A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Biomodification of lignin compounds by *Abortiporus biennis* and *Phanerochaete chrysosporium* and investigation of related enzymes by transcriptomic analysis

유관버섯과 판막버섯에 의한 리그닌 화합물의 생물학적 변환 및 전사체 분석에 의한 관련 효소 구명

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Abstract

Biomodification of lignin compounds by *Abortiporus biennis* and *Phanerochaete chrysosporium* and investigation of related enzymes by transcriptomic analysis

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The objective of the present study was to understand biomodification mechanism of lignin compounds by two white rot basidiomycetes with different enzyme system, *Abortiporus biennis* and *Phanerochaete chrysosporium*. Based on this understanding, for lignin degradation by white rot basidiomycetes, changes of enzyme system and culture condition were applied. Finally, it was investigated what kinds of enzymes were involved in degradation of lignin compounds by transcriptomic analysis. In this study, biomodification products of monolignols and synthetic lignin composed of monolignols were examined for inferring accurate biomodification mechanism.

As a result of analysis of biomodification of monolignols by white rot basidiomycetes, *A. biennis* and *P. chrysosporium* leads to not only degradation but also polymerization of monolignols. To degrade monolignols, the addition

of ascorbic acid prevented a drastic increase of the molecular weight of monolignols, furthermore, various degraded products including acid compounds were formed from monolignols by *A. biennis* and *P. chrysosporium.*

Based on these results, biomodification products of synthetic lignin were examined and changes of enzyme system of basidiomycetes for lignin degradation under the ligninolytic condition were evaluated.

At first, *A. biennis* degraded synthetic lignin during initial incubation day (5-10 days), thereafter polymerized them. This phenomenon was occurred in case of monolignols. Therefore, the optimal conditions for lignin degradation by *A. biennis* were examined through the changes of enzyme system and culture condition. Ligninolytic enzyme system of *A. biennis* was based on MnP-laccase system, thus enzyme system of *A. biennis* was changed by addition of laccase mediator. As a result, when both enzyme system and surrounding conditions were simultaneously changed by addition of laccase mediator and reducing agents, whole cell of *A. biennis* assisted considerable degradation of synthetic lignin. To verify correlation between lignin degradation and complex enzyme system of whole cell of *A. biennis*, transcriptomic analysis was carried out. As a result, genes of laccase were highly expressed as expected. Upregulation of various extracellular enzymes such as MnP and aryl alcohol oxidase were observed in *A. biennis* exposed to DHP. These results suggested strongly involvement of various extracellular enzymes functioning in bond cleavage in lignin degradation.

Catalytic enzyme system of *P. chrysosporium* is controlled by two peroxidases, LiP and MnP. Based on this catalytic system, *P. chrysosporium* also induced degradation and polymerization of synthetic lignin. To degrade synthetic lignin under the ligninolytic treatment, reducing agents, ascorbic acid and α-tocopherol during oxidative reaction were used for stabilizing unstable radicals generated. Contrary to the result of *A. biennis*, it was

noteworthy that *P. chrysosporium* with two reducing agents produced aromatic compounds (syringic acid and 2,6-dimethoxybenzodiol) and succinic acid as well as degraded lignin polymer. Transcriptomic analysis of *P. chrysosporium* provided information about various enzymes related to lignin degradation and aromatic catabolic pathway. Consequently, extracellular catalytic system of *P. chrysosporium* attacked synthetic lignin, resulting in production of aromatic compounds derived from lignin molecules. Thereafter, aromatic compounds were metabolized in short-cut TCA cycle of *P. chrysosporium* and were finally converted to succinic acid.

In conclusion, two different enzyme systems of *A. biennis* and *P. chysosporium* produced different degradation products. MnP-LAC complex system of *A. biennis* induced production of depolymerized lignin polymer. On the other hand, *P. chysosporium* with metabolic cycle related-enzymes preferentially catalyzed production of valuable lignin derived-chemical, succinic acid. Consequently, two basidiomycetes were suitable for lignin degradation with change of enzyme system and culture condition, so they can be used as novel biocatalysts for lignin application and biotechnological application of basidiomycetes.

Keywords: Lignin degradation, succinic acid, *Abortiporus biennis*, *Phanerochaete chrysosporium*, transcriptomic analysis

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List of Abbreviations

ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AAD	aryl alcohol dehydrogenase
AAO	aryl alcohol oxidase
AD	aldehyde dehydrogenase
CA	conifeyl alcohol
CDH	cellobiose dehydrogenase,
CYP 450	cytochrome P450 monooxygenase
^{13}C-NMR	carbon-13 nuclear magnetic resonance
DEGs	differentially expressed genes
DHP	dehyerogenative polymer
fc	Fold change
GC-MS	gas chromatography-mass spectrometry
GPC	gel permeation chromatography
GO annotation	gene ontology annotation
GLOX	glyoxal oxidase
GST	glutathione S transferase
KEGG	kyoto encyclopedia of genes and genomes
LC-MS	liquid chromatography-mass spectrometry
LC-MS-MS	liquid chromatography-tandem mass spectrometry
LiP	lignin peroxidase
LAC	laccase
MnP	manganese peroxidase
NBO	nitrobenzene oxidation
NGS	next generation sequencing
SA	sinapyl alcohol
SSC medium	shallow stationary culture medium
QR	1,4-benzoquinone reductase

Chapter 1

Introduction

1. Background

1.1. Lignin overview

Recently, with excessive consumption of fossil fuel and environmental problem, there has been a great interest in lignocellulosic biomass as one potential renewable source for conversion into chemicals, materials and energy (Monroe & Oxarart, 2011).

Lignin, one of lignocellulosic biomass components, is nature's second abundant aromatic polymer next to cellulose. It is composed of phenyl propane units such as ρ-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Table 1-1). And these lignin precursors are connected by both ether and carbon-carbon linkages including β-O-4, α-O-4, and β-β. β-Aryl ether substructure is the most abundant linkage (40-60%) in lignin and followed by phenylcoumaran (10%), diarylpropane (5-10%), noncyclic benzyl aryl ether (6-8%), and biphenyl (5-10%) substructures etc (Table 1-2) (Higuchi, 1990).

Lignin is considered to be complex and amorphous polymer within the lignocellulosic matrices of plant cell walls, regardless of monomeric composition. Therefore, lignin has been undervalued as a by-product of paper and lignocellulosic bioethanol industries and there have been limits on utilization of lignin as value added products (Himmel, 2009; Sahoo et al., 2011). However, some reports refered to the possibility of lignin in value added applications in these days. With biorefinery concept, lignin can be converted to useful biomaterials and chemicals for maximizing the economic value of the biomass (Kaparaju et al., 2009). Especially, in United States of America, about 227 hm^3 of bioethanol production to replace the fossil fuels by 2030 is expected to accompany around 0.225 Gt of lignin generation (Sahoo et al., 2011). However, it is still challenging to work with lignin.

Table 1-1. Chemical structures of phenyl propane units of lignin (Higuchi, 1990)

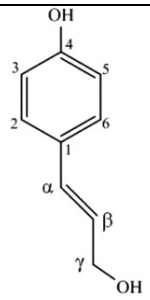
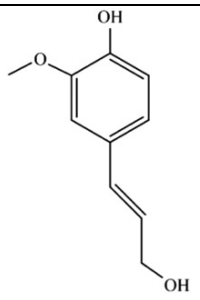
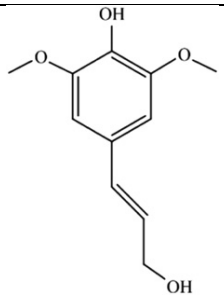
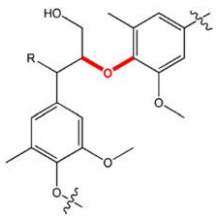
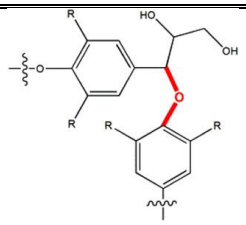
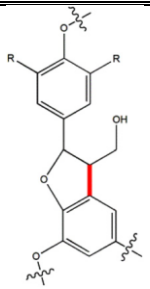
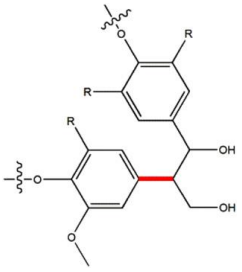
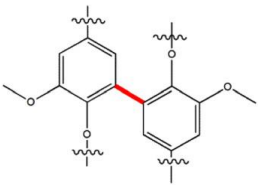
Phenyl propane units			
Types	ρ-Coumaryl alcohol	Coniferyl alcohol	Sinapyl alcohol
			

Table 1-2. Principal linkage mode between monomeric phenyl propane units in lignin (Zakzeski et al., 2010).

Ether linkage	β-Aryl ether (β-O-4)	Benzyl aryl ether (α-O-4)	
			
C-C linkages	Phenylcoumaran (β-5)	Diarylpropane (β-1)	Biphenyl (5-5)
			

1.2. White rot basidiomycetes

In nature, basidiomycetes are the best wood degrader. White rot basidiomycetes are well-known for most efficient degraders of lignocelluloses. Wood decay by white rot basidiomycetes is pale in color because of preferential attack on lignin than hemicellulose and cellulose in wood. This selective delignification occurs by extracellular enzyme systems, so called ligninolytic enzyme systems (Schmidt, 2006; Wong, 2009).

Generally, white rot basidiomycetes secrete two major types of extracellular enzymes with ligninolytic activities: peroxidases (lignin peroxidase and manganese peroxidase) and laccase. Lignin peroxidase (LiP, E.C. 1.11.1.14) is heme-containing glycoprotein which catalyzes the H_2O_2-dependent oxidative degradation of lignin. LiP was first discovered in *Phanerochaete chrysosporium*, and various isoenzymes existed in other white rot basidiomycetes. This enzyme has a catalytic cycle in the presence of H_2O_2, which leads to cleavage of C_α-C_β bond and ring fission (Figure 1-1 (A)). Manganese peroxidase (MnP, E.C. 1.11.1.13) catalyzes Mn-dependent reaction cycle. In other words, MnP oxidizes Mn^{2+} to Mn^{3+} in the presence of H_2O_2, and Mn^{3+} is a strong oxidant which oxidizes phenolic structures by single electron oxidation (Figure 1-1 (B)). Both LiP and MnP initiate free-radical reactions in which the generated radicals attack the lignin network. Laccase (Lac, E.C. 1.10.3.2) is phenol oxidase which belongs to the blue-copper family of oxidase. It catalyzes substraction of one electron from phenolic hydroxyl group of lignin in utilizing oxygen as oxidant, eventually catalyzing 4-electron reduction of O_2 to H_2O (Figure 1-1 (C)).

Ligninolytic enzymes are highly non-specific, and thus, able to degrade a wide range of recalcitrant compounds. Most white rot basidiomycetes secrete one or more of the ligninolytic enzymes in different combination as follows:

LiP-MnP group, MnP-laccase group, and LiP-laccase group (Hatakka, 1994). Accordingly, the complex ligninolytic enzyme systems have been studied for several decades in various fields such as bioremediation of pollutants as well as biodegradation of lignin. Furthermore, fungal catalysis system makes the possibilities for their biotechnological applications in numerous industries with development of proteome and genome analysis techniques. Broad understanding about white rot basidiomycetes and their enzymes will contribute to better development of new biocatalysts in various industrial field (Bouws et al., 2008; Mester & Tien, 2000).

(A)

(B)

(C)

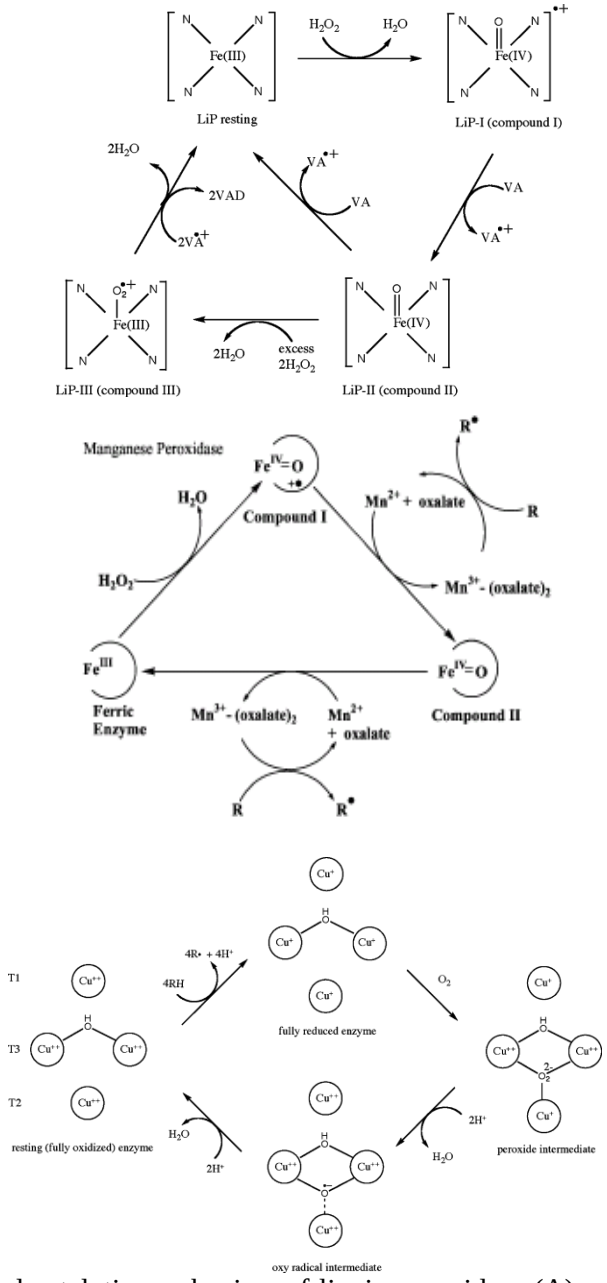


Figure 1-1. Typical catalytic mechanism of lignin peroxidase (A), manganese peroxidase (B), and laccase (C) (Wong, 2009)

1.3. Biocatalytic modification of lignin

Lignin modification is essential based on lignocellulosic biorefineries with respect to economic costs (Davis et al., 2015), but current lignin utilization is limited on combustion to produce heat and power.

Lignin modification has been studied for many decades by various approaches such as thermal, catalytic and biological modifications. Because catalytic and thermochemical modifications are most effective in lignin depolymerization, it was considered to be important industrial process for producing the bio-based chemicals and materials (Meister, 2002; Ragauskas et al., 2014). However, these processes are carried out under the energy and cost-intensive condition requiring high temperature, pressure and expensive catalyst. On the other hand, biological modification demands the mild condition compared to thermal and catalytic modification. These biocatalytic treatments have been suggested as attractive alternatives because of environmentally friendly and cost-effective process.

Very recently, new concept using the bacterial ligninolytic enzyme system has emerged for lignin depolymerization. Ligninolytic systems of microbes such as *Bacillus subtilis*, and *Amycolatopsis* sp. have been discovered (Beckham et al., 2016; Salvachúa et al., 2015; Santos et al., 2014). These researches demonstrated that bacterial ligninolytic activities lead to significant extent for degradation of lignin. However, many questions still has arisen which ligninolytic enzymes are secreted from microbes, how they act on natural lignin, and what aromatic compounds from lignin were produced. In addition, because bacteria have one or two of lignin degradation system, bacterial degradation of lignin showed low efficiency, compared with that of white rot basidiomycetes (Zhao et al., 2016).

In contrast to bacterial degradaion system of lignin, model lignin

degradation mechanism by white rot basidiomycetes has three systems, peroxidase-based, laccase-based, and Fenton reaction-based systems (Dashtban et al., 2010; Floudas et al., 2012). Accordingly, white rot basidiomycetes have been recognized to be suitable for lignin modification. Biotechnological use of white rot basidiomycetes is of considerable interest in green chemistry and biorefinery industry (Martínez et al., 2010) due to sustainability and economics for biorefinery (Ragauskas et al., 2014).

Whole cell of white rot basidiomycetes as biocatalysts provides simple bioprocessing with complex enzyme system and metabolism. In other words, biocatalytic process can induce one step conversion, which is not normally accessible by chemical process. Therefore, the development of new biocatalyst based process is recent trend. In this sense, lignin modification by biocatalysts can be a powerful tool and essential ingredient of industrial biotechnology for producing intermediates of fuels and valuable chemicals (Turner, 2009). However, it is currently unclear what the optimal condition for lignin degradation and what catabolic reactions of aromatic compounds will be under the ligninolytic condition. Therefore, combination of biological-omics tools and advanced chemical analytics is expected to offer a lot of information about fungal metabolic system for lignin degradation.

1.4. Lignin application

Lignins have different functional groups and properties depending on the source of origin and isolation process. These differences are critical in using lignin in industrial application because they have an influence on various physiochemical characteristics (Sahoo et al., 2011). On the whole, lignin is utilized for replacement of petroleum-based polymers in industry as shown in Figure 1-2. For example, kraft lignin and lignosulfonates can be utilized for production of low molecular weight chemicals (Gogotov, 2000), bio-based products like carbon fibers (Kadla et al., 2002) and adhesives (Mansouri & Salvadó, 2006). Organosolv lignins are characteristic for high purity, sulfur free, and low molecular weight distribution. These properties make organosolv lignins utilized to phenolic and epoxy resins, films, and biodegradable polymers (Lora & Glasser, 2002; Pereira et al., 2007; Vazquez et al., 1999).

Industrial applications of lignin require proper physiochemical properties, for instance, higher purity, thermo-stability and homogeneity. That is why suitable modification process of lignin is needed. Among a variety of modification processes, enzymatic approaches for lignin modification bring numerous advantages to industrial process, leading to cleaner and more environmental friendly processes and products. Enzymatic catalysts are high selective and efficient to substrate conversion, and require mild operating condition. But, enzymatic approaches require long reaction time and are difficult to reuse. Nevertheless, enzymatically modified lignin can be applied in industry as copolymers, binders, chelating complex and paintings, and there are many undoubting advantages in biotechnologies (Sena-Martins et al., 2008). To succeed in implementing enzymatic technologies, it is essential to produce industrial enzymes on a large scale and to investigate industrial biocatalysts by molecular biological approaches (Martínez et al., 2010; Sena-

Martins et al., 2008).

Although microbial modification of lignin has been extensively studied for many decades, the products obtained from degradation process are difficult to apply for industry directly due to their heterogeneous characteristics. Thus, some research groups recently studied integrated biological funnel system with the object of lignin upgrading as described in Figure 1-3 (Johnson & Beckham, 2015; Linger et al., 2014). This concept suggests microorganisms including bacteria and fungi with oxidative enzymes utilize aromatic compounds as carbon source. As a result, microbes employ “upper pathway” to produce catechol and protocatechuic acid as central intermediates. Subsequently, cleavage of aromatic rings and production of intermediary metabolites occur through “lower pathway” (Beckham et al., 2016; Fuchs et al., 2011). These metabolites can be used as polyhydroxyalkanoates (PHA) which can be converted to renewable chemicals and materials such as hydrocarbons, hydroxyacid monomers (Linger et al., 2014; Tomizawa et al., 2014). Consequently, it offers a potential biological treatment for lignin valorization to value added products.

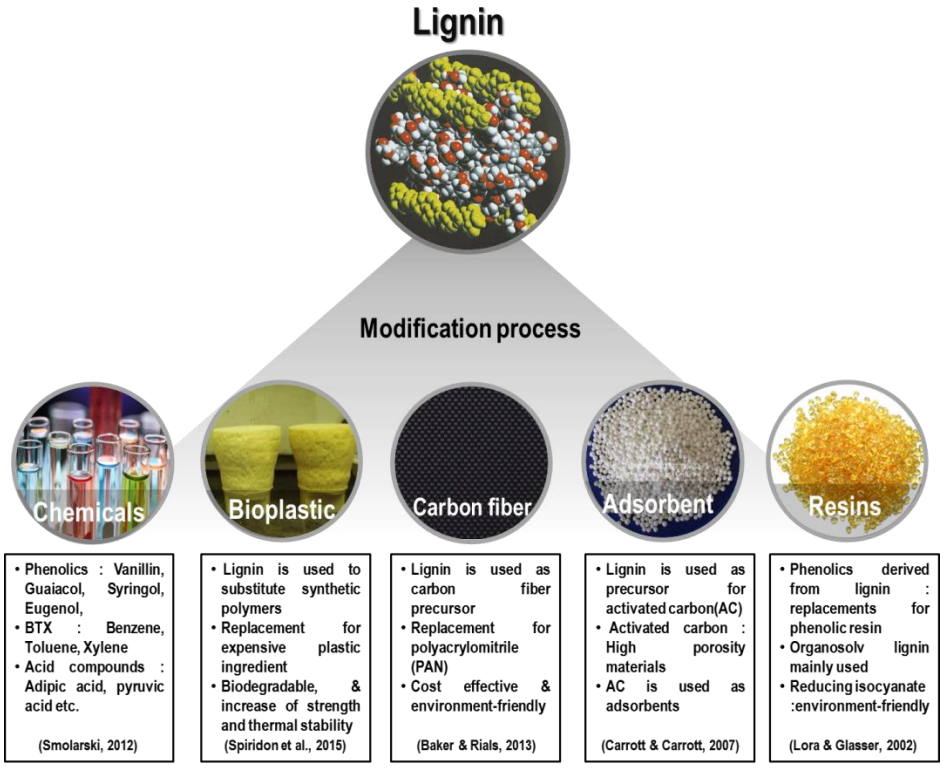


Figure 1-2. Various industrial application of lignin (Baker & Rials, 2013; Carrott & Carrott, 2007; Lora & Glasser, 2002; Smolarski, 2012; Spiridon et al., 2015)

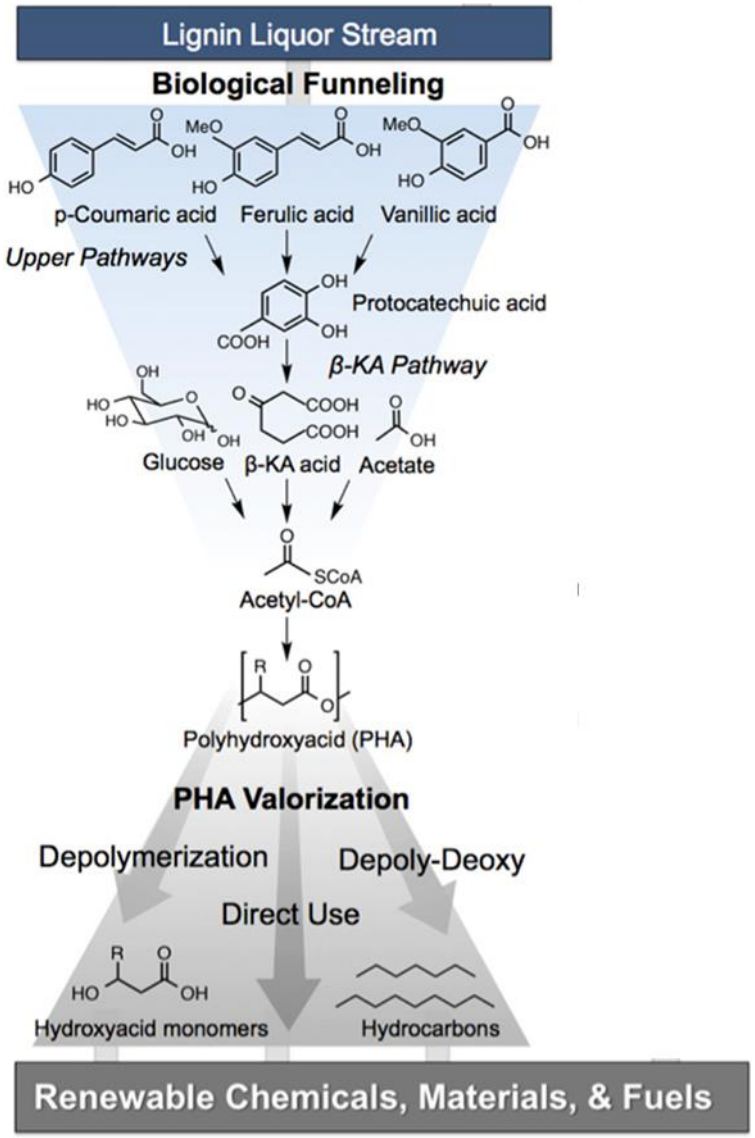


Figure 1-3. Biological funneling system for lignin valorization to value added products through aromatic catabolic pathway (Linger et al., 2014)

2. Objectives

Biomodification of lignin by white rot basidiomycetes has been investigated for many decades. Especially, basidiomycetes have been recognized as the best lignin degrader with aromatic catabolic ability. Most previous researches simply suggested that ligninolytic enzymes purified from basidiomycetes could degrade lignin. Although these findings have helped to understand biodegradation mechanism of lignin by ligninolytic enzymes, it has limits on using whole cell of white rot basidiomycetes. In addition, studies on correlation between biomodification mechanism of lignin and ligninolytic enzymes of white rot basidiomycetes are rarely conducted.

In this study, to better understand biomodification mechanism of lignin compounds by white rot basidiomycetes, *Abortiporus biennis* and *Phanerochaete chrysosporium*(ATCC 20696), biomodification products of monolignols and synthetic lignin by white rot basidiomycetes were analyzed by various analytical equipments. Enzyme system of *A. biennis* has MnP-Lac group, and *P. chrysosporium* has LiP-MnP group. Accordingly, by analyzing biomodification products of monolignols and characteristic structural changes of the synthetic lignin, different catalytic system of two basidiomycetes was evaluated.

Based on the results above, for lignin degradation under the fungal culture, enzyme system of basidiomycetes and surrounding condition were changed by addition of mediator of ligninolytic enzymes and reducing agents. In the case of *A. biennis*, laccase mediator and reducing agents such as ascorbic acid and α-tocopherol were added in culture medium. In the case of *P. chrysosporium*, reducing agents were only added for lignin degradation.

Finally, to identify extra and intracellular enzymes related to biodegradation of lignin, transcriptomic analysis was carried out using Next

generation sequencing (NGS) technique, a powerful sequencing system. Based on the results of differentially expressed genes (DEGs), GO (gene ontology) annotation and KEGG (kyoto encyclopedia of genes and genomes) pathway were analyzed to investigate function of enzymes related to lignin degradation.

Therefore, the objectives of this study were:

(1) To understand biomodification mechanism of monolignols and synthetic lignin by white rot basidiomycetes, *A. biennis* and *P. chrysosporium*.

(2) To apply the ligninolytic enzyme system of white rot basidiomycetes with change of enzyme system and surrounding condition for lignin degradation and production of lignin derived compounds

(3) To investigate complex enzymes related to biomodification of lignin compounds by transcriptomic analysis of white rot basidiomycetes.

3. Literature review

3.1. Lignin biosynthesis pathway

Lignification takes place during secondary thickening of the cell wall based on enzymatic conversion, and it helps to reinforce the plant walls of the vasculature, and provide physical barrier to pathogens and other organisms (Himmel, 2009).

Lignin composition is essentially derived from the only three monolignols such as ρ-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Studies on monolignol biosynthesis had been conducted by in vitro experiments using isotope-labeled lignin precursors since 1960's. Monolignol biosynthesis occurs by all possible enzymatic conversions. It starts with deamination and successive hydroxylation in aromatic ring of phenylalanine and tyrosine formed by shikimate pathway. Conversions of ρ-coumaric acid to ferulic acid and sinapic acid proceed via hydroxylation and methylation by enzymes. Especially, to elucidate formation mechanism of methoxyl groups of lignins, Higuchi group and Shimada group had studied on the characterization of *O*-methyltransferase. Reduction steps of acids to alcohols are mediated by coumarate CoA ligase, cinnamate CoA reductase, and cinnamyl alcohol dehydrogenase (Higuchi, 1990; Higuchi & Brown, 1963; Shimada et al., 1973).

Synthesized monolignols are dehydrogenatively polymerized by radicals of monolignol formed enzymatically in a random, so called radical coupling reaction (Figure 1-4) (Boerjan et al., 2003; Higuchi, 1990). It was found that coniferyl alcohol is polymerized to dehydrogenative polymer (DHP) *in vitro* which is closely related to spruce milled wood lignin (Freudenberg 1956). And then, Higuchi group demonstrated that mushroom enzymes such as laccase

and tyrosinase are involved in the formation of DHP, thereafter, proposed involvement of peroxidases in lignification of plant cell in presence of H_2O_2 (Higuchi, 1990). Peroxidases (EC:1.11.1.7) participate in oxidation of substrate by using H_2O_2 as oxidizing agent, dehydrogenating and generating reactive radicals in order. Laccase (EC:1.10.3.2) is also related with polymerization of monolignols with the consumption of O_2. Expression of these enzymes by genetic engineering could suggest their particular role in lignification. Nevertheless, it is still unclear whether the polymerization in lignin biosynthesis is catalyzed by peroxidase, laccase, another polyphenoloxidase, or a combination of these enzyme *in vivo* (Baucher et al., 1998).

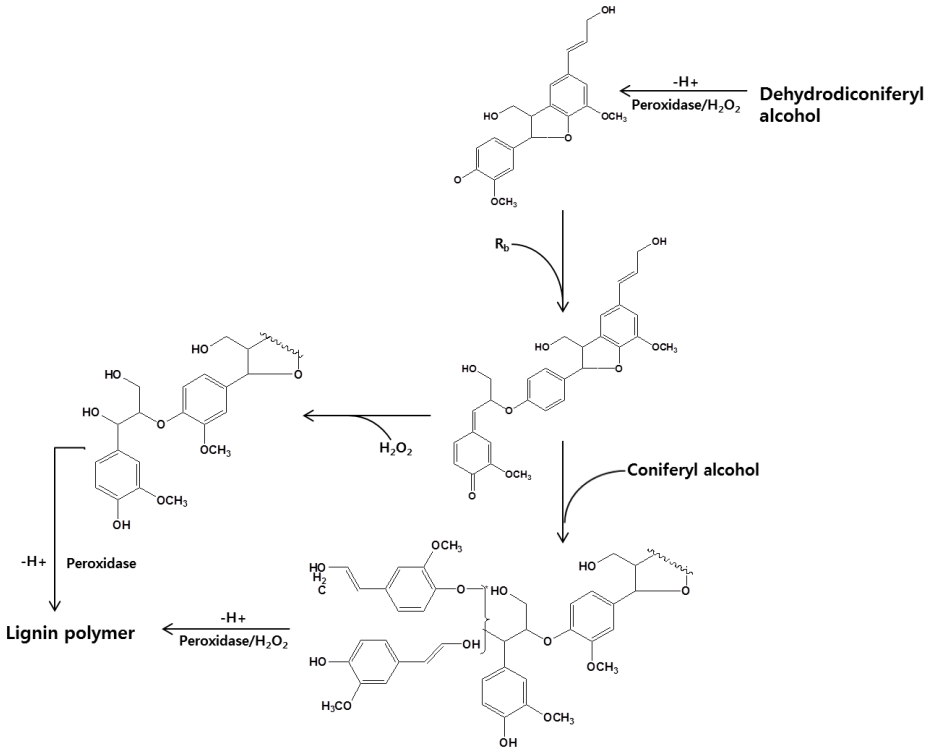


Figure 1-4. Formation of lignin via oligolignol quinone methide (R_b: C_β radical of coniferyl alcohol) (Higuchi, 1990)

3.2. Lignin degrading enzymes system of white rot basidiomycetes

3.2.1. Lignin degrading enzymes and their mediator system

From the 1960's, various researchers had tried to demonstrate correlation between lignin degrading enzymes and lignin degradation. At the beginning of 1980's, the isolation of LiP and MnP from white rot basidiomycetes elucidated that two peroxidases were involved in lignin degradation (Hatakka, 1994). However, comprehensive reactions of lignin degrading enzymes in lignin degradation are less well characterized, and thus there are still conflicting views on how the each enzymes act and how they cooperate with other enzymes.

Among the various white rot basidiomycetes, *P. chrysosporium* and *Ceriporiopsis subvermispora* have been the most extensively studied-model basidiomycetes.

P. chrysosporium was the first organism for which LiP was described, and has been reported to produce two peroxidases, LiP and MnP. Both LiP and MnP have been believed to play a key role in the biodegradation of lignin (Tien & Kirk, 1983). In particular, studies on LiP of *P. chrysosporium* have been carried out extensively by various researchers. They reported overall characteristics of LiP including the catabolic pathways for lignin degradation (Higuchi, 1986; Tien, 1987; Tien & Kirk, 1983). Besides, LiP is regulated by carbon and nitrogen sources and secreted into the environment of the fungus under conditions of nitrogen limitation (Keyser et al., 1978; Kirk et al., 1978). Based on these results, biochemistry and molecular biology of lignin degrading enzymes secreted from *P. chrysosporium* from 1970's to 1990's were mainly studied.

From the late 1990's, *C. subvermispora*, one of the strongly ligninolytic basidiomycetes, has been studied extensively in pilot scale for application in

industrial biopulping. This fungus produces mainly oxidative enzymes, MnP and laccase (Lobos et al., 1994). Although ligninolytic enzyme system of *C. subvermispora* was less characterized than that of *P. chrysosporium*, *C. subvermispora* has been evaluated as biocatalyst for useful application in industry (Ferraz et al., 2003; Fukushima & Kirk, 1995).

With powerful ability of ligninolytic enzymes, studies on their mediator system have also advanced for overcoming limitation in applying ligninolytic enzymes to widen range and expanding their catalytic activity. In case of LiP – catalyzed oxidation, veratryl alcohol (VA) acts as a redox mediator transferring oxidizing equivalents from the enzyme to lignin polymer (Huang et al., 2003; Koduri & Tien, 1994). However, mediation system of LiP may be less effective in lignin degradation because oxidation system of LiP is originally involved with mediation of VA. The MnP system was reported to be catalyzed by thiol group and carboxylic acid mediated oxidation (Forrester et al., 1988; Hofrichter, 2002a; Wariishi et al., 1989). Wariishi et al (1989) explained MnP/thiol mediated system as followings: thiol group like glutathione was oxidized to thiyl radicals by MnP-generated Mn^{3+} and thiyl radicals lead to formation of benzylic radical by substracting hydrogen of non-phenolic compounds at the α-carbon. Chelates of Mn^{3+} with carboxylic acids (oxalate, malonate, and lactate etc,) were reported to cause one-electron oxidation of various substrates, giving rise to aryl cation radicals of substrates (Figure 1-5 (A)) (Hofrichter, 2002a; Michizoe et al., 2004). Finally, laccase-mediator system is the best known case among ligninolytic enzymes and mediation systems thereof. There are several mediators in laccase system including ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), HAA (3-hydroxyantranilic acid), HBT (1-hydroxybenzotriazole), and NHA(N-hydroxyacetanilide) and so on (d'Acunzo et al., 2004; Li et al., 1999). These mediators act as electron shuttles and oxidize the lignin which does not enter the active site because of steric hindrances (Figure 1-5(B)). The presence of a

laccase-mediator complex makes a possible to exceed the redox barrier of laccase alone (Majcherczyk et al., 1999). Moreover, mediators of laccase have been recognized to be environmental-friendly and available at low cost. That was why these have drawn much attention in pulp-bleaching techniques, and biotechnological application of laccase/mediator system can develop the lignocellulose biorefinery (Cañas & Camarero, 2010).

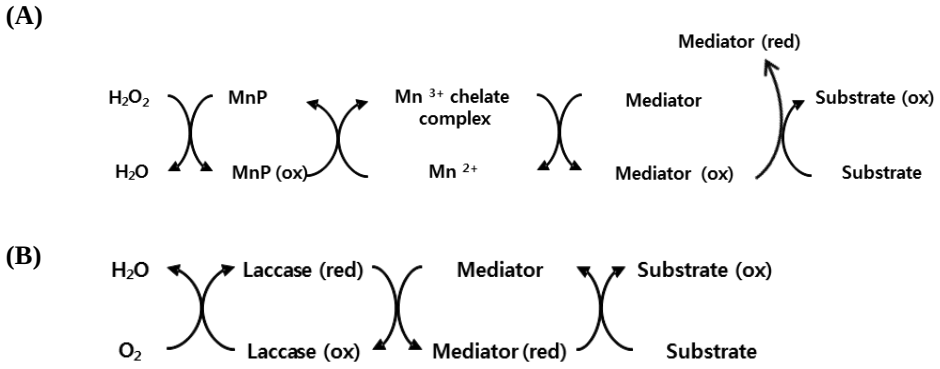


Figure 1-5. Mechanism of oxidation by the MnP (A) and laccase (B) / mediator systems (Michizoe et al., 2004; Riva, 2006)

3.2.2. Various enzymes related to lignin degradation

With development of proteome and genome analysis techniques, a variety of enzymes of white rot basidiomycetes including extra- and intra-cellular enzymes were reported to be involved in lignin degradation and metabolism of aromatic compounds (Adav et al., 2012; Kersten & Cullen, 2007; Matsuzaki & Wariishi, 2005; Ning et al., 2010).

In addition of ligninolytic enzymes, many extracellular enzymes were implicated in lignin degradation, which acts as accessory enzymes. These include oxidases generating H_2O_2 and cellobiose dehydrogenase (Dashtban et al., 2010). Oxidases generating H_2O_2 include aryl alcohol oxidase (AAO) and glyoxal oxidase (GLOX), which are found in various basidiomycetes including *P. chrysosporium* (De Jong et al., 1994; Kersten & Kirk, 1987). These studies reported that they play a key role in generating H_2O_2 for extracellular peroxidases. The activity of GLOX was highly expressed when the ligninases are expressed (Kersten & Kirk, 1987). Activity of AAO was increased when double bonds conjugated with a primary alcohol existed (GUILLÉN et al., 1992). And also, cellobiose dehydrogenase (CDH) was reported to catalyze Fenton reaction, resulting in generation of hydroxyl radicals (Wang et al., 2006).

Along with extracellular oxidative enzymes system, white rot basiodiomycetes have intracellular enzymes, which are involved in hydroxylation and oxidation of aromatics.

Cyrochrome P450 monooxygease (CYP) is representative intracellular enzyme, and its characteristics and function has been studied in many various field. In general, CYP mainly induced hydroxylation and dealkylation of substrates (Matsuzaki & Wariishi, 2004). The 154 genes of fungal CYP were founded through the whole sequence of *P. chrysosporium* genome (Martinez et al., 2004). Among them, CYPs (CYP63A1, CYP63A2, CYP63A3) related to

degradation of aromatic compounds were reported to be expreseed in transcriptional level in *P. chsosporium* (Doddapaneni et al., 2005; Doddapaneni & Yadav, 2004). As mentioned above, gene diversity of CYP contributes to their function diversity, thus molecular characteristics and functions of fungal CYP are still being studied.

Dioxygenase catalyzes ring cleavage of aromatic compounds released from lignin. Dioxygenase is divided to intradiol dioxygenase and extradiol dioxygenase, and they have completely different structures and different catalytic mechanism (Harayama et al., 1992). Intradiol dioxygenase cleave *ortho* to hydroxyl substituents of aromatics. It was reported that *P. chrysosporium* degraded vanillate and aromatic pollutants to 1,2,4-trihydroxybenzene which was an intermediate in formation of β-ketoadipate by 1,2-dioxygenase (Rieble et al., 1994). On the other hand, extradiol dioxygenase generally cleave *meta* to hydroxyl substituents. It was reported that extradiol dioxygenase activates oxygen molecule by binding both O_2 and the catecholic substrate, and induces *meta* cleavage (Lipscomb, 2008). In general, ring cleavage by dioxygenase requires catecholic substrate possessing hydroxyl substituents on two adjacent carbons. However, other researchers suggested some dioxygeases such as hydroquinone 1,2-dioxygenase and homogentisate-1,2-dioxygenase could degrade substrate which hydroxyl groups were substituted at para position such as hydroquinone and homogentisate to acid compounds (Gunsch et al., 2005; Miyauchi et al., 1999).

Glutathione S transferase (GST) was also reported to be involved in lignin degradation (Adav et al., 2012). This enzyme catalyzes the cleavage of ether bond, resulting in conjugation of glutathione to non-polar compounds (Morel et al., 2009). However, fungal GST has been poorly studied to date. Furthermore, a lot of intracellular enzymes such as aryl alcohol dehydrogenase (Delneri et al., 1999; Reiser et al., 1994) and quinone reductase (Jensen Jr et al., 2002) were involved in lignin degradation by converting aromatics.

3.3. Bimodification of lignin compounds by white rot basidiomycetes

Ligninolytic properties of white rot basidiomycetes allow the oxidation of heterogeneous lignin polymers. For elucidating the biodegradation mechanism of lignin by lignin degrading enzymes, most researchers have conducted an experiment with lignin compounds such as dimers and synthetic lignin.

First of all, ligninolytic enzymes of *P. chrysosporium* lead to degradation of various lignin model compounds. C_α-C_β, β-ether linkage and ring fission of β-O-4 model compounds were reported to be caused by *P. chrysosporium* (Enoki et al., 1981; Higuchi, 1990; Weinstein et al., 1980). Especially, side chain cleavages of β-O-4 model compounds by LiP of *P. chrysosporium* was described clearly by many researches (Higuchi, 1986; Kersten et al., 1985; Umezawa & Higuchi, 1985). LiP secreted from *P. chrysosporium* was also reported to catalyze not only C_α-C_β cleavage of β-1 compounds (Glenn et al., 1983; Kirk & Nakatsubo, 1983; Tien & Kirk, 1983) but also degradation of β-5 and β-β model compounds (Kamaya & Higuchi, 1983; Nakatsubo et al., 1981; Umezawa et al., 1982). And, laccase of *Coriolus versicolor* catalyzed C_α-C_β and O-C_β cleavages of β-O-4 model compounds, and C_α-C_β cleavage, alkyl-phenyl cleavages, and C_α oxidation in β-1 model compounds (Higuchi, 1990; Kawai et al., 1988a; Morohoshi et al., 1987). Based on these studies, we could understand how ligninolytic enzymes generate cleavage fragments from lignin model compounds.

For better comprehension of natural lignin biodegradation, studies on degradation of synthetic lignins containing similar inter monomer structures have been progressed. Especially, degradation experiments of DHP composed of β-O-4 and β-β by LiP were carried out. Gas chromatography-mass spectrometry (GC-MS) analysis showed that cyclic carbonate, formate, and

arylglycerol and so on were formed as degradation products (Umezawa & Higuchi, 1989). Most degradaion of DHPs was demonstrated by mineralization experiments of ^{14}C-labelled synthetic lignin. Rüttimann-Johnson et al. (1993) examined relationships between the mineralization rate of synthetic lignin and the activities of MnP and laccase by isotope trapping method. Various ^{14}C-volatile compounds including $^{14}CO_2$ released from ^{14}C-labeled synthetic lignin were measured (Haider & Trojanowski, 1975; Steffen et al., 2000). DHPs biodegradation was also evaluated by analyzing molecular weight of DHPs by the gel permeation chromatography (GPC) after fungal ligniniolytic treatment. Most studies showed that ligninolytic enzyme system of white rot basidiomycetes were involved in degradaion of synthetic lignin (Kawai et al., 1999; Srebotnik & Hammel, 2000; Yoshida et al., 1998).

In contrast with degradation and mineralization of synthetic lignins, some researchers found that polymerization of the lignin occurred in cultures treated with LiP (Haemmerli et al., 1986; Odier et al., 1987). These studies indicated that aryl cation and phenoxy radicals formed by ligninolytic enzymes induced repolymerization reactions as well as degradation. Thus, for preventing repolymerization of synthetic lignin during ligninolytic treatment, studies using the organic solvents and reducing agents were conducted (Yoshida et al., 1998). Ascorbic acid, one of the reducing agents, plays an important role in prevent repolymerization of lignin components (Kinne et al., 2009; Kinne et al., 2011).

Consequently, studies on biomodification of lignin model compounds and DHP by lignin degrading enzymes help to understand each catalytic enzyme system of ligninolytic enzymes and degradation mechanism of lignin. However, it is still insufficient to understand complex enzyme system of white rot basidiomycetes and apply modification process to lignin valorization. Therefore, further investigations are needed for useful application of lignin and white rot basidiomycetes.

3.4. Molecular biological approach for investigation of enzymes related to lignin modification

Due to the enormous progress in analytical equipments such as electrophoretic, genomic sequencing, and mass spectrometric techniques, studies on secretomes and protetomes of basidiomycetes have advanced. As the large amount of biological data has accumulated like gemones, transcriptomes and proteomes data, it has been easy to acquire information about enzymes and its biological function. There are two approaches for investigating function of enzymes with microorganism as follows: one is analysis at transcriptional level and the other is analysis at proteome level.

Transcriptomic profiling is carried out based on cDNA synthesized from mRNA. Investigating the transcriptomes is essential for interpreting the functional elements of the genome and understanding of phenotypic diversity (Morozova et al., 2009). This transcriptome investigation based on genome resource has been facilitated identification of various proteins using electrophoresis and mass spectrometroscopy (Shimizu et al., 2005; Wymelenberg et al., 2006a). Among basidiomycetes, genomic annotation of *P. chrysosporium* has been sequenced by the US Department of Energy's Joint Gemone Instutite, which provides high quality sequence of basidiomycetes (Martinez et al., 2004). Accordingly, studies on transcriptome analyses of *P. chrysosporium* have been progressed. As a result of analyzing the transcriptome of *P. chrysosporium*, transcripts encoding enzymes related to cellulose, hemicellulose and lignin degradation were expressed, and new transcripts that encode extracellular proteins with unknown function were revealed (Sato et al., 2009). In addition, Wymelenberg's group demonstrated distinct expression patterns of extracellular oxidoreductase-encoding genes. Under the nitrogen limited condition, expression of genes functioning in LiP

and MnP of *P. chrysosporium* were increased, and under the condition containing microcrystalline cellulose, genes encoding enzymes being active to carbohydrates were upregulated (Wymelenberg et al., 2009; Wymelenberg et al., 2010). Consequently, functions of enzymes related to lignocellulose degradation through the transcriptomic analysis were revealed.

Proteomic approach provided comprehensive identification and assessment of proteins. Among the reports concerning proteins of basidiomycetes, secretomes of *P. chrysosporium* have been nearly reported (Abbas et al., 2005; Sato et al., 2007). Most proteomic analysis was performed through the electrophoresis and mass spectrometric technique. Secretomes of *P. chrysosporium* were largely classified to enzymes groups related to cellulose hydrolysis and lignin degradation. Under the condition of ligninolytic culture, LiP and MnP secreted from *P. chrysosporium* were detected in 2 dimensional gel map. In contrast, under the condition of biopulping condition on softwood substrate, enzymes spots such as β-glucosidase, β-mannanase, and exocellobiohydrolase were mainly detected (Ravalason et al., 2008). Expression pattern of extracellular enzymes of *P. chrysosporium* was differently displayed depending on culture condition and a kind of substrate (Adav et al., 2012; Sato et al., 2007). Studies on proteomic analyses of *P. chrysosporium* also described various intracellular enzymes functioning in aromatic compounds degradation. Cytochrome P450s, glutathione S transferase, alcohol dehydrogenase, benzoquinone reductase and etc. were reported to be upregulated in lignin catabolic pathway (Adav et al., 2012; Matsuzaki et al., 2008). *P. chrysosporium* exposed to aromatic compounds such as vanillin and benzoic acid was reported that metabolic systems such as tricarboxylic acid cycle and glyoxylate cycle were activated during aromatic compounds degradation (Matsuzaki et al., 2008; Shimizu et al., 2005).

Chapter 2

Biomodification of monolignols
by white rot basidiomycetes
with reducing agent

1. Introduction

In lignin synthesis, oxidative coupling of lignin precursors proceeds via radical reaction (Higuchi, 1990; Ö nnerud et al., 2002; Russell et al., 1996). Radicals formed in lignin were polymerized randomly under the enzyme- and oxidant-controlled conditions. Some research suggested lignin polymerization occurs by oxidant which is called as redox shuttle as well as redox enzymes. They take a role to oxidize both phenolic end groups in lignin and monolignols, and manganese ion, calcium ion and superoxide anion were introduced as candidate for redox shuttle (Ö nnerud et al., 2002; Westermark, 1982). Therefore, lignin polymerization can occur differently depending on enzymatic reaction and surrounding condition.

In common with lignin synthesis, lignin degradation is also caused by redox enzymes. White rot basidiomycetes are one of microorganisms degrading lignin macromolecule through the secretion of lignin degrading enzymes. Lignin degrading enzymes are classified to oxidoreductase, and both peroxidases and laccase initiate free-radical reactions in which the generated radicals may attack the lignin network (Hofrichter, 2002b; Schmidt, 2006; Wong, 2009).

