NIOCC #15	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	+	-
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	+	-	+	+
	Aniline Blue	+	-	+	-
	Trypan Blue	+	-	+	-
NIOCC #X	Brilliant Green	-	-	-	-
	Crystal Violet	+	-	-	-
	Congo Red	+	-	-	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	+	-	-	-
	Aniline Blue	+	-	-	-
	Trypan Blue	+	-	-	-



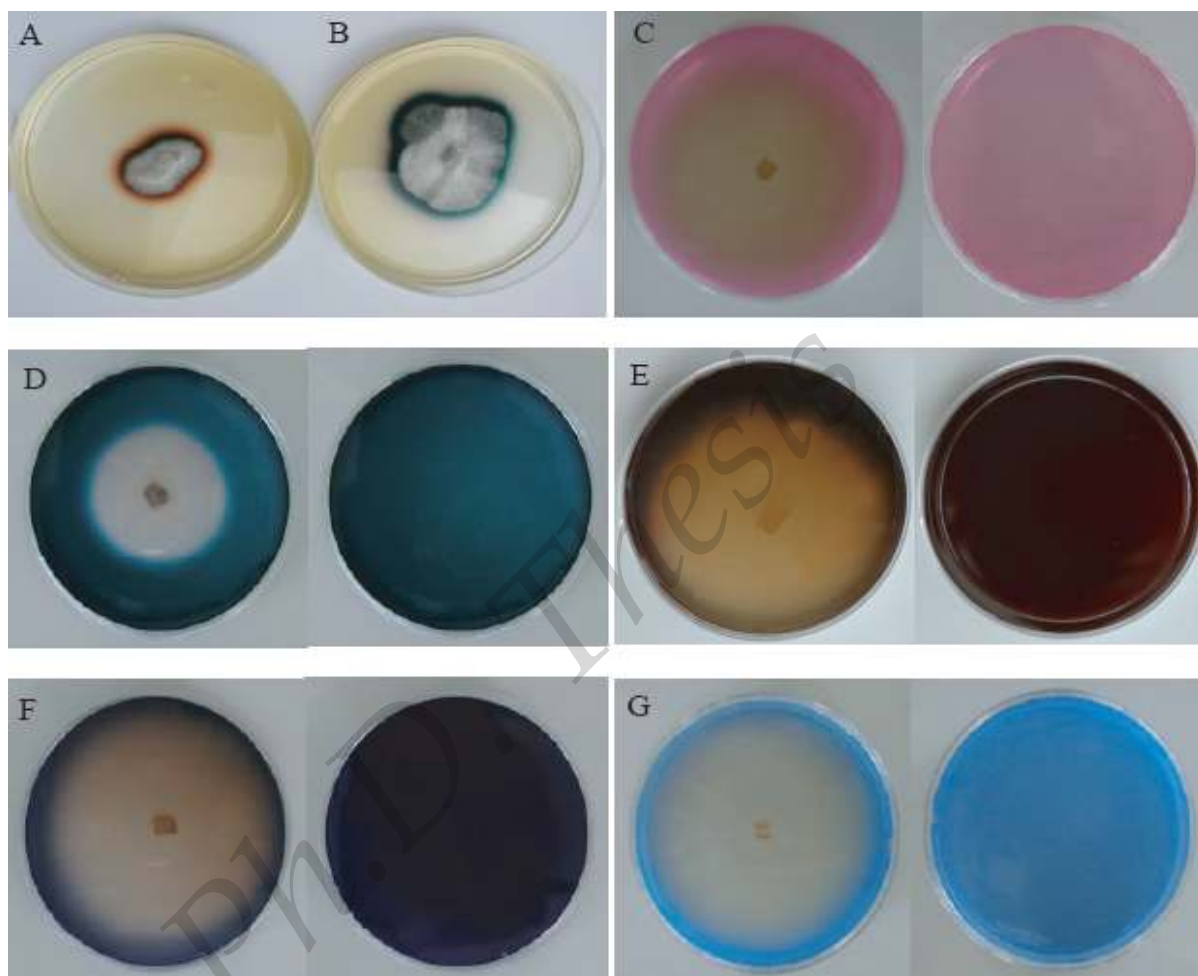
All the laccase-positive isolates have been prefixed with ‘NIOCC’ and deposited at the National Institute of Oceanography Culture Collection (NIOCC). Table 4 shows qualitatively, the decolourization potential of all the laccase positive isolates when grown on two types of solid media in the presence of various dyes. The solid media included the low nitrogen (LN) mineral medium and high nitrogen medium such as malt extract agar (MEA).

**Table 5: Production of laccase, MnP & LiP by 6 day-old cultures of the laccase-positive fungi, when grown in LN liquid medium prepared in half strength seawater.**

ISOLATE	LACCASE (U L <sup>-1</sup> )	MnP (U L <sup>-1</sup> )	LiP (U L <sup>-1</sup> )
#2a	30857	77	0.7
#Xa	16000	68	0
#7a	5714	2	0.4
#1	66	39	0.2
#4	51	2	0
#X	46	2	0
#5	40	0	0
#9	23	8	0
#10	23	0	0
#3	17	106	0.2
#15	17	2	0
#11	11	0	0.2
# 8	11	0	0
#13	6	3	1.1
# 2	6	0	0.2

Table 5 shows the quantitative production of the LDEs, laccase, MnP & LiP of all the laccase positive isolates when grown in LN medium prepared in half strength sea water. An intense brown colour obtained from the oxidation of guaiacol (Fig. 2A) and deep green colour from ABTS oxidation (Fig. 2B) indicated laccase activity. Transformation of the pink coloured Poly-R 478 dye (Fig. 2C) by NIOCC # 2a to brown, was indicative of

peroxidase activity. **Figures 2 C-E** displays qualitatively, the dye decolourization / transformation potential of NIOCC #2a.



**Fig. 2: Qualitative determination of laccase activity with (A) Guaiacol at 4 mM and (B) ABTS at 2 mM. Qualitative determination of dye decolourization potential with (C) Poly-R 478, (D) Methylene blue and (E) Congo red at 0.02 %; (F) Trypan blue and (G) Aniline blue at 0.04 % concentration.**

A 7 day-old culture of NIOCC #2a was grown in LN medium prepared in half strength seawater containing the indicators at the above given concentrations. For the dyes (**Fig. 2 C-G**), the corresponding control plate, without culture is on the right hand side of the test plate.

