Biotechnol. Bioprocess Eng. 2000, 5: 422-430

Fungal Metabolism of Environmentally Persistent Compounds
: Substrate Recognition and Metabolic Response

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Abstract Mechanism of lignin biodegradation caused by basidiomycetes and the history of lignin biodegradation studies were briefly reviewed. The important roles of fungal extracellular ligninolytic enzymes such as lignin and manganese peroxidases (LiP and MnP) were also summarized. These enzymes were unique in their catalytic mechanisms and substrate specificities. Either LiP or MnP system is capable of oxidizing a variety of aromatic substrates via a one-electron oxidation. Extracellular fungal system for aromatic degradation is non-specific, which recently attracts many people working in a bioremediation field. On the other hand, an intracellular degradation system for aromatic compounds is rather specific in the fungal cell. Structurally similar compounds were prepared and metabolized, indicating that an intracellular degradation strategy consisted of the cellular systems for substrate recognition and metabolic response. It was assumed that lignin-degrading fungi might be needed to develop multiple metabolic pathways for a variety of aromatic compounds caused by the action of non-specific ligninolytic enzymes on lignin. Our recent results on chemical stress responsible factors analyzed using mRNA differential display techniques were also mentioned.

Keywords: aromatic pollutants, basidiomycetes, bioremediation, chemical stress, lignin degradation, stress response

Lignin is the most abundant renewable aromatic polymer, and is known as one of the most recalcitrant biomaterials on earth [1-3]. Lignin is a phenylpropanoid polymer (Fig. 1) synthesized from coumaryl, coniferyl, and sinapyl alcohols. Radical condensation of these precursors, catalyzed by plant cell wall peroxidases, results in the formation of a heterogeneous, amorphous, optically inactive, random, and highly branched polymer containing almost all kinds of C-C and C-O linkages connecting aromatic nuclei [1,2]. The ether linkage has been known for showing a strong resistance to microbial decomposition. Since more than 50% of inter-unit linkages was found to be an ether-type bond in lignin, it retards the microbial decomposition of cellulose, the most prevalent natural polymer. Thus, the degradation of lignin plays a key role in the carbon cycle of the biosphere [4-6].

Only basidiomycetes have been known to be responsible for the complete mineralization of this polymer in pure culture [3-6]. Initially, much work was done to understand the chemistry of wood decayed by these fungi. Elemental and functional analyses provided the evidence that the degradation of lignin by fungi is an oxidative process [7]. Structural analysis of lignin fragments released during wood decay demonstrated that lignin degradation occurred through C-C bond cleavage

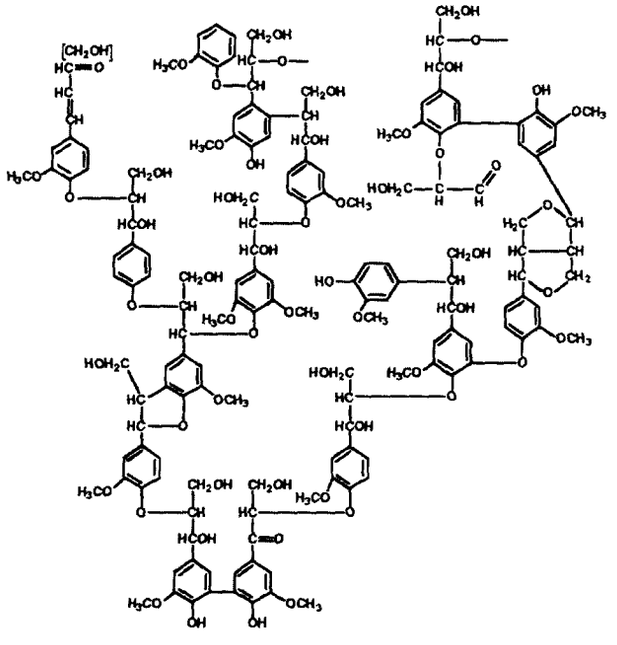

Fig. 1. Schematic structure for conifer lignin.

at C_α-C_β in propyl side chains and at alkyl-phenyl linkages as well as C-O bond cleavage at β-ether linkages in aryl ether structures [8-10]. ^{13}C-NMR study indicated that aromatic ring oxidation and fission took place

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within the polymer [11].

