ASSESSMENT OF FOLIAR ENDOPHYTIC FUNGAL DIVERSITY IN MANGROVE VEGETATION OF KOTTAYAM DISTRICT, KERALA

REPORT OF MINOR PROJECT
SUBMITTED TO THE
UNIVERSITY GRANTS COMMISSION, NEW DELHI

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Nomenclature

^{0}C Degree Celcius

DSE Dark Septate Endophytes

EtOH Ethanol

g Gram (One Thousandth of a Kilogram)

GYP Glucose Yeast Extract Peptone Medium

hrs Hours

LAF Laminar Air Flow

Mcg Microgram. One thousandth of a milligram.

min. Minute

p.s.i Pounds Per Square Inch

PA Peptone Agar Medium

PDA Potato Dextrose Agar

pH Potenz H (Where 'H' stands for Hydrogen; Negative logarithm of Hydrogen ion concentration)

UV Ultra Violet

YP Yeast Extract Peptone Agar Medium

Chapter 1

INTRODUCTION

The word 'mangrove' is derived from the combination of a Portuguese word "Mangue" for tree and a english word "Grove". Mangroves are salt tolerant plants which are mostly found in tropical and sub tropical regions of the globe (Feller et al., 2010). Plants of mangrove ecosystem grows in tidal waters that are brackish or saline. These plants inhabit mudflats, riverbanks and coastlines. These plants have ability to live in the zone inundated by the highest tides and exposed by the lowest tides. Southeast Asia and Australia have the greatest diversity of mangrove species. So far, there are about 70 species of Mangrove plants identified worldwide Spalding (1997). Since Mangrove forests are inhabited by a wide variety of animals from the sea and land, they can be considered as an ecosystem. About 85% of the total world's mangrove species population are present in the regions bordering Indian ocean region (Kathiresan and Rajendran, 2005).

Mangrove vegetation are seen along the western and eastern coastal lines of India, extending through the peninsular India. The total area of mangrove forest in India currently extends to about 6,749 km^2. When area of Mangrove forest is considered, India is the 4^{th} largest country in the world. The western coast of India has about 850km^2 of mangrove vegetation along the 3000 km of coastal line. This long coastal line and mangroves are said to protect the

coastal biodiversity (Mandal and Naskar, 2008; Naskar and Mandal, 1999).

Mangrove is an association of halophytic trees, shrubs, palms, ferns and other plants Mitsch and Gosselink (1996). Mangroves represent an ecosystem that is rich with diverse organisms which are tolerant to extremes of stresses that occurs naturally in their habitat: both biotic and abiotic. At the same time, generally, mangroves are considered to be highly productive ecosystems but are extremely sensitive and fragile. Fragile, because mangroves are under constant flux due to both natural and anthropogenic factors (Giri et al., 2011). Mangrove forests provide numerous ecosystem services, services which are beneficial for sustenance of human society, including fisheries production and nutrient cycling by way of bio-geochemical cycles (Donato et al., 2011). Other mangrove services include the filtering and trapping of pollutants and the stabilization of coastal land by trapping sediment and protection against damage from powerful storms (McLeod et al., 2006) they can also protect the wetlands located behind the mangrove zone from hurricanes (Zhang et al., 2012). Mangroves are nurseries and breeding sites for birds, fish, crustaceans, shellfish, reptiles and mammals and it provides refuge for species that have lost their original habitat (Ellison, 2004). These facts clearly portrays the importance of both mangroves and studies on mangroves.

Various researchers have presented diverse views regarding the classification of mangrove plants. According to (Tomlinson, 1986) there are two groups of which, the first one is Major element of mangroves or 'True mangroves' – which shows complete adaptation to the mangrove environment; and the second, is Minor element of mangroves – which does not display themselves conspicuously in mangrove habitats, instead they prefer the peripheral habitats of mangrove regions. The second group - the minor element of mangroves are now more appropriately called 'Mangrove associates'. These technically consist of non-arborescent plants which might be herbaceous, sub-woody or climber, and are found growing mostly in regions bordering the tidal periphery of mangrove habitats. (Mandal and Naskar, 2008).

Since mangroves are being reported to shrink at a faster phase in recent years, any study that performs survey of biodiversity, bioprospecting and germplasm establishment is of paramount importance. At a later stage, results from this kind of a baseline study would become fundamental to assess impacts on this ecosystem.

Endophytic fungi are inhabitants of plants. These cause asymptomatic infections in host plants. In recent years several studies have been conducted where endophytic fungi have been known to produce bioactive compounds; both novel and those that are already known. Since natural products are often produced by organisms in response to physiochemical milieu of the habitat and is adapted to a specific function in nature, any exploratory study conducted for novel secondary metabolites should focus on organisms that inhabit novel biotopes. Endophytic fungi inhabit such a biotope. Studies reveal that there is a correlation between the biological activity of the compounds isolated and the biotopes of the source organism. In a study, a high number of endophytes that yielded bioactive compounds were found to be inhabitants of hosts which were already identified as test organisms for antialgal and herbicidal activities, rather than in soil (Schulz et al., 2002). This emphasizes the importance of conducting bioprospecting on endophytes and specifically those that exist inside mangrove plants which are found in this unique habitat (Huang et al., 2008).

The current study attempted to assess the diversity of endophytic fungi in the mangrove plants of Kottayam District of Kerala State.

Isolates obtained were identified based on colony morphology and microscopy. Photomicrographs were taken. Biomass yield of these isolates in broth cultures were studied. This study attempted to create FT-IR fingerprint profile of the endophytic fungal isolates so as to a aid further studies and identification attempts, since identification based on morphological parameters alone, is often tedious. Germplasm comprising of the isolates have been established and is maintained for further studies. Furthermore, the study

attempted to screen the isolates for their ability to produce five industrially important enzymes *viz*: Amylase, Cellulase, Laccase, Lipase and Protease. These isolates were also subjected to preliminary screening for their antibacterial activity against a gram positive (*Bacillus* sp.) and a gram negative bacteria (*Klebsiella* sp.).

Chapter 2

REVIEW OF LITERATURE

2.1 Mangroves

Mangrove vegetation refers to those salt tolerant forest ecosystems that are seen in areas near the river mouths which fall in the coastal intertidal regions of tropical and subtropical parts of the globe; specifically between 30^0N and 37^0S (Feller et al., 2010). The mangrove plants, and their associated organisms including the microbes, fungi, animals and other plants make up the mangrove forest community. The areas of mangrove vegetation are subject not just to high salinity, but also to extreme winds and high temperatures. Furthermore, the soil is muddy and anaerobic. However, these areas are known for freshwater inflow which ensures high input of mineral nutrients and silt. This ecosystem is not only teaming with biodiversity, but also known to be one among the world's most productive ecosystems (Kathiresan and Bingham, 2001).

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display themselves conspicuously in mangrove habitats, instead they prefer the peripheral habitats of mangrove regions. The second group - the minor element of mangroves are now more appropriately called **mangrove associates**. These technically consist of non-arborescent plants which might be herbaceous, sub-woody or climber, and are found growing mostly in regions bordering the tidal periphery of mangrove habitats. (Mandal and Naskar, 2008).

In general, mangroves are considered to be highly productive ecosystems but are extremely sensitive and fragile. Mangroves are under constant flux due to both natural and anthropogenic factors (Giri et al., 2011). Mangrove forests provide numerous ecosystem services, those services which are beneficial for sustenance of human society, which includes fisheries production and nutrient cycling by way of bio-geochemical cycles (Donato et al., 2011). Other mangrove services include the filtering and trapping of pollutants and the stabilization of coastal land by trapping sediment and protection against storm damage (McLeod et al., 2006), they also have ability to protect the wetlands behind the mangrove zone from hurricanes (Zhang et al., 2012). Mangroves are nurseries and breeding sites for birds, fish, crustaceans, shellfish, reptiles and mammals and provide a refuge for species that have lost their original habitat (Ellison, 2004).

2.2 Major Factors Influencing Mangrove Ecosystem

Temperature: the suitable temperature range of well developed Mangroves is between 20^0C and 30^0C. Except for a few mangrove species like, *Avicennia marina*, *Avicennia germinans* all others are sensitive to frost and tend to disappear when temperature goes below 16^0C.

Salinity: Mangroves are seen in tidal areas where salinity ranges from 10mg/ml

to 40mg/ml, where normal fresh water plants cannot survive. Soil and water salinity influences mangrove plants in three ways: a) Osmotic inhibition of water absorption. b) Specific effects on nutrition c) Toxicity.

Soil: Mangroves are best developed in muddy, loose wet soils, comprising of fine silt and rich organic matter, which is available for growth of seedlings.

Tidal range: For the extensive growth of mangroves, wide horizontal tidal range is a required.

Rainfall: For the sequential distribution of Mangals in different zones of the tidal regions, rainfall conditions are very important Dholakia (2004).

2.3 Distribution of Mangroves

Mangrove ecosystem are found between 30^0 North and 37^0 South along the tropical coasts of Africa, Asia, Australia, America and many tropical oceanic Islands. Mangrove vegetation are seen along the western and eastern coastal lines of India, extending through the peninsular India. The total area of mangrove forest in India currently extends to about 6,749 km^2. When area of Mangrove forest is considered, India is the 4^{th} largest country in the world. The western coast of India has about $850km^2$ of mangrove vegetation along the 3000 km of coastal line. This long coastal line and mangroves are said to protect the coastal biodiversity (Mandal and Naskar, 2008; Naskar and Mandal, 1999). Its interesting to note that about 85% of the total world's mangrove species population are present in the regions bordering Indian ocean region (Kathiresan and Rajendran, 2005).

2.4 Mangrove Vegetation of Kerala

In Kerala, according to a study conducted in 1985 Kerala once had a mangrove vegetation cover of about 700km^{2}however, currently what remains is only about 10.95 km^2 of mangrove vegetation, just a relic of the once glorious past (Ramachandran and Mohanan, 1985; Kurian, 1984). Another study that was conducted six years later, concludes an approximately similar area: 16.71 km^{2}of mangrove vegetation (Basha, 1991). Out of the 16.71 km^{2}of mangrove vegetation mentioned above, 14.7 km^{2}is located in private land holdings. This amounts to about 87.97% of the total mangrove vegetation of the whole of Kerala and is therefore vulnerable to destruction.

2.5 Mangrove Vegetation of Central Kerala

Alappuzha has a mangrove cover of 90Ha (0.9 km^2). Ernakulam has 260 Ha (2.6 km^2) however it should be noted that Kottayam district currently has only 8 Ha (0.08 km^2) of Mangrove vegetation (Vidyasagaran and Madhusoodanan, 2014).

2.6 Mangrove Vegetation of Kottayam District

As mentioned in section §2.5, Kottayam currently has only 8 Ha (0.08 km^2) of Mangrove vegetation (Vidyasagaran and Madhusoodanan, 2014). Kottayam district currently has only a scattered distribution of mangrove plants. These plants grow as pockets of vegetation near Kumarakom and Vaikom areas of Kottayam district. Major share of this is located in private land holdings. Tourism and construction activities, which are primarily related to tourism is currently taking its toll on the remaining mangrove vegetation pockets. Shore areas of Vembanad Lake are currently being taken up by private land

Sl.No	Name of the Plant	Alappuzha	Kottayam	Ernakulam
1.	*Rhizophora mucronata*	Frequent	Not Found	Profuse
2.	*Rhizophora apiculata*	Rare	Not Found	Rare
3.	*Avicennia officinalis*	Frequent	Rare	Profuse
4.	*Avicennia marina*	Frequent	Threatened	Profuse
5.	*Bruguiera cylindrica*	Frequent	Not Found	Frequent
6.	*Bruguiera gymnorrhiza*	Rare	Not Found	Profuse
7.	*Kandelia candal*	Threatened	Not Found	Not Found
8.	*Bruguiera sexangula*	Threatened	Frequent	Threatened
9.	*Sonneratia alba*	Rare	Not found	Frequent
10.	*Sonneratia caseolaris*	Frequent	Rare	Profuse
11.	*Excoecaria agallocha*	Profuse	Rare	Frequent
12.	*Aegiceras corniculatum*	Frequent	Not Found	Not Found

Table 2.1: Pure Mangrove Plants of Central Kerala
(Vidyasagaran and Madhusoodanan, 2014)

holdings, buildings and are cleared for tourism related development. In addition, mangrove plants on the banks of inland water bodies are also cleared by many to aid unhindered movement of canoes. In addition to all these, Thannermukkom Salt Water Barrier that has been constructed prevents influx of salt water to a great extend, a fact that also might contribute to another reason for the sparse presence of pure mangrove plants in the area section §2.2. Earlier reports point to the presence of about five pure mangrove plants from Kottayam district however, repeated field trips conducted as a part of this study was unable to locate any more than three, viz: *Bruguiera sexangula*, *Sonneratia caseolaris*, and *Excoecaria agallocha*. Of these three, *Bruguiera sexangula* has been reported to be an endangered species which is represented only in another two districts in Kerala namely Malapuram and Kasaragod (Vidyasagaran and Madhusoodanan, 2014).

