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Anisaldehyde Production and Aryl-Alcohol Oxidase and Dehydrogenase Activities in Ligninolytic Fungi of the Genus *Pleurotus*

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A variety of simple aromatic compounds were identified in liquid cultures of the basidiomycetes *Pleurotus cornucopiae*, *P. eryngii*, *P. floridanus*, *P. pulmonarius*, *P. ostreatus*, and *P. sajor-caju* by using gas chromatography-mass spectrometry. Such compounds were detected in fungal cultures on lignin- and straw-containing media, but it was found that they were also produced in the absence of aromatic precursors. Anisylic and hydroxybenzylic compounds (such as alcohols, aldehydes, and acids) were identified, *p*-anisaldehyde being the most characteristic extracellular metabolite synthesized by these ligninolytic fungi. Small amounts of 3-chloro-*p*-anisaldehyde were also detected in several species. It is postulated that the balance between the more-or-less-oxidized aromatic compounds can be explained in terms of the activity of fungal enzymes, including aryl-alcohol oxidase and dehydrogenase. The former enzyme shows high affinity for *p*-anisyl alcohol, which is oxidized to *p*-anisaldehyde with production of H_2O_2. The aryl-alcohol dehydrogenase was detected only in the mycelium, where it reduces aromatic aldehydes in the presence of NADPH. Both enzymes could be involved in the redox cycling of these aromatic compounds, providing H_2O_2 to ligninolytic peroxidases.

Volatile aromatic compounds produced by fungi contribute to natural fragrances of mushrooms, being considered a potential source for industrial flavors and aromas. Moreover, some of these compounds could also play a role in lignin degradation by the mycelium of basidiomycetes. In spite of the recent advances in the knowledge of this biodegradation process (14, 30), the performance of the whole ligninolytic system is not completely elucidated (17, 29). The production of lignin peroxidase (LiP; EC 1.11.1.14), the enzyme presumptively responsible for lignin degradation by *Phanerochaete chrysosporium*, has also been described in *Trametes* (8, 23) and *Phlebia* (13, 24, 26) species and suggested in other fungal species. However, LiP cannot be detected in other ligninolytic fungi, e.g., in *Dichomitus squalens* (27). Among the extracellular aromatic compounds produced by fungi, veratryl alcohol has received special attention, and different roles in lignin degradation by *P. chrysosporium* have been suggested for this compound (25, 30, 31). Veratryl alcohol seems to induce LiP, to protect it from inactivation by excess H_2O_2, and to contribute to the ending of the catalytic cycle. Moreover, the veratryl radical formed after oxidation by LiP has been suggested to act as an intermediate in biodegradation.

Some *Pleurotus* species have been investigated for the biopulping of cereal straw because of their ability to remove lignin preferentially (15). No LiP has been detected in these fungi, but aryl-alcohol oxidase (AAO; EC 1.1.3.7), an extracellular enzyme generating H_2O_2 during oxidation of veratryl and other polyunsaturated primary alcohols, has been purified and characterized in *Pleurotus sajor-caju* (4), *Pleurotus eryngii* (10, 11), and *Pleurotus ostreatus* (28). In fungi producing ligninolytic peroxidases, the H_2O_2 released by AAO could be used by LiP or manganese-dependent peroxidase (MnP; EC 1.11.1.13). Moreover, the H_2O_2 can generate hydroxyl radicals which can exert a direct effect on cell wall polymers. The enzymatic production of H_2O_2 could be integrated in the biodegradation processes if the aromatic compounds (e.g., vanillin and syringaldehyde) released during lignin depolymerization could be used as substrates for the H_2O_2-generating enzymes. Along this line, Guillén et al. (12) recently reported H_2O_2 production by *P. eryngii* in the presence of aromatic aldehydes. Since an aryl-aldehyde oxidase similar to that of *Streptomyces viridosporus* (7) is lacking in this fungus, Guillén et al. suggested a mechanism involving the joint action of AAO and an aryl-aldehyde reductase. On the other hand, the substrates for this cyclic H_2O_2-generating system could also have a different origin. The biosynthesis of aromatic compounds and the presence of related oxidases and reductases in *Pleurotus* species are investigated here.