Accordingly, both lignin synthesis and degradation occur by radical reaction initiated by redox enzymes. However, because lignin degrading enzymes oxidize lignin in multi-step electron transfers with the formation of radical cation, formed radicals brought about polymerization reaction as well as degradation (Iwahara et al., 2000; Kudanga et al., 2011; Nugroho Prasetyo et al., 2010). Because the manganese peroxidase especially uses Mn^{3+} as an oxidant, that is involved in the polymerization of lignin with the generation of monolignols radical and phenolic radical (Ö nnerud et al., 2002).

Different white rot basidiomycetes were known to have enzymes that

have different specificities or mechanisms for lignin modification. In particular, whole cell of white rot basidiomycetes exhibits complex enzyme system including lignin degrading enzymes. This is why basidiomycetous secretomes comprise a wide range of oxidoreductive and hydrolytic activities (Bouws et al., 2008). Based on these characteristics, white rot basidiomycetes can be used as novel biocatalyst in the biomodification of lignin. Therefore, to apply white rot basidiomycetes to the modification process of lignin, it is essential to understand biomodification mechanism of lignin by white rot basidiomycetes.

In this study, to investigate characteristic biomodification mechanism of lignin components by the white rot basidiomycetes, *Abortiporus biennis* and *Phanerochaete chrysosporium*, biomodification mechanism of coniferyl alcohol and sinapyl alcohol was examined in a whole cell of white rot basidiomycetes. Based on this modification mechanism, the degradation of monolignols by the fungi with the addition of a reducing agent was discussed.

2. Materials and methods

2.1. Materials

2.1.1. Fungi and fungal suspension

In this study, two kinds of white rot basidiomycetes were used as biocatalyst. One was *Abortiporus biennis*, and the other was *Phanaerochaete chrysosporium*(ATCC 20696). *P. chrysosporium* was obtained from the Microbiology Chemistry Laboratory of National Institute of Forest science (NIFoS). *Abortiporus biennis* was owned by this laboratory.

Screening tests were carried out to identify a suitable biocatalyst in previous study. Among various basidiomycetes, *Abortiporus biennis* was selected as outstanding fungus for the biomodification of lignin, showing high ligninolytic enzymes activities. *P. chrysosporium* was used for biomodificaiton of lignin because lignin degradation using by *P. chrysosporium* is a well-studied model (Keyser et al., 1978).

Both *A. biennis* and *P. chrysosporium* were grown on a potato dextrose agar (PDA) medium at 28℃ for one week, and then stored at 4℃. After 7 days of fungi inoculation on PDA medium, the mycelium had fully grown. Mycelia covering the PDA medium were separated from the medium, and homogenized with 20 ml of distilled water at 5,000 rpm for 3 min in ice-water bath. Finally, fungal suspension was obtained. The dry weight of 1 ml of fungal suspension was measured after 24 hours on a dryer at 105℃.

2.1.2. Synthesis of monolignols

Coniferyl alcohol(CA) and sinapyl alcohol(SA) were synthesized from ferulic acid and sinapic acid (Sigma Aldrich Co., South Korea) using the methods of Quideau and Ralph with slight modifications (Quideau & Ralph, 1992). Methyl ferulic acid and methyl sinapic acid were prepared from ferulic acid and sinapic acid, respectively by stirring overnight with 150 ml of methanol and 15 ml of acetyl chloride. After reaction was completed, it was evaporated and dissolved in ethyl acetate. And then, evaporation was conducted again. Methyl ferulic acid and methyl sinapic acid in toluene (300 ml) was cooled in an ice-water bath under nitrogen gas and 120 ml of diisobutyl aluminum hydride (Sigma, 1M solution) was slowly added. After reation for 2 hrs, the reaction mixture was then carefully quenched with 20 ml of ethanol. The solvents were removed in vacuum condition. 100 ml of deionized water was added, and the water layer containing a gelatinous precipitate of aluminum salts was extracted with 150 ml of ethyl acetate. This was repeated 4 times. Collected ethyl acetate fraction was sequentially washed by 3% of sodium bicarbonate solution, 0.1M of hydrochloric acid, water saturated with sodium chloride and deionized water. Ethyl acetate fraction was evaporated to dryness in vacuum. Each CA and SA obtained was dried in desiccator overnight.

The yield of CA and SA obtained from ferulic acid and sinapic acid was more than 70% and 55% over, respectively. Syntheszied CA and SA were confirmed by gas chromatography-mass spectrometry (GC-MS) and carbon-13 nuclear magnetic resonance (^{13}C- NMR) analysis.

GC-MS was performed on an Agilent HP7890A GC, equipped with an Agilent HP5975A mass selective detector (MSD). The stationary phase of the GC-MS was a DB-5 capillary column (30 m x 0.25 mm ID x 0.25 um coating

thickness). Injection and detector temperatures were set to 220 ℃ and 300 ℃, respectively, and the oven temperature was increased at a rate of 5 ℃/min from 100 ℃ to 280 ℃ and the final temperature was maintained for 8 min. The split ratio was 20:1. To identify the structure of monolignols, using ^{13}C-NMR spectrometer (ADVANCE 600, Bruker, Karlsruhe, Germany), 50 mg of monolignols were dissolved in dimethyl sulfoxide-d_6 (DMSO, Sigma-Aldrich Co.) and NMR data were recorded for 1 hr at 60 ℃ using a Bruker AVANCE spectrometer at 600 MHz.

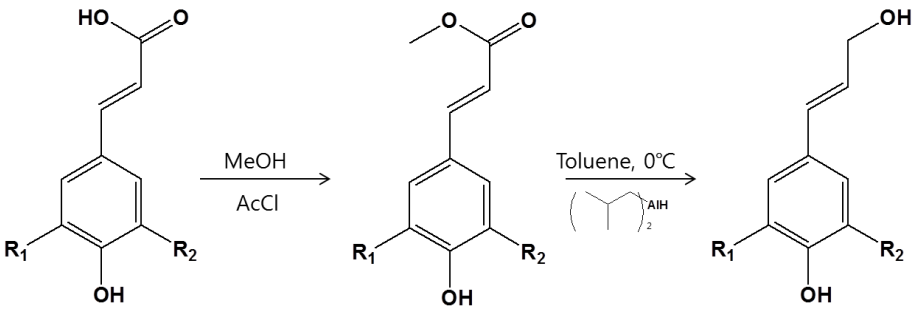


Figure 2-1. The synthesis of lignin precursors

(CA(G-unit): R_1=H, R_2=OCH_3, SA(S-unit): R_1= OCH_3, R_2=OCH_3)

2.2. Biomodification of monolignols by white rot basidiomycetes

Biomodification of monolignols was carried out focusing on analysis of metabolites by white rot basidiomycetes. The shallow stationary culture (SSC) medium was used as nitrogen limiting medium. SSC medium was proposed by Kirk group for the secretion of ligninolytic enzymes (Kirk & Farrell, 1987). To prepare a SSC medium, major components of medium were dissolved in 990 ml distilled water (Table 2-1). After the medium was autoclaved at 121℃ for 15 min, 10 ml of the trace element solution (Table 2-2) were added after filtration through 0.2 μm membrane filter. Certain volume of fungal suspension including 0.02 g of dried fungal hyphae was inoculated into 100 ml of SSC medium, and the flask was plugged with silistopper. After stationary preincubation at 28℃ for 4 days, each 40mg of CA and SA was spiked in the medium.

The flasks were withdrawn from incubator periodically. Sample treated by *A. biennis* was analyzed at incubation time 2, 4, 6, 8, 12, 24 hrs after addition of monolignols, and sample treated by *P. chrysosporium* was analyzed every 5 days during 25 days. Each sample was centrifuged at 12,000 rpm, 15 min at 4℃ to separate mycelium. After centrifugation, the supernatant was loaded to Sep-Pak C18 cartridge (Waters) slowly. Sample loaded in cartridge was eluted by passing methanol, and then methanol was evaporated under vacuum condition to remove the solvent. Finally, it was dissolved in 10 ml of methanol and extracted with 25 ml of ethyl acetate with using separating funnel at 250 rpm shaker for 15 min, and this solvent extraction was carried out 3 times. Extraction using ethyl acetate was conducted with addition of sodium chloride which caused salting-out effect. Anhydrous sodium sulfate was added in liquid sample, and then the collected ethyl acetate fractions were

filtrated, and evaporated under vacuum condition to remove the solvent. Finally, it was dissolved in 5 ml of ethyl acetate.

For the analyzing analysis of the products of monolignols separated by Sep-Pak C18 cartridge, liquid chromatography-mass spectrometry (LC-MS) was performed using Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap instrument equipped with a Dionex UltomateUltimate 3000 RSLC nano HPLC system. The mobile phase consisted of deionized water with 0.1% of formic acid (solution A) and acetonitrile with 0.1% of formic acid (solution B). Flow rate of mobile phase was 0.15 ml/min. The gradient was linearly increased from 10% to 60% of solution B over 17 min. And then it was increased to 90% for 3 min and maintained for 2 min. The gradient was returned to the initial conditions in over 30 seconds and was held for 7.5 min before the next injection. INNO C18 column (young jin biochrom, 5 um, 120 A, 2.0 mm ID x 100 mml) was used for the chromatographic separations. The injection volume was of 10 ul. Ionization of the analytes was carried out using electrospray ionization (ESI). The capillary temperature was maintained at 320℃, the ion source voltage was set at 3.5 kV and the sheath and auxiliary gas was were set at 30 and 5, respectively. The capillary voltage was set at 3.5 kV. The average scan time was 0.01 min, while the average time to change polarity was 0.02 min. The higher-energy collisional dissociation energy was generally chosen in order to maintain about 30% abundance of the precursor ion.

For the analysis of ethyl acetate fractions, gas chromatography-mass spectrometry (GC-MS) was performed on an Agilent HP7890A GC, equipped with an Agilent HP5975A mass selective detector (MSD). The stationary phase of the GC-MS was a DB-5 capillary column (30 m x 0.25 mm ID x 0.25 um coating thickness). The initial oven temperature of the GC was 50℃ for 5 min, and the temperature was then programmed to increase at a rate of 3℃/min up to 300℃, and maintained for 10 min. The temperature of injector

and detector were 220℃ and 300℃, respectively, and the carrier gas was helium at a flow rate of 1 ml/min. Peak identification was based on comparision of the mass spectra with the NIST (National Institutue of Standard and Technology) library.

To determine molecular weight distribution of the monolignols treated by white rot basidiomycetes, gel permeation chromatography (GPC) was used with Shodex KF-801, KF-802, KF-802.5 and KF-803 columns (Showa Denko, Tokyo, Japan). UV detector was used at 280 nm with a solvent of tetrahydrofuran, and the flow rate was 0.7 ml/min (Takada et al., 2004).

Table 2-1. Concentration of SSC medium components (per 1 l deionized water)

Component	Concentration	Amount (g)
Glucose ($C_6H_{12}O_{6)}$	1%	10
Ammonium tartrate ($C_4H_{12}N_2O_6$)	1.08 mM	0.2
Potassium phosphate (KH_2PO_4)	14.7 mM	2
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	2.03 mM	0.5
Calcium chloride ($CaCl_2$)	0.68 mM	0.1
Thiamine·HCl ($C_{12}H_{17}ClN_4OS \cdot HCl$)	2.97 mM	0.001
Trace element solution	1%	

Table 2-2. Concentration of each component of trace element solution (per 1 l deionized water)

Component	Concentration (mM)	Amount (g)
$C_6H_9NO_6$	7.8	1.49
$MgSO_4 \cdot 7H_2O$	0.12	3
$MnSO_4 \cdot H_2O$	2.9	0.5
NaCl	17	1
$FeSO_4 \cdot 7H_2O$	0.359	0.1
$CoCl_2$	0.775	0.08
$CaCl_2$	0.9	0.1
$ZnSO_4 \cdot 7H_2O$	0.348	0.1
$CuSO_4 \cdot 5H_2O$	0.04	0.01
$AlK(SO_4)_2 \cdot 12H_2O$	0.021	0.01
H_3BO_3	0.16	0.01
$NaMoO_4 \cdot 2H_2O$	0.041	0.01

2.3. Biomodification of monolignols with addition of reducing agent by white rot basidiomycetes

To prevent polymerization of monolignols reactants by white rot basidiomycetes, 5mM of ascorbic acid was added to the SSC medium as a reducing agent. Ascorbic acid was added 12hrs before to analyze biomodification products of lignin by *A. biennis*. In experiment using *P. chrysosporium*, addition of ascorbic acid was conducted 2 days before to extract and analyze.

The biomodification products by two fungi were analyzed as described at 2.2.

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2.4. Protein quantification and enzyme assay

Protein was determined by Bradford method with 1 mg/ml of bovine serum albumin (BSA; Sigma Chemical Co., USA) as a standard (Bradford, 1976). Calibration curve was generated using standard solution of BSA. The reaction mixture contains 1 ml of Bradford reagent and 0.1 ml of sample solution. The protein concentration was determined by using absorbance of reactants at 595 nm within 1hr and calculated as follows; y=2.6991x+0.0017 where y: aborbance at 595 nm, x: protein concentration (mg/ml).

To examine the relationship between the biomodification of monolignols and lignin degrading enzymes, the activities of the lignin degrading enzymes were measured. At the time at which monolignols were converted to other metabolites, the extracellular solution was separated at 12,000 rpm for 15 min by centrifugation. The reaction was conducted in ice-water bath.

LiP activity was measured by the rate of oxidation of veratryl alcohol to veratraldehyde. The veratryl alcohol oxidation assay was run with 100 μl of culture medium in 50 μl of 2.5 mM veratryl alcohol (veratryl alcohol; ε_{310} = 9,300 $M^{-1}cm^{-1}$), 50 μl of 0.1 mM H_2O_2, and 800 μl of 20 mM lactate buffer, pH 3.0, in a final volume of 1 ml. The reactions were started by H_2O_2 addition and were monitored at 310 nm for 3 min (Koduri & Tien, 1994). MnP and laccase activities were measured using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; ε_{420} = 36,000 $M^{-1}cm^{-1}$) as a substrate. The reaction mixture for MnP was prepared 0.8 ml of 0.2 M lactate buffer (pH 4.5) with 50 μl of ABTS (0.8g/l), 33 μl of 6mM $MnSO_4$, and 100 μl of culture medium. Next, 17 μl of 0.1 mM H_2O_2 was injected. The absorbance at a wavelength of 420 nm was measured after 30min (Krčmář et al., 1999). The reaction mixture for laccase contains 0.85 ml of 0.2 M lactate buffer (pH 4.5), 50 μl of ABTS, and 100 μl extracellular enzyme solution at a total volume of 1 ml. The

absorbance of that mixture was monitored at 420 nm after 3 min of incubation. The enzyme activity was expressed using the equation below (Hong et al., 2013; Krčmář et al., 1999).

$$\text{Ligninolytic enzyme activity (unit/mg)} = \frac{\Delta\text{Absorbance} \times 10^6 \times \text{Total volume}}{\Delta\text{Time} \times \varepsilon_{310 \text{ or } 420} \times \text{Sample weight}}$$

3. Results and Discussions

3.1. Indentification of synthesized monolignols and analysis of recovery amount of monolignols treated by white rot basidiomycetes

Synthesized CA and SA were identified by GC-MS and ^{13}C-NMR. Results of GC-MS showed purity of CA and SA was 98.2% and 99.4%, respectively (Figure 2-2). On the ^{13}C-NMR spectra, signals for aromatic carbons are visible between 150 ppm and 100 ppm and resonances for lignin side chains appear between 90 and 60 ppm (Lin & Dence, 2012; Moon et al., 2012). As shown in Figure 2-2, analysis of CA revealed G-type C3/C4 at 150–145 ppm, G-type C1 at 135–125 ppm and G-type C2/C6 at 120–110 ppm, respectively. Meanwhile, result of SA indicated that signals in the regions 150 ppm and 105 ppm were assigned to the C3/C5 and C2/C6 for S-type lignin (Figure 2-2). And the ^{13}C-NMR spectra of the monolignols had singal at 55ppm in common, which indicated methoxyl group of aromatic ring.

Recovery amount of monolignols was only analyzed in methanol extraction fractionation using Sep pak cartridge. Structural difference of two monolignols indicated different recovery rate. Amount of coniferyl alcohol remained in Sep pak cartridge was under the 15 mg, and amount of sinapyl alcohol was about 30 mg in control.

Coniferyl alcohol was degraded rapidly within 24 hr by *A. biennis*, while it treated by *P. chrysosporium* was very slowly degraded by incubation time 360 hr. Results on sinapyl alcohol were also similar to those of coniferyl alcohol (Figure 2-3).

Consequently, *A. biennis* consumed rapidly substrates, on the other hand,

substrate uptake ability of *P. chrysosporium* was very slow. *A. biennis* has MnP-LAC group as shown in Figure 2-14 and *P. chrysosporium* has LiP-MnP group Figure 2-24. Two basidiomycetes have different enzyme system, which had effect on different specificity about substrates. Especially, whole cell of basidiomycetes exhibited complex enzyme system including extracellular ligninolytic enzymes system (Bouws et al., 2008). That was why the difference of degradation rate of monolignols was showed by two fungi.

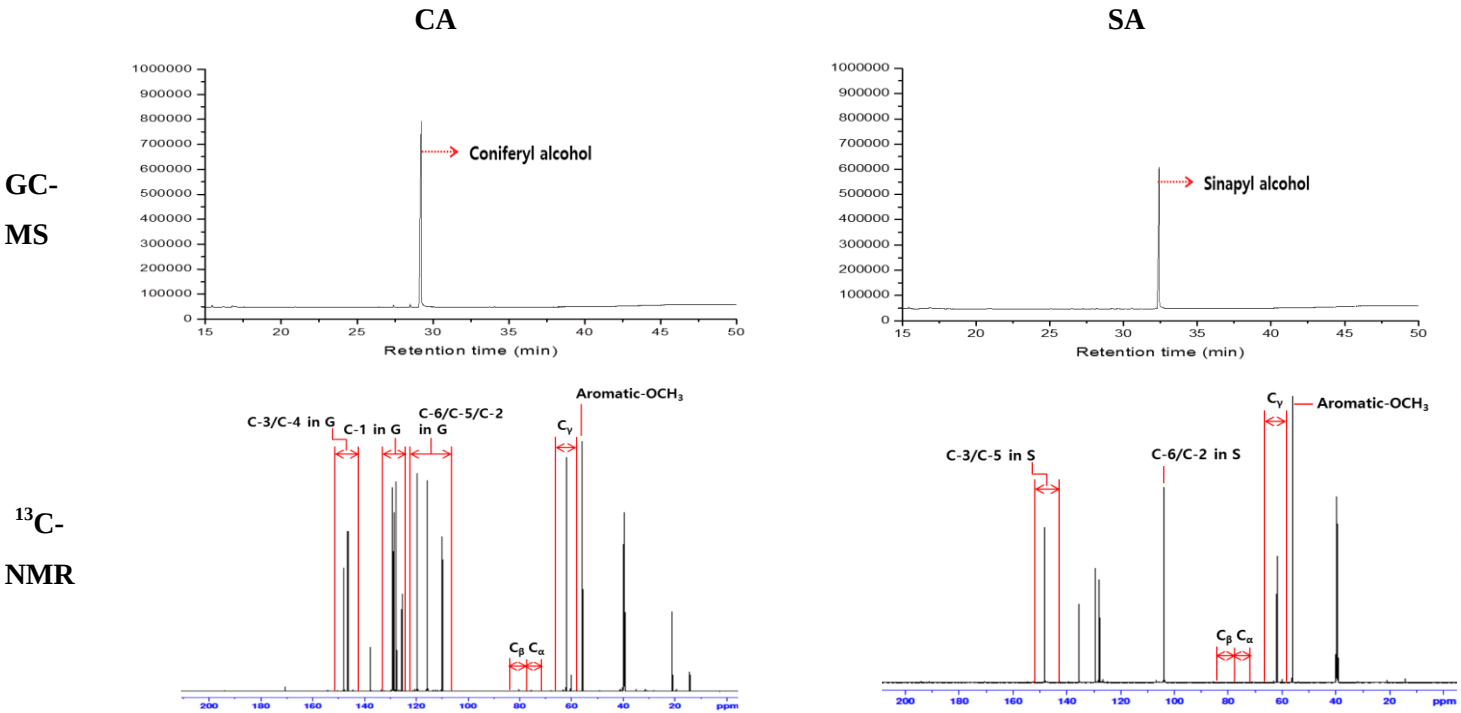
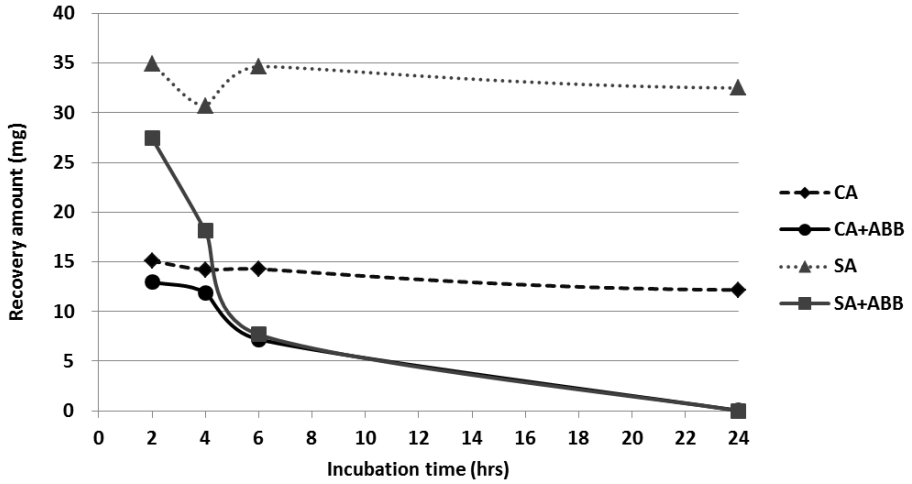


Figure 2-2. GC-MS chromatogram and ^{13}C-NMR spectrum of coniferyl alcohol (CA) and sinapyl alcohol (SA)

(A)



(B)

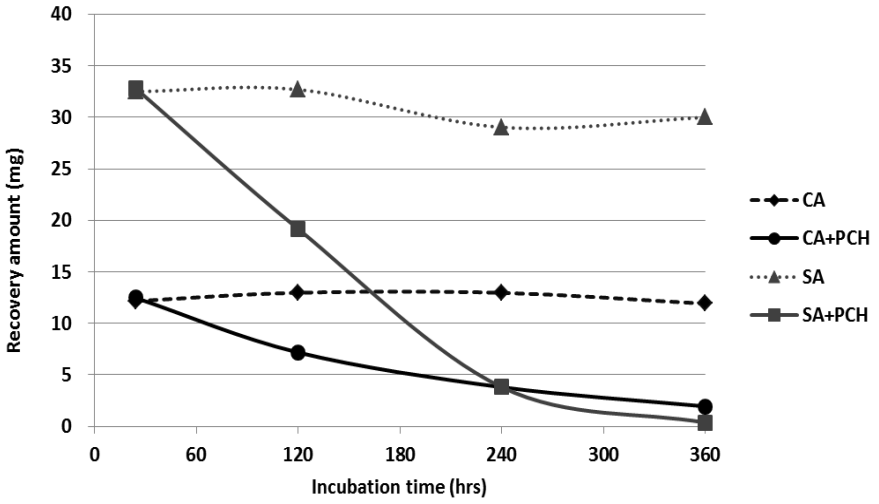


Figure 2-3. Recovery amount of monolignols treated by (A) *A. biennis* and (B) *P. chrysosporium* (CA: coniferyl alcohol in medium, CA+ABB: CA treated by *A. biennis*, SA: sinapyl alcohol in medium, SA+ABB: SA treated by *A. biennis*, CA+PCH: CA treated by *P. chrysosporium*, SA+PCH: SA treated by *P. chrysosporium*)

3.2. Degradation and polymerization mechanism of monolignols by white rot basidiomycetes

3.2.1. Modification products of monolignols by *A. biennis*

3.2.1.1. Modification products of coniferyl alcohol

The biomodification reaction of monolignols was continued for 24 hrs. At 2, 4, 6, 12 hrs after the addition of monolignols, the culture medium was extracted by methanol and ethyl acetate. As mentioned above, because *A. biennis* showed an excellent ability to uptake substrate, the time to degrade the substrates was completed within 24 hrs.

Methanol fraction extracted from Sep pak cartridge was analyzed by LC-MS and GPC, and ethyl acetate fraction was analyzed by GC-MS.

At first, the results of LC-MS analysis showed that coniferyl alcohol in the control appeared at 6.1 min (Fig.2-4(A)). In sample treated by *A. biennis*, substrate peak was reduced rapidly and new product peaks were shown at 6.3 min, 7.3 min and 7.4 min, and the peak areas increased with the incubation time (Figure 2-4(B)). The mass spectrum of these peaks was analyzed by LC-MS-MS.

The m/z of main peaks were 153, 341, and 359 in total ion chromatogram of LC-MS at 6.3 min (Figure 2-5). Basepeak 153 was additionally analyzed by LC-MS-MS, which was identified as vanillin. The peak at 6.3 min was identified as product which has a molecular formula of C20H23O6. Accordingly, it was considered that this new product was dimer containing vanillin.

The peak at 7.3 min was formed from an incubation time 2 hrs, and nearly disappeared after an incubation time of 6 hrs. It was confirmed as coniferyl

aldehyde by LC-MS-MS, indicating that precursor ion was 179 (Figure 2-6).

LC-MS analysis of the 7.4 min peak indicated that the m/z of peaks were 137, 341 and 359 in MS spectrum. This peak would be expected to dimer including the 137 peak which has a molecular formula of C8H8O2 as degradation product derived from coniferyl alcohol (data not shown).

Furthermore, as a result of molecular weights analysis of modification products of coniferyl alcohol after the fungal treatment, the weight- average molecular weight(Mw) of fungal sample increased sharply with the increase of the incubation times (Table 2-3). GPC chromatograms of fungal sample showed the difference depending on incubation time (Figure 2-7). From incubation time 4hrs, division of high molecular weight fraction and low molecular weight fraction started, and they were separated clearly on incubation time 24 hrs. On incubation time 6hrs, various modified products were generated showing separation of three peaks in chromatogram.

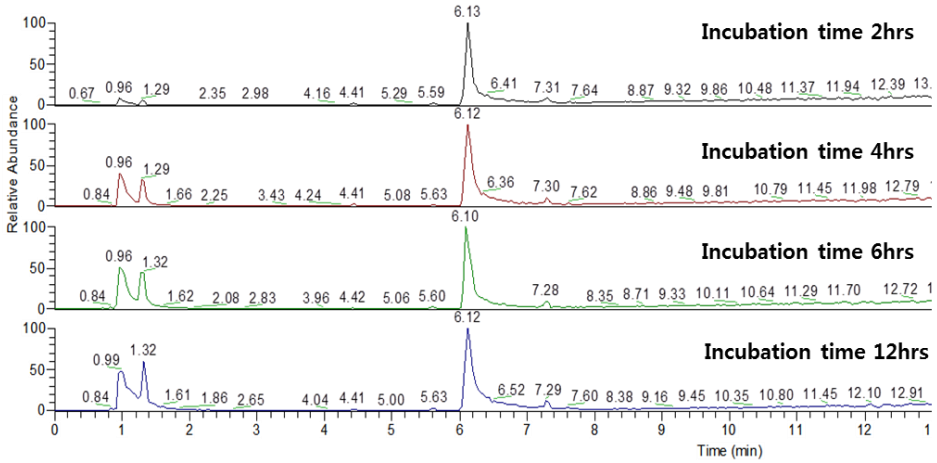
These results suggested that *A. biennis* simultaneously induced degradation and polymerization reaction. Generally, ligninolytic enzyme systems of white rot basidiomycetes were reported to bring about degradation of lignin (Higuchi, 1986; Kawai et al., 1999; Kirk & Farrell, 1987). The *A. biennis* enzyme system had an effect on C_α and C_γ oxidation by cleaving side chains, and producing vanillin and coniferyl aldehyde. These results were consistent with previous studies demonstrating that ligninolytic enzymes secreted from white rot basidiomycetes catalyzed C_α-C_β cleavage, alkyl-phenyl cleavages, and C_α oxidation in lignin model compounds (Higuchi, 1990; Kawai et al., 1988a; Morohoshi et al., 1987). Although the specific activities of the lignin degrading enzymes of *A. biennis*, manganese peroxidase and laccase were very lower than control (Figure 2-14), total activities of these enzymes were higher than those of control. Furthermore, the extracellular protein concentration increased sharply with addition of monolignols, so these results led to the possibility that complex extracellular

enzymes including lignin degrading enzymes were involved in the biomodification of monolignols.

Along with degradation products, a dimer was also formed by polymerization during the incubation, contrary to expectation. Because of the lack of a database in the mass spectrometry library, it was difficult to characterize chemical structure of dimers in detail. LC-MS and GPC analysis of methanol fractionation supported that polymerization reaction was caused by enzymes of *A. biennis*. Lignin degrading enzyme system brought about one electron oxidation of lignin molecule to form cation radicals. Reactive radicals were formed in various positions in monolignol by fungus (Ö nnerud et al., 2002), and then underwent a number of polymerization reactions that included coupling to form dimers and oligomers through various bonds including C–C, C–O and C–N (Claus, 2004; Kudanga et al., 2011) on top of the degradation of complex polymers by cleavage of covalent bonds and ring cleavage of aromatic compounds (Breen & Singleton, 1999; Kawai et al., 1988b). Many research groups have studied production of polymers using purified manganese peroxidase and laccase (Maijala et al., 2012; Rüttimann-Johnson & Lamar, 1996; Yoshida et al., 1998). As mentioned above, because total activities of extracellular enzymes were higher than control, increase of MW was affected somewhat by redox enzymes. Thus, further studies should be conducted to predict the involvement of enzymes in biomodification of monolignols through the transcriptome and proteomic approaches.

Consquently, enzyme system of *A. biennis* simultaneously catalyzed both cleavage of side chain and coupling reaction of coniferyl alcohol. As a result, unstable coniferyl alcohol-derived compounds seemed to be polymerized under the oxidation condition.

(A)



(B)

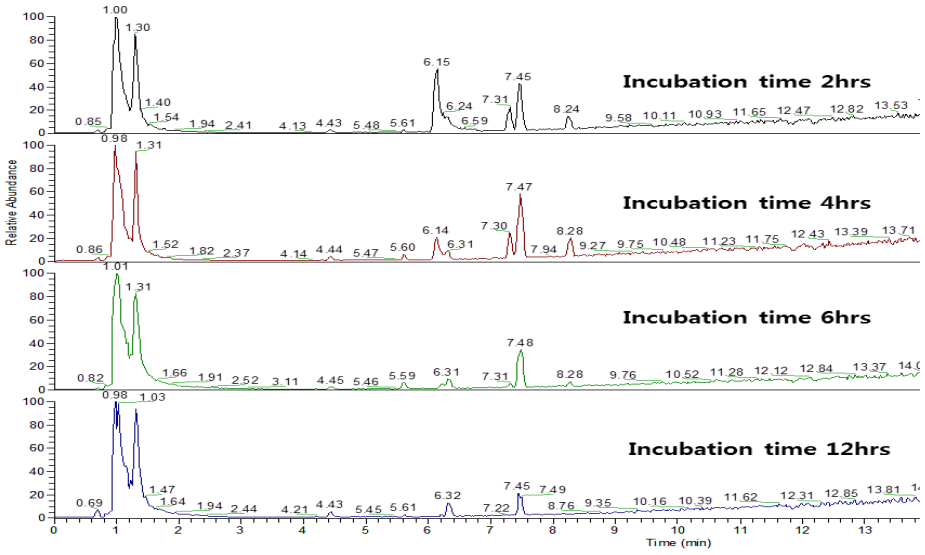


Figure 2-4. Total ion chromatograms of control, coniferyl alcohol in medium, (A) and sample treated by *A. biennis* (B) at incubation time 8hrs by LC-MS analysis

(A)

(B)

(C)

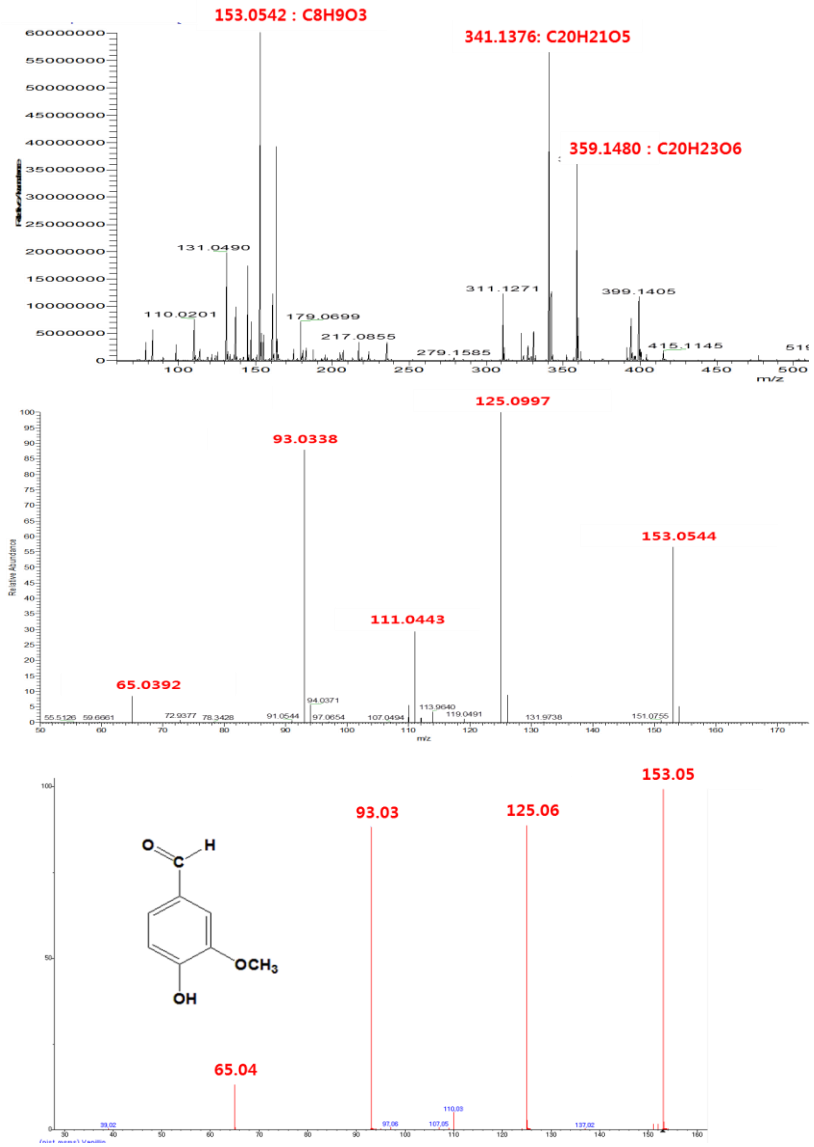
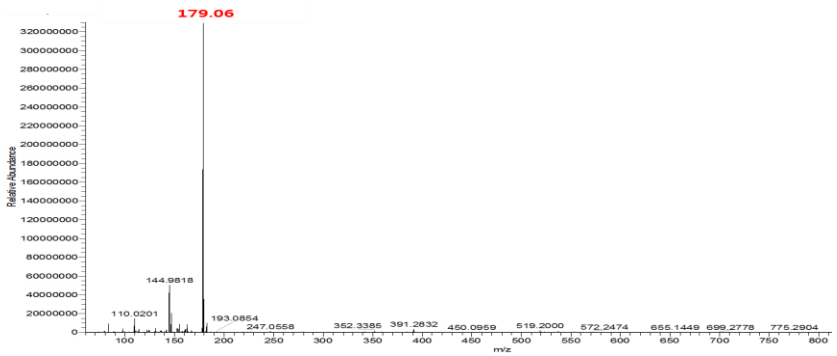
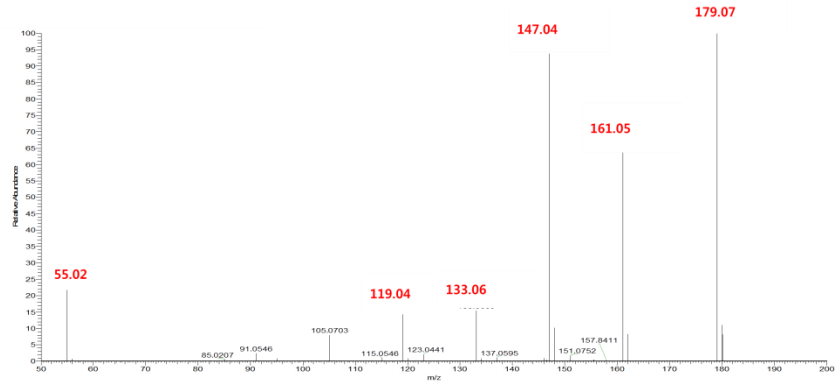


Figure 2-5. LC-MS spectrum of 6.3 min peak of biomodified product on incubation time 6 hrs (A), and LC-MS-MS spectrum of 153 (B) with MS-MS library data of vanillin (C)

(A)



(B)



(C)

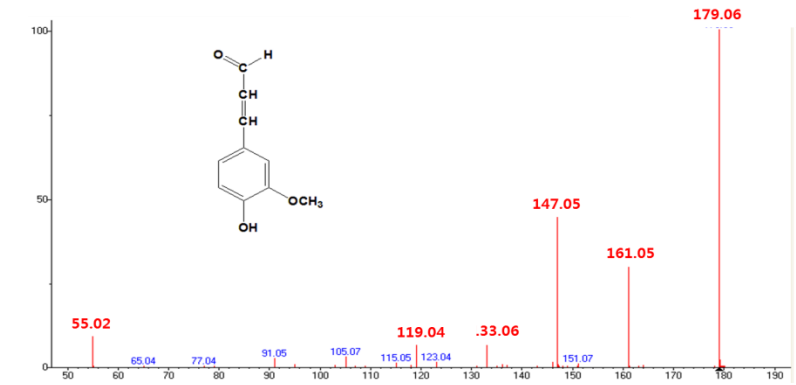


Figure 2-6. LC-MS spectrum of 7.3 min peak of biomodified product on incubation time 2 hrs (A), and LC-MS-MS spectrum of 179 (B) with MS-MS library data of coniferyl adehyde (C)

Table 2-3. Molecular weight of modification products of coniferyl alcohol by *A. biennis* ((A) Control: coniferyl alcohol in medium, (B) Sample treated by *A. biennis*)

(A)

	2hr	4hr	6hr	12hr	1d
M_n [a] (Daltons)	332	343	324	317	352
M_w [b] (Daltons)	505	536	539	491	578
Mw/Mn	1.52	1.56	1.66	1.55	1.64

(B)

	2hr	4hr		6hr			12hr		1d	
	1	1	2	1	2	3	1	2	1	2
M_n [a] (Daltons)	375	595	169	1001	328	175	985	202	1153	205
M_w [b] (Daltons)	541	768	175	1370	332	181	1336	215	1726	217
Mw/Mn	1.44	1.29	1.04	1.37	1.01	1.03	1.36	1.06	1.50	1.06

[a] number-average molecular weight

[b] weight-average molecular weight

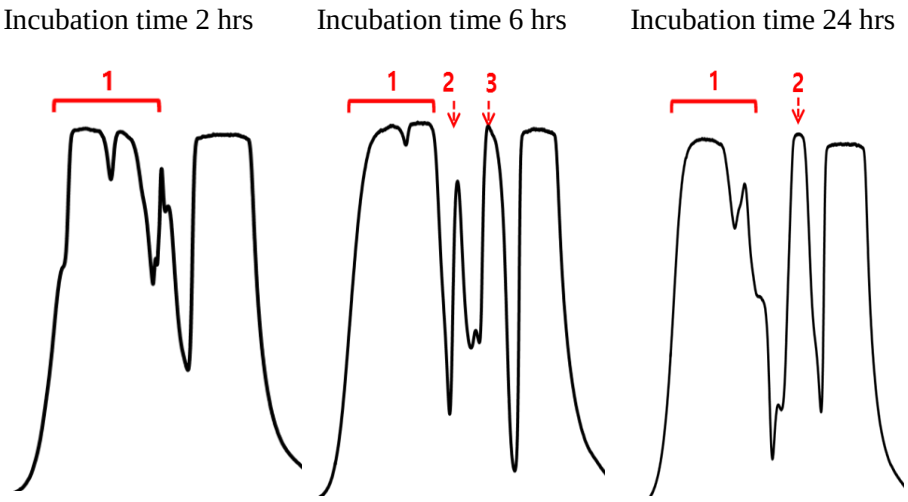


Figure 2-7. Gel permeation chromatograms of coniferyl alcohol treated by *A. biennis* during incubation time

3.2.1.2. Modification products of sinapyl alcohol

The biomodification reaction of sinapyl alcohol was also carried out similar to that of coniferyl alcohol. *A. biennis* also showed good ability to modify substrate rapidly. This result contradicted shortcoming that biomodification by basidiomycetes is time consuming process.

As a result of LC-MS analysis of methanol fraction, sinapyl alcohol was detected at 8.2 min. At incubation time of 6 hrs and 8 hrs, new modification products were detected at 7.0 min and 7.8 min (Figure 2-8). The base ion of peak at 7.0 min was 169, which had a molecular formula of C8H9O4 as determined by LC-MS-MS (Figure 2-9). However, its accurate structure was not confirmed by the MS-MS library data. Meanwhile, GC-MS analysis of ethyl acetate fraction provided modification product of sinapyl alcohol was 2,6-dimethoxy benzene-1,4-diol (Figure 2-11) which has similar formula with the substance suggested by LC-MS analysis. Therefore, 2,6-dimethoxy benzene-1,4-diol was judged to be the main degradation product of sinapyl alcohol.

Additionally, the polymerized modification product was detected by LC-MS analysis. The small peak formed at 7.8 min had 169 m/z and 433 m/z as main peaks in LC-MS spectrum (Figure 2-10). The molecular formula of 433 was identified as C22H25O9, and was expected to form dimers containing 2,6-dimethoxy benzene-1,4-diol, because the 169 peak was 2,6-dimethoxy benzene-1,4-diol.

The main degradation products, 2,6-dimethoxy benzene-1,4-diol could be formed by C_{aryl}- C_{alkyl} bond cleavage and oxidation from sinaplyl alcohol. Laccase and manganese peroxidase were reported to degrade lignin model compounds composed of syringyl type compounds such as syringic acid and syringylaldehyde and to be capable of forming 1,4-benzoquinone type compound by C_{aryl}- C_{alkyl} bond cleavage and oxidation (Faure et al., 1996;

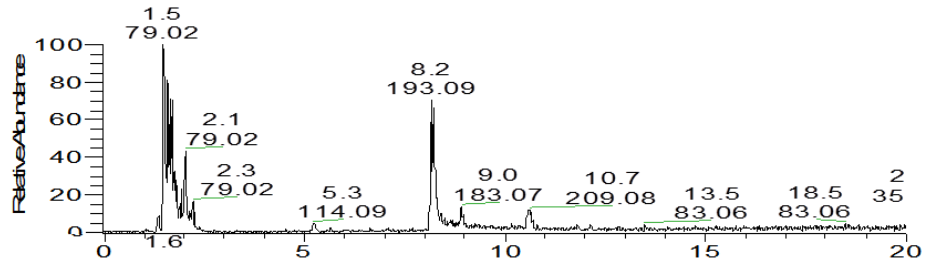
Hofrichter, 2002b). The activities of the lignin degrading enzymes were similar or slightly higher than those of control (Figure 2-14). According to this result, lignin degrading enzymes can be inferred to have some effect on the biodegradation of sinapyl alcohol.

Likewise results of coniferyl alcohol, GPC analysis suggested that *A. biennis* make an induction of polymerization of sinapyl alcohol overall. With increase of incubation time, Mw of fungal sample increased (Table 2-4), and gel permeation chromatogram also indicated similarily to that of coniferyl alcohol (Figure 2-12).

Compared with the results of coniferyl alcohol, the degree of increase of the molecular weights was less-, and the side chains of sinapyl alcohol were more highly oxidized than those of coniferyl alcohol. Because sinapyl alcohol is substituted with methoxyl groups at both C_3 and C_5 in the aromatic ring, radicals can be formed on the OH-group in the aromatic ring and at the β-position in the side chain. In contrast, coniferyl alcohol can form radicals on the OH-group, at the β-position and also at C_5 due to the absence of a methoxyl group. Accordingly, oxidation of side chain of sinapyl alcohol progressed actively, while radical coupling reactions of coniferyl alcohol were more active.

Consequently, *A. biennis* caused polymerization and degradation reaction of sinapyl alcohol at the same time, which was similarly observed in coniferyl alcohol (Figure 2-13). However, compared with results of coniferyl alcohol, side chain of sinapyl alcohol were more oxidized, and the polymerization reaction occured less frequently due to the structural difference of the two monolignols.

