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Evaluation of some wood rotting fungi for bagasse pretreatment in ethanol production

A thesis submitted to the School of Graduate Studies, Addis Ababa University in partial fulfilment of the requirements for the Degree of Master of Science in Biotechnology

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List of abbreviations

CMCase	Carboxymethyl cellulase
DNS	3,5- dinitrosalicylic acid
GC	Gas chromatography
Lac	Laccase
LiP	Lignin peroxidase
MnP	Manganese peroxidase
mV/s	Millivolt per second
OD	Optical density
PDA	Potato dextrose agar
SSF	Solid state fermentation
U	Enzyme activity unit

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Abstract

Lignocellulosic biomass is a sustainable feedstock for ethanol production compared to other substrates. In the ethanol generation process, pretreatment makes cellulose more accessible to enzymatic hydrolysis by modification of the lignin barrier. Biological pretreatment employs selective rot fungi that preferentially degrade lignin with minimum loss of polysaccharides. The system is not well developed; but is advantageous over other pretreatments because reactions are enzymatic. These fungi produce ligninolytic enzymes, predominantly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) in different combinations. Studies on biological pretreatments have shown lignin weight loss and improvement in ethanol yield. However, only few well characterised white rots have been explored. The aim of this study was to evaluate the potential of 15 wood rotting fungi isolated from Ethiopia for pretreatment during 15 days solid state fermentation, using bagasse as lignocellulosic substrate. The production of ligninolytic enzymes by *Fomitiporia aethiopica*, *F. pseudopunctata*, *Fomitopsis carnea* and *Vanderbylia vicina* were reported for the first time in this study. The white rots, *F. aethiopica, Perenniporia tephropora, Inonotus* sp. and *Pleurotus sajor-caju*, were identified as relatively highly selective based on maximum and minimum productivity of ligninolytic and polysaccharide degrading enzymes, respectively. The pretreatment by the white rots caused ligninolysis and better cellulose digestibility was obtained with higher lignin loss. Among the selective degraders, *P. tephropora* caused the highest lignin loss (7.71%) and cellulose digestibility (29.44 %) after enzyme hydrolysis of the pretreated bagasse. This digestibility showed an improvement by 38.74 % in comparison with untreated bagasse. In addition to high MnP productivity (55.87 U/g), *P. tephropora* also produced high titers of Lac (79.65 U/g) in contrast to the other selective degraders that might have attributed to better lignin loss. The ethanol yield from fermentation of cellulase enzyme hydrolysed *P. tephropora* pretreated bagasse was 1.87 g/L, which was an improvement by 27.21 % compared with untreated bagasse (1.47 g/L). Therefore, *P. tephropora* pretreatment enhances ethanol production from bagasse through partial degradation of lignin, which improves the accessibility of cellulose to enzyme hydrolysis.

Key words: bagasse, ethanol, ligninolytic enzymes, pretreatment, rot fungi

1. Introduction

Ethanol has been used as a biofuel to provide an alternative energy source and thereby reduce petroleum consumption. Simple sugars and starchy biomass are serving as feedstocks for ethanol production. However, these substrates are in limited supply and constitute animal feed and human food (Sun and Cheng, 2002). There are growing interests to use lignocellulosic biomass as raw material. It is highly abundant, cheap and ethanol production process has very low net CO_2 emission as compared to other feedstocks (Tomas-Pejo *et al*., 2008). However, the process technology for ethanol generation from lignocellulosic biomass is complicated, energy consuming and is still under development. The major challenge is in the feedstock processing due to the structural arrangement of the fibre constituents. The cellulose, which is the major sugar reservoir, is trapped by a lignin wall that becomes a barrier to enzymatic hydrolysis. Thus, in the production process, the biomass requires pretreatment to remove lignin, increase the porosity and reduce the crystallinity of cellulose (Sanchez and Cardona, 2008).

There are different pretreatment systems, which are categorised as physical, physicochemical, chemical and biological. Biological pretreatment involves the use of primarily selective lignin degrading fungi that can remove lignin with minimum loss to polysaccharides (Itoh *et al*., 2003). Selective lignin degraders preferentially break down lignin and hemicelluloses and only degrade cellulose at later stages (Hakala, 2007). Biological pretreatment is carried out under mild reaction condition with few side reactions. The system has very low chemical consumption, less energy demand and less susceptibility to pressure and corrosion as compared to other pretreatments (Lee, 1997; Samsuri *et al*., 2008).

Selective degradation is the characteristics of some white rot fungal species. These fungi degrade lignin by the action of extracellular oxidative enzymes predominantly, lignin peroxidase, manganese peroxidase and laccase (Hakala, 2007). These enzymes are produced in different combinations by different fungi (Tuor *et al*., 1995). The mechanism of degradation is by oxidation of lignin to form cation radicals that initiate a non enzymatic chain reaction and eventually mineralize the polymer (Hakala, 2007).

Studies on biological pretreatment of different lignocellulosic biomasses obtained lignin loss with low degradation of polysaccharides, resulting in improvement in ethanol production (Itoh *et al*., 2003; Munoz *et al*., 2007; Samsuri *et al*., 2008; Bak *et al*., 2009). Although these studies show promising results, only few well characterised white rots species have been

explored for pretreatment. Thus, there is a need to assess the potential of different rot fungi in search for a better lignin degradation system that exposes cellulose without significant self-consumption.

Sugar cane bagasse can be used as a lignocellulosic biomass substrate for ethanol production. Bagasse is the fibre residue left over after mechanical extraction of the juice from cane stalks. It has high cellulose composition, 30- 43 % (Kadam, 2000), which makes it suitable for ethanol production (Buaban *et al*., 2010). Sugar factories burn the bagasse as fuel to generate steam and yet produce 15-25 % in excess. This production can be further increased if steam generators are improved to reduce consumption, facilitated by solar energy and if the bagasse is substituted with cane trash (Kadam, 2000). In the Ethiopian context, with the development of hydroelectric power projects, electric power supply might be surplus in the near future. Thus, there is a possibility for sugar industries to substitute the bagasse fuel with electrical energy considering the higher demand for the liquid fuel. With surplus supply of bagasse and integration of appropriate ethanol process technology with the existing molasses to ethanol fermentors in the sugar industry, ethanol production from bagasse could be feasible. Like any other lignocellulosic biomasses, accessibility of polysaccharides in the bagasse biomass is limited. Therefore, there is a need to explore different pretreatment system to develop bagasse to ethanol conversion process.

2. Objectives

2.1. General objectives

The aim of this study was to evaluate the potential of some wood rotting fungi for pretreatment of bagasse in ethanol production.

2.2. Specific objectives

The specific objectives of this study were to:

a. determine the ligninolytic enzyme production profiles of 15 genera of wood rotting fungi isolated from Ethiopia during SSF of bagasse;
b. screen the wood rotting fungi for preferential ligninolysis based on maximum and minimum productivity of ligninolytic and polysaccharide degrading enzymes, respectively during pretreatment of bagasse under SSF conditions;
c. screen the selective degraders for effective ligninolysis based on lignin loss and improvement in cellulose digestibility of pretreated bagasse and
d. compare the ethanol yield from untreated bagasse and bagasse pretreated by the rot fungi showing the most effective ligninolysis.

3. Literature review

3.1. Ethanol as a biofuel

Evidences suggest that, half of the world's accessible petroleum oil reserve has been depleted. In the near future, oil supplies will be insufficient while the demand of growing economies such as China, India and other developing countries, continues to increase. Intensive use of petroleum oil is also contributing to green house gas emissions, which has had its influence on the global climate change over the years (Yang and Wyman, 2008).

About two third of the oil is used in transportation where, liquid fuels such as gasoline, diesel and jet fuel have been dominant. Thus, developing liquid fuels derived from other sustainable resource would significantly reduce petroleum consumption and green house gas emissions. Plant biomass is a viable feedstock as it is cheap and it can be converted to biofuels on a large scale. Liquid fuels can be produced from biomass by gasification to syngas for conversion to synthetic diesel; pyrolysis to oils; conversion of plant oil to biodiesel and fermentation of sugars to ethanol (Yang and Wyman, 2008).

Ethanol (ethyl alcohol, CH_3CH_2OH) is the most common liquid biofuel used in internal combustion engines. The physical and chemical properties of ethanol allow the biofuel to have advantages over gasoline. As ethanol is comprised of approximately 35 % oxygen, there is efficiency in combustion, reduction in carbon monoxide, NO_x and hydrocarbon emissions. It also has high octane number (108), which measures anti-knocking properties crucial for controlled combustion in cylinders. Despite these advantages, there are few setbacks, which include low energy yields (66 % of the energy generated from gasoline), corrosiveness, low vapour pressure (makes cold starts difficult), miscibility with water and toxicity to the ecosystems (Balat *et al*., 2008).

Ethanol has been blended with gasoline since 1980 for powering internal combustion engines. Brazil uses 24 % ethanol: gasoline blend while USA, Canada, Colombia, Thailand (Sanchez and Cardona, 2008) and Ethiopia (WIC, 2011) are using 10 % blends. Flexible fuel vehicles have also been developed that can run at higher ethanol concentrations, up to 85 % (Balat *et al*., 2008). In addition to transportation fuel, Ethiopia plans to develop stoves and other equipments, which operate on the combustion of ethanol (MOME, 2007).

3.2. Current processes in industrial production of ethanol

In the 1940s, a petrochemical process of hydrating ethylene was economically feasible for industrial production of ethanol in some nations. Oil was cheaper and biomass for microbial fermentation was not available. However, the rise in oil prices since 1970 forced the oil importing countries to revert to microbial fermentation process (Smith, 2004).

Plant biomasses serve as substrates for microbial fermentation. These include sucrose containing feedstock such as sugar cane, sugar beet and sweet sorghum; starchy materials including corn, wheat and barley grains and lignocellulosic biomass. The choice of feedstock is dependent on availability (affected by seasons and geographical location), price (which accounts for 1/3 of the production cost) (Glazer and Nikaido, 2007; Soccol *et al*., 2010) and the process technology required.

Industrial processes are well established for fermentation of simple sugars derived from cane, beet, grains and molasses. There is less cost in feedstock preparation as the sugars are readily available for yeast fermentation (Caylak, 1998). Industrial production from starchy biomass needs further processing as the polysaccharide cannot be fermented by yeast directly. Starch is composed of a water soluble fraction, amylose (20 %) and water- insoluble higher molecular weight fraction, amylopectin (80 %). To release the sugars, the starch is first cooked to make it soluble and susceptible to enzyme hydrolysis. α- amylase and glucoamylase enzymes liquefy the starch and catalyse the hydrolysis of the polymers, respectively (Glazer and Nikaido, 2007).

Although the process technologies have been established for fermenting simple sugars and starchy substrates, these feedstocks are found in insufficient supply as they are human food and or animal feed. Consequently, there are controversies over their use in fuel generation (Sun and Cheng, 2002; Tomas-Pejo *et al*., 2008).

3.3. Ethanol production from lignocellulosic biomass

An alternative and promising feedstock for ethanol production is lignocellulosic biomass. It is the most abundant biopolymer on earth accounting to about 50 % of the world's biomass with an estimated annual production of 10 – 50 billion tons (Sanchez and Cardona, 2008). Thus, there is sustainable supply of the raw material at costs lower than conventional agricultural feedstocks (Tomas-Pejo *et al.*, 2008). The production process of ethanol has nearly zero net carbon dioxide emission if there is sustainable replanting that balances the amount of carbon

dioxide released from combustion (Samsuri *et al*., 2008; Tomas-Pejo *et al*., 2008). In comparison with grain derived ethanol, there is lower requirement of fossil fuel for energy because, heat for boilers can be generated from burning the lignin byproduct (Elnashaie and Garhyan, 2004).

Different lignocellulosic biomasses have been studied for ethanol production and they are divided in to 6 groups. These include: crop residues (corn stover, wheat straw, rice straw, barley straw, sweet sorghum bagasse, cane bagasse, olive stones and pulp); hardwood (aspen, poplar); softwood (pine, spruce); cellulose wastes (newsprint, office paper waste, recycled paper sludge); herbaceous biomass (alfalfa hay, switch grass, reed canary grass, coastal Bermuda grass, timothy grass) and municipal solid waste (house hold garbage, paper products and food processing wastes) (Sanchez and Cardona, 2008; Wyman, 2008).

In the ethanol generation process, the lignocellulosic biomass is first reduced in size by grinding (for agricultural residues) or chipping (for wood feedstock). A pretreatment step follows to make cellulose more accessible for enzymatic attack. This procedure also breaks down hemicelluloses to release the constituent hexose and pentose sugars and small proportion of the cellulose to glucose. The cellulose is then exposed to cellulase enzymes, to release glucose for yeast fermentation. Pentose sugars such as xylose from hemicelluloses hydrolysis are also fermented by *Zymomonas mobilis* or other genetically engineered bacteria. The fermentation product (ethanol broth) is purified to separate the ethanol from the broth and dehydrated to remove water. Lignin and other by products are used to generate the electricity required for the production processes. Since the energy from lignin combustion is excessive, it can be sold to support the process economics (USDOE, 2009).

3.4. Pretreatment of lignocellulosic biomass

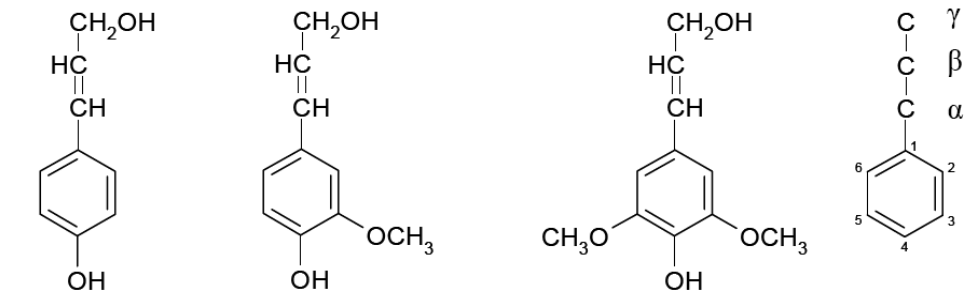
3.4.1. Structural composition and the need for pretreatment

The structural arrangement of the polysaccharides in the secondary cell wall of lignocellulosic biomasses presents a major challenge in ethanol production. Lignocellulosic biomasses are composed of about 40 – 50 % cellulose and 20 – 30 % hemicelluloses with lesser composition of lignin and other compounds such as sugars, oils and minerals (Wyman, 2008).

Cellulose is a polymer of cellobiose, D- glucopyranosyl-β- 1, 4- D- glucopyranose, which is the major carbon source for fermentation. The cellulose in wood has about 10,000 glycosyl

units in the chain, forming long bundle of molecules (fibrils). The chains are stabilized by numerous strong intermolecular hydrogen bonds between adjacent hydroxyl groups. The cellulosic material has crystalline domains as well as less ordered, amorphous regions that are targets for catalytic attacks. The hemicelluloses are highly branched heteropolymers of xylose, glucose, mannose, galactose, arabinose and uronic acids. The polymer is named after the predominant sugar as mannan, xylan or galactan. The five carbon and six carbon sugars are linked through 1,3, 1,6 and 1,4 glycosidic bond and could be acetylated. The sugars form loose hydrophilic structures that link cellulose to lignin (Bon and Ferrara, 2007). As compared to cellulose, hemicelluloses have shorter chains with numerous branching, which makes its hydrolysis easier. Softwood hemicelluloses are composed mostly of mannose while xylose is predominant in hard wood and herbaceous plants (Tomas-Pejo *et al*., 2008).

