Demethylation and Reduction of Veratic Acid by Selected White-rot Fungi

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Abstract

The white-rot basidiomycete fungi Inonotus obliquus, Phlebia radiata, Stereum rugosum and Trametes versicolor grown as non-agitated low nitrogen cultures transformed veratic acid in a variety of ways. I. obliquus reduced veratic acid effectively to verateraldehyde and veratryl alcohol but did not demethylate or decarboxylate it. P. radiata reduced veratic acid to the same products and also decarboxylated and demethylated it to guaiacol. S. rugosum and T. versicolor reduced veratic acid as in the case of I. obliquus and they also demethylated it to vanillic acid and vanillin. Decarboxylation was not observed. During fungal growth the activity of extracellular laccase and lignin peroxidase were also detected. Veratic acid as well as vanillyl alcohol stimulated laccase production in the early stage of P. radiata and S. rugosum growth and laccase activity increased to a higher level by veratic acid addition. When the cultures were flushed with oxygen, the activity of lignin peroxidase increased, whereas laccase activity decreased. The lignin peroxidase activity of P. radiata was stimulated similarly by veratic acid as laccase activity was, whereas vanillyl alcohol caused a complete inhibition of lignin peroxidase production. I. obliquus as well a T. versicolor showed neither laccase nor lignin peroxidase activity. The involvement of both enzymes in demethylative and reductive processes is discussed.

Introduction

Demethylation of veratic acid to vanillic acid by the soil fungi Haplographium sp., Hormodendrum sp. and Penicillium sp. was demonstrated for the first time by Henderson (1960). Farmer et al. (1959) and later Fukuzumi et al. (1965) showed also the reduction of this compound to verateraldehyde and veratryl alcohol by Polystictus (Trametes) versicolor, and (to veretaldehyde) by Trametes sanguineus. Henderson’s results were confirmed by Ishikawa et al. (1963) who used the wood-rotting basidiomycete Fomes fomentarius. Minami et al. (1965) apart from demethylation of veratic acid by T. sanguinea demonstrated its

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demeth(ox)ylation to p-hydroxybenzoic acid as well. Zenk and Gross (1965) confirmed the reductive activity of *T. versicolor* on veratric acid. Gold et al. (1982) showed that a pleiotropic mutant of *Phanerochaete chrysosporium* lacking phenol oxidase activity also demethylates veratric acid at both 3- and 4- positions. Later Hatakka (1985) demonstrated demethylation (at 4-position) and reduction of veratric acid in the white-rot fungus *Pycnoporus cinnabarinus*.

The majority of white-rot fungi produce extracellular laccase (Bollag and Leonowicz, 1984) and some of them produce lignin peroxidase as well (Kirk et al., 1986, Hatakka et al., 1987). The production of both enzymes may be stimulated by methoxylated phenolic compounds (Bollag and Leonowicz, 1984, Hatakka et al., 1987). These enzymes are involved in demethylation (Leonowicz et al., 1984, Kersten et al., 1985) but probably not in reduction. Laccase and lignin peroxidase operate in concert in lignin transformation processes (Ander et al., 1983, Leonowicz et al., 1988), where demethylation is a very important step (Leonowicz et al., 1988). These reports led us to examine the appearance of both the demethylative and reductive activities in four selected white-rot fungi two of which are commonly studied (*Trametes* (syn. *Coriolus*, *Polyporus* *versicolor* and *Phlebia radiata*) and two arbitrarily chosen fungi (*Stereum rugosum* and *Inonotus obliquus*). We also aimed to identify some of the enzymes that are involved in these processes.

**Experimental**

**Materials and Methods**

**Organisms.** *Inonotus obliquus* (Pers. ex Fr.) Pil. no. 19, *Trametes versicolor* (L. ex Fr.) Pil. no. 7 (ATCC 44308) and *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) were from the culture collection of the Department of Biochemistry, University of Lublin, Poland, and *Phlebia radiata* Fr. no. 79 (ATCC 64658) and *Stereum rugosum* (Pers. ex Fr.) Fr. no. 10 (ATCC 64657) were originally isolated at the Department of Microbiology, University of Helsinki, Helsinki, Finland (Hatakka and Uusi-Rauva, 1983; Hatakka and Pirhonen, 1985). The fungi were maintained on 2% (wt/vol) malt agar slants.