MATERIALS AND METHODS

Fungi and culture conditions. The following *Pleurotus* strains were obtained from the Centraalbureau voor Schimmelcultures (CBS) and the Mycothèque de l'Université Catholique de Louvain (MUCL): *P. cornucopiae* CBS 383.80, *P. floridanus* MUCL 28518, *P. pulmonarius* CBS 507.85, *P. ostreatus* CBS 411.71, and *P. sajor-caju* MUCL 29757. *P. eryngii* IJFM A169 was isolated from the mycelium of a fruiting body cap. Modified Czapek-Dox medium (11) (100 ml in 1,000-ml flasks) was used for inoculum preparation, and, after 15 days of stationary incubation at 28°C, the mycelia were washed and homogenized.

The N-limited glucose medium used for studying the production of enzymes and aromatic compounds was a modification of the inoculum medium containing 30 g of glucose and 0.6 g of ammonium tartrate per liter. The lignin- and straw-containing media were prepared by the addition of straw alkali-lignin (0.1%) (19) or milled wheat straw (1%) to the

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N-limited glucose medium. Duplicate 1-liter flasks, containing 400 ml of the glucose, straw, or lignin medium, were inoculated with mycelium suspensions of the six *Pleurotus* species. Aromatic compounds and enzymatic activities were analyzed after 15 days of incubation at 28°C in a rotary shaker at 200 rpm. To monitor the production of enzymes during fungal growth, 25-ml samples were taken periodically from the same culture. For analyzing the mycelium-associated aromatic compounds, the total contents of additional flasks were collected at different incubation times.

Analytical methods. The samples were centrifuged at 4°C, and the supernatant (culture liquid) was collected for analysis. For mycelium analysis, the pellet was washed (4°C) with water and 200 mM phosphate buffer (pH 6), homogenized in liquid N_2, suspended in 5 ml of the buffer, and centrifuged, and the supernatant (mycelium extract) was collected for analysis. Ammonium was quantified by using an ammonium electrode, and mycelium dry weight was determined at 100°C after homogenization. Lignin degradation in the alkali-lignin medium was monitored by the decrease in A_{280}.

Enzymatic activities. AAO activity was estimated as the veratraldehyde (ε_{310}, 9,300 M^{-1} cm^{-1}) formed from 5 mM veratryl alcohol in 100 mM phosphate buffer (pH 6). Aryl-alcohol dehydrogenase (AAD; EC 1.1.1.91) was quantified by 0.2 mM NADPH (ε_{365}, 3,510 M^{-1} cm^{-1}) oxidation during reduction of 0.25 mM veratraldehyde in 100 mM phosphate buffer (pH 6). Laccase (EC 1.10.3.2) was measured with 5 mM ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] in 100 mM acetate buffer (pH 5; ε_{436} of ABTS oxidation product, 29,300 M^{-1} cm^{-1}). MnP was estimated by NADPH oxidation (1). One activity unit was defined as the amount of enzyme releasing 1 μmol of reaction product per min.

Chromatographic analysis of aromatic compounds. Samples of culture liquid (25 ml) and mycelium extract (25 ml, obtained from the mycelium of a whole flask) were adjusted to acid pH and extracted three times with 30 ml of ether. Water was removed with Na_2SO_4, the ether was evaporated under an N_2 stream, and the extracted compounds were resuspended in 12.5 μl of pyridine (with 4 μg of ethylvanillin as the internal standard) and derivatized with 20 μl of bis(trimethylsilyl)-trifluoroacetamide for 10 min at 50°C. The silanized compounds were analyzed by gas chromatography-mass spectrometry (GC-MS), using an SPB1 column (30 m by 0.25 mm) programmed for the temperature to increase from 100 to 280°C at 4°C min^{-1} and an ion-trap detector. They were identified by computer comparison of the mass spectra with those in the NBS library and with those obtained from standard compounds. The retention times of the corresponding standards allowed identification of isomers with similar mass spectra. The yields were calculated from the peak areas of the different aromatic compounds, versus that of the internal standard, in chromatograms obtained by using a flame ionization detector. In the case of anisylic and *p*-OH-benzylic compounds, the quantitations were corrected with response factors obtained from the corresponding standards.