Lignin is an aromatic, amorphous and three dimensional polyphenolic network. It is biosynthesised by polymerisation of phenyl propanoid precursors, which include *p*-coumaryl, coniferyl and sinapyl alcohols. The precursors consist of an aromatic ring and a three carbon side chain (Fig. 1). They form three types of subunits, which include the hydroxyphenol (non-methoxylated), guaiacyl (monomethoxylated) and syringyl (dimethoxylated). These subunits polymerise by forming variable ether and C-C bonds in a random fashion by free radical reaction to form the lignin structure (Fig. 2). In wood, the lignin is connected to the carboxyl group of the hemicelluloses through ether and benzyl ether linkages (Lankinen, 2004). The complex and non-repetitive irregular structure makes lignin resistant to enzymatic attack (Webster and Weber, 2007). Thus, it provides protection for the plant against microbial degradation of the polysaccharides (Sanchez and Cardona, 2008).



p-coumaryl alcohol coniferyl alcohol sinapyl alcohol

Fig. 1. The structures of the phenyl propanoid precursors of lignin polymers

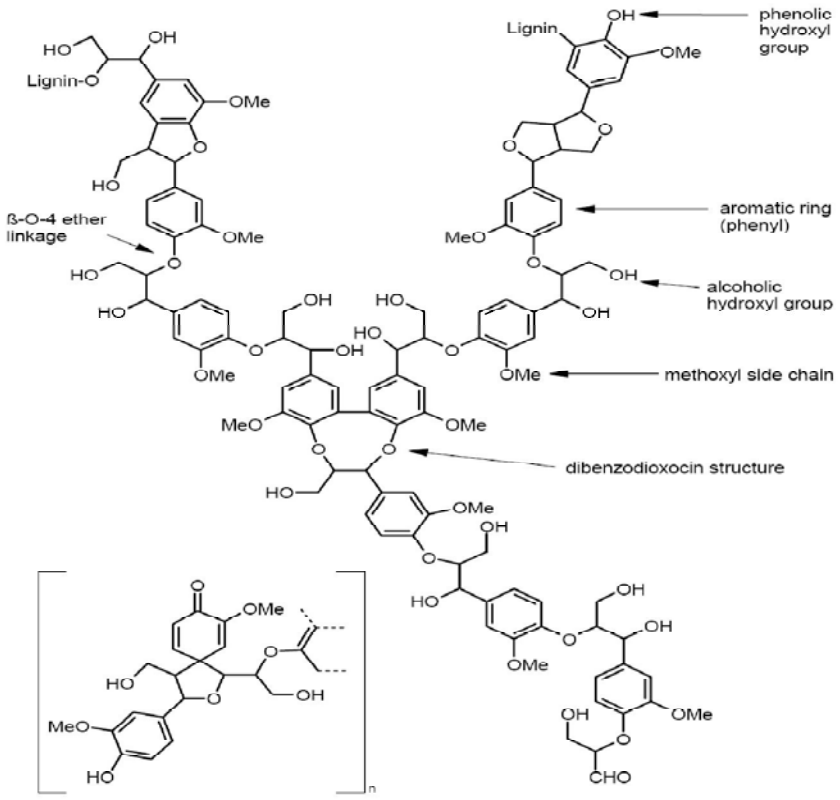


Fig. 2. A structural model of a lignin polymer

An amorphous matrix is formed in the secondary wall of a wood tissue because of the link between cellulose and lignin. This structural arrangement traps the cellulose, the major sugar reservoir, making its susceptibility to enzyme hydrolysis step in ethanol production difficult. Therefore, a pretreatment step is required to remove lignin, reduce cellulose crystallinity and increase porosity of the lignocellulosic biomass. Cellulose hydrolysis without pretreatment gives less than 20 % of the theoretical glucose yield while with pretreatment, over 90 % yield can be achieved (Sanchez and Cardona, 2008).

Pretreatment systems include physical, physico-chemical, chemical and biological mechanisms. These systems either reduce cellulose crystallinity, hydrolyse hemicelluloses and cellulose sequentially to release fermentable sugars, degrade the lignin and free the cellulose. The choice of an appropriate pretreatment depends on the feedstock; enzyme used in hydrolysis; the fermentative organism; sugar release pattern; solid concentration and overall process compatibility (Yang and Wyman, 2008). Physicochemical and chemical pretreatment systems have been well studied and the process designs have been developed for industrial scale.

3.4.2. Biological pretreatment

Biological pretreatment involves the use of microorganisms, particularly fungi such as white rot, brown rot and soft rot that are able to degrade lignin and hemicelluloses (Sun and Cheng, 2002). It is advantageous over physicochemical and chemical processes because of the mild reaction condition and thus fewer side reactions, less chemical consumption, less energy demand and less susceptibility to pressure and corrosion in the process (Lee, 1997; Samsuri *et al*., 2008). The limitation is that microbial degradation is too slow to apply on an industrial scale (Sanchez and Cardona, 2008).

The rot fungi have the natural ability to degrade wood by different mechanisms, which divides them in to 3 groups. White rots degrade all wood polymers including lignin leaving a white fibrous appearance, while the brown rots can only slightly alter lignin (Hakala, 2007) and prefer the polysaccharides. Soft rots also mostly attack the polysaccharides, with slight degradation of lignin and make the wood to soften. Degradation of lignin, by other microbes including bacteria and actinomycetes, is relatively poor (Kirk and Farrell, 1987; Glazer and Nikaido, 2007). Thus, white rots are the best candidates as the focus of pretreatment is on degradation of lignin (Samsuri *et al*., 2008).

3.5. White rot fungi and wood degradation

The taxonomy of fungi does not assign white rots to a certain clade. Generally, white rots have been identified in the phylum Basidiomycotina, classes Homobasidiomycete and Heterobasidiomycete according to a classification scheme adopted by Webster and Weber (2007). White rots are also found rarely in ascomycetes such as *Kretzschmaria deusta* and *Xylaria hypoxylon* (Schmidt, 2006).

White rots decay wood by the penetration of the microhyphae in to the lumen (cavity bounded by cell wall) and degrade the secondary wall in the proximity. This produces holes that enlarge as the hypha grows to form grooves or pockets. Eventually, as the middle lamella and the primary wall are degraded, the wood becomes fibrous with most of the cellulose remaining intact. The fibrous wood has a bleached appearance because lignin, which is slightly brown, is degraded either primarily or along with polysaccharides (Schmidt, 2006).

White rots can be divided in to simultaneous and selective degraders based on pattern of degradation. Simultaneous degraders hydrolyse all the wood polymers at similar rates represented by *Phanerochaete chrysosporium and Trametes versicolor*. Selective degraders

hydrolyse preferentially lignin and hemicelluloses and only degrade cellulose at later stages. These preferential degraders include *Ceriporiopsis subvermispora* and *Pleurotus* sp. (Hakala, 2007). It has been postulated that selective white rots produce metabolites that inhibit the formation of cellulolytic oxidants (Fackler *et al*., 2007). Selective white rots are potentially viable for pretreatment of lignocellulosic biomasses because the lignin barrier is oxidised with minimal loss to the polysaccharides (Samsuri *et al*., 2008).

3.6. White rots in pretreatment

Studies on white rot pretreatment reveal partial degradation of lignin and low polysaccharide weight loss. This improved the accessibility of polysaccharides to enzyme hydrolysis, which consequently increased the ethanol yield as compared with untreated lignocellulosic biomass. Samsuri *et al*. (2008) studied the pretreatment of bagasse with *C. subvermispora*. They reported 15.7 % lignin loss, 2.94 % cellulose loss and 6.09 % hemicelluloses loss after pretreatment and an increment in ethanol production by 12.96 %. Itoh *et al*. (2003) studied the combination of organosolve (using ethanol) and *C. subvermispora* pretreatments of beech wood chips. After the pretreatments, there was 1.6 folds increment in ethanol yield and 15 % reduction in electricity consumption. The biological pretreatment brought 13 % weight loss of lignin and only 5 % weight loss of α-cellulose. A similar pretreatment combination with *C. subvermispora* and *Ganoderma austral*e by Munoz *et al*. (2007) showed that pretreatment of *Acacia dealbata* and *Pinus radiata* resulted in 9.5 % and 11.7 % lignin weight loss, respectively with equal glucan loss of 3.8 % and improved ethanol yield. Bak *et al*. (2009) studied the degradation of rice straw by *P. chrysosoporium*. Pretreatment resulted in 21 % lignin loss, 64.9 % glucose recovery and an increase in ethanol production.

3.7. Ligninolytic enzymes

White rots degrade lignin using oxidative extracellular enzymes. The molecular size of lignin (100 KD) is too large to be taken up by the cells. Six enzymes namely, lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac), versatile peroxidases, glyoxal oxidase and aryl alcohol oxidase have been identified from lignin degrading white rots and litter decomposing fungi in different combinations. These enzymes are non-specific for oxidative reaction, which leads to the breakdown of aromatic rings and the different bonds between the subunits (Lankinen, 2004). LiP, MnP and Lac are the predominant enzymes studied for lignin degradation.

Based on the expression pattern of the oxidative enzymes, white rots can be classified in to five groups as producers of LiP, MnP and Lac; MnP and Lac; LiP and either of MnP or Lac; LiP only and Aryl alcohol oxidase and other phenol oxidase (Tuor *et al*., 1995). MnP and Lac producers are the most common (Hatakka, 2001) and those that produce LiP are efficient lignin degraders (Hakala, 2007). Among the ligninolytic enzymes, MnP is produced by most fungi (Kaal *et al*., 1995) and has been the most studied enzyme from degradation of natural substrates (Vares *et al.,* 1995). White rots producing Lac in combination with the other ligninolytic enzymes have been linked to better degradation capacity. Nevertheless, it is difficult to give higher credits to an individual enzyme as degradation has been reported in fungi producing different combinations of the enzymes (Arora *et al*., 2002).

3.7.1. Characteristics of ligninolytic enzymes

3.7.1.1. Lignin peroxidase

LiP and MnP are peroxidase globular proteins, which harbour a heme group bound to histidine residues between the cavities of the domains. Both enzymes are dependent on primary oxidation by H_20_2 to degrade lignin. The H_20_2 is produced by other extracellular enzymes of the white rot including glyoxal oxidase, glucose oxidase, veratryl alcohol oxidase and methanol oxidase. LiP catalyses the oxidation of veratryl alcohol, phenolic and non phenolic alcohol substrates (Gold and Alic, 1993; Hakala, 2007).

The primary reaction of LiP involves two step oxidation of the resting ferric enzyme (Fe^{III}) by H_20_2 to a two electron oxidised state, Fe^{4+}- oxo- porphyrin radical complex Compound I ($Fe^{IV}=O^{+}$). Compound I oxidises the aromatic substrates by two-step reduction. In the first step, the substrate is oxidised by one electron to form a cation radical leaving the enzyme in one electron oxidised state, Fe^{4+}-oxo-prophyrin complex Compound II ($Fe^{IV}=O$). Compound II further oxidises another substrate to form another cation radical. Upon reduction of compound II, the resting ferric enzyme is retained (Kirk, 1988; Gold and Alic, 1993; Hofrichter, 2002). The oxidised substrate becomes an aryl cation radical and when the substrate is non-phenolic lignin, the cation radicals spontaneously initiate unspecific and non-enzymatic chain reactions. This results in cleavage of C_α-C_β bonds, demethoxylation, ring opening (by addition of molecular oxygen to carbon centred radicals) and ultimately depolymerisation of the complex lignin structure to various products (Hakala, 2007). Ring cleavage plays the key role in lignin mineralization. For phenolic substrates, oxidation leads

to polymerisation. However, due to the high redox potential, LiP has affinity towards the non-phenolic methoxyl substituted lignin subunits (Tuor *et al.*, 1995).

3.7.1.2. Manganese peroxidase

MnP and Lac oxidise phenolic substrates to depolymerise lignin and thus are called phenol oxidases. MnP enzyme has three amino acids binding Mn^{2+}, which serves as diffusible and reducible substrate, conserved only in MnP and VP. MnP catalyse the degradation of lignin, lignin derivatives and phenolic lignin model compounds (Gold and Alic, 1993).

In the catalytic cycle, the primary oxidation of the ferric enzyme by H_2O_2 to form Compound I, is similar to LiP catalysis. Then, Mn^{2+} donates an electron to Compound I to form Mn (III) and Compound II. Compound II is again reduced back to the resting enzyme state by another Mn^{2+} to form an additional Mn^{3+} (Gold and Alic, 1993). These oxidised Mn^{3+} states are stabilized by chelating with organic acids (lactate, tartarate, manolate etc) or lipids and undergo oxidative reactions with phenolic lignin substrates. Oxidation forms phenoxyl radical intermediates, which trigger demethoxylation, quinone formation and C_α-C_β bonds cleavage that similarly degrade lignin to various products as in LiP. In the presence of lipid chelator, the MnP can also oxidize recalcitrant non-phenolic substructures. Unsaturated lipids may be produced by the fungus or derived from enzymatic cleavage of triglycerides present in wood extractives (Hakala, 2007).

3.7.1.3. Laccase

Lac is a glycoprotein with blue copper on its active site. It catalyses the oxidation of aromatic amines and phenolic compounds. Although the catalytic activity is not well understood, it has been suggested that, Lac catalyses the one electron oxidation of phenols to form phenoxy radicals, which similarly initiates unspecific reactions resulting in C_α hydroxyl oxidation to ketone, alkyl- aryl cleavage, demethoxylation and C_α-C_β bonds cleavage of phenolic lignin substructures. It also catalyses the oxidation of non-phenolic substructures in the presence of mediators such as hydroxybenzotriazole or 2,2’ azinobis- 3- ethylbenzthiazoline- 6- sulphonate (ABTS) derived naturally from the substrates or produced by the fungus (Hakala, 2007).

3.7.2. General applications

Ligninolytic enzymes have wide industrial applications due to their non-specific reaction with different substrates. The application of Lac enzyme has been well developed in pulp and paper industry and in olive oil mill wastewater treatment. Heterologous peroxidases have also been blended in commercial bleaching agents. Yet, large-scale production of the peroxidases is still under development. Studies have revealed potential of Lac in decolourization of industrial dyes, LiP in kraft pulp mill effluent treatment and MnP in biobleaching. Other applications include biodegradation of toxic compounds such as trinitrotoluene (TNT), polychlorinated bisphenyls (PCBs), organochlorines, polycyclic aromatic hydrocarbons (PAHs) and wood preservatives; improvement in digestibility of lignocellulosic biomass for animal feed (Lankinen, 2004) and in ethanol production (Hakala, 2007). Despite these potentials, commercial production of enzymes is underexploited due to low productivity, low stability and requirement of H_2O_2 for peroxidase activity (Lankinen, 2004).

