Physiological and molecular characterization of laccase gene in *Morchella* spp.

A Dissertation
Submitted in partial fulfillment of the requirement
For the award of degree of
Masters of Science in Biotechnology

Under the guidance of
Dr. M.S. Reddy
Professor

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DECLARATION

I hereby declare that the work which is being presented in this thesis **"Physiological and molecular characterization of laccase gene in *Morchella* spp."** Submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

Date: 15.07.11

Place: PATIALA

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(Divya Gupta)

CERTIFICATE

This is to certify that the thesis entitled **"Physiological and molecular characterization of laccase gene in *Morchella* spp."** submitted by Divya Gupta (Roll no: 300901005) in partial fulfillment of the requirement for the award of Degree of **Master of Sciences in biotechnology, to Thapar University (Deemed University), Patiala,** is a record of Student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Date: 15.07.11
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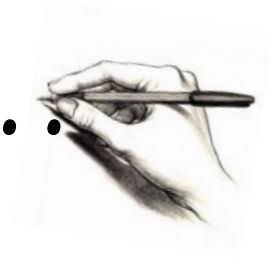
Dedicated to my parents…



Abstract

Morchella, the true morels, is a genus of edible mushrooms having economic importance. But cultivation of *Morchella* on commercial scale is yet not successful. Many reasons have been asigned for this; main reasons being complex nature of sclerotia in life cycle of morels, requirement of definite nutritional and environmental parameters for sclerotia formation or/and variation in ligninolytic enzyme level during mycelial growth, sclerotia formation and fruiting body formation. In the present study two *Morchella* spp. namely *Morchella spongiola* MR 17 and *Morchella sp*. MR 2 were used. Growth study were conducted in different medium, differnt carbon-sources and different nitrogen- sources. Maximum growth in *Morchella spongiola* MR 17 was recorded in malt extract while in *Morchella sp*. MR 2 maximum growth was measured in yeast malt extract. MSB medium containing mannose as carbon source and sodium nitrate as nitrogen source served as the best source for growth study. Increase in growth was recorded in MSB medium supplemented with zinc and manganese ions, while decrease in growth was recorded in MSB medium supplemented with copper and cadmium ions. From the sclerotial studies conduted using various lignocellulosic substrates it can be concluded that laccase enzyme plays an important role in sclerotia formation. Maximum number of sclerotia were recorded in *Morchella sp*. MR 2 using wheat grains as lignocellulosic substrate after 15 days of incubation. More growth and higher laccase activity was recorded in *Morchella sp*. MR 2. Laccase gene of 300bp was detected in *Morchella sp*. MR 2 using Cu1A F and Cu2 R set of primers and it was cloned in pTZ57 R/T cloning vector and sequenced. The present study points out the important role of laccase in sclerotia formation in morels. This may help in understanding the cultivation aspects in morels.

Chapter I

1 Introduction

The Himalayan morel popularly known as "Guchhi" is an economically important edible wild mushroom. ***Morchella,*** the true morels, is a genus of edible mushrooms belonging to class Ascomycetes. The morels are amongst the most highly prized fungi in the world (Pegler, 2003). Their harvesting represents a seasonal employment niche in the non-timber forest product (NTFP) sector for people throughout much of the world. There are a number of publications that contain recipes for morels (Weber, 1995; Lonick, 1999; Ratzloff, 1990) as well as a plethora of websites with recipes. Morels are considered to be one of the high value NTFP and the most desirable edible mushrooms known.

1.1 Importance of morels

When considering *Morchella* from the ethnological perspective, the genus offers two intertwined values, those being directly used as a prized food product and source of income from sale.

1.1.1 Morels as food

All the true morels are known to be edible (Arora, 1986; Groves, 1979; Weber, 1995). Morels have an excellent flavor and are reputed to be superior to that of other mushrooms. Not only morels, but even their mycelia have same nutritive value (Cochrane, 1958; Robinson and Davidson, 1959; Singer, 1961; Hayes and Hadded, 1976).

1.1.2. Nutritive value

Mushrooms are low calorific but protein rich and hence a good source of protein for human consumption, mushrooms contain 20-40 % protein on dry weight basis and thus surpass many foods including milk in terms of food content (Kurtzman, 1975). On average fruiting bodies of *Morchella sp.* contains 30% crude protein, 2.5 % fat, 15% ash and 85 % moisture (Kaul, 1978; Shad, 1989; Sharma, 1993). *Morchella* spp. contains essential amino acid including sulphur containing amino acid as cystine and vitamins including thiamine, riboflavin, niacin, pantothenic acid, pyridoxine and cyanocobalamine (Samajpati, 1978).

1.1.3.. Medicnal properties

Anti tumor activity

Extract *Morchella esculenta* has antitumor activity against both ascites and solid tumours (Nitha *et al*., 2007).

Antioxidant activity

Methanolic extracts of mycelia of *M. esculenta* posses high antioxidant activity (85.4%) at 25 mg/ml. The relatively high content of total phenols contributes to the morels anti-oxidative capabilities (Mau *et al*., 2004).

Anti-inflammatory activity

Mycelium of *Morchella* spp. shows 66.6% and 64.2% inhibition of acute and chronic inflammation, respectively and its activity is comparable to that of the standard reference drug, Diclofenac (Nitha *et al*., 2007).

Immune enhancers

An immune stimulatory galactomannan polysaccharide accounts for about the immune stimulatory activities of various morel extracts. At a concentration of 3.0 µg/ml, the

galactomannan polysaccharide increased NF-kappa B directed luciferase expression in THP-1 human monocytic cells to levels 50% of those achieved by maximal activating concentration (10 µg/ml) of lipopolysaccharide (Duncan *et al*., 2002).

1.4 Production and trade

An estimated 60 ± 5 tonnes of dry morels are exported every year from India to the international markets. Himachal Pradesh, Jammu and Kashmir and Uttar Pradesh are the main exporters of morels in India. The morel production during 1981-82 from Himachal Pradesh was around 11000 Kg, whereas during 1982-84 it was stated to be 48,000 Kg (information collected from Forest Department). The production figures for 1986-87 have been estimated to be 55,000 Kg. Sociobiological studies conducted in morel producing area of Himachal Pradesh by Singh and Rawat in 2000 revealed that upto 1 kg of morels are collected from Himachal Pradesh per day. The average Morels are sold at a price of Rs.10000-15000 per Kg ($750 a kilo in western countries) Prasad *et al*., 2002

Fig1.1*Morels* in landscaped area. Photo by Fred stevens.(Source: http:// www.mykoweb.com)

1.5. Occurence of morels

Species of *Morchella* in India occur primarily in Northern Western Himalayas. Species of *Morchella* have, however, been recorded/collected during the rainy season (Wakode,1983; Lakhanpal and Shad,1986; Kamal, 2005; Kaviyasaran *et al*., 2006) as well in the month of September and October, repeatedly from some parts of Himachal Pradesh including lower hills which do not experience snowfall (Lakhanpal and Shad, 1986). *Morchella* fruiting bodies have also been reported in paddy field bunds (in Jammu) and maize fields (in Solan) (Jandaik and Sharma, 1995).

Majority of the *Morchella* spp. have been collected from Northern-Western Himalayan region, especially Jammu & Kashmir (Cooke, 1870; Sydow and Butler, 1911; Ghosh and Pathak, 1962; Batra and Batra, 1963; Waraitch, 1976; Kaul, 1981), Himachal Pradesh; (Sohi *et al*., 1965; Lakhanpal and Shad, 1986) and Uttar Pradesh (Hennings, 1901; Theissen, 1911).

1.6. Life cycle of morels (Volk and Leonard, 1989; Volk and Leonard, 1990)

In morel's life cycle, plasmogamy between strain variants creates a heterokaryotic mycelium (secondary mycelium) which later on forms heterokaryotic sclerotia, which can either revert back to secondary mycelium or lead to ascocarp formation. The ascocarp is lined with tiny, microscopic elongated sacs, each of which is called an ascus. Inside each ascus are microscopic spores lined up like small eggs, approximately eight spores per ascus. These spore escapes from the lid of ascus and taken to the air, marking the beginning of the life cycle of the morels. When these spores land on appropriate food source such as moist dead, rotting or decaying plant life, the spores will hatch producing small hair like structures called hyphae. The hyphae begans to spread throughout the food source producing an interwoven mat or feeding network called mycelium. Under certain, unfavourable conditions, this mycelium contracts to form hardened protective bodies called sclerotia. The sclerotia then lie dormant until favourable conditions, the sclerotia develops ascocarps.

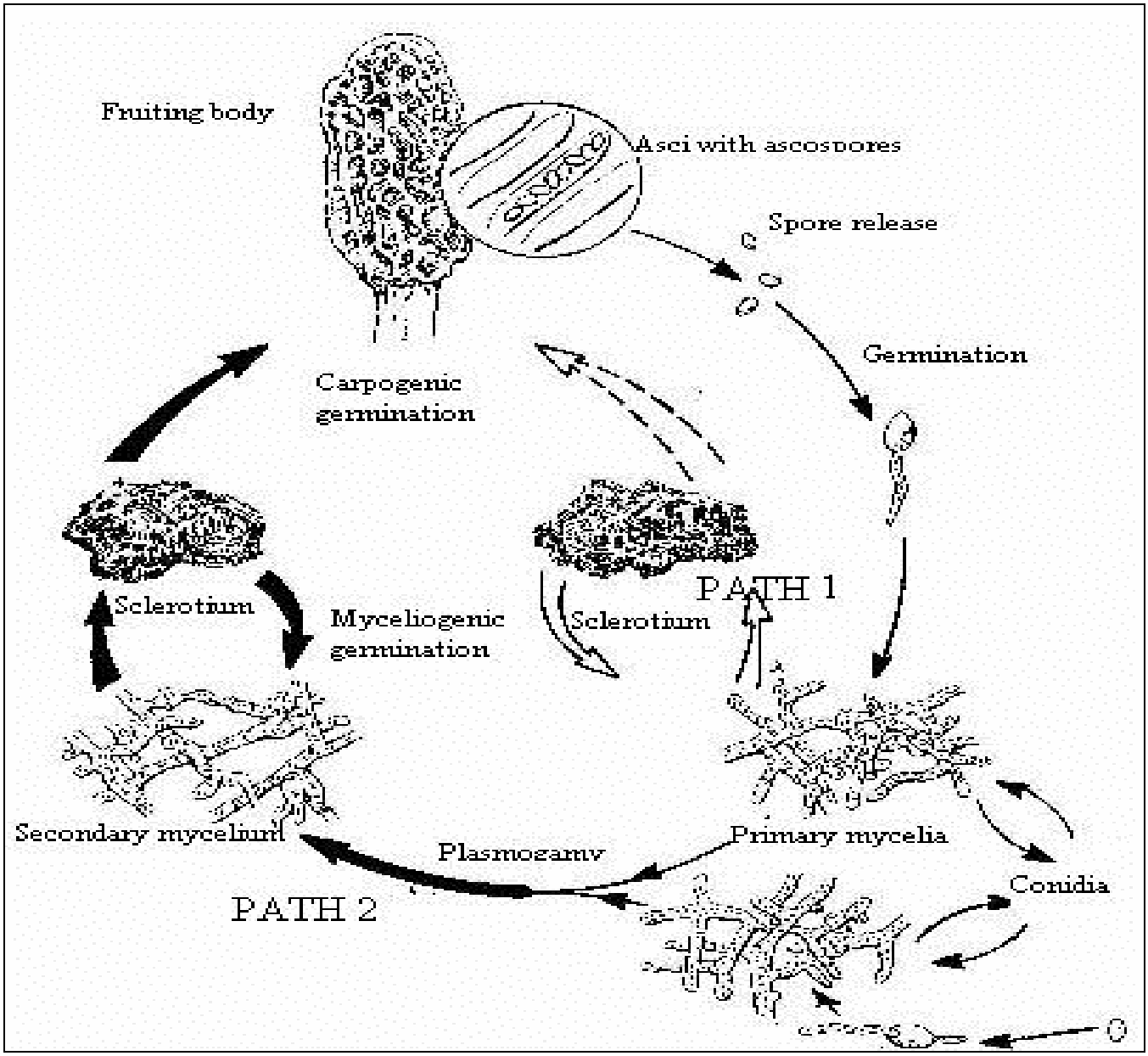


Figure 1.2.: Representation of the morel life cycle (Volk and Leonard 1989, Volk and Leonard 1990,)

1.6.1 Fruiting bodies

The fruiting bodies of *Morchella* spp. is stipitate, hollow and differentiated into pileus (cap) and stipe (stalk). The colour of the pileus varies from dirty grayish-white to dark-brownish depending on the species, age of fruiting body, and the type of vegetation. Pileus constitutes the fertile portion of the ascocarp. It is hollow and fleshy. The honey-combed surface of pileus gives the

appearance of a sponge. When young, the surface of pileus is quite smooth, and as it grows, there appears a network of ridges and grooves or depressions (pits) on the surface due to unequal growth of the hymenial surface. The ridges are sterile while the pits are fertile.

1.6.2 Ascospores and mycelium

The hymenium which lines the pits bears numerous asci. These asci are cylindrical, sub-cylinderical with obtuse apex. Each ascus contains eight spores. The ascospores are ellipsoid, unseriate, yellowish, in mass. The asci are positively phototrophic. The ascospores are wind disseminated. Falling on a suitable substratum (moist humus), each ascospores germinates to produce new mycelium germinates to produce new mycelium.

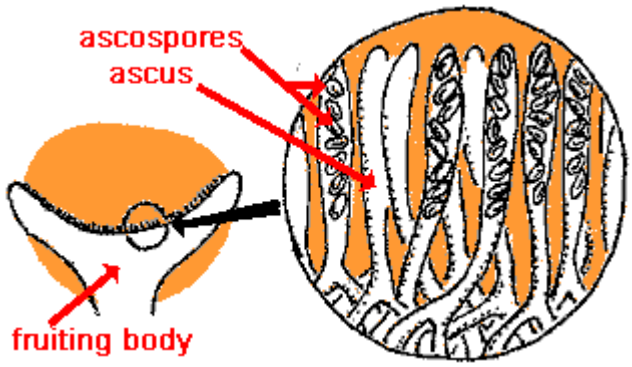


Fig1.3. Internal structure of fruiting bodies of morels

1.6.3. Sclerotial stage

Sclerotia, as a nutrient sink is critical in the study of the morel life cycle. Sclerotia is a big structure, with large cells and thick cell walls, a hard surfaced resting body of fungal cells which acts as a nutrient reservoir and is resistant to unfavorable environmental conditions, allows the organism to survive in adverse conditions. Nutrients mainly neutral lipids in the form of

triglycerides are stored in the sclerotia and during the sexual cycle, substantially all of the nutrients for fruitbody development are drawn from sclerotia. Sclerotia remain dormant for long periods of time and resume growth on the return of the favorable environmental conditions.

Sclerotium plays an important part in reproduction of morels and helps to explain why they have been so difficult to cultivate. Sclerotia are not found in other cultivated mushrooms, thus complicating their cultivation process, as sclerotia can either germinate as new mycelium or fruiting bodies, and the former is far easier to develop (Volk, 2004). Even when one is successful in cultivating the development of primordia, they are prone to abort (Volk, 2004)

1.7. Ligninolytic enzymes

White rot fungi variously secrets one or more extracellular enzymes, that are essential for lignin degradation, and combine with other processes to effects lignin mineralization. They are referred to as Lignin-Modifying enzymes or LMEs. The three enzymes comprise two glycosylated heme-containing peroxidases, **Lignin peroxidase** (*lip*, E.C. 1.11.1.14) and **Mn dependent peroxidase** (Mnp, E.C. 1.11.1.13) (Orth and Tien, 1993), and a copper containing phenoloxidase, **Laccase** (*lcc*, E.C. 1.10.3.2) (Thurston, 1994). LME production occurs during secondary metabolism and is subject to complex regulation. The lignin degrading system depends on low molecular weight metabolites and cofactors. The secondary metabolite veratryl alcohol (3, 4-dimethoxybenzene) is the redox mediator for LiP, Mn^{2+}; metal ion naturally present in the wood is the redox mediator for MnP, when properly chelated with fungal organic metabolites (e.g oxalic acid). LiP appears to be the key enzyme in the oxidation of nonphenolic phenlypropanoid units. This enzyme oxidizes aromatic nuclei into aryl cationic radicals including the cleavage of C-C and C-O linkages (Hammel and Kirk, 1985; Kirk and Farrel, 1987). MnP oxidizes Mn^{2+} to Mn^{3+}, which

in turn may attack phenolic structures in lignin as long it is stabilized by suitable metal chelators secreted by the fungus (Kuan and Tien, 1993) and also may attack nonphenolic structures via lignin peroxidation (Bao *et al*., 1997).

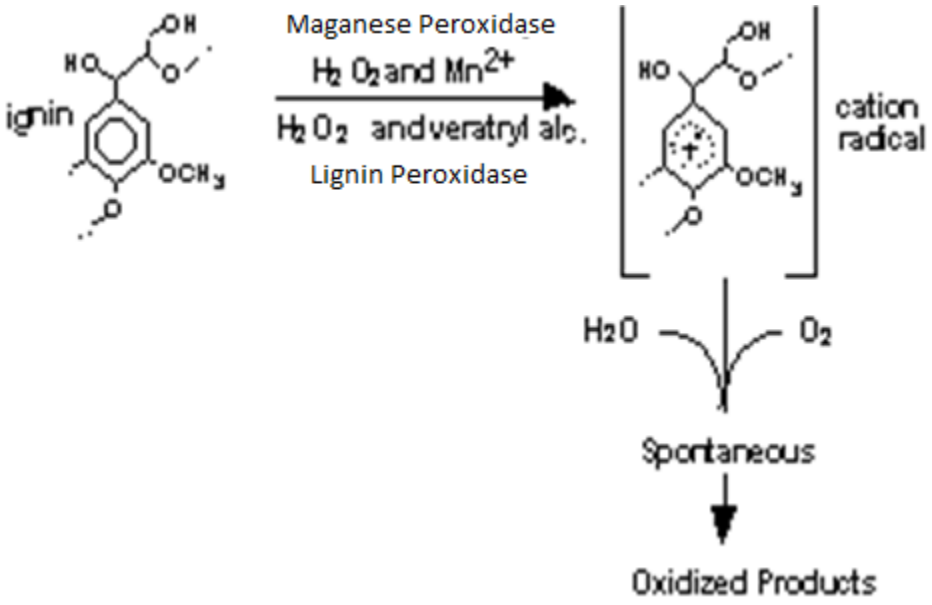


Fig 1.5 Single electron oxidation of an aromatic ring in lignin by extracellular peroxidases and subsequent oxidation cation radical by oxygen.

1.7.1. Laccase (p-diphenol-O2 oxidoreductase; EC 1.10.3.2)

Laccases are members of the blue copper oxidase enzyme family. The typical reaction of laccase is oxidation of a phenolic compound with the concurrent reduction of molecular oxygen to water. After four cycles of single electron oxidations forming free radicals, the enzyme reduces one molecule of O_2 generating two molecules of water. This mechanism requires the protein to store four electrons before the reduction of O_2. The enzyme is characterized by having four Cu^{2+} ions coordinated such that each of the known magnetic species (Type 1, Type2 and Type 3) is associated with a single polypeptide chain. Three types of copper atoms can be distinguished by

their spectroscopic and magnetic properties: Type 1 (T1), Type 2 (T2) and Type 3 (T3). Laccase contains one T1 Cu bound as a mononuclear center, one T2 and two T3 Cu atoms 12 forming a trinuclear center. In the resting stage of the enzyme all four Cu atoms are likely to exist in Cu II state.

1.7.2. Substrate for Laccase: Lignin

An important part of microbial carbon cycling is the biodegradation of plant polymers. Plants are responsible for principal input of organic carbon into soils, and soil microorganisms are largely responsible for the transformation of their structural polymers. Biogenic polymers recycled primarily by microbial degradation in the soil include cellulose, hemicellulose and chitin. Another polymer that is almost as abundant as cellulose in higher plants is **lignin,** but its overall turnover rate is much slower than any other biopolymer. Lignin has a unique structure. In wood and other lignified structures such as grass stems, lignin occurs in intimate association with cellulose and hemicelluloses, adding structural strength and protecting the polysaccharides by its biodegradation resistant barrier. Lignin have an aromatic structure consisting of polypropane subunits linked together by carbon-carbon (C-C) or ether (C-O-C) bonds into highly complex three-dimensional structure (Ander and Eriksson, 1978; Kirk *et al*., 1980; Zeikus, 1981; Kirk, 1984).

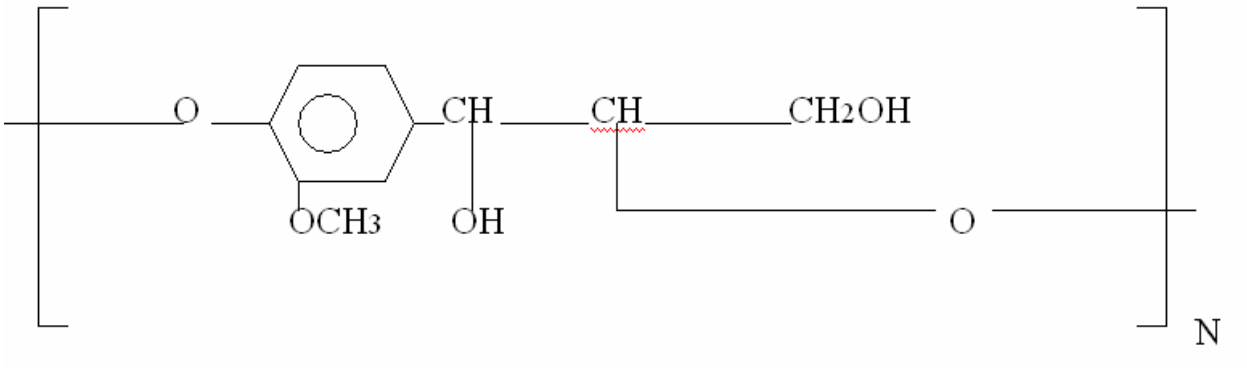


Fig 1.4: Phenylpropane subunit of lignin

Lignin is formed by removal of water from sugars to create aromatic structures. These reactions are not reversible. There are many possible monomers of lignin, and the types 10 and properties depend on the source of nature. The biosynthesis of lignin starts from phenylalanine. Synthesis of lignin is unusual in that the polymerization does not take place on the enzyme surface. Instead oxidases and peroxidases produce reactive quinon methid radicals that polymerize spontaneously (Kirk, 1989). Lignin and its subunits are, therefore, are not optically active and the randomness of polymerization process makes subsequent enzymatic degradation of the product much more difficult. Hence, biodegradation rates for lignin are much lower than for either cellulose or hemicelluloses compounds (Kirk, 1984).

