The Lignocellulolytic System in *Lentinula edodes*

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Declaration

The experiments described in this dissertation were carried out in Molecular Biotechnology Programme, The Chinese University of Hong Kong, between August 2004 and June 2009. This work is solely that of the author. No part of this dissertation is being concurrently submitted for any other degree, diploma or other qualification at this or any other institutions.

Thesis Committee

Professor JIANG, Liwen (Chair)

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Abstract

The shitake mushroom, *Lentinula edodes*, is one of the most commonly consumed edible mushrooms in Asian countries. It is a saprophyte that naturally colonizes dead wood. As a member of wood-decaying white rot basidiomycete, *L. edodes* is able to depolymerize lignin and hydrolyze wood polysaccharides. However, the enzymatic mechanism for its lignocellulolytic system is poorly understood. Examination on the *L. edodes* genome and transcriptome revealed a unique lignocellulolytic system. *L. edodes* has a diverse enzymatic arsenal for lignin degradation. The enzymes include laccase, manganese peroxidase, cellobiose dehydrogenase and various lignin degrading auxiliary enzymes. When compared to another white rot fungus *Phanerochaete chrysosporium*, *L. edodes* possesses more hemicellulase- and pectinase-coding genes, and fewer genes encoding cellulases, suggesting that it preferentially attacks non-cellulosic polysaccharides. The transcription analysis on genes related to antioxidative mechanisms also offers insights to the oxidative stress encountered by mycelium during the free radical-mediated lignin degradation.

Being the most abundant carbon-containing terrestrial biopolymer, lignocellulose serves as one of the best candidate feedstocks for biofuel production. The current cost-ineffective method for lignocellulose pretreatment is one of the major barriers that hinder the development of biofuel production. This leads to an exploration in the potential application of lignocellulolytic enzymes in this biorefinery process. Taking advantage of the strong activity of ligninolytic enzymes in *L. edodes*, we aimed at cloning and heterologously

expressing these enzymes. The present project applied a yeast expression system, *Pichia pastoris*, as a laboratory-scale platform for heterologous expression of one of our target ligninolytic enzymes, manganese peroxidase (MnP). We successfully cloned and expressed recombinant MnP. Its enzymatic activity was the highest when grown in the presence of hemoglobin. Our long-term goal is to establish a platform for the large-scale production of recombinant lignocellulotyic enzymes at low-cost, which would strengthen their application in biofuel production.

論文摘要

香菇（*Lentinula edodes*）是一種在亞洲國家最普遍的食用菌。它是一個天然枯木腐生物。作爲一種能分解木質的白腐擔子菌，香菇能夠解聚木質素和水解木材裏的多醣。然而，我們對這系統的機制了解甚少。研究香菇基因組和轉錄組發現了香菇一個獨特的木質纖維素酶系統，當中有不同的木質素降酶解，包括漆酶，錳過氧化物酶，纖維二糖脫氫酶和各種輔助木質素降解酶。與另一隻白腐菌孢原毛平革菌（*Phanerochaete chrysosporium*）相比，香菇擁有大量的半纖維素酶和果膠酶編碼基因，但編碼纖維素的基因則較少，這表示它可能優先攻擊非纖維素多醣。分析與抗氧化機制有關基因的轉錄還提供了在自由基介導的木質素降解過程中，菌絲體遇到氧化應激的一些認知。

作爲最豐富的含碳陸地生物聚合物，木質纖維素乃其中一個用作生物燃料生產的最佳原料。現今，低成本效益的木質纖維素預處理方法常阻礙了生物燃料生產的發展。這就導致了探索木質素降酶解於這生物煉製過程裏的可用性。利用香菇木質素降解酶的強大活性，我們旨在克隆和異源地表達這些酶。本研究採用了畢赤酵母這個酵母表達系統，作爲一個實驗室規模的平台，異源表達了我們的一個目標木質素降解酶 – 錳過氧化物酶（MnP）。我們成功地克隆和表達 rMnP ，並發現加入血紅蛋白時，其酶活性是最高的。我們的長遠目是建立一個低成本、可大量生產木質纖維素酶的平台，這將肯定有利於生物燃料生產的應用。

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Abbreviations

Abbreviations used in this thesis without definition include:

a.a.	Amino acid(s)
bp	Base pair(s)
cDNA	Complementary DNA
CTAB	Hexadecyl trimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ESTs	Expressed sequence tags
HIS	Histidine
Kb	Kilobase(s)
kDa	Kilodalton(s)
mRNA	Messenger RNA
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PVP40	Polyvinyl pyrrolidone (vinylpyrrolidine homopolymer), Mw 40,000
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl) aminomethane
YPD	Yeast peptone dextrose medium

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Chapter 1: Literature Review

1.1 Introduction

The shiitake mushroom, *Lentinula edodes* (Berk.) Pegler, is one of the most cultivated and popularly consumed mushrooms in Asian countries, especially in China and Japan. It is highly appreciated due to its nutritional and medicinal values, e.g. in lowering arterial blood pressure and blood cholesterol levels and preventing heart diseases and cancers (Wasser and Weis, 1999).

L. edodes is a good wood-rotter, and its lignocellulolytic activity was demonstrated just over twenty years ago (Leatham, 1985). Due to the production of significant amounts of lignocellulolytic enzymes during its vegetative mycelial growth (Buswell et al., 1995; Elisashvili et al., 2008), *L. edodes* has been applied in dye decolorization during industrial waste treatment (Hatvani and Mecs, 2002). In addition, waste mushroom logs after shiitake cultivation have recently been used as feedstock material in bioethanol production (Lee et al., 2008).

In this project, I studied the genes involved in lignocellulolytic system of *L. edodes* during vegetative mycelial growth by means of genomics and transcriptomics analysis. Comparisons on genome sequence of *L. edodes* and other model fungi, *Coprinopsis cinerea*, *Phanerochaete chrysosporium* and *Postia placenta*, were performed, allowing us to broaden the understanding on lignocellulolytic systems in wood-rotting fungi. Besides, I also heterologously expressed two ligninolytic enzymes, manganese peroxidase and laccase, in yeast system. Our long term goal is to establish a stable system for synthesizing lignocellulolytic enzymes at a low production cost, so as to encourage the industrial applications of these enzymes in waste water

treatment and biofuel production in the near future.

1.2 Life cycle of *L. edodes*

L. edodes belongs to the class Basidiomycetes, which has a unique sexual process. Meiosis of diploid nucleus takes place in basidium, forming haploid basidiospores. This class of fungi also possesses two unique characteristics. The first one is the presence of clamp connections, which is important for the maintenance of dikaryotic state. The second feature is the active launching of basidiospores, which also termed ballistospores (Carlile et al., 1994).

L. edodes possesses a typical life cycle as other basidiomycetes do (Fig. 1.1). Two compatible primary monokaryotic mycelia fuse to form secondary dikaryotic mycelium with clamp connections. During vegetative growth, dikaryotic mycelium propagates as filamentous form. Under certain stressful conditions, such as cold shock, dikaryotic mycelium aggregates to form primordium which eventually develops into fruiting body with basidium. Basidiospores are released when the fruit body is mature (Carlile et al., 1994).

1.3 Lignocellulolytic system in wood-rotting fungi

1.3.1 Structures of lignin, cellulose and hemicellulose

Wood is a major source of carbon in plant biomass (Perez et al., 2002). It is made up of lignocellulose with compositions of 20%-30% lignin, 40%-50% cellulose, and 20%-30% hemicellulose, depending on the types of wood and tissue (source from Department of energy, US). Lignin, cellulose and hemicellulose associate together in plant cell wall (Fig. 1.2)

Lignin is found in higher plants, including ferns (Kirk and Farrell, 1987). It is a rigid and complex biopolymer that protects plants from microbial attacks. It is formed by random polymerization of phenylpropanoids, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Kirk and Farrell, 1987). Due to its

heterogeneity, lignin is very resistant to enzymatic hydrolysis (Fig. 1.3).

Cellulose microfibrils are composed of linear chains of D-glucose units with β-1, 4-glucosidic bonds. Chains of cellulose are packed in highly regular and parallel crystalline structure that constitutes the core component of plant cell walls, conferring rigidity and protection to each plant cell. Cellulose microfibrils also form a central scaffold for the deposition of hemicellulose and lignin within the cell wall (Mohnen et al., 2008). A schematic diagram illustrating the structure of cellulose is shown in Fig. 1.4.

Hemicellulose is a collective term referring to non-cellulosic polysaccharides that can be extracted by dilute alkali (Mohnen et al., 2008). Different from cellulose, hemicelluloses is heterogeneous in structure, comprising of various kinds of sugar monomers, e.g. xylose, arabinose, mannose, glucose and galactose, together with some sugar acids (Kumar et al., 2008). The majority of polymers in hemicellulose are xylans, xyloglucan, mixed-linkage glucans and mannans (Mohnen et al., 2008) while galactans and arabinans occur less frequently (Broda et al., 1996). Extensive branching usually present on the sugar backbone of hemicellulose biopolymers resulting in amorphous structure, so it is much more susceptible to hydrolysis than cellulose. Fig. 1.5 illustrates the structures of xylan and glucomannan.

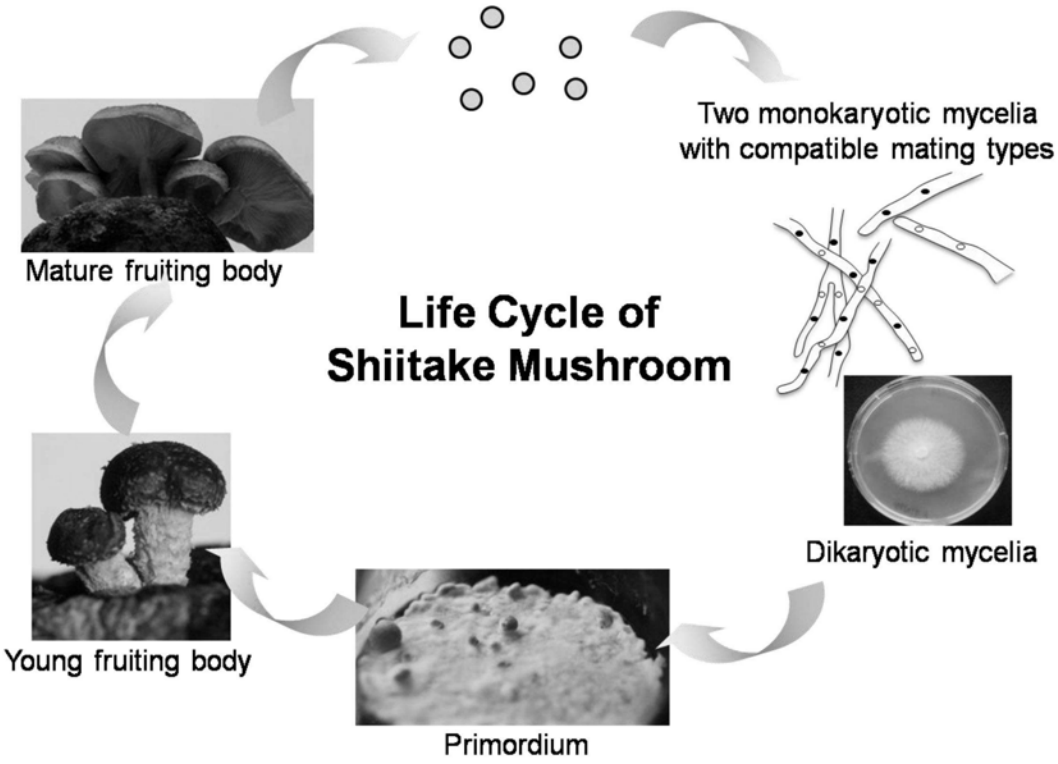


Fig. 1.1: Life cycle of shiitake mushroom, *Lentinula edodes*.

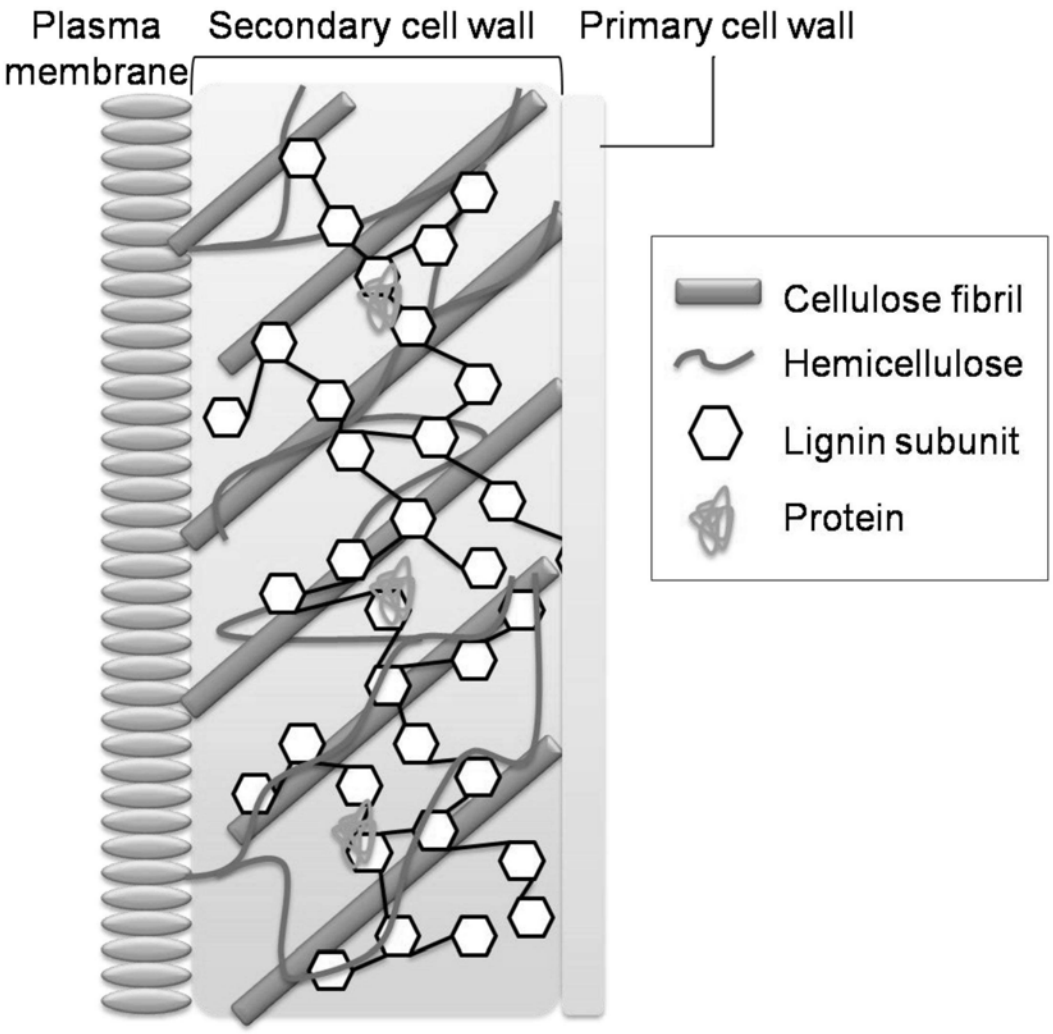


Fig. 1.2: Schematic diagram showing the association of lignin and polysaccharides in secondary cell wall.

**Modified from Mohnen et al., 2008.*

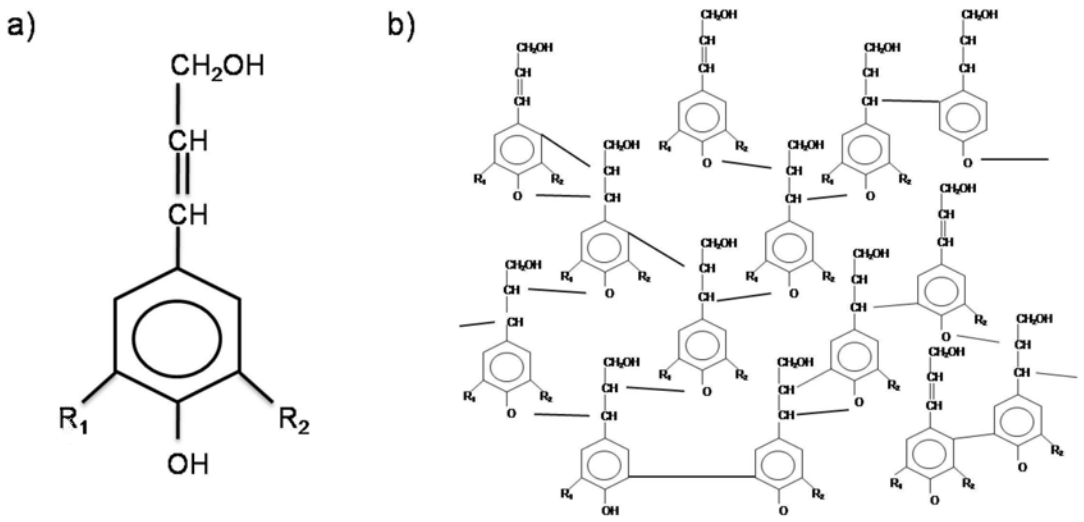


Fig. 1.3: a) The Structure of lignin precursor alcohol (p-coumaryl alcohol, R_1=R_2=H; sinapyl alcohol, R_1=R_2=OCH_3; coniferyl alcohol, R1=OCH_3, R_2=H). b) Schematic diagram shows part of the complex and heterogeneous lignin biopolymer.

**Modified from Kirk and Farrell, 1987.*

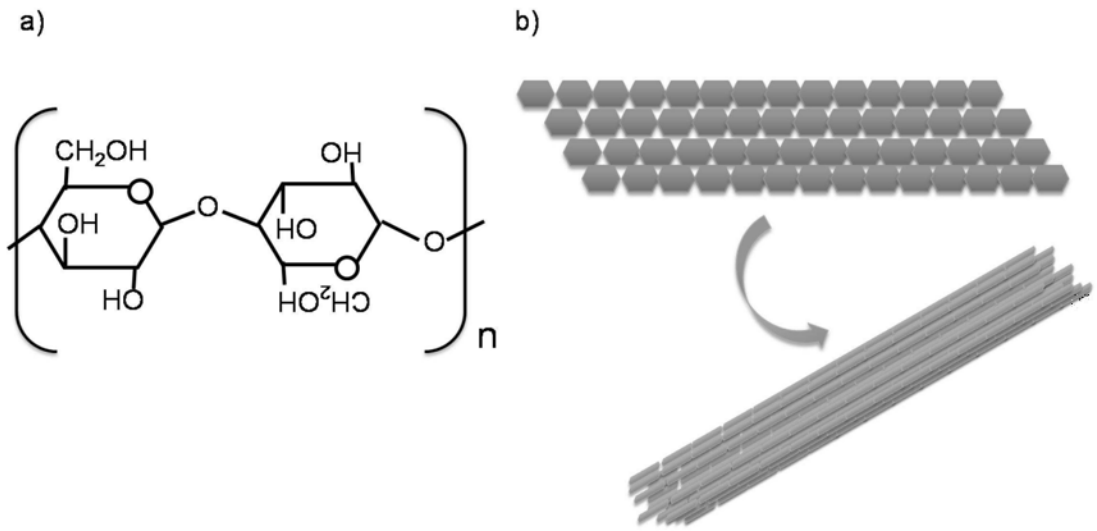


Fig. 1.4: Cellulose structure. a) Schematic diagram showing the building block of cellulose. Glucose monomer is linked with each other by β-1, 4-glucosidic bond and the number of glucose unit (n) can be ranged from 6,000 to 14,000. b) The extened chains of cellulose are tightly packed to form three-dimensional microfibril.

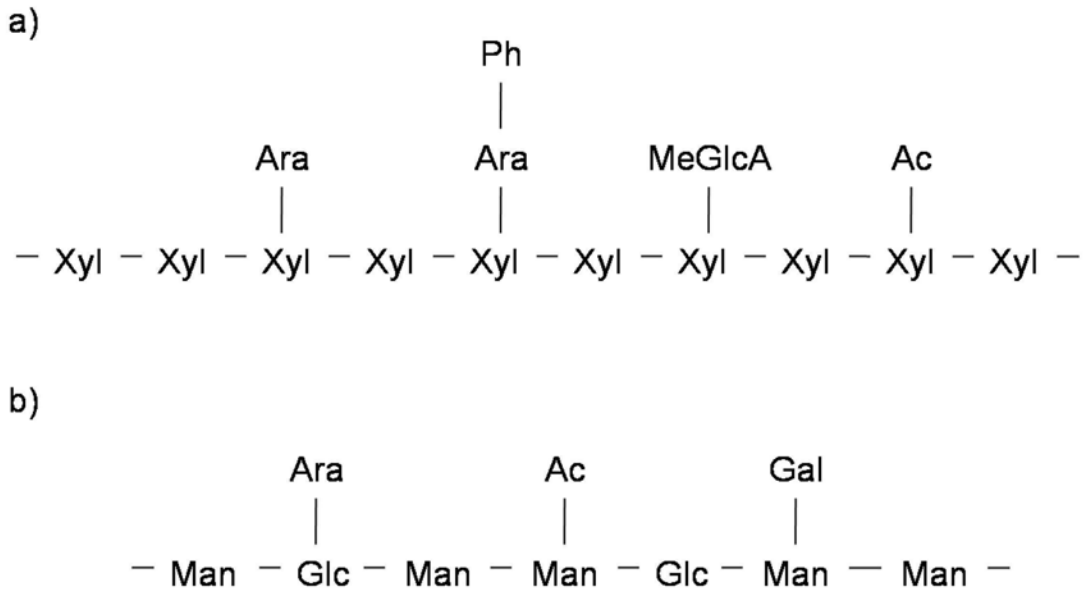


Fig. 1.5: Schematic diagram showing structures of two commonly occurred hemicelluloses, a) xylan; b) glucomannan. (Xly = xylose; Ara = arabinose; Ph = phenolic groups; MeGlcA = methylglucuronic acid; Ac = acetyl; Man = mannose; Glc = glucose; Gal = galactose.)

**Modified from Decker et al., 2008*

1.3.2 Wood-rotting fungi

Degradation of lignocellulose is important for carbon recycling in the ecosystems. Fungi, especially Basidiomycetes, are known to be efficient wood-rotters due to their capability to degrade lignocellulose. There are mainly two groups of wood-rotting fungi, the white rot and brown rot. White rot fungi, including *L. edodes*, *Phanerochaete chrysosporium* and *Pleurotus* spp., can degrade cellulose and hemicelluloses in plant cell walls, and at the same time, it can oxidize lignin completely into carbon dioxide (Martinez et al., 2005), so the wood after decay appears as white and brittle due to the removal of lignin. On the contrary, brown rot fungi, including *Postia placenta* and *Gloeophyllum trabeum*, can only modify, but not oxidize lignin, thus the decayed wood remains brown in color. It is still unclear about the mechanism of how brown rot fungi can get access to the polysaccharides protected by lignin (Perez et al., 2002).

1.3.3 Ligninolytic enzymes

As mentioned above, white rot fungi can degrade lignin completely into carbon dioxide and water (Martinez et al., 2005). The degradation process is initiated by synergistic actions of various oxidoreductases and propagated with the aid of highly oxidative molecules and cationic radicals. Important ligninolytic enzymes include lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lcc). LiP and MnP are heme-containing peroxidases, which require hydrogen peroxide to start their catalytic cycles. Resting states are restored through oxidation of their respective substrates, non-phenolic aromatics (e.g. veratryl alcohol) for LiP and Mn^{2+} for MnP (Fig. 1.6). Lcc is a copper-containing phenoloxidase. With relatively lower redox potential, Lcc can oxidize phenolic

lignin subunits directly, but only to a limited extent (Martínez et al., 2005). Mediators, such as 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), are required for Lcc to oxidize non-phenolic lignin units. MnP and Lcc are produced by almost all wood-rotted basidiomycetes. On the contrary, a number of lignin-degrading fungi, including the shiitake mushroom, do not secrete LiP (Buswell et al., 1995).

1.3.4 Ligninolytic enzymes in *L. edodes*

L. edodes is a good lignin degrader, and its ligninolytic activity was demonstrated just over twenty years ago (Leatham, 1985). *L. edodes* produces significant amounts of MnP and Lcc, but no LiP activity has been detected (Buswell et al., 1995; Elisashvili et al., 2008). Several ligninolytic enzymes have been identified in the past few years, including MnP (Kwok et al., 2006b; Sakamoto et al., 2009) and Lcc (Zhao and Kwan, 1999). Owing to the efficient ligninolytic ability, *L. edodes* has been applied in waste water treatment and dye decolorization (Hatvani and Mecs, 2002). Waste mushroom logs after the cultivation of *L. edodes* were also considered as potential feedstock for bioethanol production (Lee et al., 2008).

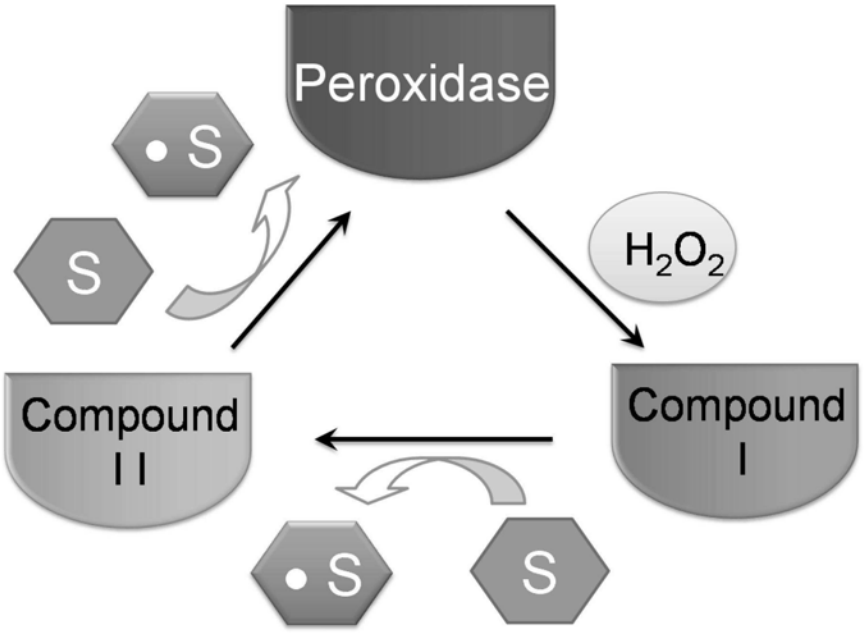
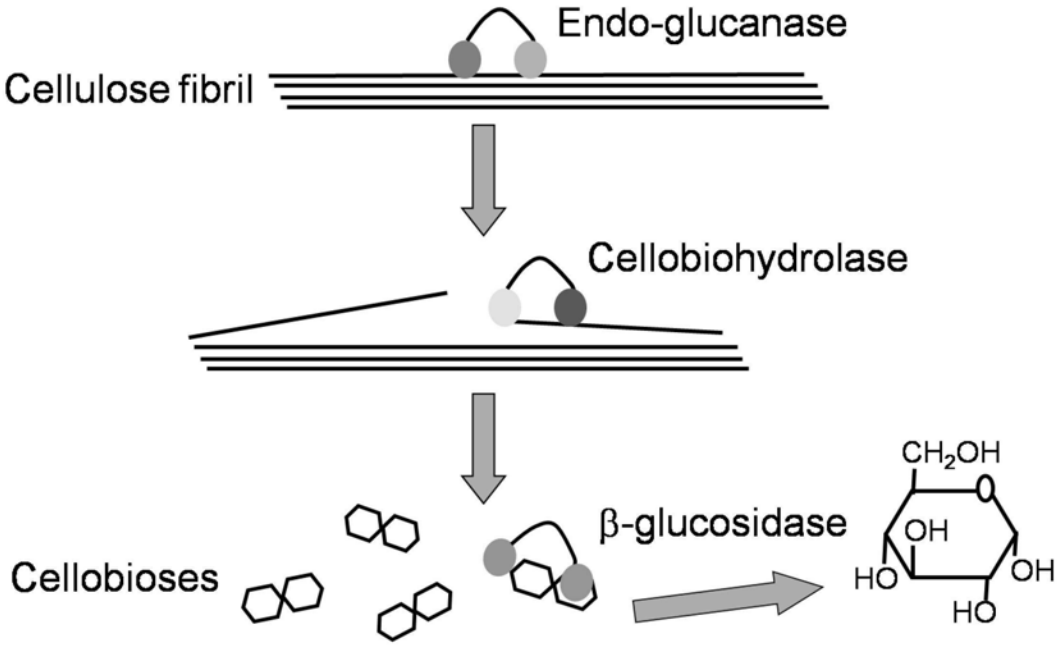


Fig 1.6: Catalytic cycle of lignin peroxidase (LiP) and manganese peroxidase (MnP). (S: Substrate for the peroxidase; •S: Oxidized form of the substrate. See text for details.)

1.3.5 Cellulolytic enzymes

Many microorganisms, including fungi, are able to secrete cellulases to completely hydrolyze the cellulose fibrils. These fungal cellulases have been studied for decades (Duenas et al., 1995; MargollesClark et al., 1996; Bhat and Bhat, 1997; Blum et al., 1999; Baldrian and Valaskova, 2008). The typical hydrolysis of cellulose involves the synergistic actions of endoglucanase, cellobiohydrolase and β-glucosidase (Baldrian and Valaskova, 2008). Endoglucanase breaks down internal glucosidic bonds within the β-1, 4-chains, generating free non-reducing ends. Cellobiohydrolase releases cellobiose from the non-reducing ends, whilst β-glucosidase hydrolyzes cellobiose into monomeric glucose units (Fig. 1.7).

Fungi can also hydrolyze hemicellulose efficiently, xylans in particular (Haltrich et al., 1996). As mentioned in chapter 1.3.1, hemicellulose is heterogeneous and extensively branched biopolymers. There are a large variety of hemicellulase, each of which is functional specific to its substrate, e.g. xylosidase is targeted to release xylose from xylobise (Decker et al., 2008). In general, hemicellulase can be classified into two major categories, depolymerizing and debranching. Depolymerizing hemicellulase acts on sugar backbones, which are usually xylans, mannans and glucans. Similar to cellulase, these types of hemicellulase can be divided into endo- and exo-acting, depending on the position of sugar chains they attack. Debranching enzymes can be grouped into two sub-classes, one acts on glycosidic linkages (e.g. α-L-arabinofuranosidase and α-glucuronidase) while another acts on ester-linkages (e.g. acetyl xylan esterase and feruloyl esterase) (Decker et al., 2008).


Endo-glucanase
Cellulose fibril
Cellobiohydrolase
β-glucosidase
Cellobioses
CH2OH
OH
HO
OH
HO

1.3.6 Cellulolytic enzymes in *L. edodes*

As early as 1985, Leatham demonstrated cellulolytic activity in *L. edodes*. According to his finding, shiitake is only moderately cellulolytic due to its low extent of cellulose degradation (Leatham, 1985). So far, only two cellulase genes, *cbhI* and *cbhII*, have been identified in *L. edodes* (Lee et al., 2001)

Despite the active production of hemicellulases in *L. edodes*, particularly xylanase (Leatham, 1985; Silva et al., 2005), the identity and characteristics of genes encoding hemicellulase remains poorly understood. Up to date, xylanase gene, *xyn11a*, is one of the few that has been cloned and characterized (Lee et al., 2005).

1.3.7 Future prospect of research on lignocellulolytic enzymes in *L. edodes*

Significant breakthrough in sequencing technology facilitates and enhances genomics and transcriptomics analysis of *L. edodes*. Currently, more than ten thousands ESTs (Expressed Sequence Tags) are available in NCBI and some of them are potential candidate genes that encode lignocellulolytic enzymes (will be discussed in chapter 2). Together with the ongoing genome sequencing project (will be discussed in chapter 3), it is expected that there will be more genes to be identified and characterized in the near future.

