Ph. D. Thesis

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STUDIES ON EXRACELLULAR ENZYME PRODUCTION DURING GROWTH OF *PLEUROTUS* SP. ON LIGNOCELLULOSIC AGRIWASTE AND THE UTILIZATION OF SPENT MUSHROOM SUBSTRATE

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Studies on extracellular enzyme production during growth of *Pleurotus* sp. on lignocellulosic agriwaste and the utilization of spent mushroom substrate

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Studies on extracellular enzyme production during growth of *Pleurotus* sp. on lignocellulosic agriwaste and the utilization of spent mushroom substrate"** is based on the original research work carried out by Ms. Jasmine Koshy under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

Cochin-22, **Dr. Padma Nambisan**

03-12-2012.

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Studies on extracellular enzyme production during growth of *Pleurotus* sp. on lignocellulosic agriwaste and the utilization of spent mushroom substrate"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin, under the guidance of Dr. Padma Nambisan, Associate Professor, Cochin University of Science and Technology, and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

Cochin 22, **Jasmine Koshy**

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TRUST IN THE LORD WITH ALL YOUR HEART, AND LEAN NOT ON YOUR OWN UNDERSTANDING; IN ALL YOUR WAYS ACKNOWLEDGE HIM, AND HE SHALL DIRECT YOUR PATHS.

PROVERBS 3: 5, 6 (BIBLE)

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Dedicated to my
Lord and Saviour
Jesus Christ

ABBREVIATIONS

%	-	Percentage
$(NH_4)_2SO_4$	-	Ammonium sulphate
”	-	Inch
°C	-	Degree Celsius
°F	-	Degree Fahrenheit
A°	-	Angstrom
A_{260}	-	Absorbance at 260 nm
A_{280}	-	Absorbance at 260 nm
bp	-	Base pair
C / N	-	Carbon / Nitrogen
$Ca(OH)_2$	-	Calcium hydroxide
cm	-	Centimetre
CO_2	-	Carbon dioxide
conc.	-	Concentration
CTAB	-	Cetyl Trimethyl Ammonium Bromide
$CuSO_4$	-	Copper sulphate
DNA	-	Deoxyribo Nucleic Acid
DNS	-	Dinitrosalicylic acid
dNTP	-	Deoxy Nucleotide Tri Phosphate
EC	-	Enzyme classification
EDTA	-	Ethylene Diammine Tetra Aceticacid
FPU	-	Filter Paper Units
g	-	Gram
GPa	-	Giga Pascal
h	-	Hour
H_2O_2	-	Hydrogen peroxide

H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
$HgCl_2$	-	Mercuric chloride
HNO_3	-	Nitric acid
IU	-	International unit
KBr	-	Potassium bromide
Kg	-	Kilogram
L	-	Litre
LiP	-	Lignin peroxidase
M	-	Molar
mg	-	Milligram
$MgCl_2$	-	Magnesium chloride
min.	-	Minutes
ml	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
MPa	-	Mega Pascal
N	-	Normal
Na_2CO_3	-	Sodium carbonate
NaCl	-	Sodium Chloride
NaOH	-	Sodium hydroxide
ng	-	Nanogram
nm	-	Nanometres
N - P - K	-	Nitrogen - Phosphorous - Potassium
θ	-	Theta
O_2	-	Oxygen
P. eous	-	*Pleurotus eous*
P. ostreatus	-	*Pleurotus ostreatus*

PCR	-	Polymerase Chain Reaction
psi	-	Pound per square inch
red.	-	Reduction
rpm	-	Rotations per minute
rRNA	-	Ribosomal Ribo Nucleic Acid
S. cerevisiae	-	*Saccharomyces cerevisiae*
sec.	-	Second
SMS	-	Spent Mushroom Substrate
sp.	-	Species
spp.	-	Species
sq. cm	-	Square centimetre
SS	-	Spent Substrate
TAE	-	Tris Acetate EDTA
Taq		*Thermus aquaticus*
U	-	Units
UV - VIS	-	Ultra Violet - Visible
V	-	Volt
w / v	-	Weight / volume
WHO	-	World Health Organisation
Z. mobilis	-	*Zymomonas mobilis*
µ	-	Micron
µg	-	Microgram
µl	-	Microlitre
µmol	-	Micromolar

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Chapter 1

INTRODUCTION

Mushrooms are multicellular structures formed from the differentiation of vegetative mycelial cells. They belong to kingdom fungi. A typical mushroom has a cap (pileus) and a stem (stipe). Basically there are three ecological groups of mushrooms: mycorrhizal mushrooms which grow in a symbiotic association with the root of host plants; parasitic mushrooms which affect living plants; and, saprophytic mushrooms which recycle dead and decaying plant material.

Mushrooms include edible / medicinal and poisonous species. According to fungal classification system proposed by Ainsworth, followed by J. Webster (Sharma and Jandaik, 1989), edible mushrooms are members of the family Tricholomataceae and subdivisions Basidiomycotina and Ascomycotina (Dung et al., 2012). Out of 5000 different species of known mushrooms, at least 1220 are reported to be edible which could be employed for foods and medicines (Muhammad et al., 2006).

Mushrooms are considered to be healthy food that can reduce malnutrition and help the country deliver the global commitment of achieving the Millennium Development Goals (MDG) on health, poverty and hunger (United Nations, 2000). They are a good source of protein and 9 essential amino acids, carbohydrates, dietary fibre, β-glucan, vitamins (folic acid, biotin, niacin, thiamine and riboflavin), and minerals (phosphorus, potassium, calcium, zinc, iron

and magnesium); but low in sodium (Manzi et al., 1999; Moa and Jica, 2000; Oei, 2003). Mushrooms are also low in fat (8 %), mainly linoleic acid, and have little starch and no cholesterol, and hence are ideal for diabetic and hypertensive patients.

Mushrooms are also considered to have medicinal value. In China alone, more than 700 medicinal and health products containing mushrooms as the main ingredient have been commercialized. Certain edible mushroom extract can be used as dietary supplements as they have several bioactive compounds with therapeutic activity such as anti-carcinogenic, anti-inflammatory, immuno-suppressor, and antibiotic effects (Longvah and Deosthale, 1998; Kapoor, 2010). The extractable products can also be classified into the category of mushroom nutriceuticals (Chang and Buswell, 1996).

Mushrooms, as an emerging high value crop, are gaining popularity in the world today with great opportunities for income generation. Mushroom cultivation is a profitable agribusiness. World production of mushrooms is estimated as 12 million tons with China taking the lead and producing 86.6 % of the world production (Chang, 2004; APEMTC, 2007). Global commercial production of edible mushrooms is increasing by 35.9 % since 1995. Mushroom cultivation has great scope in developing countries because of the cheap and easily available raw materials needed for this activity. From a dietary point of view, mushrooms are favorable food for a vegetarian-predominant country like India.

India has great potential to cultivate mushrooms. Her future share in the world production and trade of oyster mushroom is rated high for a variety of reasons. India has a very large availability of various types of raw substrate material such as wheat straw, paddy straw, bagasse and these materials are relatively inexpensive when compared with international prices. Enormous amount of

wastes are generated by the agricultural sector, forest exploitation and food industries, which are either composted or sent to sanitary landfills. Wastes such as cereal straws are largely burnt by the farmers, which causes air pollution.

Searching the equilibrium between the social, economic and environmental aspects, the reuse of wastes has taken on an extremely important dual purpose: elimination or reduction of wastes from the environment and giving them added value through the production of low cost food (Villas-Boas et al., 2002). Although, the crop residues have several uses, almost 50 % of the crop residues are still potentially available for the growing of mushrooms, as they form the potential renewable resources for mushroom cultivation. Mushrooms can not only convert these lignocellulosic biomass wastes into human food, creating a pollution-free environment, but also produce notable immune enhanced products, which have many health benefits. Hence, mushroom cultivation is an eco-friendly method of solid waste disposal / management.

Among many kinds of edible mushrooms, oyster mushrooms of *Pleurotus* spp. has been commercialized and consumed. It is a saprophytic fungus, consumed as a delicacy and appreciated for its excellent taste, ample nutrition and enticing flavour. *Pleurotus* spp. has been intensively studied for its high gastronomic value, ability to colonize and degrade lignocellulosic residues, shorter growth time when compared to other edible mushrooms; little environmental control, very few disease and pest attack for fruiting bodies, simple and cheap cultivation technique (Jwanny et al., 1995; Patrabansh and Madan, 1997). *Pleurotus* spp. can make use of the largest variety of waste substrates with its fast mycelial growth and its multilateral enzyme system. A range of about 200 different wastes is available as oyster mushroom substrates (Joseph, 2004). *Pleurotus* spp. is a common primary degrader of wood and vegetable residues (Zadrazil and Kurtzman, 1981).

Chapter 1

India has many agro-climatic regions that offer favorable climatic conditions for mushroom cultivation. In India, white button mushroom (*Agaricus* spp.) has the highest growth rate and potential for production, in the cool climatic regions. Also, button mushroom is grown mostly by large scale farmers due to its high capital requirements and sophisticated technology. *Pleurotus* spp. of mushroom is the second most common mushroom produced in India, as well as worldwide (Adejoye et al., 2006). They are preferred by small scale producers due to low investments costs, adaptability to a wide range of temperatures (22-30 °C), easy availability of substrate materials and high yields (Farm and Gtz, 2005; Moa and Jica, 2000; Gateri et al., 2008). Capital money requirement is very less for oyster mushrooms when compared to button mushrooms. Its cultivation is economically efficient for the farmers of other crops, who have crop residues as raw materials and can use low cost structures for mushroom houses.

In the early days, mushrooms were grown outdoors in most parts of the world. But, most modern mushroom farmers cannot rely on the natural environment, and hence, build temporary mushroom growing houses and provide good conditions for higher yield of mushrooms. Oyster mushrooms can be cultivated in bags, trays or plastic bottles on lignocellulosic substrates. They need substrates abundant in polysaccharides and lignin for their growth. Mycelial growth of oyster mushrooms makes use of soluble carbohydrates, glucose, as well as mineral sources such as ammonium sulphate.

During vegetative growth, mushroom mycelia secrete enzymes that degrade different components of plant material such as cellulose and lignin, present in the substrate. The degraded compounds are then absorbed by the hyphae, and the mycelium enlarges. Environmental factors such as temperature and light are known to be critical to stimulate the fruiting body formation during reproductive growth stage. Matured fruiting bodies are harvested on time.

After harvest, a considerable amount of mushroom substrate (spent mushroom substrate / spent substrate) remains as residual material. It is acidic in nature and high in organic matter. Presently, spent substrate is used for cultivation of other mushroom species or vegetables, for preparation of vermiculture or animal feed, or as compost for application in farm fields (Rinker, 2002). But, it needs heat treatment before being removed from the mushroom house. Being expensive, some mushroom growers discard the spent substrate far from the farm. But, without proper treatment, spent substrate can pose health problems. Conversely, recycling of spent substrate can increase sustainability and help farm economy (Rinker and Castle, 2005). Spent substrate can be utilized for various other value added purposes.

Spent substrate is reported to be a rich source of plant fibre. Plant fibres are long, stretched, thick-walled cells, mainly made up of cellulose. They are weaker than other synthetic fibres, but have several applications if mixed with plastics, glass, metals or synthetics. Also, unlike synthetic fibres, plant fibres have several advantages as they are biodegradable, available annually, renewable, non-carcinogenic and therefore health-friendly. Common methods to extract fibres from plant are fairly straight forward processes, though long, smelly and labour intensive. The basic principle is to encourage the softer parts (non-cellulosic) of the plant to rot so that only the stronger cellulose fibres remain. *Pleurotus* spp. has the capacity to preferentially remove lignin, with limited degradation of cellulose (Koshy and Nambisan, 2011).

Currently, ethanol, a colourless liquid fuel, intended for industrial use, is produced either through the hydration of ethylene or by fermentation using yeast. About 90 % of ethanol is derived from sugar or starch crops by fermentation (Hamelinck et al., 2010). But these crops have a high value for food application, and their sugar yield per hectare is low. Conversion of lignocellulosic biomass into ethanol

is a promising alternative. Second generation ethanol is derived from lignocellulosic materials (Taherzadeh and Karimi, 2007). Lignocellulosics have a chemically stable robust structure and the reducing sugar present in it is much harder to release than those in starchy biomass. The root cause for the recalcitrance of lignocellulosic biomass is the presence of lignin (Zhang and Lynd, 2004). Lignin acts as a rigid physical barrier and prevents cellulase from accessing the cellulose in the substrate for releasing sugar.

Lignocellulosic biomass can be saccharified for high yield of ethanol by pre-treatment for the removal of non-cellulosic part. Currently, mechanical, chemical or biological pre-treatments, or their combinations, are employed to make lignocellulosics more accessible to enzymes via. delignification and reduction of cellulose crystallinity. Literature shows that, mere mechanical treatment such as milling, cannot improve the yield of ethanol. Also, chemicals such as sulphuric acid and sodium hydroxide, used in the pre-treatments for releasing sugars, produce by-products that inhibit fermentation. If the pretreatment is very harsh, liberated sugars can be degraded to enzyme- and / or yeast-inhibiting compounds, lowering the overall yields. But, if the pretreatment is too weak it will result in low enzyme accessibility and the same drawbacks as above (Cardona et al., 2010). Biological pretreatment with microbes or microbial enzymes are currently employed, to reduce the effects of pollution. It is a safe and environmentally friendly method and is increasingly being advocated as a process that does not require high energy for lignin removal from a lignocellulosic biomass (Okano et al., 2005).

Spent substrate contains many extracellular enzymes. Several studies have shown the potential use of the spent substrate in purification of water and soil (Rinker and Castle, 2005). It was also reported that extracellular ligninases such as Lignin Peroxidase (LiP) and Laccase produced by certain *Pleurotus* spp. can degrade phenol (Kirk and Farrell, 1987). Hence, it has been hypothesized that spent

substrate of *Pleurotus* spp. can be utilised to reduce the concentration of phenolic compounds in wastewater. Phenol is a toxic aromatic hydrocarbon which exists as a colorless or white solid in its pure state. It is constantly ingested into our environment and affect aquatic life even at relatively low concentration (5-25 mg / L), causing ecological imbalance. It is found in the water effluent of industries like coke oven units, oil refineries, plastics, leather and paint industries and paper and pulp industries (Haghseresht and Lu, 1998). Phenol has high bioaccumulation rate along the food chain. Our body absorbs this chemical through respiratory organ, skin or alimentary canal. It can restrain the central nervous system and interact with the liver and nephridium (Huang et al., 2008).

Over the past several decades, there is growing concern about wide spread contamination of surface and ground water by phenol due to rapid development of chemical and petrochemical industries. In 1985, WHO imposed a stringent effluent discharge limit of 0.2 mg / L (Mishra and Bhattacharya, 2006). Therefore its removal is essential for environmentally sustainable existence. Conventional treatments to remove phenol from water include solvent extraction, microbial degradation, adsorption on activated carbon and chemical oxidation. These methods although effective and useful, suffer from serious drawbacks such high costs, low phenol removal efficiency and formation of hazardous by-products. Enzymes are known to be highly selective catalysts and therefore have been proposed as a means of removing targeted substances from wastewaters.

Chapter 1

Objectives of the present study

Commercially, *Pleurotus* spp. of mushroom are cultivated in bags. After mushroom cultivation, spent substrate remains as residual material. Proper recycling of spent substrate is beneficial for our economy. Spent substrate can be utilized for various other value added purposes through the proper knowledge of its components. Composition of various components depends on the activity of extracellular enzymes in the spent substrate. The present study was conducted to know the enzyme profile of some major extracellular enzymes - cellulase, hemicellulase (xylanase), pectinase and ligninase (lignin peroxidase and laccase) and to estimate cellulose, hemicellulose, pectin and lignin in the substrate. The use of spent substrate as a source of fibre and ethanol, and in the biodegradation of phenol by *Pleurotus* spp. was also investigated.

Specific objectives of the study

1) To study the production of extracellular enzymes cellulase, xylanase, pectinase, lignin peroxidase and laccase during cultivation of *Pleurotus* spp. on different lignocellulosic substrates: paddy straw, banana pseudo stem, pineapple leaves, sugarcane bagasse and coconut leaflets
2) To test the quality of fibre obtained from spent mushroom substrate
3) To test the feasibility of production of ethanol from spent mushroom substrate
4) To test the ability of spent mushroom substrate to degrade phenol from waste water

Chapter 2

REVIEW OF LITERATURE

2.1 *Pleurotus*

Pleurotus (oyster) mushrooms belong to class Basidiomycetes and family Tricholomataceae (Martinez, 1998; Syed et al., 2009). They are commonly called as 'Dhengri' in India, and have about 40 well-recognized species, out of which 12 species are cultivated in different parts of country (Syed et al., 2009). *Pleurotus* mushroom is fleshy and normally oyster shell-like (about 5 - 20 cm in diameter), and their colour can be white, cream, yellow, pink, brownish, or dark gray (Martinez, 1998).

Pleurotus mushrooms show the typical life cycle of Basidiomycetes while growing on lignocellulosic substrates. Life cycle has two stages: the vegetative stage and the reproductive stage. Generally, some kinds of stimuli are needed for the shift from mycelial (vegetative) growth to the fruit body formation (reproduction) phase. When environmental conditions are appropriate (temperature, light, relative humidity), the mycelium will differentiate into fruit bodies (Martinez, 1998).

2.2 Lignocellulosic biomass

Lignocellulosic biomass mainly consists of lignin, cellulose, hemicellulose, pectin and other components (Bruce, 1987). Crop residues are high in cellulose,

hemicellulose and lignin, but low in pectin (Parveen et al., 2009). Chemical composition of some common lignocellulosic materials is given in table 2.1.

Table 2.1 Chemical composition of some common lignocellulosic biomass

Components	**Corn stover**	**Rice straw**	**Wheat straw**	**Switch grass**	**Sugarcane bagasse**
Cellulose (%)	15	35	30	45	40
Hemicellulose (%)	35	25	50	30	24
Lignin (%)	8	12	20	12	25
Pectin (%)	2	1	1	2	1

*Source: Badal, 2003.

Lignocellulosic biomass are generally considered as a low - grade domestic fuel and burnt negligently or thrown away. About 50 % of this biomass source is burned for cooking and heating and left or combusted directly on the land so that a lot of crop straw and stalk resources are lost and serious environmental pollution occurs. Agricultural residues represent an abundant, inexpensive, and readily available source of renewable lignocellulosic biomass (Ruigang et al., 2003). Therefore the development of a biotechnological process to convert agro-industrial wastage up to a non-harmful as well as useful level will have environmental importance (Zadrazil, 2000; Peixoto-Nogueira et al., 2009). Increased awareness

of global deforestation has raised the demand of alternatives to wood as a raw material for pulp and paper. Hence, rather than destroying this valuable raw material, using it for value-added purposes would be more environmentally and economically beneficial (Ruigang et al., 2003).

2.3 Components of lignocellulosic biomass

2.3.1 Cellulose

Cellulose is the principle component of lignocellulosic biomass and its concentration ranges from 40 to 50 % of dry weight. Cellulose is a homopolysaccharide composed of repeating β – D - glucopyranose units (Bishnu et al., 2011). The degree of polymerization and crystallinity of cellulose varies from species to species and this is shown to have a significant impact on hydrolytic process (acidic and enzymatic) (Zhang and Lynd, 2004). Cellulose is one of the most important natural polymers in terms of its annual production and in its industrial applications (Ruigang et al., 2003). Crystalline structure of cellulose also is highly resistant to breakdown by enzymes (Langer et al.,1980, Henics, 1987)

2.3.2 Hemicellulose

Hemicellulose is less complex, its concentration in lignocellulosic biomass is 25 to 30 % and it is easily hydrolysable to fermentable sugars (Saha et al., 2007). Hemicellulose is a heteropolysaccharide composed of pentoses (D - xylose and D - arabinose), hexoses (D - mannose, D - glucose and D - galactose) and sugar acids (Bishnu et al., 2011). Softwood hemicellulose mainly contains mannose as a major constituent whereas hardwoods mainly contain Xylans (Balan et al., 2009).

2.3.3 Lignin

Lignin is the third major component of lignocellulosic biomass and its concentration ranges for 20 to 35 %. It is a complex polymer of phenyl propane units (coumaryl, coniferyl and sinapyl alcohol) (Bishnu et al., 2011). Lignin acts as cementing agent and an impermeable barrier for enzymatic attack (Howard et al., 2003). Lignin provides plants with the structural support and impermeability they need as well as resistance against microbial attack and oxidative stress (Bishnu et al., 2011). These properties of lignin may be attributed to its amorphous nature, water insolubility and optical inactivity. The latter properties also make it tough to degrade (Fengel and Wegener, 1984). Lignin forms a lignocellulosic comlex with the carbohydrates and proteins (Oziel et al., 2008)

2.3.4 Pectin

In some biomass types, such as sugar beet pulp and citrus peel, pectin can also comprise a significant portion of the lignocellulose structure (Grohmann and Baldwin, 1992; Micard et. al., 1996; Peterson et. al., 2008). In lignocellulosic substrates, pectin interacts with lignin, hemicellulose, and cellulose. The lignin – hemicellulose - pectin complex forms one of the most stringent seals around cellulose (Bishnu et al., 2011). Hence the degradation of pectin is necessary for the disintegration of these cell wall components (Edwards et al., 2011).

2.4 Recalcitrance of lignocellulosic biomass

The digestibility of lignocellulosic materials is very low (Kinfemi et al., 2009). Cell wall lignification of lignocellulosic materials is the major factor that limits the availability of cell wall structural carbohydrates (cellulose and hemicellulose) for utilization (Kerley et al., 1988). Lignin forms a lignocellulolic complex with the carbohydrates and proteins (Oziel et al., 2008). Crystalline structure of

cellulose also is highly resistant to breakdown by enzymes (Langar et al., 1980; Henics, 1987).

Utilization of lignocellulosic biomass as a carbohydrate source for glucose and ethanol production, and as a metabolic energy source in ruminant feeds, has been severely hampered by the low efficiency with which organisms and enzymes are able to convert the polysaccharide portion of the residue into monomeric sugars. This is again attributed to the lignin component of the cell wall and its association with other cell wall polysaccharides (Gould, 1989; Sun et al., 2000).

2.5 Advantages of *Pleurotus* cultivation over other fungi with respect to lignocellulosic substrate degradation

Several studies have addressed the degradation of lignocellulosics by direct cultivation of fungi (Tellez et al., 2008; Elisashvili et al., 2008). The white-rot basidiomycetes are strong decomposers of lignocellulosic wastes, when compared to other fungi, due to their capability to synthesize the relevant and unique oxidative and hydrolytic network of lignocellulolytic extracellular enzymes (Maganhotto, 2005; Eichlerova et al., 2006). *Pleurotus*, a versatile genus of white-rot basidiomycetes fungi, is well known for its complexity of enzymatic system and prominent lignocellulolytic property, and can colonize a wide range of natural lignocellulosic wastes (Elisashvili et al., 2006; Naraian et al., 2010).

The cultivation of oyster mushrooms offers one of the most feasible and economic method for the bioconversion of agro-lignocellulosic wastes (Rajarathnam and Bano, 1989; Cohen et al., 2002). *Pleurotus* spp. have a unique ability to produce xylanase (Elisashvili et al., 2008), carboxy methyl cellulase, β - glucosidase, β – xylosidase, and extracellular lignocellulolytic enzymes including laccase and lignin peroxidase (Stajic et al., 2006).

2.6 Cultivation of *Pleurotus* spp. on lignocellulosic substrates

Cultivation of the oyster mushroom has increased significantly throughout the world during the last few decades (Chang, 1999; Royse, 2002). In the recent times, the cultivation of *Pleurotus* spp. had excelled next to *Agaricus bisporus* (Erkel, 1992; Chang and Miles, 1991). At present, *Pleurotus* spp. is the second most important cultivated mushroom in terms of world production. It is a very simple and low cost production technology and gives consistent growth with high biological efficiency (Rajarathnam and Bano, 1989; Cohen et al., 2002). Cultivation is with a slight variation in the range and combination of the substrates based on their availability and cost in the respective region (Royse, 1985; Schmidt, 1986). New technologies and production techniques are being constantly developed as the number of required controllable environment parameters increases (Holker et al., 2004; Syed et al., 2009). A good quality spawn is essential for the stable production of mushrooms, because, spawn quality enhances mushroom yields (Zhang et al., 2002). Modern methods of spawn preparation use cereal grains (e.g. wheat, millet, rye), which are sterilized in glass jars or polypropylene plastic bags, inoculated with a selected strain, and incubated at appropriate temperatures for complete colonization.

Pleurotus spp. is commonly cultivated on pasteurized wheat or rice straw, but it can be also cultivated on a wide variety of lignocellulosic substrates, enabling it to play an important role in managing organic wastes whose disposal is problematic. Rice straw was appeared to be the best substrate for *Pleurotus* mushroom cultivation when compared to banana leaves, maize stover, corn husks, rice husks and elephant grass (Obodai et al., 2003). If properly sterilised, several substrates can be made suitable for cultivation - wood shavings, banana pseudo stem, waste paper, diverse plant leaves, sawdust mixtures, pulp mill sludges etc. (Gregori, 2007).

Prepared substrates are homogeneously inoculated with the spawn, either by hand or mechanically, at the rate of 0.5 - 3 % of fresh substrate weight. The spawned substrate is placed in a variety of containers. Different containers like bottle, tray, jar, grid-frame, wall-frame, horizontal trays, shelves, vertical plastic sacks, pressed rectangular blocks and others can be used for mushroom cultivation (Stamets, 2000). In practice, the most used are bag, bottle and shelf cultivation (Choi, 2003). Containers are placed inside growing rooms for incubation. Light, ventilation and watering are increased in the growing rooms, after complete colonization of the substrate by the mushroom mycelium (15 - 40 days), to promote fruiting (Martinez, 1998).

Carbon dioxide, temperatures, composition of the growing substrate etc. are some of the factors which affect the growth and fruiting of mushrooms. Cellulose / lignin ratios in substrates were correlated to mycelial growth rates and mushroom yields of *Pleurotus* spp. (Diamantopoulou, 2001). Production of fruit bodies varies according to each species, spawn quality, substrate quality, environmental conditions (temperature, light, relative humidity, concentration of O_2 / CO_2), and impact of pests (flies, mites) and diseases (fungi, bacteria, viruses). Average biological efficiencies (yield of fresh mushrooms as a percentage of the dry weight of substrate at spawning) reported from diverse substrates range from 35 - 159 %, considering a whole production cycle of about 70 - 80 days (Kong, 2004).

2.7 Production of extracellular enzymes during growth of *Pleurotus* spp. on lignocellulosic substrates

During the colonization of the lignocellulosic substrate, *Pleurotus* spp. converts easily digestible carbohydrates into simpler sugars by a process known as primary metabolism (Yamakama et al., 1992; Oziel et al., 2008). Once these sugars are

totally consumed by the fungus, and then begin the secondary metabolism, which consists of the breakdown, decomposition and mineralisation of structural carbohydrates and lignin from substrates by the extracellular enzymes like cellulase, xylanase, lignin peroxidase, manganese peroxidase and laccase (Moyson and Verachtert, 1991; Karunanandaa et al., 1995; Cohen et al., 2002).

The lignolytic system of *Pleurotus* spp. has been extensively studied in recent years by fungal cultivation on different substrates. During cultivation on lignocellulosic substrates enzyme activities change when shift between substrate colonization and fructification stages of mushroom growth (Oziel et al., 2008).

As in *Agaricus* spp, laccase and endoglucanase activities have been associated with the colonization and fructification stages of the *Pleurotus* mushroom, observing that the spawn running on the substrates is a critical period for the cultures. On the other hand, the laccase has shown to act not only in the biodegradation of lignin, but also in the detoxification of the substrate and defence from antagonic moulds. Therefore the strains with high production of this enzyme could have advantages during the colonization and biodegradation of the substrate by the mushroom (Cohen et al., 2002).

2.7.1 Factors affecting extracellular enzyme production during growth of *Pleurotus* spp. on lignocellulosic substrates

Substrate is a key component in *Pleurotus* mushroom cultivation. Substrate must be suitable for the growth and fruiting of fungus. But, the utilization of insoluble lignocellulosic substrates by edible *Pleurotus* mushrooms is dependent upon the production and secretion by these fungi of enzymes (cellulases, hemicellulases, and ligninases) that bring about hydrolysis/oxidation of the macromolecular

cellulose, hemicellulose and lignin components, respectively, thereby liberating low molecular growth nutrients (Buswell et al., 1996).

