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De-novo biosynthesis of chlorinated aromatics by the white-rot fungus *Bjerkandera* sp. BOS55

Formation of 3-chloro-anisaldehyde from glucose

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The white-rot fungus *Bjerkandera* sp. BOS55 produced de-novo several aromatic metabolites. Besides veratryl alcohol and veratraldehyde, compounds which are known to be involved in the ligninolytic system of several other white-rot fungi, other metabolites were formed. These included anisaldehyde, 3-chloro-anisaldehyde and a yet unknown compound containing two chlorine atoms. Additionally GC/MS analysis revealed the production of small amounts of anisyl alcohol and 3-chloro-anisyl alcohol. After 14 days, the extracellular fluid of *Bjerkandera* BOS55 contained 100 μM veratraldehyde and 50 μM 3-chloro-anisaldehyde. This is the first report of de-novo biosynthesis of simple chlorinated aromatic compounds by a white-rot fungus. Anisaldehyde and 3-chloro-anisaldehyde were also produced by *Bjerkandera adusta* but not by *Phanerochaete chrysosporium*.

Veratryl alcohol; Lignin degradation; *Bjerkandera*; *Phanerochaete chrysosporium*; Anisaldehyde; 3-Chloro-anisaldehyde

1. INTRODUCTION

White-rot fungi, which belong to the basidiomycetes, are the most effective lignin degraders in nature. The best characterized white-rot fungus is *Phanerochaete chrysosporium* [1]. Besides the complex and heterogeneous aromatic biopolymer lignin, this fungus is also able to degrade a wide range of xenobiotics, including polycyclic aromatic hydrocarbons and penthachlorophenol [2,3]. Lignin biodegradation results from the combined action of several enzymes working together in close co-operation.

An interesting aspect of the lignin degrading system of *P. chrysosporium*, is the involvement of an aromatic metabolite, veratryl alcohol. Addition of veratryl alcohol to whole cultures of *P. chrysosporium* increases the lignin degrading activity [1]. Veratryl alcohol is an important substrate for one of the key enzymes in lignin degradation, lignin peroxidase [1,4]. Furthermore, veratryl alcohol protects this enzyme from inactivation by H_2O_2 [5,6]. Veratryl alcohol is synthesized de-novo by *P. chrysosporium* [7] and various other white-rot fungi including *Bjerkandera adusta* [8], *Pycnoporus cinnabarinus [9], Phlebia radiata* [10] and *Trametes versicolor* [11]. Phenylalanine, 3,4-dimethoxycinnamyl alcohol, and veratrylglycerol are intermediates during the biosynthesis of veratryl alcohol by *P. chrysosporium* [12].

Many simple, aromatic compounds derived from intermediates of the shikimic acid pathway are produced by fungi [13]. However, veratryl alcohol is the only metabolite known to play a definite role in lignin biodegradation. In the present paper we report the de-novo biosynthesis of other aromatic metabolites, 3-chloro-anisaldehyde, and anisaldehyde by white-rot fungi of the genus *Bjerkandera*.

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2. MATERIALS AND METHODS

2.1. *Microorganisms*

Bjerkandera adusta (Willd.:Fr.) Karsten CBS 595.78 was obtained from the Centraal Bureau voor Schimmelcultures (SBS, Baarn, The Netherlands). *Phanerochaete chrysosporium* Burds. BKM F-1767 (ATTC 24725) was a kind gift from Prof. T.K. Kirk. *Bjerkandera* sp. BOS55 was isolated and determined as described before [14]. Strains were maintained at 4°C on hemp (*Cannabis sativa* L.) stem wood (0.2%) - BIII [4,14] medium from which they were transferred to malt extract plates (1.5% agar, 0.35% malt extract, 0.5% glucose) and experiments were inoculated with one agar plug as described before [14].