1.4 Expression systems for fungal lignocellulolytic enzymes

Due to the high commercial value of lignocellulolytic enzymes, particularly laccases and cellulases, there is an increasing interest in large scale production of these enzymes. However, the time required for cultivation of whole mushroom and the high cost for protein purification limit the use of wood-rotting fungi in industrial scale. Other microorganisms have been identified as alternative hosts for lignocellulolytic enzyme production. Yeast is

one of the most frequently used eukaryotic host system, which bears several advantages over prokaryotic organisms (e.g. *Escherichia coli*). It has a capability of protein processing, including protein folding and glycosylation, which are important for producing enzymes with native conformations and lignocellulolytic activities. Meanwhile, comparing to other eukaryotic systems, such as cultured cells from insects and plants (Porro and Mattanovich, 2004), the ease in genetic manipulation and its high growth rate allow yeast to serve as a promising heterologous expression systems for lignocellulolytic enzymes. In the past decades, a number of lignocellulolytic enzymes were successfully expressed in yeasts (Jonsson et al., 1997; Conesa et al., 2001; Conesa et al., 2002; Gu et al., 2003; Guo et al., 2005; Piscitelli et al., 2005; Bohlin et al., 2006; Colao et al., 2006; Bleve et al., 2008; Faraco et al., 2008).

Starting from early 1980s, *Saccharomyces cerevisiae*, has been used for expressing the majority of recombinant proteins produced in yeasts due to the deep understanding of its molecular genetics, biochemistry, physiology and fermentation process (Porro and Mattanovich, 2004). Also, it is non-pathogenic and recognized as GRAS organism (generally regarded as safe) by the US Food and Drug Administration (FDA) allowing its usage in food industry. However, the undesirable characteristics of *S. cerevisiae* in retaining foreign proteins within periplasmic space and hyperglycosylating proteins hinder its application for large scale protein production. This leads to the search for alternative yeast expression systems, so-called “non-conventional” yeasts (Porro and Mattanovich, 2004).

Pichia pastoris and *Kluyveromyces lactis* are two non-conventional, non-*Saccharomyces* yeast systems that have been well-developed and commonly employed in heterologous expression.

Pichia pastoris is a methylotrophic yeast that can utilize one-carbon compounds, e.g. methanol, as the sole carbon and energy source (Porro and Mattanovich, 2004). There is a strong promoter that tightly regulates the expression of a methanol-oxidizing enzyme, alcohol oxidase (AOX), which accounts for 30% of total proteins (Couderc and Baratti, 1980). There are two different AOXs produced in *P. pastoris*, AOX1 and AOX2, while there are three phenotypes depending upon methanol utilization, Mut^+ (methanol utilization wild type), Mut^S (methanol utilization slow) and Mut^- (methanol utilization deleted). With both *AOX1* and *AOX2* being intact, Mut^+ strain utilizes methanol normally. Mut^S strain has *AOX1* (stronger one) being interrupted, so it metabolizes methanol in a lower rate. For Mut^-, methanol utilization is limited due to the deletion of both *AOX1* and *AOX2* (Porro and Mattanovich, 2004). Methanol-induced *AOX1* promoter system in *P. pastoris* has been a promising expression system for lignocellulolytic enzymes (Cereghino and Cregg, 2000; Bohlin et al., 2006; Colao et al., 2006; Duruksu et al., 2009; Zhou et al., 2009). Unlike *S. cerevisiae*, retention of recombinant protein within periplasmic space is rare in *P. pastoris*. This eases the subsequent purification process and thus reduces the production cost (Porro and Mattanovich, 2004). However, the limited knowledge on its genetics and molecular biology hampered the development of *P. pastoris* on recombinant protein technology (Porro and Mattanovich, 2004). There is recent advancement in understanding unfolded protein response, which is one of the major factors affecting secreted product yield (Graf et al., 2008). In addition, *P. pastoris* strain has been engineered to code for unnatural amino acids, which expression is otherwise impossible (Young et al., 2009). The recent completion of genome sequence of *P. pastoris* (De Schutter et al., 2009) will definitely facilitate the improvement on strain

properties, production yield and also post-translational modifications on the protein being expressed.

Kluyveromyces lactis is non-methylotrophic. It was the first microorganism used to produce protein (bovine chymosin) that originated from higher eukaryote (Swinkels et al., 1993). It has been widely used for protein production in various food industries, not only because of its promising expression system, but also its GRAS status from FDA. Methods in genetic manipulation and transformation system have been well-established since 1980s (Das and Hollenberg, 1982). Its full genome sequence was completed (Dujon et al., 2004), making ease on genetic tools development and strain engineering. For industrial production, *K. lactis* has an advantage over *P. pastoris* that it does not require explosion-proof fermentation equipment. On the contrary, such apparatus is essential as the majority of protein expression in *P. pastoris* is driven by methanol. Unlike *S. cerevisiae*, *K. lactis* is a "true secretor", which secretes extracellular proteins into the culture medium that allows simpler downstream purification process (van Ooyen et al., 2006). One of the most frequently used promoters in *K. lactis* expression system is LAC4 promoter, which drives the expression of β-galactosidase. It is induced by the presence of lactose or galactose and has been employed in heterologous expression of bovine prochymosin on an industrial scale (van den Berg et al., 1990).

1.5. Industrial applications of lignocellulolytic enzymes

Lignocellulosic biomass is a major terrestrial carbon source, which confers its potential use as feedstock in biofuel production (Kumar et al., 2008). Many countries worldwide (e.g. US and Brazil) started to replace gasoline with biofuel, which is a renewable and sustainable energy source. Ninety percent of

the biofuels today is produced from sugar or starch-rich crops (i.e. sugar cane or corn), which is so-called "first generation feedstock". However, using this crop-based feedstock brings negative impacts on economy. To meet the increasing demand of biofuel, the potential application of "second generation feedstock" has been proposed. One of the possible candidates is lignocellulosic materials, ranging from left-overs or residues from crops (e.g. corn stover), forest harvests (e.g. sawdust) and grasses (Galbe and Zacchi, 2007). US Department of Energy (DOE) and US Department of Agriculture (USDA) are investing approximately $25 million in the development of biofuel, bioenergy and high-value biobased products from the above mentioned second generation feedstock (http://www.eere.energy.gov/).

However, the application of lignocellulosic materials as "second generation feedstock" is limited by the conversion of lignocellulose into simple sugars, through a complicated process that can be divided into three parts: (1) pretreatment of lignocellulosic biomass; (2) cellulose hydrolysis; and (3) fermentation of sugars. Pretreatment step functions to disrupt lignin and expose underneath polysaccharides for enzymatic hydrolysis. Cellulose hydrolysis requires cellulases to convert polymeric cellulose fibers into monomeric sugars. Finally, sugars, mainly glucose, released are fermented to form ethanol by yeast, e.g. *Saccharomyces cerevisiae* (Gray et al., 2006).

The most challenging and rate-limiting steps in biofuel production is the pretreatment of biomass. In the past, a number of physical (e.g. grinding), chemical (e.g. alkaline/ dilute acid) and physiochemical (e.g. steam) methods have been used but none of them have satisfactory performance. For example, dilute acid pretreatment results in toxic substances that cause inhibitory effects in the downstream fermentation step. To this end, the ability of ligninolytic

enzymes in degrading lignocellulosic materials into simple sugars provide a promising alternative approach for developing biofuel productions (Hatakka, 1984).

Lignocellulolytic enzymes are also widely used in other aspects. With the ability to degrade phenolic compounds, ligninolytic enzymes have been employed in industrial wastewater treatment, particularly azo dyes (Wesenberg et al., 2003; Hao et al., 2007), which are the largest chemical group of synthetic colorants, constitute the major pollutants discharged by textile and dyestuff manufacturing industries (Wong et al., 2006).

Cellulase and laccase are also used to create stonewashed appearance on denim fabrics. Laccase can be applied in food industry to reduce formation of off-flavor compounds during brewing of beer (Couto and Herrera, 2006).

1.6. Long-term significance

With the endowed lignocellulolytic ability, wood-rotting fungi have drawn many attentions for their potential industrial applications, especially biofuel production. The draft genome and transcript profiles of *L. edodes* will help elucidating the lignocellulolytic machinery driven by wood-rotting fungi and discovering more new lignocellulolytic enzymes, which may confer better properties than those currently used. The ability of these enzymes in efficiently degrading lignocellulosic materials, thereby providing the raw materials for biofuel production, certainly meet the global trend in developing a renewable and sustainable energy source.

In long term, the present study also aims at establishing a stable system for large scale production of lignocellulolytic enzymes at a low cost, which, in turn, will provide insight in relieving energy crisis, one of the global problematic issues suffering many countries nowadays.

Chapter 2: Transcriptomic analysis on the lignocellulolytic system in *Lentinula edodes*

2.1 Introduction

Lignocellulose is the most abundant terrestrial biomass. It is the major component in plant cell wall, conferring rigidity and protection against microbial attacks. (Buswell and Odier, 1987; Mohnen et al., 2008). The constituents of this recalcitrance biopolymer include lignin, cellulose and hemicellulose in various compositions (Martinez et al., 2005) depending on the type of cell wall (primary or secondary cell wall) and the category of vascular plant (gymnosperm or angiosperm) (Davin et al., 2008; Harris and Stone, 2008). Lignin is composed of three-dimensional network of phenylpropanoids, forming a physical barrier to woody cell walls (Kirk and Farrell, 1987). Cellulose consists of parallel linings of β-D-glucose microfibrils in a highly ordered and crystalline structure, providing a high degree of resistance against biodegradation (Martinez et al., 2005). Hemicellulose, made up of different pentose and hexose residues, is more susceptible to enzymatic hydrolysis (Martinez et al., 2005).

Being the most efficient wood degraders among microorganisms, white rot fungi can secrete lignocellulolytic enzymes to degrade wood components (Baldrian and Valaskova, 2008). Lignocellulolytic enzymes can be subdivided into two categories, ligninolytic and cellulolytic.

Based on the classification of Fungal Oxidative Lignin enzymes (FOLy) database, ligninolytic enzymes can be classified into lignin oxidase (LO) and lignin degrading auxiliary enzyme (LDA). LOs generate reactive oxygen species (ROS) and free radicals that can randomly breakdown lignin. LDAs

remove oxidized lignin intermediates to facilitate depolymerization. LOs and LDAs act in synergy (Levasseur et al., 2008).

Cellulolytic enzymes include glycoside hydrolase (GH) with or without carbohydrate binding module (CBM), polysaccharide lyase (PL) and carbohydrate esterase (CE), designated by CAZy (Carbohydrate Active enzymes) database (Cantarel et al., 2009). Cellulolytic enzymes cleave target glcosidic bonds in substrates.

Lentinula edodes is a white rot fungus that can efficiently colonize on wood log causing wood decay (Leatham, 1985). In spite of a few lignocellulolytic enzymes previously identified and characterized (Zhao and Kwan, 1999; Lee et al., 2001; Lee et al., 2005; Sakamoto et al., 2009), the enzymatic machinery involved in degrading lignocellulose remains largely unknown. In order to have a better understanding on genes involved in lignocellulolytic system, a large-scale, high throughput sequencing of mycelial cDNA was performed. Together with all *L. edodes* ESTs (Expressed Sequence Tags) available in NCBI, I categorized these mycelial cDNA sequences into FOLy and CAZy families.

ROS and free radicals are crucial for lignin depolymerization in white rot fungi (Buswell and Odier, 1987; Kirk and Farrell, 1987; Broda et al., 1996; Martinez, 2002), resulting in an oxidative environment. Therefore, I postulated that during lignin degradation, fungal cellular structures may be damaged. Belinky et al. (2003) showed that when growing *Phanerochaete. chrysosporium* in oxygenated environment, the activities of cellular antioxidative enzymes was elevated, compared to that growing in atmospheric air. In order to have a better understanding on the protective mechanism employed by *L. edodes* against oxidative stress during lignin degradation, I aimed at comparing the gene

expression levels and activities of antioxidative enzymes in lignocellulose-grown and non-lignocellulose-grown mycelia.

2.2 Materials and Methods

2.2.1 Strain cultivation

Lentinula edodes dikaryotic strain L54 was used in all the experiments. Mycelium was maintained in PDA (Potato Dextrose Agar, Difco). Inocula (about $1cm^2$ mycelia cubes) were prepared from this plate. For high-throughput cDNA sequencing, mycelia were cultivated on artificial logs (60% dry sawdust, 30% dry wood chips and 10% dry wheat bran) at 25°C in dark environment for one month to allow colonization of mycelia on the whole log. For quantitative RT-PCR and enzyme activity assays, mycelia were inoculated on 20g of lignocellulose medium (60% dry sawdust, 30% dry wood chips and 10% dry wheat bran with 100% moisture) and non-lignocellulose medium [PDB (Potato Dextrose Broth, Difco)], respectively, in 90mm-diameter glass pot for two weeks.

2.2.2 RNA extraction, mRNA isolation and cDNA synthesis

Total RNA was extracted from mycelia using TRI Reagent® (Molecular Research Center, Inc.). Poly (A^+) RNA was isolated from 500μg total RNA using mRNA isolation kit (Promega™) according to manufacturer's manual. First strand cDNA was synthesized by cDNA synthesis kit (GibcoBRL, Invitrogen) according to manufacturer's instructions. To examine the quality of the newly synthesized cDNA, 2μl of cDNA was analyzed in 1.5% agarose gel electrophoresis. The remaining cDNA was applied in high-throughput cDNA sequencing.

2.2.3 High-throughput cDNA sequencing

Mycelial cDNA was sequenced using the Genome Sequencer 20 system by 454 Life Sciences, Roche.

2.2.4 Data analysis

All sequences were aligned into contigs, which were then annotated by BLASTx homology search in NCBI sequence database. They were categorized into groups according to the Gene Ontology (http://www.geneontology.org/). In-house BLASTx was performed with all HKLC1 contigs and all *L. edodes* ESTs from NCBI against all entries in FOLy (http://foly.esil.univ-mrs.fr) and CAZy (http://www.cazy.org/) databases.

2.2.5 Quantitative RT-PCR

Total RNA was extracted as described above. DNase I treatment was performed to eliminate contamination of genomic DNA. First strand cDNA was synthesized using TaqMan® Reverse Transcription Reagents (Roche, Applied Biosystems) according to the manufacturer's instructions. Quantitative RT-PCR reaction was performed on Bio-Rad MiniOpticon™ real time system (Bio-Rad). 2µl of 10X diluted first strand cDNA was mixed with 0.45µl of each 10µM sequence specific primers (Table 2.1), 10µl of 2X iQ™ SYBR® Green Supermix (Bio-Rad) and brought up to 20µl with nuclease-free water according to manufacturer's manual. The reaction for each primer set was performed in duplicate and no-template-control (NTC). The PCR program was 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, 60°C for 45 seconds and 72°C for 15 seconds. Melting curve analysis was also performed by increasing the temperature from 50°C to 90°C. Data analysis was performed on Opticon Monitor™ Version 3.0 (Bio-Rad). The experiments were repeated for three times with independent biological samples.

2.2.6 Enzyme activity assays

Mycelial protein was extracted based on method described by Kwon and Anderson, (2001) with a few modifications. Mycelia were ground in liquid nitrogen to fine powder by pestle and mortar. Powder was suspended in 50mM

sodium phosphate buffer (pH7.0) containing 0.01% DTT. The mixtures were centrifuged at 20,200 x g for 15 minutes at 4°C. Supernatant was immediately used for enzyme activity assays. Total superoxide dismutase (SOD) activity was determined using SOD determination kit (Sigma-Aldrich) according to manufacturer's instructions. SOD activity was expressed in terms of % inhibition on the reduction of tetrazolium salts by 50% in a coupled system with xanthine oxidase. Percentage of inhibition was translated into units of SOD activity by means of calibration curve prepared with 0 to 200U of SOD from *E. coli* (Sigma-Aldrich) per milliliter. Catalase (CAT) activity was monitored by absorbance at 240nm as the index of H_2O_2 degradation. The reaction was carried out by adding 10μl protein extract mixture containing 657μl 50mM sodium phosphate buffer (pH7.0) and 333μl 30mM H_2O_2 at room temperature. One unit of CAT was defined as the amount of enzyme required for decomposing 1μmole of H_2O_2 per minute. Protein concentration was determined by Quick Start™ Bradford Protein Assay (Bio-Rad) using γ-globulin as standard. The experiments were repeated for three times with independent biological samples.

Table 2.1: Primer list for quantitative RT-PCR of HKLC1 contigs related to lignocellulose degradation and oxidative stress response.

Contig ID	Upper primer	Upper primer sequence (5' to 3')	Lower primer	Lower primer sequence (5' to 3')
Related to lignin degradation				
HKLC13598	HKLC13598U	GTCTTATTTATCGGCAAGGT	HKLC13598L	TTCCAGTCCTTCTACAGATT
HKLC15473	HKLC15473U	CTCTGCAACATCGACGGTTTTC	HKLC15473L	TGAGGTGGACAGTATGCGTGAG
Related to cellulose or hemicellulose degradation				
HKLC13423	HKLC13423U	TCAACCTGGGAAACGACCTG	HKLC13423L	TTGGAGAAATTATCACCGAG
HKLC13717	HKLC13717U	AACGGCGAATGGCAGATAGT	HKLC13717L	TGGCCCAGGAATGACTGTCG
HKLC13801	HKLC13801U	TACTTCTTCAGGGTCTCAAG	HKLC13801L	TGTTCAGCAGCACTCATAAG
HKLC15236	HKLC15236U	TGCTTCGTCTGGTGACAGTT	HKLC15236L	GGAACCTGAAGAACCTGAAC
HKLC15289	HKLC15289U	TTCGTTTCCGCTCCCACCAT	HKLC15289L	CGTCAAACTTCCAGGTGTGT
HKLC15389	HKLC13598U	GTCTTATTTATCGGCAAGGT	HKLC13598L	TTCCAGTCCTTCTACAGATT
HKLC15887	HKLC15887U	ACTGGCTCCGCTTCAACCTT	HKLC15887L	CCATTGAATCCAACCGCTT
Related to ROS metabolism				
HKLC10220	SOD_00220_118U	ATTCAACACGACGACTGCTGG	SOD_00220_198L	CCTGGTTAGCAGTGGTCGTGAT
HKLC10805	Cat_00805_75U	TTGTAGGCCAGAGGTTGAATTG	Cat_00805_166L	GAATGCACCCATCGTACCAA
HKLC13877	Cat_03877__49U	GAACGACTGCCAGGTTTTCC	Cat_03877_147 L	TCATTACCCAGTCCCAGTTGC
HKLC14771	HKLC14771U	ACCGATGACCTTGATGTTCCTC	HKLC14771L	ACCCAGGTGATTTTACTCCCGT
HKLC15419	HKLC15419U	GGTGCCGAAAAGGATGAAAAG	HKLC15419L	CCATAAGGTCCATCAATCCGAA
Related to cell redox homeostasis				
HKLC10129	HKLC10129U	CGCATTTCCTACCTCTACGAC	HKLC10129L	CGATGACATAGACACTGCGGA
HKLC10309	Grx-real-62u	CAACACATTGGTGGTAACGA	Grx-real-107L	CGATGAGAGTCTGGAGCTTGCC

Table 2.1 (continued)

HKLC11509	Trx-real-15	GTATCAAGAACGTCGAGACT	Trx-real-79L	AAGTAGCGGGTTCATGTCCA

2.3 Results

2.3.1 High-throughout mycelial cDNA sequencing

The cDNA synthesized from mycelial mRNA appeared as smear in gel electrophoresis with size ranging between 100bp and several kilobases (Fig. 2.1). I obtained more than 40,000 raw reads (cDNA fragments). Alignment of these raw reads resulted in 5,894 contigs (designated as HKLC1 contigs), sizes vary from 100bp to 2kb. Using BLASTx, I annotated approximately one thousand contigs with significant E-value (< 10^{-5}), in which 757 were categorized into different groups according to GO classification. All HKLC1 contigs were also subjected to sequence similarity searched against FOLy and CAZy databases, where 22 and 218 contigs could be significantly matched with entries in FOLy and CAZy families, respectively. Results for high-throughput cDNA sequencing analysis were summarized in Table 2.2.

2.3.2 HKLC1 contigs against SAGE (Serial Analysis of Gene Expression) libraries

All HKLC1 contigs were matched to SAGE tags in libraries of mycelia grown in lignocelluloses (L) and non-lignocellulose (NL) mycelia. Seven contigs were matched to SAGE tags that differentially expressed in mycelium grown in L while ten contigs were matched to SAGE tags that differentially expressed in mycelium grown in NL (Table 2.3). Among these 17 contigs, only 3 are homologous to genes with known identities (E-value < 10^{-5}).

2.3.3 Transcript level analysis on genes encoding lignocellulolytic enzymes

According to BLASTx annotations and GO classifications, I selected nine contigs related to lignocellulose degradation (Table 2.4), for quantitative RT-PCR analysis. Contigs HKLC13589 (laccase precursor), HKLC13717

(chloroperoxidase) and HKLC15236 (cellulase) were differentially expressed in mycelium grown in lignocellulose. Six contigs classified as cellulose or hemicellulose degradation related genes did not show significant differences in expression levels (Fig. 2.2 and Fig. 2.3).

2.3.4 HKLC1 contigs and all *L. edodes* ESTs against FOLy and CAZy databases

2.3.4.1 FOLy

I performed sequence homology search for all HKLC1 contigs against all entries in FOLy database and I selected those matched contigs with significant E-value (< 10^{-5}). The distribution of matched contigs was summarized in Fig 2.4. Twenty contigs were categorized into FOLy families LO1, LO3, LDA3, LDA6 and LDA7, in which LO1 and LDA3 accounted for 80% of the contigs.

When applying all *L. edodes* ESTs) available in NCBI (denoted as NCBI ESTs to sequence similarity search against all entries in FOLy, I identified 42 ESTs in 7 FOLy families with similar distribution pattern. LO1 and LDA3 were still the largest groups, followed by LDA6, LO3 and LDA7. There were two and one ESTs in LDA1 and LDA2, respectively. No sequence in HKLC1 contig or NCBI ESTs was identified as LDA4 or LDA5.

2.3.4.2 CAZy

Nineteen representative CAZy families, involving in plant cell degradation, were chosen based on Martin et al. (2008), 13 were related to cellulose or hemicellulose degradation while 6 were responsible for pectin degradation (Fig. 2.5).

For HKLC1, 23 contigs were classified into 6 CAZy families. Twenty-two of them were identified in 5 families related to cellulose or hemicellulose degradation while only one contig were detected in GH28, related to pectin

degradation. The largest families were CBM1 and GH5 (Fig. 2.5).

For NCBI ESTs, 56 ESTs were assigned into 10 CAZy families. CBM1, GH5 and GH43 were the most represented groups.

I also summarized all the information for sequence similarity search results as supplementary information (FOLy: Table 2.5 and Table 2.6; CAZy: Table 2.7 and Table 2.8).

2.3.5 Analysis on oxidative stress response in *L. edodes*

According to the BASTx annotations and GO classifications, I selected 7 contigs related to ROS metabolism (Fig. 2.6) and cell redox homeostasis (Fig. 2.7) for quantitative RT-PCR analysis. All of them showed lower expression levels in mycelium grown in lignocellulose.

For activity measurement, I observed a lower specific SOD activity in mycelium grown in lignocellulose and a similar CAT activity in the two conditions (Fig. 2.8).

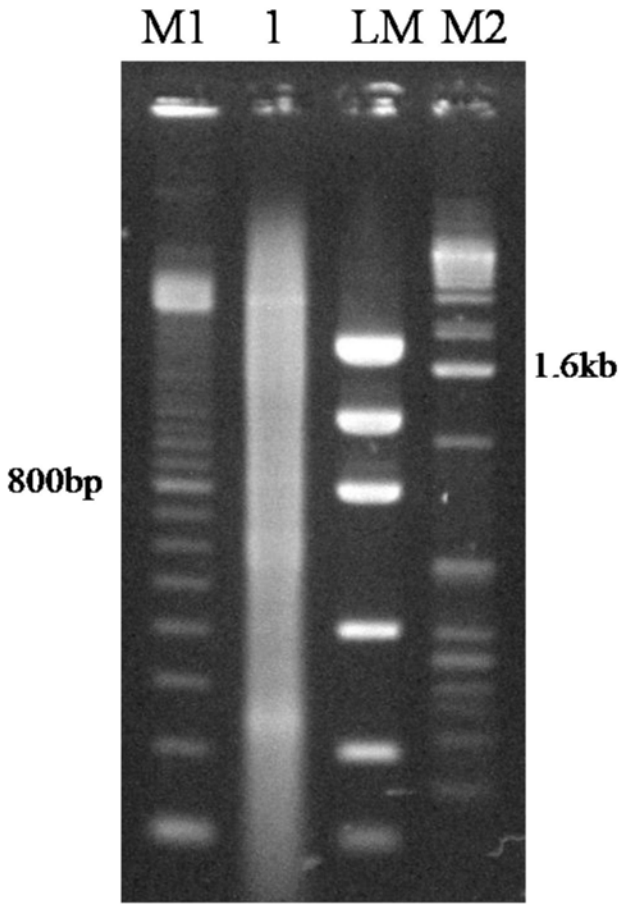


Fig. 2.1: Examination of the quality of mycelial cDNA in 1.5% agarose gel. M1: 100bp ladder; 1: 2μl of mycelial cDNA; LM: 4μl of low DNA mass ladder (Invitrogen); M2: 1kb ladder.

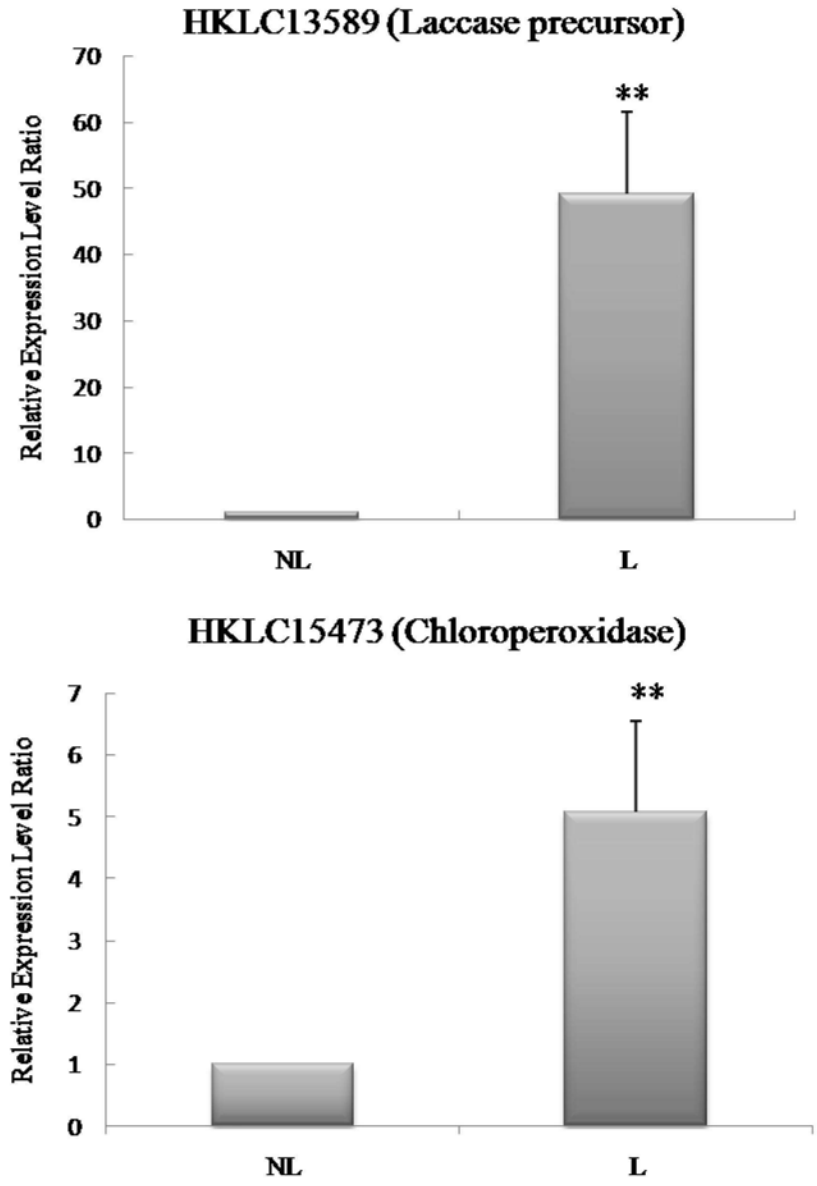


Fig. 2.2: Quantitative RT-PCR analysis on contigs related to lignin degradation in mycelia grown in non-lignocellulose (NL) and lignocellulose (L). Data represent means and standard deviations of three independent experiments and statistical significance was indicated by * $p < 0.05$; ** $p < 0.01$.

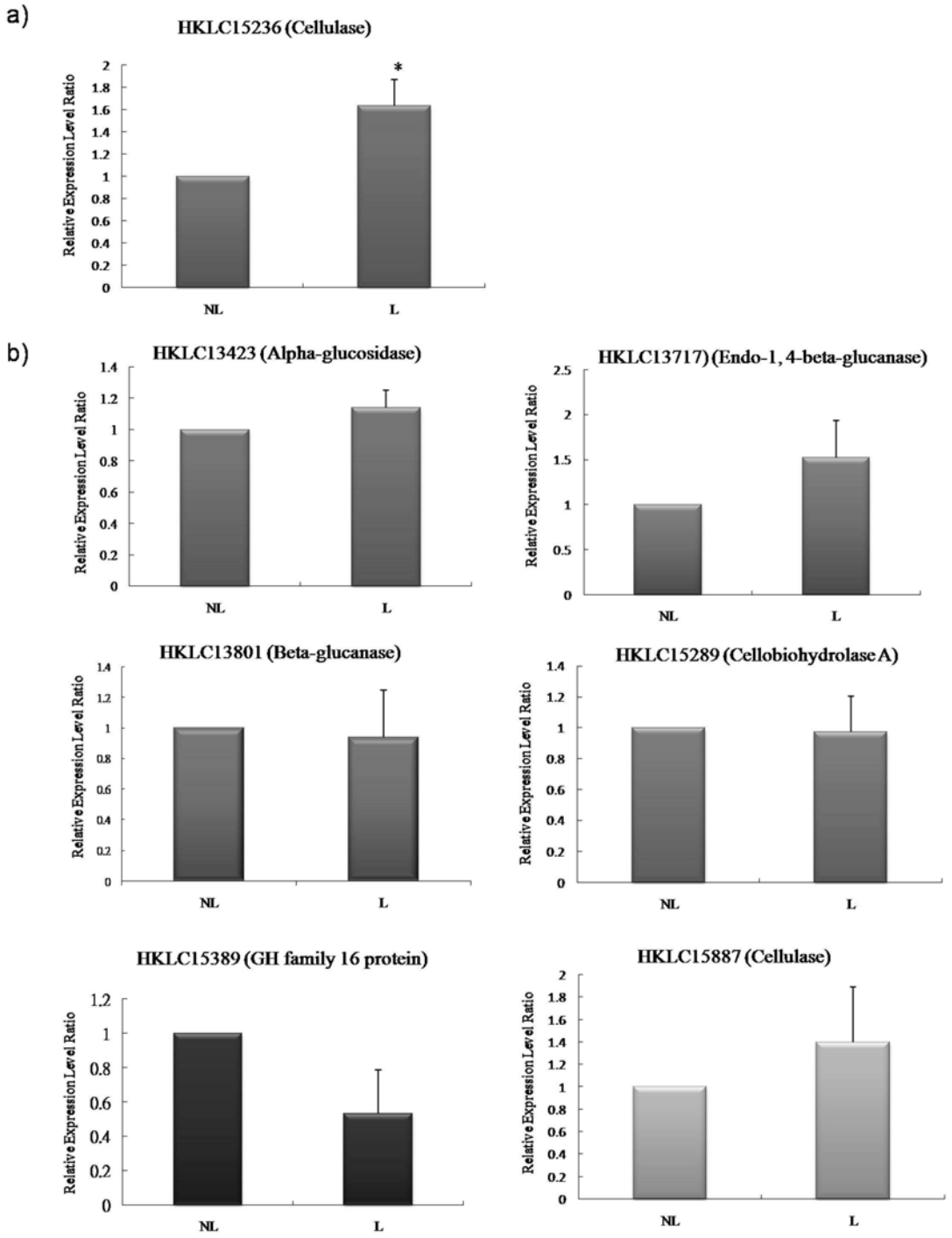


Fig. 2.3: Quantitative RT-PCR analysis of contigs related to cellulose or hemicellulose degradations with a) differential expression in mycelium grown in lignocellulose (L); and b) similar expression levels in mycelia grown in non-lignocellulose (NL) and L. Data represent means and standard deviations of three independent experiments and statistical significance was indicated by * $p < 0.05$

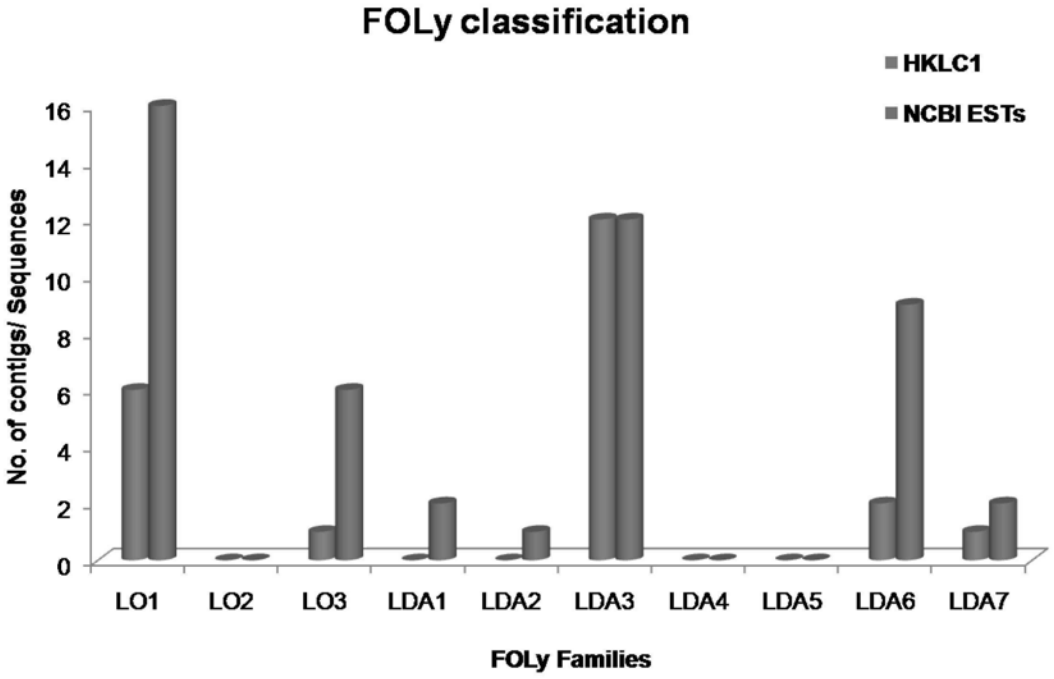


Fig. 2.4: Distribution of contigs from high-throughput cDNA sequencing (HKLC1) and all *L. edodes* ESTs (NCBI ESTs) available in NCBI into FOLy families.