Sl. No.	Name of the Plant	Kottayam	Current Status as per observation
1.	*Avicennia officinalis*	Rare	Plant Not Found
2.	*Avicennia marina*	Threatened	Plant Not Found
3.	*Bruguiera sexangula*	Frequent	Available
4.	*Sonneratia caseolaris*	Rare	Available
5.	*Excoecaria agallocha*	Rare	Available

Table 2.2: Pure Mangrove Plants of Kottayam District (Vidyasagaran and Madhusoodanan, 2014)

2.6.1 IMPACT OF THANEER MUKKAM BUND ON MANGROVE VEGETATION OF KOTTAYAM DISTRICT

2.7 Fungi

Fungi are among the most widely distributed organisms on earth. Fungi are found in an enormous diversity of habitats and the presence of fungi in these are influenced by the characteristics of these habitats (Jahiri, 2013). Fungi are eukaryotic, heterotrophic, achlorophyllous organisms which typically grows as filaments termed as hyphae, aggregate to form a network of structure called mycelium. Many of the fungal species are well known symbionts (mycorrhiza), epiphytes and saprophytes. They reproduce both sexually and asexually and produce different types of spores (Deacon, 2013). Filamentous fungi play an important ecological role in nature as decomposers and thus they have critical role in nutrient cycling. They have been employed in the production of pharmaceuticals, enzymes, organic acids and food, and some of them are useful in numerous other areas (Hageskal et al., 2009). In addition to this, many of the fungi are known to be harmful pathogens which cause severe damage and destruction to crops and animal life (Deacon, 2013).

2.7.1 Fungal Endophytes

Term endophyte (Greek: *Endo* = within + *phyte*= plant) include all organisms that lives in the internal tissue of their host (Sathish et al., 2012). Endophytes are important components of microbial biodiversity. Inside aerial tissues of plant hosts they exist asymptomatically (Clay, 1993). Endophytic fungi are mainly found in root, leaves and stems of living tissue of different plants, establishing a mutual relationship with the host, without showing any symptom of diseases. Endophytic fungi mainly consist of members of the Ascomycota as well as some taxa of the Basidiomycota, Zygomycota and Oomycota (RuYong et al., 1995). Endophytes traditionally have been considered as plant mutualists, mainly by reducing herbivory *via* production of mycotoxins, such as alkaloids. However, the vast majority of endophytes, especially horizontally-transmitted ones commonly found in woody plants, are said to have little or no effect on herbivores which feed on them (Faeth and Fagan, 2002). However, endophytic fungi have impacts on the survival and fitness of plants in all terrestrial ecosystems and play a significant role in plant biogeography, evolution, and community structure (Rodriguez et al., 2009).

Fungal endophytes are reported to possess ability to modify plants at genetic, physiologic, and ecologic levels (Weishampel and Bedford, 2006).

2.7.2 Types of Fungal Endophytes

Two major groups of endophytic fungi are recognized, based on the differences in evolutionary relatedness, taxonomy, plant hosts that are inhabited and ecological functions 1. The clavicipitaceous endophytes (C-endophytes), which infect some grasses 2. The nonclavicipitaceous endophytes (NC-endophytes), which are found asymptomatically in tissues of nonvascular plants, ferns and their allies, conifers, and also in angiosperms. C-endophytes are few in number but they are phylogenetically related clavicipitaceous species that are

fastidious in culture. These endophytes are limited to some cool and warm season grasses (Dighton et al., 2005). Usually these endophytic fungi are found within shoots of host plants, where they form systemic intercellular infections (Rodriguez et al., 2009). (Clay and Schardl, 2002) recognized three types of clavicipitaceous endophytes,1. Symptomatic and pathogenic species (Type I) 2. Species with Mixed interaction (Type II) and 3. Asymptomatic endophytes (Type III).

2.7.2.1 Clavicipitaceous endophytes (Class 1 endophytes)

Clavicipitaceous endophytes are known to be defensive mutualists of host grasses (Clay, 1993). Many of them produce bioactive compounds. These bioactive compounds confers resistance of host plants and protects them from herbivory. However it should be noted that some Class 1 endophytes do not provide insect or nematode resistance to host plants (Saikkonen et al., 1999). Several C-endophytes produce compounds that inhibit the growth of other fungi in vitro. The mechanism of enhanced disease resistance in hosts is not known: It could be due to the production of antifungal compounds produced by the endophyte or by the activity of those compounds produced by the plant in response to the endophytes. Class 1 endophytes may enhance the general ecophysiology of host plants and enable plants to counter abiotic stresses such as drought (Arachevaleta et al., 1989) and metal contamination (Malinowski and Belesky, 2000).

2.7.2.2 Nonclavicipitaceous endophytes

Class 2 endophytes Class 2 endophytes are distinct from the other NC-endophytes because generally they colonize roots, stems and leaves and are capable of forming extensive infections within plants. Some Class 2 endophytes increase plant root and shoot biomass. They commonly increase plant biomass under stressful conditions but the cellular mechanisms involved in stress tolerance and growth enhancement are poorly characterized. Physi-

ological studies have indicated that certain biochemical processes correlate with symbiotically conferred stress tolerance. In the absence of pathogen exposure, Class 2 endophyte colonized plants do not activate host defenses.

Class 3 endophytes Class 3 endophytes are distinguished on the basis of their occurrence in above ground tissues of host plants. Class 3 endophytes include the endophytic fungi associated with leaves of tropical trees (Lodge et al., 1996). Several studies indicate that Class 3 endophytes may be mutualistic. (Schulz et al., 1998) demonstrated that some Class 3 endophytes have negative impact on plant growth.

Class 4 endophytes Peyrone reported that more than 135 species of angiosperms are associated with dark pigmented fungi in root tissues (Peyronel, 1924). Presently, these fungi are referred to as dark septate endophytes (DSE) and are grouped together as Class 4 endophytes. DSE have little host or habitat specificity and are distinguished as a functional group based on the presence of darkly septate hyphae and their localization to plant roots.

2.7.3 Foliar Fungal Endophytes

Endophytic fungi exist mainly in root, leaves & stem of living tissue of different plants establishing mutual relationship without showing any symptom of diseases. Fungal endophytes associated with leaves of woody angiosperms are especially diverse & they reduce leaf damage & loss due to major pathogens (Herre, 2003). Species richness and beneficial effects of endophytes increase significantly with leaf age and it reveals the ecological and evolutionary importance of endophyte mediated protection (Arnold et al., 2003). *Acremonium, Alternaria, Cladosporium, Colletotrichum, Fusarium* spp. etc are some of the common foliar endophytes found in Mangroves (Sridhar, 2008; Kumaresan and Suryanarayanan, 2001). The composition of endophytes varies with leaf region. Several studies have shown that old leaves support

more endophytes than relatively younger leaves (Hilarino et al., 2011).

2.7.4 Foliar endophytes of Mangroves

Mangrove plants are rich source of fungal endophytes (Ananda and Sridhar, 2002). Mangrove fungi is a group that includes lower fungi (oomycetes and thraustochytrids) and also higher fungi (ascomycetes and basidiomycetes). Manglicolous fungi is the term given to those mangrove fungi associated in mangroves. This includes mostly marine fungi; however, a small group of terrestrial fungi also occurs in mangrove environment (Thatoi et al., 2013). Optimum conditions for growth of terrestrial fungi is within a pH range of 4.5 - 6.0. On the other hand, facultative marine fungi are known to grow and produce various extracellular enzymes at a pH range of 7.0 - 8.0. Diverse fungal flora together contribute to stress adaptiveness & productivity of Mangrove plant. They contribute significantly to growth of its hosts & by secreting antimicrobial compounds & thereby inhibiting the invasion by pathogen (George et al., 2015). Endophytic fungi found in mangrove roots are actually a consortium of soil, marine, and freshwater fungi. Association of fungi as endophytes with mangrove roots offers not only protection from adverse environmental conditions, it also allows plants to successfully compete with saprophytic fungi that decompose senescent roots.

There are reports of more than 200 species of endophytic fungi from mangrove plants. Some of the mangrove endophytic fungi reported hitherto are: *Cladosporium* sp., *Colletotrichum* sp. *Paecilomyces* sp. *Phoma* sp., *Phyllosticta* sp., *Trichoderma* sp., *Penicillium chermesinum* (ZH4-E2), *Irpex hydnoides* VB4, *Fusarium oxysporum*, *Pestalotiopsis microspora* VB5, *Halorosellinia* sp. (No. 1403), *Guignardia* sp. (No. 4382), *Sporothrix* sp., *Phomopsis* sp., (Zhang et al., 2010; Thatoi et al., 2013). It has been observed that most endophytic fungi have a wide range of hosts and only a few have single host.

2.7.5 Bioactive compounds of Mangrove fungi

Bioactive compounds from endophytic fungi might play a role in identifying lead compounds for drug designing in pharmaceutical industry. Endophytic fungi may be a vast source of bioactive metabolites which can be used in treatment of various microbial infections. They also hold promise as a research area that requires further investigation (Verma et al., 2014.). Mangrove endophytic fungi have been most extensively studied for their ability to produce antibacterial, anticancer, antioxidants, antidiabetic and immuno suppressve composition (Senthilmurugan et al., 2013). Bioactive compounds obtained from endophytic fungi has been reported to be active against a number of human pathogenic bacteria (Gupta et al., 2014). Mangrove endophytic fungi is a prolific source of unique and novel secondary metabolites with fascinating structural features that also exhibit biological activities that holds promise in medical research (Elavarasi et al., 2014). Diversity of antagonistic properties of endophytic fungi is important in attempts of screening for new antifungal agents (Powthong et al., 2013). The antifungal compound griseofulvin was derived from Mangrove endophytic fungi *Nigrospora* sp., was reported to exhibit activity against dermatophytic fungi (Xia et al., 2011). An endophytic fungus *Fusarium oxysporum* was isolated from *Rhizophora annamalayana*, a mangrove plant, and analyzed for taxol production, which may be used for anticancer treatment (Elavarasi et al., 2012).

Enzyme production differs between fungi and often corresponds to the requirements of its habitat (Sunitha et al., 2013). Fungal enzymes are gaining importance in agriculture, industry and human health as they are often more stable (at high temperature and extreme pH ranges) than the enzymes derived from plants and animals (Raghukumar et al., 1994). Endophytes might involve in decomposition when the tissue become senescent or die. Hence they produce and store those enzymes which are necessary for degradation of lignocellulosic materials (Maria et al., 2005). Fungi produce a wide range of extracellular enzymes that break down complex organic polymers

into simpler forms that can then be taken up by the fungi or other organisms (Peay et al., 2008). A number of soil fungi have been isolated from Mangalvanam mangrove ecosystem of Cochin and were screened for their cellulase production and found that majority of the fungi could able to produce cellulase. Apart from that, they could also produce amylase, pectinase, lipase, and caseinase (Prabhakaran and Gupta, 1990). The most economical important fungal metabolites represent antilipidemic drugs collectively known as statins, with their parent compounds mevastatin and lovastatin isolated from *Penicillium citrinum* and *Aspergillus terreus* respectively (Kjer, 2010).