RESULTS

Aromatic compounds produced by different *Pleurotus* species. The six *Pleurotus* species showed strong ability to decolorize the lignin-containing medium and removed lignin with degradation rates between 75% (*P. eryngii*) and 90% (*P. ostreatus*) in 15 days. After this period, different low-molecular-weight aromatic compounds were found in the culture liquid and identified by GC-MS. Anisaldehyde and anisic acid were among the most abundant compounds in the different cultures (Fig. 1A), but comparatively large amounts of *ortho-* and *para*-hydroxybenzyl alcohols were found in some samples (Fig. 1B). Furthermore, most of these compounds were also detected in the straw and glucose media, although the peak patterns exhibited some differences (Table 1). In addition to the aromatic compounds described above, several peaks, including aliphatic dicarboxylic acids, furancarboxylic acids, and dialkyl phthalates, were also obtained. On the other hand, some lignin-derived compounds (i.e., vanillin, syringaldehyde, vanillic acid, syringic acid, *trans-p*-coumaric acid, and *trans*-ferulic acid) found in the lignin medium before inoculation were lacking, or appeared in low amounts, after the fungal growth. This suggests that the simple aromatic compounds formerly present in the medium, in addition to those derived from lignin degradation, were almost completely transformed by the fungi, being substituted by compounds from fungal metabolism.

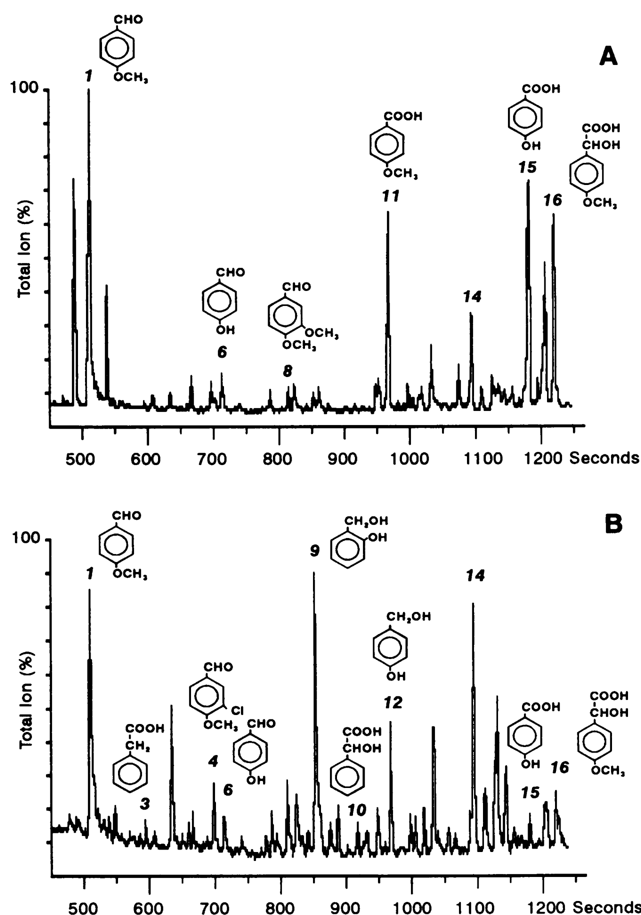

FIG. 1. GC-MS of aromatic compounds produced by *P. pulmonarius* (A) and *P. floridanus* (B) in lignin medium (after 15 days of incubation; as trimethylsilyl derivatives). Peaks: 1, *p*-anisaldehyde; 3, phenylacetic acid; 4, 3-chloro-*p*-anisaldehyde; 6, *p*-hydroxybenzaldehyde; 8, veratraldehyde; 9, *o*-hydroxybenzyl alcohol; 10, mandelic acid; 11, *p*-anisic acid; 12, *p*-hydroxybenzyl alcohol; 14, ethylvanillin (internal standard); 15, *p*-hydroxybenzoic acid; 16, *p*-methoxymandelic acid.