Phanerochaete chrysosporium has become the model for the lignin degrading fungus, because (a) it efficiently degrades both lignin and cellulose, (b) it is thermotolerant (an optimal temperature of 38°C), (c) it produces asexual (conidia) spores prolifically —an advantage for genetic manipulation—, and (d) it has been shown to form sexual fruiting structures in culture [12,13]. Besides *P. chrysosporium*, *C. versicolor* is a popular fungus in Japan because it is a JIS standard strain for wood deterioration, as well as several other edible mushrooms, such as *Lentinus edodes* (Shiitake) and *Pleurotus ostraeatus*. In this paper, the data obtained using *P. chrysosporium* and *C. versicolor* are mainly mentioned.

By determining $^{14}CO_2$ released from ^{14}C-DHP (^{14}C-labeled chemically synthesized lignin) under various physiological conditions, it has been established that the ligninolytic activity in *P. chrysosporium* is not inducible by exogenously added lignin, but rather by the limitation of nutrient carbon, sulfur, or nitrogen, which triggers the onset of secondary metabolism [7,14]. In the middle of 1980s, it had been established that the biodegradation of lignin was oxidative, extracellular, nonspecific with regard to substrate, and a secondary metabolic activity.

In 1983, two research groups in US announced simultaneous discovery of lignin peroxidase (LiP), an extracellular heme peroxidase involved in lignin degradation in *P. chrysosporium* [15-18]. Subsequently, manganese peroxidase (MnP) was isolated from the extracellular culture fluid of *P. chrysosporium* [19]. These two enzymes, LiP and MnP, along with H_2O_2-generating system [4,5] appear to be the major components of the extracellular lignin degradation system, a non-specific and strong one-electron oxidizing system.

Although basidiomycetes extracellularly utilize nonspecific oxidation system for aromatic compounds metabolism, they seemed to have highly regulated and very specific metabolic systems for each substrate. For example, it has been recently shown that *C. versicolor* metabolizes sulfur-containing heterocyclic compounds [20]. Then, we prepared a series of structurally similar thiophene derivatives and determined their detailed metabolic pathways, strongly suggesting that the fungus had the system for substrate recognition and metabolic response. These abilities might be needed for fungi to achieve a complete mineralization of lignin, because a variety of aromatic fragments must be produced via non-specific oxidation of lignin. In this article, a fungal extracellular ligninolytic system and some of a recent information on intracellular metabolic response as well as application studies for bioremediation using fungi will be summarized.

EXTRACELLULAR LIGNIN DEGRADING ENZYMES

The relationship between ligninolytic activity and Bavendamm's reaction has been known for 70 years. Bavendamm showed that most white-rot fungi, which are capable of degrading lignin, produce a colored zone around mycelium on agar plates containing tannin or phenolic compounds and that the colorization is caused by phenoloxidases secreted by the fungi [21,22]. Traditionally, three distinct types of enzymes have been considered as phenoloxidases. Tyrosinase uses oxygen to oxidize monophenols, yielding *o*-diphenols or *o*-quinones. They can also oxidize catechols to *o*-quinones. This enzyme, however, has relatively narrow substrate specificity and is found intracellularly, so that tyrosinase has not been considered to be a key enzyme in non-specific oxidation of lignin. Laccase catalyzes the oxidation of a large variety of phenolic compounds by abstraction of an electron and a hydrogen ion from the phenolic hydroxyl group to form phenoxyl radicals. The free radicals produced by the enzyme undergo disproportionation or polymerization via radical coupling. Laccase uses oxygen as an electron acceptor which is ultimately reduced to water. Peroxidases perform the same reaction as laccase, but use hydrogen peroxide rather than oxygen as the cosubstrate. It has recently been reported that laccase is capable of oxidizing substrates, which cannot be oxidized by itself, in the presence of a certain organic compound (so-called mediator) [23-25]. Although this laccase/mediator reaction recently attracts many researchers' interest, this system is not summarized in the present review because the mechanism has not been elucidated yet. In this chapter, the mechanism and role of fungal peroxidases are summarized.

Several peroxidases have been identified, isolated, and cloned from basidiomycetes. Lignin and manganese peroxidases (LiP and MnP) are most studied fungal peroxidases. Recently, *Coprinus cinereus* peroxidase (CIP or ARP) is also well characterized. CIP shows a high reactivity against luminol but is thought not to play an important role in lignin degradation.

