The promotion of laccase expression induced by interspecies interaction and its application to biomass pretreatment

（微生物間相互作用により誘導されるラッカーゼ発現促進とそのバイオマス前処理への応用）

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Abbreviations

Ds	*Dichomitus squalens*
Tr	*Trichoderma reesei*
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
MEPA	Malt Extract Peptone Agar
MEA	Malt Extract Agar
SC media	Soundar and Chandra media (1998)
H	*p*-hydroxyphenyl group
G	Guaiacyl group
S	Syringyl group
LiP	Lignin peroxidase
MnP	Manganese peroxidase
CMCase	Carboxymethyl cellulase
ABTS	2,2′-Azinobis(3-Ethylbenzthiazoline-6-Sulfonate)
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
LN	Low nitrogen
HN	High nitrogen
HPLC	High performance liquid chromatography
GC	Gas chromatography
LME	Lignin-modifying enzyme
PCR	Polymerase chain reaction
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
NDF	Neutral Detergent Fiber
Supernatant Tr	Cell-free supernatant from pure culture *T.reesei*'s 5-day cultivation in rice straw

1 Introduction

Now we can feel the climbing panic of increasing fuel prices, but these are just harbingers of the inevitable excess of growing demands over dwindling supplies of geological reserves that eventually deplete fossil energy leading to the point of complete exhaustion. Before we freeze in the dark, we must prepare to make the transition from nonrenewable carbon resources to renewable alternative energy resources. Solar energy based on photovoltaic nanometer materials, nuclear energy from light nucleus fusion reaction, and renewable bioenergy will be uplifted and can become the three kinds of main substitutes as a road map for such an endeavor. However, fossil oil provides not only energy supply but as well as industrial raw materials. Any recourse to solar energy and nuclear energy seems not sufficient enough to help solving the predicament scarce of fossil materials. Advances in genetics, biotechnology, process chemistry, and engineering are leading to a new manufacturing concept for converting renewable biomass to both valuable fuels and many industrial raw materials, which are generally referred to as the biorefinery (Ragauskas et al 2006).

Because biomass represents an abundant carbon-neutral renewable resource for the production of bioenergy and biomaterials, the integration of biorefinery manufacturing technologies and lignocellulosics from agricultural crops or other woody and herbaceous plants provide the potential for the development of sustainable biofuels and biomaterials. It will lead to a new manufacturing paradigm.

Among various biomaterials, lignin is the unutilizable polymer as yet, unlike cellulose and hemicelluloses, the two main components in the lignocellulosic matrix. Laccase can decompose lignin to a series of degradative products at different extent, which will enrich available industrial materials. Thus, lignin is expected to play an important role as major raw material for the world's biobased economy for the production of bioproducts and biofuels (Buranov, 2008). What is more important is that biodelignification can be also acted as pretreatment of saccharification in fuel fermentation activities.

This study aims to develop a simple process to increase laccase production and recovery using renewable materials.

1.1 Lignin and Rice straw: their nature and distribution

1.1.1 Lignin construction

Lignin is the third most abundant natural polymer present in nature after cellulose

and hemicelluloses. The estimated amount of lignin on earth is 300 billion metric tonnes with an annual biosynthetic production rate of 20 billion metric tons (Argyropoulos and Menachem, 1998).

There are 3 main groups of lignins:

(1) the lignins of softwoods (gymnosperms),

(2) the lignins of hardwoods (angiosperms) and

(3) the lignins of grasses (non-woody or herbaceous crops).

Lignin is polymerized from three hydroxycinnamyl alcohol subunits, p-coumaryl, coniferyl and sinapyl alcohol (Fig. 1-1), resulting in hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin, respectively (Sanna Koutaniemi et al., 2007). The apparent complexity of the biosynthetic pathway of monolignols above has been resolved during the last decade (Boerjan et al. 2003).

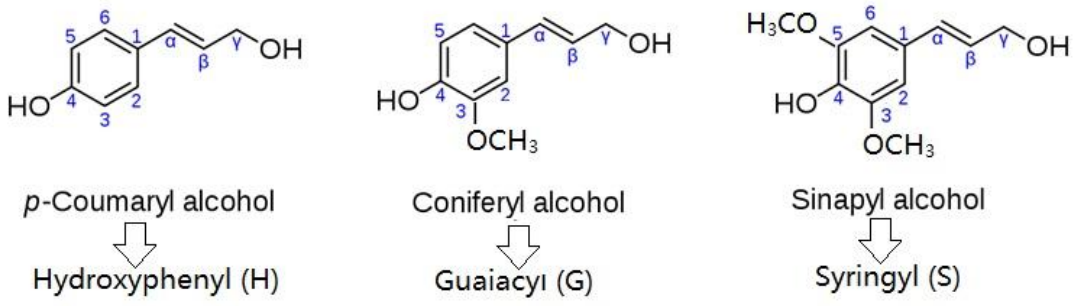


Fig.1-1. Biosynthetic monolignols and their corresponding generative lignin units

Softwood lignins are composed mostly of guaiacyl-type lignin, while hardwood lignins consist of syringyl and guaiacyl units, and herbaceous plants contain all three types of units. The distribution and relative ratio of each lignin components were summarized in Table 1-1.

Table 1-1. Lignin components of various plants

Plant group	Woody gymnosperms (softwoods)	Woody angiosperms (hardwoods)	Non-woody angiosperms (herbaceous plant)
Component of Lignin units	Mostly G units	G and S units	G , S and H units
H/G/S ratio(%)	2-18/82-98/trace	0/22-66/44-86	5-26/27-54/23-67

(Obst, 1982, 1986, Lapierre et al 1993, 1995 and Klinke et al. 2004)

1.1.2 Rice straw composition

The total yield of rice straw all over the world is 525 million tons per year. About 90% of rice straw is produced in Asia (Kim and Dale, 2004), among which, almost half of it is produced in China. Due to its higher silica content (~10%), rice straw is unique relative to other cereal straws and contains lower lignin but higher silica (ash) (Table 1-2).

Table1-2. Percent (%) chemical composition of rice straw (%) from different countries.

Country	lignin	cellulose	hemicellulose	ash	moisture	References
China	10.2 (klason)	33.9	25.6	11.8	6.9	Jin et al 2007
Europe	9.3 (klason)	48.9	30.4	11.2	0	Dohnani et al 2001
Thailand	18	44	26	12	0	Sangnark et al 2004
Japan	10 (klason)	30.3	18.5	14.5	0	This work

A recent comprehensive review on the lignin composition of rice straw was published by Buranov (2008), and a review on silica composition of rice straw was published by Van Soest (2006).

Rice straw lignin is also called *p*-hydroxyphenyl–guaiacyl–syringyl (H–G–S) lignin and contains all three monolignol units in significant amounts. The *p*-hydroxyphenyl (H unit) content in rice straw is significantly higher than that of corn and wheat straw (Lapierre 1993 and Lapierre et al. 1995). By alkaline nitrobenzene oxidation analysis, H units in soluble and extractable lignins were even much more than G units or S units in this work. Analysis of degradation products following cleavage of ether linkages by thioacidolysis indicates the respective proportions of H, G, and S units in lignins are 15, 45, and 40% for rice straw (Buranov 2008).

In rice straw, the lignins in the compound middle lamella and parenchema were abundant in G and S units, whereas those in the middle lamella of protoxylem vessels and outer layers of secondary walls of metaxylem were rich in G and H moieties (He and Terashima, 1989). Different woody lignin contents and S/G ratios among genetic variants within a single species can influence the hydrolyzability of the biomass, moreover, with the woods aging, the lignin contents varied from 22.7% to 25.8% and the S/G ratio from 1.8 to 2.3 (Davison 2005). During maturation of grasses, the syringyl content increases, too and this make the plant more resistant to fungal infection (Ride 1975).

Silica is a cell wall component in rice straw resistant to fungal diseases and from grazing by slugs. It occupies about 10% of straw mass weight absorbed in the form of orthosilicic acid ($Si(OH)_4$). The identification of organically bound silicon in biological tissues is an extremely difficult area of investigation because of its great geological abundance and low concentrations of metabolically active silicon. Active concentrations of 400–800 ppm as SiO_2 have been reported in xylem sap of rice (about 120–140 ppm as SiO_2 at 25^oC) (Jones and Handreck, 1967). Unlike lignin which protects cell wall carbohydrates through bonding and sets an ultimate limit to digestion, silica appears to operate by incrustation (Van Soest, 2006). The plant organisms that cumulate silica do so through an active transport and spend one ATP per silicon atom. The cost of synthesizing an equivalent amount of lignin is about 27 ATP (Raven, 1983).

The analysis of lignin-carbohydrate complexes (lignin and hemicellulose) from rice straw has indicated that it contains 63.9% carbohydrates (xylose, 80.1%; arabinose, 13%; glucose, 4.3%; galactose, 2.3; mannose, 0.4%), 2.8% of uronic acid, 27.7% Klason lignin, 5.6% acid-soluble lignin, 4.2% acetyl group, 4% *trans*-*p*-coumaric acid (4%), 0.8% *trans*-ferulic acid (Azuma and Koshimjima, 1988). Furfural that restrains cellulase activity was the most abundant product compound, followed by vanillin, 4- methylphenol and 4-vinylguaiacol (Garrote et al., 2007).

1.2 Current technologies for pretreatment of lignocellulosics

Different lignocellulosic biomass has different physico-chemical characteristics, so it is necessary to adopt appropriate pretreatments technologies based on the lignocellulosic biomass properties of each crude material. Universal pretreatment process is difficult to envision owing to the diverse nature of different biomass feedstocks and various applied aims. In sum, to lower cost and improve the pretreatment efficiency, the pretreatment method should be considered according to the following criteria:

a) Minimum heat and power requirements,
b) No significant sugars degradation and obtaining high sugar concentration,
c) Highly digestible pretreated solid,
d) Minimum amount of toxic compounds,
e) Operation in reasonable size and moderate cost reactors,
f) Non-production of solid-waste residues,
g) Effectiveness at low moisture content,
h) Biomass size reduction not required,
i) Fermentation compatibility,

j) Lignin recovery.

In addition, the feedstock handling and processing also effects the biochemical conversion to biofuel such as harvest time, harvested component, biomass storage and processing before pretreatment (Inman et al, 2010).

In the past decades, many pretreatment methods have been developed and suggested to be adopted. They can be classified into physical, physic-chemical, chemical, and biological pretreatments. Of course combination of these ways had been also suggested.

1.2.1 Physical pretreatments

Physical pretreatments have been carried out that include the following:

a) Mechanical comminution: a combination of chipping, grinding or milling depending on the final particle size of the material (10–30 mm after chipping and 0.2–2 mm after milling or grinding (Sun and Cheng, 2002).
b) Extrusion: an integrated method of heating, mixing and shearing, and last extrusion resulting in physical and chemical modifications during the passage through the extruder to disrupt the lignocellulose structure causing defibrillation (Karunanithy et al., 2008).
c) Ultrasound pretreatment: mechanical impacts, produced by the collapse of cavitation bubbles, provide an important benefit of opening up the surface of solid substrates to the action of enzymes at 50 ^{o}C (Yachmenev et al., 2009).
d) Liquid hot water: the liquid hot water (160~240^{o}C) solubilize mainly the hemicellulose, to make the cellulose more accessible and to avoid the formation of inhibitors, obtaining two fractions: one solid cellulose-enriched fraction and a liquid fraction rich in hemicellulose derived sugars (Mosier et al., 2005a).

1.2.2 Physic-chemical pretreatment

Physic-chemical pretreatment have been enriched in recent decades using the following:

a) Steam or SO_2-steam explosion: pressurised steam for a period of time ranging from seconds to several minutes, and then suddenly depressurised. And combination of SO_2-catalyzed steam will dramatically improve the efficiency of pretreating softwood materials.

b) Ammonia fiber explosion (AFEX) and ammonia recycle percolation (ARP):

AFEX is treatment that uses liquid anhydrous ammonia at temperatures between 60 and 100^{o}C and high pressure for a variable period of time (Kumar et al, 2009).

ARP is a method that involved the use of aqueous ammonia (5~15% wt) which passes through a reactor packed with biomass at 140~210^{o}C for 90 min and percolation rate is about 5 mL/min (Sun and Cheng 2002).

c) Microwave pretreatment: immersing the biomass in dilute chemical reagents and exposing the slurry to microwave radiation for residence times ranging from 5 to 20 min (Keshwani 2009).

d) Wet oxidation: an oxidative pretreatment method which employs oxygen or air as catalyst for 10~15 min at temperatures from 170 ~200 ^{o}C and at pressures from 10 to 12 bar O_2 (Olsson et al. 2005).

e) CO_2 explosion: based on the utilization of CO_2 in a gaseous form above its critical point to a liquid-like density by compression as a supercritical fluid. Addition of co-solvents such as ethanol can improve delignification. (Schacht et al. 2008).

1.2.3 Chemical pretreatments

Chemical pretreatments are divided into alkali, acidic and neutral treatment.

a) Alkali pretreatment:

Alkali pretreatments have a very long history for common use, and chemicals like sodium, potassium, calcium and ammonium hydroxides are suitable alkaline pretreatments for efficient application. Jeung-yil Park et al (2010) developed a novel lime pretreatment process (CaCCO) that did not require a solid-liquid separation step in which after pretreatment the residual lime was neutralized by carbonation.

b) Acidic pretreatment

Acidic pretreatment uses diluted acid pretreatments to solubilize the hemicellulosic fraction of the biomass 110~180^{o}C for 5~90 min and to make the cellulose more accessible to enzymes.

c) Neutral treatment

Ionic Liquids pretreatment: ionic liquids called “green solvents” are salts typical of large organic cations (often 1-butyl-3-methylimidazolium or 1-ethyl-3-methylimidazolium) and small inorganic anions with many interesting properties including chemical and thermal stability, non-flammability, low vapour pres-

sure requirements and a tendency to remain liquid in a wide range of temperatures (Hayes, 2009, Li et al, 2009).

Organosolv process: a combination with previous acid hydrolysis to separate hemicellulose and lignin in a two-stage fractionation. Numerous organic or aqueous solvent mixtures can be utilized, including methanol, ethanol, acetone, ethylene glycol and tetrahydrofurfuryl alcohol, in order to solubilize lignin, and relatively pure lignin can be recovered as a by-product (Zhao et al, 2009).

In summary, alkali pretreatment has mainly the action of delignification and partial hemicellullosic degradation while acid pretreatment process solves most hemicellulose and little lignin. Neutral solvent mainly depolymerize lignin, but hot liquid or steam treat works on degradation of hemicelluloses and a small quantity of lignin. The effects of different pretreatment techniques on lignocelluloses are summarized in Table1-3.

Table1-3. Effect of different pretreatment technologies on the structure of lignocellulose

Pretreatment methods *	Increases accessible surface area	Cellulose decrystallization	Hemicelluloses solubilization	Lignin removal	Lignin structure alteration	Generation of toxic compounds	Cost of energy • reagent• instrument
Milling	H	H	-	L	M-	-	H
Extrusion	H	H	M	L	M	L	H
Ultrasound	H	M	L	L	M	L	M
Liquid hot water	H	n.d.	H	L	M	L	H
Steam explosion	H	-	H	M	H	H	H
Microwave	H	H	M	M	M	M	M
AFEX	H	H	M	H	H	L	H
ARP	H	H	M	H	H	M	M
Wet oxidation	H	n.d.	-	M	H	L	H
CO_2 explosion	H	-	H	-	-	-	M
Alkali pretreatment	H	-	M	H	H	M	H
CaCCO	H	n.d.	M	H	H	L	M
Acid pretreatment	H	-	H	M	H	H	H
Ionic Liquids	H	H	H	H	H	-	M (if recycling)
Organosolv	H	H	H	H	H	M	H

Note: H: high effect; M: moderate effect; L: low effect; n.d.: not determined

* Referring to references all above in this section, and Alvira et al 2010, Mosier et al 2005.

1.2.4 Biological pretreatment

Biological pretreatment is suitable for herbaceous plant and agricultural biomass because (1) Herbaceous plants and agricultural biomass are physically smaller and structurally weaker and lighter than woody biomass. Chemically, lignin contents of herbs are lower than woody biomass (about 10% and 30%, respectively, Zhu et al, 2010). As a result, herbaceous plants are more liable to enzymatic actions than woody biomass.

(2) Moreover, hemicelluloses content (mainly pentose polymer) of herbaceous plant (about 20~30%) exhibit higher than woody biomass (hardwood about 20%, softwood about 10%), and pentose recovery yield is often low due to its decomposition to furfurals in thermal-chemical pretreatments, in which the produced furfurals represent fermentation inhibitors.

Favorably, biological pretreatments have no need for thermal energy, high pressure and chemical addition; besides, it has little toxic products or fermentation inhibitors produced.

However, current biological pretreatment works reported are very few, and most of them pretreated biomass with fungi, but not with delignifying enzymes, besides, it took so much long time as 30d to 60d to treat the biomass, which made it applications impossible that suitable for *in situ* industrial pretreatment for biomass fermentation. The degradation of cellulose and hemicelluloses is an important drawback when pretreated with fungi. As to lignolytic enzymes (mainly laccase, lignin peroxidase, and manganese peroxidase), their high production cost limits their applications for biomass pretreatment.

Thus, this study aims to upgrade laccase production and develop a biological pretreatment means by laccase, the main lignin-degrading enzyme.

The following section reviews of current biological pretreatments:

Fungal pretreatment has been previously developed to upgrade lignocellulosic materials for feed applications, decolorization and biopulping. Recently, this environmentally friendly approach has been focused as a pretreatment method for enhancing enzymatic saccharification in biorefinery.

White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocelluloses such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass showing high delignification efficiency on the basis of quantitative and struc-

tural changes (Kumar et al, 2009b, Taniguchi et al, 2005, and Shi et al., 2008). When rice straw was pretreated with *P. ostreatus* for 60 d, the total weight loss and the degree of Klason lignin degraded were 25% and 41%, respectively (Taniguchi et al, 2005). Fred A. Keller et al (2003) showed a three- to five-fold improvement in enzymatic cellulose digestibility of corn stover after pretreatment with *Cyathus stercoreus*; and a 10- to 100-fold reduction after pretreatment with *Phanerochaete chrysosporium*.

Biological pretreatment by white-rot fungi (fungal isolate RCK-1) has been combined with organosolv pretreatment in an ethanol production process by simultaneous saccharification and fermentation (SSF) from beech wood chips (Itoh et al., 2003).

In general, low energy requirement, no chemical requirement, and mild environmental conditions are the main advantages of biological pretreatment (Taherzadeh, 2008). However, the main drawback to develop biological methods is the low degrading rate and slow lignolytic speed obtained in most biological materials compared to other technologies based on heat and chemicals (Sun and Cheng, 2002).

Table 1-4 shows a comparative analysis on the advantages and disadvantages among different pretreatment.

Table 1-4. Summary of the advantages and disadvantages of different methods for pretreating lignocellulosic biomass

Pretreatment methods	Advantages	Disadvantages
Biological pretreatment	-Degrades lignin and little hemicelluloses -Low energy consumption -Low toxic products and fermentation inhibitors -No recalcitrant Black Wastewater to be treated	-Low rate of degrading lignin
Milling	-Reduces cellulose crystallinity	-High power and energy consumption
Steam explosion	-Causes lignin transformation and solubilize hemicellulose -cost-effective -Higher yield of glucose and hemicellulose in the two-step method	-Generation of toxic compounds -partial hemicelluloses degradation
AFEX	-Increases accessible surface area -Low formation of inhibitors	-Not efficient for raw materials with high lignin content -High cost of large amount of ammonia
CO_2 explosion	-Increases accessible surface area -Cost-effective -Do not imply generation of toxic compounds	-Does not affect lignin and hemicelluloses -Very high pressure requirements
Wet oxidation	- Efficient removal of lignin - Low formation of inhibitors -Minimizes the energy demand (exothermic)	-High cost of oxygen and alkaline catalyst
Orangosolv	-Causes lignin and hemicellulose hydrolysis	-High cost -Solvents need to be drained and recycled
Diluted acid	-Less corrosion problems than concentrated acid -Less formation of inhibitors	-Formation of inhibitors -Generation of degraded products from hemicellulose -Low sugar concentration in exit stream

(Based on the review by Alvira et al, 2010)

1.3 *Dichomitus squalens*

Given the condition in Table 1-4, it is comparative to push forward a cost-competitive and efficient biological pretreatment of lignocelluloses. Therefore, there is a need of continued studies and testings of more white-rot fungi for their competence to delignify lignocellulosics rapidly and efficiently.

And then, this study focuses on other efficient basidiomycetes like *Dichomitus squalens.*

1.3.1 Physiologic and ecological habits

Dichomitus squalens (synonym *Polyporus anceps*) is among effective lignin-degrading white-rot basidiomycetes, usually found on old coniferous trunks fallen by storm and on charred tree after forest fire with its basidiocarps form (Makela, 2009). It is a decayer of slash (a saprophyte) as well as a heart rot in live trees (Sinclair et al. 1987).

It produces a flat fruiting body on the underside of dead branches or stems with intact bark. The pore surface is white when fresh and ages to yellow. The fungus causes a white pocket rot (Fairweather et al., 2006). The spores are dispersed by wind, land in cracked bark crevices of dead branches, then germinate to colonize the area between the bark and the wood and eventually the dead wood, provided it has intact bark (Sinclair, 1987).

Fig. 1-2. Fruiting body of *Dichomitus squalens* (Photo by D. Reid, 1965)

Nomenclature and taxonomy

Dichomitus squalens's taxonomic rank is described in the system of nomenclature and taxonomy as follows: (Referred to http://zipcodezoo.com/Fungi/D/Dichomitus_squalens/)

Domain: *Eukaryota*
Kingdom: *Fungi*
Subkingdom: *Dikarya*
Phylum: *Basidiomycota*
Subphylum: *Agaricomycotina*
Class: *Basidiomycetes*
Subclass: *Agaricomycetidae*
Order: *Polyporales*
Family: *Polyporaceae*
Genus: *Dichomitus*
Specific descriptor: *squalens*

The scientific name of *Dichomitus squalens* was given by (P. Karst.) D.A. Reid in 1965.

1.3.2 Enzymatic system involving degradation of lignocellulosics

D. squalens has a complete extracellular enzymatic system degrading lignocellulosics. It not only expresses a set of laccases and manganese peroxidases (MnP) as their Lignin-Modifying Enzymes (LMEs), but also secrets a group of cellulase and hemicellulase.

In previous studies, laccases, MnPs, had been purified and characterized (Elke et al, 1998, Petroski et al, 1980, Li et al 1999, Perie et al, 1998 and 1996), but no lignin peroxidase (LiP) activity is detectable in *D. squalens* cultures grown under a variety of conditions, including the presence of various concentrations of carbon, nitrogen, and Mn in both agitated and stationary cultures (Perie, 1991).

Many results (Galliano, 1988, Muheim, 1990) suggest that lignin peroxidase may not be an essential component of the lignin degradation system of every white rot fungus and that alternative oxidative enzymes, such as MnP, are capable of degrading lignin.

*D. squalen*s also represents efficient cellulolytic activities. Three endo-glucanase (EnI, En Ⅱ and EnⅢ) have been purified and characterized (Rouau, 1985a, and Rouau et al, 1985b), β -D-glucosidase, exo-cellobiohydrolase, D-xylanase and exopoly-D-galacturonase has been found (Rouau et al, 1985b), thereinto, cellobiohydrolase and xylanase have been purified and characterized (Rouau et al, 1985c).

Table 1-5 shows a summarized reported enzymatic system degrading lignocellulosics.