The activities of some enzymes acting on aromatic compounds were estimated in the different media. After 15 days of growth in the glucose medium, the following AAO activities were found: ca. 110 U $liter^{-1}$ in *P. pulmonarius* and *P.*

TABLE 1. Extracellular aromatic compounds produced by six *Pleurotus* species in lignin, straw, and glucose media

Aromatic compound	Media[a] in which compound is produced by:					
	P. pulmonarius	*P. ostreatus*	*P. cornucopiae*	*P. eryngii*	*P. sajor-caju*	*P. floridanus*
p-Anisaldehyde	L, S, G	L, G	L	G	L, S, G	L, S
Benzoic acid	G	L, G	L, S, G	G	L, S, G	G, S
Phenylacetic acid	G		L, S			L
3-Chloro-*p*-anisaldehyde				L, G	L	L
p-OH-benzaldehyde	L, S, G	L, G	L, S, G	L, G	L, G	L, G
p-Anisyl alcohol	G				S	
Veratraldehyde	L					
o-OH-benzyl alcohol	S, G	L, G	L, S, G	L, G	G	L, G
Mandelic acid					L	L
p-Anisic acid	L, G		L			
p-OH-benzyl alcohol	G	L, G	G	L, G	G	L, G
p-OH-benzoic acid	L, S, G		L, S	L	L	L, G
p-Methoxymandelic acid	L, S, G				L, S	L, S
di-OH-benzoic acid					L	

[a] L, lignin; S, straw; G, glucose.

cornucopiae, 40 U liter^{-1} in *P. ostreatus*, and ca. 10 U liter^{-1} in *P. eryngii*, *P. sajor-caju*, and *P. floridanus*. The levels of laccase were considerably lower: ca. 15 U liter^{-1} in *P. sajor-caju* and *P. floridanus*, 8 U liter^{-1} in *P. pulmonarius*, and ca. 5 U liter^{-1} in *P. cornucopiae* and *P. ostreatus*. The AAO activity in *P. pulmonarius* was higher in the lignin medium (170 U liter^{-1}) and, especially, in the straw medium (870 U liter^{-1}). However, the effect of straw or lignin addition on the above enzymatic activities was not significant in the other fungi.

Among the six species studied, *P. pulmonarius* produced the highest levels of aromatic compounds and enzyme activities, which were studied in detail during fungal growth, as described below.

Enzymes and aromatic compounds in *P. pulmonarius*. Nitrogen was the limiting nutrient in the glucose medium, and secondary metabolism commenced when ammonium was exhausted by *P. pulmonarius* after 6 days (Fig. 2A). The evolution of extracellular (Fig. 2A) and mycelium-associated (Fig. 2B) AAO, AAD, and laccase in this fungus is presented. AAO activity occurred first in the mycelium and later in the extracellular medium, attaining nearly 500 U liter^{-1} after 9 days. AAD activity was detected only in the mycelium collected during the first days of incubation. Extracellular laccase showed two maxima, the first during the growth phase, whereas a continuous decline was observed in the mycelium. MnP appeared in the culture liquid when the ammonium was exhausted and attained the maximum activity after 9 days, simultaneously with the AAO maximum.

Low-molecular-weight aromatic compounds identified by GC-MS from the culture liquid of *P. pulmonarius* grown on glucose medium are shown in Fig. 3A. In this medium, the amount of *p*-anisaldehyde produced was greater than that obtained in the lignin medium (Fig. 1A). The yield of aromatic compounds extracted from the mycelium was comparatively low, and a different pattern (Fig. 3B), characterized by the presence of hydroxybenzyl alcohols, was obtained after GC-MS analyses. The evolution of different aromatic compounds during fungal growth and a comparison of their concentrations in culture liquids and mycelium extracts are presented in Table 2. As mentioned above, *p*-anisaldehyde attained much higher concentrations than the other extracellular aromatic compounds. Most of the compounds detected had already been detected in this and other *Pleurotus* species (Table 1), but the methyl ester of the *p*-anisic acid was found for the first time in the culture liquid of *P. pulmonarius*.

