Metabolism of Lignin-derived Aromatic Acids by Wood-rotting Fungi

By PAUL ANDER,* KARL-ERIK ERIKSSON AND HUI-SHENG YU†

Swedish Forest Products Research Laboratory, Box 5604, S-114 86 Stockholm, Sweden

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Metabolism of ^{14}C -labelled methoxyl groups in vanillic and syringic acid to $^{14}\text{CO}_2$ by various white-rot, brown-rot and soft-rot fungi was investigated in the presence of different amounts of glucose and nitrogen. In general, methoxyl metabolism in these fungi was repressed by 1% (w/v) glucose and stimulated by nitrogen. The soft-rot fungus *Petriellidium boydii* was the exception and metabolized the methoxyl group of vanillic acid without glucose repression. Generally, brown-rot and soft-rot fungi metabolized the methoxyl groups of syringate faster than the vanillate methoxyl group.

INTRODUCTION

Demethylation and demethoxylation are important reactions in fungal degradation of lignin as well as lignin-related phenols such as vanillic and syringic acid (Henderson, 1963; Haider & Trojanowski, 1975, 1980; Ander & Eriksson, 1978; Crawford, 1981; Kirk, 1981; Ander *et al.*, 1983*a*). Considerable data concerning the ability of different wood-rotting fungi to demethoxylate vanillic, syringic or ferulic acids have been obtained in this laboratory in recent years (Ander *et al.*, 1980; Gupta *et al.*, 1981; Buswell *et al.*, 1982*a*; Ander *et al.*, 1983*b*; Eriksson *et al.*, 1983). However, the metabolism of methoxyl groups required more detailed study using different media. Furthermore, apart from the studies by Henderson (1961, 1963) and by Haider & Trojanowski (1975, 1980), very little information is available on the fungal metabolism of syringic acid. Work in this laboratory (K.-E. Eriksson, J. K. Gupta, A. Nishida & M. Rao, unpublished results) indicated that syringate was rapidly metabolized by two soft-rot fungi and by *Sporotrichum pulverulentum*, but only slowly by two brown-rot fungi.

The metabolism of ^{14}C -labelled methoxyl groups of vanillate and syringate to $^{14}\text{CO}_2$ have here been studied further using different glucose and nitrogen concentrations. Two common features associated with the metabolism of methoxyl groups by wood-rotting fungi seem to be repression by glucose and stimulation by nitrogen. These results have important implications for the use of wood-rotting fungi in biotechnical applications.

METHODS

Organisms. *White-rot fungi:* *Sporotrichum pulverulentum*, Nov. (ATCC 32629). This strain is the anamorph of *Phanerochaete chrysosporium* Burds. (S. C. Johnsrud, unpublished results). *Pycnoporus cinnabarinus* (Jacq. ex Fr.) Karst A-360. *Pleurotus ostreatus* (Jacq. ex Fr.) Kummer IPB no. 53.

Brown-rot fungi: *Gleophyllum trabeum* (Pers. ex Fr.) Murril A-576 (synonym *Lenzites trabea*). *Poria placenta* (Fr.) Cke. *sensu* J. Eriksson, synonym *Poria monticola* Murr.

Soft-rot fungi: *Chaetomium globosum* Kunze ex Fr. F 171-1 (ATCC 34152). *Phialophora mutabilis* (van Beyma) Schol-Schwarz 203-E-15-y-1. *Petriellidium boydii* (Shear) Maltoch SP 31-4.

For sources of these fungi see Buswell *et al.* (1982*a*). *Poria placenta* was obtained from T. Highley, FPL, Madison, Wis., U.S.A., as strain Mad. 698.

Labelled substances. Methoxyl-labelled vanillic acid [^{14}C -OCH₃-vanillate] (1.1×10^6 d.p.m. mg⁻¹) and syringic acid, ^{14}C -labelled in the methoxyl groups (1.0×10^6 d.p.m. mg⁻¹) were gifts of Dr Konrad Haider, Institut für Pflanzenernährung und Bodenkunde, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, FRG.

† Present address: Tianjin Pulp and Paper Research Institute, Tianjin, People's Republic of China.

