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Biodegradation of Vanillate Derivatives by White–rot Fungus, *Phlebia Radiata*

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The decomposition of vanillic and benzylvanillic acids labeled with ^{14}C either at carboxyl, methoxyl, or aromatic ring was studied during the 10–day cultivation of *Phlebia radiata*. Seventy percents of carbon dioxide evolved from carboxyl–labeled vanillate and about 40% from ring carbon–labeled vanillate and carboxyl–labeled benzylvanillate. The degradation of methoxyl group from vanillic and benzylvanillic acids reached a similar level, yielding about 25% after one week growth of *P. radiata* in these conditions. The radioactivity inside mycelium reached two maximas, the first on the 2[nd]–3[rd] day after the induction and the second on the 7[th] day after the induction. The first peak could be derived from high polymerization which can accompany high laccase activity. These polymers of decarboxylated quinones probably attach themselves to the mycelium and can be subsequently degraded by the *P. radiata* extracellular enzymatic system. These enzymes can demethylate monomeric and polymeric structures as well as cleave the aromatic ring. The late second peak inside the mycelium can be attributed to the cleavage of monomers originating from the degradation of polyquinones or from the secondary metabolites.

INTRODUCTION

Vanillic acid is widely reported to be present in extracts of wood that has undergone varying degrees of microbial degradation; it is generally accepted as a breakdown product of the lignin component (Ishikawa *et al.*, 1963; Kirk *et al.*, 1977). The catabolism of vanillic acid may therefore be of importance in the utilization of lignocellulose. The best–known degraders of lignin are white–rot fungi belonging to *Basidiomycete* (Ander and Eriksson *et al.*, 1977; Crawford, 1981; Eriksson *et al.*, 1990).

Reports in the literature indicate that white–rot fungi metabolize vannilate through protocatechuate (Tenneson *et al.*, 1979) or through methoxyhydroquinone (Nishida and Fukuzumi, 1978; Buswell *et al.*, 1979). The metabolism pathways for vanillic acid degradation by the white–rot fungus *Sporotrichum pulverulentum* have been investigated (Buswell *et al.*, 1981). It was shown that vanillic acid was both decarboxylated to methoxyhydroquinone and reduced to vanillin and vanillyl alcohole. Ayers and Eriksson (1982) report decarboxylation of vanillate by white–rot fungi as *Polyporus dichrous, Poria ambigua, Pycnoporus cinnabarinus* and *Pleurotus ostreatus* to methoxyhydroquinone oxidatively. Mycelium extracts of all these fungi, except for *Pleurotus ostreatus*, contained high levels of NAD (P) H– dependent vanillate hydroxylase. *Pleurotus ostreatus* also released $^{14}CO_2$ from $^{14}COOH$–vanillate but using a different mechanism possibly involving phenoloxidases. The white–rot fungus *Phlebia radiata* is an efficient degrader of lignin which preferentially attacks lignin (Hatakka *et al.*, 1983; Hatakka *et al.*, 1989; Hatakka, 1994).

As it was shown in previous paper, *Phlebia radiata* can synthesize *de`novo* a lot of aromatic compounds from glucose (Rogalski *et al.*, 1996). Additionally, after the induction by vanillate and ferulate components in the initial process of the synthesis *de`novo* is stopped, and only the methylation process of vanilate and ferulate components was observed (Rogalski, 1992). This study was attempted to investigate the degradation of vanillate derivatives through the assay of evolved $^{14}CO_2$ radioactivity by means of radiorespirometry.

MATERIALS AND METHODS

Organism and cultural conditions

Phlebia radiata Fr no 79 [ATCC 64658] was isolated at the Department of Microbiology, University of Helsinki (Hatakka *et al.*, 1989; Hatakka and Uusi–Rauva, 1983) and was maintained on 2% (w/v) malt agar slants. The preparation of inoculum was obtained according to (Hatakka and Uusi–Rauva, 1983). After 6 days of growth at 28 ˚C the mycelial mats were collected and homogenized in a Warning Blender. After inoculation with 4% (v/v) of the homogenate, 100 ml conical flasks, each containing 10 ml of ADMS LN medium with 1% glucose as a carbon source, were incubated stationary at 28 ˚C. On the 3[rd] day of growth, vanillic acid in the concentration of 1 mM and about 1 kBq of vanillic acid isotopes specifically labeled on different positions were added to each inoculated flask. The flasks were then fitted with polypropylene stoppers (Kartel, Italy).

Radiorespirometric analysis

Radiorespirometric methods to collect evolving $^{14}CO_2$

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and the assay of radioactivity were applied according to (Hatakka *et al.*, 1989; Hatakka and Uusi–Rauva, 1983). Sterile synthetic air (20% oxygen) was used for aeration and $^{14}CO_2$ collection purposes. Culture flasks (10 ml sample) were taken in duplicate every 24 h. Cultures were filtrated by Whatman No. 4 filter paper on a glass filter (Schott No. 4, Duran, FRG), and the filter paper plus mycelium was combusted as described by Hatakka and Uusi–Rauva (1983) method to determine the mycelial ^{14}C activity. The radioactivity was measured using liquid scintilation counters (LKB– Wallac Oy, Finland; and Beckman – type LS 5000TD).