2.2. *Culture conditions*

The standard basal medium used in the experiments was N-limited liquid BIII medium [4] with 10 $g \cdot l^{-1}$ glucose in 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. The medium was autoclaved and a filter-sterilized thiamine solution (200 $mg \cdot l^{-1}$) was added (10 $ml \cdot l^{-1}$) afterwards. For measuring the time course of aromatic metabolite production, 5-ml aliquots of BIII medium were placed in 30 ml loosely capped serum bottles and experiments were incubated statically (unshaken) under an air atmosphere at 30°C. For the analysis of mass and ^{1}H-NMR spectra 500 ml aliquots of BIII medium were placed in 5 l

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Erlenmeyers closed with a cotton plug. After inoculation the bottles were incubated statically for 30 days at 30°C.

2.3. *Isolation and identification of aromatic metabolites*

To measure the amount of aromatic metabolites the entire culture (5 ml) was utilized for the extraction procedure. Consequently, separate cultures were prepared and analyzed in quadruplet on each sampling day. Sterile medium was incubated as an abiotic control. Acetonitrile (10 ml) was added to the culture bottles (5 ml medium, corrected for water evaporation losses), sealed with Teflon-lined silicon septa and extracted for one hour in a shake table (300 strokes/min; stroke=2 cm). A 2-ml subsample of the extract was centrifuged (10 min, 13,000 rpm) and 50 μl was injected into the HPLC.

For mass and ^{1}H-NMR spectra analysis, 500 ml of culture broth was filtered [14]. The supernatant was saturated with NaCl and extracted with 2 × 10 ml dichloromethane. The organic phase was evaporated and this crude fraction was used for total mass spectra analysis. HPLC purified unknown compounds (20 combined injections) were used, after evoporation of the solvent with N_2, for mass and ^{1}H-NMR spectra analysis.

2.4. *Synthesis of authentic compounds*

3-Chloro-anisyl alcohol (3-chloro-4-methoxybenzyl alcohol) was prepared from 3-chloro-4-methoxybenzoic acid by reduction with borane (BH_3) in tetrahydrofuran [15].

^{1}H-NMR ($CDCl_3$) δ (ppm): 3.03 (br s, -OH); 3.82 (s, $-OCH_3$); 4.46 (s, $-CH_2-$); 6.81 (d, J = 6.5 Hz, aromatic-H_5); 7.10 (dd, J = 2.0, 6.5 Hz, aromatic-H_6); 7.27 (d, J = 2.0 Hz, aromatic-H_2).

^{13}C-NMR ($CDCl_3$) δ (ppm): 55.92 (q); 63.74 (t); 111.73 (d); 121.99 (s); 126.26 (d); 128.79 (d); 133.79 (s); 154.03 (s).

3-chloro-anisaldehyde (3-chloro-4-methoxybenzaldehyde) was prepared from 3-chloro-anisyl alcohol by a gentle oxidation with pyridinium dichromate in dry dichloromethane [16]. Melting point 61–62°C.

^{1}H-NMR ($CDCl_3$) δ (ppm): 3.96 (s, $-OCH_3$); 7.02 (d, J = 8.5 Hz, aromatic-H_5); 7.75 (dd, J = 1.9, 8.5 Hz, aromatic-H_6); 7.87 (d, J = 1.9 Hz, aromatic-H_2); 9.82 (s, -CHO).

^{13}C-NMR ($CDCl_3$) δ (ppm): 56.26 (q), 111.44 (d), 123.43 (s), 130.00 (s), 130.29 (d), 130.93 (d), 159.54 (s), 189.42 (d).

2.5. *Instruments*

Supernatant (50 μl) was routinely analyzed on a Hewlett-Packard HPLC Chemstation (Pascal series) (Waldbronn, Germany) equipped with a HP1050 pumping station, a HP1040 M series II diode array detector and a HP9000-300 data processor. The column (200 × 3 mm) filled with ChromSpher C18-PAH (5-μm particles) was from Chrompack (Middelburg, The Netherlands). Aromatic metabolites were analyzed with the following gradient (0.4 ml/min, 30°C): 90:10, 0:100 and 0:100 H_2O/CH_3CN at 0, 15 and 20 min, respectively. The UV absorbance was monitored at 2 nm wavelength intervals from 210 to 350 nm.

