Ethylene production from α -oxo- γ -methylthiobutyric acid is a sensitive measure of ligninolytic activity by *Phanerochaete chrysosporium*

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Production of hydroxyl radical in ligninolytic cultures was determined by measuring the α -oxo- γ -methylthiobutyric acid-dependent production of ethylene gas. The results showed that the pattern of ethylene production was very similar to that of ligninolytic activity ($[^{14}\text{C}]\text{lignin} \rightarrow ^{14}\text{CO}_2$). Furthermore, nutritional parameters, which are known to affect ligninolytic activity, affected $\text{OH}^\bullet$ -radical-dependent ethylene production in a similar fashion. The results indicate that assay for ethylene production from α -oxo- γ -methylthiobutyric acid is a simple and sensitive measure of ligninolytic activity by *Phanerochaete chrysosporium*.

Lignin is a highly complex, amorphous, aromatic polymer and, next to cellulose, is the most abundant organic compound in Nature (Sarkanen & Ludwig, 1971). A better understanding of the biodegradation of lignin is needed to develop procedures for the biotransformation of lignin into aromatic chemical feedstock (Drew *et al.*, 1978) and to increase the efficiency of various lignocellulosic biocinversion processes (Reddy, 1978; Crawford, 1981). As a result, research on lignin biodegradation has accelerated in recent years (Kirk *et al.*, 1980; Crawford, 1981; Amer & Drew, 1980; Ander & Eriksson, 1978). A number of white-rot fungi, as exemplified by *Phanerochaete chrysosporium*, have been shown to degrade lignin extensively (Kirk, 1981), and several nutritional and cultural parameters that affect ligninolytic activity have been described (Kirk *et al.*, 1978; Jeffries *et al.*, 1981; Fenn & Kirk, 1981; Reid, 1979). However, little is known about the biochemistry of lignin degradation.

The lack of sensitive and definitive assays for monitoring ligninolytic activity by micro-organisms has long been a major problem for research in this area. Development of procedures for the synthesis of ^{14}C -labelled synthetic (Kirk *et al.*, 1975) and natural lignins (Crawford & Crawford, 1978) and their use as substrates in various biodegradation studies has greatly alleviated this problem. However, ^{14}C -labelled synthetic lignins are not commercially available and their synthesis is relatively complicated and expensive. Hence, the use of ^{14}C -labelled synthetic lignin has been restricted to relatively few laboratories. Thus there is a need for a simple, sensitive and relatively inexpensive assay for lignin degradation.

Recent results of Forney *et al.* (1982b) showed that substantial amounts of H_2O_2 are produced by ligninolytic cultures of *P. chrysosporium* and that H_2O_2 -derived hydroxyl radical ($\text{OH}^\bullet$) plays an integral role in lignin degradation, as evidenced by the fact that lignin degradation was severely inhibited by $\text{OH}^\bullet$ -trapping agents such as mannitol and benzoate. Since the reaction of $\text{OH}^\bullet$ with α -oxo- γ -methylthiobutyric acid is known to result in the formation of ethylene gas (Cohen, 1978), it appeared that quantification of the ethylene produced from α -oxo- γ -methylthiobutyric acid in cultures of *P. chrysosporium* could serve as a convenient assay for ligninolytic activity, and this possibility was investigated in the present study.

Materials and methods

P. chrysosporium Burds. ME 446 (A.T.C.C. 34541) was obtained from Dr. T. K. Kirk (Forest Products Laboratory, Madison, WI, U.S.A.) and was maintained on malt-agar plates. Inocula consisting of conidial suspensions in water were prepared as previously described (Kirk *et al.*, 1978).

The composition of the low-N medium containing 2.4 mM-N (as 0.6 mM- NH_4NO_3 and 0.6 mM-asparagine), glucose (56 mM) and mineral salts was previously described (Kirk *et al.*, 1978). The low-carbohydrate medium was the same as the low-N medium, except that the glucose concentration was 8.8 mM and that the N concentration was 24 mM (NH_4NO_3 and asparagine, 6 mM each). All media were buffered with 2,2-dimethylsuccinate (0.01 M, pH 4.5), dispensed (10 ml/125-ml Erlenmeyer flask for assaying ligninolytic activity or

5 ml/50-ml Wheaton serum bottles for OH[•] assay) and were autoclaved for 15 min at 121°C. The flasks were inoculated with the conidial suspension (~1.25 × 10⁶ conidia/ml; 0.5 ml of suspension/10 ml of medium) and were incubated at 39°C without agitation.

Ligninolytic activity was assayed as previously described (Forney *et al.*, 1982a) by determining the ¹⁴CO₂ produced after the addition of a sterile aqueous suspension of 2-¹⁴C-labelled synthetic lignin (3.6 × 10⁴ d.p.m.) to the cultures.

The assay for the OH[•] consisted of quantification of the ethylene produced from α-oxo-γ-methylthiobutyric acid after the reaction with OH[•]. After the organism was grown for various periods of time, the Wheaton bottles were closed with rubber septa and sealed with aluminum caps. α-Oxo-γ-methylthiobutyric acid was then injected through the rubber septum to give a final concentration of 3.3 mM. After 5 h of additional incubation, the reactions were stopped with 1 ml of 3 M-H₂SO₄. The ethylene in the head space was then analysed with a Varian 2440 gas chromatography fitted with a Porapak N (Waters Associates, Milford, MA, U.S.A) column and a flame-ionization detector. The carrier gas was N₂ at 30 ml·min⁻¹. The detector-gas flow rates were 300 ml of air·min⁻¹ and 30 ml of H₂·min⁻¹. The chromatograph oven was maintained at 120°C, with the injector and detector both set at 104°C.