Chemicals

Carboxyl–labelled vanillate (^{14}COOH–vanillate; 20×10^3 Bq/mg); methoxyl–labelled vanillate (O$^{14}CH_3$–vanillate; 38.7×10^3 Bq/mg); carboxyl–labelled benzylvanillate (^{14}COOH–benzylvanillate; 50×10^3 Bq/mg); methoxyl–labelled benzylvanillate (O$^{14}CH_3$–benzyl–vanillate; 23.3×10^3 Bq/mg) were kindly supplied by Dr. Konrad Haider and Dr. Jerzy Trojanowski, Institute fűr Pflanzenernschrung und Bodenkunde, Bundesforschungsanstalt fűr Landwirtschaft, Braunschweig, FRG. Vanillic acid uniformly labeled in the aromatic ring carbons (^{14}C–ring–vanillate; 3.96×10^4 Bq/mg) were gifts from Dr. K. Haider *via* Dr. P. Ander, Swedish Forest Products Research Laboratory, Stockholm, Sweden.

RESULTS AND DISCUSSION

The dynamics of $^{14}CO_2$ release by *P. radiata* was measured in a standing culture containing as the sole carbon source: a) unlabeled vanillate and separately, carboxyl, methoxyl or ring labeled vanillate; b) unlabeled benzylvanillate and separately, carboxyl or methoxyl labeled benzylvanillate.

Fig. 1 demonstrates that $^{14}CO_2$ release from carboxyl–labeled vanillate and benzylvanillate reached the maximum after 24 hrs. $^{14}CO_2$ evolution from the vanillate and benzylvanillate methoxyl carbons reached a peak after the 7th–8th day of growth (e.g. 5th–6th day after induction). The evolution of carbon dioxide from ring–labeled vanillate reached two peaks, the first 2 days after induction a little after the maximum coming from carboxyl carbons and the second time some higher value after the maximum coming from methoxyl–labeled carbons. A slight breaking of the lines as a maxima on the 7th day of growth for all used isotopes was also observed. As it was shown earlier the activities of lignolytic enzymes were observed for laccase on the second day after induction, the maximum for Mn–peroxidase activity was observed on the 5th day, and ligninase reached the maximum of its activity on the 6th day after induction of *P. radiata* growing in the same conditions as vanillic acid (Rogalski *et al.*, 1991; Rogalski *et al.*, 1996). The results also show that *P. radiata*, unlike *Fusarium oxysporium* (Targonski *et al.*, 1986), *Aspergillus terreus* (Fiedurek et al., 1986) and *Lignobacter sp.* (Rogalski *et al.*, 1982), do not require a free OH group in position 4 for intensive

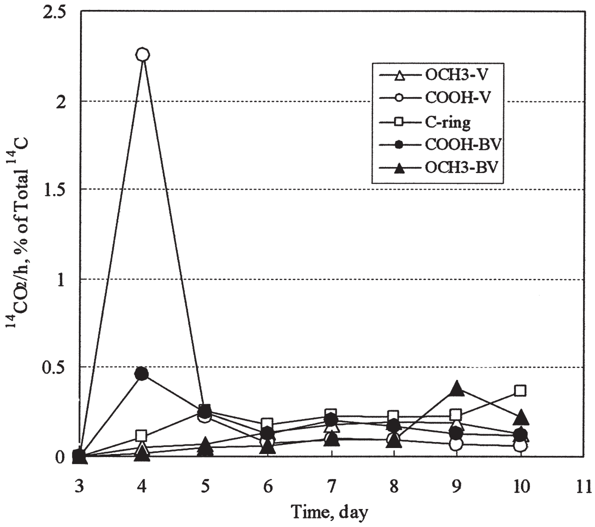

Fig. 1. $^{14}CO_2$ evolution from O$^{14}CH_3$–vanillate (△); ^{14}COOH–vanillate (○); ^{14}C–ring–vanillate (□); ^{14}COOH–benzylvanillate (●); O$^{14}CH_3$–benzylvanillate (▲) by *Phlebia radiata* grown on aromatic acids (1 mM) as the sole carbon source.

demethylation and decarboxylation of vanillic acid (see results with benzylvanillate). The recovery of ^{14}C–labeled elements coming from ^{14}C labeled aromatic acids isotopes is presented in Table 1.