MnP (EC: 1.11.1.13) oxidizes Mn^{II} to Mn^{III} and the latter acts as a freely diffusible one-electron oxidizer, nonspecifically reacting with terminal organic substrates such as phenols, thiols and lignin [4,26-30]. This nonspecific manner is advantageous for lignin degradation since lignin is such a heterogeneous polymer. Many kinetic and spectroscopic studies as well as the X-ray crystallographic data has revealed Mn^{II} binding site of MnP [27,28,31,32]. Since most lignin-degrading basidiomycetes have known to secrete MnP, this enzyme may be the most important ligninolytic enzyme [33].

LiP (EC: 1.11.1.14) is another unique heme peroxidase secreted by *P. chrysosporium* and several other fungi. It catalyzes a one-electron oxidation of nonphenolic aromatic compounds through a typical peroxidative catalytic cycle (Fig. 2) forming the aryl cation radical [34-37], suggesting that oxidized intermediates of the enzyme possess a very high reduction potential. Since only 20% of aromatic nuclei contain free phenolic groups, the ability to attack nonphenolic aromatic compounds is advantageous for the biodegradation of lignin. LiP is also unique in its pH optimum (<3) which is extremely low as a peroxidase. Veratryl (3,4-dimetho-

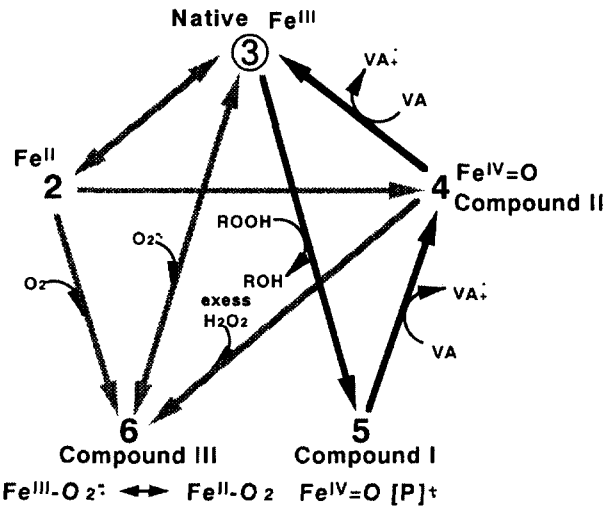

Fig. 2. Catalytic mechanism of lignin peroxidase. Solid lines show the peroxidative catalytic cycle.

xybenzyl) alcohol (VA), a preferable substrate for LiP, is synthesized *de novo* by *P. chrysosporium* under ligninolytic conditions [38]. The VA cation radical has been once proposed as a radical mediator to oxidize polymeric substrates with which LiP presumably cannot interact directly [39-43]. However, it has been shown that ferrocytochrome *c* is oxidized by LiP/H_2O_2 in the absence of VA [44], suggesting that LiP is capable of oxidizing polymeric substrates at the protein surface via a long-range electron transfer mechanism [42,44]. Also, a long-range electron transfer mechanism has been proposed for cytochrome *c* peroxidase [45,46].

To better understand the LiP reaction mechanism, people are now put their efforts on elucidating a substrate binding site and a one-electron transfer mechanism of this enzyme. Site-directed mutagenesis and X-ray crystallographic studies strongly suggested that VA binding site might be on the surface of LiP protein at Trp171 [47-51]. We have recently reported that His239 may play an important role for binding of polymeric lignin, using a surface plasmon resonance spectroscopy [52]. More recently, a genetically engineered LiP has been reported where Ser168Trp variant of MnP was prepared and the mutant enzyme clearly showed LiP activity [53].

These findings that the substrate binding site exists on the surface of LiP protein, may explain the unique properties of this enzyme, such as a higher redox potential and a lower pH optimum. In the case of horseradish peroxidase, pH optimum is in the neutral region [54]. This pH dependency is due to the acid-dissociation of distal His which is a component of the substrate oxidation architecture [54,55]. This residue acts as an acid-base catalyst during the formation of compound I and a proton acceptor from phenolic substrates during the oxidation reactions [55-57]. In the case of LiP, a proton can be released to solvent, since the substrate binding site exists on the protein surface. The cation radicals of phenolic compounds are strongly acidic, showing pKa of ~0 [58], which can be easily deprotonated at any pH. Therefore, LiP might be able to keep its high activity in a very low pH without any proton acceptors like distal His. It has been also demonstrated that the redox potential would be higher at lower pH. If the reactivity of a peroxidase with peroxides is high enough even at low pH, the higher enzymatic activity, in other words, the higher reduction potential could be expected for compounds I and II at the lower pH. It has been known that the formation of LiP compound I is pH independent over the range from 2 to 7.5 [59,60]. We would expect that the driving force for the molecular evolution of LiP is the adaptation of acidic environment to obtain a higher reduction potential to degrade any types of aromatic compounds.