Cultivation. The medium used was the dimethylsuccinate (DMS) medium described by Ander *et al.* (1980) and Fenn & Kirk (1979) and modified to contain 30 mM-DMS to maintain constant pH. Trace elements (Ander & Eriksson, 1976) and 0.1% (w/v) yeast extract (Difco), probably giving 40–50 mg amino nitrogen (l medium)⁻¹, were used in all cultivations. Glucose and nitrogen concentrations were varied as follows: LH medium contained 0.25% (w/v) glucose and 20.6 mM-nitrogen as asparagine and NH₄NO₃, HH medium contained 1.0% glucose and 20.6 mM-N, while HL medium contained 1.0% glucose and 2.6 mM-N. The low nitrogen medium with 2.6 mM-N contained (l⁻¹) 0.10 g asparagine, H₂O and 0.05 g NH₄NO₃ while the high nitrogen medium contained eight times more of these nitrogen sources. Media were adjusted to pH 4.2, filter sterilized and inoculated with 1×10^6 spores (about 1 mg) of *S. pulverulentum* per 10 ml culture medium in triplicate 125 ml flasks. Labelled vanillic or syringic acid (about 10 µl in ethanol) was added simultaneously to give about 30000 d.p.m. per culture. Inocula of the other fungi were prepared as follows. After growth on HL medium for 7–14 d, mycelium from one or two flasks per fungus was washed with water, suspended in 50 ml distilled water and disintegrated in a turmix. A sample (0.5 ml) of the resulting suspension (4–9 mg mycelium) was then added to each culture flask. *Sporotrichum pulverulentum* was grown at 39 °C and all other fungi at 28 °C. For all cultures, except *Pleurotus ostreatus*, the release of ¹⁴CO₂ from triplicate standing cultures was determined after absorption in NaOH as described by Ander *et al.* (1980). *Pleurotus ostreatus* was grown in 100 ml flasks which were flushed with air every third day to measure ¹⁴CO₂ using the same technique adopted for measuring ligninolytic activity (Buswell *et al.*, 1982*b*). Air was used rather than oxygen for comparison of results obtained with the other fungi. If NaOH-filled tubes were used to absorb respiratory CO₂ (Ander *et al.*, 1980), no growth was obtained with *Pleurotus ostreatus*.

Glucose determination. Glucose in the duplicate culture solutions was determined with GLOX, glucose oxidase reagent (Kabi Diagnostica, Stockholm, Sweden). In this method glucose is oxidized to gluconic acid with formation of H₂O₂, which reacts together with peroxidase and an aromatic amine to give a coloured product. After 60 min, absorbance was read at 450 nm (Kjellman & Rådeström, 1981) and glucose concentrations were read off against a standard curve prepared with reagent grade D-glucose.

RESULTS

Metabolism of methoxyl-labelled vanillic and syringic acids

Different wood-rotting fungi were used to study the influence of varying glucose and nitrogen concentrations on the metabolism of ¹⁴C-methoxyl-labelled vanillic and syringic acids to ¹⁴CO₂. The results are shown in Figs 1–3.

The general pattern for all the fungi tested was that LH medium containing 0.25% glucose and 20.6 mM-nitrogen (as asparagine and NH₄NO₃) was best suited for rapid ¹⁴CO₂ evolution from the methoxyl groups of both vanillate and syringate. For two of the fungi, namely *S. pulverulentum* and *Pleurotus ostreatus*, HH or HL media in the later stages of the experiments gave higher yields of ¹⁴CO₂ from syringate.

The amounts of ¹⁴CO₂ liberated by the different fungi in these media varied widely. *Sporotrichum pulverulentum* released as much as 70% ¹⁴CO₂ in 14 d from vanillate and 46% ¹⁴CO₂ from syringate in the HH medium. In the case of *Pleurotus ostreatus*, if ¹⁴CO₂ (+ respiratory CO₂) was absorbed in NaOH-filled tubes according to Ander *et al.* (1980), very little fungal growth was obtained. However, flushing with air every third day (see Methods) gave good growth, and under these conditions 26% ¹⁴CO₂ was released from vanillate in the LH medium after 14 d (Fig. 1).

Results for brown-rot and soft-rot fungi (Figs 2 and 3) show that syringate methoxyl groups are metabolized faster than vanillate methoxyl groups. For example, *Chaetomium globosum* released 35.6% ¹⁴CO₂ from syringate and only 3.4% ¹⁴CO₂ from vanillate in the LH medium.