From the Table 1, it can be observed that 70% of carbon dioxide evolved from carboxyl–labeled vanillate and about 40% from ring carbon–labeled vanillate and carboxyl–labeled benzylvanillate. The degradation of methoxyl group from vanillic and benzylvanillic acids reached a similar level, yielding about 25% after one week of growth of *P. radiata* in these conditions. The radioactivity inside mycelium reached two maximas, the first on the 2nd–3rd day after the induction and the second on the 7th day after the induction. The first peak can be connected to high polymerization which can accompany high laccase activity, which must protect the mycelium from the toxic quinones substances. These polymers of decarboxylated quinones probably attach themselves to the mycelium and can be subsequently degraded by the *P. radiata* extracellular enzymatic system. These enzymes can demethylate monomeric and polymeric structures as well as cleave the aromatic ring. The late second peak inside the mycelium can be attributed to the cleavage of monomers originating in the degradation of polyquinones or to the secondary metabolites which in both cases must be transported inside the cell.

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Table 1. ^{14}C–activities measured during 10 days after addition of labeled vanillic acid to submerged cultures of *Phlebia radiate*

Day	(^{14}COOH)–vanillic acid				($O^{14}CH_3$)–vanillic acid				(^{14}C–ring)–vanillic acid			
	$^{14}CO_2$ evolved [%]	^{14}C in mycelium [%]	^{14}C in cult. Liq. [%]	Total [%]	$^{14}CO_2$ evolved [%]	^{14}C in mycelium [%]	^{14}C in cult. Liq. [%]	Total [%]	$^{14}CO_2$ evolved [%]	^{14}C in mycelium [%]	^{14}C in cult. Liq. [%]	Total [%]
4	54.00± 1.61	5.77± 0.79	41.03± 2.41	100.8± 3.77	1.18± 0.28	29.90± 1.41	70.02± 3.11	101.1± 3.96	2.55± 0.87	41.30± 2.02	57.05± 1.81	100.90± 3.21
5	59.60± 2.01	8.63± 0.89	31.87± 2.73	100.1± 4.33	3.24± 0.53	25.70± 1.71	71.02± 3.32	100.20± 4.62	8.91± 1.12	38.02± 2.22	53.17± 2.04	100.10± 4.83
6	61.60± 2.12	10.40± 1.13	27.80± 2.43	99.80± 5.04	6.89± 0.69	32.74± 1.24	60.47± 2.83	100.10± 3.96	13.21± 1.13	46.40± 2.14	40.29± 1.52	99.92± 4.15
7	64.10± 1.92	5.78± 0.59	29.82± 2.75	99.70± 3.92	11.35± 1.05	24.56± 1.73	63.79± 3.63	99.70± 5.22	18.92± 1.41	39.63± 1.85	40.95± 1.83	99.50± 4.08
8	66.50± 2.11	4.60± 0.82	28.12± 3.08	99.21± 5.14	16.59± 1.21	17.43± 1.12	65.33± 2.44	99.31± 4.47	24.30± 2.00	23.51± 2.02	51.28± 2.24	99.11± 5.64
9	68.70± 2.23	5.41± 1.02	24.51± 2.22	98.63± 4.74	21.71± 1.51	14.49± 1.33	62.78± 2.63	99.0± 4.92	30.38± 1.92	21.68± 3.33	46.89± 1.32	99.02± 5.54
10	70.40± 2.51	4.81± 0.93	23.11± 2.82	98.31± 4.92	25.45± 1.40	23.82± 1.62	49.43± 2.11	98.73± 4.23	39.46± 2.42	54.45± 2.92	4.10± 1.09	98.41± 5.05

Day	($O^{14}CH_3$)–benzylvanillic acid				(^{14}COOH) –benzylvanillic acid			
	$^{14}CO_2$ evolved [%]	^{14}C in mycelium [%]	^{14}C in cult. Liq. [%]	Total [%]	$^{14}CO_2$ evolved [%]	^{14}C in mycelium [%]	^{14}C in cult. Liq. [%]	Total [%]
4	0.45± 0.11	17.58± 2.12	83.56± 2.64	101.60± 4.15	11.09± 0.61	15.79± 1.09	73.71± 3.02	100.50± 3.82
5	1.96± 0.52	18.97± 2.01	79.97± 2.42	100.90± 3.72	17.27± 0.82	17.75± 1.42	65.28± 2.51	100.31 ± 3.33
6	3.75± 0.72	19.68± 1.76	76.67± 3.08	100.11± 4.04	20.73± 1.22	9.67± 1.13	69.30± 3.30	99.70± 4.44
7	6.32± 1.01	21.68± 2.23	71.60± 2.11	99.50± 4.61	26.12± 1.50	19.11± 1.52	54.17± 2.82	99.41± 4.26
8	8.71± 1.11	17.94± 1.78	72.45± 3.33	99.11± 4.90	30.51± 1.41	15.71± 2.22	52.78± 1.94	99.00± 3.88
9	18.36± 1.64	7.65± 1.93	78.49± 3.11	98.50± 5.33	33.83± 2.21	11.59± 1.62	52.98± 2.23	98.40± 5.16
10	24.17± 1.52	12.68± 2.22	61.15± 2.46	98.00± 5.15	37.78± 2.04	13.72± 2.00	46.70± 2.55	98.20± 5.75

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