Production of these enzymes by the fungal mycelium is a crucial part of the colonization process and an important determinant of mushroom yields. Decay rate of plant debris is proportional to its lignin content. Lignin degradation appears to be associated with the vegetative phase of fungal growth, while cellulose degradation is associated with fruit-body formation. Several studies show that substrate composition does influence enzymatic activity (Karunanandaa et al., 1995). Lignin content of the substrate affects cellulase activity and consequently cellulose utilization. *Pleurotus* spp. grown on lignin-rich substrates show more laccase activity much greater than the activity of cellulases and hemicellulases. Climate is another factor for successful mushroom cultivation. Indoor cultivations are done for precise climate control. But it is very costly.

2.7.2 Factors affecting extracellular enzyme production during growth of *Pleurotus* spp. in solid state fermentation systems

Various lignocellulosic substrates and white-rot fungi have been used successfully in submerged and solid-state fermentation for lignocellulolytic enzyme production. The data obtained process that the type and composition of lignocellulosic substrate appear to determine the type and amount of enzyme produced by basidiomycetes (Elisashvili et al., 2006). Compared with submerged fermentation, solid-state fermentation provides certain advantages of fungal enzyme production with the aspect of application in bioprocesses such as biobleaching, biopulping, bioremediation etc (Stajic et al., 2006). Moreover, it has been shown that, during solid-state fermentation of lignocellulosic materials, some fungi produce a different set of enzymes compared with synthetic liquid cultures.

The type of fungal strain and cultural conditions can significantly affect the content of different cell components, which in turn may influence the fungal growth and metabolite production in a fermentation process. In a fermentation process, the measurement of microbial biomass is often necessary since metabolic activity is strongly related to the growth rate and the actual biomass present. Owing to the difficulty in estimating the mycelial growth in SSF involving fungi, indirect method of measurement was employed. Since *P. ostreatus* is a potent producer of cellulase, the cellulolytic activity was determined along with total protein yield. Sugars in general facilitate the growth and proliferation of saprophytic fungi and thus result in an increase in biomass (Erkel, 1992).

The other factors important for SSF are moisture content, carbon, and nitrogen sources in the fermentation media. Cellulosic wastes themselves acted as the carbon source, so no additional carbon source was required in the fermentation medium. L-asparagine was added as a source of organic nitrogen. The whole fermentation medium worked as a complex medium for growth of fermenting fungus. To put biological delignification processes into practice, it is essential to maximize both the rate and the specificity of lignin breakdown by providing conditions that favor lignin degradation and discourage carbohydrate consumption (Stajic et al., 2006).

Moisture in the culture medium greatly affects the mycelial growth rate and metabolic activity. In case of solid state fermentation with filamentous fungi, the available moisture in the substrate provides much needed turgor pressure which enables better penetration of hyphal tip into solid substrate. The yields of cellulase and protein progressively increased with an increase in initial moisture ratio, probably because availability of adequate moisture content initiated rapid uptake of water leading to hyphal cell elongation and elaborate mycelial run within the saturated straw fibres. The good water holding capacity of rice straw also

facilitated better mycelial development (Edwards et al., 2011). This observation may be further explained by a previous study on microbial biomass and its activity in birch litter which reported a strong correlation between moisture content and respiration. An initial increase in the respiration rate was recorded in the continuously wet samples which became relatively constant thereafter (Karunanandaa et al., 1995).

Effect of different nitrogen sources on ligninolytic enzyme production by *Pleurotus* spp. have been studied during solid-state fermentation of grapevine sawdust, showing a promising potential in biotechnological applications. Organic nitrogen sources have been shown to stimulate enzyme production more than inorganic sources. Also, trace elements that can interact with the enzymes or participate in gene regulation processes are necessary for synthesis and function of the ligninolytic enzymes, while their higher amounts present potential inhibitors of enzymatic reactions (Cohen et al., 2002).

The nature of the substrate as well as the cultivation method affects the expression of lignocellulolytic enzymes. The study conducted by Elisashvili et al. revealed that SSF of tree leaves by *Pleurotus* spp. was favourable for laccase and manganese peroxidase (MnP) production. Furthermore, co-culturing can be an effective method for biopulping and improvement of lignin degradation. It was found that co-culturing *P. ostreatus* with *Ceriporiopsis subvermispora* significantly stimulated lignin degradation when compared to monocultures. Laccase production and MnP activity were stimulated in co-cultures of *P. ostreatus* with *C. subvermispora* or *Physisporinus rivulosus* and a change in the isoform composition of those enzymes was also observed (Elisashvili et al., 2006).

Banana leaf waste was a better substrate than banana pseudostem waste in the production of extracellular enzymes by *P. ostreatus* and *P. sajor-caju* in SSF and

is a potential alternative to other agro-waste substrates. The yields were, however, too low and commercially not viable. It was suggested that a larger surface area of banana leaf waste could be a determining factor for better enzyme production. This is in agreement with Zhang et al. who reported that *P. sajor-caju* grew faster and provided better yields on ground straw than on chopped straw. There is, however, a substrate particle size limit, as more finely ground straw inhibited growth. Optimal particle sizes should therefore be determined for all applications (Zhang et al., 2002).

Stajic et al. reported the maximum activity of lignocellulose degrading enzymes at 16-24 days of solid state fermentation, in 2006. They used banana pseudostem as lignocellulosic biomass. Cohen et al. (2002) found the maximum enzyme activity at 10 days in pseudostem and at 20 days on leaf biomass fermentation in both *P. ostreatus* and *P. sajor-caju*. They also reported the highest laccase enzyme activity at 10 days and carboxy-methyl cellulase activity at 5-8 days on solid state fermentation of saw dust and straw in *P. ostreatus* and *P. sajor-caju*. Therefore, fewer days required for maximum enzyme activities, and the increased sugar and protein production on saw dust, straw, and baggase, compared to banana leaves or pseudostems may be due to the effect of NaOH pretreatment, or lignocellulolytic enzyme activities being higher on these substrates (Berlin et al., 2006).

2.8 Major extracellular enzymes of *Pleurotus* spp.

2.8.1 Cellulase

Pleurotus spp. can synthesize and release appreciable amounts of cellulolytic enzymes. Fungal cellulases are multi-enzyme complexes. There is a complete set of hydrolytic enzymes involved in the biodegradation of cellulose. It is composed of three main components; exo-β-l,4-glucanase, endo- β-1,4-glucanase, and β-

glucosidase. The enzymatic degradation of cellulose is a complex process. The above said components have been shown to act synergistically in the hydrolysis of cellulose. During degradation, Exo-1, 4-β-glucanase, splits of either cellobiose or glucose from the non-reducing end of cellulose chains, Endo-1, 4-β-glucanase randomly attacks and splits glycosidic linkages over the length of cellulose chain, and β-glucosidase hydrolyse cellobiose and other water soluble cellodextrins to glucose (Oziel et al., 2008).

2.8.2 Hemicellulase

Xylan, the major component of hemicellulose in plant cell walls, is the second most abundant polysaccharide after cellulose. Xylan polymer consists of a main chain of β-1,4- linked D-xylose residues or some substitutes including arabinose, galactose, mannose etc. The complete degradation of this complex structure depends on different enzymes acting in synergism (Buswell et al., 1996). Endo-β-1,4-xylanases hydrolyze β-1,4-bonds between D-xylose residues in the main chain producing xylo-oligosaccharides, and β-Dxylosidases convert xylo-oligosaccharides to xylose monomers.

Some other specific enzymes such as α-Larabnofuranosidase, α-glucuronidase, as well as several esterases have also a cooperative function into the complete degradation of xylan (Karunanandaa et al., 1995). Xylan is degraded by fungi through the production of a full complement of enzymes. The genera *Trichoderma*, *Aspergillus*, *Fusarium* and *Pichia* are considered great producers of xylanases and basidiomycetes usually secret large amount of enzymes to degrade lignocellulosic materials: white-rot fungus *Pleurotus ostreatus*, *Pleurotus sajor-caju* and *Pleurotus florida* are also producer of xylanase enzymes.

The white-rot fungi basidiomycetes have been studied nowadays as xylanase sources, once its extracellular system is produced to act in a wide range of lignocellulosic materials, and because they are considered edible, these mushrooms is highly nutritional and safe being source of important metabolites of interest to the pharmaceutical, cosmetic and food industries (Cohen et al., 2002).

2.8.3 Pectinase

Pectinotytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances. In relation to their activity, polygalacturonase (PG) cleaving á- (1, 4) glycosidic bonds of non- esterified residues and is classified as endo-PG (E.C.3.2.1.15) and exo-PG (E.C.3.2.1.67). A few reports were recorded about the production of endo and exo-polygalacturonase from a white rot fungi . In most industrial applications, fungal PGS prove to be the most useful because of higher enzyme activity and optimum activity at a lower pH range, suited to most fruit and vegetable processing applications (Rashad et al., 2009).

Recently there has been increased interest in the production of microbial polygalacturonase from food processing wastes. For industrial use, polygalacturonase can be produced from several agricultural pectin containing wastes such as apple pomace, but the main source remains citrus peel, lemon peel, coffee pulp and sugar cane bagasse. Rashad et al. studied the preparation and optimization of *Pleurotus ostreatus* medium using lemon peel as a food processing waste.

2.8.4 Ligninases

Pleurotus fungi are known to employ a variety of extracellular oxidative enzymes

to cleave lignin. The main enzymes of fungi taking part in lignin degradation are phenoloxidases such as lignin peroxidase and laccase. Lignin Peroxidase oxidises non-phenolic lignin substructure which leads to extensive degradation of lignin model compounds (Kirk and Farrell, 1987).

Laccases are extracellular phenol oxidases that catalyse the oxidative degradation of aromatic compounds in lignin, while reducing oxygen to water. They are relatively non-specific enzymes. Laccase isoforms vary between species and within species (Oziel et al., 2008). The broad substrate specificity of laccases permits their use in multiple biotechnological and industrial applications as inexpensive biologically and environmentally friendly tools for the pretreatment of lignocellulose for bioethanol production, pulp bleaching, dye degradation, and xenobiotic transformation and detoxification.

2.9 Spent mushroom substrate

After mushroom crops are harvested, millions of tonnes of spent (used) mushroom substrate (SMS) becomes available for other uses (Rinker, 2002). Mushroom industry needs to dispose off more than 50 million tons of used mushroom substrate each year (Fox and Chorover, 1999). According to Rinker (2002), more than 10 million tons of spent *Agaricus bisporus* substrates are annually produced in the world. Hence, nearly 40 % of the spent material is from *Agaricus* spp..

Mushroom substrate is considered 'spent substrate' when one full crop of mushroom has been taken and further extension becomes unremunerative (Wuest and Fahy, 1991). But, in actual case, the used growing medium is far from spent. Recently, the term spent compost or spent mushroom substrate has been replaced by a more appropriate term, 'post mushroom substrate' because it is not 'spent' and is ready to be further attacked by a new set of microorganisms (Ahlawat et al., 2009).

The bacterial population in spent mushroom substrate ranges between lowest of 14.33 x 10^{-7} in *Volvariella volvacea* (paddy straw mushroom) to highest of 59.33 x 10^{-7} in oyster mushroom spent substrate. Spent substrate from *P. florida* (oyster mushroom) harbours 5 to 23 fold higher fungal population than other spent substrates (Ahlawat et al., 2009). Among different fungi, *Trichoderma* spp. followed by *Aspergillus* spp. and *Mucor* spp. dominate in different spent substrates (Rinker, 2002). Hence, spent substrate needs heat treatment before being removed from the growing chamber (Rinker, 2002).

Various treatments adds to the cost of cultivation, under normal circumstances, mushroom growers throw away the contaminated SMS far from the farm, without considering environmental repercussion (Rinker, 2002). Without proper treatment, contaminated SMS can cause re-contamination to the mushrooms in the mushroom house. Also, improper disposal of SMS can pose a problem to the environment (Lopez et al., 2008). Conversely, recycling the SMS can increase sustainability and also help farm economy (Rinker, 2002).

During recent years, environmental legislations have forced the mushroom growers to think about better ways of SMS disposal (Ahlawat et al., 2009). The mushroom industry has been considering problems with SMS from an environmental standpoint concerning its effective disposal and recycling (Oh et al., 2000). At the same time the demand for organic residues and compost has also increased several folds considering the ill effects of synthetic pesticides and fertilizers (Ahlawat et al., 2009).

2.10 Weathering of spent mushroom substrate and its environmental impact

Spent mushroom substrate contains high amount of salts and other elements which are harmful to plant growth. Hence, in order to make the spent substrate suitable for agriculture and other purposes, it is exposed to natural climatic conditions like

varied temperature and rainfall, which is called as weathering (Rinker, 2002). Also, large dumped piles of spent mushroom substrate become anaerobic and give off offensive odour and affects air quality (Beyer et al., 1996). Hence, it is often spread onto land and allowed to weather for several years.

The impact of storage and leaching has been explored (Rinker, 2002). The volume of spent substrate decreases over the time (Ahlawat et al., 2009). Weathering causes a slow decrease in the organic matter contents (volatile solids), and makes required improvement in the characteristics of spent substrate because of on-going microbial activity, but at the same time it also releases leachate containing salts and nitrates and other nutrients (Beyer et al., 1996; Rinker, 2002). The run-off from such piles contaminates nearby water sources and pollutes them (Beyer et al., 1996).

There are contradictory reports regarding the pH of fresh and weathered spent substrate. According to Wuest and Fahy (1991), spent substrate has an initial pH of around 7.28, which increases during weathering, while, Chong et al. (1988) found a decrease in pH from its initial value 7.9 to 7.0 on weathering. Devonald (1987) reported pH of the fresh spent substrate in the range of 7.01 and 8.04.

2.11 Uses of spent mushroom substrate

Many beneficial uses for spent mushroom substrate are currently being implemented or evaluated internationally (Rinker, 2002). Spent substrate from *Agaricus bisporus* production is already in wide use as follows: in horticulture as a component of potting soil mixes or as a fertilizer; in agriculture lands to enrich soil; as a casing material in the cultivation of subsequent *Agaricus* crops, in vermiculture as a growing medium, in wetlands for remediation of contaminated water, in the bio-remediation of contaminated soils and as an animal feed (Rinker, 2002; Kim et al., 2011).

Spent substrate from other mushroom species has found acceptance as food for animals, as fuel, and as a matrix for bio-remediation (Rinker, 2002). During cultivation of mushrooms on fresh substrate, there is a gradual depletion of substrate nutrients due to the subsequent utilization by mushroom mycelium. Hence, the substrate which is already 'spent' does not support good yield when re-spawned over it (Siddhant, 2009). Good growth and better yield of mushroom can be achieved when spent substrates are supplemented with starch, peptone and wheat bran before re-spawning (Sharma and Jandaik, 1989).

Sharma and Jandaik (1989) also reported that the recycling of *Pleurotus* waste, supplemented with starch, peptone and wheat bran, for the cultivation of *Pleurotus sajor-caju* showed a significant yield of mushrooms. Nakaya et al., (2000) recycled *Pleurotus cornucopiae* waste for the cultivation of two oyster species viz., *P. cornucopiae* and *P. ostreatus*. Production of second crop of mushroom from the spent substrate can prove more efficient utilization of the substrate ingredients and can also ameliorate the problem of solid waste disposal in the mushroom industry (Fahy and Wuest, 1984).

Studies have revealed that the spent substrate is rich in organic matter helps in neutralizing acidic soils, adds nutrients to the soils and facilitates plant growth in barren areas (Rinker, 2002). Spent mushroom substrate improves soil health by improving the texture, water holding capacity and nutrient status (Kaddous and Morgans, 1986; Maher, 1991; Beyer et al., 1996). Incorporation of spent substrate in soil leads to an increase in pH as well as the organic carbon content (Kaddous and Morgans, 1986). Shukry et al. (1999) reported that addition of straw in the soil caused an increase in the number of total bacteria, actinomycetes and fungi of the rhizosphere.

The spent mushroom substrate has been found to be a good nutrient source for agriculture, mainly because of its rich nutrient status, high cation exchange

capacity and slow mineralization rate which retain its quality as an organic matter (Ahlawat et al., 2009). During growth on straw substrate, *Pleurotus* releases humic acids like fractions, which when added to the soil, increase its fertility, thus making the soil suitable for raising vegetables (Zadrazil and Brunnert, 1980; Kaddous and Morgans, 1986).

Spent *Pleurotus* substrate contains high percentage of three primary nutrients – nitrogen (N), phosphorus (P) and potassium (K), for use as a fertilizer (Rinker, 2002). Spent mushroom substrate normally contains 1.9 : 0.4 : 2.4 %, N – P - K before weathering, and 1.9 : 0.6 : 1.0 %, N – P - K after weathering for 8 - 16 months (Gupta, 2004). The phosphorus and potassium requirements of the crop plants can be fully met by incorporating 5 % of spent substrate by volume, while nitrogen requirement by 25 % of spent substrate by volume (Maher, 1991).

Spent substrate is a nutrient-rich organic by-product of the mushroom industry (Adamovic et al., 1998). Several studies have shown the feasibility of using mushroom waste to produce animal feed (Bae et al., 2006). The *Pleurotus* spp. has capability of converting lignocellulosic material into more digestible protein rich cattle feed (Zadrazil and Kurtzman, 1981). Dietary use of spent mushroom substrate in animal feed could also be feasible from an economic point of view (Oh et al., 2000). Kim et al. (2011) suggested spent substrate as an appropriate forage source for ruminants, due to high levels of fiber. Langar et al. (1982) reported that spent *Agaricus bisporus* substrates could be used as sources of minerals for animals, as they are rich in major and trace minerals. However, Bakshi et al. (1985) reported that spent *Agaricus bisporus* substrates had limited use as animal feed due to their high crude ash content (380 - 530 g / kg).

Spent mushroom substrate originated from different edible mushrooms possesses unique physicochemical and biological properties, which make it an ideal bioremediative agent for various environmental protection activities (Ahlawat et

al., 2009). Spent substrate harbours diverse category of microbes, which have the capability to adsorb organic xenobiotic compounds and other inorganic pollutants and to biologically break it down to harmless substances (Ahlawat and Singh, 2007).

Spent substrate can be mixed with different materials for removal of H_2S (Shojaosadati and Siamak, 1999) or volatile organic compounds (Mohseni et al., 1998) from air. There are reports for the use of spent substrate of *Agaricus* spp. in the treatment of metal-contaminated water from coal mines (Anon, 1997; Dvorak et al., 1992; Stark et al., 1994), biological treatment of sewage (International Organic Solutions Corp. 1996) and treatment of waters polluted with radioactive elements and heavy metals (Groudev et al., 1999). Acid mine drainage can be treated using the spent substrate of *Agaricus* spp. and *Lentinula edodes* (Chang, 1999). Spent substrate of *Pleurotus* spp. and *Lentinula edodes* can be used in the reduction of phenol content and toxicity in olive mill waste (Martirani et al., 1996; D'Annibale et al., 1998).

Studies have proved that spent mushroom substrate helps in the bioremediation to reclaim contaminated soils / industrial sites (Rinker, 2002; Ahlawat et al., 2009). The microbes, especially actinomycetes (*Streptomyces* spp. and *Thermomonospora* spp.) present in spent mushroom substrate have strong pollutants catabolising capabilities which result in decreased level of pollutants in contaminated soil after incubation with SMS (Ahlawat and Singh, 2007). The spent mushroom substrate also has the decontamination potential for land sites used for disposal of hazardous wastes (Buswell et al., 1996). Fermor et al. (2000) reported the significant potential of spent mushroom substrate in remediation of lands contaminated with xenobiotic pollutants like pentachlorophenols (PCP), polycyclic aromatic hydrocarbons (PAHS) and aromatic monomers.

Spent substrate of *Agaricus bisporus* can reduce zinc toxicity, and the distribution

of cadmium and lead (Shuman, 1999) among soil fractions. It can degrade polycyclic aromatic hydrocarbons or aromatic monomers (Semple et al., 1998, Fermor et al., 2000, Staments, 2001), inhibit nitrification (Bazin et al., 1991); treat hazardous wastes (Buswell et al., 1996); and stabilise disturbed commercial sites (Rupert, 1995). Spent substrate of *Pleurotus* spp. can degrade polycyclic aromatic hydrocarbons in age-creosote contaminated soil (Eggen, 1999). Spent substrate of *Lentinula edodes*, *Pleurotus* spp. and *Agaricus bisporus* can degrade pentachlorophenol (Chiu et al., 2000; Semple et al., 1998).

The use of hydrolytic and oxidative enzymes from *Pleurotus* spp. is one of the most important aspects for the biodegradation of organo-pollutants, xenobiotics and industrial contaminants (Cohen et al., 2002). The spent substrate from oyster mushroom shows high activity of laccase, manganese peroxidase and arylalcohol oxidase in comparison of spent substrate of button and paddy straw mushrooms (Ahlawat et al., 2009). Button mushroom spent substrate shows higher activity of lignin peroxidase only (Ahlawat et al., 2004). These enzymes can be used for various biotechnological and environmental applications. Lignocellulolytic enzymes have significant potential applications in the chemical, fuel, textile, agriculture and pulp and paper industries (Elisashvili et al., 2006).

2.12 Applications of spent mushroom substrate

2.12.1 Source of plant fibre

Plant fibres are long, stretched, thick-walled cells and are mainly made up of cellulose. Vegetable fibres are fibres produced from bast, seed, leaf and sheath of plants. They are discrete single entities as in cotton; lignocellulosic meshy as in jute; long as in pineapple leaf; or short as in areca nut. Some of them are strong and fine with high length to breadth aspect ratio for good spinnability into yarn for fabric (Das and Mukherjee, 2008). Primarily, cotton is used for apparel; jute for packaging; ramie for currency paper blanks; sisal for rope; sun hemp for tissue

paper etc. Ramie is the strongest amongst all the vegetable fibres and therefore, it has specialised applications (Das and Mukherjee, 2008). Unlike plastics, vegetable fibres are biodegradable, annually renewable, non-carcinogenic and therefore health-friendly.

Several industrial sectors, such as the paper, textile and composite industries are making persistent and ever increasing demands for cellulose fiber (Focher et al., 2001). Also, lignocellulosic raw material is in abundance. Common methods to extract fibres from plant material are fairly straight forward processes, though long, smelly and labour intensive. The basic principle is to encourage the softer parts (non-cellulosic) of the plant to rot, so that only the stronger cellulose fibres remain. Cellulose and hemicellulose components of the plant cell walls are intimately associated with lignin moiety. The individual cellulose fibres are bonded together with a lignin-rich region known as middle lamella. Hemicelluloses (mainly xylan) are also intimately associated with the cellulose fibrils, embedding the cellulose in a matrix (Olesen, 1997).

Fibres are separated from each other by the dissociation of lignin, pectin and xylan from the middle lamella and primary wall of plant material. White-rots of *Pleurotus* sp. can produce extracellular enzymes to degrade all the major components of plant material, at the same time (Eriksson et al., 1990). However, these enzymes are secreted in different proportions. They remove lignin preferentially, with limited degradation to cellulose. Hence, spent substrate of *Pleurotus* sp. is reported to be a good source of fibre.

2.12.2 Source of ethanol

Ethanol is an oxygenated fuel with high octane value like that of petroleum fuels (Bishnu et al., 2011). It is known to run combustion engines at higher compression ratios and thus provides superior performance (Wheals et. al., 1999). The world

population is estimated to increases from 6.7 billion to 8 billion by 2030 (USCB, 2008). On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell and Laherree, 1998; Kumar et al., 2011). Demand for transportation fuels across the globe is increasing. This demand is abnormally affecting developing countries in particular.

Countries that totally depend on the import of fossil fuels cannot ignore the potential of bioethanol derived from lignocellulosic biomass (Bishnu et al., 2011). The blending of ethanol into petroleum-based automobile fuels can significantly decrease petroleum use and release of greenhouse gas emissions to an extent of 85 % (Perlack et al., 2005; Bishnu et al., 2011). Further, ethanol can be a safer alternative to the common additive, methyl tertiary butyl ether (MTBE), in gasoline. MTBE is toxic and is a known contaminant in ground water (McCarthy and Tiemann, 1998; Wang et al., 1999). The US Environmental Protection Agency recently announced the beginning of regulatory action to eliminate MTBE in gasoline (Browner, 2000). Owing to depleting reserves and competing industrial needs of petrochemical feed stocks, there is global emphasis in ethanol production by microbial fermentation process (Kumar et al., 2011).

Ethanol may be produced either from petroleum products, or from biomass through fermentation. Currently, most of the ethanol produced from renewable biomass resources comes from sugarcane and starchy grains. This is partly due to ease of substrate handling and processing. On the other hand, use of sugarcane and food grains to produce bio-ethanol has caused significant stress on food prices and food security. The long-term viability of this process is in question because it will require significantly increased amounts of cultivatable land and significant hike in food prices that will ultimately lead to food insecurity (Mitchell, 2008). Accordingly, the recent focus has been on lignocellulosic materials as a source for bio-ethanol.

Lignocellulosic materials are in abundance and they are renewable (Bishnu et al., 2011). Hence, bioethanol production from lignocellulosic biomass holds tremendous potential in terms of meeting energy needs, and providing environmental benefits (Bishnu et al., 2011). Significant efforts are being made to produce ethanol from lignocellulosic biomass (almost 50 % of all biomass in the biosphere) such as agriculture residues (Bothast and Saha, 1997). The technological advances in recent years are promising to produce ethanol at low cost from lignocellulosic biomass. Ethanol production generally involves hydrolysis of lignocellulosic biomass to fermentable sugars followed by fermentation of such sugars to ethanol (Bishnu et al., 2011).

The improvements in pretreatment processes, improvement in efficacy of enzymatic hydrolysis, development of efficient fermentation processes, efficient technologies to recover ethanol and removal of toxic byproducts will decrease the operating and capital costs (Bishnu et al., 2011). The reduction in cost of ethanol production can be achieved also by reducing the cost of raw material and cofermenting hexose and pentose sugars in the same tanks (Bishnu et al., 2011).

Many countries are moving towards developing or have already developed technologies to exploit the potential of lignocellulosic materials for the production of bioethanol. The leading nations in bioethanol production are Brazil and the USA, and USA is the world's largest producer of bioethanol (Carere et al., 2008). The US fuel ethanol industry produced more than 6.2 billion litres of ethanol in 2000, most of which was produced from corn (MacDonald et al., 2001). Asian countries altogether account for about 14 % of world's bioethanol production (Bishnu et al., 2011).

2.12.2.1 Pretreatment of lignocellulosic biomass

The first step in the overall process of lignocellulosic fermentation is the pretreatment of biomass. This is the most important and rate limiting step in the overall process. Pre-treatment (i) breaks the lignin-hemicellulose-pectin complex around cellulose, (ii) disrupt / loosen-up the crystalline structure of cellulose and (iii) increase the porosity of the biomass (Bishnu et al., 2011). Biomass pre-treatment technologies can change / remove structural and compositional constraints to improve hydrolysis rate and increase yields of fermentable sugars from cellulose and will have a significant impact on the overall process (Sun and Cheng, 2002; Hamelinck et al., 2010; Mosier et al., 2005; Huber et al., 2006; Yang and Wyman, 2008). An ideal pretreatment process should yield high levels of pentoses and the hydrolysates should not have any inhibitory substances and the process is cost effective (Lynd, 1996). The current methods in practice include mechanical, physical, chemical and biological processes or their combination.

2.12.2.1.1 Mechanical pre-treatment

Waste materials can be pre-treated by a combination of chipping, grinding and milling to reduce cellulose crystallinity (Millet et al., 1976). The power requirement of mechanical pre-treatment of agricultural materials depends on the final particle size and the waste biomass characteristics (Cadoche and Lopez, 1989).

2.12.2.1.2 Physical pretreatment

Hydrothermal processes use water, steam or both, and heat, for biomass pre-treatment (Carvalheiro et al., 2008). Hydrothermal treatments mainly include

liquid hot water (autohydrolysis) and steam explosion between 150 °C and 230 °C (Garrote et al., 1999; Carvalheiro et al., 2008). It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate in lignocellulosics. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment.

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (McMillan, 1994; Duff and Murray, 1996; Mackie et al., 1985). Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation, pre-treated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose (McMillan, 1994). The water wash decreases the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose (Mes-Hartree et al., 1988).

In pyrolysis, the materials are treated at temperatures greater than 300 °C and cellulose rapidly decomposes to produce gaseous products and residual char (Kilzer and Broido, 1979; Shafizadeh and Brad-bury, 1979). The decomposition is much slower and less volatile products are formed at lower temperatures (Fan et al., 1987; Shafizadeh et. al. , 1979). AFEX is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is swiftly reduced. The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment and acid-catalyzed steam explosion (Mes-Hartree et al., 1988; Vlasenko et al., 1997; Holtzapple et al., 1992).