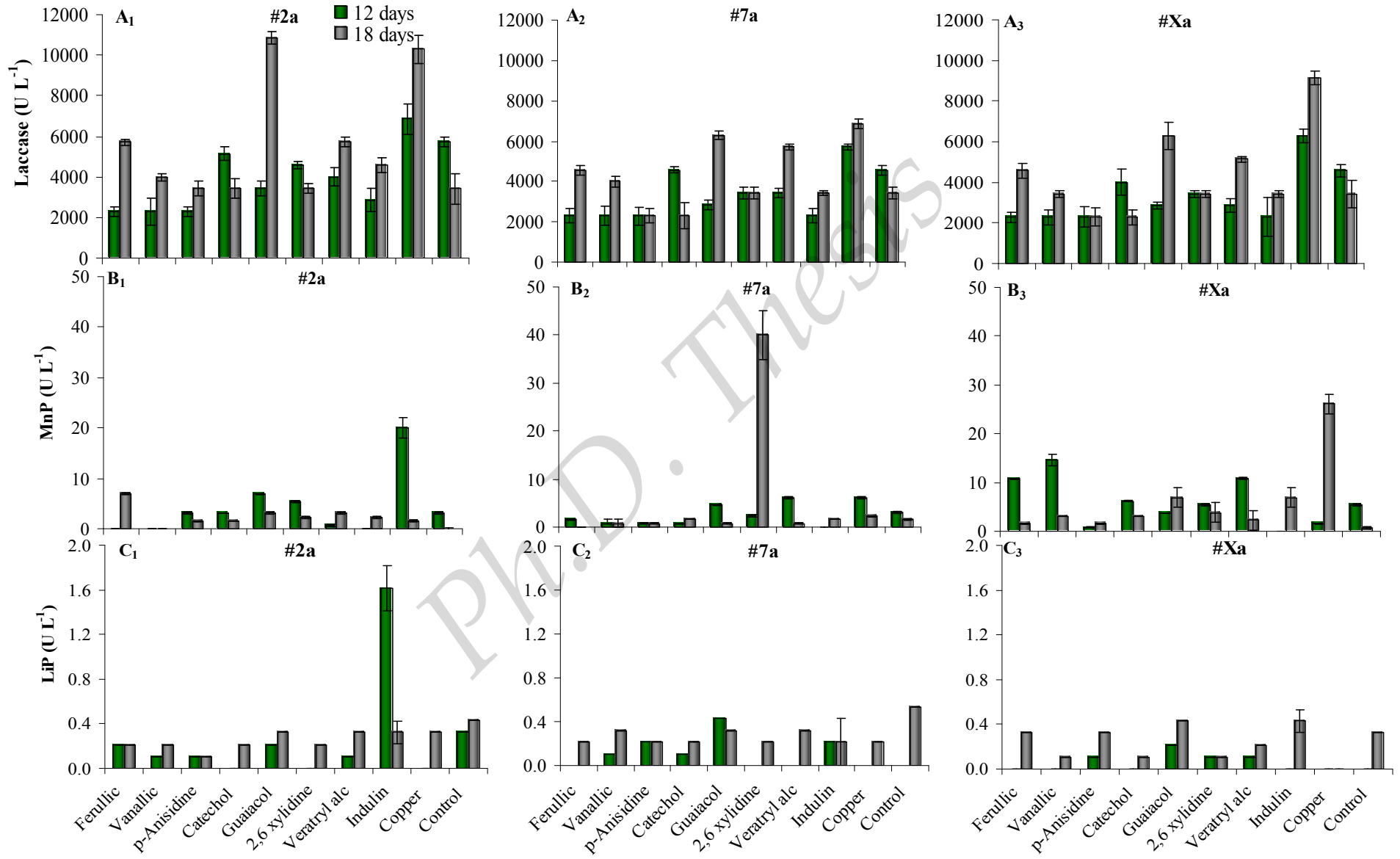


Fig. 3: LDE production by NIOCC #2a, #7a & #Xa in LN medium supplemented with various inducers estimated on day 12 & 18.

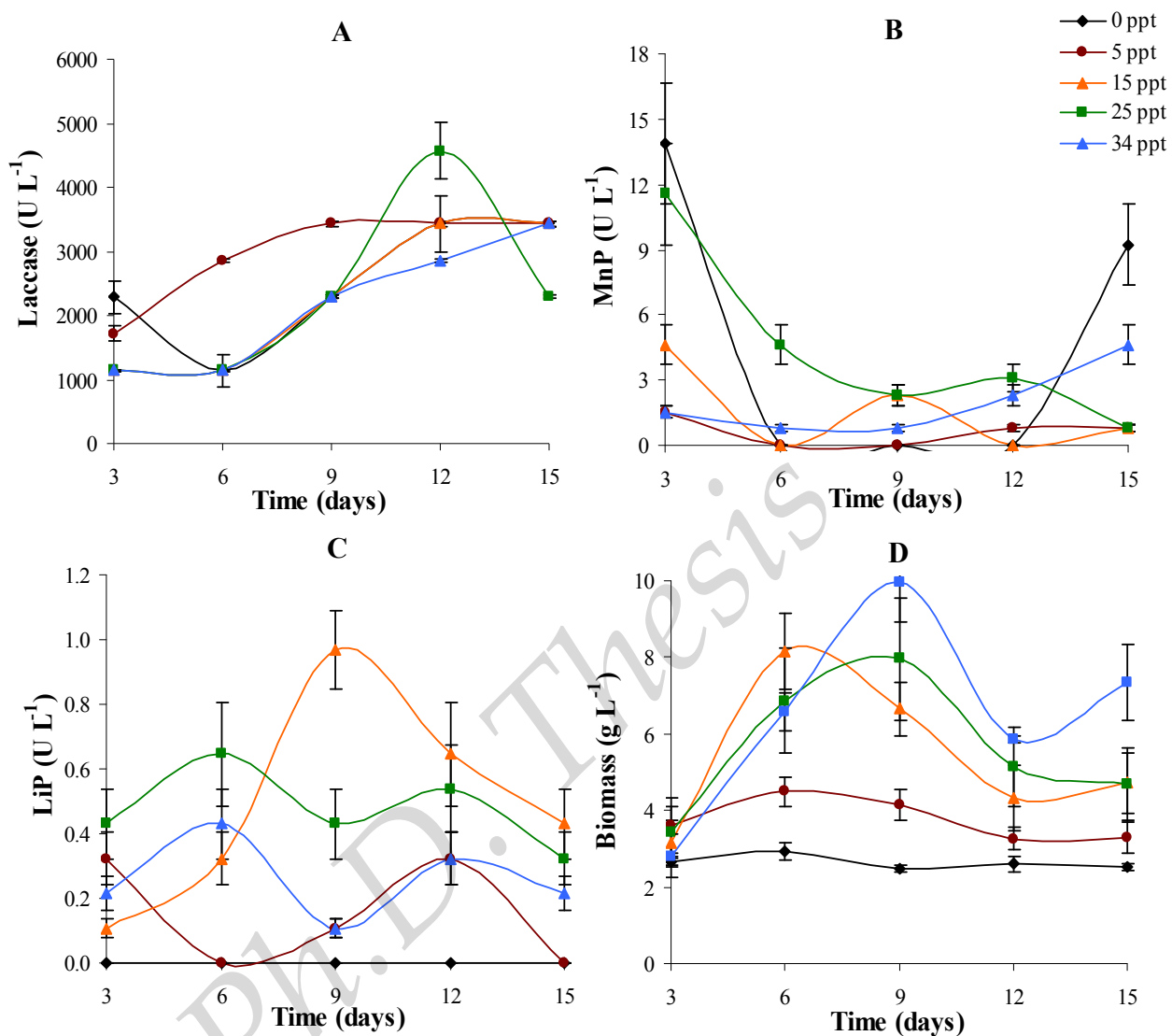
The best laccase-producers; NIOCC #2a, #7a & #Xa were all anamorphs. Growth of these fungi in LN medium in the presence of various inducers, was compared and the production of the LDEs was determined as shown in Fig. 3. Figure 3A<sub>1</sub> - A<sub>3</sub> represented laccase production by NIOCC #2a, #7a and #Xa respectively; Fig. 3B<sub>1</sub>- B<sub>3</sub> represented MnP production by NIOCC #2a, #7a and #Xa respectively whereas Fig. 3C<sub>1</sub> - C<sub>3</sub> represented LiP production by NIOCC #2a, #7a and #Xa respectively.