Table1-5. Reported enzymatic system degrading lignocellulosics from *D. squalens*

Enzymes	source	Molecular Mass (kD)	pI	Gene sequence	N-terminal amino acid sequence	Reference
laccase						
Lac 1&2	protein	66	3.5 3.6	nd	G**IGPVTDL**TITT**N**AD**I**A**PD**aF	Perie et al. 1998
Lac 3	gDNA	nd	nd	*Ds-lac 3* ns	A**IGPVTDL**TVA**N**AN**IS****PD**GY	Makela 2009
Lac 4	gDNA	nd	nd	*Ds-lac 4* ns	S**IGPVTDL**IIA**N**KD**IS****PD**GS	Makela 2009
Lac I	protein	60	nd	*Ds-lac I* ns	G**IGPVTDL**TITT**N**AD**I**A**PD**DF	This work
Lac Ⅱ	protein	60	3.8	*Ds-lac II* ns	G**IGPVTDL**TITT**N**AD**I**A**PD**DF	This work
Lc 1	protein	68	3.2	nd	nd	Susla et al. 2007
Lc 2	protein	68	3.1	nd	nd	Susla et al. 2007
MnP						
MnP1	gDNA protein	48	4.1	*mnp1* (3107 bp)	369 amino acids predicted from DNA sequence (7 introns).	Perie et al. 1996 Li et al. 1999
MnP2	gDNA protein	48.9	3.8-3.9	*mnp2* (2941 bp)	365 amino acids predicted from DNA sequence (7 introns).	Perie et al. 1996 Li et al. 1999
MnP3-5	protein	50	3.5	nd	nd	Susla et al. 2008
Cellulase						
Endo-glucanase 1	protein	42	4.8	nd	nd	Rouau et al. 1985a,b
Endo-glucanase2	protein	56	4.3	nd	nd	Rouau et al. 1985a,b
Endo-glucanase3	protein	47	4.1	nd	nd	Rouau et al. 1985a,b
Exo-glucanase1	protein	39	4.6	nd	nd	Rouau et al. 1985b,c
Exo-glucanase2	protein	36	4.5	nd	nd	Rouau et al. 1985b,c
β-D-glucosidase	Enzyme activity	nd	nd	nd	nd	Rouau et al. 1985b
Xylanase						
D-xylanase	Enzyme activity	nd	nd	nd	nd	Rouau et al. 1985b,c
Exopoly-D-galacturonase	Enzyme activity	nd	nd	nd	nd	Rouau et al. 1985b

Note: nd: not determined; ns: gene has been cloned but sequence was not determined

1.4 Laccase inducers

Fungal laccases synthesis is associated with the onset of secondary growth. They are secreted in the basidiomycete life cycle stages of vegetative mycelium, fruit body and sporophore development. It is produced by fungal mycelium, and the youngest hyphae at the margins of the colony represents usually the maximum producers, whereas older hyphae produce less or none at all (Schanel et al. 1966), and most of basidiomycetes produces laccase increasingly in the latter phase of substrate colonization and raise sharply at the onset of fruit body initiation, reaching maximum levels until the fruit body morphogenesis, and then suffer a decline throughout the subsequent stages of sporophore development (Chen et al. 2004, Chen et al. 2003, Chakarabory et al. 2000, Ohga 1999, and Largeteau et al. 2010).

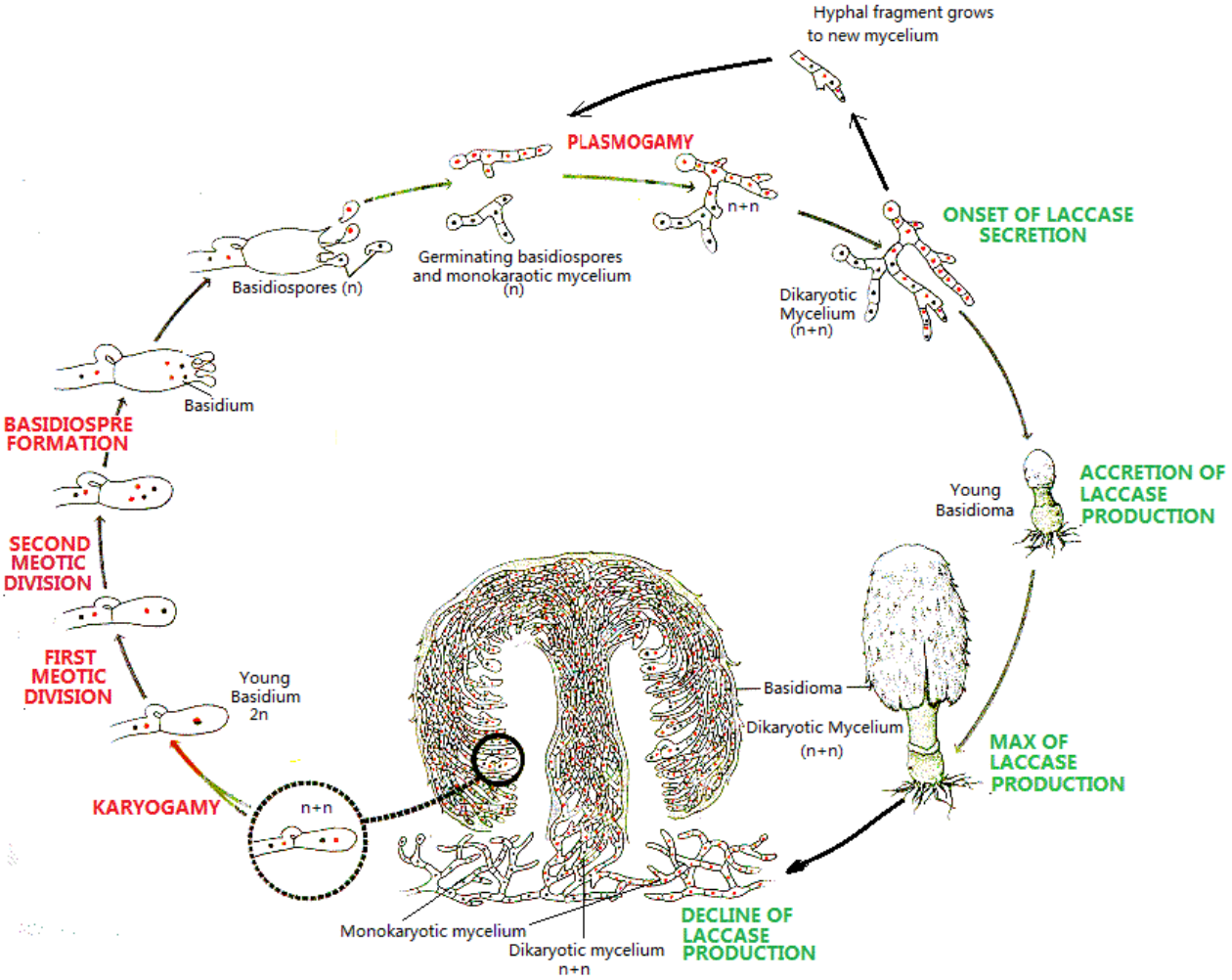


Fig1-3. Life cycle of basidiomycetes growth and laccase secretion

In the laccase expression, it can be positively regulated by various compounds and biological metabolites or affected by other microorganisms.

In earlier reports, many attempts were made to accelerate the production of laccase by various approaches. The inducers of laccase production can be divided by seven following groups:

a) Metal salts: Ca^{2+}, Li^{+}, Cu^{2+}, Mn^{2+}.

Calcium is found as a ubiquitous secondary messenger in fungi (Gadd 1994) and has been directly linked to laccase induction in the fungus. Li^{+} induced laccase by effecting Ca^{2+} metabolisms (Crowe et al. 2001). Cu^{2+} or Mn^{2+} can cause oxidative stress via well-established mechanisms of free radical formation (Halliwell et al. 1989, Ingram et al. 1984, Chen et al. 2003). Laccase yield can be increased by salinization with seawater (Niladevi 2008).

b) Organic alcohol: ethanol, isopropanol, etc.

Ethanol and isopropanol can strongly induce laccase production (Valeriano et al. 2009, Crowe et al. 2001) and the induction was rapid (< 24h) compared to other ways. Alcohols destabilize membrane and protein structures (Ingram et al. 1984), and the cellular response to this is closely correlated with that of heat shock (Piper 1995), which regulates laccase and peroxidase genes in some fungi (Li et al. 1995, Saloheimo et al. 1991).

c) Yeast extract and N nutrient at low nitrogen concentration (LN) and high nitrogen concentration (HN).

Nutrients can increase laccase yield in some specific fungi. Manjunathan (2010) have selected peptone and yeast extract to induce laccase production *by Lenitinus tuberregium*, and Niladevi (2008) found that yeast extract is a very efficient laccase inducer for *Streptomyces psammoticus.*

d) Aromatic compounds.

Aromatic compounds, especially aromatic amines, induce laccase efficiently. 2,5-Xylidine (2,5-dimethylphenylamine) is the most powerful inducers of laccase to most fungi, such as *Trametes versicolor* (Xavier 2007 and Minussi 2007), *Rhizoctonia solani* (Crowe et al. 2001), *Volvariella vovacea* (Chen et al. 2003) and so on. *p*-Anisidine (4-Methoxyaniline) (Niladevi 2008), and pyrogallol (1,2,3 -Trihydroxybenzene) (Niladevi 2008) exhibited fairly active ability of inducing laccase. It is considered that such specific induction may operate via receptor-mediated transcriptional activation (Fernandez-Larrea et al. 1996) and this induction was not linked to any stress effects at the concentration used.

e) Aromatic heterocyclic organic compound containing nitrogen.

Aromatic heterocyclic compounds type of pyridinium and azole, such as paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), caffein (1,3,7- trimethyl-xanthine), 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (NHPI), 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO), etc (Niladevi et al. 2008, Galli and Gentili, 2004, D' Acunzo et al. 2003) by effecting cAMP signaling. cAMP is another important secondary messenger known to drive many processes including sclerotial formation in *R. solani* and other fungi (Gadd et al. 1994 and Crowe et al. 2001). Crowe (2001) found that *R. solani* laccase activity and sclerotization were strongly induced by both cAMP itself and caffeine, an antagonist of cAMP phosphodiesterase. These results point to the regulation of laccase by cAMP and the involvement of both in sclerotial morphogenesis (Thurson 1994).

f) Lignin and its derivatives.

Solid lignin, veratryl alcohol (3,4-dimethoxyphenyl alcohol), three hydroxycinnamyl alcohol subunits, p-coumaryl, coniferyl and sinapyl alcohol and their corresponding aldehyde and acid forms have been verified that they can induce laccase to certain extent for different fungi (Xavier et al. 2007, Chen et al. 2003 and Gnanamani et al. 2006). Xavier (2007) had also reported laccase induction with by-products of pulp and paper industry. And author has found that the partially degraded lignin residual can induce laccase more powerful than the compounds from lignin component in this study.

g) Laccase induction by interspecific interactions.

Several reports have shown increased laccase production by lignolytic fungi during interspecific interactions, especially with *Trichoderma spp*. Laccase from *Pleurotus spp., Lentinula edodes, Trametes spp.* were increased by co-culture with different *Trichoderma* strains (Flores et al. 2009, Mata et al. 2005, Hatvani et al. 2002, Savoie et al. 1999, Zhang et al. 2006 and Baldrian 2004), and laccase activity in *Rhizoctonia solani* can be induced by *Pseudomonas fluorescens* strains (Crowe et al. 2001).

But the inductive reasons are still unclear. Some reports above proposed that antagonistic action resulted in laccase induction by a metabolites such as viscosinamide, an antifungal depsipeptide metabolite (Crowe et al 2001), but the purified material can only increase induced laccase 1.1 fold. Some suggested that some lytic enzyme, (chitinases, proteases, glucanases, N-acetyl- β -glucosaminidase and laminarinase) may be responsible for the degradation of fungal cell walls (Hatvani et al 2002 and Howell 2003), however, the inducers

proved to be heat stable.

The study has found that a group of lignolytic fungi can promote laccase production, and take *Dichomitus squalens* as an example to clarify the inductive reasons.

1.5 Laccase in biotechnological applications

In recent years, laccases have attracted much attention from researchers due to their ability to oxidize both phenolic and nonphenolic compounds even highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases have been reviewed several times up to date (Morozava et al. 2007 and Petr Baldrian 2006), and its applications was reviewed by Couto (2006).

The application of fungal laccase is mainly useful in biopulping on paper industry, decolorization on textile, food and cosmetic, bioremediation, new materials.

The direct use of white-rot fungal lignin-modifying enzymes (LMEs) in pulp and paper industry, and bleaching textile dye, could represent easier optimization and applicability as yet. In fact, commercial laccase and laccase-mediator applications, e.g. for pulp and paper, process water treatment and textile industry are already available (Morozova et al. 2007b, Widsten and Kandelbauer 2008).

Furthermore, laccases have great potentials in food industry to improve dough properties, and in cosmetic industry laccases may be added in products intended for skin lightening and hair dyeing. In biotechnological applications, laccases have also potentials as biosensors and as enzyme-electrodes in biofuel cells (Couto and Toca Herrera 2007). Attention is also paid to the development of laccases in synthesis of new biomaterials and polymers (Mikolasch and Schauer 2009).

Laccase can be also applied to pretreatment of lignocellulosic biomass recently. The global demand for the use of renewable materials for production of energy and consumables has been expanded. The “biorefinery” concept aims at the co-production of various value-added end products like biofuels and chemicals in advanced biotechnological processes from renewable biomass. Woods, grasses and agricultural crops lignocelluloses form a massive source of renewable biomass that can be used as a feedstock for biorefining.

And because the pretreatment methods of lignocellulosics based on thermo-chemical reactions produce toxic chemical fermentation inhibitors and recalcitrant "black" wastewaters, and cost much energy have limits to their application, the biological pretreatment is of prospect, this study will have a trial to pretreat lignocelluloses with induced laccase.

1.6 The significance of the study

The main focus of this work is to evaluate 10 kinds of ecologically and biotechnologically interesting white-rot fungi to increase lignin-modifying enzymes, laccase. It was postulated that the synergies between efficient lignolytic fungi and active cellulolytic fungi would promote the production of enzymes and lignocellulose breakDown. While the simultaneously inoculated co-culture did not exhibit any symbiotic or mutual relations, but the co-culture with *D. squalens* inoculated before *T. reesei* can strongly induced laccases. With the selectively lignin-degrading species *Dichomitus squalens* and *Trichoderma reesei*, two distinct strains (DSMZ 9615 and NBRC31326) were investigated as an example of interspecies interactions.

Naturally, there was a special necessity and interests to deepen the understanding of the inductive expressions of laccases during the co-culture in succession on inductive materials and induced laccase characteristics.

Compared to mostly laccase inducers mentioned above such as heavy metal ions, aromatic compounds and so forth, with toxic characters and environmental impacts, it is easy to manipulate and control that laccases are induced by interspecies interactions among microorganisms. Moreover, it proved to be more efficient in this following work.

As the significance of biodelignification of lignocellulose more and more noticed, as yet, the practices on biodelignification in some published reports were carried out using fungi, which will degrade cellulose and consume the target oligosaccharides, and what is more unrealistic for the practice of industrial biorefinery is that, it last a long pretreatment span of 30d to 60d. However, to date there is no successful trial to delignify biomass with lignin-modifying enzymes.

This study has made a practice on pretreatment of biomass with crude induced laccase and assessed the pretreated effects by constituent changes, exposed cellulose surface, extractable solid lignin, soluble lignin concentration and saccharified cellulose.

1.7 Objectives of this research

The specific aims of this study were:

a) To screen active lignolytic fungi and efficient cellulolytic fungi from selected famous white-rot fungi.

b) To determine synergic relationship between the active lignolytic fungi and the efficient cellulolytic fungi.

c) To optimize laccase expression by co-culture and other inducers.

d) To evaluate the inductive materials produced during co-culture between *D. squalens* and *T. reesei*.

e) To purify and characterize the laccase from pure culture and induced by co-culture.

f) To develop a novel biological pretreatment of biomass and comprehensive pretreated performance evaluation by many-sided different angles of practical applications.

2 Materials and Methods

The fungal strains used in this study were obtained from the culture collection situated at NBRC (NITE Biological Resource Center, Japan), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH-German Collection of Microorganisms and Cell Cultures) and ATCC (The Global Bioresource Center, US) and they are listed in Table 2-1. All these fungi have powerful competence of laccase production (Baldrian, 2006 and Morozava et al. 2007).

My study had screened out some highly efficient cellulolytic fungi and highly ligninolytic fungi from these10 species shown below:

Table 2-1. White-rot fungus strains with highly ligninolytic activities that were used in this study.

No.	Fungus Code	Species of fungus	No.	Fungus Code	Species of fungus
1	NBRC 30776	*Pleurotus ostreatus*	6	NBRC 4340	*Aspergillus nidulans*
2	NBRC 30388	*Trametes versicolor*	7	DSMZ 1016	*Panus tigrinus*
3	NBRC 9076	*Cyathus stercoreus*	8	DSMZ 9615	*Dichomitus squalens*
4	ATCC 90467	*Ceriporiopsis subermispora*	9	DSMZ 6909	*Phanerochaete chrysoporium*
5	NBRC 31326	*Trichoderma reesei*	10	NBRC 7037	*Armillaria mellea*

2.1 Laccase induction and inductive materials

Media, Cultivation and Sampling

(a) Screening of highly efficient cellulolytic fungi

The fungus was grown on a modified medium based Soundar and Chandra (1998) (SC medium), containing 1% avicel (or 1% xylan, or 0.5% avicel and 0.5% xylan), 0.2% peptone, 0.2% urea, 15.3 g/L $Ca(NO_3)_2$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2% Tween 80 and 1 ml of trace metal solution consisting of 9.9 ml distilled water, 0.1 ml conc. HCl, 0.05 g $FeSO_4$, 0.0196 g $MnSO_4 \cdot H_2O$, 0.0166 g $ZnCl_2$, 0.02 g $CoCl_2 \cdot 6H_2O$.

After sterilization at 121 ℃ for 15 min, the culture in 100-ml Erlenmeyer conical flask was inoculated with fungal inoculum with rotary shaking at 150 rpm in 26.5 ℃ for 16d.

Samples were obtained four times in the 4d, 8d, 12d and 16d during the course of cultivation. Each fungus had been incubated with shaking. This was followed by taking

avicel as goal substrates to assess carboxymethyl cellulase (CMCase) activity, or taking xylan to obtain xylanase activity, and taking mixture of CMC and xylan to get both CMCase and xylanase activities.

The liquid sample was centrifuged at 15000 rpm for 1 min to remove mycelia and spore contaminants and the supernatant was obtained for enzyme activities.

(b) Screening of highly efficient lignolytic fungi

The experiment of screening white-rot fungi for its lignolytic characters was conducted by cultivation under a submerged rice straw (10% w/v). After being chopped into small pieces of about 1 cm long, 5 g air-dried rice straw with 50 ml distilled water was sterilized at 121 ℃ for 15 min. The cultivation in 200-ml Erlenmeyer conical flask was started by adding fungal inoculum, with rotary shaking at 150 rpm in 26.5 ℃ for 48d (De Pereira et al. 2003, Soundar and Chandra 1988).

The study had sampled three times in the 12d, 24d and 36d during the course of cultivation. Each fungus had been incubated with shaking, then followed by taking rice straw as goal substrates, and was measured for enzymatic activities of laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), carboxymethyl cellulase (CMCase) and xylanase.

The liquid samples were centrifuged at 15000 rpm for 1 min to remove mycelia and spore contaminants and the supernatants were obtained for enzyme activities.

(c) Co-culture and laccase induction

The experiment of Co-culture and laccase induction was performed by cultivation in submerged rice straw (10% w/v). Substrate preparations were the same as in (b), but sampling was performed at 10d, 20d, 30d and 40d during the course of cultivation. Compared to *D. squalens* pure culture, simultaneously inoculated co-culture of *D. squalens* and *T. reesei* from start and the successively inoculated co-culture with *T. reesei* after *D. squalens* was incubation for 20d were measured for various enzyme activities. All the supernatant preparation for enzyme assay also followed the steps in (b).

Enzymatic activity assay

a) Laccase, MnP, LiP

The laccase, LiP and MnP assay were measured by continuous spectrophotometric determination of the increase in absorbance at certain wavelength resulting from the formation of chromogenic matters. All assays and absorbance peak determinations were done at 25 ℃ by using a Perkin-Elmer X3 UV/vis scanning spectrophotometer.

Laccase activity was assayed at the wavelength of 436 nm, with 0.2 mM 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate(ABTS) (final concentration).

The activity assay of MnP and LiP from submerged rice straw culture was moni-

tored at 310 nm and 270 nm, a wavelength at which phenolics and other aromatics typical of fungi and lignin-containing materials absorbed very strongly making assays in complex systems difficult.

So the assays of LiP and MnP from rice straw culture were taken by the methods (LiP2 and MnP2) at visible wavelength.

The parameters were shown in Table 2-2 below.

Table 2-2. The parameters of laccase , LiP and MnP assay

Enzyme Parameters	laccase	LiP1	LiP2	MnP1	MnP2
Wavelength (nm)	436nm	310	651	270	431
Absorbance coeffient $\varepsilon(M^{-1}cm^{-1})$	29,300	9,300	48,800	11,590	27,250
Reaction reagents	ABTS	Veratryl alcohol, H_2O_2	H_20_2 Azure B	$MnCl_2$, H_2O_2	$MnSO_4$, H_2O_2 phenol red
Buffer	Sodium acetate	Sodium tartrate	Sodium tartrate	Sodium malonate	Na malonate Na lactate Na succinate
pH	5.0	3.0	4.5	4.5	4.5

Note: laccase assay referred to Johannes et al. 2000b, Bourbonnais et al. 2000

MnP assay 1 referred to Hofrichter et al. (1998). Wariishi et al. (1992).

MnP assay 2 referred to Roy and Archibald (1993) and Frederick (1992)

LiP assay 1 referred to Schoemaker et al. (1994). Tien and Kirk (1984).

LiP assay 2 referred to Roy and Archibald (1993)

Test samples were pipetted into a suitable quartz cuvette and the enzyme activities was calculated using the equation below:

Volume activity =Δ A/min * reaction mixture volume/ (ε* enzyme solution volume)

Δ A/min represent the change of optical density per minute, and ε is the molar absorbance coefficient. One unit of lignolytic activity was defined as the amount of enzyme that oxidizes 1.0 μ mol of substrate reagent per minute at 25 ℃.

b) CMCase, avicelase and xylanase

Firstly, a 100 μl 1.0% CMC solution, avicel or xylan suspension (in 160 mM sodium acetate pH 5.0) was added to 100 μl enzyme solution in 1.5 mL tube, respectively and was allowed to react in 50 ℃ for 0~120 min (60 min).

This was followed by the addition of 600μ l DNS reagent and heating for 15min in 100 ℃ boiler and then rapidly cool down (if necessary, 15000 rpm×1 min centrifuge). Lastly, its absorbance at 600 nm (T=0, check to sugar concentration) was determined. One unit of cellulase activity was defined as 1 μmol of reducing sugar (glucose) released by enzyme per min at 50 ℃. A standard curve was prepared with 0~200μ l of 5 mM glucose solution (Ghose 1987, Adney and Baker 1996 and Thomas and Zeikus1988).

c) β -D-glucosidase, β -D-xylosidase, α -L-arabinose

The activity assay of the enzymes above was carried out by determining *p*-nitrophenol produced in the following assay reactions.

50 μ l of 50 mM KPi buffer (pH 6.5) that contained

20 mM p-nitrophenyl-β -D-glucopyranoside (Barry and Harrington 1988)

20 mM p-nitrophenyl-β -D-xylopyranoside (Michael, J. and Schmidt, J., 1988) or

15 mM p-introphenyl-α -L-arabinofuranoside, respectively, was used for measurement ofβ -D-glucosidase, β -D-xylosidase or α -L-arabinose.

The mixture was allowed to react with 150μ l of enzyme solutions after vortex for 10~1400 min (60 min) in 50 ℃ water bath, followed by addition of 1 ml 2 M Na_2CO_3 to end the reaction. Lastly, optical density at 410 nm wavelength compared to the results with no incubation was measured. The standard curves were prepared by a series of 0~60 nmol glucose solution in 200μ l buffer. One unit of the enzymatic activity was defined as 1 nmol *p*-nitrophenol produced per minute.

Determination of Fungal quantity, Protein and D-glucose

a) Fungal quantity determination

Cell wall chitin which is remarkably constant was determined in the dry mass mycelia of white-rot fungi, so the universal glucosamine in chitin can act as a reliable indicator of fungal quantity.

(1) Desiccation and Grinding:

The mixture specimen of rice straw and fungal bodies were dried to constant weight at 60 ℃ for about 3 days for a freezing-milling-drying process with liquid nitrogen, thoroughly grinded to get full homogeneity using mortar and pestle.

(2) Hydrolysis:

At least 4 mg of dry cell wall material (100 mg rice straw) from four replicate cultures of each fungal species was refluxed at 100℃ for 4 h with 6 N hydrochloric acid. The ratio of cell wall to acid was 2 mg of wall to 1 ml of acid. After cooling to room temperature, the hydrolysates were centrifuged (13000 rpm×5 min) and two 0.2- to 1-ml samples of supernatant were withdrawn and evaporated to dryness at 45 to 50℃ under reduced pressure.