2.12.2.1.3 Chemical pretreatment

In these methods, lignocellulosic materials were pre-treated with powerful oxidizing agents such as per-acetic acid or hydrogen peroxide (Gould, 1984). Teixeira and associates observed ethanol yields of 98 % when the lignocellulosic biomass was pretreated with 21 % peracetic acid (Teixeira et al., 1999). More recent processes have employed treatment with hydrogen peroxide (Saha and Cotta, 2007), sulfite (Kuhad et al., 1999; Azzam, 1989) etc.

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts is used to break the internal lignin and hemicellulose bonds (Chum et al., 1988; Thring et al., 1990). Recovery process has been shown to isolate lignin as a solid and carbohydrates as syrup (Lora and Aziz, 1985; Johansson et al., 1987; Aziz and Sarkanen, 1989). The disadvantages are that the process requires expensive high pressure equipment (Pan et al., 2005; Yamashita et al., 2010; Sarkanen, 1980; Lora and Aziz, 1985; Johansson et al., 1987; Ben-Ghedalia and Miron, 1981). Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia and Miron, 1981) and bagasse (Neely, 1984). The degradation was essentially limited to lignin, and hemicellulose was slightly attacked, but cellulose was hardly affected.

2.12.2.1.4 Biological pre-treatment

In biological pretreatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin, hemicellulose and pectin in waste materials (Schurz, 1978; Cardona et al., 2010). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic

materials (Fan et al., 1987). One of the readily available and cheap source of hydrolytic and oxidising enzymes for the pre-treatment as well as hydrolysis of lignocellulosic biomass, is the spent substrate of commercially grown edible white-rot (Avneesh et al., 2011).

Spent mushroom substrate is a suitable raw material for ethanol production. Hatakka (1994) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35 % of the straw was converted to reducing sugars by *Pleurotus ostreatus* in five weeks. The advantages of biological pre-treatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low.

2.12.2.2 Hydrolysis

Post pretreatment, the recalcitrant lignocellulosic biomass becomes susceptible to acid and/or enzymatic hydrolysis as the cellulosic microfibrils are exposed and / or accessible to hydrolyzing agents (Bishnu et al., 2011). Further steps involve isolation and hydrolysis of cellulose to generate fermentable sugars (saccharification) (Bishnu et al., 2011). This process is mainly accomplished by enzymatic methods using cellulases (Bishnu et al., 2011). Mild acid hydrolysis using sulfuric and hydrochloric acids is an alternative procedure (Bishnu et al., 2011). The goal of this process is to generate fermentable monomeric sugars from hemicellulose and cellulose content of lignocellulosic biomass. This can be accomplished by two different processes, namely, acid hydrolysis and enzymatic hydrolysis.

Although several detoxification methods, such as activated charcoal adsorption and lime treatment process, have been devised, an appropriate strategy for efficient hydrolysis of cellulose to fermentable sugars is still lacking (Kaya et al.,

2000; Aden et al., 2002). Following pretreatment, hydrolysis process is applied to lignocellulosic biomass in a two-step (stage) process because the pentose sugars (first stage hydrolysis) degrade / decompose more rapidly than hexose sugars (second stage hydrolysis). Solid fraction of cellulose and lignin are subjected to second stage hydrolysis (Bishnu et al., 2011).

2.12.2.2.1 Hydrolysis of hemicellulose

The pre-treated biomass is subjected to filtration to separate liquids (hemicellulose hydrolysate) and solid (lignin + cellulose) (Bishnu et al., 2011). In the pretreatment process, small amounts of cellulose and most of hemicellulose is hydrolyzed to sugar monomers; mainly D - xylose and D - arabinose. Hemicellulose is usually treated as a secondary stream due to lack of efficient fermentation of hemicellulosic sugars to ethanol (Florbela et al., 2008). The liquid portion is sent to a xylose (pentose) fermentation column for ethanol production (Bishnu et al., 2011). The pentoses (D - xylose and D - arabinose) from hemicellulose hydrolysis are not easily utilized by saccharomyces strains; therefore, genetically modified strains of *Pichia stipitis, Zymomonas mobilis*, are used for their fermentation (Bishnu et al., 2011).

2.12.2.2.2 Hydrolysis of cellulose

Acid hydrolysis

Mineral acids such as sulfuric acid, hydrochloric acid, hydrofluoric acid and nitric acid are widely employed for the hydrolysis of lignocellulosic biomass. Among these, the oldest and best understood process utilizes sulphuric acid. Use of hydrochloric acid, although not commonly used, has technical advantages over the sulfuric acid process, given it is relatively volatile and can be recovered by

vacuum stripping methods (Bishnu et al., 2011). The sulfuric acid-based hydrolysis process is operated under two different conditions; (í) a process that uses high sulfuric acid concentration that operates at a lower temperature and, (íí) a process that uses lowsulfuric acid concentration and operates at a higher temperature. Among the two, the latter is most commonly used.

Use of concentrated sulfuric acid alone has been shown to (i) yield very high levels of sugar (90 %), (ii) can handle diverse feedstock, (iii) is relatively rapid (10 to 12 hours), and (iv) causes less degradation (Graf and Koehler, 2000; USDOE, 2003; Hamelinck et al., 2010). Concentrated acid-based processes are very expensive and cause significant operational problems (Sun and Cheng, 2002; Galbe and Zacchi, 2002; Hamelinck et al., 2010). Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion (Shleser, 1994). In addition, the concentrated acid must be recovered after hydrolysis (Sivers and Zacchi, 1995; Gupta et al., 2009). Acid recovery is a key step for economic viability of concentrated acid pre-treatments, and these neutralization costs have hampered general use of these pre-treatments (Goldstein et al., 1983).

Dilute sulphuric acid processes have been most favored for industrial application, because it achieves reasonably high sugar yields from hemicellulose (Galbe and Zacchi, 2002; Hamelinck et al., 2010). Compared to concentrated acid hydrolysis, this pretreatment generates lower degradation products as well much less corrosion problems in hydrolysis tanks, pipes, etc (Carvalheiro et al., 2008). The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997). At moderate temperature, direct saccharification suffers from low yields because of sugar decomposition. But high temperature in dilute acid treatment is favorable for cellulose hydrolysis (McMillan, 1994).

Recently developed dilute acid hydrolysis processes use less severe conditions and achieve high xylan to xylose conversion yields. Achieving high xylan to xylose conversion yields is necessary to achieve favorable overall process economics because xylan accounts for up to a third of the total carbohydrate in many lignocellulosic materials (Hinman et al., 1992). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or AFEX. Also, a neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. This process produces relatively large number of undesirable byproducts as compared to the concentrated acid process (Bishnu et al., 2011).

Alkaline hydrolysis

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (Fan et al., 1987; McMillan, 1994). Alkali pretreatment increases cellulose digestibility (Carvalheiro et al., 2008). Most commonly used alkali in the alkali pretreatment processes are NaOH and $Ca(OH)_2$. This process results in (i) the removal of all lignin and part of hemicellulose, and (ii) increased reactivity of cellulose in further hydrolysis steps (Hamelinck et al., 2010), especially, enzymatic hydrolysis. Between NaOH and $Ca(OH)_2$, pretreatment with $Ca(OH)_2$ is preferable because it is less expensive, more safer as compared to NaOH and it can be easily recovered from the hydrolysate by reaction with CO_2 (Mosier et al., 2005).

The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the

lignocellulosic materials increases with the removal of the cross-links (Tarkow and Feist, 1969). Conversely to acid or hydrothermal processes, alkaline-based methods are more effective for lignin solubilisation exhibiting only minor cellulose and hemicellulose solubilisation, excepting ammonia recycling percolation treatment, which yield biomass solids mostly containing cellulose (Sun and Cheng, 2002; Hamelinck et al., 2010; Wyman et al., 2005).

Pretreatment with NaOH increases the digestibility cellulose from 14 to 55% while decreasing the lignin content from 25 to 20 % (Kumar et al., 2011). Alkali pretreatment process shows decreased sugar degradation and is more effective on agriculture residues as compared to wood materials (Kumar et al., 2011). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10 - 18 % (Bjerre et al., 1996). Dilute NaOH treatment of lignocellulosic materials causes swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987; Taherzadeh and Karimi, 2008). However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26 % (Millet et al., 1976).

Enzymatic hydrolysis

During enzymatic hydrolysis, cellulose is degraded by enzymes known as cellulases that are able to hydrolyse the cellulose polymer to its monomer (Fatma et al., 2010). The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions such as temperature, pH, as well as other parameters (Cantwell et al., 1988; Durand et al., 1988; Orpin, 1988).

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber randomly and cleave the cellulose chains to form glucose, cellobiose and cellotriose, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which attack the non-reducing end of cellulose and degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) or cellobiase which hydrolyzes cellobiose to produce D-glucose (Coughlan and Ljungdahl, 1988).

In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, b-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). The factors affecting activity of cellulases include enzyme source and the concentration of enzyme. To improve the rate of the enzymatic hydrolysis and the yield of sugar, research has focused on optimizing the hydrolysis process and enhancing cellulase activity (Cantwell et al., 1988; Durand et al., 1988; Orpin, 1988).

Bacteria and fungi produce cellulases that hydrolyze of lignocellulosic materials. These microorganisms can be aerobic or anaerobic and mesophilic or thermophilic. Bacteria belonging to genera of *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Erwinia, Acetovibrio,* and *Streptomyces* are known to produce Cellulase (Bisaria and Chandrakant, 1998). Anaerobic bacterial species such as *Clostridium phytofermentans, Clostridium thermocellum* and *Clostridium papyrosolvens* produces cellulases with high specific activity (Duff and Murray, 1996; Joshi, 1997; Bisaria and Chandrakant, 1998).

Fungi known to produce cellulases include *Sclerotium rolfsii, Phanerochaete chrysosporium* and various species of *Trichoderma, Aspergillus* and *Penicillium* (Sternberg, 1976; Fan et al., 1987; Duff and Murray, 1996). Among the fungi, *Trichoderma* species have been extensively studied for cellulase production (Sternberg, 1976). Most commercial glucanases (cellulases) are produced by *Trichoderma ressei* and β-D-glucosidase is produced from *Aspergillus niger* (Kaur et al., 2007).

Cellulase enzyme loadings in hydrolysis vary from 7 to 33 FPU / g substrate, depending on the type and concentration of substrates. Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulase. Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process.

Cellulase dosage of 10 FPU / g cellulose is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48 - 72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996). The yield of fermentable sugar levels obtained from pre-treated biomass increases as the enzyme load increases (Yang and Wyman, 2008). According to Kim and his associate (2005), effective concentration of enzyme for cellulose hydrolysis has been determined to be 10 to 60 FPU (filter paper units) per gram of dry cellulose or glucan- glucanase- β - D - glucosidase ratio of 1 – 75 - 2 IU.

A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman

et al., 1984; Berlin et al., 2007). Non-ionic surfactants are believed to be suitable for enhancing the cellulose hydrolysis. The rate of enzymatic hydrolysis was improved by 33 % using Tween 80 as a surfactant in the hydrolysis of newspaper (Castanon and Wilke, 1981). Cellulase activity is inhibited by cellobiose and to a lesser extent by glucose. Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of β - glucosidases during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and solid state fermentation (SSF).

The SSF process has been extensively studied to reduce the inhibition of end products of hydrolysis (Takagi et al., 1977; Blotkamp et al., 1978; Szczodrak and Targonski, 1989; Saxena and Rai, 1992; Philippidis et al., 1993; Zheng et al., 1998). Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Cellulases can be recovered from the liquid supernatant or the solid residues. Enzyme recycling can effectively increase the rate and yield of the hydrolysis and lower the enzyme cost (Mes-Hartree et al., 1988).

2.12.2.3 Fermentation

During fermentation, both pentose and hexose sugars are fermented to ethanol under anaerobic/aerobic conditions. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Brooks, 2008).

Economical production of ethanol from lignocellulose hydrolysates requires high concentrations of fermentable sugars in it. High osmolality of the media prevents the action of ethanologens, as most of these microorganisms used for ethanol

production are incapable of performing at high sugar and salt concentrations (Kurian et al., 2010). Common problems associated with fermentation of sugar are the high temperatures (35 - 45 °C) and high ethanol concentration (over 20 %). Tolerance to high temperatures and ethanol concentrations are important factors of microorganisms for increasing efficiency at industrial scale (Jimenez and Benitez, 1986; Mehdikhani et al., 2011). Industrial production of ethanol from lignocellulosic hydrolysates requires the use of microorganism capable of utilizing the different types of sugars present in it (Balat et al., 2008; Bettiga et al., 2009)

Saccharomyces cerevisiae is the most favored organism for ethanol production from hexoses. It is tolerant to temperature and high sugar and ethanol concentrations (Osho, 2005). High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol (Swiecilo et al., 2000). Invertase is one of the important extracellular enzymes in *Saccharomyces* that is responsible for converting sucrose to its subunits, glucose and fructose (Sengupta et al., 2000). Despite its extensive use, it has a number of disadvantages, such as high aeration cost, high biomass production and low temperature and ethanol tolerances (Saigal, 1993). *Zymomonas mobilis* is a potential bacterium for ethanol production (Parmjit et al., 2006). But, despite various efforts undertaken worldwide, *Zymomonas* is not yet ready to compete successfully with the yeast at industrial scale (Parmjit et al., 2006). Yeasts have higher ethanol tolerance than bacteria (Kurian et al., 2010). *P. stipitis* and *Candida shehatae* are capable of fermenting both hexose (glucose) and pentose (xylose) sugars to ethanol (Parekh and Wyman, 1986). Genetically engineered strains of *Escherichia coli, S. cerevisiae,* and *Z. mobilis* have been developed to ferment xylose (Kim and Holtzapple., 2005).

An optimal process for fermentation uses a broth containing *Saccharomyces cerevisiae* supplemented with 22 % (w/v) sugar, 1 % (w/v) of each of ammonium sulphate and potassium dihydrogen phosphate, and fermented at pH 5.0 and 30 °C

(Junior et al., 2009). Under such conditions a typical strain of *S. cerevisiae* is capable of producing 46.1 g ethanol / L broth (Maziar, 2010). Molasses medium is the most commercially used medium for ethanol production (Parmjit et al., 2006).

2.12.3 Bioremediation of phenol

Phenol is an aromatic hydrocarbon which exists as a colorless or white solid in its pure state. It is a toxic organic compound which is constantly introduced into our environment and found to affect aquatic life even at relatively low concentration (5 - 25 mg / L), causing ecological imbalance. It is found in the water effluent of industries like coke oven units, oil refineries, plastics, leather and paint industries and paper and pulp industries (Haghseresht and Lu, 1998). It has high bioaccumulation rate along the food chain. Phenol can be absorbed by our body through the respiratory organ, skin and alimentary canal. It can restrain the central nervous system and interact with the liver and nephridium (Huang et al., 2008).

Over the past several decades, there is growing concern about wide spread contamination of surface and ground water by phenol, due to rapid development of chemical and petrochemical industries. In 1985, WHO imposed a stringent effluent discharge limit of 0.2 mg / L (Mishra and Bhattacharya, 2006). Thereafter, new and tighter regulations coupled with increased enforcement concerning wastewater discharges have been forced in many countries (Khalil et al., 2009). Thus its removal is essential for environmentally sustainable existence.

Conventional treatments to remove phenol from water include solvent extraction, microbial degradation, adsorption on activated carbon and chemical oxidation. These methods although effective and useful, suffer from serious drawbacks such high costs, low phenol removal efficiency and formation of hazardous by-products. Enzymes are known to be highly selective catalysts. They have shown enormous biotechnological potential as they can be used at a wide level for of degradation (Ren and Buschle-Diller, 2007) for removing targeted substances

from wastewaters and detoxification of agro-industrial residuals with high phenolic contents (Mata, 2005).

It has been hypothesized that the addition of LiP or Laccase enzyme can reduce the concentration of phenolic compounds in wastewater. The white rot fungi, including *Pleurotus* species, have been reported on several occasions as good producers of extracellular ligninolytic enzymes and as active strains for textile dye decolorisation and other pollutants (Fu and Viraraghavan, 2001; Yonni et al., 2004; Nilsson et al., 2006; Zhao et al., 2008). It was also reported that extracellular ligninases such as Lignin Peroxidase (LiP) and Laccase produced by certain *Pleurotus* sp. can degrade phenol (Kirk and Farrell,1987). One of the readily available and cheap source of enzymes is the spent substrate of commercially grown edible white-rot fungi (Avneesh et al., 2011). Several studies have shown the potential use of the spent substrate of mushrooms in purification of water and soil.

Chapter 3

MATERIALS AND METHODS

3.1 Microorganism

Pleurotus sp. namely *P. eous* and *P. ostreatus* were selected for the work. *P. eous* spawn was procured from Kerala Agricultural University, Vellanikkara, Thrissur. *P. ostreatus* culture (NCIM - 1200) was collected from NCIM, Pune. Both fungal cultures were maintained on potato dextrose agar (PDA) medium, for further studies. The fungal strains were grown at 28 ºC for 15 days, stored at 4 ºC and sub cultured periodically.

3.2 Medium

Potato dextrose agar medium obtained from Sisco Research Laboratories (SRL, Mumbai, India) and prepared according to the instructions of manufacturer was used throughout the study for both the fungal strains.

3.3 Spawn production

Spawn was prepared using *Sorghum vulgare* grains. Carrier medium consisted of sorghum grains (1 kg) mixed with Calcium carbonate (30 g). Good quality sorghum grains, free from pest and moulds, were selected. Grains were submerged in clean water and boiled for 20 – 30 min.. When the grains became soft, they were spread evenly on a cotton cloth to drain out the water and cool the grains. Calcium carbonate was mixed for adjusting the pH and to keep the grains loose. 300 g of

grain was filled in polypropylene bags and the mouth of the cover was plugged with non absorbent cotton. The bags were sterilized by autoclaving at 121 °C for 15 min. and cooled to room temperature. A well grown mushroom with membrane covering the gills was selected. Cap (pileus) of the mushroom was surface sterilised with 70 % ethanol. A piece of mushroom (2 sq. cm size) was cut and soaked in 0.5 % $HgCl_2$ for 30 sec. and after, washed in sterile water 2-3 times to remove traces $HgCl_2$. The edges were cut off and a piece of 1 sq. cm size was placed at the centre of a petridish with PDA medium under aseptic condition for 15-20 days at room temperature. During spawn preparation an agar piece (1 sq.cm size) with fungal mycelium was placed in the sterile carrier medium and incubated for 15-20 days at room temperature.

3.4 Substrate for cultivation

Mushrooms were cultivated on the following lignocellulosic substrates procured locally.

- Banana pseudo stem
- Pineapple leaves
- Coconut leaflets
- Sugar-cane bagasse
- Paddy straw

3.5 Mushroom cultivation

Mushrooms were cultivated in bags at room temperature and 70 % humidity**.** Dried substrate was cut into 5-8 cm bits and soaked in water for 12 h. The excess water was drained and the substrate autoclaved for 30 min. at 120 ºC and 15 psi pressure. The remaining water was drained and the substrate cooled to room temperature. Alternate layers of spawn were packed in polythene bags (60 cm x 30 cm size) and

the mouth tied. Holes were punched on the bag at a distance of 2". The bags were incubated in the dark for 15 days. The room was kept cool and humid by hanging gunny screens with periodical sprinkling of water. Diffused light and ventilation was required from 16th day onwards. Mushrooms were harvested thrice within 35 days. After harvesting the mushrooms, the spent substrate was further incubated for other experiments, till 70th day. All experiments were done in triplicate.

3.6 Biological efficiency (BE)

Biological efficiency is a term frequently used in the mushroom industry to describe the potential of the macro fungus to yield fruiting body (mushroom) from a known weight of substrate. It was computed using the formula:

$$BE = \frac{\text{Fresh weight of mushroom}}{\text{Dry weight of substrate}} \times 100\%$$

3.7 pH

pH of the mushroom bed was checked using pH strips.

3.8 Recovery of enzyme

After harvest, crude enzyme extract was extracted by incubating the substrate with distilled water (1:1 w/v) and 0.05 % Tween 80 with intermittent shaking for 30 min**.** As the pH of the mushroom beds were found to vary with days of incubation, the enzyme extraction was done at the same pH as that of the bed. The mixture was centrifuged at 6000 rpm and the supernatant was treated as crude enzyme for all assays**.**

3.9 Analytical methods

3.9.1 Enzyme assays

Quantitative assays were done for the five extracellular enzymes – cellulase, xylanase, pectinase, lignin peroxidase and laccase present in the spent mushroom substrate from 7^{th}-70^{th} day of cultivation at 7 day intervals.

3.9.1.1 Cellulase assay

The cellulase activity was estimated according to the method of Ghose (1987) based on the formation of chromogen between glucose (released by the action of cellulase on filter paper) and 3,5 - dinitro salicylic acid.

Procedure

i. A Whatman # 1 filter paper strip of 0.025 mg was rolled and placed in each assay tube. The filter paper strips were saturated with 250 µl distilled water and were equilibrated for 5 min. at room temperature.
ii. 250 µl of an appropriately diluted crude enzyme was added to the tube and incubated at room temperature for 60 min. Appropriate blanks and controls were also run along with the test.
iii. At the end of the incubation period, the reaction was stopped by addition of 1 ml of DNS reagent.
iv. The tubes were incubated for 5 min. in a boiling water bath for colour development and were cooled rapidly by transferring into a cold water bath.
v. The orange red colour developed was measured against a reagent blank at 540 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).

vi. The concentration of glucose released by the enzyme was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of glucose.

vii. One unit of filter paper activity is defined as the amount of enzyme releasing 1 µg of glucose from filter paper per ml per min.. Enzyme activity was expressed as U / ml.

3.9.1.2 Xylanase assay

The Xylanase activity was estimated according to the method of Miller (1989) based on the formation of chromogen between xylose (released by the action of xylanase on xylan) and 3,5 - dinitro salicylic acid.

Procedure

i. 250 µl of 1 % xylan in distilled water was taken in each assay tube.

ii. 250 µl of an appropriately diluted crude enzyme was added to the tube and incubated at room temperature for 60 min.. Appropriate blanks and controls were also run along with the test.

iii. At the end of the incubation period, the reaction was stopped by addition of 1 ml of DNS reagent.

iv. The tubes were incubated for 5 min. in a boiling water bath for colour development and were cooled rapidly by transferring into a cold water bath.

v. The orange red colour developed was measured against a reagent blank at 540 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).

vi. The concentration of xylose released by different dilutions of the enzyme was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of xylose.

vii. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µg xylose from xylan per ml per min.. Enzyme activity was expressed as U / ml.

3.9.1.3 Pectinase assay

The Pectinase activity was estimated according to the method of Miller (1989) based on the formation of chromogen between galacturonic acid (released by the action of pectinase on pectin) and 3,5 - dinitro salicylic acid.

Procedure

i. 250 µl of 1 % pectin in distilled water was taken in each assay tube.
ii. 250 µl of an appropriately diluted crude enzyme was added to the tube and incubated at room temperature for 60 min.. Appropriate blanks and controls were also run along with the test.
iii. At the end of the incubation period, the reaction was stopped by addition of 1 ml of DNS reagent.
iv. The tubes were incubated for 5 min. in a boiling water bath for colour development and were cooled rapidly by transferring into a cold water bath.
v. The orange red colour developed was measured against a reagent blank at 540 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).
vi. The concentration of xylose released by different dilutions of the enzyme was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of D-galacturonic acid.
vii. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µg D-galacturonic acid from xylan per ml per min.. Enzyme activity was expressed as U / ml.

3.9.1.4 Ligninase assay

3.9.1.4.1 Lignin Peroxidase (LiP)

The LiP activity was estimated according to the method of Tien and Kirk (1988) based on the rate of oxidation of Veratryl alcohol to Veratraldehyde.

Procedure

i. The enzyme reaction mixture was prepared by the addition of 500 µl distilled water, 250 µl Veratryl alcohol and 250 µl enzyme extract. Appropriate blanks and controls were also run along with the test.
ii. The reaction was started by the addition of 250 µl H_2O_2 and was monitored at 310 nm against a blank in a UV - VIS spectrophotometer (Shimadzu, Japan).
iii. One unit of enzyme activity is defined as the amount of enzyme oxidising 1µmol of veratryl alcohol per min..

3.9.1.4.2 Laccase

The Laccase activity was estimated according to the method of Buswell and Odier (1987) based on the rate of oxidation of ABTS (2,2-azinobis-3-ethylbenz-thiazoline-6-sulphonate) to its corresponding cation radical, $ABTS^+$.

Procedure

i. The enzyme reaction mixture was prepared by the addition of 300 µl distilled water and 600 µl enzyme extract. Appropriate blanks and controls were also run along with the test.

ii. The reaction was started by the addition of 100 µl ABTS and the dark green colour was monitored at 420 nm against a blank in a UV - VIS spectrophotometer (Shimadzu, Japan).
iii. One unit of enzyme activity is defined as the amount of enzyme oxidising 1µmol of ABTS per min..

3.9.2 Estimations

Total reducing sugar and total protein of the crude enzyme extract were estimated simultaneously with the enzyme assays. Estimation of cellulose, lignin, hemicelluloses and pectin was done for 28, 49 and 70 day old spent substrates. Untreated substrate was taken as control.

3.9.2.1 Reducing sugar estimation - Total reducing sugar was estimated according to the method of Miller (1989).

Procedure

250 µl of appropriately diluted sample and 250 µl distilled water were taken in each tube. 1ml of DNS reagent was added and vortexed. The tubes were incubated for 5 min. in a boiling water bath for colour development and were cooled rapidly by transferring into a cold water bath. The orange red colour developed was measured against a reagent blank at 540 nm in a UV - VIS spectrophotometer (Shimadzu, Japan). The concentration of reducing sugar was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of glucose and was expressed as mg / ml.

3.9.2.2 Protein estimation - Total protein was estimated according to the method of Lowry et al. (1951).

Reagents

1) Solution A : 0.2 N NaOH
   Solution B : 4 % Na_2CO_3
   Solution C : 2 % Sodium potassium tartarate
   Solution D : 1 % $CuSO_4$
   Working reagent (100 ml): 49 ml Solution A + 49 ml Solution B + 1 ml Solution C + 1 ml Solution D

2) Folin Ciocalteau's phenol reagent:
   Prepared fresh at 1:1 dilution with distilled water

Procedure

To 200 µl of appropriately diluted sample, 1 ml of working reagent was added. Mixed thoroughly and incubated. After 10 min., 100 µl of Folin Ciocalteau's phenol reagent was added to the mixture and incubated for 30 min.. The dark blue colour developed was measured against a blank at 640 nm in a UV - VIS spectrophotometer (Shimadzu, Japan). The concentration of protein was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of Bovine Serum Albumin (BSA) and was expressed as mg / ml.

3.9.2.3 Specific activity

Specific activity was calculated by dividing the enzyme units with protein content and was expressed as U / mg protein.

$$\text{Specific activity} = \frac{\text{Enzyme activity (U / ml)}}{\text{Protein (mg / ml)}}$$

3.9.2.4 Cellulose estimation

Cellulose was estimated according to the method of Updegroff (1969) based on the formation of chromogen between hydroxyl methyl fufural (released by the action of acetic / nitric reagent and H_2SO_4, on cellulose) and anthrone reagent.

Reagents

1) Acetic / nitric reagent: 150 ml of 80 % acetic acid and 15 ml of concentrated HNO_3
2) Anthrone reagent: 200 mg dissolved in 100 ml of concentrated H_2SO_4. Prepared fresh and chilled for 2 h before use.

Procedure

i. To 0.5 g of the sample in a test tube added 3 ml acetic / nitric reagent and vortexed well.
ii. Tubes were placed in a water bath at 100 °C for 30 min..
iii. Contents were cooled and then centrifuged for 20 min..
iv. Supernatant was discarded and the residue was washed with distilled water.
v. 10 ml of 67 % H_2SO_4 was added to the residue and allowed to stand for 1 h.
vi. 1 ml of this solution was diluted to 100 ml.
vii. To 1 ml of the diluted solution added 10 ml of anthrone reagent and mixed well.
viii. Tubes were kept in a boiling water bath for 10 min. for colour development and were cooled rapidly by transferring into a cold water bath.

ix. The dark green colour developed was measured against a reagent blank at 630 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).

x. The concentration of cellulose was determined by comparing the absorbance against a standard curve constructed similarly with known concentrations of cellulose and was expressed as percentage.