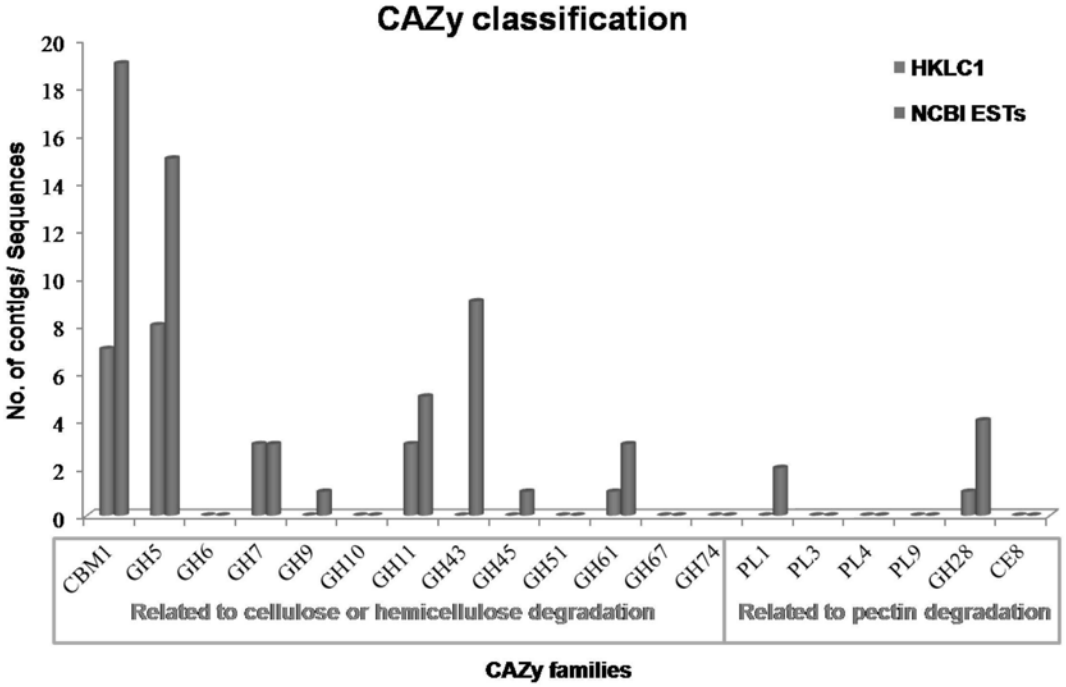


Fig. 2.5: Distribution of contigs from high-throughput cDNA sequencing (HKLC1) and all *L. edodes* ESTs (NCBI ESTs) available in NCBI into CAZy families.

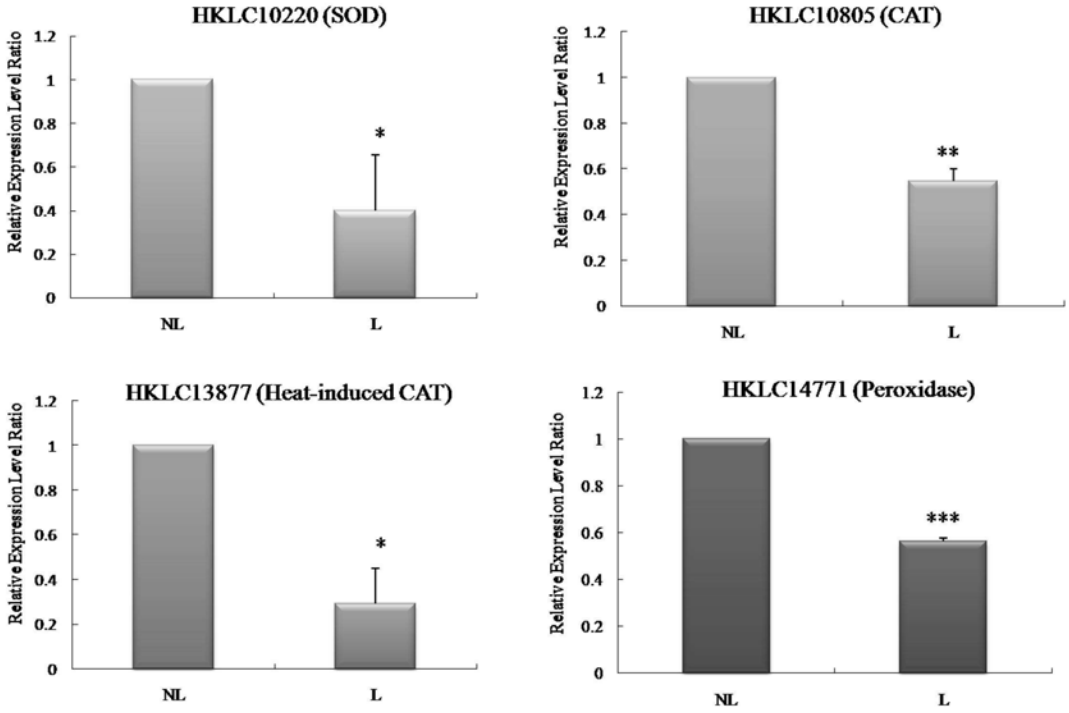


Fig. 2.6: Quantitative RT-PCR analysis of contigs related to ROS metabolism in mycelia grown in non-lignocellulose (NL) and lignocellulose (L). Data represent means and standard deviations of three independent and statistical significance was indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

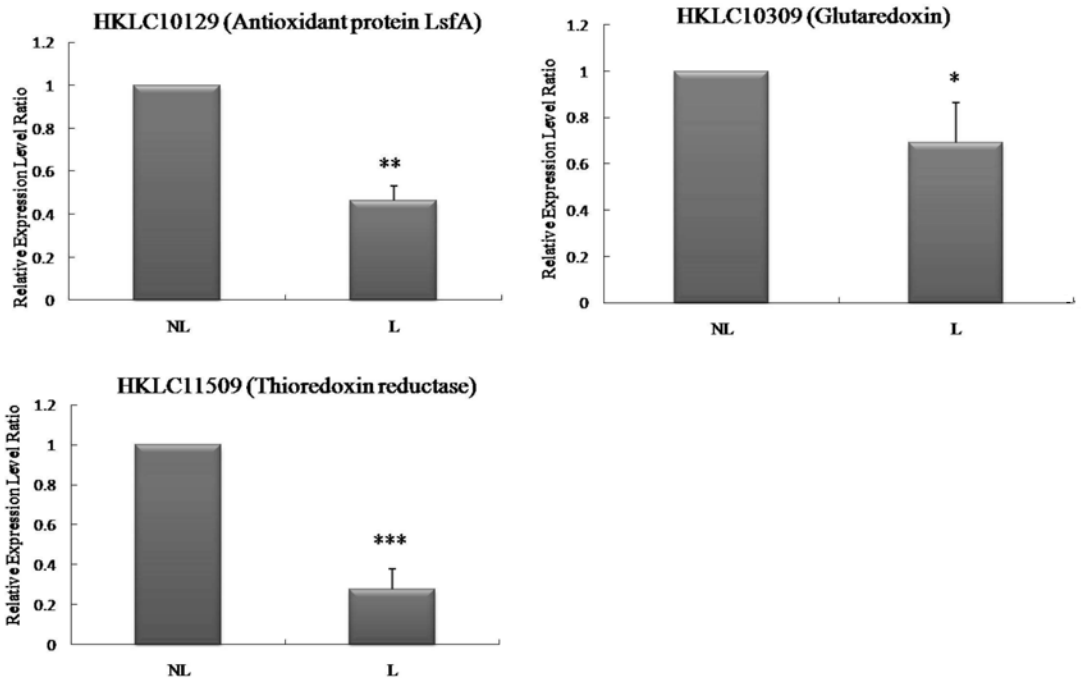


Fig. 2.7: Quantitative RT-PCR analysis of contigs related to cell redox homeostasis in mycelia grown in non-lignocellulose (NL) and lignocellulose (L). Data represent means and standard deviations of three independent experiments and statistical significance was indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

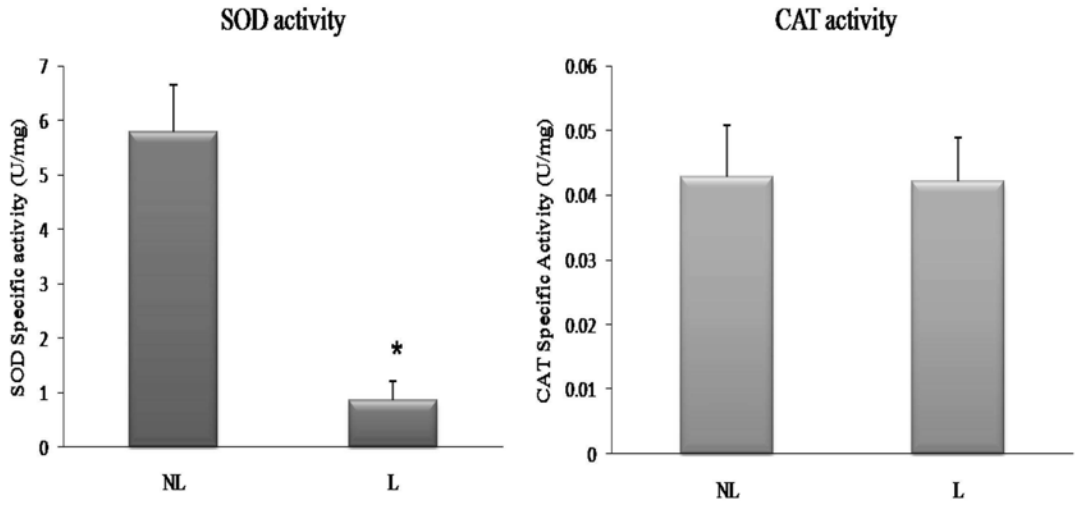


Fig. 2.8: Activity assays for SOD (superoxide dismutase) and CAT (catalase). Data represent means and standard deviations of three independent experiments and statistical significance was indicated by * $p < 0.05$.

Table 2.2: Summary of results for high-throughput cDNA sequencing analysis.

Total no, of reads (cDNA fragments)	42377
Total no. of contigs	5894
Length	~100bp to ~2kb
No. of BLASTx annotated contigs	2608
No. of annotated contigs with E-value < 10^{-5}	1017
No. of annotated contigs with GO classification (Biological process)	757
No. of contigs classified into FOLy families	544
No. of contigs classified into FOLy families with E-value < 10^{-5}	20
No. of contigs classified into CAZy families	688
No. of contigs classified into CAZy families with E-value < 10^{-5}	179

Table 2.3: HKLC1 contigs that matched to SAGE tags with differential expression in mycelium grown in lignocellulose.

Contig ID[a]	SAGE tag[b]	NL[c]	L[d]	Accession no.[e]	Annotation[f]	Organism	E-value
Differentially expressed genes in mycelium grown in lignocellulose (L)[#]							
HKLC15660	GCTTGACGAG	12	83	XP_679255	Hypothetical protein	*Plasmodium berghei*	2.4
HKLC15695	GAAGACGAAA	45	61	YP_002093220	3-oxoacyl-(acyl-carrier-protein) synthase	*Burkholderia cenocepacia*	6e-03
HKLC15319	CCACTCCTTT	2	23	XP_002225643	Hypothetical protein	*Branchiostoma floridae*	0.21
HKLC15289	TAGCTGGTCG	8	16	YP_527744	Cellobiohydrolase A	*Saccharophagus degradans*	6e-12
HKLC15610	TAGTTTTCAA	0	10		Unknown		
HKLC15542	ACATTGAAGA	1	9	XP_001879765	Predicted protein	*Laccaria bicolor*	5e-25
HKLC15517	GGAAGAATGC	0	5		Unknown		

[a]Contig obtained from high-throughput cDNA sequencing.

[b]Sequence information of SAGE tag

[c]Number of SAGE tag count for the corresponding contig in mycelium grown in non-lignocellulose (NL). The total number of tag count in NL was 4008.

[d]Number of SAGE tag count for the corresponding contig in mycelium grown in lignocelluloses (L). The total number of tag count in L was 3241.

[e]Accession number of the matched transcript from NCBI (http://www.ncbi.nim.nih.gov/).

[f]Annotation of the matched transcript from BLASTx.

[#]Difference between the numbers of SAGE tag counts were verified as statistically significant by Fisher's exact test. (p-value <0.05).

Table 2.4: HKLC1 contigs that related to lignocellulose degradations.

Contig ID[a]	Length[b]	Read[c]	Accession no.[d]	Annotation[e]	Organism	E-value[f]	GOP[g]
Related lignin degradation							
HKLC13598	99	2	LAC1_PHLRA	Laccase precursor	*Phlebia radiata*	3.77e-06	N/A
HKLC15473	222	12	CAC03461	Chloroperoxidase	*Agaricus bisporus*	2e-05	N/A
Related to cellulose or hemicellulose degradation							
HKLC13423	108	3	AGLU_ASPOR	Alpha-glucosidase precursor	*Aspergillus oryzae*	5.44e-08	Carbohydrate metabolism
HKLC13717	117	2	XP_746778	Endo-1,4-beta-glucanase	*Aspergillus fumigatus*	1.57e-07	Carbohydrate metabolism
HKLC13801	104	2	XP_569544	Beta-glucosidase	*Cryptococcus neoformans*	1e-10	N/A
HKLC15236	152	55	ZP_00683852	Cellulase	*Xylella fastidiosa*	3.52e-07	Carbohydrate metabolism
HKLC15289	746	176	YP_527744	Cellobiohydrolase A	*Saccharophagus degradans*	2.9e-12	Carbohydrate metabolism
HKLC15389	284	14	XP_001875146	Glycoside hydrolase family 16 protein	*Laccaria bicolor*	1e-30	N/A
HKLC15887	437	36	XP_570869	Cellulase	*Cryptococcus neoformans*	2.07e-36	Glucan metabolism

[a]Contig ID from high-throughput mycelial cDNA sequencing.

[b]Length of the cDNA contig.

[c]Number of cDNA fragments aligned to the cDNA contig.

[d]Accession number of the matched transcript in NCBI BLASTx.

[e]Annotation of the matched transcript from BLASTx.

[f]Cut-off at E-value < 10^{-5}.

[g]Gene Ontology (biological process) classification.

Table 2.5: Classification of HKLC1 contigs into various FOLy families.

FOLy family[a]	Contig[b]	FOLy annotation[c]	Organism	E-value[d]
LO1	HKLC13598	Laccase 1 DVT	*Lentinula edodes*	3e-14
	HKLC13709	Laccase 1 BVT	*Lentinula edodes*	4e-14
	HKLC12651	Laccase	*Auricularia polytricha*	3e-13
	HKLC13880	Laccase	*Auricularia polytricha*	1e-09
	HKLC10109	Laccase 4	*Pleurotus pulmonarius*	3e-05
	HKLC12419	Putative laccase	*Termitomyces sp.*	5e-05
LO3	HKLC15703	Unnamed protein product	*Aspergillus oryzae*	2e-06
LDA3	HKLC10080	Copper radical oxidase	*Phanerochaete chrysosporium*	4e-85
	HKLC10392	Copper radical oxidase	*Phanerochaete chrysosporium*	8e-56
	HKLC15518	Copper radical oxidase	*Phanerochaete chrysosporium*	6e-53
	HKLC10016	Copper radical oxidase	*Phanerochaete chrysosporium*	1e-41
	HKLC13941	Copper radical oxidase	*Phanerochaete chrysosporium*	1e-35
	HKLC15424	Copper radical oxidase	*Phanerochaete chrysosporium*	3e-25
	HKLC13087	Copper radical oxidase	*Phanerochaete chrysosporium*	7e-20
	HKLC10373	Copper radical oxidase	*Phanerochaete chrysosporium*	5e-14
	HKLC11914	Copper radical oxidase	*Phanerochaete chrysosporium*	1e-06
	HKLC15587	Copper radical oxidase	*Phanerochaete chrysosporium*	5e-06
	HKLC11135	Glyoxal oxidase precursor, putative	*Cryptococcus neoformans*	6e-06
LDA6	HKLC13201	Mala s 12 allergen precursor	*Malassezia sympodialis*	9e-05
LDA7	HKLC13775	NADH-quinone oxidoreductase	*Gloeophyllum trabeum*	1e-11

[a]Classification according to FOLy database.

[b]Contig ID designated for high-throughput mycelial cDNA sequencing.

[c]Annotation of the matched entry in FOLy database.

[d]Cut-off at E-value < 10^{-5}.

Table 2.6: Classification of NCBI ESTs into various FOLy families.

FOLy family[a]	EST[b]	FOLy annotation[c]	Organism	E-value[d]
LO1	LeESTcombined_09305	Fet3 protein	*Phanerochaete chrysosporium*	9e-32
	LeESTcombined_06792	Laccase 1 BVT; LAC1BVT	*Lentinula edodes*	8e-24
	LeESTcombined_05993	Laccase 2	*Lentinula edodes*	5e-19
	LeESTcombined_01147	Unnamed protein product	*Aspergillus oryzae*	5e-17
	LeESTcombined_03204	Laccase 1 DVT; LAC1DVT	*Lentinula edodes*	6e-17
	LeESTcombined_03580	Fet3 protein	*Phanerochaete chrysosporium*	1e-16
	LeESTcombined_02377	Laccase 1 DVT; LAC1DVT	*Lentinula edodes*	2e-15
	LeESTcombined_05221	Fet3 protein	*Phanerochaete chrysosporium*	4e-14
	LeESTcombined_03595	Laccase 1 BVT; LAC1BVT	*Lentinula edodes*	2e-13
	LeESTcombined_02423	Fet3 protein	*Phanerochaete chrysosporium*	8e-11
	LeESTcombined_02659	Laccase 3 VT; LAC3VT	*Lentinula edodes*	4e-09
	LeESTcombined_03726	Laccase 1 BVT; LAC1BVT	*Lentinula edodes*	6e-09
	LeESTcombined_04962	Putative laccase	*Termitomyces sp.*	1e-05
	LeESTcombined_09209	Putative laccase	*Termitomyces sp.*	5e-05
LO3	LeESTcombined_00463	Unnamed protein product	*Aspergillus oryzae*	2e-10
	LeESTcombined_06515	Unnamed protein product	*Aspergillus oryzae*	7e-09
	LeESTcombined_00193	Unnamed protein product	*Aspergillus oryzae*	1e-07
	LeESTcombined_00746	Unnamed protein product	*Aspergillus oryzae*	3e-07
LDA1	LeESTcombined_08266	Hypothetical protein CC1G_01540	*Coprinopsis cinerea*	4e-31
	LeESTcombined_07140	Aryl-alcohol oxidase precursor	*Pleurotus pulmonarius*	2e-19

Table 2.6 (continued)

LDA2	LeESTcombined_07904	Vanillyl-alcohol oxidase (Aryl-alcohol oxidase) (4-allylphenol oxidase)		4e-10
LDA3	LeESTcombined_11358	Copper radical oxidase	*Phanerochaete chrysosporium*	3e-178
	LeESTcombined_04004	Copper radical oxidase	*Phanerochaete chrysosporium*	3e-74
	LeESTcombined_10771	Copper radical oxidase	*Phanerochaete chrysosporium*	5e-40
	LeESTcombined_10773	Copper radical oxidase	*Phanerochaete chrysosporium*	1e-31
	LeESTcombined_11052	Copper radical oxidase	*Phanerochaete chrysosporium*	4e-29
	LeESTcombined_05817	Copper radical oxidase	*Phanerochaete chrysosporium*	1e-26
	LeESTcombined_04571	Glyoxaloxidase 1	*Ustilago maydis*	8e-22
	LeESTcombined_01469	Copper radical oxidase	*Phanerochaete chrysosporium*	6e-15
	LeESTcombined_06860	Copper radical oxidase	*Phanerochaete chrysosporium*	7e-13
	LeESTcombined_07284	Copper radical oxidase	*Phanerochaete chrysosporium*	3e-08
	LeESTcombined_02330	Copper radical oxidase	*Phanerochaete chrysosporium*	2e-06
	LeESTcombined_10540	Glyoxal oxidase precursor, putative	*Cryptococcus neoformans*	6e-06
LDA6	LeESTcombined_07938	Unnamed protein product	*Aspergillus oryzae*	2e-47
	LeESTcombined_11490	Mala s 12 allergen precursor	*Malassezia sympodialis*	6e-35
	LeESTcombined_01155	Glucose oxidase	*Botryotinia fuckeliana*	3e-23
	LeESTcombined_11075	Mala s 12 allergen precursor	*Malassezia sympodialis*	3e-23
	LeESTcombined_00569	Unnamed protein product	*Aspergillus oryzae*	8e-18
	LeESTcombined_09229	Glucose oxidase	*Penicillium expansum*	2e-13
	LeESTcombined_06712	Mala s 12 allergen precursor	*Malassezia sympodialis*	1e-10

Table 2.6 (continued)

LDA7	LeESTcombined_05033	1,4-benzoquinone reductase	*Phanerochaete chrysosporium*	4e-77
	LeESTcombined_01315	NADH-quinone oxidoreductase	*Gloeophyllum trabeum*	2e-76

[a]Classification according to FOLy database.

[b]ID designated for all *L. edodes* ESTs available in NCBI.

[c]Annotation of the matched entry in FOLy database.

[d]Cut-off at E-value < 10^{-5}.

Table 2.7: Classification of HKLC1 contigs into various CAZy families.

CAZy family[a]	Contig[b]	CAZy annotation[c]	Organism	E-value[d]
CBM1	HKLC13717	Cellulase CEL7A	*Lentinula edodes*	9e-10
	HKLC13801	Cellulose-binding beta-glucosidase	*Phanerochaete chrysosporium*	2e-09
	HKLC14682	Unnamed protein product	*Exidia glandulosa*	6e-08
	HKLC12846	Unnamed protein product	*Exidia glandulosa*	1e-07
	HKLC13873	Cellulose-binding beta-glucosidase	*Phanerochaete chrysosporium*	2e-07
	HKLC15209	Mannanase	*Armillariella tabescens*	9e-07
	HKLC13648	Beta-mannase		7e-05
GH5	HKLC15292	Exo-beta-1,3-glucanase	*Lentinula edodes*	1e-53
	HKLC15887	Exo-beta-1,3-glucanase	*Lentinula edodes*	3e-47
	HKLC15355	Exo-beta-1,3-glucanase	*Lentinula edodes*	1e-37
	HKLC11674	Hypothetical protein CNF01760	*Cryptococcus neoformans*	7e-07
	HKLC15035	Hypothetical protein MGG_09433	*Magnaporthe grisea*	9e-07
	HKLC15209	Mannanase	*Armillariella tabescens*	9e-07
	HKLC12611	Unnamed protein product	*Aspergillus niger*	1e-06
	HKLC13648	Beta-mannase		7e-05
GH7	HKLC13717	Unnamed protein product	*Talaromyces emersonii*	1e-10
	HKLC14682	Unnamed protein product	*Exidia glandulosa*	6e-08
	HKLC12846	Unnamed protein product	*Exidia glandulosa*	1e-07
GH11	HKLC11208	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	5e-08
	HKLC11389	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	6e-08

Table 2.7 (continued)

GH11	HKLC10251	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	6e-06
GH61	HKLC15002	Unnamed protein product	*Thielavia terrestris*	7e-05
GH28	HKLC11858	Hypothetical protein	*Vitis vinifera*	3e-05

[a]Classification according to CAZy database.

[b]Contig ID designated for high-throughput mycelial cDNA sequencing.

[c]Annotation of the matched entry in CAZy database.

[d]Cut-off at E-value < 10^{-5}.

Table 2.8: Classification of NCBI ESTs into various CAZy families.

CAZy family[a]	EST[b]	CAZy annotation[c]	Organism	E-value[d]
CBM1	LeESTcombined_08569	Xylanase	*Lentinula edodes*	7e-88
	LeESTcombined_08489	Cellulose-binding beta-glucosidase	*Phanerochaete chrysosporium*	1e-19
	LeESTcombined_00496	Family 61 endoglucanase	*Phanerochaete chrysosporium*	2e-19
	LeESTcombined_11490	Hypothetical protein	*Neurospora crassa*	9e-13
	LeESTcombined_05979	Unnamed protein product	*Aspergillus oryzae*	3e-12
	LeESTcombined_09564	Cellulase CEL7A	*Lentinula edodes*	9e-10
	LeESTcombined_08810	Cellulose-binding beta-glucosidase	*Phanerochaete chrysosporium*	4e-09
	LeESTcombined_07074	Endoglucanase V	*Trichoderma viride*	1e-08
	LeESTcombined_07391	Hypothetical protein	*Neurospora crassa*	4e-08
	LeESTcombined_09861	Unnamed protein product	*Exidia glandulosa*	6e-08
	LeESTcombined_04295	Unnamed protein product	*Exidia glandulosa*	1e-07
	LeESTcombined_08998	Endo-1,4-beta-mannosidase, putative	*Aspergillus fumigatus*	7e-07
	LeESTcombined_10413	Man5C	*Phanerochaete chrysosporium*	2e-06
	LeESTcombined_04655	Hypothetical protein	*Neurospora crassa*	3e-06
	LeESTcombined_07172	Cellulose-binding beta-glucosidase	*Phanerochaete chrysosporium*	3e-06
	LeESTcombined_02857	Endoglucanase IV precursor	*Neurospora crassa*	2e-05
	LeESTcombined_09542	Beta-mannase		7e-05
GH5	LeESTcombined_10191	Exo-beta-1,3-glucanase	*Lentinula edodes*	7e-131
	LeESTcombined_11113	Exo-beta-1,3-glucanase	*Lentinula edodes*	2e-75
	LeESTcombined_03873	Hypothetical protein CNF01760	*Cryptococcus neoformans*	6e-56

Table 2.8 (continued)

GH5	LeESTcombined_00007	Exo-beta-1,3-glucanase	*Lentinula edodes*	1e-53
	LeESTcombined_07521	Cytoplasm protein, putative	*Cryptococcus neoformans*	7e-37
	LeESTcombined_08016	Cytoplasm protein, putative	*Cryptococcus neoformans*	1e-32
	LeESTcombined_09076	Exo-beta-1,3-glucanase	*Cryptococcus neoformans*	6e-24
	LeESTcombined_03846	Exo-beta-1,3-glucanase	*Lentinula edodes*	1e-16
	LeESTcombined_08156	Glycoside hydrolase family 5, candidate beta-glycosidase	*Bacteroides vulgatus*	5e-09
	LeESTcombined_02878	43 kda secreted glycoprotein precursor	*Paracoccidioides brasiliensis*	1e-08
	LeESTcombined_08998	Endo-1,4-beta-mannosidase, putative	*Aspergillus fumigatus*	7e-07
	LeESTcombined_09022	Hypothetical protein CNF01760	*Cryptococcus neoformans*	7e-07
	LeESTcombined_10413	Man5C	*Phanerochaete chrysosporium*	2e-06
	LeESTcombined_09542	*Beta-mannase*		7e-05
GH7	LeESTcombined_09564	Unnamed protein product	*Talaromyces emersonii*	1e-10
	LeESTcombined_09861	Unnamed protein product	*Exidia glandulosa*	6e-08
	LeESTcombined_04295	Unnamed protein product	*Exidia glandulosa*	1e-07
GH9	LeESTcombined_04363	Family 9 glycosyl hydrolase	*Phanerochaete chrysosporium*	3e-14
GH11	LeESTcombined_08569	Xylanase	*Streptomyces thermoviolaceus*	1e-54
	LeESTcombined_01815	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	3e-17
GH11	LeESTcombined_08883	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	1e-12
	LeESTcombined_09111	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	1e-11
	LeESTcombined_10276	Endo-1,4-beta-xylanase; xylanase D	*Cellulomonas fimi*	3e-05

Table 2.8 (continued)

GH43	LeESTcombined_04970	Hypothetical protein MG04376.4	*Magnaporthe grisea*	9e-37
	LeESTcombined_02797	Hypothetical protein MG04376.4	*Magnaporthe grisea*	2e-35
	LeESTcombined_05122	Arylsulfatase	*Rhodopirellula baltica*	4e-24
	LeESTcombined_08113	Hypothetical protein MG04376.4	*Magnaporthe grisea*	4e-07
	LeESTcombined_00061	Hypothetical protein	*Sorangium cellulosum*	8e-07
	LeESTcombined_06658	Hypothetical protein MG04376.4	*Magnaporthe grisea*	1e-06
	LeESTcombined_10597	Hypothetical protein MG10628.4	*Magnaporthe grisea*	1e-05
GH45	LeESTcombined_07074	Hypothetical protein AN6786.2	*Aspergillus nidulans*	5e-11
GH61	LeESTcombined_00496	Hypothetical protein AN1041.2	*Aspergillus nidulans*	4e-23
	LeESTcombined_05979	Unnamed protein product	*Aspergillus oryzae*	3e-12
	LeESTcombined_02857	Hypothetical protein MG05364.4	*Magnaporthe grisea*	5e-08
PL1	LeESTcombined_01815	Polysaccharide deacetylase	*Bacillus cereus*	8e-13
GH28	LeESTcombined_02085	Hypothetical protein	*Vitis vinifera*	6e-40
	LeESTcombined_04003	Unnamed protein product	*Aspergillus oryzae*	3e-22
	LeESTcombined_11126	Hypothetical protein	*Vitis vinifera*	1e-08
	LeESTcombined_10218	Putative protein	*Arabidopsis thaliana*	5e-07

[a]Classification according to CAZy database.