The dry hydrolysates were then redissolved in distilled water to make a solution containing 5 to 15 μg of glucosamine hydrochloride per ml of solution.

(3) Color development

The dilute hydrolysate solution (1 ml) was added to 0.25 ml of 4% acetylacetone (in 1.25 N sodium carbonate [vol/vol]) and heated at 90℃ for 1 h in a test tube covered with a Teflon-lined screwcap.

After cooling, 2 ml of ethanol was added, with shaking to dissolve precipitates. This was followed by addition of 0.25 ml of Ehrlich reagent (1.6 g of N-N-dimethyl-p-aminobenzaldehyde in a 30:30 ml mixture of ethanol and concentrated HCl).

(4) Determination

The color formed in the solution was measured using a 1 cm cuvette at 530 nm with a spectrophotometer. Duplicate determinations were performed for each hydrolysate. The glucosamine hydrochloride content of cell wall hydrolysates was measured by comparing the absorbance of glucosamine hydrochloride with that of a standard curve of D-(+)-glucosamine hydrochloride of the following concentrations (5, 10, 15, 20 and 30 μg/ml).

The chitin content of the cell walls was then calculated as the 1,4-anhydro-N-acetyl-2-deoxy-D-glucopyranose equivalent with an adjustment of 7% for loss due to hydrolysis (Chen and Johnson 1983, Nilsson and Bjurman, 1998).

b) Protein assay

Protein concentrations were measured using a BCA protein assay kit (Pierce). A calibration curve was plotted using bovine serum albumin as a standard protein.

c) D-glucose determination

The D-glucose concentration in the cultivated liquids was tested by D-Glucose F-kit (Roche) and the absorbance was checked at 340 nm.

Determination of Lignin constructional Unit-H, G, S

Alkaline nitrobenzene oxidation was applied to the lignin constructional unit. Samples (11~15 mg of the original specimen) were added to 8 ml of 2 M NaOH and 0.5 ml

nitrobenzene and the mixture was kept at 170 ℃ for 2 h. As the internal standard solution (EV), a 0.1 M NaOH solution containing 3-ethoxy-4-hydroxybenzaldehyde (about 30 mg/100 ml) was added. The reaction mixture was extracted three times with 15 ml dicholoromethane. The aqueous phase was acidified with 4 M HCl to pH~1 and extracted twice with 20 ml dichoromethane and once with 15 ml ethylether. The combined organic solvent phase was washed once with 20 ml water and then dried by adding anhydrous Na_2SO_4. After the removal of the insoluble inorganic materials by filtration, the solution was evaporated to dryness and silylated with N,O-bis (trimethylsilyl) acetamide at 100 ℃ for 10 min. The silylated compounds were analyzed by gas chromatography (GC-FID: Shimadzu 17A) equipped with NB-1(GL science) fused-silica capillary column (length 30 m, 0.25 mm i.d.).

The GC condition is shown as follows;

Injection volume: 1μ l

Column temperature: 150 ℃ for 15 min, and elevated to 180 ℃ at rate of 3 ℃/min, and elevated to 280 ℃ at rate of 10 ℃/min.

Injection temperature: 250 ℃

Detector temperature: 280 ℃

Column flow rate of He gas: 1.9 mL/min

Splitting ratio: 60:1.

2.2 Purification and characterization of laccase

Laccase purification

About 500 mL liquid cultures were separated from mycelia (15-day-old *D. squalens* pure culture or co-culture with *T. reesei*) in rice straw media with centrifugation at 10000×g (KUBOTA 6900 with RA-1500 rotor) for 10 min at 4 ℃ to remove mycelia, spores and impurities.

Crystalline ammonium sulfate was added to the suspension to 40% (or 35%) saturation and the mixture was incubated for 1 h with gentle mixing followed by a 10000 g × 10 min centrifugation at 4 ℃, then the supernatant was added with ammonium sulfate to 80% (or 85%) saturation and centrifuged at 10000×g for 10 min. The precipitate fractions were resuspended and were transferred to dialysis for overnight at 4 ℃. All dialysises in purification procedures were carried out using dialysis membrane (MWCO14K, Viskase Companies, Inc.).

The enzyme liquid was applied to successive open columns of DE52 (by 15 mm×20 cm; whatman), Q-sepharose (by 10 mm×10 cm; Pharmacia Biotech),

Q-sepharose (by 10 mm×10 cm; Pharmacia Biotech) and DE52 (by 10 mm×10 cm; Whatman) equilibrated with 20 mM Na-acetate buffer (pH 5.0) and eluted with 0.25 M NaCl in 20 mM Na-acetate buffer (pH 5.0) to remove the soluble lignin and other impurities.

The subsequent chromatography steps were performed by AKTA purifier system (GE Healthcare) using Q-sepharose Fast Flow HR10/10 column (bed volume 10 ml, Pharmacia Biotech) and MonoQ HR5/5column (bed volume 1 ml: GE Healthcare) twice. Q-sepharose column was eluted with a gradient of 1 M sodium chloride from 0 to 30% in 20 mM Na-acetate buffer (pH 5.0) at the flow rate of 1.5 mL/min, Fractions containing laccase activity were found only in one peak and MonoQ column was eluted with a gradient of 1 M sodium chloride for 0-25% in 20 mM Na-succinate buffer (pH 4.5) at the flow rate of 0.5 mL/min. Fractions containing laccase activity were found in two chromatographic peaks, called Lac1 and Lac2. The fractions containing the highest laccase enzyme activities were isolated by SDS-PAGE and pooled. The peaks containing laccase isozymes were collected and stored at -20 ℃.

Laccase purification was performed following the procedure shown in Fig.2-1.

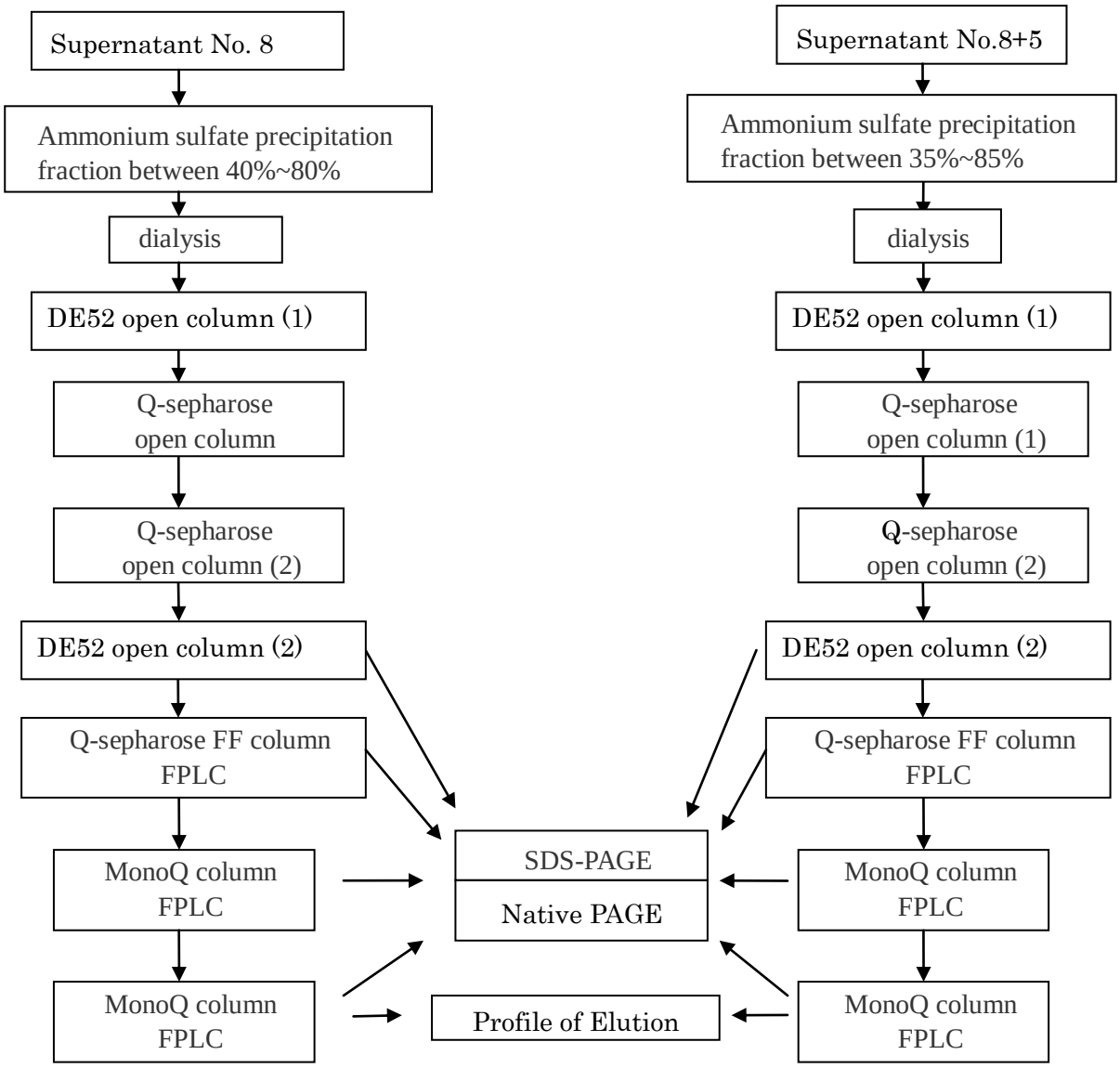


Fig. 2-1. Procedure of laccase purification

SDS-PAGE

SDS-PAGE was performed on polyacrylamide gel (13% and 7.5%) in a Mini protean electrophoresis chamber at 4 ℃. Proteins were stained using coomassie brilliant blue R250 (CBB).

Native PAGE and stain methods

Native PAGE was performed on polyacrylamide gel (7.5%) and 1.5 mol/l Tris buf-

fer (pH 8.8) in a Mini protean electrophoresis chamber at a constant current of 30 mA at 4 ℃. Two samples were prepared: one for CBB staining to exhibit all proteins, and the other for laccase active staining.

Active staining: gels were initially soaked in pH 5.0 160 mM Na-acetate buffer to adjust its pH to 5.0, and then stained with 3 mM ABTS in 0.32 mM Na-acetate buffer until the green band appeared (after about 1~10 min).

N-terminal amino acid sequencing

The N-terminal amino acid sequences of purified laccases were determined by Procise 492HT (Applied Biosystems) from a blotted membrane (0.2 μm Sequi-Blot PVDF; Bio-Rad).

Native molecular mass by gel filtration

For the estimation of the native molecular mass, gel filtration was performed using HPLC equipped with a Superose 6 HR 10/30 column (GE Healthcare) or a Shim-pack Diol-300 column (Shimadzu) equilibrated with 20 mM Tris-HCl (pH 8.0) buffer containing 1 mM $MgCl_2$ and 150 mM NaCl, at flow rate of 0.5 ml/min or 1 ml ml/min, respectively. Gel Filtration Standard (Bio-Rad) was used as molecular markers for the calibration. Each measurement of standards or samples was performed in triplicate.

2.3 Pretreatment with laccase and assessment

Pretreatment with cell-free crude laccase supernatant

Rice straw was smashed for 1 min by TESCOM juice mill and pretreated for 5 days by supernatant from *D. squalens* pure culture or supernatant from co-culture with *T. reesei*. Each incubation vial (2 g straw/20 ml supernatant) contain 1 mM $CuSO_4$, 1% Tween 20, and 1 mM syringic acid (4-hydroxy-3,5-dimethoxybenzoid acid) as laccase mediator system (Johannes et al. 2000a, Camarero 2004, Elegir et al. 2005 and Minussi et al. 2007).

To obtain a precise pretreatment result, 40μl of 10 g/l tetracycline (in 70% ethanol) and 30μl of 10 g/l cycloheximide were added to pretreating system to prevent other microorganisms from degrading lignin. Oxygen was flushed for one minute once a day into vials to ensure that oxygen was enough to laccase oxidation. Rice straw treated by 2% NaOH at 90 ℃ for 2 h was used as positive control and untreated rice straw soaked in deionized water as negative control for laccase-pretreated straw.

Exposed Surface Determination by Simons' stain

Preparation of the Dye Solutions.

Chicago Sky Blue 6B (Direct Blue 1) and Direct Orange 15 dyes were obtained from Sigma-Aldrich. The blue dye was dissolved in nanopure water to a final concentration of 1% (w/v) and kept in a sealed container at room temperature.

The orange dye contains high and low molecular weight (HMW and LMW) molecules and has to be fractionated to isolate the higher MW fraction that is known to have a higher affinity for cellulose. Fractionation was carried out by filtering a 1% (w/v) solution of orange dye through a 100 K ultrafiltration membrane (Millipore Corp., Bedford, MA) at 5000×g. The orange dye solution was poured into the Amicon container (full) and filtered through until about 20% of the original volume remained. To determine the concentration of the top fraction (retentate), a known volume (1 ml) of the retentate was lyophilized and the weight of the solid residue was measured. This value was then used to dilute the HMW fraction to a final concentration of 0.2% (w/v). Equal volumes of the blue and orange dye solutions were mixed to produce the "1:1 staining solution" and sodium chloride was added to a final concentration of 2%.

Staining. A 25 mg sample of pulp (dry weight) was placed in 100 mL of the 1:1 staining solution in 125-ml Erlenmeyer flasks and incubated in a water bath with shaking at 75 ℃ for 48 h. The mixture was filtered through a sintered glass filter under vacuum and the stained pulp was washed with 5 ml of cold water (4 ℃) and pressed gently between two pieces of filter paper to remove any unabsorbed dye. The stained pulp was then stripped using a 25% pyridine solution as described below.

Stripping. The pulp samples that had been stained, washed with cold water, and blotted with filter paper were placed in about 80 mL of 25% pyridine solution in 125-mL Erlenmeyer flasks and incubated at 45 ℃ for 18 h. Each mixture was then filtered through a sintered glass filter, and the filtrate was collected and volumed to exactly 100 mL with 25% pyridine solution. The absorption of this final solution was measured at 450 and 621.5 nm using a spectrophotometer.

Calculations. To determine the concentrations of orange and blue dye in the stripping solution (CO and CB), the following two equations (Lambert-Beer law for a binary mixture) were solved simultaneously:

$$A_{450nm}=\varepsilon_{O450}\bullet L\bullet C_O+\varepsilon_{B450}\bullet L\bullet C_B \quad (1)$$

$$A_{621.5nm}=\varepsilon_{O621.5}\bullet L\bullet C_O+\varepsilon_{B621.5}\bullet L\bullet C_B \quad (2)$$

Where A is the absorption of the mixture at 450 or 621.5 nm, ε is the extinction coefficient of each component at the respective wavelength, and L is the length traveled by light through the solution (cuvette width). In this study, L=1 cm and the extinction coefficients were as follows: ε_{O450}=50.67 L g^{-1} cm^{-1}; ε_{B450}=1.97 L g^{-1} cm^{-1}; $\varepsilon_{O621.5}$= 0.075 L g^{-1} cm^{-1}; $\varepsilon_{B621.5}$=15.65 L g^{-1} cm^{-1}. The method was revised and performed based

on references (Esteghlalian et al 2001 and Xu et al 2010).

Partial depolymerized lignin extraction by dioxane

The process is to determine the extractable lignin, mostly derived from partial decomposition of lignin matrix by laccase pretreatment. With some bonds between lignin units cleaved, the partial depolymerized lignin in matrix can become soluble and nomadic by extraction. Therefore, the changes of extractable lignin can exhibit the partly degraded extent of lignin. This method was modified based on Aya Fujimoto et al. (2005).

The pretreated rice straw was dried in 70 ℃ oven for overnight. About 100 mg rice straw was extracted with 10 ml 92% dioxane/water (v/v) on a 150 rpm shaker for 48h. After centrifugation, the amount of extracted lignin from rice straw (if necessary, dilute it with 92% dioxane) was estimated based on the UV absorbance at 280 nm of the solution by the use of 13 $g^{-1}cm^{-1}$ as absorbance coefficient. The changes of the extractable lignin were compared to assess partially-depolymerized lignin, that is, extractable lignin derived from its unextractable polymer precursor before pretreatment.

Saccharification

All contents were converted into 300 ml vial for saccharification, following a test that sugar concentration in pretreated solution was determined to measure the sugar released by the cellulase in supernatant. All experimental protocol of saccharification was made referred to NERL protocol (Selig at al. Mar. 2008, NREL Technical Report).

60 FPU (30 FPU/g straw) cellulase (from Acremonium spp., 1 g powder contains 377FPU, 214U avicelerase, 47789 U CMCase, 436U β -glucosidase, 17754 U xylanase) was added to each vial. And 3.2 mg tetracycline and 2.4 mg cycloheximide were complemented to prevent the growth of organisms during the digestion. Lastly, 0.1 M, sodium citrate buffer (pH 4.8) was added till to reach 100 ml digestion volume. All contents of each vial were brought to the incubator set at 50.0 ±1 ℃. At the same time, a substrate blank contains buffer, antibiotics, and the identical amount of enzyme in 100.0 ml volume was prepared.

Shaking or rotation was retained sufficiently to keep solids suspended for a period of 48 hours. Sampling was taken at the interval of 24 hours. Sugar analysis was carried out using appropriate HPLC method according to NREL Technical Report (Sluiter et al. 2006).

Component analysis

The analyses of rice straw constituent were performed by ANKOM Technology

methods. These methods determine Acid Detergent Fiber (ADF), Acid Detergent Lignin (ADL) and Neutral Detergent Fiber (NDF).

NDF is the residue remaining after digesting with Neutral Detergent Solution (Add 30.0 g Sodium dodecyl sulfate; 18.61 g Ethylenediaminetetraacetic disodium salt, dihydrate; 6.81 g Sodium borate 4.56 g Sodium phosphate dibasic, anhydrous; and 10.0 ml Triethylene glycol, in 1 L distilled H_2O; pH range from 6.9 to 7.1. The solution was agitated and heated to aid dissolve compositions) in the digestion instrument (ANKOM^{2000}, ANKOM Technology—capable of performing the digestion at 100±0.5℃ and maintaining a pressure of 10-25 psi with 65 rpm agitation). When the NDF extraction and rinsing process was complete, the bags were placed in a 250 ml beaker and enough acetone was added to cover bags and soak for 3-5 min. After air-dry, samples were completely dried in oven at 102±2℃ (2-4 hrs) and weighed. The fiber residues were predominantly cellulose, hemicelluloses, lignin and ash with soluble constituent removed.

ADF is the residue remaining after digesting with Acid Detergent Solution (20 g cetyl trimethylammonium bromide (CTAB) in 1 L 1.00N H_2SO_4) in ANKOM^{2000}, When the ADF extraction and rinsing process was complete, the bags were placed in a 250 mL beaker and enough acetone was added to cover bags and soak for 3-5 min. After air-dry, samples were completely dried in oven at 102±2℃ (2-4 hrs) and weigh. The fiber residues were predominantly cellulose, lignin and ash by dissolving the hemicelluloses constituent.

ADL is the residue remaining after ADF is extracted by 72% sulfuric acid (1634 g/l at 20℃) for three times. Following a complete rinse, samples were dried in oven at 102±2℃ (2-4 hrs) and weighed. The residues were predominantly lignin and ash by dissolving the cellulose constituent.

The original rice straw, NDF or ADL bags had been placed in pre-weighed beaker (30 or 50 ml) at 525℃ for 3 hours or until C-free, cooled and weighed to obtain the whole ash, NDF ash or ADL ash weight. The weight loss of ADL is the lignin constituent burned. Blank ash correction was calculated using a blank bag sequentially ran through ADF and lignin steps.

3 Laccase expression induced by co-culture and inductive materials

3.1 Preface

Laccase expression can be positively regulated by various compounds, such as metal ions, organic alcohols, aromatic compounds and lignin derivatives as well as other constituents similar to it as mentioned earlier.

In nature, we can observe various occurrences of symbioses, synergies or competitions between or among diverse microorganisms, and interestingly, the secretion of fungal laccase is also involved in the biocontrol phenomenon. It is found that some fungi can promote the production of laccase during interactions with other organisms.

Several articles have reported that laccase activities were increased by lignolytic fungi during interspecific interactions, especially with *Trichoderma spp*. (Flores et al. 2009, Mata et al. 2005, Hatvani et al. 2002, Zhang et al. 2006, Baldrian 2004 and Crowe et al. 2001), most of which proposed that antagonistic action resulted in laccase induction by metabolites, such as viscosinamide, an antifungal depsipeptide metabolite, which can only increase induction of laccase by 1.1 fold when the material is purified, others suggested that some lytic enzyme, (chitinases, proteases, glucanases, *N*-acetyl-β-glucosaminidase and laminarinase) may be responsible for the degradation of fungal cell walls, however, the inducers of *D. squalens* proved to be heat stable. However, in this context, the inductive reasons are still unclear.

Therefore, the aim of this section is to screen out the active lignolytic fungi and laccase-promoting interspecies interactions among white-rot fungi and compare its inductive effects to other laccase inducers. Moreover, inductive materials were also analyzed in this section.

3.2 Screening of highly cellulolytic and efficient lignolytic fungi

Screening of highly cellulolytic fungi

This section was carried out to screen the highly efficient cellulolytic fungi using test assays of CMCase and xylanase representing cellulase and hemicellulase, respectively. Sampling was conducted in a four-day interval for four times on 4d, 8d, 12d and 16d, in the whole 16d's course of cultivation.

Fig. 3-1 showed a parallel result in the CMCase activities in each sampling supernatant. Fungi No.5 (*Trichoderma reesei*) and No.6 (*Aspergillus nidulans*) exhibited much more extraordinary CMCase than other fungi. The same results in xyla-

nase activities were observed, No.5 (*Trichoderma reesei*) and No.6 (*Aspergillus nidulans*) showed stand-out xylanase activities compared to the other fungi (Fig. 3-2).

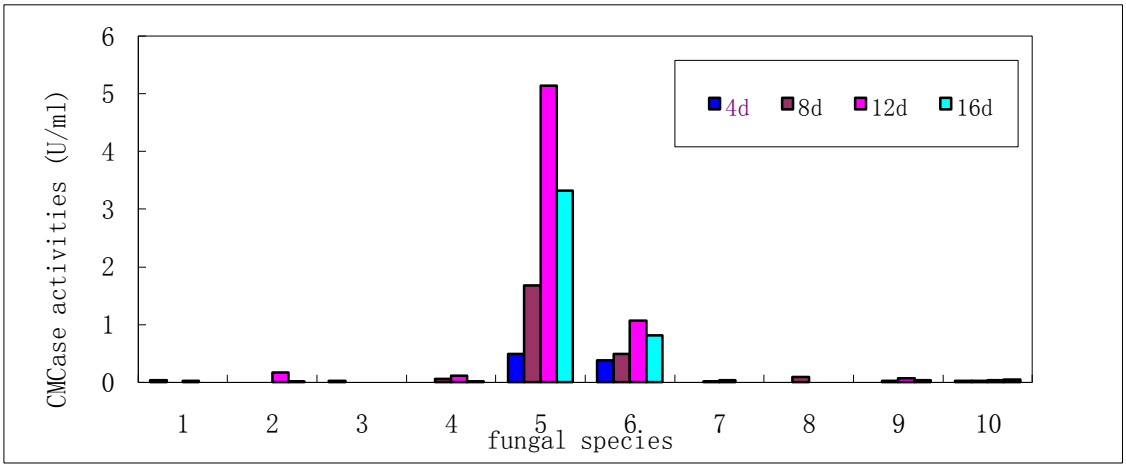


Fig. 3-1. Results of the screening of cellulase activities from 10 species of fungi

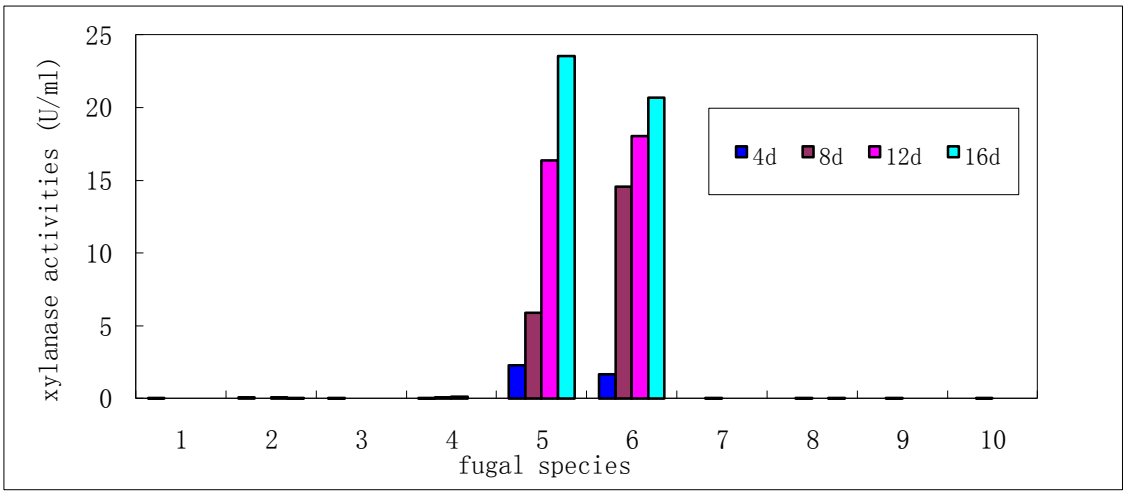


Fig. 3-2. Results of the screening of hemicellulase activities from 10 species of fungi

Thus, *Trichoderma reesei*, and *Aspergillus nidulans* were chosen as highly cellulolytic fungi in the experiment.