2.8 Host endophyte interaction

Study of broad characters of different endopyte-plant associations may provide greater insight into the evolution of mutualism (Rodriguez et al., 2009). Endophytes form inconspicuous infections within tissues of healthy plants. This might persist for all or nearly all of their life cycle and their host tissues appear symptomless and they remain asymptomatic for many years and only become parasitic when their hosts are stressed (Firáková et al., 2007). The relationship between the host plant and its endophyte shows symbiotic characteristics since the endophytic fungi usually obtains nutrients and protection from the host plant and in return, it profoundly enhances the fitness of the host by producing certain functional metabolites. Still, if the host plant is weakened, the endophyte can also become aggressive and thereby reveal the smooth transition between symbiont and opportunistic pathogen (Kjer, 2010). The mode of endophyte transmission (vertical or horizontal) is thought to significantly influence the evolution and sustainability of mutualisms (Sachs et al., 2004). For vertically transmitted endophytes, the fitnesses of the two partners are connected, the outcome of the association is predictable and mutualism is strongly selected. On the other hand, horizontal transmission provides opportunities for plant colonization by a variety of

fungi that may express different symbiotic lifestyles.

2.9 FT-IR

Rapid and simple identification of microorganisms is an essential task. Traditional methods such as conventional plating, biochemical tests, and immunological methods have several steps and may take a long time to get confirmatory results. FT-IR methods have been reported to provide biochemical fingerprints of bacteria within a short time frame (some analyses take only minutes) in a simple and economical way (Davis and Mauer, 2010). For this reason, FT-IR is increasingly gaining importance in the field of microbiology.

Molecular spectroscopy was introduced as a possible identification approach in the 1950s with limited success (Levine et al., 1953). Infrared signals of microorganisms are highly specific and the fingerprint-like patterns can be used for probing the identity of microorganisms (Naumann et al., 1991), thus FTIR-spectroscopy is suitable for the identification of microorganisms and presents a new addition to taxonomic and genetic methods (Schmitt and Flemming, 1998). Fourier transform infrared spectroscopy (FT-IR) is a powerful technique for characterizing the chemical composition of very complex probes such as microorganisms (Fischer et al., 2006). Erukhimovitch and co-workers used this method of spectroscopic study for the identification of various fungal genera which were known to be responsible for causing serious damage to agriculture (Erukhimovitch et al., 2005) and Fischer identified some air borne fungi such as *Aspergillus* and *Penicillium* (Fischer et al., 2006) using FT-IR spectroscopy. FT-IR has a wide range of applications in the field of pharmaceutical industry (drug related studies (Kazarian and Chan, 2006)), medicine, researches (protein analysis (Glassford et al., 2013)) etc. FT-IR is also used to measure the process of biodegradation.

Chapter 3

MATERIALS AND METHODS

3.1 Collection and Identification of Plant materials

For the isolation of foliar endophytic fungi, healthy twigs of mangrove plants (*Excoecaria agallocha*, *Sonneratia caseolaris* and *Bruguiera sexangula.*) were collected during repeated field trips. Plants of each species were randomly selected for the study. Twigs from each plant were collected, tagged and processed as separate units. The twigs were placed in polybags that were sterilized beforehand by swabbing with 70% EtOH.. Mouth of the polybags were sealed to prevent loss of water and further contamination during transportation. The collected specimens were transported by placing over ice pack, brought to the laboratory and were subsequently processed in the laboratory by about 12 hours.

3.2 Standardization of Surface Sterilization Procedure

The procedure for surface sterilization was standardized. Plant materials were first washed under tap water. They were then washed in 4% Extran^{TM} and subsequently with water to remove traces of the detergent. This was followed by treatment with 0.1% $HgCl_2$. Treatment with mercuric chloride was done for a period of 3 minutes. Leaves were then washed repeatedly three to four times, with sterile water to remove traces of mercuric chloride.

Prior to inoculation, surface prints of the leaves were prepared. This was done by pressing both upper and lower surface of the uncut explant on the surface of sterile PDA. These preparations were labeled as "surface print" and cultured along with other cultures under aseptic conditions to understand the efficacy of the surface sterilization process. During the above mentioned procedures, extreme care was taken to ensure that no fresh wounds or damages are made on the explant after surface sterilization programme. After drying the surface of the explant with the help of autoclaved blotting paper placed in the sterile petri-dishes, the explant was trimmed into small bits with the help of sharp sterile surgical blade and forceps. One sterile petri-dish with blotting paper was used for each leaf. Once the surface sterilization process was standardized, and efficacy of which was counter checked with the help of surface prints, the same procedure was adopted for other plants as well.

3.3 Collection of Isolates

Surface sterilized leaves prepared as mentioned in 3.2 After making each incisions or cuts, the surgical blade was flame sterilized. Trimmed explants were transferred aseptically onto sterile petri-dish containing media (prepared beforehand and labelled as "Culture") with the help of forceps in such a way

that, freshly cut edges come in contact with media surface. Name of the plant, date of inoculation were marked on these petri-dishes. All the metal instruments such as forceps, scalpel and surgical blades were flame sterilized prior to use. All the works conducted inside the LAF chamber were done in the vicinity of flame. Before starting the work in LAF, UV lamp was kept lit for 30min. This was done to make the chamber aseptic. After switching off the UV lamp, 20min was allowed to pass before inoculation process began. The working table of the LAF and hands of the operator were swabbed with 70% alcohol prior to work.

The fungal isolates were obtained from three mangrove plants *Excoecaria agallocha*, *Sonneratia caseolaris* and *Bruguiera sexangula.* The isolates had been obtained in an earlier study by placing surface sterilized leaf segments on Potato Dextrose Agar (PDA) media taken in a petriplate. Hyphal tips were isolated and cultured to obtain pure isolates. The initial isolates were subcultured on PDA. A total of five different isolates thus obtained were subjected to further investigations as described below.

3.4 Identification of Isolates

Endophytic fungal isolates were identified by culturing them on petriplates and subjecting the samples to microscopic observation, combined with studies on colony morphology. These information were used together with reference literature (Gilman and Joseph, 1998) to identify the isolates. Photomicrographs were taken.

3.5 Preparation of culture media

3.5.1 Potato Dextrose Agar (PDA).

Potato ⋯ 200g

Dextrose ··· 20g

Agar ··· 15g

Distilled water ··· 1000ml

pH ··· 5

Infusion of 200g potato was taken in a 1000ml beaker and filtered through a muslin cloth. 20g of Dextrose was added to the filtrate. Total volume was made up to 995ml. pH was adjusted to 5. Final volume was then made up to 1000ml. 15g of Agar was added for solidification. The preparation was then boiled to dissolve agar. After ensuring that agar has perfectly dissolved, the media was transferred to multiple conical flasks each having a capacity of 250ml. The conical flasks were then plugged with cotton, and mouth covered with aluminum foil prior to autoclaving. After autoclaving, the media contained in conical flasks were taken into Laminar Air Flow chamber. When temperature of the prepared media gradually dropped to 35-30^{0}C, Ambistryn-S was added into the media and stirred well. The media was then poured into petri dishes and allowed to cool down. .

3.5.2 Potato Dextrose Broth Medium (PDB).

Potato Broth Medium was prepared by the same method described above, except that agar was not added to effect formation of a solid consistency. The media prepared were poured into four different conical flask of 250 ml volume. The conical flasks were capped with cotton plugs and covered with brown paper and autoclaved at 121^0C at 15psi pressure for 15 minutes.

3.5.3 Glucose Yeast Extract Peptone medium (GYP)

Glucose ··· 1.0 g

Yeast extract ··· 0.1g

Peptone ··· 0.5g

Agar ··· 16 g

Distilled water ··· 1000 mL

pH ··· 6

For the preparation of GYP, above listed chemicals were weighed properly and each chemical was added to distilled water one by one after ensuring that the previous one has dissolved completely. The solution was prepared in a 1000ml beaker and the final volume was made up to 995 ml. pH of the solution was adjusted to 6. Final volume was made up to 1000 ml. 16g of agar was added and boiled till the agar dissolved completely. After dissolving the agar, the media was transferred into conical flask and then plugged with cotton and autoclaved. Petri dishes were prepared aseptically in Laminar Air Flow Chamber.

3.5.4 Yeast Extract Peptone Agar Medium (YP)

Yeast extract ··· 0.1g

Peptone ··· 0 .5 g

Agar ··· 16 g

Distilled water ··· 1000 mL

pH ··· 6

The above listed chemicals were weighed properly and each chemical was added to distilled water one by one after ensuring that the previous one has dissolved completely. The solution was prepared in a 1000ml beaker and the final volume was made up to 995 ml. pH of the solution was adjusted to 6. Final volume was made up to 1000 ml. 16g of agar was added and boiled

till the agar dissolved completely. After dissolving the agar, the media was transferred into conical flask and then plugged with cotton and autoclaved. Petri dishes were prepared aseptically in Laminar Air Flow Chamber.

3.5.5 Peptone Agar medium (PA)

Peptone ··· 10 g

NaCl ··· 5 g

$CaCl_2.2H_2O$ ··· 0.1 g

Agar ··· 16 g

Distilled water ··· 1000 ml

pH ··· 6

For the preparation of PA ,the above listed chemicals were weighed properly and each chemical was added to distilled water one by one after ensuring that the previous one has dissolved completely. The solution was prepared in a 1000ml beaker and the final volume was made up to 995 ml. pH of the solution was adjusted to 6. Final volume was made up to 1000 ml. Added 16g of agar and boiled till the agar dissolved completely. After dissolving the agar, the media was transferred into conical flask and then plugged with cotton and autoclaved. Petri dishes were prepared aseptically in Laminar Air Flow Chamber.

3.5.6 Czapek's Dox Broth

Sucrose ··· 30 g

Sodium nitrate ··· 3 g

Dipotassium phosphate ··· 1 g

Magnesium Sulphate ··· 0.5 g

Potassium chloride ··· 0.5 g

Ferrous sulphate ··· 0.01 g

Distilled water ··· 1000 ml

pH ··· 7.3

The above listed chemicals were weighed properly and each chemical (except Dipotassium phosphate) was added to distilled water one by one after ensuring that the previous one has dissolved completely. The solution was prepared in a 1000ml beaker. Dipotassium phosphate was dissolved in 25ml distilled water separately and this solution was then added to the former. Total final volume was made up to 995 ml. pH of the solution was adjusted to 6. Final volume was made up to 1000 ml. add 16g of agar and boiled till the agar dissolved completely. Petri dishes were prepared aseptically in Laminar Air Flow Chamber.

3.5.7 Nutrient agar

Peptic digest of animal tissue ··· 5 g

Sodium chloride ··· 5 g

Beef extract ··· 1.5 g

Yeast extract ··· 1.5 g

Agar ··· 2%

pH ··· 7.4

3.6 Inoculation

Prior to inoculation, all the metal instruments such as forceps, scalpel and inoculation loop were flame sterilized prior to use, using a spirit lamp. All the works conducted inside the Laminar Air Flow chamber were done in the vicinity of flame. Before starting the work in LAF, UV lamp was kept lit for 15min. This was done to make the chamber aseptic. After switching off the UV lamp, 20min was allowed to pass before inoculation process began. The working table of the LAF and hands of the operator were swabbed with 70% alcohol prior to work.

Rims of petriplates containing the initial isolates were flamed before opening them. Petriplates and other culture vials were always kept in the vicinity of the flame while they were opened. Using a flame sterilized inoculation loop, an aliquote was transferred into fresh medium taken in a sterile culture vial.

3.7 Culturing and Incubation

3.7.1 Establishment of Cultures

Cultures of the isolates were incubated at 28^0C for 7 days. In the case of slow growing cultures, more time was allowed as appropriate. Petriplates were observed regularly to understand growth of fungi.

3.7.2 Establishment of Culture for FT-IR

Biomass for FT-IR was prepared by culturing on Potato Dextrose Broth medium taken in a 250ml conical flask. 50ml of PDB was taken in each conical flask. Ambistryn-S was added into the medium to prevent bacterial growth. These cultures were incubated for six days. The biomass was separated by filtering through a filter paper.