3.9.2.5 Lignin estimation

Lignin was estimated according to the method of KCL (1982), based on the formation of acid insoluble lignin (obtained by the hydrolysis of sample with concentrated H_2SO_4).

Procedure

i. Sample was kept in 105 °C for 2 h.

ii. Crucibles were ignited at 575 ± 25 °C (in a muffle furnace) to achieve constant weight of ± 0.3 mg and kept in a desiccator until use.

iii. 0.5 g (W_1) of sample is weighed and kept in a boiling test tube.

iv. 7.5 ml of 72 % H_2SO_4 (chilled to 4 °C) was added to the sample and mixed well with a glass rod. Sample was stirred well in every 15 min. and was allowed to stand for 2 h for complete hydrolysis.

v. Hydrolysed solution was transferred to an Erlenmeyer flask and diluted to a 3 % acid concentration with 280 ml of deionised water. All the residual solids along with hydrolysis liquid must be transferred.

vi. Flask was placed on a heating manifold and attached to the reflux condenser. Hydrolysate was heated to boiling and was refluxed for 4 h.

vii. At the end of 4 h, the condenser was rinsed with a small amount of deionised water before disassembling the reflux apparatus.

viii. Hydrolysed solution was vacuum filtered through one of the previously ignited filtering crucibles.

ix. All the particles clinging to the flask was washed into the crucible and the filtered residue was washed until get free of acid.

x. Crucibles and the contents were dried at 105 ± 3 °C for 2 h, until constant weight was achieved and then cooled and recorded the weight (W_2 - crucible + acid insoluble lignin + acid insoluble ash).

xi. Crucibles with the content were ignited at 575 ± 25 °C (in a muffle furnace) for 3 h. Cooled in a desiccator and recorded the weight (W_3 - crucible + acid insoluble ash).

xii. The concentration of lignin was calculated as weight of acid insoluble lignin expressed in percentage.

$$\text{Percentage acid insoluble lignin} = \frac{W_2 - W_3}{W_1} \times 100$$

3.9.2.6 Hemicellulose estimation

Hemicellulose was estimated according to the method of Goering and Vansoest (1975).

Reagent

1) Neutral detergent solution:

   18.61 g Disodium-EDTA (Ethylene diamine tetra acetic acid) and 6.81 g Sodium borate decahydrate was weighed and transferred to a beaker. This was dissolved in about 200 ml of distilled water by heating and added 100 ml of solution containing 30 g of Sodium Lauryl Sulphate and 10 ml of 2-ethoxy ethanol. Again added about 100 ml of solution containing 4.5 g of

disodium hydrogen phosphate and made up the volume to 1 L. pH was adjusted to 7.

2) Acid detergent solution: 20 g of CTAB (cetyl trimethyl ammonium bromide) in 1 L of 1 N sulphuric acid

Procedure

Neutral detergent fibre (NDF)

i. To 1 g of the powdered sample in a refluxing flask added 10 ml of cold neutral detergent solution.
ii. Again added 2 ml of decahydronaphthalene and 0.5 g sodium sulphite. Boiled and refluxed for 1 h.
iii. Contents were filtered through sintered glass crucible by suction, twice washed with hot water and finally with acetone.
iv. Crucible with contents was dried at 100 °C for 8 h.
v. Cooled in a desiccator and weighed.

Acid detergent fibre (ADF)

i. 1 gm of the powdered sample was placed in a round bottom flask and added 100 ml of acid detergent solution. Refluxed for 1 h.
ii. Container was removed and the contents were filtered through sintered glass crucible by suction, twice washed with hot water and finally with acetone.
iii. Crucible with contents was dried at 100 °C for 8 h. Cooled in a desiccator and weighed.

Percentage of hemicellulose = Neutral detergent fibre – Acid detergent fibre

3.9.2.7 Pectin estimation

The pectin was estimated according to the method of Ranganna (1979), based on the formation of Calcium pectate by the addition of Calcium chloride to an acidic solution of the sample.

Procedure

i. 5 g of blended sample was weighed into a 100 ml beaker and added 30 ml 0.01 N HCl.
ii. Boiled for 30 min. and filtered under suction. Residue was washed with hot distilled water and the filtrate was collected.
iii. To the residue added 10 ml 0.05 N HCl. Boiled for 20 min., filtered, washed and collected the filtrate.
iv. To the residue again added 10 ml 0.3 N HCl. Filtered, washed and collected the filtrate.
v. Filtrates were pooled, cooled and made to a volume of 50 ml.
vi. 10 ml of the diluted filtrate was taken in a 100 ml beaker and added 25 ml water.
vii. Acidic solution obtained was neutralized with 1 N NaOH, using Phenolphthalein indicator. An excess of 1 ml 1 N NaOH was added with constant stirring and allowed to stand overnight.
viii. After incubation added 5 ml 1 N Acetic acid. After 5 min. added 2.5 ml 1 N Calcium chloride solution with stirring and allowed to stand for 1 h.
ix. Solution was boiled for 1-2 min. and was filtered through a pre-weighed Whatmann no.1 filter paper. Precipitate was washed with hot water 8-10 times.

x. Filter paper along with the precipitate (Calcium pectate) was dried overnight at 100 °C in a weighing dish. Cooled in a desiccator and weighed.

xi. Pectin content is expressed as percentage calcium pectate.

$$\text{Percentage calcium pectate} = \frac{\text{Weight of Calcium pectate} \times 50}{\underset{(10\ ml)}{\text{Volume of filtrate taken}} \times \underset{(5\ gm)}{\text{Weight of sample for estimation}}} \times 100$$

3.10 Substrate analysis

3.10.1 Strength analysis of fibres obtained from untreated substrate and spent mushroom substrate

i. Spent mushroom substrate obtained after the harvest was dried overnight at 50 ± 2 °C.

ii. Spent substrate fibre was cut into an equal size (10 cm x 0.5 cm). Untreated substrate fibre was used as control.

iii. Fibre strength was analysed by Shimadzu Autograph AG-I series Universal Testing Machine (UTM) at cross head speed 5 mm / min. and gauge length 40 mm.

iv. Tensile strength and elastic modulus were recorded.

3.10.2 FT-IR (Fourier Transform Infrared) spectroscopic analysis of untreated substrate and spent mushroom substrate

Fourier-Transform infra red spectroscopy is a technique that provides information about the chemical bonding or molecular structure of materials whether organic or inorganic. The bonds and group of bonds vibrate at characteristic frequencies. A molecule that is exposed to infra red rays absorbs infrared energy at frequencies which are characteristics to that molecule. During FT-IR analysis a spot on specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of infrared rays at different frequencies is translated to an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analysed and matched with known signatures of identified materials.

The parameters used in FT- IR analysis were: spectral range 4000-400 cm^{-1} and 4 cm^{-1} resolution. The untreated substrate sample and spent substrate sample were subjected to FT-IR spectroscopic analysis (Thermo Nicolet, Avatar 370), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulphate) detector (7800-350 cm^{-1}), at Sophisticated Test and Instrumentation centre, Cochin University of Science and Technology, Cochin, Kerala.

3.10.3 (X-ray diffraction) analysis of untreated substrate and spent mushroom substrate

X-ray Diffraction is a non-destructive analytical technique which provides detailed information about the internal lattice of crystalline substances, including unit cell dimensions, bond-lengths, bond-angles, and details of site-ordering. Data generated from the X-ray analysis is interpreted and refined to obtain the crystal structure. Untreated and biologically treated samples were analysed for crystallinity using X-ray diffractometre (Bruker Kappa Apex II) at a wavelength of

1.5406 Aº. Samples were scanned over the angular range 3-80°, 2θ. The crystalline index of cellulose, CrI, was determined based on the empirical method,

$$CrI\,(\%) = \frac{(I_{002} - I_{am})}{I_{002}} \times 100$$

I_{002} is the counter reading at peak intensity at a 2θ angle close to 22.5° corresponding to crystalline cellulose

I_{am} is the counter reading at peak intensity at a 2θ angle close to 18.7° representing the amorphous fraction of cellulosic fibres.

3.10.4 SEM-EDS (Scanning Electron Microscope –Energy Dispersive Spectrometer) analysis of untreated substrate and spent mushroom substrate

Scanning Electron Micrographs (SEM) is a method for high resolution surface imaging using an electron beam. The advantages of SEM over light microscopy are greater magnification and much larger depth of field. Different elements and surface topographies emit different quantity of electrons, due to which the contrast in a SEM micrograph (picture) is representative of the surface topography and distribution of elemental composition on the surface. Surface imaging of untreated and biologically treated samples were recorded using SEM-EDS combination in SEI mode, under low vacuum resolution (4 nm).

3.11 Production of ethanol from spent mushroom substrate

3.11.1 Source of microorganism

To isolate the microorganism for fermentation studies, toddy (a natural fermented beverage) samples were collected from two different regions of Kerala (Ernakulam and Thrissur), from the fruit and inflorescence of the palm tree, *Cocos nucifera*,

under the assumption that it contains effective strain of yeast. Commercial yeast also was selected for the study.

- Toddy from Nadathara, Thrissur
- Toddy from Nedumbasseri, Ernakulam
- Commercial yeast

3.11.2 Isolation of microorganism

Microorganisms were isolated by serial dilution. 4-6 dilutions were done for the toddy samples and commercial yeast preparation (5 % yeast dissolved in distilled water). For growth of the microbe, YEPD (yeast extract peptone dextrose) medium was selected. 0.1 ml of diluted samples was plated on YEPD agar medium (YEPD) supplemented with 0.1 mg / ml streptomycin. The plates were incubated at 30 °C for 48 h. Morphologically distinguished colonies were then selected and purified by subsequent streaking on YEPD medium. Pure culture of each strain was kept on YEPD agar slants and stored at 4 $^{\circ}$C until needed.

3.11.2.1 YEPD Media composition (for 1L)

Yeast extract - 10 g
Peptone - 20 g
Dextrose - 20 g
Agar - 20 g

3.11.3 Screening of microorganism

Microorganisms isolated from three sources were screened for fermentation based on the ethanol tolerance and sugar tolerance.

3.11.3.1 Ethanol tolerance - Ethanol tolerance was estimated according to the method of Ross (1982).

Procedure

i. Microbes were inoculated in YEPD media at different ethanol concentrations (10 %, 12 %, 14 %, 16 % and 18 %).
ii. Incubated at 30 °C for 48 h.
iii. Turbidity of media was measured at 615 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).
iv. Concentration of ethanol at which the growth of microbe was just inhibited was asserted as its ethanol tolerance.

3.11.3.2 Sugar tolerance - Sugar tolerance was estimated according to the method of Ekunsanmi and Odunfa (1990).

Procedure

i. Microbes were inoculated in YEPD media at different sugar concentrations (50 mg / ml, 150 mg / ml, 250 mg / ml and 350 mg / ml)
ii. Incubated at 30 °C for 48 h.
iii. Turbidity of media was measured at 540 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).
iv. Concentration of sugar at which the growth of microbe was just inhibited was taken as its sugar tolerance.

3.11.4 Genetic characterisation of microbe BTPJ-1 screened for ethanol production

3.11.4.1 DNA isolation - Isolation of DNA was done according to the method of Rogers and Bendich (1994)

Reagents

1) CTAB buffer

   CTAB – 10 %

   Tris HCl (pH 8) – 0.1 M

   NaCl – 1.4 M

   EDTA – 0.02 M

Procedure

i. Microbe was grown in YEPD media at 30 °C for 48 h.
ii. Fungal mycelium was directly collected from culture medium and allowed to freeze at 4 °C for 1 h.
iii. 1 g of frozen tissue (wet weight) was powdered using a pre-cooled and sterile mortar and pestle.
iv. Powder was transferred into 50 ml capacity centrifuge tube containing 16 ml of pre-heated (65 °C) CTAB buffer and incubated at 65 °C for 30 min. in a water bath followed by incubation at room temperature.
v. Equal volume of chloroform-isoamyl alcohol (24:1) was added to the tubes. Mixed thoroughly to form an emulsion and centrifuged at 10,000 rpm for 10 min..
vi. Aqueous layer was collected and transferred to a new centrifuge tube using a cut tip.

vii. Chloroform-isoamyl alcohol extraction was repeated twice and the aqueous phase was collected.

viii. Sodium acetate (pH 5.2) was added to the aqueous phase for obtaining a final concentration of 300 mM.

ix. DNA was precipitated by the addition of two-third volume of ice cold propenol and kept at -20 °C overnight.

x. After incubation, DNA strand was pooled out using a glass rod, washed with 70 % ethanol and air dried

xi. DNA is finally dissolved in Milli Q water and kept at -20°C for further manipulation.

xii. The purity of DNA was checked by reading the absorbance ratio A_{260} / A_{280}. The quantification of DNA was done using DNA / Protein pack® software of Shimadzu UV - VIS spectrophotometer.

3.11.4.2 Agarose gel electrophoresis

Reagents

1) TAE (Tris acetic acid EDTA) buffer (50 X)
   Tris base - 242 g
   Glacial acetic acid - 57.1 ml
   0.5 M EDTA - 100 ml
   Made up to 1 L using distilled water

Procedure

The agarose gel electrophoresis was carried out for the visualization of isolated DNA. 1 % (w / v) agarose in 1 X TAE buffer was melted in a microwave oven. The molten agarose was poured into a gel casting tray and allowed to solidify at

room temperature. 5 µl of DNA (in 6 X loading buffer) was loaded on to gel and electrophoresis was carried out at 80 V for 1 h. 100 bp DNA ladder was used as marker. The gel was stained in freshly prepared 0.5 mg / ml ethidium bromide solution for 10 min. and viewed on an UV transilluminator and the image was captured with the help of gel documentation system (Bio rad, U.S).

3.11.4.3 Gene amplification

A PCR was performed in a total reaction mix of 20 µl of the isolated genomic DNA from BTPJ-1 for amplification. The PCR was performed with the universal primers ITS1 and ITS4.

Table 3.1 Primers for gene amplification

Forward primers	**Sequence (5' - 3')**
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

Table 3.2 PCR reaction mix (for 20 µl)

Components	**Concentration**
Taq buffer	1x
$MgCl_2$	1.5 mM
dNTP	0.25 mM
ITS1	0.5 µM
ITS2	0.5 µM
Taq polymerase	1 unit
DNA	100 ng

The PCR reaction was conducted with 35 cycles of denaturation. The PCR product was electrophoresed in a 1 % (w / v) agarose gel stained with ethidium bromide, observed on an UV transilluminator and the image was captured with the help of gel documentation system (Bio rad, U.S).

Table 3.3 PCR programme

Initial	94˚C, 5 min.
Denaturation	94˚C, 30 sec.
Annealing	58˚C, 2 min.
Extension	72˚C, 3 min.
Elongation	72˚C, 10 min.

3.11.5 Substrate for ethanol production

a) Untreated substrate: Substrate was dried at 50 ± 2°C, and powdered to 425 µ.

b) Chemically treated substrate:

i. Substrate was mixed with 1 % NaOH in the ratio 1:20 w / v and was autoclaved for 30 min. at 120 °C and 15 psi for 30 min.
ii. After autoclaving, substrate was cooled and washed 8 - 10 times to make it free of alkali.
iii. Substrate was dried and treated with commercial cellulase of 21.79 FPU for 1 h.
iv. After enzyme treatment, substrate was again dried at 50 ± 2 °C, and powdered to 425 µ.

c) Biologically treated substrate: Spent substrate (28^{st}, 49^{th} and 70^{th} day) obtained after the harvest, was dried at 50 ± 2 °C, and powdered to 425 µ.

3.11.6 Fermentation medium

Fermentation medium consisted of substrate suspended in distilled water at 1:40 w / v (2.5 %), supplemented with 0.3 % ammonium sulphate, 0.15 % potassium di-hydrogen phosphate and 0.5 % yeast extract.

3.11.7 Inoculum

Microbe BTPJ-1 was grown in a medium containing 6.0 % sucrose, 0.5 % yeast extract and 0.5 % peptone for 24 h at 28 ˚C. Cells were pelleted out at 8000 rpm for 15 min. and used as inoculum (2.5 %, wet weight) for fermentation.

3.11.8 Ethanol production

i. Powdered spent substrates of were utilised for ethanol production. Untreated substrate was used as control.
ii. Fermentation medium was sterilized and total reducing sugar was estimated.
iii. Medium was inoculated and incubated for 72 h at 30 ˚C with intermittent shaking.
iv. The fermented medium was checked for ethanol content colorimetrically.

3.11.9 Ethanol estimation

Ethanol was estimated according to the method of Bennette (1971) and Pilone (1985) based on the oxidation of ethanol in the sample by potassium dichromate in a strongly acidic medium. Reduction of dichromate results in the formation of chromogen that can be monitored.

Reagents

1) Potassium dichromate

3.4 g potassium dichromate was dissolved in 32.5 ml concentrated H_2SO_4 and made up to 100 ml.

Procedure

i. 100 µl of sample was taken in each test tube.
ii. 1 ml of Potassium dichromate reagent was added to the tube and incubated at room temperature for 30 min..
iii. The orange red colour developed was measured against a reagent blank at 540 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).
iv. The concentration of ethanol was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of ethanol.

3.11.10 Statistical analysis

Correlation analysis was carried out to find the relation between the reducing sugar present in the biologically treated substrate and the yield of ethanol.

3.12 Degradation of phenol

Procedure

i. Spent substrates which gave maximum lignin peroxidase and laccase activity were selected for the experiment.
ii. Initial concentration of phenol in the water sample was estimated.

iii. Sample was incubated with the spent substrate in the ratio 1:1 w / v, for 1 day / 7 days.
iv. Final concentration of phenol in the sample was estimated.

3.12.1 Phenol estimation

Phenol was estimated according to the method of Box (1981). Folin Ciocalteau's phenol reacts with reducing compounds like phenol, in presence of sodium carbonate, to give a coloured complex which can be monitored.

Reagents:

1) Folin Ciocalteau's phenol reagent
2) Sodium carbonate reagent:
   20 g of anhydrous sodium carbonate was dissolved in 80 ml distilled water. Boiled and cooled. A few more crystals were added and made up to 100 ml. Incubated for 24 h and filtered.

Procedure

i. To 20 µl of the sample in a test tube added 1.6 ml of water, 100 µl of Folin Ciocalteau's phenol reagent and incubated for 5 min.
ii. After incubation, 300 µl of sodium carbonate reagent was added.
iii. Incubated at 40 °C for 30 min.
iv. The blue colour developed was measured against a reagent blank at 765 nm in a UV - VIS spectrophotometer (Shimadzu, Japan).
v. The concentration of phenol was determined by comparing absorbance against a standard curve constructed similarly with known concentrations of gallic acid and was expressed as mg / ml.

Chapter 4

RESULTS

4.1 Cultivation of *Pleurotus eous* and *Pleurotus ostreatus* on lignocellulosic substrates

Pleurotus eous and *Pleurotus ostreatus* differed in the rate of growth on the five lignocellulosic substrates: banana pseudo stem (BP), pineapple leaves (PL), coconut leaflets (CL), sugarcane bagasse (SB) and paddy straw (PS). In the first phase, the spawn run period, the mycelia completely covered the substrates (Fig. 4.1). Spawn run period of the two species was different on 5 lignocellulosic substrates as given in the table (Tab. 4.1). In banana pseudo stem, spawn run was found restricted to some regions. Sugarcane bagasse was found to be very prone to contamination during this period, hence holes were punched on the bag only after the spawn run. However coconut leaflets were found to be resistant to contamination. Primodia / pin heads appeared by the 16^{th} day and the cultures entered the reproductive phase. Oyster shaped fruiting bodies were produced by 18^{th} day. Fruiting body of *P. eous* was pink in colour while that of *P. ostreatus* was creamy white (Fig. 4.2). On paddy straw, the number of fruiting bodies produced was higher, while on the other substrates the fruiting bodies produced were fewer in number but larger in size. Increase in temperature, carbon dioxide and decrease in humidity were observed to reduce the size of fruiting body. Depending on the substrate, the date of harvest differed. In case of paddy straw, the first three flushes of mushrooms were harvested by the 24^{th} day. For other substrates, harvest continued till the 35^{th} day.

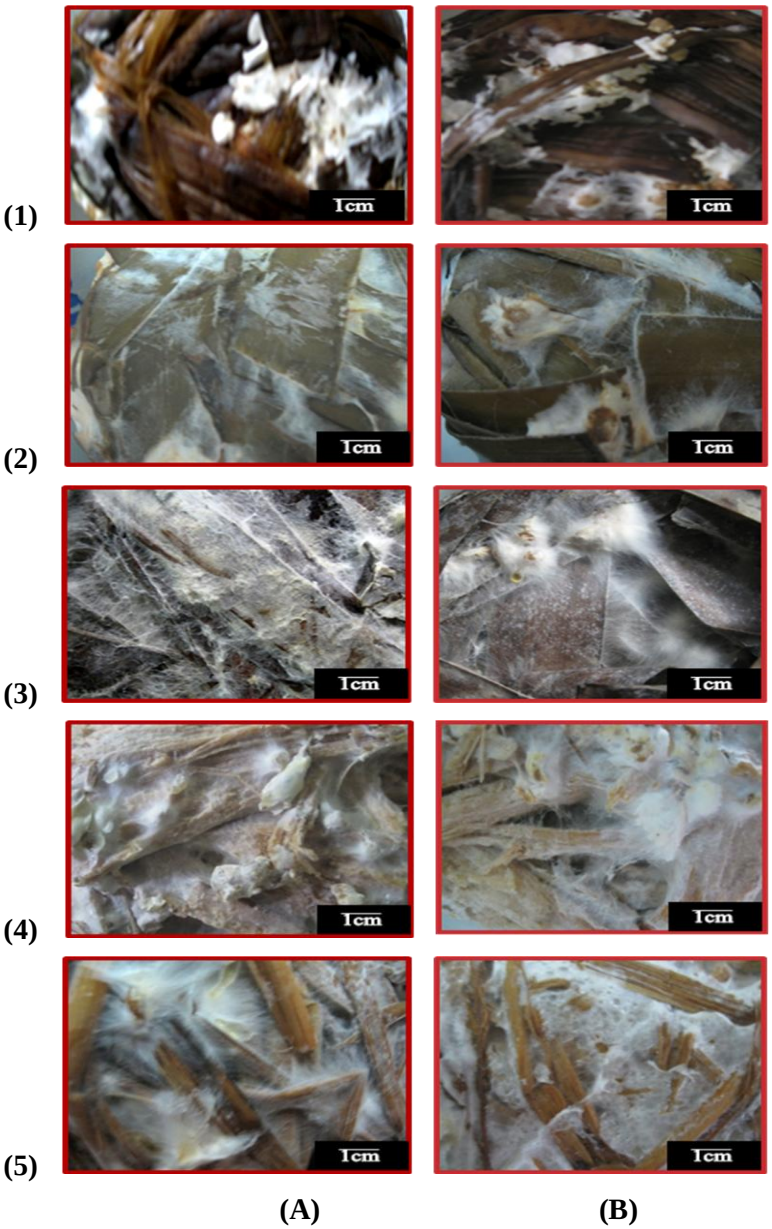
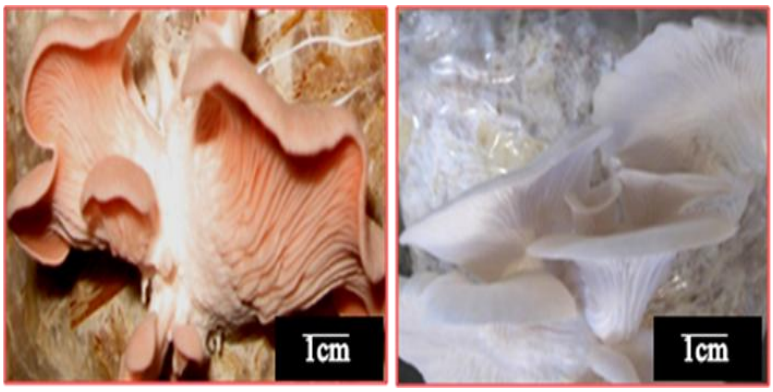


Figure 4.1 Spawn run of (A) *Pleurotus eous* and (B) *Pleurotus ostreatus* on lignocellulosic substrates: (1) banana pseudo stem (2) pineapple leaves (3) coconut leaflets (4) sugarcane bagasse and (5) paddy straw



A **B**

Figure 4.2 Fruiting body of (A) *Pleurotus eous* (B) *Pleurotus ostreatus*

Table 4.1 Spawn run period of *Pleurotus* sp. on lignocellulosic substrates

Substrate	**Spawn run period (days)**
Banana pseudo stem	15-16
Pineapple leaves	14
Coconut leaflets	14
Sugarcane bagasse	15-16
Paddy straw	10-12

4.2 Biological efficiency (BE) of *Pleurotus* sp.

Biological efficiency obtained for the first three flushes of *P. eous*, while growing on different substrates (Tab. 4.2), was 60-80 %, while *P. ostreatus* showed 70-90 % efficiency. Highest BE was obtained while growing on paddy straw.

Table 4.2 Biological efficiency shown by *Pleurotus* sp. while growing on ligno-cellulosic substrates

Substrate	Biological efficiency of *P. eous* (%)	Biological efficiency of *P. ostreatus* (%)
Banana pseudo stem	60±5	70±5
Pineapple leaves	60±5	70±5
Coconut leaflets	60±5	70±5
Sugarcane bagasse	70±5	80±5
Paddy straw	80±5	90±5

4.3 pH of mushroom bed during growth of *Pleurotus* sp. on different lignocellulosic substrates

No change in pH was observed for the first 7 days when *Pleurotus* sp. was grown on the 5 lignocellulosic substrates. The growth of *P. eous* made the substrate acidic by the 21st day (in the range of 4-6.5), while *P. ostreatus* raised the pH of the substrate to neutral or alkaline range. During growth of *P. eous*, pH of all substrates was lowest on 21st day (Tab. 4.3a). Banana pseudo stem and pineapple leaf substrates were initially more acidic than other substrates, but became close to neutral pH after fructification, while the other substrates remained acidic. pH of all substrates was found to remain unchanged after 35th day, up to the end of the experiment at 70th day.

Table 4.3a pH of mushroom bed during growth of *Pleurotus eous* on different lignocellulosic substrates

Substrate	Initial pH	Age of culture (days)									
		7	14	21	28	35	42	49	56	63	70
		Spawn run		Fructification			Spent mushroom substrate				
Banana pseudo stem	5	5	4.5	4	5	6.5	6.5	6.5	6.5	6.5	6.5
Pineapple leaves	5	5	4.5	4	5.5	6	6	6	6	6	6
Coconut leaflets	5.5	5.5	5	4	4.5	5	5	5	5	5	5
Sugarcane bagasse	5.5	5.5	5	4	4.5	5.5	5.5	5.5	5.5	5.5	5.5
Paddy straw	5.5	5.5	5	4.5	5	5.5	5.5	5.5	5.5	5.5	5.5

The pH of substrates used for growing *P. ostreatus* ranged from acidic to neutral / alkaline (pH 4.5-9), during cultivation (Tab. 4.3b). The highest pH during growth was observed on 21st day. Pineapple leaves and paddy straw substrates became alkaline (pH 9) in the initial stages (21st day) of fructification. Although all the substrates initially had an acidic pH, paddy straw became basic after fructification, banana pseudo stem and pineapple leaves became neutral, while sugar-cane bagasse and coconut leaflets remained acidic in nature. pH of the substrates was unchanged from 42nd - 70th day.