Results show that one the whole NIOCC #2a performed better than NIOCC #7a and NIOCC #Xa, especially with regard to laccase production.

<b>Day</b>	<b>Source of Variation</b>	<b>df</b>	<b>F</b>	<b>P-value</b>	<b>F critical</b>
12	Inducer	9	59	1.7E-11	2
	Culture	2	13	0.00037	4
18	Inducer	9	17	5E-07	2
	Culture	2	6	0.00954	4

Since laccase was the focus of study and the peroxidase titer as compared to laccase in all cases was negligible, a two-way ANOVA was carried out for only laccase production on day 12 and 18 separately between the different inducers used and the 3 isolates (NIOCC #2a, #7a & #Xa). From the above *p*-values, it was clear that both, the type of inducer as well as the isolate used, showed significant difference in laccase production. From the average values from Fig. 3 as well as from Table 4, it is obvious that NIOCC #2a was better than the other two anamorphs and thus was selected for further investigation in this thesis.

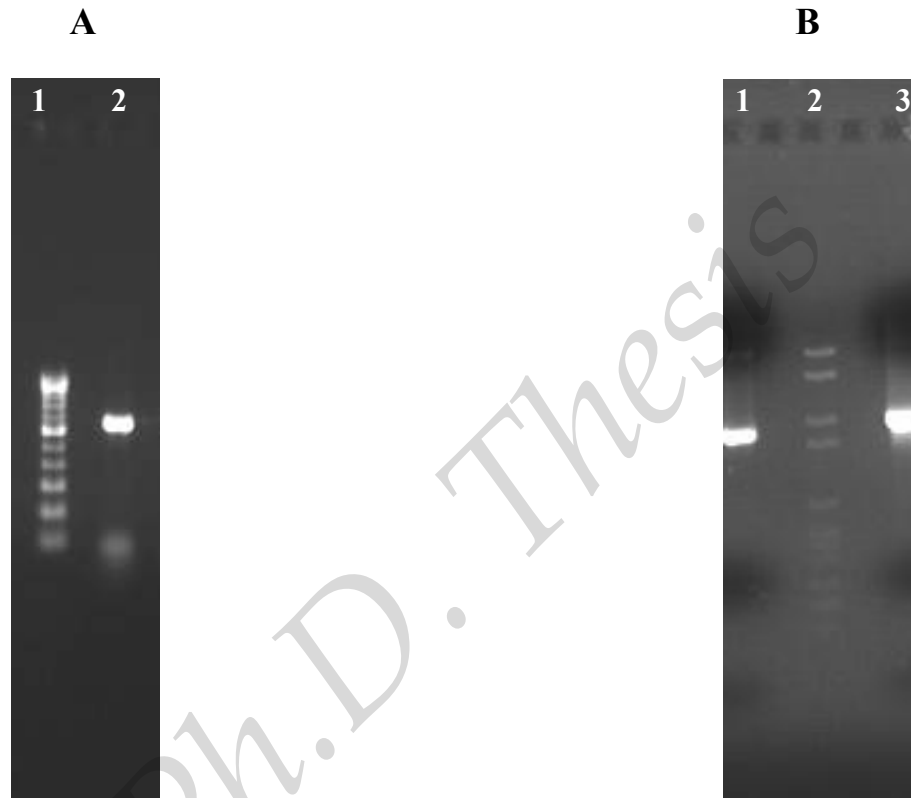
The temporal production of biomass and the LDEs of NIOCC #2a was determined in B&K medium prepared in varying salinities (calculated in terms of parts per thousand, ppt) (Fig. 4). The MnP (Fig. 4 B) and LiP (Fig. 4 C) production was very low. However, from Fig. 4 A, it is observed that maximum laccase production occurred on day 12 in 25 ppt seawater and maximum biomass (Fig. 4 D) was attained in full strength seawater for most of the days sampled.



**Fig. 4: Temporal production of (A) Laccase; (B) MnP; (C) LiP and (D) Biomass.**

NIOCC #2a was grown in B&K medium prepared in seawater of varying salinities from 0 to full strength sea water (34 ppt).

Having confirmed that the marine-derived NIOCC #2a was a good laccase producer, it was deposited in the Microbial Type Culture Collection (MTCC, Chandigarh, India) under the accession number MTCC 5159. It was also deposited under the Budapest treaty for patent culture deposition (Raghukumar & D'Souza, 2004). Henceforth, NIOCC #2a is referred to as MTCC 5159.