3.7.3 Screening of Anti-microbial potential

Antimicrobial activity of all the five different isolates of endophytic fungi were done according to the protocol of (Zhang et al., 2009) with slight modifications. Bacterial inoculum ($100\mu L$) was spread over sterile nutrient agar media taken in petriplates using sterile cotton swab. These initial cultures were placed in incubator for six hours after which, 9mm diameter discs of fungal biomass taken from actively growing fungal cultures maintained on PDA were placed on the aforementioned nutrient agar plates inoculated with test bacteria. They were placed in refrigerator for 12 hrs to permit antimicrobial compounds from fungal biomass to diffuse into the medium. These cultures were incubated for a period of 12 hrs at room temperature after sealing the rims of the petriplates with parafilm. One test organism each were chosen from both gram positive and gram negative groups of bacteria viz: *Klebsiella* sp. (Gram negative) and *Bacillus* sp. (Gram positive). After incubation, the diameter of the inhibition zone was measured in millimeter by using ruler.

3.7.4 Measurement of Biomass Yield

After culturing fungi on PDB for a period of six days, the biomass was filtered from the liquid broth using an oven dried and pre-weighed filter paper. The biomass thus obtained were dried at 40^0C for 2 hours in a hot air oven. When moisture was completely removed from biomass, final weight of biomass along with filter paper was taken. The weight of dried biomass alone was calculated by subtracting initial weight of the filter paper from final weight.

3.8 Observation of Initial Cultures

Petridishes were observed regularly for observing the growth of fungus. Colony morphology of the fungal cultures were also noted and photographs were taken. The fungal hyphae were picked aseptically and placed on fresh petri

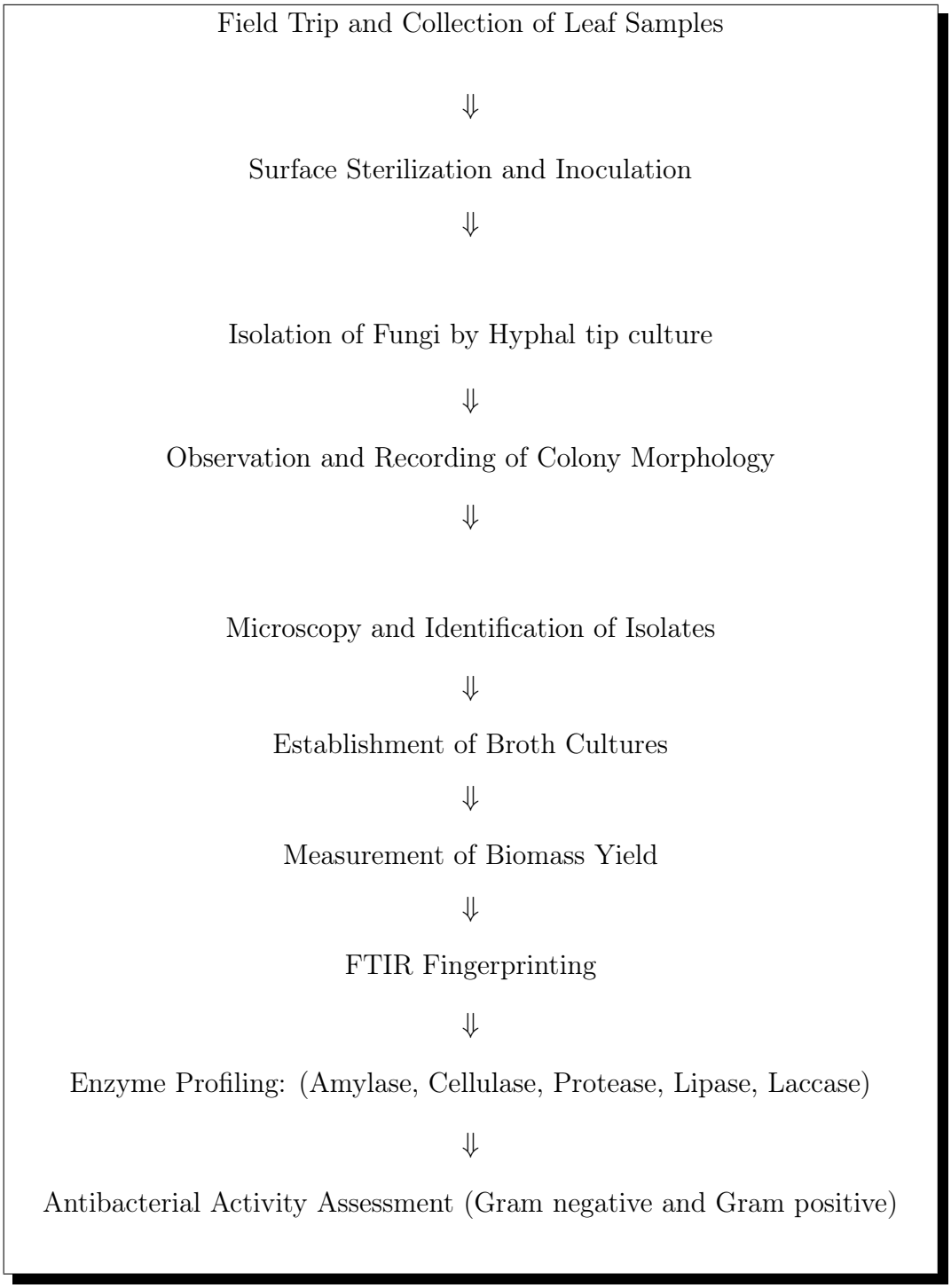


Figure 3.1: Overall Work Flow

dishes containing media; for sub-culturing. After 8-14 days, slides were prepared to identify the fungus. Staining was done with Lactophenol cotton blue. 3-4 drops of Lactophenol cotton blue was poured onto a small amount of mycelium taken on a clean glass slide. The duration for staining was 5-15 min.. The slides were observed under a compound microscope and photomicrographs were taken.

3.9 FT-IR

FT-IR fingerprints of fungal biomass was prepared by Shimadzu IR Affinity-1 FT-IR Spectrophotometer, with ATR attachment. Only the fingerprint region was scanned for observing variation in the patterns.

3.10 Preparation of Chemicals for enzyme profiling

1. **1% Iodine in 2% Potassium Iodide:** Weigh 2g potassium iodide and dissolve it in little amount of water and thoroughly mixed, 1 g iodine was then added and made up to 100 ml using distilled water.
2. **0.5% Na-carboxymethyl cellulose (CMC):** Weigh 0.5 g Na-carboxymethyl cellulose (CMC) and made up to 100ml using distilled water.
3. **0.2% aqueous Congo red:** 0.2 g Congo red powder is dissolved in 100 ml distilled water.
4. **1 Molar NaCl:** Weighed 14.61 g of NaCl in a final volume of 250 ml.
5. **1-Naphthol (α-Naththol), 0.005% (pH, 6):** 0.005 g of 1-Naphthol is weighed and made up to 100 ml using distilled water and checked the pH of the solution using pH meter.

3.11 Enzyme Profiling Studies

3.11.1 Amylase activity

The amylase activity of each isolate was assessed by growing them on Glucose Yeast extract Peptone (GYP) agar medium with 2% soluble starch and it is placed in the incubator for sufficient growth. After 5 days of incubation, the plates were flooded with 1% iodine in 2% potassium iodide and wait for 10 minutes. A clear zone formed surrounding the colony of fungi were considered positive for amylase.

3.11.2 Cellulase activity

Isolates were cultured on Yeast extract Peptone agar medium (PA) supplemented with 0.5% Na-carboxymethyl cellulose (CMC) and kept for 3 days. After incubation, the plates were flooded with 1% aqueous Congo red for 15 minutes and then destained with 1M NaCl for 15 minutes.A clear zone formed surrounding the colony were considered positive for cellulase.

3.11.3 Laccase activity

It was assessed by growing the fungi on GYP agar medium amended with 1- naphthol, 0.005% (pH 6) and incubated. On oxidation of 1-naphthol by laccase, the medium changed from clear to blue.

3.11.4 Protease assay

Protease assay was done by growing isolates in 250ml flasks containing 50ml of GYP agar medium amended with 0.4% gelatin (sterilized separately and mixed with sterile GYP agar medium) adjusted the pH to 6 and kept for 5 days and then plates were flooded with saturated aqueous ammonium sulphate. A clear zone formed surrounding the colony of isolates were considered

as positive for protease activity.

3.11.5 Lipase activity

The fungi were grown on peptone agar medium supplemented with Tween 20 (separately sterilized and added 1 mL to 100 mL medium).At the end of the incubation period, a clear zone formed around the active colony indicates lipase activity. A clear zone formed surrounding the colony in agar plates was considered positive for lipase.

Chapter 4

RESULTS

4.1 Hosts and Isolates

Earlier reports state the presence of five different species of true mangroves, however, only three of the listed plants were located from the area under study. The distribution of mangroves in Kottayam district is mainly restricted to some isolated pockets of land area in and around Kumarakom and Vaikom. These areas harbor mangroves primarily in private land holdings. The once luxurious vegetation is now under serious threat due to habitat destruction which is in turn related to encroachment and development of infrastructure for tourism. The presence of Thaneermukkam bund (Salt Water Barrier) also has its impact on mangrove vegetation which usually grows in places with salinity. The construction and utilization of the aforementioned bund is meant to help local agriculture by reducing inflow of saline water from sea. This could be one added reason for the reduction in the distribution and diversity of mangrove plants compared to reports obtained earlier. Out of five true mangrove plants reported earlier from the area, only three were located during the study. The three plant species located from study area are: *Sonneratia caseolaris, Bruguiera sexangula and Excoecaria agallocha.*

Sl.No	Host Plant	Fungi
1.	*Sonneratia caseolaris*	*Alternaria fasciculata*
2.	*Sonneratia caseolaris*	*Cylindrocarpon* sp.
3.	*Sonneratia caseolaris*	*Cladosporium sphaerospermum*
4.	*Excoecaria agallocha*	*Rhizoctonia* sp.
5.	*Bruguiera sexangula*	Sterile mycelium

4.2 Colony Morphology of Fungal Isolates

4.2.1 *Alternaria fasciculata*

On PDA, off-white colonies were observed initially and during sporulation it turned to dark brown on verso side and black on recto side.

4.2.2 *Cylindrocarpon* sp.

On PDA, *Cylindrocarpon* sp. produced colonies with white cottony mycelia, initially. During sporulation, the white coloured colonies turn brown from center to periphery. On verso side and recto side of the colony appeared black.

4.2.3 *Cladosporium sphaerospermum*

On PDA, *Cladosporium sphaerospermum* produced greenish brown velvety colonies on the verso side and recto side appeared brownish black.

4.2.4 *Rhizoctonia* sp.

Young colonies appear in a light shade of brown and they later become darker with age. It is tough to separate from the media easily. Brown coloured on the verso side and blackish brown on the recto side.

Sl.No	Fungi	Source Plant
1.	*Alternaria fasciculata*	*Sonneratia caseolaris*
2.	*Cylindrocarpon* sp.	*Sonneratia caseolaris*
3.	*Cladosporium sphaerospermum*	*Sonneratia caseolaris*
4.	*Rhizoctonia* sp.	*Excoecaria agallocha*
5.	Sterile mycelium	*Bruguiera sexangula*

Table 4.1: Isolates obtained from the study

Sl.No	Isolate	Biomass Yield (g)
1.	*Alternaria fasciculata*	0.72
2.	*Cylindrocarpon* sp.	0.52
3.	*Cladosporium sphaerospermum*	0.39
4.	*Rhizoctonia* sp.	0.60
5.	Sterile mycelium	0.32

Table 4.2: Biomass Yield on PDB

4.2.5 Sterile mycelium

It produced white cottony mycelia on PDA with faster growth rate than the other isolates, and appeared white on both verso and recto side. There was no sporulation and therefore was considered as sterile under the given culture conditions.

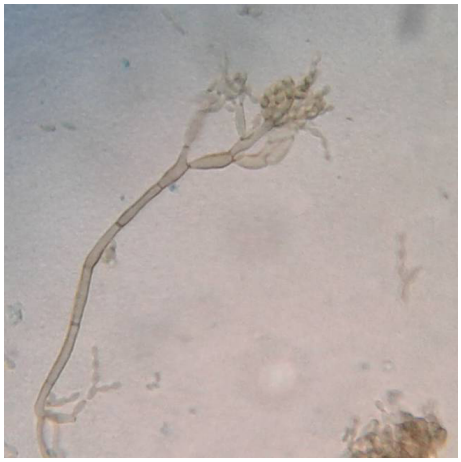
4.3 Biomass yield

Results of biomass yield when the isolates were cultured on 50 ml of PDB for 7 of days at 28^0C are givien in table 4.2.