Mass spectra were measured with a Hewlett-Packard HP5890 GC with 30 m DB17 column and a HP5970 MSD. Injection temperature was 200°C. The starting temperature of the column was 80°C, gradient 7°C/min, final temperature 240°C. Helium was used as carrier gas (20 ml/min).

^{1}H-NMR spectra were obtained with a Bruker AC-E200 spectrometer (Rheinstetten-Forchheim, Germany). Synthesized compounds and HPLC purified metabolite were dissolved in $CDCl_3$. Tetramethylsilane was used as an internal standard.

2.6. *Chemicals*

Veratryl alcohol, veratraldehyde, anisyl alcohol (4-methoxybenzyl alcohol), anisaldehyde (4-methoxybenzaldehyde) were from Janssen Chimica (Tilburg, The Netherlands). 3-Chloro-4-methoxybenzoic acid was from Heraeus (Karlsruhe, Germany).

3. RESULTS

3.1. *Identification of aromatic metabolites*

When grown in a synthetic medium with glucose as the sole source of carbon and energy, *Bjerkandera* sp. BOS55 produced several aromatic metabolites. A typical HPLC plot of a 14-day-old supernatant is shown in Fig. 1. In the 14-day-old sterile synthetic medium none of the aromatic metabolites were detected. The aromatic metabolites were identified by extracting the supernatant with dichloromethane and analyzing the organic phase on GC/MS. In addition to veratraldehyde and veratryl alcohol, *Bjerkandera* BOS55 produced anisaldehyde and 3-chloro-anisaldehyde. Furthermore a yet unknown peak was detected (Fig. 1) which, based on GC/MS analyses, contained 2 chlorine atoms. With the GC/MS, small amounts of anisyl alcohol and 3-chloro-

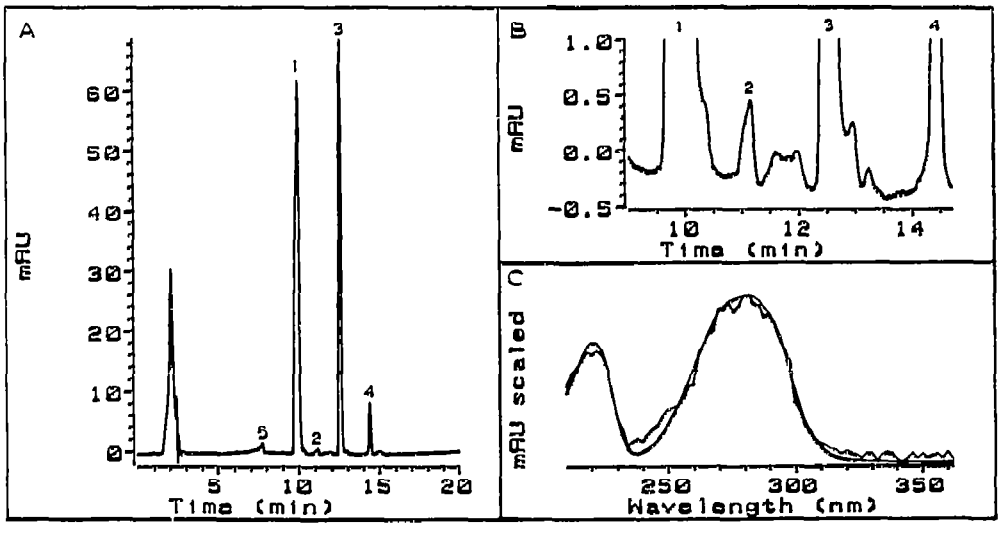

Fig. 1. (A) Typical HPLC chromatogram (λ 265 nm) of a 14-day-old supernatant of *Bjerkandera* BOS55. (B) Enlargement of the chromatogram. (C) UV spectrum of peak 2 (rough line) and reference spectrum of anisaldehyde (smooth line). Peaks are: (1) veratraldehyde, (2) anisaldehyde, (3) 3-chloro-anisaldehyde, (4) unknown compound, (5) veratryl alcohol.