FUNGAL DEGRADATION OF AROMATIC POLLUTANTS

Environmentally persistent aromatic compounds found in various categories of the pollutants have been reported to be degraded by basidiomycetes, especially lignin-degrading fungi. For example, polychlorinated phenols, polychlorinated dibenzo-*p*-dioxins, polychlorinated biphenyls, nitorophenols, nitorobenzens, and polyaromatic hydrocarbons have been applied to fungal treatment, showing a complete mineralization or at least degradation to some extent [61-74]. In this chapter, degradation pathways and mechanisms for several selected compounds are summarized.

Polychlorinated phenols and dibenzo-*p*-dioxins constitute significant categories of environmentally persistent pollutants. Over the past decade, the degradation of those compounds by *P. chrysosporium* has been reported. The mineralization of ^{14}C-labeled 2,4-dichloro- and 2,4,5-trichlorophenols by *P. chrysosporium* occurred only under secondary metabolic conditions, suggesting that the ligninolytic system is (at least in part) responsible for the degradation of polychlorinated phenols [62, 63]. Fig. 3 shows the metabolic pathway for the degradation of 2,4,5-trichlorophenol. All three chlorines were removed from the aromatic ring of 2,4,5-trichlorophenol before ring cleavage occurred. The pathway involves several cycles of oxidative dechlorination catalyzed by peroxidases, which were followed by reduction and methylation reactions. 2,4,5-Trichlorophenol and phenolic intermediates are effectively oxidized by MnP. Thus, the reduction of quinone products to phenolic compounds is thought to be the reactivation of the substrate for fungal peroxidases. This oxidative dechlorination caused by the cycle of redox reactions has also been observed in the fungal degradation of 2,4-dichlorophenol and pentachlorophenol [61, 62].

It has been reported that *P. chrysosporium* degrades polychlorinated dibenzo-*p*-dioxins under ligninolytic conditions [67,73-75]. Fig. 4 shows the proposed pathway for the degradation of 2,7-dichlorodibenzo-*p*-

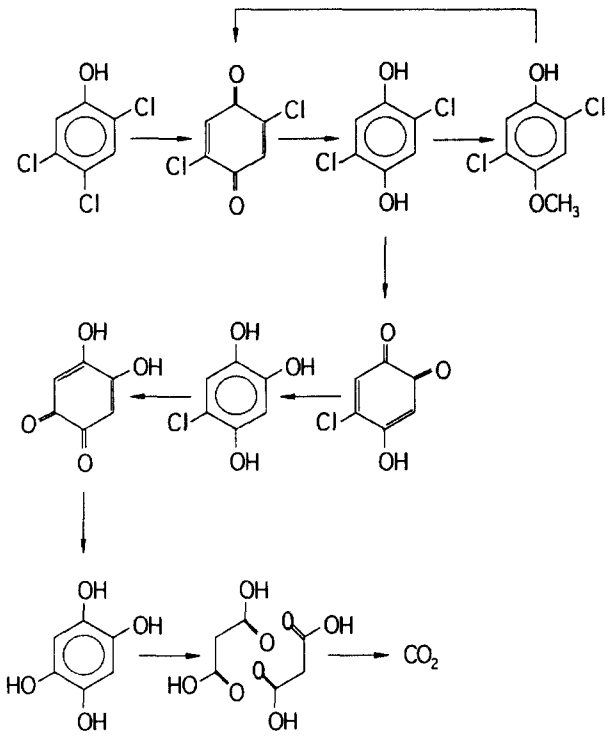

Fig. 3. Proposed pathway for the degradation of 2,4,5-trichlorophenol by *P. chrysosporium*.