**Chemicals.** Veratric acid, vanillyl alcohol and aromatic compounds used as references for HPLC and TLC were purchased from Fluka (Buchs, Switzerland) and Aldrich (Milwaukee, Wisconsin, USA). Syringaldazine was obtained from Aldrich.

**Culture conditions.** For inoculum of *I. obliquus*, *P. radiata*, *S. rugosum* and *T. versicolor* pieces of agar (ca. 0.5 cm²) were cut and grown in ADMS medium (Hatakka and Uusi-Rauva, 1983) containing 2.0 mM of nitrogen (LN) and 56 mM of glucose in nonagitated conical flasks for 6 days at 28°C. The mycelial mats were collected and homogenized in a Waring Blender. The stationary cultivations after inoculation with 4% (vol/vol) of the homogenate, were carried out in 100 ml conical flasks containing 10 ml of LN-ADMS medium at 28°C. Part of the culture flasks were flushed with 100% oxygen every third day. Veratric acid or vanillyl alcohol were added 48 h after inoculation in final concentrations of 0.5, 1.0 or 2.0 mM. Veratric acid and vanillyl alcohol were dissolved in a small amount of 0.1 M NaOH. The pH of the solutions was adjusted to pH 5.5 with 0.1 M HCl and the solutions were sterilized by filtration through Sterivex-GS 0.2 μm filters (Millipore, Bedford,
Massachusetts). *P. chrysosporium* was cultivated in C-limited cultures on a rotary shaker as described by Haemmerli et al., (1986).

**Enzyme activities.** Laccase and lignin peroxidase activities from culture filtrates were determined from 4 parallel flasks using Beckman DU-8 spectrophotometer. Syringaldazine was used as substrate for laccase (Leonowicz and Grzywnowicz, 1981) but using 0.1 M citrate-phosphate buffer, pH 5.0. Veratryl alcohol oxidation method was used to determine lignin peroxidase activity (Tien and Kirk, 1988). Both activities are expressed as nanokatalas (i.e. nanomoles of substrates oxidized in one second) per litre. Peroxidase activity was determined with p-aminidin as a substrate at pH 5.5, and expressed according to Lobarzewski (1981).

**Isolation of extracellular enzymes.** Cultures of *P. radiata* which were stimulated by veratric acid were harvested at the peaks of laccase and lignin peroxidase activity. From these culture filtrates, extracellular laccase and lignin peroxidase enzymes were purified by the methods of Rogalski et al., 1991a and Leisola et al., 1985, respectively. Lignin peroxidase from the filtrate of *P. chrysosporium* culture which was stimulated by veratryl alcohol was isolated as described Leisola et al., 1985. Peroxidase from *T. versicolor* culture filtrate was isolated and purified according to Lobarzewski and Paszczyński, 1985. The enzymes were stored under nitrogen at 4°C, or frozen at −20°C.

**Extration of aromatic compounds from culture filtrates.** 5 ml portions of culture filtrates were acidified to pH 2.0 with 1M HCl and extracted three times with equal volumes of dichloromethane.

**Detection of phenolic compounds.** The presence of phenolic compounds in the extracts was ascertained with diazosulfanilamide (DASA) test according to Małarczyk et al., 1989. Veratric acid as a non-phenolic compound does not react with DASA.

**Thin-layer chromatography.** Extracts were evaporated to small volumes and analyzed by thin-layer chromatography using silica gel F-254 plates (Merck) according to Leonowicz et al., 1984. The chromatograms were developed in a solvent system containing benzene-methanol-propanionic acid (22:2:1) and visualised either in UV light or by DASA reaction as described Leonowicz et al., 1968, i.e by successive spraying of plates with butanol saturated with diazosulfanilamide and with 20% aqueous solution of sodium carbonate. Compounds were identified using authentic reference compounds.