(A)



(B)

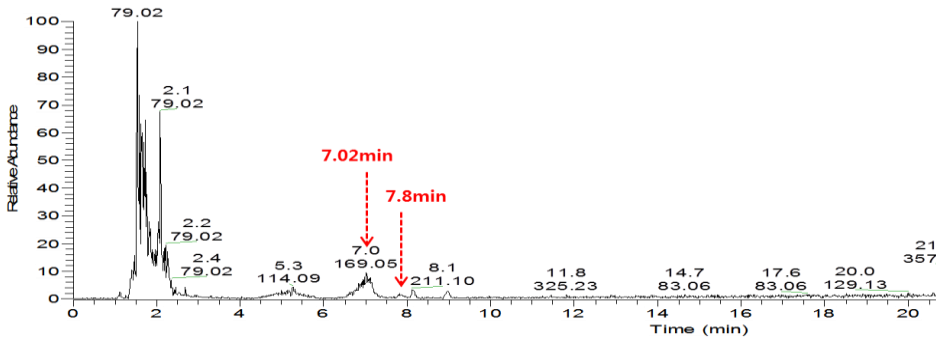
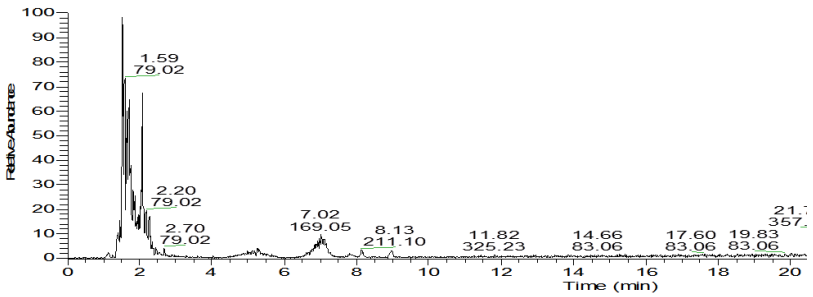
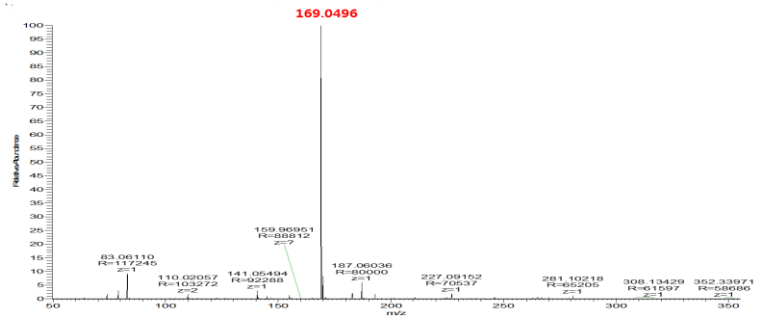


Figure 2-8. Total ion chromatograms of control, sinapyl alcohol in medium, (A) and sample treated by *A. biennis* (B) at incubation time 8 hrs by LC-MS analysis

(A)



(B)



(C)

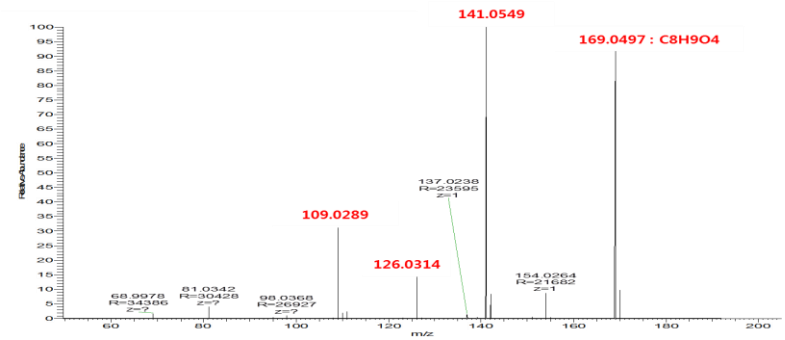
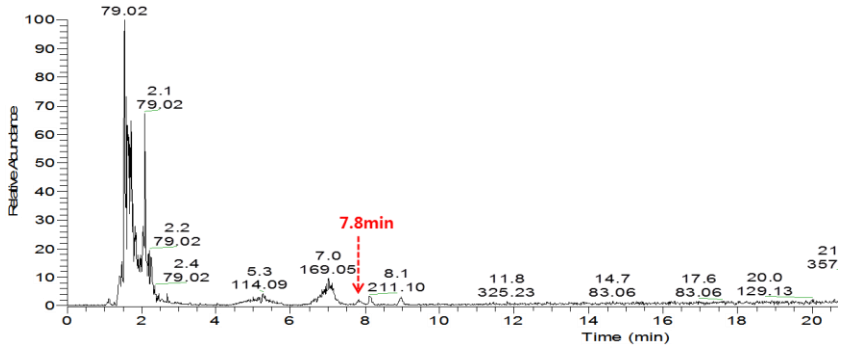
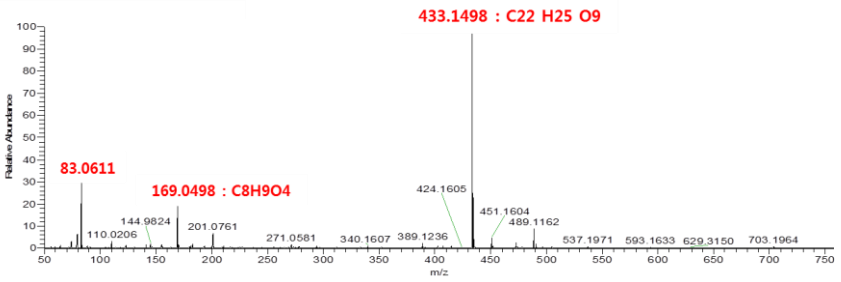


Figure 2-9. Total ion chromatogram of biomodified product on incubation time 8 hrs (A), and LC-MS spectrum of 7.0 min peak (B) with MS-MS spectrum of 169 (C)

(A)



(B)



(C)

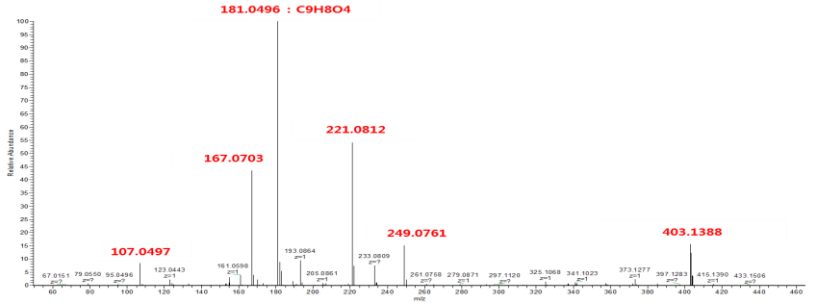


Figure 2-10. Total ion chromatogram of biomodified product on incubation time 6 hrs (A) by LC-MS analysis, and mass spectrum of 7.8 min peak (B) with MS-MS spectrum of 433 peak (C)

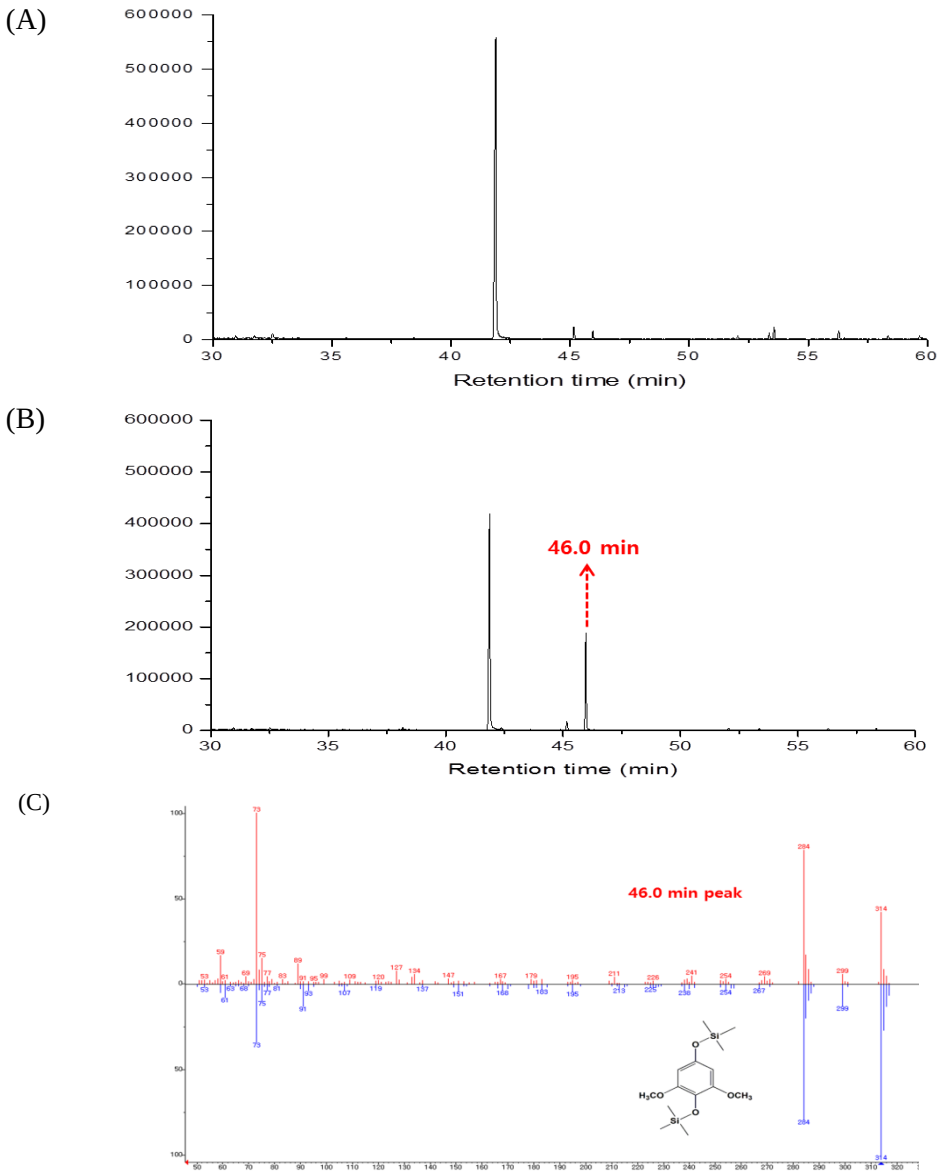


Figure 2-11. Total ion chromatogram of control (A) and biomodified product on incubation time 6 hrs (B) by GC-MS analysis, and mass spectrum of 46.0 min peak with mass library data of 2,6-dimethoxy benzene-1, 4 bis (trimethylsiloxyl)- (C)

Table 2-4. Molecular weight of modification products of sinapyl alcohol by *A. biennis* ((A) Control: sinapyl alcohol in medium, (B) Sample treated by *A. biennis*)

(A)

	2hr	4hr	6hr	12hr	1d
M_n [a] (Daltons)	295	318	340	334	328
M_w [b] (Daltons)	437	479	527	510	498
Mw/Mn	1.48	1.51	1.55	1.53	1.52

(B)

	2hr		4hr		6hr		12hr		1d	
	1	2	1	2	1	2	1	2	1	2
M_n [a] (Daltons)	725	190	754	200	771	196	848	195	795	198
M_w [b] (Daltons)	830	208	876	211	896	207	1093	206	983	209
Mw/Mn	1.14	1.09	1.16	1.06	1.16	1.06	1.29	1.06	1.24	1.06

[a] number-average molecular weight

[b] weight-average molecular weight

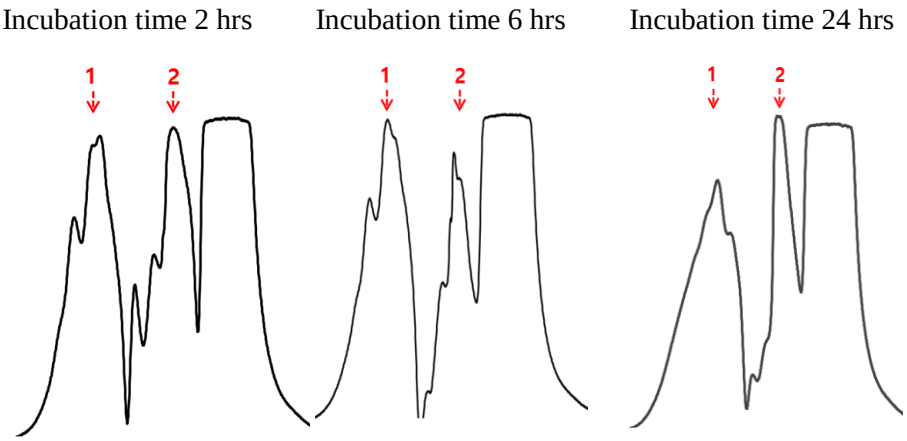


Figure 2-12. Gel permeation chromatogram of sinapyl alcohol treated by *A. biennis* during incubation time

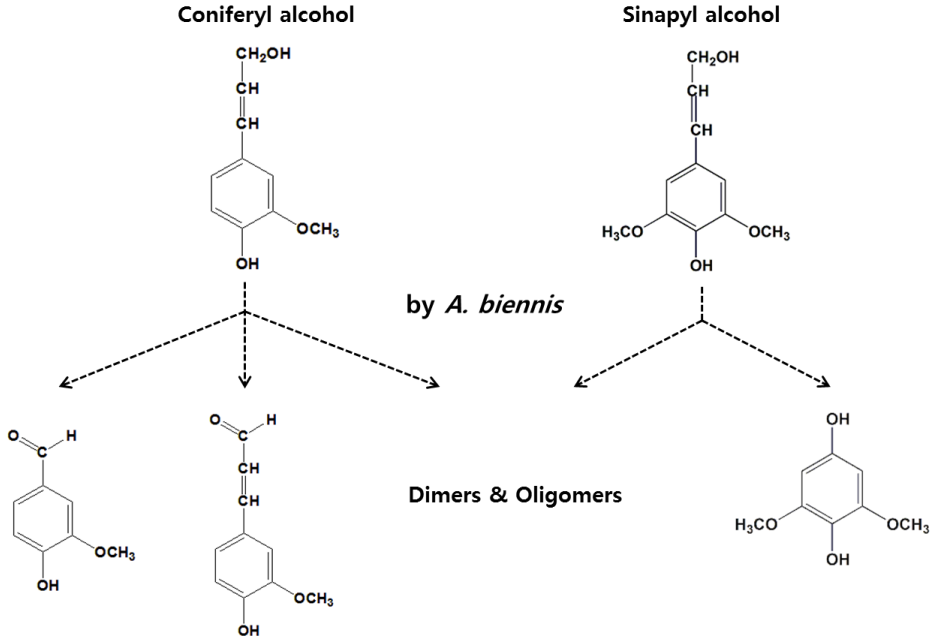
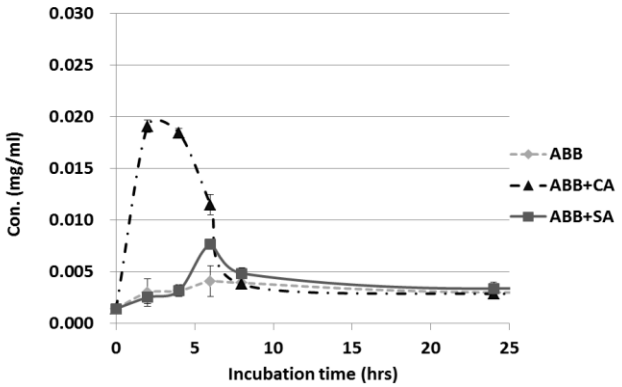
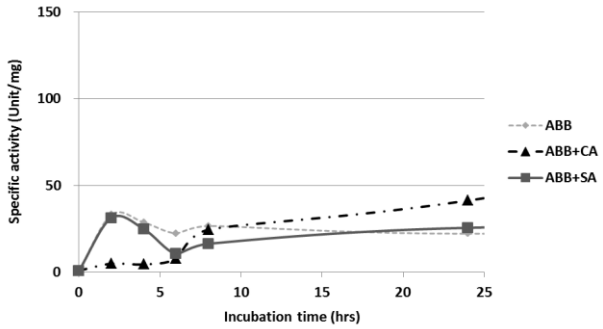


Figure 2-13. Modification pathway of monolignols by *A. biennis*

(A)



(B)



(C)

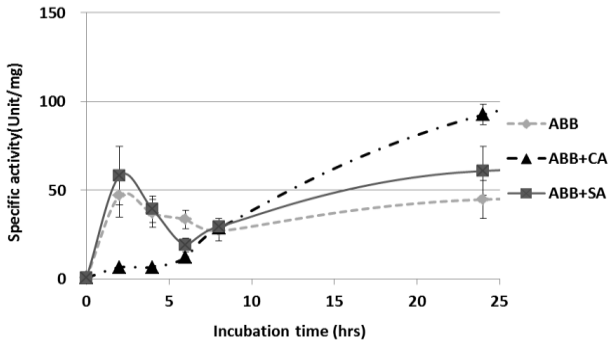


Figure 2-14. Protein concentration (A) and activities of manganese peroxidase (B) and laccase (C) of *A. biennis* with addition of monolignols (ABB: *A. biennis* in medium, ABB+CA: fungal sample adding coniferyl alcohol, ABB+SA: fungal sample adding sinapyl alcohol)

3.2.2. Modification products of monolignols by *P. chrysosporium*

3.2.2.1. Modification products of coniferyl alcohol

The change of substrate by *P. chrysosporium* was progressed slowly as suggested in 3.1. Every 5 days, modification products were analyzed during 25 days.

Result of LC-MS analysis showed polymerized products from confieryl alcohol. In comparison to control, the peaks of new products were detected at 13.7, 14.8, and 15.1 min (Figure 2-15).

The m/z of peak at 13.7 min had 163, 259, and 685 as main peak in mass spectrum (Figure 2-16 (A)). MS-MS spectrum of 163 was identified to be same with that of coniferyl alcohol (Figure 2-16 (B)). 259 had C15H15O4 composition, which was expected as dimer composed of coniferyl alcohol derived compounds. Finally, 685 peak was analyzed to be made up C24H45O15N8. Because nitrogen was included, this compound was predicted to contain metabolites of fungus. Thus, modification product presented at 13.7 min was expected to be oligomer which was polymerized by compounds derived from coniferyl alcohol and metabolites of fungus.

As a result of analysis of 14.8 min and 15.1 min peaks, mass spectrum of both was showed in Figure 2-17. Peak at 14.8 min had 505 as basepeak, which composition was C14H33O12N8, and peak at 15.1 min had 163 and 540 as main peaks. 163 of mass spectrum was identified as confieryl alcohol derived compound as mention above, therefore, this peak was considered to be oligomer containing confieryl alcohol derived compound.

Polymerization of coniferyl alcohol by *P. chrysosporium* was also verified by results of GPC analysis. Weight-average molecular weight (Mw) of modified products increased with incubation period till 20 days, showing Mw was 790 (Table 2-5). Increase of degree of molecular weight

demonstrated that coupling reaction of degradation products of coniferyl alcohol had an effect on increase of molecular weight.

In contrary with result of LC-MS and GPC analysis, degradation product was detected through GC-MS analysis of ethyl acetate fraction. New product was detected at 44.4 min, and the intensities of peaks detected at 50.8 min and 54.1 min were increased relatively higher than that of control (Figure 2-18). Peak at 44.4 min was identified as vanillyl alcohol-trimethysilylated, and 50.8 min and 54.1 min peak were identified as 3-vanilpropanol-trimethylsilyated and vanillylmandeic acid-trimethylsilylated, respectively (Figure 2-19). Although two products detected at 50.8 min and 54.1 min were comprised in control, they were considered as main modification products of coniferyl alcohol because of sizeable increases in quantity by fungus, compared to control. *P. chrysosporium* lead to mainly cleavage of side chain of coniferyl alcohol.

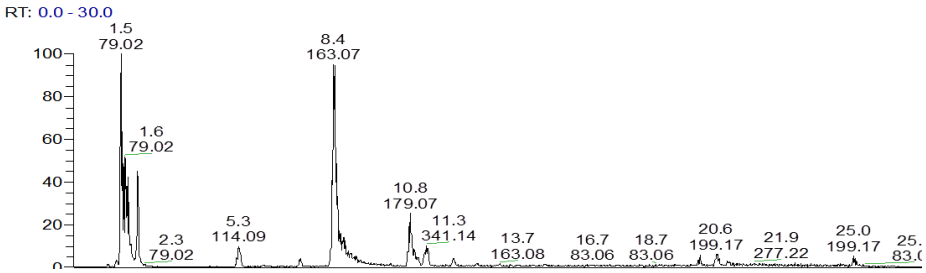
P. chrysosporium was reported to produce variable amounts of lignin peroxidase and manganese peroxidase isoenzymes and lack laccase (Hatakka, 1994). As mentioned above, lignin peroxidase purified from *P. chrysosporium* catalyzed carbon-carbon bond cleavage, side chain oxidation and hydroxylation (Tien, 1987). This result presented that specific activities of lignin degrading enzymes of *P. chrysosporium* were very lower than that of control (Figure 2-24). However, in terms of total activities of enzymes, it was considered LiP and MnP were involved in biomodification of coniferyl alcohol.

Along with degradation process, catalysis reaction of lignin peroxidase was reported to cause polymerization through the free radical formation by various researches (Pollegioni et al., 2015; Ward et al., 2001). Coniferyl alcohol oxidized by extracellular enzymes were unstable because ligninolytic enzymes catalyzed one electron oxidation of lignin molecule to form cation radicals (Ö nnerud et al., 2002) . Reactive radicals have a tendency to move to

large molecules. That was why polymerization reaction occurred initiatively, so molecular weight of fungal sample increased.

In conclusions, enzymes system of *P. chrysosporium* catalyzed not only sidechain cleavage of coniferyl alcohol but also coupling reaction (Figure 2-23). Although precise structure of oligomers could not be suggested because of lack of LC-MS database, this study demonstrated distinguishing microbial modification mechanism of coniferyl alcohol by *P. chrysosporium.*

(A)



(B)

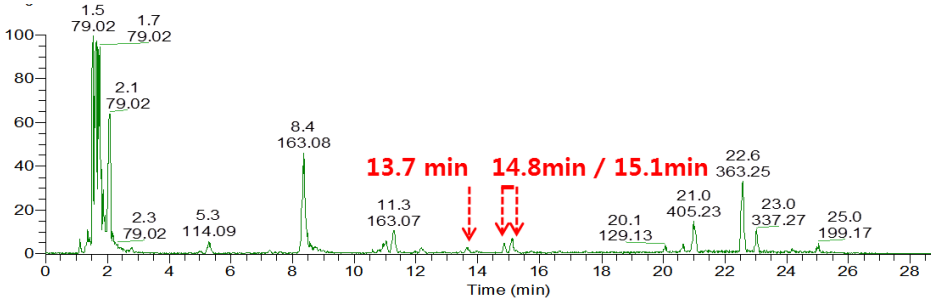
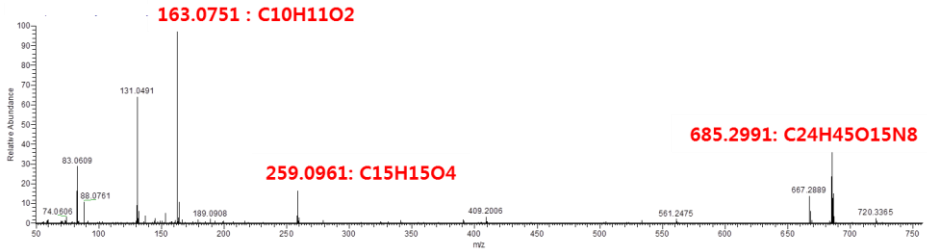


Figure 2-15. Total ion chromatograms of control, coniferyl alcohol in medium, (A) and sample treated by *P. chrysosporium* (B) on incubation day 10

(A)



(B)

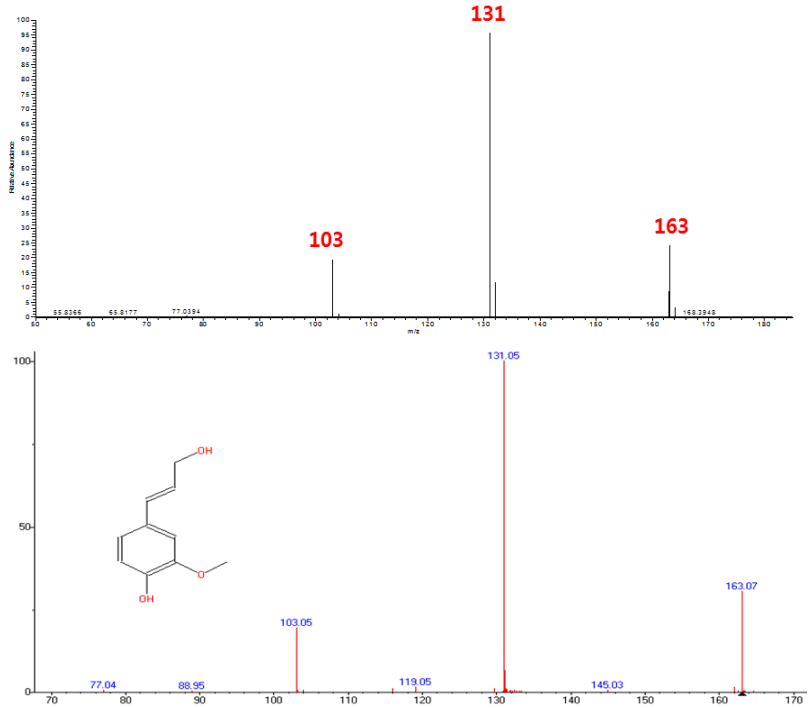
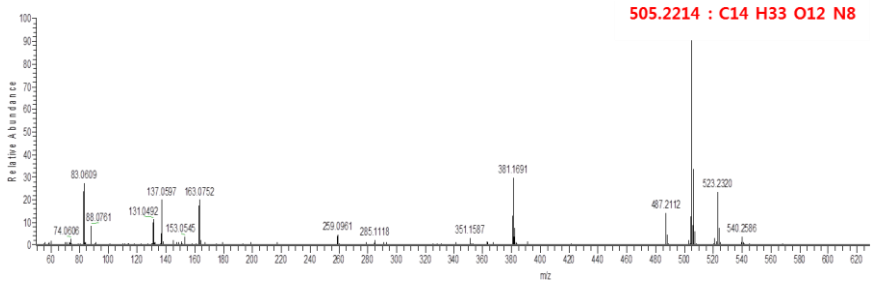


Figure 2-16. Mass spectrum of 13.7 min peak (A) and MS-MS spectrum of 163 and library data of coniferyl alcohol (B)

(A)



(B)

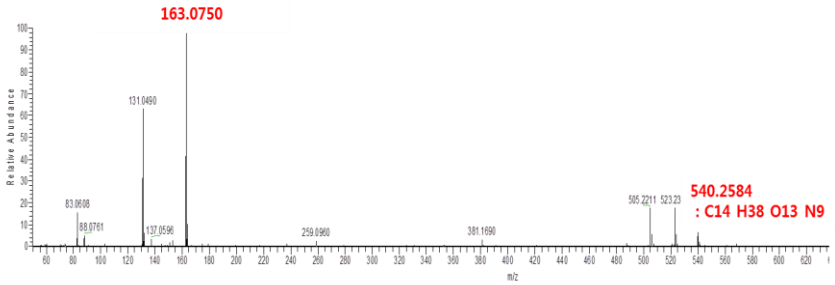


Figure 2-17. Mass spectra and molecular composition of main peak of 14.8 min peak (A) and 15.1 min peak (B)

Table 2-5. Molecular weight of modification products of coniferyl alcohol by *P. chrysosporium* ((A) Control: coniferyl alcohol in medium, (B) Sample treated by *P. chrysosporium*)

(A)

	1d	5d	10d	15d	20d
M_n [a] (Daltons)	330	351	358	362	360
M_w [b] (Daltons)	520	564	583	595	590
Mw/Mn	1.58	1.61	1.63	1.64	1.64

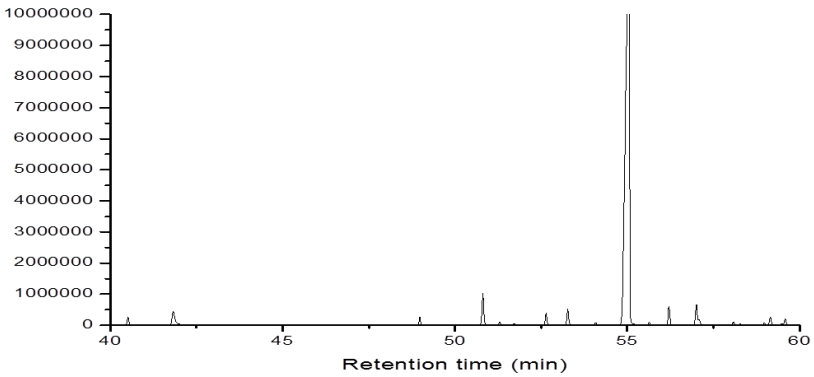
(B)

	1d	5d	10d	15d	20d
M_n [a] (Daltons)	339	410	378	403	402
M_w [b] (Daltons)	564	664	684	781	790
Mw/Mn	1.66	1.62	1.81	1.94	1.97

[a] number-average molecular weight

[b] weight-average molecular weight

(A)



(B)

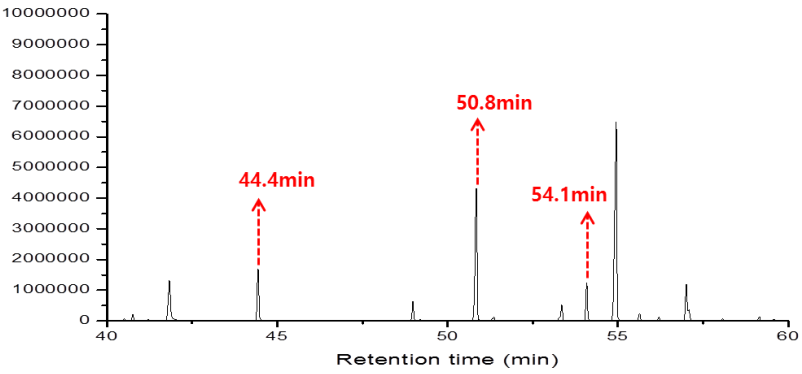


Figure 2-18. Total ion chromatograms of control, coniferyl alcohol in medium, (A) and fungal sample (B) on incubation day 10

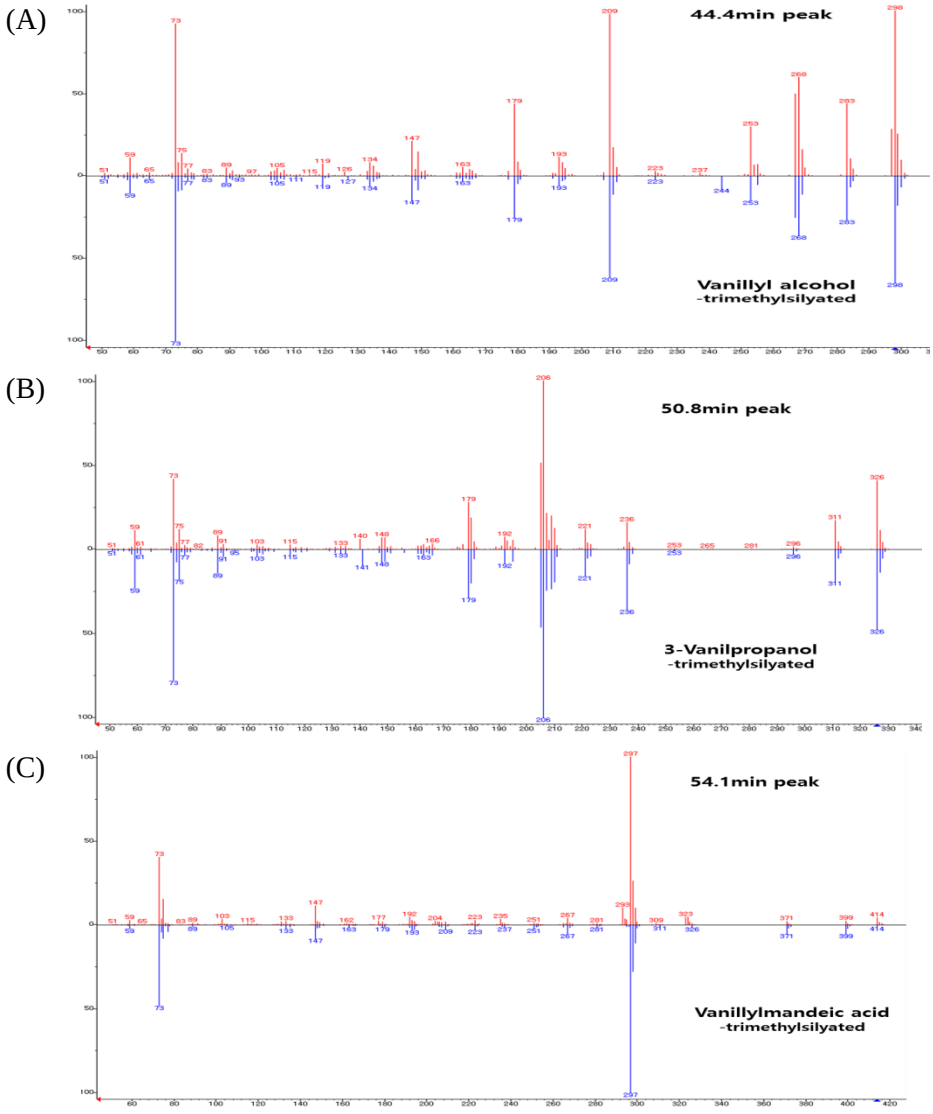


Figure 2-19. Mass spectra of modification products with library data ((A) 44.4 min, (B) 50.8 min, (C) 54.1 min)

3.2.2.2. Modification products of sinapyl alcohol

In case of sinapyl alcohol, polymerization products were detected by LC-MS, and degradation products were identified by GC-MS.

Based on analysis of LC-MS, two peaks were detected newly in fungal sample at 13.6 min and 13.8 min (Figure 2-20). At first, peak at 13.6 min had 385.16 as basepeak in LC-MS-MS analysis, and the basepeak was composed of C22H25O6. Basepeak of peak at 13.8 min was 193, which was identified to sinapyl alcohol. Accordingly, new product formed at 13.8 min was predicted to polymerized product composed of sinapyl alcohol-derived compound (Figure 2-20). GPC results also supported that *P. chrysosporium* induced polymerization of sinapyl alcohol overall. Mw of fungal sample increased with incubation days. On incubation day 20, Mw of fungal sample was 869 which was larger than that of control by 300 (Table 2-6).

GC-MS analysis showed various degradation products. Syringaldehyde from methanol fraction was detected at 47.1 min (Figure 2-21), and acid compounds, lactic acid and succinic acid, were detected at 19.2 min and 31.4 min from ethyl acetate fraction, respectively (Figure 2-21).

P. chrysosporium exhibited degradation and polymerization mechanism of sinapyl alcohol. In degradation process, lignin degrading enzymes of *P. chrysosporium* appeared to catalyze Cα oxidation by cleavage of side chain, even though specific activities of lignin degrading enzymes were lower than that of control (Figure 2-24). And also, it was considered that radicals formed by these enzymes lead to polymerization reaction between unstable monomers.

The interesting result of this study was that lactic acid and succinic acid from sinapyl alcohol were formed by ring fission. For formation of acid compounds from phenolic compounds, various processes were required such as demethylation, ring fission and C-C cleavage (Pollegioni et al., 2015). In detail, demethylation process was essential to generate substrates for the ring

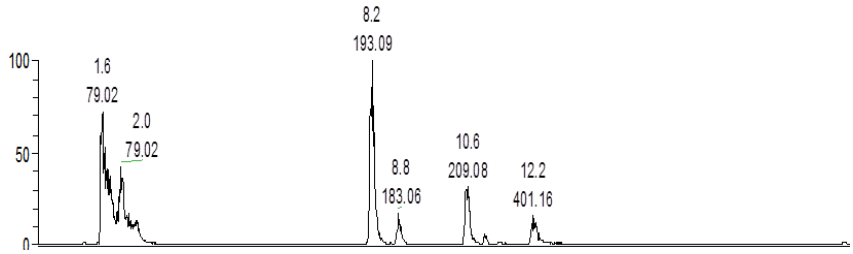
cleavage steps. O-demethylase was involved in demethylation of lignin related compounds. It was well known that ring cleavage of aromatic compound was catalyzed by dioxygenase species like 3,4- dioxygenase, 4,5- dioxygenase, and 2,3-dioxygenase (Masai et al., 1999). Dioxygenase purified from *P. chrysosporium* were reported to cleave aromatic ring oxidatively. The enzyme catalyzes an intradiol cleavage of the substrate aromatic ring to produce acid compounds (Rieble et al., 1994).

After these degradation processes, fungal metabolic mechanism seemed to be concerned in formation of acid compounds. Succinic acid and lactic acid were composed of C4 and C3. Generally, acid compounds formed by ring fission have C6 or more. Accordingly, the cleavage products are degraded through distinct pathways and enter the TCA cycle. Based on this catabolic pathway, succinic acid, lactic acid and pyruvic acid etc. could be produced from lignin related compounds (Johnson & Beckham, 2015).

These results suggested that enzymes system of *P. chrysosporium* were thought to be powerful tools for utilization of the aromatic biomass. That was because acid compounds produced from lignin can be converted into myriad products including polyhydroxyalkanoate. Recently, studies on aromatic catabolism using microorganism has been carried out intensively for formation of lignin derived acid compounds. However, to date, researches on enzymes related to ring cleavage of aromatic compounds has primarily focused on bacterial enzymes (Johnson & Beckham, 2015; Tomizawa et al., 2014; Vardon et al., 2015).

Consequently, whole cell of white rot basidiomycete produced various oxidative products with complex enzymes system. Accordingly, *P. chrysosporium*, aromatic catabolizing fungus, can be attractive alternative for lignin upgrading as potential biocatalyst.

(A)



(B)

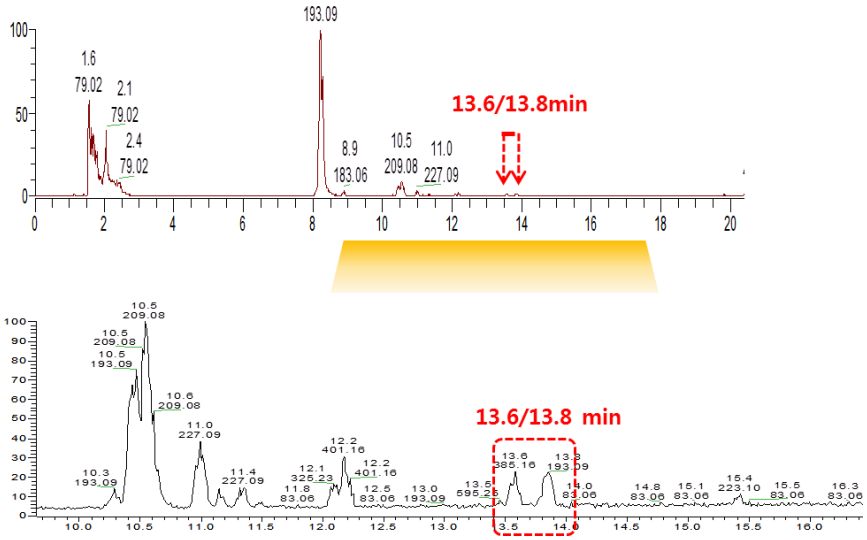
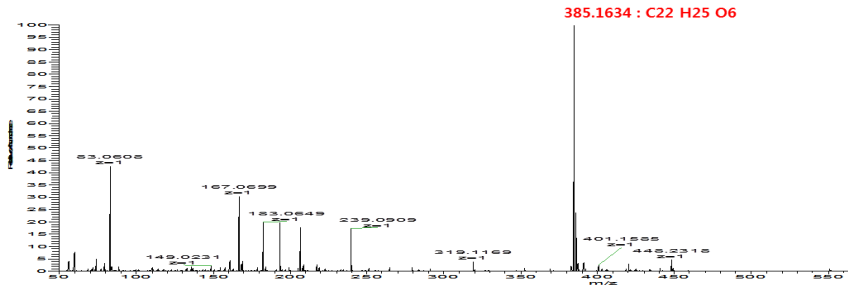
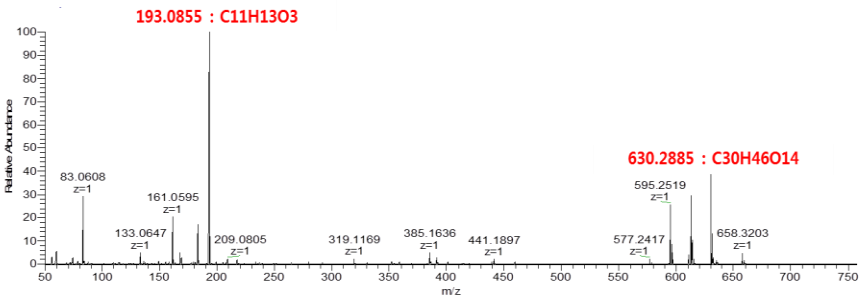


Figure 2-20. Total ion chromatograms of control, sinapyl alcohol in medium, (A) and sample treated by *P. chrysosporium* (B) on incubation day 5

(A)



(B)



(C)

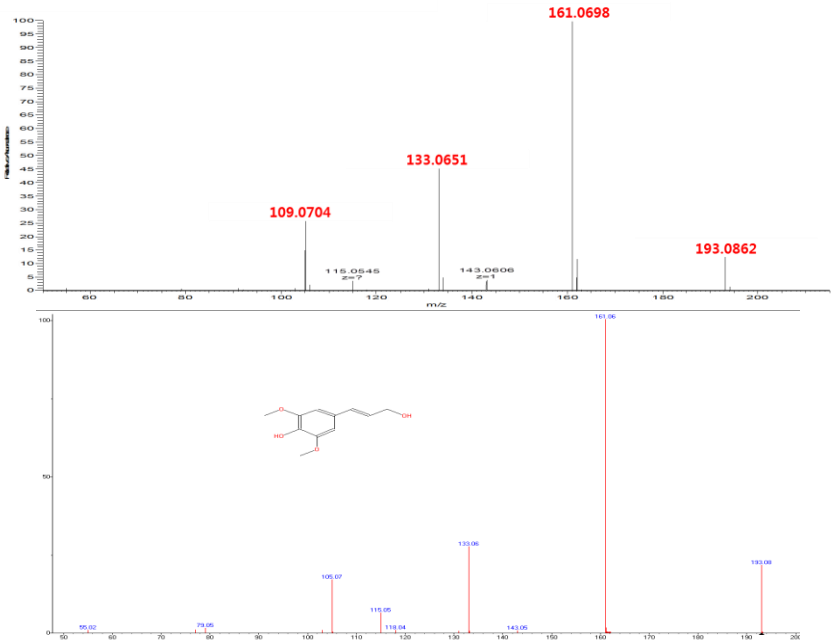


Figure 2-21. Mass spectra of 13.6 min peak (A) and 13.8 min peak (B), MS-MS spectrum of 193 with library data of sinapyl alcohol (C)

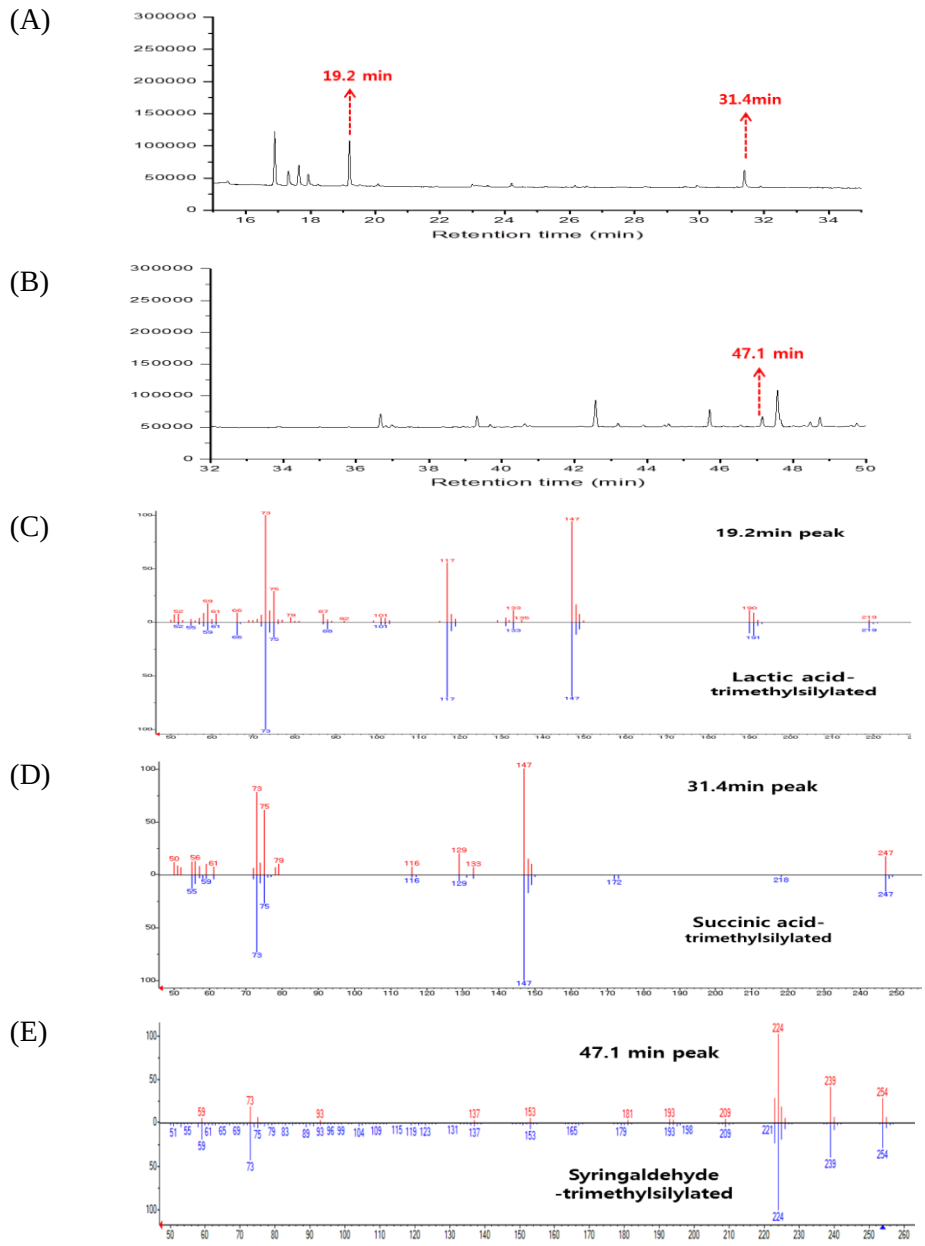


Figure 2-22. Total ion chromatograms of sample treated by *P. chrysosporium* (A, B), and GC-MS spectrum of 19.2 min peak (A), 31.4 min peak (B), and 47.1 min peak (C) in fungal sample with library data

Table 2-6. Molecular weight of modification products of sinapyl alcohol by *P. chrysosporium* ((A) Control: sinapyl alcohol in medium, (B) Sample treated by *P. chrysosporium*)

(A)

	1d	5d	10d	15d	20d
M_n [a] (Daltons)	328	390	373	381	380
M_w [b] (Daltons)	498	560	549	569	569
Mw/Mn	1.52	1.44	1.47	1.49	1.50

(B)

	1d	5d	10d	15d	20d
M_n [a] (Daltons)	406	415	442	449	452
M_w [b] (Daltons)	587	653	755	810	869
Mw/Mn	1.44	1.57	1.71	1.80	1.92

[a] number-average molecular weight

[b] weight-average molecular weight

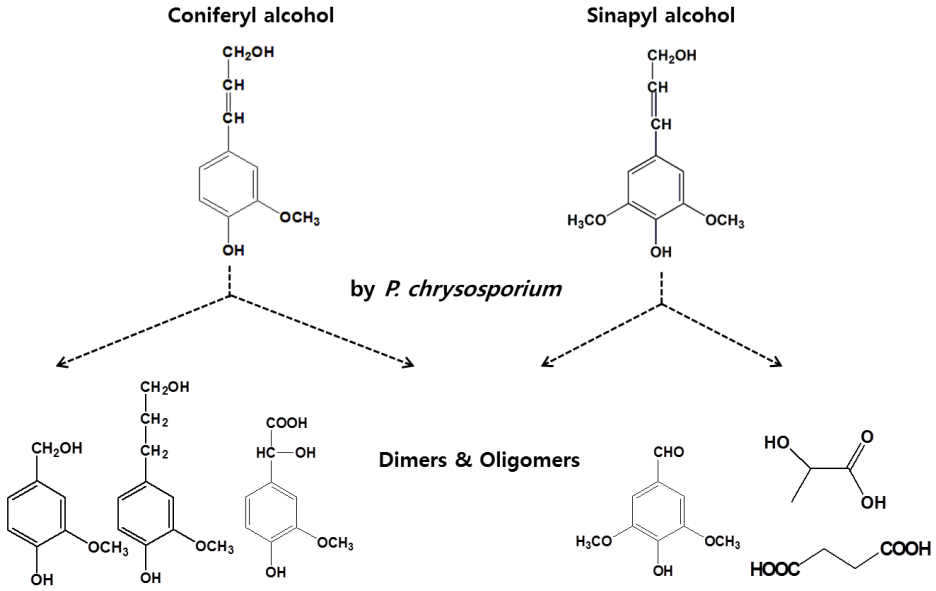
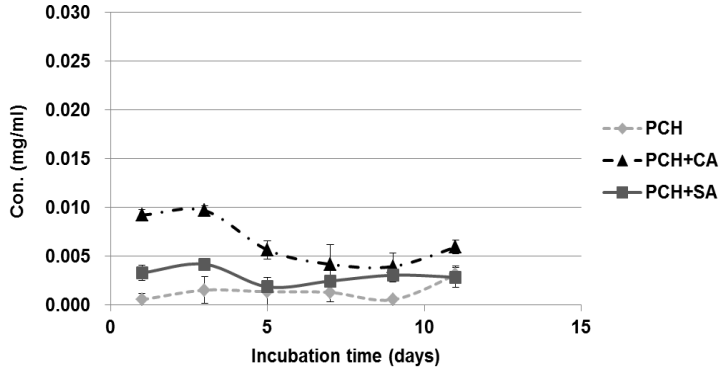
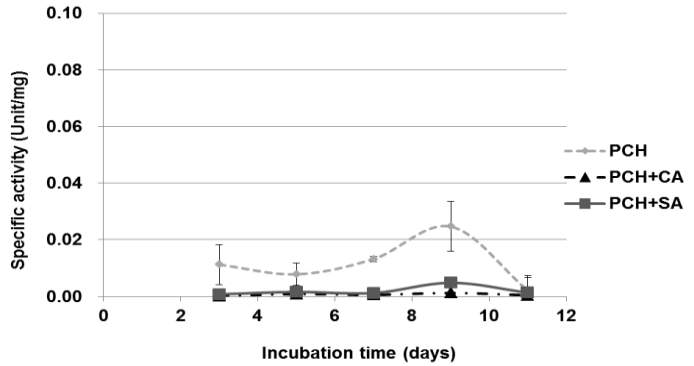


Figure 2-23. Modification pathway of monolignols by *P. chrysosporium*

(A)



(B)



(C)

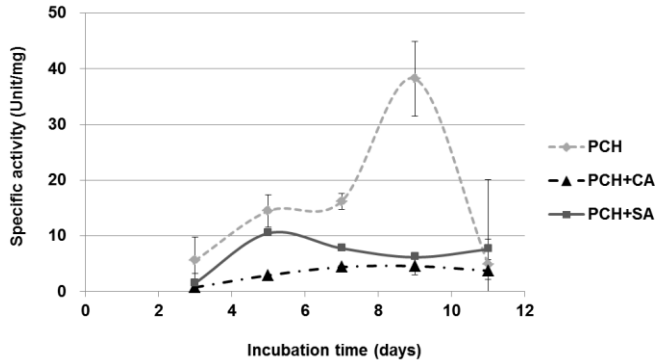


Figure 2-24. Protein concentration (A) and activities of manganese peroxidase (B) and lignin peroxidase (C) of *P. chrysosporium* with addition of monolignols (PCH: *P. chrysosporium* in medium, PCH+CA: fungal sample adding coniferyl alcohol, PCH+SA: fungal sample adding sinapyl alcohol)

3.3. Degradation mechanism of monolignols by white rot basidiomycetes with reducing agent

3.3.1. Degradation of monolignols by *A. biennis* with reducing agent

This experiment was conducted to degrade monolignols, and modification products were analyzed by GPC and GC-MS. GPC analysis indicated that molecular weights of monolignols treated with *A. biennis* was lower than those of reducing agent-free experiment (Table 2-7). In case of coniferyl alcohol, Mw and polydispersity ranged between 707 Da to 1,113 Da and between 1.67 to 2.15 at an incubation time 4 hrs and 6 hrs, respectively. In the case of sinapyl alcohol, the molecular weight was similar with that of the control. In reducing agent free experiment, polymerization reaction took place on and around the large molecules. As the addition of a reducing agent blocked unstable monolignols from migrating into oligomers, polymerization of monolignols seemed to be inhibited. Ascorbic acid as reducing agent is one of the most extensively studied antioxidant and can directly scavenge O_2, O_{-2} and OH· (Shao et al., 2008). Therefore, ascorbic acid were used to inhibit further oxidation of the phenolic products in enzymatic reaction (Kinne et al., 2009). As a result, these GPC observations supported the hypothesis that ascorbic acid had a significant role in preventing the increase of molecular weight, thus stabilizing radicals within monolignols.

