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Vanillic Acid Metabolism by Selected Soft-rot, Brown-rot, and White-rot Fungi

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Abstract. Metabolism of vanillic acid, a product of lignin degradation, has been studied in selected representatives of soft-rot, brown-rot and white-rot fungi. All of the brown- and white-rot species examined decarboxylated vanillate to methoxyhydroquinone oxidatively. Mycelium extracts of all these fungi, except *Pleurotus ostreatus* contained high levels of an NAD(P)H-dependent vanillate hydroxylase. *P. ostreatus* also released $^{14}CO_2$ from $^{14}COOH$-vanillate but by a different mechanism possibly involving phenoloxidases. Most of these fungi also contained a dioxygenase which catalysed the *intra*-diol cleavage of hydroxyquinol (1,2,4-trihydroxybenzene) to form maleylacetate. No 3-O-demethylase activity was detected, and data indicate that in some of the fungi examined cleavage of the aromatic ring occurs without prior removal of the methoxyl group. None of the soft-rot fungi tested contained vanillate hydroxylase or hydroxyquinol 1,2-dioxygenase, but very low levels of protocatechuate 3,4-dioxygenase were detected in mycelium extracts. Vanillate catabolism among members of this group occurs via a different route which may involve ring demethylation although no 3-O-demethylase activity was detected in this study. The enzyme NAD(P)H-quinone oxidoreductase was demonstrated to exist in all the studied groups of fungi.

Key words: Vanillic acid metabolism – White-rot fungi – Brown-rot fungi – Soft-rot fungi – Vanillate hydroxylase – Aromatic ring cleavage – Quinone oxidoreductase

It is generally recognized that the major decay groups of wood decomposing fungi, the soft-rot, brown-rot and white-rot fungi, vary in their ability to degrade the lignin component (Kirk 1971; Higuchi 1971; Ander and Eriksson 1978; Crawford and Crawford 1980). Of these groups, only certain members of the white-rot fungi are known to totally decompose the lignin polymer.

Recently, an investigation into the metabolism of the vanillic acid, a well-established product of lignin degradation (Chen et al. 1981) by the white-rot fungus *Sporotrichum pulverulentum* has been undertaken in this laboratory (Ander et al. 1980). Previous reports in the literature indicate that white-rot fungi metabolize vanillate through protocatechuate (Flaig and Haider 1961; Cain et al. 1968) or through methoxyhydroquinone (Kirk and Lorenz 1973; Nishida and Fukuzumi 1978; Buswell et al. 1979a). When *S. pulverulentum* is grown in the presence of vanillate, mycelial extracts have been shown to contain induced levels of: (I) vanillate hydroxylase, which catalyses the oxidative decarboxylation of vanillate to methoxyhydroquinone (Buswell et al. 1979a; Yajima et al. 1979); (II) hydroxyquinol 1,2-dioxygenase, which catalyses the intradiol ring-cleavage of hydroxyquinol to maleylacetate (Buswell and Eriksson 1979); and (III) a quinone oxidoreductase which reduces several quinones to the corresponding phenols using NADH or NADPH as electron donor (Buswell et al. 1979b). In this paper we have examined the distribution of these enzymes, together with dioxygenases catalysing the ring cleavage of protocatechuate, catechol and gentisate, among selected representatives of each decay group. Evolution of $^{14}CO_2$ from carboxyl-, methoxyl- and ring-labelled vanillic acids has also been determined in an effort to correlate cell-free enzymic activities with the sequence of reactions involved in vanillate degradation.

Materials and Methods

Organisms

The following fungi were used in this study: (I) soft-rot fungi: *Chaetomium globosum* (Kunze ex Fr.) F 171-1 (ATCC 34152); *Humicola grisea* Traaden SP 37-22; *Petriellidium boydii* (Shear) Maltoch SP 31-4; (II) brown-rot fungi: *Daedalea quercina* (L. ex Fr.) 69346; *Lenzites trabea* (Pers. ex Fr.) Fr. Karst. A-576; *Fomes pinicola* (Sw. ex Fr.) Karst. B.H.; (III) white-rot fungi: *Phanerochaete* sp. L1; *Polyporus dichrous* Fr.; *Poria ambigua* Bres. BB3-1; *Pycnoporus cinnabarinus* (Jacq. ex Fr.) Karst. A-360; *Pleurotus ostreatus* (Jacq. ex Fr.) Kummer IPB No. 53.

All isolates, except *P. ostreatus* and *P. dichrous*, were kindly provided by Dr. Thomas Nilsson, The Swedish University of Agricultural Sciences, Uppsala, Sweden. *P. ostreatus* was obtained from Dr. R. Sopko, North Carolina State University, Department of Plant Pathology, Raleigh, USA. The source of *P. dichrous* is unknown.

Media

Cultures of soft-rot and white-rot fungi were grown on a modified Norkrans medium, pH 5.5, with 1% (w/v) cellulose (Eriksson and Pettersson 1975) in which $NH_4H_2PO_4$ was

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replaced by 1.15 g/l L-asparagine. Representatives of the brown-rot fungi did not grow on cellulose under the growth conditions described above and cellobiose (0.25 %, w/v), sterilized separately, was substituted as carbon source. Yeast extract (Difco), 0.1 % (w/v), was added to cultures of brown-rot fungi, and at a concentration of 0.01 % (w/v) to all other fungal cultures. After an appropriate mycelium density for all fungi had been attained (see below) vanillic acid (1 mM) was added as a filter-sterilized solution of its sodium salt, together with a further addition of 0.25 % (w/v) cellobiose.