**High performance liquid chromatography.** Extracts were evaporated to dryness and dissolved in 1.0 ml acetonitrile. HPLC analyses were carried out with a Hewlett-Packard 1090M liquid chromatograph equipped with a diode-array UV-VIS detector and a fluorescence detector (Hewlett-Packard). Before injection the samples were filtered through 0.2 μm Acro LC-13 membrane filter units (Gelman Sciences). The column was a NovaPak C18 3.9 × 150 mm (Waters). All analyses were run by linear gradient elution using acetonitrile and 1.0 mM H₃PO₄, pH 3.2, from 11%–89% (vol/vol) to 50%–50%, analysis time was 12 min, flow rate 0.75 ml min⁻¹ and injection volume 10 l. Compounds were identified by comparing the retention and UV spectra of eluted peaks to authentic references. External standard method was used for quantitative determination of the identified compounds.

**Results**

**Demethylation of veratric acid.** The cultures of *T. versicolor*, *S. rugosum* and *P. radiata* turned yellow after addition of veratric acid and the extracts displayed a positive result of DASA test, which may suggest the demethylative activity of these cultures. Vanillyl compounds, i.e. suspected primary products of veratric acid demethylation, were found only in the cultures of *S. rugosum* and *T. versicolor* (Tables I and II). *S. rugosum* showed clear demethylation since both vanillic acid and vanillin were detected in small quantities, whereas *T. versicolor*
produced only a trace amount of vanillin (Table II). In *P. radiata*, traces of guaiacol were observed (Table I). This suggests that both decarboxylation and demethylation of veratric acid occurred. *I. obliquus* did not show any demethylative activity. Isolated enzyme preparations of *P. radiata* laccase, *P. radiata* and *P. chrysosporium* lignin peroxidase and *T. versicolor* peroxidase (see Materials and Methods) did not demethylate veratric acid since no vanillyl compounds were found.

Table I

<table>
<thead>
<tr>
<th>Fungus</th>
<th>DASA test</th>
<th>Products detected by TLC and HPLC</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>VALD</td>
</tr>
<tr>
<td><em>I. obliquus</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>P. radiata</em></td>
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<td>+</td>
</tr>
<tr>
<td><em>S. rugosum</em></td>
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<td>+</td>
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<tr>
<td><em>T. versicolor</em></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

DASA = diazosulfamidamide; TLC = thin-layer chromatography; HPLC = high performance liquid chromatography; VALD = veratraldehyde; VALC = veratryl alcohol; VAN ACID = vanillic acid; VAN = vanillin; GUA = guaiacol

Reduction of veratric acid. All fungal cultures reduced veratric acid to veratraldehyde and veratryl alcohol (Table I), although in different amounts depending on the fungus (Table II). *T. versicolor* and *I. obliquus* reduced veratric acid most readily. Other fungi reduced veratric acid much less efficiently (Table II). Isolated enzyme preparations of laccase, lignin peroxidases and peroxidase did not reduce this acid at all.

Sequence of demethylative and reductive processes. In *S. rugosum* already after 4 h from the addition of veratric acid, a small amount of vanillic acid was detected and simultaneously the amount of veratraldehyde remained relatively low. After 10 h demethylation and reduction were clearly established, the former being distinctly stronger (Table II). In comparison with the other fungi *T. versicolor* showed the strongest reductive effect on veratric acid: in 4 h almost 30% of the initial amount of veratric acid was reduced to veratraldehyde (Table II). At the same time a trace amount of vanillin was detected, which indicated that both the demethylative and reductive processes were operating. The absence of vanillic acid, the expected product of demethylation of veratric acid, indicated that it was rapidly reduced to vanillin.