[b]ID designated for all *L. edodes* ESTs available in NCBI.

[c]Annotation of the matched entry in CAZy database.

[d]Cut=off at E-value < 10^{-5}.

Table 2.9: Contigs related to oxidative stress response.

Contig ID[a]	Length[b]	Read[c]	Accession no.[d]	Annotation[e]	Organism	E-value[f]	GOP[g]
Related to ROS metabolism							
HKLC10220	335	9	AAK82369	Manganese superoxide dismutase	*Phanerochaete chrysosporium*	2e-41	N/A
HKLC10805	221	13	XP_001889495	Catalase	*Laccaria bicolor*	2e-23	N/A
HKLC13877	205	11	AAK15159	Heat-induced catalase	*Pleurotus sajor*	5e-24	Response to oxidative stress
HKLC14771	231	16	ZP_00687656	Peroxidase	*Burkholderia ambifaria*	1.32e-22	N/A
HKLC15419	882	59	XP_751211	NADPH oxidase NoxA	*Aspergillus fumigatus*	3.5e-61	Reactive oxygen species metabolism
Related to cell redox homeostasis							
HKLC10129	411	40	XP_747511	Antioxidant protein LsfA	*Aspergillus fumigatus*	5.64e-34	Regulation of cell redox homeostasis
HKLC10309	205	10	GLRX_LYCES	Glutaredoxin	*Lycopersicon esculentum*	1.72e-06	Cell redox homeostasis
HKLC11509	99	3	XP_001875741	Thioredoxin reductase	*Laccaria bicolor*	2e-08	N/A

[a]Contig ID from high-throughput mycelial cDNA sequencing.

[b]Length of the cDNA contig.

[c]Number of cDNA fragments aligned to the cDNA contig.

[d]Accession number of the matched transcript in NCBI BLASTx.

[e]Annotation of the matched transcript from BLASTx.

[f]Cut-off at E-value < 10^{-5}.

[g]Gene Ontology (biological process) classification.

2.4 Discussion

In the present study, I obtained a transcription profile for *L. edodes* during vegetative mycelial growth in lignocellulosic medium. I assembled 5,894 contigs from the high-throughput mycelial cDNA sequencing. With the BLASTx annotations and GO classifications, 9 contigs were selected for the transcript level analysis in mycelia grown in lignocellulose (L) or non-lignocellulose (NL). In order to have a catalog of genes involved in lignocellulolytic degradation, I employed FOLy and CAZy databases to classify the contigs into various families. From the assembled contigs, I chose 7 related to oxidative stress response for transcriptional studies and activity assays to address the effect of increased oxidative stress on the antioxidative enzymes in mycelium.

2.4.1 Contigs with differential expression in lignocellulose-grown mycelium

In order to identify genes that are important for lignocellulose degradation, I mapped all HKLC1 contigs to our in-house SAGE libraries (Chum, 2006). Seven candidates with differential expressions in lignocellulose-grown mycelium were selected. However, most of them did not show significant sequence similarity (E-value < 10^{-5}) to any of the known proteins in NCBI.

HKLC15289 was the only SAGE tag-matched contig with known identiy, Cellobiohydrolase A (CBH). CBH is an exo-acting cellulase that cleaves cellobiose from the ends of cellulose microfibrils. In *L. edodes*, two CBH-coding genes have been cloned, *cel7A* and *cel6B*, which transcript levels were elevated when growing in cellulosic media (Lee et al., 2001). However, the SAGE result obtained for HKLC15289 was inconsistent with quantitative RT-PCR analysis, where HKLC15289 was expressed at similar levels in non-lignocellulose-grown and lignocellulose-grown mycelia, so I postulate that

HKLC15289 may be differed from Cel7A and Cel6B.

2.4.2 Transcript level analysis on contigs related to lignocellulolytic enzymes

The expression levels of HKLC13589 (laccase precursor) and HKLC15473 (chloroperoxidase) in mycelium grown in lignocellulose was 50-fold and 5-fold higher than that grown in non-lignocellulose, respectively, indicating they are probably participated in lignocellulose degradation during vegetative mycelial growth in *L. edodes*.

HKLC13589 shared a high sequence similarity to laccase precursor. Laccase is a copper-containing phenoloxidase that catalyzes one electron oxidation of phenolic compounds while in the presence of mediators, it can also oxidize non-phenolic residues (Martinez, 2002). It has been isolated in a broad range of fungi, including *L. edode* (Zhao and Kwan, 1999; Sakamoto et al., 2008; Yano et al., 2009). Apart from lignin degradation, laccase is also implicated in morphogenesis (Baldrian, 2006) and pigmentation (Collins and Dobson, 1997). At least 3 groups of laccases are identified in *L. edodes* (Zhao and Kwan, 1999; Sakamoto et al., 2006; Yano et al., 2009) and this will be further discussed in Chapter 5.

HKLC15473 was homologous to chloroperoxidase-coding gene, a novel haloperoxidase in *L. edodes*. Chloroperoxidase (CPO), like lignin peroxidase and manganese peroxidase, is a heme-containing peroxidase that chlorinates and cleaves lignin (Ulrich et al., 2004; Kwok et al., 2006b). To date, little is known about the role of CPO in white-rot basidiomycetes, but it was suggested to mediate lignin depolymerization owing to its ability of chlorinating and cleaving lignin model compounds (Ortiz-Bermudez et al., 2003).

Among all 7 contigs related to cellulose or hemicellulose degradation, only

HKLC15236 showed an increased expression in mycelium grown in L. The low extend of cellulose degradation in *L. edodes* (Leatham, 1985) may explain the similar expression of cellulytic enzyme-coding contigs in mycelia grown in lignocellulose and non-lignpcellulose media.

2.4.3 Classification of genes involved in lignocellulolytic degradation

LO1 and LDA3 were the most represented FOLy families in *L. edodes.* LO1 contain mainly laccases (Levasseur et al., 2008), which are blue copper phenoloxidases and described in the section above. I found 14 candidates of LO1 in *L. edodes*, comparing to 17 candidate genes in the genome of *Coprinopsis cinerea*, a model fungus (Levasseur et al., 2008). The action of laccase is often cooperated with LDA1, aryl-alcohol oxidase, e.g. veratryl alcohol oxidase in *Pleurotus ostreatus* (Marzullo et al., 1995). LDA1 inhibit the repolymerization of oxidized lignin intermediates by reducing laccase-generated quinonoids and phenoxy radicals (Fig. 2.9) (Levasseur et al., 2008). In *L. edodes*, I detected 2 candidates of LDA1, comparing to 18 LDA1 in *C. cinerea*, suggesting there may be certain differences between the process of laccase-mediated ligninolysis in these two fungi.

I also identified 12 candidates of LDA3 [10 copper radical oxidases (CRO) and 2 glyoxal oxidases (GLX)] in *L. edodes,* while only 7 LDA3 (6 CROs and 1 GLX) were detected in the genome of *P. chrysosporium,* another well-established model of white rot fungi (Levasseur et al., 2008). This observation suggested *L. edodes* may be active in H_2O_2 production. LDA1 and LDA3 are suggested to be the main enzymes producing H_2O_2 in ligninolytic fungi (Kersten and Cullen, 2007) and they are physiologically connected with LO2, the heme-peroxidases (Kersten and Kirk, 1987). To the best of our knowledge, at least two LO2 have been discovered in *L. edodes* (CPO that was described in the section above

and manganese peroxidase that will be discussed in Chapter 4).

I detected 4 candidate ESTs in LO3, which is cellobiose dehydrogenase (CDH) according to FOLy classification (Levasseur et al., 2008). Only a single CDH has been identified in *C. cinerea* and *P. chrysosporium.* Like lignin peroxidase and manganese peroxidase, CDH is a heme-containing protein involving in carbohydrate metabolism and lignin degradation by generating hydroxyl radicals through Fenton reaction pathway (Kersten and Cullen, 2007).

LDA2, LDA4, LDA5 and LDA6 are oxidases responsible for generating H_2O_2 in the presence of molecular oxygen. LDA7 are benzoquinone reductase, which reduces benzoquinone to hydroquinone. In *L. edodes*, I identified 1 LDA2, 7 LDA6 and 2 LDA7, but no LDA4 and LDA5.

I found out a large portion of ESTs were CBM1, GH5 and GH43 in CAZy classification. CBM (carbohydrate-binding module) associates with other CAZymes and promotes interaction with substrates (Cantarel et al., 2009). They may also present independently without coupling with any enzyme but functions in such form remained unclear (Cantarel et al., 2009). Various groups of CBM differ in amino acid sequences. CBM1 are exclusively identified in fungi that related to cellulose hydrolysis (http://www.cazy.org/index.html). In *L. edodes*, I identified 17 candidates of CBM1. In genomes of *C. cinerea* and *P. chrysosporium*, high copy numbers of CMB1 were detected as well, indicating CBM1 plays an important role in cellulose degradation (Martin et al., 2008). Numerous activities, including β-1, 3-glucosidase and cellulase, have been described for GH5 members. GH5 are usually abundant among genomes in fungi (Martin et al., 2008). I also found 7 ESTs in GH43 (related to hemicellulose degradation) but in *C. cinerea* and *P. chrysosporium*, only 4 have been reported (Martin et al., 2008).

The classification of FOLymes and CAZymes using *L. edodes* ESTs provided new insights for the genes differentially expressed during lignocellulose degradation. However, as ESTs were used instead of full length protein sequences, there may be over-estimation on the number of candidates in each FOLy or CAZy families. In Chapter 3, a genomic comparison on the distributions of FOLymes and CAZymes in *L. edodes*, *C. cinerea* and *P. chrysosporium* will give us a better understanding on the enzyme arsenals in the lignocellulolytic system of wood-rotting fungi.

2.4.4 Oxidative stress response in *L. edodes*

There is limited information regarding the influence of oxidative stress on fungal cellular protective mechanism during lignin degradation. Malarczyk et al. (1995) demonstrated that in wood-rotting fungus, *Pleurotus sajor-caju*, SOD activity remained low when the activities of ligninolytic enzymes were high. On the contrary, SOD activity peaked when the activities of ligninolytic enzymes were low, suggesting the radicals might be fully utilized in lignin depolymerization during high activities of ligninolytic enzymes (Leonowicz et al., 1999; Leonowicz et al., 2001).

In the present study, the expression levels of all 7 contigs, related to either ROS metabolism or cellular homeostasis, were significantly lower in mycelium grown in lignocellulose, indicating they were not actively transcribed in response to the high oxidative stress environment during lignin degradation. This could also be further supported by the lower SOD activity in lignocellulose-grown mycelium. Combining the current observations and the results obtained by Malarczyk et al. (1995), I postulate the ROS and free radicals generated are efficiently utilized and scavenged during lignin degradation and thus extracellular oxidative stress is not elevated.

Moreover, atmospheric O_2 can be transformed into ROS when exposed to high energy source, such as UV (Bergamini et al., 2004). During lignin degradation, O_2 is converted to H_2O_2 by LDAs (Levasseur et al., 2008). Presuming there are constant levels of intracellular ROS in lignocellulose-grown and non-lignocellulose-grown mycelia, the reduction of extracellular O_2 during lignin degradation may result in a net decrease of ROS level in lignocellulose-grown mycelium. As a result, lignocellulose-grown mycelium may encounter lower oxidative stress than non-lignocellulose-grown mycelium and thus there are down-regulations in both expression and activity of antioxidative enzymes in lignocellulose-grown mycelium (Fig. 2.10).

Concerning the deviation between CAT transcript levels and specific activities in mycelia grown in the two conditions, I postulate there may be several CAT isozymes in *L. edodes*. As demonstrated by Kwon and co-worker (2003), there were four CAT isozymes expressed during lignin degradation in *P. chysosporium*.

Different from SOD, I observed similar CAT specific activities in lignocellulose-grown and non-lignocellulose-grown mycelia. This can be explained by the fact that large amount of H_2O_2 is produced during lignin degradation to activate ligninolytic peroxidases (Levasseur et al., 2008). However, there may be a chance that these H_2O_2 diffuse and penetrate through the fungal membrane, damaging cellular components (Kwon and Anderson, 2001). Therefore, CAT activity may be required to remain at a constant level to deal with excessive H_2O_2 (Fig. 2.10).

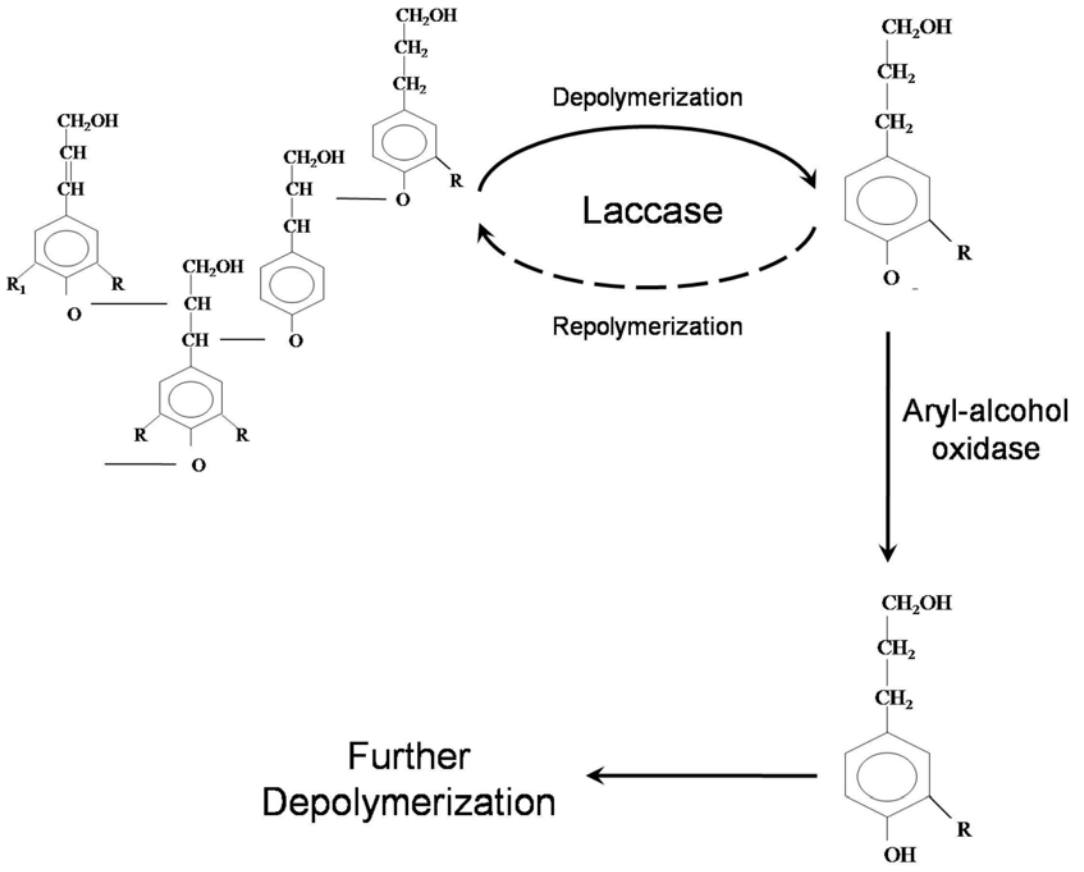


Fig. 2.9: Schematic diagram showing the laccase-mediated depolymerization of lignin and reduction of phenoxy radical to phenolic compound by aryl-alcohol oxidase. Repolymerization of phenoxy radical to lignin is inhibited.

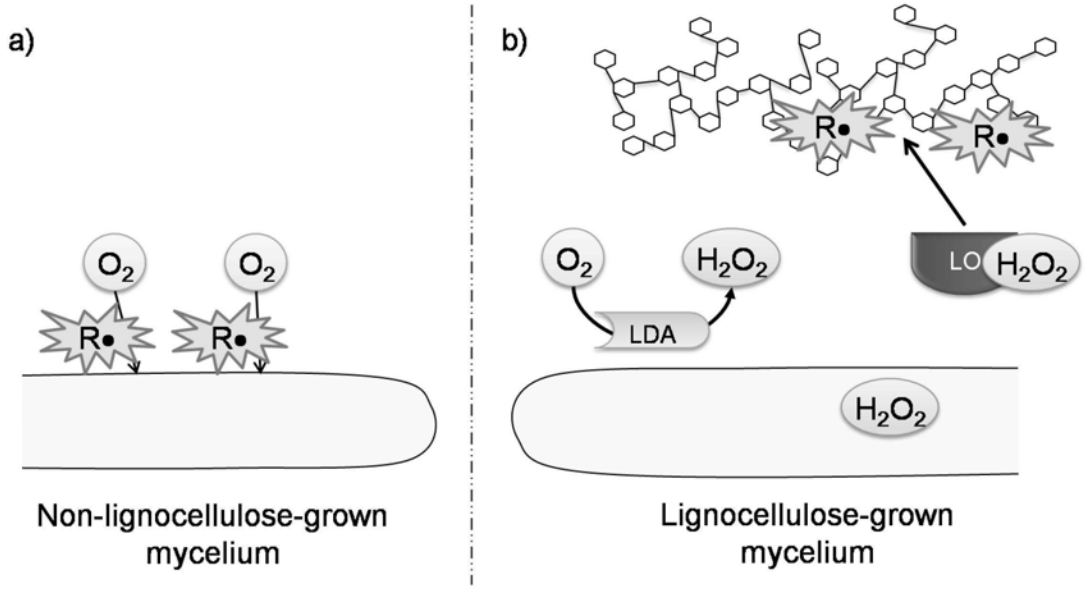


Fig. 2.10: A proposed model illustrating differences in the degree of oxidative stress encountered by mycelia grown in a) non-lignocellulose (NL) and b) lignocellulose (L). a) In NL, extracellular ROS, resulted from O_2 transformation, attacks mycelium. b) In L, with the catalysis of LDAs, O_2 is converted to H_2O_2, activating LOs (peroxidases) for lignin degradation. Due to the efficient scavenging of ROS by lignin and reduction in atmospheric O_2 level, lower oxidative stress is encountered by mycelium. On the other hand, H_2O_2 generated may permeate the mycelial membrane, so catalase (CAT) activity remains at constant level. (See text for details)

2.5 Conclusion

By means of high-throughput cDNA sequencing, I obtained a collection of genes expressed during vegetative mycelial growth in lignocellulosic medium. The transcript levels of contigs related to lignocellulose degradation were examined. Together with all *L. edodes* ESTs in NCBI, classifications of genes into various FOLy and CAZy families were performed. LO1 and LDA3 were the most strongly represented FOLymes while CBM1, GH5 and GH43 were the largest groups of CAZymes. In addition, based on the transcription analysis and enzyme activity measurements, I postulate that during lignin degradation, mycelia may encounter lower oxidative stress despite the production of ROS and free radicals. These findings provided new insights on the genes involved in lignocellulolytic system of wood-rotting fungi.

Chapter 3: Genomic analysis on the lignocellulolytic system in *Lentinula edodes*

3.1 Introduction

Wood-rotting basidiomycetes, such as *Lentinula edodes*, play an essential role in facilitating carbon recycle in ecosystem. They possess complex and unique extracellular enzyme systems that can efficiently destruct the recalcitrant biopolymers in wood (Perez et al., 2002; Kumar et al., 2008). Lignin depolymerization is undertaken by a collection of extracellular oxidases, including peroxidases and laccases, with the aid of low molecular weight radicals (Faison and Kirk, 1983; Cullen, 1997; Leonowicz et al., 1999). Cellulose and hemicellulose hydrolysis are resulted from the synergistic actions of various cellulases and hemicellulases (Perez et al., 2002).

In order to get accessible to plant cell wall polysaccharides, different wood-rotting fungi adopt unique strategies to "counteract" with the physical barrier lignin. White rot fungi completely depolymerize lignin to CO_2 and H_2O with the aid of ligninolytic enzymes. They can be further divided into 2 sub-classes, simultaneous rot and selective delignification (Martinez et al., 2005). The former simultaneously degrades lignin, cellulose and hemicellulose while the latter preferentially degrades lignin and hemicellulose first, then cellulose (Martinez et al., 2005). Brown rot fungi only modify lignin with polymeric residues remained *in situ*. However, little is known about the differences in the enzymatic moieties for lignocellulose degradation in different fungal species.

The shiitake mushroom, *Lentinula edodes*, is a common edible mushroom consumed in Asian countries. As a member of white rot fungi, *L. edodes* has

strong ligninolytic ability but relatively weak cellulolytic activity (Leatham, 1985). The enzymatic arsenal for lignocellulose degradation in *L. edodes* remains poorly understood, only a few lignocellulolytic enzymes that were previously identified, such as laccases (Zhao and Kwan, 1999; Sakamoto et al., 2008; Yano et al., 2009), manganese peroxidase (Sakamoto et al., 2009), cellobiohydrolases (Lee et al., 2001) and xylanase (Lee et al., 2005). Besides, it is still controversial about the rotting pattern of *L. edodes*. It preferentially degrades lignin (Leatham, 1985; Faix et al., 1991) while it has also been suggested to degrade lignin and polysaccharides simultaneously (Vane et al., 2003).

Here I analyzed the draft genome sequence of *L. edodes* based on FOLy (Fungal Oxidative Lignin enzymes) and CAZy (Carbohydrate Active enzymes) classifications (mentioned in Chapter 2). I compared the genomic distributions of FOLy and CAZy families in *L. edodes, Phanerochaete chrysosporium* (the most intensively studied white rot fungus), *Coprinopsis cinerea* (a model fungus) and *Postia placenta* (a model brown rot fungus). By doing so, I can have a better understanding on both the diversity of lignocellulolytic enzymes and the wood-rotting pattern of *L. edodes*. In addition, I can elucidate, on a genetic basis, the differences in enzymatic arsenals among different wood-decaying fungi.

3.2 Materials and Methods

3.2.1 Strain cultivation and DNA preparation

L. edodes monokaryon L54-A mycelia were cultured on PDA (Potato Dextrose Agar; Difco) plates at 25°C for about 60 days. Mycelia were harvested, lyophilized and disrupted using TissueLyser (QIAGEN) for DNA extraction using DNeasy® Plant Mini Kit (QIAGEN) according to manufacturer's instructions. The concentration and purity of the DNA preparations were assayed by spectrophotometry measurements (OD_{260} and OD_{280}). The quality of DNA was examined by 1.5% agarose gel electrophoresis in 1X TBE buffer (prepared from 10X TBE stock solution which contains 0.9M Tris base, 0.9M boric acid and 2mM EDTA), visualized by ethidium bromide staining.

3.2.2 High-throughput sequencing

Sequencing of *L. edodes* monokaryon L54-A was performed using the Roche GS-FLX/ GS-FLX Titanium sequencer (454 Life Sciences Corporation) according to manufacturer's instructions.

For paired-end sequencing, DNA was sheared into fragments of about 3kb. *Eco*RI restriction sites were protected by methylation, and adaptors containing an *Eco*RI site were ligated to the fragment ends. The fragments were subjected to *Eco*RI digestion and circularized by ligation of the compatible ends, and subsequently randomly sheared. All fragments were sequenced by GS-FLX system, creating paired-end reads and also shotgun reads.

3.2.3 Sequence assembly

GS-FLX shotgun and paired-end sequencing reads were assembled using GS-FLX-provided Newbler assembler (Roche). Contiguous stretches of sequence (contigs) were ordered and linked together into larger supercontigs (scaffolds) by the paired-end reads lying in different contigs.

3.2.4 Data analysis

In-house BLASTp was performed using all predicted gene models in *L. edodes* genome against all entries in FOLy (http://foly.esil.univ-mrs.fr) and CAZy (http://www.cazy.org/) databases.

3.3 Results

3.3.1 *L. edodes* genome

I obtained approximately 600Mbp sequences from GS-FLX shotgun and paired-end sequencing. It was estimated to be ~15x coverage of *L. edodes* genome. I obtained 9754 contigs, which were assembled into 1358 scaffolds. A total of 12,585 gene models were predicted from the genome and I classified 127 and 1113 genes (E-value < 1e-5) into FOLy and CAZy families, respectively. To be stringent enough, a cut-off at E-value < 1e-50 was used to select potential FOLymes and CAZymes in the analysis (Table 3.1 and Table 3.2).

3.3.2 FOLy classification

There were 49 unique gene models (E-value < 1e-50) predicted to encode potential FOLymes, of which 23 were classified as lignin oxidases (LOs) while 26 were categorized to lignin degrading auxiliary enzymes (LDAs).

In distinct contrast to *P. chrysosporium*, I predicted 15 laccases (LO1) in *L. edodes*, which was comparable to the number of LO1 (17) in *C. cinerea*. The number of peroxidases (LO2) was in between *P. chrysosporium* (16) and *C. cinerea* (1). There was 1 cellobiose dehydrogenase (LO3) more in *L. edodes* than in the other two basidiomycetes (Table 3.3).

L. edodes possessed the highest number of LDAs among the three fungal species. Compared to *P. chrysosporium* and *C. cinerea*, the numbers of putative glucose oxidase (LDA6) and glyoxal oxidase (LDA3) were extremely high in *L. edodes*. The other potential LDAs included 7 aryl-alcohol oxidases (LDA1), 1 pyranose oxidase (LDA4) and 2 benzoquinone reductase (LDA7). No vanillyl-alcohol oxidase (LDA2) and galactose oxidase (LDA5) was found in *L. edodes* genome (Table 3.3).

Detailed information for all gene models predicted as potential FOLymes were summarized in Table 3.4.

3.3.3 CAZy classification

In *L. edodes* genome, there were 317 gene models (E-value < $1e^{-50}$) classified as CAZymes, including 8 carbohydrate binding modules (CBM), 221 glycoside hydrolases (GH), 18 carbohydrate esterases (CE), 3 polysaccharide lyases (PL) and 67 glycosyltransferases (GT) (Table 3.5).

The overall number of gene models related to cellulose or hemicellulose degradations in *L. edodes* (45) was higher than that in *P. placenta* (27), but far lower than those in *P. chrysosporium* (95) and *C. cinerea* (131) (Table 3.6). I found a large reduction in numbers in CBM1, GH families 5 and 61 (cellulases). There was no GH11 (xylanases), GH45 (cellulases) or GH61 (cellulases) in *L. edodes* genome.

I also predicted that there were 24 gene models encoding pectin degrading enzymes, in which GH28 (polygalacturonase) represented the majority. The number of pectin degrading enzymes in *L. edodes* was 3 fold higher than those in other 3 fungi (Table 3.6).

Detailed information for all gene models predicted as potential CAZymes were listed in Table 3.7.

Table 3.1: The number of candidate gene models in FOLy families at different cut-off E-values.

	Maximum E-value[a]			
	1e-05	1e-10	1e-20	1e-50
LO1	21	20	18	15
LO2	11	7	6	6
LO3	55	18	4	2
LDA1	9	9	9	7
LDA2	1			
LDA3	11	9	8	5
LDA4	1	1	1	1
LDA6	16	16	14	11
LDA7	2	2	2	2
Total	127	82	62	49

[a]Number of candidate gene models at that particular cut-off E-value.

Table 3.2: The number of candidate gene models in CAZy families at different cut-off E-values.

	Maximum E-value[a]			
	1e-05	1e-10	1e-20	1e-50
CBM	163	114	42	8
GH	532	440	340	221
CE	136	110	75	18
PL	29	26	21	3
GT	253	159	104	67
Total	1113	849	582	317

[a]Number of candidate gene models at that particular cut-off E-value.

Table 3.3: The numbers of potential FOLymes in various fungi.

FOLy families	*L. edodes*	*P. chrysosporium*[a]	*C. cinerea*[a]
LO1	15	0	17
LO2	6	16	1
LO3	2	1	1
Sub-total	*23*	*17*	*19*
LDA1	7	3	18
LDA2	0	0	0
LDA3	5	1	0
LDA4	1	1	0
LDA5	0	0	0
LDA6	11	1	1
LDA7	2	4	2
Sub-total	*26*	*10*	*21*
Total	**49**	**27**	**40**

[a]The number of candidate genes in each FOLy family was adapted from Levasseur et al., 2008.

Table 3.4: Classification of *L. edodes* gene models into various FOLy families.

FOLy families[a]	Gene model[b]	FOLy annotation[c]	Organism	E-value[d]	HKLC1[e]	Other databases[f]
LO1	606_g	Laccase	*Trametes versicolor*	0		NUC, EST
	616_g	Hypothetical protein	*Termitomyces sp.*	0		
	673_g	Multicopper oxidase 2A	*Phanerochaete chrysosporium*	2e-62	HKLC11678	
	1449_g	Laccase 3 VT; LAC3VT	*Lentinula edodes*	0		NUC
	1813_g	Laccase 1 AVT; LAC1AVT	*Lentinula edodes*	0	HKLC13598 HKLC13709	NUC
	2073_g	Laccase 2 VT; LAC2VT	*Lentinula edodes*	2e-173		NUC
	2074_g	Laccase	*Lentinula edodes*	3e-79		
	3659_g	Laccase	*Lentinula edodes*	0		HKLC0
	4080_g	Hypothetical protein	*Termitomyces sp.*	0		
	7012_g	Laccase	*Termitomyces sp.*	0		
	7559_g	Laccase	*Lentinula edodes*	7e-168	HKLC10109	
	7837_g	Hypothetical protein	*Termitomyces sp.*	0		
	7846_g	Hypothetical protein	*Termitomyces sp.*	0		
	7904_g	Laccase	*Lentinula edodes*	0		
	12088_g	Fet3 protein	*Phanerochaete chrysosporium*	0	HKLC12651 HKLC13880 HKLC12419	HKLC0
LO2	4282_g	Manganese dependent peroxidase 1	*Lentinula edodes*	0		NUC

Table 3.4 (continued)

LO2	4368_g	Manganese peroxidase isoform 1	*Dichomitus squalens*	6e-144		
	4369_g	Manganese peroxidase isoform 2	*Dichomitus squalens*	2e-133		
	4683_g	Manganese peroxidase isoform 2	*Dichomitus squalens*	5e-136		
	4684_g	Manganese peroxidase isoform 2	*Dichomitus squalens*	1e-140		
	7322_g	Unnamed protein product	*Debaryomyces hansenii*	7e-73	HKLC13907	
LO3	1761_g	Unnamed protein product	*Aspergillus oryzae*	1e-55	HKLC10457	NUC, HKLC0
	4562_g	Cellobiose dehydrogenase	*Grifola frondosa*	0		
LDA1	118_g	Aryl-alcohol oxidase precursor	*Pleurotus pulmonarius*	1e-56		EST
	6258_g	Hypothetical protein CC1G_01542	*Coprinopsis cinerea*	5e-101		
	6267_g	Aryl-alcohol oxidase precursor	*Pleurotus eryngii*	3e-111		HKLC0
	7558_g	Aryl-alcohol oxidase precursor	*Pleurotus eryngii*	2e-139		EST
	8143_g	Aryl-alcohol oxidase precursor	*Pleurotus eryngii*	2e-104		EST
	9007_g	Aryl-alcohol oxidase precursor	*Pleurotus eryngii*	1e-125		
	9018_g	Hypothetical protein CC1G_09790	*Coprinopsis cinerea*	5e-103		
LDA3	1380_g	Copper radical oxidase	*Phanerochaete chrysosporium*	0	HKLC10080	EST
					HKLC10392	
					HKLC15518	
					HKLC10016	
					HKLC15424	
					HKLC10373	

Table 3.4 (continued)

LDA3	2899_g	Copper radical oxidase variant A	*Phanerochaete chrysosporium*	0	HKLC13941	
					HKLC13087	
					HKLC11914	
					HKLC11135	
					HKLC13549	
					HKLC12954	
					HKLC15405	
	3126_g	Glyoxal oxidase precursor	*Phanerochaete chrysosporium*	1e-168		
	10680_g	Copper radical oxidase	*Phanerochaete chrysosporium*	0		HKLC0
	10879_g	Copper radical oxidase variant A	*Phanerochaete chrysosporium*	0		EST, HKLC0
LDA4	1127_g	Pyranose 2-oxidase	*Tricholoma matsutake*	0		
LDA6	55_g	Unnamed protein product	*Aspergillus oryzae*	3e-68	HKLC13201	EST
					HKLC10209	
					HKLC12516	
					HKLC14291	
					HKLC13472	
					HKLC11363	
	1177_g	Unnamed protein product	*Aspergillus oryzae*	8e-96		HKLC0
	1415_g	Mala s 12 allergen precursor	*Malassezia sympodialis*	1e-133		EST
	1630_g	Unnamed protein product	*Aspergillus oryzae*	5e-74		EST
	1687_g	Unnamed protein product	*Aspergillus oryzae*	6e-59		HKLC0

Table 3.4 (continued)

LDA6	2610_g	Putative oxidoreductase	*Fusarium oxysporum*	2e-56		
	3158_g	Unnamed protein product	*Aspergillus oryzae*	9e-94		
	3168_g	Unnamed protein product	*Aspergillus oryzae*	1e-88		
	6673_g	Unnamed protein product	*Aspergillus oryzae*	7e-86		
	7239_g	Unnamed protein product	*Aspergillus oryzae*	2e-84		
	11331_g	Mala s 12 allergen precursor	*Malassezia sympodialis*	1e-129	HKLC15139 HKLC13006	EST, HKLC0
LDA7	7662_g	1,4-benzoquinone reductase	*Phanerochaete chrysosporium*	7e-73		EST, HKLC0
	8889_g	1,4-benzoquinone reductase	*Phanerochaete chrysosporium*	3e-81	HKLC13775	EST, HKLC0

[a]Classification according to FOLy database.