Preparation of Fungal Inocula

Since spore production and germination times differed widely among the different fungi, test cultures were inoculated with fungal mycelia. Mycelia were produced in standing culture from spores in 50 ml of medium, contained in 250 ml flasks, with cellobiose as carbon source. Cultures were incubated at 28° C for up to 6 weeks prior to use. Depending on a visual estimation of the amount of fungal mycelium contained in each flask, material from 2–5 flasks was harvested by decantation and the combined mycelial mass washed twice with 50 ml of sterile distilled water. The mycelial mass was transferred to a stoppered sterile flask containing 50 ml distilled water and glass beads (7 mm dia), and agitated on a wrist-action shaker for periods up to 20 min. Aliquots (2–8 ml) of the fungal suspension were then used to inoculate test culture flasks.

The brown-rot fungi, on first cultivation from a spore inoculum, produced a strong dark brown colour which disappeared after re-inoculation (one to three times) of the mycelium into fresh medium. Cultures which no longer produced this brown colouration were used for inoculation.

Cultivation of Test Mycelia

To allow for direct comparisons, attempts were made to standardize culture conditions as much as possible even though the fungi used in this study would clearly be expected to have different requirements for optimal growth. A major problem encountered with the white-rot fungi, in particular those species reported to produce high levels of phenol-oxidases under conditions of laboratory culture, i.e. *P. cinnabarinus* and *P. ostreatus* (Ander and Eriksson 1977) was the polymerization of vanillic acid to form coloured products. In an effort to alleviate this difficulty the fungi were grown under conditions known to result in cellobiose: quinone oxidoreductase production. This extracellular enzyme, which catalyses the reduction of quinones to the corresponding phenols, is strongly induced by cellulose and requires cellobiose as electron donor (Westermark and Eriksson 1974a, b). Even so, formation of coloured products was still observed in cultures of high phenoloxidase-producing fungi.

Fungal cultures were grown at 28°C in shaken 1 l Erlenmeyer flasks containing 300 ml of medium until visual inspection showed good mycelium production. At this point cellobiose, followed by vanillate, was added. Uninoculated flasks and flasks without vanillate served as controls. For all except the brown-rot fungi, vanillate utilization was determined by measuring the decrease in absorbance at 254 nm and mycelia were harvested when a decrease of 30–70 % was observed. Vanillate utilization by brown-rot fungi could not be monitored in this way due to the presence in the culture medium of metabolites which absorbed strongly in the UV range. Vanillate disappearance, as measured by gas chromatography, also occurred very rapidly in these cultures. For brown-rot rungi, therefore, a standard procedure was adopted whereby mycelia were harvested after 10 h incubation with vanillate.

Mycelia were collected by filtration through a nylon cloth (30 µm mesh size), washed with 50–100 ml water and excess liquor squeezed out by hand. After washing with a further 50–100 ml water excess liquid was removed as before and the fungal material frozen at −15° C until required for enzyme analysis.

Preparation of Mycelial Extracts

For the preparation of extracts, thoroughly washed mycelia were suspended in 2–3 vol buffer, pH 7.0, and broken in a homogenisator at 0–5° C. Extracts for vanillate hydroxylase assays were prepared in 0.1 M K-phosphate buffer, otherwise Tris-HCl buffer was used. The cell break was initially clarified by centrifugation at 3,000 ×*g* for 10 min and the highly turbid extract was then further centrifuged at 30,000 × *g* for 30 min and the supernatant retained. Assays for ring-cleavage enzymes were performed using the supernatant fraction obtained after a single centrifugation at 30,000 × *g* for 30 min. Efforts to detect demethylase activity were made using mycelial extracts clarified by centrifugation at 3,000 × *g* for 5 min ($^{14}CO_2$ evolution assay) and with the supernatant fraction obtained after centrifugation at 30,000 × *g* for 30 min (polarographic assay).

Enzyme Assays

Vanillate hydroxylase was assayed at 30° C by measuring $^{14}CO_2$ evolution from $^{14}COOH$-vanillate for 1 h as previously described (Buswell et al. 1979a; Ander et al. 1980). Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) was measured by monitoring the disappearance of substrate at 290 nm (Cain et al. 1968). Catechol 1,2-dioxygenase (EC 1.13.11.1) was assayed as described by Hegeman (1966). Gentisate 1,2-dioxygenase (EC 1.13.11.4) was assayed by measuring the increase in extinction at 334 nm caused by maleylpyruvate formation (Buswell and Clark 1976). Oxidation of hydroxyquinol to maleylacetate (i.e. *ortho*-ring-cleavage) was observed by running repeat scans over the absorbance range 240–300 nm. Cleavage of the aromatic ring was accompanied by the appearance of an absorbance peak at 243 nm which disappeared when the reaction mixture was acidified to pH 3 (Buswell and Eriksson 1979). NAD(P)H: quinone oxidoreductase was assayed according to Buswell et al. (1979b) using 2-methoxyquinone. Demethylase activity was determined by measuring the release of $^{14}CO_2$ from $O^{14}CH_3$-vanillate and with the Clark oxygen electrode (Rank Bros, Bottisham, England).

Extraction of Culture Filtrate

The culture fluid (300 ml) was adjusted to pH 2 and extracted with 3 × 200 ml distilled ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness at 40° C under vacuum.

Gas Chromatography (GC)

GC was performed using a Packard model 427 with flame ionization detector and fitted with a glass capillary column SE-30 (25 m × 0.36 mm). Operating conditions: injection 200°C, detection 200°C, programme 6 min 150°C, rise 5°C/min to 170°C, final time 5 min. Carrier gas N_2 at 30 ml/min.