As a result of GC-MS analysis of the modification products of coniferyl alcohol, both polymerization products and degradation product were detected at an incubation time 8 hrs. 3- Hydroxy-3-(4'-hydroxy-3'-methoxyphenyl)propionic acid-tri TMS and matairesinol-di TMS were

detected at 84.6 min and 89.2 min (Figure 2-25). 3- Hydroxy-3-(4'-hydroxy-3'-methoxyphenyl)propionic acid could be formed by Cα and Cγ oxidation from coniferyl alcohol, whereas matairesinol could be formed by coniferyl alcohol-derived compounds with C2C6 structure (Figure 2-25). In this study, because the reaction time with ascorbic acid was 1 hr, the polymerization reaction already appeared to partially occur before the addition of ascorbic acid, which is why various degradation products were not detected even though degree of increase of Mw decreased.

On the other hand, a siginificant amount of degraded products from sinapyl alcohol were detected by GC-MS analysis. Syringaldehyde and various acid compounds were detected in small quantities as well as 2,6-dimethoxy benzene-1,4-diol (Figure 2-26). The addition of ascorbic acid mainly induced the degradation reaction from sinapyl alcohol, contrary to that of coniferyl alcohol. Acid compounds such as glycolic acid and succinic acid are originally generated by basidiomycete as primary metabolites. It indicated *A. biennis* scarcely generated acid compounds as metabolites (Figure 2-26 (A)), however *A. biennis* reacted with sinapyl alcohol generated glycolic acid and succinic acid (Figure 2-26(B)). This result means that acid compounds derived from sinapyl alcohol might be secondary metabolites of fungus and it could be produced by demethylation and ring cleavage of sinapyl alcohol (Pollegioni et al., 2015; Tomizawa et al., 2014). These results supported that ascorbic acid stablized radicals formed in monolignol for making an induction more oxidation and degradation, not being polymerization. Furthermore, *A. biennis* was suggested to have great ability to catalyze aromatics, which was meaningful in terms of lignin valorization because studies on aromatic catabolism by microorganism have been carried out intensively for formation of lignin derived acid compounds (Johnson & Beckham, 2015; Linger et al., 2014).

In conclusion, as novel biocatalyst, *A. biennis* had the ability to degrade

monolignols, to produce valuable acid compounds with the addition of ascorbic acid, and to rapidly modify lignin compounds. Therefore, *A. biennis* could be used in database to perform studies on the biomodification of lignin by fungus in future.

Table 2-7. Molecular weight of modification products of monolignols by *A. biennis* with addition of ascorbic acid (Control: monolignols in medium, ABB: fungal sample treated by *A. biennis*)

Coniferyl alcohol

	Control			ABB		
	4hr	6hr	8hr	4hr	6hr	8hr
M_n [a] (Daltons)	358	355	355	423	517	533
M_w [b] (Daltons)	506	494	492	707	1113	1088
Mw/Mn	1.41	1.39	1.39	1.67	2.15	2.04

Sinapyl alcohol

	Control			ABB		
	4hr	6hr	8hr	4hr	6hr	8hr
M_n [a] (Daltons)	369	368	369	395	409	381
M_w [b] (Daltons)	521	522	517	603	606	562
Mw/Mn	1.41	1.42	1.40	1.53	1.48	1.48

[a] number-average molecular weight

[b] weight-average molecular weight

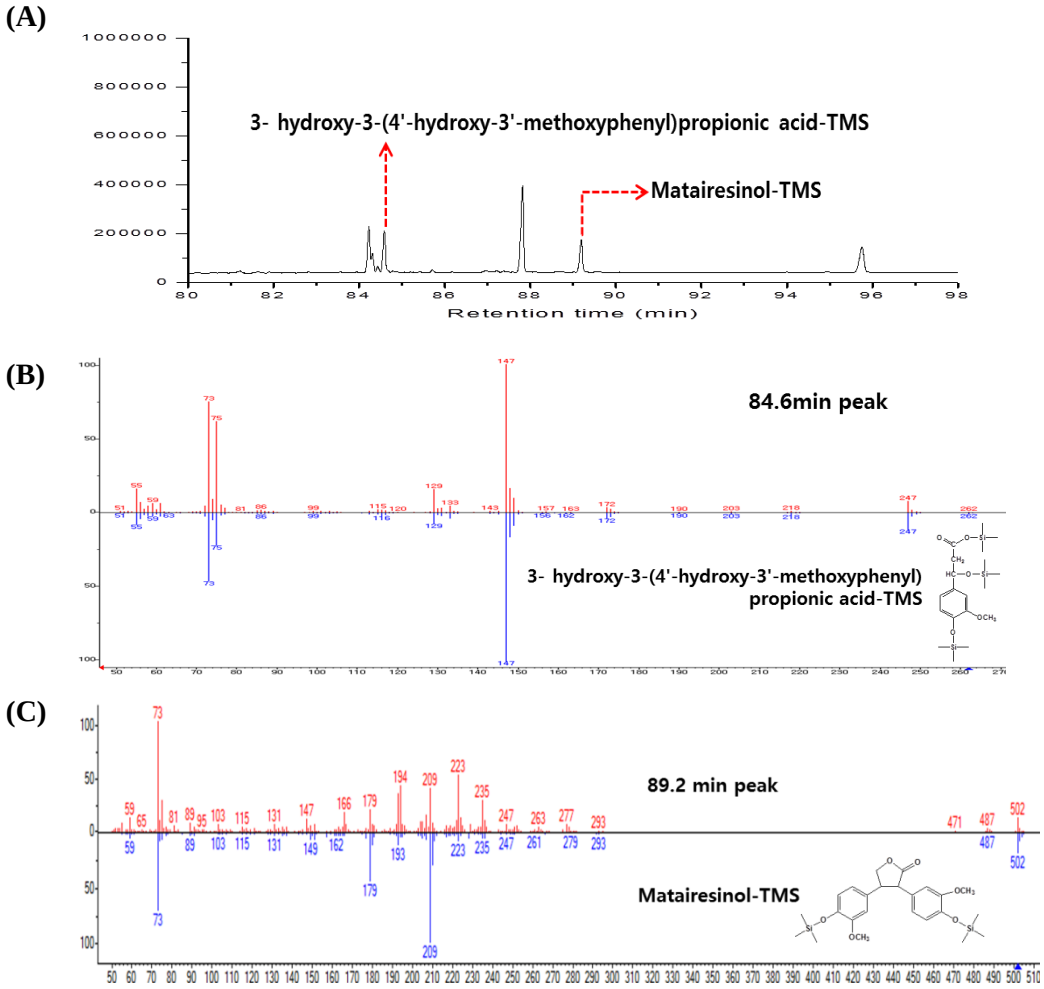
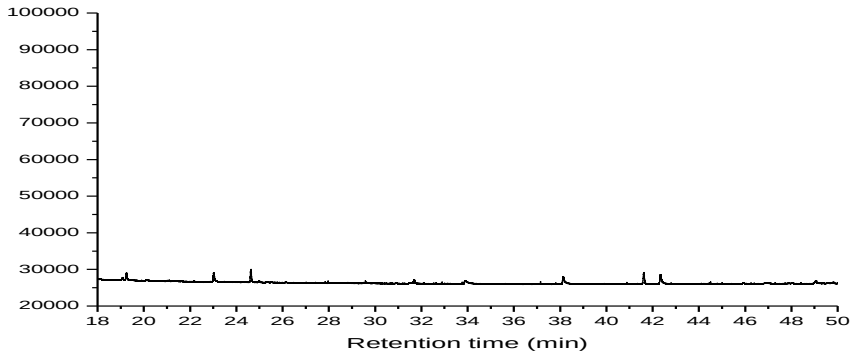


Figure 2-25. Total ion chromatogram of modification products of coniferyl alcohol by *A. biennis* with ascorbic acid (A), and mass spectra of new products with mass library data (B&C)

(A)



(B)

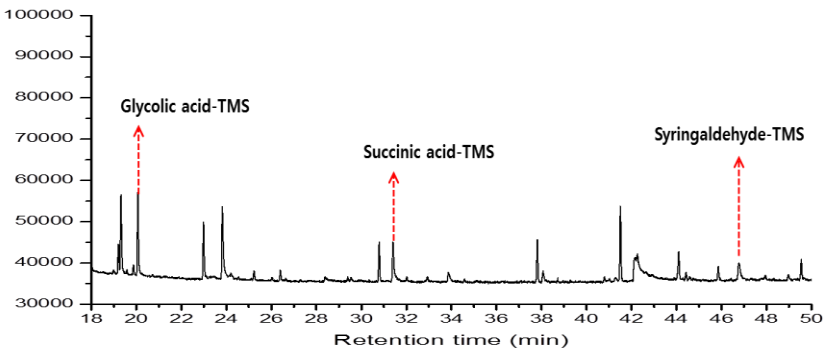


Figure 2-26. Total ion chromatograms of fungal metabolites (A) and modification products of sinapyl alcohol by *A. biennis* with ascorbic acid (B)

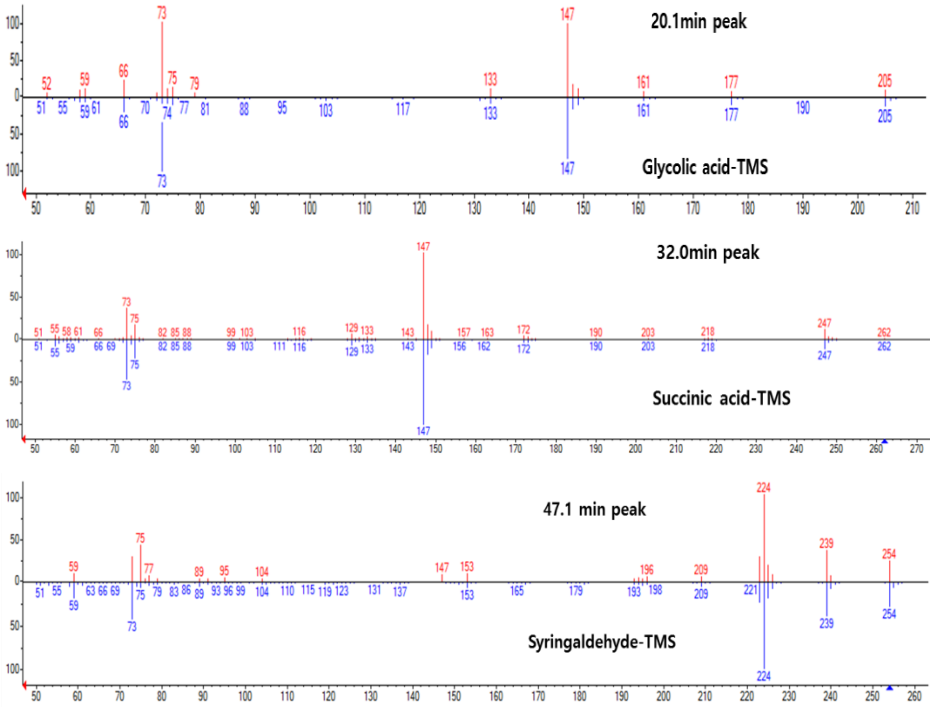


Figure 2-27. Mass spectra of new products derived from sinapyl alcohol with mass library data

3.3.2. Degradation of monolignols by *P. chrysosporium* with reducing agent

Ascorbic acid as reducing agent affected on primarily inducing degradation reaction of monolignols by *P. chrysosporium.*

Table 2-8 presented that Mw of sample treated by fungus was scarcely increased. This means ascorbic acid inhibited polymerization between unstable radicals formed by one electron oxidative system of enzymes (Kinne et al., 2009).

Along with these results, various degradation products were detected through GC-MS analysis. New products were detected at 45.0, 49.4, 51.3, 53.7 min in methanol extract on day 5 (Figure 2-28), and these were identified as vanillyl alcohol, vanillic acid, 3-vanilpropanol and 3-(4-hydroxy-3-methoxy phenyl) propanoic acid, respectively. In addition, analysis result of ethyl acetate extract showed succinic acid at 32.0 min and trihydroxybenzene at 43.8 min were formed from conifeyl alcohol from 10 days to 25 days (Figure 2-29).

Sinapyl alcohol was degraded to hydroquinone and succinic acid by *P. chrysosporium* under the presence of ascorbic acid (Figure 2-30).

These compounds were not detected in free-ascorbic acid experiment. As a result, enzyme system of *P. chrysosporium* vigorously catalyzed oxidation of monolignols while ascorbic acid played a role to block the formation of oxidative radicals from monomers. That was why degradation products were more oxidized structures. Especially, demeth(ox)ylation and hydroxylation within aromatics make ring fission easier, which was attributed to produce succinic acid. Dioxygenase was one of enzymes involved in ring cleavage. Dioxygenase activity has been detected in *P. chrysosporium* (Rieble et al., 1994; Valli & Gold, 1991). For enzymatic ring cleavage of aromatic compounds, ring dihydroxylation are required (Bugg & Winfield, 1998;

Pollegioni et al., 2015; Rieble et al., 1994). Accordingly, degraded products such as hydroquinone and trihydroxybenzene detected in this study facilitated formation of succinic acid. Finally, hydroxylated aromatics were metablized by fungus, which means that acid compounds from monolignols was considered to be secondary metabolites of fungus.

In conclusion, *P. chrysosporium* can be used as novel biocatalyst for lignin application due to enzymes system related to ring cleavage and production of acid compounds.

Table 2-8. Molecular weight of modification products of monolignols by *P. chrysosporium* with addition of ascorbic acid (Control: monolignols in medium, PCH: fungal sample treated by *P. chrysosporium*)

Coniferyl alcohol

	Control					PCH			
	5d	10d	15d	20d		5d	10d	15d	20d
M_n [a] (Daltons)	354	377	423	422		343	375	371	360
M_w [b] (Daltons)	626	644	724	764		591	679	676	686
Mw/Mn	1.77	1.71	1.71	1.81		1.72	1.81	1.82	1.91

Sinapyl alcohol

	Control					PCH			
	5d	10d	15d	20d		5d	10d	15d	20d
M_n [a] (Daltons)	353	376	390	411		380	386	398	406
M_w [b] (Daltons)	564	621	651	705		585	596	705	710
Mw/Mn	1.60	1.65	1.67	1.72		1.54	1.54	1.77	1.75

[a] number-average molecular weight

[b] weight-average molecular weight

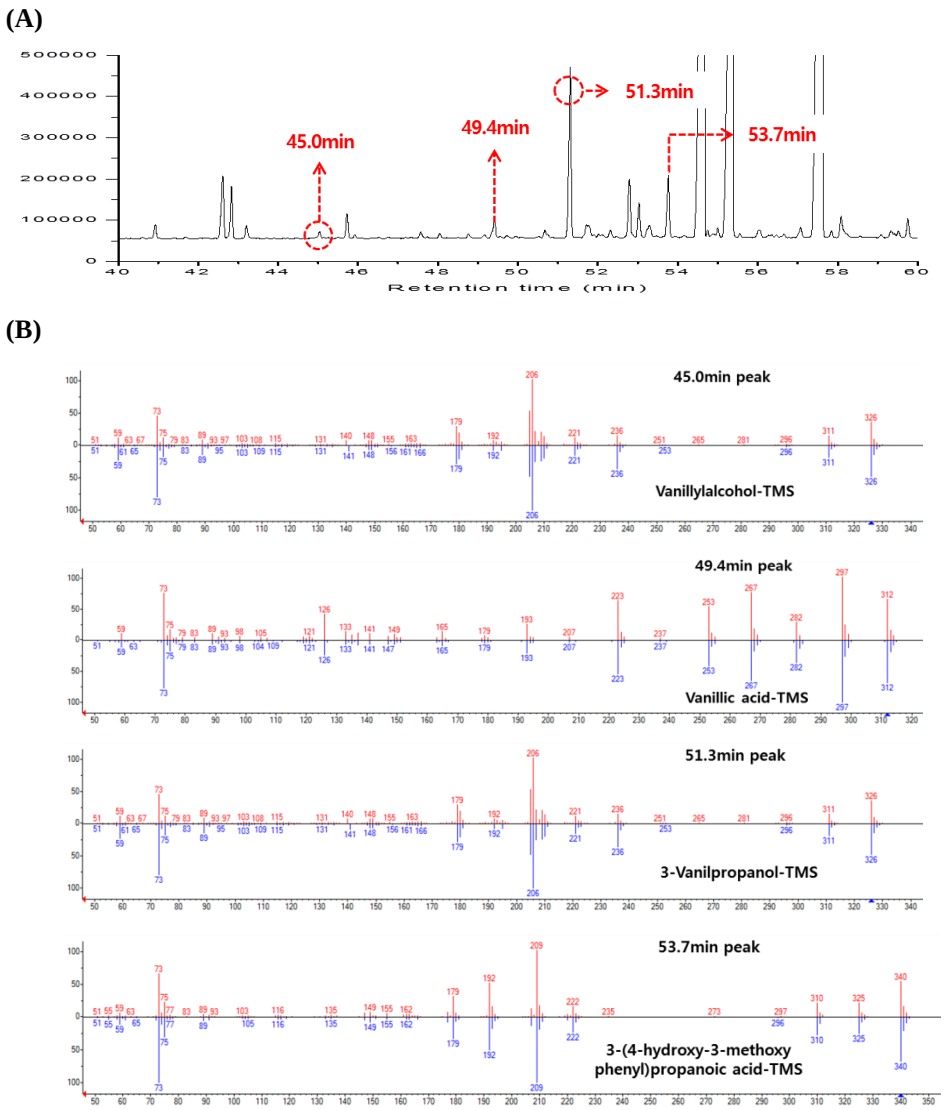


Figure 2-28. Total ion chromatograms of modification products in methanol extract of coniferyl alcohol by *P. chrysosporium* with ascorbic acid (A), and mass spectra of new products with mass library data (B)

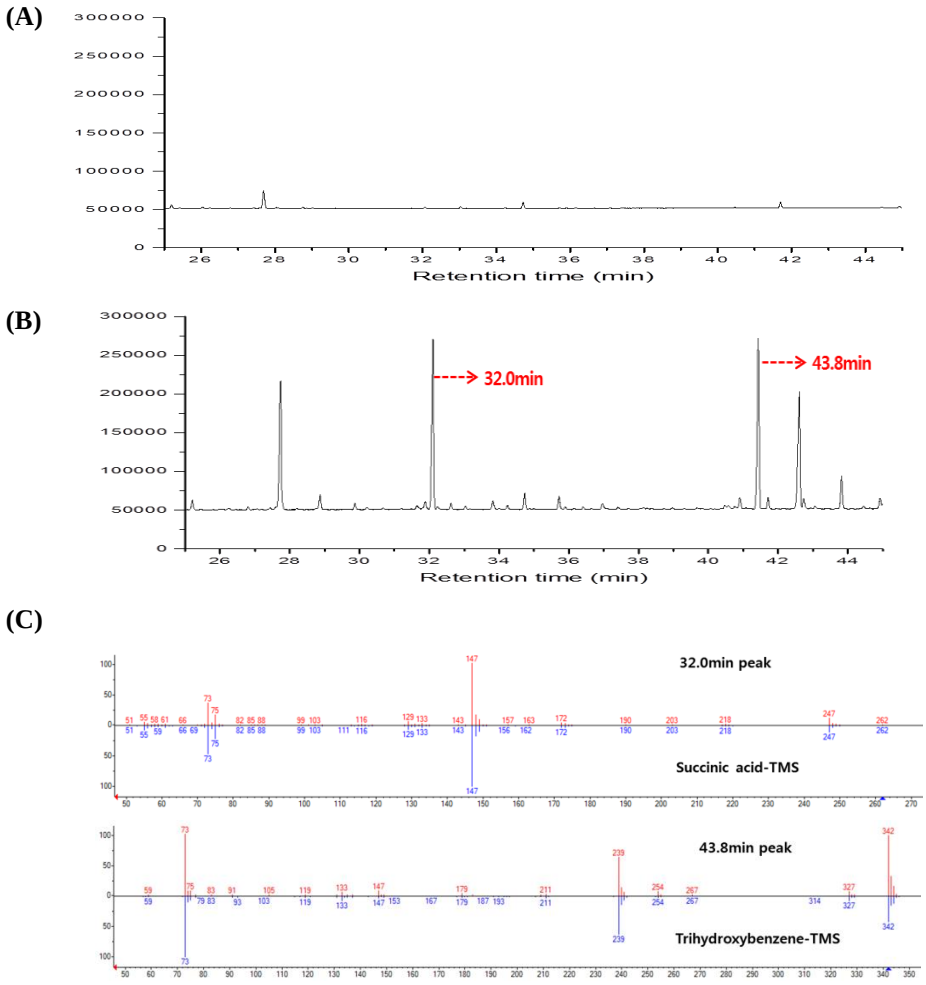


Figure 2-29. Total ion chromatograms of fungal metabolites (A) and modification products in ethylacetate extract of coniferyl alcohol by *P. chrysosporium* with ascorbic acid (B), and mass spectra of new products with mass library data (C)

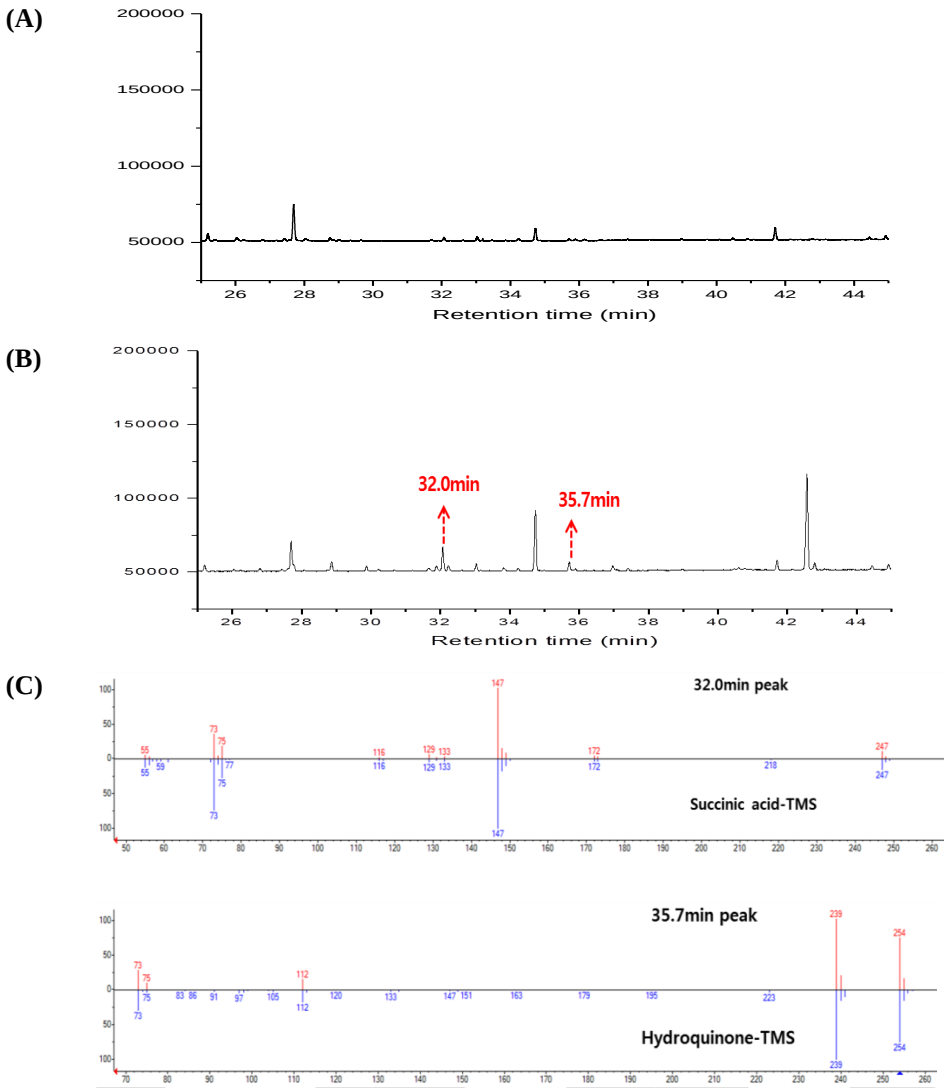


Figure 2-30. Total ion chromatograms of fungal metabolites (A) and modification products of sinapyl alcohol by *P. chrysosporium* with ascorbic acid (B), and mass spectra of new products with mass library data (C)

4. Conclusions

A. biennis and *P. chrysosporium* leads to not only degradation but also polymerization of monolignols.

A. biennis degraded coniferyl alcohol to vanillin and coniferyl aldehyde while simultaneously producing oligomers. Sinapyl alcohol was degraded to 2,6-dimethoxy benzene-1,4-diol and was polymerized by *A. biennis*. The enzyme systems of *A. biennis* had more influence on the oxidation and degradation of sinapyl alcohol.

P. chrysosporium degraded coniferyl alcohol to vanillyl alcohol, 3-vanilpropanol and vanillylmandeic acid, and sinapyl alcohol to syringaldehyde, lactic acid and succinic acid while simultaneously producing oligomers. *P. chrysosporium* catalyzed not only side chain cleavage but also ring cleavage of sinapyl alcohol.

Polymerization of monolignols in two fungal medium was an unexpected result. Thus, for the induction of degradaion of monolignols, ascorbic acid was used as a reducing agent in monolignol biomodification. The addition of ascorbic acid prevented a drastic increase of the molecular weight of monolignols, especially via preferential degradaion of sinapyl alcohol. Furthermore, various degraded products, including acid compounds, were formed from monolignols by *A. biennis* and *P. chrysosporium.* Especially, more intermediates such as trihydroxybenzene, hydroquinone, and succinic acid from monolignols were generated under the ligninolytic treatment by *P. chrysosporium.* As a result, ascorbic acid stabilized radicals generated by enzymes of basidiomycetes. The results obtained in this study may be exploited in several lignin applications using microorganisms. This also open up possibilities for lignin biomodification by a novel biocatalyst..

Chapter 3

Degradation of synthetic lignin by
Abortiporus biennis with mediator and reducing agents
and analysis of its related enzymes
by transcriptomic analysis

1. Introduction

Interest of lignin utilization as a value-added source has increased in recent years with generation of over 50 million tons/year of lignin in the US pulping/paper industry (Ragauskas et al., 2006). However, because lignin is one of most recalcitrant biomaterials, modification process was required.

Among the various lignin modification processes, biological treatment using white rot basidiomycetes have been suggested as new design for lignin modification with its powerful ability to degrade lignin. Biotechnological use of white rot basidiomycetes is of considerable interests in green chemistry and biorefinery industry with the selective and non-specific ligninolytic enzymes systems (Martínez et al., 2010). Generally, lignin degrading enzymes are known to catalyze oxidation of lignin in multi-step electron transfers with the formation of radical cation (Wong, 2009). With powerful ability of ligninolytic enzymes, their mediator system have also advanced for overcoming limitation in applying ligninolytic enzymes to widen range and expanding their catalytic activity. Thus, catalytic ability with mediators has been evaluated as new biocatalyst for biotechnological application in industry (Cañas & Camarero, 2010; Munk et al., 2015).

Until now, most studies on lignin degradation by white rot fungi have been carried out focusing on biodegradation mechanism of lignin model compounds by purified lignin degrading enzymes. It was demonstrated the involvement of extracellular peroxidases and laccase in biodegradation of lignin (Hammel et al., 1985; Higuchi, 1990; Kawai et al., 1999; ten Have & Teunissen, 2001). In addition, several studies on synthetic lignin represented merely change of molecular weight of DHPs after fungal ligninolytic treatment (Kawai et al., 1999; Srebotnik & Hammel, 2000; Yoshida et al., 1998).

To improve the usefulness of white rot basidiomycetes in lignin modification, it was necessary to understand characteristic biomodification mechanism of lignin by whole cell of white rot basidiomycetes. Furthermore, proteomic and metabolic approaches to investigate enzymes related to lignin modification will contribute to improve industrial applicability of basidiomycetes for lignin modification.

Results of above chapter suggested white rot basidiomycetes polymerized monolignols under the ligninolytic condition. Therefore, objective of this study was to degrade lignin under the fungal culture of *Abortiporus biennis* by changing enzyme system and surrounding condition. Above all, biodmodification mechanism of synthetic lignin by *A. biennis* was understood, and then addition of enzyme mediator and reducing agent treatments were conducted for enhancement of lignin degradation. Finally, to identify enzymes related to biodegradation of lignin depending on the addition enzyme mediator and reducing agents, transcriptomic analysis was carried out.

2. Materials and methods

2.1. Materials

2.1.1. Preparation of fungus and fungal suspension

In this chapter, *A. bienni*s was used for biomodificatin of synthetic lignin. The fungus and fungal suspension were prepared as described in section 2.1.1. of Chapter 2.

2.1.2. Synthesis of dehydrogenative polymer(DHP)

Dehydrogenative polymer (DHP) was used as synthetic lignin. For synthesis of DHP, HRP (horseradish peroxidase; 199 U/mg, MW= 44,000 Da) was purchased from Sigma, and Three solutions were prepared to synthesize dehydrogenative polymers (DHP) as follows: Solution 1, potassium phosphate buffer (0.025 N, pH 6); Solution 2, mixture of dioxane (10 ml) and buffer solution (40 ml) containing 1.43 mmol of monolignols (molar feed ratios of SA /CA, 6:4); and Solution 3, hydrogen peroxide (0.140 ml ;28 wt.%) in buffer (50 ml). 5,000 units of HRP was added to Solution 1 (100 ml), and then Solution 2 and Solution 3 were added drop-wise to Solution 1 for 5 hrs at room temperature with stirring at 400 rpm (End-wise polymerization). The mixture was then stirred for an additional 5 hrs. The precipitates were separated from the buffer solution by centrifugation (12,000 rpm, 15 min), and lyophilized to obtain DHP (Moon et al., 2012)

2.2. Analysis of structural changes and degradation products of DHP

The 200 ml of SSC medium was prepared as mentioned in Chapter 2, and then fungus (dried weight: 0.04 g) was inoculated into SSC medium. After preincubation for 4 days, 200 mg of DHPs were spiked in the medium. Each sample was taken by centrifugation at 12,000 rpm, 15 min at 4℃ to separate DHP in media. After centrifugation, 50 ml of dioxane was added to the precipitates containing mycelia to facilitate the extraction of DHP adsorbed onto mycelia. DHP in dioxane was lyophilized.

2.2.1. Structural changes of DHP

Structural changes of precipitated DHP were carried out as follows.

2.2.1.1. Gel permeation chromatography (GPC)

Molecular weight distribution of the DHP treated with white rot basidiomycetes was analyzed as described in section 2.2 of Chapter 2.

2.2.1.2. Content of phenolic hydroxyl group

Analysis of phenolic hydroxyl group contents was carried out as by Månsson's method (Månsson, 1983). DHP acetylated by pyridine and acetyl chloride (1:1, v/v) at 105℃ for 3hrs was dissolved in 2ml of a dioxane/pyrrolidine mixture (1:1, v/v) and 10μl of 1-methylnaphthalene was added as an internal standard. The aminolysis reaction was conducted in 30℃ for 1hr, and then the amount of 1-acetylpyrrolidine was measured using GC

analysis (Agilent 7890A) on an instrument equipped with a capillary column DB-5 (30 m × 0.32 mm × 0.25 μm, Agilent) using the following temperature program: injection and detector temperatures were set to 220˚C and 300˚C, respectively. The oven temperature program consisted of 100˚C for 5 min, followed by a heating rate of 30 ˚C/min to 173 ˚C for 5min, 5˚C/min to 180˚C for 5min, and finally 20˚C/min to 200˚C for 3min (Kim et al., 2012).

2.2.1.3. Nitrobenzene oxidation (NBO)

Change of DHP structure after fungal treatment was anlalyzed by measuring nitrobenzene oxidation products. At first, 30mg of DHP was mixed with 4ml of 2M NaOH and 250μl of nitrobenzene in glass bomb. The mixture was reacted at 170˚C for 2hr. 3-ethoxy-4-hydroxybenzaldehyde was used as an internal standard. (Iiyama & Lam, 1990). The reaction products were trimethylsilylated with 100 μl pyridine and 100 μl of N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) at 105 ℃ for 2 h, and analyzed with an Agilent 7890A GC equipped with a 5974C mass selective detector and FID. A DB-5 capillary column (60 m × 0.25 mm × 0.25 μm film thickness) was used for analysis of products. The injector and detector temperature were 200 ˚C and 250 ˚C, respectively. The oven temperature program consisted of 120 ˚C for 10 min, followed by heating at a rate of 10 ˚C/min to 280 ˚C (Kim et al., 2012).

2.2.2. Analysis of degradation products of DHP

After centrifugation, the supernatant was extracted with 50ml of ethyl acetate in triplicate, evaporated and finally, dissolved in 10ml of ethylacetate. For analysis of characteristics of liquid fraction, GPC was carried out as described in section 2.2.1, and GC-MS was performed as described as section 2.3 in Chapter 2.

2.3. Analysis of structural changes and degradation products of DHP with addition of mediator and reducing agents

To degrade DHP during biomodification by *A. biennis*, reducing agents and mediator of enzyme were added in culture medium.

1mM of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as laccase mediator was added in medium with addition of DHP. After that, ABTS was added again on incubation day 12.

5mM of ascorbic acid and 1mM of α-tocopherol as reducing agent were added in SSC medium. Additions of these reducing agents were conducted periodically on incubation day 2, 8, 13, 17 after preincubation.

Structural changes of DHP and degradation products by fungus were analyzed as described at 2.2.

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2.4. Effect of laccase and its mediator system on biomodification of lignin compounds

To better understand laccase mediator system, change of molecular weight of lignin compounds was examined by using laccase purchased from Sigma.

In vitro reaction were carried out at 30℃ in 5.0 ml of 0.05M sodium lactate buffer (pH5) containing 2 unit/ml of laccase, substrates and mediator. 4mg of Monolinols and 10 mg of DHP were added, respectively. Laccase mediator, 1mM of ABTS was added. Additional ascorbic acid was added at various intervals. After incubation for the desired period, monolignols were extracted by Sep pak C 18 cartridge with methanol, and DHP was obtained by lyophilization after reaction was finished. Analysis of molecular weight followed as described in 2.2.1 of this chapter.

For examining oxidation of substrate by laccase depending on addition of ABTS, it was determined spectrophotometrically using reaction mixtures containing 14mM of guaiacol and 2,6-dimethoxyphenol as substrate. Reaction were started by adding ABTS and measured in absorbance at 465nm(substrate : guaiacol) or 469nm(substrate : 2,6-dimethoxyphenol).

2.5. Transcriptomic analysis of *A. biennis* exposed to DHP

For investigating enzymes of *A. biennis* related to lignin degradaion, transcriptomic analysis was carried out using Illumina HiSeq ™ 2500.

To analyze DEGs between fungal samples, fungal samples were divided into 3 groups (Table 3-1).

Table 3-1. Fungal samples of which transcriptomic analysis was performed

	Fungal sample groups	Abbreviation
1.	*A. biennis* on 20 days	ABB
2.	*A. biennis* treated with reducing agents and ABTS on 20 days	ABB+ABTS+R.A
3.	*A. biennis* treated with DHP under the addition of reducing agents and ABTS on 20 days	ABB+DHP+ABTS+R.A

*ABB: *Abortiporus biennis*

*ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

*DHP: dehydrogenative polymer

*R.A: Reducing agents

2.5.1. Total RNA extraction and NGS library preparation

Mycelim of fungal samples were ground to fine powder with liquid nitrogen. Total RNA was isolated using the Hydrid-R™ extraction kit. The quality and quantity of total RNA was analyzed by Agilent Bio analyzer with RNA nano chip and Perkin Elmer Victor 3 with utilizing of Quant-iT ™ RiboGreen assay kit, respectively.

After total RNA extraction, NGS library preparation for transcriptomic analysis was carried out by BIOO Scientific kit. mRNA was purified from total RNA extracted by fungal cell, and then cDNA was synthesized using the NEXflex Rapid Directional mRNA Seq kit (BIOO Scientific). Quantification of cDNA library was checked by qPCR protocol. Sequencing was carried out using Illumina HiSeq ™ 2500 platform. Transcriptome data produced from Illumina HiSeq ™ 2500 were filtered by removing the adapter sequences, empty reads, low quality reads, and the reads with more than 20% Q < 20 bases. Transcriptome de novo assembly was carried out with three assemblers such as CLC Genomics Workbench (ver. 5.1) and Trinity (release 2.1.1). A default k-mer value (25-mer) was used for assembly with CLC while Trinity was performed with various k-mer lengths. As Trinity or CLC Genomics Workbench does not cluster assembled contigs, CD-HIT-EST was used to cluster the contigs with identity more than 95% and coverage of 100%. All resulting data sets were merged into a single assembly by collapsing identical or near-identical contigs into single unigenes.

New gene set of *A. biennis* was made through de novo assembly with RNA-seq data obtained by preprocessing. Mapping process was performed using the TOPHat program, and then transcript assembly was performed by cufflinks program. Depending on read mapping on new gene set, FPKM levels were determined, after normalization process. All experiments related to transcriptomic analysis were performed in National Instrumentation Center for

Environmetal Management(NICEM).

2.5.2. Analysis of Differently Expressed Genes (DEGs)

To identify the differentially expressed genes between the three samples as described in Table 3-1, edgeR package was used based on results of FPKM level. Fold change (fc) value was determined by statistics process. Absolute value of the log2 ratio >1 were used as threshold to determine significant differences in gene expression between the samples.

2.5.3. GO annotation

For investigating function of genes and genes products of fungus, GO (gene ontology) annotation was analyzed. Based on sequence homology search against the NCBI NR database using an E-value cutoff of 0.0001, unigenes were annotated on the GO subcategories by using Blast2GO program.

2.5.4. KEGG annotation

KEGG (kyoto encyclopedia of genes and genomes) metabolic pathways analysis was performed by aligning genes with sequences from the NCBI NR database and automatically assigning gene functions to corresponding KEGG terms.

3. Results and Discussions

3.1. Biomodification mechanism of DHP by *A. biennis*

3.1.1. Structural change of DHP

To investigate biomodification mechanism of DHP by *A. biennis*, structural characteristics of DHP were investigated through the analysis of phenolic hydroxyl(OH) groups, ether bonds and molecular weights.

Table 3-2 indicates the molecular weight change of DHP during incubation days. Although the weight-average molecular weight (M_w) of control were similar with that of DHP, the Mw of DHP treated by *A. biennis* decreased by incubation day of 10, thereafter increased by incubation day of 25. M_w of DHP modified by *A. biennis* was 3,203 on 10 days and 3,785 on 25 days.

The amount of phenolic OH group of DHP under the ligninolytic treatment by *A. biennis* was higher than that of control (Figure 3-1). On incubation day 10, phenolic OH content reached on 11.0%, which is significant increase compared with previous researches on lignin biomodification (Revin et al., 2002). During incubation days, phenolic OH contents of DHP modified by fungus kept in higher content than that of control. Especially, there was high content of phenolic OH of DHP on incubation day 10 with low molecular weight. One of the great advantages for replacement of phenolics by lignin was high phenolic OH contents within lignin (Stewart, 2008). Accordingly, high phenolic OH groups of DHP on day 10 provided opportunities for modification in attempts to develop novel applications for lignin.

NBO can produce phenolic acids and aldehydes compounds from lignin

such as vanillin, syringaldehyde, vanillic acid and syringic acid (Kim et al., 2012). NBO is important method for cleavage of ether bonds such as α-O-4 and β-O-4 within lignin for quantitatively determining lignin units (Chen, 1992; Lawther et al., 1996).

After fungal treatment, the amount of NBO products decreased with increase of incubation day (Table 3-3). These results mean that *A. bennis* catalyzed ether bonds cleavages of DHP.

Based on the results above, it was revealed that catalytic enzyme system of *A. bennis* caused cleavage of ether bonds, which contributed to high phenolic OH contens of DHP and decrease of molecular weight in initial incubation day. With increase of incubation day, polymerization reaction occurred by *A. bennis* despite of decrease of amount of NBO products in fungal sample. In latter part of incubation day, DHP seemed to be polymerized by other type linkages not ether bonds, which was considered to be condensed form than DHP modified on 5 days (Baucher et al., 1998).

Degradation of lignin has been recognized to occur based on lignin degrading enzymes of white rot basidiomycetes (Higuchi, 1990; Kirk & Farrell, 1987). In this study, it was observed that fungal dried weight increased with addition of DHP (Figure 3-3), and extracellular protein concentration and specific activities of lignin degrading enzymes were higher than those of control (Figure 3-4). This result presented that *A. biennis* seemed to use DHP as carbon source for growth as well as glucose in medium, which had positive effect on secretion of ligninolytic enzymes. Previous study reported that lignin not only served as substrate for growth but was degraded in presence of carbohydrates (Kirk et al., 1976). As a result, activities of ligninolytic enzymes of *A. biennis* were enhanced by addition of synthetic lignin, and had an impact on simultaneously degradation and polymerization of DHP. Especially, polymerization in later incubation day seemed to occur by increase of unstable radicals generated during degradation process. In addition, taking

into account the increase of extracellular protein concentration in fungus exposed to DHP**,** it suggested a variety of extracellular oxidative enzymes such as H_2O_2-generating enzymes, and Fenton reaction-related enzymes were implicated in lignin modification (Dashtban et al., 2010; Kersten & Cullen, 2007).

Consequently, biomodification mechanism of DHP by *A. biennis* was similar with that of monolignols. Especially, because DHP is macromolecular polymer, it was difficult to degrade lignin through only ligninolytic treatment by *A. biennis.* Therefore, for lignin degradation by *A. biennis*, both enzyme system of fungus and culture condition were changed through the addition of laccase mediator and reducing agents, which presented in section of 3.2.

Table 3-2. Molecular weight change of dehydrogenative polymer (DHP) treated by *A. biennis* (Control: DHP in medium. ABB+ DHP: DHP treated by *A. biennis*)

Treatment periods		0d	5d	10d	15d	20d	25d
Control	M_w [a] (Daltons)	3686	3527	3676	3701	3457	3563
	M_n [b] (Daltons)	1832	2038	2393	2426	1978	1988
	M_w/M_n	2.01	1.73	1.54	1.53	1.75	1.79
ABB+DHP	M_w [a] (Daltons)	3686	3440	3203	3581	3403	3785
	M_n [b] (Daltons)	1832	1836	1714	1800	1498	1825
	M_w/M_n	2.01	1.87	1.87	1.99	2.27	2.07

*milled wood lignin: M_w: 9919, M_n: 4240, Polydispersity: 2.34

[a] weight-average molecular weight

[b] number-average molecular weight

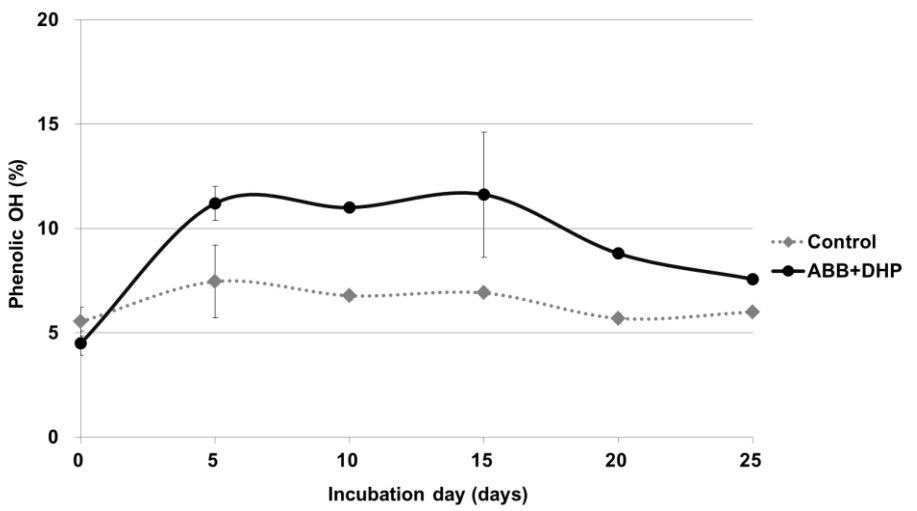


Figure 3-1. Phenolic hydroxyl group content of dehydrogenative polymer (DHP) treated by *A. biennis* (Control: DHP in medium, ABB+DHP: DHP treated by *A. biennis*)

*Phenolic OH content of DHP: 4.6%

Table 3-3. Nitrobenzene oxidation products (μmol/g sample) of dehydrogenative polymer (DHP) treated by *A. biennis* (Control: DHP in medium, ABB+DHP: DHP treated by *A. biennis*)

Incubation days	Control			ABB+DHP		
	G unit	S unit	Sum of G&S units	G unit	S unit	Sum of G&S units
0d	161.2±16.8	861.0±123.5	1022.3±140.3	149.3±2.0	773.7±48.2	923.0±50.2
5d	171.7±5.8	701.0±23.7	872.7±29.5	129.6±53.7	698.1±15.4	827.7±207.4
10d	236.8±71.4	1037.4±273.6	1274.2±345.1	99.0±22.5	421.7±98.4	520.7±120.9
15d	159.1±10.7	681.7±33.1	840.8±22.4	105.0±1.4	650.9±92.7	755.9±94.1
20d	173.4±48.3	937.9±50.4	1111.3±98.8	75.0±12.8	424.7±82.5	499.7±95.3
25d	112.3±15.6	845.6±40.7	957.9±56.3	45.9±12.1	254.3±88.2	300.3±7.6

***** DHP: G unit: 219.2±36 μmol/g $_{sample}$, S unit: 1081.8±102.5 μmol/g $_{sample}$,
Sum of G & S units: 1301.1±138.5 μmol/g $_{sample}$

3.1.2. Characteristics of lignin oligomers derived from DHP

To evaluate characteristics of lignin oligomers derived from DHP, GPC and GC-MS analysis were conducted.

As a result of GC-MS analysis, monomeric compounds newly formed by fungus were not detected during incubation day. This result suggested that *A. biennis* did not lead to formation of monomeric compounds from DHP.

The molecular weight distribution of lignin oligomers dissolved in culture medium was divided into group 1 and group 2 (Figure 3-2(A)). Mws of group 1 were about 1,000 during incubation period. These distributions of lignin oligomers became more obvious by *A. biennis* (Figure 3-2(B, C)). The Mw of group 1 slightly declined with increase of incubation day indicating 1,057 and 970 on incubation day 5 and 25, respectively (Table 3-4).

As a result, *A. biennis* catalyzed degradation of lignin oligomers dissolved in medium during incubation day. Degraded products seemed to have an effect on polymerization of DHP after 15 days. Therefore, degradation of lignin oligomers was intimately interconnected with polymerization reaction of DHP by catalytic system of *A. biennis*.