**Fig. 5: PCR amplicon of the region of (A) ITS 1 - 5.8 S - ITS 2 and (B) 18 S rDNA of MTCC 5159**

Lane 1: 100 bps – 1 kb marker

Lane 2: ITS 1-5.8S-ITS 2 rDNA PCR amplicon (~ 650 bps)

Lane 1: 'NS<sub>1</sub>- NS<sub>4</sub> region' of 18S rDNA amplicon (~1033 bps)

Lane 2: 150 bps – 2.2 kb marker

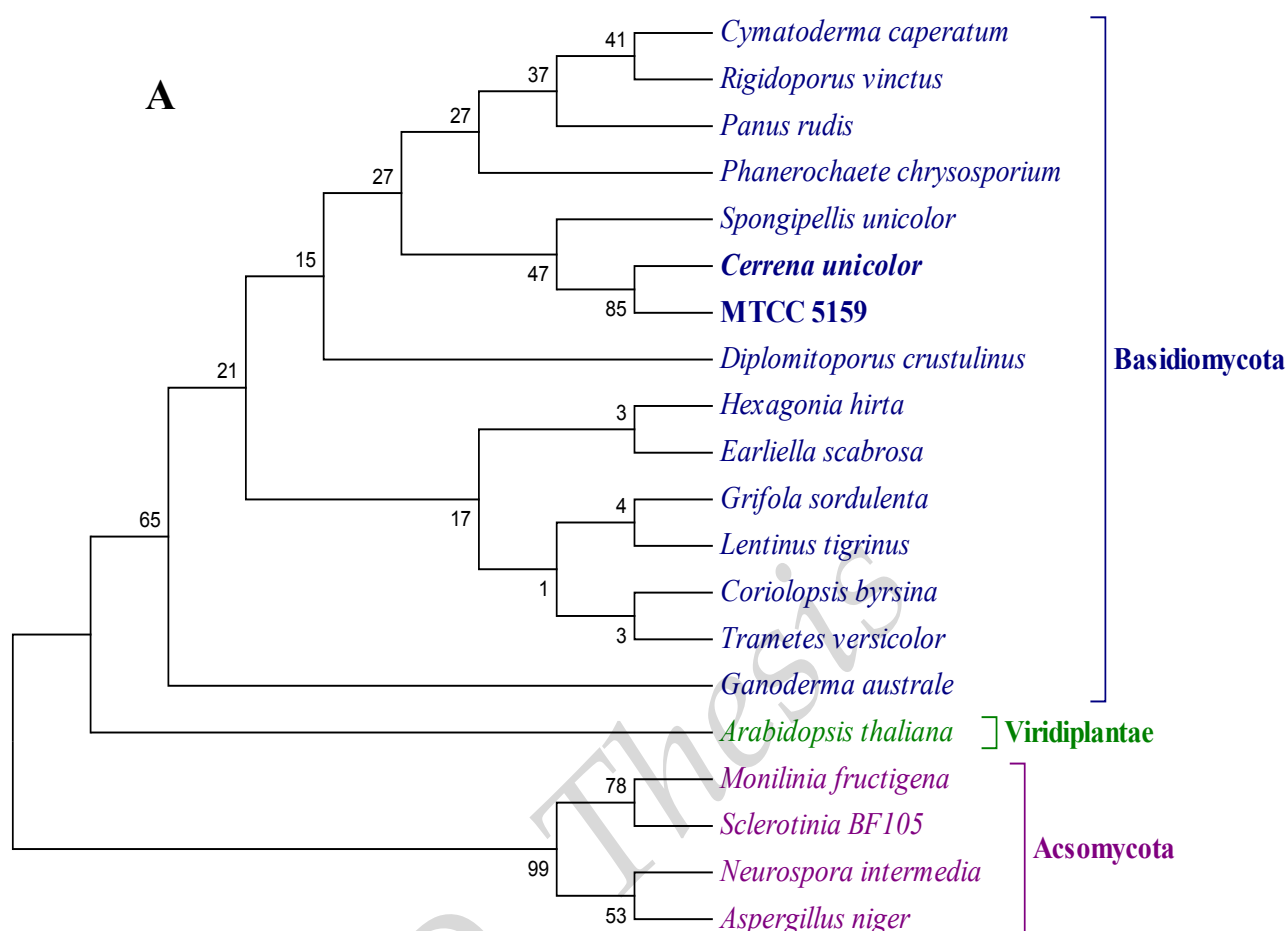
Lane 3: 'NS<sub>3</sub>- NS<sub>8</sub> region' of 18S rDNA amplicon (~1230 bps)

Pure cultures of MTCC 5159 on MEA were all of identical appearance. The colony consisted of rich white cottony aerial mycelium. The reverse of the colonies was colourless; no pigments were produced even after 1 month of cultivation. Several attempts to bring MTCC 5159 to sporulate such as the use of carrot juice agar and wood pieces in carrot juice agar (Appendix 8.1.4), were not successful. As the morphology was uninformative, rDNA sequence comparison was used for the identification. (Fig. 5A & B). Here the rDNA sequence of MTCC 5159 was compared with the rDNA sequences of other fungi present in the database. To confirm the identification, three separate stretches of rDNA from MTCC 5159 were used namely, the 18S, the D1/D2 region of the 25-28 S and the ITS1-5.8S-ITS2 rDNA regions. The sequences for the same have been deposited in the gene bank under the following accession numbers; EF059806, AY939878 & AY939879 respectively.

The phenograms reflecting the phylogenetic relationship between MTCC 5159 constructed using data from the BLAST analysis of the various rDNA regions of MTCC 5159 are shown separately in Figs. 6A, B & C and the details along with the accession numbers of the sequences used to construct the trees are given in Tables 6A, B & C.

In Tables 6A, B & C, some hits have not been listed due to the following reasons:

1. Once the closest hit (both genus & species name required) to MTCC 5159 was listed, the other hits denoting the same genus but different species were not included.
2. When the species name was missing. (An exception is *Sclerotinia* BF 105, since the species name is not available for this genus and this belongs to only the out-group). Note the out-group genera have been kept common within the 18S, ITS1-5.8S-ITS2 & D1/D2 phenograms. Another exception was the phenograms drawn with ITS1-5.8S-ITS2 rDNA sequence data, since unidentified fungi showed greater homology than the first positively identified hit, *Cerrena unicolor*, these fungi have been listed.