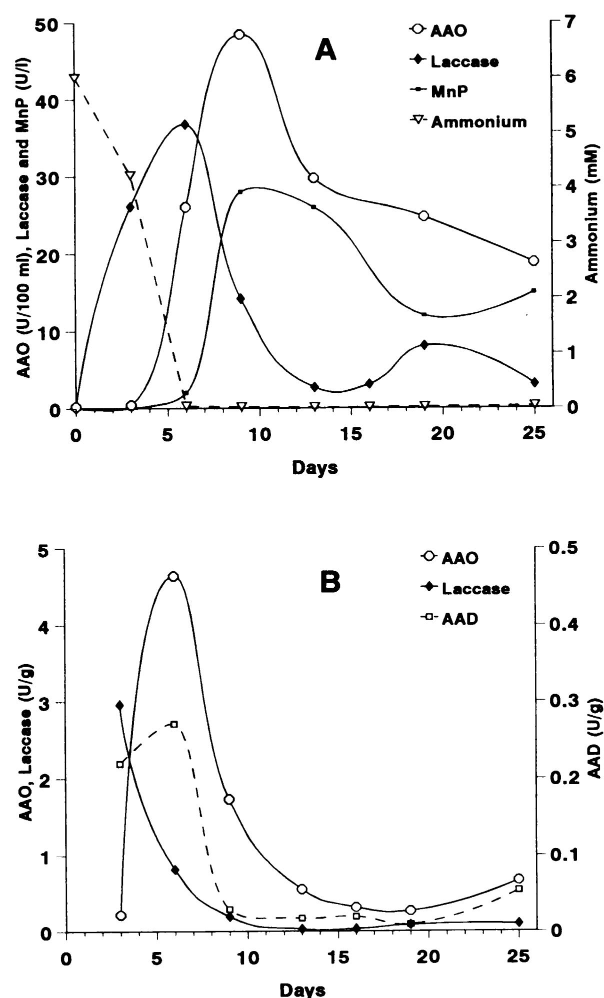

FIG. 2. Evolution of AAO, AAD, laccase, and MnP activities and ammonium levels in culture liquid (A) and mycelium extract (B) from *P. pulmonarius* grown on glucose medium.

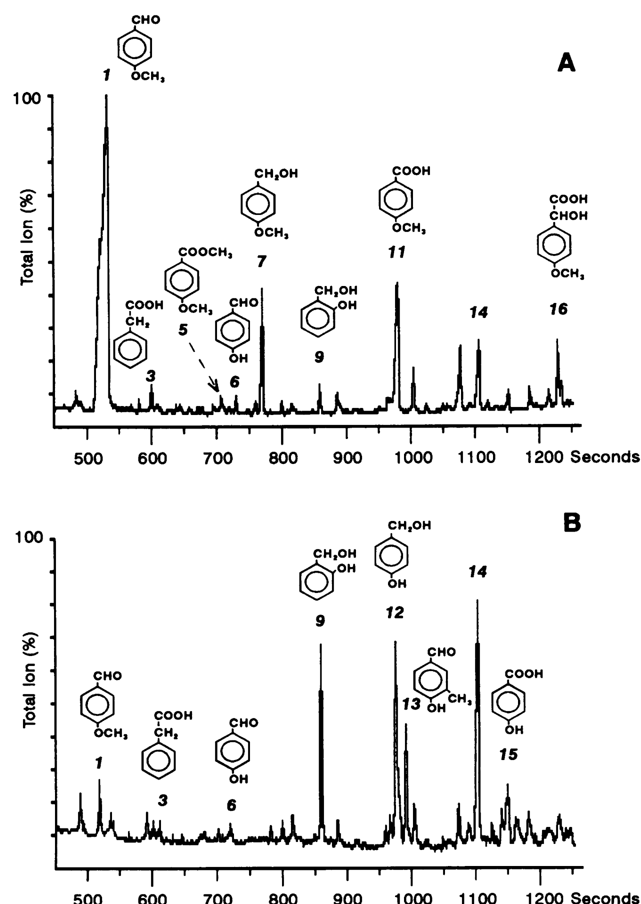

FIG. 3. GC-MS of aromatic compounds recovered from the extracellular medium (A) (5-day culture liquid) and the mycelium (B) (12-day mycelium extract) of *P. pulmonarius* grown on glucose medium (as trimethylsilyl derivatives). Peaks: 1, *p*-anisaldehyde; 3, phenylacetic acid; 5, methyl *p*-anisate; 6, *p*-hydroxybenzaldehyde; 7, *p*-anisyl alcohol; 9, *o*-hydroxybenzyl alcohol; 11, *p*-anisic acid; 12, *p*-hydroxybenzyl alcohol; 13, vanillin; 14, ethylvanillin (internal standard); 15, *p*-hydroxybenzoic acid; 16, *p*-methoxymandelic acid.