Stimulation of laccase and lignin peroxidase. Of the four fungi studied, only *P. radiata* and *S. rugosum* produced laccase when veratric acid was added to the medium (Table III). Besides laccase, *P. radiata* also produced lignin peroxidase.
Table II
The yield of veratic acid demethylation and reduction by fungal cultures. Aromatic compounds were determined by high performance liquid chromatography

<table>
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<tr>
<th>Fungus</th>
<th>Growth time (hrs)</th>
<th>VER ACID (mol%)</th>
<th>VALD (mol%)</th>
<th>VALC (mol%)</th>
<th>VAN ACID (mol%)</th>
<th>VAN (mol%)</th>
<th>VAN ALC (mol%)</th>
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<td>12.7</td>
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<td>P. radiata</td>
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<tr>
<td>T. versicolor</td>
<td>4</td>
<td>12.4</td>
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<td>0.1</td>
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<td>8.6</td>
<td>30.2</td>
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</table>

* mol% = (mol compound recovered/mol veratic acid added) × 100%

However, I. obliquus and T. versicolor showed neither inducible laccase nor lignin peroxidase activities (Table III). Laccase produced by S. rugosum showed its maximum after six days of growth, i.e. in a very similar way as P. radiata (Figs. 2 and 4). Results shown in Figs. 1, 2 and 4 indicate that laccases produced by P. radiata and S. rugosum were considerably stimulated by veratic acid. In P. radiata cultures, the higher concentration of veratic acid (1.0 and 2.0 mM) had a positive influence on laccase production (Fig. 2) whereas lignin peroxidase activity was not enhanced (Fig. 3). Vanillyl alcohol (1 mM) stimulated only laccase production in P. radiata and in S. rugosum (Figs. 2 and 4) but to a much smaller extent than veratic acid did. In P. radiata lignin peroxidase activity was completely inhibited by vanillyl alcohol (Fig. 3). In cultures of P. radiata which were flushed with 100%
oxygen (Figs 1, 2 and 3) oxygen atmosphere clearly increased lignin peroxidase production and the highest activities (>1500 nkat l⁻¹) were obtained when 1.0 mM veratric acid was added to the medium (Fig. 3). On the contrary, under similar conditions laccase production by *P. radiata* was apparently repressed by high oxygen concentration (Fig. 2).

**Table III**
The appearance of extracellular laccase and lignin peroxidase in fungal culture grown on ADMS (LN) medium and stimulated with 1mM veratric acid

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Laccase</th>
<th>Lignin peroxidase</th>
</tr>
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<tbody>
<tr>
<td><em>I. obliquus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. radiata</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. rugosum</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>T. versicolor</em></td>
<td>-</td>
<td>-</td>
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</table>

+, activity found; −, activity not detected

**Discussion**

Our results confirm the results of *Hatakka* (1985) concerning demethylation and reduction of veratric acid by *Pycnoporus cinnabarinus*. In the present study the prevalent metabolic pathway of veratric acid transformation largely depended on the fungal species. Vanillyl compounds arising from veratric acid could not be detected in *I. obliquus* culture filtrates. However, this fungus readily reduced veratric acid: after 24 h, over 25 mol% of veratric acid was converted to veratraldehyde. Guaiacol was detected in *P. radiata* culture liquors which points to both demethylative and decarboxylative activity of this fungus. As such a product was not proven in *P. cinnabarinus* culture (*Hatakka*, 1985), *P. radiata* may indicate yet another mechanism of veratric acid demethylation. Formation of guaiacol has earlier been detected by *Streptomycetes sp.* degrading vanillin (*Crawford* and *Olson*, 1978). *P. radiata* reduced veratric acid to veratraldehyde and veratryl alcohol in a similar way as *I. obliquus* (Tables I and II) and *P. cinnabarinus* (*Hatakka*, 1985) did.