[b]ID designated for each gene model predicted from *L. edodes* draft genome.

[c]Annotation of the matched entry in FOLy database.

[d]Cut-off E-value at 1e< 10^{-50}.

[e]Mapping of HKLC1 contigs to predicted gene models.

[f]Mapping of *L. edodes* NCBI core nucleotide sequence database (NUC), NCBI EST database (EST) and in-house EST(fruiting body and fruiting body after sporulation) database (HKLC0) to predicted gene models. NUC, EST and HKLC0 indicate at least one sequence in the databases mapped to the corresponding gene model.

Table 3.5: The numbers of potential CAZymes in various fungi.

CAZy families	*L. edodes*	*P. chrysosporium*[a]	*C. cinerea*[a]	*P. placenta*[a]
CBM	8	45	89	6
GH	221	180	211	144
CE	18	19	51	10
PL	3	4	13	6
GT	67	68	72	75
Total	**317**	**316**	**436**	**241**

[a]The number of candidate genes in each CAZy family was adapted from Levasseur et al., 2008 and Martinez et al., 2009.

Table 3.6: The numbers of potential candidate genes in CAZy families related to cellulose, hemicellulose or pectin degradation in various fungi.

	CAZy families	*L. edodes*	*P. chrysosporium*[a]	*C. cinerea*[a]	*P. placenta*[a]
Cellulose or hemicellulose degradations	CBM1	7	29	45	0
	GH5	18	24	23	20
	GH6	1	1	5	0
	GH7	4	9	7	0
	GH9	1	1	1	0
	GH10	3	6	5	3
	GH11	0	1	6	0
	GH43	4	4	4	1
	GH45	0	0	0	0
	GH51	3	2	1	1
	GH61	3	14	33	2
	GH67	0	0	0	0
	GH74	1	4	1	0
	Sub-total	**45**	**95**	**131**	**27**
Pectin degradation	PL1	1	0	1	0
	PL3	0	0	2	0
	PL4	1	0	2	0
	PL9	0	0	0	0
	GH28	21	4	3	7
	CE8	1	2	0	2
	Sub-total	**24**	**6**	**8**	**9**

[a]The number of candidate genes in each CAZy family was adapted from Levasseur et al., 2008 and Martinez et al., 2009.

Table 3.7: Classification of *L. edodes* gene models into various CAZy families.

CAZy families[a]	Gene model[b]	CAZy annotation[c]	Organism	E-value[d]	HKLC1[e]	Other databases[f]
CBM1	2135_g	Acetyl xylan esterase	*Volvariella volvacea*	1e-93	HKLC12617	
	2271_g	Hypothetical protein	*Neurospora crassa*	6e-51		EST
	4562_g	Cellobiose dehydrogenase	*Thielavia heterothallica*	1e-120		
	6091_g	Hypothetical protein MGG_08408	*Magnaporthe grisea*	3e-60		EST
	6316_g	Xylanase	*Lentinula edodes*	9e-141	HKLC12169	NUC, EST, HKLC0
	6323_g	Cip2	*Hypocrea jecorina*	1e-122		HKLC0
	6458_g	Xylanase	*Lentinula edodes*	3e-86		NUC, EST
GH5	294_g	Man5D	*Phanerochaete chrysosporium*	3e-108		
	1759_g	Mannanase	*Armillariella tabescens*	2e-147		
	2103_g	Hypothetical protein MGG_09433	*Magnaporthe grisea*	2e-134		HKLC0
	2132_g	Exo-beta-1,3-glucanase	*Cryptococcus neoformans*	5e-133	HKLC11674	
	2272_g	Exo-beta-1,3-glucanase	*Lentinula edodes*	0		NUC, HKLC0
	2273_g	Exo-beta-1,3-glucanase	*Lentinula edodes*	3e-157	HKLC15292	NUC, EST, HKLC0
					HKLC15355	
					HKLC15887	
	3112_g	Endoglucanase 1	*Robillarda sp.*	1e-132		
	3547_g	Expressed protein	*Cryptococcus neoformans*	4e-106	HKLC15035	EST
	4457_g	Cytoplasm protein, putative	*Cryptococcus neoformans*	0	HKLC14207	HKLC0
	5210_g	Hypothetical protein CNF01760	*Cryptococcus neoformans*	1e-162		EST
	5879_g	Cytoplasm protein, putative	*Cryptococcus neoformans*	7e-63		HKLC0

Table 3.7 (continued)

	6942_g	Cytoplasm protein, putative	*Cryptococcus neoformans*	8e-89		
	7913_g	Exo-beta-1,3-glucanase	*Cryptococcus neoformans*	8e-141	HKLC12611	
					HKLC15318	
GH5	8594_g	Cytoplasm protein, putative	*Cryptococcus neoformans*	4e-170		EST
	9320_g	Endoglucanase	*Trametes hirsute*	3e-124		
	9342_g	Exo-1,3-beta-glucanase	*Agaricus bisporus*	2e-103	HKLC10198	HKLC0
	11229_g	Endoglucanase	*Phanerochaete chrysosporium*	6e-117		
	11510_g	Endoglucanase	*Polyporus arcularius*	6e-155		
GH6	3628_g	Cellulase CEL6B	*Lentinula edodes*	0		NUC
GH7	2000_g	Cellulase CEL7A	*Lentinula edodes*	3e-178	HKLC14682	
	3475_g	Cellulase CEL7A	*Lentinula edodes*	2e-164		
	9013_g	Cellulase CEL7A	*Lentinula edodes*	0	HKLC12846	
					HKLC13717	
	11496_g	Cellulase CEL7A	*Lentinula edodes*	0		NUC
GH9	2049_g	Family 9 glycosyl hydrolase	*Phanerochaete chrysosporium*	1e-152	HKLC12029	
GH10	2345_g	Endo-1,4-B-xylanase A	*Phanerochaete chrysosporium*	1e-152		
	6381_g	Endo-1,4-B-xylanase A	*Phanerochaete chrysosporium*	6e-103		
	6478_g	Endo-1,4-B-xylanase A	*Phanerochaete chrysosporium*	2e-119		
GH43	2566_g	Putative secreted arabinase	*Streptomyces coelicolor*	2e-59		
	5418_g	Unnamed protein product	*Aspergillus niger*	1e-74		
	5617_g	Hypothetical protein MG07520.4	*Magnaporthe grisea*	1e-62		

Table 3.7 (continued)

GH43	6154_g	Galactan 1,3-beta-galactosidase	*Phanerochaete chrysosporium*	3e-153	
GH51	7005_g	Unnamed protein product	*Aspergillus niger*	2e-152	HKLC0
	9199_g	Unnamed protein product	*Meripilus giganteus*	2e-167	
	12462_g	Unnamed protein product	*Meripilus giganteus*	0	
GH61	2520_g	Cellulose-growth-specific protein	*Agaricus bisporus*	8e-88	
	2521_g	Cellulose-growth-specific protein	*Agaricus bisporus*	1e-81	
	4388_g	Unnamed protein product	*Botryosphaeria rhodina*	2e-64	
GH74	11005_g	Glycoside hydrolase family 74	*Phanerochaete chrysosporium*	0	
PL1	5988_g	Pectate lyase plya	*Aspergillus niger*	8e-71	
PL4	10710_g	Rhamnogalacturonan lyase	*Emericella nidulans*	3e-129	HKLC0
GH28	86_g	Hypothetical protein	*Vitis vinifera*	1e-81	
	125_g	Hypothetical protein	*Vitis vinifera*	8e-52	
	1195_g	Hypothetical protein	*Vitis vinifera*	1e-62	
	1714_g	Hypothetical protein	*Vitis vinifera*	1e-57	
	2304_g	Hypothetical protein	*Vitis vinifera*	7e-79	
	2451_g	Endopolygalacturonase	*Chondrostereum purpureum*	3e-90	
	2506_g	Exo-rhamnogalacturonase B	*Aspergillus niger*	4e-91	
	2593_g	Unnamed protein product	*Aspergillus oryzae*	8e-63	EST
	3399_g	Hypothetical protein	*Vitis vinifera*	1e-88	
	3427_g	Endopolygalacturonase	*Chondrostereum purpureum*	2e-89	HKLC0
	3737_g	Endopolygalacturonase	*Chondrostereum purpureum*	2e-74	

Table 3.7 (continued)

GH28	3836_g	Endo-xylogalacturonan hydrolase	*Aspergillus tubingensis*	1e-98		
	3967_g	Hypothetical protein	*Vitis vinifera*	7e-82		
	4123_g	Hypothetical protein	*Vitis vinifera*	5e-58	HKLC11858	EST
	5421_g	Hypothetical protein	*Vitis vinifera*	3e-64		
	5503_g	Exo-polygalacturonase	*Botryotinia fuckeliana*	9e-81		
	9635_g	Hypothetical protein	*Vitis vinifera*	2e-62		
	9637_g	Endopolygalacturonase	*Chondrostereum purpureum*	1e-89		
	10023_g	Endopolygalacturonase	*Chondrostereum purpureum*	3e-77		
	11462_g	Polygalacturonase, putative	*Cryptococcus neoformans*	6e-51		
	11485_g	Exo-rhamnogalacturonase B	*Aspergillus niger*	6e-69		
CE8	3440_g	Pectin methylesterase	*Vitis riparia*	9e-63		HKLC0

[a]Classification according to CAZy database.

[b]ID designated for each gene model predicted from *L. edodes* draft genome.

[c]Annotation of the matched entry in CAZy database.

[d]Cut-off at E-value < 1e-50.

[e]Mapping of HKLC1 contigs to predicted gene models.

[f]Mapping of *L. edodes* NCBI core nucleotide sequence database (NUC), NCBI EST database (EST) and in-house EST(fruiting body and fruiting body after sporulation) database (HKLC0) to predicted gene models. NUC, EST and HKLC0 indicate at least one sequence in the databases mapped to the corresponding gene model.

3.4 Discussion

I decoded a draft genome sequence of *L. edodes* with approximately 15x coverage and I predicted the potential lignocellulolytic enzyme-coding genes by homology search against FOLy (Levasseur et al., 2008) and CAZy (Cantarel et al., 2009) databases. The whole genome distribution of FOLy and CAZy families in *L. edodes* is compared with 3 other fungi, *Phanerochaete chrysosporium*, *Coprinopsis cinerea* and *Postia placenta*.

3.4.1 FOLymes distribution in *L. edodes* genome

Laccase can be found in a wide spectrum of fungi (Baldrian, 2006). FOLy classification revealed there were large number of laccase-coding genes in *L. edodes* and *C. cinerea.* No putative laccase-coding genes were found in "non-mushroom forming" basidiomycetes, *P. chrysosporium* and *Ustilago maydis* while only 1 was detected in each of the Ascomycota *Aspergillus nidulans* and *Trichoderma reesei* (Levasseur et al., 2008). The abundance of putative laccase-coding genes in the genomes of mushroom-forming basidiomycetes, *L. edodes* and *C. cinerea* suggested laccase may play important role in mushroom development, which has been discussed previously (Zhao and Kwan, 1999; Nagai et al., 2003).

In contrast to *P. chrysosporium*, *L. edodes* possesses a number of laccases and peroxidases (LO2) in the genome, implying its ligninolytic system is composed of a diversified enzymatic arsenal. According to FOLy annotation, no potential lignin peroxidase-coding gene was identified, which further confirms the previous reports that *L. edodes* probably does not produce lignin peroxidase (Buswell et al., 1995; Silva et al., 2008).

There is a large number of putative glucose oxidase (LDA6) encoding genes in *L. edodes*. Glucose oxidase catalyzes the oxidation of glucose with

concomitant reduction of molecular oxygen into H_2O_2. Glucose oxidase cooperates with H_2O_2-dependent LO2 peroxidases to degrade lignin (Green, 1977; Levasseur et al., 2008). Glucose oxidase mutation in *P. chrysosporium* lowered its ligninolytic activity (Kelley et al., 1986), indicating the essential role of glucose oxidase in lignin degradation. Besides LDA6, *L. edodes* also possesses a large number of genes encoding aryl-alcohol oxidases (LDA1) and glyoxal oxidases (LDA3) than *P. chrysosporium* and *C. cinerea*, suggesting *L. edodes* may rely on these 3 kinds of enzymes for the continuous H_2O_2 generation during lignin degradation.

3.4.2 CAZymes distribution in *L. edodes* genome

The overall number of CAZymes encoding genes in *L. edodes* genome (317) is fairly typical and the numbers of carbohydrate esterases, polysaccharide lyases and glycosyltransferases are similar to those in white rot *P. chrysosporium*. However, the number of carbohydrate binding modules (CBM) is particularly lower when compared with *P. chrysosporium* and *C. cinerea* (Martin et al., 2008). There are only 2 CBM more than that in brown rot *P. placenta* (Martinez et al., 2009). *L. edodes* possessed only a few CBM1. Besides, there is also a reduced number of cellulases, of GH families5, 6, 7 and 61, in *L. edodes* when compared to *P. chrysosporium* and *C. cinerea.* These observations may explain the weak cellulolytic activity in *L. edodes* (Leatham, 1985).

L. edodes preferentially attacks hemicellulose (Leatham, 1985; Elisashvili et al., 2008). CAZy annotation showed that the number of hemicellulase encoding genes in *L. edodes* genome is comparable to that in *P. chrysosporium* or *C. cinerea*, except that GH11 xylanase completely missing in the *L. edodes* genome.

It is surprising that the number of candidate genes for polygalacturonase (GH28) is the most highly represented group among all GH families. Polygalacturonase degrades pectin, which is a structurally complex polysaccharide found in plant cell wall. It comprises ~35% of the polysaccharides in primary cell walls and ~5% in secondary cell walls (Mohnen et al., 2008). The abundance of polygalacturonase encoding genes in the genome is in agreement with the high polygalacturonase activity during the vegetative cultivation of *L. edodes* mycelium (Leatham, 1985).

3.4.3 Implication of FOLymes and CAZymes distributions in *L. edodes* genome

The degradation pattern for *L. edodes* on wood logs is still unclear. Lignin composition decreased more rapidly than polysaccharides during the initial inoculation of *L. edodes* (Leatham, 1985; Faix et al., 1991). Besides, low cellulase activity but high hemicellulase activity was also observed throughout the mycelial cultivation (Leatham, 1985). Based on our findings on the genomic distribution of FOLymes and CAZymes and the transcript levels of several lignocellulolytic enzyme-coding genes (Chapter 2), it appears that *L. edodes* has a rot pattern of selective delignification that preferentially attacks lignin and hemicellulose before cellulose.

3.4.4 Mapping the genome with transciptome

Although I have predicted a number of protein-coding gene models in *L. edodes* genome, I cannot exclude the presence of pseudogenes. With the availability of our in-house EST database from high throughput cDNA sequencing, including HKLC1 contigs mentioned in Chapter 2, and *L. edodes* ESTs from NCBI, I can identify some predicted gene models that are expressed. I estimate that at least 11 candidate gene models of FOLymes and

13 candidate gene models of CAZymes (only included the 19 families involving in cellulose, hemicellulose and pectin degradation) are transcribed in lignocellulose-grown mycelium. Of the total 118 candidate gene models identified in FOLy and CAZy, 53 can be mapped with at least 1 *L. edodes* EST.

3.5 Conclusion

Our laboratory obtained a draft genome sequence of *L. edodes* and predicted over 12,000 gene models. FOLy classification of the gene models revealed a diversified enzymatic arsenal for lignin degradation. From CAZy annotation, I found a reduced number of cellulase but abundant hemicellulase and pectinase encoding genes in *L. edodes*. The genome sequence offers insights of the lignocellulolytic system and the wood-rotting pattern of *L. edodes,* and provides a better understanding on the diversification of lignocellulose degrading mechanism in wood-decaying fungi.

Chapter 4: Isolation, characterization and heterologous expression of *Lentinula edodes* manganese peroxidase gene, *lemnp2*, in the methylotrophic yeast *Pichia pastoris*

4.1 Introduction

Next to cellulose, lignin is the second most abundant carbon-containing biopolymers on earth (Davin et al., 2008). It has a complex chemical structure deriving from free radical-mediated polymerization of phenylpropanoids (Kirk and Farrell, 1987). Due to the heterogenic, hydrophobic and macromolecular configuration of lignin, plant cell walls are highly resistant to microbial attacks (Buswell and Odier, 1987). White rot basidiomycete is one of the few wood invaders found in nature. It can degrade lignin completely to carbon dioxide and water. The major degradative enzymes involved in lignin depolymerization are laccases (Lcc) and peroxidases, especially lignin peroxidase (LiP) and manganese peroxidase (MnP) (Cullen, 1997). Although the mechanism of lignin degradation by wood-rotting fungi is still not fully understood, a number of studies revealed that it involves free radicals and small molecules with high oxidative state, thus it is a process of "enzymatic combustion" (Buswell and Odier, 1987; Kirk and Farrell, 1987; Martinez et al., 2005).

Lentinula edodes is one of the most popular edible mushrooms in Asia. It is a white-rot fungus that naturally grows on wood log. Its ligninolytic activity has been demonstrated as early as 1980s (Leatham, 1985). Studies showed that *L. edodes* actively secretes Lcc and MnP but not lignin peroxidase (Buswell et al., 1995; Elisashvili et al., 2008; Silva et al., 2008). MnP belongs to heme-containing peroxidase family II and its reaction is manganese (II) ion-dependent (Martinez, 2002). Mn^{3+} ions, generated from the oxidation of

Mn^{2+} ions by H_2O_2-oxidized MnP, is a freely diffusible oxidant that attacks lignin biopolymer in cell wall matrix (Wariishi et al., 1992). In order to make industrial applications of MnP possible, a number of eukaryotic expression systems have been used to produced recombinant *Phanerochaete chrysosporium* MnPs, including *Aspergillus oryzae* (Stewart et al., 1996) and *Aspergillus niger* (Conesa et al., 2000). Conesa and co-workers demonstrated that with an addition of 5g/L hemoglobin, MnP activity was 10 fold higher than those without heme-supplementation (Conesa et al., 2000). The methylotrophic yeast *Pichia pastoris* could also produce active MnP with an activity up to 2,500U/L in fed-batch culture supplemented with 0.1g/L hemin (Jiang et al., 2008a).

In the present study, I aimed at isolating gene encoding MnP in *L. edodes*, by random sequencing of a normalized mycelia cDNA library. The gene structure and mRNA expression level in mycelia cultivated in lignocellulose or non-lignocellulose were characterized. In order to study the enzyme properties, the MnP coding gene was heterologously expressed in the yeast *P. pastoris*.

4.2 Materials and Methods

4.2.1 Strain cultivation

Lentinula edodes dikaryotic strain L54 was used in all the experiments. Mycelium was maintained in PDA (Potato Dextrose Agar, Difco), from which inocula (about 1cm^2 mycelia cubes) were made. For cDNA library construction, mycelia were cultivated on artificial logs (60% dry sawdust, 30% dry wood chips and 10% dry wheat bran) at 25°C in dark environment for one month to allow colonization of mycelia on the whole log. For quantitative RT-PCR, mycelia were inoculated on 20g of lignocellulose medium (60% dry sawdust, 30% dry wood chips and 10% dry wheat bran with 100% moisture) and non-lignocellulose medium [PDB (Potato Dextrose Broth, Difco)], respectively, in 90mm-diameter glass pot for two weeks.

4.2.2 cDNA library construction

Total RNA was extracted from mycelia using TRI Reagent® (Molecular Research Center, Inc.). DNaseI treatment was performed to eliminate genomic DNA contamination. cDNA library was constructed using Creator™ SMART™ cDNA Library Construction Kit (Clontech). A novel normalization method was used to enrich genes with low abundance (Fig. 2.1). Reverse transcription of cDNA was performed using customized CDS III/3' PCR primer (5'-ATTCTAGAGGCCGAGGCGGAAGACATG-d(T)$_{16}$N$_8$-3') in first strand cDNA synthesis with restricted primer concentration of 0.04μM. Apart from the modification stated above, the general procedures followed the manufacturer's instructions.

4.2.3 Random sequencing of cDNA library

Clones were randomly picked from the cDNA library constructed. PCR was performed using M13 (-20) forward primer and Oligo-dT$_{16}$-VN+adaptor primer

(5'-ATCTAGAGCGGCCGC-d(T)$_{16}$VN-3'). PCR products were sequenced by Macrogen Inc., Seoul. Sequences were annotated using BLASTx homology search in NCBI sequence databases. They were classified into groups manually according to their biological functions.

4.2.4 Cloning and sequencing of *lemnp2*

Total RNA was extracted as described above. The 5' RACE (Rapid Amplification of cDNA Ends) were performed using FirstChoice® 5' RLM-RACE kit (Ambion) according to manufacturer's instructions. Nested PCR was performed with *mnp* specific primers, Mnp_nest_530 (5'-TCGCTCCATTCCTTGCCTCAG3') and Mnp_nest_483 (5'-ACCCAACTTCGCTGCCAACCTT-3'). RACE products were cloned using TOPO Cloning kit (Invitrogen) and sequenced.

Mycelia were cultured in PDA for two weeks before being ground in liquid nitrogen. Genomic DNA was isolated by grinding mycelia in liquid nitrogen, followed by the addition of 500μl CTAB buffer, pH5 (2g CTAB, 0.1M Tris, 0.02M EDTA, 1.4M NaCl and 1g PVP40) and incubation at 55°C for 15 minutes. The mixture was centrifuged at 12,000 x g for 5 minutes and the supernatant was then transfer to new microfuge tube. 250μl of chloroform:Isoamylalcohol was added (24:1) to the supernatant, mixed and centrifuged at 13,000rpm for 1 minute. Upper aqueous phase was transferred to new tube and DNA was recovered by ethanol precipitation. PCR was performed using genomic DNA with primers MnpATG (5'-ATGGCATTTTCGAGTATTGTCC-3') and MnpLow (5'-TTAAGAAGTGCAAGTGGCTTCGT-3').

Sequence alignment was done by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Signal peptide prediction was performed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/)

while protein molecular weight was predicted using ExPASy Protparam tool (http://www.expasy.ch/cgi-bin/protparam).

4.2.5 Quantitative RT-PCR of *lemnp2* expression level

Total RNA was extracted as described above. DNase I treatment was performed to eliminate contamination of genomic DNA. First strand cDNA was synthesized using TaqMan® Reverse Transcription Reagents (Roche, Applied Biosystems) according to the manufacturer's instructions. Quantitative RT-PCR reaction was performed on Bio-Rad MiniOpticon™ real time system (Bio-Rad). 2µl of 10X diluted first strand cDNA was mixed with 0.45µl of each 10µM of Mnp_rt_737_up (5'-GCAGTTGACGGTTTGATCCCT-3') and Mnp_rt_828_low (5'-GCGATACGAGCAGAGAGACAA3'), 10µl of 2X iQ™ SYBR® Green Supermix (Bio-Rad) and brought up to 20µl with nuclease-free water according to manufacturer's manual. The reaction for each primer set was performed in duplicate and no-template-control (NTC). The PCR program was 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, 60°C for 45 seconds and 72°C for 15 seconds. Melting curve analysis was also performed by increasing the temperature from 50°C to 90°C. Data analysis was performed on Opticon Monitor™ Version 3.0 (Bio-Rad). The experiments were repeated for three times with independent biological samples.

4.2.6 *Pichia pastoris* cultivation and expression vector construction

P. pastoris strain KM71 and plasmid pPIC9K were obtained from Invitrogen. *P. pastoris* was cultured on YPD medium (1% yeast extract, 2% peptone and 2% dextrose). Full length *mnp* cDNA was obtained from RT-PCR using MnpATG and MnpLow primers (described previously). Two expression vectors, designated as pPIC9K/MnpATG and pPIC9K/Mnp58, were constructed (Fig. 4.8a). For the construction of pPIC9K/MnpATG, reamplification of *lemnp2*

cDNA using gene specific primers with *EcoR*I (MnpPicEcoRI-ATGU; 5'-TTTGAATTCATGGCATTTTCGAGTATTGTCC-3') and *Not*I (MnpPicNotI-L; 5'-ATAATAATGCGGCCGCTTAAGAAGTGCAAGTGG-3') restriction sites was performed. For the construction of pPIC9K/Mnp58, upper primer MnpPicEcoRI-58U (5'-TTTGAATTCGCTCCCGCTTCTCAGAATGCGG-3') was used instead of MnpPicEcoRI-ATGU. After digestion with *EcoR*I and *Not*I, the resulting PCR products were ligated into pPIC9K, which has been digested with the same enzymes. The two ligated mixtures were transformed into *E. coli* TOP10 (Invitrogen) by electroporation. Transformants were selected on LB medium containing 100μg/ml ampicillin. Positive clones were screened by PCR using α-mating factor (αMF) and 3'AOX primers.

4.2.7 Transformation of *P. pastoris* and selection of transformants

P. pastoris transformation was carried out using Multi-copy *Pichia* Expression Kit (Invitrogen) according to manufacturer's instructions. Expression vectors were purified by Wizard®Plus Minipreps kit (Promega) and linearized at *Bsp*EI sites. Plasmids from all positive clones were mixed to generate two library pools of pPIC9K/MnpATG and pPIC9K/Mnp58. Ten microgram of each linearized plasmid pool was used in each transformation. *P. pastoris* KM71 was also transformed with linearized pPIC9K with no insert to serve as control. Transformants were selected on regeneration dextrose medium (RD; 1M sorbitol, 2% dextrose, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4 x 10^{-5} % biotin and 0.005% amino acids lacking histidine). The selected positive transformants were cultivated at 30°C in 10ml buffered induction medium (1% yeast extract, 2% peptone, 0.1M potassium phosphate, pH6 and with 0.5% methanol) for rMnP activity screening. Hemoglobin (Sigma-Aldrich) was added to the culture medium at

concentration 5g/L. One milliliter of culture supernatant was collected daily for MnP activity measurement. The clone with the highest MnP activity was selected for further characterization.

4.2.8 Enzyme activity assay

MnP activity was assayed by measuring the oxidation of 2, 6-dimethoxyphenol (2, 6-DMP) at 469nm (ε_{469} = 49.6mM^{-1}cm^{-1}). The reaction mixture contained 0.75ml of 0.4mM $MnSO_4$ in 50mM sodium malonate (pH4.5), 0.3ml of 20mM 2, 6-DMP and 50μl culture supernatant. Reaction was initiated by the addition of 75μl of 0.1mM H_2O_2. One unit was defined as the amount of enzyme producing 1μmol of product per minute. Protein concentration was determined by Quick Start™ Bradford Protein Assay (Bio-Rad) using γ-globulin as standard.

4.2.9 SDS-PAGE

Proteins were separated in 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using MiniProtean III apparatus (Bio-Rad). Coomassie blue staining was used to visualize proteins on SDS-PAGE.

4.2.10 Analysis of rMnP activity in different heme-supplemented media

Single colonies of rMnP secreting clones were cultivated in 20ml buffered induction media described above containing 1g/L hemin (Sigma-Aldrich)) and 5g/L hemoglobin at 30°C. Aliquots (1ml) of culture supernatant was collected at 0, 3, 6, 9, 24 and 48 hours for OD600 and MnP activity measurements.

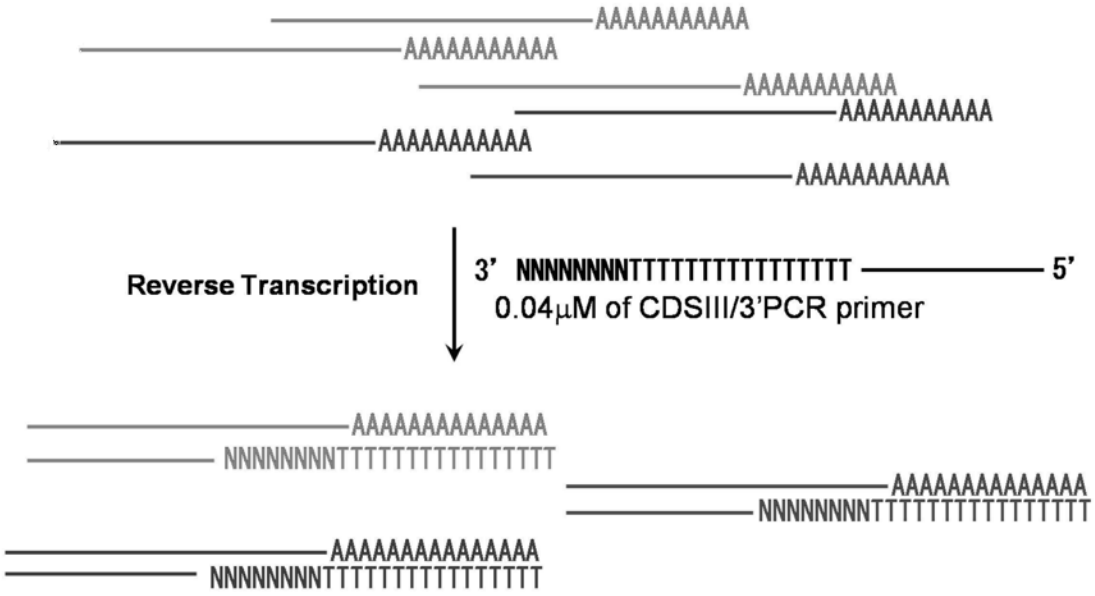


Fig. 4.1: Schematic diagram illustrating the principle of the novel normalization method. Low concentration (0.04μM) of customized primer, CDSIII/3' PCR, was used in cDNA synthesis. There would be equal chances on primer annealing for genes that are abundantly expressed (red), moderately expressed (blue) and rarely expressed (green). Therefore, low abundant genes would be enriched in the cDNA pool.

4.3 Results

4.3.1 Random sequencing of cDNA library

The percentage of recombinant clones in the cDNA library was estimated to be greater than 88% (Fig. 4.2) and the calculated titer was 3 x 10^6 cfu/ml.

Classification of the 224 randomly sequenced clones was summarized in Table 4.1. The list only retained clones with E-value < 10^{-5} in BLASTx homology search. About one third of the clones (71) were categorized as structural protein-coding genes, while 30% did not show any homology to known proteins. Approximately 20% of the clones were classified as genes related to metabolism, in which 5 clones had high sequence similarities to genes encoding lignocellulolytic enzymes (Table 4.2). Clone O3 was selected for further characterization.