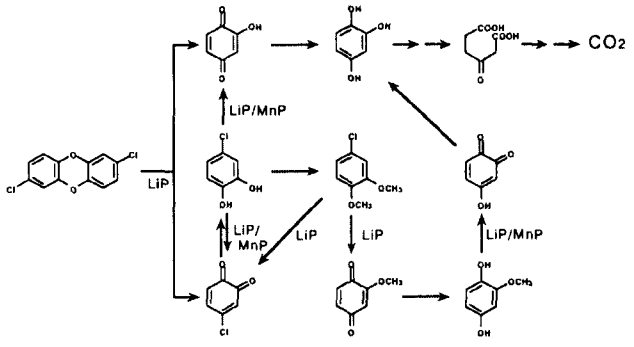

Fig. 4. Proposed pathway for the degradation of 2,7-dichlorodibenzo-*p*-dioxin by *P. chrysosporium*.

dioxin by *P. chrysosporium*. The first and the most important step in the pathway is the oxidative cleavage of the dioxin ring by LiP, generating two quinone products. Quinones were reactivated for LiP and MnP by the reduction as discussed above. The key metabolic intermediate in this pathway was 1,2,4-trihydroxybenzene, which is intracellularly cleaved to form, after subsequent reduction, β-ketoadipic acid. The oxidative dechlorination observed in the metabolic pathways for polychlorinated phenols and dioxin resulted in the release of Cl^-, which is advantageous because of its non-toxicity. It has recently been reported that *P. chrysosporium* brought about the reductive dechlorination in

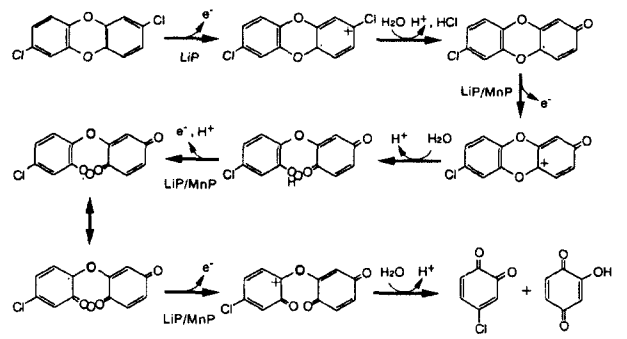

Fig. 5. Proposed mechanism for the lignin peroxidase-catalyzed dioxin ring cleavage of 2,7-dichlorodibenzo-*p*-dioxin.

tracellularly [76]. Since a chloro-substituent is an electron-exdrawing group, causing a higher redox potential for aromatic ring, fungal peroxidases may not be able to oxidize highly chlorinated aromatic substrates. The reductive dechlorination may be the key reaction for the fungal degradation of highly chlorinated aromatic compounds.

The mechanism of LiP-catalyzed C-O-C bond cleavage has also been studied [73]. The nature of the products generated during the oxidation of 2,7-dichlorodibenzo-p-dioxin by LiP and our understanding of the mechanism of LiP enables us to propose a mechanism for dioxin ring cleavage (Fig. 5). The cleavage reaction occurred under argon; furthermore, ^{18}O from $^{18}O_2$ was not incorporated into the primary monomeric products during LiP reaction. These results strongly suggested that C-O-C bond cleavage brought about via the attack of H_2O on cation intermediates (Fig. 5). LiP-catalyzed initial formation of the cation radical was followed by the attack of water and the release of one chloride. The carbon-centered radical intermediate was oxidized by LiP/MnP to the cation which was attacked by water with the cleavage of the first C-O-C bond. An effective one-electron oxidation of carbon-centered radicals to corresponding cations by MnP has been suggested for MnP oxidation of a lignin model dimers [28]. After similar cycle of oxidation and water attack, the second C-O-C bond was cleaved to form 4-chloro-1,2-benzoquinone and 2-hydroxy-1,4-benzoquinone (Fig. 5).