Table 4.3b pH of mushroom bed during growth of *Pleurotus ostreatus* on different lignocellulosic substrates

Substrate	Initial pH	Age of culture (days)									
		7	14	21	28	35	42	49	56	63	70
		Spawn run		Fructification			Spent mushroom substrate				
Banana pseudo stem	5	5	5.5	7.5	7	6.5	7	7	7	7	7
Pineapple leaves	5	5	5.5	9	7	6.5	7	7	7	7	7
Coconut leaflets	5.5	5.5	6	6.5	5	4.5	5	5	5	5	5
Sugarcane bagasse	5.5	5.5	6	6.5	4.5	4	4.5	4.5	4.5	4.5	4.5
Paddy straw	5.5	5.5	6	9	8.5	7	8.5	8.5	8.5	8.5	8.5

4.4 Assays and estimations

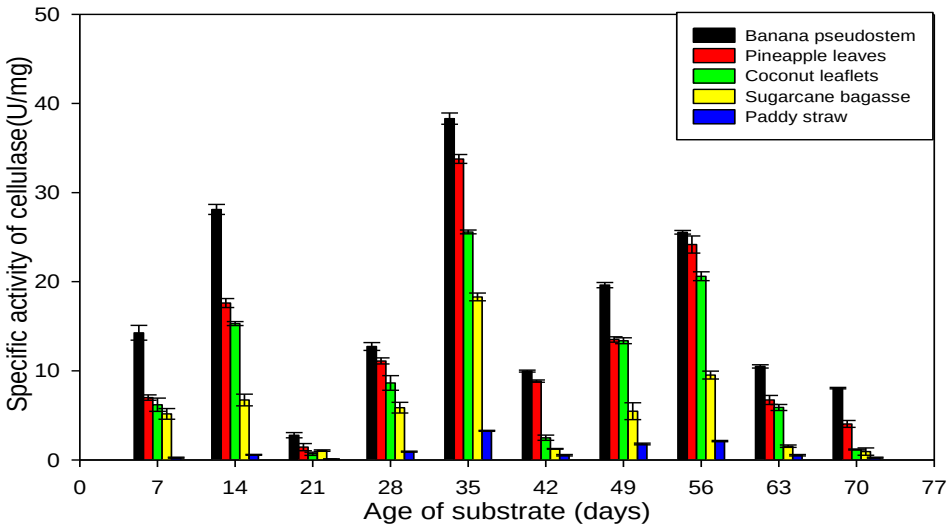
Quantitative assays for cellulase, xylanase, pectinase, lignin peroxidase and laccase present in the substrate of *P. eous* and *P. ostreatus* were done. Both mushrooms showed higher enzyme activity while growing on banana pseudo stem and least activity on paddy straw. Cellulose, hemicellulose, lignin and pectin present in the mushroom substrate were estimated. Mushrooms degraded these components more efficiently while growing on banana pseudo stem.

4.4.1 Production of cellulases and degradation of cellulose by *Pleurotus* sp. growing on lignocellulosic substrates

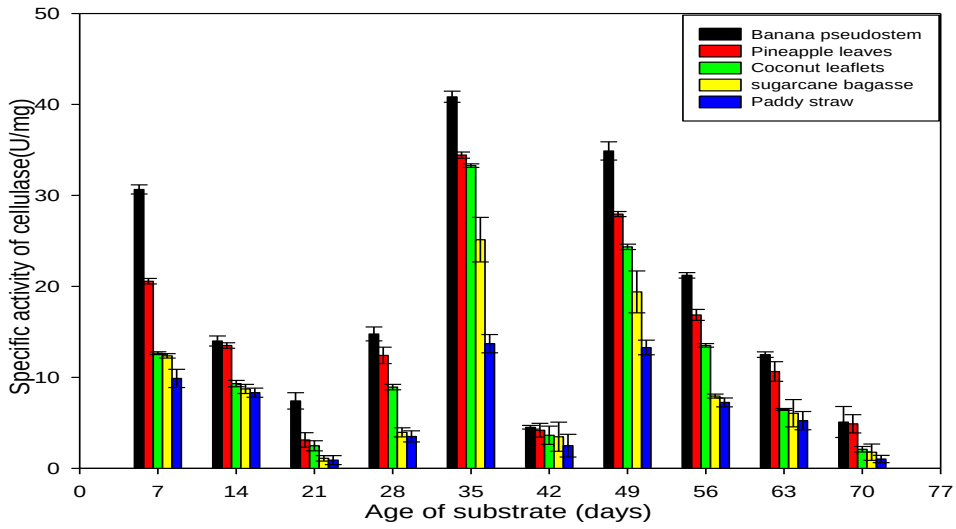
Production pattern of cellulase by *Pleurotus* sp. showed three distinct peaks, during each of the three stages of cultivation – spawn run, fructification and in spent mushroom substrate (Fig. 4.3). *P. eous* produced more cellulase in the final stages (14th day) of spawn run, while cellulase production by *P. ostreatus* was more in the initial stages (7th day). Both species showed a decrease in the cellulase production on 21st day, and maximum cellulase production was observed on 35th day. Even after harvest (35th day), cellulase activity was observed in the spent mushroom substrate and cellulose degradation occurred.

Cellulose content was more in banana pseudo stem, when compared to the other four substrates (Tab. 4.4). *Pleurotus* sp. produced more cellulase while growing on banana pseudo stem and brought about 80-89 % reduction in the cellulose. Production of cellulase by *P. eous* while growing on banana pseudo stem was nearly 15 times more than the production on paddy straw, and the, degradation of cellulose in banana pseudo stem was three times more than the degradation in paddy straw.

Among the two mushrooms, *P. ostreatus* produced more cellulase. Production of cellulase by *P. ostreatus*, while growing on paddy straw, was nearly 5 times more than the production by *P. eous*. Also, degradation of cellulose in paddy straw by *P. ostreatus* was twice that of *P. eous.*



a) ***Pleurotus eous***



b) ***Pleurotus ostreatus***

Figure 4.3 Production of cellulase by *Pleurotus* sp. growing on lignocellulosic substrates

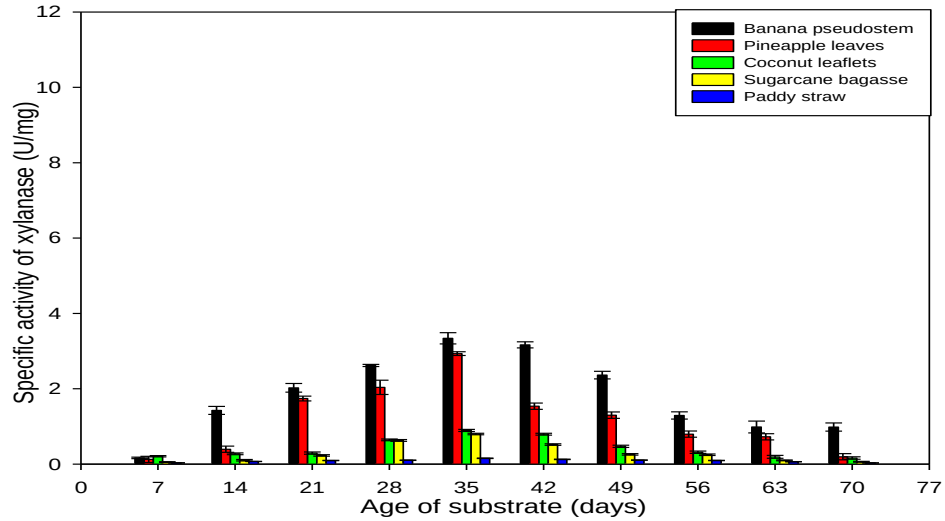
[Note: Error bars represent ± standard deviation from a triplicate average]

Table 4.4 Degradation of cellulose by *Pleurotus* sp. growing on lignocellulosic substrates

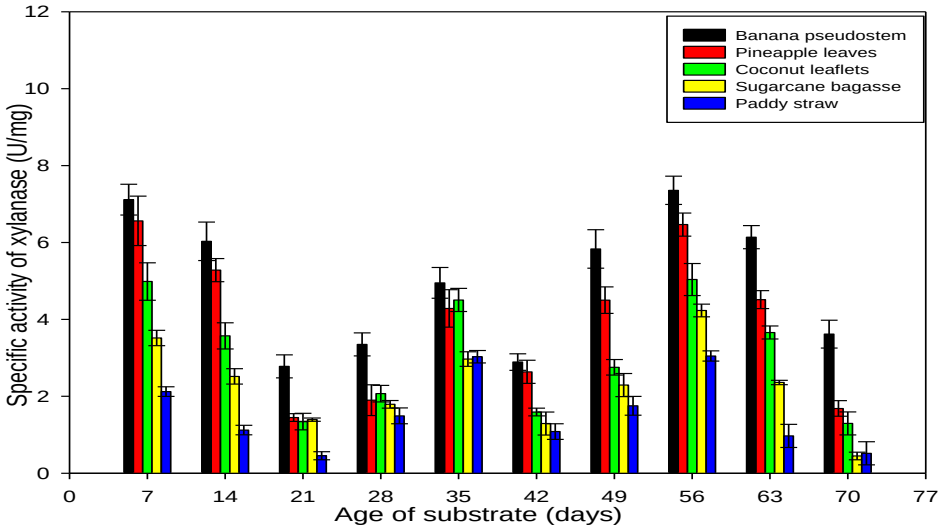
Substrate	Cellulose content in untreated substrate % dry weight	Cellulose content in 70 day old spent substrate of *P. eous*		Cellulose content in 70 day old spent substrate of *P. ostreatus*	
		% dry weight	Percentage reduction	% dry weight	Percentage reduction
Banana pseudostem	71.13±1.06	14.81±0.41	80.28	8.06±0.31	88.97
Pineapple leaves	60.94±0.96	19.23±0.62	68.31	10.26±0.21	83.33
Coconut leaflets	41.53±0.82	20.76±0.41	51.98	11.34±0.58	73.17
Sugarcane bagasse	37.75±0.61	21.66±0.59	43.98	12.55±0.43	67.96
Paddy straw	30.17±0.73	23.91±0.63	24.97	16.46±0.63	47.86

4.4.2 Production of xylanase and degradation of hemicellulose by *Pleurotus* sp. while growing on lignocellulosic substrates

Pattern of xylanase produced by the two mushroom species under study was very different from each other. *P. eous* and *P. ostreatus* showed maximum production of xylanase on 35th and 56th day of cultivation, respectively (Fig. 4.4). Production of xylanase was several fold higher in *P. ostreatus* than in *P. eous*. Also, both species produced more xylanase while growing on banana pseudo stem and degraded hemicellulose by 53 % (*P.* eous) and 80 % (*P. ostreatus*) (Tab. 4.5). In the case of *P. eous*, production of xylanase was low during spawn run period and in the spent mushroom substrate. *P. eous* produced more xylanase while growing on banana pseudo stem and pineapple leaves, when compared to the other three substrates. Least enzyme production was observed while growing on paddy straw and hemicellulose degradation was only 3 %. In *P. ostreatus*, higher xylanase activity was observed in the spent mushroom substrate compared to the other growth phases. Production of xylanase by *P. ostreatus*, while growing on paddy straw, was nearly 20 times higher than that of *P. eous*, and hemicellulose degradation was about 20 %.



a) ***Pleurotus eous***



b) ***Pleurotus ostreatus***

Figure 4.4 Production of xylanase by *Pleurotus* sp. growing on lignocellulosic substrates

[Note: Error bars represent ± standard deviation from a triplicate average].

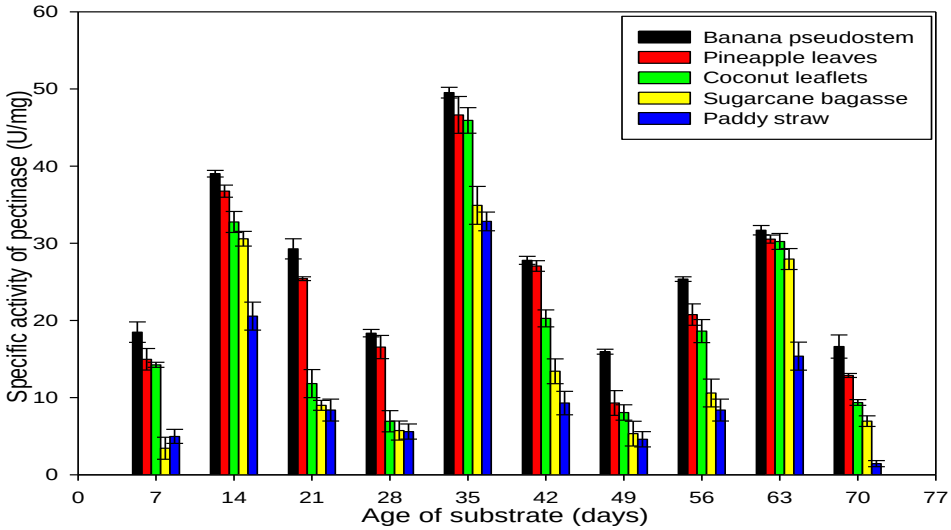
Table 4.5 Degradation of hemicellulose by *Pleurotus* sp. growing on lignocellulosic substrates

Substrate	Hemi-cellulose content in untreated substrate % dry weight	Hemicellulose content in 70 day old spent substrate of *P. eous*		Hemicellulose content in 70 day old spent substrate of *P. ostreatus*	
		% dry weight	Percentage reduction	% dry weight	Percentage reduction
Banana pseudo stem	15.16±0.16	7.14±0.31	53.33	3.11±0.61	80.11
Pineapple leaves	18.39±0.24	12.52±0.27	34.33	5.13±0.53	71.22
Coconut leaflets	28.21±0.35	18.39±0.51	35.71	13.21±0.32	53.17
Sugarcane bagasse	20.65±0.65	18.16±0.43	10.11	14.32±0.47	30.13
Paddy straw	23.12±0.42	22.22±0.62	3.34	18.14±0.32	20.62

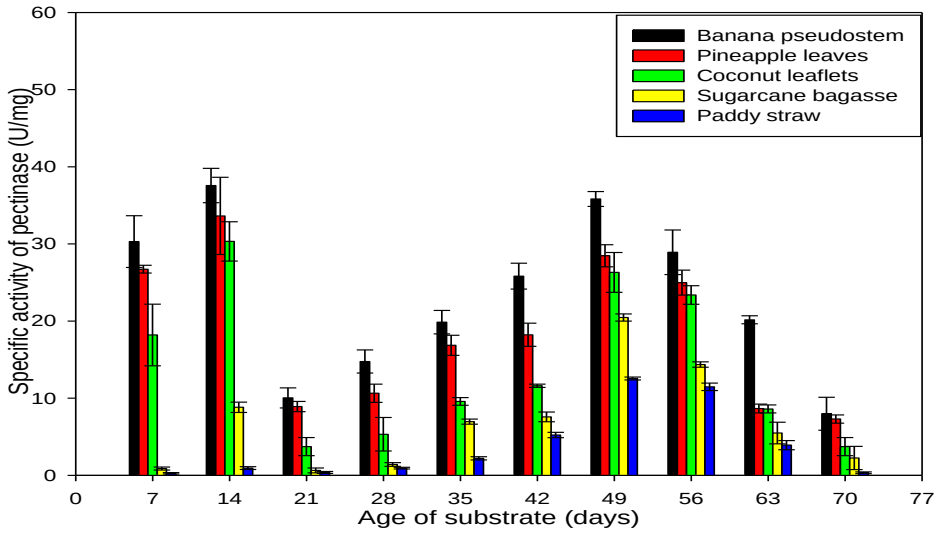
4.4.3 Production of pectinase and degradation of pectin by *Pleurotus* sp. while growing on lignocellulosic substrates

P. eous and *P. ostreatus* showed maximum pectinase production on 35th and 14th day of cultivation, respectively (Fig. 4.5). *P. eous* produced more pectinase than *P. ostreatus*, (2-4 times more while growing on sugarcane bagasse and paddy straw), and degradation of pectin by *P. eous* was twice that of *P. ostreatus* (Tab. 4.6).

Nearly 10 times higher pectinase activity was seen in the SMS of *P. ostreatus* compared to the vegetative and reproductive stages. Pectin content was higher in banana pseudo stem compared to the other substrates. *P. eous* and *P. ostreatus* produced more pectinase while growing on banana pseudo stem and degraded pectin by 88 % and 78 % respectively.



a) *Pleurotus eous*



b) *Pleurotus ostreatus*

Figure 4.5 Production of pectinase by *Pleurotus* sp. growing on lignocellulosic substrates

[Note: Error bars represent ± standard deviation from a triplicate average]

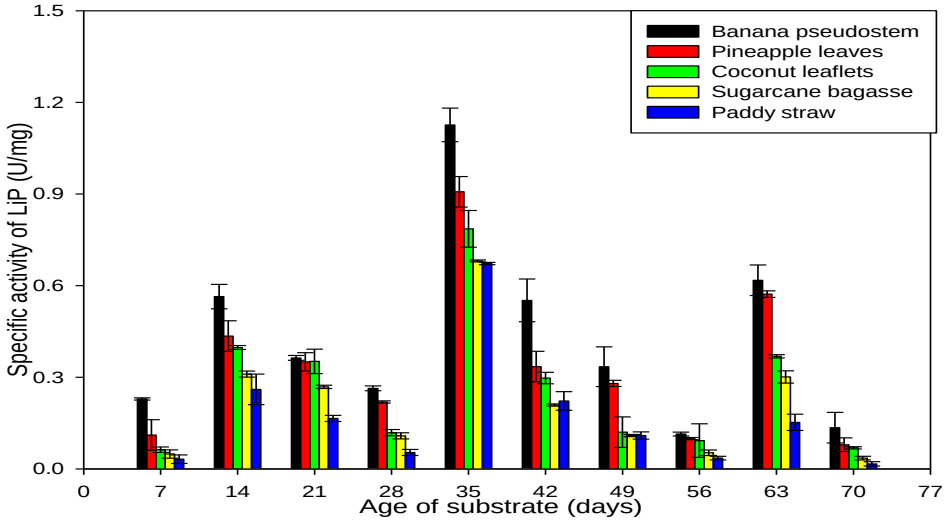
Table 4.6 Degradation of pectin by *Pleurotus* sp. growing on lignocellulosic substrates

Substrate	Pectin content in untreated substrate % dry weight	Pectin content in 70 day old spent substrate of *P. eous*		Pectin content in 70 day old spent substrate of *P. ostreatus*	
		% dry weight	Percentage reduction	% dry weight	Percentage reduction
Banana pseudo stem	1.05±0.04	0.12±0.01	88.17	0.23±0.03	78.09
Pineapple leaves	0.88±0.03	0.18±0.02	79.14	0.22±0.04	75.01
Coconut leaflets	0.45±0.01	0.12±0.01	73.33	0.14±0.01	68.18
Sugarcane bagasse	0.51±0.05	0.21±0.03	58.82	0.34±0.05	34.33
Paddy straw	0.69±0.06	0.44±0.04	36.23	0.59±0.06	14.89

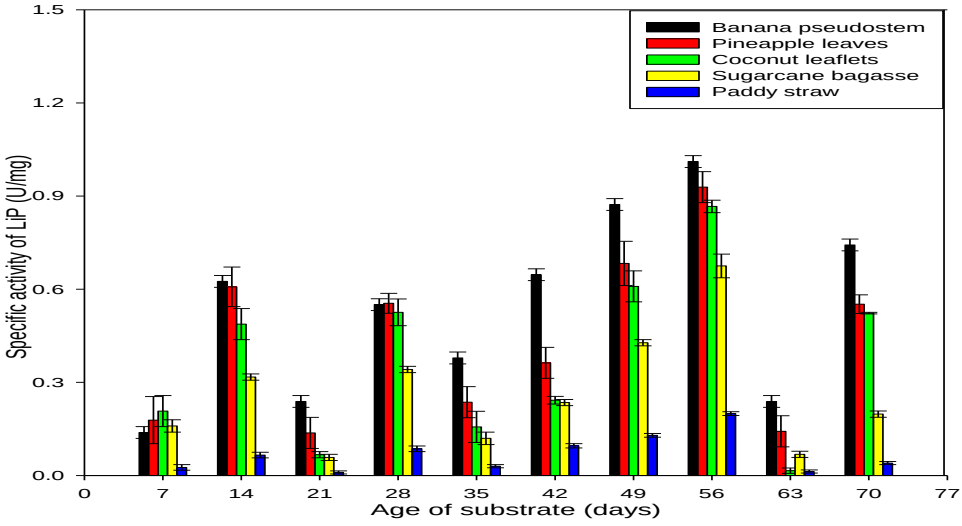
4.4.4 Production of ligninases and degradation of lignin by *Pleurotus* sp. growing on lignocellulosic substrates

Pleurotus sp. produced more ligninases while growing on banana pseudo stem compared to other substrates. Laccase and lignin peroxide (LiP) produced by *P. eous* and *P. ostreatus* brought about 64 % and 70 % respectively of lignin degradation in banana pseudo stem (Tab. 4.7). *P. eous* and *P. ostreatus* showed maximum LiP production on 35^{th} and 56^{th} day of cultivation, respectively (Fig. 4.6). While growing on paddy straw, *P. eous* produced more LiP than *P. ostreatus*. *P. ostreatus* produced more LiP after the vegetative and reproductive stages of cultivation.

Among the two ligninases, both species produced more laccase than LiP (Fig. 4.7). *P. eous* showed maximum laccase production on 42^{nd} day of cultivation, while *P. ostreatus* produced more laccase during vegetative and reproductive stages of cultivation. Both species produced lesser amount of laccase on paddy straw.



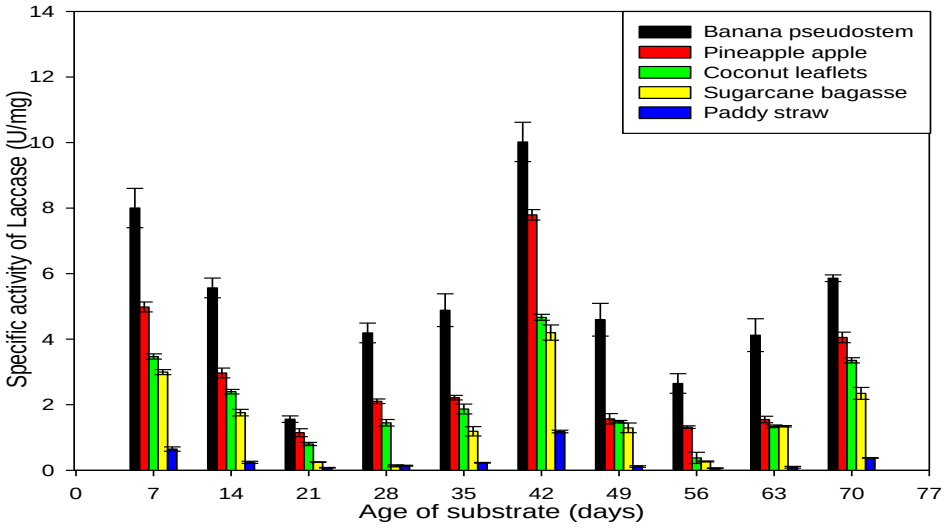
a) ***Pleurotus eous***



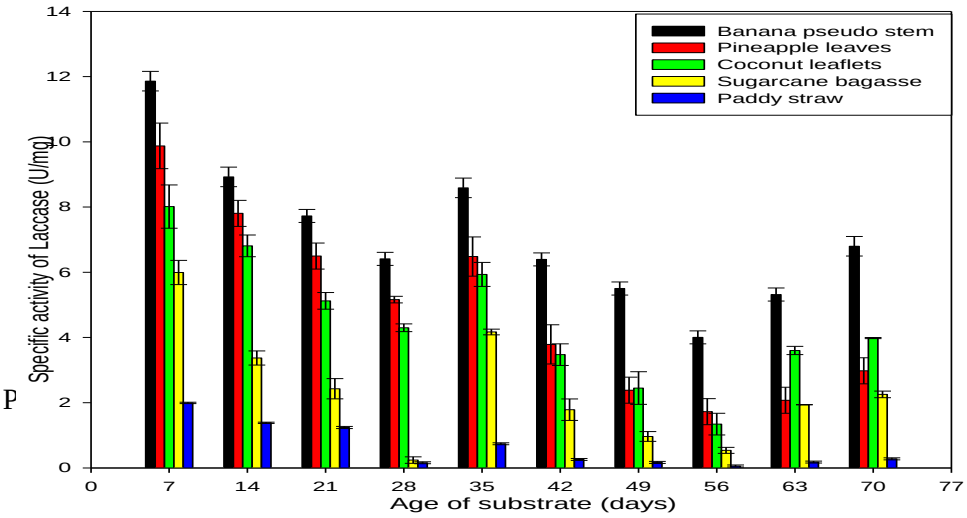
b) ***Pleurotus ostreatus***

Figure 4.6 Production of lignin peroxidase by *Pleurotus* sp. growing on lignocellulosic substrates

[Note: Error bars represent ± standard deviation from a triplicate average].



a) ***Pleurotus eous***



P

b) ***Pleurotus ostreatus***

Figure 4.7 Production of laccase by *Pleurotus* sp. growing on lignocellulosic substrates

[Note: Error bars represent ± standard deviation from a triplicate average].

Table 4.7 Degradation of lignin by *Pleurotus* sp. growing on lignocellulosic substrates

Substrate	Lignin content in untreated substrate % dry weight	Lignin content in 70 day old spent substrate of *P. eous*		Lignin content in 70 day old spent substrate of *P. ostreatus*	
		% dry weight	Percentage reduction	% dry weight	Percentage reduction
Banana pseudo stem	25.30±0.35	9.11±0.15	64.01	8.22±0.18	69.97
Pineapple leaves	20.21±0.24	11.36±0.17	46.97	9.32±0.81	56.87
Coconut leaflets	40.27±0.46	23.41±0.37	42.50	20.46±0.74	49.95
Sugarcane bagasse	27.54±0.37	18.52±0.20	35.33	16.18±0.91	39.94
Paddy straw	15.31±0.25	11.19±0.14	26.96	10.13±0.36	30.33

4.5 Use of *Pleurotus* sp. for pretreatment of lignocellulosic substrates for fibre extraction

4.5.1 Tensile strength and elastic modulus of fibres obtained from spent substrate of *Pleurotus* sp.

Untreated and treated fibres of banana pseudo stem, pineapple leaves, coconut leaflets, sugarcane bagasse and paddy straw were analysed for their tensile strength (Tab. 4.8) and elastic modulus (Tab. 4.9).

Among the untreated substrates, tensile strength was greater in banana fibres (10.23 MPa) and lowest in paddy straw. Growth of mushrooms for 49 days brought about 2-4 times increase in the tensile strength when compared to untreated substrate, however fibres from 70 day old spent substrates were weaker than the untreated fibres. Spent substrate (49^{th} day) of pineapple leaves showed more tensile strength when compared to fibres obtained from other spent substrates**.** They were nearly 2 times stronger than other spent substrate fibres. But among spent substrate fibres of 28^{th} day and 70^{th} day, banana pseudo stem fibres were found to be stronger. Fibre obtained from the spent substrate of *P. ostreatus* showed more tensile strength when compared to the fibres of *P. eous* substrate.

Elastic modulus was greater in fibres from coconut leaflets and least in those from sugarcane bagasse, irrespective of treatment. Among the spent substrate fibres, elastic modulus was higher in fibre from 49^{th} day spent substrate. Elastic modulus of 70^{th} day spent substrate fibre was lesser than the untreated fibre. Fibre obtained from the spent substrate of *P. eous* showed higher elastic modulus when compared to the fibres of *P. ostreatus* substrate.

Table 4.8 Tensile strength of fibres from untreated substrate and from 28 day old (SS-28), 49 day old (SS-49) and 70 day old (SS-70) spent substrates obtained after growth of *Pleurotus* sp.

Substrate	Untreated substrate (MPa)	Tensile strength of spent substrate of *P. eous* (MPa)			Tensile strength of spent substrate of *P. ostreatus* (MPa)		
		SS-28	SS-49	SS-70	SS-28	SS-49	SS-70
Banana pseudo stem	10.23	14.44	17.80	8.95	17.51	22.23	6.63
Pineapple leaves	4.92	6.38	12.76	3.17	8.26	13.04	2.94
Coconut leaflets	6.84	13.33	24.30	6.14	17.38	26.89	5.16
Sugarcane bagasse	3.77	4.24	6.82	0.89	4.87	7.32	0.65
Paddy straw	8.05	8.19	10.53	6.91	10.98	11.01	2.02

Table 4.9 Elastic modulus of fibres from untreated substrate and from 28 day old (SS-28), 49 day old (SS-49) and 70 day old (SS-70) spent substrates obtained after growth of *Pleurotus* sp.

Substrate	Untreated substrate (MPa)	Elastic modulus of spent substrate of *P. eous* (MPa)			Elastic modulus of spent substrate of *P. ostreatus* (MPa)		
		SS-28	SS-49	SS-70	SS-28	SS-49	SS-70
Banana pseudo stem	225.91	310.25	400.40	137.94	301.67	363.33	115.96
Pineapple leaves	176.14	294.47	382.03	127.56	214.29	315.82	97.67
Coconut leaflets	651.57	748.81	879.52	150.45	714.53	801.78	127.45
Sugarcane bagasse	130.99	152.72	367.45	78.24	143.43	249.99	21.93
Paddy straw	150.67	181.76	374.56	82.18	176.50	313.74	73.45

4.5.2 Surface and chemical characterization

Fourier Transform Infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM) were done for untreated banana pseudo stem, pineapple leaves and coconut leaflets and for the 70 day old spent substrates obtained after growth of *Pleurotus* sp. on these substrates, for observing the changes in functional groups, cellulose crystallinity and surface morphology.