The residue from ethyl acetate extracts was dissolved in pyridine and silylated with bis-trimethylsilyltrifluoracetamide (BSTFA) for 1 h. Syringol or veratric aldehyde were used as internal standards; these were never found as products. Identification of products was based on comparison with authentic reference compounds. The standard deviation of the quantitative determinations was 20%.

Evolution of $^{14}CO_2$ from Fungal Cultures After Addition of Labelled Substrates

In experiments using ^{14}C-labelled vanillic acids, formaldehyde and methanol the medium was the same Norkrans' medium as described above but cellobiose (0.25%, w/v) was the carbon source in all cases. Test mycelia were grown up on glucose (0.25%, w/v) for 5–10 days and a cell suspension prepared as above. Aliquots (0.5 ml) of the fungal suspension were then added aseptically to 25 ml Norkrans' medium + cellobiose in 125 ml flasks. After 24 h growth in standing culture, 1.0 mM unlabelled vanillic acid, adjusted to pH 5.5 and filter-sterilized, was added followed by one ^{14}C-labelled compound. Labelled methanol and formaldehyde were added to a concentration of 3 nM and the ring-, carboxyl- and methoxyl-labelled vanillic acids to 30 nM. This corresponds to about 30,000 dpm in each culture. Incubations were set up in triplicate and samples taken daily. Uninoculated flasks containing separately each of the labelled compounds served as controls.

Estimations

Reducing sugars were estimated by the Somogyi-Nelson method (Nelson 1944; Somogyi 1952). Protein was determined spectrophotometrically and by a modified biuret method (Gornall et al. 1949).

Radioactively Labelled Substances

Carboxyl-labelled vanillic acid (^{14}COOH-vanillate) (6.2 $\times 10^6$ dpm/mg), methoxyl-labelled vanillic acid ($O^{14}CH_3$-vanillate) (0.9 $\times 10^6$ dpm/mg) and ring-labelled vanillic acid (^{14}C-ring-vanillate) (2.38 $\times 10^6$ dpm) were the gifts of Dr. Konrad Haider, Inst. für Pflanzenernährung und Bodenkunde, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, FRG. The ^{14}C-labelled methanol and ^{14}C-labelled formaldehyde were purchased from New England Nuclear. Stock solutions of both were prepared to contain 5.55 $\times 10^6$ dpm/ml.

Chemicals

Methoxyquinone was a gift from Dr. Göran Gellerstedt of this Institute. All other chemicals were obtained from commercial sources and shown by gas chromatography (GC) to be free of impurities which could interfere with the analytical determinations. Munktell's Cellulose Powder No. 400 from Grycksbo Pappersbruk, Stora Kopparsbergs Bergslags AB, Grycksbo, Sweden, and cellobiose (BDH, England) were used as carbon co-substrates.

Results

Vanillate Degradation and Production of Metabolites

Despite a fast growth rate and good mycelium production, soft-rot fungi were generally slowest at utilizing vanillate. No metabolic products were detected in spent culture liquids apart from low levels of vanillin produced by *Chaetomium globosum* (Table 1). One other feature of this group were the relatively high final pH values (5.0–7.3) observed in cultures irrespective of whether vanillate was added to the medium.

Brown-rot fungi grew poorly, even when cellobiose was substituted for cellulose as a carbon source. However, vanillate was rapidly depleted in all cases and methoxyhydroquinone was readily detectable as a reaction product, accumulating to very high levels in cultures of *Fomes pinicola* (Table 1).

Generally, vanillate was quickly metabolized by white-rot fungi and GC analyses of spent culture liquids showed the presence of both methoxyhydroquinone and the reduced compounds, vanillin and vanillyl alcohol (Table 1). An unidentified peak, with the same relative retention in both cases, was also observed in culture filtrates of *Poria ambigua* and L-1.

Evolution of $^{14}CO_2$ from ^{14}COOH-vanillate by Extracts of White-rot, Brown-rot and Soft-rot Fungi

High levels of an enzyme catalysing the evolution of $^{14}CO_2$ from ^{14}COOH-vanillate were detectable in mycelial extracts of all the brown-rot and white-rot fungi used in this study. In most cases, $^{14}CO_2$ evolution was dependent on the presence in reaction mixtures of reduced pyridine nucleotide (Tables 2 and 3). In brown-rot fungi both forms of the coenzyme appeared equally effective while amongst the white-rot representatives *P. ambigua* showed a preference for NADH (Tables 2 and 3). Considerable $^{14}CO_2$ evolution was observed in unsupplemented reaction mixtures containing extracts of either *Daedalea quercina*, *F. pinicola* or *Polyporus dichrous*. However, if extracts were passed through a Sephadex G-25 column prior to assay this $^{14}CO_2$ evolution decreased, suggesting that it was dependent on endogenous NAD(P)H (Table 4). Extracts of *Sporotrichum pulverulentum*, grown in the presence of vanillate with glucose as a co-substrate, readily reduce NAD and NADP (Yajima et al. 1979).

As previously reported for *S. pulverulentum* (Buswell et al. 1979a) enzymic activity resided mainly in the soluble fraction and although basal levels were detectable in extracts of mycelia grown on cellulose/cellobiose, these levels were greatly increased by the presence of vanillate in the growth medium. One exception to the aforementioned observations was *Pleurotus ostreatus*, where large quantities of $^{14}CO_2$ was released from carboxyl labelled vanillate without coenzyme supplementation (Table 2). Indeed, inclusion of NADH or NADPH in reaction mixtures greatly reduced the levels of $^{14}CO_2$ evolution.