1.7.3. Biodegradation of lignin

Lignin resists attack by most microorganisms, and anaerobic processes tend not to attack the aromatic rings at all. Aerobic breakdown of lignin is slow and may take many days. Lignin is nature's cement along with hemicellulose to exploit the strength of cellulose while conferring flexibility. Some organisms, particularly fungi, have developed the necessary enzymes to break lignin apart. The initial reactions are mediated by extra cellular lignin peroxidases, manganese peroxidases and laccases produced by white rot fungi (Kirk and Farrel, 1987). Actinomycetes also degrade lignin but typically degrade less than 20% of the total lignin present (Crawford 1986; Basglia *et al*., 1992). Lignin degradation is primarily an aerobic process carried out by mainly white rot fungi during secondary metabolism (Boominathan and Reddy, 1992) and in an anaerobic environment lignin can persist for very long periods (Van Soest, 1994). This recalcitrance, possession of lignolytic ability among relatively few species at 20.3 * 1012 kg annually (Bassham, 1975) contribute to lignin degradation being regarded as the rate limiting

step to carbon turnover in lignocellulose dominated environments. Intact wood is attacked first by brown rot and white rot fungi, which are both basidiomycetes. Brown rot fungi, by a mechanism not clear, bypass the protective lignin barrier and attack the cellulose and hemicellulose components of the wood directly. Logs decomposed in this manner fall apart into brown powder consisting of mainly enzymatically-liberated lignin. In contrast, white rot fungi degrade preferentially,leavinga soft fibrous cellulose residue.

1.7.4. Catalytic mechanism of laccases

Laccase have very broad substrate specificities and can couple 4 one-electron oxidations to a variety of substrates such as di and polyphenols, aromatic amines, and a considerable range of other components, to the irreversible 4-electron reduction of O_2 to water.The mononuclear T1 Cu site functions as the primary electron acceptor, extracting the electrons from the reducing substrates and delivering them to the trinuclear T2/T3 site.The trinuclear T2/T3 centre, the reduction. Reduction of oxygen most likely takes place in two steps, since bound oxygen intermediates are involved. In laccase-catalyzed oxidation, the substrate loses a single electron and forms a free radical. The unstable free radical may further undergo laccase-catalyzed oxidation or nonenzymatic reaction such as hydration and polymerzation.

Aryloxy radicals formed by laccases may further undergo non-enzymatic oxidation/reduction or couple to other phenolic structures and produce intensely colored products. Laccases, LiPs, and MnPs can oxidize phenolic compounds thereby creating phenoxy radicals, while non-phenolic compounds are oxidized via cation radicals. Laccases oxidize

aromatic compounds with relatively low ionization potentials, whereas LIPS readily oxidize compounds with high ionization potentials.

Laccase has low redox potential therefore is able to catalyze single electron oxidation steps only with the easy to oxidize phenolic compounds of lignin, with concurrent reduction of O_2 to water. However the activity of laccase can be fostered and expanded towards more difficult to oxidize non-phenolic substrates by use of mediators. The oxidative enzymes cause one-electron oxidations of aromatic moieties in lignin. The resulting cation radicals are susceptible to further oxidation in presence of O_2. The lignolytic system is nonselective, consequently other aromatic substrates, such as black listed pollutants are oxidized and degraded by white rot fungi. Important substrates that can be directly degraded by LME are pentachlorophenol, dioxins, polycyclic aromatic hydrocarbons and azo dyes also.

1.5 Aim of the study

At present, morels comprising the genus *Morchella,* is one of the costliest and most sought mushroom from the edible fungi in the world. The morels are highly appreciated for their culinary aspects, gastronomical delights and excellent flavor. Unlike other mushrooms, the cultivation of morels on commercial scale is a difficult task, may be due to the complex nature of intermediate stage of sclerotium in morel life cycle and requirement of definite nutritional and environmental parameters for sclerotia formation. Commercial cultivation of mushrooms is generally done on lignocellulosic substrates; wherein ligninolytic enzymes are secreted. Also, variations have been found in the ligninolytic enzyme levels of many fungi during mycelial growth, sclerotia formation and fruiting body formation, too. We need to improve the understanding of the mechanisms that underlie the cultivation of morels on commercial scale.

So, in an attempt to cultivate morels, *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 were studied for their growth pattern and ligninolytic enzyme activities (laccase, manganese peroxidase, lignin peroxidase, cellulase, dinitrosalicylic assays) in different media, different carbon sources, different nitrogen sources and under different metal stress conditions of copper, cadmium, zinc and manganese. Sclerotial studies were carried out in different substrates and ligninolytic enzymes activities were studied. Laccase gene (300 bp) involved in the sclerotia formation in *Morchella sp.* MR 2 was identified. Laccase gene was cloned and studied.

Chapter-II

Review of Literature

2.1 Morels

Among the various types of wild mushrooms, morels *(Morchella* spp.*)* occupy a special position. It is the excellent flavor that the morels posses, which has fascinated man and made him interested in collecting morels for food (Rofle and Rofle, 1925; Lambert, 1983; Gray, 1959). Over the past ten or fifteen years, mycologists have made some important discoveries regarding the morels. Through laboratory experiments, much has been learnt about how morels grow. Mycologists want to cultivate morels just like button mushroom and there have been numerous attempts to achieve success ever since morels have been consumed. There are various reports in literature which attempts to cultivate morels.

2.1.1 Physiological studies in morels

Brock, (1951) studied nutrition and physiology of *Morchella sp.* using various carbon and nitrogen sources. He concluded sodium nitrate to be a moderate source of nitrogen for *Morchella esculenta*, nitrates considered as excellent sources of nitrogen for many fungi, though inability to utilize it has been reported for higher Basidiomycetes, Saprolegniaceae and Blastocladiales.

Kaul, (1977) conducted studies on carbon utilization in 6 species of *Morchella*. He used 26 different carbon compounds, starch, maltose, fructose, glucose, glycogen and sucrose were for all strains tested ribose, sorbose, rhamnose, tartaric acid and malic acid were not utilized by any ands dulcitol, insulin, melinose and arabinose gave poor growth in all cases. Other compounds gave intermediate to poor growth. Kaul, (1977) also studied the nitrogen utilization nine morel strains on 30 different nitrogen compounds and observed that asparagines, urea,

peptones, glutamic acid, ammonium salts, Sodium nitrate and Potassium nitrate were good sources of nitrogen for all species, while hydroxylamine-HCl and hydrazine-2HCl were not utilized. Intermediate to poor growth was obtained with cystine, methionine and histidine, glycine, valine, serine, phenylalanine, leucine, isoleucine and arginine.

Dijkstra *et al*., (1972) reported casein's positive effects on *Agaricus sp.*, but no such positive effects were observed on *Morchella* types.

Kaul (1981) recorded 25°C as the best temperature for the growth of *Morchella* species. Dizbay and Karaboz (1986) raised submerged cultures of various *Morchella* species for microbial protein production. They used various agricultural products and waste material, olive black water, beet molasses, potato and pumpkin as substrate in sub merged cultures. The best mycelia development was observed in beet molasses medium. *M. elata* was found to be the most productive species among the *Morchella* strains used in subculture experiment of the cytoplasm stream from the mycelium to the sclerotium.

Guler and Arkan, (1999) have studied the cultural characteristics of *M. esculenta* on a variety of nutritional media. They investigated the cultural characteristics of *Morchella esculenta* mycelium in potato dextrose agar, malt extract agar and complete medium yeast plates prepared with casein, casamino Acids, peptone and sodium nitrate ($NaNO_3$). They determined aerial and vegetative mycelium types in the agar media with casein, casamino and peptone. The third colonial characteristic was observed. They determined that in the agar media with $NaNO_3$ the colonization increase in circular forms spreading from the centre to the edges and mycelium colonization periods were 20-24 days in agar media with $NaNO_3$.

2.1.2 Cultivation attempts of Morels

Costantin and Matruchot, (1883) were first to start experiments on the cultivation of *Morchella esculenta* in France in 1883, Costantin obtained a yield of 350 g of fruiting bodies/sqm of bed surface. Yet Baron d´Yvorie, (1989) was first to succeed in growing these fungi. He obtained fruiting bodies of *Morchella esculenta* on apple rinds plus humus plus pieces of ripe morel carpophores taking clue from Roze, (1883) that *M. esculenta* parasitizes the rhizomes of *Helianthus tuberosus.*

Hervey *et al*., (1978) crossed several hundred pairs of single ascospore isolates of *Morchella esculenta*. They observed the build up aerial hyphae at the interaction interface in all non-self combination and termed this as "barrage-like build up". They observed that either ascus fusion nuclei must be heterozygous for certain genetics factors or that asci of a single ascocarp do not at all arise from a single pair of nuclei.

Schmidt, (1983) reported that ascospores of *M .esculanta* germinated and developed extensive mycelium within 24 hours, (after one year of storage) at temperature above 15°C and was slower at lower temperature, but spore germinated within one week at temperatures of 2°C or above on malt extract agar.

Repin, (1901) got fruiting bodies around a bed composed of dry leaves rendered alkaline with Sodium carbonate in a trench in which apple residue had been deposited. He also established that morels can also grow saprophytically.

Ower *et al.,* (1986, 1989) studied cultivation of *Morchella* and got registered two patents "Cultivation of *Morchella. U.S. Patent No. 4,594,809" and "*Cultivation of *Morchella. U.S. Patent No. 4,866,878"* for the commercial production of morels **.**They firstly developed sclerotial spawn for morel production. For this they filled a 50 ml container with wheat (or any

other vegetable material) between about 40-80% of its volume .Then they covered wheat with perforated liner, typically plastic film or metal film and remaining 20-60% of container was filled by moist soil. Then they covered and autoclaved the container to kill the possible contaminating organism. The soil layer of sterilized container was inoculated with ascospores, vegetative hyphae or with small pieces of sclerotia and jar was sealed again, incubated at a temperature of between 18°C - 22°C.They observed that hyphae from inoculum were grown through the soil layer and colonized the grain. After about a week, a loosely compacted mass appeared in the soil, when they viewed it microscopically, a highly branched hyphal cells that swell to a barrel shape having septa were observed. They concluded that it was the sclerotial hypal cells that adhere to adjacent cells to form a solid mass that was visible to naked eye, these cell stored the material obtained from colonized grains. They also observed that cell of the total soil layer could become enmeshed in the sclerotia. At this point they harvested sclerotia and used them as spawn. They used the above developed mature sclerotia in two ways to inoculate substratum. In the first way they divided mature sclerotia into pieces and used them to inoculate substratum. These pieces produced hyphae, which upon addition of nutrients produced additional sclerotial mass with in the substratum before induction of sexual cycle. In the second method, sclerotia that have been developed in the jars were directly inoculated into substratum and the mycelia which grow there form were induced to the sexual cycle, without addition of nutrients. Their studies concluded that ascocarps or fruiting bodies of *Morchella sp.* could be cultured by providing their mycelium with nutrients and subsequently produce nutrient-primed mycelia such as nutrient rich sclerotia or nutrient rich hyphae in which sufficient nutrient were stored and were supplied to ascocarps that would develop later. The fungus was induced to give rise to ascocarp by initially maintaining the fungus in an environment that is poor in exogenous

nutrients and by exposing the fungus to high level of water .After induction, primordial appeared. The peroid from primordial appearance until midway to maturation of the fruiting bodies were prone to abort. During this critical period, particular attention was directed to maintain favourable conditions. The fruit bodies which were grown to maturation were ultimately harvested.

Volk and Leonard, (1989) studied the cytology of the life-cycle of *Morchella* .The cytological studies of various stages of the morel life-cycle were done and demonstrates that the average number of nuclei per cellular compartment in vegetative hyphae of *Morchella* is 10–15 and that hyphal fusions are quite frequent. The resting structures, the sclerotia, are actually pseudosclerotia which form from the repeated branching and enlargement of terminal hyphae from either primary (homokaryotic) or secondary (heterokaryotic) hyphae. They also depicted the development of fruiting body primordia. Photomicrographs of ascus development demonstrate autogamy rather than *de novo* heterokaryo. They have introduced a comprehensive life-cycle diagram of the morel for the first time.

Stewart C. Miller, (2005) also studied cultivation of *Morchella* and got registered a patent "Cultivation of *Morchella. U.S. Patent No. 6,907,691 B2. U.S. Patent No. 4,866,878"* for the production of morels. He used *Morchella sp.* Mycelium and a tree seedling (having a root system and shoot system) for cultivation of *Morchella* ascocarps. He firstly inoculated the root system with the mycelium to produce an inoculated tree seedling, then the mycelium was stimulated to form sclerotia by severing the shoot system from the root system and sclerotia was induced to produce ascocarps by providing conditions conducive to the formation of ascocarps (as providing spring like conditions).

There is another references of Masaphy 2005-external fruitbody dev in morchella-chck n write that Sehgal and Sharma, (2007) studied forced heterokaryons formation in *M.esculenta* and observed pairing of nuclei in the mycelia derived from ascocarp and generated heterokaryons, indicating a possible role of heterokaryosis in the life cycle of *Morchella* in formation by hyphal fusion in the sub-hymenial layer of the fruiting body.

Masaphy, (2010) provided the first report on successful fruit body formation and development in a soilless system. He successfully initiated and developed the fruiting body of *M. rufobrunnea* in laboratory scale experiment. He inoculated the fungal mycelium into a sterile of potting soil buffered with limestone at 120 °C for 1 hour. Then placed the inoculated medium over a layer of nutritionally rich medium based on wheat grains and incubated this at 18-25 °C for 2-3 weeks. After this incubation sclerotia formed, which were subjected to continous watering for 5-24 hours. Then he incubated the induced sclerotia at 16-22° C for 2-4 weeks for carpogeneic initiation and fruiting body development. Scmidt, 1983; Buscot, 1989; Volk and Leonard (1990) reported that freezing and thawing associated with the winter and early spring lead to the formation of ascocarps.

2.2 Sclerotial studies

Ower, (1982) and Ower *et al*., (1986) observed that accomplishment of small-scale laboratory production and secretion of extracellular enzymes by *Morchella esculenta* and *M. angusticeps* enhances their possibilities of domestication.

Volk and Leonard, (1989) have studied physiological and environmental studies of sclerotium formation and maturation in isolates of *Morchella crassipes*. There study provided a set of nutritional and environmental parameters suitable for the growth of morel *(Morchella crassipes*) sclerotia in the laboratory, using a modification of the jar method of Ower *et al*., (U.S. patent 4,594,809, June 1986). The optimum nutritional and environmental conditions for morel sclerotium formation and maturation as determined in there study consisted of a layer of rye grain supplemented with peptone, yeast extract, trace elements, and casamino acids overlaid with perforated aluminum foil and covered with a layer of nutrient-poor soil medium in an 8-oz. (ca. 237-ml) glass jar in the dark.

The formation of sclerotia was first reported in morels almost simultaneously by Molliard, (1905) and Fron, (1905).The former observed their formation on sterile moistened bread and considered them to be imperfectly developed fruiting bodies, whereas the latter reported them to be large, looking and tasting like fruiting bodies. Both of them, however, did not realized the significance of these structure in the life cycle of the morel.

Mehta and Sharma, (1992) reported that among the ten natural and semi-synthetic media, sclerotia were formed only in Malt Extract, Glucose, Asparagine, Perti´s mineral, Richards and Leonian Agar medium. In liquid solutions under in vitro conditions, no correlation could be worked out between mycelia growth and sclerotial formation, and the formation of sclerotia was specific to the basal media used in all species.

Amir *et al*., (1993) studied the morphology and physiology of *Morchella esculenta* during sclerotial formation. They have identified six major stages in the growth of *Morchella esculenta*, using the split-plate method of cultivation, up to sclerotial maturation. One side of the plate contained Noble agar (NA), amended with 0·5 M glucose, on which the sclerotia formed. The other contained PDA, on which mycelium formed. They placed the inoculum on the NA side of the plate and the hyphae grew towards the PDA side. They noticed that when the hyphae reached the end of the plate, the direction of the cytoplasm stream reversed, translocating carbohydrates from the young part of the colony (mycelium) to the older part, at which stage initials formed. As the sclerotia developed, the soluble carbohydrates changed quantitatively and qualitatively, mannitol, arabitol and trehalose appearing in addition to glucose. Total carbohydrates decreased from 50 to 3·2% of the dry weight of the sclerotia. Meanwhile, the mycelial biomass decreased and the soluble carbohydrates virtually disappeared, probably moving to the sclerotia. The hyphal morphology on the NA side developed into pipes to suit the conduction.

Singh *et al*., (1999) studied the sclerotic variability produced by *Morchella* mycelium on different substrates such as saw dust, paddy straw, sand, farmyard manure and temperate garden. Several agricultural substrates or byproducts that are rich in ligninolytic inducers have been successfully used for ligninolytic enzyme production in submerged and solid state fermentation for ligninolytic enzyme production (Papinutti and Lechner, 2008; Zhang *et al*., 2009).

Kanwal and Reddy, (2011) have studied the effect of different carbon and nitrogen sources on the sclerotia formation in *M. crassipes* **.**They used Cultural assays to compare the effect of various carbon (C) and nitrogen (N) sources on in vitro sclerotial formation and development, specifically in mycelial cultures of black and yellow morels (*Morchella*

elata and *Morchella crassipes*, respectively).They concluded while different C and N sources supported abundant mycelia growth, these nutritional parameters also influenced sclerotial formation. Carbon sources such as ribose, cellobiose, galactose, xylose, sucrose and mannitol produced many (18–125) large-sized (diameter 0.16–0.43 cm) and cream-colored sclerotia in *M. crassipes*; in *M. elata*, small-sized (diameter 0.16–0.28 cm) and brown-pigmented sclerotia were formed in media containing ribose, galactose, sorbose and mannitol. Among the nitrogen sources, sodium nitrate and yeast extract caused both morel species to produce significantly fewer sclerotia (6–24) of significantly smaller size (diameter 0.11–0.27 cm). Carbon sources such as mannitol and ribose and N sources, sodium nitrate and yeast extract produced numerous large-sized sclerotia in morels.

2.3 Ligninolytic enzymes

Dhouib *et al*., (2005) have reported that autochithonous fungi from Tunisia produces lignolytic enzymes . Their work represents the first report on the ability of autochthonous fungi of Tunisia to produce ligninolytic enzymes. They have isolated three hundred fifteen fungal strains from different Tunisian biotopes. These fungal strains were first screened for lignin-modifying enzymes on solid media containing Poly R-478 or ABTS. Of the 315 tested strains, 49 exhibited significant ABTS-oxidation activity, expressed within the first week of incubation and only 18 strains decolourised the Poly R-478. They have further screened positive strains in liquid culture and laccase, and lignin and Mn^{2+}-oxidizing peroxidases activities were assayed. They have concluded of the 67 strains grown on liquid medium, 28 produced at least one of these 3 enzymes. They have identified 8 highest producers of ligninolytic activities by molecular techniques and 3 among them produced Lac, MnP and LiP simultaneously. New isolates reported in there work as fungi with significant ligninolytic activities includes Oxyporus,

Stereum and Trichoderma. The isolated Trametes trogii CTM 10156 was the best Lac producer.They have optimised culture conditions and medium composition for the the above strain and this resulted in high Lac production of 110 U ml^{-1} within 15 days of incubation (367 times higher than control medium).

Papinutti and Lechner, (2008) reported that laccase was the only enzyme from the group of aromatic and phenolic compounds have been widely used to elicit enhanced ligninolytic enzyme production by different organisms and the nature of compound that induces these enzyme activities differs greatly with the species (Leonowicz *et al*., 2001).

Zhang *et al*., 2009 produced and characterized novel laccase with cold adaptation and high thermal stability from new white root fungus *Pycnoporus sp. SYBC-L1*. They have recorded highest laccase activity of 24.1U/ml in optimal culture medium in submerged fermentation, which was approximately 40 fold than in basal medium. They found laccase produced was not only cold adapted with relative catalytic activity of 30.2 % at 0°C but also a high thermostable enzyme and the half lives at 60, 70, 80 °C were 85.5, 37.2, and 2.6 hour respectively. they recorded that laccase effectively decolourised weak acid blue AS and diamond black PV upto 88% and 74.7% respectively, within 2 hour in the absence of any redox mediators.

Kanwal and Reddy, (2010) studied the effect of different carbon, nitrogen sources and agrowastes on ligninolytic enzyme production of *M. crassipes* .They have concluded that the maximum growth was observed in mineral salts broth containing glucose as the carbon source and sodium nitrate as the nitrogen source. Among the inducers, chemical inducers inhibited the growth whereas in natural substrates, growth was not affected much. Manganese peroxidase and lignin peroxidase activity were not detected in the medium with different carbon and nitrogen sources, whereas laccase activity varied depending on carbon source (0.7-3.48 U/ml). Among the

inducers, natural inducers resulted in an increase in the enzyme activities. Maximum laccase activity was observed in rice straw (12. 6 U/ml) followed by ABTS (11.6 U/ml); Manganese peroxidase activity was maximum in rice straw (14.32 U/l) wheat straw (12.16 U/l) and phenol red (15 U/l) as the inducers, whereas for Lignin peroxidase activity, rice straw (22 U/l), wheat straw (16 U/l) and veratryl alcohol (20 U/l) served as the best inducers. They reported that addition of asparagine or aspartic acid as a nitrogen source to the rye also had a beneficial effect on sclerotium formation, while addition of carbon sources had no significant effect.