Table 3-4. Molecular weight change of oligomers derived from dehydrogenative polymer (DHP) treated by *A. biennis* (Control: DHP in medium. ABB+ DHP: DHP treated by *A. biennis*)

Control

	5d		10d	
	1	2	1	2
M_w [a] (Daltons)	1021	225	1009	232
M_n [b] (Daltons)	846	208	793	217
M_w/M_n	1.21	1.08	1.27	1.07

ABB+DHP

	5d		10d		15d		20d		25d	
	1	2	1	2	1	2	1	2	1	2
M_w [a] (Daltons)	1057	206	1036	213	988	222	993	228	970	224
M_n [b] (Daltons)	877	193	852	198	837	206	844	210	828	201
M_w/M_n	1.21	1.07	1.22	1.08	1.18	1.08	1.18	1.09	1.17	1.11

[a] weight-average molecular weight

[b] number-average molecular weight

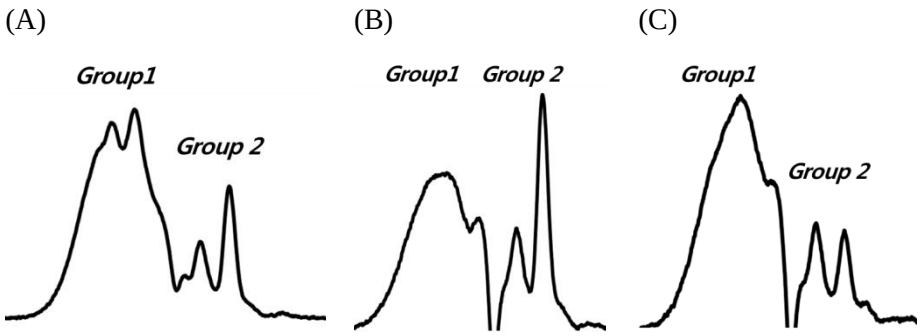


Figure 3-2. Gel permeation chromatogram of oligomers derived from DHP during incubation time ((A) Control on day 5, (B) ABB+DHP sample on day 5, (C) ABB+DHP sample on day 20)

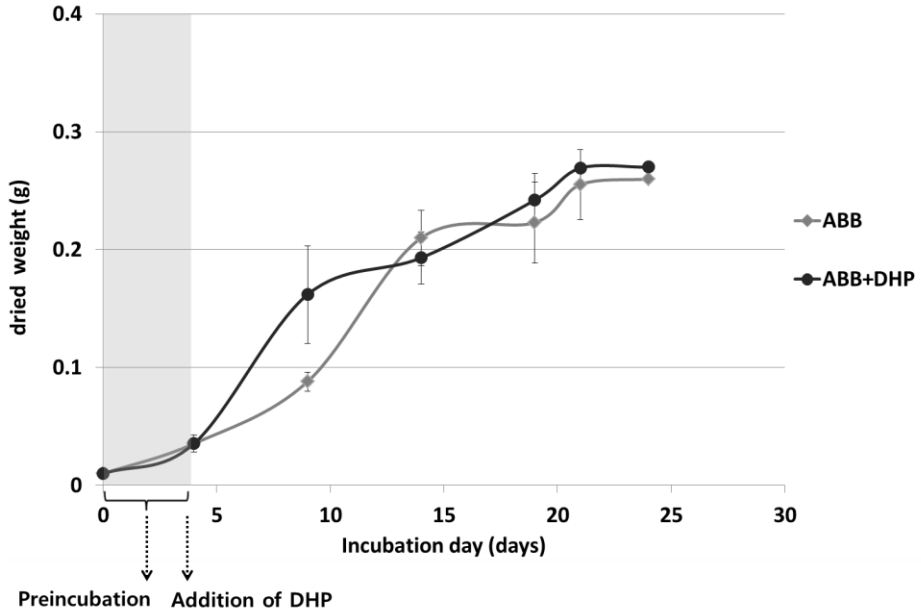
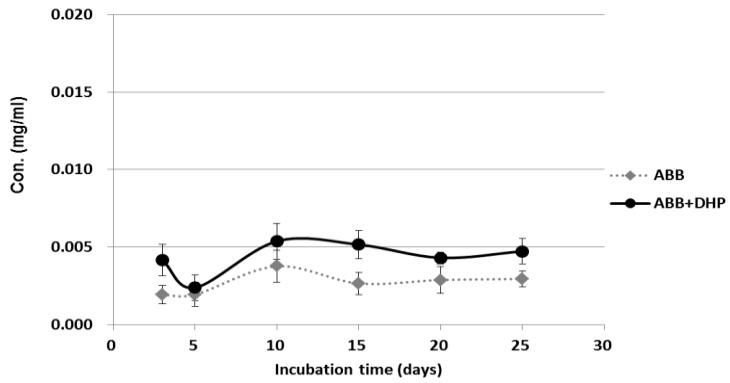
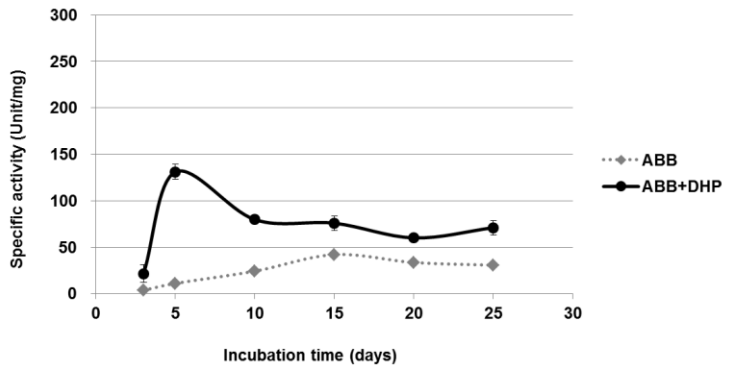


Figure 3-3. Mycelium dried weight of *A. biennis* depending on addition of DHP during incubation days (ABB: *A. biennis*, ABB+ DHP*: A. biennis* exposed to DHP)

(A)



(B)



(C)

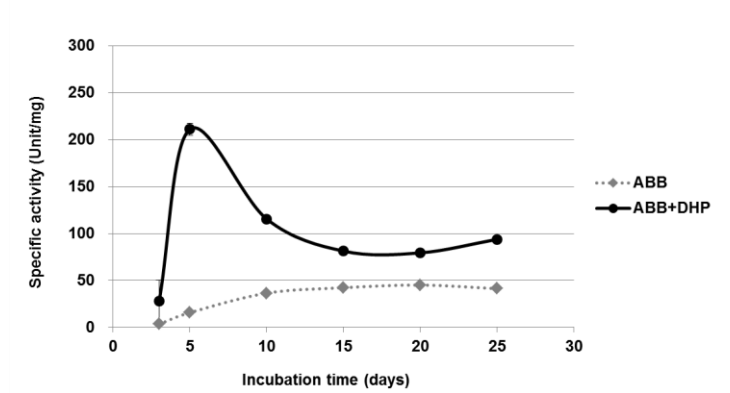


Figure 3-4. Protein concentration (A) and activities of manganese peroxidase (B) and laccase (C) of *A. biennis* with addition of DHP (ABB: *A. biennis* in medium, ABB+DHP: fungal sample adding DHP)

3.2. Degradation of synthetic lignin by *A. biennis* with mediator and reducing agents

3.2.1. Structural change of DHP depending on addition of each mediator and reducing agents

In this study, to degrade DHP, enzymes system of *A. biennis* and culture condition was regulated through the addition of mediator and reducing agents. Above all, to regulate enzymes system of *A. biennis*, mediator system of lignin degrading enzyems was used. Laccase-mediator system is best known case among ligninolytic enzymes and their mediation system. There are several mediators in laccase system including ABTS, HAA (3-hydroxyzntranilic acid), HBT (1-hydroxybenzotriazole), and NHA(N-hydroxyzcetanilide) and so on (d'Acunzo et al., 2004; Li et al., 1999). Mediators of laccase have been recognized to be environmental-friendly and available at low cost. That was why this have drawn much attention in pulp-bleaching techniques, and these days, biotechnological application of laccase mediator system can develop the lignocellulose biorefinery (Cañas & Camarero, 2010). Fundamentally, laccase mediator system is able to overcome the limitation of laccase alone to phenolic compounds (Rochefort et al., 2004). Action of laccase catalyzes polymerization of lignin compounds as well as degradation (Gouveia et al., 2013; Maijala et al., 2012), while their mediator system catalyze depolymerization of lignin (Fernaud et al., 2006; Shleev et al., 2006). However, the effect of laccase mediator system on lignin is equivocal because polymerization and depolymerization occurs differently depending on a kind of basidiomycetes and surrounding conditions (Munk et al., 2015). Accordingly, biomodification mechanism of DHP by *A. biennis* was observed under the addition of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic

acid)). ABTS oxidized by laccase formed cation radical and dication, resulting in oxidation of aromatic compounds (Majcherczyk et al., 1999).

This study showed molecular weight of DHP treated by *A. biennis* was lower than that of control, and reached on 2,676 on incubation day 5 (Table 3-5 (A)). Amount of phenolic OH group of DHP modified was higher than that of control from 1day and 10 days, thereafter it had a similar tendency with control (Figure 3-5). Amount of NBO products of DHP was lower than that of control. After incubation day 10, it was declined sharply, and total amount of G and S unit was 131.5 μmol/g sample on incubation day 15 (Table 3-6).

Based on the results above, the mediator system of *A. biennis* catalyzed degradation of DHP through the cleavage of ether bonds in initial incubation day. The difference with results of 3.1 was that polymerization did not occur despite of cleavage of ether bonds. It explained catalytic system of *A. biennis* with laccase mediator, ABTS, assisted degradaion of DHP.

Along with enzyme system of of *A. biennis*, it was investigated how change of surrounding condition influences on biomodificaiton of lignin in present study. Ascorbic acid and and α-tocopherol were used as reducing agents in this study. Ascorbic acid and α-tocopherol is representative hydrophilic and hydrophobic reducing agent, respectively. Preliminary study indicated that addition of ascorbic acid alone was effective to degradaion of DHP in initial day. α-Tocopherol was reported to scavenge and quench reactive oxygen species and peroxy radicals (Huang et al., 1996; Shao et al., 2008). Furthermore, because ascorbic acid regenerate tocopherol from tocopheroxyl radical (Niki, 1991), the presence of two reducing agents is predicted to be effective to depolymerize DHP. Therefore, experiment with α-tocopherol as well as ascorbic acid was carried out for degradaion of DHP.

As a result of GPC analysis, molecular weights of DHP were decreased slightly (Table 3-5(B)). Mw of control was 3,132 and 3,034 Da on incubation day on 10 and 20, respectively. Mw of DHP treated by *A. biennis* ranged from

2,839 to 3,003 during incubation day, which was very stable variation.

Phenolic OH contents of DHP declined with increase of incubation day (Figure 3-5). After 10 days, phenolic OH contents of fungal sample were similar with that of control.Compared with the NBO products of control, those after fungal treatment was generally lower. Amount of NBO products of DHP modified by *P. chrysosporium* decreased from 1579.2 μmol/g on day 1 to 404.5 μmol/g on day 25 (Table 3-7).

Results of phenolic OH contents and NBO products were indicative that α-tocopherol played a significant role to stabilize DHP, which was supported by decrease of phenolic OH group content despite of cleavage of ether bonds.

Consequently, *A. biennis* assisted degradaion of DHP by the change of enzyme system and culture condition through the addition of laccase mediator and reducing agents. These findings could expand catalytic system of *A. biennis* towards various industrial applications. Especially, enzyme system of *A. biennis* possess biotechnological potential in lignin modification.

Table 3-5. Molecular weight change of dehydrogenative polymer (DHP) treated by *A. biennis* depending on addition of each ABTS and reducing agents (Control: DHP in medium, ABB+ DHP: DHP treated by *A. biennis*)

(A) DHP+Mediator

		1d	5d	10d	15d	20d
Control	M_w [a] (Daltons)	3260	-	3109	-	3352
	M_n [b] (Daltons)	1591	-	1705	-	1773
	M_w/M_n	2.05	-	1.94	-	2.00
ABB+DHP	M_w [a] (Daltons)	3307	2676	2991	2918	3151
	M_n [b] (Daltons)	1581	1316	1425	1396	1471
	M_w/M_n	2.09	2.03	2.10	2.09	2.14

(B) DHP+Reducng agents

		1d	5d	10d	15d	20d
Control	M_w [a] (Daltons)	3077	-	3132	-	3034
	M_n [b] (Daltons)	1128	-	1201	-	1134
	M_w/M_n	2.72	-	2.61	-	2.68
ABB+DHP	M_w [a] (Daltons)	3156	2839	2943	3003	2952
	M_n [b] (Daltons)	1466	1357	1311	1364	1353
	M_w/M_n	2.15	2.09	2.24	2.20	2.18

DHP : : M_w: 3421, M_n: 1476, Polydispersity: 2.32

[a] weight-average molecular weight

[b] number-average molecular weight

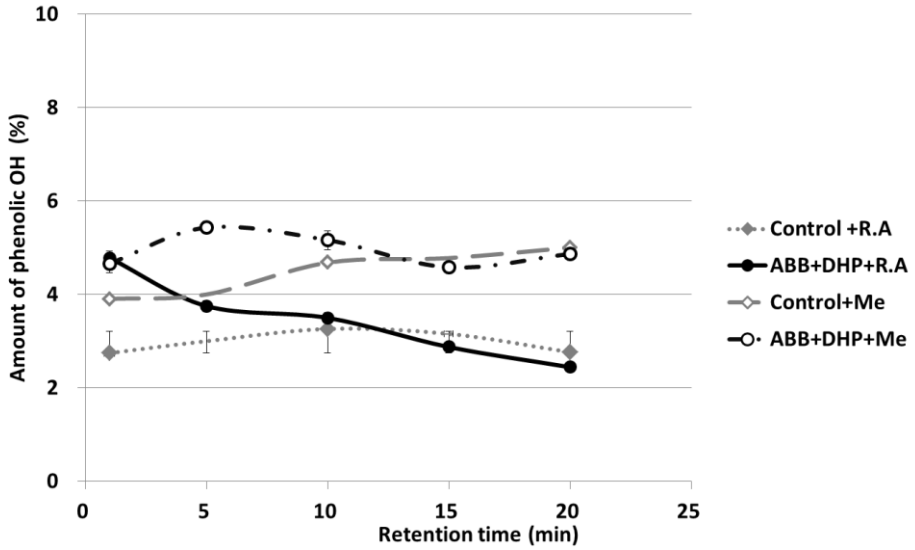


Figure 3-5. Phenolic hydroxyl group content of dehydrogenative polymer (DHP) treated by *A. biennis* depending on addition of each ABTS and reducing agents

(Control+R.A: DHP in medium with reducing agents, ABB+DHP+R.A: DHP treated by *A. biennis* with reducing agents, Control+Me: DHP in medium with mediator, ABB+DHP+Me: DHP treated by *A. biennis* with mediator)

*Phenolic OH content of DHP: 5.0%

Table 3-6. Nitrobenzene oxidation products (μmol/g sample) of dehydrogenative polymer (DHP) treated by *A. biennis* with mediator (Control: DHP in medium, ABB+DHP+ABTS: DHP treated by *A. biennis* with ABTS)

Incubation days	Control			ABB+DHP+ABTS		
	G unit	S unit	Sum of G&S units	G unit	S unit	Sum of G&S units
1d	245.8	1487.4	1733.2	174.1	255.3	429.4
5d	-	-	-	76.6	479.5	556.
10d	182.7	1205.9	1388.6	110.8	548.8	659.7
15d	-	-	-	36.3	95.1	131.5
20d	132.6	1463.9	1596.5	48.8	186.6	235.4

***** DHP: G unit: 310.7±120.8 μmol/g $_{sample}$, S unit:1461.9±346.4 μmol/g $_{sample}$,
Sum of G & S units: 1772.6±.467.2 μmol/g $_{sample}$

Table 3-7. Nitrobenzene oxidation products (μmol/g sample) of dehydrogenative polymer (DHP) treated by *A. biennis* with reducing agents (Control: DHP in medium, ABB+DHP+R.A : DHP treated by *A. biennis* with reducing agents)

Incubation days	Control			ABB+DHP+R.A		
	G unit	S unit	Sum of G&S units	G unit	S unit	Sum of G&S units
1d	70.0±10.2	1500.1±89.2	1570.1±99.4	87.2±0.6	1491.9±9.7	1579.2±10.3
5d	-	-	-	43.6±5.2	860.7±51.0	904.2±45.8
10d	73.7±5.1	1596.1±260.2	1669.8±265.2	37.4±4.8	1086.6±334.4	1124.0±329.5
15d	-	-	-	146.4±63.2	636.7±381.8	783.0±445.0
20d	62.1±10.1	1061.7±202.3	1123.8±212.4	68.1±9.2	336.4±42.1	404.5±51.3

***** DHP: G unit: 310.7±120.8 μmol/g $_{sample}$, S unit: 1461.9±346.4 μmol/g $_{sample}$,
Sum of G & S units: 1772.6±.467.2 μmol/g $_{sample}$

3.2.2. Structural change of DHP with mediator and reducing agents at the same time.

Based on results above, each addition of reducing agent and mediator induced degradaion of DHP by *A. biennis*. Laccase mediator, ABTS played a key role to oxidation of non-phenolic lignin compounds via electron transfer route (Baiocco et al., 2003). Reducing agents have an important effect on inhibition of polymerization by stabilizing reactive radicals (Huang et al., 1996; Shao et al., 2008). As a result, change of enzymes system and surrounding condition depolymerized DHP. Accordingly, two conditions in biomodification of DHP were simultaneously applied, looking forward to synergetic effects.

ABTS was added with DHP at the same time, and reducing agents, ascorbic acid and α-tocopherol were added periodically during incubation day.

At first, molecular weight of DHP under the changed ligninolytic condition decreased rapidly after 10 days. The Mw of DHP in fungal sample was 2,654 and 2,514 on incubation day 15 and 20, respectively (Table 3-8). DHP on day 20 was significantly degraded by fungus with decrease of polydispersity index. Many studies reported laccase mediator system of basidiomycetes showed excellent ability to depolymerize synthetic lignin (Eggert et al., 1996; Kawai et al., 1999; Srebotnik & Hammel, 2000). However, most previous studies were conducted by using the purified laccase from basidiomycetes. Therefore, it was very meaningful in inducing lignin degradaion by using whole cell of basidiomycete.

Structural changes of DHP modified by fungus had a similar tendency with above mentioned results. Phenolic OH contents of DHP were declined with increase of incubation day (Figure 3-6) although amounts of NBO products was fairly low (Table 3-9). This means that reducing agents stabilized phenolic OH group of lignin created by breaking of ether bonds.

One addressable result was that decrease of molecular weight occurred after 10 days although ether bonds cleaved by *A. biennis* from initial incubation day. In present study, addition of ABTS was simultaneously conducted with DHP. In previous experiments adding the only ABTS, ABTS was added on preincubation 3 days, and then DHP was spiked in culture medium on preincubation 4 days. At that time, DHP was degraded from initial incubation day. However, this study showed different results. Degradaion of DHP occurred when sufficient mediator and reducing agents existed in medium. More studies were required for better understanding of detailed modification mechanism of DHP depending on addition point of mediator and reducing agents.

Consequently, whole cell of *A. biennis* assisted considerable degradaion of DHP under the simultaneous addition of both laccase mediator and reducing agents. This result could suggest optimal condition for degradaion of lignin by using *A. biennis*. Furthermore, lignin degradation system of *A. biennis* could be effectually used in database to perform biomodification of lignin by fungus in future.

Table 3-8. Molecular weight change of dehydrogenative polymer (DHP) treated by *A. biennis* with addition of both ABTS and reducing agents (Control: DHP in medium, ABB+ DHP: DHP treated by *A. biennis*)

Control			
	5d	10d	20d
M_w [a] (Daltons)	3302	3438	3349
M_n [b] (Daltons)	1644	1578	1498
M_w/M_n	2.01	2.18	2.24

ABB+DHP				
	5d	10d	15d	20d
M_w [a] (Daltons)	3759	3240	2654	2514
M_n [b] (Daltons)	1514	1508	1332	1323
M_w/M_n	2.48	2.15	1.99	1.90

DHP: M_w: 3498, M_n: 1721, Polydispersity: 2.03

[a] weight-average molecular weight

[b] number-average molecular weight

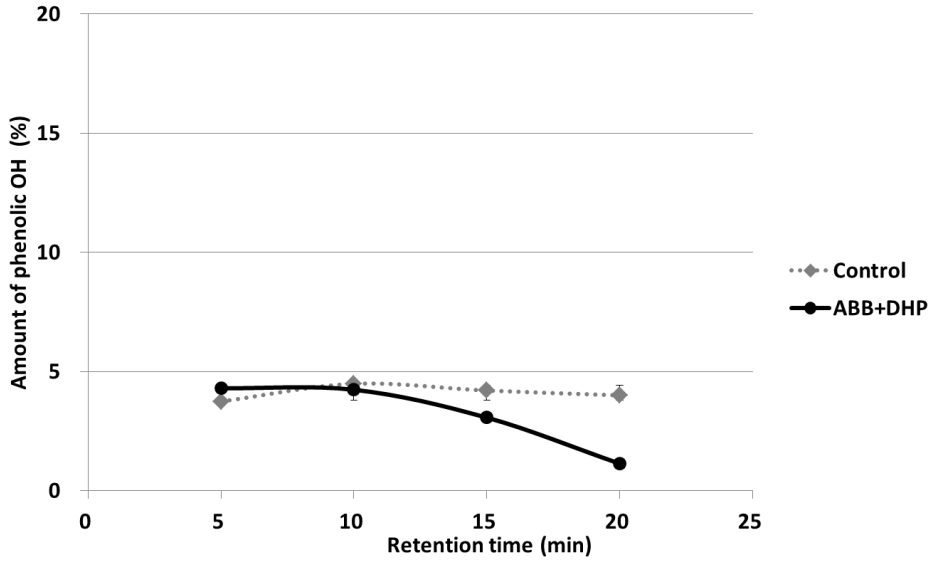


Figure 3-6. Phenolic hydroxyl group content of dehydrogenative polymer (DHP) treated by *A. biennis* with addition of both ABTS and reducing agents (Control: DHP in medium, ABB+DHP: DHP treated by *A. biennis*)

*Phenolic OH content of DHP: 5.3%

Table 3-9. Nitrobenzene oxidation products (μmol/g sample) of dehydrogenative polymer (DHP) treated by *A. biennis* with addition of both ABTS and reducing agents

Incubation days	**ABB+DHP**		
	G unit	S unit	Sum of G&S units
5d	83.2±15.5	371..0±69.0	454.2±84.5
10d	43.2±13.7	212.5±69.9	255.7±83.6
15d	33.2±2.4	156.4±3.4	189.7±5.8
20d	56.9±26.9	272.0±127.4	328.9±154.3

3.2.3. Degradation of lignin oligomers derived from DHP depending on addition of mediator and reducing agents.

Along with structural changes of DHP, characteristics of lignin oligomers derived from DHP were examined as showed in Table 3-10 and Figure 3-7.

Lignin oligomers were degraded by *A. biennis* when each reducing agents and mediator were added (Table 3-10). In results mentioned above, lignin oligomers were divided to large molecular weight fraction (group 1) and low molecular weight fraction (group 2) as described in Figure 3-2. On the other hand, addition of reducing agents and mediator caused these oligomers division to be disappeared.

At first, GPC results indicated that lignin oligomers were degraded slightly under the ligniolytic treatment wih ABTS. The Mw on incubation day 20 was 840 (Table 3-10).

Next, in the case of reducing agents, degradation of lignin oligomers occurred actively, and the Mw reached on 606 and 657 on incubation day 15 and 20 (Table 3-10).

Finally, when both reducing agents and mediator were simultaneously added in medium, the Mw of lignin oligomers was the lowest compared to those of other conditions.

On the whole, lignin oligomers consisted of group 1 and group 2 in initial incubation day. With increase of incubation day, *A. biennis* catalyzed degradation of lignin oligomers. Accordingly, degraded oligomers existed in medium after fungal treatment with reducing agents and mediator (Figure 3-7). Catalytic system of *A. biennis* also exhibited great ability to degrade lignin oligomers dissolved in medium by addition of both reducing agents and mediator.

Table 3-10. Molecular weight change of oligomers derived from dehydrogenative polymer (DHP) after treated by *A. biennis*

		5d		**10d**		**15d**	**20d**
DHP +Mediator	M_w [a] (Daltons)	1057	165	909		910	840
	M_n [b] (Daltons)	751	161	568		586	543
	M_w/M_n	1.41	1.02	1.60		1.55	1.55
DHP +Reducing agents	M_w [a] (Daltons)	1117	225	668		606	657
	M_n [b] (Daltons)	874	218	376		348	365
	M_w/M_n	1.27	1.03	1.78		1.74	1.80
DHP +Mediator +Reducing agents	M_w [a] (Daltons)	887	199	990	231	842	634
	M_n [b] (Daltons)	761	185	840	222	487	375
	M_w/M_n	1.17	1.08	1.18	1.04	1.73	1.69

[a] weight-average molecular weight

[b] number-average molecular weight

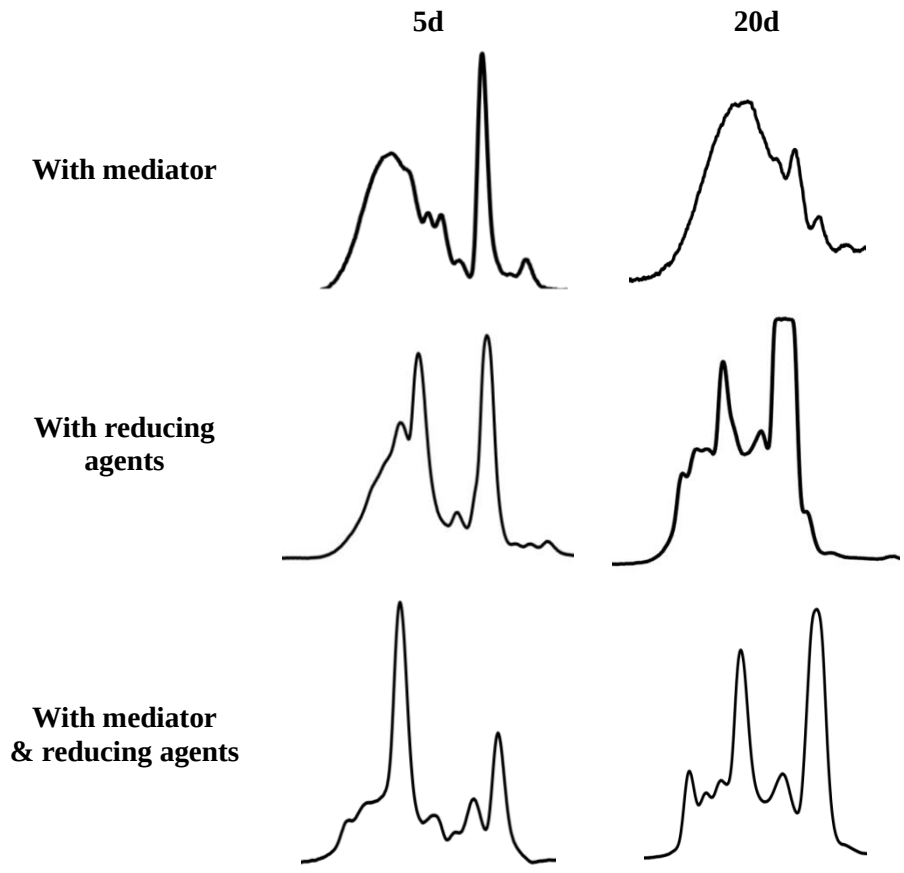


Figure 3-7. Gel permeation chromatogram of oligomers derived from DHP depending on mediator and reducing agents

3.3. Effect of laccase mediator on biomodification of lignin compounds

3.3.1. Effect of laccase and laccase mediator system on biomodification of monolignols and DHP

Laccase mediator system of *A. biennis* showed outstanding ability to degrade DHP. To demonstrate clearly involvement of laccase and its mediator system in lignin degradaion, function of laccase and its mediator system depending on a kind of substrates was evaluated.

Coniferyl alcohol was rapidly polymerized by laccase, and their molecular weight after reaction with laccase reached on 1,582 on incubation time 12 hrs (Table 3-11). Addition of ABTS lead to decrease of molecular weight of coniferyl alcohol showing their Mw was 708 on incubation time 12 hrs (Table 3-11). Furthermore, ascorbic acid as reducing agent brought about degradaion of coniferyl alcohol.

Likewise modification of coniferyl alcohol by laccase, modification of sinapyl alcohol showed similar tendency. The molecular weight of sinapyl alcohol after laccase treatment with ABTS and ascorbic acid reached on 691, the lowest value, along with decrease of polydispersity index (Table 3-12).

As a result, monolignols were polymerized by laccase within short time because laccase easily attack phenolic compounds. The degree of increase of the molecular weights of coniferyl alcohol was more highly polymerized than those of sinapyl alcohol. Because coniferyl alcohol is substituted with methoxyl groups at only C5 in the aromatic ring, radicals can be form on the various positions including OH-group in the aromatic ring (Kudanga et al., 2011). Accordingly, radical coupling reactions were more active in coniferyl alcohol.

In contrast to monolignols results, DHP was degraded by laccase. The Mw of DHP by laccase declined by 2,004 on incubation time 48 hrs (Table 3-13). Addition of mediator and reducing agent also lead to degradaion of DHP. Laccase preferentially broke ether bonds of DHP, not polymerization. Because the redox potential of monolignols and DHP are fundamentally different, it was assumed that catalytic mechanism of laccase acted on DHP differently, compared with monolignols. That was the reason why the redox potentials is very crucial factor in degradation of lignin (Li et al., 1999). And also, this result suggested that laccase of *A. biennis* was implicated in lignin degradation in initial incubation time.

In present study, laccase mediator system degraded DHP and declined the rate of polymerization of monolignols. Because the non-phenolic compounds were reported to have high redox potential, it was difficult to oxidize those by laccase alone (Cañas & Camarero, 2010; Li et al., 1999). Accordingly, laccase mediator system showed capability to more degrade lignin.

Consequently, synergetic effect of laccase mediator and reducing agent existed in lignin degradaion. This finding demonstrated above results which laccase mediator system of *A. biennis* played a crucial role in degradaion DHP with reducing agents.

Table 3-11. Molecular weight distributions of coniferyl alcohol after laccase treatment (CA: coniferyl alcohol in buffer, LAC+CA: coniferyl alcohol treated by laccase, LAC+CA+ABTS: coniferyl alcohol treated by laccase mediator system, LAC+CA+ABTS+Ascorbic acid: coniferyl alcohol treated by laccase mediator system with ascorbic acid)

		2hr	**4hr**	**6hr**	**12hr**
CA	M_w [a] (Daltons)	371	368	349	296
	M_n [b] (Daltons)	361	359	342	270
	M_w/M_n	1.03	1.03	1.02	1.09
LAC+CA	M_w [a] (Daltons)	1527	1509	1539	1582
	M_n [b] (Daltons)	1000	932	944	1158
	M_w/M_n	1.53	1.62	1.63	1.37
LAC+CA +ABTS	M_w [a] (Daltons)	1348	1000	746	708
	M_n [b] (Daltons)	885	730	574	570
	M_w/M_n	1.52	1.37	1.30	1.24
LAC+CA +ABTS +Ascorbic acid	M_w [a] (Daltons)	996	744	703	674
	M_n [b] (Daltons)	738	585	537	531
	M_w/M_n	1.35	1.27	1.31	1.27

[a] weight-average molecular weight

[b] number-average molecular weight

Table 3-12. Molecular weight distributions of sinapyl alcohol after laccase treatment (SA: sinapyl alcohol in buffer, LAC+SA: sinapyl alcohol treated by laccase, LAC+SA+ABTS: sinapyl alcohol treated by laccase mediator system, LAC+SA+ABTS+Ascorbic acid: sinapyl alcohol treated by laccase mediator system with ascorbic acid)

		2hr	**4hr**	**6hr**	**12hr**
SA	M_w [a] (Daltons)	385	399	300	291
	M_n [b] (Daltons)	377	391	287	242
	M_w/M_n	1.02	1.02	1.14	1.20
LAC+SA	M_w [a] (Daltons)	1007	938	1175	1080
	M_n [b] (Daltons)	884	846	781	754
	M_w/M_n	1.14	1.11	1.50	1.43
LAC+SA +ABTS	M_w [a] (Daltons)	1019	1172	916	813
	M_n [b] (Daltons)	772	809	643	609
	M_w/M_n	1.32	1.45	1.42	1.33
LAC+SA +ABTS +Ascorbic acid	M_w [a] (Daltons)	853	989	910	691
	M_n [b] (Daltons)	665	742	661	540
	M_w/M_n	1.28	1.33	1.38	1.28

[a] weight-average molecular weight

[b] number-average molecular weight

Table 3-13. Molecular weight distributions of DHP after laccase treatment (DHP: DHP in buffer, LAC+ DHP: DHP treated by laccase, LAC+DHP+ABTS: DHP treated by laccase mediator system, LAC+DHP+ABTS+Ascorbic acid: DHP treated by laccase mediator system with ascorbic acid)

		6hr	**12hr**	**24hr**	**48hr**
DHP	M_w [a] (Daltons)	2816	3124	3148	2965
	M_n [b] (Daltons)	1122	1206	1147	1196
	M_w/M_n	2.51	2.59	2.74	2.48
LAC+DHP	M_w [a] (Daltons)	2617	2189	2330	2004
	M_n [b] (Daltons)	1153	1012	1111	1037
	M_w/M_n	2.27	2.16	2.10	1.93
LAC+DHP +ABTS	M_w [a] (Daltons)	2153	1840	1712	1525
	M_n [b] (Daltons)	1077	940	1001	948
	M_w/M_n	2.00	1.96	1.71	1.61
LAC+DHP +ABTS +Ascorbic acid	M_w [a] (Daltons)	1619	1946	1380	1520
	M_n [b] (Daltons)	867	1409	756	931
	M_w/M_n	1.87	1.38	1.83	1.63

*DHP : : M_w: 2996, M_n:1573, Polydispersity: 1.90

[a] weight-average molecular weight

[b] number-average molecular weight

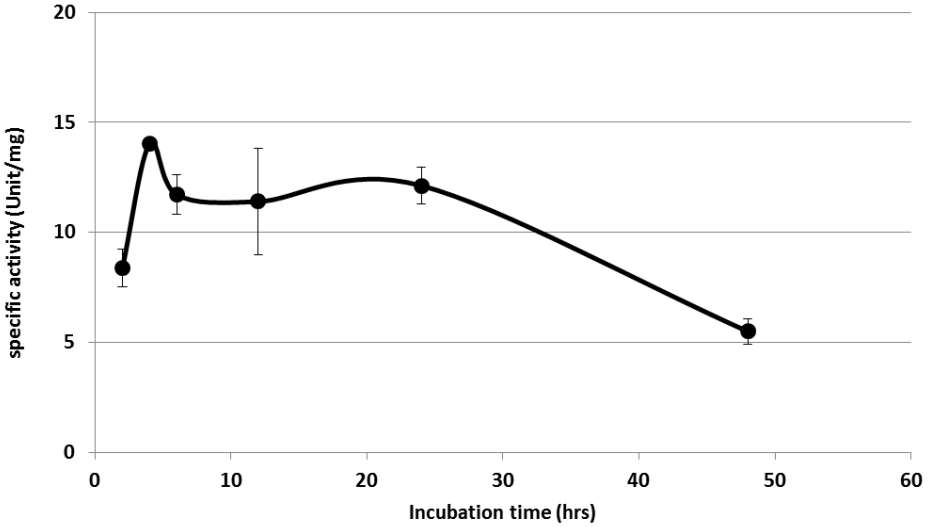


Figure 3-8. Laccase activity during incubation time

3.3.2. Comparison of substrate oxidation by laccase and laccase mediator system

To investigate the stability of laccase depending on a kind of substrate, the increase in absorbance due to oxidation of substrate were monitored for 20 min. As shown in Figure 3-9, increases of absorbance were observed in both guaiacol and syringol. The initial oxidations of monolignols by laccase were higher than those of monolignols with ABTS (Figure 3-9). Especially, in the case of guaiacol, addition of ABTS caused decrease of oxidation rate of guaiacol. High oxidation rate of substrate was reported to lead to polymerization of substrates, because transient increase of lignin phenoxy radical induce polymerization (Hammel et al., 1993; Yoshida et al., 1998). Accordingly, ABTS contributed to make a reduction of oxidation rate of substrate. That was why it led to degradation of lignin polymers.

When compared with difference of oxidation rate between guaiacol and syringol, initial oxidation rate of syringol was higher than that of guaiacol. It was reported that sinapyl alcohol among three monolignols was the most easily oxidized because of electron donating effect of methoxyl group substituted in S unit groups (Kobayashi et al., 2005). As a result, laccase catalyzed more oxidation of syringol than guaiacol.

However, increase of molecular weight of G unit by laccase was higher than that of S unit as shown in 3.3.1. This result suggested that possession of the number of positions which radical can be formed is more important than oxidation rate of substrate in polymerizing lignin. In addition, presence of side chain in aromatic compounds seemed to influence on polymerization of lignin precursors with oxidation of substrate.

In conclusion, laccase mediator system degraded lignin, resulting in lowering oxidation rate of substrates. Especially, ABTS was effective in reducing oxidation of G unit, which seemed to affect on degradaion of lignin.

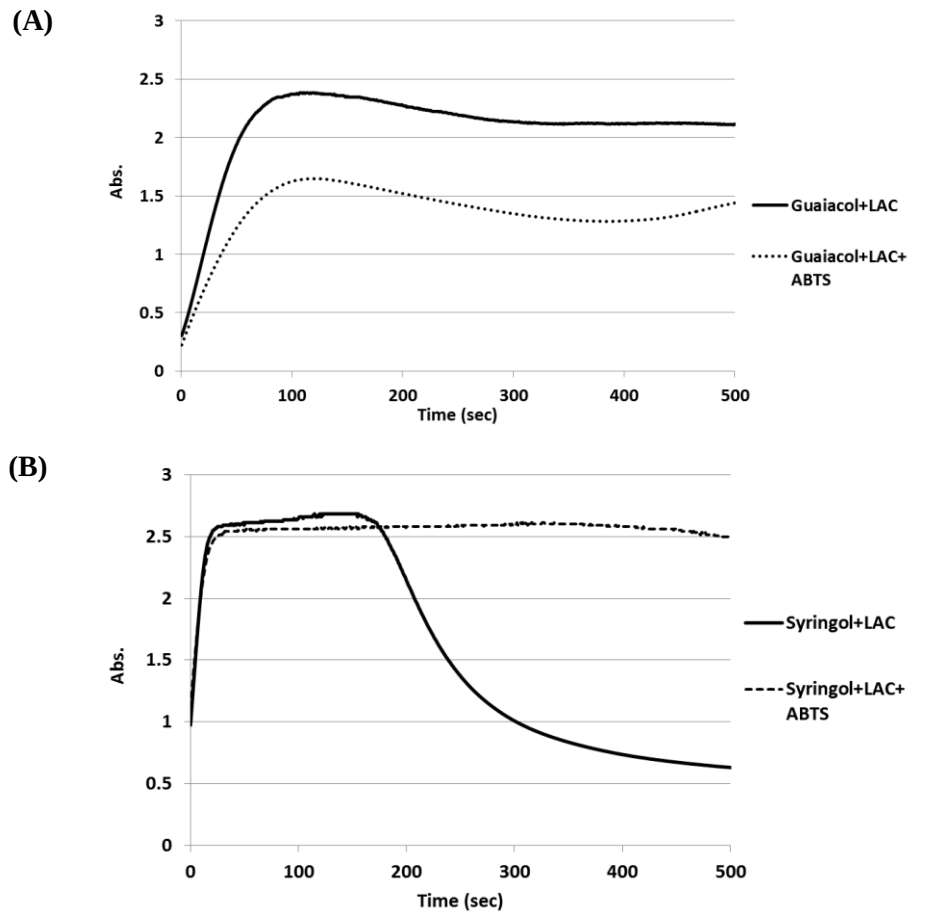


Figure 3-9. Oxidation of phenolics by laccase depending on the presence or absence of mediator ((A) Guaiacol, (B) Syringol)

3.4. Transcriptomic analysis during biomodification of synthetic lignin by *A. biennis*

3.4.1. Sequencing and assembly of cDNA libraries

To obtain information about overall DEGs of *A. biennis* during lignin degradaion, normalized cDNA libraries were sequenced by Illumina HiSeq 2500. Table 3-14 shows data information between raw data and trimmed data of each sample. After filtering of low quality reads, trimmed data showed high Q30 value (Table 3-14).

Assembly of the clean reads was performed by assembly program. Mapping ratio ranged from 78.1% to 80.6% (Table 3-14).

In summary, high-throughput sequencing technology resulted in high quality sequence of transcribed *A. biennis* genes. Based on this result, GO annotation and DEGs analysis was carried out.

Table 3-14. Data information of samples

		ABB	ABB+ABTS +R.A	ABB+DHP +ABTS+R.A
Raw data	Total reads No.	23,185,825	23,774,488	22,458,249
	Total reads length	3,501,059,575	3,589,947,688	3,391,195,599
Trimmed data	Total reads No	19,620,650	19,832,566	20,098,504
	Total reads length	2,892,584,955	2,931,081,167	2,977,637,048
	%	82.74%	81.54%	87.50%
	Q30	98.31%	98.29%	98.32%
Mapping data	Total reads No.	19,620,650	19,832,566	20,098,504
	Mapped reads No.	15,470,674 (78.9%)	15,492,334 (78.1%)	16,206,599 (80.6%)

*Total read length = Total reads X Read length

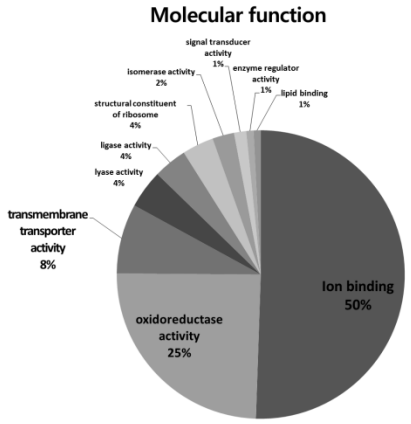
3.4.2. GO annotation

Gene ontology(GO) describes the roles of genes and genes products in microorganism. It is divided to three categories, biological process, molecular function, and cellular component. Biological process describes biological goal accomplished by ordered assemblies of molecular function. Molecular function is defined as biochemical activities such as catalytic or binding activities. Cellular component refers to location which genes were expressed including membrane, cell and organelle and so on (Ashburner et al., 2000; Consortium, 2004).

Molecular function is indicative of the biochemical activity. That of *A. biennis* showed that ion binding and oxidoreductase acitivities were mainly expressed (Figure 3-10(A)). In cellular component, genes and genes products related to “extracellular region” and “cell” components were highly expressed indicating 63% and 24%, respectively (Figure 3-10(B)). Biological process of genes expressed in *A. biennis* was “biosynthetic process” and “catabolic process” (Figure 3-10(C)). These results demonstrated extracellular response with oxidoreductase activity occurred in *A. biennis*, which purposes to catabolize and metabolize the substrates.

In conclusion, GO annotation supported that extracellular oxidative enzymes of *A. biennis* play a crucial role on catabolic and degradation reaction of substrates.

(A)



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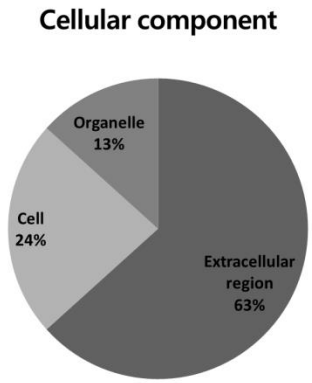

(C)

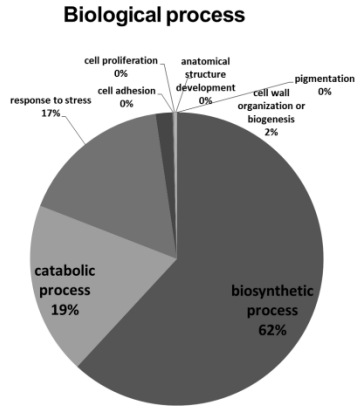

Figure 3-10. Molecular function (A), cellular component (B), and biological process (C) of *A. biennis* by GO annotation

3.4.3. Functional analysis of extracellular enzymes related to lignin degradaion

Catalytic system of *A. biennis* is based on MnP-LAC group. Above results presented that laccase mediator system of *A. biennis* induced significant degradation of lignin, which was supported by results of transcriptomic analysis.

Figure 3-12 and 3-13 show expression level of MnP and LAC. Contigs identified as MnP were 46. Amont them, 17 contigs were highly expressed in sample exposed DHP with mediator and reducing agents than fungus exposed only ABTS and reducing agents (Figure 3-11). In the case of laccase, 38 contigs identified as laccase were discovered in *A. biennis*. High expression of 23 contigs in ABB+DHP+ABTS+R.A sample was observed, indicating overall high fold change (*fc*). These results mean that ligninolytic enzymes, MnP and LAC, were involved in degradaion of synthetic lignin. Especially, many genes of laccase were highly expressed overall compared with that of MnP, which demonstrated laccase mediator system of *A. biennis* had a significant effect on lignin degradation. Because the laccase-mediator complex makes a possible to exceed the redox barrier of laccase alone, it has a outstanding ability to degrade lignin (Majcherczyk et al., 1999). And also, addition of DHP induced high expression of laccase under the condition of laccase mediator system. Fungus recognized DHP as carbond source as shown in above result, which enhanced secretion of laccase with growth of tip cell of mycelium. As a result, laccase secreted from fungus degraded lignin polymer (Moore-Landecker, 1996). These findings suggested that biotechnological application of laccase mediator system can develop the lignocellulose biorefinery (Cañas & Camarero, 2010).

Along with MnP and laccase, a variety of extracellular enzymes were highly expressed in DEG analysis. (Figure 3-14). These enzymes were

classified to three categories as fallows: H_2O_2–generating enzyme group, Fenton reaction group and bond cleavage group. At first, copper radical oxidase and aryl alcohol oxidase belong to H_2O_2 –generating enzyme group. These generate H_2O_2 for LiP and MnP in lignin degradation process (Dashtban et al., 2010; Kersten & Cullen, 2007). As a result, discovery of these enzymes emphasized involvement of MnP in lignin degradation in this study. Next, cellobiose dehydrogenase and multicopper oxidase belong to Fenton reaction group. FPKM level of two enzymes increased in ABB+DHP+ABTS+R.A sample (Figure 3-14). Cellobiose dehydrogenase is redox enzyme generating hydroxyl radicals in Fenton reaction (Adav et al., 2012). It was reported to be also implicated to lignin degradation by cleaving the β-ether bonds and inducing the demethoxylation and hydroxylation (Henriksson et al., 2000). Hydroxyl radicals generated by these enzymes attack lignin polymers, resulting in degrading lignin. In this study, one of interesting results was that esterases related to bond cleavage were upregulated, showing that *fc* ranged from 0.1 to 2.0. Esterase observed in this study was carboxylesterase, which was reported to break ester bond and hydrolyze substrate (Zorn et al., 2005). Accordingly, esterase also seemed to affect on breaking bonds of lignin. Genes of alcohol oxidase and multiple oxidase were highly expressed in *A. biennis* exposed to DHP (Figure 3-14), which indicated that *A. biennis* degraded DHP through various oxidases.

Consequently, addition of laccase mediator induced high expression of ligninolytic enzymes genes. Especially, expression of laccase genes was enhanced in *A. biennis* exposed to DHP. Various extracellular enzymes including H_2O_2 generating enzymes and enzymes related to Fenton reaction were also expressed, which mean involvement of these enzymes in lignin degradation. In conclusion, *A. biennis* degraded synthetic lignin with peroxidase-based, laccase-based, and Fenton reaction-based systems under the addition of laccase mediator and reducing agents.