TABLE 2. Anisylic and hydroxybenzylic compounds in culture liquid and mycelium of *P. pulmonarius* after 5, 12, and 24 days of growth in glucose medium

Compound	Concn of compound in:					
	Culture liquid (μM)			Mycelium (nmol/g)		
	5 days	12 days	24 days	5 days	12 days	24 days
p-Anisaldehyde	66.8	198.1	134.1	0	5.1	83.5
p-OH-benzaldehyde	0	0.2	2.0	16.1	2.0	19.2
p-Anisyl alcohol	2.2	0	0	0	0	0
o-OH-benzyl alcohol	0.3	0.9	0	18.2	31.8	0
p-OH-benzyl alcohol	0	0	0	15.4	53.2	0
p-Anisic acid	4.1	6.7	4.9	0	1.6	95.6
p-OH-benzoic acid	0.1	3.0	1.4	0	0	0

DISCUSSION

The fact that most of the simple aromatic compounds detected in media containing lignin or straw were also found in glucose medium suggests that they can be synthesized by *Pleurotus* species. Moreover, these fungi displayed high levels of ability to degrade not only polymeric lignin but also simple aromatic compounds released during degradation. Of the species studied, *P. pulmonarius* synthesized the largest quantity and greatest diversity of simple aromatic compounds. Considerably smaller amounts of aromatic compounds were found in the mycelium than in the extracellular medium. The highest concentrations were found during secondary metabolism, although a decline was observed at the end of the incubation period. Synthesis of veratryl alcohol paralleling ligninolytic activity was also detected during secondary metabolism of *P. chrysosporium*. The addition of straw increased fungal growth, as already described in *P. pulmonarius* (20). However, limited enhancement of enzymatic activities was observed, except for *P. pulmonarius* AAO activity, which showed an eightfold increase.

Of the compounds synthesized by *P. pulmonarius*, the *p*-anisylic compounds (alcohol, aldehyde, and acid) were the most representative (Fig. 4). Since the different isomers of these compounds present similar mass spectra, their identification was completed by comparison of retention times. *p*-Anisaldehyde was the most abundant aromatic metabolite detected in the culture liquid (near 0.2 mM), being also in appreciable amounts in the mycelium at the end of the incubation period, when reductase activity was low. The production of *p*-anisaldehyde has been reported previously in basidiomycetes, being present in fruiting bodies and cultures of *Ischnoderma benzoinum* (2, 3). During the screening of basidiomycetes carried out by Gallois et al. (9), anisaldehyde was

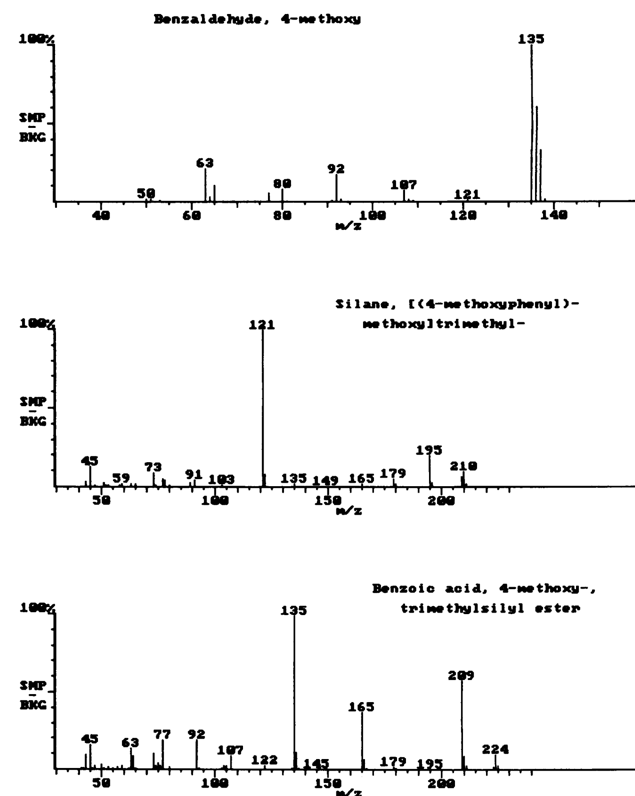

FIG. 4. Mass spectra of *p*-anisaldehyde, *p*-anisyl alcohol, and *p*-anisic acid from a gas chromatogram (Fig. 3A) of extracellular aromatic compounds synthesized by *P. pulmonarius* (as trimethylsilyl derivatives).