4.5.2.1 Fourier Transform Infrared spectroscopy

4.5.2.1.1 Banana pseudo stem

The FTIR absorption for untreated banana pseudo stem and 70 day old spent substrates of *Pleurotus* sp. after growth on banana pseudo stem showed strong bands in the range 3432-3417 cm^{-1} which represent the O=H bonds of alcohol (Fig. 4.8). Spent substrate of *P. ostreatus* showed wider band than that of untreated banana pseudo stem and the spent substrate of *P. eous*. C=O stretch of alcohol was observed in the spent substrates of *P. eous* and *P. ostreatus* at 1258.49 cm^{-1} and 1252.92 cm^{-1} respectively. Substrate of *P. ostreatus* showed sharper peak than that of *P. eous*. In the untreated banana pseudo stem, this stretch was not observed.

Peak for lignin monomers at 1731.53 cm^{-1} was found only in the spent substrate of *P. ostreatus.* Aromatic ring stretch for the lignin monomers, guaiacyl and syringyl also was observed only in the spent substrate of *P. ostreatus*, at 1510.71 cm^{-1}. C=O vibration in the range 1047–1052 cm^{-1} for lignin monomers was seen both in the untreated substrate and the spent substrates. Vibration obtained for the untreated substrate was less compared to that of spent substrates. C=O vibration in the spent substrate of *P. ostreatus* was more prominent compared to that of

P. eous. A sharp C-H bend was observed at 778.69 cm^{-1} in the untreated banana pseudo stem which showed the phenyl rings in lignin polymer. However, this peak was very small in the spent substrates.

Peak for crystalline cellulose in the range 1426-1419 cm^{-1} was present in all the three substrates. C_1 group frequency at 893.40 cm^{-1} represented amorphous cellulose which was seen only in the untreated banana pseudo stem. Hemicellulose peaked at 1731.53 cm^{-1} for C=O vibration, was present in three substrates. Peaks in the untreated substrate and the spent substrate of *P. eous* were small compared to that of *P. ostreatus*. Peak in the range 1377-1373 cm^{-1} showed sugars which was present in the three substrates, but the peak in the spent substrate of *P. ostreatus* was large. Vibrations obtained in the range 1047–1052 cm^{-1} also denoted the O=H group of sugars, which was more prominent in the spent substrates. Peak at 897.01 $cm^{-1}$and 897.92 cm^{-1} showed the ß-glycosidic linkages between the sugar units in the spent substrate of *P. eous* and *P. ostreatus* respectively

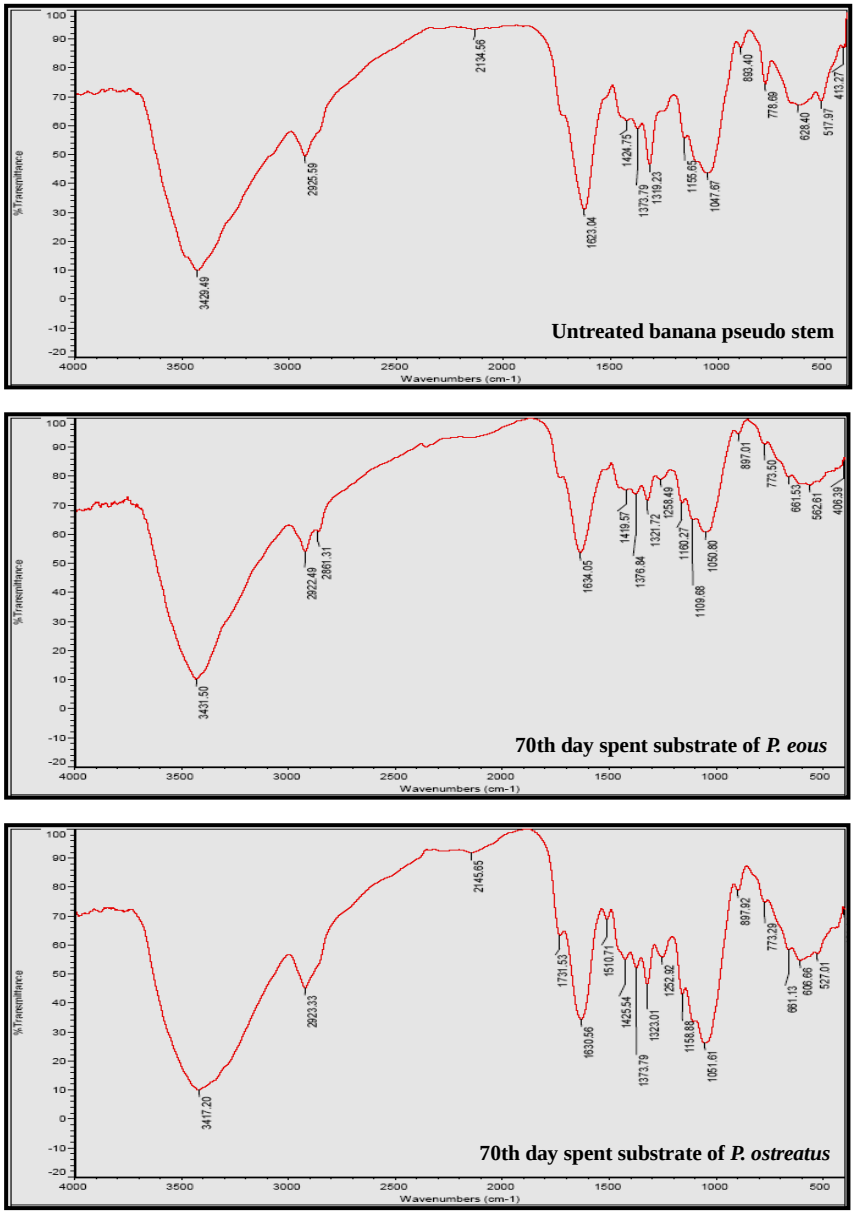


Figure 4.8 FTIR pattern of banana pseudo stem and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.1.2 Pineapple leaves

In the case of pineapple leaves, O=H stretch for alcohols in the range 3496-3291 cm^{-1} was seen only in the 70 day old spent substrates of *Pleurotus* sp. (Fig. 4.9). But, C=O stretch for alcohol in the range 1264-1240 cm^{-1} was observed in all the three substrates. C=O stretch in untreated substrate was very small. Spent substrate of *P. ostreatus* showed larger peak than that of *P. eous*.

Untreated pineapple leaves and the spent substrate of *P. eous* showed an aromatic ring stretch in the range 1570-1547 cm^{-1}, for lignin monomers, which was absent in the spent substrate of *P. ostreatus*. Stretch found in the spent substrate was prominent than that in the untreated substrate. Although, C=O vibration in the range 1069–1055 cm^{-1} for guaiacyl and syringyl alcohols was observed in all the substrates, peaks in the spent substrates were very prominent.

Similar peaks were obtained for the spent substrates of *Pleurotus* sp. in the range 1428-1424cm^{-1}, for crystalline cellulose. C_1 group frequency for amorphous cellulose in the range 902-897 cm^{-1} also was seen only in the spent substrates. Peak observed in the spent substrate of *P. eous* was larger.

Spent substrates of *Pleurotus* sp. showed similar peaks for sugars in the range 1382-1362 cm^{-1}. Vibrations in the range 1070–1063 cm^{-1}, which represented the O=H group of sugars, were seen in all the three substrates. Vibrations in the spent substrates were more compared to the untreated pineapple leaves. Among the spent substrates, 70 day old substrate of *P. ostreatus* gave sharper peak. Peak for ß-glycosidic linkages between the sugar units in the range 902–897 cm^{-1} was visible only in the FTIR pattern of spent substrates. Larger peak was observed in the substrate of *P. eous*

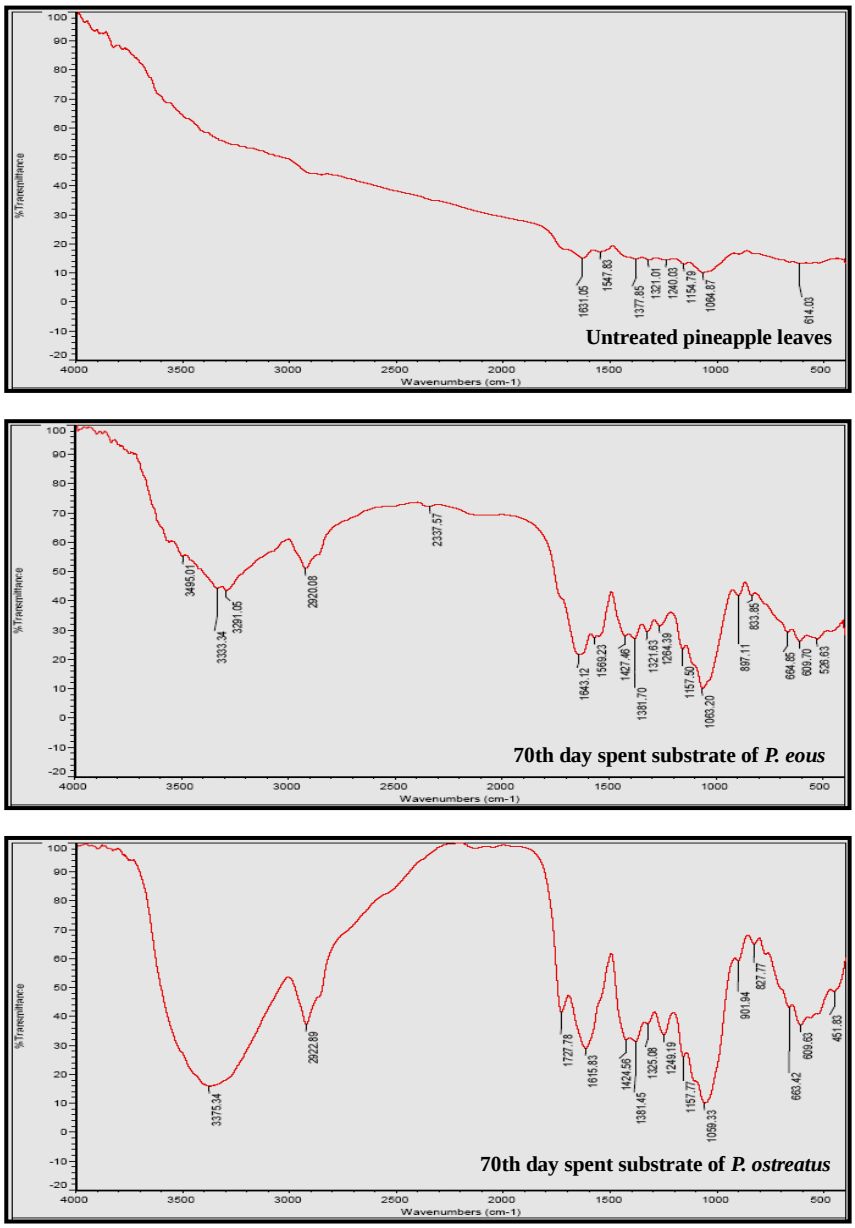


Figure 4.9 FTIR pattern of pineapple leaves and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.1.3 Coconut leaflets

Untreated coconut leaflets and 70 day old spent substrates of *Pleurotus* sp. gave strong O=H stretch for alcohol in the range 3405-3301 cm^{-1} (Fig. 4.10). Spent substrate of *P. ostreatus* showed wider band than the other two substrates. C=O stretch of alcohol was observed in the untreated substrate and spent substrates of *Pleurotus* sp. in the range 1247-1233 cm^{-1}. Untreated substrate and spent substrate of *P. ostreatus* had similar peaks, while substrate of *P. eous* showed larger stretch.

Lignin monomers in the three substrates gave the aromatic ring stretch in the range 1518-1514 cm^{-1}, but, substrate of *P. eous* showed the larger peak. C=O vibration in the range 1062.17 cm^{-1} for lignin monomers was seen only in the spent substrate of *P. eous*. A small C-H bend was observed at 788.53 cm^{-1} in the untreated coconut leaflets which showed the phenyl rings in lignin polymer. This peak was absent in the spent substrates.

Hemicellulose peaked in the range 1730-1721 cm^{-1} for C=O vibration, was present in three substrates. Peaks in the untreated substrate and the spent substrate of *P. ostreatus* were small compared to that of *P. eous*. Although the peak for crystalline cellulose in the range 1453-1446 cm^{-1} was present in all the three substrates, peak present in the spent substrate of *P. eous* was prominent.

Peak in the range 1375-1372 cm^{-1} showed sugars which was present in the three substrates, but the peak in the spent substrate of *P. ostreatus* was large. Vibrations at 1062.17 cm^{-1} which denoted the O=H group of sugars, was found only in the spent substrate of *P. eous*.

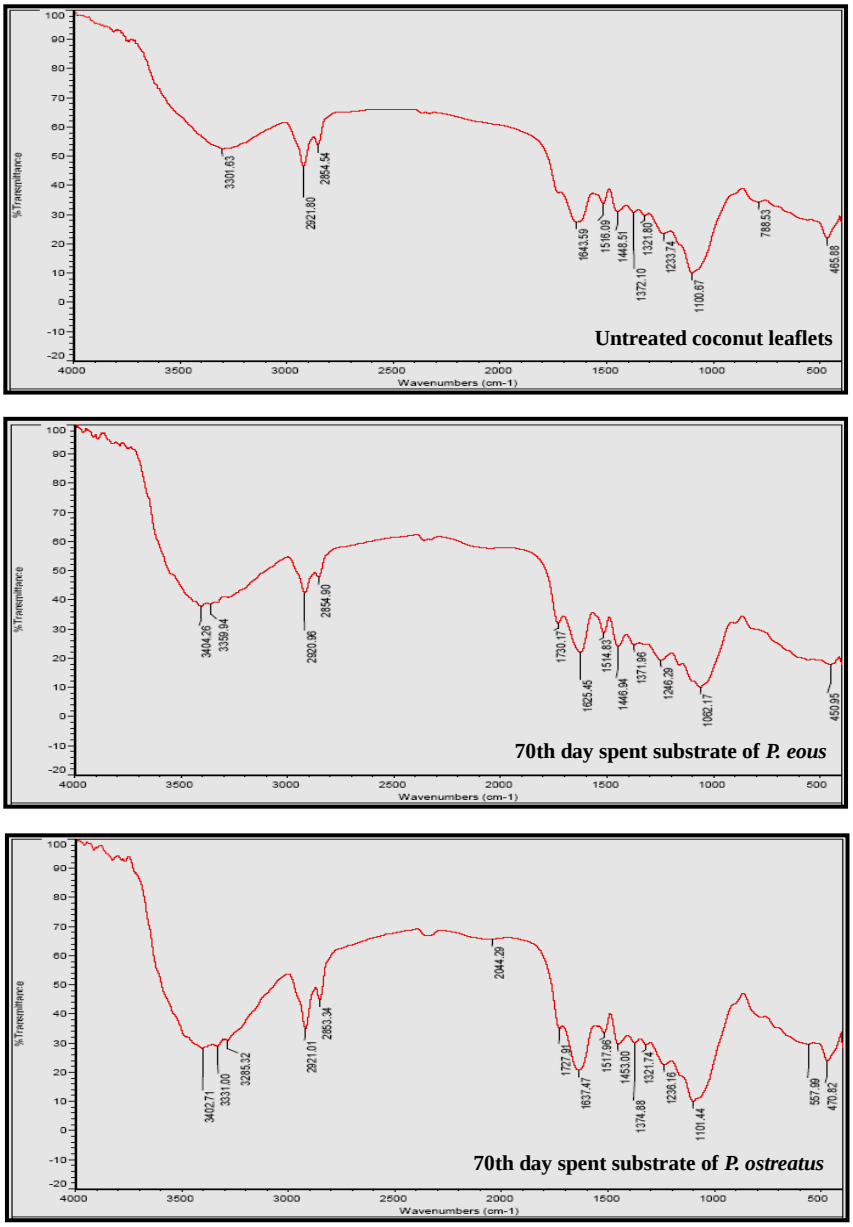


Figure 4.10 FTIR pattern of coconut leaflets and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.2 X-ray diffraction crystallography

4.5.2.2.1 Banana pseudo stem

Crystallinity index of cellulose in untreated banana pseudo stem was found to be 97.34 % (Fig. 4.11). Growth of *P. eous* and *P. ostreatus* in the substrate for 70 days brought about a reduction in the crystallinity by 3% and 4% respectively.

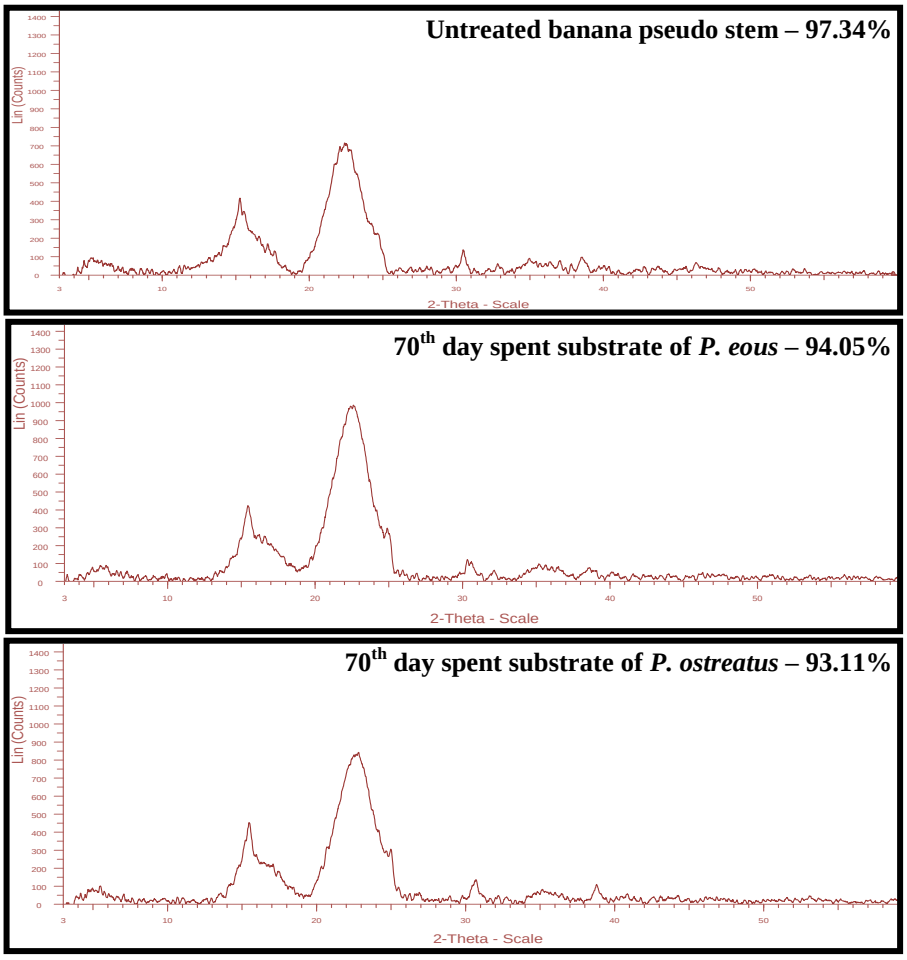


Figure 4.11 XRD pattern of banana pseudo stem and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.2.2 Pine apple leaves

Cellulose crystallinity index for untreated pineapple leaves was 96.80 % (Fig. 4.12). Growth of *P. eous* could reduce the crystallinity only by 1 %, while *P. ostreatus* brought about 12 % reduction in cellulose crystallinity after 70 days of growth.

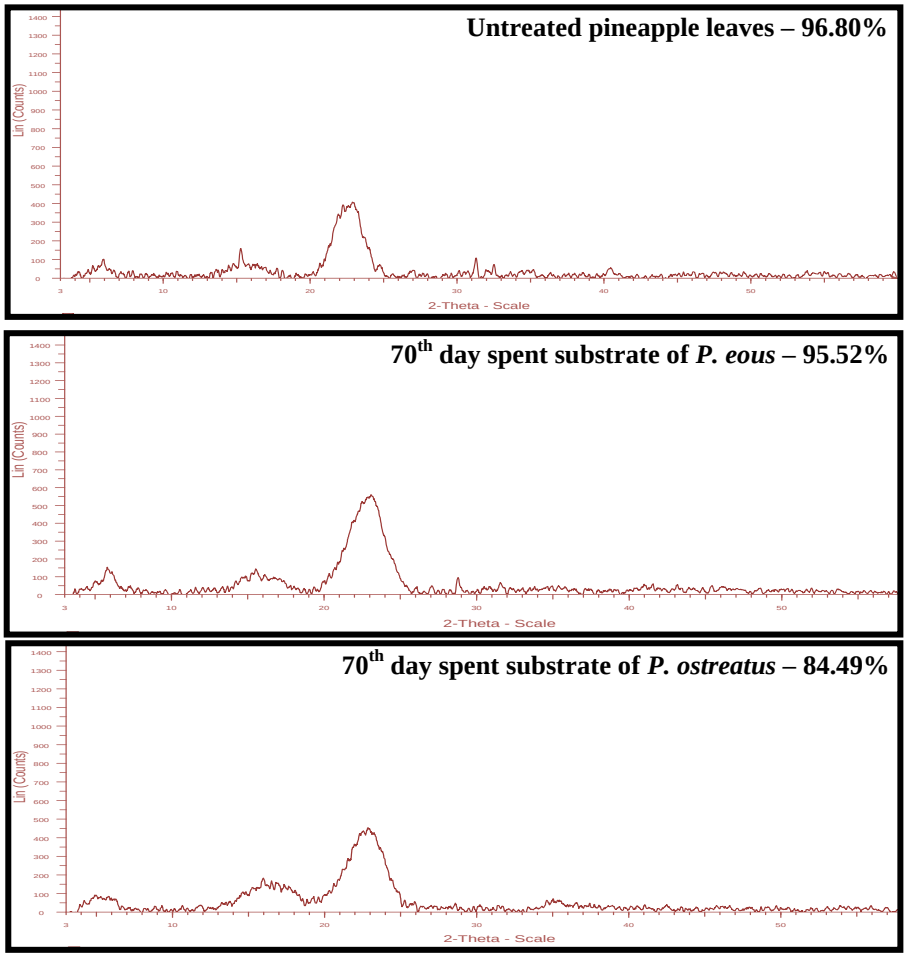


Figure 4.12 XRD pattern of pineapple leaves and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.2.3 Coconut leaflets

Crystallinity of cellulose in untreated coconut leaflets was found 97.70 % (Fig. 4.13). *P. eous* and *P. ostreatus* reduced the crystallinity of the substrate by 3% .

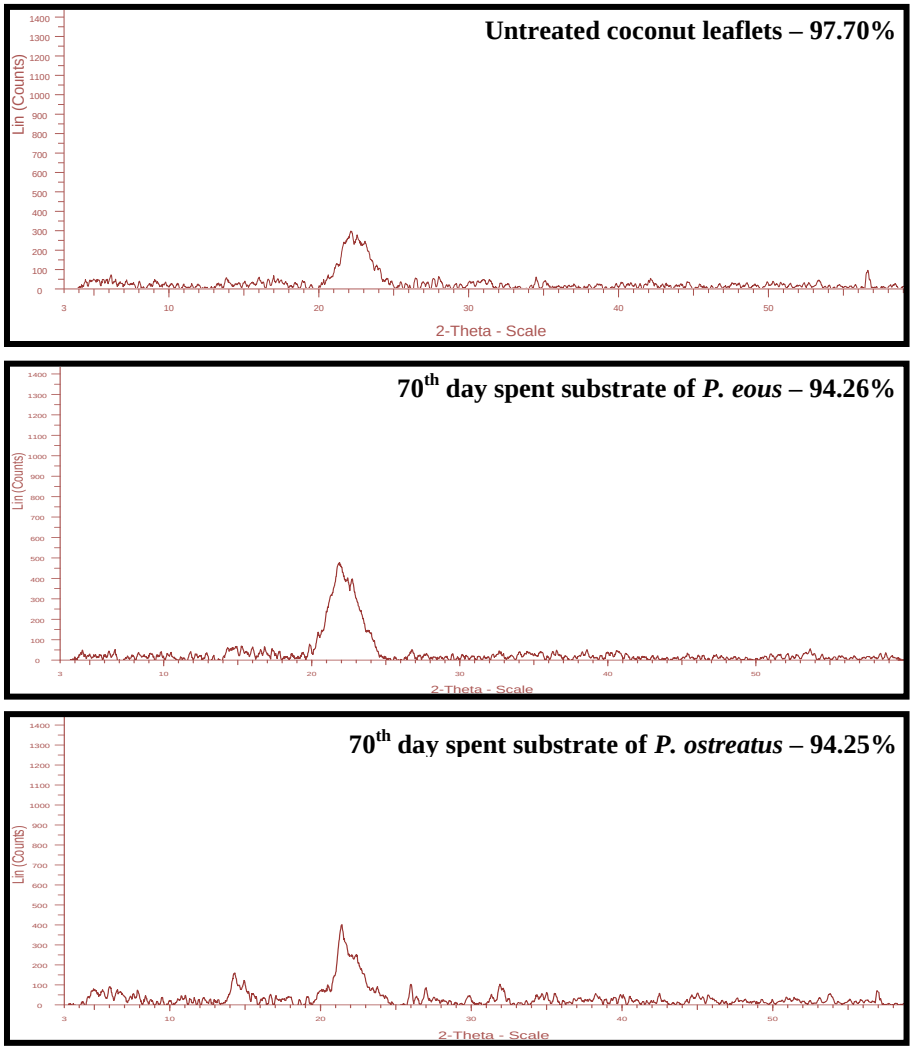


Figure 4.13 XRD pattern of coconut leaflets and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.3 Scanning Electron Microscopy

4.5.2.3.1 Banana pseudo stem

SEM images revealed the significant differences occurred in the fibre morphology due growth of *Pleurotus* sp. In the SEM image of untreated banana pseudo stem (Fig. 4.14), fibres were very smooth and intact in appearance. In the 70 day old spent substrates, the fibres were rough and uneven.

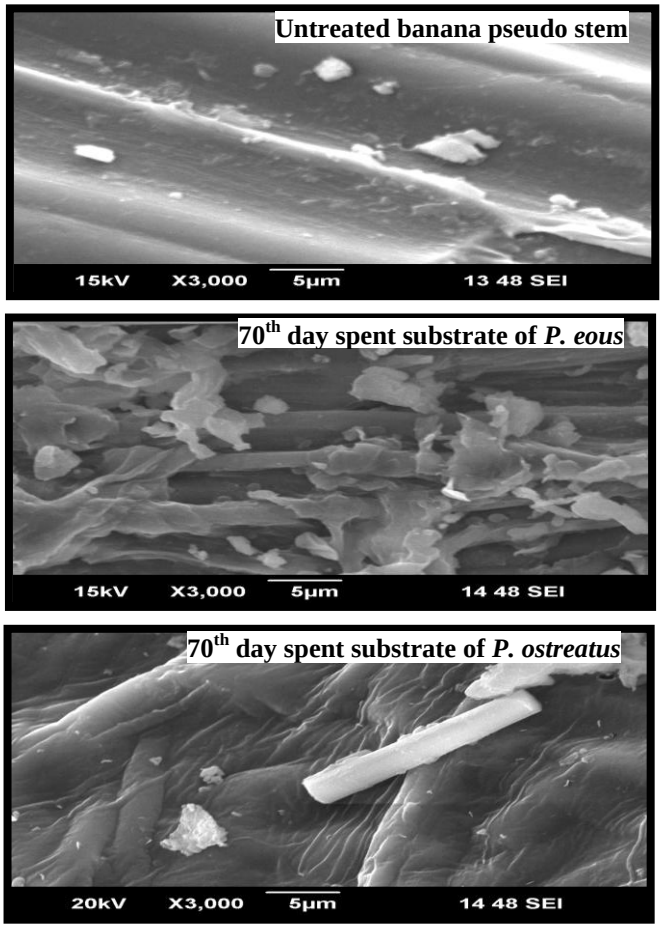


Figure 4.14 SEM image of banana pseudo stem and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.3.2 Pineapple leaves

Surface topography of untreated pineapple leaves was very irregular (Fig. 4.15). Subsequent to the growth of *Pleurotus* sp., leaf fibres were exposed.