Extracts of soft-rot fungi uniformly catalysed only very low levels of $^{14}CO_2$ evolution in both the presence and

Table 1. Metabolic intermediates detected in culture filtrates after growth of rot fungi on a medium containing 1 mM vanillate

Fungi	Vanillate remaining (%)	Incubation before vanillate addition (h)	Harvesting time from start (h)	Product concentration[a] (mM)		
				Vanillin	Vanillyl alc.	MHQ
Soft-rot						
Chaetomium globosum	54	26	59	0.008	–	–
Humicola grisea	73	26	140	–	–	–
Petriellidium boydii	60	26	46	–	–	–
Brown-rot						
Daedalea quercina	70	38	48	–	–	0.110
Lenzites trabea	65	49	59	–	–	0.066
Fomes pinicola	5	49	59	–	–	1.0
White-rot						
L-1	31	49	65.5	0.23	0.034	0.013
Polyporus dichrous	61	72	83.5	–	Trace	Trace
Poria ambigua	46	29.5	51.5	–	0.015	–
Pycnoporus cinnabarinus	50	29.5	55.5	0.14	–	–
Pleurotus ostreatus	10	41	55.5	–	–	–

[a] Analysis was made only with respect to the listed compounds

Table 2. Vanillate hydroxylase activity in extracts (with and without added co-factors) of white-rot fungi grown in the presence and absence of vanillate

	Co-factors		
	None	NADH	NADPH
Group I fungi (Low phenoloxidase)			
1. *Poria ambigua*	6	2,575	877
	(327)	(674)	(307)
2. *Polyporus dichrous*	250	5,437	4,541
	(4)	(103)	(71)
3. L-1	0	430	331
	(3)	(178)	(222)
Group II fungi (High phenoloxidase)			
4. *Pleurotus ostreatus*	51,784	1,019	944
	(7,115)	(313)	(305)
5. *Pycnoporus cinnabarinus*	44	4,864	5,162
	(2)	(194)	(70)

Values are expressed as dpm $^{14}CO_2$/h · mg protein
Figures in parentheses refer to levels of enzyme recorded in extracts of fungi grown without vanillate

Table 3. Vanillate hydroxylase activity in extracts (with and without added co-factors) of brown-rot and soft-rot fungi grown in the presence and absence of vanillate

	Co-factors		
	None	NADH	NADPH
Brown-rot fungi			
1. *Daedalea quercina*	93	4,084	3,952
	(0)	(351)	(281)
2. *Fomes pinicola*	1,597	8,211	7,948
	(52)	(1,825)	(1,256)
3. *Lenzites trabea*	7	1,886	1,368
	(0)	(132)	(64)
Soft-rot fungi			
1. *Chaetomium globosum*	22	36	53
	(0)	(4)	(21)
2. *Petriellidium boydii*	50	43	84
	(37)	(2)	(0)
3. *Humicola grisea*	57	27	12
	(9)	(2)	(1)

Values are expressed as dpm $^{14}CO_2$/h · mg protein.
Figures in parentheses refer to levels of enzyme recorded in extracts of fungi grown without vanillate

absence of NAD(P)H (Table 3) and enzyme activity was regarded as negligible.

Aromatic Ring-cleavage Activities in Mycelial Extracts of White-rot, Brown-rot, and Soft-rot Fungi

No significant ring-cleavage activity against protocatechuate or gentisate was detectable in mycelial extracts of any of the brown-rot or white-rot fungi used in this study (Table 5). Extracts of *P. ostreatus* catalysed a conversion of protocatechuate and catechol as indicated by a decrease in

Table 4. Vanillate hydroxylase activity in mycelial extracts of *Fomes pinicola* and *Daedalea quercina* after passage through Sephadex G25

	None	NADH	NADPH
D. quercina	0	3,803	3,350
F. pinicola	226	7,987	6,581

Values are expressed as dpm $^{14}CO_2$/h · mg protein
Extract used was the supernatant fraction obtained after centrifugation at 30,000 × *g* for 30 min
Fungi were grown in the presence of vanillate

Table 5. Distribution of enzymic activities among selected representatives of soft-rot, brown-rot, and white-rot fungi

	Vanillate hydroxylase	Protocatechuate[a] 3,4-dioxygenase	Catechol[a] 1,2-dioxygenase	Hydroxyquinol[b] 1,2-dioxygenase	NAD(P)H : quinone[c] oxidoreductase	
					NADPH	NADH
A. Soft-rot						
1. *Petriellidium boydii*	–	0.001	<0.001	ND	13.01	4.96
					(2.18)	(1.46)
2. *Chaetomium globosum*	–	0.002	<0.001	ND	0.58	0.53
					(0.14)	(0.10)
3. *Humicola grisea*	–	0.001	<0.001	ND	0.13	<0.01
					(0.15)	(<0.01)
B. Brown-rot						
1. *Daedalea quercina*	+	ND	<0.001	+	2.47	1.07
					(1.87)	(1.27)
2. *Fomes pinicola*	+	ND	<0.001	+	0.043	0.029
					(0.017)	(0.014)
3. *Lenzites trabea*	+	<0.001	<0.001	+	18.62	23.46
					(2.14)	(0.89)
C. White-rot						
1. *Polyporus dichrous*	+	<0.001	<0.001	+	5.98	5.68
					(0.94)	(0.89)
2. *Poria ambigua*	+	ND	ND	+	0.08	0.07
					(0.036)	(0.031)
3. L-1	+	ND	ND	ND	1.98	1.88
					(1.04)	(1.01)
4. *Pycnoporus cinnabarinus*	+	ND	ND	+	9.19	15.99
					(0.65)	(0.72)
5. *Pleurotus ostreatus*	?	?	?	ND	<0.01	<0.01
					(<0.01)	(<0.01)

[a] Expressed as micromol of substrate used (or product formed) per minute per milligram of protein
[b] No quantitative determination was made; + equals activity detected
[c] Expressed as micromoles of NADPH or NADH oxidized per minute per milligram of protein. Values in parenthesis refer to levels found in extracts of non-induced mycelia
ND = not detected

absorbance at 290 nm and an increase at 260 nm, respectively. However, these extracts contained phenoloxidases and, since both catechol and protocatechuate are substrates for these enzymes, it is not clear if cleavage by dioxygenases is involved.