Metabolism of vanillate and syringate in relation to glucose concentration and mycelial weights

Glucose concentrations and mycelial dry weights of the various fungi after 3, 6 and 9 d are also shown in Figs 1–3. In the LH medium, little or no glucose was left after 3 d and a decrease in mycelial weight was observed for many of the fungi after 9 d. If this low amount of mycelium is taken into consideration, the suitability of the LH medium for rapid ¹⁴CO₂ evolution is still more apparent.

The direct influence of glucose on ¹⁴CO₂ evolution from the methoxyl groups of vanillate and syringate is obvious for all fungi, although they differed in this respect. *Sporotrichum pulverulentum*, the two brown-rot fungi and the two soft-rot fungi *C. globosum* and *Phialophora mutabilis* started to evolve ¹⁴CO₂ at a rather low glucose concentration of about 0.1% or lower.

Fig. 1. Accumulated $^{14}\text{CO}_2$ evolution from [$^{14}\text{C}\text{-OCH}_3$] vanillate (VA) and [$^{14}\text{C}\text{-OCH}_3$] syringate (SA), by the white-rot fungi *S. pulverulentum*, *Pycnoporus cinnabarinus* and *Pleurotus ostreatus* on LH (■) (0.25%, w/v, glucose and 20.6 mM-nitrogen), HL (▲) (1% glucose and 2.6 mM-N), or HH (●) (1% glucose and 20.6 mM-N) media after 14 d. Glucose concentrations in these media are shown after 3, 6 and 9 d cultivation. $^{14}\text{CO}_2$ data are mean values from three replicate cultures. Glucose values are from duplicate cultures. Final standard deviation (δ_{n-1}) for the $^{14}\text{CO}_2$ values:

<i>S. pulverulentum</i>	on VA: LH, 4.2%; HL, 1.2%; HH, 5.5%
	on SA: LH, 11.6%; HL, 6.6%; HH, 5.9%
<i>Pycnoporus cinnabarinus</i>	on VA: LH, 0.1%; HL, 0.83%; HH, 1.0%
	on SA: LH, 0.6%; HL, 2.6%; HH, 2.2%
<i>Pleurotus ostreatus</i>	on VA: LH, 10.4%; HL, 5.8%; HH, 3.5%
	on SA: LH, 7.4%; HL, 4.9%; HH, 2.6%

Pycnoporus cinnabarinus and *Pleurotus ostreatus* were not so sensitive to glucose and began to evolve $^{14}\text{CO}_2$ at 0.4–0.6% glucose. *Petriellidium boydii* was unusual in that the rate of $^{14}\text{CO}_2$ evolution from vanillate was highest during the first 3 d in all three media. After 3 d, about 13% $^{14}\text{CO}_2$ had accumulated using both LH and HH media, and glucose repression of vanillic acid methoxyl group metabolism was not apparent in this fungus. However, the metabolism of the syringate methoxyl groups was clearly repressed by glucose.

Chaetomium globosum was the best growing fungus and more than 50 mg mycelium was obtained on HH and HL media after 6 d. This, however, made $^{14}\text{CO}_2$ release per mg mycelium smaller as compared with, for example, *S. pulverulentum* which yielded a maximum of 20 mg mycelium.

Fig. 2. Accumulated $^{14}\text{CO}_2$ evolution from methoxyl-labelled vanillate (VA) and syringate (SA) by the brown-rot fungi *G. trabeum* and *Poria placenta* on LH (■), HL (▲) and HH (●) media. Other conditions are as in Fig. 1. Final standard deviations (δ_{n-1}) for the $^{14}\text{CO}_2$ values:

<i>G. trabeum</i>	on VA: LH, 3.4%; HL, 2.0%; HH, 0.86%
	on SA: LH, 7.5%; HL, 15.4%; HH, 7.2%
<i>Poria placenta</i>	on VA: LH, 0.35%; HL, 0.15%; HH, 1.0%
	on SA: LH, 2.7%; HL, 0.15%; HH, 4.1%

DISCUSSION

The data presented here indicate that for many wood-rotting fungi, the systems involved in the metabolism of lignin degradation products, such as vanillic and syringic acid, are repressed by glucose and stimulated by nitrogen. Similar results were reported earlier by Haider & Trojanowski (1980) using *Gleophyllum trabeum* (*Lenzites trabea*) and several methoxyl-labelled phenols, among them vanillic and ferulic acids. These authors proposed that the further oxidation of the C_1 -compound obtained from the methoxyl group was retarded by glucose. Recent results in our laboratory (Ander *et al.*, 1983b) support this suggestion and, in the case of *Sporotrichum pulverulentum*, the C_1 -compound has been identified as methanol.