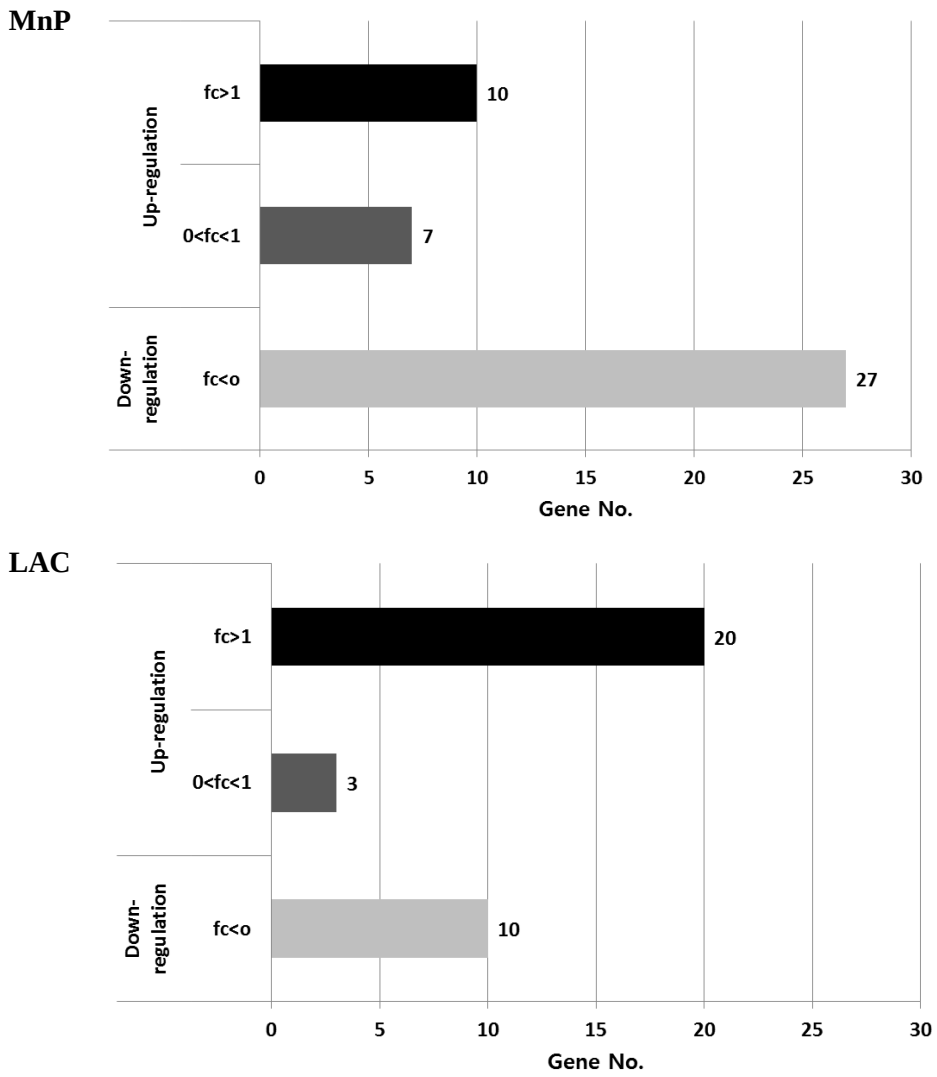

Figure 3-11. Number of up and down regulated genes when *A. biennis* was exposed to synthetic lignin with mediator and reducing agents

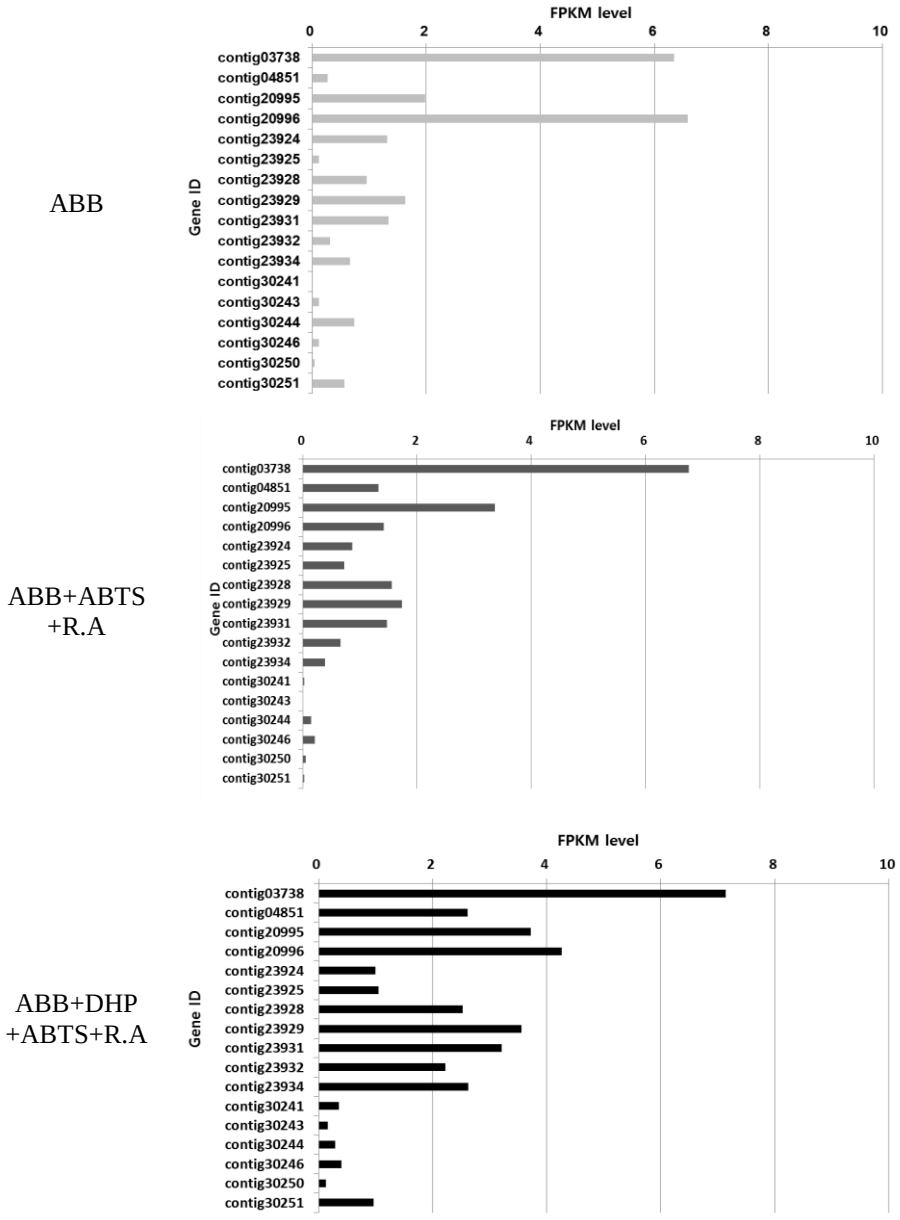

Figure 3-12. Expression level of MnP by FPKM analysis

*ABB: *Abortiporus biennis*, *DHP: dehydrogenative polymer

*ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

*R.A: Reducing agents

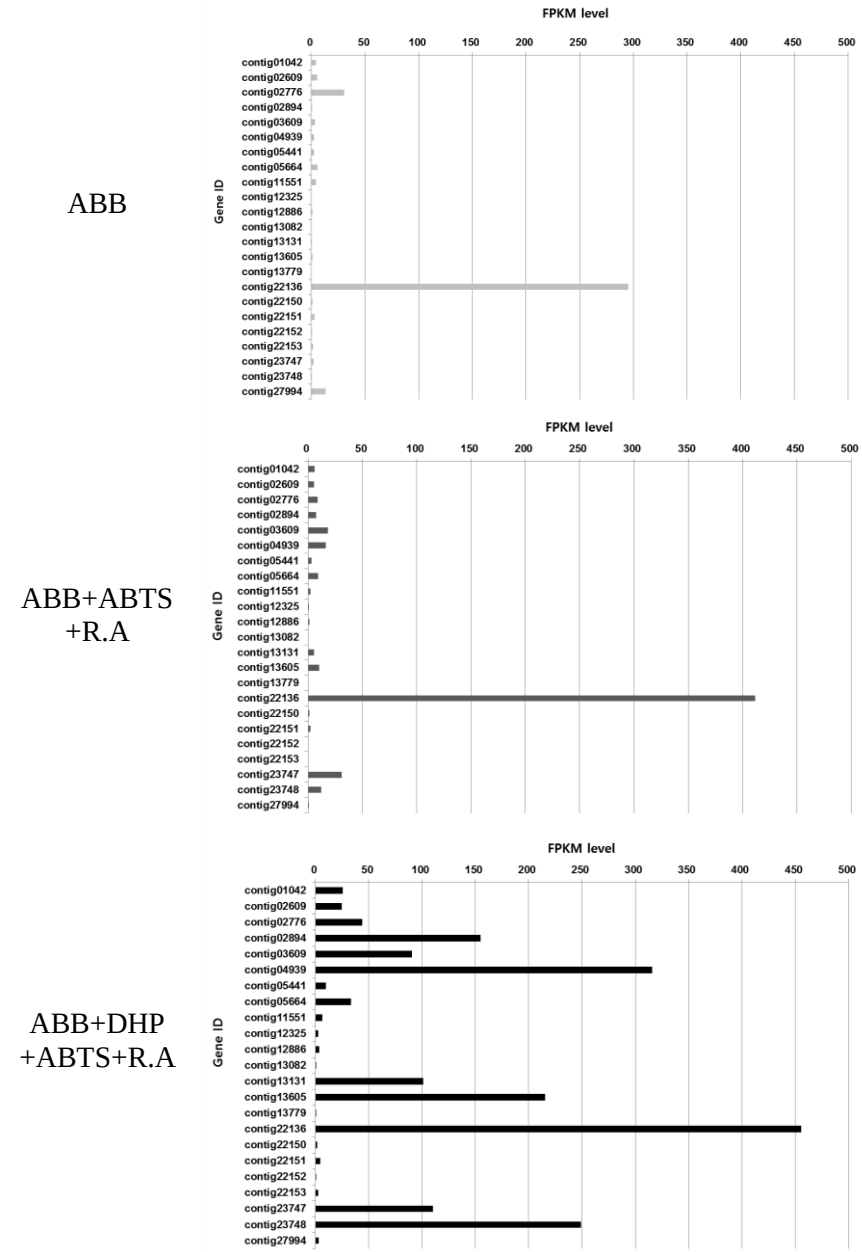

Figure 3-13. Expression level of LAC by FPKM analysis

*ABB: *Abortiporus biennis*, *DHP: dehydrogenative polymer

*ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

*R.A: Reducing agents

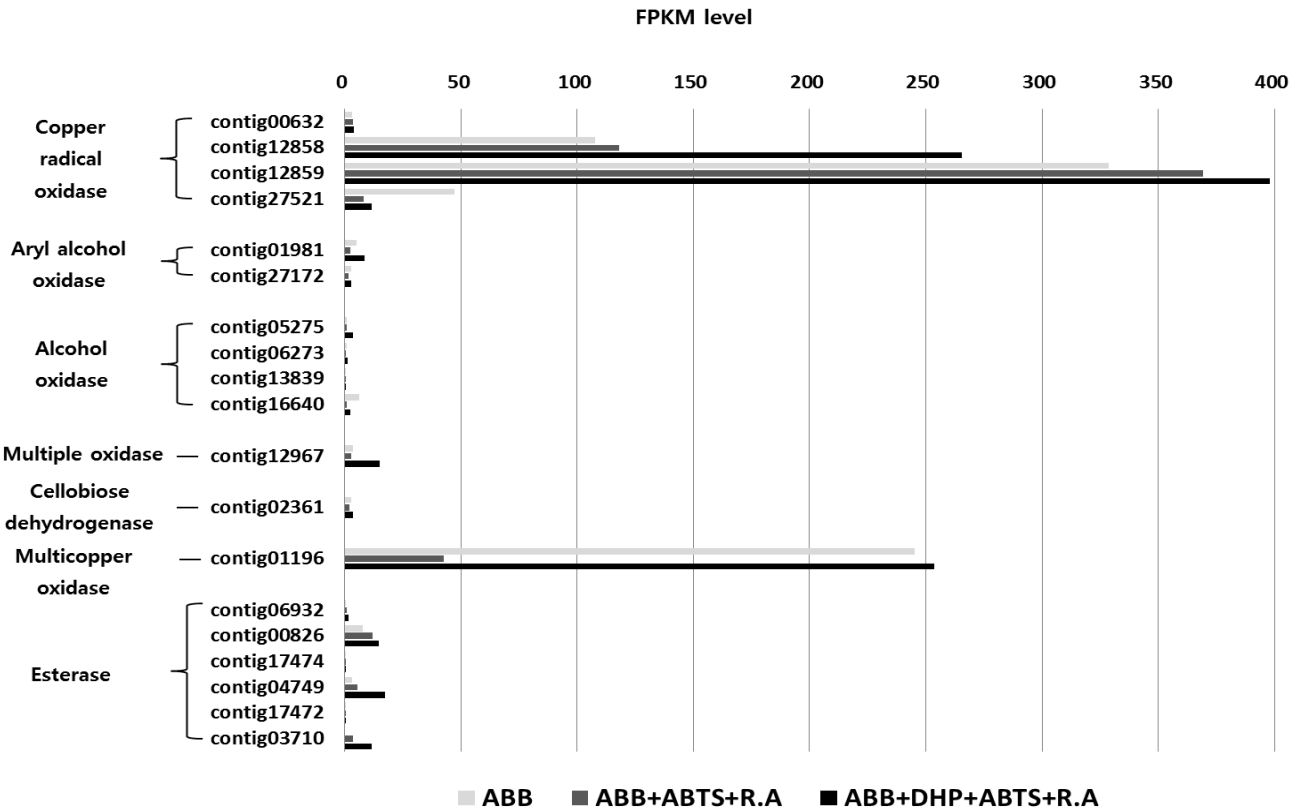

Figure 3-14. Expression level of other extracellular enzymes by FPKM analysis

*ABB: *A. biennis*, *DHP: dehydrogenative polymer, *ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), *R.A : Reducing agents

4. Conclusions

A. biennis showed similar catalytic reaction mechanism with synthetic lignin, compared with that of monolignols. Catalytic enzyme system of *A. biennis* induced degradation of synthetic lignin in initial day and polymerization in latter part of incubation day. Although *A. biennis* cleaved ether bonds in lignin during incubation days, DHP seemed to be polymerized by other type linkages. Molecular weight of lignin oligomers dissolved in medium was higher than that of control during initial incubation days (5-10days), thereafter it was declined. These results mean that degradation and polymerization reaction of lignin oligomers and DHP were interacted on each other by catalytic system of *A. biennis.*

Based on these results, for lignin degradation by *A. biennis,* the changes of enzyme system and culture condition were performed. At first, when laccase mediator, ABTS, was only added, the weight average molecular weight of DHP treated by *A. biennis* reached on 2,676 on incubation day 5, which was very low compared to that of DHP. Amount of NBO products of DHP treated by *A. biennis* was lower that that of control. *A. biennis* considerably catalyzed cleavage of ether bonds and it contributed to decrease of molecular weights. And then, change the culture condition by adding the reducing agents, ascorbic acid and α-tocopherol, was conducted. Molecular weights of DHP were decreased slightly indicating 3,034 Da on incubation day 20. Reducing agents played an important role in preventing the polymerization by the unstable radicals, leading to stabilization of unstable radicals within synthetic lignin. Finally, changes of two conditions were simultaneously applied for more degraded DHP, looking forward to synergetic effects. It showed significant decrease of molecular weight and amount of NBO products in DHP after fungal treatment.

Consequently, whole cell of *A. biennis* assisted considerable degradation of DHP under the simultaneous addition of both laccase mediator and reducing agents.

Laccase mediator system of *A. biennis* showed an excellent ability to degrade synthetic lignin. Accordingly, to demonstrate the mechanism of laccase mediator system in lignin degradation, function of laccase and its mediator system depending on a kind of substrates was examined. As a result, laccase mediator system using ABTS degraded lignin compounds by lowering the oxidation rate of substrates.

Transcriptomic analysis was indicative of involvement of various extracellular enzymes in lignin degradation. As expected, genes of laccase were very upregulated in *A. biennis* exposed to DHP. MnP was also highly expressed. In addition, a lot of extracellular enzymes related to lignn degradation were found including H_2O_2 and hydroxyl radicals–generating enzymes. Accordingly, this result demonstrated that complex laccase mediator system of *A. biennis* played a crucial role in production of degraded lignin polymer.

In conclusions, both catalytic enzyme system of *A. biennis* and culture conditions were an important factor for lignin degradation. Especially, laccase mediator system of *A. biennis* had a considerable impact on lignin degradation. Invovlement of other extracellular enzymes in lignin degradation were also observed through the transcriptomic analysis. Additionally, if further studies on laccase mediator system of *A. biennis* are conducted by using the various mediators, it will serve as a good biocatalyst for lignin valorization in biorefinery.

Chapter 4

Production of succinic acid derived from synthetic lignin by *Phanerochaete chrysosporium* and analysis of its related enzymes by transcriptomic analysis

1. Introduction

Among white rot basidiomycetes, lignin degradation by *Phanerochaete chrysosporium* is a well-studied model. *P. chrysosporium* secretes LiP-MnP group, and optimizes in a nitrogen-limiting environment for lignin degradation (Keyser et al., 1978). In addition to LiP and MnP, *P. chrysosporium* come into lignin degradation with various enzymes such as aryl alcohol oxidase (Asada et al., 1995), glyoxal oxidase (Kersten & Kirk, 1987), and cellobiose dehydrogenase (Henriksson et al., 2000) and so on. In general, ligninolytic enzymes catalyze oxidation of lignin in multi-step electron transfers with assistance of other accessory enzymes including enzymes generating H_2O_2 (Dashtban et al., 2010; Wong, 2009).

This fungal catalysis system makes the possibilities for their biotechnological applications as novel biocatalyst in numerous industries with development of proteome and genome analysis techniques (Bouws et al., 2008). Especially, lignin modification using microorganism is promising research area. Very recently, National Renewable Energy Laboratory of USA has supported studies on production of acid compounds like adipic acid and pyruvic acid as intermediate of polyhydrocarbon from lignin by microorganism with the object of lignin upgrading (Johnson & Beckham, 2015; Linger et al., 2014; Vardon et al., 2015).

Actually, biomodification process has some disadvantages which is time consuming process compared with chemical modification, and is difficult to control enzymes system of microorganisms to produce value added products. Furthermore, because whole cell of basidiomycetes has complex enzymes systems including extracellular and intracellular enzymes, it was not easy to apply basidiomycetes to modification process for lignin application. However, enzyme system of white rot basidiomycetes has a good ability to catabolize

aromatic compounds, based on ring fission enzyme process (Pollegioni et al., 2015; Tomizawa et al., 2014). Extracellular enzymes of white rot basidiomycetes attack lignin polymers, which results in formation of aromatic compounds by bonds cleavages. Thereafter, those were metabolized intracellularly (Kirk & Farrell, 1987; Shimizu et al., 2005). This unique metabolic system of lignin degrading basidiomycetes can be advantageously applied to produce value added chemicals from lignin, which was not easy by thermo-chemical modification process. For forging the biomodification process by basidiomycetes, it is necessary to understand biomodification mechanism of lignin by white rot basidiomycetes.

In this study, firstly, biomodification mechanism of synthetic lignin by *P. chrysosporium* was investigated. To degrade lignin and produce valuable chemicals from lignin, optimal conditions were examined. Finally, a transcriptomic analysis was utilized to investigate complex enzyme systems of *P. chrysosporium* related to biomodication of DHP when exposed to synthetic lignin.

2. Materials and methods

2.1. Materials

2.1.1. Preparation of fungus and fungal suspension

In this chapter, *Phanerochaete chrysosporium* (ATCC 20696) was used for biomodificatin of synthetic lignin. *P. chrysosporium* (ATCC 20696) was obtained from the Microbiology Chemistry Laboratory of National Institute of Forest Science (NIFoS).

The fungus and fungal suspension were prepared as described in section 2.1.1. of Chapter 2.

2.1.2. Synthesis of dehydrogenative polymer (DHP)

DHP, one of synthetic lignins, was synthesized according to the methods described in section 2.1.2. of Chapter 3.

2.2. Analysis of structural changes and degradation products of DHP

Fungal suspension of *P. chrysosporium* ((dried weight: 0.04 g)) was inoculated in SSC medium. After pre-incubation for 4 days, 200 mg of DHP was added in medium.

Structural changes of DHP treated by *P. chrysosporium* were analyzed as described in section 2.2. of Chapter 2 and section 2.2.1. of Chapter 3.

Degradation products from DHP dissolved in medium was also analyzed as described in section 2.2.2. of Chapter 3.

2.3. Analysis of structural changes and degradation products of DHP with addition of reducing agents

To degrade DHP during biomodification by *P. chrysosporium* reducing agents were added in culture medium.

5 mM of ascorbic acid and 1 mM of α-tocopherol as reducing agent was added in SSC medium, periodically on incubation day 2, 8, 13, 17 after preincubation.

As mentioned above, structural changes of DHP and degradation products in medium by fungus were analyzed as described in section 2.2.

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2.4. Transcriptomic analysis of *P. chrysosporium* exposed to DHP

For investigating biomodification related enzymes of *P. chrysosporium*, transcriptomic analysis was carried out using Illumina HiSeq ™ 2500.

To analyze DEGs between fungal samples, fungal samples were divided into 8 groups, depending on addition of reducing agents and incubation days (Table 4-1).

Table 4-1. Fungal samples of which transcriptomic analysis was performed

Fungal sample		Abbreviation
Non addition of reducing agents	*P. chrysosporium* on 5 days	5PCH
	P. chrysosporium treated with DHP on 5 days	5PCH+DHP
	P. chrysosporium on 25 days	25PCH
	P. chrysosporium treated with DHP on 25 days	25PCH+DHP
Addition of reducing agents	*P. chrysosporium* treated with reducing agents on 5 days	5PCH+R.A
	P. chrysosporium treated with DHP and reducing agents on 5 days	5PCH+DHP+R.A
	P. chrysosporium treated with reducing agents on 25 days	25PCH+R.A
	P. chrysosporium treated with DHP and reducing agents on incubation 25 days	25PCH+DHP+R.A

*PCH: *Phanerochaete chrysosporium*

*DHP: dehydrogenative polymer

*R.A: Reducing agents

2.4.1. Total RNA extraction and NGS library preparation

Total RNA extraction, cDNA synthesis and library preparation were conducted according to the methods described in section 2.5.1. of Chapter 3. Unlike *A. biennis*, RNA-seq data obtained by the mycelium of *P. chrysosporium*, were mapped on reference dataset through the re-sequencing method. Other analysis including FPKM analysis was identically performed.

2.4.2. Analysis of Differently Expressed Genes (DEGs)

DEG analysis was performed equally as described in section 2.5.2. of Chapter 3.Fold change (*fc*) values were determined depending on addition of substrate this study. Absolute value of the log2 ratio >2 were used as threshold to determine significant differences in gene expression between the samples.

2.4.3. GO annotation

GO annotation was performed equally as described in section 2.5.3. of Chapter 3.

2.4.4. KEGG annotation

KEGG metabolic pathways analysis was as described in section 2.5.4. of Chapter 3.

3. Results and Discussions

3.1. Biomodification mechanism of DHP by *P. chrysosporium*

3.1.1. Structural change of DHP

For examining the structural changes of DHP by *P. chrysosporium*, phenolic hydroxyl (OH) group, amount of NBO products, and molecular weight were analyzed.

Table 4-2 shows the molecular weight change of DHP during incubation days. Compared to the weight-average molecular weight (M_w) of DHP, Mw of control were similar with DHP during incubation days. Mw of DHP treated by *P. chrysosporium* had a tendency to decrease by incubation day 10. The M_w was 3,106 and 3,240 on incubation day 5 and 10, respectively. After that, the M_w was increased between 15 days and 25 days and reached peak values with 15 days (3,897 Da), 20 days (3,580 Da), and 25 days (3,930 Da).

Phenolic OH content of DHP was 4.6%, which was similar compared with that of other lignins such kraft lignin (4.5%), soda lignin (4.4%), and lignosulfonate (2.0%) (Mansouri & Salvadó, 2006). Phenolic OH content of control was 6.6% during incubation days which was slight higher than DHP. Phenolic OH content of DHP treated by fungus was increased 10.5% on incubation day 10 (Figure 4-1). Phenolic OH contents of DHP modified by fungus kept high value than that of control.

The amount of NBO products of DHP during fungal treatment was shown in Tables 4-3. Amounts of NBO products of DHP modified by *P. chrysosrporium* declined than that of control except for incubation day 20.

As for these results, *P. chrysosporium* lead to cleavage of ether bonds, so DHP was degraded with high phenolic OH group in initial incubation days. However, in latter part of incubation days, polymerization occurred with increase of molecular weight despite of decrease of ether bonding in DHP. It implied that DHP was condensed by other type linkages not ether bonds like α-O-4 and β-O-4 under the enzyme controlled condition, which was predicted to be rigid form (Baucher et al., 1998). With respect to the structure of lignin, phenolic OH groups are the most reactive sites (Sarkanen & Ludwig, 1971). Because DHP modified by fungus could be very unstable with high phenolic OH content, DHP structure could be changed easily by attack of enzymes of *P. chrysosporium*. That was why structural changes of DHP occurred during incubation days.

In the present study, *P. chrysosporium* simultaneously induced degradation and polymerization of DHP during incubation days. Ligninolytic enzyme system of *P. chrysosporium* were reported to catalyze cleavage of C_{α}-C_{β}, *β*-ether linkage, and ring fission (Enoki et al., 1981; Higuchi, 1990). In this study, extracellular protein concentration of fungus exposed to DHP was higher than that of control (Figure 4-4), and mycelium dried weight was increased with addition of DHP (Figure 4-3). Likewise *A. biennis*, *P. chrysosporium* also grew by recognizing DHP as nutrient source. During growing period, a lot of extracellular proteins were secreted from *P. chrysosporium,* which was considered to influence on biodegradation of DHP in initial incubation day. However, because activities of ligninolytic enzymes were little difference between control and sample adding DHP (Figure 4-4), it was difficult to explain correlation between biodegradation of DHP and ligninolytic enzymes secreted from *P. chrysosporium.* With development of proteome and genome analysis equipment, *P. chrysosporium* were reported to degrade lignin through the extra and intracellular enzymes system. Oxidase generating H_2O_2, aryl alcohol dehydrogenases, cytochrome P450

monooxygeanse and quinone reductases etc. were involved in lignin degradation (Dashtban et al., 2010; Kersten & Cullen, 2007; Shimizu et al., 2005).

Consequently, complex extra and intra-cellular enzymes seemed to be implicated in degradation and polymerization of DHP. Analysis of various enzymes related to biomodification of synthetic lignin was conducted through the transcriptomic anlaysis as described in section 4.4. of this chapter.

Table 4-2. Molecular weight change of dehydrogenative polymer (DHP) treated by *P. chrysosporium* (Control: DHP in medium, PCH+ DHP: DHP treated by *P. chrysosporium*)

		0d	5d	10d	15d	20d	25d
Control	M_w [a] (Daltons)	3686	3527	3676	3701	3457	3563
	M_n [b] (Daltons)	1832	2038	2393	2426	1978	1988
	M_w/M_n	2.01	1.73	1.54	1.53	1.75	1.79
PCH+DHP	M_w [a] (Daltons)	3686	3106	3240	3897	3580	3930
	M_n [b] (Daltons)	1832	1603	1656	1627	1717	1877
	M_w/M_n	2.01	1.94	1.96	2.40	2.08	2.09

milled wood lignin: M_w: 9919, M_n:4240, Polydispersity: 2.34

DHP: M_w: 3686, M_n: 1832, Polydispersity: 2.01

[a] weight-average molecular weight

[b] number-average molecular weight

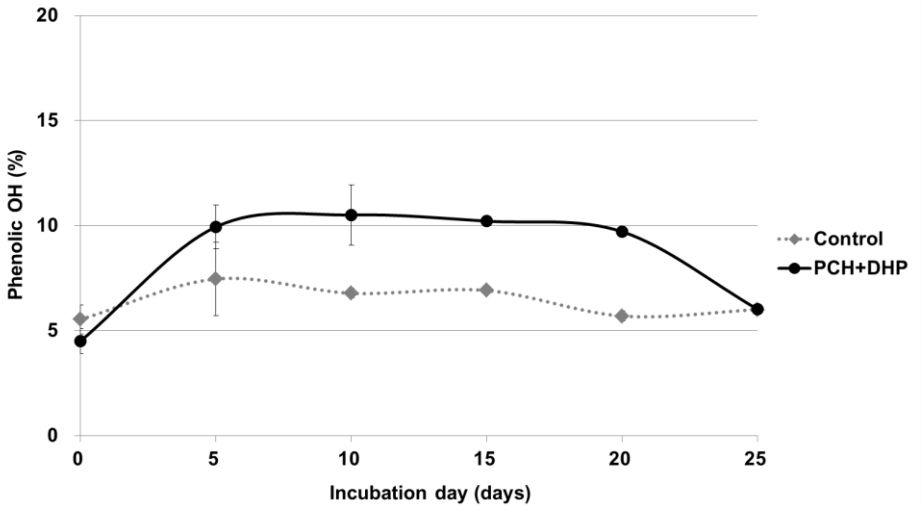


Figure 4-1. Phenolic hydroxyl group content of dehydrogenative polymer (DHP) treated by *P. chrysosporium* (Control: DHP in medium, PCH+DHP: DHP treated by *P. chrysosporium*)

*Phenolic OH content of DHP: 4.61%

Table 4-3. Nitrobenzene oxidation products (μmol/g sample) of dehydrogenative polymer (DHP) treated by *P. chrysosporium* (Control: DHP in medium, PCH+DHP: DHP treated by *P. chrysosporium*)

Incubation days	Control			PCH+DHP		
	G unit	S unit	Sum of G&S units	G unit	S unit	Sum of G&S units
0d	161.2±16.8	861.0±123.5	1022.3±140.3	137.4±5.0	686.37±45.2	823.8±50.2
5d	171.7±5.8	701.0±23.7	872.7±29.5	76.9±37.0	508.9±22.6	585.7±59.6
10d	236.8±71.4	1037.4±273.6	1274.2±345.1	60.1±6.5	254.7±50.8	314.8±57.3
15d	159.1±10.7	681.7±33.1	840.8±22.4	93.0±5.8	706.2±185.6	799.2±191.3
20d	173.4±48.3	937.9±50.4	1111.3±98.8	160.9±45.0	1248.4±765.3	1409.3±810.2
25d	112.3±15.6	845.6±40.7	957.9±56.3	40.7±0.4	678.6±7.2	719.3±7.6

***** DHP: G unit: 219.2±36 μmol/g $_{sample}$, S unit: 1081.8±102.5 μmol/g $_{sample}$,
Sum of G & S units: 1301.1±138.5 μmol/g $_{sample}$

3.1.2. Characteristics of oligomers derived from DHP

Lignin oligomers derived from DHP were dissolved in culture medium. These oligomers were extracted with ethylacetate. GPC and GC-MS analysis were conducted.

At first, results of GPC analysis was showed in Table 4-4 and Figure 4-2. Oligomers distribution of control was divided to group 1 and group 2 (Figure 4-2(A)). Mw of group 1 was 1,021 and 1,009 on 5 and 10 days, respectively. On the other hands, sample treated with *P. chrysosporium* showed different distribution. Low molecular weight compounds were degraded by *P. chrysosporium* (Figure 4-2). Mw of lignin oligomers was increased by incubation day 15, and then decreased to 25 days (Table 4-4). Change of molecular weight of oligomers depending on incubation time was contrast to that of DHP. DHP was degraded by incubation day 10, thereafter was polymerized. As a result, degradation of DHP by fungus released low molecular weight compounds in culture medium, which reacted with oligomers dissolved in medium.

Consequently, degradation and polymerization reaction of lignin oligomers derived from DHP were also caused by catalytic system of *P. chrysosporium*, which was intimately connected with DHP modification process.

Table 4-4. Molecular weight change of oligomers derived from dehydrogenative polymer (DHP) after treated by *P. chrysosporium* (Control: DHP in medium. PCH+ DHP: DHP treated by *P. chrysosporium*)

Control				
	5d		10d	
	1	2	1	2
M_w [a] (Daltons)	1021	225	1009	232
M_n [b] (Daltons)	846	208	793	217
M_w/M_n	1.21	1.08	1.27	1.07

PCH+DHP					
	5d	10d	15d	20d	25d
M_w [a] (Daltons)	941	978	1028	966	949
M_n [b] (Daltons)	565	631	646	643	643
M_w/M_n	1.66	1.55	1.59	1.50	1.48

[a] weight-average molecular weight

[b] number-average molecular weight

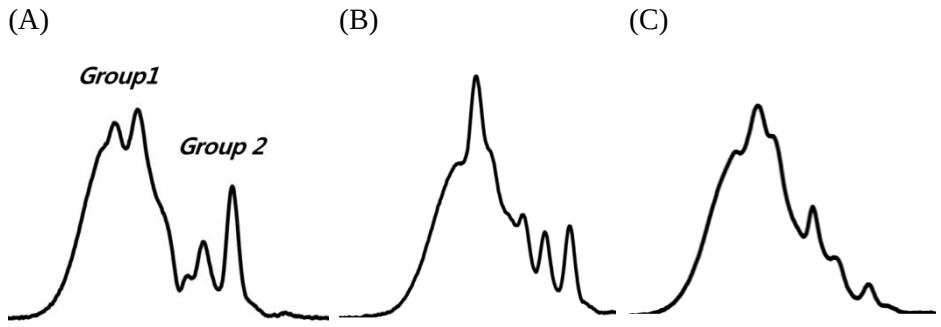


Figure 4-2. Gel permeation chromatogram of oligomers derived from DHP during incubation time ((A) Control on day 5, (B) PCH+DHP sample on day 5, (C) PCH+DHP sample on day 20)

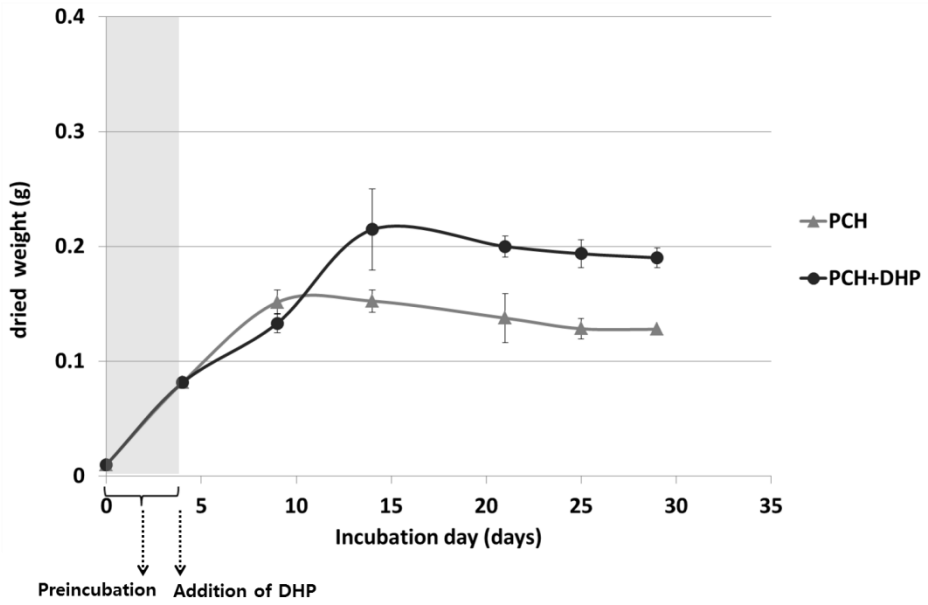
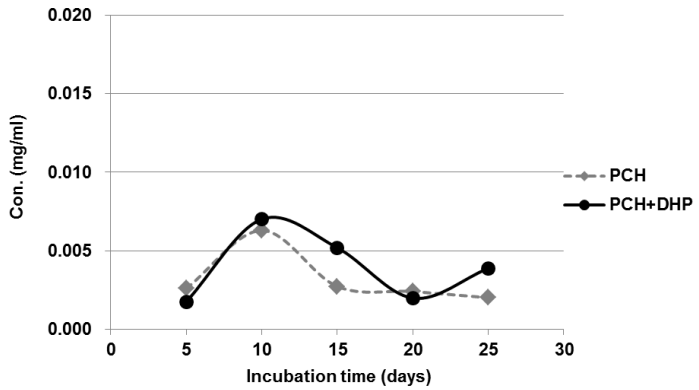
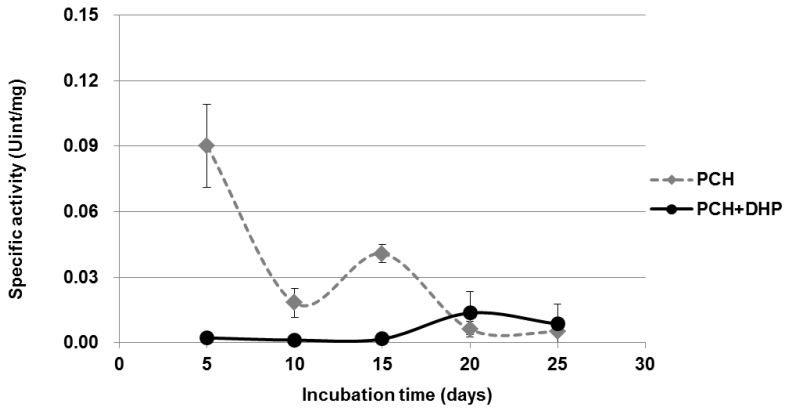


Figure 4-3. Mycelium dried weight of *P. chrysosporium* depending on addition of DHP during incubation days (PCH: *P. chrysosporium*, PCH+ DHP: *P. chrysosporium* exposed to DHP)

(A)



(B)



(C)

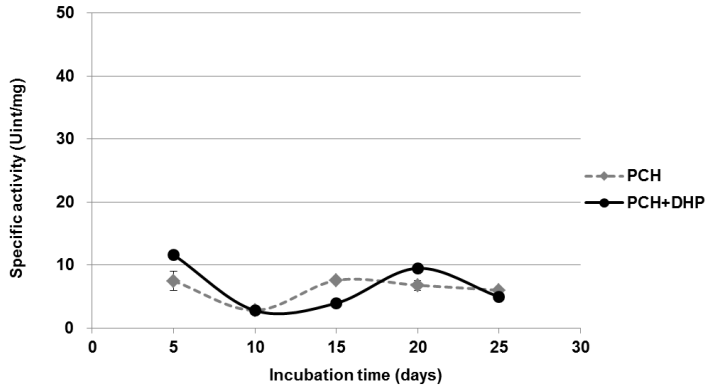


Figure 4-4. Protein concentration (A) and activities of manganese peroxidase (B) and lignin peroxidase (C) of *P. chrysosporium* with addition of DHP (PCH: *P. chrysosporium* in medium, PCH+DHP: fungal sample adding DHP)

3.2. Production of lignin derived compounds and degradation of synthetic lignin by *P. chrysosporium* with reducing agents

3.2.1. Structural change of DHP by *P. chrysosporium* with ascorbic acid and α-tocopherol.

Preliminary experiment presented that ascorbic acid affected only degradation of lignin oligomers derived from DHP. Therefore, for degradation of DHP, the other reducing agent, α-tocopherol was used. α-Tocopherol was well known as lipophillic reducing agent. It was reported to scavenge and quench reactive oxygen species and peroxy radicals (Huang et al., 1996; Shao et al., 2008). Furthermore, ascorbic acid regenerate tocopherol from tocopheroxyl radical, which were reported to increase effectiveness of antioxidant (Niki, 1991). Accordingly, in this study, for degradation of DHP with production of DHP derived chemical, two reducing agents were used.

As a result of GPC analysis, molecular weights of DHP were decreased slightly (Table 4-5). Mw of control was 3,132 and 3,034 Da on incubation day on 10 and 20, respectively. On incubation day 25, molecular weight of DHP treated by fungus was 2,919 Da.

Phenolic OH contents of DHP declined with increase of incubation day (Figure 4-5). Addition of α-tocopherol affected on decrease of phenolic OH contents of DHP, which was supported by decrease of that of control, compared with result of experiment adding only ascorbic acid. This suggested that α-tocopherol lead to degradation of polymer by stablizing phenolic OH group within DHP.

Compared with the NBO products of control, those after fungal treatment was generally lower, and decreased rapidly between incubation day 10 and 15

(Table 4-6). Amount of NBO products of DHP modified by *P. chrysosporium* ranged from 254.7 μmol/g to 1076.4 μmol/g (Table 4-6).

Results of phenolic OH contents and NBO products were indicative that α-tocopherol played a significant role to stabilize DHP containing high phenolic OH group by cleavage of ether bonds. Accordingly, the Mw of DHP treated by *P. chrysosporium* with α-tocopherol showed no significant fluctuation throughout incubation days overall. Consequently, when α-tocopherol together with ascorbic acid was added as reducing agent, it could degrade DHP.

Previous studies on biomodification of synthetic lignin has been reported that synthetic lignin was degraded by lignin degrading enzymes of white rot basidiomycetes, suggesting only molecular weight change of DHPs (Kawai et al., 1999; Srebotnik & Hammel, 2000; Yoshida et al., 1998) or detection of various ^{14}C-volatile compounds released from ^{14}C- labelled synthetic lignin (Haider & Trojanowski, 1975; Steffen et al., 2000). On the other hand, this study presented that *P. chrysosporium* simultaneously induced degradation and polymerization of lignin with structural change. Furthermore, one of the most interesting results was that reducing agents was necessary for degradation of synthetic lignin under the enzymatic-controlled condition. Therefore, above showed findings were very meaningful in applying catalysis system of *P. chrysosporium* to lignin modification process.

Table 4-5. Molecular weight change of dehydrogenative polymer (DHP) treated by *P. chrysosporium* with ascorbic acid and α-tocopherol (Control: DHP in medium. PCH+ DHP: DHP treated by *P. chrysosporium*)

		1d	10d	20d
Control	M_w [a] (Daltons)	3077	3132	3034
	M_n [b] (Daltons)	1128	1201	1134
	M_w/M_n	2.72	2.61	2.68

		1d	5d	10d	15d	20d	25d
PCH+DHP	M_w [a] (Daltons)	2987	3290	3507	3445	3300	2919
	M_n [b] (Daltons)	1187	1333	1285	1489	1391	1198
	M_w/M_n	2.51	2.47	2.73	2.31	2.37	2.44

DHP: M_w: 3421, M_n: 1476, Polydispersity: 2.32

[a] weight-average molecular weight

[b] number-average molecular weight

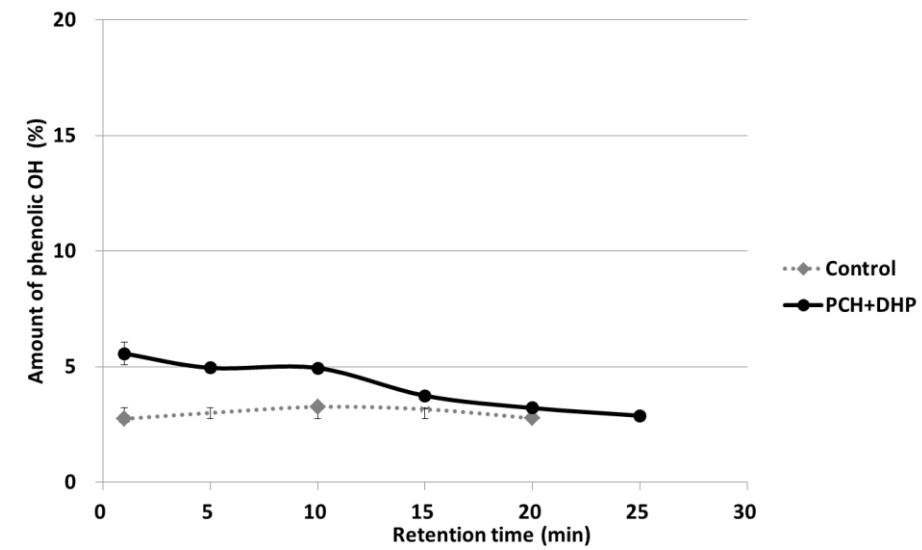


Figure 4-5. Phenolic hydroxyl group content of dehydrogenative polymer (DHP) treated by *P. chrysosporium* with ascorbic acid and α-tocopherol (Control: DHP in medium, PCH+DHP: DHP treated by *P. chrysosporium*)

*Phenolic OH content of DHP: 5.0%

Table 4-6. Nitrobenzene oxidation products (μmol/g sample) of dehydrogenative polymer polymer (DHP) treated by *P.chrysosporium* with ascorbic acid and α-tocopherol (Control: DHP in medium, PCH+DHP: DHP treated by *P. chrysosporium*)

Incubation days	Control			PCH+DHP		
	G unit	S unit	Sum of G&S units	G unit	S unit	Sum of G&S units
1d	70.0±10.2	1500.1±89.2	1570.1±99.4	47.6±17.2	93.7. ±20.0	985.4±37.1
5d	-	-	-	56.0±2.2	1020.4±175.5	1076.4±177.7
10d	73.7±5.1	1596.1±260.2	1669.8±265.2	34.8±4.4	777.3±0.1	812.1±4.3
15d	-	-	-	42.1±12.1	212.7±156.3	254.7±168.4
20d	62.1±10.1	1061.7±202.3	1123.8±212.4	57.4±1.4	319.4±0.9	376.8±2.2
25d	-	-	-	65.2±0.6	339.6±15.6	404.8±16.2

***** DHP: G unit:310.7±120.8 μmol/g $_{sample}$, S unit:1461.9±346.4 μmol/g $_{sample}$,

Sum of G & S units : 1772.6±.467.2 μmol/g $_{sample}$

*** - : Not detection**

3.2.2. Degradation of oligomers derived from DHP and production of lignin derived compounds by *P. chrysosporium* with ascorbic acid and α-tocopherol.

The molecular weight of lignin oligomers decreased with increase of incubation day (Table 4-7). On incubation day 25, Mw of lignin oligomers was 854 Da after fungal treatment. This result was similar with that of experiment adding only ascorbic acid. As mentioned above, ascorbic acid, hydrophillic reducing agent, had a positive effect on degradation of lignin oligomers derived from DHP.

As a result of analyzing degradation products of DHP by using GC-MS, various degradation products, aromatic compounds and acid compounds, were detected (Figrue 4-6). It was most interesting observation in studies on lignin degradation.

On incubation day 10, succinic acid, 2,6-dimethoxy-1,4-benzodiol and syringic acid were detected at 31.4 min, 45.8 min and 53.4 min, respectively (Figrue 4-6). Because α-tocopherol functioned as stabilizing DHP, catalytic enzyme system of *P. chrysosporium* induced preferentially not polymerization but formation of various degradation intermediates from DHP. Syringic acid released from DHP was oxidized to 2,6-dimethoxy-1,4-benzodiol. Thereafter, succinic acid was formed through the metabolic pathway of *P. chrysosporium*. *P. chrysosporium* secereted about 0.02 mg of succinic acid as primary metabolite in 200ml of medium for incubation days (Figure 4-8(A)), and the fungus also secreted maximum 0.3 mg of succinic acid with reducing agents for incubation days. On the other hand, *P. chrysosporium* exposed to DHP secreted 0.1 mg to 3.7 mg of succinic acid from incubation day 1 and 25 (Figure 4-8(A)). Concentration of succinic acid ranged from 0.75 to 18.51 mg/g $_{\text{of substrate}}$ on incubation day 1 and 25, respectively (Figure 4-8(B)). Yield of succinic acid from DHP by *P. chrysosporium* was low yet compared with

other studies on acid compound production derived from lignin by biological treatment (Johnson & Beckham, 2015; Vardon et al., 2015). However, above results indicated potential of basidiomycetes as biocatalyst for lignin application.

Lignin degrdading enzymes of white rot basidiomycetes were reported to cause C_{aryl}- C_{alkyl} bond cleavage and oxidation of lignin monomers (Hofrichter, 2002b; Kirk & Nakatsubo, 1983). Various intracellular enzymes including dioxygenase were also reported to affect on degradation of lignin compounds. With development of analysis equipment, proteomic differential display techniques were used for analyzing of enzymes related to lignin degradation. Some studies reported that various intracellular metabolisms such as TCA cycle and glyoxylate cycle were concerned with formation of degradation products when *P. chrysosporium* was exposed to aromatic compounds (Matsuzaki et al., 2008; Shimizu et al., 2005; Tomizawa et al., 2014).

In this study, *P. chrysosporium* has an excellent ability to catalyze DHP extra- and intra-cellularly. Accordingly, *P. chrysosporium* were thought to be powerful tools for utilization of the aromatic biomass. That was because acid compounds produced from lignin can be converted into myriad products including polyhydroxyalkanoate. Therefore, formation of acid compounds derived from lignin was promising research area in these days. Especially, studies on aromatic catabolism using microorganism has been carried out intensively for production of lignin derived acid compounds (Johnson & Beckham, 2015; Tomizawa et al., 2014; Vardon et al., 2015).