3.7.3. Role in pretreatment

As discussed in section 3.6, the increase in accessibility of cellulose by biological pretreatment is a result of delignification. This mineralisation of lignin is carried out by the action of the ligninolytic enzymes (Bak *et al*., 2009). Improved productivity of these enzymes has resulted in more effective biological pretreatment. Bak *et al*. (2009) optimised the production of MnP by *P. chrysosporium* and studied its effect on cellulose digestibility. Their result shows an increase in glucose yield from cellulase hydrolysis at the time of maximal MnP production.

3.8. Solid state fermentation and conditions for pretreatment

Solid state fermentation refers to the transformation of a biological material in the absence of free water to obtain useful products. Submerged fermentation (SmF) on the other hand is carried out in dilute solutions or in slurries (Lee, 1997). SmF reactor designs are well developed and widely used for enzyme production and other products, while there is growing interest on SSF (Papinutti and Forchiassin, 2007).

SSF is more advantageous over SmF for biological delignification because it: resembles the natural growing environment of fungi; is less exposed to bacterial contamination; does not require large volume fermentors; requires low sterilization energy cost; has better aeration and has reduced energy requirement for stirring and drying (Lee, 1997). Novel isozymes of

ligninolytic enzymes that were not produced in liquid cultures have been identified during SSF (Datta *et al.*, 1991; Lobos *et al.*, 1994).

Delignification of lignocellulosic biomass during SSF can be maintained under the right eco-physiological conditions. The major factors that need consideration include particle size of lignocellulosic biomass; moisture content; aeration; nutrient supplementation; inoculum type and amount; incubation time; mixing and heat removal (Lee, 1997).

With regard to nutrient supplementation, nitrogen source and composition is critical for ligninolytic enzyme production. It was assumed that these enzymes were secreted as secondary metabolites when nutrients such as N, C and S were depleted (Kirk and Farrell, 1987). Under such limited nitrogen composition, high titres of the enzymes were not produced (Bonnarme and Jeffries, 1990). However, studies have revealed that organic nitrogen (specifically peptone) stimulates production of LiP in *Bjerkandra* sp. (Kimura *et al.*, 1990), MnP and Lac in *Pleurotus ostreatus* and *Lentinus edodes* (Kaal *et al.*, 1995). The peptides in peptone simulate peptides released during autolysis of mycelia after growth phase. This triggers a shift towards secondary metabolism by the fungus (Martinez *et al.*, 1996).

Increase in enzyme productivity can also result from improved growth due to nitrogen supplementation. Kaal *et al.* (1995) suggested that increase in biomass of *P. ostreatus* and *L. edodes* stimulated productivity of MnP. Although the data has not been presented here, growth of an *Inonotus* sp. supplemented with peptone was significantly intense compared to growth with inorganic nitrogen and consequently, higher titres of MnP were detected. Nevertheless, the impact of N starvation or supplementation varies among different white rots and needs a case by case study approach. This can be attested by the high productivity of LiP observed in *P. chrysosporium* in N limited cultures in contrast to *Bjerkandra* sp. (Kaal *et al.*, 1995).

White rots cannot use lignin as the sole carbon source during delignification but require the presence of cellulose and hemicelluloses in the lignocellulosic biomass medium (Kirk and Farrell, 1987). Glucose supplement can serve as a substitute carbon source. There are suggestions that glucose suppresses production of polysaccharide degrading enzymes (Levonen-Munoz and Bone, 1985) and to a small extent, stimulates the production of ligninolytic enzymes (Lee, 1997).

3.9. Bagasse as feedstock for ethanol production

Sugar cane bagasse is the fibre residue left over after mechanical extraction of the juice from sugarcane stalks. It is among the largest agro-industrial by-products in tropical countries (Pandey *et al*., 2000).

Processes have been developed for using bagasse as raw material in SSF both as carbon source and as inert support, where the former is the most common. Products from bioconversion include chemicals, metabolites, protein enriched animal feed (by cultivating fungi) and enzymes (mostly cellulase and xylanase). Bioconversion of bagasse is economically feasible for producing enzymes, amino acids and drugs (Pandey *et al*., 2000).

Bagasse is composed of 30- 43 % cellulose, 26- 30 % hemicelluloses and 18- 26 % lignin (Kadam, 2000). Due to its high polysaccharide composition, bagasse is suitable for generation of ethanol (Buaban *et al*., 2010). However, production faces the same problem of accessibility to cellulose and cost for pretreatment and hydrolysis. Association with other technologies that produce value added product has been suggested to make the process economic feasibile (Pandey *et al*., 2000). In Brazil, a demonstration unit, producing 5000 L/day has been set up whereby bagasse undergoes organosolve pretreatment followed by dilute acid hydrolysis (Dias *et al*., 2009).

Supply of bagasse must be surplus to consider commercial ethanol generation. Currently, sugar industries utilise the bagasse as fuel to generate steam and power. Yet, the bagasse produced usually exceeds the requirement for fuelling boilers and about 15- 25 % of bagasse is surplus. It has been suggested that this percentage can be increased by improving the efficiency of the current boilers; adopting energy conservation measures that reduce steam consumption; substituting bagasse with cane trash and using solar energy to generate steam (Kadam, 2000).

In Ethiopia, the sugar factories consume most of the bagasse because the boilers are outdated and inefficient. What is left as excess is usually sold as animal feed. Therefore, improvement in the boilers would significantly increase the excess bagasse. With the development of mega-hydroelectric power stations in the country, sufficient electric power might be available for local consumption and export. Considering the weight in demand for liquid fuel, a shift towards electric power usage for heating boilers may become economically feasible in the near future.

Setting up ethanol production plants in sugar factories would be feasible as the raw material and operational fermentation systems are available on site. Currently, Ethiopia is producing ethanol from molasses for blending with gasoline. Fincha sugar factory is supplying 8 million litres per annum, while the others factories Metehara and Wonjii are in the preparation stage and Tendaho, is under construction (MOME, 2007). Therefore, if an appropriate pretreatment technology is employed to unlock the polysaccharides, the prospect of ethanol production from bagasse is viable.

3.10. Some white rots and brown rots isolated from Ethiopia

Previous studies have described some wood inhabiting fungi in Ethiopia (Hjortstam and Ryvarden, 1996). Dr. Adane Bitew (Department of Medical Laboratory Technology, School of Medicine) has also collected some wood rotting fungi from some forests of Ethiopia (Table 1). The collection includes 12 white rot and 3 brown rot fungi namely *Tyromyces* sp., *Laetiporus sulphureus* and *Fomitopsis carnea*. These rot fungi are grouped under 5 families based on the classification scheme followed by Kirk *et al*. (2001). These isolates have not been characterised for their role in lignin degradation or ligninolytic enzyme production. The ligninolytic enzyme reported on some of these rot fungi from previous studies are summarised in Table 1. Currently, there are no publications on the production of ligninolytic enzymes by 4 species namely *Fomitiporia aethiopica*, *F. pseudopunctata*, *Vanderbylia vicina* and *Fomitopsis carnea*. Among these, a distinct species isolated from the Ethiopian highlands, previously classified in the *Phellinus* sp., was re-described as *Fomitiporia aethiopica* after morphological and molecular studies by Decock *et al.* (2005). *L. edodes* is the only rot fungus that has been studied for pretreatment by Samsuri *et al*. (2008) and is reported to have improved the ethanol yield.

Table 1. Classification of some rot fungi genera isolated from Ethiopia and ligninolytic enzymes detected in these taxa from previous studies

Order	Family	Genus/ species	Ligninolytic enzymes
Agaricales	Schizophyllaceae	*Schizophyllum commune*	LiP, MnP, Lac (Asgher *et al*., 2008)
	Pleurotaceae	*Pleurotus sajor-caju*	MnP, LiP, Lac (Tuor *et al.,* 1995)
Hymenochaetales	Hymenochaetaceae	*Fomitiporia aethiopica*	NPF[a]
		F. pseudopunctata	NPF
		Inonotus sp.	MnP (Palma *et al*., 2011), LiP and Lac (Risna and Suhirman, 2002)
		Phellinus sp.	MnP, LiP, Lac (Risna and Suhirman, 2002) and other phenol oxidases (Tuor *et al.*, 1995)
Polyporales	Polyporaceae	*L. edodes*	MnP, Lac (Tuor *et al.*, 1995)
		Microporus sp.	MnP (Song, 1997)
		Perenniporia tephropora	MnP (Ralph *et al*., 1996) and Lac (Younes *et al*., 2007)
		Perenniporia sp.	MnP, LiP and Lac (Risna and Suhirman, 2002)
		Polyporus sp.	LiP and either of MnP/ Lac or LiP only in some (Tuor *et al.*, 1995)
		Vanderbylia vicina	NPF
		Tyromyces sp.[b]	MnP (Eichlerova *et al*., 2006)
		Laetiporus sulphureus[b]	LiP and MnP (Mtui and Masalu, 2008)
	Fomitopsidaceae	*Fomitopsis carnea*[b]	NPF

[a] no publications were found on ligninolytic enzyme activity, [b] brown rot fungus

4. Materials and methods

Most of the experiments were carried out in the Microbial Biotechnology Laboratory, Biotechnology Program Unit, Addis Ababa University. Certain instruments and reagents were utilised from Mycology Laboratory, Cellular, Microbial and Molecular Biology Program Unit; UV spectrophotometer was facilitated by the Polymer Physics Laboratory, Department of Physics and GC analysis was carried out in the Chromatography Laboratory, Department of Chemistry. All experiments were conducted according to the following protocols.

4.1. Culture preparation

The 15 rot fungi in Table 1 were generously provided by Dr. Adane Bitew (Department of Medical Laboratory Technology, School of Medicine), as slant or plate cultures. These rot fungi were subcultured on 41 g/L potato dextrose agar (PDA) plates and were incubated for 5- 7 days at 27 ^{0}C.

4.2. Solid state fermentation

Production of ligninolytic enzyme and pretreatment by the rot fungi were studied during SSF of bagasse substrate. The SSF conditions were prepared as follows.

4.2.1. Substrate preparation

Sugar cane bagasse residue from mechanical crushing of cane stalks was purchased from a local cane juice shop. The bagasse was sundried, chopped in to smaller pieces by a cleaver, milled using a blender and passed through 1 mm sieve (to remove smaller particle sizes). Residual sucrose on the fibre was removed by soaking in water (approx. 1:100 w/v) for 1 hr in two rounds with frequent stirring. This bagasse preparation was sundried and used as the lignocellulosic substrate.

4.2.2. Seed culture preparation

A mineral salt solution was prepared from Basal III medium and trace elements (10:1 v/v) (Tien and Kirk, 1988; Kumar *et al*., 2006), with additional carbon and nitrogen supplements. The Basal III medium was prepared from KH_2PO_4, 20 g/L; $MgSO_4$, 5 g/L and $CaCl_2$, 1 g/L. The trace element solution was composed of (g/L) $MgSO_4$, 3; $MnSO_4$, 0.5; NaCl, 1; $FeSO_4.7H_20$, 0.1; $CoCl_2$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4$, 0.1; $AlK(SO_4)_2.12H_2O$, 0.01; H_3BO_3, 0.01 and $Na_2MoO_4.2H_2O$, 0.01 dissolved in nitrilotriacetate solution, 1.5 g/L adjusted

to pH 6.5 using 3 % KOH. Glucose (1 g/L) and peptone (10 g/L) were added as carbon and nitrogen supplement, respectively.

The bagasse preparation (5 g), in 500 ml Erlenmeyer flask, was autoclaved at 121 ^{0}C for 30 minutes. After cooling, 15 ml of the mineral salt solution with the supplements was membrane filtered (0.2 µm) on to the substrate.

Each flask was inoculated with four approximately 6 x 5 mm^2 agar cube blocks (Samsuri *et al.*, 2008) cut from the peripheral sides of the mycelia on the PDA cultures. The SSF culture was incubated stationary at 27 ^{0}C for 15 days. The growth characteristics of each rot fungi on PDA and SSF media were compared by visual observation of the mycelia.

4.3. Enzyme extraction and assay

The SSF substrate was soaked in 50 ml sodium acetate buffer (25 mM, pH 4.5) for 90 minutes on a rotary shaker (120 rpm) at room temperature. The liquid homogenate was filtered through cotton gauze. The extract was centrifuged at 3000 rpm for 5 minutes to remove solid particulate matter and maintained in aliquots at -20 ^{0}C.

The crude extract was directly used as the enzyme in the assay tubes. Enzyme to substrate concentration was maintained at 1:10 (v/v). The absorbance of each tube was measured on 1 cm (width), 1 ml cuvette using Jenway 6300 visible spectrophotometer (Jenway Ltd., England). Enzyme productivity (U/g) was determined using equation 1 where, U (U/ml) is the unit expressing activity per ml of enzyme used, *e* (ml) is the volume of the enzyme extract collected from the SSF substrate and *s* (g) is the weight of the SSF substrate. All enzyme reactions were carried out in triplicates following the respective protocols.

$$\text{Enzyme productivity} = \frac{\text{U x } e}{s} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

4.3.1. Manganese peroxidase assay

MnP activity was measured according to Kuwahara *et al*. (1984) based on the oxidation of phenol red characterised by change in colour from red to pinkish purple, under alkaline conditions. The reaction mixture was composed of phenol red (0.01 %), sodium lactate (25 mM), $MnSO_4$ (100 µM) and egg albumin (0.1 %) in 20 mM sodium succinate buffer (pH 4.5). To initiate the reaction, H_2O_2 (100 µM, final concentration) was added and the tubes were incubated for 5 minutes at 30 ^{0}C. The reaction was terminated with 2 N NaOH (40

µl/ml) and the absorbance was measured at 610 nm. One unit (U) was defined as the amount of enzyme required to increase 0.1 optical density (OD) units per minute.

4.3.2. Laccase assay

Lac activity was detected based on the oxidation of guaiacol ($C_6H_4(OH)(OCH_3)$), which turns the colourless substrate brown (Coll *et al*., 1993). The reaction mixture was composed of 1 mM guaiacol in 50 mM sodium acetate buffer (pH 4.5). The tubes were incubated at 37 ^{0}C for 1 hour and the absorbance was measured at 465 nm. One unit was defined according to Arora and Sandhu (1985) as the amount of enzyme required to increase 1 OD unit per hour.