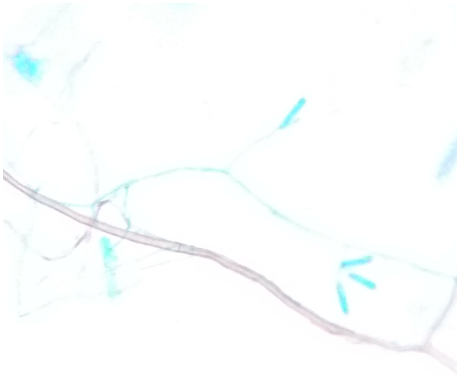
4.4 FT-IR Fingerprints

FT-IR fingerprints of all the five isolates have been obtained and are presented in Figures: 4.2 and 4.3 on page 35, 4.4 and 4.5 on page 36.

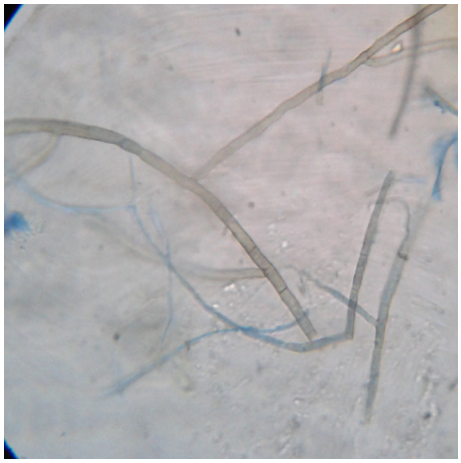
(a) *Alternaria fasciculata*



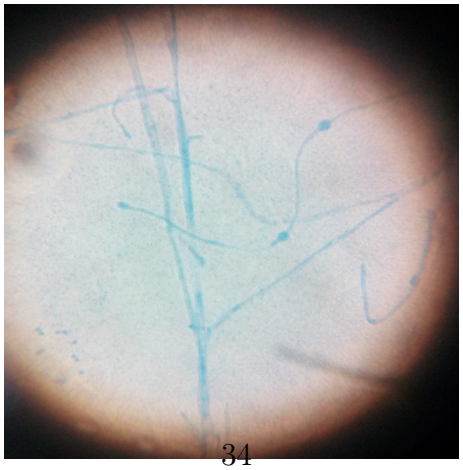
(b) *Cladosporium sphaerospermum*



(c) *Cylindrocarpon* sp.



(d) *Rhizoctonia* sp.



(e) Sterile Mycelium

Figure 4.1: Photomicrographs of Fungal Isolates

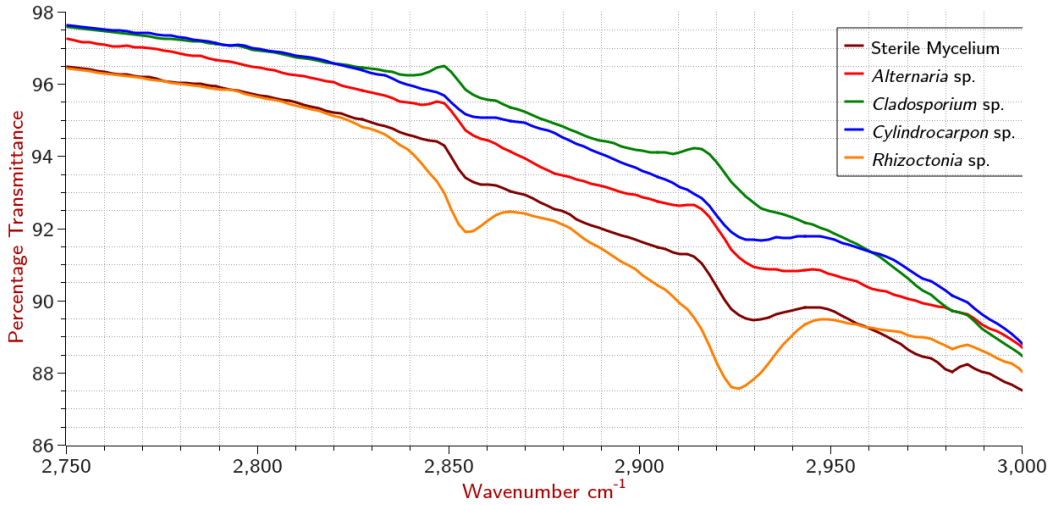


Figure 4.2: FT-IR Spectrum: Area 1

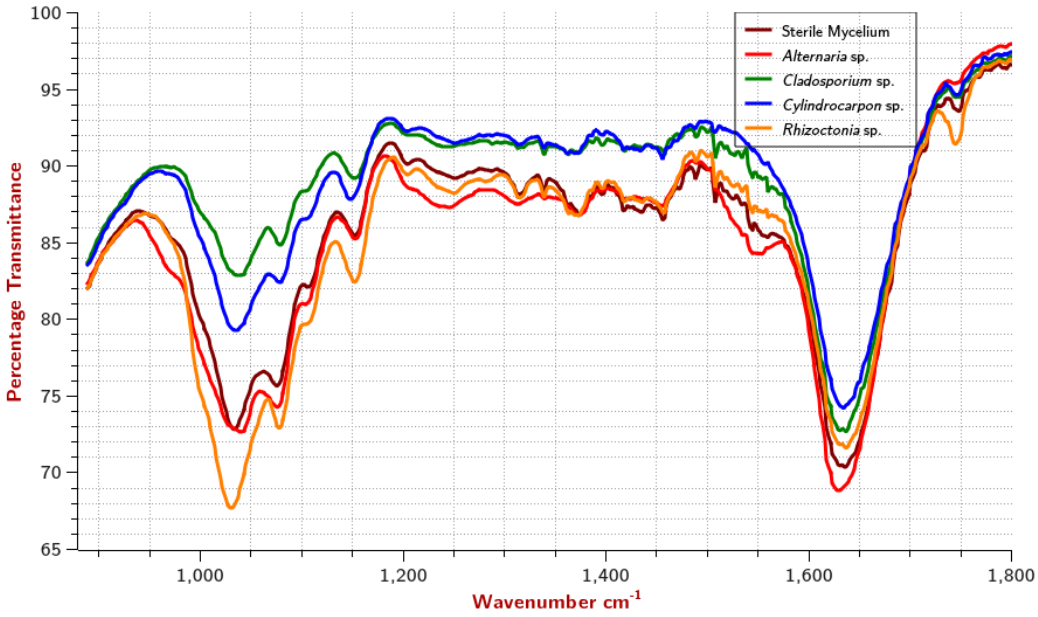


Figure 4.3: FTIR Spectrum: Area 2

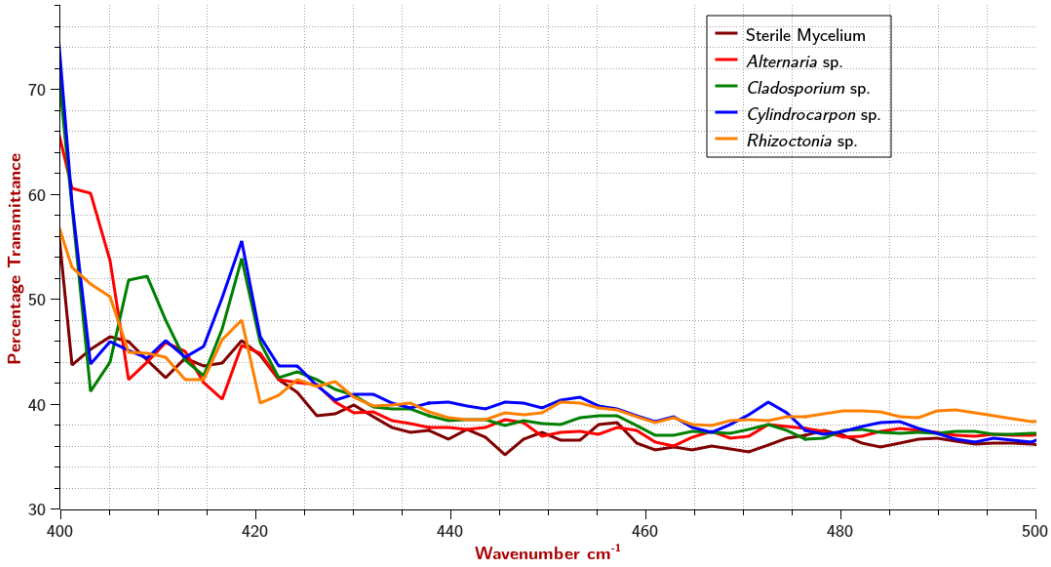


Figure 4.4: FTIR Spectra: Area 3

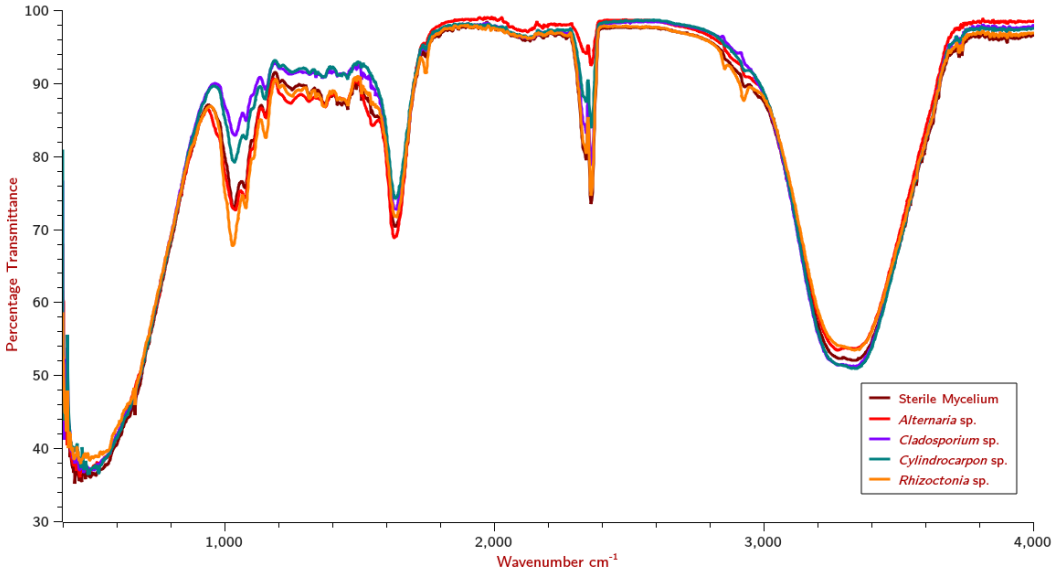


Figure 4.5: FTIR Spectra: Overview of all isolates

SlNo	Fungal Isolate	Amylase	Cellulase	Laccase	Lipase	Protease
1.	Sterile Mycelia	-	-	+	-	-
2.	*Rhizoctonia* sp.	-	-	-	+	-
3.	*Cladosporium sphaerospermum*	+	+	-	-	-
4.	*Cylindrocarpon* sp.	+	+	+	+	-
5.	*Alternaria fasciculata*	-	+	+	-	-

Table 4.3: Exoenzymes in Fungal Isolates Screened

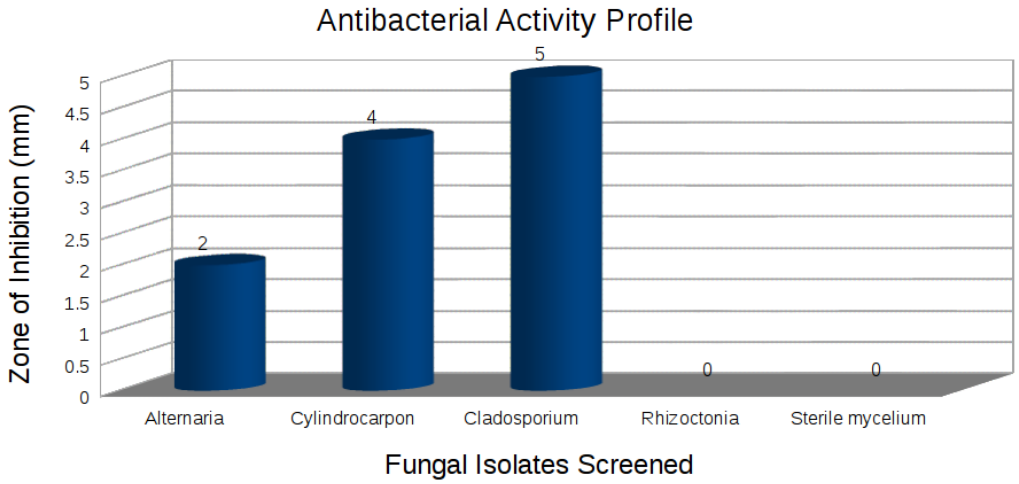


Figure 4.6: Antibacterial Activity Profile Tested against *Bacillus* sp.