All brown-rot and several white-rot fungi were able to catalyse the intradiol cleavage of hydroxyquinol (1,2,4-trihydroxybenzene) (Table 5) to yield a product with the same spectral characteristics as maleylacetate (Chapman and Ribbons 1976; Gaal and Neujahr 1979; Buswell and Eriksson 1979). Hydroxyquinol readily autooxidizes to 2-hydroxy-1,4-benzoquinone (Corbett 1970) and, since quinones are known to inhibit ring-cleaving enzymes (Bilton and Cain 1968; Varga and Neujahr 1972) it was necessary in some cases to use low concentrations of substrate (i.e. 50 nmol) in order to demonstrate hydroxyquinol 1,2-dioxygenase. Furthermore, extracts of *D. quercina* and *F. pinicola* were passed through a Sephadex G-25 column prior to assay. This removed endogenous levels of reduced pyridine nucleotide which serve as coenzymes for maleylacetate reductase (Chapman and Ribbons 1976; Gaal and Neujahr 1979; Buswell and Eriksson 1979) and thus prevented the accumulation of the ring fission product on which the spectrophotometric assay was based.

The three soft-rot fungi showed no activity against hydroxyquinol but extracts contained very low but detectable levels of protocatechuate 3,4-dioxygenase activity. No gentisate 1,2-dioxygenase was found in these fungi.

Intracellular Quinone Oxidoreductase Activity in White-rot, Brown-rot, and Soft-rot Fungi

Extracts of most fungi tested contained NAD(P)H:quinone oxidoreductase activity although levels varied greatly between fungi (Table 5). Very high activities were found in *Petriellidium boydii, Lenzites trabea* and the white-rot fungi *P. dichrous* and *Pycnoporus cinnabarinus*. Appreciable levels were also found in extracts of several fungi grown on cellulose/cellobiose although, in most cases, these were increased when vanillate was included in the culture medium.

Metabolism of Labelled Formaldehyde, Methanol and Vanillic Acids

The release of $^{14}CO_2$ by *C. globosum, L. trabea, P. cinnabarinus, P. ostreatus* and *S. pulverulentum* was measured in standing cultures containing unlabelled vanillic acid and, separately, carboxyl-, methoxyl-, and ring-labelled vanillate, $^{14}CH_3OH$ and $H^{14}CHO$.

Table 6. ^{14}C-activities measured 10–15 days after addition of unlabelled vanillate and labelled vanillate, CH_3OH or HCHO to cultures grown for 24 h on 0.25% cellobiose, 0.01% yeast extract and modified Norkrans medium at pH 5.5

Organism	Type of label	%^{14}C in culture solution	%^{14}C in mycelium	%$^{14}CO_2$ evolved	Total
Chaetomium globosum (after 14 days)	$^{14}CH_3OH$	14.6	5.0	69.8 (22.0)	89.4
	$H^{14}CHO$	27.1	31.7	32.9	91.7
	^{14}COOH-VA	5.8	2.2	81.1	89.1
	$O^{14}CH_3$-VA	28.5	25.4	38.3	92.2
	^{14}Ring-VA	11.6	21.7	57.3	90.6
Lenzites trabea (after 10 days)	$^{14}CH_3OH$	69.2	0.2	20.9 (16.0)	90.3
	$H^{14}CHO$	54.9	3.1	31.0	89.0
	^{14}COOH-VA	73.4	1.7	9.8	84.9
	$O^{14}CH_3$-VA	92.6	2.4	0.7	95.7
	^{14}Ring-VA	85.2	2.4	3.4	91.0
Pycnoporus cinnabarinus (after 14 days)	$^{14}CH_3OH$	18.4	19.1	56.5 (22.0)	94.0
	$H^{14}CHO$	45.6	31.6	20.5	97.7
	^{14}COOH-VA	8.9	0.8	68.2	77.9
	$O^{14}CH_3$-VA	24.5	20.1	49.8	94.4
	^{14}Ring-VA	22.3	13.5	48.2	84.4
Pleurotus ostreatus (after 14 days)	$^{14}CH_3OH$	3.8	21.0	63.5 (22.0)	88.3
	$H^{14}CHO$	28.0	58.7	15.2	101.9
	^{14}COOH-VA	11.4	11.0	64.6	87.0
	$O^{14}CH_3$-VA	38.6	48.7	9.8	97.1
	^{14}Ring-VA	15.1	45.5	5.5	66.1
Sporotrichum pulverulentum (after 14.8 days)	$^{14}CH_3OH$	5.0	2.6	84.9 (23.0)	92.5
	$H^{14}CHO$	47.3	16.5	32.5	96.3
	^{14}COOH-VA	13.6	2.5	79.5	95.6
	$O^{14}CH_3$-VA	14.2	13.6	61.9	89.7
	^{14}Ring-VA	18.2	20.2	59.8	98.2

Figures in parenthesis indicate the levels of radioactivity detected in the NaOH-filled tubes in uninoculated control flasks