Screening of efficiently lignolytic fungi

This aspect considered three kinds of lignin-modifying enzymes, laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) as indicators to screen lignolytic fungi from the selected 10 species of famous wood-rot fungi. Sampling was carried out in a 12-day interval for three times on 12d, 24d and 36d, in the whole 36d's course of incu-

bation.

From Fig.3-3, Fig.3-4 and Fig.3-5, lignolytic enzyme activities had been investigated, and it was obviously showed that laccase activities took the main action in the initial period and exhibited weaker effects with the incubation going along while the other lignolytic enzyme, MnP and LiP, brought their weak degradation effect on lignin in the initial culture stage and occupied the main process and function in late stage.

Generally, most of basidiomycetes produces laccase increasingly in the the latter phase of substrate colonization and raise sharply at the onset of fruit body initiation, reaching maximum levels until the fruit body morphogenesis, and then suffer a decline throughout the subsequent stages of sporophore development (Chen et al. 2004, 2003, Chakarabory et al. 2000 and Largeteau et al. 2010). The youngest hyphae at the margins of the colony represent usually the maximum producers, whereas older hyphae produce less or none at all (Schanel et al. 1966). These were confirmed by experimental phenomenon in this work.

Fig.3-3 exhibited the screening results of laccase activities when cultivated in submerged rice straw media. Fungus No.8 (*Dichomitus squalens*) exhibited predominant laccase activities, reaching highest activity of 368.6 U/L during the incubation period, which was much higher than the other fungi including the famous wood-rotting fungi, *Pleurotus ostreatus*, *Trametes versicolor*, *Phanercochaete chrysoporium*. Three of them were focused by a great cluster of concentrated researches due to their extraordinary lignolytic abilities in recent decades. Although the Fungus No.4 (*Ceriporiopsis subermispora*) (Fukushima and Kirk 1995), No.5 (*T. reesei*) (Martinez 2008), No.6 (*A.nidulans*) (Scherer and Fischer 1998) and No.9 (*P. chrysoporium*) (Srinivasan et al. 1995) showed high laccase activities from them reported, there were no detected laccase activities cultivated in this rice straw condition.

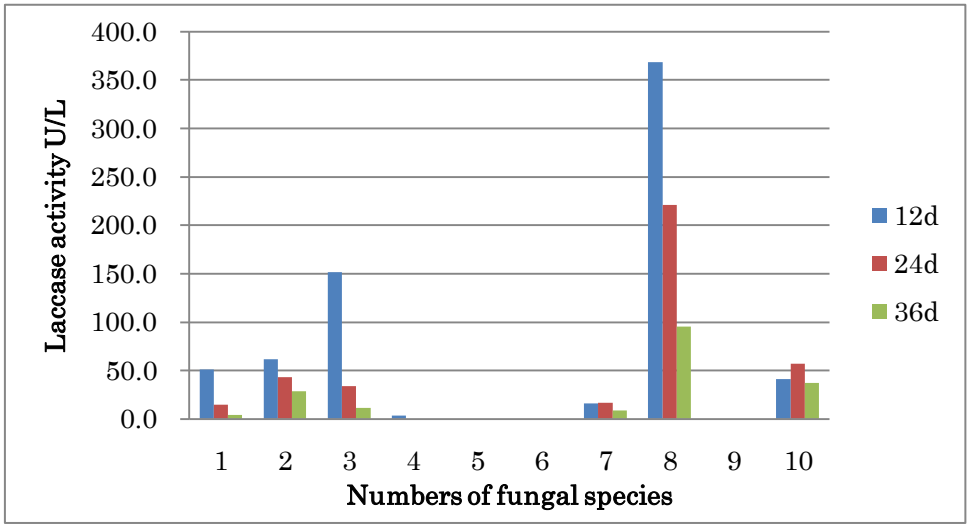


Fig. 3-3. Results of the screening of fungi by laccase activities in rice straw media

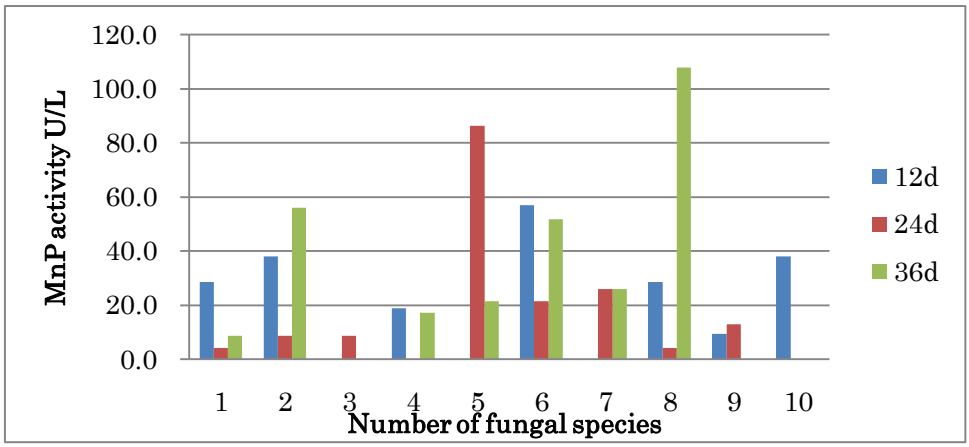


Fig. 3-4. Results of the screening of fungi by MnP activities in rice straw media

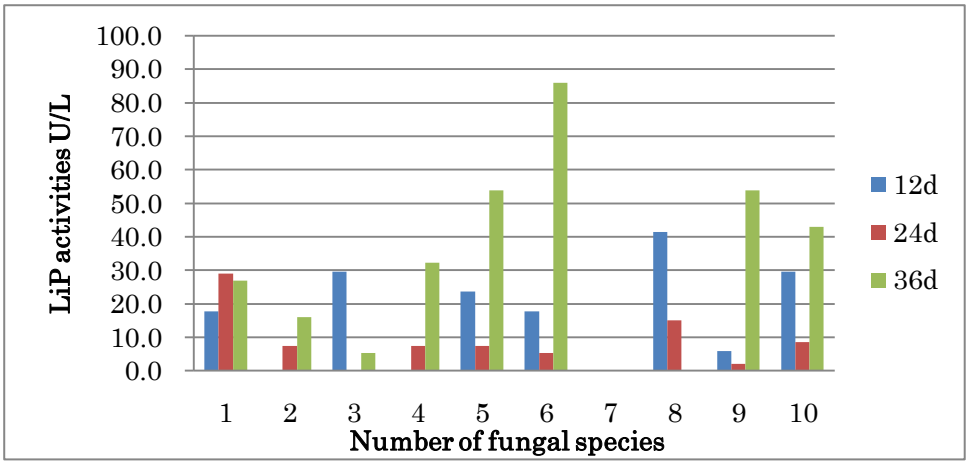


Fig. 3-5. Results of the screening of fungi by LiP activities in rice straw media

Another interesting strain was Fungus No. 10, *Armillaria mellea*, because of its fairly efficient and stable laccase activity (Fig.3-3) but weak cellulase and hemicellulase activities (Fig.3-1, Fig. 3-2). This can be considered as a favorable enzyme characteristic taking as the fungus for biopretreatment of lignocelluloses due to its ideal ability of high lignolytic but low cellulolytic behaviors.

Fig. 3-4 showed the screening results of MnP activities. *D. squalens* still prevailed in MnP activity during incubation, valued 107.9 U/L at its top activity. Interestingly, the No.5 (*T. reesei*) and No.6 (*A. nidulans*) having predominant cellulolytic competences showed efficient MnP activities and LiP activities (Fig. 3-4 and Fig. 3-5). *A. mellea* also had certain MnP and LiP activities.

Based on the above results, this study took the Fungus No.8 (*D. squalens*) and No.10 (*A. mellea*) as the efficient lignolytic fungi and No.5 (*T. reesei*), No.6 (*A. nidulans*) as active cellulolytic fungi.

Optimization of cultivation conditions for *D. squalens*

This section investigated the lignolytic enzyme production as effected by culture conditions focusing on the most lignolytic fungus *D. squalens*.

Laccase could be secreted both shaking and static cultures at the same level but grew more with mass fungal quantities at the static culture (from outer observation). Submerged culture produced most laccase activities compared to solid and hemi-solid culture (data not shown).

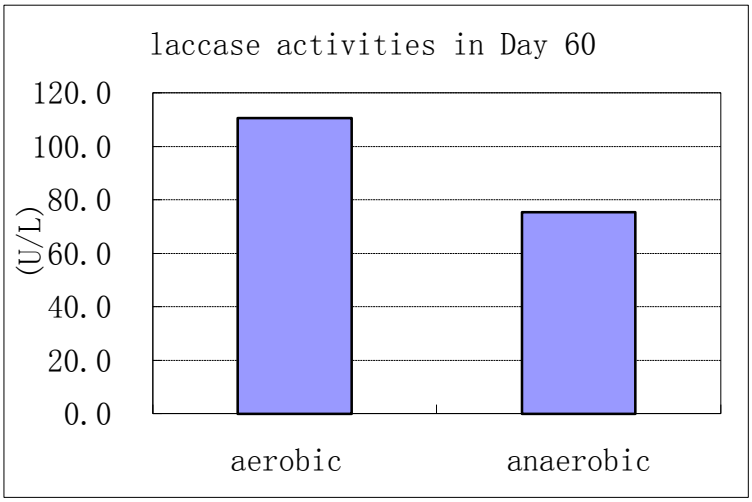


Fig 3-6. Laccase production in aerobic and anaerobic culture

More laccases were produced in aerobic conditions than in anaerobic conditions, but

anaerobic conditions can stimulate the MnP and LiP activities. Probably, anaerobic environment deactivated oxygen-needing laccase activities (Fig. 3-6, Fig. 3-7). Because the laccase activities were much more than MnP and LiP under aerobic condition, this work considered the aerobic environment as cultivation conditions.

Then, the study was performed in submerged culture with shaking, to set an aerobic cultivation conditions.

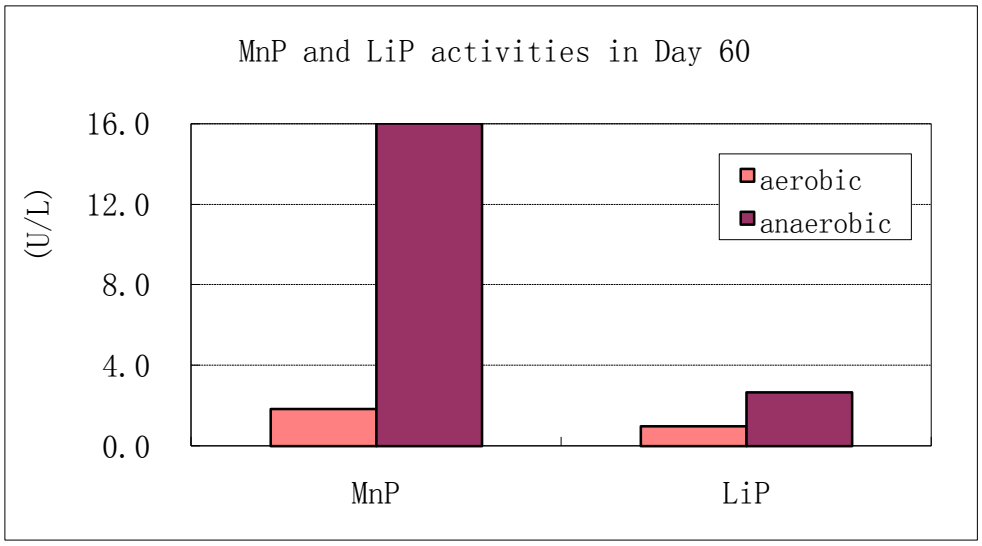


Fig. 3-7. MnP and Lip production in aerobic and anaerobic culture

3.3 Laccase expression induced by co-culture between lignolytic fungi and cellulolytic fungi

It was postulated that the two cellulolytic fungi and two lignolytic fungi mentioned above would cooperate each other because the action that lignolytic fungi delignify lignin from lignocellulosic matrix may do some favor to cellulolytic fungi to easily saccharify cellulose and hemicelluloses, and in return, the promoted saccharification provides more sugar to lignolytic fungi to grow.

To construct the synergic combination between two classes of fungi, four pairs of two-species groups, *D. squalens* and *T. reesei*, *D. squalens* and *A. nidulans*, *A. mellea* and *T. reesei* or *A. mellea* and *A. nidulans* had been incubated for 40d in rice straw by simultaneous inoculation or successive inoculation (the latter was inoculated after the former had been cultivated for 20d). And two-single pure culture fungi were cultivated at the same time as controls, respectively. The changes of laccase, MnP and CMCase activities were observed during co-cultures incubation.

Laccase activities in co-culture

As Fig. 3-8, 3-9 and 3-10 shows, it was unexpected that laccase activities were

hardly detected in all simultaneous inoculated co-cultures during the whole incubation, but in successive inoculated co-cultures, the laccase activities in pure culture phase (for 20 days) can be amplified by inoculating the latter fungus in three two-species groups, *D. squalens* and *T. reesei*, *D. squalens* and *A. nidulans*, or *A. mellea* and *T. reesei*.

Significantly, laccase activity was upgraded over 20 folds in successive inoculated co-culture of *D. squalens* and *T. reesei*, reaching 137.3 U/L (30d) from 5.0 U/L in the pure culture phase (20d) (Fig. 3-8).

Likewise, laccase activity was also increased over 10 folds in successive inoculated co-culture of *D. squalens* and *A. nidulans* reaching 67.4 U/L (30d) from 5.0 U/L in the pure culture phase (20d) (Fig. 3-9).

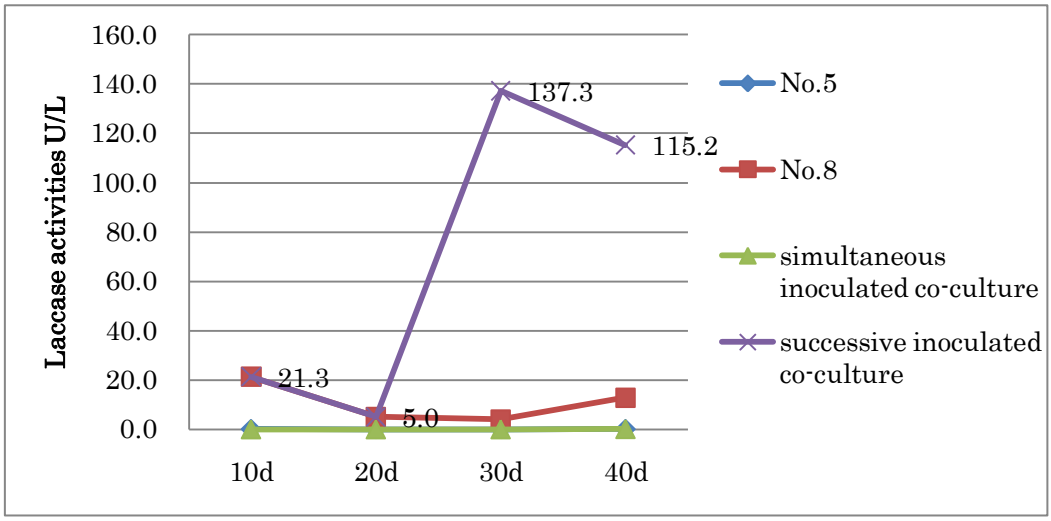


Fig. 3-8. Laccase activities in co-culture of *D. squalens* (No.8) and *T. reesei* (No.5)

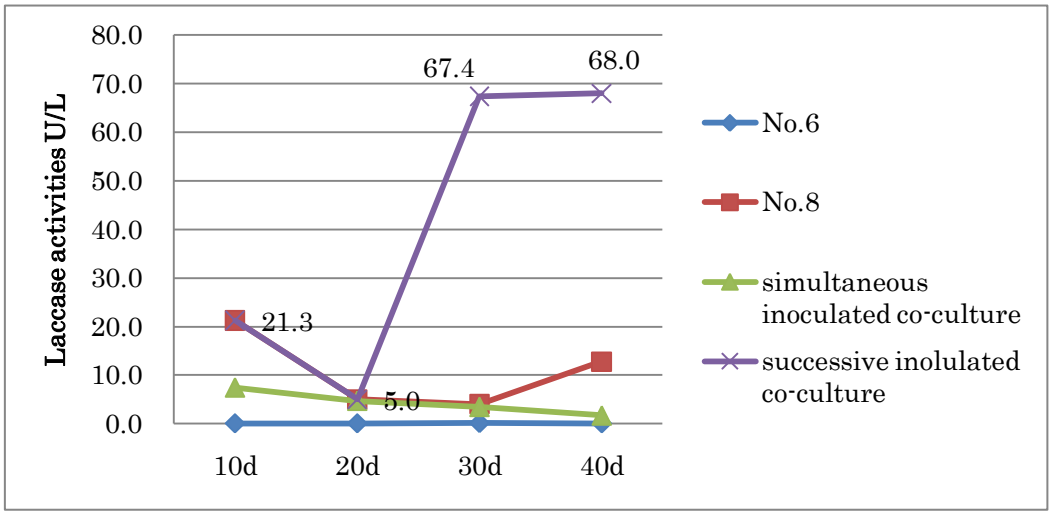


Fig. 3-9. Laccase activities in co-culture of *D. squalens* (No.8) and *A. nidulans* (No.6)

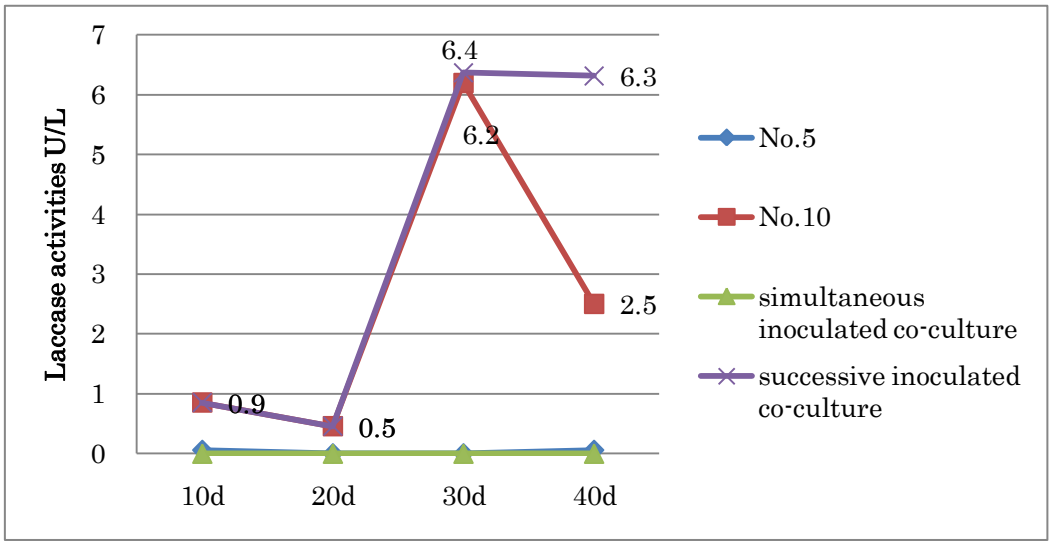


Fig. 3-10. Laccase activities in co-culture of *A. mellea* (No.10) and *T. reesei* (No.5)

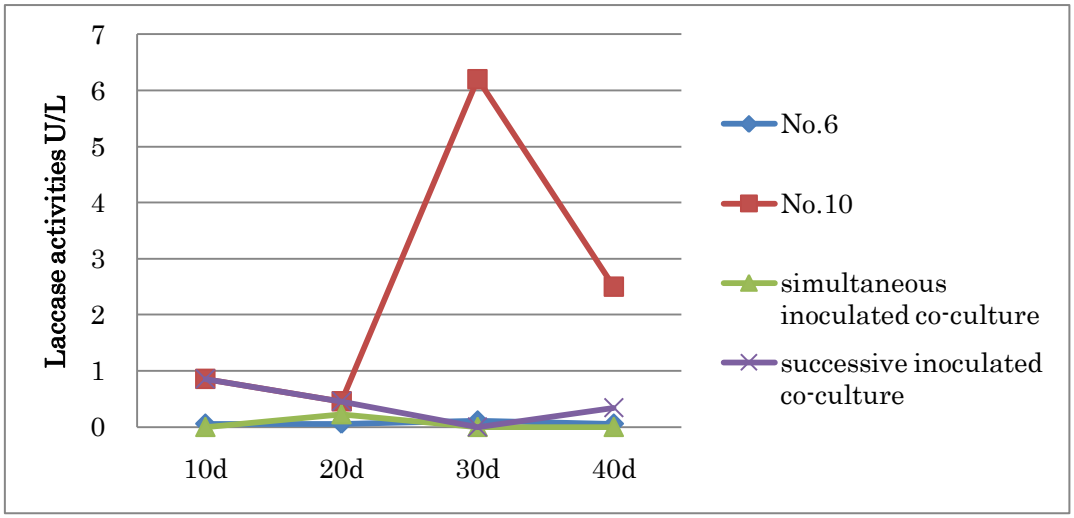


Fig. 3-11. Laccase activities in co-culture of *A. mellea* (No.10) and *A. nidulans* (No.6)

As Fig.3-10 shows, although laccase activities from successive inoculated co-culture of *A. mellea* and *T. reesei* increased only a few compared to the laccase activity of *A. mellea*'s pure culture, the laccases from successive inoculated co-culture were more stable.

Based on Fig.3-11, the group of *A. mellea* and *A. nidulans* showed no increase in laccase activities but suppressed effects on laccase production between the two fungi appeared by two ways of co-cultures, simultaneous inoculated co-culture and successive inoculated co-culture.

Because the pair of *D. squalens* and *T. reesei* showed the most significant increase of laccase activities during co-culture among all designed pairs of fungi, this work selected the pair of *D. squalens* and *T. reesei* as an example to study the laccase increase.

The following parameters, MnP and CMCase activities, fungal quantities and glucose accumulation during the co-culture incubation were also investigated.

MnP and CMCase in the co-culture of *D. squalens* and *T. reesei*

T. reesei showed remarkable MnP activities in its pure culture, so both simultaneous inoculated co-culture and successive inoculated co-culture had a higher laccase activity than *D. squalens*'s pure culture as predicted.