2.4 Laccase gene

Laccases have been investigated since 1883, when Yoshituda reported upon a " diastase like" activity requiring air, for polymerization of *Rhus vernicifera* extracts. In the intervening years large number of publications dealt with laccase obtained form plant, fungal and insect sources and even from bacteria.

The presence of LiPs and MnPs in *P. chrysosporium* is well established, but *P. chrysosporium* is widely quoted as an example of a white rot fungus that does not produce laccase (Thurston 1994). It was not known whether the inability to demonstrate the presence of laccase in *P. chrysosporium* cultures was due to the use of culture conditions, which are not favorable for laccase production, by this organism or whether the organism lacks the genetic machinery for producing laccase. However, studies carried out by Srinivasan *et al.,* (1995) showed that on regulation of expression of *lip* and *mnp* genes in cellulose- and wood-grown cultures of *P.chrysosporium*, this fungus produced low but consistent levels of laccase.

Claudia *et al.,* (1998) have studied the lignolytic system of *Pycnoporus cinnabarius*, a basidiomycetes that produces an unusual set of lignolytic enzymes. They showed that this

organism under conditions that stimulated lignin degradation produced just a single isoform of laccase, but no LiP or MnP. Laccase cDNA isolated from this organism was 1,828bp long without the poly(A) Tail and contained a 1,554bp open reading frame. It was predicted that the mature laccase polypeptide secreted by *P.cinnabarius* contained 497 amino acid residues and had a composite molecular mass of 53,871 Da. All of the expected Cu^{2+} ligands (10 His residues and one Cys residue) were present in the laccase gene coding sequence. But in addition to these residues they had found that this sequence contained a phenyalanine residue that interacts with the type 1 copper center and it was located nearest to the C-terminal of the protein, which varies in laccase from different sources and is considered as a residue that is probably important in governing the reduction-oxidation potential of Type 1 copper centers. This laccase polypeptide sequence when compared with all laccases already submitted in the databank showed closest relation with *Trametes villosa lcc*1 (83%). Some wood-degrading fungi contain all three classes of the lignin-modifying enzymes,while others contain only one or two of these enzymes. Several investigations indicate that laccase is widely distributed in many genera of white rot fungi. *Phanerochaete chrysosporium* has been extensively studied as a model organism for fungal lignin degradation (Kirk and Farrell 1987; Boominathan and Reddy 1992; Hattaka 1994).

Chefetz *et al*., (1998) focused on the laccase of a thermophilic strain of *Chaetomium thermophilium* isolated from composted municipal solid waste. The optimal temperature range for fungal laccase activity is 30 to 60°C (Munoz *et al*., 1997). None of the laccases studied previously was produced by thermophilic fungi. The laccase examined in this study had a temperature optimum between 50 to 60°C and was stable at higher temperatures than the laccases purified from mesophilic fungi, such as *Pyncoporus cinnabarinus* (Eggert and Eriksson 1996). Fungal laccases are generally active at low pH values (pH 3 to 5). In contrast, the *C.*

thermophilium laccase exhibited maximum activity at pH 6 to 8 and resembled the laccases isolated directly from composted MSW. The N-terminal sequence of the *C. thermophilium* laccase did not exhibit homology to other fungal laccase N-terminal sequences, although similar oxidative activities were observed. They could conclude that laccase produced by thermophilic fungi such as *C .thermophilium* could be involved in polymerization that yields humic macromolecules. In this process, natural and xenobiotic phenols are oxidized by laccase present in thecompost environment, resulting in the formation of free radicals, which can be spontaneously bound to soluble high-molecular-weight compounds, which results in humic macromolecules.

As stated above laccases may be involved in polymerization processes that lead to the formation of humic substances, similarly certain other researchers have studied about the physiological functions of laccases. Laccases have been implicated in pigmentation, fruiting body formation and pathogenecity (Eggert, 1998) . Williamson *et al.,* (1998) confirmed that laccases of animal pathogen *Cryptococcus neoformans* oxidizes dihydroxyphenylalanine into a melanin like pigment. Similarly, Tsai *et al.,* (1999) reported that laccase produced by *Aspergillus nidulans* is required for pigment biosynthesis during conidial development and maturation. It has been found that laccases play an important role in morphogenesis of fungus.

Many laccases have been identified in plant and fungal species. Enzymes of prokaryotic origin are poorly known. Endo *et a*l., (2003) have reported enzymological characterization of EpoA, laccase-like extracytoplasmic phenol oxidase produced by *Streptomyces griseus*. EpoA was expressed and purified with an *E. coli* host vector system as a recombinant protein fused with a C- terminal histidine tag (rEpoA). EpoA was found to be a stable homotrimer containing all 3 types of Cu (Type 1,2 and 3). It also oxidizes various laccase substrates while neither

syringaldazine nor guiaiacol served as substrates. Laccases are also known to be produced by bacteria such as in the endospore coat of *Bacillus subtilis* (Solano and Sanchez-Amat, 1999, Martins *et al*., 2002) and *Azospirillum lipoferum* (Givaudan *et al*., 1993). Extensive work has been done on the production of laccases by Basidiomycetes especially white rot and brown rot fungi.

2.5 Fruiting body formation and ligninolytic enzymes

Fruit body formation in fungus may involve phenol oxidase catalyzed formation of extracellular pigments coupled to oxidative polymerization of cell wall components strengthening cell-to-cell adhesion. Leatham and Stahmann, (1981) reported in *Lentinula edodes* a strong laccase activity in the fruiting stage, which indicated that laccases might catalyze the formation extracellular pigments by oxidative polymerization.

Trojanowski *et al*., (1984) reported that some ectomycorrhizal (ECM) fungi have been shown to partly mineralize lignin and/or dehydrogenative polymers of lignin monomers in axenic culture. This feature of the ECM shows that some ECM seen to have undergone evolutionary reversal to saprotrophy and have retained genes for saprophytic enzyme systems (Hibbertt *et al*., 2000).

Chen *et al.,* (2003) produced evidence that the ECM *Piloderma* spp. have multiple laccase like genes. They also hypothesized that as laccases have different functions under different conditions in various fungi similarly, laccases may play roles in not only nutrient mobilization, but also in the development of multihyphal components of the symbiosis, such as rhizomorphic extramatrical mycelium or the hyphal mantle. There is also a convincing evidence of extracellular laccase production by the ECM basidiomycete *Thelephora terrestris* (Kanunfree and Zuncan, 1998).

Among the edible mushrooms *Agaricus bisporus* and *Pleurotus ostreatus* have known to show strong laccase activity and are easy to culture organisms. Most of the studies on laccase have done in white rot and brown rot fungi. Reports are available which show laccase production by *Neurospora crassa* (Germann *et al.*, 1988), *Aspergillus nidulans* (Aramayo and Timberlake, 1990) and *Podospora anserine* (Fernandez- Larrea and Stahl, 1996). But the levels produced by all these organisms are too low.

Laccase gene from various basidiomycetes have been cloned and characterized: such as from *Phlebia raidata* (Saloheima *et al*., 1991), Basidiomycete PM1 (Coll *et al*., 1993), *Agaricus bisporus* (Perry *et al*, 1993), *Trametes villosa* (Yaver *et al*. 1996), *P. cinnabarius* (Eggert *et al*.,1998), *Lentinula edodes* (Zhao and Kwan, 1999) and few others.

The amino acid composition in the region of pentapeptide segment located downstream of second conserved histidine residue T1 site was found to be unique for all the three deduced laccases in *Gaeumannomyces graminis* var *tritici*, which implies that the enzymes have different substrate specificities and probably different functions in the fungus. Multiple genes that encode for different laccase isozymes have been identified in several decomposer fungi. Two allelic variants of *L. edodes lac*1 gene were cloned and characterized by Zhao and Kwan 1(994). Similarly allelic forms of laccase gene of *Trametes villosa* (Yaver *et al*., 1996), *Pycnoporus cinnabarius* (Temp *et al*., 1999) have been characterized. *Rhizoctonia solani* has four laccase genes (Wahleithner *et al*., 1996). Similarly *Agaricus bisporus* has two (Smith and Thruston, 1998) and *P. ostreatus* has at least three laccase genes (Giardina, 1999).

Eggert and Eriksson, (1995) described the importance of laccase in the synthesis of phenoxazinone pigments, which give the fruiting bodies of *P.cinnabarius* a red color. They also reported that the phenoxazinone pigments and consequently the laccase activity could also be

linked to the antimicrobial activity (biosynthesis of cinnabarinic acid) of the fungus. Heterobasidon a known confer pathogen shows to produce laccase as reported by Frederick *et al*., (2003). Laccase production by this plant pathogen can be linked to pathogenecity for degrading the cell wall of the host tissue and for detoxifying the host chemical and structural differences. The cell wall components degradation provides the fungus with required nutrients for its growth and aids penetration of the host tissue allowing the survival and spreading of the fungus.

PCR strategy used in this study carried out by D'Souza *et al*., (1996) was based on the use of degenerate primers corresponding to the consensus sequences conserved in the copperbinding regions in the N-terminal domains of known basidiomycete laccases.The primers were as designated as Cu F1 (5' CAT TGG CAT GGC TTC TTT CA 3') and Cu 2R (5' GGC TGT GGT ACC AGA AGG TTC C 3'). The work was carried on various white rot and brown rot fungi. The results showed that this was a useful and valid approach for screening wood rot fungi for the presence of laccase gene specific sequences. However, *Fomes fomentarius* and *Pleurotus ostreatus*, two white rot fungi previously reported to produce laccases, failed to yield laccase PCR products in three separate experiments in which different genomic DNA preparations were used as templates. The reason for this failure was attributed to the possible presence of an intron in either one or both DNA template regions corresponding to the primers, thereby resulting in low binding of either one or both primers to the DNA template. The results indicated that the PCR primers used in this study are useful in isolating laccase gene sequences from previously uncharacterized white rot and brown rot basidiomycetes. That the PCR products represent laccase gene fragments is suggested by the following lines of evidence: (i) the expected; 200-bp PCR amplified product was obtained in 8 out of the 11 wood rot fungi tested; (ii) the sequences

of the PCR products analyzed (including some smaller PCR products) had a high degree of similarity to corresponding regions of previously published laccase gene sequences; and (iii) the PCR products were identified as laccase gene fragments by the BLASTN computerized analysis program (1). They suggested that the strategy outlined in this study could also be useful in isolating the individual laccase genes and cDNAs from different wood rot fungi with the PCR products as probes. This approach can be used for isolation and characterization of laccase genes from selected wood rot basidiomycetes included in this study.

Many fungi have shown to produce different isozymes of the laccase enzymes. It has been proposed that laccase gene that encode for different isozymes may have evolved in different fungi and that these might underpin different functions in different fungi and under different conditions (Temp *et al*., 1999; Soden and Dobson, 2001).

Laccase genes isolated from different fungi are known to be of different sizes. The *lac*1 gene in *Lentinula edodes* is about 1.5 – 1.6 Kb in size coding for 526 amino acid residues with 13 introns (Zhao and Kwan, 1999). In *Pleurotus ostreatus lcc*K gene identified is 1.59 Kb in size with 19 introns coding for 533 amino acid residues (Okamoto *et al.,* 2003). Though different organisms have laccase genes of different sizes and different number of copies there still exist a common pattern that is displayed by these sequences. All code for a 520 to 220 amino acid polypeptide including an N- terminal signal peptide. In addition, the single cysteine residue and the 10 histidine residues involved in binding the four catalytic cupric ions found in each laccase molecule are conserved together with a small amount of sequence around the four regions in which the copper ligands are clustered.

Luis *et al*., (2004) carried out PCR amplifications of laccase genes of wide range of basidiomycetes from the forest soil. A degenerate PCR- primer pair Cu F1/Cu 2R, specific for

basidiomycetes, was designed to assess directly the diversity of laccase genes in the forest soils. PCR ampilfications of mycelial cultures and fruiting bodies of wide spectrum of basidiomycetes, covering all functional groups (saprophytes, symbionts and pathogens) produced multiple DNA fragments around 200bp. A neighbor joining tree analysis of the PCR amplified laccase sequences showed a clear species specificity, but also revealed that most fungal taxa possess several laccase genes showing a large sequence divergence. This supported the result of different laccase gene families by Mansur *et al*., (1997).

Chapter III

Materials and Methods

3.1 Organism and culture conditions

Pure fungal cultures of *Morchella* spp. (isolated from fruiting bodies) were used for this study. Pure culture of *Morchella* spp. were maintained on 2% Malt extract (ME) at 25 °C under static conditions in dark. Details of the locations of their collection are given in table 3.1

Table 3.1: Location of *Morchella* fruiting bodies, collected from Himalayan region of India.

Isolate code	Location	State	Collection origin
MR2	Rohru	Himachal Pradesh	31.2167N,77.7500E
MR17	Chambaghat	Hiamchal Pradesh	30.923506N,77.098768E

Physiological Work

3.2 Growth study on different media

Fifty ml of different fungal media such as MMN (Modified Melin Norkrans), MSB (Mineral Salt Broth), MGB (Morel Growth Broth), ME (Malt Extract), YME (Yeast and Malt Extract) and PDB (Potata Dextrose Broth) (Appendix) were dispensed into flasks and autoclaved at 121 °C at 15 psi for 15 minutes. Cooled and inoculated with two (7-mm diameter) discs cut from actively growing cultures of *Morchella spongiola* MR 17 and *Morchella sp.* MR 2. The flasks were incubated at 25 °C for 10 days under static conditions in dark. Control groups of the media without any culture, too, were placed. After incubation, the mycelia were harvested. The mycelia

were dry at 60°C for 48 hrs. Dry biomass, pH, protein conc., reducing sugar and enzyme activities were studied.

3.2.1 Dry Biomass: The mycelia harvested in each case was dry and weighed to determine dry biomass.

Enzyme activities study with the culture filtrate

3.2.2 Laccase Activity

Principle: Laccase activity was measured by monitoring the oxidation of 500µM ABTS(2,2´-Azino-bis-3[3-ethyl benzothiazoline-6-solfonic acid]) buffered with 0.1M sodium acetate buffer (pH 5) at 420 nm (Eggert *et al*.,1996).

Procedure:

1. In a test tube, 2.5 ml of 0.1M Na-acetate buffer (pH 5) was taken.
2. Added 0.33 ml of 5mM 2,2'-Azino-bis(3-ethylbenzthiazolin-6-suphonate) (ABTS).
3. All were incubated at 25°C for 5 minutes.
4. To start the reaction 0.17 ml of culture filtrate was added.
5. The test tubes were incubated at 25°C for 15 minutes.
6. The increase in absorbance was monitored from 0 min to 15 min at 420nm spectrophotometrically.

The average of the 15 determinations was calculated to get ΔA, increase in absorbance/min.

$\Delta A = (A15 – A1)/15$

One unit of enzyme activity was defined as 1 µM of substrate oxidized per min under the described conditions.

1U/ml = 1 m**M/ml.min = ΔA * (1000/**e**420 * d) * V/v * F**

where

The resulting equation is: = Δ**A** * **0.490** * **F**

Δ**A** -- Increase in absorbece /min at 420nm

(1000/e420 * d) -- Conversion to mmol converted substrate/ml using the

molar extinction coefficient (εABTS, 420 = 3,600[l * mol-1 * mm])

d -- Length of the light path in mm (10mm)

V -- Total volume (3000 µl) **v** -- Volume of sample (170 µl)

V/v -- Dilution in assay (17.647)

F -- Dilution factor of stock

3.2.3 Bradford Assay

Principle: This method was used for determination of protein concentration. It relies on the binding of the dye Coomassie Brilliant Blue to protein. At low pH the free dye has absorption maxima at 470 and 650 nm, but when bound to protein has an absorption maximum at 595 nm.

Procedure:

1. In a test tube, 5 ml of Bradford reagent was taken.
2. Added 0.1 ml of culture filterate.
3. Absorbance was monitored at 595 nm spectrophotometercially.
4. Bovine Serum Albumin (BSA) was used to prepare standard.

3.3 Growth studies on different carbon

To study the effect of different carbon sources on growth and enzyme activities, glucose in mineral salts broth (50 ml, pH 5.6) was replaced with equal amounts (10 g/L) of other carbon sources as fructose, sucrose, mannose, cellobiose, ribose, sorbose and mannitol. The media were

autoclaved at 121 °C, 15 psi for 15 minutes. Cooled and inoculated the media with two discs (7-mm diameter) cut from actively growing culture of *Morchella spongiola* MR 17 and *Morchella* sp. MR 2. The flasks were incubated at 25 °C for 10 days under static conditions in dark. Control groups of the media without any culture, too, were placed. The mycelia were harvested and culture filterate was analysed for pH and enzyme activities such as laccase activity, DNS assay and Bradford assay as explained above. The mycelia harvested in each case was dry at 60 °C for 48 hours and dry weight was measured.

3.4 Growth studies on different nitrogen sources

As with the carbon sources, nitrogen sources were also evaluated similarly by replacing sodium nitrate ($NaNO_3$) (0.2 g/L) with other nitrogen sources such as, ammonium nitrate (NH_4NO_3), sodium nitrite ($NaNO_2$), peptone, yeast extract, casein and tryptone. Fifty ml of each media, dispensed in flasks, was autoclaved at 121 °C at 15 psi for 15 minutes, cooled and inoculated with 7 mm (diameter) disc cut from actively growing culture of *Morchella spongiola* MR 17 and *Morchella* sp. MR 2.Incubated them at 25°C for 10 days under static conditions in dark. . Control groups of the media without any culture, too, were placed. The mycelia were harvested and culture filterate was analysed for pH and enzyme activities such as laccase activity, DNS assay, Anthrone assay and Bradford assay as explained above. The mycelia harvested in each case was dried at 60 °C for 48 hours and dry weight was measured for biomass.

3.5 Growth studies under metal stress conditions

The response of *Morchella spongiola* MR 17 *and Morchella sp.* MR 2 to various concentrations of copper, cadmium, zinc and manganese was assessed by growing pure mycelial culture in liquid MSB medium. MSB liquid medium was prepared (pH 5.6), dispensed 25 ml in 250 ml flaks and autoclaved. Two mycelial discs (7 mm diameter) cut from actively growing culture of

test fungus was inoculated and incubated in the dark at 25°C for 3 days. To avoid immediate metal stress and also to allow fungus to initiate growth, the metals were added after three days of fungal inoculation. The concentration (0-50 µM) of different metals Cu^{2+} ($CuSO_4.5H_2O$), Cd^{2+} ($3CdSO_4.8H_2O$), Zn^{2+} ($ZnSO_4$) and Mn^{2+} ($MnSO_4$) were used. The flasks were incubated at 25°C in dark. Control groups of the media without any culture, too, were placed. The mycelium was harvested after 10 days, dry biomass was measured and culture filterate was analysed for laccase activity, lignin peroxidases activity, manganese peroxidases activity and pH studies.

Sclerotial studies

3.6 Sclerotia formation on different substrates

Ligninocellulosic substrates such as wheat straw, rice straw, saw dust, wheat grains and pine needles were finely chopped into small pieces, water soaked over night, drained the next day. Filled the tissue culture jars (Kasablanky, Mumbai) with these substrates, added 5 ml of water, sealed them with caps, autoclaved at 121°C at 15 psi for 1hour. Cooled, inoculated with two 7mm (diameter) discs cut from actively growing culture of *Morchella spongiola* MR 17 and *Morchella* sp. MR 2 and incubated at 25°C for 10 days under static conditions in dark. Control groups of the substrate without any culture, too, were placed.

The culture were analyzed daily. The number of sclerotian formed was counted. For supernatant collection, of 50 ml of autoclaved distilled water was added to the jars, followed by shaking at 120 rpm for an hour. The solution was centrifuged at 5000 rpm at room temperature for 10 mins and the supernatant was analysed for laccase activity, lignin peroxidases activity, manganese peroxidases activity and pH studies.

3.6.1 Manganese peroxidase activity

Principle: Manganese peroxidase activity was measured by monitoring the rate of Mn(III)-malonate complex formation at 270 nm(Warishi et al., 1992). One unit of enzyme activity was defined as 1µM of substrate oxidized per minute.

Procedure:

1. In a test tube, 200 µl of 50mM Na- malonate buffer (pH 4.5) was taken.
2. Added 200µl 0.5 mM $MnCl_2$.
3. To start the reaction 1.4ml of culture filtrate and 0.2 mM of hydrogenperoxide (H_2O_2) were added.
4. The increase in absorbance was monitored from 0 min to 15 min at 270nm spectrophotometrically.

3.6.2 Lignin peroxidase activity

Principle: Lignin peroxidase activity was determined by the hydrogen dependent veratrylaldehyde formation from Veratryl alcohol (Tien and Kirk, 1984). One unit of enzyme activity was defined as 1µM of substrate oxidized per minute.

Procedure:

1. In a test tube, 1.2 ml of 125 mM sodium tartrate buffer (pH 2.5) was taken.
2. Added 2ml of autoclaved water and 380 µl of culture filterate.
3. To start the reaction 300µl of 20mM veratryl alcohol and 2mM of hydrogenperoxide (H_2O_2) were added.
4. The increase in absorbance was monitored from 0 min to 2 min at 370nm spectrophotometrically.