**Fig. 6: (A) Phenogram constructed from 18S rDNA sequence homologues of MTCC 5159.**

The accession numbers along with other details of the members belonging to Class Basidiomycota in the above figure are given in Table 6A. The accession numbers of the out-group members are given below:

Viridiplantae: *Arabidopsis thaliana* (X16077)

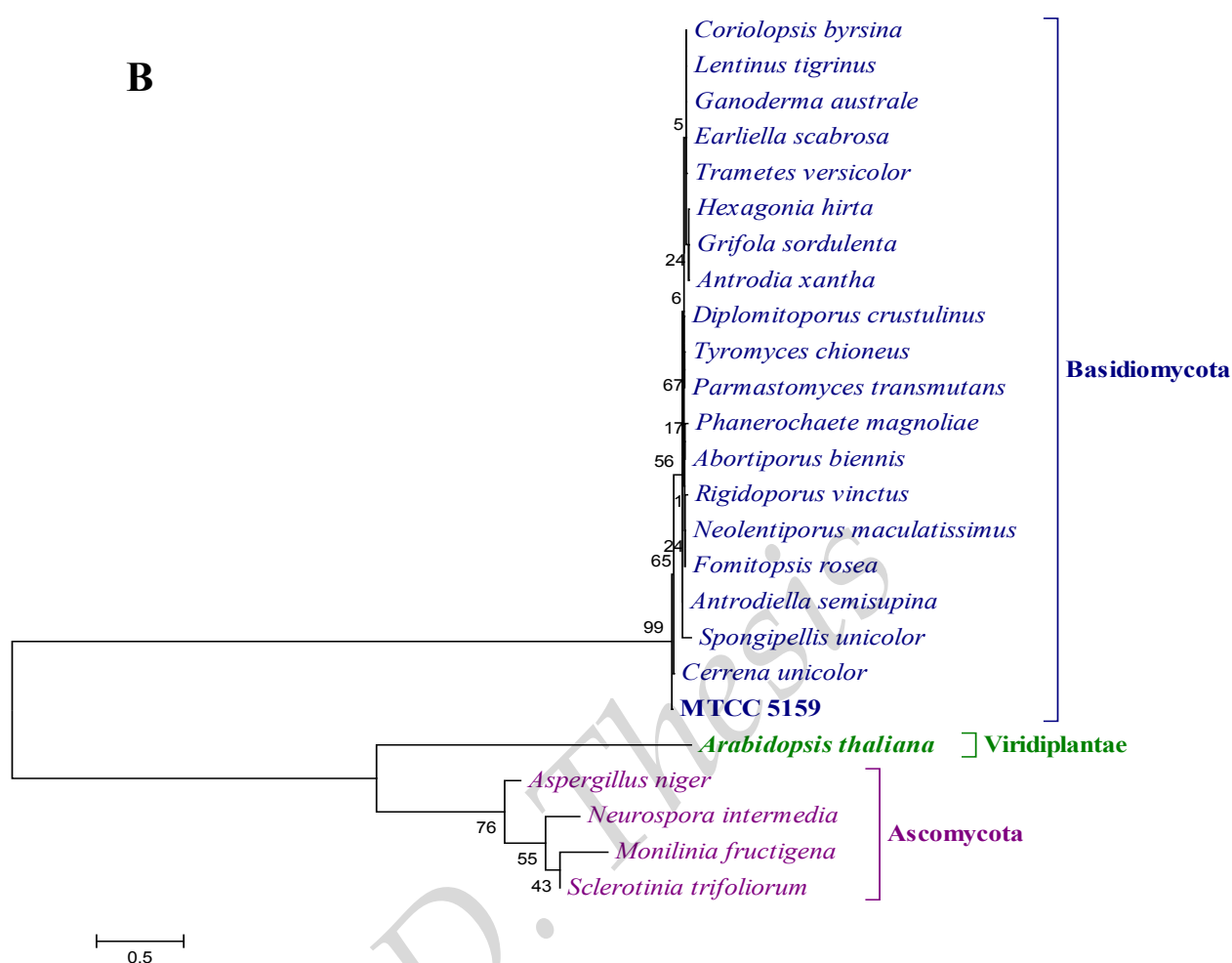
Ascomycota: *Monilia fructigena* (EF207429)  
*Sclerotinia BF 105* (AM901701)  
*Neurospora intermedia* (EF197071)  
*Aspergillus niger* (AB305102)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are not significant. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 359 positions in the final dataset.



**Table 6A: Sequences producing significant alignments with MTCC 5159 18S rDNA (EF059806.1)**

<b>Accession No.</b>	<b>Description</b>	<b>Max score</b>	<b>Total score</b>	<b>Query coverage</b>	<b>E value</b>	<b>Max identity</b>
AY850007.1	<i>Cerrena unicolor</i>	2058	2058	100 %	0.0	99 %
M59760.1	<i>Spongipellis unicolor</i>	2025	2025	100 %	0.0	98 %
AF082849.1	<i>Cymatoderma caperatum</i>	2025	2025	100 %	0.0	98 %
AY665780.1	<i>Grifola sordulenta</i> isolate AFTOL-ID 562	2013	2013	100 %	0.0	98 %
AF026569.1	<i>Panus rudis</i>	2010	2010	100 %	0.0	98 %
	:					
AY336766.1	<i>Earliella scabrosa</i> strain Wu 9704-83	2008	2008	100 %	0.0	98 %
	:					
AY336773.1	<i>Coriolopsis byrsina</i> strain CRM-46	1997	1997	100 %	0.0	98 %
AY336770.1	<i>Diplomitoporus crustulinus</i> strain FP 101824	1997	1997	100 %	0.0	98 %
	:					
AY336763.1	<i>Ganoderma australe</i> strain Wu 9302-56	1997	1997	100 %	0.0	98 %
AY336760.1	<i>Hexagonia hirta</i> strain CBS 515.96	1997	1997	100 %	0.0	98 %
	:-----:-----:-----:-----:-----:					
AY946269.1	<i>Lentinus tigrinus</i>	1997	1997	100 %	0.0	98 %
AY336751.1	<i>Trametes versicolor</i> strain Wu 9507-7	1980	1980	100 %	0.0	97 %
AY336752.1	<i>Rigidoporus vinctus</i> strain Chen 674	1953	1953	100 %	0.0	97 %
AF026593.1	<i>Phanerochaete chrysosporium</i>	1947	1947	100 %	0.0	97 %