4.3.2 Genomic and cDNA sequences of *lemnp2*

A fragment of about 1.1kb was generated from 5'RACE. Together with the 3' sequence obtained from random cDNA library sequencing (clone O3), the full length cDNA sequence of manganese peroxidase encoding gene, designated as *lemnp2* by Sakamoto et al. (2009), was approximately 1.4kb with an open reading frame of 1128bp*. The length of deduced polypeptide was 376 amino acids, leading by a 19 amino-acid signal peptide sequence. The predicted molecular weight was 39kDa with pI value of 4.18. Its amino acid sequences show high similarity to MnP1 of *Dichomitus squalens* (66%) and MnP3 of *Phanerochaete chrysosporium* (65%). Conserved binding sites for Mn^{2+} and heme were also found in *lemnp2* (Fig. 4.3). Using *lemnp2* gene specific primers, a fragment of ~1.9kb genomic sequence was amplified (Fig. 4.4). Thirteen introns were predicted along the genomic sequence of *lemnp2** (Fig. 4.5 and 4.6).

**The genomic and cDNA sequences have been published recently by Sakamoto et al. (2009) and they have DDBJ accession numbers of AB306944 and AB306943, respectively.*

4.3.3 Analysis on transcript levels of *lemnp2* in mycelia grown in different media

Quantitative RT-PCR of *lemnp2* was performed with mycelia grown on lignocellulose (L) and non-lignocellulose (NL). The transcript level of *lemnp2* in mycelium grown in L was more than 10 fold higher than that grown in NL (Fig. 4.7).

4.3.4 Construction of expression vectors and selection of transformants in *P. pastoris*

Two expression vectors, pPIC9K/MnpATG and pPIC9K/Mnp58, were constructed for the expression of rMnP in *P. pastoris*. In the first construct, a full length cDNA of *lemnp2* was ligated immediately downstream of αMF, so there were two different signal peptides, yeast and native. The second construct was generated with a deletion of the predicted signal sequence in *lemnp2* and thus only α-mating factor (αMF) was present (Fig. 4.8).

Twenty positive transformants were selected from RD media for each expression vectors. Three *P. pastoris* transformants containing pPIC9K/MnpATG (KM71/pPIC9K/MnpATG#1, KM71/pPIC9K/MnpATG#2 and KM71/pPIC9K/MnpATG #3) produced the highest MnP activity. No detectable MnP activity was observed in transformants containing pPIC9K/Mnp58.

4.3.5 Expression of rMnP in *P. pastoris*

The molecular weight of rMnP in *P. pastoris* transformants KM71/pPIC9K/MnpATG#1, KM71/pPIC9K/MnpATG#2 and KM71/pPIC9K/MnpAT#3 was estimated to be 45kDa by SDS-PAGE (Fig. 4.9).

4.3.6 Recombinant MnP activity in different heme-supplemented media

Recombinant MnP activity for strain KM71/pPIC9K/MnpATG#1 cultured in media with no heme-supplementation and with 1g/L hemin exhibited similar patterns that decreased in first 9 hours, peaked at 24 hours and then remained stable. The maximum rMnP were 0.4U/L and 0.3U/L, respectively, in media with no supplement and with hemin. When cultivated in 5g/L hemoglobin supplemented medium, rMnP activity increased in first 9 hours, reaching a maximum of 350U/L, and then remained stable (Fig. 4.10).

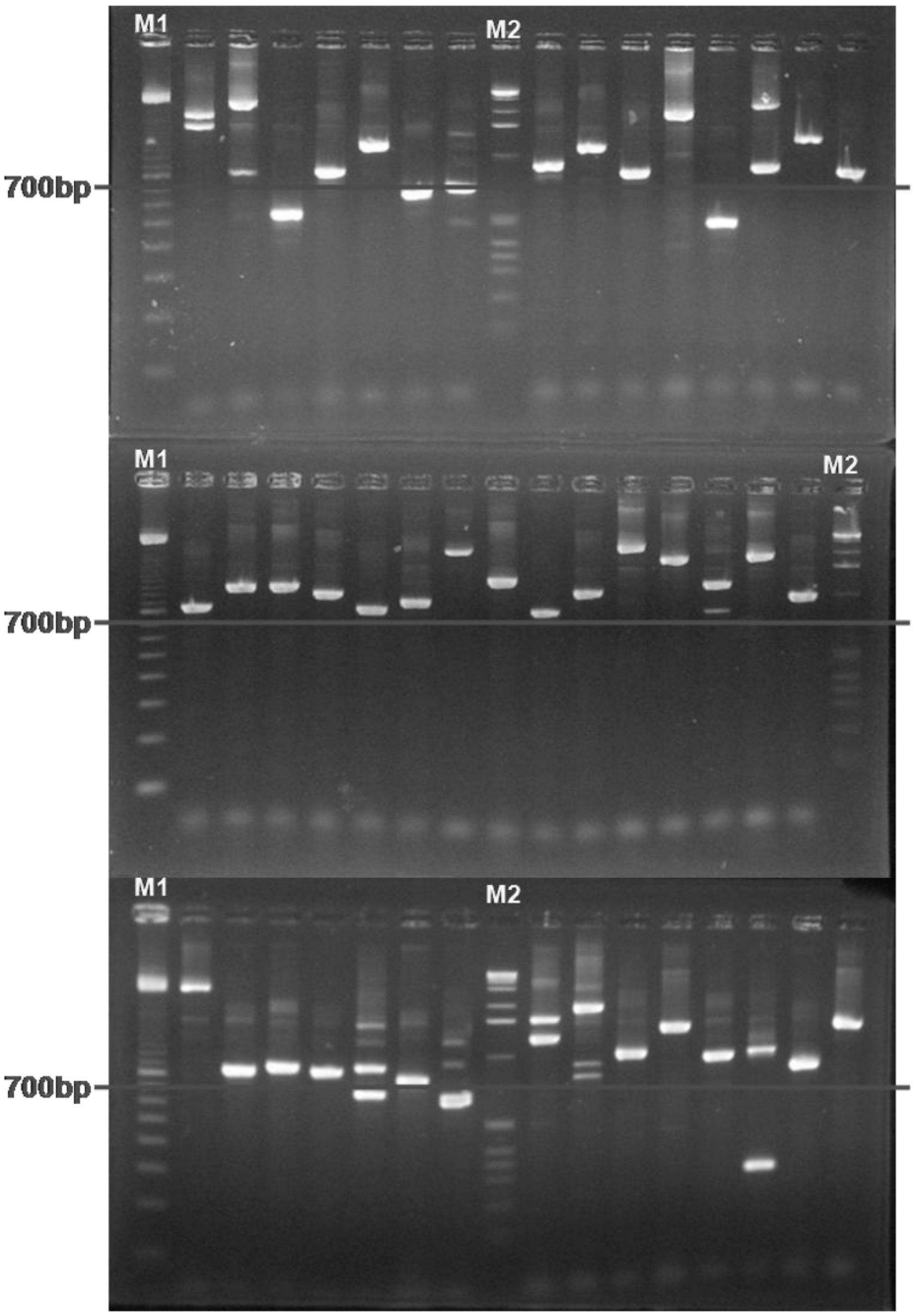


Fig. 4.2: Determination of percentage of recombinant clones in cDNA library. PCR amplification was performed by M13 forward and M13 reverse primers. Size of each clone was determined by 1.5% agarose gel electrophoresis. Band size > 700bp was regarded as clone containing insert. It was estimated that the percentage of recombinant clones was > 88%. (Lane M1: 100bp ladder; M2: 1kb ladder)

Table 4.1: Classification of clones from random cDNA library sequencing according to their biological functions.

Biological function[a]	No. of clones[b]
Cell division	1
Mitochondrial structural genes	3
Transport	6
Stress response	7
Cell signaling/ cell communication	7
Gene expression/ RNA synthesis	24
Metabolism	44
Cell structure/ cytoskeleton	71
Unclassified	61
Total	**224**

[a]Classification was performed manually.

[b]Only included clones with significant E-value (< 10^{-5}) in BLASTx homology search.

Table 4.2: Clones from random cDNA sequencing with high sequence homology (E-value < 10^{-5}) to genes related to lignocellulose degradation.

Clone ID[a]	Length[b]	Accession no.[c]	Annotation[d]	Organism	E-value
O3	746	BAG72079	Manganese peroxidase*	*Lentinula edodes*	7e-98
N84	255	BAG72081	Manganese peroxidase*	*Lentinula edodes*	1e-05
A120	681	YP_527744	Cellobiohydrolase A	*Saccharophagus degradans*	2e-10
N42	255	BAC20641	Cellobiose dehydrogenase	*Grifola frondosa*	5e-08
O22	875	EED55723	Endo-1,3(4)-β-glucanase	*Aspergillus fumigatus*	4e-31

[a]Clone ID from random cDNA library sequencing.

[b]Length of sequence of cDNA clones.

[c]Accession number of the matched transcript from NCBI (http://www.ncbi.nim.nih.gov/).

[d]Annotation of the mathched transcript from BLASTx.

** Updated in March 2009.*

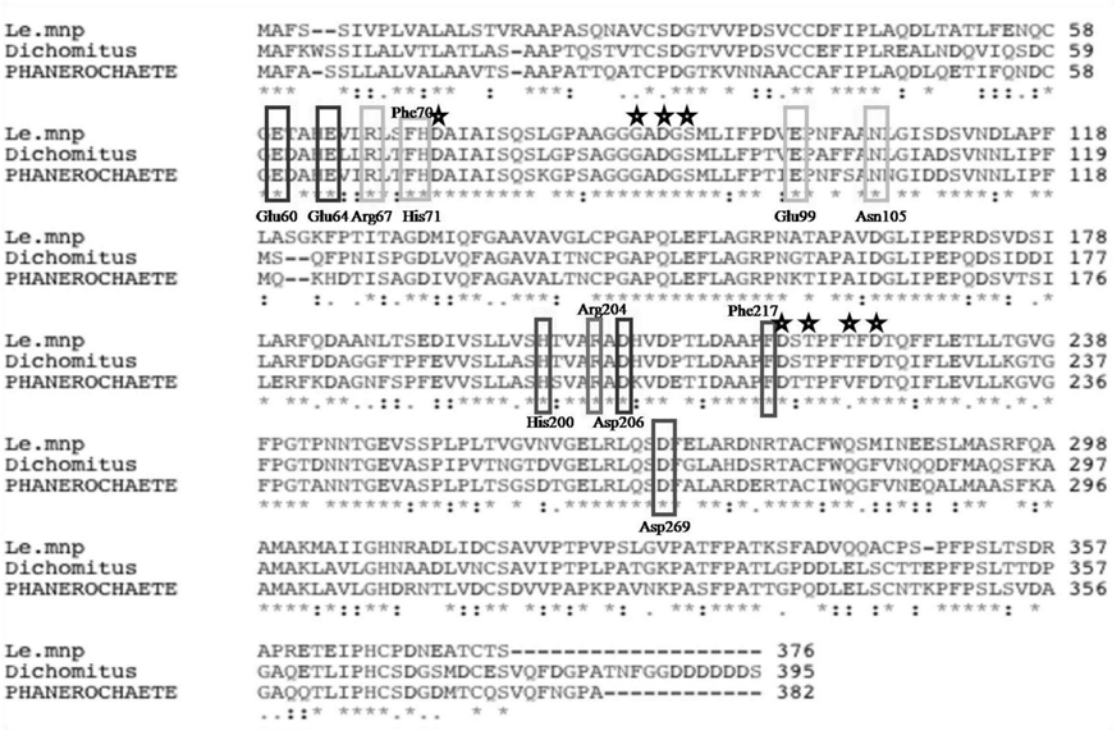


Fig. 4.3: Sequence alignment of *lemnp2* from *L. edodes* against other fungal *mnp*, *Dichomitus squalens* and *Phanerochaete chrysosporium*. Blue boxes indicated the conserved sites for Mn^{2+} binding; Green box indicated the site required for stabilization of Mn^{2+} binding; Orange boxes indicated the distal binding residues for heme-binding cavity; Red boxes indicated the proximal residues for heme-binding cavity; ★ indicated the conserved Ca^{2+} binding pockets. (Glu: glutamic acid; Arg: arginine; Phe: phenylalanine; His: histidine; Asn: asparagines; and Asp: aspartic acid)

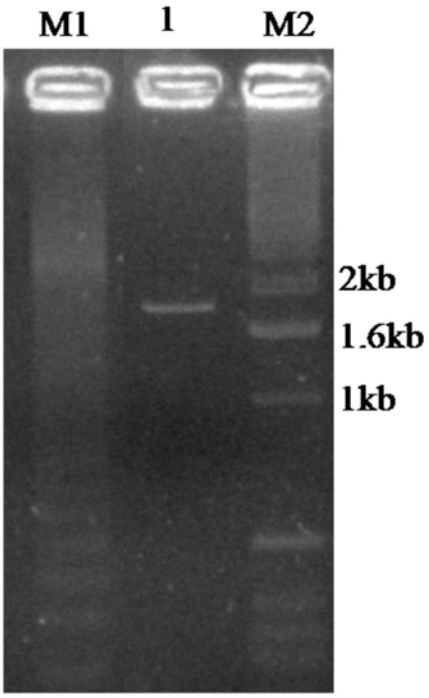


Fig. 4.4: PCR amplification of *lemnp2* genomic sequence. Size was estimated to be ~1.9kb. Sequencing was performed to identify introns and exons of genomic *lemnp2*. Lane M1: 100bp ladder; 1: *lemnp2* genomic fragment; M2: 1kb ladder.

```
gLemnp          ATGGCATTTTCGAGTATTGTCCCCCTTGTCGCTCTCGCTCTGTCCACCGTCAGAGCTGCT 60
cDNA            ATGGCATTTTCGAGTATTGTCCCCCTTGTCGCTCTCGCTCTGTCCACCGTCAGAGCTGCT 60
                ************************************************************

gLemnp          CCCGCTTCTCAGAATGCGGTTTGTTCTGACGGCACTGTTGTTCCCGACAGCGTTTGCTGT 120
cDNA            CCCGCTTCTCAGAATGCGGTTTGTTCTGACGGCACTGTTGTTCCCGACAGCGTTTGCTGT 120
                ************************************************************

gLemnp          GATTTTATTCCGGTGAGTTCGGTCCAATCATATTCGATATATTGTGTATATTGATGAACT 180
cDNA            GATTTTATTCCG------------------------------------------------ 132
                ************

gLemnp          TTCCAAGCTTGCCCAAGATCTTACCGCAACTCTATTCGAGAACCAGTGTGGCGAAACTGG 240
cDNA            -------CTTGCCCAAGATCTTACCGCAACTCTATTCGAGAACCAGTGTGGCGAAACTG- 184
                       ***************************************************

gLemnp          CAAGTGTTGGGAGCGTATGTAATCTTGACTGAAGCGCTAACTTTGATTGGTTTTAGCCCA 300
cDNA            ------------------------------------------------------CCCA 188
                                                                     ****

gLemnp          TGAGGTACTTCGTCTGAGCTTCCGTATGTTATAGCGATATCTTTGTCTTCAATTTCTTCT 360
cDNA            TGAGGTACTTCGTCTGAGCTTCC----------------------------------- 211
                ***********************

gLemnp          TACTGATTTGAATGTATCTCAGACGATGCTATCGCTATCTCCCAATCTCTTGGACCTGCG 420
cDNA            ----------------------ACGATGCTATCGCTATCTCCCAATCTCTTGGACCTGCG 249
                                       ************************************

gLemnp          GCGCTAAGATTATCACAGTCTTCATCTTACACTAGACTCACGATCCTTTCACGAAAGTGG 480
cDNA            GC--------------------------------------------------------TGG 254
                **                                                        ***

gLemnp          AGGAGGTGCTGACGGCTCTATGTTGATTTTCCCGGACGTCGAACCCAACTTCGCTGCCAA 540
cDNA            AGGAGGTGCTGACGGCTCTATGTTGATTTTCCCGGACGTCGAACCCAACTTCGCTGCCAA 314
                ************************************************************

gLemnp          CCTTGGTATCTCCGACAGTGTCAATGACCTCGCTCCATTCCTTGCCTCAGGAAAATTCCC 600
cDNA            CCTTGGTATCTCCGACAGTGTCAATGATCTCGCTCCATTCCTTGCCTCAGGAAAATTCCC 374
                ************************** ********************************

gLemnp          GACTATCACTGCTGGAGATATGATCCAGTTCGGTGCTGCCGTCGCTGTCGGCCTTTGCCC 660
cDNA            GACTATCACTGCTGGAGATATGATCCAGTTCGGTGCTGCCGTCGCTGTCGGCCTTTGCCC 434
                ************************************************************

gLemnp          TGTATGTGATCTCCCCGAAAACAAATTTTACTTTAGTTGATACTCTTCACTTTCTCAAGG 720
cDNA            TG---------------------------------------------------------- 436
                **

gLemnp          GCGCACCTCAGCTGGAGTTTTTGGCTGGCCGACCCAATGCCACTGCGCCTGCAGTTGACG 780
cDNA            GCGCACCTCAGCTGGAGTTTTTGGCTGGCCGACCCAATGCCACTGCGCCTGCAGTTGACG 496
                ************************************************************

gLemnp          GTTTGATCCCTGAACCTCAAGACTCGGTCGACTCAATCTTGGCTCGTTTCCAGGATGCGG 840
cDNA            GTTTGATCCCTGAACCTCGAGACTCGGTCGACTCAATCTTGGCTCGTTTCCAGGATGCGG 556
                ****************** ******************************************

gLemnp          TTACGCCGTTTATCATTTTACTACCACCTTTACTCATTTACTAATTCTTTCTTCCGTAAC 900
cDNA            ------------------------------------------------------------


gLemnp          AGGCAAACTTGACCTCTGAAGACATTGTCTCTCTGCTCGTATCGGTATGTCCGGCAATTA 960
cDNA            ---CAAACTTGACCTCTGAAGACATTGTCTCTCTGCTCGTATCG---------------- 597
                   ****************************************

gLemnp          CTTGGAACTGGCTCGGTCCATCTTAATATTATGATTTGTTATGCAGCACACAGGTCATTC 1020
cDNA            ---------------------------------------------CACACAG------- 604
                                                             *******

gLemnp          TTTCATGAATCATCGATAACTCACAGATCTCTCACCCGTTTTACTTTTCAGTCGCCCGAG 1080
cDNA            ------------------------------------------------TCGCCCGAG 613
                                                                 *********
```

```
gLemnp          CAGACCATGTCGACCCCACTCTGGATGCTGCGCCTTTCGACTCGGTAAGCAGACTCATCC 1140
cDNA            CAGACCATGTCGACCCCACTCTGGATGCTGCGCCTTTCGACTCG---------------- 657
                ****************************************

gLemnp          TCATTATTGACCGAAGTTCTAAAATTGACATCTATTATCAGACTCCATTCACCTTTGACA 1200
cDNA            ----------------------------------------ACTCCATTCACCTTTGACA 676
                                                        ******************

gLemnp          CACAGTTCTTCCTTGAAACTCTCTTGACTGGTGTCGGATTCCCGGGAACTCCTAGTATGT 1260
cDNA            CACAGTTCTTCCTTGAAACTCTCTTGACTGGTGTCGGATTCCCGGGAACTCCTA------ 730
                ********************************************************

gLemnp          ACAAATGCTTTTCGACCTCCTTCAAGAACTCTGAATTGGACTTGGTACAGACAACACTGG 1320
cDNA            ------------------------------------------------ACAACACTGG 740
                                                                 **********

gLemnp          TGAAGTGTCCTCCCCTCTTCCCCTCACTGTCGGTGTAAACGTTGGAGAGCTGCGACTTCA 1380
cDNA            TGAAGTGTCCTCCCCTCTTCCCCTCACTGTCGGTGTAAACGTTGGAGAGCTGCGACTTCA 800
                ************************************************************

gLemnp          ATCCGACTTTGAGCTCGCTCGTGACAACCGGACAGCCTGCTTCTGGCAAAGCATGATCAG 1440
cDNA            ATCCGACTTTGAGCTCGCTCGTGACAACCGGACAGCCTGCTTCTGGCAAAGCATGATCA- 859
                ************************************************************

gLemnp          TATGTGGAAAAGATAAGCAATCAATATAGCACACTGATCTCTTGTATCCTTCTGCAACTG 1500
cDNA            ------------------------------------------------------------


gLemnp          CAACAGACGAAGAATCCTTGATGGCATCGAGGTTCCAGGCTGCTATGGCCAAAATGGCTA 1560
cDNA            ------ACGAAGAATCCTTGATGGCATCGAGGTTCCAGGCTGCTATGGCCAAAATGGCTA 913
                      ******************************************************

gLemnp          TCATTGGACACAACCGTGCTGACTTGATTGACTGCTCCGCTGTTGTTCCGACTCCCGTTC 1620
cDNA            TCATTGGACACAACCGTGCTGACTTGATTGACTGCTCCGCTGTTGTTCCGACTCCCGTTC 973
                ************************************************************

gLemnp          CTTCTTTGGGCGTCCCCGCGACGTAAGTCCGGAATGACTATGAGATTGACGCTTTGATCT 1680
cDNA            CTTCTTTGGGCGTCCCCGCGAC---------------------------------- 995
                **********************

gLemnp          GACAAGTTTTGCAGTTTCCCGGCGACGAAGAGTTTCGCTGATGTTCAGCAAGCGTGCCCT 1740
cDNA            -------------TTTCCCGGCGACGAAGAGTTTCGCTGATGTTCAGCAAGCGTGCCCT 1041
                             *********************************************

gLemnp          TCGCCCTTCCCGAGTCTCACCTCCGACCGTGAGTGTTTTCTACACACCTTTCACCTTACC 1800
cDNA            TCGCCCTTCCCGAGTCTCACCTCCGACCGTG---------------------------- 1072
                ******************************

gLemnp          ACTCAATACAATTAACCCGATTTCTAGGTGCTCCCAGGGAGACTGAGATTCCTACACTGC 1860
cDNA            ----------------------------CTCCCAGGGAGACTGAGATTCCT-CACTGC 1101
                                            ********************** ******

gLemnp          CCCGACAATGAAGCCACTTGCACTTCTTAA 1890
cDNA            CCCGACAACGAAGCCACTTGCACTTCTTAA 1131
                ******** **********************
```

Fig. 4.5: Sequence alignment of genomic and cDNA sequences of *lemnp2* using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Thirteen introns were predicted along genomic *lemnp2*.

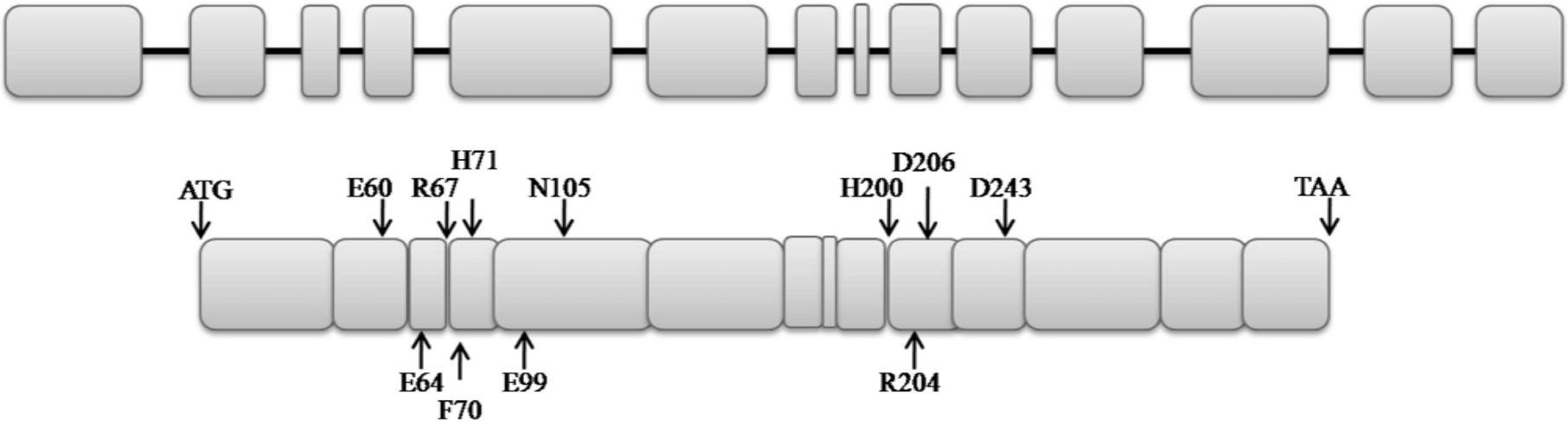


Fig. 4.6: Schematic diagram showing structures of genomic and cDNA sequences of *lemnp2*. Sites indicated are conserved for Mn^{2+} ion binding and heme binding. (E: glutamic acid; R: arginine; F: phenylalanine; H: histidine; N: asparagines; and D: aspartic acid)

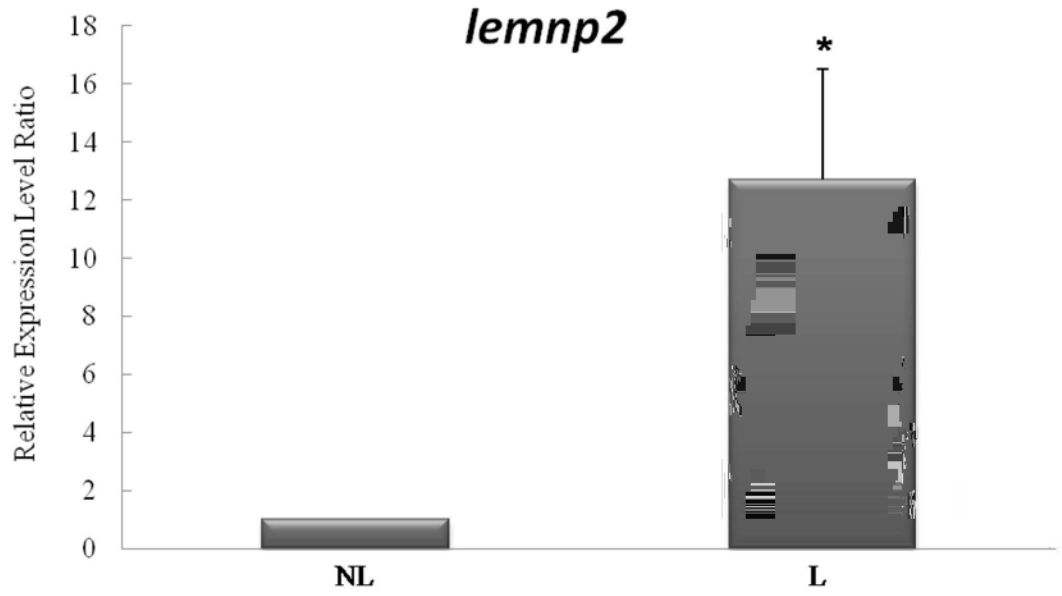


Fig. 4.7: Quantitative RT-PCR of *lemnp2* in mycelia grown in non-lignocellulose (NL) and lignocellulose (L). The expression level of *lemnp2* in mycelium grown in L was >10 fold higher than that grown in NL. Data represented means and standard deviations of three independent experiments. Statistical significance was indicated by *$p<0.05$.

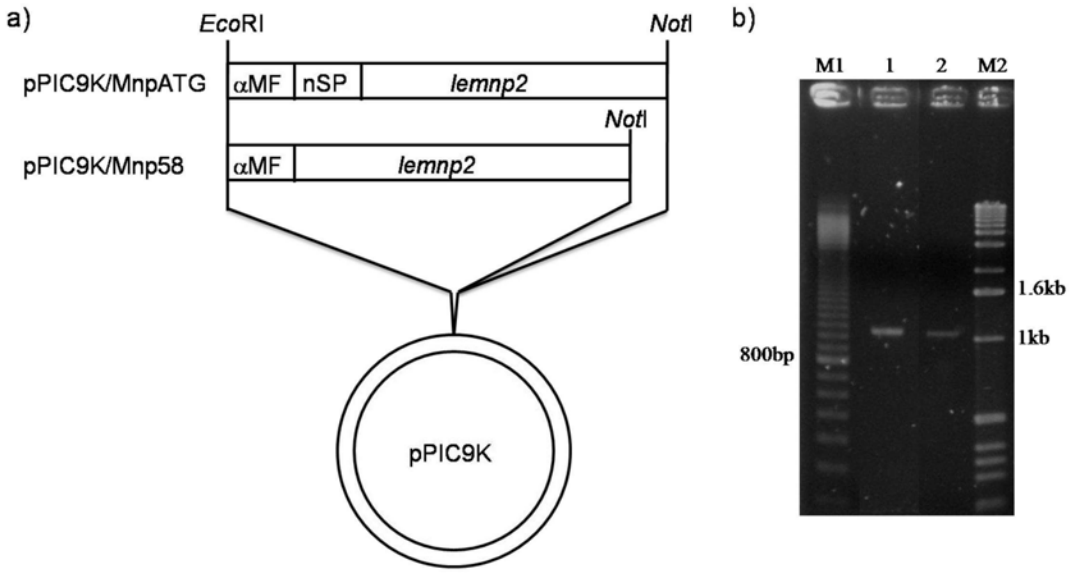


Fig. 4.8: a) Schematic diagram showing the two different expression cassettes. pPIC9K/MnpATG contained full length cDNA of *lemnp2*, including native fungal signal peptide (nSP), following the α-mating factor (αMF) derived from *Saccharomyces cerevisiae*. Native signal peptide was eliminated in pPIC9K/Mnp58, so only αMF was present. All *lemnp2* cDNA were cloned into pPIC9K vector, downstream of AOX promoter through *Eco*RI and *Not*I sites. b) PCR amplification of *lemnp2* cDNA. Lane M1: 100bp ladder; 1: full length *lemnp2* cDNA; 2: *lemnp2* cDNA with deletion of nSP; M2: 1kb ladder.