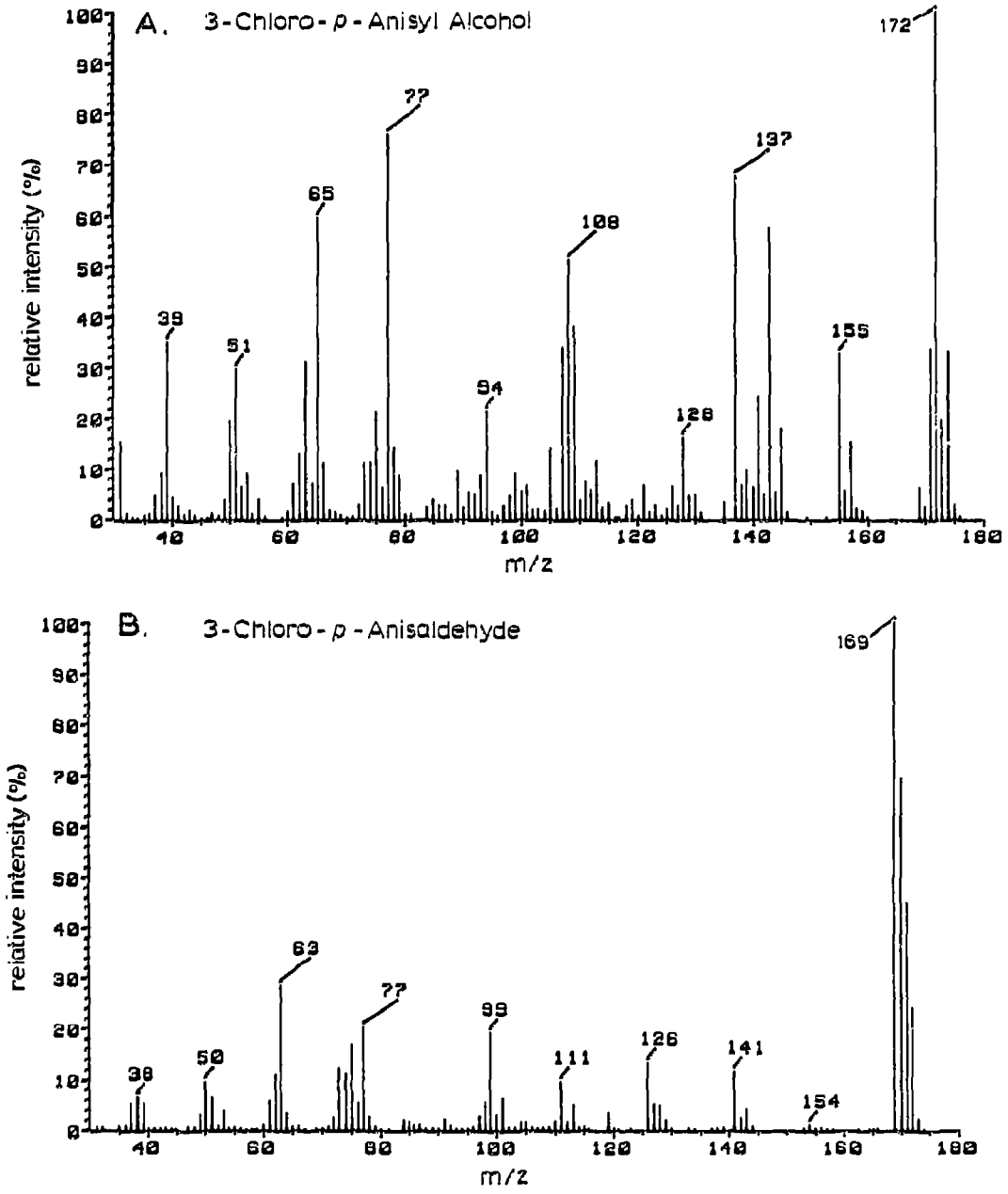

Fig. 2. Mass spectra of 3-chloro-anisyl alcohol and 3-chloro-anisaldehyde.