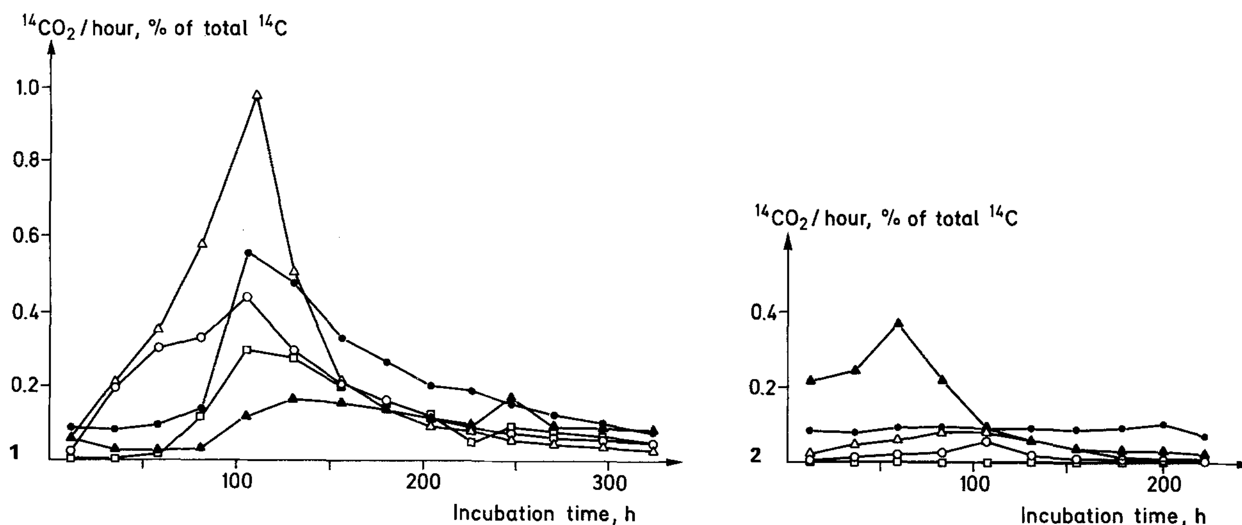
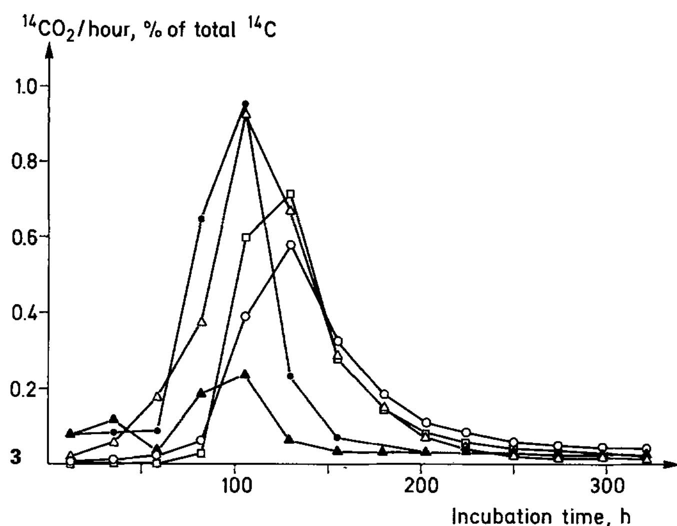


Fig. 1. ^{14}CO evolution from ^{14}COOH-vanillate (△——△), $O^{14}CH_3$-vanillate (□——□), ^{14}C-ring-vanillate (○——○), $^{14}CH_3OH$ (●——●) and $H^{14}CHO$ (▲——▲) by *Chaetomium globosum*. $^{14}CO_2$ data are mean values from 3 replicate cultures

Fig. 2. ^{14}CO evolution from ^{14}COOH-vanillate (△——△), $O^{14}CH_3$-vanillate (□——□), ^{14}C-ring-vanillate (○——○), $^{14}CH_3OH$ (●——●) and $H^{14}CHO$ (▲——▲) by *Lenzites trabea*. $^{14}CO_2$ data are mean values from 3 replicate cultures

Figure 1 shows that with the soft-rot fungus *C. globosum* the release of $^{14}CO_2$ from all three specifically labelled vanillic acids reached a peak about 4–5 days after addition. Release of $^{14}CO_2$ from carboxyl- and ring-labelled vanillate occurred rapidly but was delayed for about 60 h in the case of the methoxyl-labelled compound. However, peak release of $^{14}CO_2$ from labelled methanol and formaldehyde, likely products of a demethylation reaction, was also delayed indicating that demethylation may have taken place rapidly but that conversion of the released C-1 moiety to carbon dioxide occurred more slowly. Alternatively, since a relatively high percentage (31.7%) of labelled formaldehyde was found in the fungal mycelium (Table 6), the initial low detectable levels of $^{14}CO_2$ from methoxyl-labelled vanillate may reflect a



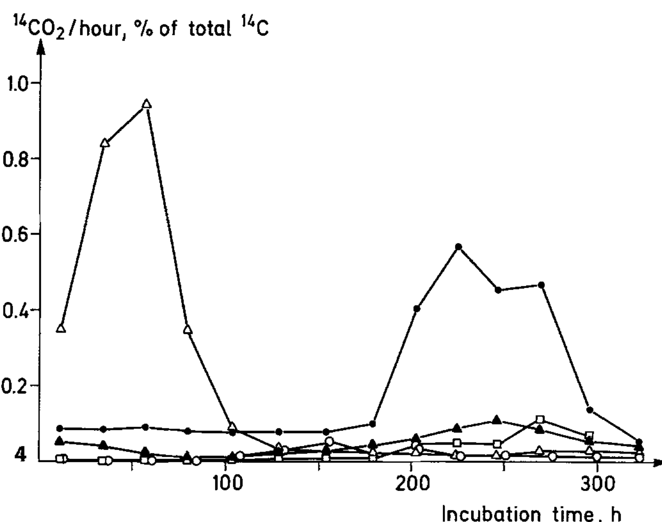


Fig. 3. ^{14}CO evolution from $^{14}COOH$-vanillate (△——△), $O^{14}CH_3$-vanillate (□——□), ^{14}C-ring-vanillate (○——○), $^{14}CH_3OH$ (●——●) and $H^{14}CHO$ (▲——▲) by *Pycnoporus cinnabarinus*. $^{14}CO_2$ data are mean values from 3 replicate cultures