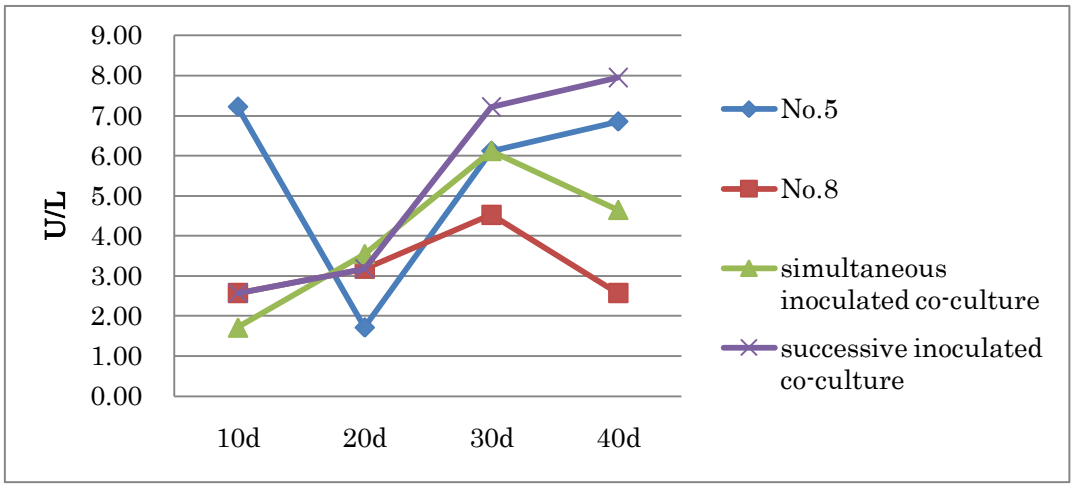


Fig.3-12. MnP activities in co-culture of *D. squalens* and *T. reesei*

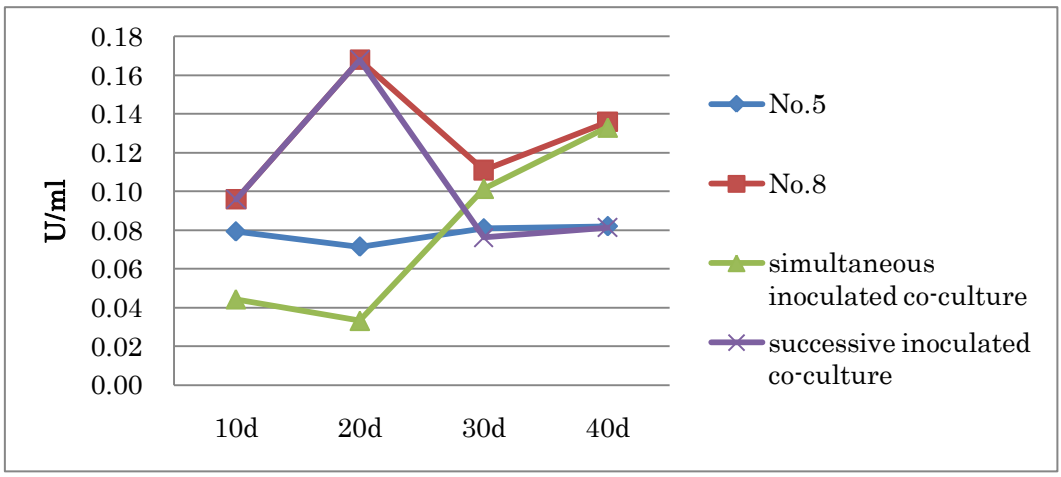


Fig.3-13. CMCase activities in co-culture of *D. squalens* and *T. reesei*

In the submerged rice straw media, *D. squalens* exhibited higher CMCase activities even more than the active cellulolytic fungi, *T. reesei*. But in the successive inoculated

co-culture, the laccase activities were partially suppressed and could be good to pre-treatment process of rice straw because any state of diminishing cellulase activities and maximizing the lignolytic activities will always be favorable in the biopretreatment to prevent sugar consumption during the pretreatment stage.

The changes of fungal quantities of co-culture during incubation

From Fig.3-14, it can be seen that although the laccase activities were amplified over 20 folds from 20d to 30d, the microbial quantities of *D. squalens* and *T. reesei* had not been magnified conspicuously. Besides it, *T. reesei* itself produced no laccases in rice straw media. Thus it could be concluded that the great increased laccase activities was not derived from synergic combination between fungi due to no obvious fungal growth but from the increment of laccase expression regulated by some inductive effects produced by interspecies interactions.

In addition, Fig. 3-14 showed that *T. reesei* can reproduce much more rapidly than *D. squalens* in state of pure culture before 20 days, which may be the reason that no laccase were produced in the simultaneous inoculated co-culture because *T. reesei*'s growth overwhelmed *D. squalens*'s growth thus limiting the laccase production of *D. squalens* when grown in co-culture with *T. reesei*.

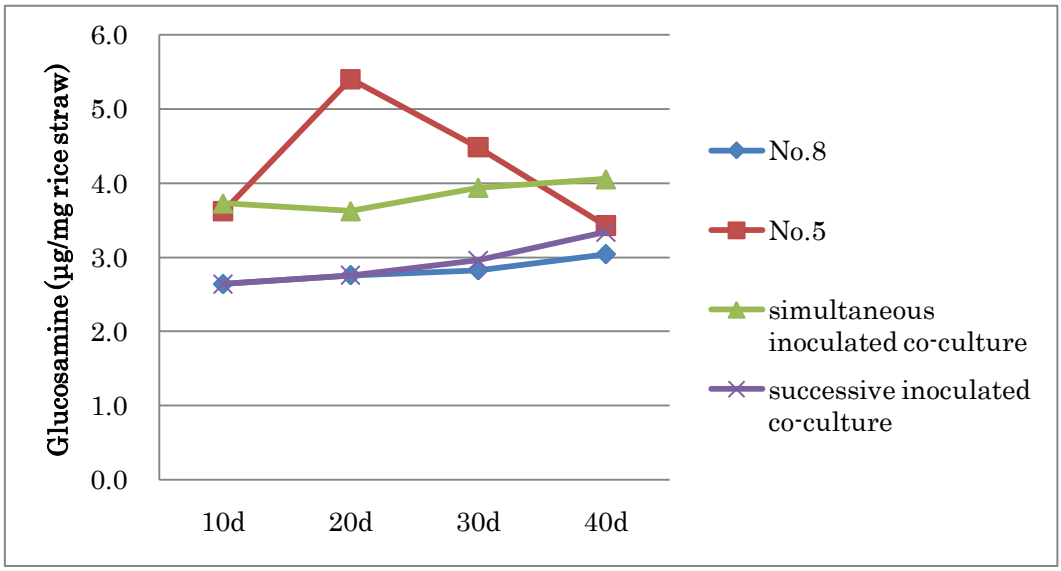


Fig.3-14. Changes in fungal quantities in co-culture of *D. squalens* (No.8) and *T. reesei* (No.5)

Fig. 3-15 shows that the culture of *D. squalens* with slow growth also accumulated little quantity of glucose since it degraded small amount of rice straw than *T. reesei*.

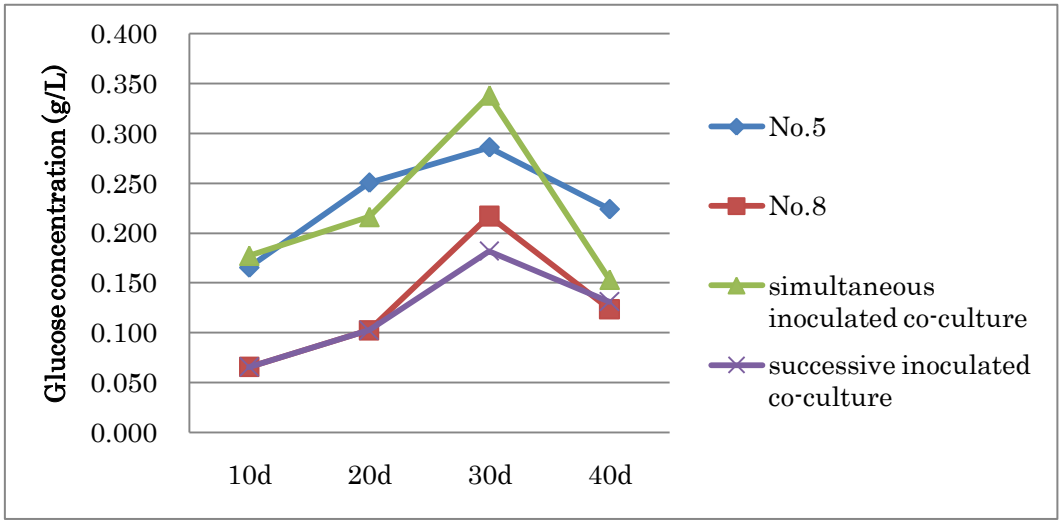


Fig.3-15. Glucose accumulation during incubation of *D. squalens* (No.8) and *T. reesei* (No.5)

From the results above, it could be seen that *D. squalens* was a fairly well fungus suitable for lignocellulosic pretreatment owing to its relatively low growth, high laccase and MnP activities, and its capability to be strongly induced by *T. reesei*.

3.3 Induction timing and maximization of laccase production

The timing of induction

Fungal laccases synthesis is associated with the onset of secondary growth, produced by fungal mycelium. They could be secreted in all basidiomycete life cycle stages of vegetative mycelium, fruit body and sporophore development except its initial spore stage (Fig. 1-3).

After *D. squalens* has been inoculated to rice straw using mycelium inoculation, it can develop vegetative mycelia and fruit body into shape for 8-9 days by observation of static plate cultivation. As Fig.3-16, the screening experiment had showed the changes of laccase activities during the incubation of *D. squalens*. Some reports indicated that most of basidiomycetes produced laccase increasingly in the latter phase of substrate colonization and raised sharply at the onset of fruit body initiation, reaching maximum levels until the fruit body morphogenesis, and then suffered a decline throughout the subsequent stages of sporophore development (Chen et al. 2004, 2003, Chakarabory et al. 2000, Ohga, 1999, and Largeteau et al 2010). Because *D. squalens* developed its fruit body into shape from inoculated mycelia in 8-9 days by static culture, and secreted most laccase on 12d in a 36d's cultivation, it can be known that *D. squalens* also produced laccase at maximum levels until the fruit body morphogenesis like most other basidiomycetes.

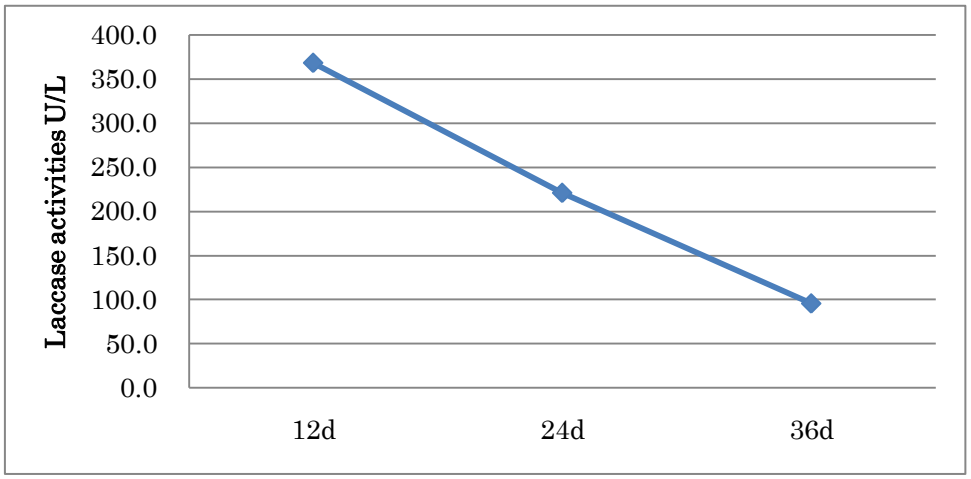


Fig. 3-16. Changes of laccase activities during the incubation of *D. squalens*

Generally, a dikaryon mycelium ultimately gives rise to either fruiting bodies with basidia or directly to basidia without fruiting bodies. In shaking conditions of this experiment, the dikaryon mycelium may become directly basidia without fruitbodies. Laccase secretion in this occasion has been reported by Schanel et al. (1966) that the young hyphae at the margins of the colony represented usually the maximum producers of laccases, whereas aged hyphae produced less or none at all.

D. squalens had the most active ability of producing laccase at about 9-12 days. So the most feasible timing of inducing laccase would be about 10d. The length of induction was decided by a preparative experiment as shown in Fig.3-17. According to the results, a 5-day length of induction by co-culture was adopted.

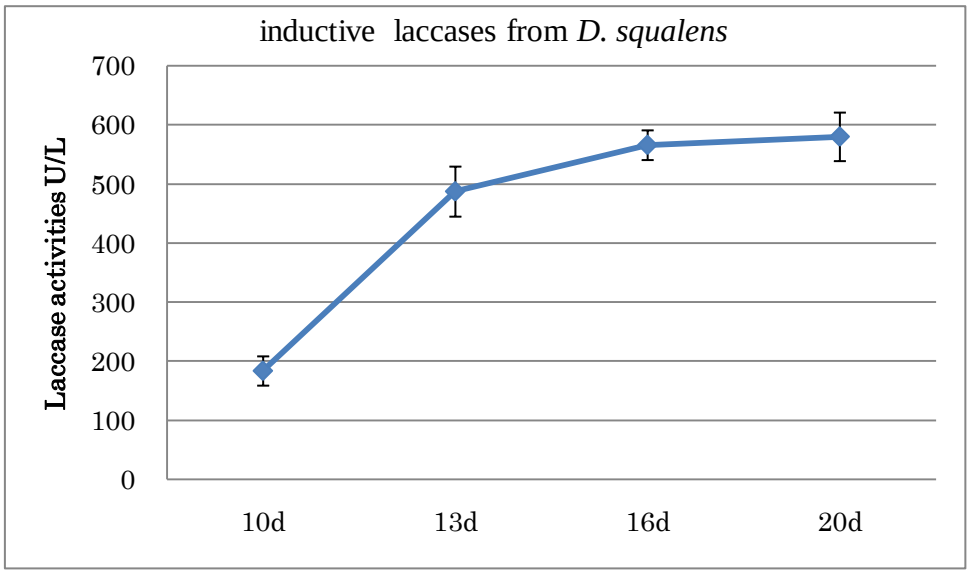


Fig.3-17. Changes in laccase activities induced by *T. reesei* after its inoculation on 10d

The effects of laccase production in 10-day incubation followed by a 5-day co-culture with *T. reesei* were shown in Fig.3-18. Results showed an induced laccase activity of 555 U/L, which was significantly higher than the laccase activity of 137 U/L at 30d as shown in Fig. 3-8 at 20-day incubation followed a 10-day co-culture.

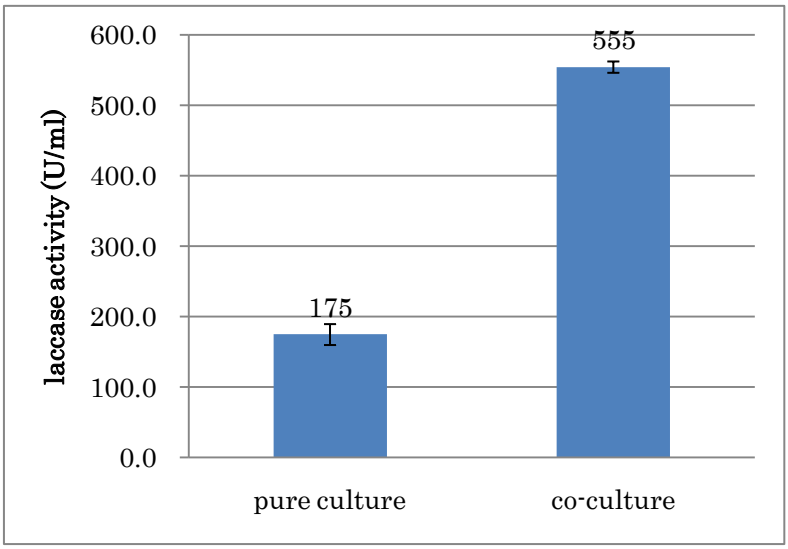


Fig.3-18. Laccase activities from co-culture induction. The pure culture of *D. squalens* was previously cultivated for 10d followed by the addition of *T. reesei* to form a co-culture with *D. squalens* for 5d. A 15d-cultivated pure culture of *D. squalens* was conducted as control.

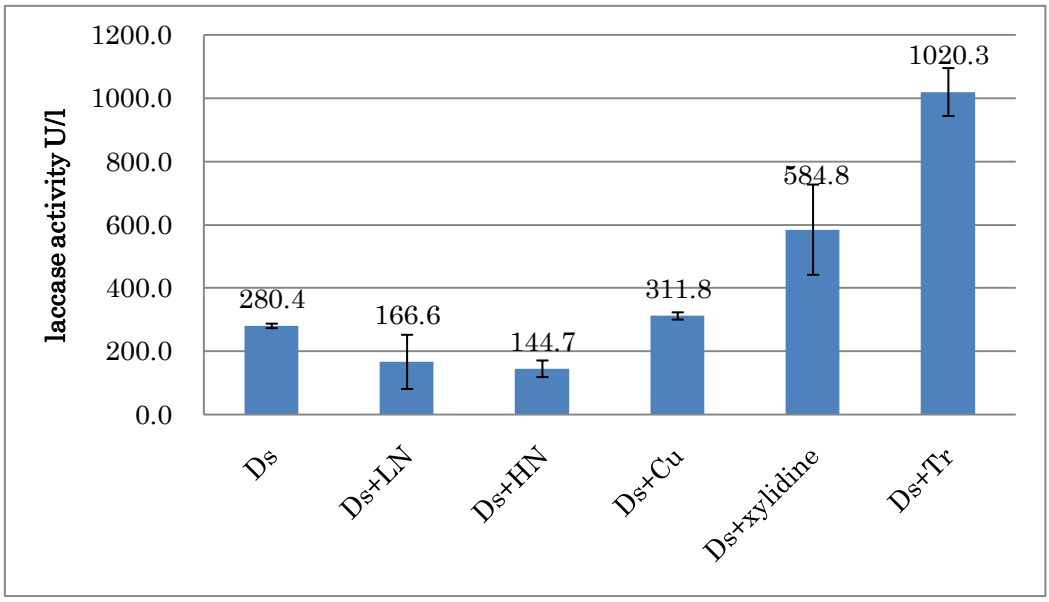
Other inductive materials and maximization of laccase induction

The section compared the inductive effects from other common inducers of laccases to the effects from co-culture. Then various combinations of them were tried to maximize laccase induction.

The study investigated the inductive effects by N nutrient (high nitrogen and low nitrogen), Cu^{2+}, and 2,5-xylidine. N nutrient addition can induce laccase in many basidiomycetes. Cu^{2+} can nearly induce laccases of all fungi with genes encoding laccases for it is necessary to form the active centers of laccase with four copper ions at low concentration. But it can become an inhibitor to laccases at high concentration of over 1 mM. 2,5-Xylidine was the most powerful inducer of laccase than other materials in most laccase-producing fungi.

As shown in Fig.3-19, N nutrient exhibited no inductive effects of laccases, even conversely suppressed laccase production from *D. squalens* and copper ion showed weak induction with *D. squalens*, laccase increased was only about 1.1 folds. As ex-

pected, 2,5-xylidine exhibited its vigorous competence of inducing D. squalens laccase with about 2.5 folds yield upgrade while it was overshadowed by the laccases activities of 1020 U/L induction of about 4-folds by co-culture with *T. reesei*.



Note: LN-low nitrogen, HH- high nitrogen, Ds-*D. squalens*, Tr-*T. reesei*

Fig.3-19. Comparison on laccase induction effect of inductive materials

To create more fruitful inductive laccase, a combination between co-culture and a series of N and Cu^{2+} concentration has been performed to assess their cooperative inductive effects and it was unfortunate that no conspicuous increase was observed (Fig.3-20).

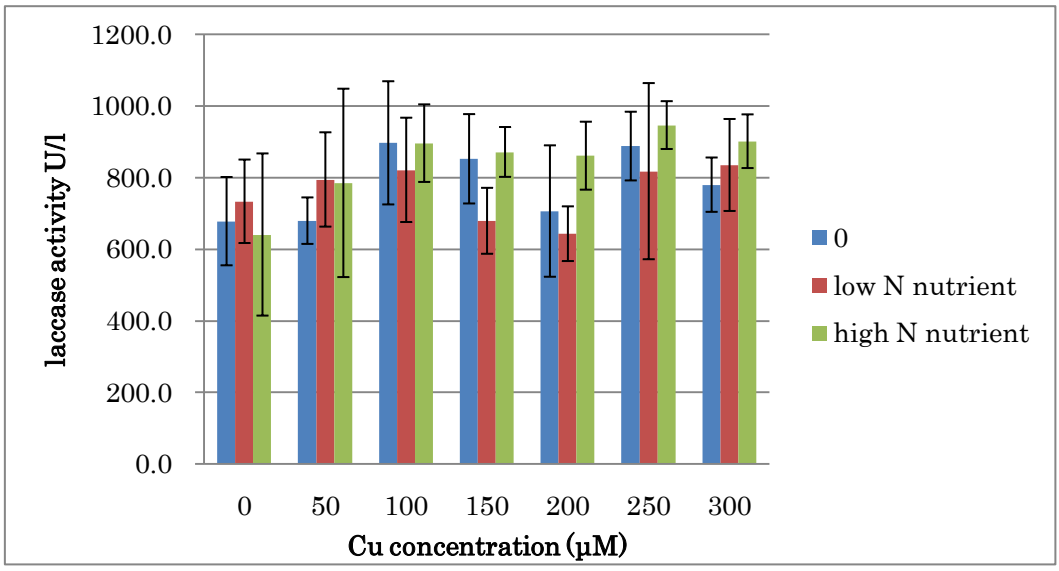


Fig. 3-20. Combination of inductive effects between co-culture and a series of N and Cu

concentration.

Fig.3-21 showed the combination of two most efficient inducers, 2,5-xylidine and co-culture with *T. reesei* to further promote laccase induction and more prolific induced laccases (1340 U/L) were achieved.

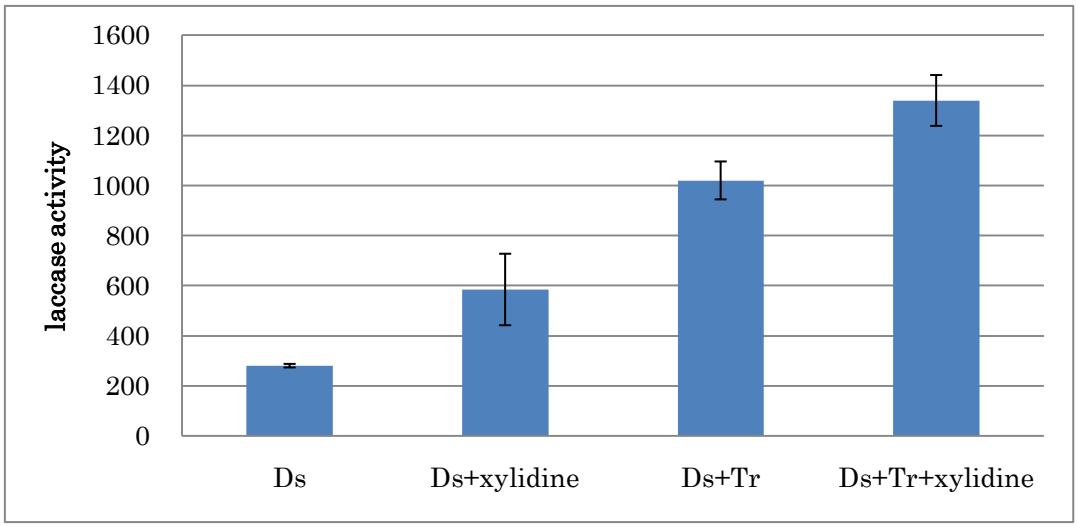


Fig.3-21. Laccase production induced by combination of xylidine and *T. reesei* strain

3.4 Materials for the induction of laccase expression by co-culture

Naturally, the inductive materials produced by co-culture were focused on this study and the inductive mechanisms were analyzed.

To simplify the solution content during cultivation and induction, rice straw media was replaced with a non-lignin PDB (potato dextrose broth) media to incubate *D. squalens* for 10 days and then cultivated for 5 days in co-culture with *T. reesei* but it was found that no induction appeared in rice straw media and furthermore the laccase yields were very poor (about 10 U/L) in both pure culture and co-culture.

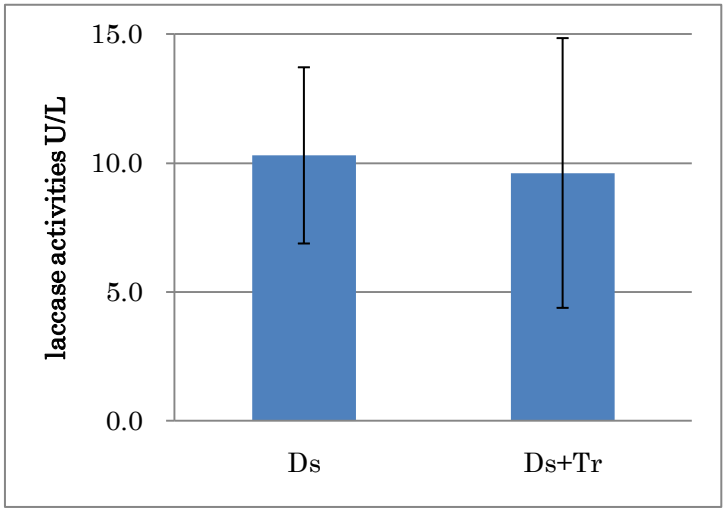


Fig. 3-22. Laccase activities from *D. squalens* and its co-culture with *T. reesei*.