3.7 Sclerotia formation on the combination of soil and substrate (Ower *et al.*, 1986)

Ligninocellulosic substrates such as wheat straw, rice straw, saw dust, wheat grains and pine needles were finely chopped into small pieces, water soaked over night,, drained the next day. Filled the tissue culture jars (Kasablanky, Mumbai) with these substrates, added 5ml of water. Placed aluminium foil with fine holes above these substrates, followed by a soil layer. Jars were sealed with caps, autoclaved at 121°C at 15 psi for 1hour. Cooled, inoculated with two 7mm (diameter) discs cut from actively growing culture of *Morchella spongiola* MR 17 and *Morchella* sp. MR 2 and incubated at 25°C for 10 days under static conditions in dark. Control groups of the soil and substrates without any culture, too, were placed.

The cultures were analyzed daily. The number of sclerotian formed was counted. For supernatant collection, of 100 ml of autoclaved distilled water was added to the jars, followed by shaking at 120 rpm for an hour. The solution was centrifuged at 5000 rpm at room temperature for 10 mins and the supernatant was analysed for laccase activity, cellulase activity and pH studies.

Cloning laccase gene

3.8 Total RNA isolation

Morchella sp. MR 2 was grown in MSB medium for 10 days in presence of ABTS as inducer. The mycelia was crushed into fine powder using liquid N_2 and stored at -70°C. RNA was isolated from the mycelial powder by CTAB (N, N, N′, N′-cetyl trimethyl ammonium bromide) method.

Procedure for total RNA isolation

1. Powdered mycelial sample was taken in the falcon tube. To this 15ml CTAB extraction buffer and 400µl 2- mercaptoethanol was added, mixed well and kept at 65 °C for 5-10 minutes.

2. Added 15 ml of Choloroform: Isoamylalcohol (24:1 v/v) and centrifuged at 20°C at 5500 rpm for 20 minutes.

3. The upper phase was transferred to a new tube with 15 ml of chloroform: isoamylalcohol and centrifuged again at 20°C at 5500 rpm for 20 minutes.

4. The upper phase was again transferred to a new tube and then 3 ml of 10M LiCl was added. It was then kept on ice in the cold room for overnight.

5. The next day, it was centrifuged at 10,000 rpm at 4°C for 30 minutes and supernatant was discarded.

6. Pellet was resuspended in 700µl SSTE solution (1M sodium choride, 0.5% SDS, 10mM Tris-HCl [pH8], 1mM EDTA) and then transferred to a 2 ml eppendorf tube and kept at 60°C for 2-3 minutes.

7. Added 700µl of chloroform: isoamylalcohol and centrifuged at 10,000 rpm at 4°C for 10 minutes.

8. The upper phase was transferred to a new tube with 700µl of chloroform: isoamylalcohol and centrifuged at 10,000 rpm at 4°C for 10 minutes.

9. The upper phase was again transferred to a new eppendorf tube.

10. Then 1.2 ml of ethyl alcohol was added to precipitate RNA and incubated for 2 hour at -80°C and after incubation centrifuged at 13000 rpm at 4°C for 30 minutes.

11. Washed the pellet with 70% ethanol .

12. The RNA pellet was briefly dry and dissolved in RNase-free and DEPC (Diethylpyrocarbonate) treated water and stored at -70°C.

3.9 Molecular analysis of nucleic acids

3.9.1 Electrophoresis of nucleic acids on non-denaturing agarose gels

Nucleic acids were loaded on agarose gels (0.7%- 2 %(w/v)) prepared in 0.5X TBE buffer pH 8.0 (Appendix) using a 6X loading dye (Appendix). Ethidium bromide (EtBr) (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then migrated and visualized on a U.V. transilluminator (312 nm) and equipment used for RNA samples was washed in detergent and rinsed in autoclaved double distilled water to eliminate ribonucleases contamination before to use.

3.9.2 Nucleic acid quantification

DNA and RNA were quantified using Nanodrop 1000 Spectrophotometer (Thermo scientific, USA). The purity of the sample from contaminating polysaccharides and proteins was evaluated by the ratio between $OD_{260/230}$ and $OD_{260/280}$ respectively. Pure samples were indicated by a value closer to or higher than 1.8 for DNA and 2.0 for RNA.

3.10 Amplification of mRNA by reverse transcription PCR (RT-PCR)

cDNA was synthesized from total RNA by reverse transcription PCR method (The Reverse AID^{TM} First Strand cDNA Synthesis Kit, Fermentas Life Sciences, USA). Two µg of total RNA was denatured at 65°C for 5 minutes in the presence of 0.5 µg of oligodT18 primer and immediately cooled on ice. The first strand cDNA was synthesized in the presence of 200U MMLV reverse transcriptase, 20U ribonuclease inhibitor, 10 mM of each dNTPs and 1X reaction buffer supplied by the manufacturer. The reaction was carried out at 42°C for 1 hour. The enzyme was inactivated by incubating at 70°C for 10 minutes and immediately placed on ice and then it was stored at -20°c for further use.

3.11 Amplification of cDNA using Laccase gene specific primers

cDNA prepared above was used for the amplification of laccase gene using laccase gene specific primers Cu1AF and Cu2R. The PCR program, reaction mixture and annealing temperature were as follow:

Table3.2. PCR oligonuclotide primers used in this work

Primer	**Sequence**
Cu1AF	5'-ACATCGGTTCATTGGCATGG -3'
Cu2R	5'-GGCTGTGGTACCAGAAGGTTCC-3'

Table 3.3.PCR reaction mixture used in this work

Component	**Volume(µl)**
Template DNA (0.1-10ng)	1
PCR Buffer 10X (Invitrogen)	2.5
$MgCl_2$ (50mM)	1
dNTPs (2mM)	2.5
ForwardPrimer(Cu1AF) (10µM)	1.5
Reverse Primer**(**Cu2R) (10 µM)	1.5
TaqDNA Polymerase (5U/µl)	0.3
Autoclaved MQ water	14.7
Total reaction volume 25 µl	

Table 3.4.PCR program used in this work

S.NO	PCR step	Temperature	Time
1	Initial denaturation	94°C	3 min
2	Denaturation	94°C	30 sec
3	Annealing	50°C	1 min
4	Elongation	72°C	2 min
5	Final extension	72°C	10 min

Step 2 to 4 was repeated for 35 cycles.

3.12 Cloning of laccase gene products

The PCR products amplified from cDNA was purified using PCR product purification kit (Geneaid Biotech Ltd, USA) according to the manufacturer's instructions, quantified using Nanodrop 1000 Spectrophotometer(Thermo scientific, USA), (44.8 ng/µl) and cloned into the pTZ57R/T cloning vector system (2886 bp) provided in the PCR cloning kit(Fermentas Life Sciences, USA).

Volume (in ul) of insert(PCR Purified Product) required for a 10 µl ligation reaction:

$$\frac{\text{Size of insert}}{\text{Conc. Of insert}} \times \frac{\text{Conc. Of vector}}{\text{Size of vector}} \times \frac{3}{1}$$

Table 3.5. Ligation reaction mixture used in this work

Component	**Volume(** µl)
Vector pTZ57R/T(55 ng/µl)	1.5
5X Ligation Buffer	3.0
PCR Product	0.6
Water, Nuclease-free	8.9
T4 DNA Ligase	1.0

Total reaction volume 15µl

The ligation reaction mixture was prepared in a autoclaved eppendrof in the composition mentioned above and incubated overnight at 4°C.

The ligated products were transformed into *E.coli* DH5αcells (using heat shock method) for sequencing.

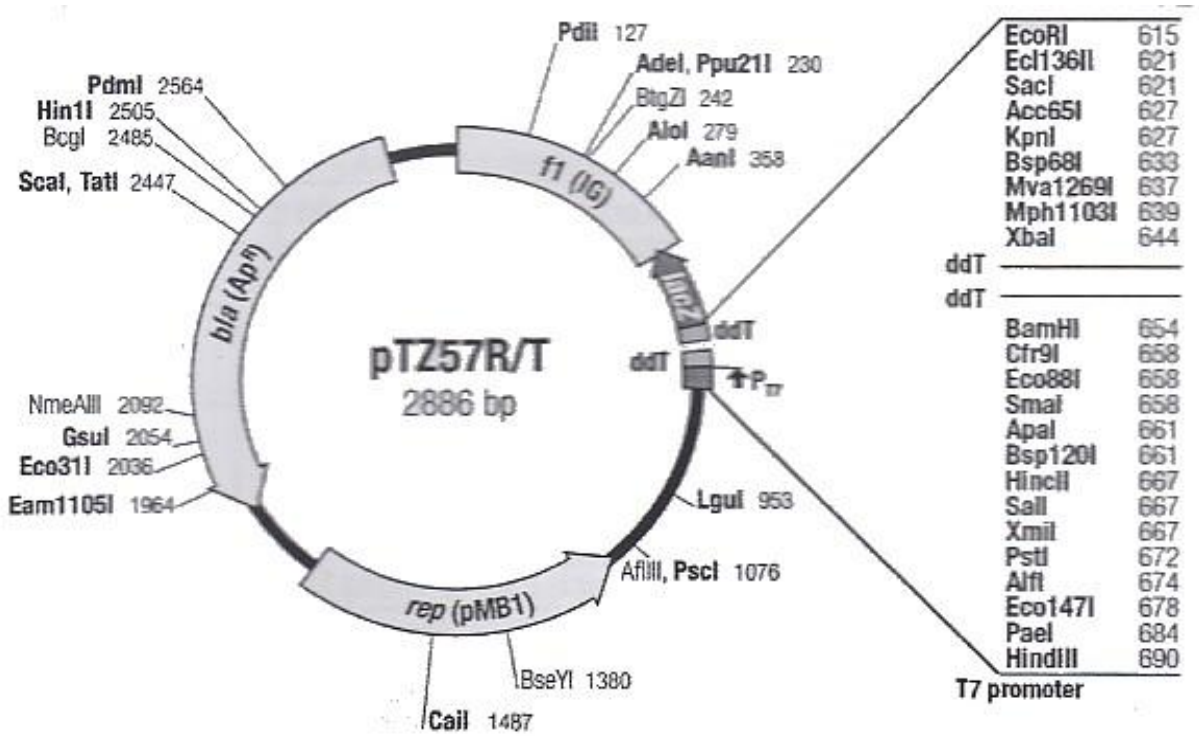


Fig 3 .1: Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated.

3.13 Transformation of DH5α by heat shock method

1. *E.coli* DH5α cells were taken from glycerol stock and streaked on Luria Agar (LA) plates and incubated at 37°C overnight.

2. Single colonies were isolated and inoculated in 20ml Luria Broth (LB) and incubated at 37°C for 16-20 hrs with vigorous shaking.

3. 200 µl of the overnight grown culture was inoculated in 20 ml LB and incubated with vigorous shaking at 37°C for 2-3 hours.

4. The above culture was then transferred to sterile, ice-cold 50 ml propylene tubes and centrifuged at 5000 rpm for 10 minutes at 4°C.

5. The supernatant was discarded and 10 ml of ice-cold sterilized 0.1M $CaCl_2$ was added and the tubes were incubated in ice for 15 minutes.

6. The cells were again centrifuged and the supernatant was discarded and 1 ml of 0.1M $CaCl_2$ was added and the tubes were incubated in ice for 2 hours to make competent cells.

7. To 100 µl of competent cells, 5 µl of ligated product was added to pre-chilled microfuge tube and mixed gently and kept in ice for 30 minutes for binding of the plasmid to the cells.

8. Then the cells were given a heat shock treatment for exactly 2 minutes at 42°C in a still water bath.

9. The cells were rapidly transferred into ice and kept for 2-3 minutes. 1 ml of LB + Ampicillin (50 µg/ml) was added to each tube and the tubes were then kept at 37°C for 1 hour for expression of Amp^r gene of the transformed cells.

10. 100 µl of the transformed cells were plated on LA + Amp + X-Gal + IPTG plates (Appendix).

11. The plates were incubated at 37°C for 16-20 hours and checked for appearance of white and blue colonies. The plates were kept in a refrigerator overnight to intensify the blue color of the colonies and differentiate between recombinants and non- recombinants.

12. Positive clones were screened by plasmid amplification using insert specific primers.

3.14 Bacterial Plasmid DNA isolation

1. The recombinant white colonies were picked up from the plates with the help of autoclaved toothpicks and inoculated in 2 ml of LB + Amp (Appendix) and incubated overnight at 37°C.

2. 1.5 ml of the culture was taken in a tube and centrifuged the cells and discarded the broth.

3. 100 µl of Solution 1 (Appendix) was added and the cells were dissolved in the solution by vortexing and kept at room temperature for 5 minutes.

4. 200 µl of freshly prepared Solution 2 (Appendix) was added to the cells and mixed the contents by inverting the tubes 10-12 times rapidly and at kept for 2 minutes.

5. 150 µl of Solution 3 (Appendix) was added and the tubes were inverted slowly10-12 times and kept in ice for 10-15 minutes.

6. 400 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added to the contents and inverted the tubes for 2 minutes and centrifuged at 12,000 g for 10 minutes.

7. The upper aqueous layer was transferred to a fresh tube.

8. Added equal volume of ice cold isopropanol and precipitated at -20°C for 1 hour and centrifuged at 12,000 g for 10 minutes for precipitation of DNA.

9. The supernatant was discarded and the pellet was washed with 400 µl of 70% ethanol and centrifuged 12,000 g for 5 minutes.

10. The supernatant was removed and the pellet was air-dried and dissolved in 50 µl of autoclaved MQ (Milli Q) water.

3.15 Screening for positive clones

The plasmid DNA of the recombinant bacterial cells was isolated to confirm the presence of the plasmid in the cells. These plasmids were used for PCR reaction by insert specific primes Cu1AF and Cu2R using the same PCR program, reaction mixture as mentioned above to check that the plasmid DNA contained the insert.

Chapter IV

Results and Discussion

4.1 Growth studies on different media

To study the effect of different media on growth and enzyme activities, *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 were grown in fungal growth media such as MMN (Modified Melin Norkrans), MSB (Mineral Salt Broth), MGB (Morel Growth Broth), ME (Malt Extract), YME (Yeast and Maltose Extract) and PDB (Potata Dextrose Broth) (Appendix). The cultures were harvested after 10 days of incubation. Biomass of mycelia, pH, protein concentration (Bradford Assay), laccase Activity were studied from culture filtrate.

4.1.1 Biomass: Among the various media used for growth studies in *Morchella spongiola* MR 17 maximum biomass was recorded in PDB, MSB, MGB and YME media. Whereas in *Morchella sp.* MR 2 maximum dry biomass was recorded in YME medium, followed by MSB and ME media and minimum in MMN medium. Optimum dry biomass was recorded in MGB and PDB media. Among *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 highest dry biomass was recorded in *Morchella sp.* MR 2 in YME medium. (Table 4.1 and Figure 4.1)

Table 4.1 Effect of different fungal growth media on dry biomass (in mg/50ml) of *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD)

S.No.	Media	*Morchella spongiola* MR 17	*Morchella sp.*MR 2
1.	MMN	4 ± 0.7	7 ± 2.8
2.	ME	115 ± 3.5	67 ± 2.1
3.	PDB	38 ± 0.7	24 ± 2.8
4.	YME	70 ± 2.8	142 ± 6.3
5.	MSB	60 ± 0.7	84 ± 2.8
6.	MGB	50 ± 1.4	29 ± 2.8

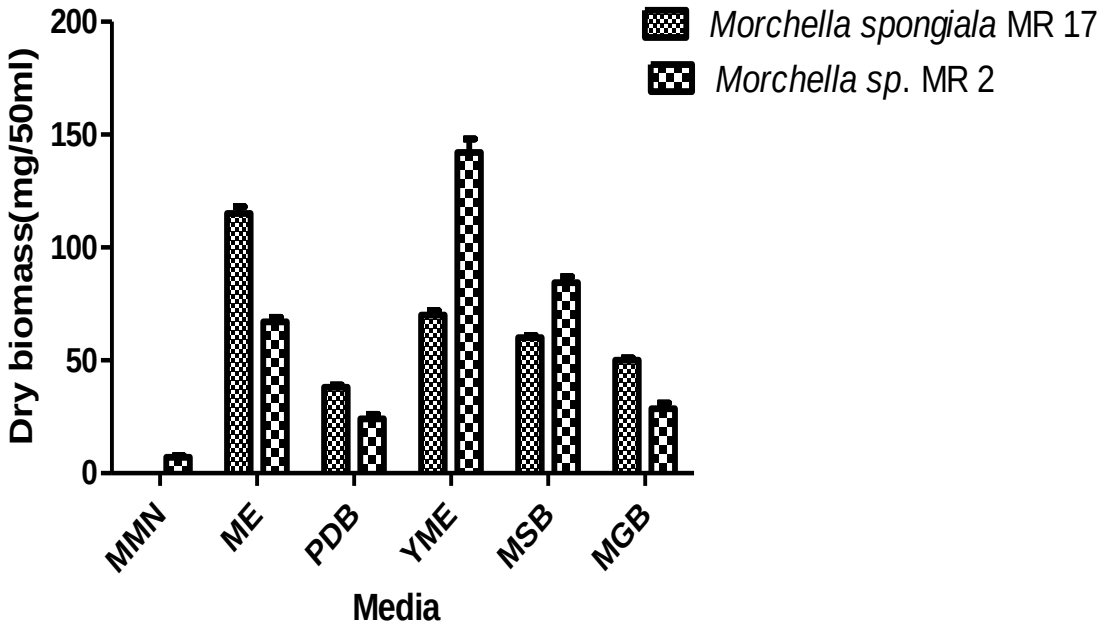


Fig 4.1 Influence of different fungal growth media on dry biomass of *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.1.2 pH: After 10 days of incubation in both the fungi, increase in pH was observed. Maximum increase in pH was observed in ME medium and in YME medium in *Morchella Spongiola* MR 17 and in *Morchella sp.* MR 2 respectively, whereas minimum increase in both was observed in MMN media.(Table 4.2 and Figure 4.2)

Table 4.2 Effect of different fungal growth media on pH in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD)

S.No.	Media	Control	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	MMN	5.63 ± 0.049	5.94 ± 0.014	6.06 ± 0.014
2.	ME	5.93 ± 0.021	6.29 ± 0.028	6.46 ± 0.049
3.	PDB	3.56 ± 0.084	3.75 ± 0.014	4.18 ± 0.014
4.	YME	5.83 ± 0.098	6.25 ± 0.049	8.95 ± 0.028
5.	MSB	5.85 ± 0.014	6.00 ± 0.021	7.01 ± 0.021
6.	MGB	5.99 ± 0.014	6.16 ± 0.021	6.83 ± 0.021

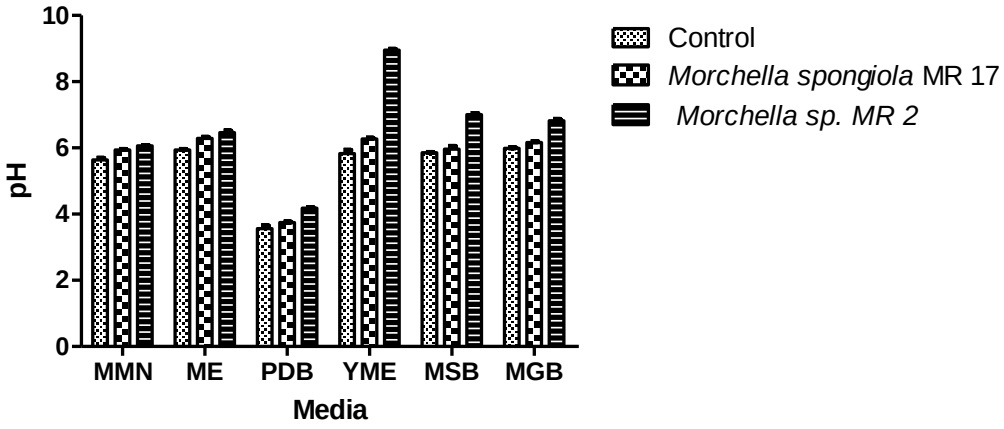


Fig 4.2 Influence of different fungal growth media on pH in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.1.3 Protein content

Maximum protein content was observed in YME medium by *Morchella sp.* MR 2. Malt extract medium was observed to be the best for high protein content by *M. spongiola* MR 17. Potato dextrose broth acted as the moderate source of media for protein content. Media such as MMN, MSB and MGB acted as poor media for protein content. (Table 4.3 and Figure 4.3)

Table 4.3 Effect of different fungal growth media on protein concentration (mg/ml) in *Morchella spongiola* MR 17 and *Morchella sp*. MR 2*(*Mean ± SD)

S.No.	Media	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	MMN	0.26 ± 0.136	0.27 ± 0.001
2.	ME	1.01 ± 0.141	0.66 ± 0.032
3.	PDB	0.55 ± 0.002	0.51 ± 0.028
4.	YME	0.64 ± 0.001	1.12 ± 0.014
5.	MSB	0.28 ± 0.070	0.35 ± 0.002
6.	MGB	0.32 ± 0.001	0.21 ± 0.001

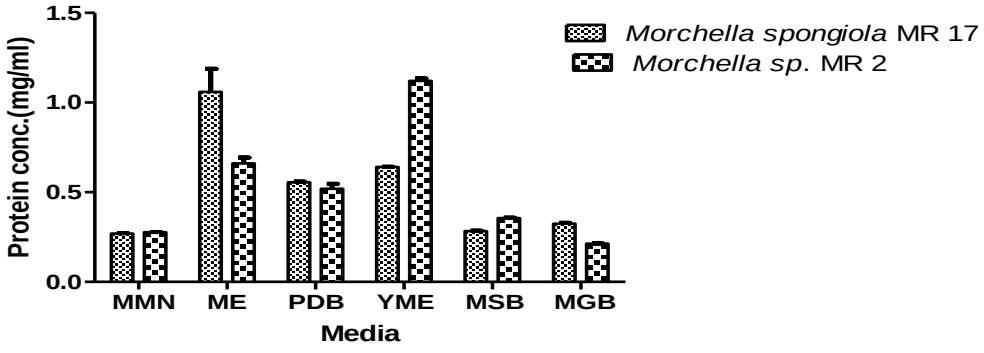


Fig 4.3 Influence of different fungal growth media on protein concentration in *Morchella spongiola* MR 17 and *Morchella sp.* MR2.