**Fig. 6: (B) Phenogram constructed from D1/D2 domains of the 25-28S rDNA sequence homologues of MTCC 5159**

The accession numbers along with other details of the members belonging to Class Basidiomycota in the above figure are given in Table 6B. The accession numbers of the out-group members are given below:

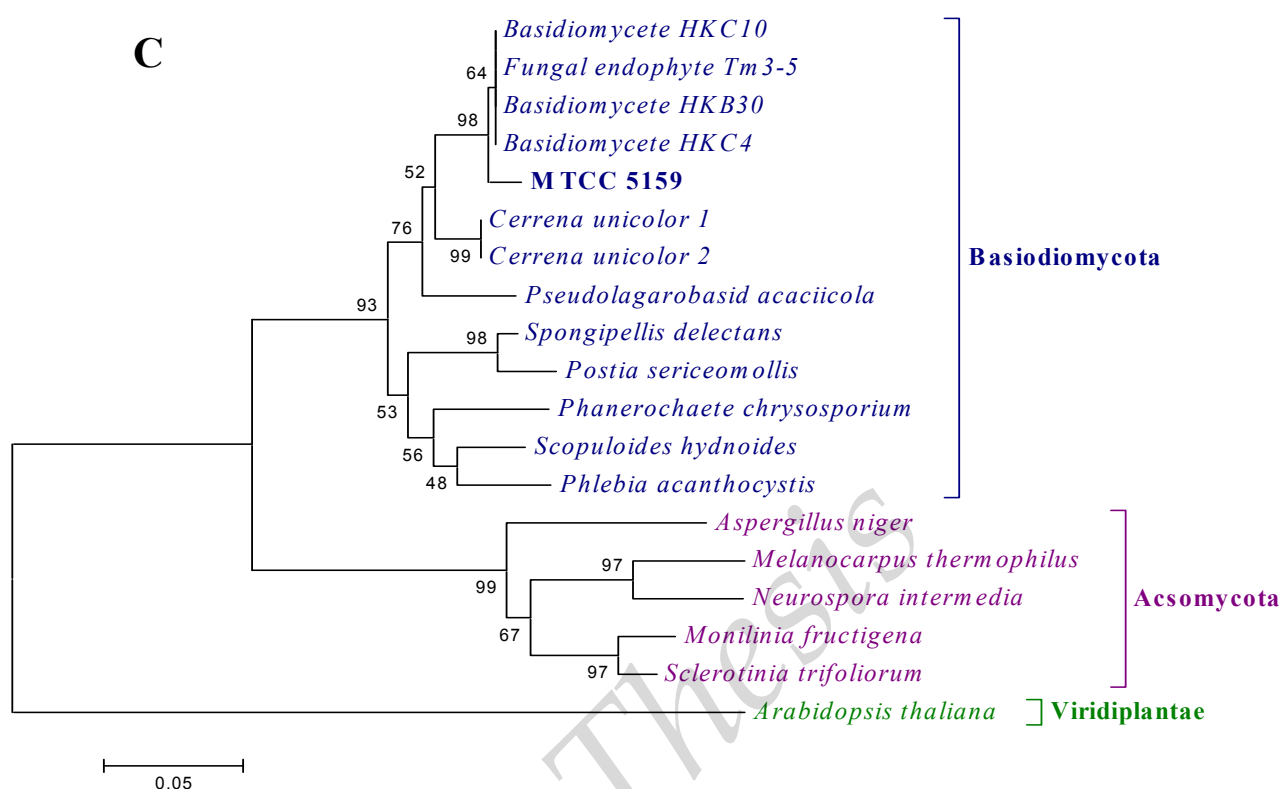
Viridiplantae: *Arabidopsis thaliana* (X52320)

Ascomycota: *Aspergillus niger* (AB305102)  
*Neurospora intermedia* (EF197071)  
*Monilia fructigena* (EF207429)  
*Sclerotinia trifoliorum* (DQ904361)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are not significant. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 409 positions in the final dataset.

**Table 6B: Sequences producing significant alignments with MTCC 5159 25-28S rDNA (AY939878.2)**

<b>Accession No.</b>	<b>Description</b>	<b>Max score</b>	<b>Total score</b>	<b>Query coverage</b>	<b>E value</b>	<b>Max identity</b>
AY850007.1	<i>Cerrena unicolor</i>	1875	1875	100 %	0.0	98 %
AF334899.1	<i>Abortiporus biennis</i>	1851	1851	100 %	0.0	98 %
	:					
M59760.1	<i>Spongipellis unicolor</i>	1842	1842	100 %	0.0	98 %
AF334902.1	<i>Antrodia xantha</i>	1840	1840	100 %	0.0	98 %
AY336770.1	<i>Diplomitoporus crustulinus</i>	1829	1829	100 %	0.0	98 %
	:					
AY336766.1	<i>Earliella scabrosa</i> strain Wu 9704-83	1829	1829	100 %	0.0	98 %
AY336764.1	<i>Fomitopsis rosea</i> strain RLG 6954	1829	1829	100 %	0.0	98 %
AF334938.1	<i>Tyromyces chioneus</i>	1829	1829	100 %	0.0	98 %
	:					
AY336773.1	<i>Coriolopsis byrsina</i> strain CRM-46	1823	1823	100 %	0.0	97 %
AY665780.1	<i>Grifola sordulenta</i> isolate AFTOL-ID 562	1823	1823	100 %	0.0	97 %
AY293143.1	<i>Parmastomyces transmutans</i>	1821	1821	100 %	0.0	97 %
	:					
AY336774.1	<i>Antrodiella semisupina</i> strain FCUG 1216	1818	1818	100 %	0.0	97 %
	:					
AY336760.1	<i>Hexagonia hirta</i> strain CBS 515.96	1818	1818	100 %	0.0	97 %
AF334921.1	<i>Neolentiporus maculatissimus</i>	1816	1816	100 %	0.0	97 %
AY336763.1	<i>Ganoderma australe</i> strain Wu 9302-56	1812	1812	100 %	0.0	97 %
	:-----:-----:-----:-----:-----:					
AB084596.1	<i>Phanerochaete magnoliae</i>	1812	1812	100 %	0.0	97 %
AY946269.1	<i>Lentinus tigrinus</i>	1812	1812	100 %	0.0	97 %
AY336752.1	<i>Rigidoporus vinctus</i> strain Chen 674	1807	1807	99 %	0.0	97 %
AY309019.1	<i>Trametes versicolor</i> strain BCRC 36387	1807	1807	100 %	0.0	97 %



**Fig. 6: (C) Phenogram constructed from ITS1-5.8S-ITS2 rDNA sequence homologues of MTCC 5159.**

The accession numbers along with other details of the members belonging to Class Basidiomycota in the above figure are given in Table 6C. The accession numbers of the out-group members are given below:

Viridiplantae: *Arabidopsis thaliana* (U43225)

Ascomycota: *Aspergillus niger* (AB305102)  
*Melanocarpus thermophilus* (AJ271586)  
*Neurospora intermedia* (EF197071)  
*Monilia fructigena* (EF207429)  
*Sclerotinia trifoliorum* (DQ904361)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 1.04232160 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 212 positions in the final dataset.