Fig. 4. ^{14}CO evolution from $^{14}COOH$-vanillate (△——△), $O^{14}CH_3$-vanillate (□——□), ^{14}C-ring-vanillate (○——○), $^{14}CH_3OH$ (●——●) and $H^{14}CHO$ (▲——▲) by *Pleurotus ostreatus*. $^{14}CO_2$ data are mean values from 3 replicate cultures

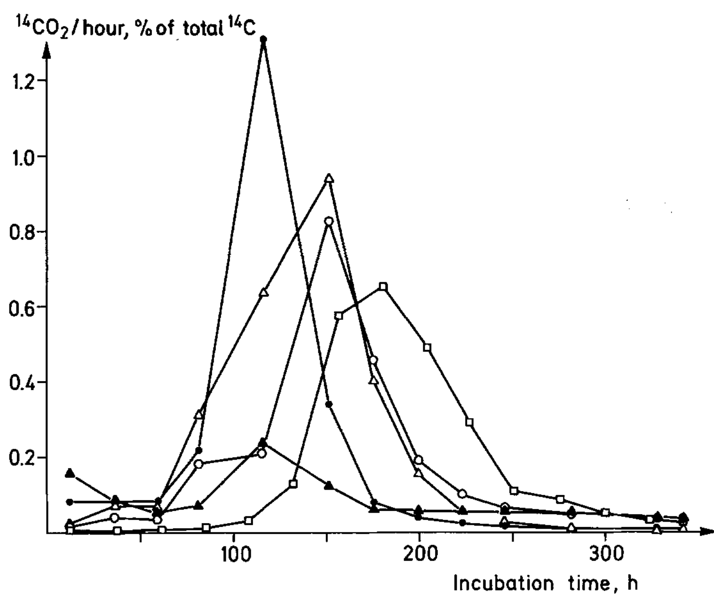


Fig. 5
^{14}CO evolution from $^{14}COOH$-vanillate (△——△), $O^{14}CH_3$-vanillate (□——□), ^{14}C-ring-vanillate (○——○), $^{14}CH_3OH$ (●——●) and $H^{14}CHO$ (▲——▲) by *Sporotrichum pulverulentum*. $^{14}CO_2$ data are mean values from 3 replicate cultures

high rate of incorporation of released C-1 compound into cell material. This is supported by the similar level of incorporation found with methoxyl-labelled vanillate.

Amounts of $^{14}CO_2$ evolved from labelled substrates by the brown-rot fungus *L. trabea* were much smaller compared with other fungi (Fig. 2). Small peaks were observed between 4–4.5 days with carboxyl- and ring-labelled vanillate (Fig. 2) but total $^{14}CO_2$ evolved was only 9.8% and 3.4% respectively of the radioactive carbon available (Table 6). No release of $^{14}CO_2$ from methoxyl-labelled vanillate was observed when the experiment was terminated after 10 days even though significant levels were evolved in flasks which contained labelled formaldehyde (Table 6).

The rate of $^{14}CO_2$ release from carboxyl- and ring-labelled vanillate in cultures of *P. cinnabarinus* was slow initially but increased rapidly after 2 and 3.5 days respectively reaching a peak after 4.5 and 5.5 days respectively (Fig. 3). Evolution of $^{14}CO_2$ in flasks containing methoxyl-labelled vanillate was not detected until the 4th day even though a rapid release was observed from $H^{14}CHO$. However, a large proportion (31.6%) of labelled carbon from $H^{14}CHO$ was again incorporated into fungal mycelium (Table 6).

Very different patterns of $^{14}CO_2$ evolution from carboxyl-, methoxyl- and ring-labelled vanillate were obtained with *P. ostreatus* (Fig. 4). Over 50% of the total available radioactive carbon located in the carboxyl group was converted to $^{14}CO_2$ within 3 days whereas less than 10% of the methoxyl- and ring-carbon appeared as carbon dioxide. Instead, between 45% and 50% of the methoxyl- and ring-carbon was found associated with the fungal mycelium.

Figure 5 shows the amounts of $^{14}CO_2$ released from the various labelled substrates by cultures of *S. pulverulentum*. After 4 days the quantities of $^{14}CO_2$ from carboxyl- and ring-labelled vanillate greatly increased reaching a peak after about 6 days. Evolution of $^{14}CO_2$ from the methoxyl groups was not detected until after 4.5 days and peaked after 7.5 days.

Maximum release rate from $^{14}CH_3OH$ and $H^{14}CHO$ occurred after 4.5 days.

No radioactivity appeared in the NaOH-filled tubes located in uninoculated control flasks except in the case of $^{14}CH_3OH$ where between 21% and 23% of the total ^{14}C was recorded over a period of 14 days.