Several reports have shown that increased laccase production by lignolytic fungi during interspecific interactions, especially with *Trichoderma spp.* and proposed that antagonistic action resulted in laccase induction by a metabolites, such as viscosinamide, an antifungal depsipeptide metabolite (Crowe et al. 2001).

But antagonistic action was not sufficient to explain the encounter in PDB media between *D. squalens* and *T. reesei* because no laccase induction took place and its laccase activities was far below from that in rice straw.

What are the inductive materials? Some reports suggested that some lytic enzyme, (chitinases, proteases, glucanases, *N*-acetyl-β-glucosaminidase and laminarinase) may be responsible for the degradation of fungal cell walls leading to laccase secretion to resist the degradation (Hatvani et al. 2002 and Howell 2003).

From Fig.3-23, the addition of cell-free supernatant from pure culture *T. reesei*'s 5-day cultivation in rice straw (Supernatant Tr) to PDB media cultivating *D. squalens* could strongly induce laccase, and the autoclaved supernatant can also bring laccase induction. Thus, the inducers proved to be heat-stable, indicating that they were not the lytic enzymes mentioned above. The results strongly suggested that they may be related to lignin or its derivatives.

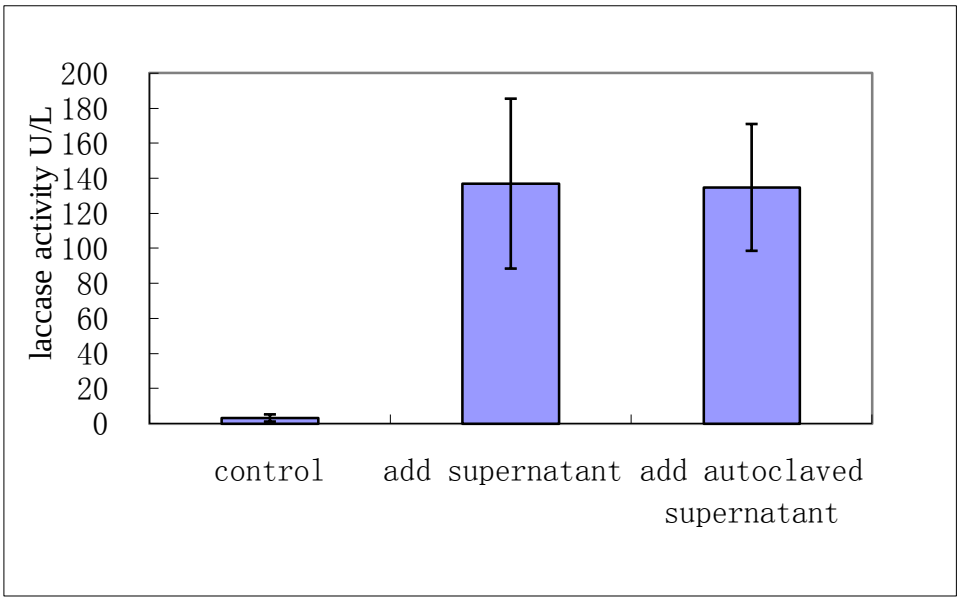


Fig. 3-23. Laccase activities of *D. squalens* in non-lignin media (PDB) induced by Supernatant Tr. The induction was performed by adding 5 ml of Supernatant Tr into 25 ml of *D. squalens* PDB media.

It is therefore necessary to analyze further the Supernatant Tr. Fig. 3-24 showed that *D. squalens* culture in PDB media were induced using sterile distilled water, more than 5 kD fractions and less than 5 kD fractions of Supernatant Tr divided by ultrafilter, and Supernatant Tr, and it was seen that both more than 5 kD fractions and less than 5 KD fractions could induce laccases as their Supernatant Tr precursor. It turned out that the inductive material must be those materials with a molecular mass across 5 k.

Then it was shifted to 10 k ultrafilter with the same design, as shown in Fig. 3-25, then similar results appeared that all fractions of Supernatant Tr could induce laccase from *D. squalens* cultivated in PDB media.

The results above indicated that not only a sole component but a series of materials with continuous molecular sizes from 0 to over 10 kD induced *D. squalens* laccases which may be related to lignin.

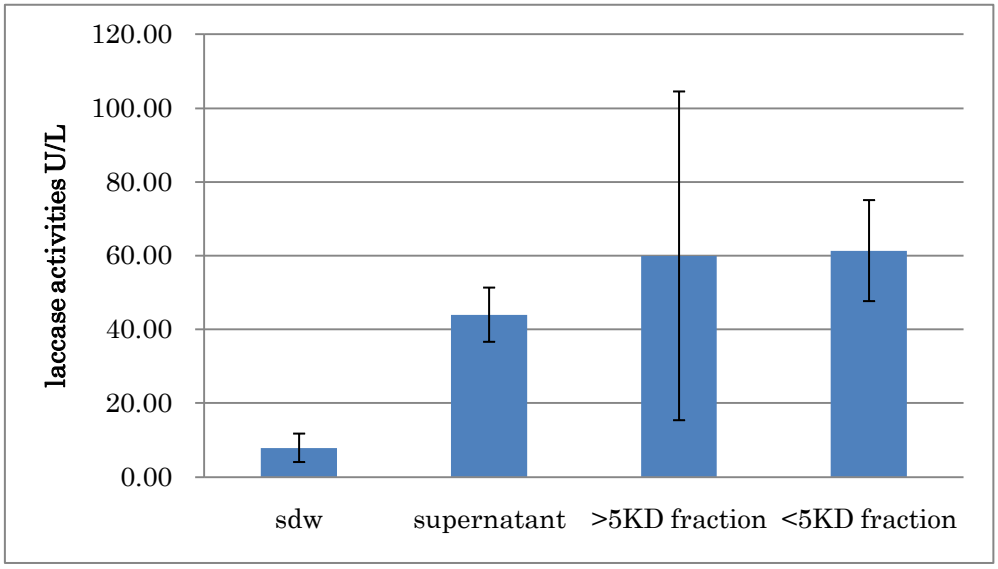


Fig.3-24. Laccase activities induced by Supernatant Tr and its fractions divided by 5 kD.

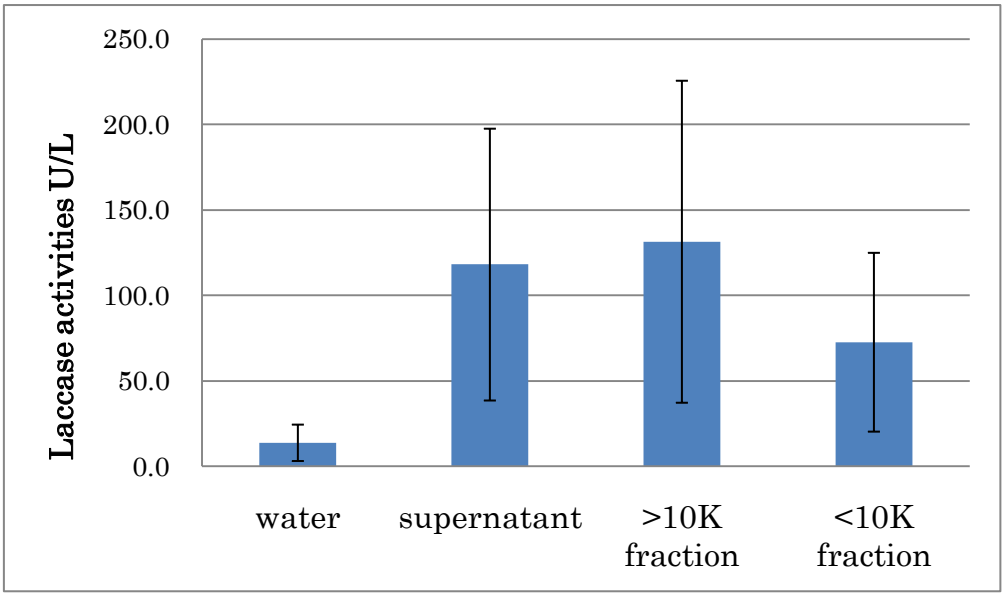


Fig.3-25. Laccase activities induced by Supernatant Tr and its fractions divided by 10kD.

To investigate the relations between inductive effects and lignin, three basic lignin component Units, p-hydroxyphenyl (H) acid, Guaiacyl (G) acid and syringyl (S) acid would be added to *D. squalens* non-lignin PDB media, respectively.

Because the chemicals as basic units of rice straw lignin have certain toxic action to fungi, a tolerance experiment was pursued. Based from Table 3-1, when the substrate

concentration of basic units reached to over 0.05 g, most fungi growth were restrained, while at the substrate content of 0.01 g, *D. squalens* and *T. reesei* were both fecund. So, the substrate concentration in the inductive experiment was fixed on the level of 0.01 g/25 ml PDB media.

Table 3-1. The tolerance concentration of basic units of lignin by fungal growth

D . squalens	g/25 ml PDB culture			
	0.01 g	0.03 g	0.05 g	0.1 g
G	+++	+	none	none
H	++++	++++	+++	+++
S	+++	+	none	none
T. reesei				
G	+++	+	none	none
H	++++	+++	+++	++
S	+++	++	+	++

Note: + denotes poor growth, ++ denotes moderate growth, +++ denotes good growth, and ++++ denotes abundant growth

Furthermore, we can find the difference that *T. reesei* grew well in the substrate of S nearly at any level, but *D. squalens* suffers a stiff development in this substrate except at the content level of 0.01 g. *D. squalens* had a low tolerance to S unit but *T. reesei* had a high tolerance, thus it was expected that the S unit would have some relations to *D. squalens* laccase secretion during co-culture with *T. reesei.*

Moreover, an amount of 0.01 g of H, G and S (their acid form) units were added to *D. squalens* non-lignin PDB media (25 ml), respectively and S unit was found to be able to induce laccase at the same extent as rice straw as expected (Fig.3-26).

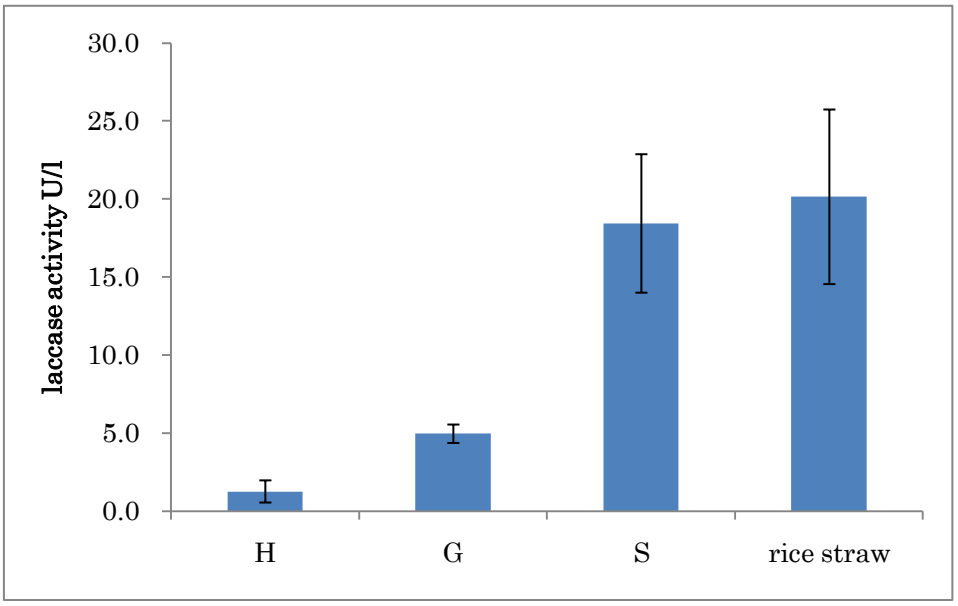


Fig.3-26. *D. squalens* laccase in PDB media induced by the addition of H, G and S lignin component unit. A 0.01 g of H, G and S (acid form) units were added to *D. squalens* non-lignin PDB media (25 ml), respectively, and a 0.3 g of rice straw powder was added as control (rice straw contains about 10% lignin and 3% of H, G, S were assumed).

Thus, it was concluded that the partially degraded lignin with 0 to over 10 kD molecular mass degraded by *T. reesei* had induced laccase expression from *D. squalens.*

To verify this postulation, soluble lignin in Supernatant Tr was completely depolymerized to monomolecular lignin component units, H, G, S component units, by alkaline nitrobenzene oxidation.

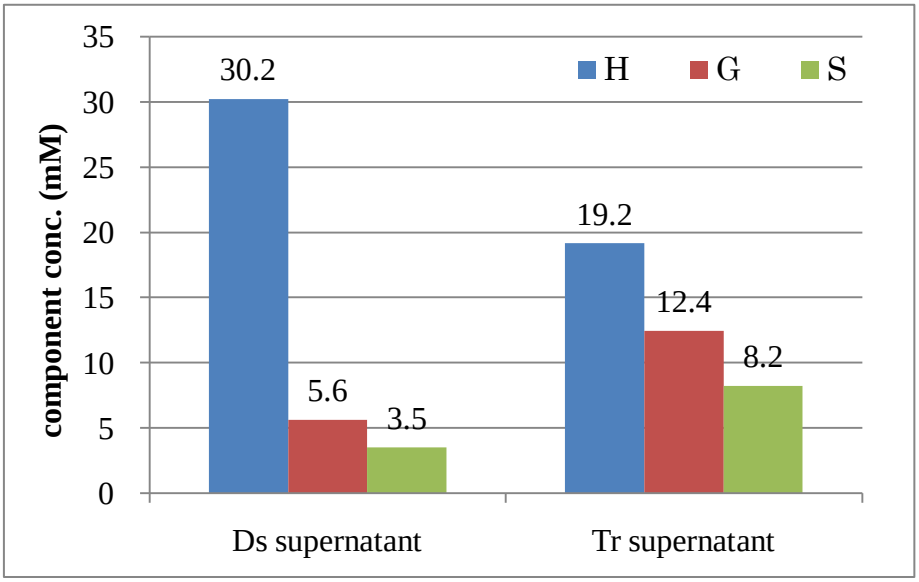


Fig. 3-27. Analysis of lignin basic components in Supernant Tr and Ds supernatant of pure culture.

As shown in Fig. 3-27, about 3 folds of S component unit concentration in Supernatant Tr was detected than in supernatant from *D. squalens* culture in rice straw.

It was coincident that 5 ml of Supernatant Tr containing a concentration of 8.2 mM S unit (1.624 g/l) (Fig. 3-27) was added into 25 ml *D. squalens* PDB media (Fig.3-23), its concentration (1.624/6=0.321 g/L) was very near to the permissible tolerance concentration of S unit (0.01 g/25 ml=0.4 g/l) in Table 3-1 and Fig. 3-26. This indicated that S unit may have the same action both in rice straw and PDB media.

Moreover, from Fig.3-28, in non-lignin PDB media, few laccases could be found from *D. squalens* culture cultivated for 15 days and no laccase inductions appeared in 10d cultivated *D. squalens* induced by 5d co-culture with *T. reesei*, under submerged powder media of red pine, a softwood species, in which lignin do not contains the S unit.

Based on the results above, it was undoubted that the conclusion that the partially degraded lignin with 0 to over 10kD molecular mass degraded by *T. reesei* had induced laccase expression from *D. squalens* has been proved.

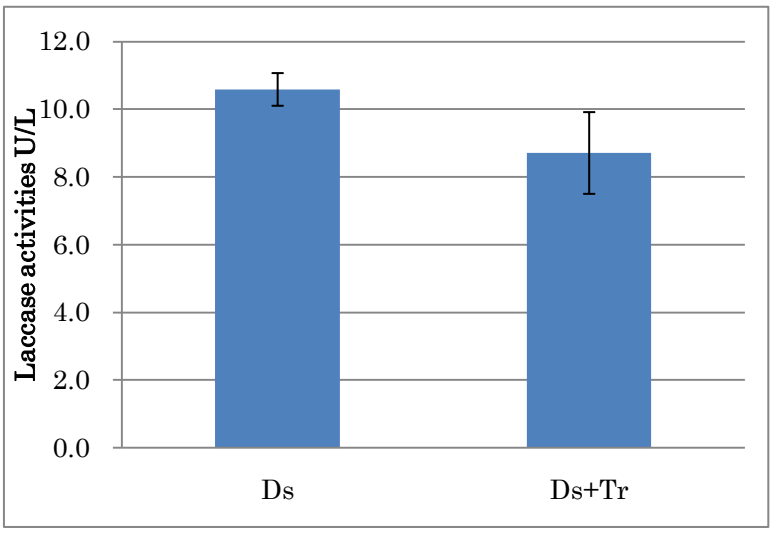


Fig. 3-28. Laccase activities cultivated in submerged red pine softwood powder media. A 2.5 g of red pine powder (40 mesh) was added into 25 ml deionized water and then autoclaved at 121 ℃ for 20 min.

4 Purification and characterization of laccases

4.1 Preface

D. squalens holds a group of completed extracellular enzymatic system degrading lignocellulosics. It does not only produce a set of Lignin-Modifying Enzymes (LMEs) consisting of laccases and manganese peroxidases (MnP) but also secretes a number of cellulases and hemicellulases.

In previous studies, two chromatographic isoforms of *D. squalens* laccases had been purified and characterized by Perie (1998) and Susla et al. (2007) and two laccases with different N-terminal amino acid sequences have been predicted from *D. squalens* gDNAs (Makela 2009) but no lignin peroxidase (LiP) activity is detectable in *D. squalens* cultures grown under a variety of conditions (Perie, 1991).

Although many researches concerning laccase inductions by various chemicals and biological interspecies interactions were reported, most of them concluded laccase induction only by laccase assay, but few performed a further analysis to the changes of laccase isoforms during the inductions.

This section aimed to investigate the make-up of laccase isoforms from *D. squalens* pure culture and co-culture with *T. reesei*, to find out which novel laccase(s) have/has been induced or not, and which of laccase isoforms had been increased by comparing the characteristics of isozymes.

4.2 Purification of laccases

In an attempt to purify laccase isoforms, the procedures were designed as follows:

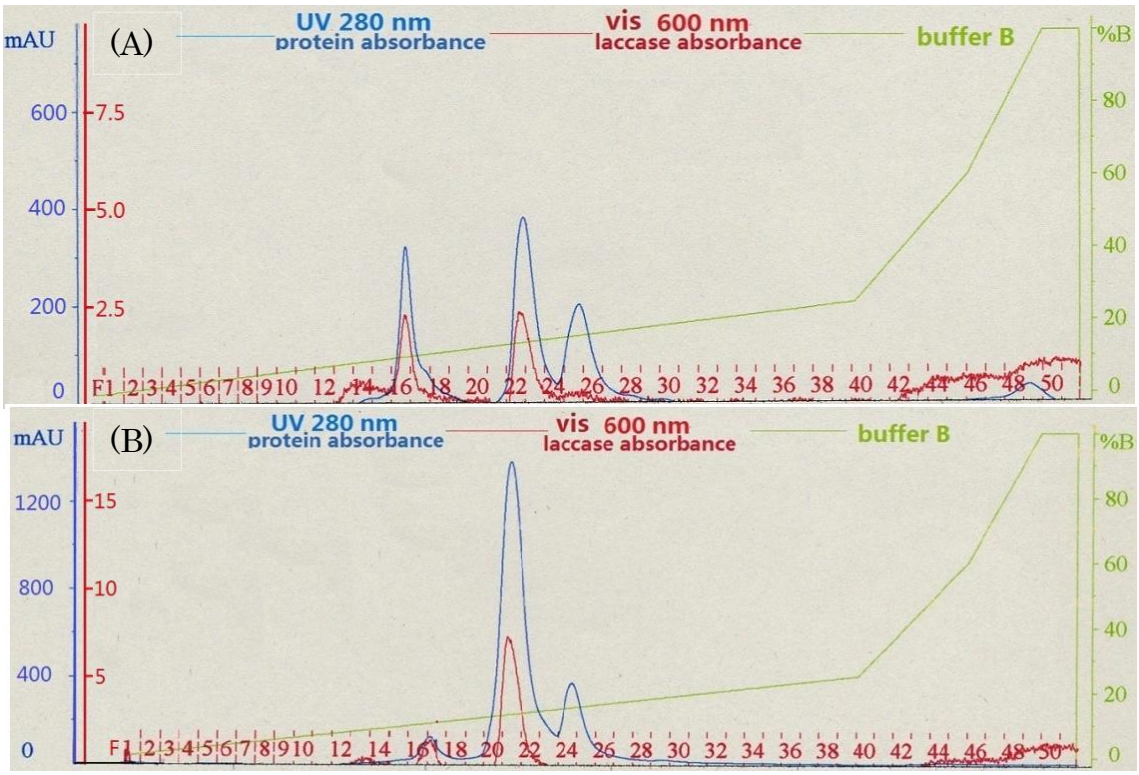
Ammonium sulfate precipitation →Open column →FPLC purifier (Q-sepharose FF column, MonoQ column) (Fig.2-1).

The supernatant after centrifugation to separate fungal spore and mycelia as well as other solid contaminants still contained a great amount of colloidal components, especially, soluble lignin, which had a series of continuous molecular masses with positive charge so that the physico-chemical properties coming from some of them were very similar to laccase, moreover, they had close affinities to laccase. These made it difficult to separate laccase from soluble lignin (They could not be separated by resin column separation or SDS-PAGE). Thus, processes such as ammonium sulfate precipitation and four open columns loading anion exchanger were used to remove the soluble lignins. A

35% saturation of ammonium sulfate could precipitate most of soluble lignins. The precipitates between 35%~85% were resolved and dialyzed in a 0.02 M Na-acetate buffer (pH 5.0), and then passed an open column (DE52), and diluted with 0.25 M NaCl in 0.02 M Na-acetate buffer (pH 5.0). The soluble lignins were partially retained in the column where the laccases were eluted out. The open column separation was repeated further for three times with the order of Q-sepharose FF, Q-sepharose FF and DE52 for the final purification step.

The purified laccases were applied to AKTA purifier (FPLC) to further separate the laccase isoforms. The laccase supernatants were loaded into a Q-sepharose Fast Flow column equilibrated with 20 mM Na-acetate buffer (pH 5.0) after dialysis, and eluted laccases with a gradient of 0~30% 1 M NaCl (in 20 mM Na-acetate buffer, pH 5.0). The Q-sepharose chromatography yielded a single peak with laccase activities but the peak showed an asymmetrical shape with a small shoulder (data not shown), which indicated that the laccases may have not been completely purified.

Then after dialysis equilibrated with 20 mM Na-succinate buffer (pH 4.5) (buffer A) to desalt, laccases were transferred and loaded into a monoQ column equilibrated with buffer A, and eluted by 15 ml (30 volumes of 0.5 ml fractions) of 1 M NaCl in 20 mM Na-succinate buffer (pH 4.5) (buffer B) with a gradient of 0~30%. Two peaks of laccases, Lac1 and Lac2 appeared, but the peak of Lac2 was still mixed with an unknown impurity. So the pooled laccases were loaded into monoQ column once again and eluted by 20 ml (40 volumes of 0.5 ml fractions) of buffer B with a gradient of 0~25%. The Lac2 peak was eventually separated from the impurity peak (Fig.4-1).



Note: Buffer A: 0.02M Na-succinate pH 4.5 Buffer B: 1M NaCl in buffer A

Fig.4-1. Elution profiles of laccases from pure culture (A) and laccases from co-culture (B).

Based from Fig4-1, there were two kinds of laccase isoforms, Lac1 and Lac 2 that were both in pure culture and co-culture, and Lac 1 was the minor laccase while Lac 2 represented the major laccase in *D. squalens* laccase systems. And the peak of Lac 2 was apparent to be amplified by induction, but it is unclear whether Lac 1 was induced or not.

Table 4-1 and 4-2 shows the summary of the laccase isozymes purification.