4.3.3. Lignin peroxidase assay

LiP assay based on Azure B decolourization method, according to Archibald (1992) by absorption spectrophotometer, failed in the present study. Readings from the reagent blank containing the dye were unstable. Similar problems on the protocol were encountered by Vares *et al*. (1995). Thus, a qualitative assay was conducted based on decolourization of azure B on agar plates by inoculating the rot fungi on solid media according to Zhao *et al*. (1996). The composition of the medium was prepared so that it simulates the SSF condition, to predict the actual LiP productivity in the bagasse substrate. The SSF simulating media contained peptone, 10 g/L; glucose, 10 g/L; azure B, 0.1 g/L and agar, 15 g/L dissolved in a mineral salt solution prepared as described in section 4.2.2 and cultures were incubated for 10 days at 27 ^{0}C. However, under certain conditions, MnP could decolourize azure B and create bias (Archibald, 1992). Thus, the rot fungi showing dye decolourization on the SSF simulating media were confirmed for LiP activity on Mn deficient agar plates (confirmation media), supplemented with veratryl alcohol (Zhao *et al*., 1996; Levin *et al*., 2004). The media was composed of mineral salt solution (without $MnSO_4$); glucose, 10 g/L; NH_4NO_3, 26 mM; aspartic acid, 15 mM; thiamine, 1.68 mg/L; sodium succinate, 0.1 M; veratryl alcohol, 2 mM; agar, 15 g/L and azure B, 0.02 g/L. The cultures were incubated for 10 days at 27 ^{0}C. All detections of decolourization were confirmed by visual observation of a cube sections cut out from regions very close to the mycelial growth.

4.3.4. Carboxymethyl cellulase and xylanase assay

Extracellular carboxymethyl cellulase (CMCase) and xylanase enzyme activities were determined by DNS (dinitrosalicylic acid, $C_7H_4N_2O_7$) method according to Bernfeld (1955). The assay is based on the reduction of 3,5- DNS (yellow) to 3-amino, 5-nitrosalicylic acid (red brown) by the respective reducing sugars released from the polysaccharides. The DNS reagent was prepared from DNS, 10 g/L; C_6H_6O, 2 g/L; Na_2SO_3, 0.5 g/L and NaOH, 5 g/L. The substrates contained carboxymethyl cellulose (0.5 %) and birch wood xylan (1%) in 50 mM citrate phosphate buffer (pH 5.0) for CMCase and xylanase assays, respectively. The tubes were incubated at 40 ^{0}C for 15 minutes. The reaction was terminated by adding DNS reagent to the reaction solution (2:1 v/v) followed by boiling in water bath for 5 minutes. The absorbance of each reaction tube was measured at 540 nm after cooling. The CMCase and xylanase activities were expressed in international units where, 1 U is the amount of enzyme required to release 1 µmol of glucose (0.18 mg/ml) (Ghose, 1987) and xylose (0.15 mg/ml) (Ghose and Bisaria, 1987), respectively per minute. Standard curves were prepared using known concentrations of glucose and xylose (Appendix 1A and 1C, respectively).

4.4. Screening for selective rot fungi

Highly selective rot fungi that showed maximum and minimum productivity of ligninolytic and polysaccharide degrading enzymes, respectively were screened. Aggregate metrics index analysis, similar to multimetric approach by Barbour *et al.* (1999) for environmental data analysis, was used as the screening methodology. Productivity of enzymes was used as metrics. Each metric was categorised in to 4 levels of productivity as: high, moderate, low and marginal. The interval (scoring range) between levels was determined by dividing the range (the difference between maximum and minimum productivity) by 4 (the number of levels). These levels were then assigned unitless scores. The values of scores range from maximum 4, for high, to a minimum 1 for marginal productivity for ligninolytic enzymes. Scoring criterion was reversed for CMCase and xylanase and thus the values of the scores range from maximum 4, for marginal, to a minimum 1 for high productivity. The level of each rot fungus was identified based on the value of the metric (U/g) and scores were assigned accordingly. Aggregate index for each rot fungi was determined by summation of scores across all metrics. These indices were divided in to 4 levels of selectivity as: high, moderate, low and poor. The interval between levels was determined as described for the metrics and each rot fungus was categorised based on the value of its index.

Minimum xylanase activity was set as selection criterion in the present study even though residual xylan was not exploited as a substrate for saccharification and fermentation. This was done to consider its potential as a significant bagasse constituent.

4.5. Screening for effective ligninolysis

Lignin compositions and cellulose digestibility of bagasse, pretreated by the highly selective rot fungi during the SSF were compared with untreated bagasse to screen for effective ligninolysis.

4.5.1. Lignin degradation

Acid insoluble lignin or Klason lignin was determined according to Munoz *et al*. (2007) by hydrolysing the polysaccharides with concentrated acid. Acid soluble lignin was also analysed by UV absorption (Ferraz *et al*., 2000; Sluiter *et al*., 2008) of the acid hydrolysed filtrate. Before hydrolysis, non-structural materials or residual extractives (as some of its composition was removed during soaking in section 4.2.1) were removed to facilitate acid penetration. Mineralised lignin components, water-soluble inorganic materials, non-structural sugars and nitrogenous materials were removed by autoclaving in water suspension, at neutral pH to avoid hydrolysis of hemicelluloses. Chlorophyll, waxes and other minor components were removed according to Sluiter *et al*. (2005) by Soxhlet extraction using ethanol.

Primarily, the fungal biomass on the pretreated bagasse was removed by soaking in 2 L distilled water twice for 30 minutes with continuous stirring. The floating low density fungal biomass was decanted and the bagasse was recovered (Kumar *et al*., 2006). The bagasse was suspended in water (1:100 w/v), adjusted to pH 7 – 7.5 and autoclaved at 121 ^{0}C for 15 minutes. The liquid was discarded and the bagasse was rewashed with 200 ml boiling water. Other extractives were removed with ethanol (96 %) in a Soxhlet extractor (Glassco laboratory equipments Ltd., UK) set to reflux for 9 hours. After extraction, the fibre was rewashed with 80 ml fresh ethanol (96 %) and air-dried.

The extract free bagasse (0.1 g) was treated with 1.5 ml H_2SO_4 (72 %) in a test tube. The tube was vortexed vigorously initially and then continuously every 10 minutes for 2 hours. The resulting acid hydrolysate was diluted in 56 ml distilled water to bring the concentration to 1.88 %. The hydrolysate was autoclaved at 121^{0}C for 1 hour and filtered through 0.45 µm

tared Whatman glass fibre filter. The residue on the filter (the acid insoluble lignin) was washed with 100 ml water and oven dried at 105 ^{0}C to constant weight.

The filtrate collected from the acid hydrolysis was used to determine the acid soluble lignin. The absorbance of the filtrate was analysed at 205 nm using Lambda 19 UV-Vis spectrophotometer (Perkin Elmer Inc., Germany). The concentration of acid soluble lignin (%) was determined using equation 2, where, *A* is the absorbance, *V* (L) is the volume of the filtrate and ε (105 L/ g cm) is the absorptivity of soluble lignin, l (1 cm) is the path length of the cuvette and *w1* (g) is the initial weight of the extract free bagasse.

$$\text{Acid soluble lignin} = \frac{A \times V}{\varepsilon \times l \times w1} \times 100 \ldots\ldots\ldots\ldots\ldots (2)$$

The lignin composition of the extract free bagasse was the sum of acid insoluble and acid soluble lignin. Lignin weight losses caused by the selected rot fungi pretreatments were determined from the difference in composition with the lignin content of the untreated extract free bagasse.

4.5.2. Cellulose digestibility

Cellulose digestibility was defined as percentage of the theoretical maximum of glucose obtained from hydrolysis of cellulose (Bak *et al*., 2009). The glucose released from enzyme hydrolysis and the cellulose composition of untreated bagasse were determined as follows.

4.5.2.1. Enzyme hydrolysis

Bagasse substrate for enzyme hydrolysis was prepared by removing fungal biomass and water-soluble mineralised lignin (to prevent enzyme inhibition) as described in section 4.5.1 without the ethanol extraction. Cellulase enzyme (Endoglucanase, EC 3.2.1.4) extracted from *Aspergillus niger* (Sigma chemical group, USA) was used to hydrolyse cellulose. The enzyme (60 U/g of cellulose) was dissolved in 50 mM citrate-phosphate buffer (pH 5.0) and centrifuged at 3000 rpm for 5 minutes to precipitate particulate matters. The bagasse (1 g) was hydrolysed by filter sterilised (0.2 µm) enzyme under solid state conditions (1:10) at 37 ^{0}C for 5 days. Glucose was extracted by soaking the hydrolysate with 10 ml water for 20 minutes on a rotary shaker (120 rpm), at room temperature. The amount of glucose was estimated by the method described in section 4.3.4 using the standard curve in Appendix 1B. Slight modification was made to the composition of the DNS reagent, with the addition of sodium potassium tartarate, 200 g/L to increase the stability of reduced DNS (Miller, 1959).

4.5.2.2. Cellulose composition

Water and ethanol soluble extractives were removed from an untreated bagasse according to Sluiter *et al*. (2005) by Soxhlet extraction, set to reflux for 18 and 9 hrs, respectively. The bagasse was air-dried and the composition of the residual extractives was estimated.

The extractive free residue (1 g) was delignified according to Ferraz *et al.* (2003) in a solution containing 0.1 ml acetic acid and 0.6 g sodium chlorite in 32 ml water solution. The reaction was carried out at 80 ^{0}C in a water bath for 1 hour. Equal amounts of sodium chlorite and acetic acid were re-added every hour in 4 rounds. The mixture was filtered through porous glass filter number 4 and washed with cold water and acetone, respectively. The filtered residue was extracted with 95 % ethanol for 4 hours in the Soxhlet extractor. Finally, the residue was washed with cold water and oven dried at 105 ^{0}C to constant weight.

The oven dried holocellulose residue (0.5 g) was treated with 2.5 ml NaOH (17.5 %) at 20 ^{0}C. NaOH (5 ml) was re-added 3 times at 5 minutes interval. The mixture was allowed to stand for 30 minutes and then was diluted with 8.25 ml distilled water and again allowed to stand for 1 hour at 20 ^{0}C. The fibre was filtered on a sintered glass crucible number 4 and soaked in 50 ml NaOH (8.3 %) for 2 minutes. The crucible was then soaked in 3.25 ml acetic acid (10 %) for 3 minutes and washed with 300 ml water. The residual cellulose was oven dried at 105 ^{0}C to a constant weight (Rowell *et al*., 2005). The cellulose composition *C* (%) was calculated using equation 3, 4 and 5 where *c'* (%) is the cellulose composition in the extractive free holocellulose, *w2* (g) is the weight of extractive free holocellulose residue, *w3* (g) is the weight of cellulose in the extractive free holocellulose, *H* (%) is the composition of holocellulose in the bagasse, *L* (g) is the extractive free lignin composition per gram of bagasse obtained from section 4.5.1 and *E* (g) is the composition of residual extractives per gram of bagasse. Cellulose digestibility (%) was estimated using equation 6 where, *g* (g) is the amount of glucose per gram of substrate obtained from section 4.5.2.1.

$$c' = \frac{w3}{w2} \times 100 \quad \text{(3)}$$

$$H = (1- L)(1-E) \times 100 \quad \text{(4)}$$

$$C = c' \times H \times 100 \quad \text{(5)}$$

$$\text{Cellulose digestibility} = \frac{g}{c} \times 100 \quad \text{(6)}$$

4.6. Ethanol fermentation and GC analysis

The substrates for ethanol fermentation were cellulase hydrolysate from a pretreatment that caused the highest lignin loss and cellulose digestibility from section 4.5; cellulase hydrolysate of untreated bagasse and standard glucose. *Saccharomyces cerevisiae* strain TAYI 4-2, was obtained from Amare Gessese (Ph.D.). Stock yeast cells for inoculum were cultured aerobically in 50 ml broth composed of (g/L) glucose, 10; yeast extract, 1; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.1 and $(NH_4)_2SO_4$, 0.1 (Samsuri *et al*., 2008). Fermentation broth was prepared from 5 ml substrate and additional nutrients (g/L): yeast extract, 1; NaH_2PO_4, 1.2; $MgSO_4.7H_20$, 0.025 and NH_4HPO_4, 0.5. The pH of the broth was adjusted to 5.5 with NaOH (Vaithanomsat *et al*., 2011). Yeast inoculum 10 % (v/v) was added and anaerobic fermentation of 5 ml broth was carried out in 7 ml tubes for 72 hours at 30 ^{0}C.

Fermented broth samples (1 ml) were centrifuged at 10,000 rpm for 5 minutes to precipitate cells and other particulate matter. The supernatant was diluted 10:1 with isopropanol (1 g/L, internal standard). Gas chromatography (GC) was conducted on DANI GC 1000 (DANI Instruments Ltd., Italy) with flame ionisation detector (FID) under optimised operational condition. Alltech Econo-Cap EC^{TM}-5 capillary (30 m length, 0.32 mm diameter) coated with 95 % methylpolysiloxane (stationary phase) was used as column. The flow rates of nitrogen (carried gas) and hydrogen (FID fuel) were adjusted to 5 and 0.65 bar, respectively. The GC was conditioned as splitless and the temperatures of the inlet, oven and detector were set at 210, 155 and 250 ^{0}C, respectively. The starting temperature of the oven was 50 ^{0}C, 1 minute hold time at a heating rate of 30 ^{0}C per minute. After 4.5 minutes run time, the peak areas (mV/s) of ethanol and isopropanol were recorded. The data was used to determine ethanol concentration using the equation from the standard curve in Appendix 1D, which was developed by the same procedure using known concentration of ethanol. Fermentation broth samples were also analysed for unfermented glucose composition by the DNS method described in section 4.5.2.1.

5. Results

5.1. Growth characteristics of the rot fungi

Intense growth was observed from 8 rot fungi on the SSF media (Table 2). Only 3 rots had poor growth; but this was also evident on the PDA. In addition, 11 rot fungi changed the faded yellow colour of the bagasse to red or light red.

Table 2. Growth characteristics of the rot fungi based on visual observation on PDA and SSF media

	Growth on		Bagasse colour
	PDA	SSF	
Fomitiporia pseudopunctata	++++[c]	++++	R[f]
Fomitiporia aethiopica	++[a]	++++	R
Fomitopsis carnea	++++	++++	-[d]
Inonotus sp.	+++[b]	++++	R
Perenniporia tephropora	++	++++	R
Pleurotus sajor-caju	++++	++++	R
Polyporus sp.	++++	++++	-
Vanderbylia vicina	++	++++	R
Lentinus edodes	++++	+++	LiR[e]
Phellinus sp.	++++	+++	R
Schizophyllum commune	++++	+++	-
Microporus sp.	+++	+++	LiR
Perenniporia sp.	++	++	LiR
Tyromyces sp.	++	++	LiR
Laetiporus sulphureus	++	++	-

[a] poor, [b] medium, [c] intense,
[d] no colour change observed, [e] light red, [f] red

5.2. Production of ligninolytic and polysaccharide degrading enzymes

The productivity of MnP and Lac enzymes are shown in Fig. 3A and 3B, respectively. These enzymes were equally prevalent in 11 rot fungi. However, in terms of productivity, MnP was produced in relatively high titres by many rot fungi than Lac (Appendix 7 and 8). *L. sulphureus* and *S. commune* did not show any activities while, *Polyporus* sp. and *Inonotus* sp. did not produce MnP and Lac, respectively. The activities of MnP and Lac enzymes by *Perenniporia* sp. and *P. sajor-caju*, respectively were also negligible. The highest productivity of MnP and Lac were recorded from *F. aethiopica* (55.87 ± 0.37 U/g) and *P. tephropora* (79.65 ± 1.78 U/g), respectively. *P. tephropora* also showed high MnP productivity (51.73 ± 1.12 U/g). Marginal productions of MnP and Lac were obtained from *V. vicina* (5.56 ± 0.26) and *L. edodes* (2.37 ± 1.11 U/g), respectively.