As described above, only one strain of the basidiomycetes shows the ability to degrade a variety of chemical structures, based on the ability of LiP and MnP for the non-specific oxidation of aromatic compounds. However, the metabolism of aromatic compounds by fungi, not assisted by LiP and MnP, has been also reported. In the fungal degradation of certain PAHs and biphenyl ether compounds, the hydroxylation reaction, most likely catalyzed by cytochrome P450 type enzymes, seemed be the key reaction [77]. We have recently reported that a series of sulfur-containing heterocyclic compounds are metabolized by lignin-degrading fungus *C. versicolor*, which has been known to produce LiP and MnP, without help of those extracellular enzymes. We also found that structurally similar substrates were

Table 1. Matabolic response for thiophene derivatives in *C. versicolor*

Substrate	Redox Reactions on Hydroxylmethyl Group	S oxidation	Hydroxylation	Glycosylation	C-S Cleavage	Final Product
2-HMT	○	×	×	×	◉ (γ-thiobuthylolactone)	Nutrient
2-HMBT	○	×	×	○	×	2-HMBT-Xyl
4-HMDBT	○	×	×	○	×	4-HMDBT-Xyl
2-MBT	—	●	×	×	×	2-MBT-1-dioxide
7-MBT	—	●	×	×	×	7-MBT-1-dioxide
DBT	—	●	×	×	×	DBT-5-dioxide
4-MDBT	× (4-HMDBT-5-oxide)	●	◉ (4-MDBT-5-oxide)	◉ (4-HMDBT-5-dioxide)	×	4-HMDBT-5-dioxide-Xyl

○ : Effective conversion under HN (non-ligninolytic) conditions — : No such substitution group on the substrate
● : Effective conversion under LN (ligninolytic) conditions × : No such reaction occurred.
◉ : Conversion occurred on methabolic intermediates shown in parenthesis

HMT : hydroxymethylthiophene, HMBT : hydroxymethylbenzothiophene, HMDBT : hydroxymethyldibenzothiophene, MBT : methylbenzothiophene, DBT : dibenzothiophene, MDBT : methyldibenzothiophene, Xyl : xyloside

metabolized through different pathways, suggesting the existence of the substrate recognition and metabolic response systems in fungi. In the next chapter, chemical stress responsible system found in fungi will be summarized.

SUBSTRATE RECOGNITION AND METABOLIC RESPONSE

P. ostraeatus has been known to convert dibenzothiophene (DBT) and PAH to their oxidized products probably catalyzed by intracellular P450 (77); however, this fungus has been also known not to produce LiP. Then, we attempted the fungal metabolism of DBT and its related compounds by *C. versicolor*, LiP/MnP/ laccase-producing fungus.

Twenty-three thiophene derivatives were utilized as a substrate. Those were categorized into the compounds containing the thiophene-, benzothiophene-, and dibenzothiophene-skeletons with several different substituents at C_4 position of aromatic ring or at the vicinal position to the sulfur of thiophene ring. Detailed analysis on metabolites enabled us to draw the proposed metabolic pathways. Table 1 shows typical results obtained from the metabolic study [78]. Fig. 6 summarizes fungal response against the structure of added sulfur-containing heterocyclic compounds. *C. versicolor* first distinguishes whether the substrate contains aromatic moiety or not. If it is non-aromatic, the driving force for the metabolism is an acquisition of a sulfur nutrient. Therefore, this pathway is more active when cultured in no sulfur-containing medium. It has been reported that thiodiglycol was metabolized in that manner [79]. If the substrate is aromatic, it is recognized as xenobiotics. Then, the fungus distinguishes whether the aromatic moiety contains the hydroxymethyl group, for converting to xyloside conjugate [20]. Interestingly, *P. chrysosporium* has a tendency to catalyze the glucosylation and *P. ostraeatus* and *C. versicolor* to catalyze the

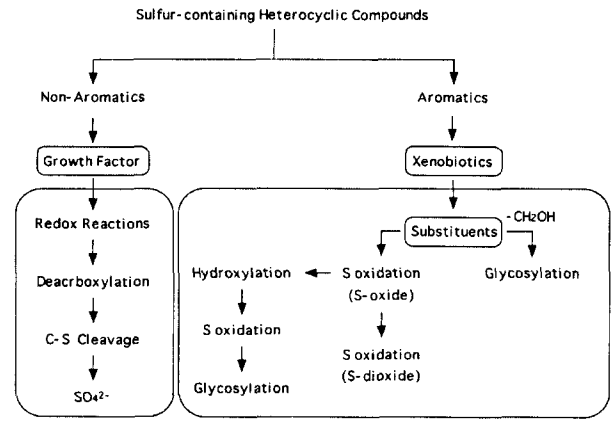

Fig. 6. Substrate recognition and metabolic response for fungal degradation of sulfur-containing heterocyclic comounds.

xylosylation [77]. When the fungus could not find the hydroxyl group for the glycosylation, it converts the substrate to more hydrophilic products via sulfur oxidation or hydroxylation reactions (Fig. 6). We also found that the position of substituents on biphenyl skeleton exhibited the strong effect on the substrate recognition and metabolic response in the basidiomycete, *Tyromyces palustris*.