4.1.4 Laccase activity: Laccase activity was recorded in all the media in both *Morchella spongiola* MR 17 and *Morchella* sp. MR 2. In *Morchella spongiola* MR 17, maximum laccase was measured in ME medium followed by YME medium whereas in *Morchella* sp. MR 2 maximum laccase activity was recorded in YME medium followed by MSB and ME media. Minimum laccase activity was recorded in MMN medium in both the fungi(Table 4.4 and Figure 4.4)

Table 4.4 Effect of different fungal growth media on laccase activity (U/ml) in *Morchella spongiola* MR 17 and *Morchella* sp. MR 2 *(*Mean ± SD).

S.No.	Media	*Morchella spongiola* MR 17	*Morchella* sp. MR 2
1.	MMN	0.016 ± 0.002	0.020 ± 0.001
2.	ME	0.231 ± 0.002	0.352 ± 0.003
3.	PDB	0.160 ± 0.001	0.249 ± 0.004
4.	YME	0.200 ± 0.004	0.365 ± 0.000
5.	MSB	0.188 ± 0.003	0.359 ± 0.001
6.	MGB	0.180 ± 0.002	0.255 ± 0.002

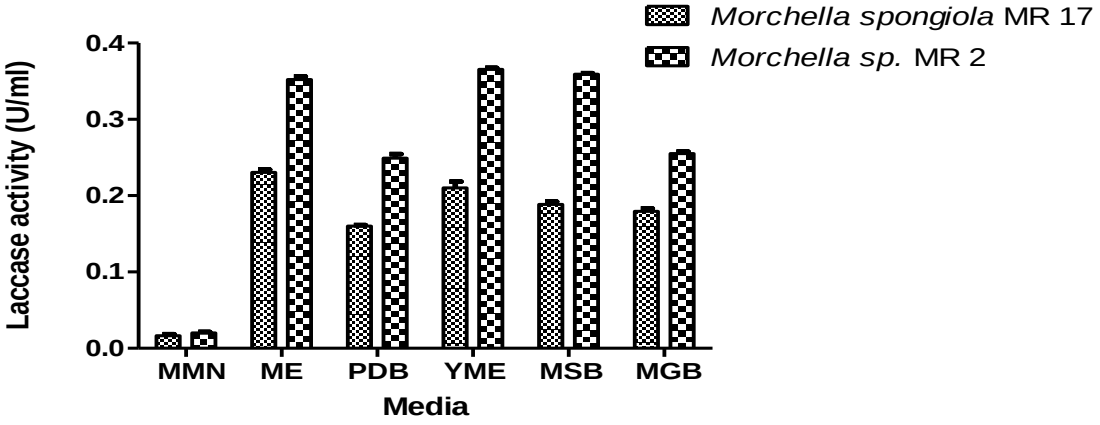


Fig 4.4 Influence of different fungal growth media on laccase activity of in *Morchella spongiola* MR 17 and *Morchella sp.* MR2.

4.2 Growth studies on different carbon sources

To study the effect of different carbon sources on growth and enzyme activities, *Morchella. spongiola* MR 17 *and Morchella sp*. MR 2 were grown in mineral salts broth with different carbon sources. Biomass of mycelia, pH and enzyme activities were studied.

4.2.1 Biomass: The pattern of carbon source utilization was found to be similar in both *Morchella spongiola* MR 17 *and Morchella sp*. MR 2. In both the fungi maximum dry biomass was recorded in mannose, followed by glucose and mannitol; optimum dry biomass was recored in sucrose, fructose and ribose; and least biomass was recorded in sorbose.

Higher dry biomass was recorded in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17 , in all different carbon sources used. (Table 4.5 and Figure 4.5)

Table 4.5 Effect of different carbon sources (in mineral salts broth) on dry biomass (mg/50ml) of *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD)

S.No.	Carbon Source	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Glucose	59 ± 1.02	64 ± 0.79
2.	Fructose	44 ± 1.09	50 ± 1.20
3.	Sorbose	1 ± 0.00	2 ± 0.01
4.	Sucrose	48 ± 1.20	56 ± 1.80
5.	Mannose	66 ± 1.60	69 ± 1.05
6.	Ribose	35 ± 1.82	32 ± 1.27
7.	Mannitol	54 ± 0.77	60 ± 0.59

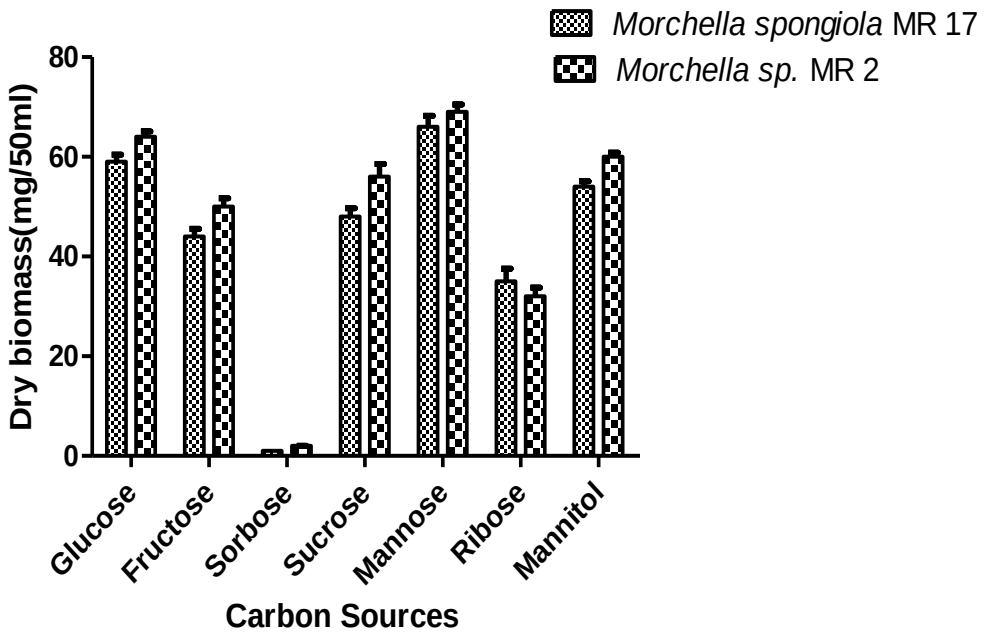


Fig 4.5 Influence of different carbon sources (in mineral salts broth) on dry biomass of *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.2.2 pH: Increase in pH (as compared to control) was observed in both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 in all the carbon sources tested. Maximum increase was recorded in mannose followed by glucose and mannitol, optimal increase was recorded in sucrose and fructose, whereas in sorbose least increase in pH was recorded in both the fungi.

Higher increase in pH was recorded in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17 in all different carbon sources used. (Table 4.6 and Figure 4.6)

Table 4.6 Effect of different carbon sources on pH in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD)

S.No.	Carbon Sources	Control	*Morchella spongiola* MR 17	*Morchella sp*. MR 2
1.	Glucose	5.81 ± 0.014	6.95 ± 0.014	8.18 ± 0.00
2.	Fructose	5.73 ± 0.021	6.00 ± 0.014	8.01 ± 0.014
3.	Sorbose	5.58 ± 0.028	5.62 ± 0.028	5.59 ± 0.042
4.	Sucrose	5.65 ± 0.014	6.50 ± 0.070	8.03 ± 0.042
5.	Mannose	5.84 ± 0.028	7.60 ± 0.048	8.41 ± 0.084
6.	Ribose	5.70 ± 0.014	5.93 ± 0.070	6.79 ± 0.015
7.	Mannitol	5.85 ± 0.035	6.61 ± 0.010	8.07 ± 0.028

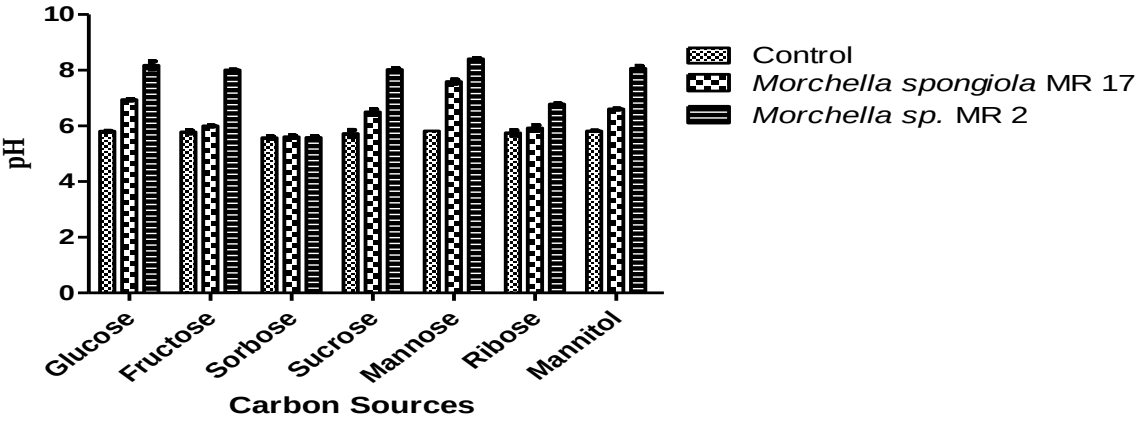


Fig 4.6 Influence of different carbon sources (in mineral salts broth) on pH in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.2.3 Protein content: In both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2, maximum protein content was recorded in MSB medium with mannose as carbon source followed by glucose and mannitol, optimum protein content was recorded in sucrose and fructose, whereas in sorbose as carbon source had the least protein content.

More protein concentration was recorded in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17, in all MSB medium with different carbon sources used.(Table 4.7 and Figure 4.7)

Table 4.7 Effect of different carbon sources (in mineral salts broth) on protein concentration (mg/ml) in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2*(*Mean ± SD)

S.No.	Carbon Source	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Glucose	0.114 ± 0.001	0.390 ± 0.003
2.	Fructose	0.055 ± 0.002	0.322 ± 0.002
3.	Sorbose	0.001 ± 0.000	0.029 ± 0.002
4.	Sucrose	0.083 ± 0.002	0.326 ± 0.001
5.	Mannose	0.212 ± 0.002	0.482 ± 0.001
6.	Ribose	0.030 ± 0.001	0.255 ± 0.002
7.	Mannitol	0.183 ± 0.002	0.340 ± 0.001

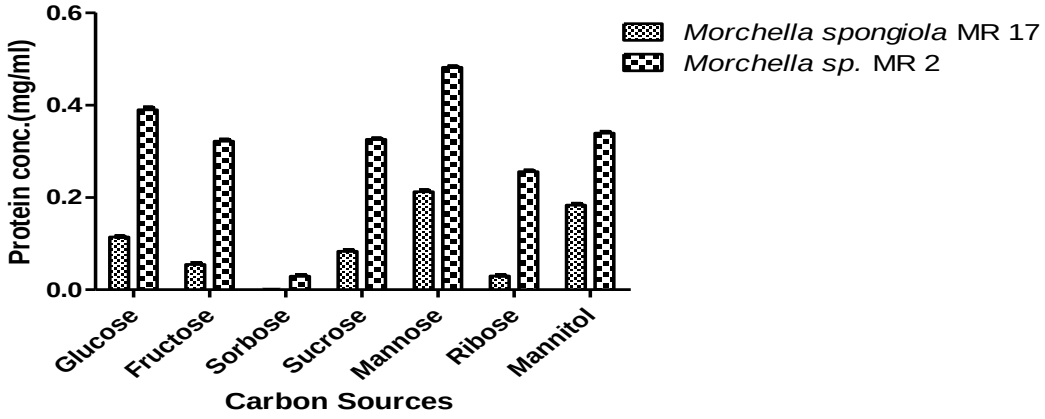


Fig 4.7 Influence of different carbon sources (in mineral salts broth) on protein concentration (mg/ml) in *Morchella spongiola* MR 17 and *Morchella sp*. MR2.

4.2.4 Laccase activity: Highest laccase activity was recorded in mannose, followed by glucose and mannitol in both the fungi. Optimal sources of carbon for laccase activity were observed to be sucrose and fructose, whereas in sorbose least laccase activity was detected in both the fungi.

Maximum laccase activity was present in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17 , in all different carbon sources used(Table 4.8 and Figure 4.8)

Table 4.8 Effect of carbon sources (in mineral salts broth) on laccase activity (U/ml) in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 (Mean ± SD)

S.No.	Carbon Source	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Glucose	0.190 ± 0.002	0.357 ± 0.003
2.	Fructose	0.142 ± 0.001	0.301 ± 0.001
3.	Sorbose	0.005 ± 0.001	0.009 ± 0.001
4.	Sucrose	0.168 ± 0.002	0.339 ± 0.002
5.	Mannose	0.207 ± 0.001	0.360 ± 0.003
6.	Ribose	0.100 ± 0.003	0.282 ± 0.001
7.	Mannitol	0.189 ± 0.001	0.350 ± 0.002

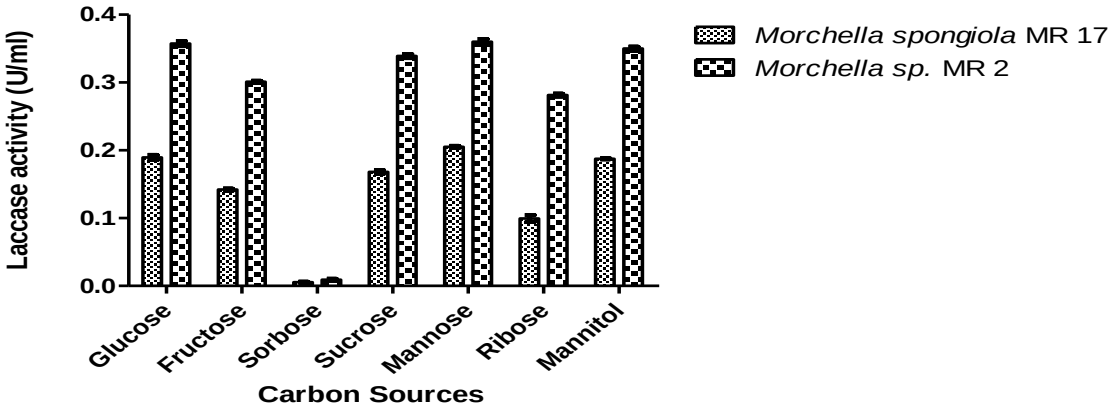


Fig 4.8: Influence of carbon sources (in mineral salts broth) on laccase activity in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2.

4.3 Growth studies on different nitrogen sources

To study the effect of different nitrogen sources on growth and enzyme activities *Morchella spongiola* MR 17 *and Morchella sp.* MR 2 were grown in MSB in which sodium nitrate ($NaNO_3$) was replaced with equal amounts (0.2 g/L) of other nitrogen sources. Biomass of mycelia, pH and enzyme activities were studied.

4.3.1 Biomass: Both *Morchella spongiola* MR 17 *and Morchella sp*. MR 2 showed the similar pattern of nitrogen utilization. In both the fungi, maximum dry biomass was recorded in sodium nitrate, followed by tryptone and casein; optimum dry biomass was recorded in sodium nitrite and peptones and least biomass recorded in urea. (Table 4.9 and Figure 4.9)

Higher dry biomass was recorded in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17, in all different nitrogen sources used.

Table 4.9 Effect of different nitrogen sources (in mineral salts broth) on biomass (mg/50ml) in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD).

S.No.	Nitrogen source	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Sodium nitrate	62 ± 04	70 ± 02
2.	Sodium nitrite	46 ± 02	49 ± 01
3.	Peptone	32 ± 01	37 ± 03
4.	Urea	20 ± 03	25 ± 01
5.	Casein	52 ± 02	56 ± 02
6.	Tryptophan	56 ± 01	62 ± 02

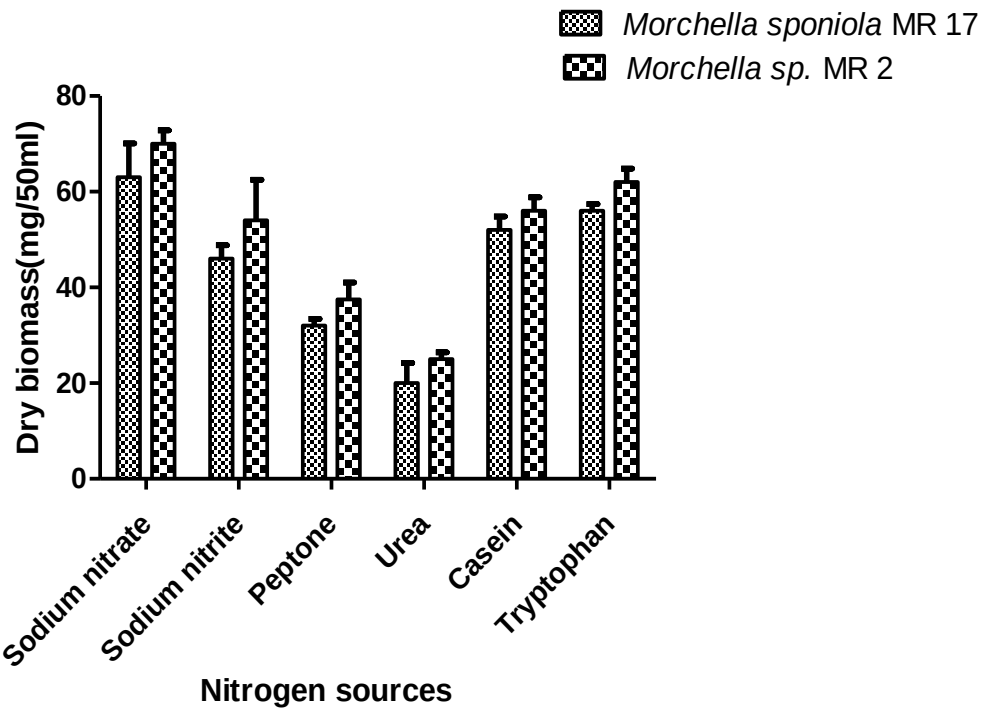


Fig 4.9 Influence of different nitrogen sources (in mineral salts broth) on biomass in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.3.2 pH: Increase in pH (as compared to controls) was observed in both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 in all the nitrogen sources under study. Maximum increase was recorded in sodium nitrate and minimum increase in pH was recorded in urea as nitrogen source in both the fungi.

Higher increase in pH was recorded in *Morchella sp*. MR 2 compared to *Morchella. spongiola* MR 17 ,in all different nitrogen sources used.(Table 4.10 and Figure 4.10)

Table 4.10 Effect of different nitrogen sources (in mineral salts broth) on pH culture filterate harvested from *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD).

S.No.	Nitrogen source	Control	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Sodium nitrate	5.80 ± 0.01	6.90 ± 0.02	8.18 ± 0.02
2.	Sodium nitrite	5.82 ± 0.02	6.19 ± 0.01	6.69 ± 0.01
3.	Peptone	5.84 ± 0.03	5.90 ± 0.03	6.02 ± 0.01
4.	Urea	6.13 ± 0.02	6.20 ± 0.01	6.24 ± 0.02
5.	Casein	5.83 ± 0.01	6.21 ± 0.02	7.68 ± 0.03
6.	Tryptophan	5.82 ± 0.01	6.61 ± 0.01	8.00 ± 0.01

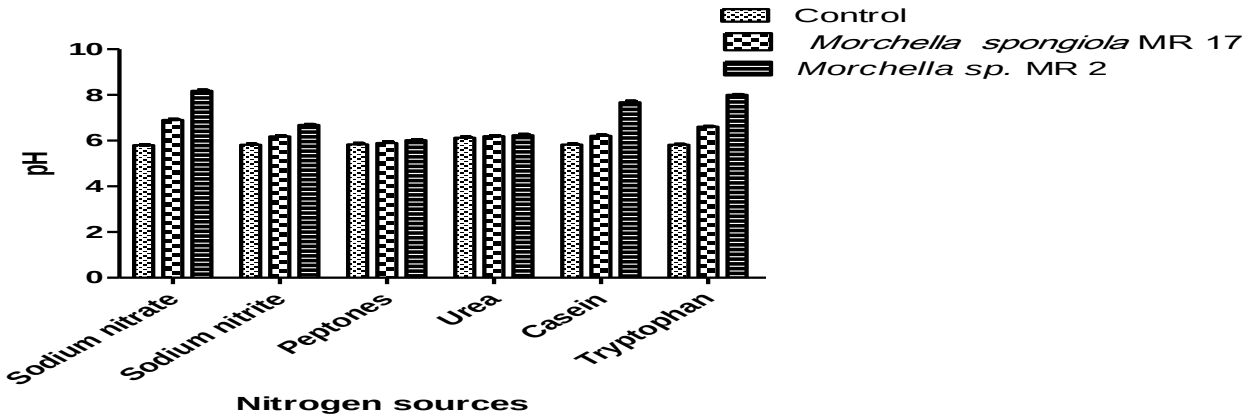


Fig.4.10 Influence of different nitrogen sources (in mineral salts broth) on pH in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.3.3 Protein content: In both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2, maximum protein content was recorded in MSB medium with sodium nitrate as nitrogen sources followed

by tryptone and casein, optimum protein content was recorded in sodium nitrite and peptone, whereas in urea as nitrogen source has least protein content.

More protein concentration was recorded in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17, in all MSB medium with different nitrogen sources used(Table 4.11 and Figure 4.11)

Table 4.11: Effect of different nitrogen sources (in mineral salts broth) on protein concentration (mg/ml) in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD).