In their studies of vanillic acid metabolism by different wood-rotting fungi, Buswell *et al.* (1982a) obtained 50% and 38% $^{14}\text{CO}_2$ in 14 d from the methoxyl group of vanillate by *Pycnoporus cinnabarinus* and *Chaetomium globosum*, respectively. In this investigation, only 10% and 3.4% $^{14}\text{CO}_2$ was obtained from $^{14}\text{OCH}_3$ -vanillate over the same period. Further, Buswell *et al.* (1982a) reported less than 1% $^{14}\text{CO}_2$ evolution from the vanillate methoxyl groups in cultures of *Gleophyllum trabeum* after 10 d, whereas here 15% CO_2 was obtained in 14 d. These

Fig. 3. Accumulated $^{14}\text{CO}_2$ evolution from methoxyl-labelled vanillate (VA) and syringate (SA) by the soft-rot fungi *C. globosum*, *Phialophora mutabilis* and *Petriellidium boydii* on LH (■), HL (▲) and HH (●) media. Other conditions are as in Fig. 1. ---, Approximate decrease. Final standard deviations (δ_{n-1}) for the $^{14}\text{CO}_2$ values:

<i>C. globosum</i>	on VA: LH, 1.2%; HL, 0.72%; HH, 3.8%
	on SA: LH, 2.5%; HL, 1.4%; HH, 0.96%
<i>Phialophora mutabilis</i>	on VA: LH, 1.7%; HL, 0.99%; HH, 0.25%
	on SA: LH, 3.0%; HL, 0.28%; HH, 1.6%
<i>Petriellidium boydii</i>	on VA: LH, 7.1%; HL, 5.3%; HH, 1.1%
	on SA: LH, 2.5%; HL, 2.0%; HH, 3.0%

differences almost certainly reflect differences in inocula preparation, and in media and cultural conditions. The vanillate and syringate demethylating ability reported here for white-rot fungi, and also for brown-rot and soft-rot fungi, are in good agreement with the known ability of these fungi to demethylate lignin (Seifert, 1966; Ander & Eriksson, 1978; Crawford, 1981), although due caution should be exercised when extrapolating results obtained with lignin model compounds to include the intact polymer. Thus, our unpublished results indicate that the soft-rot fungi used in this study do not release $^{14}\text{CO}_2$ from ring- or methoxyl-labelled synthetic lignin (DHP), although some of the soft-rot fungi used by Haider & Trojanowski (1975) released small amounts of $^{14}\text{CO}_2$ from such lignin. On the other hand, degradation of lignin in wood by different soft-rot fungi, among them *C. globosum*, has been reported after prolonged incubation (Savory & Pinion, 1958; Eslyn *et al.*, 1975).

The physiology of growth by *P. ostreatus* clearly requires further investigation. Our findings show that this fungus grows better in the presence of increased levels of CO₂. This may explain the observed higher yield of ¹⁴CO₂ (26%) from methoxyl-labelled vanillate as compared to 10% ¹⁴CO₂ reported by Buswell *et al.* (1982*a*) for *Pleurotus ostreatus* on a similar medium. Results by Scháněl & Dovrtěl (1980) further indicate that when *Pleurotus ostreatus* grows on wood, CO₂ can stimulate peroxidase production. This may increase growth of the fungus.

In conclusion, the white-, soft- and brown-rot fungi tested were similar to each other in that the initial release of ¹⁴CO₂ from the methoxyl groups of vanillic and syringic acids in almost all cases occurred most rapidly in the presence of low amounts of glucose and high amounts of nitrogen. If wood-rotting fungi are used in the future for biotechnical applications in the pulp and paper industry, the glucose and nitrogen concentrations are clearly important parameters to consider.

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