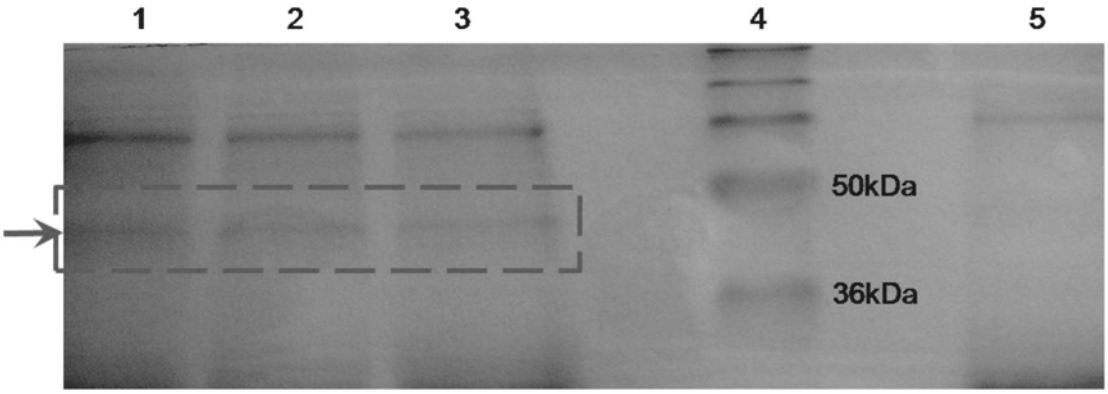


Fig. 4.9: SDS-PAGE showing expression of recombinant MnP from the culture supernatant of *P. pastoris* strain KM71/pPIC9K/MnpATG#1 (Lane 1), KM71/pPIC9K/MnpATG#2 (Lane 2), KM71/pPIC9K/MnpATG#3 (Lane 3) and KM71/pPIC9K as control (Lane 5). The media were supplemented with 5g/L hemoglobin. The molecular weight of rMnP was shown to be ~ 45kDa. (Lane 4: Molecular marker)

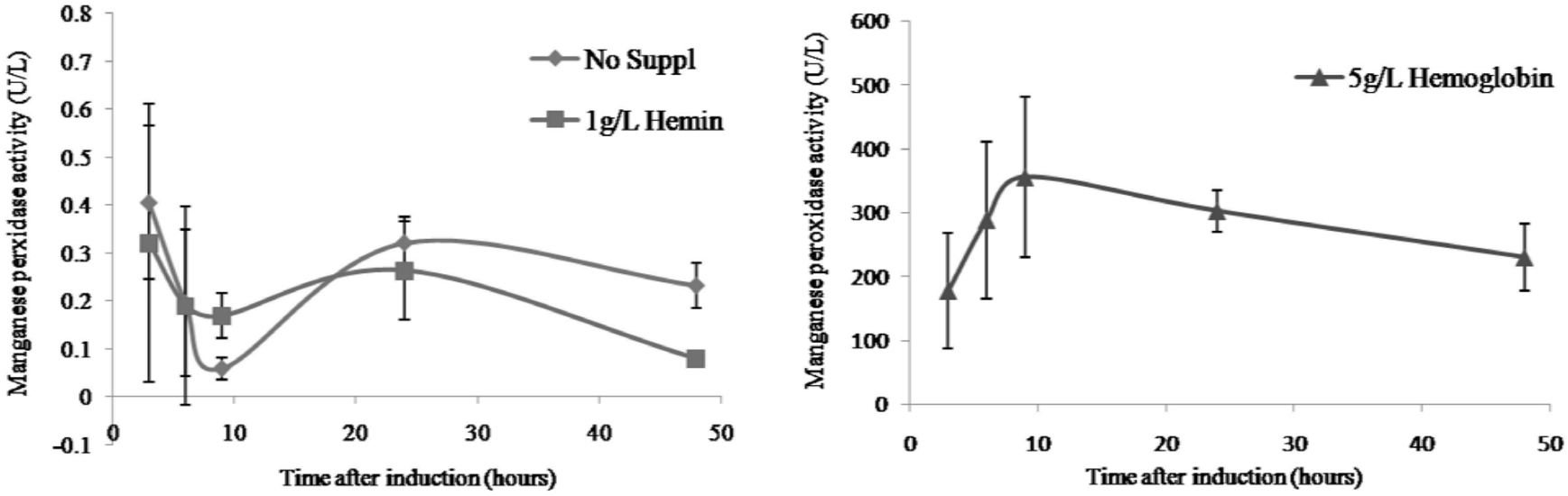


Fig. 4.10: Activity assays of rMnP expressing strain KM71/pPIC9K/MnpATG#1. Two heme supplements were tested, 1g/L hemin and 5g/L hemoglobin. The highest MnP activity (350U/L) was observed at 9 hours after methanol induction in medium with hemoglobin supplementation. Data represent means and standard deviations of three independent experiments.

4.4 Discussion

In the present study, a gene coding for manganese peroxidase in *L .edodes*, *lemnp2*, was isolated from a mycelia cDNA library and its genomic sequence was identified. The transcript level of *lemnp2* in lignocelluloses and non-lignocellulose grown mycelia was studied. In addition, heterologous expression of LeMnP2 was performed using yeast *P. pastoris*.

4.4.1 Random sequencing of cDNA library

A large portion of the clones identified (65 out of 224) was hydrophobin which is abundantly expressed during vegetative mycelia growth (Ng et al., 2000; Suizu et al., 2008). This frequent occurrence of hydrophobin in the normalized cDNA library indicates that the normalization method could not effectively reduce the copy number of abundantly expressed genes. However, it might, to certain extent, enrich some rarely expressed genes, e.g. *lemnp2*, which has never been detected in our SAGE libraries (Chum, 2006).

4.4.2 Genomic and cDNA sequences of *lemnp2*

According to Martinez (2002) for the classification of lignin-degrading peroxidases based on amino acid sequence (refer to Fig. 4.3), LeMnP2 was categorized as group I peroxidase as it contained typical Mn^{2+} ion binding site (E_{60}, E_{64} and D_{206}) but no tryptophan near the proximal histidine (H_{200}), which is conserved for lignin peroxidase and versatile peroxidase. R_{204} was identified in LeMnP2 and it is known to be important for stabilization of Mn^{2+} binding (Martinez, 2002). Distal (R_{67}, F_{70}, H_{71}, E_{99} and N_{105}) and proximal (H_{200}, F_{217} and D_{269}) residues in heme-binding cavity could also be identified, in which R_{67} and H_{71} are involved in the binding of peroxide (Martinez, 2002). In addition, all amino acid residues involved in the two Ca^{2+} ion binding pockets (proximal: D_{218}, T_{220}, T_{223} and D_{225}; distal: D_{72}, G_{87}, D_{89} and S_{91}) were conserved, except

for the proximal S_{201}, but it may vary among fungal MnPs (Martinez, 2002).

There were 13 introns identified in genomic *lemnp2*, which was agreed with the finding of Sakamoto et al. (2009). They also found that there was a metal response element (MRE) motif in the promoter region of *lemnp2*. MRE has been observed in other fungal *mnp* suggesting that it is a possible regulatory component for the transcription of MnP encoding gene in fungi (Martinez, 2002).

Nitrogen depletion has also been suggested to trigger ligninolytic activity, which is a secondary metabolic function in many of the white-rot fungi (Martinez, 2002). Buswell and co-workers (1995) proposed that nitrogen deficiency would be a signal for the production of MnP in *L. edodes*. In high nitrogen condition, MnP production was suppressed (Buswell et al., 1995). In other white-rot fungi, including *Phanerochaete chrysosporium* and *Pleurotus ostreatus*, high nitrogen content in media would also down-regulate MnP at transcriptional level (Gold and Alic, 1993; Kamitsuji et al., 2005).

4.4.3 Analysis of transcript levels of *lemnp2* in mycelia grown in different media

The transcript level of *lemnp2* in mycelium grown in L was significantly higher than that grown in NL. *Lemnp2* was the dominant transcript responsible for MnP activity throughout the cultivation process of *L. edodes* mycelia in sawdust media (Sakamoto et al. 2009). It is reasonable to postulate the contributory role of LeMnP2 in lignin degradation.

4.4.4 Expression of rMnP in *P. pastoris*

The measurement of MnP activity assay using 2, 6-DMP proved the recombinant LeMnP2 produced in *P. pastoris* was a functionally active enzyme having the typical MnP properties. The present finding also demonstrated that

P. pastoris could effectively process rMnP with proper protein folding and other posttranslational modifications, such as glycosylation.

Strain KM71/pPIC9K/MnpATG, containing 2 signal peptides (αMF and nSP) in the expression cassette, could produce active rMnP. Previous studies showed that *P. pastoris* was able to utilize not only αMF (Jiang et al., 2008a) but also signal sequence from other fungal protein (Gu et al., 2003). In the present study, the successful production of active rMnP suggested *P. pastoris* might be capable to identify and correctly cleave the signal peptide originated from *L. edodes*.

Hyperglycosylation has been one of the major feedbacks for the recombinant protein produced from yeast, especially *Saccharomyces cerevisiae* (Porro and Mattanovich, 2004). However, the molecular mass of rMnP produced by strain KM71/pPIC9K/MnpATG was estimated to be 45kDa, which was similar to the mass of native LeMnP2 (44.8kDa) (Sakamoto et al., 2009), suggesting that rMnP produced was not hyperglycosylated.

4.4.5 Effect of different heme-supplementations

With 5g/L hemoglobin supplementation, the activity of rMnP produced by strain KM71/pPICK9K/MnpATG#1 reached a maximum of 350U/L, which was much higher than that without heme-supplementation or with 1g/L hemin. Conesa and co-workers (2000) demonstrated that with hemoglobin and hemin supplementation, the activity of rMnP produced using *A. niger* increased by 10 fold and 7 fold, respectively, when compared to non-supplemented medium. Heme availability was known to be an important factor for the production of heme-containing proteins (Conesa et al., 2000).

Hemoglobin supplementation resulted in significantly higher rMnP activity than the addition of hemin. As suggested by Conesa et al. (2000), the added

hemoglobin might not only supply heme to rMnP, but might function as a kind of protein excess that protect rMnP from proteolytic degradation.

Recombinant MnP activity produced by strain KM71/pPIC9K/MnpATG#1 in the present study (350U/L) was higher than that reported by Gu et al. (2003) with hemin supplementation (120U/L) but it was only comparable to that in *A. oryzae* (330U/L). However, the production of active rMnP can be further enhanced by up-scale fermentation, as demonstrated by Jiang et al. (2008) that fed-batch cultivation of *P. pastoris* could increase rMnP activity from 200U/L to 2,500U/L.

4.5 Conclusion

In the present study, a MnP encoding gene, *lemnp2*, in *L. edodes* was identified and characterized. The cDNA of lemnp2 encoded a polypeptide of 376 amino acids with all of the conserved binding sites for Mn^{2+}, heme and Ca^{2+}. The expression level of *lemnp2* in lignocellulose-grown mycelium was significantly higher that in non-lignocellulose-grown mycelium. Recombinant LeMnP2 was successfully expressed using *P. pastoris* with maximum rMnP activity of 350U/L in the presence of 5g/L hemoglobin supplementation. To our knowledge, it is the first report of heterologous expression of MnP from *L. edodes* in yeast system, which may benefit to the industrial applications of MnP.

Chapter 5: Characterization and heterologous expression of *Lentinula edodes* laccase, *lcc1*, in yeast *Kluyveromyces lactis*

5.1 Introduction

Laccase, a multicopper-containing phenoloxidase, is found in a wide range of fungi, including *Lentinula edodes* (Zhao and Kwan, 1999; Sakamoto et al., 2008; Yano et al., 2009). As a member of ligninolytic enzymes, laccases catalyze the single electron oxidation of phenolic compounds, a process accompanied with a reduction of molecular oxygen (Leonowicz et al., 2001). Laccase contains a catalytic center with 4 copper atoms. Type 1 (blue copper) center is the site where oxidation of substrate takes place while Type 2 and Type 3 coppers form a trinuclear cluster where reduction of oxygen takes place (Riva, 2006).

Owing to its capability to degrade aromatic compounds, laccase has a high industrial value and can be applied in food, textile and paper manufacturing processes. (Couto and Herrera, 2006). There are increasing efforts to heterologously express of laccases at bioreactor scales in order to establish stable and low-cost production pipelines (Guo et al., 2005; Jolivalt et al., 2005; Bohlin et al., 2006; Couto and Toca-Herrera, 2007; Bleve et al., 2008; Faraco et al., 2008; Sakamoto et al., 2008).

The first laccase encoding gene in *L. edodes* was cloned by Zhao and Kwan (1999). Several laccase genes were subsequently identified and two of them (*lcc1* and *lcc4*) were expressed heterologously in tobacco BY-2 cells (Sakamoto et al., 2008) and *Aspergillus oryzae* (Yano et al., 2009). However, the long cultivation time required for producing the recombinant proteins in a high yield is the major limitation for their industrial applications (Porro and

Mattanovich, 2004). Comparing to the above mentioned expression systems, yeast is an advantageous host for a large scale production of recombinant proteins. In addition to a high growth rate, *Kluyveromyces lactis* secretes the recombinant proteins and largely eases the down-stream purification process (Porro and Mattanovich, 2004). Therefore, *K. lactis* is commonly used in industrial scales (van Ooyen et al., 2006).

In the present study, I categorized the laccase-coding genes in *L. edodes* into various groups based on their protein sequence homology. I examined the expression levels of one representative gene from each group of laccases in mycelia grown in lignocellulose or non-lignocellulose media in order to compare the roles of these laccases in lignin degradation. Finally, I tried heterologous expression of *lcc1* in *K. lactis* to understand its enzymatic properties. A better understanding on the expression and enzymatic properties may benefit the industrial applications of laccases.

5.2 Materials and Methods

5.2.1 Strain cultivation

Lentinula edodes dikaryotic strain L54 was used. Mycelium was maintained in PDA (Potato Dextrose Agar, Difco), from which an inocula (about 1cm^2 mycelia cubes) were prepared. For cDNA library construction, mycelia were cultivated on artificial logs (60% dry sawdust, 30% dry wood chips and 10% dry wheat bran) at 25°C in darkness for one month to allow colonization of mycelia on the whole log. For quantitative RT-PCR, mycelia were inoculated on 20g of lignocellulose medium (60% dry sawdust, 30% dry wood chips and 10% dry wheat bran with 100% moisture) and non-lignocellulose medium [PDB (Potato Dextrose Broth, Difco)], respectively, in 90mm-diameter glass pots for two weeks.

5.2.2 Alignment of laccase sequences

Fifteen amino acid sequences of *L. edodes* laccases were obtained from NCBI. After eliminating those partial sequences, 10 full length laccases encoding genes were selected for sequence alignment by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

5.2.3 Quantitative RT-PCR

Total RNA was extracted from mycelia using TRI Reagent® (Molecular Research Center, Inc.). DNase I treatment was performed to eliminate contamination of genomic DNA. First strand cDNA was synthesized using TaqMan® Reverse Transcription Reagents (Roche, Applied Biosystems) according to the manufacturer's instructions. Quantitative RT-PCR reaction was performed on Bio-Rad MiniOpticon™ real time system (Bio-Rad). 2μl of 10X diluted first strand cDNA was mixed with 0.45μl of each 10μM of gene specific primers (Table 5.1), 10μl of 2X iQ™ SYBR® Green Supermix (Bio-Rad)

and brought up to 20µl with nuclease-free water according to manufacturer's manual. The reaction for each primer set was performed in duplicate and no-template-control (NTC) was included. The PCR program was 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, 60°C for 45 seconds and 72°C for 15 seconds. Melting curve analysis was performed by increasing the temperature from 50°C to 90°C. Data analysis was performed on Opticon Monitor™ Version 3.0 (Bio-Rad). The experiments were repeated for three times with independent biological samples.

5.2.4 Cloning of native laccase

Total RNA was extracted as described above. For pKLAC1/Lcc1 contruct, full length cDNA of *lcc1* were amplified using primers Lcc_XhoI_47U (5'-TTTCTCGAGAAAAGAATGTTTTACTTCTCATCTTTC) and Lcc_KpnI_1621L (5'-TTTGGTACCTAGACTGTATTTTGGAACTTGT-3'), based on the *lcc1* sequence (accession no.: AB055157) from NCBI. After digestion with *Xho*I and *Kpn*I, the PCR products were ligated to pKLAC1 (New England Biolabs). The ligated mixture was introduced into *E. coli* TOP10 (Invitrogen) by electroporation. Transformants were selected on LB medium containing 100µg/ml ampicillin. Positive clones were screened by PCR using LAC4 promoter sequencing primer (5'-GCGGATAACAAGCTCAAC-3') and pKLAC1 reverse sequencing primers (5'-TTATCGCACAAGACAATC-3').

5.2.5 Transformation of *Kluyveromyces lactis*

K. lactis transformation was performed by *K. lactis* Protein Expression Kit, including competent cell GG799 (New England Biolabs) according to manufacturer's instructions. Expression vectors were purified by Wizard®Plus Minipreps kit (Promega) and linearized at *Sac*II sites. Plasmids from all positive clones were mixed to generate a library pool of pKLAC1/Lcc1. Four

micrograms of the linearized plasmid from the pool was used in transformation. *K. lactis* GG799 was also transformed with linearized pKLAC1 with no insert as control. Transformants were selected on YCB medium containing 5mM acetamide (supplied from the kit). Integration of expression cassette pKLAC1/Lcc1 into *K. lactis* GG799's genome was verified by PCR with integration primer 1 and 2 supplied from the kit. Positive transformants with active enzyme secretion was screened on YPGal agar plates (1% yeast extract, 2% peptone, 2% galactose and 2% agar) containing 0.5mM ABTS and supplemented with different concentrations of $CuSO_4$ (0.1mM, 0.2mM and 0.3mM). The plates were cultivated at 30°C until blue/ purple color was detected.

5.2.6 RNA extraction and transcription analysis of *lcc1* in *K. lactis*

Total RNA extraction from *K. lactis* was prepared by making spheroplasts. Single colony was inoculated in 30ml YPGal medium and cultivated overnight at 30°C with shaking. Cells were collected by centrifugation at 1,500 x g for 5 mins. One milliliter of sodium phosphate/ sorbitol buffer (1.2M sorbitol, 10mM sodium phosphate, pH7.2) was added to resuspend cell pellet. Then cells were collected by centrifugation at 24,000 x g for 3mins, pellet was resuspend in 1ml sodium phosphate/ sorbitol buffer. Three microliter of β-mercaptoethanol and 320μl of 1mg/ml lyticase (Sigma-Aldrich) were added to the cell, followed by incubation in shaker at 30°C for 15mins to make spheroplasts. Then the spheroplastss were collected by centrifugation at 12,000 x g for 3mins and washed by 500μl of sodium phosphate/ sorbitol buffer twice. Afterwards, the spheroplasts were resuspended in 800μl of TRI Reagent® (Molecular Research Center, Inc.) and pre-chilled glass beads were added. The spheroplasts were broken by vortexing for 30sec, followed by 30sec on ice,

and repeated for 10 cycles. The broken cells were chilled on ice for 2mins and then centrifuged for 12,000 x g for 10mins at 4°C. Equal volume of chloroform was added to the supernatant and mixed thoroughly. The mixture was incubated at room temperature for 15mins before centrifuging at 12,000 x g at 4°C. Upper aqueous layer was transferred into new tube and were mixed with 250μl of isopropanol and 250μl of 0.8M sodium citrate/ 1.2M sodium chloride solution. The mixture was incubated at room temperature for 10mins and total RNA was pelleted by centrifugation at 12,000 x g for 8mins. After washing with 70% ethanol and air-dry, the pellet was resuspended in 50μl RNase-free water. The transcription of *lcc1* was verified by RT-PCR using *lcc1* specific primers, Lcc_XhoI_47U and Lcc_KpnI_1621L.

5.2.7 Codon optimization of *lcc1* for heterologous expression in *K. lactis*

Condon optimization and gene synthesis were performed by GenScript Corporation (Piscataway, US). The gene was then subcloned into pKLAC1 via *Xho*I and *Kpn*I restriction sites to create construct pKLAC1/SynLcc.

Table 5.1: Primer list for quantitative RT-PCR of laccase genes.

Laccase	Upper primer	Sequence (5' to 3')	Lower primer	Sequence (5' to 3')
Lcc1	Lcc1-210U	CACTAACGGAACG ATGCTCTT	Lcc1-400L	TCCACGCAATCCATC GCAATA
Lac3VT	Lac3VT-303U	CTACTCACAGACC CAACGATG	Lac3VT-408L	TGAATGGTTTGTTGC GATAGG
Lac1B	Lac1B-102U	GAATTTATTCATCG TCAACAAG	Lac1B-201L	GCCCTTATTTCCTGA GATTAGT

5.3 Results

5.3.1 Sequence alignment of laccases

Ten selected laccase amino acid sequences shared high similarity. Based on the sequence alignment, I categorized the sequences into 3 distinct groups: 2 in Group 1; 6 in Group 2; and 2 in Group 3 (Fig. 5.1). Four conserved copper binding sites were found in all laccase sequences.

5.3.2 Analysis on transcription levels of laccases in mycelia grown in different media

Since the cDNA sequences of genes within each laccase group are highly similar, I randomly selected one member from each group and compared their expressions in lignocellulose-grown and non-lignocellulose-grown mycelia: Group 1 (*lcc1*; accession no.: BAB84354); Group 2 (*lac1B*; accession no.: AAF13038) and Group 3 (*lac3VT*; accession no.: AAT99291). From the quantitative RT-PCR results, I found that 1) *lcc1* differentially expressed (~2-fold) in non-lignocellulose-grown mycelium; 2) *lac1B* was differentially expressed (>40-fold) in lignocellulose-grown mycelium; 3) *lac3VT* showed similar expressions in mycelia grown in both media (Fig. 5.2).

5.3.3 Vector construction, transformation and screening of *K. lactis* transformants

The full length cDNA of *lcc1* was amplified with a size of ~1.6kb (Fig. 5.3a) and cloned into pKLAC1 vector to construct pKLAC1/Lcc1 expression cassette (Fig. 5.3b).

Over 80% *K. lactis* transformants contained pKLAC1/Lcc1 expression cassette integrated in the genome as indicated by 1.9kb PCR products amplified from primer 1 and primer 2 (Fig. 5.4). I screened the enzyme-secreting strain on YPGal plates containing 0.5mM ABTS and $CuSO_4$ in different concentrations.

No laccase activity was detected on the plates after screening for about 80 transformants. Purple color development was observed when growing *L. edodes* L54 dikaryotic mycelium on PDA plates with 0.5mM ABTS, serving as a positive control for plate assay.

From RT-PCR of *K. lactis* RNA, I verified *lcc1* was transcribed (Fig. 5.5).