Succinic acid was one of value added chemicals which was mentioned in DOE report (Department of Energy, USA). It was well known as building block chemicals available from biochemical transformation of biorefinery sugar (Bozell & Petersen, 2010). Succinic acid is utilized in surfactant/detergent agent, food additives, and heath-related agents, and regonized as industrial potential chemical. In addition, this is a common

metabolite fromed by microorganism (Zeikus et al., 1999). Therefore, formation of succinic acid from DHP by *P. chrysosporium* in this study was noticeable and noteworthy result.

To better understand the fungal cellular responses against DHP, DEGs and KEGG pathway were analyzed by transcriptomic analysis using next generation sequencing (NGS) technology.

Table 4-7. Molecular weight change of oligomers derived from dehydrogenative polymer (DHP) after treated by *P. chrysosporium* with ascorbic acid and α-tocopherol (Control: DHP in medium. PCH+ DHP: DHP treated by *P. chrysosporium*)

Control			
	1d	10d	20d
M_w [a] (Daltons)	848	852	920
M_n [b] (Daltons)	490	510	476
M_w/M_n	1.73	16.7	1.93

PCH+DHP						
	1d	5d	10d	15d	20d	25d
M_w [a] (Daltons)	1276	1061	1015	1049	930	854
M_n [b] (Daltons)	741	682	609	577	541	512
M_w/M_n	1.72	1.56	1.67	1.82	1.72	1.67

[a] weight-average molecular weight

[b] number-average molecular weight

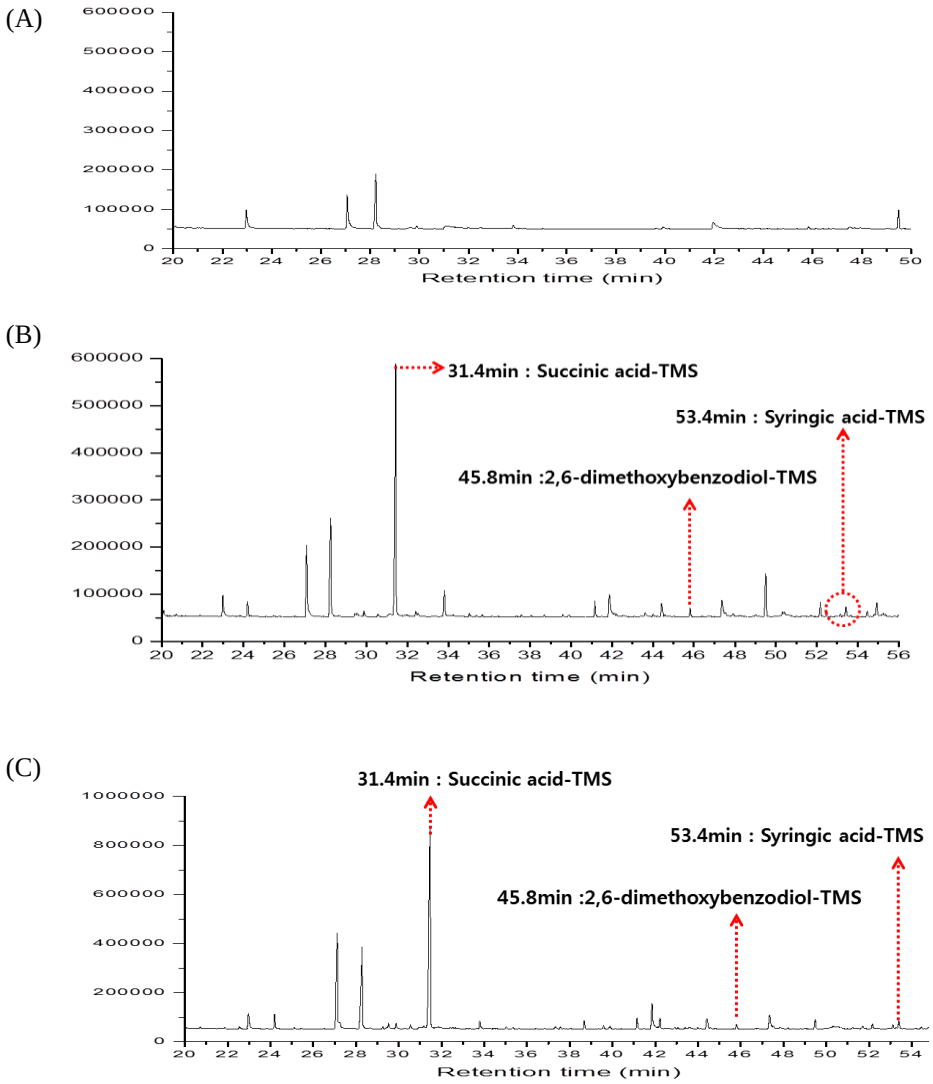


Figure 4-6. Total ion chromatograms of control (A) and sample treated by *P. chrysosporium* on incubation day 10 (B) and 20 (C)

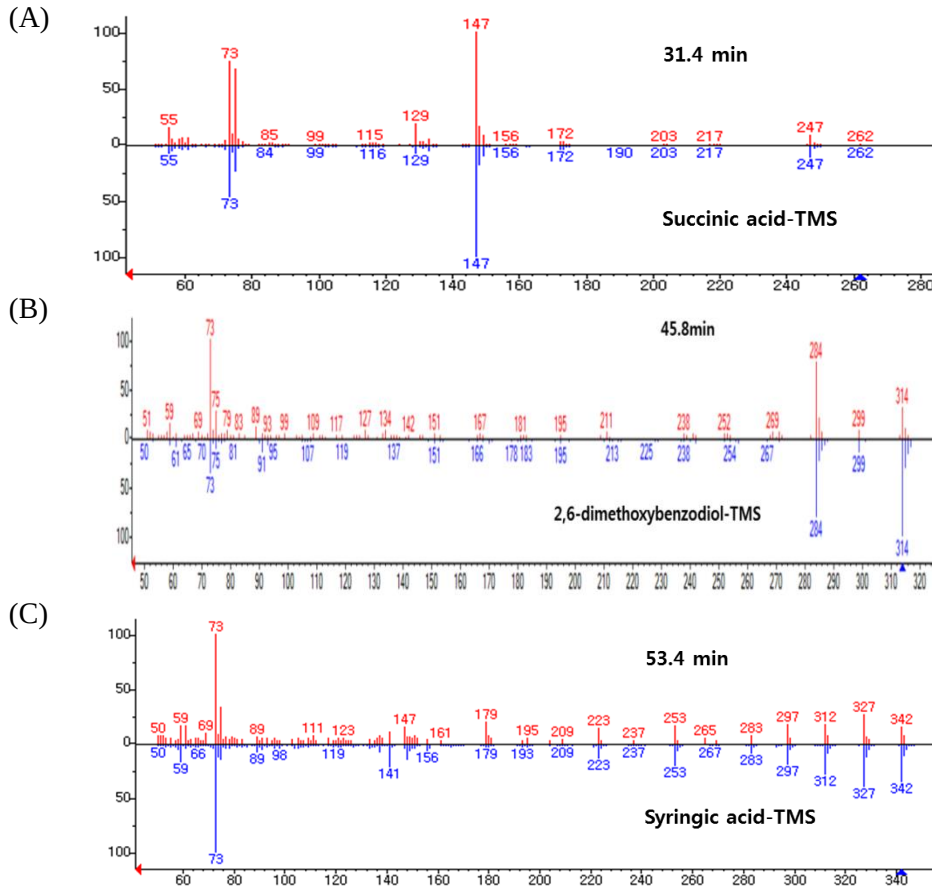
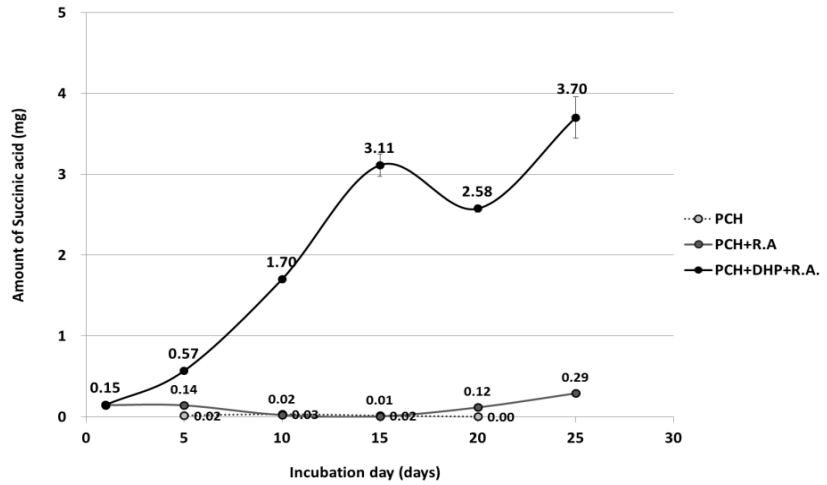


Figure 4-7. Mass spectra of degradation products with library data ((A) 31.4 min, (B) 49.8 min, and (C) 53.4 min)

(A)



(B)

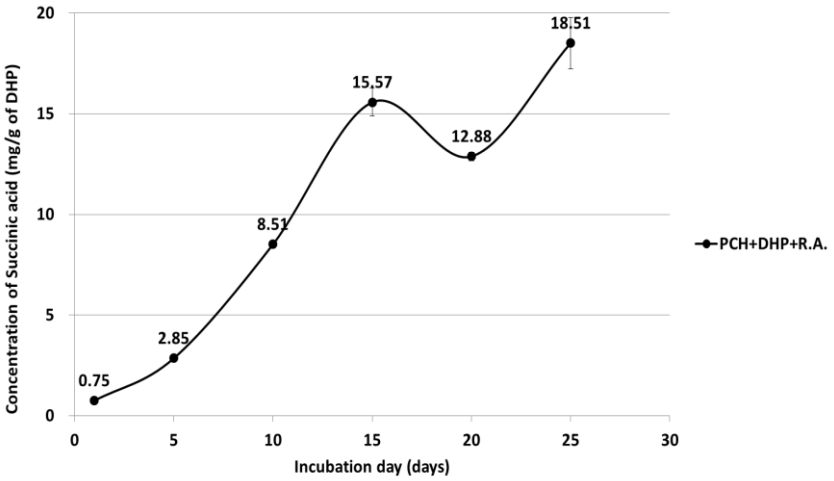


Figure 4-8. Amount of succinic acid by *P. chrysosporium* in 200 ml of culture medium (A), and concentration of succinic acid from DHP by fungus with reducing agent (B)

*PCH: *Phanerochaete chrysosporium*

*DHP: dehydrogenative polymer

*R.A: Reducing agents

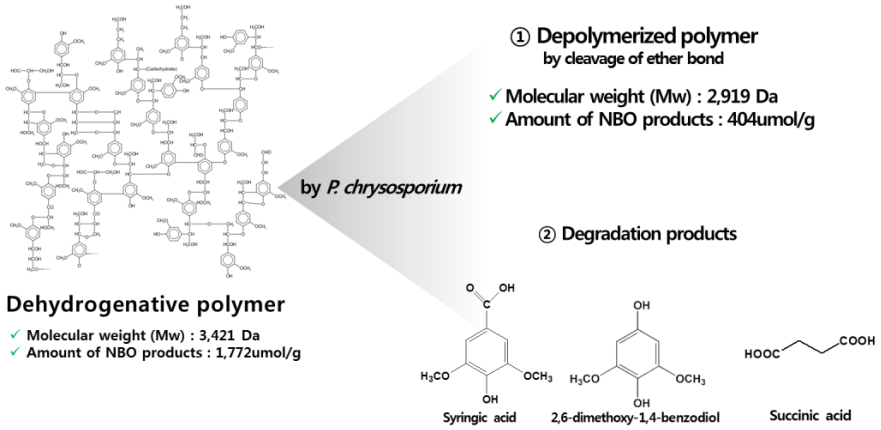


Figure 4-9. Degradation mechanism of DHP by *P. chrysosporium* with addition of ascorbic acid and α-tocopherol

3.3. Degradation mechanism of synthetic lignin derived compounds by *P. chrysosporium*

3.3.1. Degrdation products of syringic acid by *P. chrysosporium*

Syringic acid, 2,6-dimethoxy-1,4-benzodiol, and succinic acid were detected as main degradation products of DHP, as showed in section of 3.2. It was assumed that succinic acid was produced from aromatic compounds released from DHP. Based on these results, for verifying formation mechanism of succinic acid from aromatic compounds, degradation mechanism of syringic acid and hydroquinone were investigated in this section.

Figure 4-10 shows that new degraded products of syringic acid were detected at 32.0, 35.5, and 44.2 min. These peaks were identified as succinic acid, hydroquinone, and 4-hydroxybenzoate, respectively (Figure 4-11). Syringic acid was transformed to 4-hydroxybenzoic acid by demethoxylation reaction. 4-Hydroxybenzoic acid was transformed to hydroquinone by C_{α} oxidation reaction. And then, after ring cleavage process, succinic acid was formed through fungal metabolism. Figure 4-12 shows degradation mechanism of syringic acid by *P. chrysosporium*.

From these results, degradation mechanism of syringic acid was suggested, which was similar with sinapyl alcohol degradation mechanism by *P. chrysosporium* as described in section 3.3 of Chapter 2. In this study, one unique phenomena is demethoxylation process by *P. chrysosporium* when compared with previous researches. In general, methoxylated substrates were transformed to hydroxylated products by one electron oxidation. It was reported that peroxidases were involved in demethoxylation of lignin related substances (Ander & Eriksson, 1985; Ander et al., 1983; Ander et al., 1985; Kersten et al., 1985). This transformation was accompanied with oxidative

activity as well as demethoxylant activity of microorganism (Lopretti et al., 1998). However, results of this study indicated only removal of methoxyl group in aromatic compounds. This reaction could be accompanied with only demethoxylant activity without oxidative activity (Lopretti et al., 1998). In addition, cytochrome P 450 enzymes was reported to have a key role in demethylation of lignin (Kelly et al., 2003; Warrilow et al., 2008). Accordingly, in present study, *P. chrysosporium* catalyzed demeth(ox)ylation, which had an effect on removal of methoxyl groups without accompanying oxidation. To better understand this phenomenon, more detailed studies are necessary.

For verifying degradation mechanism of aromatic compound precisely, degradation mechanism of hydroquinone was observed in next section.

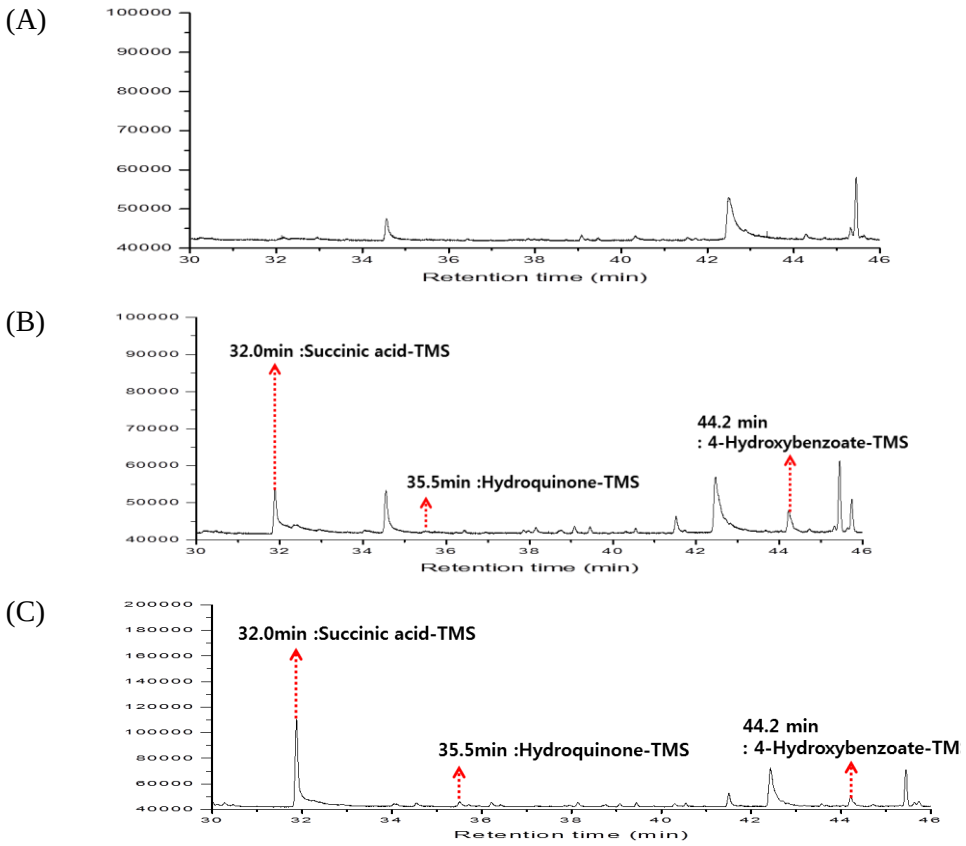


Figure 4-10. Total ion chromatograms of of control (A) and sample treated by *P. chrysosporium* on incubation day 10 (B) and 20 (C)

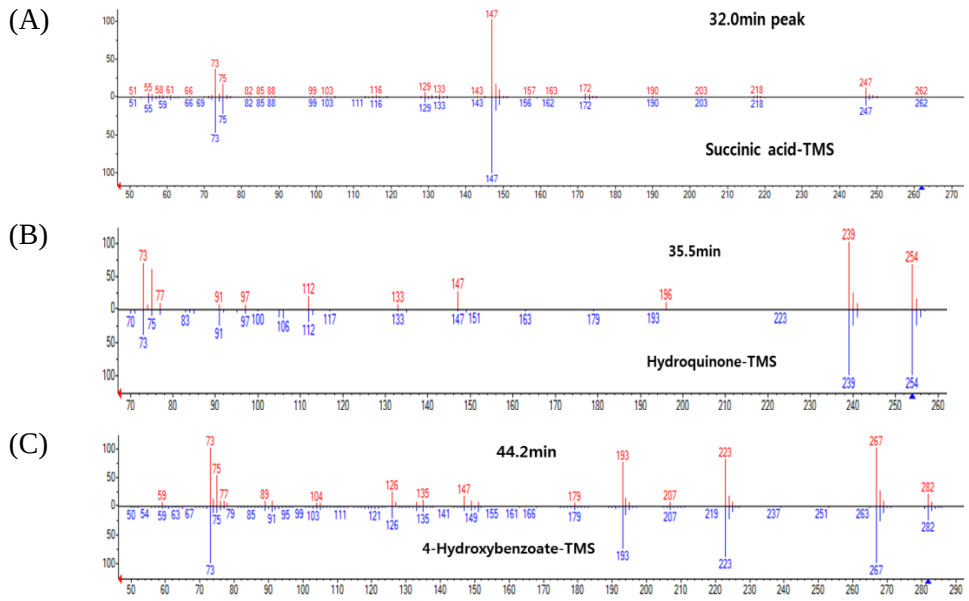


Figure 4-11. Mass spectra of degradation products with library data ((A) 31.4 min, (B) 35.5 min, and (C) 44.2 min)

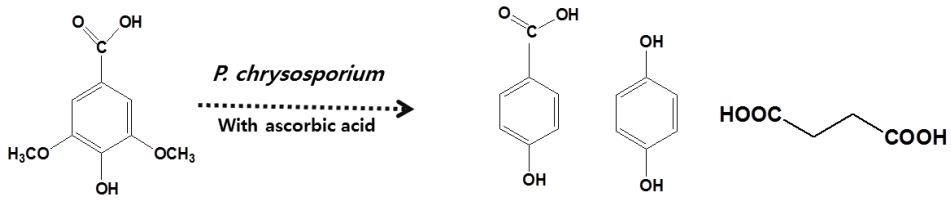


Figure 4-12. Degradation mechanism of syringic acid by *P. chrysosrporioum* with addition of ascorbic acid

3.3.2. Degrdation products of hydroquinone by *P. chrysosporium*

Based on results of 3.3.1, it was assumed that hydroquninone as degradation product of syringic acid was metabolized intracellularly by *P. chrysosporium* and succinic acid was formed finally. Accordingly, degradation mechanism of hydroquinone by *P. chrysosporium* was examined for better understanding of degradation mechanism of aromatic compounds. This experiment was conducted under the same condition as section of 3.3.1.

Figure 4-13 shows that new peak was detected at 32.0 min in fungal sample. *P. chrysosporium* converted hydroquinone to succinic acid (Figure 4-13).

Succinic acid was major degradation product of hydroquinone. Compared with result of 3.3.1, more amount of succinic acid was formed from hydroquinone. From incubation day 5, amount of succinic acid increased, and concentration of succinate on incubation day 20 reached 56.8 mg/g $_{\text{of substrate}}$ (Figure 4-14). Amount of succinic acid formed from hydroquinone by fungus was much more than that from syringic acid. This result suggested that hydroquinone was intermediate in degradation process of syringic acid. Although intermediates formed by ring fission for entering of metabolic pathway were not detected, unique aromatic metabolism in fungal system was observed in this study.

To form succinic acid from hydroquinone, various processes were required. In general, dioxygenase was involved in ring cleavage of aromatic compounds. Intradiol dioxygenase cleave *ortho* to the hydroxyl substituents, on the contrary, extradiol dioxygenases cleave *meta* to the hydroxyl substituent (Eltis & Bolin, 1996). Accordingly, because hydroquinone has *para* hydroxyl substituent, additional hydroxylation seemed to occur on aromatic ring. Cytochrome P 450 monooxygenase reportedly have a role in intracellular monooxygenation reaction in biodegration and bioremediation of

aromatic compounds (Hirosue et al., 2011; Wong, 1998; Yadav et al., 2003). Ring fission of hydroxylated aromatic compounds by dioxygenase has been extensively described (Eltis & Bolin, 1996; Masai et al., 1999; Rieble et al., 1994). Transcript analysis was performed to investigate enzymes related to aromatic catabolic pathway from lignin additionally. This result was shown in section of 3.4.

Lignin is degraded under the nutrient limited conditions when *P. chrysosporium* enters secondary metabolism (Jeffries et al., 1981; Shimada et al., 1981). Above observations proposed that formation of succinic acid was enhanced with metabolism of aromatic compounds by fungus. Consequently, it was concluded that *P. chrysosporium* catalyzed lignin degradation and production of succinic acid from aromatic compounds by entering the secondary metabolism.

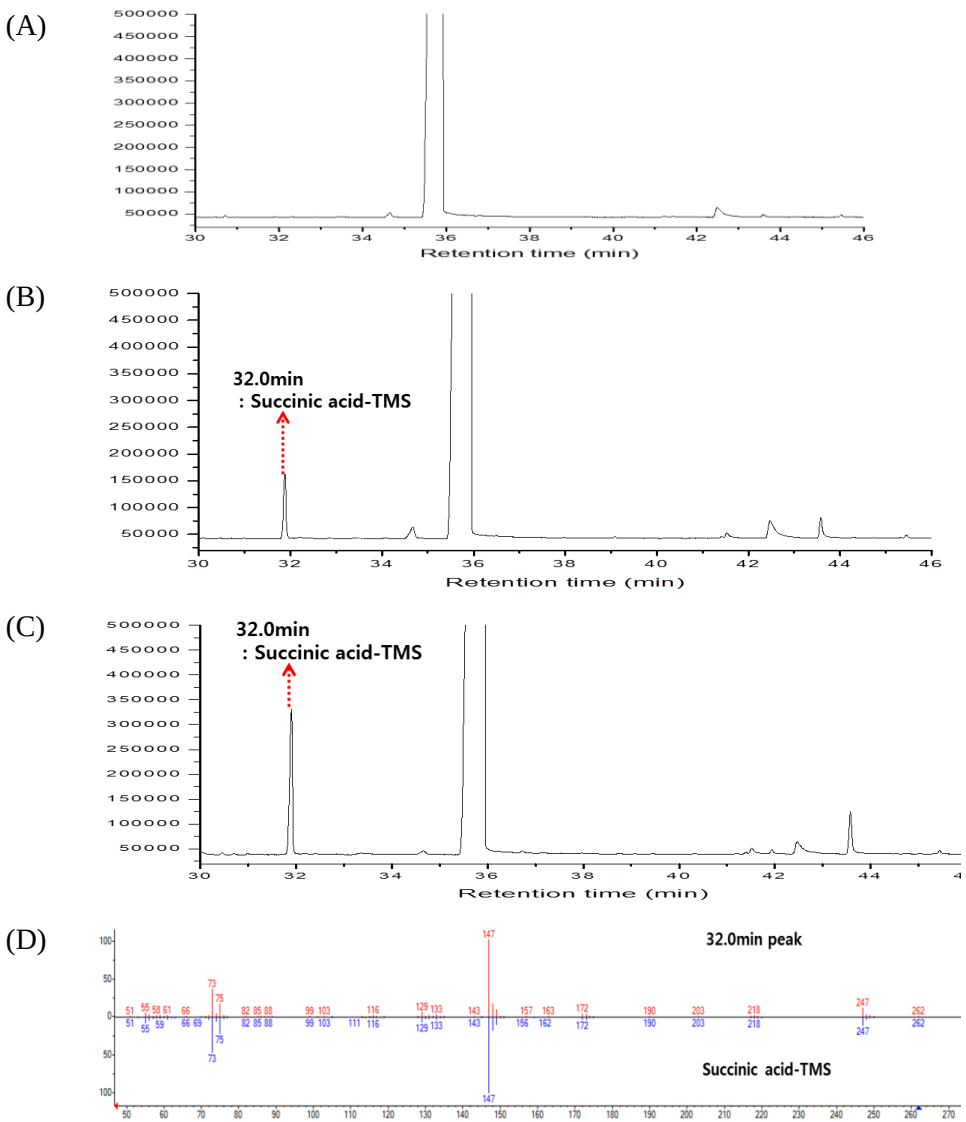


Figure 4-13. Total ion chromatograms of control (A) and sample treated by *P. chrysosporium* on incubation day 15 (B) and 25 (C), and mass spectrum of degradation products with library data (D)

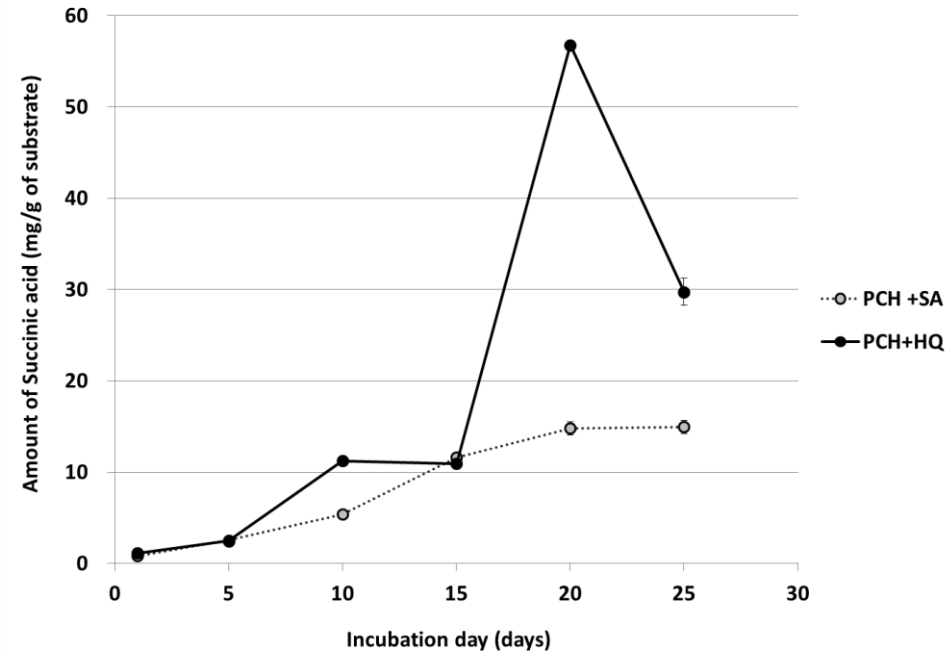


Figure 4-14. Quantitative analysis of succinic acid from syringic acid and hydroquinone under the ligninolytic treatment by *P. chrysosporium* (PCH+SA: succinated derived from syringic acid by fungus, PCH+HQ: succinated derived from hydroquinone by fungus)

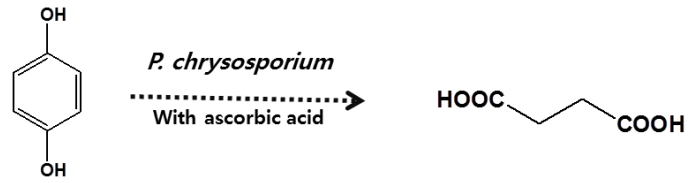


Figure 4-15. Degradation mechanism of hydroquinone by *P. chrysosrporioum*

3.4. Transcriptomic analysis during biomodification of synthetic lignin by *P. chrysosporium*

3.4.1. Sequencing and assembly of cDNA libraries

Transcriptomic analysis of *P. chrysosporium* provided new insights in expressed genes of this fungus under the different culture condition by applying high throughput sequencing. Normalized cDNA libraries were sequenced by Illumina HiSeq 2500. Table 4-8 shows data information between raw data and trimmed data of each sample. After filtering of low quality reads, trimmed data showed high Q30 value (Table 4-9). This means that high quality sequences were obtained and assembled in this study.

RNA-Seq data obtained through the preprocessing was mapped on reference dataset of cDNA library. Mapping ration was showed in Table 4-9. Mapping ratio ranged from 58.7% to 74.6%.

In summary, high-throughput sequencing technology resulted in high quality sequence of transcribed *P. chrysosporium* gene although mapping ratio was not high. Subsequently, DEGs analysis was performed based on dataset mapped on reference sequence. And then, annotation and functional interpretation of genes expressed in *P. chrsosporium* were carried out in this study.

Table 4-8. Data information of samples

			Raw data		Trimmed data		Mapping		
			Total read bases	Total reads No.	Total read bases	Total reads No.	Processed reads	Mapped reads	Overall read mapping ratio
Absence of reducing agents	5d	PCH	4,443,054,640	43,990,640	4,246,918,908	42,800,504	21,400,252	12,776,208	58.70%
		PCH+DHP	4,014,834,638	39,750,838	3,838,384,369	38,666,482	19,333,241	11,471,439	59.30%
	25d	PCH	3,785,599,382	37,481,182	3,337,341,205	34,503,172	17,251,586	12,077,129	70.00%
		PCH+DHP	4,897,457,478	48,489,678	4,290,840,142	44,454,902	22,227,451	15,388,150	69.20%
Presence of reducing agents	5d	PCH	3,749,992,236	37,128,636	3,581,777,177	36,103,290	18,051,645	12,370,629	68.50%
		PCH+DHP	4,496,933,090	44,524,090	4,291,485,498	43,276,006	21,638,003	15,215,244	70.30%
	25d	PCH	6,275,636,212	62,135,012	5,557,584,905	57,438,664	28,719,332	21,420,553	74.60%
		PCH+DHP	4,903,479,098	48,549,298	4,254,880,869	44,170,288	22,085,144	15,122,574	68.50%

*Total read base = Total reads X Read length

*processed reads: Amount of cleaned reads after trimming

*mapped reads: Number of read mapped on reference sequence

*overall read mapping ratio: total mapped reads / total processed reads

Table 4-9. Percentage of sequence having Q30 (%) of samples

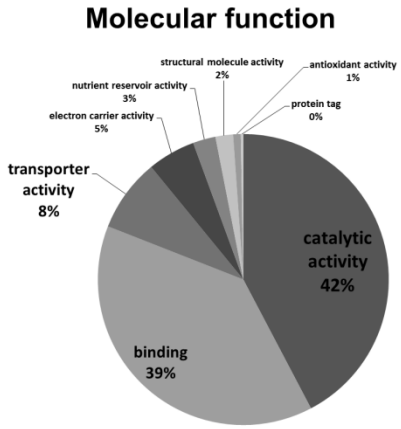
		Non addition of reducing agents		Addition of reducing agents	
		Raw data	Trimmed data	Raw data	Trimmed data
5d	PCH	92.26	94.74	91.89	94.44
	PCH+DHP	92.33	94.81	91.83	94.42
25d	PCH	81.88	88.52	81.82	88.29
	PCH+DHP	81.35	88.18	80.55	87.82

*Q30 (%): Sequence with value of phred quality score≥30

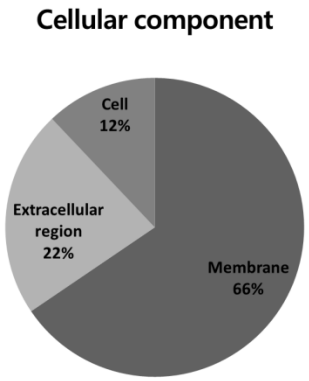
3.4.2. GO annotation

Analysis of molecular function of *P. chrysosporium* showed that catalytic activity, binding and transport activity were mainly expressed in genes of fungus (Figure 4-16 (A)). This result suggested that *P. chrysosporium* has excellent ability to catalyze substrates by biochemical activities of specific enzymes. In cellular component reflecting understanding of eukaryotic cell structure, genes and genes products related to “membrane”, “extracellular region”, and “cell” components were expressed. Especially, a lot of genes in membrane category were indicated as much as 65.5% in cell components (Figure 4-16 (B)). Expression of genes related to “extracellular region” and “cell” demonstrated extra and intra cellular response intimately occurred in *P. chrysosporium* because ontology of cellular component refer to the place where the gene product is active (Consortium, 2001). Biological process of genes expressed in *P. chrysosporium* was “coupled to transmembrane movement of substances” (Figure 4-16 (C)). Biological process refers to a biological goals which gene product contributes (Consortium, 2001; Consortium, 2004). In conclusion, *P. chrysosporium* metabolizes substrate intracellularly by movement of substrates through the transmembrane. This was why succinic acid was formed from DHP by *P. chrysosporium*.

(A)



(B)



(C)

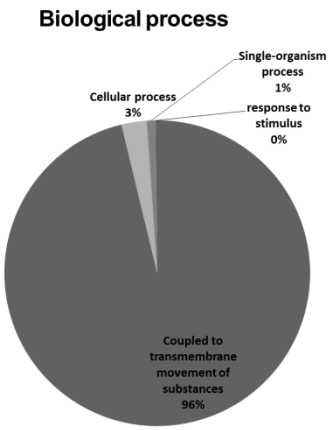


Figure 4-16. Molecular function (A), cellular component (B), and biological process (C) of *P. chrysosporium* by GO annotation

3.4.3. Functional analysis of extracellular enzymes related to lignin degradation of *P. chrysosporium*

P. chrysosporium is one of the best studied white rot basidiomycetes. Extracellular LiP and MnP have been isolated from *P. chrysosporium.* These representative enzymes play a key role in lignin degradation (Higuchi, 1990; Kirk & Farrell, 1987; Tien, 1987). Transcriptomic analysis was carried out to investigate characteristics of enzymes when *P. chrysosporium* degraded synthetic lignin under the presence or absence of reducing agents.

Figure 4-17 and 4-18 show expression level of extracellular enzymes related to lignin degradation depending on presence or absence of reducing agents. Each unigenes of enzymes were identified to have high identification compared with annotated enzyme as a result of NCBI BLAST search.

At first, in case of absence of reducing agents, LiP and MnP genes were highly expressed in fungus treated by DHP on incubation day 25 (Figure 4-17). Furthermore, copper radical oxidase (CRO), multicopper oxidase (MCO), cellobiose dehydrogenase (CDH) and glyoxal oxidase (GLOX) activities were enhanced with increase of incubation day. The *fc* thereof was higher on incubation day 25 than 5 (Figure 4-19). CRO expressed in *P. chrysosporium* is glyoxal oxidase with broad substrate specificity for the oxidation. The main role of glyoxal oxidase is generation of H_2O_2 for LiP and MnP in lignin degradation process (Kersten & Cullen, 2007; Wymelenberg et al., 2006b). MCO secreted from *P. chrysosporium* has been reported to modulate Fenton reaction through the Fe^{2+} oxidation with ferroxidase activity (Kersten & Cullen, 2007; Larrondo et al., 2003). In general, MCO secreted from fungi was considered as laccase. However, *P. chrysosporium* was well known for not secreting laccase. Therefore, these results will be very meaningful if more studies are conducted to investigate difference of gene sequence and its function between laccase of other fungus and MCO of *P. chrysosporium.* CDH

is also involved in generation reactive hydroxyl radicals via Fenton reaction (Dashtban et al., 2010) and play an important role in lignin degradation by breaking ether bonds and demethoxylating aromatic compounds (Henriksson et al., 2000). Accordingly, *P. chrysosporium* attack lignin polymer utilizing these extracellular enzymes. Thus, DHP was degraded with formation of a variety of aromatic fragments during incubation day. With increase of incubation day, unstable aromatic fragments formed by one electron oxidizing enzymes seemed to move large macromolecules. Therefore, polymerization of DHP occurred on incubation day 25.

DEG analysis of fungus exposed to reducing agents showed involvement of extracellular enzymes in lignin modification (Figure 4-18). Expression levels of extracellular enzymes by FPKM analysis were increased with addition reducing agent, compared with that of absence of reducing agent. Especially, FPKM level of LiP gene reached 22.9 with 7.9 of *fc* (Figure 4-18 & 19). The *fc* of copper radical oxidase and multicopper oxidase reached 2.89 and 2.92, respectively (Figure 4-19). Because copper radical oxidase and multicopper oxidase are accessory enzyme which help peroxidases to catalyze substrates, increase of LiP expression attribute to increase expression of accessory enzymes (Dashtban et al., 2010).

On the whole, addition of reducing agents induced similar or high expression of extracellular enzymes. However, lignin was degraded with reducing agent as described in section 3.2. These results provided extacellular enzymes of *P. chrysosporium* catalyzed H_2O_2 dependent oxidative degradation and polymerzation of synthetic lignin depending on incubation day, resulting in generating high active and unstable radicals. The most interesting observation was that reducing agents such as ascorbic acid and α-tocopherol facilitated predominantly lignin degradation by stabilizing radicals despite of enhancement of extracellular lignin degrading enzymes acitivities.

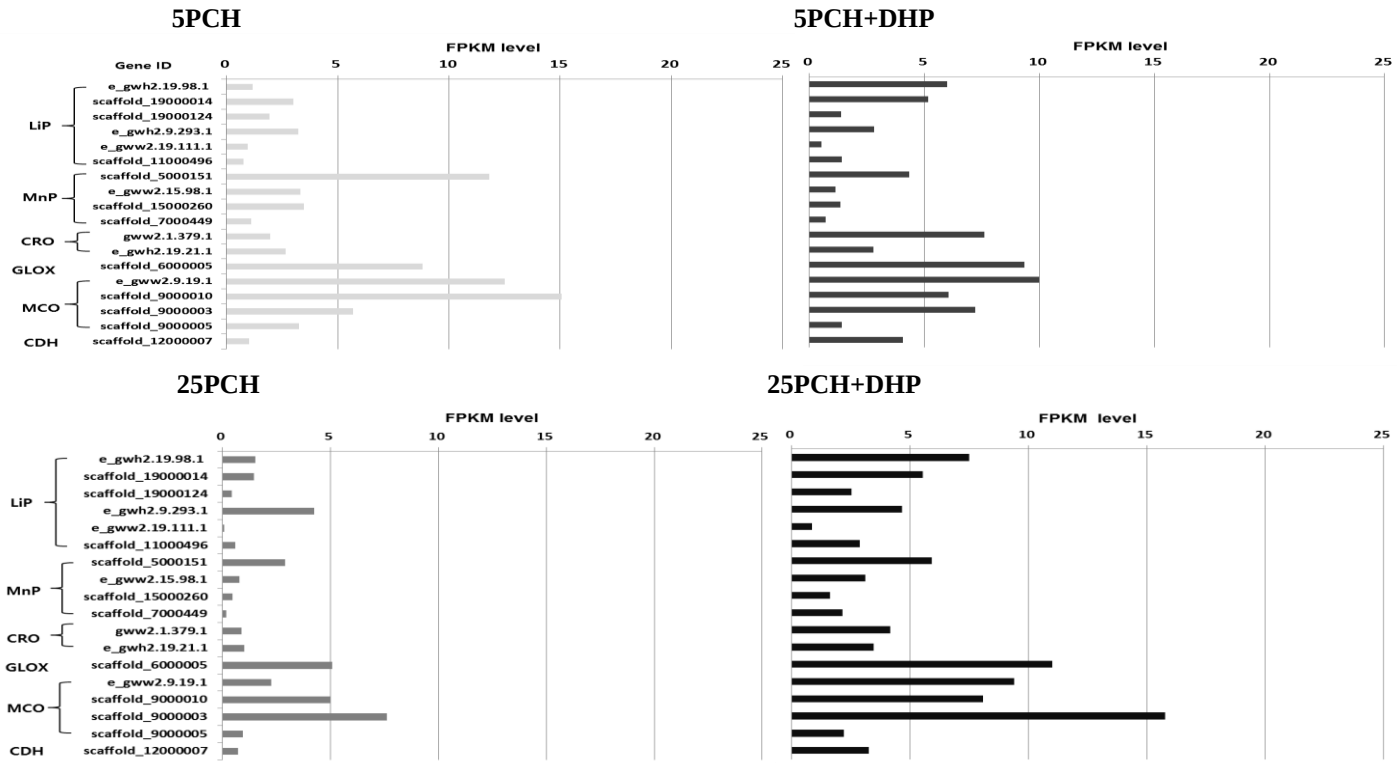


Figure 4-17. Expression level of extracellular enzymes related to lignin modification by FPKM analysis without reducing agents *LiP: lignin peroxidase, *MnP: manganese peroxidase, *CRO: copper radical oxidase, *GLOX: glyoxal oxidase, * MCO: multicopper oxidase, * CDH: cellobiose dehydrogenase

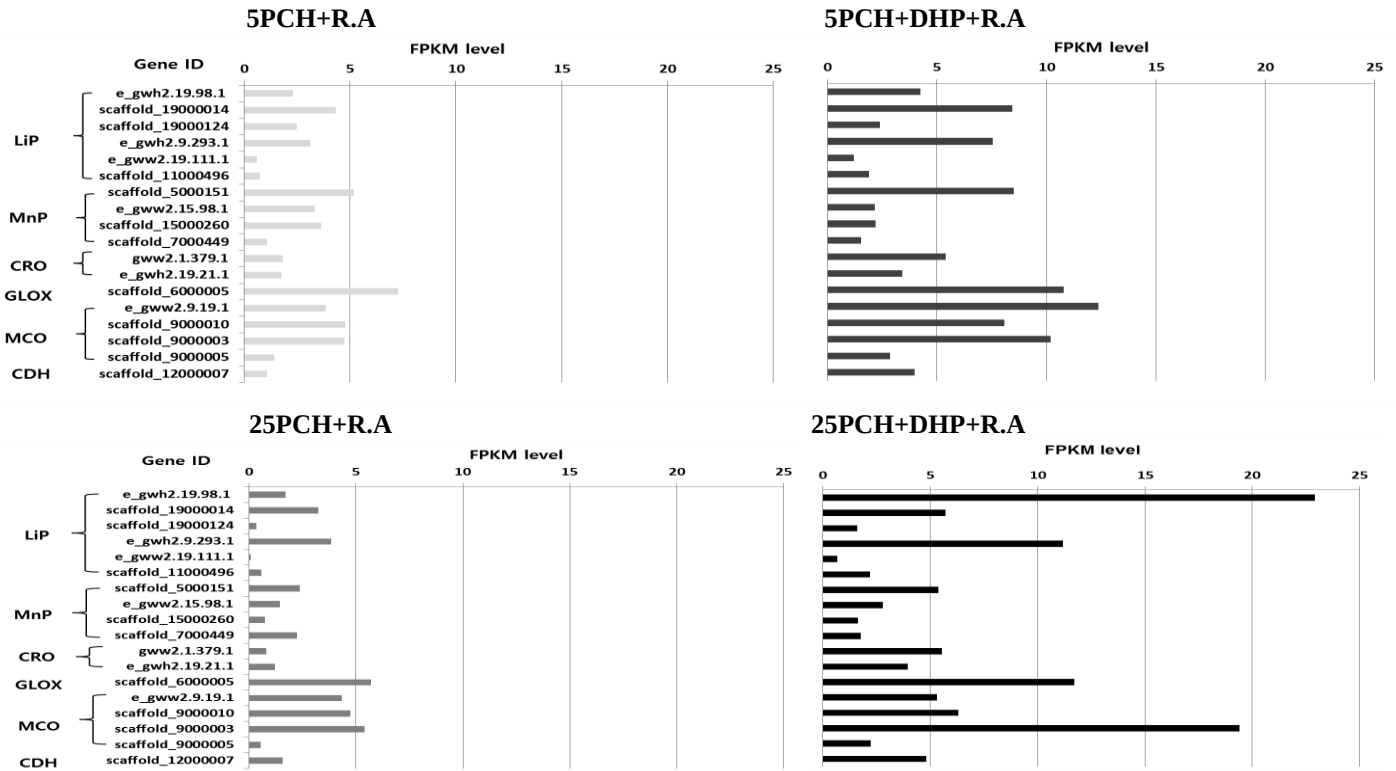


Figure 4-18. Expression level of extracellular enzymes related to lignin modification by FPKM analysis with reducing agents *LiP: lignin peroxidase, *MnP: manganese peroxidase, *CRO: copper radical oxidase, *GLOX: glyoxal oxidase, * MCO: multicopper oxidase, * CDH: cellobiose dehydrogenase

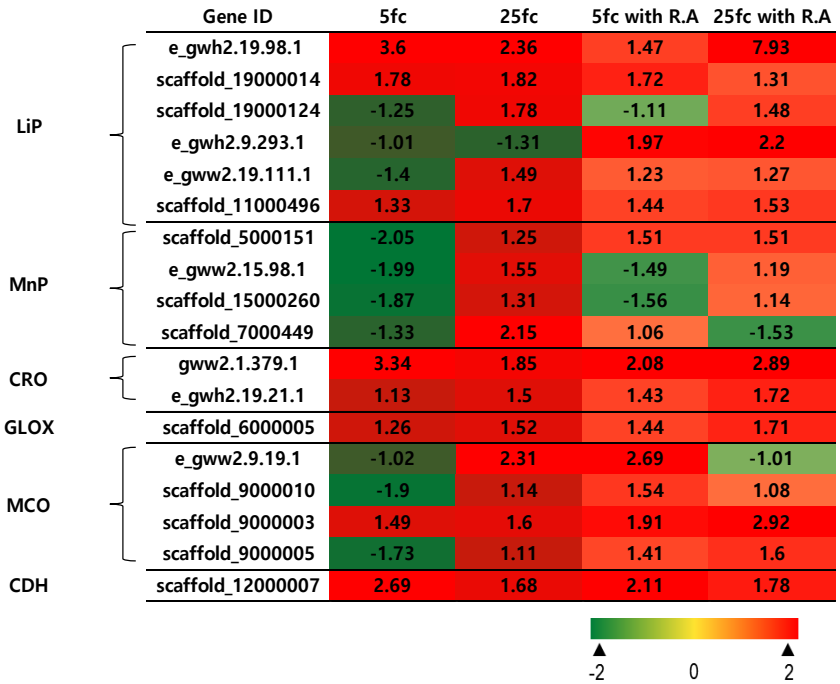


Figure 4-19 Heat map of genes of extracellular enzymes of *P. chrysosporium* depending on addition and non-addition of reducing agents

*fc: fold change (fc) is difference of gene's expression between *P. chrysosporium* and *P. chrysosporium* exposed in DHP

*5fc: fold change on incubation day 5

*5fc with R.A: fold change on incubation day 5 under the condition of presence of reducing agent

*25fc: fold change on incubation day 25

*25fc with R.A: fold change on incubation day 5 under the condition of presence of reducing agent

3.4.4. Functional analysis of intracellular enzymes related to lignin degradation of *P. chrysosporium*

Transcriptomic analysis using next generation sequencing technology has advantage to be able to analyze function of extra and intra cellular enzymes at once because it provides multidimensional examinations of cellular transcriptomes (Morozova et al., 2009).

In this study, DEG results demonstrated involvement of a variety of intracellular enzymes in lignin degradation. Cytochrome P450 monooxygenase, glutathione S transferase, 1, 4-benzoquinone reductase, and aryl alcohol dehydrogenase were expressed (Figure 4-20, 21).

Cytochrome P450 monooxygenase (CYP450) was expressed highly on incubation day 5 (Figure 4-20). The *fc* increased when reducing agents were added (Figure 4-21). Accordingly, CYP450 was implicated in lignin degradation because CYP450 activity was enhanced when degradation of DHP occurred. Intracellularly, monooxygenation reaction by CYP450 is well known to play an important role in biodegradation of lignin and aromatic compounds (Bezalel et al., 1996; Matsuzaki & Wariishi, 2005; Teramoto et al., 2004). CYP450 is implicated in the oxidative elimination of hydrophobic substances (Wislocki et al., 1980).