anisyl alcohol were also detected in 30-day-old cultures. The mass spectra of synthesized 3-chloro-anisyl alcohol and synthesized 3-chloro-anisaldehyde are given (Fig. 2). In Fig. 3, the normalized UV-spectra of both 3-chloro-anisyl alcohol and 3-chloro-anisaldehyde are presented. For all aromatic metabolites detected with HPLC (Fig. 1), the HPLC retention times, the UV spectra , the GC retention times and the mass spectra were identical to authentic compounds. 3-Chloro-anisaldehyde biosynthesized by *Bjerkandera* BOS55 was purified with HPLC. Its ^{1}H-NMR spectrum (^{1}H-NMR ($CDCl_3$) δ (ppm): 3.98 ($-OCH_3$); 7.03, 7.77, 7.90 ring protons; 9.84 (−CHO)) was identical to the spectrum of the synthesized compound.

3.2. *Biosynthesis of aromatic metabolites by Bjerkandera BOS55*

A typical time course of aromatic metabolite production by *Bjerkandera* BOS55 is given in Fig. 4. Although the amount of anisaldehyde decreases to very low concentrations after 14 days (Figs. 1 and 4) it was still detectable. This is shown with the UV spectrum which fits quite well with the spectrum of the standard (Fig. 1C). For most compounds, maximum concentration was

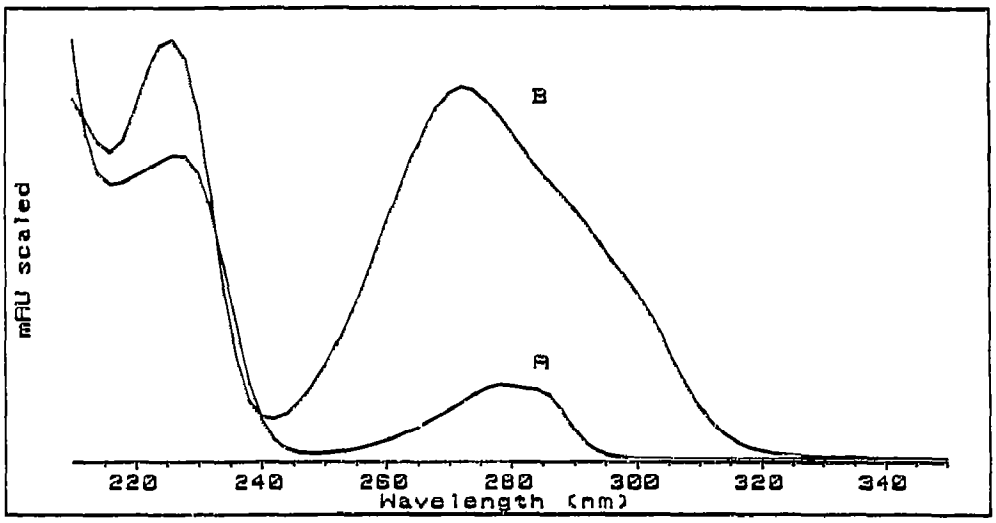

Fig. 3. Scaled UV spectra recorded in acetonitrile/H_2O (80:20) of 3-chloro-anisyl alcohol (A) and 3-chloro-anisaldehyde (B).

reached after 10 (3-chloro-anisaldehyde (50 μM) and anisaldehyde (3 μM) to 14 (veratraldehyde (100 μM)) days of incubation (Fig. 2), only the amount of veratryl alcohol showed a significant increase during the rest of the experiment.