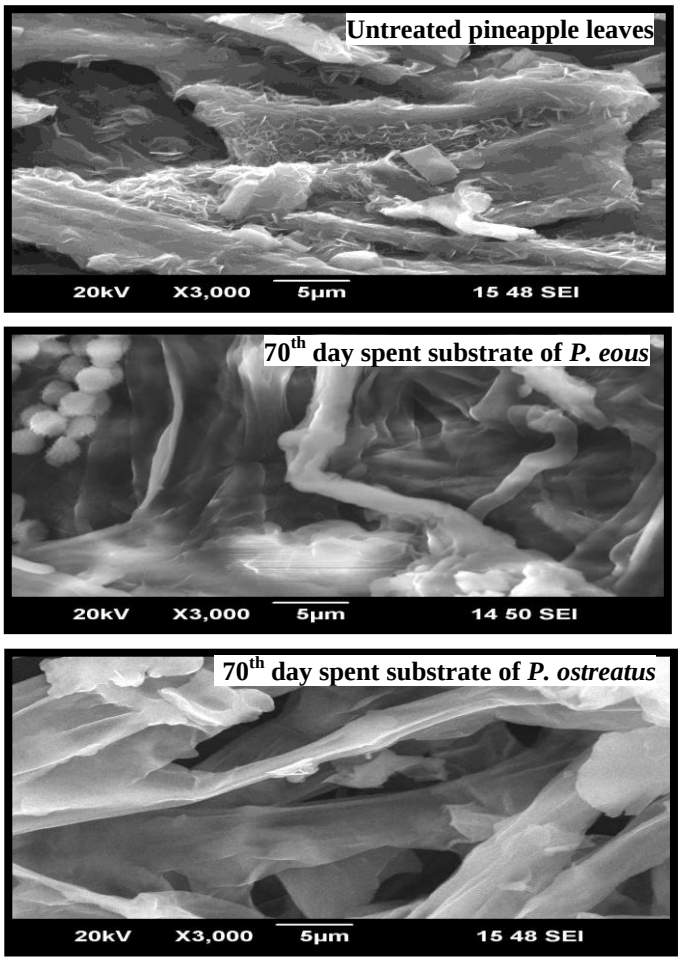


Figure 4.15 SEM image of pineapple leaves and the 70 day old spent substrates of *Pleurotus* sp.

4.5.2.3.3 Coconut leaflets

From the micrograph it was possible to observe that due to the growth of *Pleurotus* sp. on coconut leaflets, the tissues had partially disintegrated, while the surface of untreated coconut leaflets was to be found more intact (Fig. 4.16).

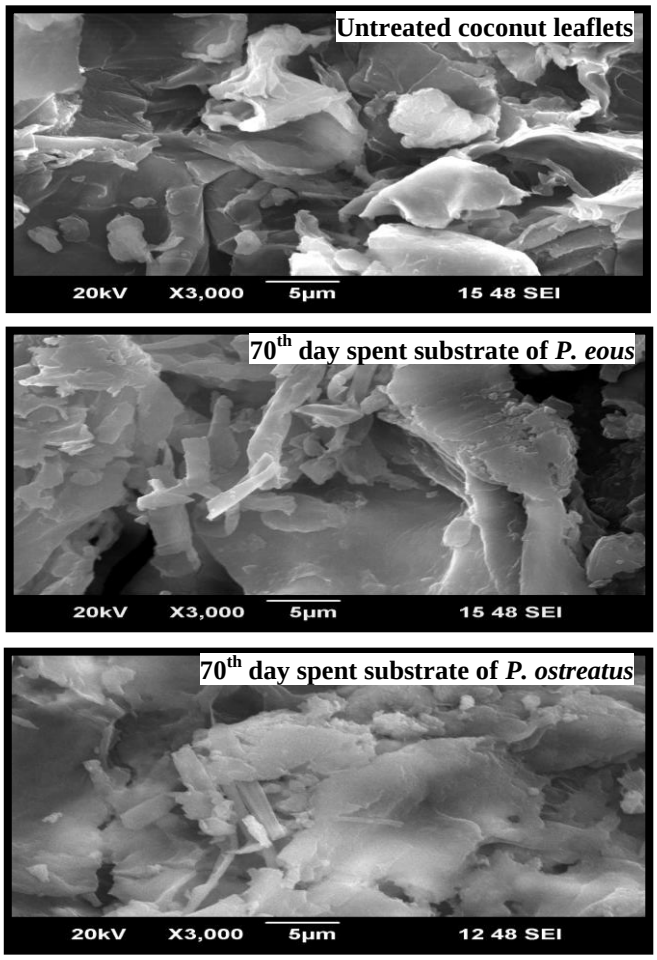
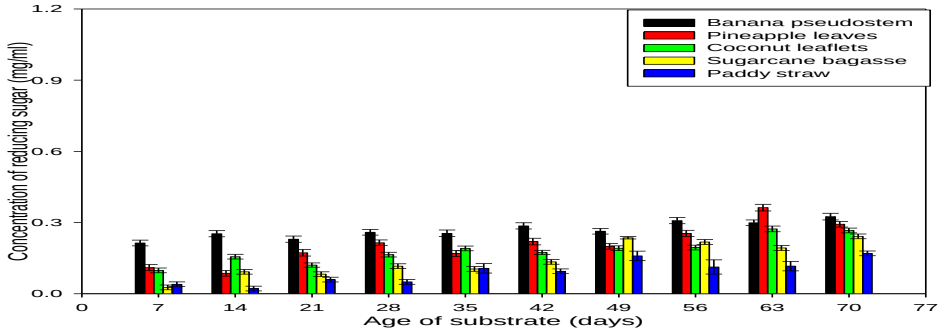


Figure 4.16 SEM image of coconut leaflets and the 70 day old spent substrates of *Pleurotus* sp.

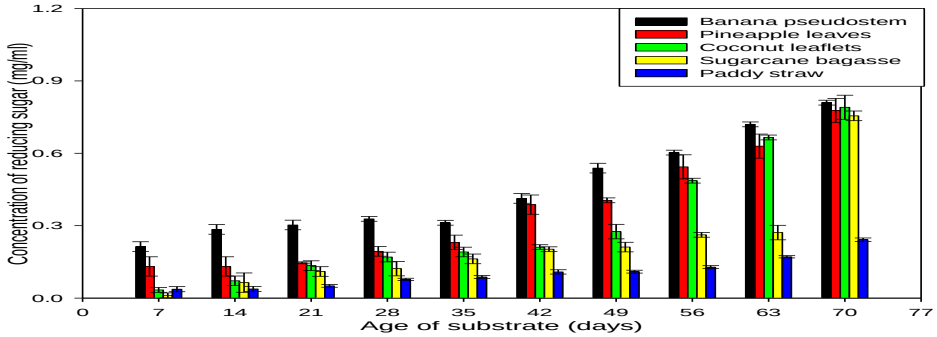
4.6 Use of *Pleurotus* sp. for pretreatment of lignocellulosic substrates for ethanol production

4.6.1 Yield of reducing sugars from substrate of *Pleurotus* sp.

The yield of reducing sugars from the substrate of *P. ostreatus* was two fold higher than that of *P. eous* (Fig.4.17). Concentration of reducing sugars increased in direct proportion to the days of incubation and was more in the spent mushroom substrate of both species. Both mushrooms released higher amount reducing sugars while growing on banana pseudo stem while paddy straw had the least amount of reducing sugars.



a) *Pleurotus eous*



b) *Pleurotus ostreatus*

Figure 4.17 Amount of reducing sugar in the substrate of *Pleurotus* sp.

[Note: Error bars represent ± standard deviation from a triplicate average].

4.6.2 Isolation and screening of microbial strains for fermentation of lignocellulosic substrates to ethanol

26 microbial strains were isolated from toddy obtained from Nadathara, Thrissur, while 14 strains were isolated from toddy obtained from Nedumbassery, Ernakulam and 14 from commercial yeast preparation. The isolates were screened for use in ethanol production based on their ethanol tolerance and sugar tolerance. Among the isolates from toddy obtained from Thrissur, strain BTPJ-1 was observed to be more tolerant to ethanol (16 %) when compared to other isolates (Tab. 4.10). From 14 isolates from toddy obtained from Nedumbassery, TYE-1 showed more tolerance to ethanol (12 %). Strain C-11 isolated from commercial yeast preparation, as also a bacterial strain *Zymomonas mobilis* obtained from NCIM, Pune, showed up to 10 % tolerance to ethanol.

Table 4.10 Ethanol tolerance of microbes isolated from toddy and commercial yeast preparation

Organism	Ethanol concentration				
	10 %	12 %	14 %	16 %	18%
Strain BTPJ-1	+	+	+	+	-
Strain TYE-1	+	+	-	-	-
Commercial yeast	+	-	-	-	-
Zymomonas mobilis	+	-	-	-	-

The strain BTPJ-1 was observed to be more tolerant to sugar (up to 250 mg / ml) when compared to other isolates (Tab. 4.11). Commercial yeast and TYE-1 showed same tolerance to sugar. *Zymomonas mobilis* was the least tolerant to sugar.

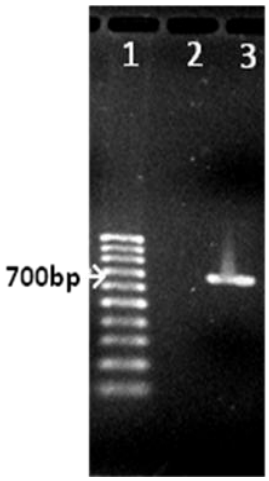
Table 4.11 Sugar tolerance of microbes isolated from toddy and commercial yeast preparation

Organism	Sugar concentration			
	50 mg/ml	**150 mg/ml**	**250 mg/ml**	**350 mg/ml**
BTPJ-1	+	+	+	-
TYE-1	+	+	-	-
Commercial yeast	+	+	-	-
Zymomonas mobilis	+	-	-	-

As the strain BTPJ-1 showed more tolerance to ethanol and sugar when compared to the other isolates, it was selected for use in the fermentation of spent substrates.

4.6.3 Genetic characterisation of microbe BTPJ-1 screened for ethanol production

Amplification of 18S rRNA gene with universal primers ITS1 and ITS4 yielded an amplicon of approximately 700 bp at an annealing temperature of 58 ºC (Fig. 4.18).



- Lane 1 – 100 bp ladder
- Lane 2 – Negative control
- Lane 3 – Amplicon

Figure 4.18 PCR amplified gene of BTPJ-1

A total of 779 bp of gene sequence was obtained from the amplicon. The sequence has been submitted to GenBank and is available as accession no. JN403044. The partial 18S rRNA gene sequence showed 99 % similarity to *Saccharomyces cerevisiae* sequences in the GenBank database <http://www.ncbi.nlm.nih.gov/blast>

Sequence obtained for the amplicon of BTPJ-1

TTTGAGGTCAACTTTAAGAACATTGTTCGCCTAGACGCTCTCTTCTTAT
CGATAACGTCCCAATACGCTCAGTATAAAAAAGATTAGCCGCAGTTGG
TAAAACCTAAAACGACCGTACTTGCATTATACCTCAAGCACGCAGAGA
AACCTCTCTTTGGAAAAAAAAACATCCAATGAAAAGGCCAGCAATTTC
AAGTTAACTCCAAAGAGTATCACTCACTACCAAACAGAATGTTTGAGA
AGGAAATGACGCTCAAACAGGCATGCCCCCTGGAATACCAAGGGGCG
CAATGTGCGTTCAAAGATTCGATGATTCACGGAATTCTGCAATTCACAT
TACGTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGAT

CCGTTGTTGAAAGTTTTTAATATTTTAAAATTTCCAGTTACGAAAATTC
TTGTTTTTGACAAAAATTTAATGAATAAATAAAATTGTTTGTGTTTGTT
ACCTCTGGGCCCCGATTGCTCGAATGCCCAAAGAAAAAGTTGCAAAGA
TATGAAAACTCCACAGTGTGTTGTATTGAAACGGTTTTAATTGTCCTAT
AACAAAAGCACAGAAATCTCTCACCGTTTGGAATAGCAAGAAAGAAA
CTTACAAGCCTAGCAAGACCGCGCACTTAAGCGCAGGCCCGGCTGGAC
TCTCCATCTCTTGTCTTCTTGCCCAGTAAAAGCTCTCATGCTCTTGCCAA
AACAAAAAAATCCATTTTCAAAATTATTAAATTTCTTTAATGATCCT-
TCCGCA

4.6.4 Ethanol yield from spent substrate of *Pleurotus* sp.

Highest yield of ethanol was obtained when spent substrate of 70th day was used for fermentation (Tab. 4.12 and 4.13). Spent substrate of *P. ostreatus* yielded more ethanol when compared to the substrate of *P. eous.* Among the five substrates, banana pseudo stem yielded more ethanol on fermentation. 70 day old substrate yielded 2-3 times more ethanol than the 28 day old substrate. Ethanol yield from banana pseudo stem was 3-6 times more than the yield from paddy straw. Sugarcane bagasse substrate of both species gave same yield of ethanol on fermentation. Ethanol produced from paddy straw substrate *P. ostreatus* was nearly 3 times more than the yield from *P. eous.* Among the five substrates, more ethanol was obtained from the fermentation of banana pseudo stem, pineapple leaves and coconut leaflets.

Table 4.12 Yield of ethanol from untreated substrate and from 28 day old (SS-28), 49 day old (SS-49) and 70 day old (SS-70) spent substrates obtained after growth of *Pleurotus eous*

Substrate	Ethanol yield from spent substrate of *P. eous* (g / L)		
	SS-28	**SS-49**	**SS-70**
Banana pseudo stem	29.56±2.03	54.54±3.03	67.63±4.01
Pineapple leaves	20.36±1.96	43.72±3.36	58.52±3.66
Coconut leaflets	18.57±0.98	38.89±2.09	55.56±3.35
Sugarcane bagasse	11.42±1.25	26.76±2.03	34.66±2.56
Paddy straw	4.32±0.25	9.53±1.23	11.23±1.25

Table 4.13 Yield of ethanol from untreated substrate and from 28 day old (SS-28), 49 day old (SS-49) and 70 day old (SS-70) spent substrates obtained after growth of *Pleurotus ostreatus*

Substrate	Ethanol yield from spent substrate of *P. ostreatus* (g / L)		
	SS-28	**SS-49**	**SS-70**
Banana pseudo stem	30.12±2.26	64.56±3.06	80.23±3.69
Pineapple leaves	23.32±2.13	50.63±3.12	67.12±3.25
Coconut leaflets	24.56±1.56	47.42±2.35	60.35±2.68
Sugarcane bagasse	11.58±1.02	26.89±2.56	35.42±2.78
Paddy straw	10.45±1.01	23.78±3.05	31.13±2.54

Biological pre-treatment using *Pleurotus* sp. could increase the ethanol yield from substrates by 4-9 times within 70 days. However, ethanol yield from chemically pre-treated substrate was more than that from biologically treated substrates (Tab. 4.14).

Table 4.14 Comparison of ethanol yield from untreated, chemically treated and 70 day old (SS-70) spent substrates obtained after growth of *Pleurotus* sp.

Substrate	Ethanol from untreated substrate (g/L)	Ethanol from treated substrate (g / L)		
		Chemically treated	Biologically treated	
		NaOH + Cellulase	SS-70 of *P. eous*	SS-70 of *P. ostreatus*
Banana pseudo stem	10±1.02	90±3.36	67.63±4.01	80.23±3.69
Pineapple leaves	8±0.68	75±3.12	58.52±3.66	67.12±3.25
Coconut leaflets	7±0.56	70±2.95	55.56±3.35	60.35±2.68
Sugarcane bagasse	4±0.23	52±2.05	34.66±2.56	35.42±2.78
Paddy straw	3±0.31	40±2.13	11.23±1.25	31.13±2.54

Table 4.15 Correlation analysis of reducing sugar and ethanol obtained from biologically treated substrates of *Pleurotus eous*

Age of substrate (in days)	Substrate	Reducing sugar (mg/ml)	Ethanol (g/L)	Correlation coefficient
28	Banana pseudo stem	0.259	29.56	**0.85**
	Pineapple leaves	0.2149	20.36	
	Coconut leaflets	0.1649	18.57	
	Sugarcane bagasse	0.1162	11.42	
	Paddy straw	0.0499	4.32	
49	Banana pseudo stem	0.2635	54.54	
	Pineapple leaves	0.1997	43.72	
	Coconut leaflets	0.1918	38.89	
	Sugarcane bagasse	0.2358	26.76	
	Paddy straw	0.1594	9.5	
70	Banana pseudo stem	0.325	67.63	
	Pineapple leaves	0.293	58.52	
	Coconut leaflets	0.2667	55.56	
	Sugarcane bagasse	0.2418	34.66	
	Paddy straw	0.1701	11.23	

Table 4.16 Correlation analysis of reducing sugar and ethanol obtained from biologically treated substrates of *Pleurotus ostreatus*

Age of substrate (in days)	Substrate	Reducing sugar (mg/ml)	Ethanol (g/L)	Correlation coefficient
28	Banana pseudo stem	0.3283	30.12	**0.84**
	Pineapple leaves	0.1937	23.32	
	Coconut leaflets	0.1701	24.56	
	Sugarcane bagasse	0.1221	11.58	
	Paddy straw	0.0776	10.45	
49	Banana pseudo stem	0.5385	64.56	
	Pineapple leaves	0.4051	50.63	
	Coconut leaflets	0.2756	47.42	
	Sugarcane bagasse	0.2114	26.89	
	Paddy straw	0.1095	23.78	
70	Banana pseudo stem	0.8101	80.23	
	Pineapple leaves	0.7773	67.12	
	Coconut leaflets	0.7907	60.35	
	Sugarcane bagasse	0.7552	35.42	
	Paddy straw	0.2418	31.13	

Statistical analysis indicated that the reducing sugar present in the spent mushroom substrates is positively correlated (directly proportional) to the yield of ethanol. Correlation between the two variables is 0.85 and 0.84 for the substrates of *P. eous* and *P. ostreatus* respectively (Tab. 4.15 and 4.16)

4.7 Use of spent substrate of *Pleurotus* sp. for degradation of phenol

P. eous showed maximum LiP and laccase production on 35th and 42nd day of cultivation, respectively. *P. ostreatus* gave more LiP and laccase activity on 56th and 35th day, respectively. Hence, spent substrate of respective days were analysed for its utilisation in the degradation of phenol. Maximum reduction in the phenol concentration by one day incubation was given by the 35 day old spent substrate of *P. ostreatus* (48 %) (Tab. 4.17) followed by the 42 day old spent substrate of *P. eous* (47 %). Seven days incubation could bring about 51 % reduction in the concentration of phenol, when 35 day old spent substrate of *P. ostreatus* was used, while 42 day old spent substrate of *P. eous* brought about 50 % reduction.

Table 4.17 Reduction in the concentration of phenol by incubating the sample with spent substrate of *Pleurotus* sp.

Organism	Age of SMS (in days)	Initial phenol conc. (mg/l)	Phenol content after 1 day incubation (mg/l)	Red. in phenol content (%)	Phenol content after 7 day incubation (mg/l)	Red. in phenol content (%)
P. eous	35	12281.7 ± 23	6820.42 ± 35.15	44.46	6439.76 ± 35.15	47.56
	42		6470.50 ± 79.03	47.32	6107.71± 105.5	50.26
P. ostreatus	35		6364.62 ± 23.4	48.17	5999.90 ± 23.40	51.14
	56		6770.70 ± 82.03	44.87	6331.47 ± 70.31	48.44

Chapter 5

DISCUSSION

Pleurotus eous and *Pleurotus ostreatus* used in this study are edible oyster mushrooms of commercial importance. Potato Dextrose Agar (PDA) medium, which is generally used for maintaining fungal culture by investigators, was used here as the medium for maintaining mushroom culture. Culture maintenance, spawn production and mushroom cultivation were carried out at 28 °C, which was observed as the temperature suitable for the growth of *Pleurotus* sp.

5.1 Growth of *Pleurotus* sp. on lignocellulosic substrates

Pleurotus spp. are reported to be efficient colonizers and degraders of lignocelluloses (Rajarathnam and Banu, 1989) and various lignocellulosic substrates are used to support the growth, development and fruiting during cultivation of oyster mushrooms. Paddy straw is the most widely used substrate for oyster mushroom cultivation in many parts of South India. However, the area under paddy cultivation is decreasing in Kerala and in coastal Karnataka. Non-availability and cost of paddy straw are major constraints in its use for oyster mushroom cultivation. In the present study, in addition to paddy straw substrate, mushrooms were cultivated on four lignocellulosic substrates, which were inexpensive and abundantly available throughout the year - banana pseudo stem, pineapple leaves, coconut leaflets and sugarcane bagasse.

5.2 Vegetative phase of growth of *Pleurotus* sp. on lignocellulosic substrates

In the life cycle of oyster mushrooms two phases are observed: the vegetative phase / spawn run phase and the reproductive phase. After inoculation of the mushroom bed, the mycelia showed a lag phase of about a week, after which the mycelia grew profusely from the spawn and permeated the substrate. Spawn run period was found to be different for each substrate, attributable to variation in the chemical composition and carbon: nitrogen ratio of substrates (Bhatti et al., 1987). Carbon and nitrogen are both needed for the growth of mushrooms which utilise about 30 parts of carbon for every part of nitrogen (Waksman and Cordon, 1938). An excess of one or the other of these elements during growth causes undesirable results. If carbon is in excess, several generations of organisms are needed to consume the additional carbon thereby extending the time necessary for spawn run and subsequent decomposition of the substrate. If nitrogen is in excess, all the carbon is consumed and the extra nitrogen is released as ammonia (Gotaas, 1956; Fuller et al., 1964).

Physical properties and morphological characteristics of substrates also affected the mycelial run of mushrooms. Higher rate of mycelium run was observed in paddy straw, when compared to the other substrates studied. This is probably due to the presence of an optimum proportion of cellulose, hemicellulose, lignin and pectin, and the physical nature including the high porosity and aeration in paddy straw (Saxena and Rai, 1992; Salmones et al., 1999). Banana pseudo stem showed the lowest mycelium run rate, and showed poor and patchy growth. This may be attributed to the presence of different kinds of poly phenolic substances in banana pseudo stem (Wang et al., 1999). Stoilova et al. (2006) reported that, phenolic groups lowered the growth of different types of microorganisms. Nevertheless, some phenolic compounds stimulated the production of lignin degrading enzymes. Banana pseudo stem is rich in nutrients, but it retains more humidity than the other

substrates. Microbes require sufficient water for metabolism to proceed. But if the moisture content is high, the water will displace the air in the voids of the substrate, causing anaerobic conditions (Gotaas, 1956). This perhaps is another factor contributing to poor mycelial run on banana pseudo stem. Among the substrates tested, coconut leaflets were found to be resistant to contamination, probably because of the high lignin content (40 %). Sugarcane bagasse was found in this study to be more prone to bacterial and fungal contamination than the other substrates tested. According to Kathe and Balasubramanya (1996), this could be due to partial breakdown of cellulose and hemicellulose by the oyster mushrooms, thus making them available to competitors. The presence of contaminants could compete with mushrooms for space, nutrients, as well as causing chemical alteration of the substrate, which hinders mushroom development (Chang and Miles, 1992)

5.3 Reproductive phase of growth of *Pleurotus* sp. on lignocellulosic substrates

During reproductive phase of the mushroom, primodia were formed and developed into oyster shaped fruiting bodies. To switch from vegetative phase to reproductive phase, mycelial tips should be retarded from growth. This was done by regulating the environmental factors like temperature, relative humidity and carbon dioxide, which are known to influence the development of mushroom fruiting bodies. A rise in the temperature inside the growing room would decrease relative humidity. Higher temperatures would also promote higher rate of metabolism and respiration in the fruiting bodies resulting in higher carbon dioxide production. This in turn would inhibit the development of mushroom fruiting bodies (Kang et al., 2004). In the initial stages of growth, the mushroom beds were incubated in dark in sealed poly bags. Increase in carbon dioxide levels within the bags suppressed fruiting until the mycelium completely penetrated the

substrate. Thereafter the mushroom beds were transferred from dark to diffused light and the bags were slit for fresh air and proper formation of fruiting bodies.
On paddy straw the spawn run period was less (10 - 12 days) and number of fruiting bodies produced was more (15 - 20), while on other substrates spawn run period was more (14 - 16 days), but fruiting bodies produced were fewer in number (2 - 5) and larger in size. According to Chang (2004) nutrient content of substrate affects the growth and formation of fruiting bodies of *Pleurotus* sp. Shah et al., (2004) reported highest yield on rice straw possibly due to comparatively better availability of nitrogen, carbon and minerals from substrate. Higher number of effective fruiting bodies may be due to the presence of glucose, fructose and trehalose in the substrate (Kitamoto et al., 1995). Poppe (2000) found that Indole Acetic Acid (IAA) increased the number of fruiting body of mushroom.

The crop of oyster mushroom is harvested in mainly three flushes, while the maximum yield is obtained in the first flush. In both Pleurotus strains studied, first flush gave the highest mean yield of 140 g, next flush gave the second highest mean yield of 100 g and third flush gave lowest mean yield of 20 g. Yields similar to this were reported by Iqbal et al. (2005) and Shah et al. (2004). In general, the number of fruit bodies recorded per flush decreased after each harvest indicating that the nature and amount of nitrogen available in a substrate after each flush influences the degree of cellulose degradation which in turn affects the yield (Zadrazil and Brunnert, 1980). Variable biological efficiency (BE) was reported when different lignocellulosic materials were used as substrates for cultivation. Highest BE was reported on paddy straw. The probable reason for higher yields of *Pleurotus* sp. on paddy straw may be that the essential nutrients required for the mushroom spawn run and fructification were supplied by paddy straw by rapid decomposition compared to other substrates. Kaya et al. (2000) reported that the yield of the mushroom was directly related to the spread of mycelium in the

substrate. This study also showed that the mycelial run and the physical and chemical properties of substrates influenced the yield and BE.

5.4 pH of mushroom bed during growth of *Pleurotus* sp. on different lignocellulosic substrates

All the substrates were acidic in nature (pH 5-5.5) before the inoculation of the mushroom bed. After inoculation, mushroom went through a lag phase for several days and no change in pH was observed for first 7 days, in the 5 lignocellulosic substrates. Several workers have observed that if the initial pH of the substrate is low, there will be an initial lag in the spawn run. After one week mycelia began to grow profusely. During the later stage (7 - 14 days) of vegetative phase, the substrate remained acidic due to the decomposition products of the easily attacked components of the substrate such as sugars and fats being simple acids (Gray, 1967). Later the substrate becomes mildly alkaline due to the production of ammonia.

5.5 Assays and estimations

Pleurotus sp. accomplish the enzymatic degradation of lignocellulosic biomass into simpler soluble compounds, by elaborating various hydrolyzing (cellulase, xylanase and pectinase) and oxidizing (ligninase) enzymes (Yolisa, 1997). Mushrooms degrade lignocellulosic polysaccharides with the help of hydrolases, which play a crucial role in supplying the culture with carbon and energy for extensive substrate colonisation and fruiting body formation and also providing microorganisms with materials for their biosynthetic activity. In the present study, it was observed that, the progressive breakdown of cellulose, hemicellulose, lignin and pectin was correlated with apparent increase in the activities of cellulase, xylanase, pectinase and ligninases, respectively.

The activity of the lignocellulolytic enzymes varied significantly depending upon the proportion of lignocellulolsic materials present in the substrate. For instance, both mushroom species produced higher amounts of cellulase when banana pseudo stem was used as substrate, probably due to the high content of cellulosic material present in it. During spawn run phase of cultivation, decomposition of plant material is quite rapid, although materials that are easily converted are preferentially degraded. Easily transformed materials include carbohydrates such as sugars and starches (Keller, 1996; Gray, 1967; Braun, 2008). As the easily decomposed material is being used up, the microbes begin to attack the more resistant parts of the substrate, such as cellulose and lignins (Fuller et al., 1964). Metabolism of all the major components is integrated and interdependent. Hemicelluloses must be degraded, at least in part, before the cellulose in plant cell walls can be effectively degraded (Blackwell, 1992). Also, metabolism of the cellulose and hemicellulose provide the energy and the hydrogen peroxide needed to degrade lignin. The lignin-degrading enzymes are inducible enzymes, and their production is dependent on the varying concentration of the accompanying materials within the lignocellulosic substrates (Muhammad et al., 2006). It has also been reported that different enzymes peaked at different fermentation periods and declined subsequently, probably due to the inactivation and / or degradation of these enzymes (Mandels and Sternberg 1976). That enzymes are not uniformly secreted at different stages of mycelial growth was also reported by Ruel et al. (1994).