also identified in *D. squalens* and *Armillaria mellea*, but it was not found in *P. ostreatus*. In the present study, appreciable amounts of *p*-anisyl alcohol were found at the beginning of growth of *P. pulmonarius*, and it disappeared later, suggesting oxidation by AAO. An aryl-aldehyde oxidase (7) is not produced by *Pleurotus* species, but AAO also shows some aldehyde oxidase activity (10), which could lead to slow oxidation of *p*-anisaldehyde to *p*-anisic acid. Moreover, 3-chloro-*p*-anisaldehyde, a major compound in *Bjerkandera adusta* cultures (5), was found in some of the samples, especially in *P. eryngii*, and traces of 3-chloro-*p*-anisyl alcohol were also detected. These findings suggest that biosynthesis of chlorinated aromatic compounds may not be an exceptional event in fungal metabolism.

Hydroxybenzylic compounds were also present in several samples. The hydroxybenzyl alcohols were the most abundant aromatic compounds in the mycelium from *P. pulmonarius*, attaining the greatest quantity at the beginning of the secondary metabolism. Such products disappeared at the end of the incubation period, when only hydroxybenzaldehyde and hydroxybenzoic acid were detected. Hydroxybenzyl alcohols were also found in the culture liquid of other *Pleurotus* species (Fig. 1B) and could originate from anisylic compounds by fungal demethylation, as reported for veratric acid (16).

In addition to benzylic, hydroxybenzylic, and anisylic compounds, several phenylethyl-type compounds (including phenylacetic, mandelic, and *p*-methoxymandelic acids) were found. The biosynthesis of *p*-anisaldehyde by fungi has not yet been established but, as proposed for other aromatic compounds (33), it could be initiated via phenylalanine, *p*-coumaric acid, and *p*-methoxycinnamic acid. If *p*-methoxymandelic acid and related compounds come from intermediates in *p*-anisaldehyde production, the final biosynthetic reactions in *Pleurotus* spp. could be different from those proposed for veratryl alcohol in *P. chrysosporium*, in which veratraldehyde is formed from veratrylglycerol after a C_2-unit elimination reaction catalyzed by LiP (30).

Intracellular and extracellular oxidases and reductases of aromatic compounds are produced by *Pleurotus* species. *Pleurotus* AAD was reported for the first time in *P. eryngii* (34), and it could be similar to the enzyme found in the mycelium of *P. chrysosporium* (22). Reduction of aromatic acids has also been reported in ligninolytic fungi (18), suggesting the presence of an additional intracellular reductase. *P. eryngii* AAO can oxidize a variety of substituted benzylic alcohols, showing the highest activity on *p*-anisyl alcohol (K_m, 0.04 mM; V_{max}, 208 U mg^{-1}) (11) but very low activity on phenolic benzyl alcohols. The enzymes mentioned above are involved in redox reactions, probably leading to an equilibrium between concentrations of aromatic alcohols, aldehydes, and acids in *Pleurotus* cultures. A more complete knowledge of enzyme kinetic properties is required to explain the balance attained for each series of compounds. However, the dominance of *p*-anisaldehyde can be a consequence of high AAO affinity for *p*-anisyl alcohol, whereas the presence of hydroxybenzyl alcohols can be due to the lack of an enzyme (6) with high activity on these compounds. In the same way, *p*-anisaldehyde and great amounts of veratraldehyde and 3-chloro-*p*-anisaldehyde (5) are produced by *B. adusta* cultures, which also produce AAO (21).

The involvement of AAO in lignocellulose degradation is supported by the presence of AAO activity and extracellular H_2O_2 during the solid-state fermentation of wheat straw with different *Pleurotus* species (15, 32). The results presented here provide evidence of the synthesis of aromatic compounds by different species of this genus. These compounds can be used as substrates for the extracellular production of H_2O_2, which is needed for lignin biodegradation, through a hypothetical cyclic system (12) including fungal oxidases and reductases.

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ADDENDUM IN PROOF

A new biosynthetic pathway, including benzylic intermediates, has been proposed recently for veratryl alcohol (K. A. Jensen, Jr., K. M. C. Evans, T. K. Kirk, and K. E. Hammel, Appl. Environ. Microbiol. **60**:709–714, 1994) and could also be applied to other methoxybenzylic compounds.

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