Table 4-1. Purification summary table of laccase isozymes from pure culture, *D. squalens*

Step		volume (ml)	Total Protein* (mg/ml)	Total activity (Unit/l)	Specific activity (unit/mg)	Yield (%)	Purity (%)
Crude supernantant		530	0.552	278	504000	100	0.06
Ammonium sulfate 40-80% cut		68	0.816	3010	3690000	138.8	0.32
open column	DE52 1	25	0.241	5147	21357000	87.3	2.97
	Q-sepharose1	13.2	0.288	6205	21579000	55.5	4.72
	Q-sepharose2	10.8	0.272	7253	26699000	53.1	6.11
	DE52 2	8.7	0.170	8519	50243000	50.3	12.15
FPLC**	Q-sepharose	14.7	0.058	4253	72741000	42.4	20.86
	MonoQ 1	6.03	0.068	3716	54647000	15.2	43.71
	MonoQ2 lac1 (Peak1)	1.261	0.009	41	45300000	0.04	100
	MonoQ2 lac2 (Peak2)	2.202	0.0814	5531	67948000	8.26	100

Note: * Protein concentration determined by Bio-Rad assay using BSA as a standard protein.

**the peak fractions from FPLC were pooled.

Table 4-2. The purification summary table of laccase isozymes from co-culture with *T. reesei*

Step		volume (ml)	Total Protein* (mg/ml)	Total activity (Unit/l)	Specific activity (unit/mg)	Yield (%)	Purity (%)
Crude supernantant		525	0.934	575	616000	100	0.05
Ammonium sulfate 35-85% cut		80	0.943	5289	5609000	130.8	0.30
open column	DE52 1	40	0.267	4246	15903000	52.5	2.09
	Q-sepharose1	20.8	0.185	6485	35096000	41.7	5.81
	Q-sepharose2	12.4	0.192	9474	49381000	36.3	9.39
	DE52 2	9.9	0.237	9611	40511000	29.4	9.51
FPLC**	Q-sepharose	12.5	0.095	8765	92206000	33.9	18.81
	MonoQ 1	6	0.089	5304	59596000	9.8	41.84
	MonoQ2 lac1 (Peak1)	0.8	0.020	1051	52853000	0.3	100
	MonoQ2 lac2 (Peak2)	0.90	0.231	11427	49505000	3.2	100

Note: as the same as Table 4-1

To find out whether Lac 2 from pure culture or Lac 2 from co-culture were of the same laccase isoform or not, many characteristics of them were determined.

4.3 Characterization of laccases

4.3.1 Molecular mass

The denatured mass determination of laccase isozymes were performed by SDS-PAGE (7.5%), as shown in Fig.4-2.

For the estimation of the native molecular mass, gel filtration was performed using Gel Filtration Standard (Bio-Rad) as molecular markers for the calibration. Each measurement of standards or samples was performed in triplicate.

The results of molecular mass were listed in Table 4-3

Table 4-3. The parallel of laccase isoform molecular mass

Laccase isoforms	denatured mass (SDS-PAGE)	native mass by HPLC
Lac 2 from pure culture	60	51.5
Lac 2 from co-culture	60	50.0

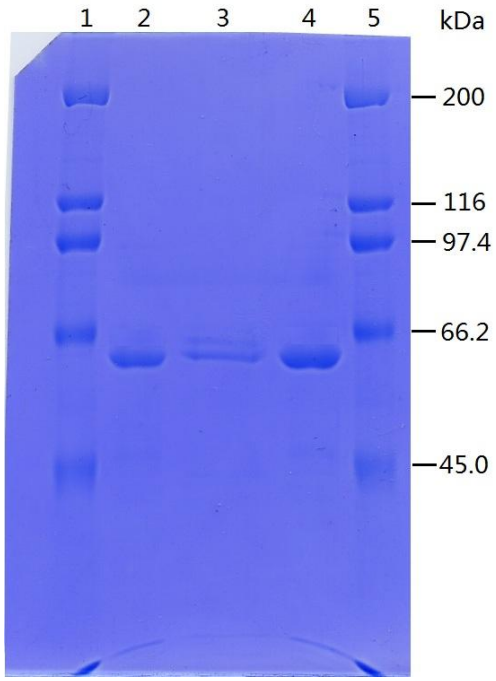


Fig. 4-2. SDS-PAGE (7.5%) of laccase isoforms from monoQ purification fractions. Lane 1 and 5, molecular mass markers; Lane 2, Lac 2 from pure culture; Lane 3, Lac 1 from co-culture; Lane 4, Lac 2 from co-culture.

4.3.2 Isoelectric focusing

A 40% ampholyte (Bio-Lyte 3/10 Ampholyte, Bio-Rad) was used for isoelectric focusing of laccase isoforms. And the pIs (denatured) of Lac2 from pure culture and co-culture were located at 3.8 and 3.7, respectively.

4.3.3 Optimal pH and temperature for laccase activities

Fig. 4-3 shows both the two origins of Lac2 exhibited by its optimal activities at pH 2.5 and the changes of their activities went through by almost same trend, which declined sharply when pH value dropped below 5.0, at which a standard method of laccase assay was adapted. The temperatures were fixed at 30 ℃ in all pH levels.

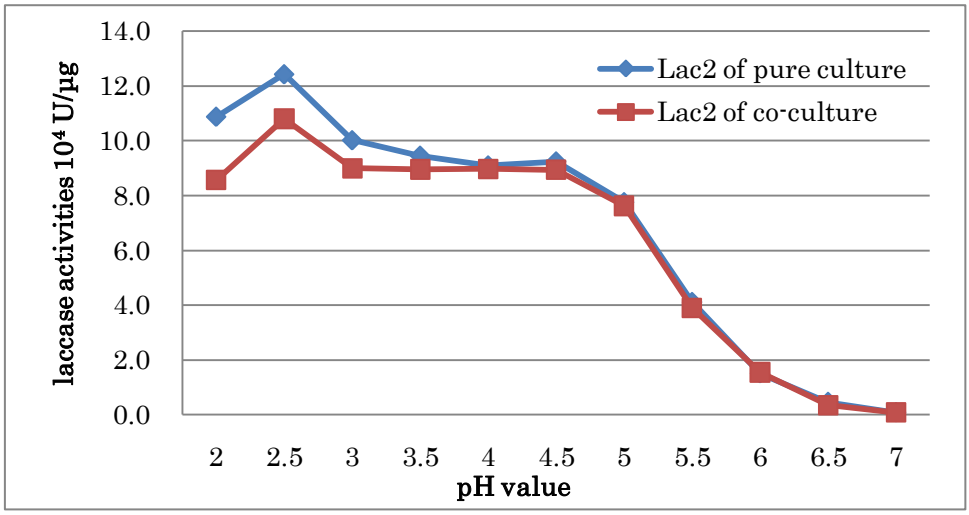


Fig. 4-3. Changes of laccase activities in response to different pH values. The temperatures were fixed at 30 ℃ in all pH levels.

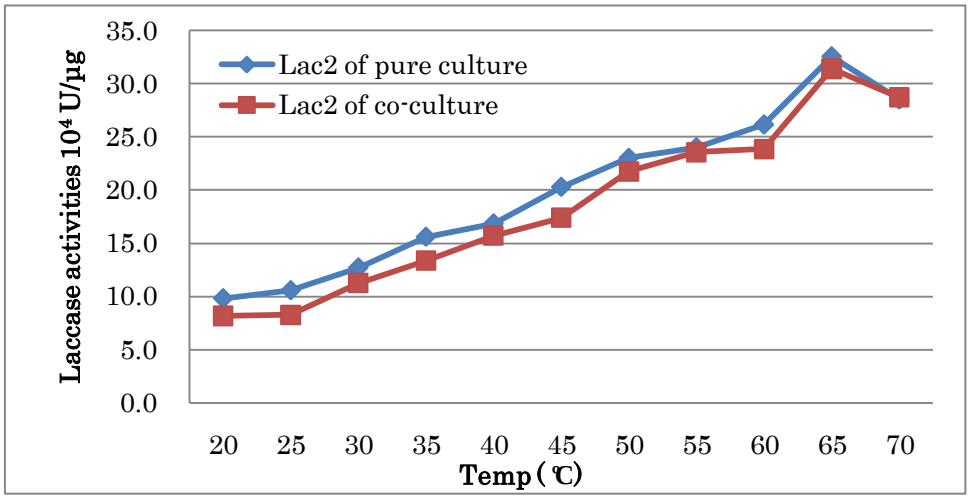


Fig.4-4. Changes of laccase activities in response to different temperature levels. The pH value was fixed at 5.0 in all temperature levels.

Fig.4-4 showed that the two origins of Lac2s had its highest activities both in 65 ℃, keeping an increasing tendency from 20 ℃ to 65 ℃ along the same trace curve.

Both Lac2s were stable for 7 d at 30 ℃ and about 4d at 40 ℃ (5% activities lost),

which were favorable for its applications. Their activities underwent a rapid decline when environmental temperatures ascent above 50 ℃. Further, it took only less than 2 days to loss 50% of their activities. Their activities could be kept only several minutes above 70 ℃.

4.4.4 N-terminal amino acid sequence

The N-terminal amino acid sequences of purified Lac2s were determined by sequencer through loading a blotted membrane from SDS-PAGE (10%).

The 20 N-terminal amino acids from two Lac2 were arranged as in the same sequence: GIGPVTDLTITNADIAPDDF.

Based on the characteristics and elution profiles between the two Lac2s (originated from pure culture and co-culture) that it can be concluded that no novel laccase was induced and the two laccases were of the same type of laccase.

5 Lignolytic pretreatment of rice straw by crude induced laccase

5.1 Preface

Compared to physico-chemical methods based on thermal or chemical pretreatments, biological pretreatments have no need for thermal energy, high pressure and chemicals addition, besides it, it has little toxic products or fermentation inhibitors produced.

Moreover, physico-chemical methods are always involved in requirements to treat the black pretreatment waste liquids containing high-concentration of aromatic compounds which are awfully recalcitrant to be treated using any common treatment technology such as anaerobic digestion, but it can only be evaporated and combusted, which, no doubt, would need to further cost more energies to abate its economic feasibility.

As mentioned above, this study took rice straw as objective biomass, biological pretreatment is suitable for herbaceous plants and agricultural biomass, because (1) Herbaceous plants and agricultural biomass are physically smaller and structurally weaker and lighter than woody biomass. Chemically, lignin content of herb is lower than woody biomass (about 10% and 30%, respectively) (Zhu et al, 2010). As a result, herbaceous plants are more liable to enzymatic actions than woody biomass. (2) Hemicelluloses content (mainly pentose polymer) of herbaceous plant (about 20~30%) exhibit higher than woody biomass (hardwood about 20%, softwood about 10%), and pentose recovery yield is often low due to its decomposition to furfurals in thermal-chemical pretreatments, in which the produced furfurals represent fermentation inhibitors.

However, current biological pretreatment works reported are much less than thermal and chemical pretreatment, and most of them pretreated biomass with fungi, but not with delignifying enzymes, besides it, it took so much time for as long as 30d to 60d to treat the biomass. This made its applications impossibly suitable for *in situ* industrial pretreatment for biomass fermentation.

As yet, there are no reports that lignocellulosic biomass was pretreated efficiently by laccase. Thus, this section has had a trial to delignify rice straw by crude laccase as pretreatment process using the alkaline pretreatment (NaOH) and without treatment as control experiments.

Researchers always concentrate their eyes in only one indicator such as change of

lignin contents to assess the pretreatment effect in biomass pretreatment process. Actually, it is not enough to evaluate the true state of the lignocellulosic biomass after pretreatment, especially for biological pretreatments. Cellulose exposure, partial degradation of residual lignin and soluble depolymerized lignin in solution are likewise important indicators for assessing the pretreatment effect. Thus, this study also aimed to develop a comprehensive method to assess pretreatment effect from various angles.

5.2 Biological pretreatment by laccase

The biological pretreatments were carried out using crude laccase from pure culture or co-culture. Laccase can directly degrade phenol-derivative compound, and indirectly degrade non-phenol compounds such as nonphenolic lignin moieties, through laccase mediator system, enabling the oxidation of compounds that are not oxidized by laccase. Furthermore, the mediators can diffuse far away from the laccase to sites that are difficult to reach by the enzyme itself (Camarero et al. 2005).

In this study, the laccase mediator system was constructed by preparing a solution containing a final concentration of 1 mM $CuSO_4$, 1% Tween 20, and 1 mM syringic acid (4-hydroxy-3,5-dimethoxybenzoid acid).

To obtain a precise pretreatment result, tetracycline and cycloheximide were added to pretreating system to prevent other microorganisms from degrading lignin or carbohydrates. Oxygen was flushed for one minute once a day into the vials to ensure that enough laccase oxidation.

Rice straw treated by 2% NaOH at 90 ℃ for 2 h was used as positive control and untreated rice straw soaked in deionized water as negative control for laccase-pretreated straw.

5.2.1 The changes of rice straw compositions

Crude laccases from Ds-pure culture and co-culture can delignify lignin of rice straws in a period of as few as 5d by 44% and 59%, respectively, which were fairly well-degraded. In view of typically low degradation rate and ratio of lignin by biological pretreatment such as fungal degradation for even as long as 30-60 days had been also reported (Taniguchi et al, 2005 and Shi et al., 2008). Alkaline pretreatment (NaOH) can remove 95% of lignin (Fig.5-3), but it also resolved over half of hemicellulose and soluble materials (such as starch). These constituents could have been utilized to sugar fermentations, while the samples treated by crude laccase retained most of solubles and hemicellulose.

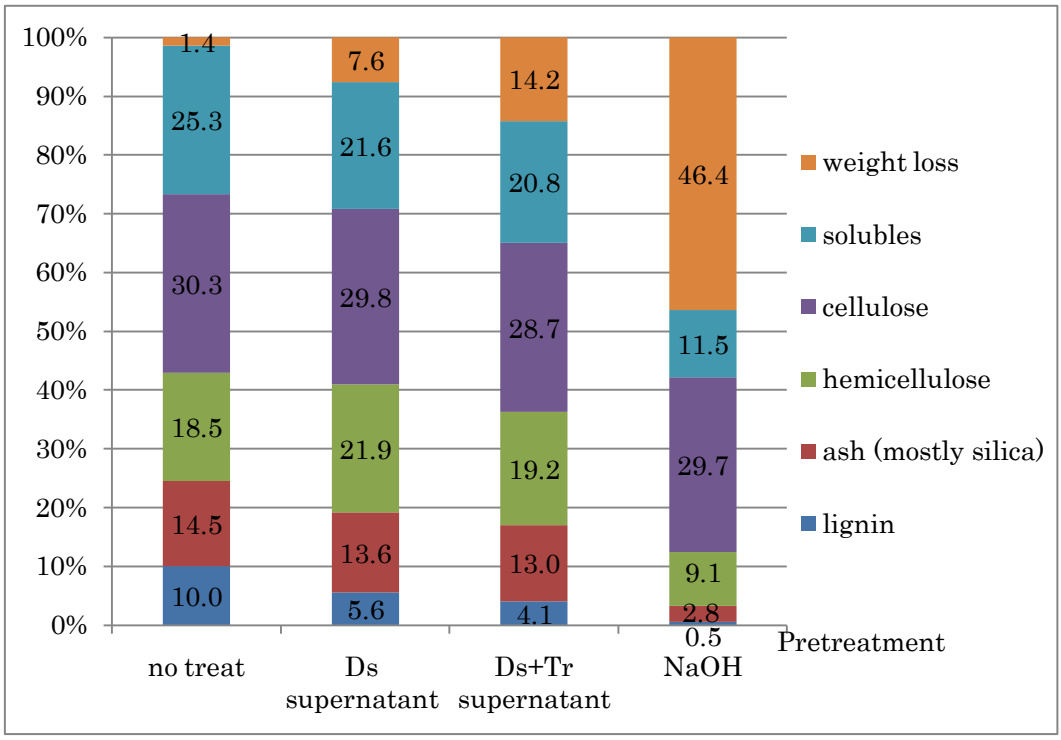


Fig. 5-1. Constituents of rice straw after pretreatment

5.2.2 Complete oxidation of soluble lignin

Fig. 5-1 shows the results of soluble lignin concentration in the liquid residues after pretreatment of rice straw. The residual aromatic compounds in pretreated liquids by NaOH showed a very high concentration (104.4 g/l), which always became the conundrum to researchers pursuing the work on biomass utilization, while, favorably, in the laccase-pretreated liquid residues, concentrations of aromatic compound pretreated by Ds pure culture supernatant or Ds+Tr co-culture supernatant were even lower than no-treated samples, especially in Ds+Tr co-culture laccases despite there was a high initial lignin concentration before pretreatment (Fig. 5-2) and remarkable dissolved lignins depolymerized from rice straw (Fig. 5-1). This showed that the considerable depolymerized lignin in liquids had completely oxidized by laccases.

For the soluble lignin concentration determinations were conducted by absorbance assay at 280 nm wavelength, at which proteins also produce absorbance. Thus it was necessary to analyze the error from protein absorbance. It is well-known that the benzene rings from the amino acids, phenylalanine, tyrosine and tryptophan, bring protein absorbance at 280 nm wavelength, which is theoretically the same as soluble lignin, the aromatic group matrix, in which all three basic units contain one benzene ring. From

Table 4-1 and 4-2, crude laccase supernatant from pure culture or co-culture contains 0.552 and 0.934 g/l proteins, respectively. The contents of benzene ring in proteins are far less in lignins, moreover, the absolute concentration of proteins in crude supernant, 0.552 and 0.934 g/l, were much less than the absolute concentrations of soluble lignins, 11.6 g/l and 10.8 g/l, respectively. Thus, it was concluded that the absorbance error brought from proteins could be ignored.

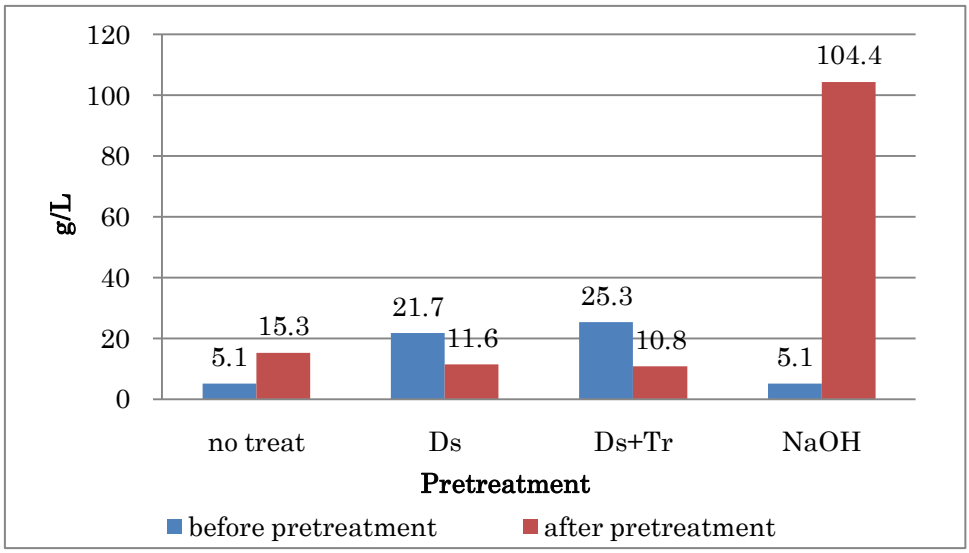


Fig. 5-2. Changes of soluble lignin concentration in pretreated liquid residues

5.2.3 Partially deploymerized lignins in solid residues

The pretreated rice straw contained partially depolymerized lignins that were still attached to the rice straw, which also represented the pretreated states to some extent. The partially depolymerized lignin was determined by extraction for 2 days using 92% dioxane, and then the extracted lignin was checked at 280 nm wavelength. The un-treated rice straw contained 10% of lignins (Fig 5-1).

From Fig.5-3, by analysis of dioxane extraction from pretreated solid residues, it can be observed that based on the lignin removed, a ratio of 44% and 59% (Fig. 5-1), 18.6% and 24.7% lignins have been partially depolymerized by laccase from Ds pure culture and co-culture, respectively. And NaOH treatment partially depolymerized 5.9% of lignins, which occupied all of the lignins (5.0%) that were not removed from rice straw (Fig.5-1).

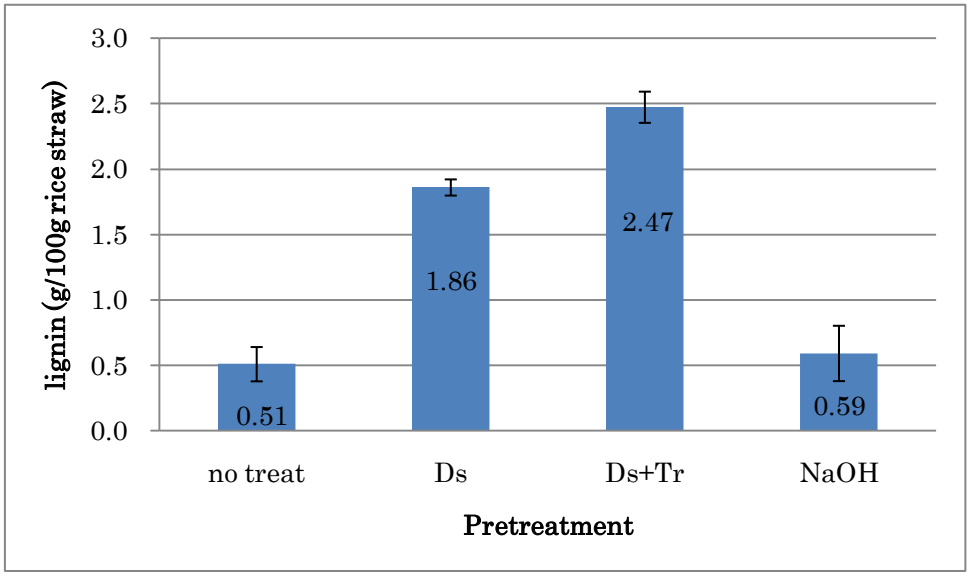


Fig.5-3. Extractable lignin in pretreated solid residue by dioxane

5.2.4 Saccharification ratio

After pretreatment of rice straw, saccharification processes were conducted and this started by the addition of cellulases for 2 days.

Based on Fig.5-4, an unsatisfactory result was observed that the glucose concentrations from samples pretreated by crude laccases hardly made any difference with that from no-treat samples.

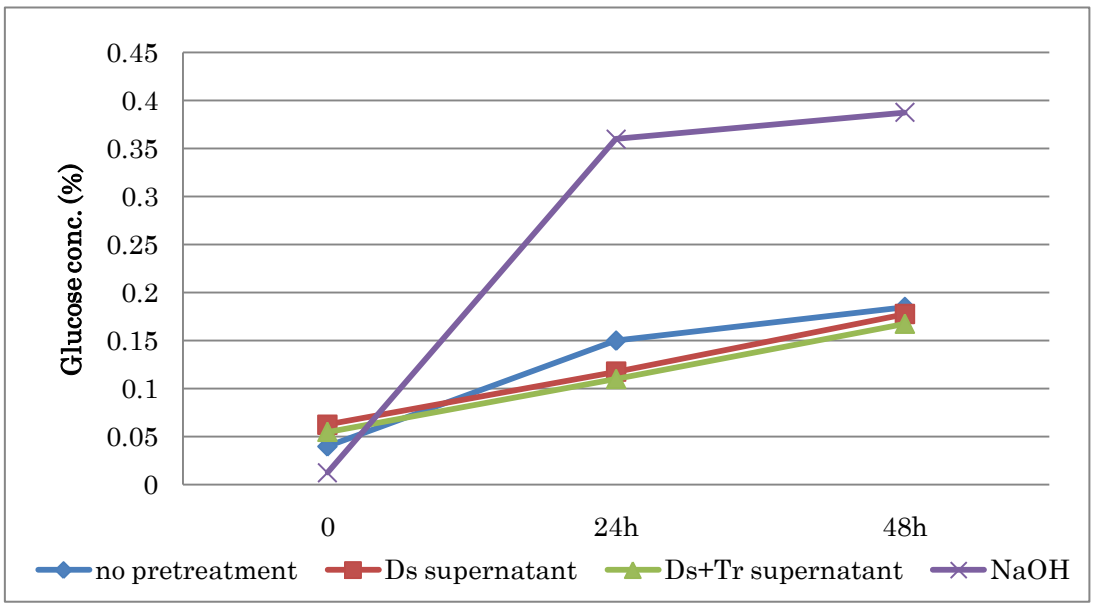


Fig. 5-4. Glucose concentration by saccharification after pretreatment

5.2.5 Increase of cellulose exposure

To investigate the phenomenon of no obvious saccharification result (Fig. 5-4) but considerable delignification from rice straw (Fig. 5-1 and 5-3), Simon's stain was performed to determine the cellulose exposure and rice straw porosity.