These specific manners for the metabolic response suggested that basidiomycetes might possess a chemical stress responsible system to maintain their homeostasis. Thus, we initiated the search for the chemical stress responsible genes utilizing a differential display method. Differential display is a mRNA fingerprinting method where the key element is to use a set of oligonucleotide primers, one being anchored to the polyA tail of a subset of mRNAs, the other being short and arbitrary in sequence [80]. To accelerate the identification of differentially expressed mRNA, fluorescent-labeled oligo dT primer was utilized [81]. Recently, a successful application of differential display method to study fungal cel-

Table 2. 4-MDBT-5-oxide responsible genes from *C. versicolor*

Gene fragment	Upstream primer	Downstream primer	Incubation period	Gene similality*
DDga07	5'GATCATGGTC3'	5'TTTTTTTTTTTTTTTGA3'	24 h	HR
DDgc16-1	5'TCGGTCATAG3'	5'TTTTTTTTTTTTTTTGC3'	24 h	AAD (84)
DDgc16-2	5'TCGGTCATAG3'	5'TTTTTTTTTTTTTTTGC3'	24 h	TF
DDgg20	5'GATCAAGTCC3'	5'TTTTTTTTTTTTTTTGG3'	24 h	HSP (85)
DDa06	5'TGGTAAAGGG3'	5'TTTTTTTTTTTTTTTTA3'	48 h	TF
DDc09	5'GTTTTCGCAG3'	5'TTTTTTTTTTTTTTTTC3'	24 h	AAD (84)

Abbreviations: HR, hormone receptor; AAD, arylalcohol dehydrogenase, TF; transcriptional factor, HSP; heat-shock protein
*Analyzed using FASTA/GDB. Numbers in parenthesis show the reference number.

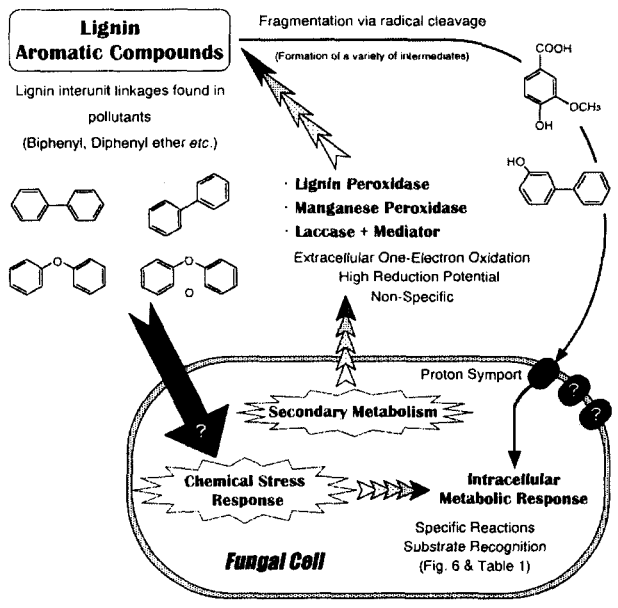

Fig. 7. Non-specific extracellular oxidation and specific intracellular metabolism.

lular biology has been reported [82,83]. When 4-methyldibenzothiophene-5-oxide was added as the chemical stress to *C. versicolor* culture, several stress responsible genes were expressed (Table 2). It is too early to draw the complete scheme for fungal stress responsible system, but at least the existence of chemical stress responsible genes strongly suggests that a sophisticated system may be involved in a specific intracellular metabolic strategy. Fig. 7 shows the schematic drawing for a non-specific extracellular system and a specific intracellular system for multiple functions of fungi to metabolize variety of aromatic compounds.

Acknowledgments This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, Kyushu University Interdisciplinary Programs in Education and Projects in Research Development, and the Proposal-Based New Industry Creative Type Technology R&D Promotion Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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[Received July 14, 2000; accepted November 23, 2000]