Discussion

Recent work from this laboratory using the white-rot fungus *Sporotrichum pulverulentum* has established that vanillic acid undergoes oxidative decarboxylation to methoxyhydroquinone, as well as reductive conversions resulting in the formation of vanillin and vanillyl alcohol (Ander et al. 1980). Oxidative decarboxylation of vanillate was originally described in *Lenzites trabea* and *Polyporus dichrous* (Kirk and Lorenz 1973) and was more recently reported in a *Trametes sp.* (Nishida and Fukuzumi 1978). The enzyme catalysing this conversion, vanillate hydroxylase, was subsequently reported in mycelial extracts of *S. pulverulentum* (Buswell et al. 1979a) and has now been purified and partially characterized (Buswell et al. 1981). It is clear that $^{14}CO_2$ evolution from $^{14}COOH$-vanillate by brown-rot and white-rot fungi (except *Pleurotus ostreatus*) was catalysed by vanillate hydroxylase and in all cases, NADH and NADPH served as coenzyme. In several fungi oxidative decarboxylation of vanillate was further supported by the presence of methoxyhydroquinone in spent culture liquids after growth in the presence of vanillate (Table 1). Decarboxylation in *P. ostreatus* appears to involve a different mechanism since NADH and NADPH reduced the level of $^{14}CO_2$ release. Decarboxylation of vanillate can also be performed by laccase and peroxidase (Haider et al. 1962; Berlin and Barz 1975; Krisnangkura and Gold 1979; Ander et al. 1980) and the reduced pyridine nucleotides are probably interacting with the radical species on which this decarboxylation mechanism depends. *P. ostreatus* is known to be a high producer of phenoloxidases under similar growth conditions used here (Ander and Eriksson 1977). *Pycnoporus cinnabarinus* is also a strong phenoloxidase producer although this fungus possesses much higher levels of NAD(P)H:quinone oxidoreductase than *P. ostreatus*. Phenoloxidase-catalysed decarboxylation of vanillate is perhaps prevented in the presence of this enzyme. *P. ostreatus*, an agaric fungus, also differs taxonomically from the other white-rot representatives which are all 'bracket' fungi. In the case of the soft-rot fungi examined here there is no evidence to suggest that vanillate is metabolized by a route involving oxidative decarboxylation to methoxyhydroquinone.

It is interesting to note that many of the fungi producing vanillate hydroxylase, oxidatively cleaved the aromatic ring of hydroxyquinol to yield maleylacetate. One exception was L-1 where no ring cleavage activity was detectable. At the same time, most of these fungi apparently lack significant levels of dioxygenases active against dihydroxy ring-fission substrates such as catechol, protocatechuate and gentisate. However, other workers have demonstrated protocatechuate 3,4-dioxygenase, as well as enzymes catalysing the further conversion of the ring-fission product, in extracts of *Polystictus versicolor* (a lignin-degrading white-rot fungus) (Cain et al. 1968). Preliminary results show this fungus to produce vanillate hydroxylase. Certainly the ability to demonstrate ring cleavage also varied between batches of *P. cinnabarinus* mycelium used in this study, probably reflecting small differences in growth conditions.

The role of a tri-hydroxylated intermediate in vanillate catabolism by *S. pulverulentum* has been discussed by Ander et al. (1980). They found that release of $^{14}CO_2$ from methoxyl-labelled vanillate occurred after oxidative decarboxylation and cleavage of the aromatic ring. This led them to suggest that hydroxylation rather than demethylation of methoxyhydroquinone preceded ring fission. We have not observed in *S. pulverulentum* the long delay in the conversion of the labelled methoxyl carbon to $^{14}CO_2$ reported by Ander et al. (1980). However, this is very likely due to the higher nitrogen content of theNorkrans' medium. The rapid metabolism of $H^{14}CHO$ and $^{14}CH_3OH$ by *S. pulverulentum* appears to rule out the possibility that the delayed $^{14}CO_2$ release from $O^{14}CH_3$-vanillate was due to slow conversion of the released C-1 unit and supports the conclusion of Ander et al. (1980) that ring fission occurs without prior demethylation. There are clear similarities between the brown-rot and white-rot fungi used in this study from the standpoint of vanillate catabolism. The data reported here would indicate the route involving methoxyhydroquinone to be a major route for vanillate dissimilation in these fungi rather than an oxidative demethylation reaction followed by ring-cleavage of the resultant dihydroxy intermediate. This is surprising perhaps as far as the brown-rot representatives are concerned since members of this group are widely reported to demethylate the lignin polymer (Ander and Eriksson 1978). However, much larger numbers of fungi from each group would need to be examined before any firm statements may be made relating to the distribution of the methoxyhydroquinone pathway.

The soft-rot fungi examined clearly differ from representatives of the other decay groups in apparently lacking vanillate hydroxylase and hydroxyquinol 1,2-dioxygenase. Vanillate may undergo an initial demethylation to yield protocatechuate and protocatechuate 3,4-dioxygenase was detectable in mycelial extracts. Specific activities were very low, however, and similar to basal (non-induced) levels recorded in other microorganisms. Attempts to demonstrate cell-free demethylase activity were also unsuccessful although a veratrate O-demethylase, able to demethylate in both the 3- and 4-positions, has been purified by affinity chromatography from another soft-rot fungus, *Chaetomium piluliferum* (Paszczyński and Trojanowski 1977). Furthermore, the observed patterns of $^{14}CO_2$ release from $^{14}CH_3OH$ and $H^{14}CHO$, the two most likely C-1 intermediates resulting from a ring-demethylation, make it difficult to establish the chronological order in which the various parts of the substrate molecule (i.e., carboxyl, methoxyl, and ring) are catabolized. However, failure to detect the levels of O-demethylase and ring-cleaving dioxygenase activity reported by other workers for soft-rot species may again reflect the growth media used here where priority was given to standardized cultivation conditions. Alternatively, since O-demethylases are notoriously unstable enzymes failure to detect activity may result from defective procedures used to prepare cell-free extracts.

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