CMCase and xylanase activities were detected from all rot fungi (Fig 3C and 3D, respectively). *P. sajor-caju* had the least CMCase productivity (0.3 ± 0.02 U/g), while *Tyromyces* sp. produced the enzyme in the highest titers (8.53 ± 0.19 U/g). *F. aethiopica* was the least xylanase producer (2.25 ± 0.14 U/g), while *L. edodes* recorded the highest productivity (19.52 ± 1.12 U/g).

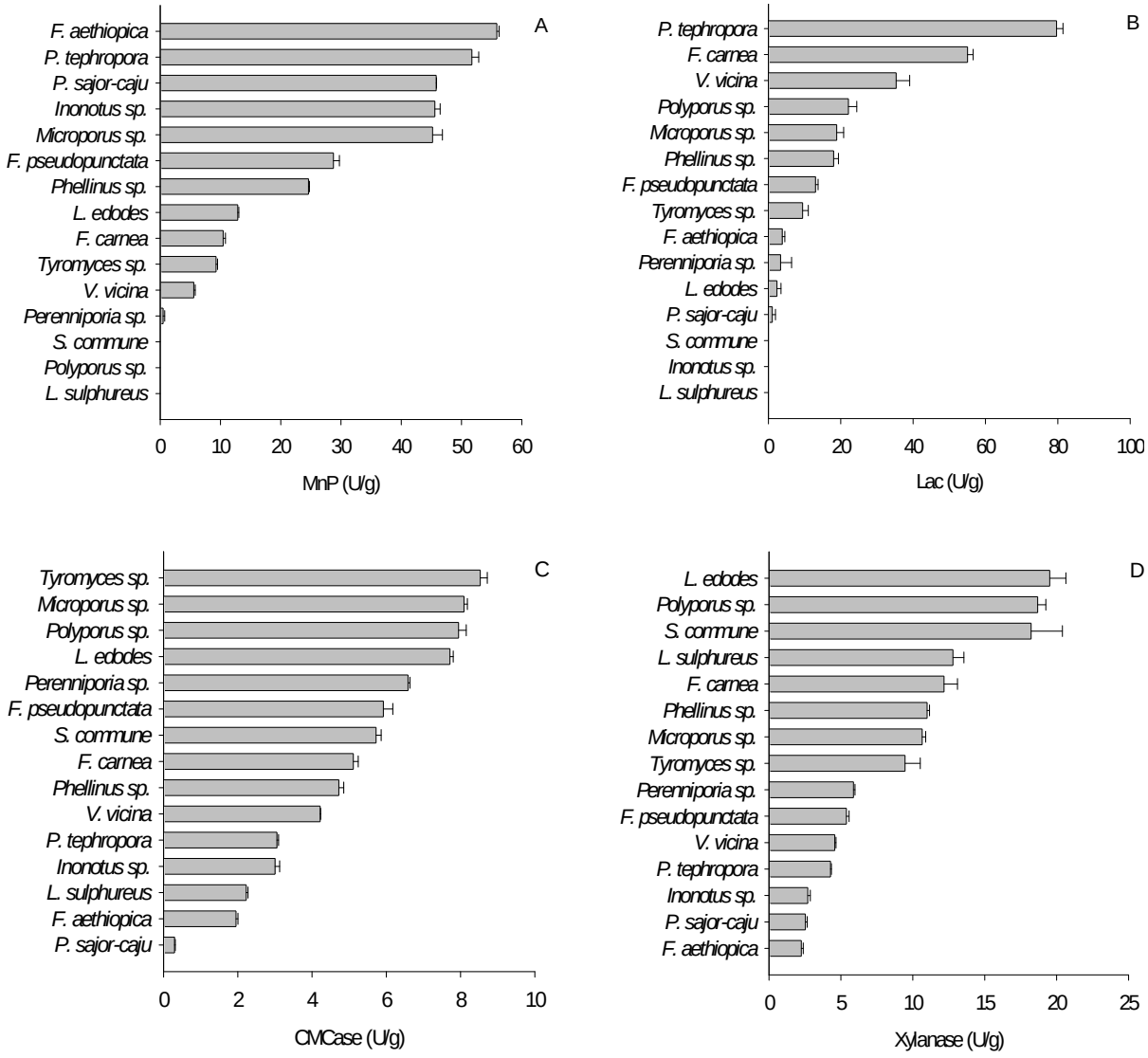


Fig. 3. Productivity of ligninolytic and polysaccharide degrading enzymes by the rot fungi. **A**: MnP, **B**: Lac, **C**: CMCase and **D**: Xylanase

LiP was produced by 7 rot fungi, showing different intensities of azure B decolourization (Table 3). Decolourization was initially observed from 8 rot fungi cultured on SSF simulating media. Among these, *Phellinus* sp. did not decolourize azure B on the confirmation media, which rules out the possibility of LiP production. Complete decolourization was observed only from *Polyporus* sp. (Appendix 13) on both media, while the other rot fungi only managed to fade or partially decolourize the dye.

Table 3. Intensity of azure B decolourization on SSF simulating and confirmation media

	SSF simulating media	Confirmation media
Polyporus sp.	++++[e]	++++
Fomitiporia aethiopica	+++	+++
Inonotus sp.	+++	+++
Tyromyces sp.	++[c]	+++[d]
Pleurotus sajor-caju	+[b]	+
Microporus sp.	+	+++
Vanderbylia vicina	+	+
Phellinus sp.	++	-[a]

[a] no decolourization, [b] mild fade, [c] fade, [d] partial decolourization, [e] complete decolourization

5.3. Categorisation by ligninolytic enzyme production profile

Based on production of ligninolytic enzymes, 13 rot fungi were categorised in to 5 groups as LiP, MnP and Lac; LiP and MnP; LiP and Lac; MnP and Lac and only Lac producers (Table 4). There were 5 rot fungi grouped under MnP and Lac producers and 4 were LiP, MnP and Lac producers. At least two of the ligninolytic enzymes were simultaneously produced by 12 rot fungi.

Table 4. Categorisation of the rot fungi by ligninolytic enzyme production

Group	Enzymes	
I	LiP, MnP and Lac	*Fomitiporia aethiopica*, *Microporus* sp. *Tyromyces* sp., *Vanderbylia vicina*
II	LiP and MnP	*Inonotus* sp., *Pleurotus sajor-caju*
III	LiP and Lac	*Polyporus* sp.
IV	MnP and Lac	*Fomitiporia pseudopunctata*, *Fomitopsis carnea*, *Lentinus edodes*, *Perenniporia tephropora*, *Phellinus* sp.
V	Lac only	*Perenniporia* sp.

5.4. Screening for selective rot fungi

F. aethiopica, P. tephropora, Inonotus sp. and *P. sajor-caju* were relatively highly selective among the rot fungi (Table 5). These fungi belonged to the white rot group. They showed high MnP, marginal xylanase and either low or marginal CMC productivity (Appendix 4-6).

Table 5. Comparison based on aggregate metrics index analysis

	Scores of mertrics					Aggregate index	Relative selectivity
	MnP	Lac	LiP	CMC	Xylanase		
Fomitiporia aethiopica	4	1	3	4	4	16	High
Perenniporia tephropora	4	4	1	3	4	16	High
Inonotus sp.	4	1	3	3	4	15	High
Pleurotus sajor-caju	4	1	1	4	4	14	High
Fomitiporia pseudopunctata	3	1	1	2	4	11	Moderate
Vanderbylia vicina	1	2	1	3	4	11	Moderate
Microporus sp.	4	1	1	1	3	10	Low
Fomitopsis carnea	1	3	1	2	2	9	Low
Laetiporus sulphureus	1	1	1	4	2	9	Low
Polyporus sp.	1	2	4	1	1	9	Low
Perenniporia sp.	1	1	1	1	4	8	Low
Phellinus sp.	2	1	1	2	2	8	Low
Tyromyces sp.	1	1	2	1	3	8	Low
Schizophyllum commune	1	1	1	2	1	6	Poor
Lentinus edodes	1	1	1	1	1	5	Poor

5.5. Screening selective rot fungi for effective ligninolysis

5.5.1. Lignin degradation analysis

The lignin composition of extract free untreated bagasse was determined to be 25.93 % (acid insoluble, 24 % and acid soluble, 1.93 %). The residual lignin compositions in the 4 pretreated bagasse were determined and lignin weight losses were observed from 3 pretreatments (Table 6). The highest lignin loss (7.71 %) was caused by *P. tephropora* pretreatment. Lignin degradation by *P. sajor-caju* could not be determined, as the lignin composition after pretreatment (32.62 %) was more than what was obtained in the untreated bagasse.

Table 6. Residual lignin compositions and weight losses of bagasse after pretreatments

	Acid insoluble lignin (%)	Acid soluble lignin (%)	Total lignin (%)	Lignin loss (%)
Perenniporia tephropora	21.90	2.03	23.93	7.71
Fomitiporia aethiopica	22.45	1.74	24.19	6.71
Inonotus sp.	23.50	1.92	25.42	1.97
Pleurotus sajor-caju	30.80	1.82	32.62	ND[a]

[a] not determined

5.5.2. Cellulose digestibility

The washed untreated bagasse was composed of 8.58 % residual extractives, 67.72 % holocellulose and 42.42 % cellulose. All pretreatments enhanced the glucose yields from enzyme hydrolysis and hence the cellulose digestibility (Table 7). *P. tephropora* pretreatment caused the highest: glucose yield (7.345 g/L), cellulose digestibility (29.44 %) and saccharification of bagasse (12.49 %, w/w). When compared with the untreated bagasse, the digestibility was improved by 38.74 %. *P. sajor-caju* pretreatment had the lowest improvement in digestibility (1.7 %).

Table 7. Comparison of glucose yield and cellulose digestibility among pretreatments and with untreated bagasse

	Glucose yield		Cellulose digestibility (%)	Improvement in digestibility (%)
	g/L	g /g of substrate		
Untreated	5.2950	0.0900	21.22	
Perenniporia tephropora	7.3450	0.1249	29.44	38.74
Fomitiporia aethiopica	7.1700	0.1219	28.73	35.39
Inonotus sp.	6.5250	0.1109	26.15	23.23
Pleurotus sajor-caju	5.3850	0.0915	21.58	1.70

Therefore, *P. tephropora* was chosen as the selective degrader, displaying the most effective ligninolysis among the rot fungi based on the results from lignin loss and cellulose digestibility.

5.6. Ethanol yield

The ethanol yield from fermentation of the enzyme hydrolysate of *P. tephropora* pretreated bagasse was 1.87 g/L (Table 8). This showed an improvement by 27.21 % compared with the untreated bagasse (1.47 g/L).

P. tephropora pretreatment hydrolysate had relatively higher content of unfermented glucose than the untreated bagasse. However, the glucose conversion efficiency (50.87 %) was lower than untreated bagasse (55.56 %). The highest conversion efficiency (83.53 %) was obtained from fermentation of the standard glucose under the same conditions.

Table 8. *S. cerevisiae* fermentation profile of pretreated and untreated bagasse enzyme hydrolysates and, standard glucose

Fermentation substrate	Ethanol yield (g/L)[a]	Glucose conversion efficiency (%)	Unfermented glucose (g/L)
Perenniporia tephropora pretreated bagasse hydrolysate	1.87	50.87	2.36
Untreated bagasse hydrolysate	1.47	55.56	1.82
Standard glucose	4.18	83.53	0.97

[a] GC analysis of ethanol is presented in Appendix 11

6. Discussion

Pretreatment during the SSF can be attributed to favourable conditions for fungal growth and ligninolytic enzyme production. Factors that might have contributed include resemblance of the solid state condition to the natural environment (Lee, 1997), stimulation of secondary metabolism by peptone (Kimura *et al*., 1990; Kaal *et al*., 1995; Martinez *et al*., 1996) and to a small extent, glucose supplement (Lee, 1997) to favour ligninolytic enzyme production.

The productions of MnP and Lac enzymes were more prevalent than LiP in the present study. The MnP and Lac producing group is common among white rots (Hatakka, 2001). MnP is produced by most white rots (Kaal *et al*., 1995) and is the most studied enzyme from decomposition of natural substrate (Vares *et al.,* 1995). LiP on the other hand is less prevalent among white rots (Hakala, 2007) and different factors in the SSF condition might have affected production. LiP activity might have been regulated by Mn^{2+} in the SSF media. Mn reduces the accumulation of veratryl alcohol (Hamman *et al*., 1999), which is an important redox mediator of LiP catalysed reaction (Hakala, 2007). Bonnarme and Jeffries (1990) reported a 2.5 fold increment in LiP activity in a media without Mn. Poor productivity of LiP makes the detection of the enzyme activity difficult (Kaal *et al*., 1995) and might have influenced the low intensity of azure B decolourization in the present study.

The production of all the predominant ligninolytic enzymes by *F. aethiopica* is the first report on such activity by this re-described species of the taxa. Its ability to produce high titres of MnP draws interests to further characterize and study the potential application of the enzyme. The other high MnP producer, *P. tephropora,* has been previously studied for its MnP activity (Ralph *et al*., 1996); however, the enzyme has not been well characterised. MnP activity has also been previously studied from *P. sajor-caju* (Boyle *et al*., 1992; Tuor *et al*., 1995; Martinez *et al*., 1996), *Inonotus* sp. (Palma *et al*., 2011) and *Microporus* sp. (Song, 1997). However, for *Microporus* sp. the role of MnP has not been linked to lignin degradation.

High productivity of Lac enzyme by *P. tephropora* has been reported by Ralph *et al*. (1996). Although the productivity of Lac were moderate and low by *F. carnea* and *V. vicina,* respectively, this is the first report of ligninolytic enzyme activity.

Different studies have identified LiP activities from different species of the *Polyporus* genus. LiP activity has been detected from the white rot species *P. tulipiferae* (Rothschild *et al*., 2002), *P. platensis, P. brumalis, P. pinsitus* and *P. varius* (Tuor *et al*., 1995) and the brown rot species, *P. ostreiformis* (Dey *et al*., 1991). Although the productivity were either low or marginal, *Microporus* sp., *Tyromyces* sp. and *V. vicina* showed LiP activity that has not been previously reported. The *Tyromyces* sp. will be an addition to the few list of LiP producing brown rot fungi (Dey *et al*., 1991; Mtui and Masalu, 2008). LiP activities have been previously reported from *P. sajor-caju* (Tuor *et al*., 1995) and *Inonotus* sp (Risna and Suhirman, 2002).