Results

Correlation between the OH[•] production and ligninolytic activity

Previous studies showed that the ligninolytic system of *Phanerochaete chrysosporium* is synthesized after the cessation of primary growth (apparently as a part of secondary metabolism) and in response to nitrogen starvation (Keyser *et al.*, 1978; Jeffries *et al.*, 1981). In view of the integral role of OH[•] in lignin degradation by *P. chrysosporium* (Forney *et al.*, 1982b), the kinetics of ligninolytic activity (¹⁴C[lignin]→¹⁴CO₂) and of OH[•]-radical-dependent ethylene production from α-oxo-γ-methylthiobutyric acid were determined in a low-N medium (Fig. 1). The pattern of ethylene production was comparable with that of ligninolytic activity. During the first 2 days of incubation, there was little or no ligninolytic activity or ethylene production. After 3 days, ethylene production and ligninolytic activity increased rapidly and reached a peak between 8 and 10 days of incubation. No ethylene was detectable in parallel control bottles with no added α-oxo-γ-methylthiobutyric acid or when the cultures were autoclaved before adding α-oxo-γ-methylthiobutyric acid.

Previous studies (Keyser *et al.*, 1978) showed that growth of *P. chrysosporium* in high-N medium

Fig. 1. Ligninolytic activity (¹⁴C-labelled synthetic lignin→¹⁴CO₂) and OH[•] production, as determined by the ethylene production from α-oxo-γ-methylthiobutyric acid, by *P. chrysosporium* grown in the low-N medium described in the text

Symbols: ■, ¹⁴CO₂; ●, ethylene. Bars represent the S.D. for three replicate cultures.

Fig. 2. Ligninolytic activity and OH[•] production (both parameters measured as described in the legend to Fig. 1) by *P. chrysosporium* grown in the low-carbohydrate medium described in the text

Symbols: ■, ¹⁴CO₂; ●, ethylene. Bars represent S.D.

resulted in a considerable delay in the onset of ligninolytic activity compared with that observed in low-N medium. Recent results (R. L. Kelley & C. A. Reddy, unpublished work) showed that the onset of OH[•] was also delayed considerably when *P. chrysosporium* was grown in a high-N medium. For example, in the first 8 days of incubation, there was no detectable OH[•] production or ligninolytic activity.

Effect of carbohydrate limitation on OH[•] production

Ligninolytic activity is known to be triggered not only in response to N-starvation but also in response to carbohydrate starvation (Jeffries *et al.*, 1981). Furthermore, ligninolytic activity appeared earlier in carbohydrate-starved cultures than in N-starved cultures (Jeffries *et al.*, 1981). The results presented in Fig. 2 show that there was an earlier appearance of both ethylene production and ligninolytic activity in these low-carbohydrate cultures as compared with that observed in cultures grown in low-N medium (Fig. 1). Also, both the activities appeared to reach a peak in 6 days, which was earlier than was observed for the corresponding activities in cultures grown in low-N medium.

Discussion

Our results are consistent with the previous reports showing that ligninolytic activity is triggered in response to N or carbohydrate starvation and that H₂O₂-derived OH[•] is involved in lignin degradation by *P. chrysosporium* (Keyser *et al.*, 1978; Jeffries *et al.*, 1981; Forney *et al.*, 1982b). The results showed that there is a positive correlation between OH[•] production as determined by ethylene production from α -oxo- γ -methylthiobutyric acid and ¹⁴CO₂ production by ligninolytic cultures of *P. chrysosporium* in low-N, high-N and low-carbohydrate media. These results indicate the suitability of ethylene assay as a measure of ligninolytic activity.

Previous studies indicated that measurement of ¹⁴CO₂ respired from ¹⁴C-labelled synthetic lignins, is a satisfactory and reliable assay for ligninolytic activity (Kirk *et al.*, 1975; Crawford & Crawford, 1978). However, measurement of OH[•] production by determining the ethylene produced from α -oxo- γ -methylthiobutyric acid, offers certain advantages over ¹⁴CO₂ assay for measuring ligninolytic activity. The assay is simple, sensitive, inexpensive and reproducible. The OH[•]-dependent production of ethylene appears to reflect the initial non-enzymic oxidative attack on the lignin polymer (Forney *et al.*, 1982b), whereas ¹⁴CO₂ assay measures the terminal event in the metabolism of ¹⁴C-labelled lignin. Thus the ethylene assay for OH[•] represents a more direct measure of a specific early event in lignin degradation. Furthermore, this assay is an indirect measure of an enzymic activity (i.e. enzymic production of H₂O₂, which undergoes one-electron

reduction to form OH[•] which is implicated in ligninolytic activity (Forney *et al.*, 1982b).

In summary, the assay for ethylene production from α -oxo- γ -methylthiobutyric acid as described in the present paper appears to be a rapid and sensitive measure of ligninolytic activity. Availability of such simple assays should give further impetus to expanded research on the biochemistry, physiology and genetics of lignin degradation by white-rot fungi.

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