Glutathione S transferase (GST) was highly expressed on incubation day 25 under the presence of reducing agents (Figure 4-21). On incubation day 25, FPKM level of GST genes reached 69.5 under the absence of reducing agent (Figure 4-20) and 295.3 under the presence of reducing agent, respectively (Figure 4-21). In particular, the *fc* value was 5.9 under the presence of reducing agents on day 25 (Figure 4-22). Furthermore, KEGG pathway analysis presented that *fc* value of glutathione reductase (EC 1.8.1.7) was 3.6 on day 25 under the presence of reducing agents (data unshown). Since this enzyme catalyzes glutathione disulfide to glutathione which is substrate of

GST, it influences on GST activity. GST was well-known to be effective to cleavage of beta-aryl ether linkage in lignin (Adav et al., 2012; Blixt et al., 2008). Accordingly, these results implied that GST had an effect on breaking ether bond of DHP, thus, DHP degradation occurred preferentially on incubation day 25 under the presence of reducing agents.

In summary, CYP450 and GST–mediated reaction intracellularly was effective on lignin degradation.

Aryl alcohol dehydrogenase (AAD) and 1,4-benzoquinone reductase (QR) were also expressed in this study (Figure 4-20, 21). AAD is able to convert aromatic aldehydes into their corresponding alcohols, and their reducing activity formed during secondary metabolism was implicated in lignin biodegradation (Delneri et al., 1999; Reiser et al., 1994). QR belongs to a family of flavoprotein quinone reductaseds. This enzyme play a role in the conversion of quinone to corresponding hydroquinone needed to produce additional Fenton reagent (Jensen Jr et al., 2002). Expression of AAD was highest in fungal sample treated by DHP with reducing agents on incubation day 25 showing FPKM level was 18.8 and *fc* was about 7.0 (Figure 4-21, 22). Expression of QR also was kept highly in 5PCH+DHP, 5PCH+DHP+R.A, and 25PCH+DHP+R.A samples which lignin degradation occurred.

In this study, high expression of intracellur enzymes, CYP450, GST, AAD and QR was observed with occurrence of DHP degradation, strongly suggesting the involvement of lignin degradation.

Consequently, synthetic lignin was degraded by intracellular enzymes of *P. chrysosporium* as well as extracellular enzymes under the presence of reducing agents stabilizing active radicals. Especially, LiP, MnP, and GST are effective to cleave ether bonds of lignin polymer. Through this process, aromatic fragments released from lignin were oxidized by intracellular enzymes such as CYP450, AAD and QR.

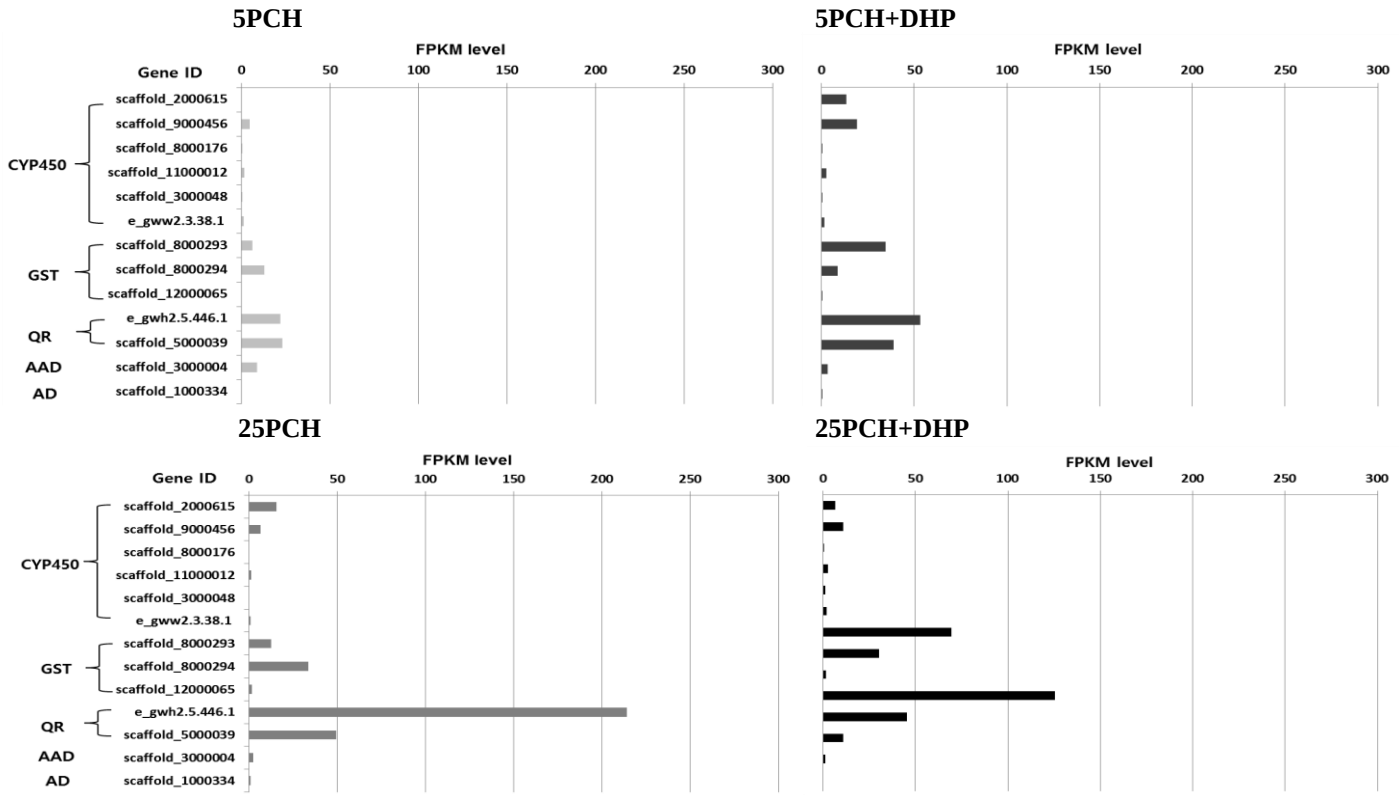


Figure 4-20. Expression level of intracellular enzymes related to lignin modification by FPKM analysis without reducing agents

* CYP450: Cytochrome P450 monooxygenase, *GST: Glutathione S transferase, *AAD: aryl alcohol dehydrogenase,
* AD: aldehyde dehydrogenase, * QR: 1,4-benzoquinone reductase

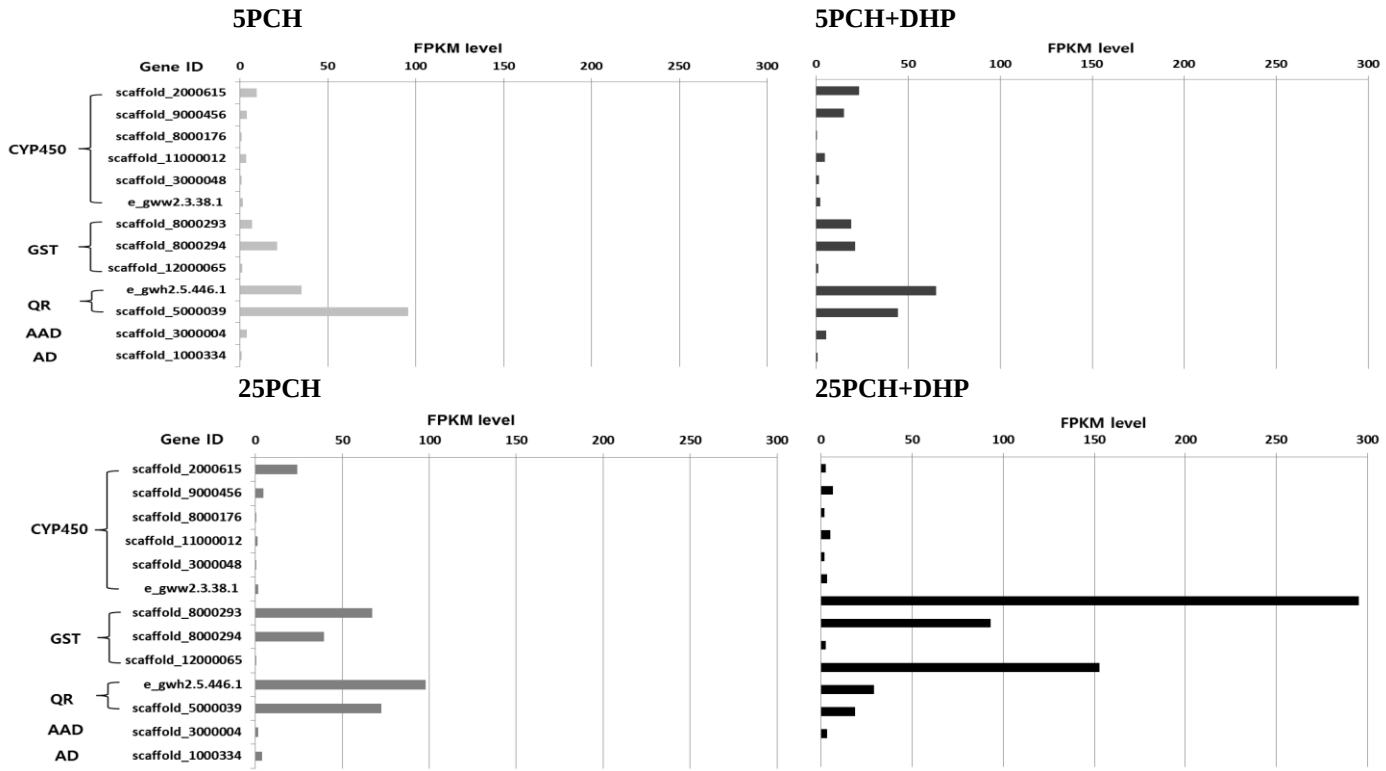


Figure 4-21. Expression level of intracellular enzymes related to lignin modification by FPKM analysis with reducing agents

* CYP450: Cytochrome P450 monooxygenase, *GST: Glutathione S transferase, *AAD: aryl alcohol dehydrogenase,
* AD: aldehyde dehydrogenase, * QR: 1,4-benzoquinone reductase

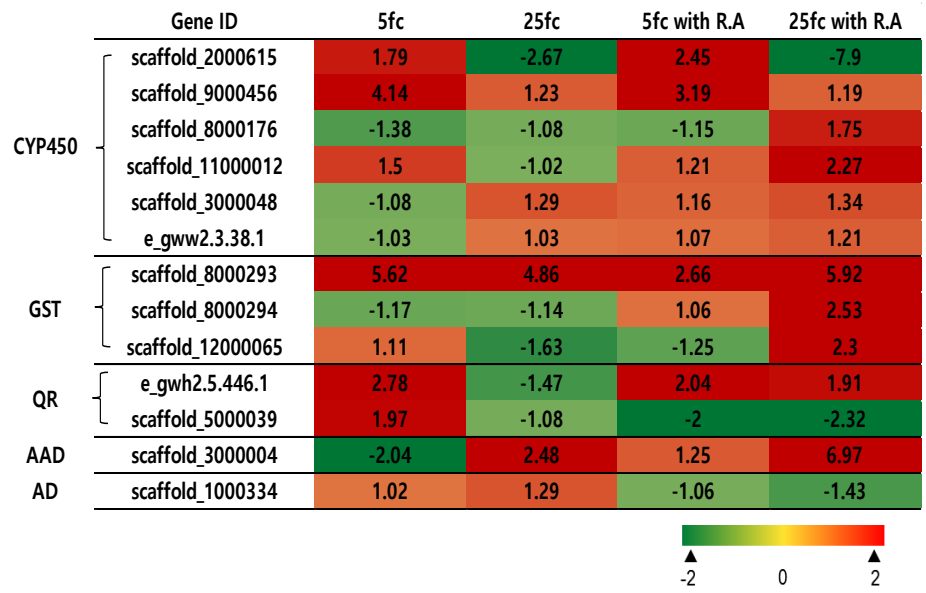


Figure 4-22. Heat map of genes of intracellular enzymes of *P. chrysosporium* depending on addition and non-addition of reducing agents

*fc: fold change (fc) is difference of gene's expression between *P. chrysosporium* and *P. chrysosporium* exposed in DHP

*5fc: fold change on incubation day 5

*5fc with R.A: fold change on incubation day 5 under the condition of presence of reducing agent

*25fc: fold change on incubation day 25

*25fc with R.A: fold change on incubation day 5 under the condition of presence of reducing agent

3.4.5. Functional analysis of enzymes related to formation of succinic acid of *P. chrysosporium*

Through the above mentioned results, it was confirmed that lignin degradation occurred by extra and intracellular enzymes.

Production of succinic acid from DHP was observed in section 3.3. We carried out KEGG pathway analysis based on DEG results to investigate enzymes related to production of succinic acid from DHP, and understand metabolic process of *P. chrysosporium* at transcriptional level.

KEGG metabolic pathway analysis presented 109 metabolic pathways in *P. chrysosporium.* Among them, results showed that ring fission pathway and metabolic pathway such as carbon fixation pathway, tricarboxylic acid (TCA) cycle, and glyoxylate metabolism were involved in production of succinate.

At first, genes related to dioxygenases were expressed (Figure 4-23). Dioxygenase play a key role in production of acid compounds from aromatic compounds. Two kinds of dioxygenases were expressed in this study. One is 1,2-dioxygenase(EC 1.13.11.1), which generally catalyzes catechol to muconate. The other is homogentisate 1,2-dioxygenase(EC 1.13.11.5), which transforms homogentisate to 4-maleyl-acetoacetate. Homogentisate 1,2-dioxygenase is responsible for the ring cleavage step in the degradation pathway and was previously reported to upregulated with ethylbenzene (Gunsch et al., 2005; Gunsch et al., 2006). Especially, FPKM level of dioxygenase annotated as homogentisate 1,2-dioxygenase reached 33.8 on day 5 and 25.6 on dauy 25, which was higher than that of EC 1.13.11.1 ((Figure 4-23). This suggested that hydroquinone derived from DHP could be degraded to succinic acid by *P. chrysosporium*. Thus, both 1,2-dioxygenase and homogentisate 1,2-dioxygenase were considered to influence on ring cleavage of aromatic compounds released from synthetic lignin in this study.

KEGG pathway results were indicative of involvement of three

metabolic pathways in producing succinic acid.

Figure 4-24 presents FPKM level of enzymes of carbon fixation pathway. Among 15 enzymes expressed in this pathway, 6 enzymes were involved in formation of succinic acid. Isocitrate dehydrogenase (EC:1.1.1.42), aconitase (EC:4.2.1.3), citrate synthase (EC:2.3.3.8), and succinate dehydrogenase (EC:1.3.5.1) were highly expressed in 25PCH +DHP +R.A sample with increase of FPKM level (Figure 4-24). The *fc* of four enzymes were higher than that of 5PCH +DHP sample and 25PCH +DHP sample although FPKM level of malate dehydrogenase and fumarase were decreased.

Figure 4-25 shows expression of enzymes in TCA cycle and glyoxylate metabolism of *P. chrysosporium.* Basidiomycetes were reported to have a unique metabolic system, a short-cut TCA/glyoxylate bicycle system (Munir et al., 2001a; Shimizu et al., 2005). Citrate synthase (EC:2.3.3.1), isocitrate dehydrogenase (EC:1.1.1.42), and 2-oxoglutarate dehydrogenase (EC:1.2.4.2), functioning in TCA cycle were reportedly enhanced when *P. chrysosporium* was exposed to vanillin. This pathway converts isocitrate to succinate producing intermediates such as 3-carboxy-1-hydroxypropyl-ThPP and succinyl-CoA (Shimizu et al., 2005). On the other hand, isocitrate can be converted to succinate and glyoxylate directly in short-cut TCA cycle, which glyoxylate is key intermediate in glyoxylate cycle (Munir et al., 2001b). Interestingly, glyoxylate cycle is shown in basidiomycetes (Munir et al., 2001a) as well as plants and certain microorganism (Cozzone, 1998).

This study showed that 7 enzymes in TCA cycle and 3 enzymes in glyoxylate cycle were up-expressed under the presence of reducing agents, especially on incubation day 25 (Figure 4-25, 26). Citrate synthase (EC:2.3.3.1) and aconitase (EC:4.2.1.3) activity were enhanced by 3-fold and 1.83-fold, respectively on day 25 under presence of reducing agent. And also, 2-oxoglutarate dehydrogenase (EC:1.2.4.2) and succinyltransferase (EC:2.3.1.61) were upregulated on day 25 with reducing agents (Figure 4-25).

The most interesting was that *fc* value of isocitrate lyase was 7.1 in glyoxylate metabolism (Figure 4-26). The addition of synthetic lignin, DHP, drastically enhanced enzymes activities functioning in TCA & glyoxylate cycle. Especially, the upregulation of three enzymes in short-cut TCA cycle is indicative of enhancement of succinate production. On the whole, FPKM level of enzymes functioning in TCA/glyoxylate bicycle increased on 25days with presence of reducing agents, and *fc* value of 25PCH+DHP+R.A sample increased rapidly compared with that of 5PCH+DHP+R.A and 25PCH+DHP (Figure 4-26).

Thus, it was assumed that aromatic compounds derived from synthetic lignin were degraded by dioxygenases (EC 1.13.11.1, EC 1.13.11.5), resulting in formation of acid compounds which was important intermediate for entering the metabolic pathway. Degradation products derived from synthetic lignin stimulated primary metabolism of fungus. Accordingly, these intermediates were metabolized by a lot of enzymes functioning in TCA and glyoxylate cycle of *P. chrysosporium.* This assumption was supported by upregulation of enzymes functioning in TCA & short-cut TCA cycle. Glyoxylate cycle has been reported to be in existence for short-cut TCA cycle in basidiomycetes (Shimizu et al., 2005).

Consequently, short-cut TCA cycle and TCA cycle is major metabolic pathway in *P. chrysosporium* in producing succinic acid (Figure 4-27). In particularly, short-cut TCA cycle in glyoxylate metabolism seemed to strongly be involved in formation of succinic acid from DHP.

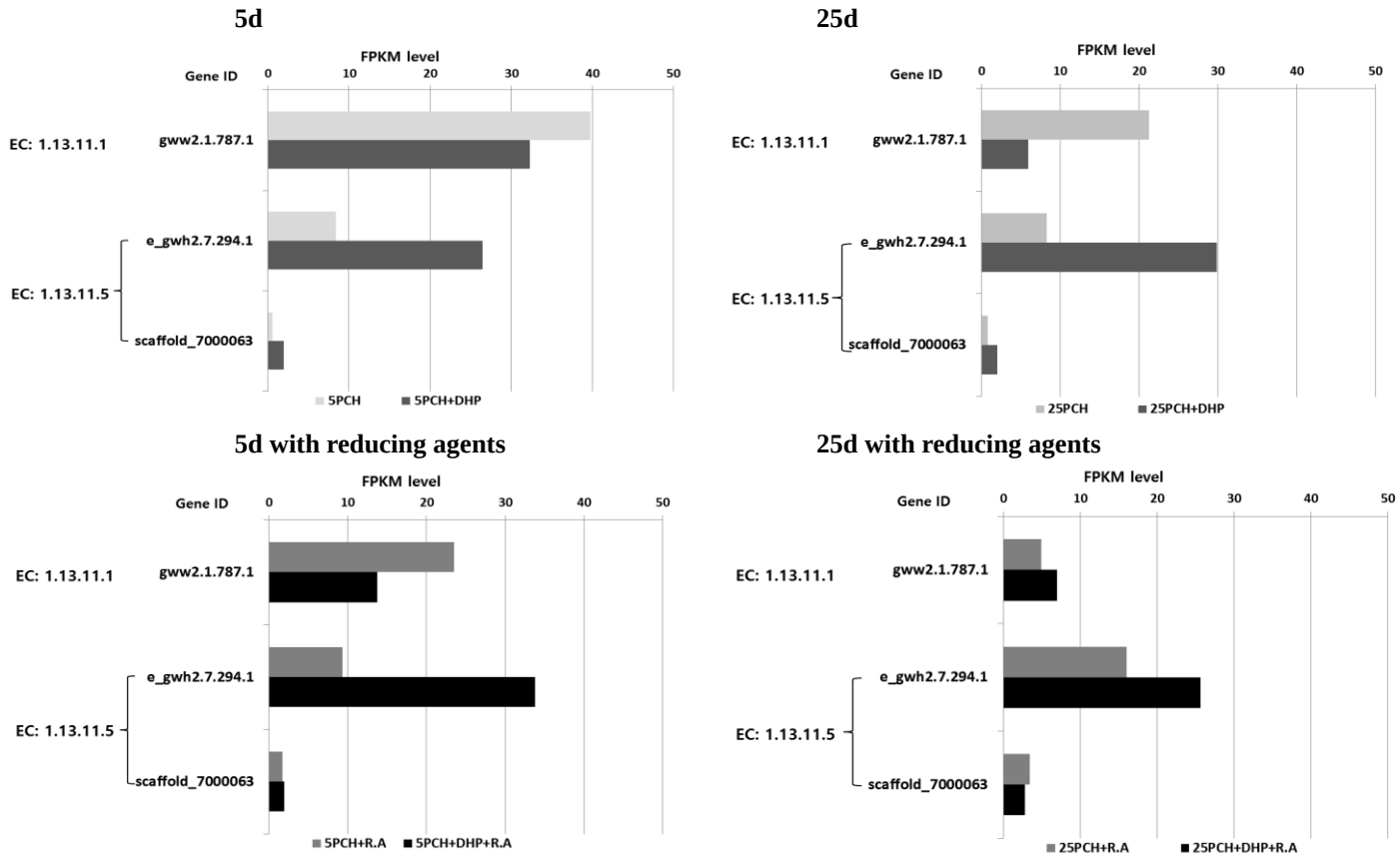
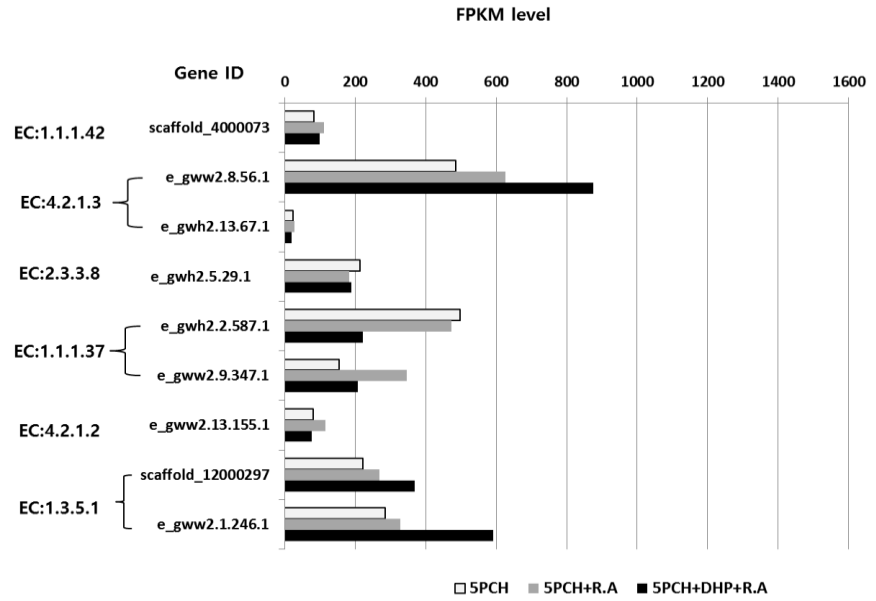


Figure 4-23. Expression level of dioxygenase by KEGG pathway analysis

5d



25d

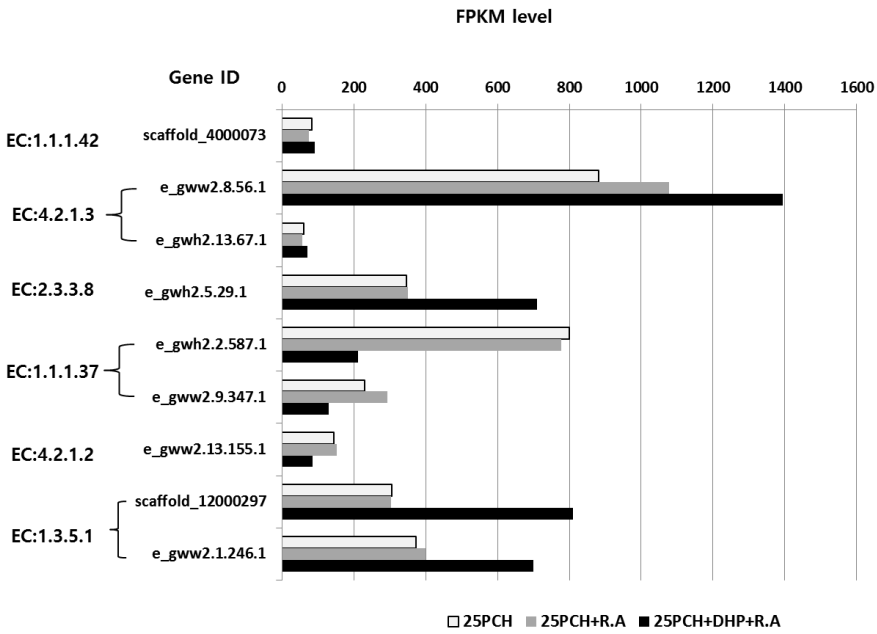


Figure 4-24. Expression level of enzymes related to carbon fixation pathway by KEGG analysis

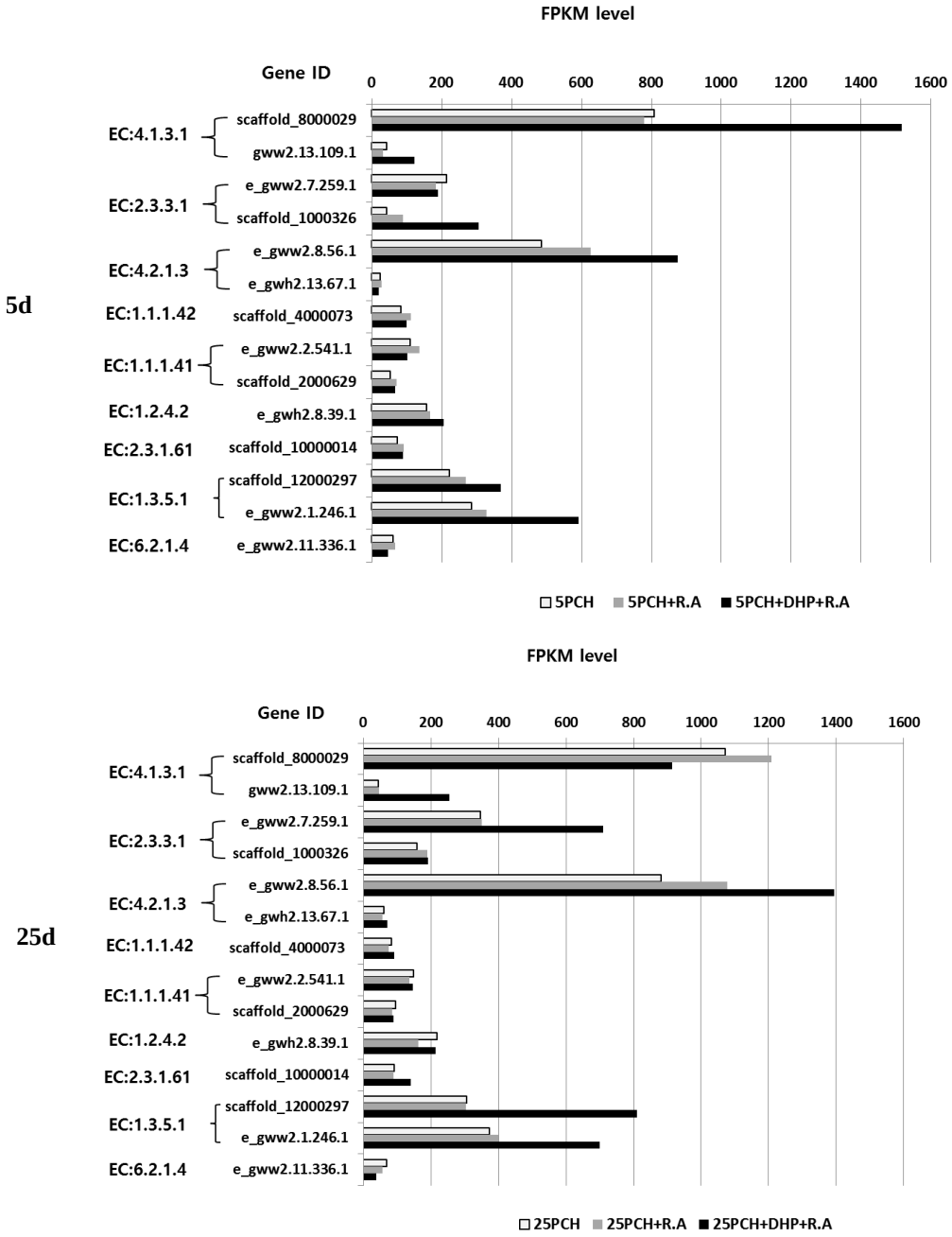


Figure 4-25. Expression level of enzymes related to glyoxylate metabolism and TCA cycle by KEGG analysis

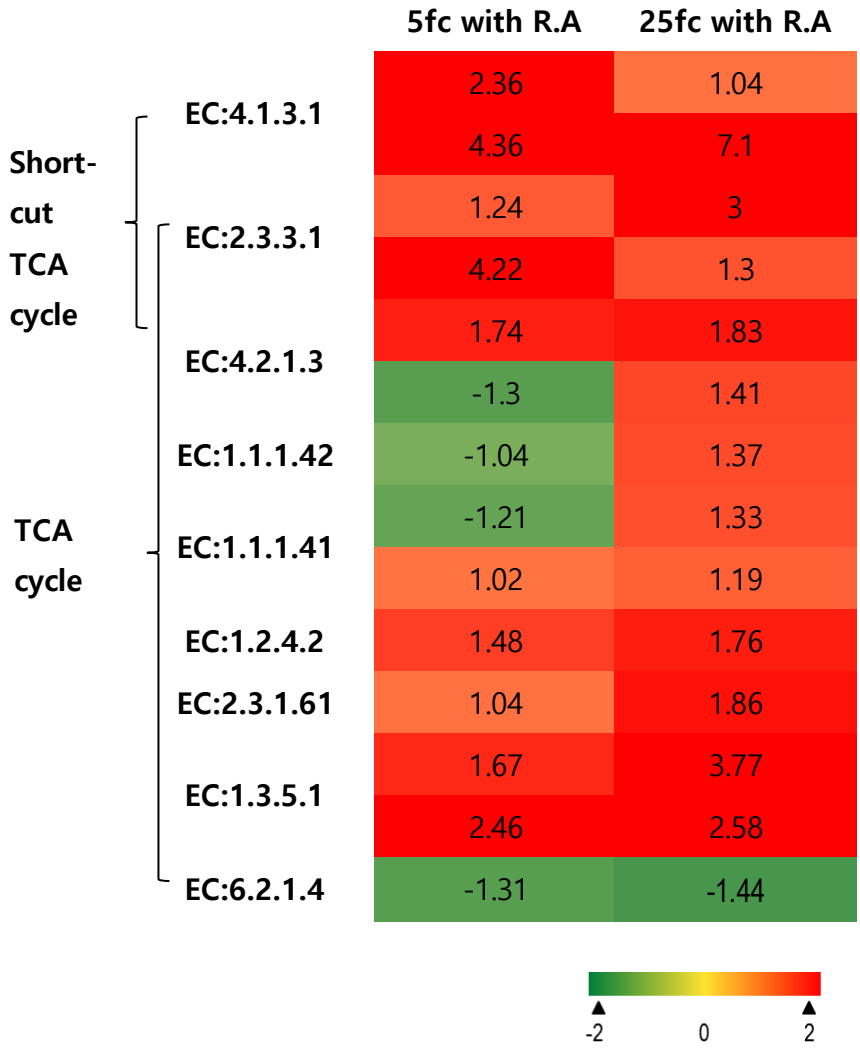


Figure 4-26. Heat map of genes of enzymes functioning in short-cut TCA cycle and TCA cycle depending on addition and non-addition of reducing agents

*5fc with R.A: fold change on incubation day 5 under the condition of presence of reducing agent

*25fc with R.A: fold change on incubation day 5 under the condition of presence of reducing agent

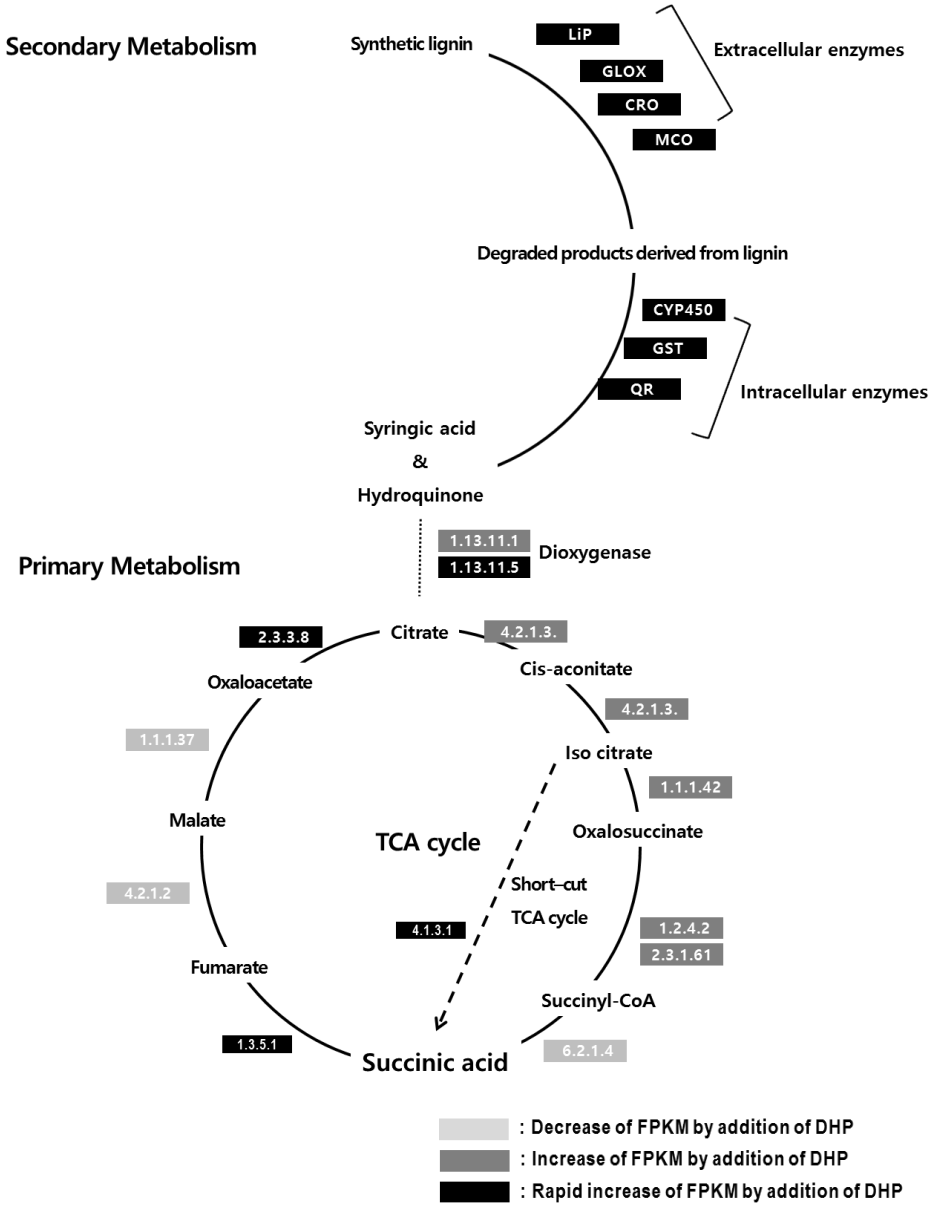


Figure 4-27. Metabolic pathway for formation of succinic acid in *P. chrysosporium*

4. Conclusions

P. chrysosporium simultaneously induced degradation and polymerization of synthetic lignin, DHP. In initial incubation day (~10 days), DHP was degraded by *P. chrysosporium*, which was supported by decrease of molecular weight and high content of phenolic hydroxyl group by ether bond cleavage. However, with increase of incubation day (15~25days), DHP was polymerized with increase of molecular weight despite of low amount of NBO produts. It implied that DHP was condensed under the enzyme controlled condition. On the other hand, lignin oligomers derived from DHP was polymerized in initial day and degraded in latter part of incubation day, which was contrast to that of DHP. Consequently, degradation and polymerization reaction of DHP and lignin oligomers were caused by catalytic system of *P. chrysosporium*.

For degradation of DHP, ascorbic acid and α-tocopherol were used as reducing agents. As a result, addition of ascorbic acid and α-tocopherol concurrently induced degradation of DHP and production of aromatic compounds (syringic acid and hydroquinone) and succinic acid under the ligninolytic treatment by *P. chrysosporium*. Because α-tocopherol functioned as stabilizing DHP, catalytic system of by *P. chrysosporium* induced preferentially degradation of DHP and production of valuable degradation product.

In present study, degradation mechanism of syringic acid and hydroquinone were investigated for verifying formation mechanism of succinic acid from aromatic compounds. It was confirmed that *P. chrysosporium* degraded syringic acid to hydroquinone and succinic acid, and hydroquinone to succinic acid. This finding suggested catalytic system of *P. chrysosporium* has excellent ability to degrade aromatic compounds to

valuable acid compounds.

Transcriptomic analysis of *P. chrysosporium* provided information about enzymes related to lignin degradation and aromatic catabolic pathway under the presence of reducing agents. As a result, bond cleavage of lignin was caused by a lot of enzymes following extracellular enzymes such as LiP and MnP, and intracellular enzymes such as cytochrome P 450 monooxygenase and glutathione S transferase. Aromatic compounds released from DHP were metabolized in short-cut TCA cycle and TCA cycle of *P. chrysosporium*. In this study, isocitrate lyase, citrate synthase and aconitase activities were enhanced when *P. chrysosporium* was exposed to DHP with reducing agents. Consequently, short-cut TCA cycle and TCA cycle is major metabolic pathway in producing succinic acid.

In conclusion, under the presence of ascorbic acid and α-tocopherol as reducing agents, DHP was degraded and succinic acid was formed from DHP by *P. chrysosporium*. Various enzymes functioning in short-cut TCA cycle and TCA cycle were involved in production of succinic acid. These findings suggest *P. chrysosporium* is novel biocatalyst for biotechnological application of basidiomycetes.

Chapter 5

Concluding remarks

Lignin valorization is the greatest challenges in lignocellulosic biorefining. In that sense, lignin modification has been performed by various approaches because it is essential with respect to economic costs. Very recently, new concept using the ligninolytic enzyme system of basidiomycetes has emerged for lignin degradation as attractive alternatives.

In this study, to better understand biomodification mechanism of synthetic lignins by white rot basidiomycetes, *Aboritporus biennis* and *Phanerochaete chrysosporium* (ATCC 20696), biomodification products of monolignols and synthetic lignin by white rot basidiomycetes were analyzed Based on results above, for the lignin degradation, enzyme system of basidiomycetes and surrounding culture condition were changed with addition of mediator of ligninolytic enzymes and reducing agents. Finally, to investigate extra and intracellular enzymes related degradation of lignin, transcriptomic analysis was carried out.

Biomodification mechanism of monolignols and synthetic lignin by *A. biennis* and *P. chrysosporium* were simultaneouly indicative of degradation and polymerization process. That was because ligninolytic enzyme of two basidiomycetes catalyzed oxidative reaction, resulting in generation of unstable and reactive radicals which induced polymerization.

Based on these results, lignin degradation was induced under the ligninolytic condition. Enzyme system of *A. biennis* has MnP-Lac group, and *P. chrysosporium* has LiP-MnP group. Accordingly, different enzyme system of two basidiomycetes and surrounding culture condition were changed for satisfying the lignin degradaion.

A. biennis induced degradaion of synthetic lignin when both laccase mediator and reducing agents were added. Therefore, simultaneous application of two changes in degradaion of synthetic lignin was tried, looking forward to synergetic effects. As a result, whole cell of *A. biennis* assisted considerable degradaion of synthetic lignin. The weight average molecular weight of

synthetic lignin in fungal sample was 2,654 and 2,514 on incubation day 15 and 20, respectively. NBO products of synthetic lignin treated by fungus declined significantly than that of control. This result means that catalytic system of *A. biennis* with laccase mediator and reducing agents broke the ether bonds in lignin, which was attributed to decrease molecular weight of synthetic lignin. Especially, laccase mediator system using ABTS degraded lignin compounds by lowering the oxidation rate of substrates. These results were supported by transcriptomic analysis. Transcriptomic analysis demonstrated that laccase mediator system of *A. biennis* had a powerful impact on lignin degradaion. The high expression of laccase was observed. Besides, other extracellular enzymes such as aryl alcohol oxidase and copper radical oxidase were highly expressed. Consequently, enhancement of activity of these enzymes implied that complex extracellular enzymes of *A. biennis* were involved in lignin degradaion.

P. chrysosporium catalyzed degradaion of synthetic lignin and lignin oligomers with addition of reducing agents. One of most interesting observation in this study was production of aromatic compounds (syringic acid and hydroquinone) and succinic acid as degraded products of synthetic lignin. Because α-tocopherol and ascorbic acid functioned as stabilizing DHP, catalytic system of by *P. chrysosporium* induced preferentially degradation of DHP and production of valuable degradation product. To verify the degradation mechanism of aromatic compounds derived from synthetic lignin, degradation products of syringic acid and hydroquinone by *P. chrysosporium* were investigated. *P. chrysosporium* degraded syringic acid to hydroquinone and succinic acid, and hydroquinone to succinic acid. This finding suggested catalytic system of *P. chrysosporium* has excellent ability to degrade aromatic compounds to valuable acid compounds. Transcriptomic analysis of *P. chrysosporium* provided information about enzymes related to lignin degradation and aromatic catabolic pathway under the presence of reducing

agents. High expression of extracellular peroxidases and glutathione S transferase mean their involvement in cleavage of ether bonds of lignin. Furthermore, cytochrome P450 monooxygenase, aryl alcohol dehydrogenase and quinone reductase were also highly expressed, which play a key role in oxidation of aromatic compounds. Finally, as a result of analysis of enzymes functioning in formation of succinic acid, enzymes functioning in short-cut TCA cycle and TCA cycle of *P. chrysosporium* were observed. Especially, isocitrate lyase, citrate synthase and aconitase activities were upregulated when *P. chrysosporium* was exposed to DHP with reducing agents. Consequently, short-cut TCA cycle and TCA cycle is major metabolic pathway in producing succinic acid.

This study investigated the degradation and polymerization mechanism of lignin compounds by *A. biennis* and *P. chrysosporium*. Based on these results, production of low molecular weight compounds was induced through lignin degradaion by basidiomycetes depending on the change of enzyme system and surrounding condition. Consequently, two basidiomycetes with different catalytic system produced different degraded products from synthetic lignin. Transcriptomic analysis suggested that laccase of *A. biennis* had excellent ability to degrade lignin and enzymes functioning in short-cut TCA cycle of *P. chrysosporium* as well as ligninolytic enzymes had a crucial impact on production of succinic acid. Although more research is required to understand the phenomena of lignin degradaion by fungal system, this result proposed that fungal catalysis system have the possibilities for their biotechnological applications in industrial application of lignin. Furthermore, biological strategy for lignin application is considerable promising for lignin valorization in lignocellulosic biorefinery.

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초 록

유관버섯과 판막버섯에 의한 리그닌 화합물의 생물학적 변환 및 전사체 분석에 의한 관련 효소 구명

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본 연구에서는 백색부후균 *Abortiporus biennis*와 *Phanerochaete chrysosporium*(ATCC20696)에 의한 리그닌 화합물의 생물학적 변환 기작을 이해함으로써 리그닌 생분해를 유도하고자 하였으며, 전사체 분석을 통해 이에 관련된 효소를 구명하고자 하였다.

우선 *A. biennis*와 *P. chrysosporium*에 의한 모노리그놀의 생물학적 변환 산물을 분석한 결과, 분해산물뿐 아니라 중합산물이 생성되었다. 따라서 백색부후균에 의한 모노리그놀의 분해반응을 유도하기 위해 환원제인 아스코르브산을 첨가한 결과, 모노리그놀의 중합을 저지하고, 다양한 분해산물의 생성을 확인되었다. 이러한 결과를 바탕으로, *A. biennis*와 *P. chrysosporium*에 의한 합성리그닌의 생물학적 변환을 시도하였으며, 최종적으로 리그닌 생분해를 유도하고자 하였다.

우선 *A. biennis*는 배양 초기 합성리그닌의 분해를 일으켰지만, 배양 후기에는 중합반응을 일으켰다. 이는 모노리그놀의 생물학적 변환 결과와 비슷한 경향으로, 합성리그닌의 분해를 유도하기 위해 laccase mediator인 ABTS 및 환원제인 아스코르브산과 알파 토코페롤을 첨가하여 합성리그닌의 구조적 변화를 분석하였다. 그 결과, ABTS 및 환원제가 동시에 첨가될 때, nitrobenzene oxidation 산물의 양이 급격히 감소하였으며, 이에 따라 합성리그닌의 분자량 역시 감소하였다. 따라서 *A. biennis*의 리그닌 생분해 관련 효소를 구명하기 위해 전사체 분석을 실시한 결과, laccase와 관련된 많은 유전자들이 높게 발현되었으며, manganese peroxidase를 비롯하여 다양한 균체 외 효소들의 높은 발현이 확인되었다. 따라서 *A. biennis*에 의한 리그닌 생분해는 laccase mediator 시스템을 비롯하여 리그닌 결합 분해와 관련된 다양한 효소들에 의해 야기된 것으로 판단된다.

*P. chrysosporium*에 의한 합성리그닌의 생물학적 변환 산물을 분석한 결과, 앞선 결과와 마찬가지로 합성리그닌은 분해 및 중합 반응이 나타났다. 따라서 본 연구에서는 앞선 *A. biennis*의 실험과 동일하게 환원제의 첨가를 통해 배양기간 동안 합성리그닌을 분해시켰다. 환원제의 주기적 첨가와 함께 *P. chrysosporium*은 합성리그닌의 저분자화뿐 아니라, 분해 산물로 syringic acid, 2,6-dimethoxybenzodiol 및 숙신산을 생성하였다. 따라서 합성리그닌 유래 분해 산물 생성에 관여하는 *P. chrysosporium*의 효소 시스템을 파악하기 위해 전사체 분석을 실시하였다. 전사체 분석 결과, lignin peroxidase와 manganese peroxidase를 비롯하여 multicopper oxidase 및 copper radical oxidase와 같은 다양한

균체외 효소를 비롯하여 cytochrome P450 monooxygenase, glutathione S transferase 등의 균체 내 효소의 발현이 증가되었다. 또한 TCA cycle 및 short-cut TCA cycle에 관여하는 다양한 효소 발현 역시 증가하는 경향을 보였다. 이를 통해, *P. chrysosporium*은 리그닌 분해 효소를 통해 리그닌 결합을 분해하고, 균체 내 대사 기작을 통해 분해 산물로 생성된 방향족 화합물로부터 숙신산을 생성한 것이라 판단된다.

결론적으로, *A. biennis*는 laccase mediator 및 환원제의 첨가와 함께 합성리그닌의 급격한 저분자화를 유도하였으며, *P. chrysosporium*는 환원제의 첨가 조건 하에 리그닌 유래 숙신산을 생성하였다. 따라서 다른 효소 시스템을 가진 두 백색부후균은 다른 분해 산물을 생성함을 구명하였다. 전사체 분석 결과, *A. biennis*의 laccase mediator 시스템은 저분자화된 리그닌 생성에 영향을 미쳤으며, *P. chrysosporium*의 short-cut TCA cycle에 관여하는 다양한 효소들은 합성리그닌으로부터 숙신산 생성에 영향을 미쳤다. 이러한 결과로부터 백색부후균에 의한 리그닌의 생물학적 변환 가능성을 확인하였으며, 백색부후균의 효소시스템 및 배양조건의 변화를 통해 리그닌의 생분해뿐 아니라 유용 화합물 생산을 유도할 수 있었다.

키워드 : 리그닌 생분해, 저분자화, 숙신산, *Abortiporus biennis*, *Phanerochaete chrysosporium*, 전사체 분석

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