5.6 Production of cellulases and degradation of cellulose by *Pleurotus* sp. growing on lignocellulosic substrates

Cellulose-rich organic materials were reported to be good substrates for cultivation of *Pleurotus* sp. (Sivaprakasam et al., 1986). The enzymatic degradation of cellulose is a complex process that requires the participation of at

least 3 types of cellulolytic activity: exo-β-l,4-glucanase, endo- β-1,4-glucanase, and β-glucosidase. Exo- and endoglucanase act synergistically to produce cellobiose, which is then degraded into glucose by β-glucosidase (Enari and Markkanen 1977). Among the microorganisms, fungi have been studied extensively because the elongated hyphae create mechanical pressure on the cellulose structure of plant cell walls triggering cellulase synthesis in the hyphae (Schwarz 2001). Cellulase converts cellulose into fermentable sugars (Mane et al. 2007). *Pleurotus* sp. produced cellulase for the degradation of cellulose, during all the phases of cultivation. During spawn run, the presence of hexose sugars is crucial for the formation of mycelium. In the present study, between vegetative phase and reproductive phase (21st day), a reduction in the production of cellulase was recorded when the substrates were highly acidic (in case of *P. eous)* or highly alkaline (in the case *P. ostreatus)*. Degradation of cellulose and presence of glucose are important factors for fruiting body development (Kitamoto et al., 1995). Cellulase production was positively correlated with yield of sporophores as reported by Ramasamy and Kandaswamy (1976). Other storage carbohydrates such as mannitol, glycogen and trehalose are also degraded during fructification and supply a significant proportion of the carbon for growth of fruiting bodies (Wannet et al., 1998). Trehalose and mannitol are major sugars in mushroom fruiting bodies (Harada et al., 2004). In the present study, it was found that, cellulase production continued in the spent mushroom substrate probably because unutilised cellulose was still available in the substrate.

5.7 Production of xylanase and degradation of hemicellulose by *Pleurotus* sp. while growing on lignocellulosic substrates

In general, the fungi are capable of hydrolyzing xylan and forming oligosaccharides and some sugar monomers (xylose, arabinose, mannose, and others). Moyson and Verachtert (1991) reported that *Pleurotus pulmonarius* and

Pleurotus sajorcaju degraded hemicellulose simultaneously with lignin. Xylanase is an induction enzyme. *Pleurotus* sp. produced very low amount of xylanase when compared to cellulase, and appeared to utilise more hexoses instead of pentoses, during growth and development. Enzyme production pattern of the two *Pleurotus* sp. studied was totally different from each other probably because, neutral pH was more favourable for xylanase production. This contradicted the finding of Bajpai (1997) that acid pH levels (5.0-6.5) generally favoured fungal xylanase production. But Quinnghe et al., (2004) observed that the xylanase of *Pleurotus ostreatus* SYJ042 was stable at a pH range of 3.0-9.0. Studies conducted by Furlan et al., (1997) also demonstrated that *P. ostreatus* showed higher xylanase activity in pH 5.4, corresponding with higher mycelial growth in pH 5.0. In the present study, higher xylanase production was in *P. ostreatus*, whose optimum pH range for growth was 4.5-9. *P. eous* degraded more hemicellulose during fructification, when pH of the substrates was close to neutral pH, but *P. ostreatus* utilised very less amount of pentoses during fructification. Also, *P. eous* produced more xylanase while growing on banana pseudo stem and pineapple straw and the pH of these substrates were observed to be close to neutral pH (35^{th} day) when maximum xylanase activity was recorded. *Pleurotus* sp. produced very low amounts of xylanase while growing on paddy straw. This is in agreement with the results obtained by Techapun et al. (2003) who reported that paddy straw was not a good inducer of xylanase.

5.8 Production of pectinase and degradation of pectin by *Pleurotus* sp. while growing on lignocellulosic substrates

Pectinase activity, in the present experiments, was high compared to the other enzymes. As in the case of cellulase, *P. eous* produced pectinase during all the phases of cultivation, while *P. ostreatus* produced low amounts of pectin during fructification. Enzyme production increased with the mycelial run and peaked in

production during late spawn run period of both *Pleurotus* sp. and late fructification of *P. eous*, after which the level of enzyme declined rapidly in the substrate of *P. ostreatus*. Acidic pH was found to be favorable for pectinase production. *P.* eous, whose substrates were acidic in nature, were observed to produce more pectinase than *P. ostreatus*. But among the 5 substrates, *P. eous* and *P. ostreatus* produced more pectinase while growing on banana pseudo stem, possibly because of the higher pectin content (1.05 %); even though the coconut leaflets substrate was most acidic.

5.9 Production of ligninases and degradation of lignin by *Pleurotus* sp. growing on lignocellulosic substrates

Lignin biodegradation is a multistep process involving an unknown number of extracellular and intracellular enzymes and a number of their isoforms (Eriksson et al., 1990; Kirk and Farrell, 1987). The exact role of individual enzymes and their relative contribution to the degradative process is still not known. Dissolved lignin has been regarded as an effective inhibitor of cellulase (Berlin et al., 2006), since lignin when dissolved becomes available to microorganisms, which then switch to the lignin degrading metabolism to gain energy. Also, the cellulosic compounds present in the substrate acts as co-substrate for lignin degradation. *Pleurotus* spp. are known to produce more ligninases than cellulases. It is due to their powerful degrading capabilities towards various recalcitrant chemicals, these white rots and their lignin degrading enzymes have long been studied for biotechnical applications (Takano et al., 2001). The ligninolytic system comprises of manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Hataka, 1994; Higuchi, 2004). *Pleurotus* sp. can degrade lignin slowly under favorable conditions to form phenolic components such as resorcinol, guaicol and catechol and thus causing an oxidative shortening of side chain.

Earlier reports indicate that a low pH is preferred for the production of ligninolytic enzymes (Kirk and Farrell, 1987, Higuchi, 2004, Cullen, 1997). However in our results a low pH did not favour the production of ligninases. *P. ostreatus* which had an optimum growth pH in neutral/alkaline range, was observed to produce more ligninases than *P. eous*. *P. eous* produced more LiP and laccase during fructification and in the spent substrate respectively, when the substrates were close to neutral pH. Also, among the five substrates, the least acidic were banana pseudo stem and pineapple leaves in which the ligninase activity was higher. *P. ostreatus* produced more LiP in the spent substrate at neutral pH. Ligninase production was low when lignin content in the substrate was low as in the case of paddy straw.

Among the ligninases, both *Pleurotus* sp. produced more laccase than lignin peroxidase and only negligible amounts of MnP. Maximum laccase production was observed to be at a pH range of 4.0-9.0 which was also the optimum growth pH of *P. ostreatus*. Maximum laccase production was recorded during spawn run and fructification of *P. ostreatus*. This is in agreement with the finding that laccase activity appears to be regulated in association with the development of fruiting bodies (Leonard & Philips 1973; Philips & Leonard 1976; Wood et al., 1991; Leatham & Stahmann 1981). Increase in laccase activity during the vegetative phase until the appearance of fruiting bodies has also been reported in *Agaricus bisporus* (Wood et al., 1991) and *Lentinus edodes* (Leatham & Stahmann 1981). Copious amounts of laccase were found in the culture supernatants of *P. sajor-caju* (Buswell et al. 1996). In case of *P. eous*, maximum laccase activity was observed in the spent substrate.

On a practical level, laccase could be used as a morphogenetic marker. A rapid drop in its activity indicates the maximum anamorphic growth that has been

achieved. This would be useful to mushroom growers in timing the opening of their spawn-run bags for fruiting; maximum vegetative growth cannot be ascertained visually with a high degree of accuracy. The decline in activity of laccase when fruiting bodies develop might be due to the increase in cellulase activity which has remained at a low level during the vegetative phase (Turner, 1975; Wood et al., 1991). In the present study also it was observed that, laccase activity was less during fructification of *P. eous* culture.

5.10 Use of *Pleurotus* sp. for pretreatment of lignocellulosic substrates for fibre extraction

High cellulose and low lignin content of a plant fibre are necessary for its various applications (Das and Mukherjee, 2008). The tensile strength of the cellulose fibres is enormous, being the strongest known material with a theoretically estimated tensile strength of 7.5 GPa. Tensile strength is measured as the amount of tensile stress that the fibre can withstand, before it breaks, while elastic modulus shows the stretchability of the fibre without breaking. Tensile strength depends on the concentration of cellulose and lignin present in the fibre.

Among the untreated substrates, tensile strength was greater in banana fibres (10.23 MPa) and lowest in paddy straw (3.77 MPa). Cellulose content of banana pseudo stem was 71 %, and that of paddy straw was only 30 %. Also, lignin content of banana pseudo stem and paddy straw were 25 % and 15 % respectively. Although the lignin content of banana pseudo stem was more, it was stronger than paddy straw, possibly because of the high cellulose content. It was observed that, fibres from the substrate of *P. eous* were stronger, although the lignin content was more. This might be because cellulase was produced more by *P. ostreatus* and hence the cellulose content was less in its substrate. Among the spent substrate fibres, 49 day old fibres were stronger. This was possibly due to the appropriate

cellulose : lignin ratio present in these fibres. 28 day old fibres might be weaker due to high lignin content, while 70 day old fibres could be weaker due to low cellulose content.

Elastic modulus was shown more by 49 day old spent fibres which had more tensile strength and least strong were 70 day old spent substrate fibres. Also, fibres obtained from the spent substrate of *P. eous* showed higher elastic modulus when compared to the fibres of *P. ostreatus* substrate. Hence, cellulose : lignin ratio also appeared to influence the elastic modulus of fibres. The cell wall of plant fibres are made up of a net-like arrangement of cellulose fribils which strengthens the fibres. Lignin gives rigidity and mechanical resistance to the fibre. Its presence reduces the elastic properties of fibres than non-lignocellulosic fibres, ex. cotton. A decrease in pectin, hemicellulose, as well as low lignin content, improves the elasticity of the fibres. But, irrespective of treatment, elastic modulus was greater in fibres from coconut leaflets and least for sugarcane bagasse fibres. This might be due the differences in fibre cell length and also due to the length to breadth ratio of cells; parameters which were not taken into account in the present study.

Plant fibres are weaker than other synthetic fibres. Surface morphology, chemical and physical characteristics and large surface area could make plant fibres ideal for the production of many commercial products, if mixed with plastics or other synthetics. The objective is to combine two or more materials in such a way that a synergism between the components results in a new material that is much better than the individual components. One application of plant fibres is in the production of filters. Unmodified plant fibres can absorb heavy metal ions and oils. Applications could include the clean-up of polluted drinking water or industrial waste waters. Another use is in the manufacture of fibre-reinforced / fibre-cement composites. It can also be the application of fibre-polymer combinations. Also, plant fibres have advantages in terms of cost, weight, and

environmental factors when compared to similar combinations based on glass fibre reinforcement.

The main characteristics of most plant fibres are described in terms of their microscopic features, chemical composition, and physical properties (Olesen and Ferquist, 1996). Fourier Transform Infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM) revealed the changes in functional groups, surface morphology and cellulose crystallinity of untreated banana pseudo stem, pineapple leaves and coconut leaflets and the 70 day old spent substrates of *Pleurotus* sp. after growth on these substrates.

In the FTIR pattern, O=H stretch in the 3500-3300 cm^{-1} and C=O stretch in the range 1300-1200 cm^{-1} was given by all the untreated substrates and the spent substrates of *Pleurotus* sp. But the wider band was observed in the spent substrates of *Pleurotus* sp.. Among the spent substrates, substrate of *P. ostreatus* gave larger and sharper peak. This indicated that the growth of mushrooms of *Pleurotus* sp. could increase the alcoholic groups present in the untreated substrates. Aromatic ring stretch in the range 1600-1500 cm^{-1} and in the range 1100-1000 cm^{-1} indicated lignin monomers (guaiacyl and syringyl) and it was observed that *Pleurotus* sp. brought about degradation of lignin in the untreated substrate which resulted in an increase in the peak for lignin monomers in the spent substrate. Also a sharp C-H bend at 778.69 cm^{-1} was observed only in the untreated banana pseudo stem which indicated the phenyl rings in lignin. Peaks obtained for spent substrates in this range were very small due to the reduction in lignin content of substrate due to fungal growth. Similar peaks were observed for crystalline cellulose (range 1500-1400 cm^{-1}) in the untreated and spent substrate, irrespective of the treatment given. In coconut leaflets, peak obtained from the spent substrate of *P. eous* was even prominent. These results showed that 70 days of fungal growth could not reduce the crystallinity of cellulose. C=O vibration at

1750-1700 cm^{-1} for hemicellulose also was present in all the three substrates. But this vibration was more prominent in the spent substrates than the untreated substrate. This might be because, the removal of other three components (cellulose, lignin and pectin) due to enzymatic degradation, resulted in more amount of hemicellulose in the spent substrate. O=H vibrations in the range 1400-1350 cm^{-1}, 1070-1040 cm^{-1} were 900-700 cm^{-1} found prominent only in the spent substrates. This confirms the formation of sugars due to the degradation of polysaccharides present in the lignocellulosic substrates.

X-ray diffraction studies pointed out that the growth of *Pleurotus* sp. could not bring much reduction in the crystallinity index of cellulose. Maximum reduction in the cellulose crystallinity was reported due to the growth of *P. ostreatus* on pineapple leaves, for 70 days. Other than this, in all the other cases, only 1 - 4 % reduction in the cellulose crystallinity was observed due to fungal growth, which corroborates the results from FTIR spectroscopy. As discussed earlier, the existing literature indicates that the white rots of *Pleurotus* sp. degrade lignin more than cellulose. SEM images revealed the morphological changes that occurred in the substrate due to the growth of *Pleurotus* sp.. Fungal growth altered the biomass structure significantly. Untreated substrates were ordered and compact due to the well-organised cell structure. But the growth of oyster mushrooms released various hydrolysing and oxidising enzymes due to which the fibres were loosened and visibly separated or peeled off from the initial connected structure and hence, the ordered structure of the lignocellulosic substrates was destroyed. That changes in surface morphology of tissues, brought about by removal of lignin and hemicellulose, was reported by Zhao et al. (2008). Partial degradation of cellulose and the removal of pectin from the intercellular lamellae (which glued the cells together) also might have added to the deformed and disintegrated appearance.

5.11 Use of *Pleurotus* sp. for pretreatment of lignocellulosic substrates for ethanol production

Pleurotus spp. are efficient degraders of lignin present in the lignocellulosic materials. Also, during growth, they could reduce the hemicellulose and pectin content present in the substrate. But, although they produced cellulase, they could not bring much reduction in the crystallinity of cellulose. During the cultivation period, *P. ostreatus* produced more cellulase and xylanase than *P. eous*. Also, the yield of reducing sugars from the substrate of *P. ostreatus* was twofold higher than that of *P. eous*. Some sugars were absorbed by the fungus for their metabolism and the remaining sugars accumulated in the substrate. So the concentration of reducing sugars was more in the spent mushroom substrate of both species. *Pleurotus* sp. produced more cellulase and xylanase while growing on banana pseudo stem which yielded more reducing sugar than the other substrates. Paddy straw yielded very less reducing sugar.

Among the spent substrates, more ethanol was produced the fermentation of 70 day old substrate, possibly because of higher concentrations of reducing sugars in it. Spent substrates of 28^{th} and 49^{th} day yielded less amount of ethanol. Spent substrate of *P. ostreatus*, which contained more amount of reducing sugars, yielded more ethanol, when compared to the substrate of *P. eous*. Among the five substrates, banana pseudo stem yielded more ethanol on fermentation, and paddy straw yielded the least. These findings suggest that, the concentration of total reducing sugar is correlated with the quantity of ethanol produced. Statistical analysis confirmed that the above variables are positively correlated to each other. *Pleurotus* sp. provided efficient pre-treatment for the lignocellulosic substrates on which they grew, as they could increase the ethanol yield from substrates by 4 - 9 times within 70 days. But ethanol yield from spent substrates was less than that from chemically pre-treated substrate, possibly because of the cellulose crystallinity and the presence of non-fermentable sugars. It was reported that

sodium hydroxide pre-treatment is the most renowned method of increasing the digestibility of cellulosic materials (Gharpuray et al. 1983; Lynd et al. 2002). Also, 1 % NaOH was reported to be more effective for pre-treatment as harsh alkali treatment dissolves lignin instead of degrading it. Alkali treatment can release some cellulose and hemicellulose by hydrolyzing ester bonds between lignin and cellulose or hemicellulose and, thus, can improve the biodegradability of lignocellulosics.

5.12 Use of spent substrate of *Pleurotus* sp. for degradation of phenol

Spent substrates of *Pleurotus* sp. with high ligninase activity were utilised for bioremediation of phenol in the waste water sample. It was found that the extracellular ligninase enzymes present in the spent substrate of *Pleurotus* sp. could react with aqueous phenolic compounds to simply remove them from the aqueous phase. Literature shows that, among ligninases, laccases oxidize phenolic residues of lignin, whereas lignin peroxidases are effective in the oxidation of the non phenolic residues of the polymer. In the present study also the same was observed, although the two ligninases present in the spent substrate contributed together for the biodegradation of phenol, among the two enzymes, laccase was found to be more efficient in degrading phenol. From the results it appears that, spent substrate of *Pleurotus* sp. can be used effectively to remove phenol from industrial effluents, hence it assures up to 50 % degradation of phenol content within 7 days.

Chapter 6

SUMMARY AND CONCLUSION

Pleurotus eous and *Pleurotus ostreatus* used in this study were obtained respectively from Kerala Agricultural University, Thrissur and NCIM, Pune. Cultures were maintained on Potato Dextrose Agar (PDA) medium. Spawn was prepared on carrier medium consisting of *Sorghum vulgare* grains mixed with Calcium carbonate. Culture maintenance, spawn production and mushroom cultivation were done at 28 °C. *Pleurotus* sp. were cultivated on four lignocellulosic substrates other than paddy straw - banana pseudo stem, pineapple leaves, coconut leaflets and sugarcane bagasse – which were available throughout the year from local region at low cost. All the five substrates were found suitable for the cultivation of *P. eous* and *P. ostreatus*

During vegetative phase, spawn run rate of both species was found to vary on different substrates. Higher rate of mycelium run was observed on paddy straw. Banana pseudo stem gave the lowest mycelium run rate during the course of study. Factors such as chemical composition, physical and morphological properties, porosity and aeration of substrate appeared to influence the spawn run rate of the *Pleurotus* sp.. Among the substrates, sugarcane bagasse was found to be prone to contamination due to the high sugar content, while coconut leaflets with high lignin content were very resistant. During reproductive phase oyster shaped fruiting bodies were formed. High temperature, low humidity and increase in carbon dioxide had a negative impact on the development of fruiting bodies. The crop was harvested in three flushes. Biological efficiency (BE) of *Pleurotus* sp. was

influenced by the spawn run rate and was different on the five lignocellulosic materials. Highest BE was obtained on paddy straw.

pH of all the substrates were initially acidic, and due to the lag growth phase of the fungus no change was observed for the first one week. Various products of fungal metabolism kept the substrate of *P. eous* acidic throughout the cultivation, while the substrate of *P. ostreatus* changed from acidic to neutral or alkaline range. pH of spent substrates of both *Pleurotus* sp. remained constant.

Pleurotus sp. produced extracellular enzymes such as cellulase, xylanase, pectinase and ligninase (lignin peroxidase and laccase) while growing on different lignocellulosic substrates, and degraded cellulose, hemicellulose, pectin and lignin respectively. Both mushrooms showed higher enzyme activity while growing on banana pseudo stem and least activity on paddy straw. Hence degradation of the above said components happened more efficiently while growing on banana pseudo stem and was the least in paddy straw. Enzymes were secreted in different proportions during each stage of mushroom growth. Different enzymes peaked at different fermentation periods and declined subsequently. Activity of the enzymes appeared to depend upon the proportion of components present in the lignocellulosic substrate. Among the *Pleurotus* sp., *P. ostreatus* produced more enzymes than *P. eous*, except in the case of pectinase.

Pleurotus sp. produced cellulase during all the phases of cultivation as glucose is essential for the growth and fruiting of *Pleurotus* sp.. Cellulase production appeared to be correlated to content of cellulose in the selected substrates. Both species produced more cellulase while growing on banana pseudo stem in which the cellulose content is high. *P. eous* produced more cellulase in the final stages of spawn run, while cellulase production by

P. ostreatus was more in the initial stages. Between vegetative phase and reproductive phase, there was a reduction in the production of cellulase. Both species showed maximum cellulase production on 35th day. After harvest, although the formation of fruiting body stopped, cellulase production continued.

Pleurotus sp. produced very less amount of xylanase when compared to cellulase. Production pattern of xylanase for both *Pleurotus* sp. was totally different from each other probably because of the pH differences in their substrate**.** Neutral and alkaline pH were found to be more favorable for xylanase production. Production of xylanase by *P. ostreatus* was several fold higher than that of *P. eous*.

Production of pectinase by *Pleurotus* sp. was high when compared to the production of the other four enzymes. Acidic pH was more favorable for pectinase production, and *P. eous*, whose substrates were acidic in nature, showed more pectinase activity than *P. ostreatus* and the degradation of pectin by *P. eous* was twice that by *P. ostreatus*. Pectinase production was more influenced by the pectin content of the substrates than the pH of the substrates. Hence, although the coconut leaflets were more acidic, *Pleurotus* sp. produced more pectinase while growing on banana pseudo stem where the pectin content was higher than the other substrates.

Pleurotus sp. degraded lignin, the most recalcitrant component of plant material, due to their powerful ligninolytic system comprising of lignin peroxidase (LiP) and laccase. Among the two ligninases, both species produced more laccase than LiP. Alkaline pH was found favorable for the production of both ligninases.

Fibres of banana pseudo stem, pineapple leaves, coconut leaflets, sugarcane bagasse and paddy straw and spent substrate fibres obtained after the growth

of *Pleurotus* sp. on these substrates were analysed for their tensile strength and elastic modulus. In the untreated controls, tensile strength was higher in banana fibres and lowest in paddy straw, while elastic modulus was more for coconut leaflets fibres and least for sugarcane bagasse fibres. Among the fibres obtained from the spent substrate of *Pleurotus* sp., 49 day old spent substrate yielded fibres of more tensile strength and elastic modulus. Hence, they have several advantages over untreated substrate fibres and can be used for various applications if mixed with synthetic materials such as plastics or glass. Tensile strength and elastic modulus of fibres from 70 day old spent substrates were even lesser than the untreated fibres. Tensile strength was higher in the fibres from the spent substrate of *P. ostreatus*, but, elastic modulus was more for the fibres from spent substrate of *P. eous*. High cellulose and low lignin content was found necessary for a plant fibre for strength and elasticity. Cellulose and lignin content of the substrates and the ratio between them appeared to influence the tensile strength and elastic modulus of the fibres obtained from them.

Fourier Transform Infrared (FTIR) pattern, X-ray diffraction (XRD) pattern and Scanning Electron Microscopic (SEM) images of the untreated fibres and spent substrate fibres obtained after cultivation were studied. Growth of *Pleurotus* sp. increased the alcoholic groups present in the substrates, hence in the FTIR pattern, wider band for alcohol was observed in the spent substrate of *Pleurotus* sp., when compared to the untreated substrate. Peaks for the phenyl rings in lignin were very small in spent substrates, when compared to the untreated substrate which showed a reduction in lignin content of the substrate due to growth of *Pleurotus* sp.. Also, an increase in the peak for monomers of lignin in the spent substrate confirmed the degradation of lignin by *Pleurotus* sp.. O=H vibrations for sugars were prominent in the spent substrates which confirms the degradation of polysaccharides present in the substrates by *Pleurotus* sp..

Pleurotus sp. could not bring any change in the cellulose crystallinity of their substrates and peaks for crystalline cellulose were found similar in untreated substrate and spent substrates. X-ray diffraction studied also confirmed this finding. SEM images revealed that the growth of *Pleurotus* sp. partially disintegrated the substrate and the fibres got visibly separated from each other in comparison to the untreated substrates which were ordered and compact with well-organised structure.

As *Pleurotus* sp. produced cellulase during growth on lignocellulosic substrates, reducing sugar yield from their substrate was more than that from the untreated substrate. Hence, spent substrates were utilized for the production of ethanol, while untreated substrates and chemically pre-treated substrates were kept as control. Among the substrates, reducing sugar content was high in banana pseudo stem, but reducing sugar yield from paddy straw was low. For the fermentation of sugars, *Saccharomyces cerevisiae* (GenBank Accession no. JN403044) isolated from coconut toddy (obtained from Nadathara, Thrissur, Kerala) was used.

Cultivation of *Pleurotus* sp. increased the yield of ethanol from lignocellulosic substrates by 4-9 times, although the ethanol yield from spent substrates was less than that from chemically pre-treated substrate. 70 day old spent substrate yielded more ethanol than the other spent substrates. Among the five substrates, fermentation of banana pseudo stem yielded more ethanol and paddy straw yielded the least. Also, production of ethanol from the spent substrate of *P. ostreatus* was more than that of *P. eous*.

Spent substrate of *Pleurotus* sp. was found to be a good source of ligninases such as LiP and laccase. Ligninases can reduce the concentration of phenolic compounds in wastewater. Hence, the possibility of using spent substrates with high ligninase activity for degradation of phenol was tested.

Extracellular ligninases were observed to remove the phenolics from the sample waste water. Both the ligninases present in the spent substrate contributed to the degradation of phenol, but, laccase was found to be more efficient. Spent substrates of *Pleurotus* sp. assured up to 50 % reduction in phenol content in the waste water within 7 days.

To conclude, the present study investigates the production of extracellular enzymes cellulase, hemicellulase, pectinase and ligninase (LiP and Laccase) during growth and the spent substrate of two edible mushrooms *Pleurotus eous* and *Pleurotus ostreatus* in five lignocellulosic agriwaste substrates (viz. banana pseudo stem, pineapple leaves, coconut leaflets, sugarcane bagasse, paddy straw). The study establishes the potential to use cheap and locally available agriwaste as substrates for mushroom cultivation and the possibility for farmers to generate additional income by utilisation of agriwaste for mushroom cultivation. The study also establishes three possible uses for the spent mushroom substrate:

i. as a source of cheap plant fibre
ii. as feed stock for fermentation to ethanol for biofuel purposes, and
iii. as a bioremediation agent for removal of phenolic compounds from waste water

Chapter 7

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LIST OF PUBLICATIONS

Papers published in peer-reviewed journals

- **Jasmine Koshy** and Padma Nambisan (2011) Biopulping of paddy straw by *Pleurotus eous*, Advanced Biotech 11(1), 45 - 47, ISSN: 0973 - 0109
- **Jasmine Koshy** and Padma Nambisan (2012) Ethanol production from spent substrate of *Pleurotus eous*, International Journal of Applied Biology and Pharmaceutical Technology 3(1), 280 - 286, ISSN: 0976 - 4550
- **Jasmine Koshy** and Padma Nambisan (2012) Pretreatment of agricultural waste with *Pleurotus* sp. for ethanol production, International Journal of Plant, Animal and Environmental Sciences 2(2), ISSN: 2231 - 4490
- **Jasmine Koshy**, Neethu Chandran and Padma Nambisan (2012) Biodegradation of phenol using spent substrate of *Pleurotus* sp., World Journal of Pharmacy and Pharmaceutical Sciences 1(2), 656 - 661, ISSN: 2278 - 4357

Papers published in seminar proceedings

- **Jasmine Koshy,** Anjana Xavier and Padma Nambisan (2010) Bioconversion of spent mushroom substrate of *Pleurotus eous* to ethanol, International conference on Green Path to Sustainability - Prospects and Challenges, Assumption college, Changanassery, Kerala, ISBN: 978-81-907269-9-3
- **Jasmine Koshy**, Neethu Chandran and Padma Nambisan. (2010) Bioremediation of phenol using spent mushroom substrate of *Pleurotus eous*, 1st Kerala Women's Science Congress (KWSC), St. Theresa's college, Ernakulam.

Posters presented

- **Jasmine Koshy** and Padma Nambisan (2011), Pretreatment of lignocellulosic agriwaste by *Pleurotus* sp. for ethanol production, National Symposium on Emerging Trends in Biotechnology, Cochin University of Science and Technology (CUSAT) - Cochin, Ernakulam.
- **Jasmine Koshy**, Anjana Xavier and Padma Nambisan (2011) Production of bioethanol from spent mushroom substrate of *Pleurotus ostreatus*, conference by International Water Association (IWA) on Microbes in Wastewater & Waste Treatment, Bioremediation and Energy Production, Birla Institute of Technology and Science (BITS) - Pilani, Goa.
- **Jasmine Koshy**, Neethu Chandran and Padma Nambisan (2011), Biodegradation of phenol using spent mushroom substrate of *Pleurotus ostreatus*, conference by International Water Association (IWA) on Microbes in Wastewater & Waste Treatment, Bioremediation and Energy Production, Birla Institute of Technology and Science (BITS) - Pilani, Goa.
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