In our work the demethylation of veratric acid was probably caused by a specific veratrate demethylase (*Pasczyński* and *Trojanowski*, 1977) and not by laccase, lignin peroxidase or peroxidase. It may be similar to that described earlier by *Farmer et al.*, (1959) and *Minami et al.*, (1965). The enzyme showing such activity was further isolated from *Chaetomium piluliferum* and *Xerocomus badius* by *Pasczyński* and *Trojanowski* (1977) and designated veratrate O-demethylase. Lignin peroxidase is able to dealkoxylate some non-phenolic compounds such as veratryl alcohol (*Haemmerli et al.*, 1987), 1, 4-dimethoxybenzene,
tetramethoxybenzene (Kersten et al., 1985) and non-phenolic beta-0-4 dimeric substructures of lignin (Kirke et al., 1986). However, veratric acid was not demethylated by pure lignin peroxidases of either P. radiata or P. chrysosporium origin. Also purified T. versicolor peroxidase did not demethylate this compound, what is in opposition to earlier results of Trojanowski et al., (1967). In this experiment, however, the authors used a nonpurified enzyme preparation, which could not exclude the activity of other demethylating enzymes. The activity of manganese-dependent peroxidase was not studied.

Also the reduction of veratric acid by various wood-rotting fungi has been observed before (Fukuzumi et al., 1965; Zenk and Gross, 1965; Hatakka, 1985). But so far the enzymes reducing veratryl compounds have been difficult to isolate from fungal material because of their lability (Zenk and Gross, 1965). Recently, Munheim et al., (1991) purified an intracellular NADPH requiring aryl-aldheyde reductase from P. chrysosporium. The enzyme consists of several subunits which may explain the low stability of the activity.

The reduction of veratric acid in all fungal cultures was rapid, much faster than demethylation. In the study with P. cinnabarinus it was found that an easily metabolized carbon source such as glucose shifted the reaction towards reduction of veratric acid while demethylation was more prominent when cellulose was used as a carbon source (Hatakka, 1985). Reduction of veratric acid to veratraldehyde and veratryl alcohol are probably more energy consuming or NADPH requiring reactions than demethylation. Energy is readily available from glucose used as a carbon source. Our results are summarized in Fig. 5 which shows how veratric acid was transformed when incubated in the culture medium of different fungi.

The appearance of laccase and lignin peroxidase activities varied with the fungi. I. obliquus, for example, showed neither laccase nor lignin peroxidase activity in the presence of veratric acid or vanillyl alcohol. Identical results were obtained with T. versicolor. The production of lignin peroxidase and laccase by T. versicolor has been thoroughly studied (Jonsson et al., 1989; Rogalski et al., 1990). T. versicolor is known as an efficient producer of both constitutive and inducible laccase (Bollag and Leonowicz, 1984). However, usually relatively high nutrient nitrogen medium is used for studies on laccase production (Bollag and Leonowicz, 1984). The production of laccase by T. versicolor requires different conditions than those applied in this work, i.e. 1) lower glucose concentration, as its excess causes repression of laccase synthesis (Grabbe et al., 1968) and 2) not less than two weeks culture growth on high nitrogen medium (Rogalski et al., 1991b; Leonowicz et al., 1978). Fahraeus and Reinhammar (1967) obtained extracellular inducible laccase of T. versicolor after 6–8 days of growth, but they used an aerated fermentor for cultivation. In addition, the stimulators used hitherto for enhancement of laccase production, like 2, 5-xylidine or various phenolic compounds, are usually toxic for fungal organisms.
Fig. 1 Production of extracellular laccase (○●) and lignin peroxidase (△▲) in *P. radiata* cultures without addition of aromatic compound: ○△ culture growth without flushing; ●▲ cultures flushed with oxygen
Fig. 2 Production of extracellular laccase in *P. radiata* cultures stimulated with veratric acid or vanillyl alcohol.

- ○ 0.5 mM veratric acid;
- △ 1.0 mM veratric acid;
- □ 2.0 mM veratric acid;
- ▲ 1.0 mM veratric acid, cultures flushed with oxygen;
- ■ 1.0 mM vanillyl alcohol;
- ● control without stimulator and flushing
Fig. 3 Production of extracellular lignin peroxidase in *P. radiata* cultures stimulated with veratric acid or vanillyl alcohol.