In general, the ligninolytic enzyme production profiles of *F. aethiopica*, *F. pseudopunctata*, *F. carnea* and *V. vicina* have not been previously reported. The profiles of *Inonotus* sp., *Phellinus* sp., *Perenniporia* sp. and *P. sajor- caju* and the absence of any activity from *L. sulphureus* and *S. commune*, did not completely match and were contradictory, respectively with previous studies (Table 1) This could be due to the variation in the species or strains of the rot fungi and or the fermentation conditions in the present study. In the case of *L. sulphureus* and *S. commune*, enzyme activities were reported by Mtui and Masalu (2008) and Asgher *et al*. (2008) in liquid cultures prepared according to Tien and Kirk (1984, 1988), respectively. These media were also composed of limited inorganic N source and veratryl alcohol as lignin substrate. The effect of media on ligninolytic enzyme production has been reported by Kimura *et al*. (1990). They obtained high productivity of LiP by the white rot *P. chrysosporium* only in cultures composed of limited inorganic nitrogen; while on the contrary *Bjerkandra* sp. produced high titres in the presence of organic nitrogen rich supplements.

During growth, the red colour formed on the bagasse substrate could be an indication of lignin degradation during SSF. Fackler *et al*. (2007) observed red colouring of wood, during short-term treatment, from the release of free phenoxy radicals and resulting quinoid structures due to lignin degradation. In this study, this evidence was observed in all the highly selective white rots.

Ligninolytic and polysaccharide degrading enzyme productivity were inversely related among the highly selective degraders. As white rots, these fungi could have follow selective degradation pattern with more preference to lignin degradation (Hakala, 2007). This physiology might have been further influenced by the SSF conditions in this study. In addition to stimulation of secondary metabolism by the supplements, glucose can suppress

the production of polysaccharide degrading enzymes (Levonen-Munoz and Bone, 1985). These white rots did not utilise the polysaccharides despite presumably having better access, after lignin modification. Thus, it was more likely that lignin was metabolised along with the supplements as carbon and energy source. This metabolic pathway was suitable for the pretreatment system.

Better lignin degradation by *P. tephropora* might be attributed to its high Lac productivity. The white rot had no LiP activity and MnP productivity was comparable with the other selective lignin degraders. Arora *et al*. (2002) observed that white rots with high productivity of Lac showed better lignin degradation and the combination of either of the ligninolytic enzyme with Lac led to higher lignin loss.

The lignin loss by *P. tephropora* (7.71 %) was comparable with *Coriolus versicolor* (6- 7 %) and *P. chrysosporium* (9 %) pretreatments of hardwood lignin (Kashino *et al*., 1993). In other studies, lignin losses up to 15.7 % (Samsuri *et al*., 2008), 21 % (Bak *et al*., 2009) and 21.7 % (Itoh *et al*., 2003) have been reported. The relatively lower lignin degradation in the present study could be due to incomplete pretreatment from the short SSF period (15 days). This was evident with red colour formation of the bagasse substrate, which is a characteristic of short term pretreatment (Fackler *et al*., 2007). Other factors might include culture conditions and or simply the natural lignin degradation capability of *P. tephropora*.

The lignin composition of bagasse after *P. sajor-caju* pretreatment was greater than the untreated bagasse. This was an indication of cellulose and hemicelluloses weight loss from degradation, which was compensated by the accumulation of the unmodified lignin. However, the white rot had only marginal productivity of CMCase and xylanase. It is therefore suggested that the white rot might have cell bound CMCase and or xylanase activity that was not detected. In a study by Valaskova and Baldrian (2006), 66 % activities of cellulose, xylan and mannan degrading enzymes by *P. ostreatus* were cell bound. Even though the weight loss could not be determined, lignin degradation by *P. sajor-caju* was still evident from the red colour formation of the bagasse substrate and high productivity of MnP.

Pretreatment that caused higher lignin loss showed better cellulose digestibility. Samsuri *et al*. (2008) and Bak *et al*. (2009) observed that as the lignin weight loss increased, glucose yield from enzyme hydrolysis was improved. Thus, the modification of the lignin barrier has increased the accessibility of the cellulose to enzyme hydrolysis.

In previous studies, ethanol yield has been improved while sustaining 2.94 % (Samsuri *et al*., 2008) and 5 % (Itoh *et al*., 2003) cellulose weight losses from pretreatments. Improvements in glucose yield were also observed in the present study, despite CMCase activities. The highest improvement in digestibility was observed from *P. tephropora* pretreatment while having low CMCase productivity, which was higher than only marginal productivity by the other highly selective degraders. Thus, the impact of lignin modification has overshadowed the effect of cellulose degradation by the enzyme.

The saccharification of *P. tephropora* pretreated bagasse (12.49 %) was higher than that of beechwood (9.5 %) pretreated by *P. chrysosporium* (Sawada *et al*., 1995). The cellulose digestibility (29.44 %) was also higher than reports from *P. chrysosporium* pretreatment of corn stover (0.3- 12 %) and was comparable with *Cyathus stercoreus* pretreatment (8.3- 35.7 %) (Keller *et al*., 2003). In other studies, cellulose digestibility reached around 44 % (Samsuri *et al*., 2008) and as high as 64.9 % (Bak *et al*., 2009). These results were obtained from white rot pretreatments that caused higher lignin losses.

P. sajor-caju pretreatment showed low improvement in cellulose digestibility (1.7 %), despite the inability to determine lignin loss. The improvement, although relatively low, indicates some form of lignin modification. It also gives an implication that the suggested cell bound polysaccharide degrading enzyme activity might have been dominated by xylanase. However, this suggestion needs to be confirmed by further analysis.

The increase in glucose yield due to *P. tephropora* pretreatment consequently enhanced the production of ethanol as compared with the untreated bagasse. The glucose conversion efficiency was slightly reduced due to the pretreatment. In general, the fermentation was highly affected by factors in the biomass saccharification process, which was evident from the large difference in conversion efficiency as compared to fermentation of a standard glucose.

7. Conclusions and recommendations

MnP and Lac enzymes are more prevalent, while LiP is less frequent and low in activity among the studied rot fungi during SSF of bagasse. The white rots, *F. aethiopica, P. sajor-caju, Inonotus* sp. and *P. tephropora* are relatively, highly selective lignin degraders. They exhibit low CMCase and xylanase activity and produce ligninolytic enzymes in high titres. Pretreatment by these selective degraders cause lignin weight loss and as the degradation of lignin increases, accessibility to cellulose is also improved. Minimal polysaccharide consumption under these pretreatment conditions does not highly affect the glucose yield (*P. sajor-caju* pretreatment is an exception).

Among these selective white rots, *P. tephropora* is the most effective lignin degrader. Better ligninolysis is attributed to the high productivity of Lac enzyme in combination with MnP. *P. tephropora* pretreatment of bagasse improves ethanol production by partially degrading lignin, which increases accessibility of cellulose to enzyme hydrolysis.

Based on observations made in the present study, the following points are recommended for fungal pretreatment.

a. MnP produced by *F. aethiopica* should be purified, characterised and its potential in dye decolourization or other applications need to be studied.
b. The SSF system for *P. tephropora* pretreatment should be optimised for better productivity of ligninolytic enzymes while maintaining low polysaccharide consumption. Nutrient composition, moisture content and duration of pretreatment are among the conditions that need to be considered.
c. Hydrolysis of hemicelluloses and fermentation of 5 carbon sugars need to be integrated in bagasse to ethanol conversion process to increase ethanol yield.
d. A conditioning system might be required to remove possible inhibitory substances that affect polysaccharide degrading enzyme and fermentation.
e. Combination of *P. tephropora* pretreatment with chemical or physicochemical pretreatments and its effect on reduction of chemical and energy consumptions should be studied.
f. The possibility of employing cell free enzymatic systems for pretreatment, preferably MnP and Lac, need to be explored.

8. References

Archibald, F.S. (1992). A new assay for lignin-type peroxidases employing the dye azure B. *Appl. Environ. Microbiol.* **58**(9):3110-3116.

Arora, D.S. and Sandhu, D.K. (1985). Laccase production by a white rot fungus *Daedalea flavida*. *Enzyme Microb. Technol.* **7**:405-408.

Arora, D.S., Chander, M. and Gill, P.K. (2002). Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. *Int. Biodeter. Biodegr.* **50**:115-120.

Asgher, M., Kausar, S., Bhatti, H.N., Shah, S.A.H. and Ali, M. (2008). Optimization of medium for decolourization of Solar golden yellow R direct textile dye by *Schizophyllum commune* IBL- 06. *Int. Biodeter. Biodegr.* **61**:189-193.

Bak, J.S., Ko, J.K., Choi, I., Park, Y., Seo, J. and Kim, K.H. (2009). Fungal pretreatment of lignocelluloses by *Phanerochaete chrysosporium* to produce ethanol from rice straw. *Biotechnol. Bioeng.* **104** (3):471-482.

Balat, M., Balat, H. and Oz, C. (2008). Progress in bioethanol processing. *Prog. Energy Combus. Sci.* **34**:551-573.

Barbour, M.T., Gerritsen, J., Synder, B.D. and Stribling, J.B. (1999). *Rapid bioassessment protocols for use in streams and wadeable rivers: Periphyton, Benthic macroinvertebrates and fish*. 2nd ed. U.S. Environmental Protection Agency, Washington DC, pp. 9-9- 9-10.

Bernfeld, P. (1955). Amylases, alpha and beta. *Method Enzymol.***1**:149-158.

Bon, E.P.S. and Ferrara, M.A. (2007). Bioethanol production via enzymatic hydrolysis of cellulosic biomass. **In**: *The Role of Agricultural Biotechnologies for Production of Bioenergy in Developing Countries.* Proceedings of a FAO Seminar, October 12, 2007, Rome.

Bonnarme, P. and Jeffries, T.W. (1990). Mn (II) regulation of lignin peroxidase and manganese dependent peroxidases from lignin degrading white rot fungi. *Appl. Environ. Microbiol.* **56**(1):210-217.

Boyle, C.D., Kropp, B.R. and Reid, I.D. (1992). Solubilization and mineralization of lignin by white rot fungi. *Appl. Environ. Microbiol.* **58**(10):3217-3224.

Buaban, B., Inoue, H., Yano, S., Tanapongpipat, S., Ruanglek, V., Champreda, V., Pichyangkura, R., Rengpipat, S. and Eurwilaichitr, L. (2010). Bioethanol production from ball milled bagasse using an on-site produced fungal enzyme cocktail and xylose fermenting *Pichia stipitis*. *J. Biosci. Bioeng.* **110**(1):18-25.

Caylak, B. (1998). Comparison of different production processes for bioethanol. *Turk. J. Chem.* **22:**351-359.

Coll, P.M., Fernandez, J.M., Villanueva, J.R., Santamaria, R. and Perez, P. (1993). Purification and characterization of a phenoloxidase (Laccase) from the lignin degrading basidiomycete PM1 (CECT 2971). *Appl. Environ. Microbiol.* **59**(8):2607-2613.

Datta, A., Bettermann, A. and Kirk, T.K. (1991). Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. *Appl. Environ. Microbiol.* **57**:1453-1460.

Decock, C., Adane Bitew and Castillo, G. (2005). *Fomitiporia tenuis* and *Fomitiporia aethiopica* (Basidiomycetes, Hymenochaetales), two undescribed species from the Ethiopian highlands: taxonomy and phylogeny. *Mycologia* **97**(1):121-129.

Dey, S., Maiti, T.K. and Bhattacharyya, B.C. (1991). Lignin peroxidase production by a brown rot fungus, *Polyporus ostreiformis*. *J. Ferment. Bioeng.* **72**:402-404.

Dias, M.O.S., Ensinas, A.V., Nebra, S.A., Filho, R.M., Rossel, C.E.V. and Maciel, M.R.W. (2009). Production of bioethanol and other bio-based materials from sugar cane bagasse: Integration to conventional bioethanol production process. *Chem. Eng. Res. Des.* **87**:1206-1216.

Eichlerova, I., Homolka, L., Lisa, L. and Nerud, F. (2006). The influence of extracellular H_2O_2 production on decolourization ability in fungi. *J. Basic Microb.* **46**(6):449-455.

Elnashaie, S. and Garhyan, P. (2004). Bioethanol production- solving the efficiency bottle neck. *TCE- The Chem. Eng.* **755:**33-34.

Fackler, K., Gradinger, C., Schmutzer, M., Tavzes, C., Burgert, I., Schwanninger, M., Hinterstoisser, B., Watanabe, T. and Messner, K. (2007). Biotechnological wood modification with selective white rot fungi and its molecular mechanisms. *Food Technol. Biotechnol.* **45**(3):269-276.

Ferraz, A., Baeza, J., Rodriguez, J. and Freer, J. (2000). Estimating the chemical composition of biodegraded pine and eucalyptus wood by DRIFT spectroscopy and multivariate analysis. *Bioresource Technol.* **74**:201-212.

Ferraz, A., Cordova, A. and Machuca, A. (2003). Wood biodegradation and enzyme production by *Ceriporiopsis subvermispora* during solid state fermentation of *Eucalyptus grandis*. *Enzyme Microb. Technol.* **32**:59-65.

Ghose, T.K. (1987). Measurements of cellulase activities. *Pure Appl. Chem.* **59**(2):257-268.

Ghose, T.K. and Bisaria, V.S. (1987). Measurement of hemicellulase activities Part 1: Xylanases. *Pure Appl. Chem.* **59**(12):1739-1752.

Glazer, A.N. and Nikaido, H. (2007). *Microbial Biotechnology: Fundamentals of Applied Microbiology*. 2nd ed. Cambridge University Press, New York, pp. 461-463.

Gold, M.H. and Alic, M. (1993). Molecular biology of lignin degrading Basidomycete *Phanerochaete chrysosporium. Microbiol. Rev.* **57**(3):605-622.

Hakala, T.K. (2007). Characterisation of the lignin modifying enzymes of the selective white rot fungus *Physisporinus rivulosus*. Ph.D. dissertation. University of Helsinki, Helsinki. (http://www.doria.fi/bitstream/handle/10024/13100/characte.pdf?sequence=1) (Accessed on April, 2010)

Hamman, O.B., Rubia, T. and Martinez, J. (1999). The effect of manganese on the production of *Phanerochaete flavido-alba* ligninolytic peroxidases in nitrogen limited cultures. *FEMS Microbiol. Lett.* **177**:137-142.

Hatakka, A. (2001). Biodegradation of lignin. **In**: *Biopolymers*, Vol.1, p. 143, (Hofrichter, M. and Steinbuchel, A. eds). Wiley- VCH, Weinheim. (http://www.wileyvch.de/books/biopoly/pdf/v01_kap05.pdf) (Accessed on March, 2011).

Hjortstam, K and Ryvarden, L. (1996). New and interesting wood-inhabiting fungi (Basidiomycotina- Aphyllophorales) from Ethiopia. *Mycotaxon* **60**:181-190.

Hofrichter, M. (2002). Review: lignin conversion by manganese peroxidase (MnP). *Enzyme Microb. Technol.* **30**:454-466.

Itoh, H., Wada, M., Honda, Y., Kuwahara, M. and Watanabe, T. (2003). Bioorganosolve pretreatment for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. *J. Biotechnol.* **103**:273-280.

Kaal, E.E.J., Field, J.A. and Joyce, T.W. (1995). Increasing ligninolytic enzyme activities in several white rot basidiomycetes by nitrogen sufficient media. *Bioresource Technol.* **53**:133-139.

Kadam, K.L. (2000). *Environmental Life Cycle Implications of Using Bagasse Derived Ethanol as a Gasoline Oxygenate in Mumbai (Bombay): Technical Report*. National Renewable Energy Laboratory, Colorado, p. 8.