**Table 6C: Sequences producing significant alignments with MTCC 5159 ITS1-5.8S-ITS2 rDNA (AY939879.1)**

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
EF029829.1	Basidiomycete sp. HKC10 (from marine sponges)	586	586	100 %	2e-164	99 %
EF029823.1	Basidiomycete sp. HKC4 (from marine sponges)	586	586	100 %	2e-164	99 %
EF029817.1	Basidiomycete sp. HKB30 (from marine sponges)	586	586	100 %	2e-164	99 %
	:					
AY456192.1	Fungal endophyte Tm3-5	586	586	100 %	2e-164	99 %
	:					
EF577058.1	<i>Cerrena unicolor</i> (from <i>Tremex fuscicornis</i> )	448	448	100 %	9e-123	91 %
DQ056858.1	<i>Cerrena unicolor</i>	442	442	100 %	4e-121	91 %
	:					
DQ517883.1	<i>Pseudolagarobasidium acaciicola</i> strain CBS 115543	416	416	100 %	3e-113	89 %
	:					
AY089734.1	<i>Postia sericeomollis</i> isolate Brunn-75	366	366	100 %	3e-98	87 %
AJ006670.1	<i>Spongipellis delectans</i> Sample 26	357	357	67 %	2e-95	95 %
AB210074.1	<i>Phlebia acanthocystis</i> TMIC 35095	342	342	97 %	4e-91	86 %
	:					
EU118665.1	<i>Scopuloides hydroides</i> voucher KHL 11916 (GB)	339	339	66 %	6e-90	94 %
	:-----:-----:-----:-----:-----:-----:					
AY854086.1	<i>Phanerochaete chrysosporium</i> isolate AFTOL-ID 776	298	298	66 %	1e-77	91 %

MTCC 5159 18S rDNA (EF059806) and D1/D2 of 25-28S rDNA (AY939878) showed 99 % and 98 % homology respectively with *Cerrena unicolor* (AY850007). MTCC 5159 (AY939879) showed 91 % identity with two *Cerrena unicolor* sequences (EF577058.1 & DQ056858.1), which were the closest completely identified sequences, from ITS1-5.8S-ITS2 rDNA homology.

## 2.5 DISCUSSION

Mangroves harbor a rich diversity of mycota. Fungi that live in these environments are mostly saprophytic in lifestyle; some of them however also live as endophytes. Lignicolous fungi are best isolated from substrates rich in lignin and in a state of decay. Mangrove detritus was collected during the low tide at all times since it has been shown that slightly higher number and type of fungal species are present compared to during high tides (Besitulo *et al.*, 2002). Detritus from such sites would have been submerged for longer periods resulting in a more diversified mycota. Presence of fouling organisms, especially the remains of calcareous shells and bore-holes on the wood samples indicate that they had been subjected to a considerable period of marine submersion. This translates into a greater chance of isolating marine-derived lignicolous fungi (Vrijmoed, 2000).

The mangrove swamps are regularly flushed with fresh water from the rivers. This causes a great variation in salinity and other physico-chemical parameters in the estuarine areas. These parameters also varied greatly due to the varying conditions brought by the pre-monsoon and monsoon periods. Together, these conditions tend to support a rich diversity of marine and marine-derived fungi and affect the abundance and diversity of species in the mangroves. Species abundance and diversity also have been influenced by other factors such as the nature of the substrata (e.g. host plant species, age of plant parts), the condition of the substrata (e.g. duration period of submergence) and the maturity and diversity of the mangrove vegetation itself (Abdel-Wahab & El-Sharouny, 2002).

Leaves were chosen due to their high lignocellulosic content (Kumaresan *et al.*, 2002) Although the particle-plating method using mangrove leaves yielded

maximum number of fungal isolates, none of them were found to be laccase-positive. Wood of tree trunks however with their greater lignin content yielded the laccase producing fungal strains. Wherever possible, morphology of fruiting bodies and spores (sexual and asexual) were used in the identification of some of fungal isolates using classical light microscopy. *Halocyphina villosa* is considered to be the most commonly occurring tropical obligate marine fungus in mangroves (Hyde & Jones, 1988). Its spores had been isolated during the first sampling in the presence of highly saline conditions but could not to be maintained *in-vitro*. Being an obligate marine form, salinity obviously has a great impact, which could account for its absence in the last two sampling when salinities were low.

From Table 4 and Table 5, it was clear that the laccase production and decolourization potential of the sporulating laccase-positive fungal isolates were much lower than the three anamorphic isolates, namely MTCC 5159 (NIOCC #2a), #7a & #Xa. Hence, no further emphasis had been given to the identification of the sporulating forms. From Table 5 it was evident that MTCC 5159 was superior to the other two anamorphs; NIOCC #7a & NIOCC #Xa. However, to alleviate the doubt whether the comparatively poor performance of these two anamorphs was only due to the medium composition, the LDE production of all the three anamorphs in LN medium, which is known to support high laccase production (Raghukumar *et al.*, 1994) prepared in half-strength seawater, in the presence of various inducers was determined (Fig. 3). The results further emphasized the superiority of MTCC 5159.

However, it was required to confirm that MTCC 5159 isolated during the 3<sup>rd</sup> sampling is actually marine-derived fungus and not a terrestrial one. Since in the last two samplings, the salinity of the estuarine area was closer to fresh water than seawater due to the combined effects of the monsoons as well as the low tide conditions prevalent at that time. For this MTCC 5159 was tested for growth and LDE production at different time intervals, in varying salinities in B&K medium. Maximum biomass in full strength seawater and maximum laccase production in 25 ppt seawater confirmed that MTCC 5159 was in fact a marine-derived or salt tolerant fungus.

MTCC 5159 was identified using rDNA homology. The 18S and D1/D2 of 25-28S rDNA of MTCC 5159 showed maximum identity with *Cerrena unicolor*

(AY850007) at 99 % and 98 % respectively. This because in the case of EF059806 as well as AY939878, the first BLAST hit was AY850007, which was an 18S rDNA sequence.