3.3. *Biosynthesis of aromatic metabolites by other strains*

Formation of the newly detected aromatic metabolites was also investigated in *B. adusta* and *P. chrysosporium*. *Bjerkandera adusta* produced the same aromatic metabolites as *Bjerkandera* BOS55 as judged by HPLC and GC/MS. The metabolites occurred at similar concentrations in both *Bjerkandera* spp., except that the anisaldehyde (20 μM) concentration was higher in the supernatant of *B. adusta*. *P. chrysosporium*, however, only produced veratryl alcohol (0.1 mM after 14 days) as observed by HPLC analysis of the supernatant. GC/MS analysis also revealed trace amounts of veratraldehyde. Anisyl alcohol, 3-chloro-anisyl alcohol or the respective aldehydes, were not detectable by GC/MS.

4. DISCUSSION

Veratryl alcohol and veratraldehyde are known aromatic metabolites excreted by white-rot fungi which play an important role in lignin degradation [1]. In the present article we show that white-rot fungi of the genus

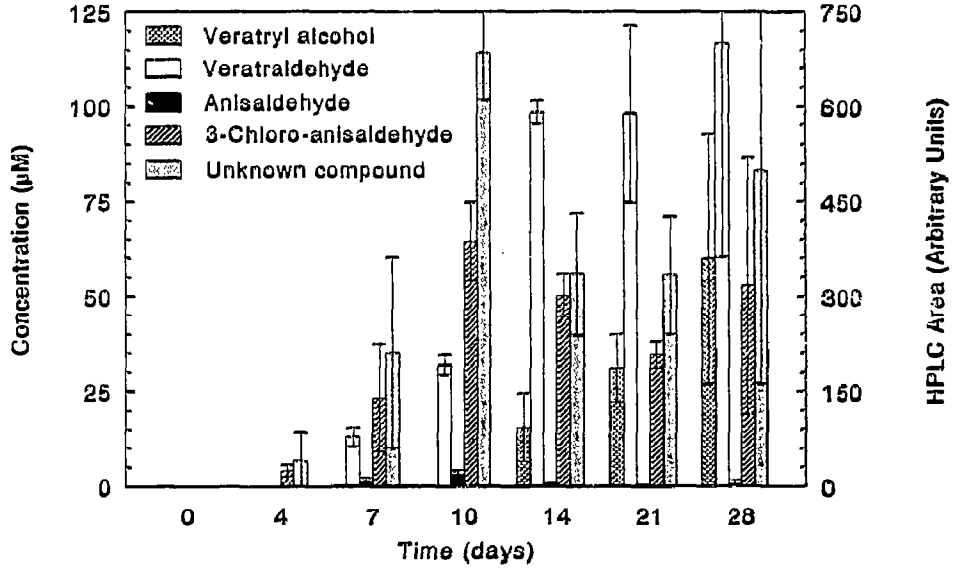

Fig. 4. Time course of the de-novo production of aromatic metabolites by *Bjerkandera* BOS55. The identified compounds are plotted in concentration (μM) units, the unknown compound containing two chlorine atoms is plotted in HPLC area units. The means of quadruplets ± S.D. are given.

Bjerkandera produce two pairs of aromatic metabolites not formed by *P.chrysosporium*: anisyl alcohol and anisaldehyde, 3-chloro-anisyl alcohol and 3-chloro-anisaldehyde. In contrast to *P. chrysosporium, B. adusta* produces an extracellular aryl alcohol oxidase [17], thought to be an important enzyme in the generation of H_2O_2. This enzyme oxidizes both veratryl alcohol and anisyl alcohol, displaying higher activity with the latter [17]. Preliminary results indicate that 3-chloro-anisyl alcohol is also a substrate for the aryl alcohol oxidase, suggesting that this alcohol is the precursor to 3-chloro-anisaldehyde.

As far as we know this is the first report of the de-novo biosynthesis of 3-chloro-anisyl alcohol by fungi. On the contrary, anisaldehyde and 3-chloro-anisaldehyde discovered in the *Bjerkandera* spp, are known aromatic metabolites of other fungi. *Lepista diemii* produces 3-chloro-anisaldehyde [18], anisaldehyde is produced by several fungi including *Camarophyllus virgineus* [19], *Trametes suaveolens* [20], *Daedalea juniperina* [21] and *Ischnoderma benzoinum* [22]. Berger et al. [8] screened many fungi for the production of flavours and fragrances. They found that *B. adusta* biosynthesizes anisaldehyde and veratraldehyde de-novo and concluded that this fungus is a potential flavour producer. In this work we have demonstrated that under certain culture conditions *Bjerkandera* spp. also form undesirable chlorinated products.