Kashino, Y., Nishida, T., Takahara, Y., Fujita, K., Kondo, R. and Sakai, K. (1993). Biomechanical pulping using white rot fungus IZU-154. *TAPPI J.* **76**(12):167-171.

Keller, F.A., Hamilton, J.E. and Nguyen, Q.A. (2003). Microbial Pretreatment of Biomass: potential for reducing severity of thermochemical biomass pretreatment. *Appl. Biochem. Biotech.* **105**:27-41.

Kimura, Y., Asada, Y. and Kuwahara, M. (1990). Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. *Appl. Environ. Microbiol.* **32**:436-442.

Kirk, P.M., Cannon, P.F., David, J.C. and Stalpers, J.A. (2001). *Ainsworth and Bisby's Dictionary of Fungi*. 9th ed. CABI publishing, Oxon.

Kirk, T.K. (1988). Lignin degradation by *Phanerochaete chrysosporium*. *ISI Atlas of Science: Biochem.* **0894-3753**:71-76.

Kirk, T.K. and Farrell, R.L. (1987). Enzymatic ''combustion'': the microbial degradation of lignin. *Annu. Rev. Microbiol.* **41**:465-505.

Kumar, A.G., Sekaran, G. and Krishnamoorthy, S. (2006). Solid state fermentation of *Achras zapota* lignocellulose by *Phanerochaete chrysosporium*. *Bioresource Technol*. **97**:1521-1528.

Kuwahara, M., Glenn, J.K., Morgan, M.A. and Gold, M.H. (1984). Separation and characterization of two extracellular H_2O_2- dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Lett.* **169**(2):247-250.

Lankinen, P. (2004). Ligninolytic enzymes of the basidomycetous fungi *Agaricus bisporus* and *Plebia radiata* on lignocellulose containing media. Ph.D. dissertation. University of Helsinki, Helsinki pp. 9-12. (https://oa.doria.fi/bitstream/handle/10024/3279/ligninol.pdf?sequence=1) (Accessed on April, 2010).

Lee, J. (1997). Biological conversion of lignocellulosic biomass to ethanol. *J. Biotechnol.* **56**:1-24.

Levin, L., Papinutti, L. and Forchiassin, F. (2004). Evaluation of Argentinean white rot fungi for their ability to produce lignin modifying enzymes and decolourize industrial dyes. *Bioresource Technol.* **94**:169-176.

Levonen-Munoz, E. and Bone, D.H. (1985). Effect of different gas environments on bench scale solid state fermentation of oat straw by white rot fungi. *Biotechnol. Bioeng.* **27**:382-387.

Lobos, S., Larrain, J., Salas, L., Cullen, D. and Vicuna, R. (1994). Isoenzymes of manganese dependent peroxidase and laccase produced by the lignin degrading basidiomycete *Ceriporiopsis subvermispora*. *Microbiology* **140**:2691-2698.

Martinez, M.J., Ruiz-Duenas, J., Guillen, F. and Martinez, A.T. (1996). Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. *Eur. J. Biochem.* **237**:424-432.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**(3):426-428.

Ministry of Mines and Energy (MOME). (2007). *The Biofuel Development and Utilisation Strategy*. Berta Printing Press, Addis Ababa.

Mtui, G. and Masalu, R. (2008). Extracellular enzymes from brown rot fungus *Laetiporus sulphureus* isolated from mangrove forests of coastal Tanzania. *Sci. Res. Essays* **3**(4):154-161.

Munoz, C., Mendonca, R., Baeza, J., Berlin, A. and Saddler, J. (2007). Bioethanol production from bioorganosolve pulps of *Pinus radiata* and *Acacia dealbata*. *J. Chem. Technol. Biot.* **82**:767-774.

Palma, C., Contreras, E., Urra, J. and Martinez, M.J. (2011). Eco-friendly technologies based on banana peel use for the decolourization of the dyeing process waste water. *Waste Biomass Valor.* **2**(1):77-86.

Pandey, A., Soccol, C.R., Nigam, P. and Soccol, V.T. (2000). Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource Technol.* **74**:69-80.

Papinutti, V.L. and Forchiassin, F. (2007). Lignocellulolytic enzymes from *Fomes sclerodermeus*. *J. Food Eng.* **81**:54-59.

Ralph, J.P., Graham, L.A. and Catcheside, D.E.A. (1996). Extracellular oxidases and the transformation of solubilised low rank coal by wood-rot fungi. *Appl. Microbiol. Biot.* **46**:226-232.

Risna, R.A. and Suhirman. (2002). Ligninolytic enzyme production by *Polyporaceae* from Lombok, Indonesia. *Fungal Divers.* **9**:123-134.

Rothschild, N., Novotny, C., Sasek, V. and Dosoretz, C.G. (2002). Ligninolytic enzymes of the fungus *Irpex lacteus* (*Polyporus tulipiferae*): isolation and characterization of lignin peroxidase. *Enzyme Microb. Technol.* **31**:627-633.

Rowell, R.M., Pettersen, R., Han, J.S., Rowell, J. and Tshabalala, M.A. (2005). Cell wall chemistry. **In**: *Handbook of Wood Chemistry and Wood Composites*, (Rowell, R.M., ed). CRC Press, Florida.

Samsuri, M., Gozan, M., Hermansyah, H., Prasetya, B., Nasikin, M. and Watanabe, T. (2008). Ethanol production from bagasse with combination of cellulase- cellubiase in simultaneous saccharification and fermentation using white rot fungi pretreatment. *J. Chem. Nat. Resource Eng.* **3**:20-32.

Sanchez, O.J. and Cardona, C.A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technol.* **99:**5270-5295.

Sawada, T., Nakamura, Y., Kobayashi, F., Kuwahara, M. and Watanabe, T. (1995). Effects of fungal pretreatment and steam explosion pretreatment on enzymatic saccharification of plant biomass. *Biotechnol. Bioeng.* **48**:719-724.

Schmidt, O. (2006). *Wood and Tree Fungi: Biology, Damage, Protection and Use*. Springer, Berlin, pp. 138-140.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. and Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedures (LAP)*. National Renewable Energy Laboratory, Colorado.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J. and Templeton, D. (2005). *Determination of Extractives from Biomass: Laboratory Analytical Procedures (LAP)*. National Renewable Energy Laboratory, Colorado.

Smith, J.E. (2004). *Biotechnology*. 4th ed. Cambridge University Press, New York, pp. 105-106.

Soccol, C.R., Vandenberghe, L.P.S., Medeiros, A.B.P., Karp, S.G., Buckeridge, M., Ramos, L.P., Pitarelo, A.P., Ferreira-Leitao, V., Gottschalk, L.M.F., Ferrara, M.A., Bon, E.P.S., Moraes, L.M.P., Araujo, J.A. and Torres, F.A.G. (2010). Bioethanol from lignocelluloses: status and perspectives in Brazil. *Bioresource Technol.* **101**:4820-4825.

Song, H. (1997). Biodegradation of aromatic hydrocarbons by several white rot fungi. *J. Microbiol.* **35**(1):66-71.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technol.* **83:**1-11.

Tien, M. and Kirk, T.K. (1984). Lignin- degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterisation, and catalytic properties of a unique H2O2 requiring oxygenase. *Proc. Natl. Acad. Sci.* **81**:2280-2284.

Tien, M. and Kirk, T.K. (1988). Lignin peroxidase of *Phanerochaete chrysosporium*. **In**: *Methods in Enzymology: Biomass, Part B, Lignin, Pectin and Chitin*, Vol. 161, p. 240, (Wood, W.A. and Kellogg, S.T., eds). Academic Press, Inc, San Diego.

Tomas-Pejo, E., Olivia, J.M. and Ballesteros, M. (2008). Realistic approach for full scale bioethanol production from lignocelluloses: a review. *J. Sci. Ind. Res. India* **67:**874-884.

Tuor, U., Winterhalter, K. and Fiechter, A. (1995). Enzymes of white rot fungi involved in lignin degradation and ecological determinants of wood decay. *J. Biotechnol.* **41**:1-17.

U.S. Department of Energy (USDOE). (2009). ABC's of biofuel. (http://www1.eere.energy.gov/biomass/abcs_biofuels.html#prod) (Accessed on April, 2010).

Vaithanomsat, P., Apiwatanapiwat, W., Chumchuent, N., Kongtud, W. and Sundhrarajun, S. (2011). The potential of coconut husk utilization for bioethanol production. *Kasetsart J. Nat. Sci.* **45**:159-164.

Valaskova, V. and Baldrian, P. (2006). Estimation of bound and free fractions of lignocellulose degrading enzymes of wood rotting fungi *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus*. *Res. Microbiol.* **157**:119-124.

Vares, T., Kalsi, M. and Hatakka, A. (1995). Lignin peroxidase and other ligninolytic enzymes produced by *Phlebia radiata* during solid state fermentation of wheat straw. *Appl. Environ. Microbiol.* **61**(10):3515-3520.

Walta Information Centre (WIC). (2011). Ethanol blended fuel price decreased by 40 cent. **In**: *WIC National News: Business*. March 16, 2011. (http://www.waltainfo.com/index.php?option=com_content&task=view&id=25763&Itemid=134) (Accessed on May, 2011).

Webster, J. and Weber, R.W.S. (2007). *Introduction to Fungi***.** 3rd ed. Cambridge University Press, New York.

Wyman, C.E. (2008). Cellulosic ethanol: a unique sustainable liquid transportation fuel. *Mater. Res. Soc. Bull.* **33**:381-383.

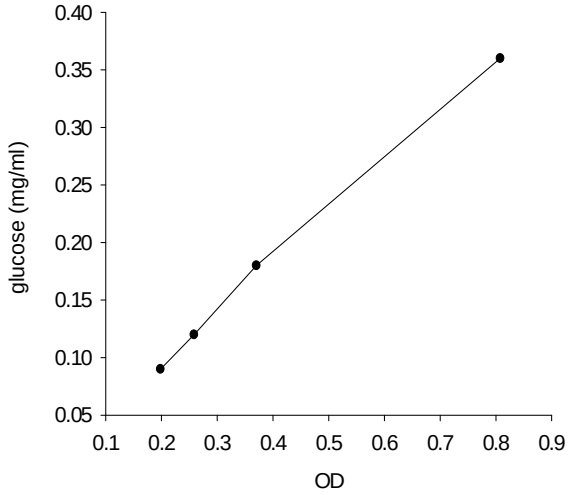
Yang, B. and Wyman, C.E. (2008). Pretreatment: the key to unlocking low cost cellulosic ethanol. *Biofuel Bioprod. Bior.* **2**:26-40.

Younes, S.B., Mechichi, T. and Sayadi, S. (2007). Purification and characterization of the laccase secreted by the white rot fungus *Perenniporia tephropora* and its role in the decolourization of synthetic dyes. *J. Appl. Microbiol.* **102**:1033-1042.

Zhao, J., Koker, H.T. and Janse, B.J.H. (1996). Comparative studies of lignin peroxidases and manganese dependent peroxidases produced by selected white rot fungi in solid media. *FEMS Microbiol. Lett.* **145**:393-399.

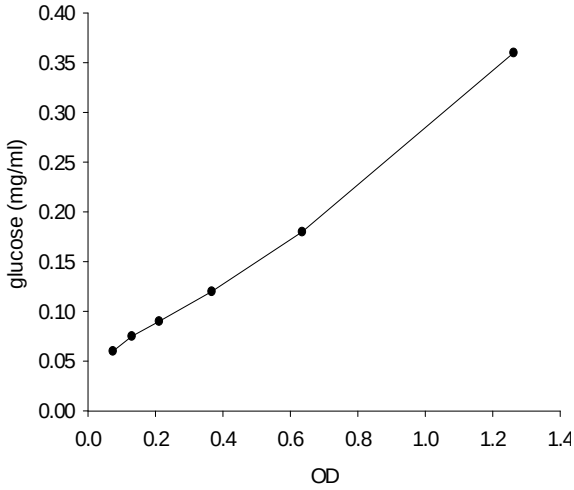
9. Appendices

Appendix 1. Standard curves of reducing sugars and ethanol



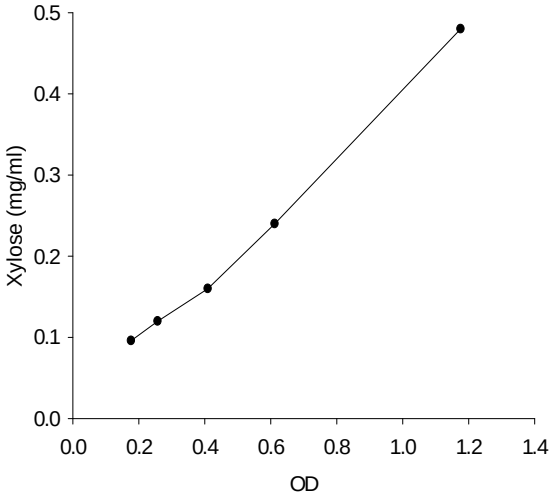
Appendix 1A. Standard curve of glucose by DNS method (Bernfeld, 1955)

Equation: y= 0.4386x + 0.082, R^2= 0.9972



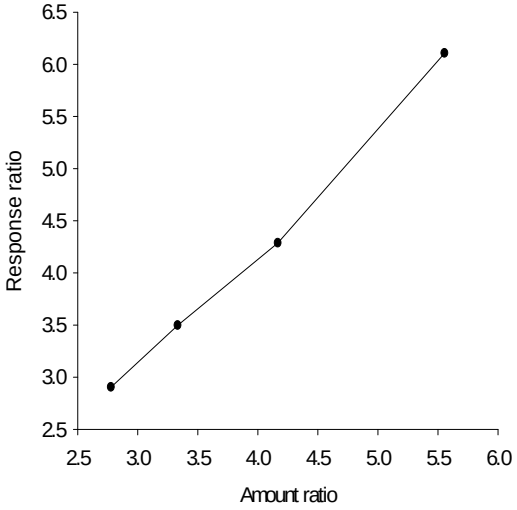
Appendix 1B. Standard curve of glucose by DNS method (Miller, 1959)

Equation: y = 0.25x + 0.035, R^2= 0.993



Appendix 1C. Standard curve of xylose by DNS method (Bernfeld, 1955)

Equation: y = 0.3889x + 0.0146
R^2= 0.9938



Appendix 1D. Standard curve of ethanol

Amount ratio = ethanol (g/L)/ propanol (g/L)
Response ratio = ethanol peak area (mV/s)/ propanol peak area (mV/s)
Equation: y = 1.149x - 0.351, R^2 = 0.994

Appendix 2. Volume of enzyme extracts from SSF

	Volume (ml)
Fomitopsis carnea	42
Laetiporus sulphureus	36
Tyromyces sp.	38
Fomitiporia aethiopica	37
Fomitiporia pseudopunctata	37
Inonotus sp.	39
Phellinus sp.	43
Pleurotus sajor-caju	36
Lentinus edodes	37
Microporus sp.	40
Polyporus sp.	41
Perenniporia sp.	40
Perenniporia tephropora	40
Vanderbylia vicina	39
Schizophyllum commune	37
Control[a]	40