Simon's stain is based on the competitive adsorption of two dyes (Direct Blue 1 and Direct Orange 15) in an aqueous environment. Blue dye molecules are small and can penetrate small pores within the biomass, while the large orange dye molecules have greater affinity for the hydroxyl groups of cellulose, thus replacing blue dye molecules in large pores. Thereby, the amount of orange and blue dyes adsorbed by the biomass can efficiently reflect the improvement of cellulose exposure and biomass porosity caused by pretreatment.

Results in Fig. 5-5 shows that the increase of exposed cellulose was little after pretreated by crude laccases, and from Fig.5-1, it was observed that there were negligible silica (ash) removals, showing that although lignins had been remarkably removed, celluloses were not exposed and still encrusted by silica.

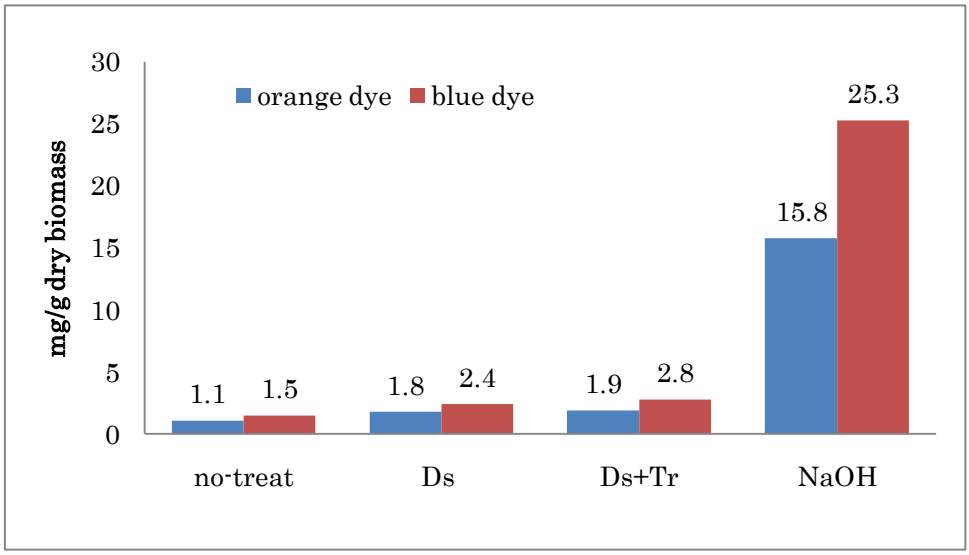


Fig. 5-5. Pigment adsorption by simon's stain method

In summary, crude laccases from Ds pure culture and co-culture can depolymerize lignin of rice straws by 44% and 59%, respectively. Moreover, it can be observed that 18.6% and 24.7% rice straw have been partially depolymerized by laccase from Ds pure culture and co-culture, respectively, and what was more favorable is that, in the laccase-pretreated liquid residues, concentrations of aromatic compound were even lower than not-treated samples. This indicates that the depolymerized lignin in liquids had

completely oxidized. Although alkaline pretreatment (NaOH) delignified 95% of lignin (Fig.3) and all the 5% lignin residue can also be extracted by dioxane as partially degraded lignin, the residual aromatic compounds in pretreated liquids showed a very high concentration (104.4 g/L). Results of saccharifying the pretreated rice straw showed that there was no change in detected glucose concentration compared to not-treated straw. Further analysis of adsorption methods showed that the increase of exposed cellulose was little and cellulose was still encrusted by silica.

6 Conclusions

This thesis described the promotion of laccase expression by microorganism interspecies interaction, analyzed the inductive materials in the induction course, and then purified the induced laccase and found out that the induced laccase and original laccase are of the same laccase, at last, had an application of the induced laccases to biomass pretreatment. The main findings and conclusions obtained from this work were:

1. Fungi No.5 (*Trichoderma reesei*) and No.6 (*Aspergillus nidulans*) exhibited much more extraordinary CMCase and xylanase than other fungi using avicel and xylan as substrates.

2. Fungus No.8 (*Dichomitus squalens*) exhibited predominant laccase and manganese peroxidase (MnP) activities, when cultivated in submerged rice straw media, which was much higher than the other fungi including the famous wood-rotting fungi, *Pleurotus ostreatus, Trametes versicolor, Phanerochaete chrysoporium*. Fungus No. 10, *Armillaria mellea*, can be considered as a favorable fungus for biopretreatment of lignocelluloses due to its ideal enzymatic characteristics of its fairly efficient and stable laccase activity but weak cellulase and hemicellulase activities.

3. To construct the synergic combination between two classes of fungi, four pairs of two-species groups, *D. squalens* and *T. reesei*, *D. squalens* and *A. nidulans*, *A. mellea* and *T. reesei* or *A. mellea* and *A. nidulans* had been incubated for 40d in rice straw by simultaneous inoculation or successive inoculation (the latter was inoculated after the former had been cultivated for 20d). Results showed that laccase activities were hardly detected in all simultaneous inoculated co-cultures during the whole incubation, but in successive inoculated co-cultures, the laccase activities in pure culture phase (for 20 days) can be amplified by inoculating the latter fungus in three two-species groups, *D. squalens* and *T. reesei*, *D. squalens* and *A. nidulans*, or *A. mellea* and *T. reesei*. Significantly, laccase activity was upgraded over 20 folds in successive inoculated co-culture of *D. squalens* and *T. reesei*, and over 10 folds in successive inoculated co-culture of *D. squalens* and *A. nidulans*.

4. Although the laccase activities were amplified over 20 folds from 20d to 30d, the fungal quantities of *D. squalens* and *T. reesei* had not been magnified conspicuously. Besides it, *T. reesei* itself produced no laccases in rice straw media. Thus it could be concluded that the great increased laccase activities was not derived from synergic combination between fungi due to no obvious fungal growth but from the increment of laccase expression regulated by some inductive effects

produced by interspecies interactions.

5. *D. squalens* produced laccase at maximum levels until the fruit body morphogenesis like most basidiomycetes, having the most active ability of secreting laccase at about 9-12 days. So the most feasible timing of inducing laccase would be about 10d. According to the results of induction length experiment, a 5-day length of induction by co-culture was adopted. 10-day incubation followed by a 5-day co-culture with *T. reesei* showed an induced laccase activity of 555 U/L, which was significantly higher than the laccase activity of 137U/L at 30d (20-day incubation followed a 10-day co-culture).

6. N nutrient exhibited no inductive effects of laccases, even conversely suppressed laccase production from *D. squalens* and copper ion showed weak induction with *D. squalens*, laccase increased was only about 1.1 folds. 2,5-xylidine exhibited its vigorous competence of inducing *D. squalens* laccase with about 2.5 folds yield upgrade while it was overshadowed by the laccases activities of 1020 U/L induction of about 4-folds by co-culture with *T. reesei*. The combination of two most efficient inducers, 2,5-xylidine and co-culture with *T. reesei* can further promote laccase induction and achieve more prolific induced laccases (1340U/L).

7. No induction appeared between *D. squalens* and *T. reesei* in PDB media as rice straw media and furthermore the laccase yields were very poor (about 10 U/L) in both pure culture and co-culture, but the addition of cell-free supernatant from pure culture *T. reesei*'s 5-day cultivation in rice straw (Supernatant Tr) to PDB media cultivating *D. squalens* could strongly induce laccase, and the autoclaved supernatant can also bring laccase induction, indicating strongly that they may be non-protein materials related to lignin or its derivatives.

   All Supernatant Tr's fractions divided by 5K or 10K cut-off ultrafilter can induce laccase yields. This indicated that not only a sole component but a series of materials with continuous molecular sizes from 0 to over 10KD induced *D. squalens* laccases and may be related to lignin. To investigate the relations between inductive effects and lignin, three basic lignin component Units, p-hydroxyphenyl (H) acid, Guaiacyl (G) acid and syringyl (S) acid were added to *D. squalens* non-lignin PDB media, respectively, and S unit was found to be able to induce laccase at the same extent as rice straw. By further component analysis of Supernatant Tr, about a 3 folds of S component unit concentration in Supernatant Tr was detected than in supernatant from *D. squalens* culture in rice straw. No laccase inductions appeared in 10d cultivated *D. squalens* induced by 5d co-culture with *T. reesei*, under submerged powder media of red pine, a softwood species, in which lignin do not contains the S unit.

Based on the results above, it was undoubted that the partially degraded lignin with 0 to over 10KD molecular mass degraded by *T. reesei* had induced laccase expression from *D. squalens* has been proved.

8. After the removal of soluble lignins by ammonia sulfate precipitation and open columns, the purified laccases were applied to AKTA purifier to further separate laccase isoforms. Results showed that there were two chromatographic laccase isozymes, Lac1 and Lac2, both in pure culture and co-culture, and Lac 1 was the minor laccase while Lac 2 represented the major laccase in *D. squalens* laccase systems. And the peak of Lac 2 was apparent to be amplified by induction, but it is unclear whether Lac 1 was induced or not.

9. By comparison of molecular masses, pIs, optimal pH and temperature for laccase activities, N-terminal amino acid sequences and elution profiles between two Lac2, it can be concluded that no novel laccase was induced and the two laccases were of the same type.

10. Although alkaline pretreatment (NaOH) can remove 95% of lignin and all of 5% residual lignin can be extracted for partially depolymerization, it also resolved over half of hemicellulose and solubles (such as starch) and residual aromatic compounds in pretreated liquids by NaOH showed a very high concentration (104.4 g/l), which always became the conundrum to researchers pursuing the work on biomass utilization. Crude laccases from Ds-pure culture and co-culture can delignify lignin of rice straws in a period of as short as 5d by 44% and 59%, respectively, which were fairly well-degraded in view of typical low degradation rate and ratio of lignin by biological pretreatment such as fungal degradation for even as long as 30-60 days. Moreover, 18.6% and 24.7% lignins have been partially depolymerized by laccase from Ds pure culture and co-culture, respectively. What was more favorable is that, in the laccase-pretreated liquid residues, concentrations of aromatic compound were even lower than no-treated samples despite there was a high initial lignin concentration before pretreatment and remarkable dissolved lignins depolymerized from rice straw. This showed that the depolymerized lignin in liquids had completely oxidized.

11. After pretreatment of rice straw, saccharification processes were conducted. An unsatisfactory result was observed that the glucose concentrations from samples pretreated by crude laccases hardly made any difference with that from no-treat samples. To investigate the phenomenon of no obvious saccharification result but considerable delignification from rice straw, Simon’s stain was performed to determine the cellulose exposure and rice straw porosity. Results shows that the increase of exposed cellulose was little after pretreated by crude laccases, and from component analysis, there were negligible silica (ash) removals, showing

that although lignins had been remarkably removed, celluloses were not exposed and still encrusted by silica.

In sum, *D. squalens* represented more efficient lignin-degrading fungus especially in rice straw media than many famous wood-rotting fungi and its laccase yields can be strongly induced by co-culture with *T. reesei*. The partially degraded lignin by *T. reesei* strongly induced the *D. squalens* laccase, Lac 2. The crude induced laccase delignified 59% of rice straw lignin, and further partially depolymerized 24.7% of lignin in a period of as few as 5d and moreover, thoroughly decomposed these fall-off lignin to non-aromatic materials.

Though it cannot remove silica affecting saccharification from rice straw, still laccase use is promising for pretreatment of herbaceous plants and woody materials with low silica content.

In future, it is prospective that the biological pretreatment of biomass by laccase can bring more considerable and rapid lignin depolymerization by the following ways:

a) Screening and combination of co-culture and other inductive factors to arouse a more fruitful laccase yields.
b) Finding out some more proper laccase mediators to construct more efficient laccase mediator system.
c) Having a trial to make a cocktail with crude induced laccases and various manganese peroxidases to get an enzymatic synergy existing in nature commonly found in plants or microorganisms.

The laccase-encoding gene and promoter sequence will be determined and its transcription in the induction course will be also investigated.

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Abstract

論文の内容の要旨

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論文題目

The promotion of laccase expression induced by interspecies interaction and its application to biomass pretreatment

（微生物間相互作用を利用したラッカーゼ生産とそのバイオマス前処理への応用）

Laccases have been paid much attention both from researchers and from industries in recent decades due to their possible involvement in the transformation of a wide phenolic and non-phenolic compounds and lignin, as well as bioremediation of highly recalcitrant aromatic environmental pollulants.

In nature, there are several symbioses, synergies or competitions between or among various microorganisms, and interestingly, the secretion of fungal laccase is also involved in the biocontrol phenomenon. It is found that some fungi can promote the production of laccase during interactions with other organisms. This leads us to a possible way to upgrade laccase production to solve environmental problems, and to make or improve pretreatment processes of lignocellulosics such as by interspecies interactions.

Therefore, the aim of this study was to screen out the active lignolytic fungi and laccase-promoting interspecies interactions among white-rot fungi and its application to the pretreatment process of lignocellulosic biomass.

Chapter I　Laccase expression induced by co-culture and inductive materials

Firstly, this section was performed to screen out the most highly efficient cellulolytic fungi and the most active ligninolytic fungi from these 10 species of fungi shown in Table 1:

It was found that *T.reesei* (No.5) and *A.nidulans* (No.6) were the most efficient to degrade avicel and xylan (as culture media), while *D. squalens* (No.8) and *A. mellea* (No.10) exhibited the most predominant ability to delignify rice straw by the indicators of laccase and MnP activity.

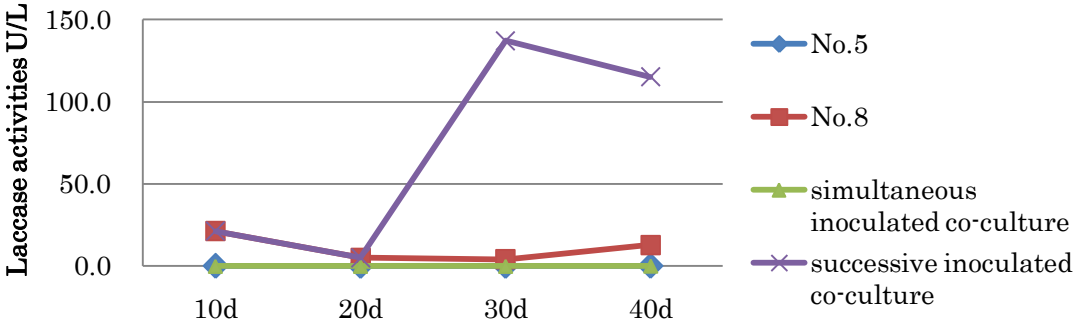
It is postulated that the two cellulolytic fungi and two lignolytic fungi would cooperate each other due to the synergy of cellulases and lignolytic enzymes in degradation of lignocellulosics. To construct the synergic combination between fungi, four sets of two-species groups, *D.squalens* and

Table1 White-rot basidiomycete strains used in this study.

No.	Fungus Code	Species of fungus	No.	Fungus Code	Species of fungus
1	NBRC 30776	*Pleurotus ostreatus*	6	NBRC 4340	*Aspergillus nidulans*
2	NBRC 30388	*Trametes versicolor*	7	DSMZ 1016	*Panus tigrinus*
3	NBRC 9076	*Cyathus stercoreus*	8	DSMZ 9615	*Dichomitus squalens*
4	ATCC 90467	*Ceriporiopsis subermisporo*	9	DSMZ 6909	*Phanerochaete chrysoporium*
5	NBRC 31326	*Trichoderma reesei*	10	NBRC 7037	*Armillaria mellea*

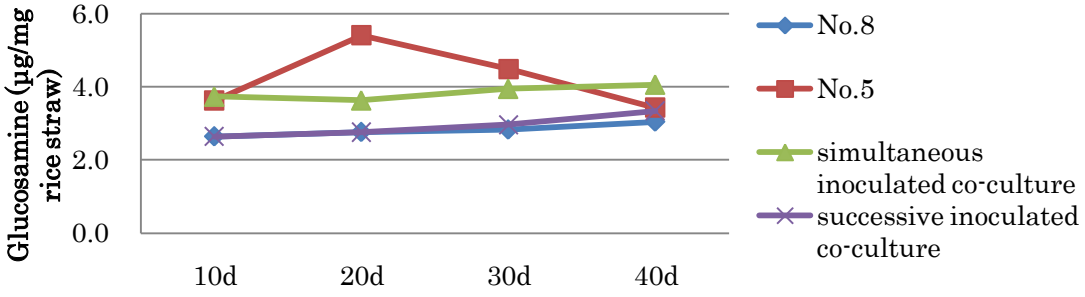
T. reesei, or *A.mellea* and *A.nidulans* have been incubated for 40d in rice straw by simultaneous inoculation or successive inoculation (the latter was inoculated after the former had been cultivated for 20d). Unexpectedly, hardly laccase activites were detected in all simultaneous inoculated co-cultures during the whole incubation, but in successive inoculated co-cultures, the laccase activities in pure culture phase (for 20 days) can be amplified by inoculating the latter fungus in three two-species groups, *D.squalens* and *T.reesei*, *D. squalens* and *A.nidulans*, or *A. mellea* and *T. reesei*, significantly, laccase activity was upgraded 20 folds in *D.squalens* and *T.reesei*, and 7 folds in *D. squalens* and *A.nidulans*.

Fig.1 Laccase activities of *D. squalens* (No.8) and *T.reesei* (No.5)



This study took the group of *D.squalens* (Ds) and *T.reesei* (Tr) as an example to verify the phenomenon of laccase increase. Obviously, it was shown that laccase from *D.squalens* was induced by inoculation of *T.reesei*. It was not because cell growth by some synergies between them brought laccase increase as *T.reesei* showed little growth in successive inoculated co-culture after vaccination in 20d and since 20d, there was no obvious changes of D.squalens's quantities, too (Fig.2), and moreover, no laccase activity was detected in *T.reesei*'s pure culture (Fig.1).

Fig.2 The microbial quantities during incubation



What are the inductive materials? No laccase induction phenomenon appeared in non-lignin media(PDB) by the same way of successive inoculated co-culture, but the addition of cell-free supernatant from pure culture *T.reesei*'s 5-day cultivation in rice straw (Supernatant Tr) to PDB media cultivating *D.squalens* could strongly induce laccase, and the autoclaved supernatant can also bring laccase induction. So the inducers proved to be heat-stable, and may relate to lignin or its derivatives. Then three basic lignin component Units, p-hydroxyphenyl (H) acid, Guaiacyl (G) acid and syringyl (S) acid were added to *D.squalens* non-lignin PDB media, respectively and S unit was found to be able to induce laccase at the same extent as rice straw.

Additionally, all the ultrafiltrate fractions from Supernatant Tr by 10K-cut-off or 5K-cut-off ultrafilter can actively induce laccase as Supernatant Tr, which shows that not only a sole component but a series of materials with continuous molecular sizes from 0 to over 10KD containing S unit can be the inductive materials, therefore it is concluded that the partially degraded lignin with 0 to over 10KD molecular mass degraded by *T.reesei* have induced laccase expression from *D. squalans*.

To verify this postulation, soluble lignin in Supernatant Tr was completely depolymerized to monomolecular lignin component units by alkaline nitrobenzene oxidation and about a 3 folds of S component unit concentration was detected than in *D.squalens* culture in rice straw. Besides it, few laccases were found in *D. squalens* culture cultivated in submerged powder media of red pine, a softwood species, in which lignin contains no S unit. Undoubtedly, the conclusion above has been proved.

Chapter II Purification and characterization of pure culture's laccase and induced laccase

In this chapter, the laccases from *D.squalens* pure culture and induced by co-culture were purified and characterized. Two kinds of laccases, Lac1 and Lac 2 have been purified, and Lac 1 was the minor laccase while Lac 2 represented the major laccase in *D.squalens* laccase systems. And the peak of Lac 2 was apparent to be amplified by induction, but it is unclear whether Lac 1 was induced or not.

Table 2. Comparison on the characteristics between laccase from pure culture or co-culture

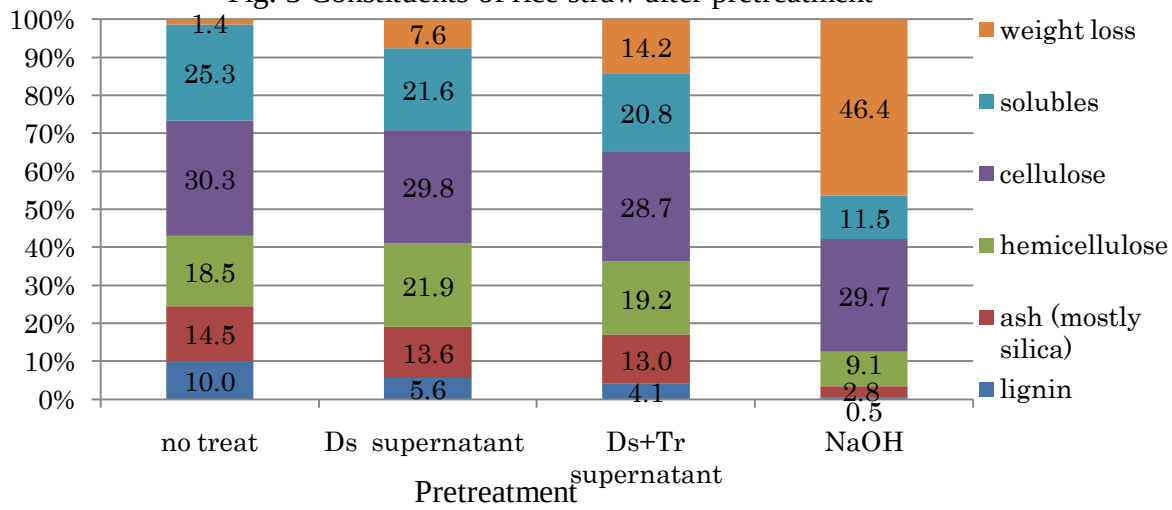
Parameters	**Lac 2 from pure culture**	**Lac 2 induced by co-culture**
Molecular mass	60 (denatured); 51.5 (native)	60 (denatured); 50.0 (native)
Isoelectric focusing	3.8	3.7
Temp. of optimal activitiy	65 °C	65 °C
pH of optimal activity	2.5	2.5
N-terminal amino acid sequence	GIGPVTDLTITNADIAPDDF	GIGPVTDLTITNADIAPDDF

Based on the characteristics between two Lac 2 (from pure culture and co-culture) shown in Table 2 and elution profiles, it was concluded that no novel laccase was induced and the two laccases are of the same laccase.

Chapter III Lignolytic pretreatment of rice straw by crude induced laccase

As yet there are no reports that lignocellusic biomass was pretreated efficiently by laccase. This work has had a trial to delignify rice straw by crude laccase as pretreatment process using the alkaline pretreatment (NaOH) and no treatment as control experiments and assessed its effect from various angles.

Fig. 3 Constituents of rice straw after pretreatment



From Fig.3, crude laccases from Ds pure culture and co-culture can depolymerize lignin of rice straws by 44% and 59% in 5d, respectively. Moreover, by further analysis of dioxane extraction from pretreated solid residues, it can be known that 18.6% and 24.7% rice straw have been partially depolymerized by laccase from Ds pure culture and co-culture, respectively, and what is more favorable is that, in the laccase-pretreated liquid residues, concentrations of aromatic compound were even lower than no-treated samples. This showed that the depolymerized lignin in liquids had completely oxidized. Although alkaline pretreatment (NaOH) delignified 95% of lignin (Fig.3) and the 5% lignin residue can also be extracted by dioxane as partially degraded lignin, the residual aromatic compounds in pretreated liquids showed a very high concentration (104.4 g/L).

Moreover, results of saccharifying the pretreated rice straw showed that there was no change in detected glucose conc. compared to not-treated straw. By further analysis of adsorption technique using two dyes having high affinity to cellulose, results showed that the increase of exposed cellulose was little, and from Fig.3, negligible silica (ash) removal was observed, showing that cellulose was still encrusted by silica.

Conlusions

D.squalens is a more efficient lignin-degrading fungus in rice straw media than many famous wood-rotting fungi and its laccase production can be further induced by co-culture with *T.reesei*. The partial degraded lignin by *T.reesei* strongly induced the *D.squalens* laccase, Lac 2. The crude induced laccase delignified 59% of rice straw lignin, and further partially depolymerized 24.7% of lignin and moreover, thoroughly decomposed these fall-off lignin to non-aromatic materials.

Though it cannot remove silica affecting saccharification from rice straw, still laccase use is promising for pretreatment of herbaceous plants with low silica content.