S.No.	Nitrogen source	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Sodium nitrate	0.220 ± 0.002	0.365 ± 0.003
2.	Sodium nitrite	0.185 ± 0.001	0.272 ± 0.002
3.	Peptone	0.102 ± 0.003	0.201 ± 0.001
4.	Urea	0.050 ± 0.002	0.062 ± 0.001
5.	Casein	0.187 ± 0.001	0.284 ± 0.002
6.	Tryptophan	0.201 ± 0.002	0.360 ± 0.002

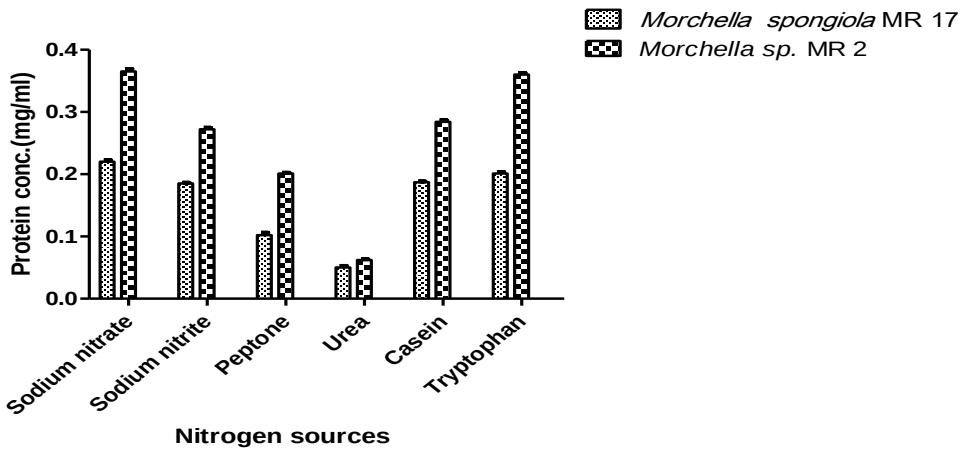


Fig 4.11 Influence of nitrogen sources (in mineral salts broth) on protein concentration (mg/ml) in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2.

4.3.4 Laccase activity: Laccase activity was present in both *Morchella spongiola* MR 17 and *Morchella sp*. MR2 in MSB medium with different nitrogen sources under study. Highest laccase activity was observed in sodium nitrate as the nitrogen source by *Morchella sp*. MR 2.

Laccase activity was also detected in considerable amounts in casein and tryptophan as the nitrogen sources in both the fungi. Urea was observed to be poor sources of nitrogen for laccase activity.

Higher laccase activity was recorded in *Morchella sp*. MR 2 as compared to *Morchella spongiola* MR 17, in all different carbon sources used(Table 4.12 and Figure 4.12)

Table 4.12 Effect of different nitrogen sources (in mineral salts broth) on laccase activity (U/ml) of *Morchella spongiola* MR 17 and *Morchella sp*. MR 2 .*(*Mean ± SD).

S.No.	Nitrogen source	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Sodium nitrate	0.189 ± 0.002	0.359 ± 0.001
2.	Sodium nitrite	0.151 ± 0.001	0.195 ± 0.002
3.	Peptone	0.090 ± 0.002	0.126 ± 0.001
4.	Urea	0.020 ± 0.002	0.072 ± 0.001
5.	Casein	0.160 ± 0.003	0.202 ± 0.002
6.	Tryptophan	0.172 ± 0.001	0.290 ± 0.002

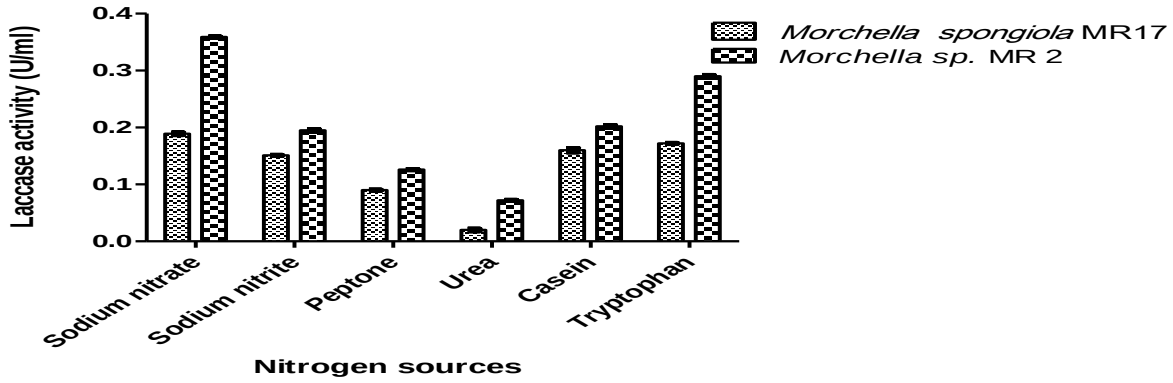


Fig. 4.12: Effect of different nitrogen sources (in mineral salts broth) on laccase activity (U/ml) of *Morchella spongiola* MR 17 and *Morchella sp*. MR 2

4.4 Growth studies under different metal stress conditions

To study the effect of different metals on growth and enzyme activities, *Morchella spongiola* MR 17 *and Morchella sp.* MR 2 were grown in MSB with varying concentrations (0-50µM) of zinc, manganese, copper and cadmium. The cultures were harvested after 10 days of incubation and dry biomass, pH and ligninocellulosic enzyme activities (laccase, manganese peroxidases and lignin peroxidases) were recorded.

4.4.1 Biomass: Both zinc and manganese had a positive effect on growth of both *Morchella spongiola* MR 17 and *Morchella sp.* MR2. In both the fungi increase in dry biomass was recorded at concentration of 30 µM. On further increasing the concentration, a decrease in dry biomass was observed by both the fungi.

Copper and cadmium both inhibited the growth, as with an increase in their conc., a decrease in dry biomass was recorded by both the fungi(Table 4.13, 4.14 and Figure 4.14, 4.15)

Table 4.13:Effect of different metals on biomass (mg/50ml) of *Morchella spongiola* MR 17 (Mean ± SD).

S.No	Conc.(µM)	Zinc	Manganese	Copper	Cadmium
1.	0	60 ± 01	61 ± 01	62 ± 01	61 ± 00
2.	10	68 ± 01	65 ± 03	59 ± 03	58 ± 01
3.	20	72 ± 02	69 ± 01	56 ± 01	56 ± 01
4.	30	75 ± 01	72 ± 01	52 ± 01	51 ± 02
5.	40	71 ± 01	68 ± 02	50 ± 02	49 ± 01
6.	50	67 ± 01	64 ± 01	50 ± 03	48 ± 01

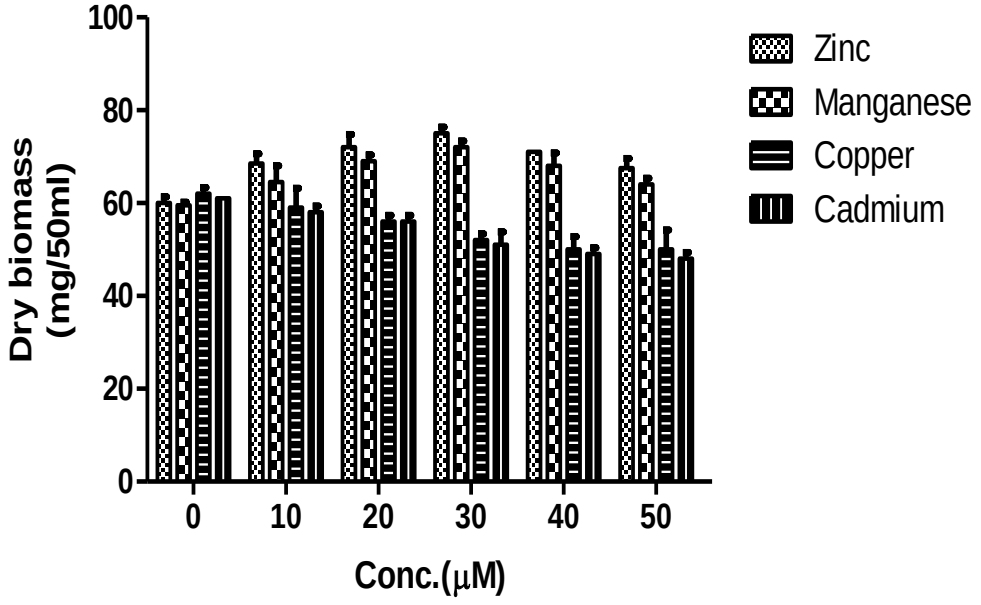


Fig 4.13 :Effect of different metals at different conc. on biomass (mg/ml) of *Morchella spongiola* MR 17(Mean ± SD).

Table4.14:Effect of different metals on biomass (mg/50ml) of *Morchella sp.* MR 2(Mean ± SD).

S.No	Conc.(μM)	Zinc	Manganese	Copper	Cadmium
1.	0	78 ± 01	80 ± 00	78 ± 01	77 ± 01
2.	10	85 ± 02	86 ± 01	75 ± 01	73 ± 02
3.	20	87 ± 02	90 ± 02	72 ± 03	70 ± 01
4.	30	90 ± 03	93 ± 02	70 ± 03	66 ± 01
5.	40	87 ± 01	89 ± 03	68 ± 02	63 ± 02
6.	50	84 ± 01	87 ± 01	66 ± 02	61 ± 01

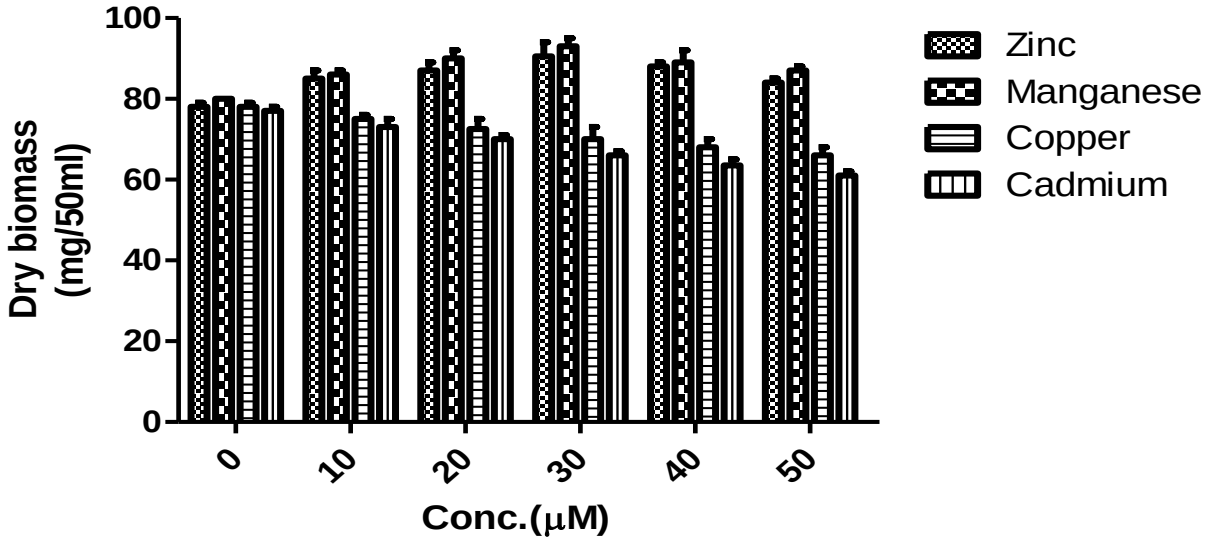


Fig. 4.14 Influence of different metals at different conc. (in mineral salts broth) on dry biomass of *Morchella sp.* MR 2

4.4.2 pH: As the concentration of zinc and manganese in MSB medium increased, an increase in pH was recorded upto concentration of 30 µM, further increasing the concentration did not give linear increase in pH, whereas in presence of copper and cadmium in MSB medium lead to a decrease in pH of medium and which further increased as concentration of both the metals increased in medium.(Table 4.15, 4.16 and Figure 4.15, 4.16)

Table 4.15 :Effect of different metals on pH of *Morchella spongiola* MR 17(Mean ± SD).

S.No	Conc.(µM)	Zinc	Manganese	Copper	Cadmium
1.	0	6.90 ± 0.01	7.12 ± 0.01	7.01 ± 0.01	6.84 ± 0.02
2.	10	7.02 ± 0.02	7.23 ± 0.01	6.68 ± 0.02	6.72 ± 0.02
3.	20	7.21 ± 0.03	7.39 ± 0.02	6.59 ± 0.02	6.66 ± 0.01
4.	30	7.78 ± 0.01	7.60 ± 0.03	6.48 ± 0.01	6.52 ± 0.01
5.	40	7.66 ± 0.02	7.48 ± 0.01	6.24 ± 0.01	6.43 ± 0.01
6.	50	7.59 ± 0.02	7.41 ± 0.02	6.18 ± 0.01	6.38 ± 0.03

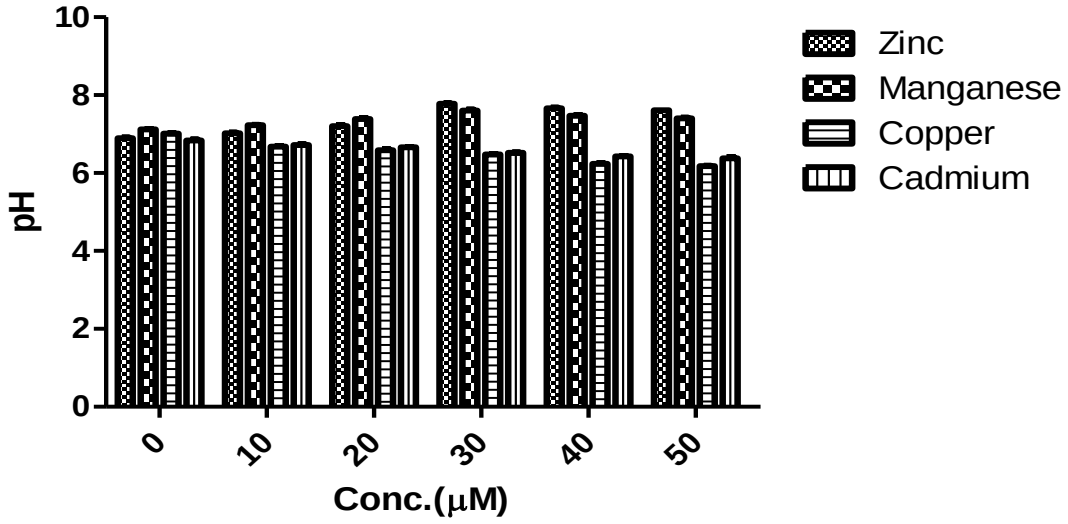


Fig 4.15 : Effect of different metals at different conc. on pH of *Morchella spongiola* MR 17(Mean ± SD).

Table 4.16 :Effect of different metals on pH of *Morchella sp.* MR 2 (Mean ± SD).

S.No	Conc.(μM)	Zinc	Manganese	Copper	Cadmium
1.	0	8.32 ± 0.01	8.29 ± 0.01	8.01 ± 0.01	7.92 ± 0.02
2.	10	8.49 ± 0.01	8.44 ± 0.01	7.75 ± 0.01	7.48 ± 0.01
3.	20	8.82 ± 0.02	8.78 ± 0.03	7.69 ± 0.02	7.35 ± 0.01
4.	30	9.19 ± 0.02	9.11 ± 0.02	7.45 ± 0.02	6.89 ± 0.03
5.	40	9.00 ± 0.01	9.02 ± 0.02	7.02 ± 0.01	6.85 ± 0.02
6.	50	8.89 ± 0.01	8.92 ± 0.01	6.98 ± 0.02	6.84 ± 0.01

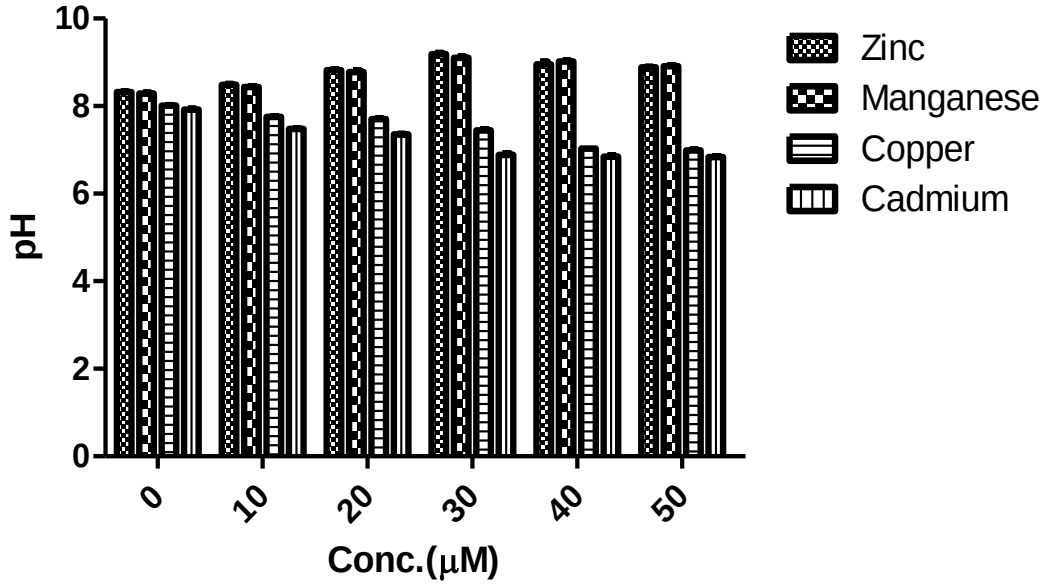


Fig 4.16 :Effect of different metals at different conc. on pH of *Morchella sp.* MR 2 (Mean ± SD).

4.4.3 Laccase activity: Not much variations were observed in the laccase activity with different metals. These metals studied in the present case did not act as positive inducers for laccase activity in both the fungi(Table 4.17, 4.18 and Figure 4.17, 4.18).

Table 4.17 Influence of different metals at different conc. (µM) present in mineral salts broth on pH of *Morchella spongiola* MR 17

S.No	Conc.(µM)	Zinc	Manganese	Copper	Cadmium
1.	0	0.220 ± 0.001	0.221 ± 0.000	0.220 ± 0.001	0.222 ± 0.001
2.	10	0.225 ± 0.002	0.224 ± 0.003	0.218 ± 0.002	0.219 ± 0.001
3.	20	0.228 ± 0.001	0.227 ± 0.002	0.212 ± 0.003	0.215 ± 0.002
4.	30	0.231 ± 0.003	0.230 ± 0.001	0.210 ± 0.002	0.212 ± 0.003
5.	40	0.225 ± 0.003	0.227 ± 0.001	0.208 ± 0.001	0.210 ± 0.001
6.	50	0.221 ± 0.001	0.225 ± 0.002	0.207 ± 0.001	0.208 ± 0.002

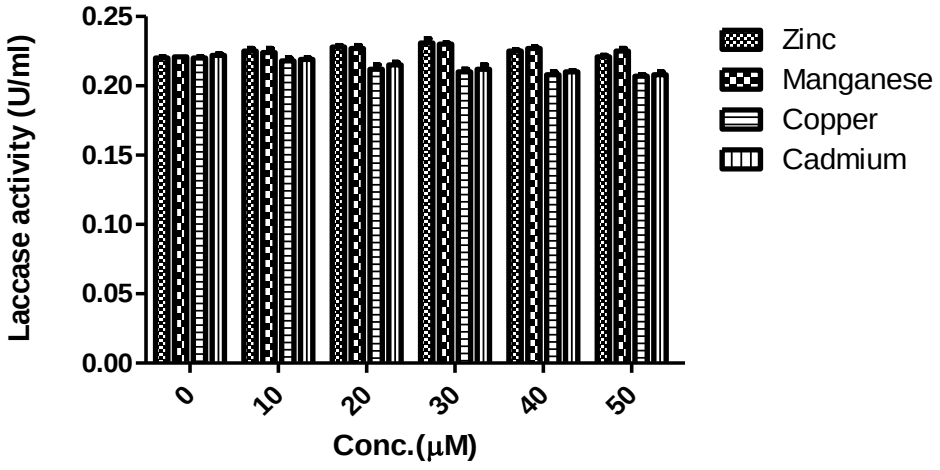


Fig 4.17: Influence of different metals at different conc.(µM) present in mineral salts broth on pH of *Morchella spongiola* MR 17

Table 4.18: Influence of different metals at different conc. (µM) present in mineral salts broth on pH of *Morchella sp.* MR 2

S.No	Conc.(µM)	Zinc	Manganese	Copper	Cadmium
1.	0	0.358 ± 0.000	0.360 ± 0.001	0.358 ± 0.001	0.359 ± 0.000
2.	10	0.364 ± 0.001	0.367 ± 0.001	0.356 ± 0.003	0.356 ± 0.002
3.	20	0.368 ± 0.002	0.370 ± 0.002	0.353 ± 0.001	0.352 ± 0.001
4.	30	0.370 ± 0.003	0.372 ± 0.003	0.349 ± 0.002	0.346 ± 0.002
5.	40	0.365 ± 0.002	0.368 ± 0.002	0.347 ± 0.001	0.345 ± 0.001
6.	50	0.363 ± 0.001	0.369 ± 0.001	0.346 ± 0.002	0.343 ± 0.003

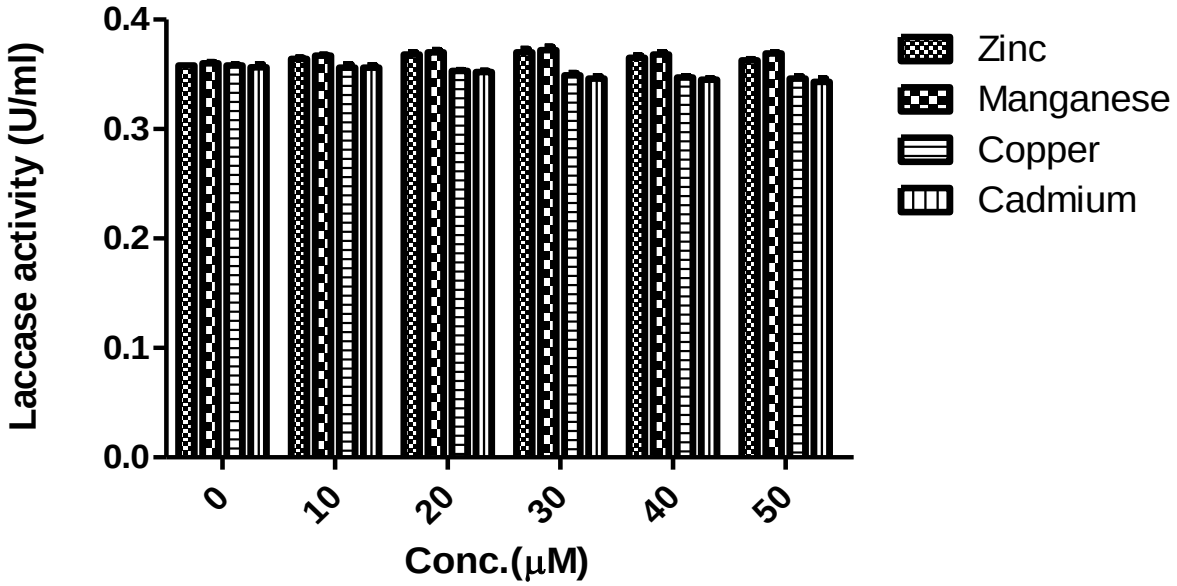


Fig. 4.18 Influence of different metals at different conc. (µM) present in MSB medium on laccase activity of *Morchella sp.* MR 2

4.4.4 Manganese peroxidase activity: Both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 showed manganese peroxidase (MnP) activity only in MSB medium having manganese ions (as Mn ions are inducers of MnP activity). With increase in concentration of manganese ions increase in MnP was recorded.