[a] SSF media without inoculum

Appendix 3. Enzyme assay conditions and unit definitions

Assay	Volume of enzyme (ml)	Volume of reaction mixture (ml)	Incubation time (minutes)	Unit (U) definitions
MnP	0.3	3	5	0.1 increase in OD[a] unit per minute per ml of enzyme used = OD/ 0.1 x 5 x 0.3
Lac	0.1	1	60	Increase in 1 OD unit per hour per ml of enzyme used = OD/ 0.1
CMCase	0.1	1	15	1 µmol of reducing sugar released per minute per ml of enzyme used = glucose concentration[b]/ (0.18 x 15 x 0.1)
Xylanase	0.1	1	15	= Xylose concentration[b]/ (0.15 x 15 x 0.1)

[a] optical density after considering enzyme blank, [b] glucose and xylose concentrations were determined from standard curves in Appendix 1A and 1C, respectively

Appendix 4. Range and intervals of metrics and aggregate indices

Metrics	Range [a]	Intervals [b]
MnP	55.87	13.97
Lac	59.74	19.91
LiP	4	1
CMCase	8.23	2.06
Xylanase	17.27	4.32
Aggregate indices	11	2.75

[a] minimum – maximum productivity (U/g) of enzymes, [b] Range/number of levels

Appendix 5. Range of levels and respective scores assigned to each metrics

Productivity					Productivity		
Level	MnP	Lac	LiP	Score	CMCase	Xylanase	Score
High	(41.90, 55.87]	(59.74, 79.65]	(3, 4]	4	(6.47, 8.53]	(15.20, 19.52]	1
Moderate	(27.94, 41.90]	(39.83, 59.74]	(2, 3]	3	(4.42, 6.47]	(10.89, 15.20]	2
Low	(13.97, 27.94]	(19.91, 39.83]	(1, 2]	2	(2.36, 4.42]	(6.57, 10.89]	3
Marginal	[0, 13.97]	[0, 19.91]	[0, 1]	1	[0.3, 2.36]	[2.25, 6.57]	4

Appendix 6. Range of indices representing different category of selectivity

Selectivity	Aggregate indices
High	(13.25, 16]
Moderate	(10.5, 13.25]
Low	(7.75, 10.5]
Poor	[5, 7.75]

Appendix 7. MnP enzyme activities, productivity and scores of the rot fungi

	OD[a]			U[c]			U/g[d]						Score
	A[b]	B[b]	C[b]	A	B	C	A	B	C	Mean	STDEV[e]	SE[f]	
F. aethiopica	1.125	1.140	ND[g]	7.50	7.60	0.00	55.50	56.24	0.00	55.87	0.52	0.37	4[h]
P. tephropora	0.988	0.994	0.928	6.59	6.63	6.19	52.69	53.01	49.49	51.73	1.95	1.12	4
P. sajor-caju	0.956	0.955	0.952	6.37	6.37	6.35	45.89	45.84	45.70	45.81	0.10	0.06	4
Inonotus sp.	0.846	0.904	0.880	5.64	6.03	5.87	43.99	47.01	45.76	45.59	1.52	0.87	4
Microporus sp.	0.788	0.889	0.866	5.25	5.93	5.77	42.03	47.41	46.19	45.21	2.82	1.63	4
F. pseudopunctata	0.584	0.617	0.547	3.89	4.11	3.65	28.81	30.44	26.99	28.74	1.73	1.00	3[i]
Phellinus sp.	0.433	0.424	0.431	2.89	2.83	2.87	24.83	24.31	24.71	24.62	0.27	0.16	2[j]
L. edodes	0.263	0.254	0.266	1.75	1.69	1.77	12.97	12.53	13.12	12.88	0.31	0.18	1[k]
F. carnea	0.185	0.176	0.199	1.23	1.17	1.33	10.36	9.86	11.14	10.45	0.65	0.37	1
Tyromyces sp.	0.182	0.175	0.192	1.21	1.17	1.28	9.22	8.87	9.73	9.27	0.43	0.25	1
V. vicina	0.116	0.106	0.099	0.77	0.71	0.66	6.03	5.51	5.15	5.56	0.44	0.26	1
Perenniporia sp.	0.008	0.018	0.001	0.05	0.12	0.01	0.43	0.96	0.05	0.48	0.46	0.26	1
Control	0.008	0.005	0.000	0.05	0.03	0.00	0.43	0.27	0	0.23	0.22	0.12	NS[l]
L. sulphureus	0	0	0	0	0	0	0	0	0	0	0	0	1
Polyporus sp.	0	0	0	0	0	0	0	0	0	0	0	0	1
S. commune	0	0	0	0	0	0	0	0	0	0	0	0	1

[a] difference in OD between sample and enzyme blank, [b] triplicates, [c] defined according to Appendix 2, [d] (U x *e*) /*s*, [e] standard deviation, [f] standard error, [g] not determined, [h,i,j,k] high, moderate, low and marginal productivity levels, respectively according to Appendix 5, [l] not scored

Appendix 8. Lac enzyme activities, productivity and scores of the rot fungi

	OD			U			U/g						Score
	A	B	C	A	B	C	A	B	C	Mean	STDEV	Error	
P. tephropora	0.958	1.035	0.994	9.58	10.35	9.94	76.64	82.8	79.52	79.65	3.08	1.78	4
F. carnea	0.631	0.643	0.692	6.31	6.43	6.92	53.004	54.012	58.128	55.05	2.71	1.57	3
V. vicina	0.369	0.454	0.535	3.69	4.54	5.35	28.782	35.412	41.73	35.31	6.47	3.74	2
Polyporus sp.	0.325	0.233	0.251	3.25	2.33	2.51	26.65	19.106	20.582	22.11	4.00	2.31	2
Microporus sp.	0.201	0.225	0.282	2.01	2.25	2.82	16.08	18	22.56	18.88	3.33	1.92	1
Phellinus sp.	0.186	0.204	0.239	1.86	2.04	2.39	15.996	17.544	20.554	18.03	2.32	1.34	1
F. pseudopunctata	0.195	0.167	0.166	1.95	1.67	1.66	14.43	12.358	12.284	13.02	1.22	0.70	1
Tyromyces sp.	0.103	0.104	0.166	1.03	1.04	1.66	7.828	7.904	12.616	9.45	2.74	1.58	1
F. aethiopica	0.035	0.068	0.052	0.35	0.68	0.52	2.59	5.032	3.848	3.82	1.22	0.71	1
Perenniporia sp.	0.118	0	0.008	1.18	0	0.08	9.44	0	0.64	3.36	5.28	3.05	1
L. edodes	0.024	0.011	0.061	0.24	0.11	0.61	1.776	0.814	4.514	2.37	1.92	1.11	1
P. sajor-caju	0	0.039	0.006	0	0.39	0.06	0	2.808	0.432	1.08	1.51	0.87	1
Control	0.028	0.001	0	0.28	0.01	0	2.24	0.08	0	0.77	1.27	0.73	NS
S. commune	0	0	0	0	0	0	0	0	0	0	0	0	1
Inonotus sp.	0	0	0	0	0	0	0	0	0	0	0	0	1
L. sulphureus	0	0	0	0	0	0	0	0	0	0	0	0	1

Appendix 9. CMCase enzyme activities, productivity and scores of the rot fungi

	OD			Glucose (mg/ml)			U			U/g						Score
	A	B	C	A	B	C	A	B	C	A	B	C	Mean	STDEV	Error	
Tyromyces sp.	0.643	0.695	0.679	0.2902	0.3130	0.3060	1.0749	1.1594	1.1334	8.1691	8.8111	8.6136	8.53	0.33	0.19	1[h]
Microporus sp.	0.618	0.598	0.596	0.2793	0.2705	0.2696	1.0343	1.0018	0.9985	8.2742	8.0143	7.9883	8.09	0.16	0.09	1
Polyporus sp.	0.608	0.569	0.557	0.2749	0.2578	0.2525	1.0180	0.9547	0.9352	8.3479	7.8284	7.6685	7.95	0.36	0.21	1
L. edodes	0.629	0.632	0.608	0.2841	0.2854	0.2749	1.0521	1.0570	1.0180	7.7859	7.8219	7.5334	7.71	0.16	0.09	1
Perenniporia sp.	0.481	0.495	0.488	0.2192	0.2253	0.2222	0.8117	0.8345	0.8231	6.4938	6.6758	6.5848	6.58	0.09	0.05	1
F. pseudopunctata	0.503	0.486	0.433	0.2288	0.2214	0.1981	0.8475	0.8199	0.7338	6.2712	6.0669	5.4298	5.92	0.44	0.25	2[i]
S. commune	0.476	0.46	0.437	0.2170	0.2100	0.1999	0.8036	0.7776	0.7403	5.9467	5.7543	5.4779	5.73	0.24	0.14	2
F. carnea	0.337	0.369	0.362	0.1560	0.1700	0.1670	0.5778	0.6298	0.6184	4.8536	5.2902	5.1947	5.11	0.23	0.13	2
Phellinus sp.	0.316	0.337	0.305	0.1468	0.1560	0.1420	0.5437	0.5778	0.5258	4.6758	4.9692	4.5221	4.72	0.23	0.13	2
V. vicina	0.314	0.313	0.316	0.1459	0.1455	0.1468	0.5404	0.5388	0.5437	4.2155	4.2028	4.2408	4.22	0.02	0.01	3[j]
P. tephropora	0.21	0.222	0.217	0.1003	0.1056	0.1034	0.3715	0.3910	0.3829	2.9720	3.1280	3.0630	3.05	0.08	0.05	3
Inonotus sp.	0.219	0.201	0.235	0.1043	0.0964	0.1113	0.3861	0.3569	0.4121	3.0118	2.7837	3.2145	3.00	0.22	0.12	3
L. sulphureus	0.178	0.173	0.164	0.0863	0.0841	0.0801	0.3195	0.3114	0.2968	2.3006	2.2421	2.1368	2.23	0.08	0.05	4[k]
F. aethiopica	0.137	0.152	0.142	0.0683	0.0749	0.0705	0.2529	0.2773	0.2610	1.8716	2.0519	1.9317	1.95	0.09	0.05	4
P. sajor-caju	0.008	0.009	0.003	0.0117	0.0121	0.0095	0.0434	0.0450	0.0352	0.3122	0.3239	0.2538	0.30	0.04	0.02	4
Control	0	0	0	0.0082	0.0082	0.0082	0.0304	0.0304	0.0304	0.2430	0.2430	0.2430	0.24	0.00	0.00	NS

[h,i,j,k] high, moderate, low and marginal productivity levels, respectively according to Appendix 5

Appendix 10. Xylanase activities, productivity and scores of the rot fungi

	OD			Xylose (mg/ml)			Units			U/ g						Score
	A	B	C	A	B	C	A	B	C	A	B	C	Mean	STDEV	Error	
L. edodes	1.569	1.585	1.314	0.6245	0.6307	0.5254	2.7757	2.8033	2.3350	20.5400	20.7446	17.2793	19.52	1.94	1.12	1[h]
Polyporus sp.	1.337	1.204	1.303	0.5343	0.4826	0.5211	2.3748	2.1450	2.3160	19.4732	17.5886	18.9914	18.68	0.98	0.57	1
S. commune	1.721	1.28	1.164	0.6836	0.5122	0.4671	3.0383	2.2763	2.0758	22.4837	16.8445	15.3612	18.23	3.76	2.17	1
L. sulphureus	0.95	0.912	1.111	0.3839	0.3691	0.4465	1.7060	1.6404	1.9843	12.2835	11.8107	14.2866	12.79	1.31	0.76	2[i]
F. carnea	0.725	0.929	0.75	0.2964	0.3757	0.3061	1.3172	1.6698	1.3604	11.0649	14.0260	11.4277	12.17	1.62	0.93	2
Phellinus sp.	0.725	0.69	0.693	0.2964	0.2828	0.2839	1.3172	1.2568	1.2619	11.3283	10.8082	10.8528	11.00	0.29	0.17	2
Microporus sp.	0.713	0.768	0.719	0.2917	0.3131	0.2940	1.2965	1.3915	1.3069	10.3721	11.1324	10.4550	10.65	0.42	0.24	3[j]
Tyromyces sp.	0.592	0.613	0.844	0.2447	0.2528	0.3426	1.0874	1.1237	1.5229	8.2644	8.5402	11.5739	9.46	1.84	1.06	3
Perenniporia sp.	0.383	0.377	0.403	0.1634	0.1611	0.1712	0.7263	0.7159	0.7608	5.8101	5.7272	6.0866	5.87	0.19	0.11	4[k]
F. pseudopunctata	0.372	0.412	0.367	0.1591	0.1747	0.1572	0.7073	0.7764	0.6986	5.2337	5.7452	5.1698	5.38	0.32	0.18	4
V. vicina	0.312	0.292	0.303	0.1358	0.1280	0.1323	0.6036	0.5690	0.5880	4.7079	4.4384	4.5866	4.58	0.14	0.08	4
P. tephropora	0.28	0.272	0.265	0.1234	0.1203	0.1175	0.5483	0.5345	0.5224	4.3863	4.2757	4.1789	4.28	0.10	0.06	4
Inonotus sp.	0.182	0.137	0.169	0.0853	0.0678	0.0802	0.3789	0.3012	0.3565	2.9557	2.3492	2.7805	2.70	0.31	0.18	4
P. sajor-caju	0.184	0.148	0.167	0.0860	0.0720	0.0794	0.3824	0.3202	0.3530	2.7533	2.3054	2.5417	2.53	0.22	0.13	4
F. aethiopica	0.132	0.159	0.124	0.0658	0.0763	0.0627	0.2925	0.3392	0.2787	2.1648	2.5101	2.0625	2.25	0.23	0.14	4
Control	0	0.072	0.04	0.0145	0.0425	0.0301	0.0644	0.1889	0.1336	0.5156	1.5109	1.0685	1.03	0.50	0.29	NS

[h,i,j,k] high, moderate, low and marginal productivity levels, respectively according to Appendix 5

Appendix 11. GC analysis of ethanol concentrations from fermentation broths

Fermentation broths	Ethanol[a] (mv/s)	Propanol[b] (mv/s)	Response[c] ratio	Amount[d] ratio	Ethanol [e] (g/L)
P. tephropora pretreated bagasse hydrolysate	2626.008	1462.335	1.795764	1.868376	1.87
Untreated bagasse hydrolysate	2005.078	1497.176	1.33924	1.471053	1.477
Standard glucose	1377.882	309.782	4.447909	4.176596	4.18

peak areas of [a] ethanol and [b] propanol, [c] peak areas of (ethanol/propanol),
[d] (Response ratio + 0.351)/1.149 (based on equation from Appendix 1D),
[e] Amount ratio x propanol concentration (0.9 g/L)/dilution factor (0.9)

Appendix 12. Growth of *Inonotus* sp. on SSF

Appendix 13. Decolourization of azure B by *Polyporus* sp.

Culture of *Polyporus* sp. (left) shows complete decolourization while *P. tephropora* (right) did not change the blue colour of azure B on confirmation media