4.5 Anti-bacterial activity profiling.

Antibacterial activity was tested against *Bacillus* sp and *Klebsiella* sp. The rationale being testing of antimicrobial activity against one gram positive and one gram negative bacteria respectively. Three of the five isolates exhibited antibacterial activity against *Bacillus* sp. There was no antibacterial activity against *Klebsiella* sp. for any of the five isolates screened. The results of the study are presented in Figure: 4.6

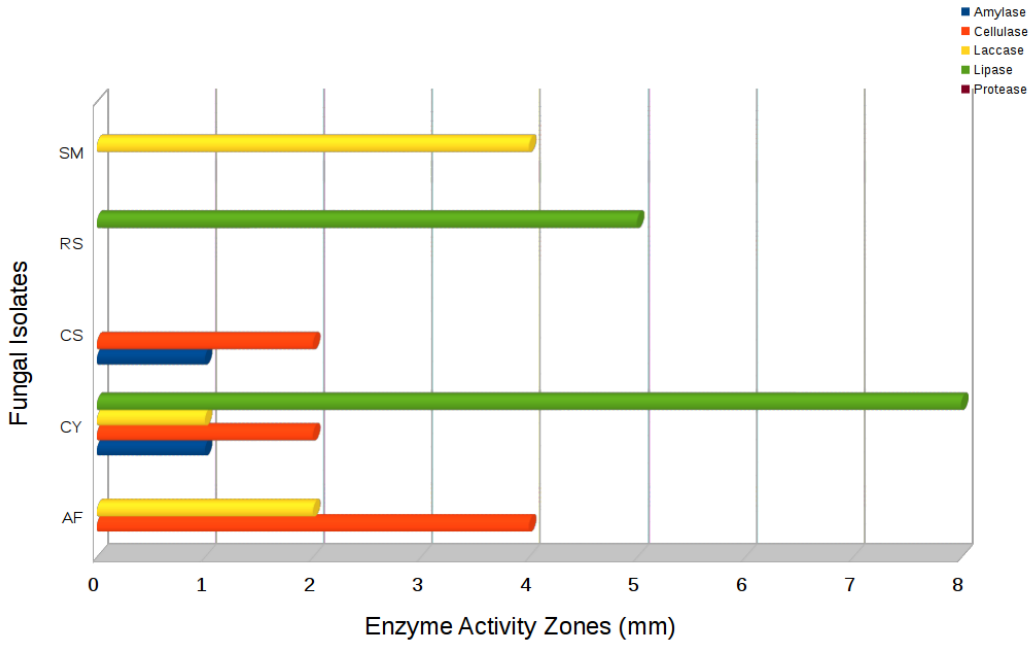
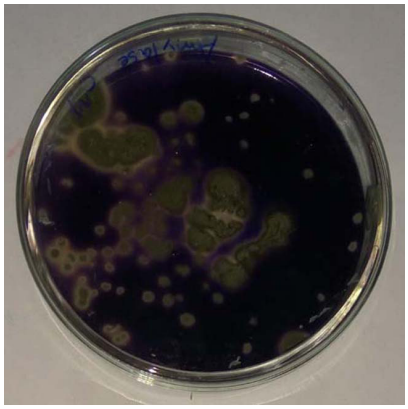


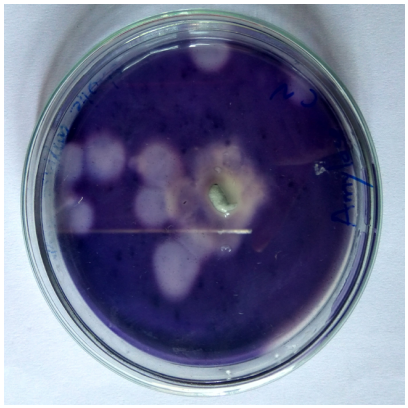
Figure 4.7: Results of Enzyme profiling
AF: *Alternaria fasciculata ;* CY: *Cylindrocarpon* sp. ; CS: *Cladosporium sphaerospermum*; RS: *Rhizoctonia* sp.; SM: Sterile Mycelium;

4.6 Enzyme Profiling

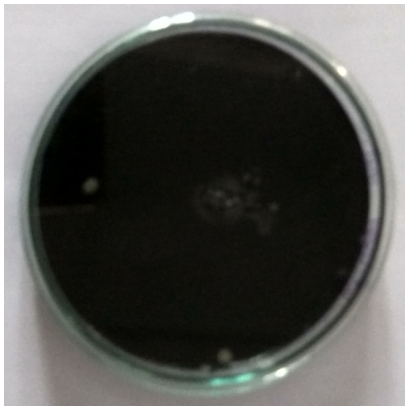
Presence of exoenzymes viz: Amylase, Cellulase, Laccase and Lipase was reported from all the fungal isolates subjected to study. The findings are presented in Figure: 4.7 and Table: 4.3 on the preceding page. None of the isolates reported the presence of Protease.



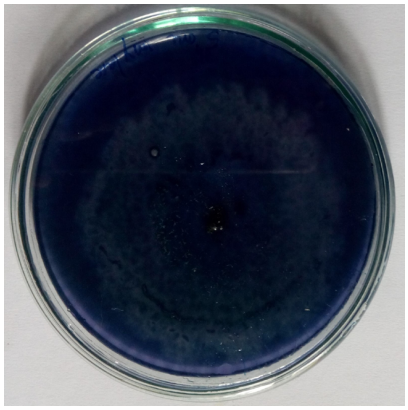
(a) Amylase Positive:
Cladosporium sphaerospermum



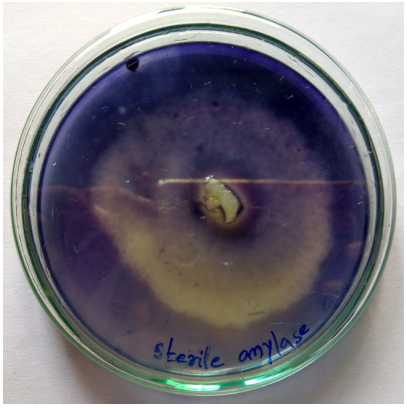
(b) Amylase Positive:
Cylindrocarpon sp.



(c) Amylase Negative:
Alternaria fasciculata

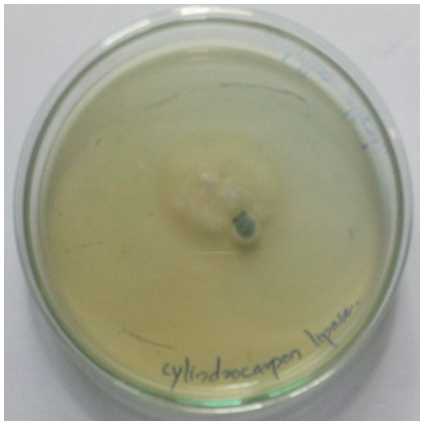


(d) Amylase Negative:
Rhizoctonia sp.

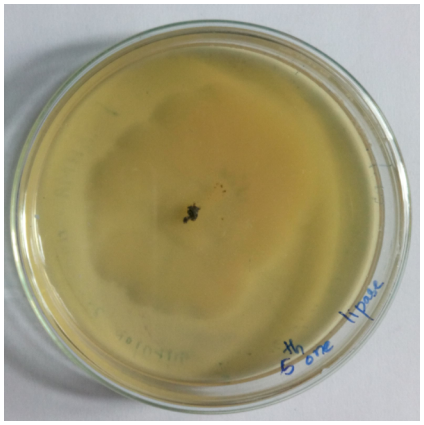


(e) Amylase Negative:
Sterile Mycelium

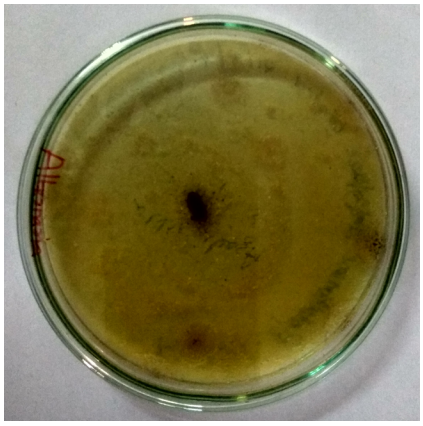
Figure 4.8: Enzyme Profiling: Amylase



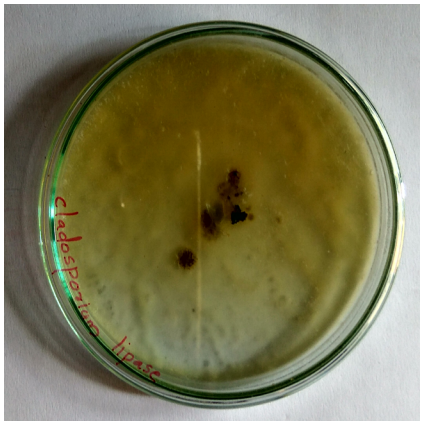
(a) Lipase Positive:
Cylindrocarpon sp.



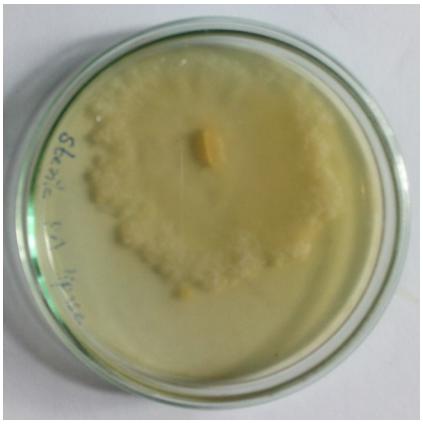
(b) Lipase Positive:
Rhizoctonia sp.



(c) Lipase Negative:
Alternaria fasciculata

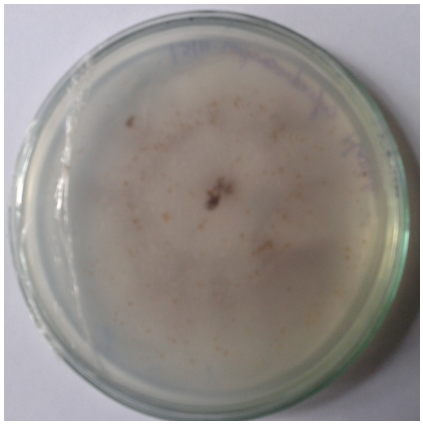


(d) Lipase Negative:
Cladosporium sphaerospermum

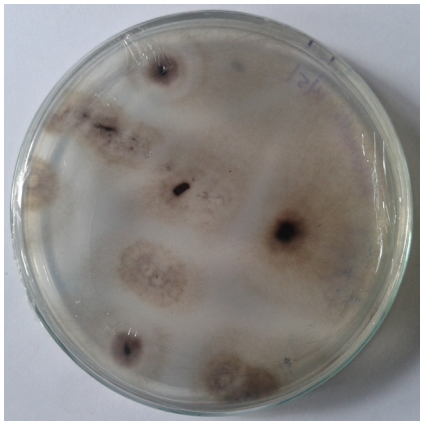


(e) Lipase Negative:
Sterile Mycelium

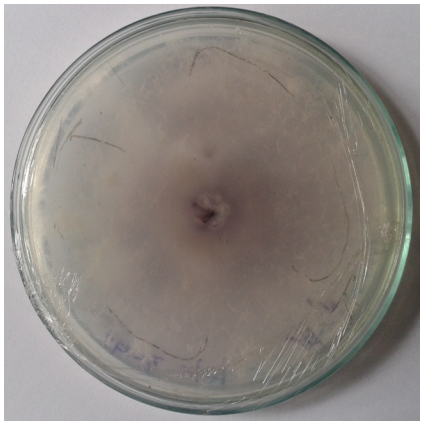
Figure 4.9: Enzyme Profiling: Lipase



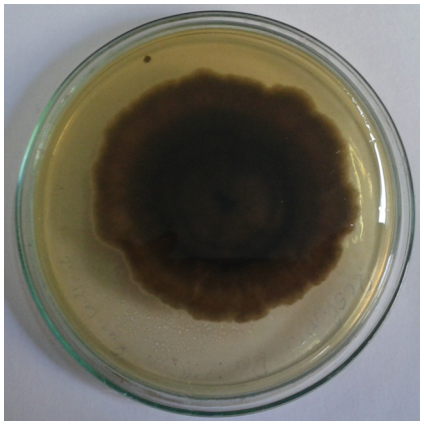
(a) Laccase Positive: *Cylindrocarpon* sp.