Higher MnP activity was recorded in *Morchella sp.* MR 2 compared to *Morchella spongiola* MR 17 (Table 4.19 and Figure 4.19)

Table 4.19 Effect of manganese ions at different conc. (µM) in MSB on MnP activity(U/L) in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD).

S.No	Conc.	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	0	0.002 ± 0.000	0.001 ± 0.000
2.	10	0.031 ± 0.002	0.073 ± 0.001
3.	20	0.049 ± 0.001	0.080 ± 0.002
4.	30	0.053 ± 0.003	0.097 ± 0.001
5.	40	0.063 ± 0.002	0.100 ± 0.003
6.	50	0.067 ± 0.001	0.103 ± 0.002

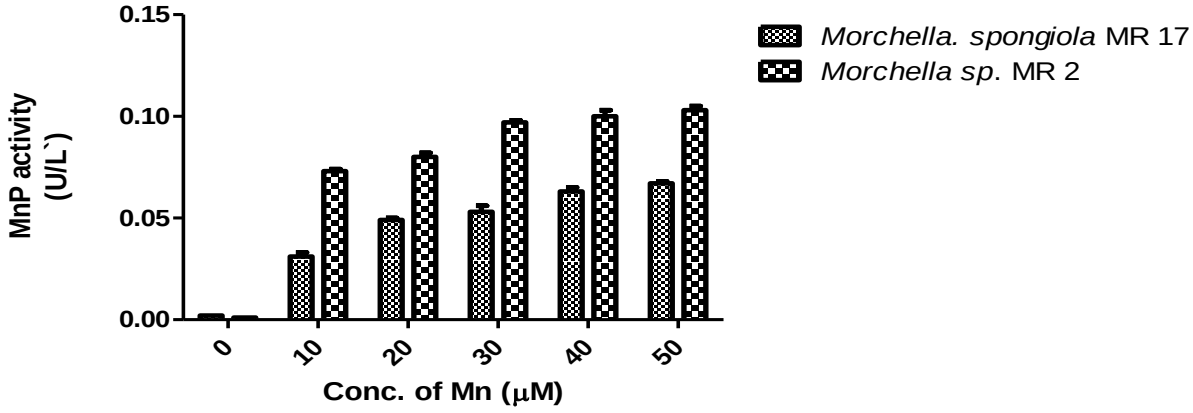
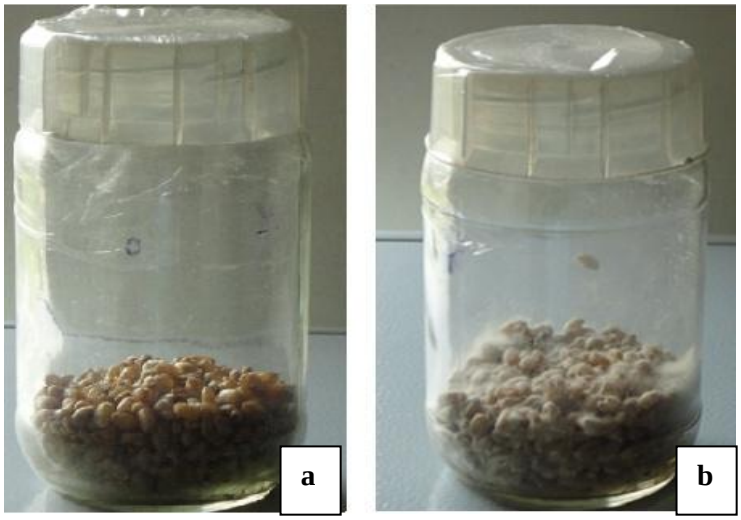


Fig. 4.19 Influence of manganese metals at different concentration in MSB medium on MnP activity in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2

4.4.5 Lignin peroxidase activity: Lignin peroxidase activity was not detected in MSB medium containing various metals under study at different concentrations in both *Morchella* spp. as no lignin rich source was present in MSB medium.

4.5 Sclerotia formation on different substrates

To study the effect of different liginnocellulosic substrates (wheat grains, wheat straw, rice straw, pine needles and saw dust) on sclerotia formation in *Morchella spongiola* MR 17 *and Morchella sp.* MR 2, their pure mycelium were grown in these liginnocellulosic substrates. pH and enzyme activities (laccase, manganese peroxidase and lignin peroxidase activities) were recorded.



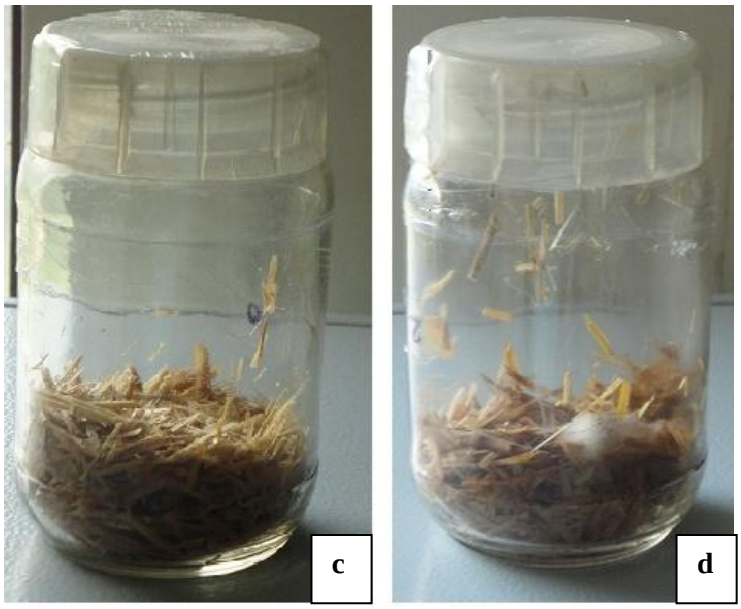


Fig 4.20 a) wheat grains as control without mycelia b) Growth of *Morchella sp.* MR 2 on wheat grains as substrate c) Control without mycelia d) Growth of *Morchella spongiola* MR 17 on wheat straw as substrate

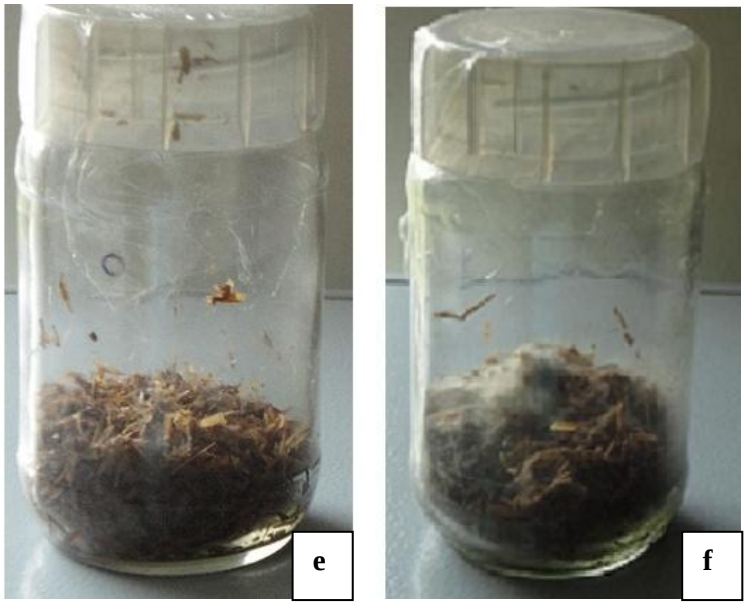


Fig: 4.21 e) Rice straw as control without mycelia f) Growth of *Morchella sp.* MR 2 in rice straw as substrate

4.5.1 Sclerotial Biomass: Sclerotia were observed in both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 in various lignocellulosic substrates used in the present study. In *Morchella sp.* MR 2, maximum biomass was observed in wheat grains as substrate, followed by rice straw. Biomass yield was observed in less quantity in both pine needles and saw dust in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2. Higher biomass was recorded in *Morchella sp.* MR2 compared to *Morchella spongiola* MR 17, in all substrates used for sclerotia formation.(Table 4.20 and Figure 4.22)

Table 4.20 Effect of different lignocellulosic substrates on biomass(in grams), during sclerotia formation in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD)

S .No.	Substrates	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Wheat grains	8.99 ± 0.07	11.54 ± 0.64
2.	Wheat straws	4.69 ± 0.57	05.53 ± 0.65
3.	Rice straw	6.62 ± 0.11	08.44 ± 0.45
4.	Pine needles	2.26 ± 0.33	02.34 ± 0.31
5.	Saw dust	2.34 ± 0.24	03.02 ± 0.17

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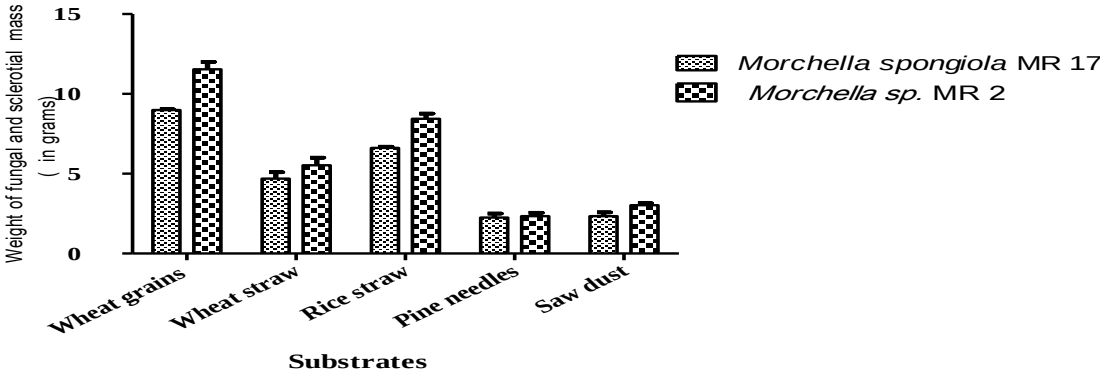

Fig. 4.22 Influence of different lignocellulosic substrates on biomass (in grams), during sclerotia formation on *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 .

4.5.2 pH: Increase in pH as compared to their respective controls was observed in all the cases. Both *Morchella spongiola* MR 17 and *Morchella sp* MR 2 preffered slightly acidic pH for growth.(Table 4.21 and Figure 4.23)

Table 4.21 Effect of different lignocellulosic substrates on pH, during sclerotia formation in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD)

S.No.	Substrates	Control	*Morchella spongiola* MR17	*Morchella sp.* MR 2
1.	Wheat grains	5.80 ± 0.01	6.29 ± 0.07	6.84 ± 0.02
2.	Wheat straws	5.36 ± 0.01	6.01 ± 0.04	6.64 ± 0.01
3.	Rice straw	5.65 ± 0.00	6.12 ± 0.02	6.70 ± 0.01
4.	Pine needles	4.15 ± 0.01	5.04 ± 0.07	4.92 ± 0.07
5.	Saw dust	6.23 ± 0.02	6.19 ± 0.02	6.75 ± 0.03

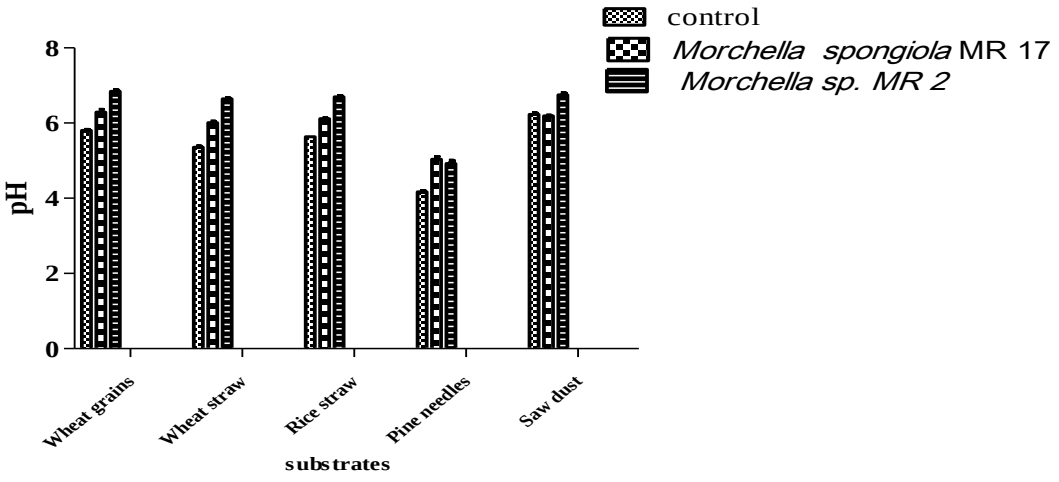

Fig. 4.23 Influence of different lignocellulosic substrates on pH, during sclerotia formation in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2.

4.5.3 Laccase activity:

In both *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 laccase activity was recorded in all the substrates. Maximum laccase activity was observed in wheat grains and rice straw, followed by wheat straw. Least laccase activity was detected in pine needles and sawdust.

Higher laccase activity was recorded in *Morchella sp.* MR 2 compared to *Morchella spongiola* MR 17 in all lignocellulosic substrates used for sclerotia formation.(Table 4.22 and Figure 4.24)

Table 4.22 Effect of different lignocellulosic substrates on laccase activity (U/ml), during sclerotia formation in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 (Mean ± SD)

S.No.	Substrates	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1 .	Wheat grains	16.29 ± 0.04	52.54 ± 0.02
2.	Wheat straw	06.24 ± 0.02	11.30 ± 0.01
3.	Rice straw	09.34 ± 0.01	34.26 ± 0.02
4.	Pine needles	00.01 ± 0.00	00.03 ± 0.00
5.	Saw dust	00.04 ± 0.00	00.06 ± 0.01

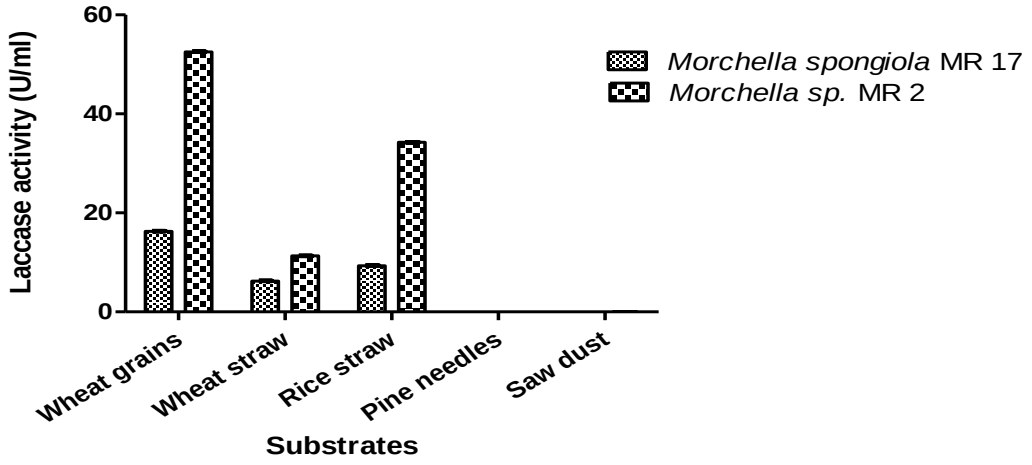

Fig.4.24: Influence of different substrates on laccase activity (U/ml), during sclerotia formation on *Morchella spongiola* MR 17 and *Morchella sp.* MR 2.

4.5.4 Lignin peroxidase activity: Lignin peroxidase activity was recorded in various lignocellulosic substrates used. Maximum LiP activity was recorded in wheat grains, followed by rice straw and then wheat straw. Both saw dust and pine needles show optimum LiP activity. Among *Morchella. spongiola* MR 17 and *Morchella sp.* MR2 maximum enzyme activity was present in *Morchella sp.* MR 2 in all the lignocellulosic substrates.(Table 4.23 and Figure 4.25)

Table 4.23 Effect of different lignocellulosic substrates on lignin peroxidases activity (U/L), during sclerotia formation in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 *(*Mean ± SD).

S.No.	Substrates	*Morchella spongiola* MR 17	*Morchella sp.* MR 2
1.	Wheat grains	0.203 ± 0.004	0.146 ± 0.002
2.	Wheat straws	0.125 ± 0.002	0.145 ± 0.001
3.	Rice straw	0.179 ± 0.004	0.150 ± 0.003
4.	Pine needles	0.044 ± 0.001	0.053 ± 0.003
5.	Saw dust	0.053 ± 0.003	0.065 ± 0.001

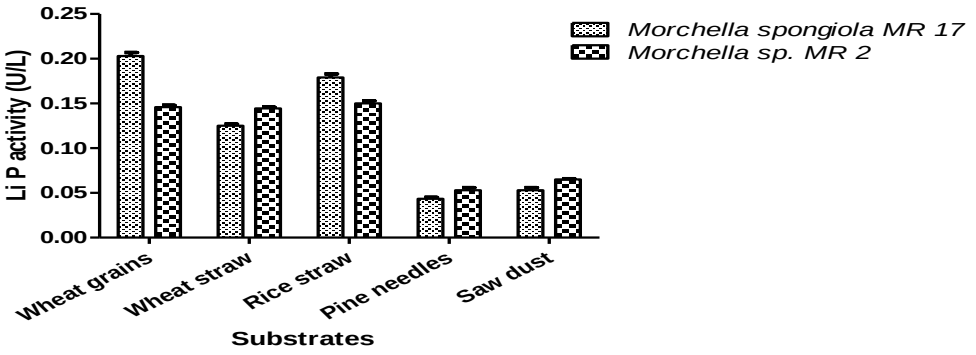

Fig 4.25 Influence of lignocellulosic different substrates on lignin peroxidase activity U/L during sclerotia formation in *Morchella spongiola* MR 17 and *Morchella sp.* MR 2.

4.5.5 Manganese peroxidase activity: Manganese peroxidase activity was not detected in various lignocellulosic substrates.

4.6 Sclerotia formation on the combination of soil and substrate (Ower et al., 1986)

To study the phenomenon of sclerotia formation in *Morchella spongiola* MR 17 *and Morchella sp.* MR 2 in various lignocellulosic substrates (wheat grains, wheat straw, rice straw, pine needles and saw dust) their pure mycelium were grown in combination of soil and these lignocellulosic substrates (Ower et al., 1986**)** and the cultures were harvested after 12 days, 15 days and 18 days of incubation. pH, number of sclerotia, enzyme activities(laccase enzyme and cellulase enzyme activity) were recorded.

4.6.1 Sclerotia formation: No sclerotia formation was observed in *Morchella spongiola* MR 17 in various lignocellulosic substrates after 12, 15 and 18 days of incubation.

Sclerotia formation was observed in *Morchella sp.* MR 2 in various lignocellulosic substrates after 12, 15 and 18 days of incubation and following results were obtained.(Fig 4.26 and 4.27)

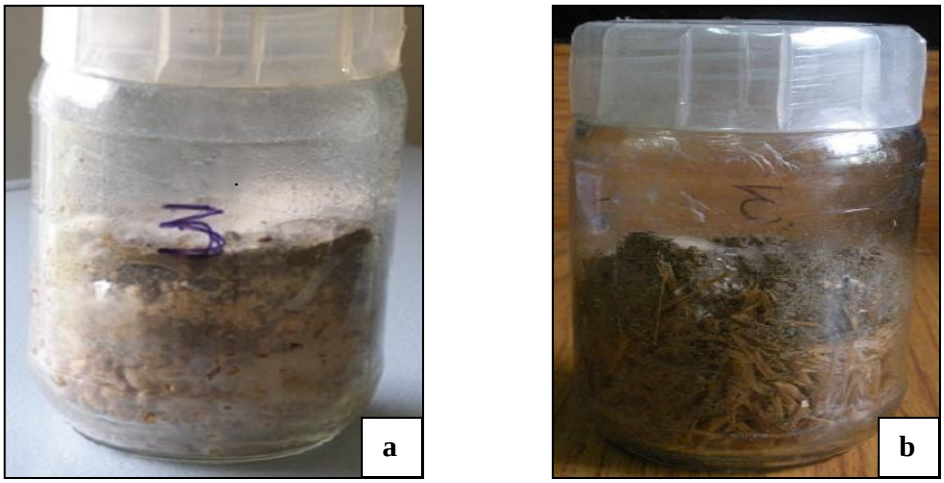

Fig 4.26: Production of sclerotia by *Morchella* sp. MR 2 in jars. Upper layer consists of nutrient-poor soil separated from a lower layer of a) wheat grains (substrate) b) wheat straw by perforated aluminum foil.