○ 0.5 mM veratic acid; △ 1.0 mM veratic acid; □ 2.0 mM veratic acid; ▲ 1.0 mM veratic acid, cultures flushed with oxygen; ■ 1.0 mM vanillyl alcohol; ● control without stimulator and flushing.
Fig. 4 Production of extracellular laccase in *S. rugosum* culture.

- O growth without stimulation;
- • culture stimulated with 1.0 mM veratic acid;
- A culture stimulated with 1.0 mM vanillyl alcohol.
Laccase production stimulated by these compounds is most probably connected with defence of the fungus against unfavourable conditions of fungal culture (Leonowicz et al., 1978). Veratric acid is probably only weakly or not toxic.
to *T. versicolor*. Moreover, as a non-phenolic compound veratric acid probably could not stimulate laccase production in this fungus.

The results obtained with *P. radiata* and *S. rugosum* differed from those with *I. obliquus* and *T. versicolor* since in the former two fungi veratric acid clearly stimulated laccase production. Vanillyl alcohol enhanced laccase activity not as much as veratric acid. The rapid disappearance of laccase was unexpected. Such an effect was not known in previous investigations, where laccases stimulated by phenolic compounds usually maintained their activity until the end of lag phase of the fungal growth (Grabbe et al., 1968; Bollag and Leonowicz, 1984). The laccases of *P. radiata* and *S. rugosum* are probably very different than in other wood-rotting fungi. Indeed, a novel combination of prosthetic groups containing both PQQ (pyrroloquinoline quinone) and two copper atoms has recently been found in *P. radiata* laccase (Kärhänen et al., 1990). The possible effect of nitrogen depletion on the rapid disappearance of laccase activity should also be investigated. The surplus of oxygen may have inhibited laccase activity in *P. radiata*.

The production of lignin peroxidase in *P. radiata* was strongly stimulated by veratric acid and even better in the presence of oxygen, but vanillyl alcohol completely repressed the activity. In *P. radiata* veratric acid is reduced to veratryl alcohol (Lundell et al., 1991), which is a well known stimulator of lignin peroxidase in this fungus (Hatakka et al., 1987) and in *P. chrysosporium* (Tonon and Odier, 1988). Vanillyl alcohol which is a phenolic compound, did probably not enhance lignin peroxidase production, because a substrate for lignin peroxidase should be fully methylated (Tien and Kirk, 1984). However, although phenolic compounds, vanillin (Rogalski et al., 1991b) and guaiacol (Hatakka et al., 1987) are efficient stimulators of lignin peroxidase activity in *P. radiata* which indicates a complicated interdependence of various factors. Laccase and lignin peroxidase may cooperate in fungal metabolism of aromatic compounds so that laccase oxidizes and polymerizes phenolic compounds. These compounds are thus removed from disturbing the action of lignin peroxidase. Such cooperation of lignin peroxidase and laccase may also affect the activity of veratrate demethylase. The cultures of *P. radiata*, *S. rugosum* and *T. versicolor* turned yellow after addition of veratric acid. This reaction which coincided with the peak of laccase activity, may indicate the appearance of phenolic products of veratrate demethylase action. The demethylated products were probably transformed further by laccase thus enabling the reaction catalyzed by veratrate demethylase to continue.

Studies on chemical composition of wood after degradation caused by white-rot fungi often show veratric and vanillic acid derivatives as products of lignin degradation (Eriksson et al., 1990). Depending on environmental demands, further transformation of aromatic acids occurs either via catabolic or anabolic pathways. The methoxyphenolic intermediates arising from the catabolic pathway, including demethylation by laccase to ortho-diphenols (Anderson et al., 1983)
and dearomatization by protocatechuate dioxygenase to ketoacids (Wojtas-Wasilewska et al., 1988), may serve as carbon and energy sources for the fungi (Leonowicz et al., 1988). Via the anabolic pathway, after oxidative polymerization by laccase (Leonowicz et al., 1984; Bollag et al., 1982) or copolymerization with nitrogen containing organic compounds, the intermediates enrich soil humus (Bollag et al., 1983; Liu et al., 1985).

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Literature