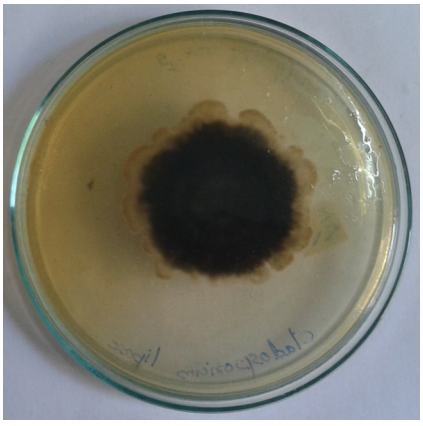
(b) Laccase Positive: *Alternaria fasciculata*



(c) Laccase Positive: Sterile Mycelium

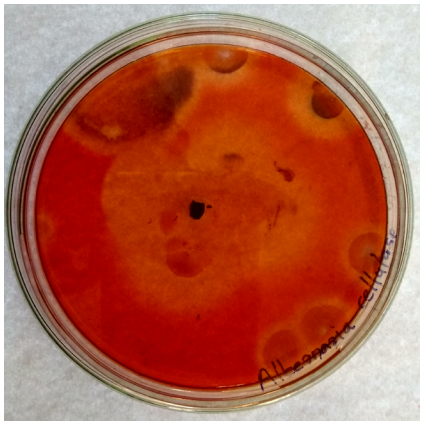


(d) Laccase Negative: *Rhizoctonia*

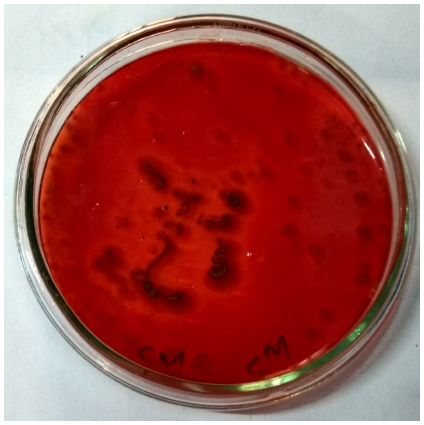


(e) Laccase Negative: *Cladosporium sphaerospermum*

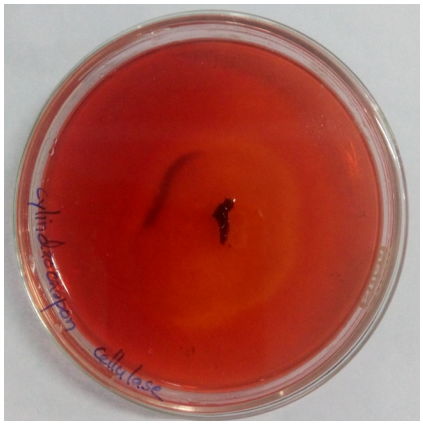
Figure 4.10: Enzyme Profiling: Laccase



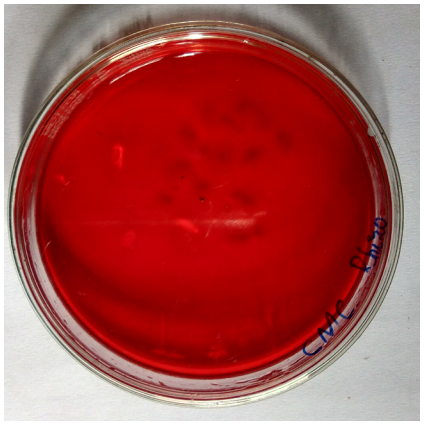
(a) Cellulase Positive: *Alternaria fasciculata*



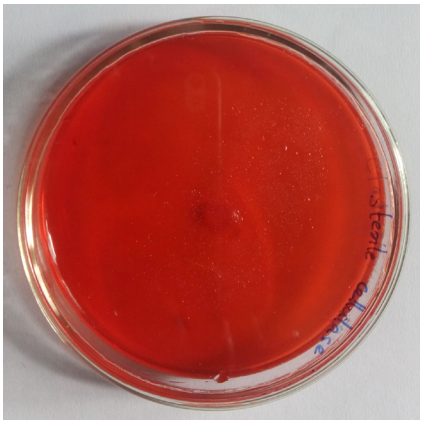
(b) Cellulase Positive: *Cladosporium sphaerospermum.*



(c) Cellulase Positive: *Cylindrocarpon* sp.

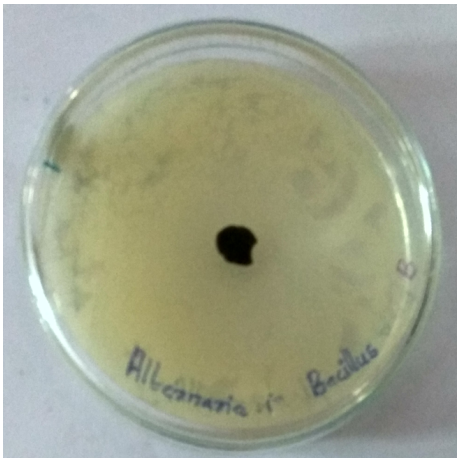


(d) Cellulase Negative: *Rhizoctonia* sp.

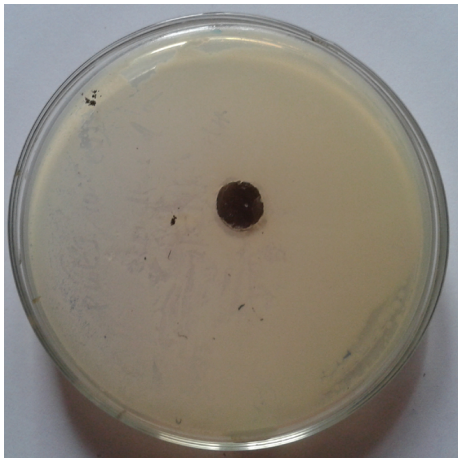


(e) Cellulase Negative: Sterile Mycelium

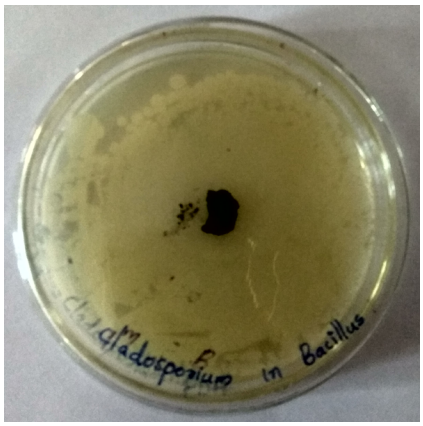
Figure 4.11: Enzyme Profiling: Cellulase



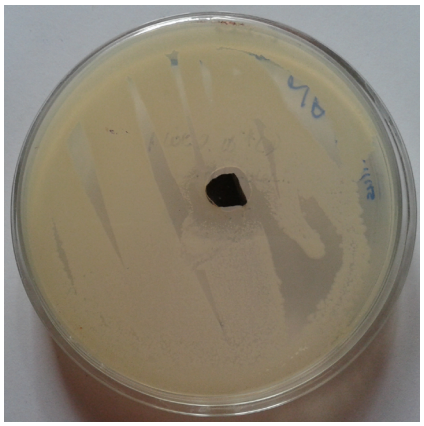
(a) Before Test: *Alternaria fasciculata*



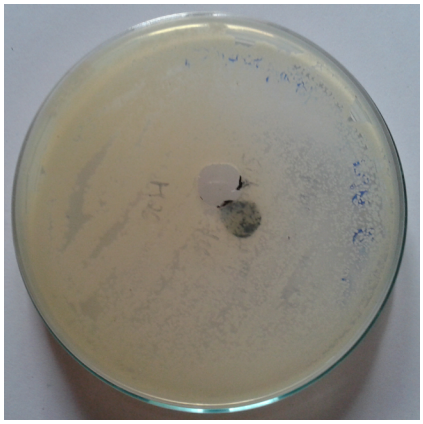
(b) After Test: *Alternaria fasciculata*



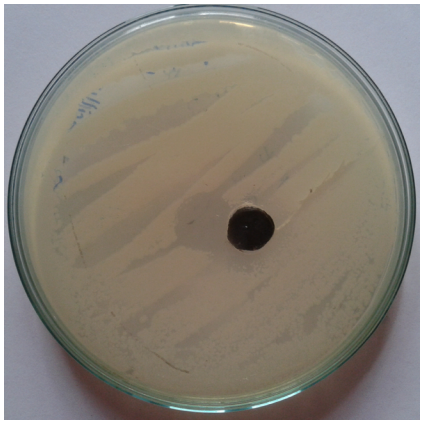
(c) Before Test: *Cladosporium sphaerospermum*



(d) After Test: *Cladosporium sphaerospermum*

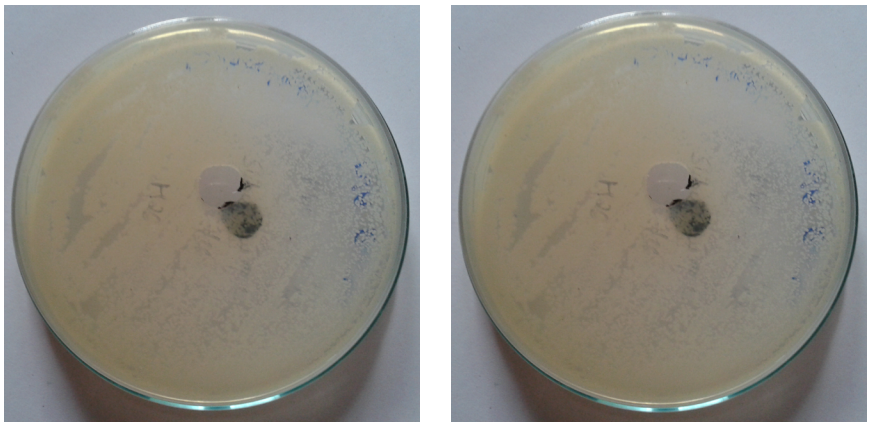


(e) Before Test: *Cylindrocarpon sp.*



(f) After Test: *Cylindrocarpon* sp.

Figure 4.12: Antibacterial Activity Profiling Against *Bacillus* sp.



(a) Before Test: *Rhizoctonia* sp. (b) After Test: *Rhizoctonia* sp.

(c) Before Test: Sterile Mycelium (d) After Test: Sterile Mycelium

Figure 4.13: Antibacterial Activity Profiling Against *Bacillus* sp.

Chapter 5

DISCUSSION

5.1 FT-IR Fingerprints of Fungal Isolates

Identification of fungi is a challenging task. In many cases identity of a fungal isolate is done by observing colony morphology and microscopic observation of micropreparations. However this approach is tough and requires expertise. An alternative approach to fungal identification is the use of molecular tools to study ITS sequences and proceed with DNA barcoding. However, this approach is expensive. Another alternative that could help in identification is FT-IR fingerprinting. This approach is comparatively better when utilizing ATR technology. This has been identified as a promising technology (Santos et al., 2010). This approach was utilized to characterize the fungal isolates used for this present study and the FT-IR finger prints reveal unique patterns in three prominent areas. The variation in transmittance of the wave, emitted from interferometer and emerging through the sample is dependent upon the overall chemical profile of the biomass.