Fig 4.27: Production of sclerotia by *Morchella* sp. MR 2 in jars. Upper layer consists of nutrient-poor soil separated from a lower layer of wheat straw (substrate) by perforated aluminum foil. (Ower *et al*., 1986**)**.

4.6.2 Sclerotia number: Number of sclerotia increased from twelth to fifteenth days of incubation and afterwards remained almost constant in all lignocellulosic substrates.(Figure 4.24 and Table 4.28)

Table 4.24 Effect of different lignocellulosic substrates on number of sclerotia formed in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation. *(*Mean ± SD)

S.No	Substrate	12 days	15 days	18 days
1.	Wheat grains	42 ± 0 2	47 ± 03	49 ± 02
2.	Wheat straw	28 ± 03	32 ± 02	34 ± 01
3.	Rice straw	34 ± 01	39 ± 02	40 ± 02
4.	Pine needles	07 ± 02	09 ± 01	09 ± 01
5.	Saw dust	06 ± 01	07 ± 01	09 ± 00

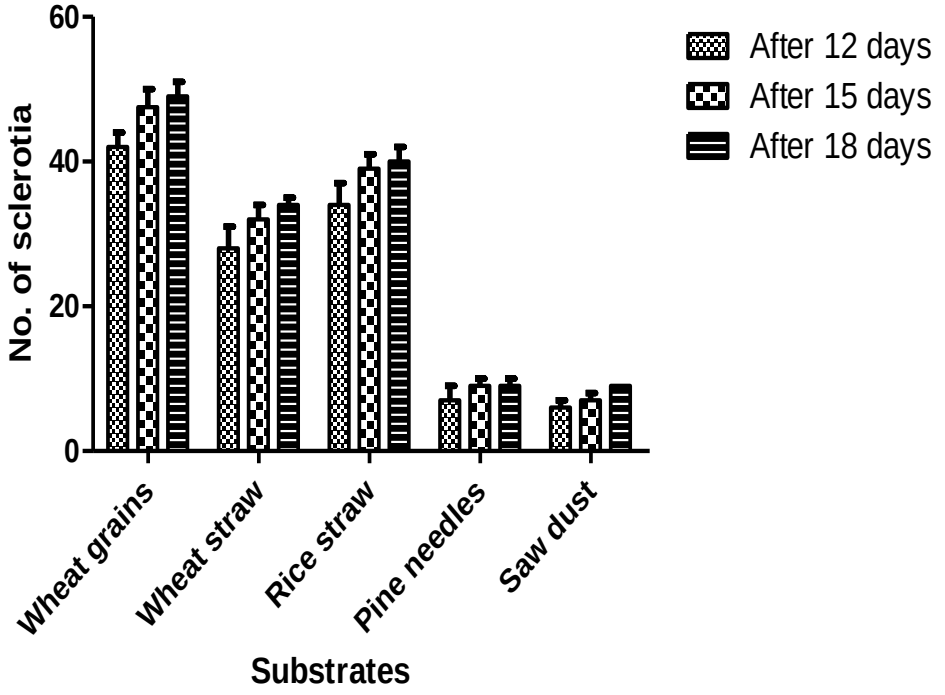

Fig. 4.28 Influence of different lignocellulosic substrates on number of sclerotia formed in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation.

4.6.3 pH: Increase in pH was measured in all lignocellulosic substrates after 12, 15 and 18 days of incubation. This increase was highest from 0 to 12 days of incubation followed by 12 to 15 days of incubation. After 15 days increase in pH remains almost constant. (Table 4.25 and Figure 4.29)

Table 4.25 Effect of different lignocellulosic substrates on pH, during sclerotia formation in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation.*(*Mean ± SD)

S.No	Substrate	Control	After 12 days	After 15 days	After 18 days
1.	Wheat grains	6.80 ± 0.02	7.32 ± 0.01	7.54 ± 0.01	7.60 ± 0.02
2.	Wheat straws	7.30 ± 0.01	7.49 ± 0.01	7.80 ± 0.02	7.85 ± 0.01
3.	Rice straw	7.32 ± 0.01	7.62 ± 0.02	7.87 ± 0.01	7.91 ± 0.03
4.	Pine needles	8.19 ± 0.02	8.25 ± 0.02	8.29 ± 0.02	8.31 ± 0.01
5.	Saw dust	8.00 ± 0.01	8.12 ± 0.01	8.18 ± 0.02	8.21 ± 0.02

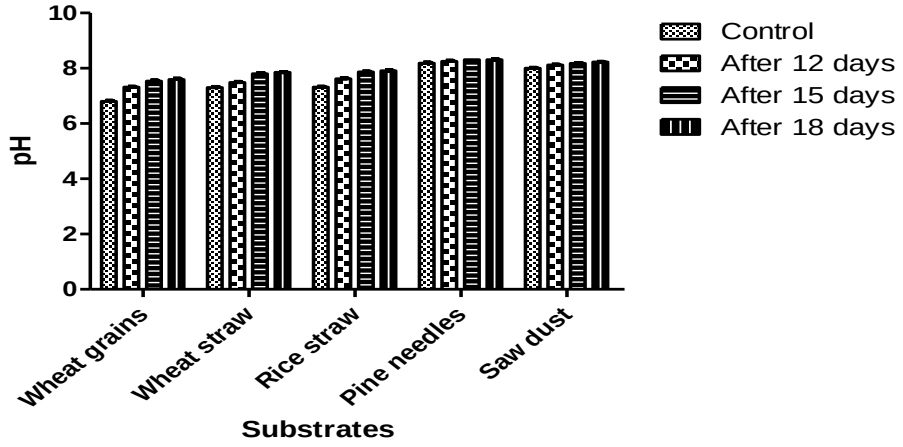

Fig. 4.29 Influence of different lignocellulosic substrates on pH, during sclerotia formation in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation.

4.6.4 Laccase activity: Laccase activity increased upto 15 days of incubation and there after decrease in laccase activity was recorded. Maximum laccase activity was measured in wheat straw followed by rice straw and wheat straw. Less laccase activity was observed in pine needles and saw dust.(Table 4.26 and Figure 4.30)

Table 4.26 Effect of different lignocellulosic substrates on laccase activity (U/ml) during sclerotia formation in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation. *(*Mean ± SD)**.**

S.No	Substrates	12 days	15 days	18 days
1.	Wheat grains	15.09 ± 0.04	18.02 ± 0.01	17.30 ± 0.02
2.	Wheat straws	06.92 ± 0.03	07.61 ± 0.02	06.99 ± 0.03
3.	Rice straw	08.95 ± 0.02	10.30 ± 0.01	09.92 ± 0.01
4.	Pine needles	00.64 ± 0.03	00.68 ± 0.02	00.65 ± 0.02
5.	Saw dust	02.29 ± 0.01	03.32 ± 0.01	03.28 ± 0.02

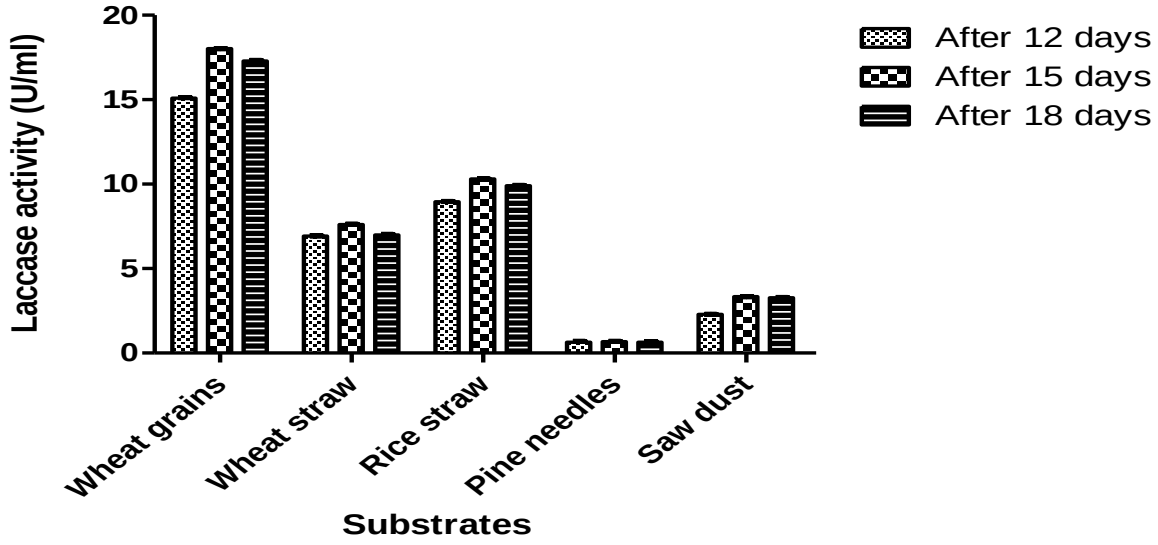

Fig. 4.30 Influence of different lignocellulosic substrates on laccase activity (U/ml) during sclerotia formation in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation.

4.6.5 Cellulase activity: Upto 15 days of incubation cellulase activity increases, there after remains almost most constant in all lignocellulosic substrates. Maximum cellulose activity was measured in wheat straw followed by rice straw and wheat straw. Less cellulose activity was observed in pine needles and saw dust.

Table 4.27 Effect of different lignocellulosic substrates on cellulase activity (U/ml) during sclerotia formation in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation. *(*Mean ± SD)**.**

S.No	Substrate	After 12 days	After 15 days	After 18 days
1.	Wheat grain	0.095 ± 0.002	0.100 ± 0.001	0.100 ± 0.001
2.	Wheat straw	0.091 ± 0.001	0.092 ± 0.002	0.090 ± 0.001
3.	Rice straw	0.093 ± 0.003	0.094 ± 0.001	0.092 ± 0.002
4.	Saw wood	0.019 ± 0.001	0.020 ±0.001	0.020 ± 0.001
5.	Pine needles	0.007 ± 0.000	0.008 ±0.001	0.007 ± 0.001

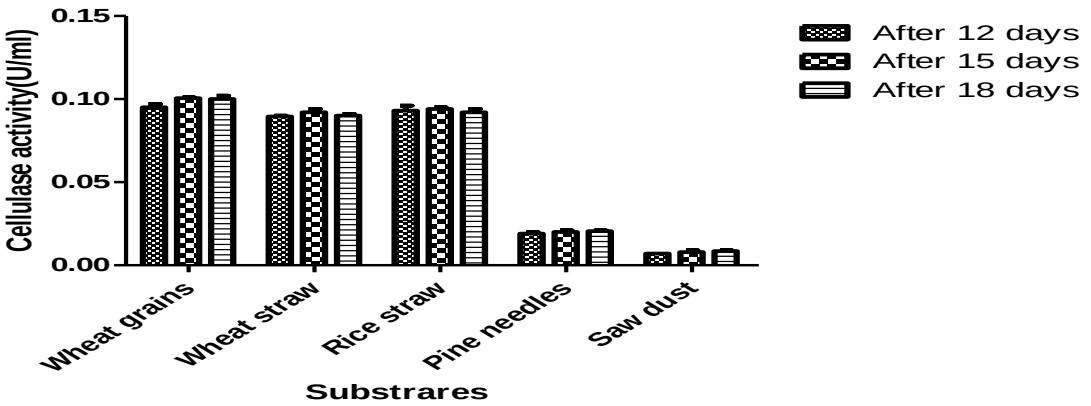

Fig 4.31 Influence of different lignocellulosic substrates on cellulase activity (U/ml) during sclerotia formation in *Morchella sp.* MR 2 after 12, 15 and 18 days of incubation.

Cloning of laccase gene

4.7 Identification of laccase gene in *Morchella sp*. MR 2

It was evidient from the physiological results that laccase activity was maximum among lignocellulosic enzymes during sclerotia formation and laccase gene was expressed higher in *Morchella sp.* MR 2 as compared to *Morchella spongiola* MR 17 therefore *Morchella sp.* MR 2 was selected for laccase gene study.

A set of primers Cu1AF and Cu2R were designed and used for the amplification of laccase gene from the cDNAs of *Morchella sp.* MR 2

4.7.1 RNA isolation, cDNA preparation by RT-PCR and PCR

To induce the laccase gene, *Morchella sp.* MR 2 was first grown on MSB medium in presence of ABTS as inducer for 10 days. Total RNA was isolated from ABTS treated mycelium using the CTAB method (Fig.4.32). First strand cDNA was synthesized from 2µg total RNA by RT-PCR method. These cDNA were used for the amplification of laccase gene with Cu1AF and Cu2R primers. An amplicon of 300 bp was obtained (Fig.4.33)

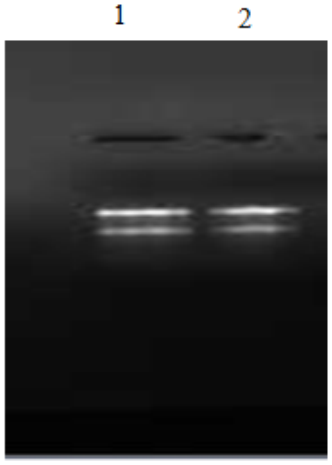

Fig:4.32 Total RNA isolation from ABTS treated *Morchella sp.* MR 2. Lane 1, 2: Total RNA

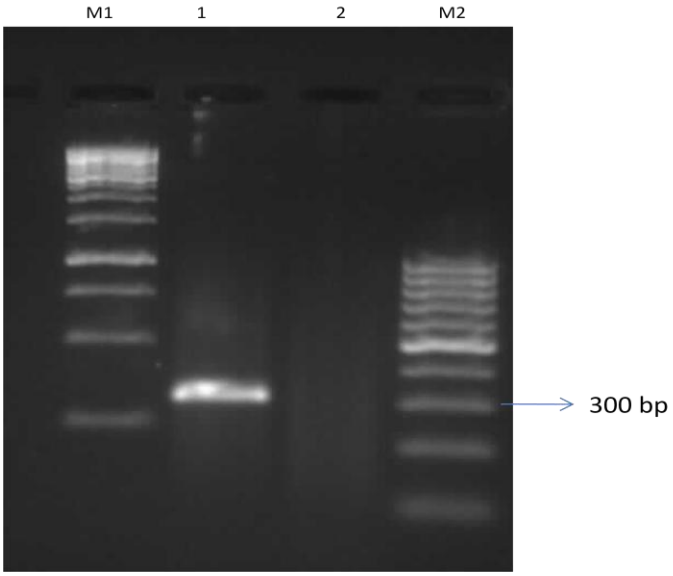

Fig 4.33: Amplification of cDNA of *Morchella sp.* MR 2 with Cu1AF and Cu2R primers, LaneM1:1Kb GeneRuler ladder, Lane 2: *Morchella sp.* MR2 cDNA amplified with primer pair, Lane 2: Negative control, Lane M2: 100 bp GeneRuler ladder.

4.7.2. Cloning and sequencing of laccase gene

The PCR products amplified from cDNA were purified using PCR product purification kit (Geneaid Biotech Ltd, USA) and ligated into pTZ57R/T vector system (InsTAclone^{TM} PCR cloning kit (Fermentas, USA). These ligated products were transformed into DH5α *E. coli* cells by a heat shock method. The positive clones were screened by α-complementation and the plasmid DNA of the recombinant cells were isolated using the alkali lysis method. Plasmid containing laccase inserts were confirmed by PCR with insert specific primers. The inserts in the plasmids were sequenced by chain termination method (Sanger *et al.,* 1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South campus, Delhi University, New Delhi, India).

Discussion

Morchella, the true morels, are known for their excellent flavor and these are amongst the most highly prized edible fungi in the world (Pegler, 2003). One kilogram of dry weight of fruiting bodies of morels fetch 15,000/- rupees in the market. (Prasad *et al*., 2002), But cultivation of *Morchella* spp.fruiting body on commercial scale is still not successful, may be due to complex nature of sclerotia, variation in levels of lignolytic enzymes during formation of sclerotia and fruiting formation.

By comparing the growth of *Morchella spongiola* MR 17 and *Morchella sp.* MR 2 in different media, carbon sources, nitrogen sources and sclerotial studies in different lignocellulosic substrates, It was observed that *Morchella sp.* MR 2 were able to grow more efficiently than *Morchella spongiola* MR 17. Sclerotia formation was absent in *Morchella spongiola* MR 17 in various lignocellulosic substrates used, using open jar method (Ower *et al* ., 1986). In all the physiological studies an increase in pH, dry biomass, protein concentration, laccase activity and decrease concentration of reducing sugar was observed. A plausible reason for such an observation could be attributed to the physiology of fungi, as the fungal growth proceed it secrets enzymes in medium and absorbs the sugar or lignin (in degraded form due to secretion of fungal lignocellulosic enzymes) from medium and substrates respectively.

Among various media used for growth studies in both *Morchella* species, optimum growth was recorded in mineral salt broth and morel growth broth. The best growth in *Morchella spongiola* MR 17 and *Morchella* sp. MR 2 was recorded in malt extract and yeast malt extract medium respectively.

Maximum growth was recorded in mineral salt broth(MSB) with sodium nitrate as nitrogen source, similar results were reported by Kanwal and Reddy, (2010). In MSB with urea as nitrogen source least growth was recorded. Optimum growth was recorded in MSB with casein and nitrite as nitrogen source.

Difference in growth of was observed under metal stress at varying concentration in both *M. spongiola* MR 17 and *Morchella sp.* MR 2. Presence of zinc and manganese in the medium have positive effect on the growth of both fungi, where maximum growth was recorded at the concentration of 30µM. Growth declined in the presence of copper and cadmium in the medium and this decline in growth increased as the concentration of metal in the medium increased.

From sclerotial studies, using various lignocellulosic substrates, maximum number of sclerotia were formed in wheat grains followed by rice straw and wheat straw, whereas in pine needles and saw dust few sclerotia were observed. In presence of these ligninocellulosic substrates, lignolytic enzymes (laccase, manganese peroxidase and lignin peroxidase) were expressed. Among these lignolytic enzymes expression of laccase was maximum and manganese peroxidases expression was almost negligible This may be due to absence of manganese ions in ligninocellulosic substrates. Similar results were reported by Claudia *et al*., (1998) in their work on ligninolytic system of *pycnoporus cinnabarius*

In open jar method (Ower *et al*., 1982), sclerotia formation was observed only in *Morchella sp.* MR 2 and maximum sclerotia formation was recorded in wheat grains. As the number of days of incubation were increased variation in laccase enzyme activity was recorded. Firstly increase and then slightly decrease in laccase activity was observed. Maximum activity was recorded after fifteen days of incubation (18U/ml). With an increase in the sclerotia number, laccase activity was observed to be increased. Similar work by Georgiou et al. (2006) and Liew

et al. (2011) have shown that laccase plays an important role in sclerotia formation in fungi. Similarly, the trend of higher laccase activity in sclerotia as compared to mycelia growth in our study was observed. Variations have been observed in the ligninolytic enzyme production during fruiting body formation as compared to mycelia growth (De Groot *et al*., 1997; Das et al., 2006). So, it can be concluded that laccase enzyme plays an important role in sclerotia formation in morels.

Laccase gene of 300 bp was detected in *Morchella sp.* MR 2 using Cu1AF and Cu2R set of primers .This gene was cloned in pTZ57R/T cloning vector and sequenced.

Chapter V

Conclusion

Inability of *Morchella* spp. to be cultivated on commercial scale successfully, necessitates to study the physiological factors that governs their growth and sclerotia formation. According to the result obtained, it can be concluded that laccase enzyme plays a key more in sclerotia formation. Among *Morchella spongiola* MR 17 and *Morchella sp*. MR 2 higher laccase activity was measured in *Morchella sp.* MR 2. In *Morchella spongiola* MR 17 maximum growth was recorded in malt extract medium while in *Morchella sp.* MR 2 maximum growth was recorded in yeast malt extract medium. Maximum growth was measured in MSB (mineral salts broth) with mannose as carbon source and sodium nitrate as nitrogen source while minimum growth was measured in MSB medium with sorbose as carbon source and urea as nitrogen source. Increase in growth was measured in MSB medium supplemented with zinc and manganese ions (maximum increase was recorded at the conc. 30µM) while decrease in growth was measured in MSB medium supplemented with copper and cadmium ions. Increase in manganase peroxidase activity was recorded due to presence of manganese ions in MSB medium, as the concentration of manganese was increased in the medium increase in manganese peroxidase activity was recorded. More number of sclerotia were formed using Wheat grain, rice straw and wheat straw as lignocellulosic substrates. Open jar method described by Ower *et al* in 1986 can be used for sclerotia formation in *Morchella sp*. MR 2 only. Laccase enzyme plays a key role in sclerotia formation and higher expression of laccase activity was recorded in *Morchella sp*. MR 2.

Maximum sclerotia formation was measured in wheat grain after 15 days of incubation From the molecular work carried on *Morchella sp*. MR 2 , it can be concluded that laccase gene of 300bp was present in *Morchella sp*. MR 2 , that can be amplified with Cu 1AF and Cu 2R primers, at annealing temperature of 50 ºC. Results obtained suggest that *Morchella sp*. MR 2 possess a high potential for sclerotia formation and could be exploited for its cultivation on commercial scale.

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