For the codon optimized construct pKLAC1/Lcc1, again, no detectable laccase activity was observed on plate assays.

```
gi|18146856|dbj|BAB83132.1|      ------MLPFVSLLAAELVCLVYGAEVIRDT---TTYGQTVS--ENLFIV 39
gi|18461104|dbj|BAB84355.1|      ------MLPFVSLLAAELVCLVYGAEVIRDT---TTYGQTVS--ENLFIV 39
gi|6466812|gb|AAF13037.1|AF153   ------MLPFVYLLAAELVCLVYGAEVIRDT---TTYGQTVS--ENLFIV 39
gi|6466814|gb|AAF13038.1|AF153   ------MLPFVSLLAAELVCLVYGAEVIRDT---TTYGQTVS--ENLFIV 39
gi|51242697|gb|AAT99286.1|       ------MLPFVYLLAAELVCLVYGAEVIRDT---TTYGQTVS--ENLFIV 39
gi|51242699|gb|AAT99287.1|       ------MLPFVYLLAAELVCLVYGAEVIRDT---TTYGQTVS--ENLFIV 39
gi|18461106|dbj|BAB84356.1|      MFLINVVFGFWLLCVPTSLRLVSGVEIVKSHPRDKIWHEKIDHHDQLFIV 50
gi|51242707|gb|AAT99291.1|       MFLINVVFGFWLLCVPTSLRLVSGVEIVKSHPRDNIWHEKTDHHDQLFIV 50
gi|18461102|dbj|BAB84354.1|      ---------MFYFSSFLLLGPIGGALAAIGP---------VT---DLHIV 29
gi|51242705|gb|AAT99290.1|       ---------MFYFSSFLLLGPIGGALAAIGP---------VT---DLHIV 29
                                          :  :     :  : *.    .              :*.**

gi|18146856|dbj|BAB83132.1|      NKQLAPDGFQRSTVLAGLTPSTGSFPGPLISGNKGVRFNLNVTDQLTDPS 89
gi|18461104|dbj|BAB84355.1|      NKQLAPDGFQRSTVLAGLTPSTGSFPGPLISGNKGVRFNLNVTDQLTDPS 89
gi|6466812|gb|AAF13037.1|AF153   NKQLAPDGFQRSTVLAGLTPSTGSFPGPLISGNKGVRFNLNVTDQLTDPS 89
gi|6466814|gb|AAF13038.1|AF153   NKQLAPDGFQRSTVLAGLTPSTGSFPGPLISGNKGVRFNLNVTDQLTDPS 89
gi|51242697|gb|AAT99286.1|       NKQLAPDGFQRSTVLAGLTPSTGSFPGPLISGNKGVRFNLNVTDQLTDPS 89
gi|51242699|gb|AAT99287.1|       NKQLAPDGFQRSTVLAGLTPSTGSFPGPLISGNKGVRFNLNVTDQLTDPS 89
gi|18461106|dbj|BAB84356.1|      NKDIAPDGFQRSTVLAGRTPQDASFPGPLITGRKGDRFTINVTDLLTDPT 100
gi|51242707|gb|AAT99291.1|       NKDIAPDGFQRSTVLAGRTPQDASFPGPLIAGRKGDRFTINVTDLLTDPT 100
gi|18461102|dbj|BAB84354.1|      NSFIQPDGFNRSGVLA-----EGVFPGPLITGNKGDNFQINVIDELTNGT 74
gi|51242705|gb|AAT99290.1|       NSFIQPDGFNRSGVLA-----EGVFPGPLITGNKGDNFQINVIDELTNGT 74
                                 *. : ****:** ***      . ******:*.** .* :** * **: :

gi|18146856|dbj|BAB83132.1|      MVRSTTVHWHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTF 139
gi|18461104|dbj|BAB84355.1|      MVRSTTVHWHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTF 139
gi|6466812|gb|AAF13037.1|AF153   MVRSTTVHWHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTF 139
gi|6466814|gb|AAF13038.1|AF153   MVRSTTVHWHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTF 139
gi|51242697|gb|AAT99286.1|       MVRSTTVHWHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTF 139
gi|51242699|gb|AAT99287.1|       MVRSTTVHWHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTF 139
gi|18461106|dbj|BAB84356.1|      MDRSTSIHWHGLFQKTTNYADGVAFVSQCPIATNHSFLYDFGVPDQAGTF 150
gi|51242707|gb|AAT99291.1|       MDRSTSIHWHGLSQKTTNYADGVAFVSQCPIATNHSFLYDFGVPDQAGTL 150
gi|18461102|dbj|BAB84354.1|      MLLSTSIHWHGLFQKTTNWADGPAFVNQCPIAANDSFLYNFNVPDQAGTF 124
gi|51242705|gb|AAT99290.1|       MLLSTSIHWHGLFQKTTNWADGPAFVNQCPIAANDSFLYNFNVPDQAGTF 124
                                 *  **::***** *****:*** ***.*****:*.****:* *******:
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gi|18146856|dbj|BAB83132.1|      WYHSHISVQYCDGLRGPLVIYDPQDPQAYLYDVDDETTVLTIADWFHNTS 189
gi|18461104|dbj|BAB84355.1|      WYHSHISVQYCDGLRGPLVIYDPQDPQAYLYDVDDETTVLTIADWFHNTS 189
gi|6466812|gb|AAF13037.1|AF153   WYHSHISVQYCDGLRGPLVIYDPQDPQAYLYDVDDETTVLTIAD-----C 184
gi|6466814|gb|AAF13038.1|AF153   WYHSHISVQYCDGLRGPLVIYDPQDPQAYLYDVDDETTVLTIAD-----C 184
gi|51242697|gb|AAT99286.1|       WYHSHISVQYCDGLRGPLVIYDPQDPQAYLYDVDDGTTVLTIADWFHNTS 189
gi|51242699|gb|AAT99287.1|       WYHSHISVQYCDGLRGPLVIYDPQDPQAYLYDVDDETTVLTIADWFHNTS 189
gi|18461106|dbj|BAB84356.1|      WYHSHLSVQYGDGLRGPIVIYDPEDPHQDLYDVDDESTIITIADWFHLTS 200
gi|51242707|gb|AAT99291.1|       WYHSHLSVQYGDGLRGPIVIYDPEDPHQDLYDVDDESTIITIADWFHLTS 200
gi|18461102|dbj|BAB84354.1|      WYHSHLSTQYCDGLRGPLVVYDPQDPYADLYDVDDDSTVITLADWYH--- 171
gi|51242705|gb|AAT99290.1|       WYHSHLSTQYCDGLRGPLVVYDPQDPYVDLYDVDDDSTVITLADWYH--- 171
                                 *****:*.** ******:*:***:**   ****** :*::*:**

gi|18146856|dbj|BAB83132.1|      TELIAAAVAPPADATLINGKGRYLGGPA-VPLAVINVQQGKRYRFRMISI 238
gi|18461104|dbj|BAB84355.1|      TELIAAAVAPPADATLINGKGRYLGGPA-VPLAVINVQQGKRYRFRMISI 238
gi|6466812|gb|AAF13037.1|AF153   TELIAAAVAPLADATLINGKGRYLGGPA-VPLAVINVQQGKRYRFRMISI 233
gi|6466814|gb|AAF13038.1|AF153   TELIAAAVAPPADATLINGKGRYLGGPA-VPLAVINVQQGKRYRFRMISI 233
gi|51242697|gb|AAT99286.1|       TELIAAAVAPLADATLINGKGRYLGGPA-VPLAVINVQQGKRYRFRMISI 238
gi|51242699|gb|AAT99287.1|       TELIAAAVAPPADATLINGKGRYLGGPA-VPLAVINVQQGKRYRFRMISI 238
gi|18461106|dbj|BAB84356.1|      LQLLAALVVPNADATLINGNGRYVGGPD-TPLTVINVEHGKRYRFRLIDM 249
gi|51242707|gb|AAT99291.1|       LQLLAALVVPNADATLINGNGRYVGGPD-TPLTVINVEHGKRYRFRLIDM 249
gi|18461102|dbj|BAB84354.1|      VPAPQAGAVPTSDATLINGLGRSVNGPADAPFAVVNVVQGSRYRFRLVSI 221
gi|51242705|gb|AAT99290.1|       VPAPQAGAVPTSDATLINGLGRSVNGPADAPFAVVNVVQGSRYRFRLVSI 221
                                      * ..* :******* ** :.**  .*::*:** :*.*****::.:

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gi|6466812|gb|AAF13037.1|AF153   ACDANHNFSIDGHRLTVIEVDGENHEPATVDNIQIFPGQRYSFVLDATQP 283
gi|6466814|gb|AAF13038.1|AF153   ACDAYHNFSIDGHRLTVIEVDGENHEPATVDNIQIFPGQRYSFVLTATQP 283
gi|51242697|gb|AAT99286.1|       ACDANHNFSIDGHRLTVIEVDGENHEPATVDNIQIFPGQRYSFVLDATQP 288
gi|51242699|gb|AAT99287.1|       ACDANHNFSIDGHRLTVIEVDGENHEPATVDNIQIFPGQRYSFVLNATQP 288
gi|18461106|dbj|BAB84356.1|      SFDAFHGFSIDNHNLTIIEVDGINTLPHTVNFIEIYPAQRYSFVLNANRP 299
gi|51242707|gb|AAT99291.1|       SFDAFHGFSIDNHNLTIIEVDGINTLPHTVNFIEIYPAQRYSFVLNANRP 299
gi|18461102|dbj|BAB84354.1|      SCDPNFLFSIDGHTFTVIEADGVNHEPIVADSIQIFAAQRYSFILTANQT 271
gi|51242705|gb|AAT99290.1|       SCDPNFLFSIDGHTFTVIEADGVNHEPIVADSIQIFAAQRYSFILTANQT 271
                                 : *. . ****.* :*:**.** *  * ..: *:*:..*****:* *.:.
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gi|18146856|dbj|BAB83132.1|     VDNYWVRALSSSGVGFSGFTGGLNSGILRYQGAPDADPTTTN---STGVV 335
gi|18461104|dbj|BAB84355.1|     VDNYWVRALSSSGVGFSGFTGGLNSGILRYQGAPDADPTTTN---STGVV 335
gi|6466812|gb|AAF13037.1|AF153  VDNYWVRALSSSGVGTSGFIGGLNSGILRYQGAPDADPTTTN---STGVV 330
gi|6466814|gb|AAF13038.1|AF153  VDNYWVRALSSSGVGFSGFTGGLNSGILRYQGAPDADPTTTN---STGVV 330
gi|51242697|gb|AAT99286.1|      VDNYWVRALSSSGVGTSGFIGGLNSGILRYQGAPDADPTTTN---STGVV 335
gi|51242699|gb|AAT99287.1|      VDNYWVRALSSSGVGFSGFTGGLNSGILRYQGAPDADPTTTN---STGVV 335
gi|18461106|dbj|BAB84356.1|     ISNYRIRALSNTGVGAHNFTNGVNSAILRYVGAPEEEPQTSQPLSSNVMI 349
gi|51242707|gb|AAT99291.1|      ISNYRIRALSNTGVGAQNFTNGVNSAILRYVGAPEEEPQTSQPLSSDVMI 349
gi|18461102|dbj|BAB84354.1|     ADNYWIRANPNN--GHTGFAGGINSAILRYSGAPVADPVTTQ---TSANL 316
gi|51242705|gb|AAT99290.1|      ADNYWIRANPNN--GHTGFAGGINSAILRYSGAPVADPVTTQ---TSANL 316
                                 .** :** ...  *  .* .*:**.**** ***  :* *::   :   :

gi|18146856|dbj|BAB83132.1|     LTESMLHPLENPGAPGLPFPGGADEVLNLTLGFNLPATFF-MNDTQYIPP 384
gi|18461104|dbj|BAB84355.1|     LTESMLHPLENPGAPGLPFPGGADEVLNLTLGFNLPATFF-MNDTQYIPP 384
gi|6466812|gb|AAF13037.1|AF153  LTESMLHPLENPGAPGLPFPGGADEVLNLTLGFNLPATFF-MNDTQYIPP 379
gi|6466814|gb|AAF13038.1|AF153  LTESMLHPLENPGAPGLPFPGGADEVLNLTLGFNLPATFF-MNDTQYIPP 379
gi|51242697|gb|AAT99286.1|      LTESMLHPLENPGAPGLPFPGGADEVLNLTLGFNLPATFF-MNDTQYIPP 384
gi|51242699|gb|AAT99287.1|      LTESMLHPLENPGAPGLPFPGGADEVLNLTLGFNLPATFF-MNDTQYIPP 384
gi|18461106|dbj|BAB84356.1|     FREGDLHPLENPGAPGNPYPGGADLVLNFTLGFDAEMVLFSMNGVVFESP 399
gi|51242707|gb|AAT99291.1|      FREGDLHPLENPGAPGNPYPGGADLVLNFTLGFDAEMVLFSMNGVVFESP 399
gi|18461102|dbj|BAB84354.1|     LQETSLVPRENPGAPGNATANGVDVDLNLVLSFVG--GRFEINGVSFVPP 364
gi|51242705|gb|AAT99290.1|      LQETSLVPRENPGAPGNATANGVNVDLNLVLSFVG--GRFEINGVSFVPP 364
                                 : *  * * ******* . ..*.:  **:.*.*      * :*.. : .*

gi|18146856|dbj|BAB83132.1|     TVPVLLQILSGAQSPQDLMPPGSVYTLPINKTIEINF--FGNATPGGPHP 432
gi|18461104|dbj|BAB84355.1|     TVPVLLQILSGAQSPQDLLPPGSVYTLPINKTIEINF--FGNATPGGPHP 432
gi|6466812|gb|AAF13037.1|AF153  TVPVLLQILSGAQSPQDLLPPGSVYTLPINKTIEINF--FGNATPGGPHP 427
gi|6466814|gb|AAF13038.1|AF153  TVPVLLQILSGAQSPQDLLPPGSVYTLPINKTIEINF--FGNATPGGPHP 427
gi|51242697|gb|AAT99286.1|      TVPVLLQILSGAQSPQDLLPPGSVYTLPINKTIEINF--FGNATPGGPHP 432
gi|51242699|gb|AAT99287.1|      TVPVLLQILSGAQSPQDLLPPGSVYTLPINKTIEINF--FGNATPGGPHP 432
gi|18461106|dbj|BAB84356.1|     TVPVLLQILSGAHHAQDLLPKGGVHTLPPNKVIEINF--FGGDAPAGPHP 447
gi|51242707|gb|AAT99291.1|      TVPVLLQILSGAHHAQDLLPKGGVHTLPPNKVIEINF--FGGDAPAGPHP 447
gi|18461102|dbj|BAB84354.1|     TVPVLLQILSGATTAAELLPSGSVYTLPLNSVIQLSFNTVAVAAVGGPHP 414
gi|51242705|gb|AAT99290.1|      TVPVLLQILSGATTAAELLPSGSVYTLPLNSVIQLSFNTVAVAAVGGPHP 414
                                 ************  . :*:* *.*:*** *..*::.*  ..  : .****
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gi|18146856|dbj|BAB83132.1|     FHLHGHSFDVVRSADNTTYNYKNPVRRDVTAVAIG-NQTTIRFVTDNPGP 481
gi|18461104|dbj|BAB84355.1|     FHLHGHSFDVVRSADNTTYNYKNPVRRDVTAVAIG-NQTTIRFVTDNPGP 481
gi|6466812|gb|AAF13037.1|AF153  FHLHGHSFDVVRSADNTTYNYENPVS--CTYVVHP-KQTTIRFVTDNPGP 474
gi|6466814|gb|AAF13038.1|AF153  FHLHGHSFDVVRSADNTTYNYKNPVS--CTYVVHP-KQTTIRFVTDNPGP 474
gi|51242697|gb|AAT99286.1|      FHLHGHSFDVVRSADNTTYNYENPVRRDVTSVAIG-NQTTIRFVTDNPGP 481
gi|51242699|gb|AAT99287.1|      FHLHGHSFDVVRSADNTTYNYENPVRRDVTSVAIG-NQTTIRFVTDNPGP 481
gi|18461106|dbj|BAB84356.1|     FHLHGHAFDVVRTSDSSEYNFKNPVRRDTTLVAMK-NQTTIRFVTDNAGP 496
gi|51242707|gb|AAT99291.1|      FHLHGHAFDVVRTSDSSEYNFKNPVRRDTTLVAMK-NQTTIRFVTDNAGP 496
gi|18461102|dbj|BAB84354.1|     FHLHGHTFDVVRSAGSTEYNYINPPRRDVVSTGAATDNVTIRFTTDNAGP 464
gi|51242705|gb|AAT99290.1|      FHLHGHTFDVVRSAGSTEYNYINPPRRDVVSTGAAIDNVTIRFTTDNAGP 464
                                ******:*****::..: **: **     . .    .:.****.***.**

gi|18146856|dbj|BAB83132.1|     WFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQ 531
gi|18461104|dbj|BAB84355.1|     WFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQ 531
gi|6466812|gb|AAF13037.1|AF153  WFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQ 524
gi|6466814|gb|AAF13038.1|AF153  WFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQ 524
gi|51242697|gb|AAT99286.1|      WFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQ 531
gi|51242699|gb|AAT99287.1|      WFLHCHIDLHLEFGLAVVMAEVPSLVKATDVTTPAWDELCPIYDSLTPAQ 531
gi|18461106|dbj|BAB84356.1|     WFLHCHIDLHLSLGLAVVLAEDTRDTKKDDMIPADWKGLCPIYNSLTPSQ 546
gi|51242707|gb|AAT99291.1|      WFLHCHIDLHLSLGLAVVLAEDTRDTKKDDMIPADWKGLCPIYNSLTPSQ 546
gi|18461102|dbj|BAB84354.1|     WFLHCHIDWHLEAGFAIVFAEDAPDVAAVNPVPDAWNQLCPTYDALTPAQ 514
gi|51242705|gb|AAT99290.1|      WFLHCHIDWHLEAGFAIVFAEDAPDVAAVNPVPDAWNQLCPTYDALTPAQ 514
                                ******** **. *:*:*:** .  .   :  .  *. *** *::***:*

gi|18146856|dbj|BAB83132.1|     LP-- 533
gi|18461104|dbj|BAB84355.1|     LP-- 533
gi|6466812|gb|AAF13037.1|AF153  LP-- 526
gi|6466814|gb|AAF13038.1|AF153  LP-- 526
gi|51242697|gb|AAT99286.1|      LP-- 533
gi|51242699|gb|AAT99287.1|      LP-- 533
gi|18461106|dbj|BAB84356.1|     LP-- 548
gi|51242707|gb|AAT99291.1|      LP-- 548
gi|18461102|dbj|BAB84354.1|     LGGN 518
gi|51242705|gb|AAT99290.1|      LGGN 518
                                *
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Fig. 5.1: Sequence alignment of all *L. edodes* laccase genes in NCBI. Based on the sequence homology, they were classified into three groups. : Group 2 laccase; : Group 3 laccase; : Group 1 laccase; : Copper ion binding sites.

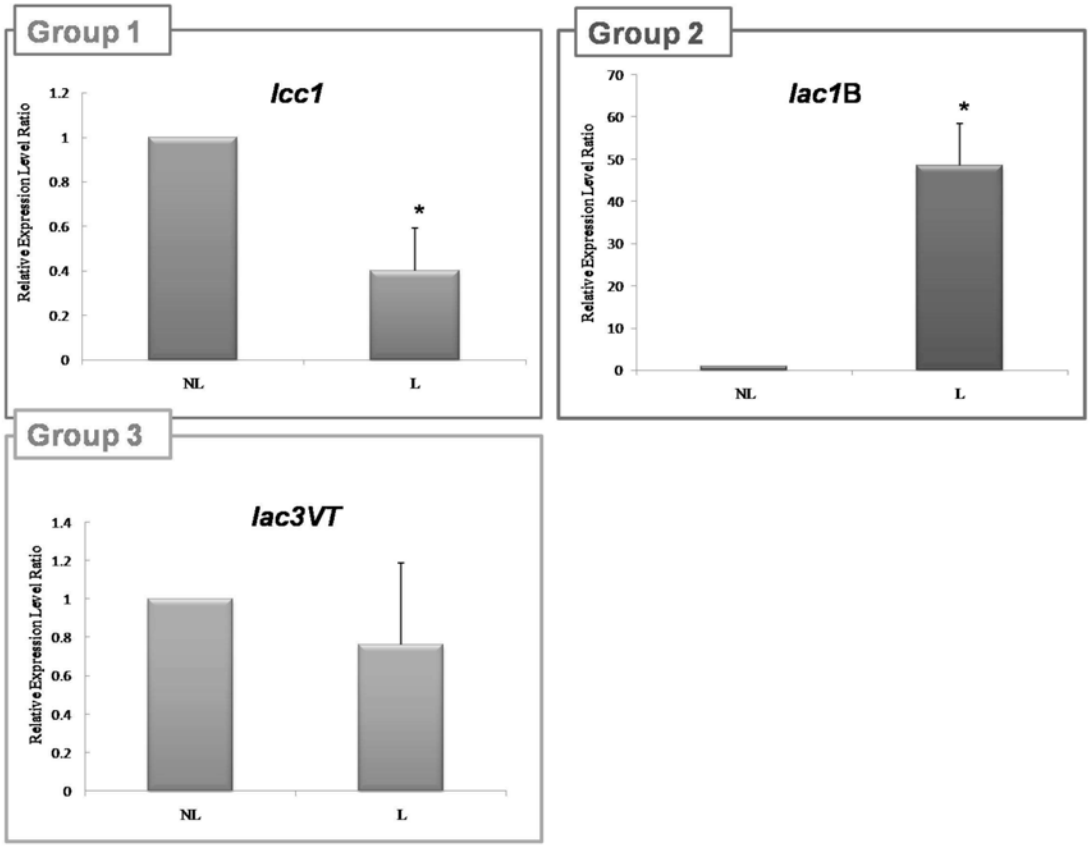

Fig. 5.2: Quantitative RT-PCR analysis on laccase-coding genes in mycelia grown in non-lignocellulose (NL) and lignocellulose (L). One representative gene from each group was chosen: Group 1: *lcc1* (accession no.: BAB84354); Group 2: *lac1B* (AAF13038); Group 3: *lac3VT* (AAT99291). Data represent means and standard deviations of three independent experiments and statistical significance was indicated by * $p < 0.05$.

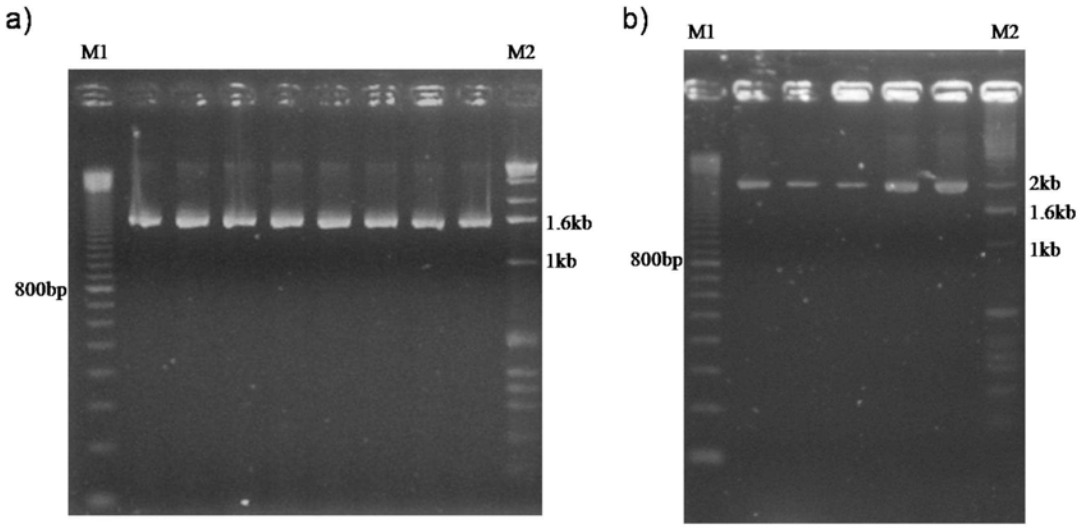

Fig. 5.3: a) PCR amplification of *lcc1* using gene specific primers. b) Screening PCR was performed to select positive clones. Plasmids from all positive transformants were pooled and used in transformation of *Kluyveromyces lactis*. M1: 100bp ladder; M2: 1kb ladder.

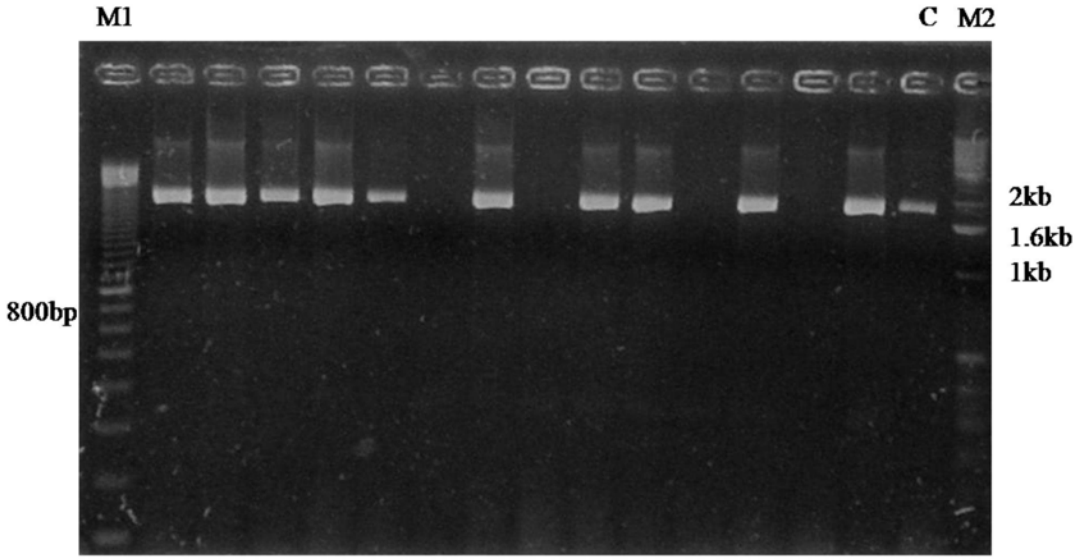

Fig. 5.4: Screening PCR for integration of expression cassette into *K. lactis* genome. Expected band size of positive transformants was 1.9kb. M1: 100bp ladder; C: control strain with parent vector only; M2: 1kb ladder.

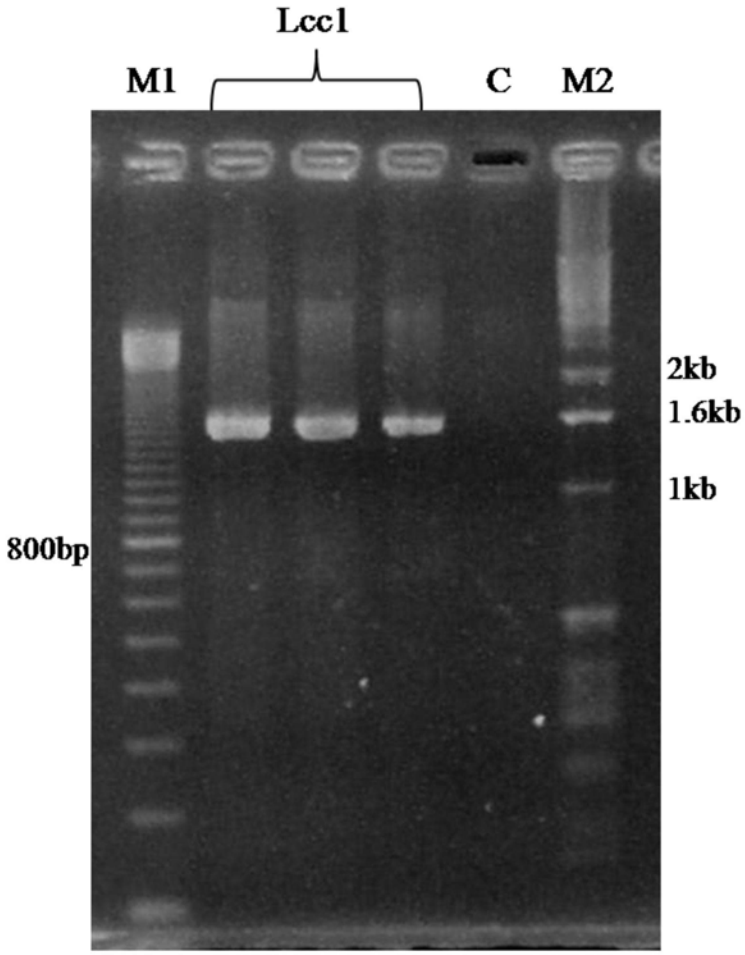

Fig. 5.5: RT-PCR of *lcc1* in *K. lactis* using *lcc1* specific primers. RNA was extracted from yeast cells after one day induction. M1: 100bp ladder; Lcc1: positive transformants containing pKLAC1/Lcc1 vector; C: control strain with parent vector only; M2: 1kb ladder.

5.4 Discussion

In the present study, I categorize 10 full length laccase sequences of *L. edodes* into 3 distinct groups based on their amino acid sequence similarity. I randomly selected one representative gene from each group and examined its transcript levels in mycelia grown in lignocellulose and non-lignocellulose media. I also tried to express a selected laccase, *lcc1*, in the yeast *Kluyveromyces lactis* but no enzyme activity was detected.

5.4.1 Grouping of laccases

The 3 groups of *L. edodes* laccases encoding genes share over 80% amino acid sequence homology. Individuals within each group are highly similar with a > 95% identity. From the genome analysis in Chapter 3, I found that all of the 15 laccase sequences in NCBI were mapped to 3 gene models, 2073_g, 1813_g and 1449_g, corresponding to Group 1, Group 2 and Group3 laccase encoding genes. These observations suggest the laccase sequences within each group are transcribed from a single gene in the genome and the small differences in the amino acids may be due to strain variations. There are 6 candidate gene models of laccases that cannot be matched with any of the existing *L. edodes* ESTs, suggesting they may represent novel laccase-coding genes.

All of the *L. edodes* laccases encoding genes contain domains for copper bindings, which are signature sequences conserved among fungal laccases (Kumar et al., 2003). It has been suggested that the axial ligand position of T1 copper center (refer to Fig. 5.1: F_{479} for Group 1; L_{496} for Group 2; and L_{511} for Group 3) influences the redox potential of laccase (Kumar et al., 2003). Having phenylalanine at this position usually results in a higher redox potential than replacing with leucine (Xu et al., 1998; Kumar et al., 2003). Therefore, I predict

that Group 1 laccase would have higher redox potential than Group 2 and Group 3.

Among 3 groups of laccases, the N-terminal amino acid sequences show a high variability, which may provide cues on the transcriptional and translational regulations of various laccase isoforms in *L. edodes*, which are still unclear.

5.4.2 Differential expressions of various laccases

The representative genes from 3 groups of laccases exhibit a different expression pattern in mycelia grown in lignocellulose and non-lignocellulose, indicating there may be a unique mechanism in transcriptional regulation for each group of laccases.

Sakamoto et al. (2008) demonstrated that *lcc1* (Group 1 laccase) was abundantly expressed in mycelium grown in non-lignocellulose. I showed that *lcc1* was up-regulated by 2-fold in non-lignocellulose-grown mycelia than that grown in lignocellulose-grown mycelium. The exact function of *lcc1* in *L. edodes* is still unclear.

I observed a large increase (> 40 fold) in the expression level of *lac1B* (Group 2 laccase) in mycelium grown in lignocellulose, suggesting a potential role of *lac1B* in lignin degradation, other than the previously reported function in gill-browning (Zhao and Kwan, 1999; Nagai et al., 2003).

There is a limited understanding on the function of *lac3VT* (Group 3 laccase). A similar transcript level of *lac3VT* was detected in mycelia grown in both conditions, indicating that it is not up-regulated in response to lignocellulose media.

5.4.3 Heterologous expression of Lcc1 in *K. lactis*

I have selected Lcc1 as a candidate for heterologous expression in the present study while cloning of other laccases have been undergoing in our lab using

either *K. lactis* or *Pichia pastoris*.

No detectable laccase activity was obtained in *K. lactis* transformants containing pKLAC1/Lcc1 construct. Recombinant Lcc1 was secreted in tobacco BY-2 cells with typical laccase activity (Sakamoto et al., 2008), suggesting that full length *lcc1* encodes a functional protein in a heterologous expression system. Besides, I also verified this failure was not related to transcription of *lcc1* as I confirmed the presence mRNA by RT-PCR.

Codon bias has a great impact on recombinant protein production. As the native *lcc1* exhibits a poor codon usage bias for *K. lactis*, I consider this may be a limiting factor for the translation of Lcc1. With codon optimization, up to 15 fold increase in recombinant protein production of β-1, 3-1, 4- glucanase in *Pichia pastoris* could be achieved (Teng et al., 2007). However, in the current study, I could not detect any enzyme activity in *K. lactis* transformants even though I have replaced the native *lcc1* with codon optimized construct.

As suggested by Yano et al. (2009), laccase may be able to oxidize the host cell metabolites under aerobic conditions, which in turns, elevate the oxidative stress encountered by the host. The higher the redox potential of laccase, the greater the oxidative stress for the host. In the current study, it is predicted that Lcc1 would have a high redox potential as indicated by the phenylalanine residue in the axial ligand position of T1 copper center. Therefore, high oxidative stress may be one reason for the undetectable laccase activity in *K. lactis* transformants.

5.5 Conclusion

In the present study, I identify 3 groups of laccases in *L. edodes* and each group shows a unique expression pattern in mycelia grown in different media. This information provided insights on the contributory roles of different groups of laccases during lignocellulose degradation.

Chapter 6: Concluding remarks

With the endowed ability to degrade wooden substances, wood-decaying fungi play an essential role in carbon recycling of the most abundant terrestrial biomass, lignocellulose. Different wood-decaying fungi have evolved unique enzymatic mechanisms to colonize different habitats. Lignocellulolytic enzymes are of great interest for green biotechnological applications, such as wastewater treatment, due to their ability to decompose phenolic compounds. They can also be applied in pretreatment and hydrolysis of lignocellulose during biofuel production. Identification, cloning and heterologous expression of lignocellulolytic enzymes can facilitate their industrial scale application. However, the time and cost for identifying and cloning lignocellulolytic enzymes hinder their applications.

Recent breakthrough in the sequencing technology not only makes genome sequencing accessible, but also allows large scale transcriptome analysis. I can simultaneously identify a whole arsenal of lignocellulolytic enzymes in a particular fungus. This certainly facilitates the study of lignocellulolytic systems among different wood-decaying fungi and broadens our understanding on the diversity of lignocellulolytic enzymes.

The genome sequencing of *Lentinula edodes*, an economically important edible mushroom, offers insights to its unique enzymatic mechanism for lignocellulose degradation. Wide spectrum of lignin degrading enzymes confers an efficient ligninolytic activity. The highly abundant hemicellulase- and pectinase-coding genes in the genome suggested its preferential attack on non-cellulosic polysaccharides. On the other hand, I detected few cellulase-coding genes and similar transcript levels of several cellulase-related

contigs in lignocellulose and non-lignocellulose-grown mycelia. These data provided possible explanation for its weak cellulolytic activity. Finally, with the aid of the transcriptome analysis, I identified a number of potential lignocellulolytic enzyme-coding genes without tedious experimental procedures.

Lignin degradation involves the generation of ROS and free radicals, which may attack mycelium. The lower transcript levels of contigs related to antioxidative mechanism and a lower SOD activity in lignocellulose-grown mycelium suggested that there is an overall reduction in oxidative stress encountered by mycelium during lignin degradation.

A stable and low-cost platform for large scale production of ligninolytic enzymes will facilitate their industrial applications. Yeast is an ideal host for the heterologous expression of these lignocellulolytic enzymes, not only because of it is an eukaryote with high growth rate, but also its genetic manipulation is easy and it does not require any sophisticated fermentation equipment. I successfully expressed recombinant manganese peroxidase (rMnP) in the methylotrophic yeast *Pichia pastoris* with an activity similar to that produced in *Aspergillus oryzae*.

More efforts are necessary for the characterization of its enzymatic properties and production of rMnP in bioreactor scale. The ultimate goal of establishing a stable, robust and low-cost yeast expression system for a large scale production of lignocellulolytic enzymes will accelerate their industrial applications, including biofuel production, wastewater treatment, wood pulping and textile industry. An overview of the present study and the contributions on the lignocellulolytic system in *L. edodes* are summarized in Fig. 6.1 and Fig. 6.2.

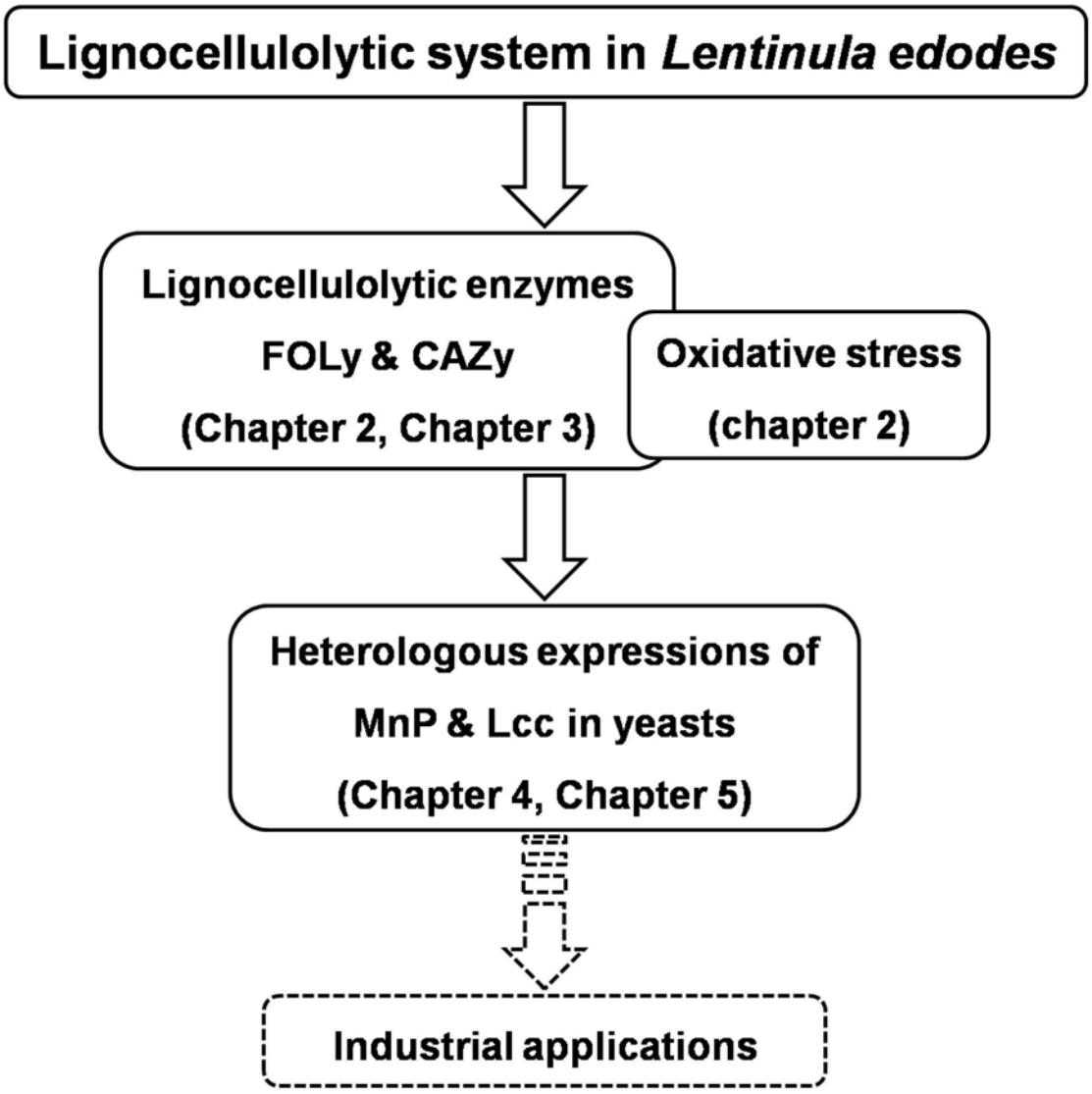

Fig. 6.1: An overview of the present study on lignocellulolytic system in *Lentinula edodes*.

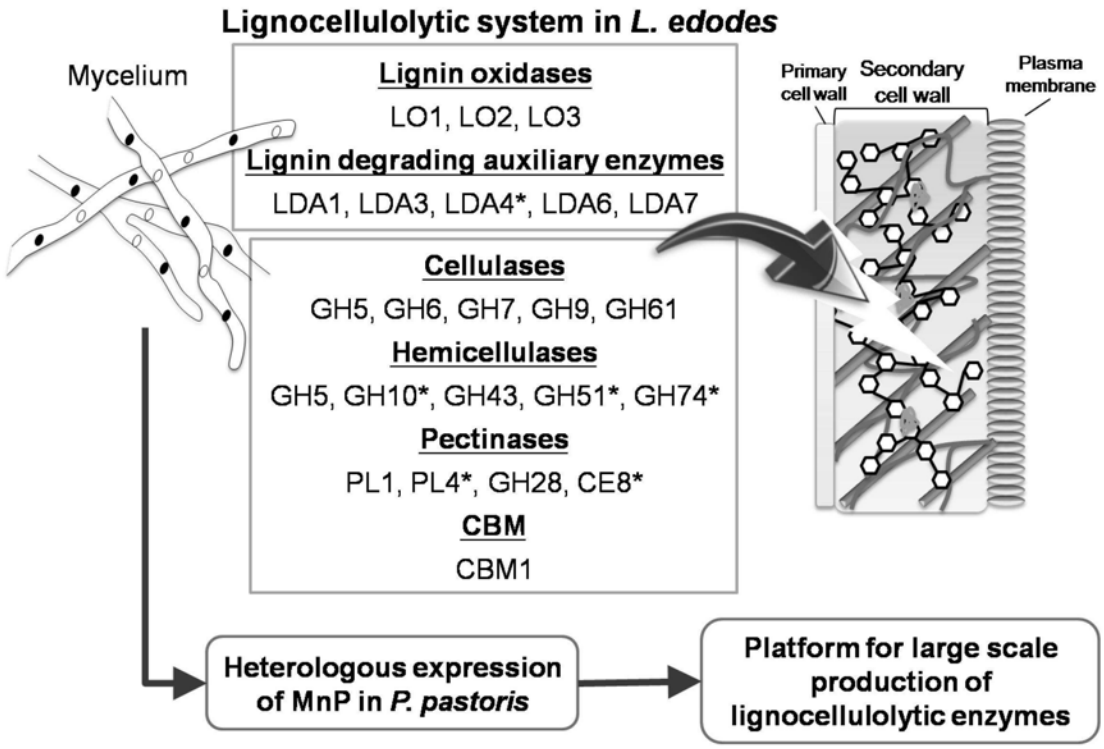

Fig. 6.2: Contribution of the present project to the understanding of lignocellulolytic system in *L. edodes*. All the FOLy and CAZy families found in the genome and ESTs were listed. They are probably secreted by *L. edodes* to attack plant cell wall. Families that could be detected in the genome but not in ESTs were marked with asterisks (*). I also heterologously expressed manganese peroxidase (MnP), one of the LO2, in *P. pastoris* and examined its activity. This serves as a platform for large scale productions of lignocellulolytic enzymes.

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