Biohalogenation is not a rare event in nature, at the moment more than 700 halogenated compounds are known [23]. This is, however, the first report of chlorinated aromatics production by a white-rot fungus.

We conclude that the ligninolytic complexes of *P. chrysosporium* and *Bjerkandera* spp. differ significantly both with respect to enzymes (aryl alcohol oxidase [17] and manganese inhibited peroxidase [14]) and aromatic metabolites produced. Further research in the physiological and ecological role of the above described aromatic metabolites in lignin degradation is therefore necessary.

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REFERENCES

[1] Buswell, J.A., in: Handbook of applied mycology. Vol. 1 (D.K. Arora, et al., Eds.) Marcel Dekker, New York, 1992, pp. 425–480.
[2] Aust, S.D. (1990) Microb. Ecol. 20, 197–209.
[3] Field, J.A., De Jong, E., Feijoo Costa, G. and De Bont, J.A.M. (1992) Appl. Environ. Microbiol., in press.
[4] Tien, M. and Kirk, T.K. (1988) Methods Enzymol. 161B, 238–248.
[5] Haemmerli, S.D., Leisola, M.S.A., Sanglard, D. and Fiechter, A. (1986) J. Biol. Chem. 261, (15) 6900–6903.
[6] Valli, K., Wariishi, H. and Gold, M.H. (1990) Biochemistry, 29, 8535–8539.
[7] Lundquist, K. and Kirk, T.K. (1978) Phytochemistry 17, 1676.
[8] Berger, R.G., Neuhauser, K. and Drawert, F. (1986) Flavour Frag. J. 1, 181–185.
[9] Hatakka, A.I. (1985) Arch. Microbiol. 141, 22–28.
[10] Kantelinen, A., Waldner, R., Niku-Paavola, M.-L. and Leisola, M.S.A. (1988) Appl. Microbiol. Biotechnol. 28, 193–198.
[11] Kawai, S., Umezawa, T. and Higuchi, T. (1986) Wood Res. 73, 18–21.
[12] Shimada, M., Nakatsubo, F., Kirk, T.K. and Higuchi, T. (1981) Arch. Microbiol. 129, 321–324.
[13] Turner, W.B. and Aldridge, D.C., Fungal Metabolites II. Academic Press, London, 1983, pp. 3–43.
[14] De Jong, E., Field, J.A. and De Bont, J.A.M. (1992) FEBS Lett. 299, 107–110.
[15] Yoon, N.M., Pak, C.S., Brown, H.C., Krishnamurthy, S. and Stocky, T.P. (1973) J. Org. Chem. 38, 2786–2792.
[16] Corey, E.J. and Schmidt, G. (1979) Tetrahedron Lett. 399–402.
[17] Muheim, A., Waldner, R., Leisola, M.S.A. and Fiechter, A. (1990) Enzyme Microb. Technol. 12, 204–209.
[18] Thaller, V. and Turner, J.L. (1972) J. Chem. Soc. Perkin, 1, 2032–2034.
[19] Farrell, I.W., Thaller, V. and Turner, J.L. (1977) J. Chem. Soc. Perkin I, 1886–1888.
[20] Birkinshaw, J.H., Bracken, A. and Findlay, W.P. (1944) Biochem. J. 38, 131–132.
[21] Birkinshaw, J.H. and Chaplen, P. (1955) Biochem. J. 60, 255–261.
[22] Berger, R.G., Neuhauser, K. and Drawert, F. (1987) Biotechnol. Bioeng. 30, 987–990.
[23] Neidleman, S.L. (1975) CRC Crit. Rev. Microbiol. 5, 333–358.