The trough observed near wavenumber 3300 cm^{-1} indicates O-H stretching band of water. The area between wave numbers 2800 to 3000 denotes lipids of the fungal biomass; Figure: 4.2 on page 35. The peaks of each fungi are different in this range revealing the difference between these fungi with

respect to their lipid profile and could be of use in characterization. Another area observed was between wavenumbers 900 and 1200 cm^{-1} which indicates the differences in profile of carbohydrates between the fungal isolates. This region also holds promise in characterization and creation of a fingerprint. However, the large band observed near wavenumber 1050 cm^{-1} denotes carbohydrates and vibrations by nucleic acids. The peaks and bands in the region between 1300 and 1500 is related to protein profile of the samples. The band near wavenumber 1050 and 1150 cm^{-1} represents C-O stretching by carbohydrates (Salman et al., 2010). The region between wavenumbers 400 to 500cm^{-1} probably indicates metal complexes which could vary according to the mineral nutrient profile and may not be of much importance for characterization, however further studies need to focus on this area because there is notable difference between all the isolates in this region.

5.2 Enzyme Profiling

5.2.1 Enzyme Profiling in the light of endophyte-host plant interaction

Filamentous fungi are known to produce exoenzymes in nature. Production of these exoenzymes enables fungi to utilize diverse substrates and perform various functions related to its niche and trophic levels. Many of these enzymes help them to lead a saprophytic or pathogenic mode of existence. Many of these exoenzymes are hydrolases like amylase and lipase.

Amylase is known to help fungi to breakdown starch from substrates. Substrates containing starch and glycogen are hydrolyzed by attacking the $\alpha-(1,4)-$glycosidic bonds into simple sugars like maltose and are absorbed and utilized as sources of carbon and energy. Fungi that produce amylase have the ability to utilize starch from plant origin. Since this study focused on profiling of several bio-degrading hydrolytic enzymes like amylase and

lipase it becomes significant in that the presence of these enzymes throws light on the mode of interaction that these endophytic fungi are having with their hosts. Amylolytic capability reveals how these fungi are capable of utilizing starch which becomes available in senescing organs (Sunitha et al., 2013). Since the current study focused on foliar endophytic fungi, the presence of amylolytic ability in two endophytic fungal isolates viz: *Cladosporium sphaerospermum* and *Cylindrocarpon* sp. indicates their ability to degrade liter and probably could indicate early colonization in these plants in-view of litter degradation (Maria et al., 2005).

Cellulase is a plant cell wall degrading enzyme produced by fungi. This enzyme has been reported from several phytopathogenic forms of fungi (King et al., 2011). The presence of this enzyme in endophytic fungi, along with amylase and others suggests that endophytic fungi could also have an existence in the form of a latent asymptomatic pathogen. In this context its interesting to note that three of the five isolates are hereby reported to produce cellulases.

Lipases are known to function in a similar way by hydrolysing fatty acids. The products of hydrolysis are then utilized for metabolic processes. Many plants produce lipid storage products. Capability of endophytic fungi to breakdown lipids is of importance probably in utilizing such lipid reserves after colonization. It might also help in digesting lipid based wax like protective coatings on leaves while establishing and infection and further degradation as part of litter degradation process. In fact its interesting to note that a few mangrove plants which were subjected to quantification of waxy coatings on leaf surface reported presence of three times more wax compared to other emergent plants (Misra et al., 1984). This lipid deposition is supposed to be an adaptive strategy that helps plants to tolerate extreme physical conditions of mangrove ecosystems. Presence of lipase activity of foliar endophytic fungi could indicate the aforementioned capabilities to invade leaves and further to degrade the waxes after senescence, as part of litter degradation.

Laccases are known to oxidize different types of substrates both organic and inorganic. Laccase production has been reported from wide range of organisms however fungal laccases have recieved a lot of attention over the past few decades due to their importance in lignin degradation. Laccases have been studied mainly from wood rotting fungi but largely, the filamentous fungi are omitted. Though this group of enzymes are involved in several roles ranging from morphogenesis and differentiation of reproductive structures in fungi, they are popularly known for their lignolytic capability. They also help fungi in formation of rhizomorphs, polyphenolic glues that bind hyphae together and helps fungus to overcome immune responses of the host plant. Antifungal polyphenols are also oxidized and thus disarmed by extracellular fungal laccases helping better colonization of host plant. Above all, they are also involved in wood material degradation, production of humus by litter degradation (Madhavi and Lele, 2009). These facts reveal the importance of the results obtained. Three of the five endophytic fungi revealed the presence of this important enzyme. Interestingly the only exoenzyme reported from the sterile fungal isolate was laccase and the production was the highest observed value among the isolates screened.

Protease was not reported from any of the isolates. This enzyme has been reported from several phytopathogens but were not present in any of the isolates subjected to study. In several cases where it has been reported earlier, it is associated with disease progression. However since the isolates were asymptomatic endophytes, it is probable that they are not producing any detectable quanitity of proteinases so as to cause disease related host response and progression of infection into a symptomatic phase.

The above aspects throws light on the intricate relationship that exists between the host and the endophyte. The aforementioned facts suggests that endophytic fungi subjected to screening are existing in delicate balance of nutrient dependance with the host and possibly symbolizes early colonization of mature leaves so that they can be degraded completely when the leaf

becomes part of litter.

Among all the isolates obtained, *Cylindrocarpon* sp. was capable of producing all enzymes except protease. This indicates its capability and adaptation to live inside the host plant by utilizing several substrates, yet without eliciting symptoms of disease. These evidences also supports the hypothesis that endophytes could be latent pathogens.

In general, the results reveal that *Cylindrocarpon* sp. produces four out of the five exoenzymes screened. On the contrary, Laccase was the only enzyme produced by Sterile Mycelium. *Cylindrocarpon* sp. holds promise for potential use in production of Lipase at industrial scale, after conducting proper quantification and reaction optimization. Sterile Mycelium has similar potential to produce Laccase while *Alternaria* sp holds promise for production of Cellulase.

5.2.2 Industrial significance of the results of enzyme profiling

Apart from the understanding the interrelationship of host plant and endophytic fungus, the study has importance with regard to the utility of these enzymes in industries. Amylases are utilized in starch processing industries and such industries as food, fermentation, textile, paper, detergent and production of pharmaceuticals (Saranraj and Stella, 2013). Though amylase production has been reported in the current study, the results indicate only low level of production. Optimization of reaction conditions might offer better results but that needs further investigation. Likewise cellulase is of industrial application in the field of agriculture, detergents, fermentation, food, paper, textile, biotechnology, brewing, reconversion of cellulosic materials to ethanol, biofuels, animal feed production etc.. Of the three isolates producing Cellulase, *Alternaria fasciculata*, seems promising because the activity zone of this isolate was remarkable Figure: 4.7 on page 38. Sterile fungal isolate screened processed highest activity profile for Laccase enzyme and holds

promise for further studies for optimization and quantification; laccases being industrially important for detoxification of phenolic pollutants, xenobiotics, biobleaching, biosensors, its also used in pulp and paper industry. Laccases are used also in food industry and soil bioremediation. Lipases obtained from microbes especially fungi are considered to be stable and industrially important. It is used in industries like diary, brewing, food, detergents, textile pharmaceutical, fat, oil and biodiesel related industries. *Cylindrocarpon* sp. exhibits very promising potential for the production of this industrially important enzyme and also warrants further studies.

5.3 Antibacterial Activity Studies

Antibacterial activity was tested against *Bacillus* sp and *Klebsiella* sp. The rationale being the prospecting the antimicrobial activity against one gram positive and one gram negative bacteria respectively. Three of the five isolates *viz: Alternaria fasciculata, Cylindrocarpon sp. and Cladosporium sphaerospermum* exhibited antibacterial activity against *Bacillus* sp. Earlier studies have reported the presence of an antibiotic compound called Altersetin from *Alternaria* sp. which is chemically related to equisetin and showed more activity against gram positive bacteria (Hellwig et al., 2002). There was no antibacterial activity against *Klebsiella* sp. for any of the five isolates screened. This could be due to the protective action of the LPS layer of gram negative bacteria that secures the contents inner to the layer, in the case of gram negative bacteria. The results of the study are presented in figure 4.6 on page 37. The interesting correlation observed is that there is no demonstrable relationship between the production of hydrolytic enzymes and antibacterial activity. Therefore there is a higher probability that these fungi are in fact producing some compounds with antibiotic activity. Further studies should focus on separating various fractions in organic solvents of varying polarity to find out the compounds responsible for antibacterial action. Since

these antibacterial activity are reported form fungal endophytes of plants, it could also suggest the existence of some degree of mutually beneficial inter-relationship between the fungal endophyte and the host plant because the antibacterial compounds produced by the endophytic fungi may in turn be conferring the host plants, resistance to bacterial infections.

Chapter 6

SUMMARY & CONCLUSIONS

Earlier reports state the presence of five different species of true mangroves, however, only three of the listed plants were located from the area under study. The distribution of mangroves in Kottayam district is mainly restricted to some isolated pockets of land area in and around Kumarakom and Vaikom. These areas harbor mangroves primarily in private land holdings. The once luxurious vegetation is now under serious threat due to habitat destruction which is in turn related to encroachment and development of infrastructure for tourism. The presence of Thaneermukkam bund (Salt Water Barrier) also has its impact on mangrove vegetation which usually grows in places with salinity. The construction and utilization of the aforementioned bund is meant to help local agriculture by reducing inflow of saline water from sea. This could be one added reason for the reduction in the distribution and diversity of mangrove plants compared to reports obtained earlier. Out of five true mangrove plants reported earlier from the area, only three were located during the study. The three plant species located from study area are: *Sonneratia caseolaris, Bruguiera sexangula and Excoecaria agallocha.* Five different endophytic fungal isolates were obtained they are: *Alternaria fasciculata*, *Cylindrocarpon* sp., *Cladosporium sphaerospermum*, *Rhizoctonia* sp. and and isolate of *Sterile mycelium.*

These five different mangrove foliar endophytic isolates were subjected to: Identification based on morphological characters, characterization of Biomass yield on PDA, characterization by FT-IR , Antibacterial assay against a gram positive and a gram negative bacteria and Profiling for Five different Exoenzymes viz: Amylase, Cellulase, Laccase, Protease and Lipase. Mangrove plants occupy areas with unique environmental factors, both physical and chemical. This makes them ideal for screening for potential for bioactive compounds with potential application in different fields of human activities. The current study helped to identify the isolates based on morphological features however one of the isolate remained sterile in culture and was labeled as Sterile Mycelia following the practice of several reports of similar kind. The remaining four fungal isolates are: *Alternaria fasciculata*, *Cylindrocarpon* sp. *Cladosporium sphaerospermum* and *Rhizoctonia sp.* Microphotographs were taken and these were further subjected to broth culture to study biomass yield. *Alternaria fasciculata* exhibited highest biomass yield however Sterile Mycelium reported the lowest biomass yield. FT-IR fingergrints were prepared as part of characterization strategy to aid identification for further analytical studies. The FT-IR fingerprints showed variation between fungal isolates and therefore reveals its utility in characterization. Enzyme profiling revealed presence of four exoenzymes. Every fungal isolate produced at least one biodegrading enzyme and it has implication in explaining the interrelationship between the host plant and endophytic fungi. These enzymes also are important industrially; therefore some of these fungal isolates could be subjected to further study in view of industrial scale isolation of these relevant enzymes. *Cylindrocarpon* sp. reported the presence of four enzymes on the contrary, Laccase was the only enzyme produced by Sterile Mycelium. *Cylindrocarpon* sp. holds promise for potential use in production of Lipase at industrial scale, after conducting proper quantification and reaction optimization. Sterile Mycelium has similar potential to produce Laccase while *Alternaria fasciculata* holds promise for production of Cellulase. Protease

was not detected in any of the fungal isolates. Antimicrobial study revealed activity of three fungal isolates against gram positive bacteria (*Bacillus* sp.). None of the isolate exhibited activity against gram negative bacteria (*Klebsiella* sp.). Further studies might lead to isolation, confirmation and charactrization of the compounds responsible for antibacterial activity.

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