From ITS1-5.8S-ITS2 rDNA homology, the closest two completely identified sequences were both *Cerrena unicolor*. Here MTCC 5159 (AY939879.1) had 91 % identity with both the *Cerrena unicolor* sequences (EF577058.1 & DQ056858.1). Since the ITS1-5.8S-ITS2 rDNA is a hyper variable region, i.e. this region reflects more variation than either the 18S or the 25-28S rDNA. The evolutionary distance resolved by ITS, is usually restricted to demarcating within the species level and cannot be completely relied above genus level, like 18S or 25-28S rDNA. Organisms showing more than 90 % similarity can be considered as belonging to the same genus (Sugita & Nishikawa, 2003). Whereas for 18S rDNA sequence homology,  $\geq 99$  % sequence identity can safely be considered as belonging to the same genus (Guarro *et al.*, 1999). The 18S, 25-28S and ITS1-5.8S-ITS2 rDNA sequence homology leads to the conclusion that MTCC 5159 belongs to the genus *Cerrena* and species *unicolor*. **MTCC 5159 is designated as *Cerrena unicolor* MTCC 5159, henceforth.**

The taxonomical hierarchy for *Cerrena unicolor* MTCC 5159 is as follows: Eukaryota, Fungi, Dikarya, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetes incertae sedis, Polyporales, Coriolaceae, *Cerrena unicolor* MTCC 5159.

A few other facts also corroborate with the source of isolation and identification of *Cerrena unicolor* MTCC 5159:

1. ***Cerrena unicolor* MTCC 5159 is an anamorphic lignicolous marine-derived fungus.** Mangicolous fungi are adapted to grow and reproduce in a wide range of salinities; this allows them to inhabit the intertidal brackish water in mangroves. This point has been observed in the genus *Halophytophthora*, some species of this genus reproduce asexually in response to fluctuating salinities and/or temperatures and to a change of dry and wet conditions. This teleomorphic to anamorphic adaptation of this species had allowed them to flourish in the mangrove



environments, where conditions fluctuate greatly according to the daily tidal rhythm (Nakagiri, 2002).

2. ***Cerrena unicolor* MTCC 5159 has maximum homology with the marine sponge-derived fungi.** From the ITS1-5.8S-ITS2 rDNA homology studies with other fungi, it is observed that MTCC 5159 is taxonomically closest (99 % identity) to fungal strains; HKC 4, HKB 30 & HKC 10, all of which are known to be associated with marine sponges. With the exception of some diseased states and the genus *Koralionastes*, fungi have not been regularly observed in marine sponges. It is probable that sponges, by their filter-feeding process, trap fungal particles/spores in their tissues. If these fungi flourish in their new habitat, they can be called 'marine-derived' since they would have had enough time to adapt to the marine environment. Marine sponge-derived fungi are mostly anamorphic (Jensen & Fenical, 2002) and are the single most significant source of new marine fungal metabolites. A terrestrial fungus *Coriolus consors*, (related to *Cerrena*) as well as an unidentified sponge-derived / associated fungus were shown to be capable of unusual modifications of hirsutane sesquiterpenes (Wang *et al.*, 1998).
3. ***Cerrena unicolor* MTCC 5159 has highest homology with a fungal endophyte.** Fungal endophytes are fungi that reside in living leaf tissues. One cannot positively conclude that MTCC 5159 is an endophyte since it was isolated from decaying wood. However, it showed 99 % identity to a fungal endophyte, Tm3 - 5 (Table 6C). This high homology to a fungal endophyte suggests the possibility of MTCC 5159 having an alternate life style as a fungal endophyte. This has been shown to be the case for many anamorphic mangicolous fungi, which are endophytic (Kumaresan *et al.*, 2002) yet retain the capacity to live like saprophytes. This is in concurrence with the source of isolation of *Cerrena unicolor* MTCC 5159.

The genus '*Cerrena*' has been known for its symbiotic relationship with both plants and animals such as the horntail *Tremex fuscicornis* (wood wasp). Woodwasps maintain a

close association with a fungal symbiont such as *Cerrena unicolor*, both as larvae and adults. Adult female wood wasps oviposit in dying and dead standing or trees exposed to stress. As the eggs are laid, the wood is simultaneously inoculated with a mass of fungal oidia which are maintained in special pouches associated with the mycangia (egg-laying apparatus). The fungus permeates the surrounding wood and the larvae tunnel into this, ingesting both wood and fungal hyphae (Kukor & Martin, 1983). The fungal symbiont is indispensable for larval development especially beyond the first instar (Pazoutova & Srutka, 2007). These authors isolated a fungus from *T. fuscicornis*, the fungus had a very similar morphology to *Cerrena unicolor* MTCC 5159 and was also unable to form basidiocarps *in vitro*. It too was identified using ITS1-5.8S-ITS2 and 18S rDNA homology. The closest match was *Cerrena unicolor* (EF577058.1). The original, *Cerrena unicolor* (Bull) Murrill was identified from basidiospores. This was successfully mated with the fungus isolated from the mycangia of *T. longicollis* (Tabata & Abe, 1995), using the dikaryotization technique, indicating that this fungus too, was a *Cerrena unicolor* strain. The above indicates that *Cerrena unicolor* is capable of living as an endosymbiont, an endophyte as well as a 'free-living form'. The reason for this symbiosis between fungus and animal is the large amount of lignin-degrading enzymes (such as laccases) and proteases (data not shown) produced by this fungus, which helps it in breaking down wood components and making it palatable for the larvae and in return fresh hosts for the fungus are found, via *Tremex*.

D'Souza *et al.*, (2006) have shown that MTCC 5159 produces a high laccase titer. Thus, *Cerrena unicolor* MTCC 5159 is a mangicolous, lignicolous marine-derived saprophytic basidiomycete probably capable of retaining an endophytic as well as endosymbiotic life style. This is in concordance with the source of isolation of this fungus which was decaying mangrove wood (with holes made by wood borers) at the intertidal region. Besides, this fungus shows greater genetic homology with the marine sponge-derived fungal strains than the terrestrial endosymbiont *C. unicolor* from *Tremex*. This implies that *Cerrena unicolor* MTCC 5159 may have been subjected to the marine influence long enough to alter its genetic makeup. This makes the strain lucrative, in terms of isolation of novel marine metabolites.