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Rapid Solubilization and Depolymerization of Purified Kraft Lignin by Thin Layers of *Phanerochaete chrysosporium*

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Summary. *Phanerochaete chrysosporium* was not able to degrade purified Kraft lignin under nitrogen limitation when the secondary growth phase was limited by slow feeding of glucose. The formation of new mycelia was found to be the primary phenomenon during the secondary phase. Lignin was degraded only when excess energy was available. Using a homogeneous, thin layer of cells, the physical contact between the new cells and lignin was improved and the local oxygen-limited conditions were avoided, resulting in a rapid and total bioalteration of lignin. Completely water- and acid-soluble products, which comprised about 30–35% of the original lignin absorbance, were formed.

Introduction

Degradation of lignin by the white rot, wood-decomposing fungus *Phanerochaete chrysosporium,* is a process that does not occur during the primary growth of the organism. The ligninolytic phase in the life cycle of this fungus has been defined as a phenomenon that is typically secondary metabolism (Kirk et al. 1980). The unique conditions for this phase involve nitrogen limitation, oxygen atmosphere, and non-agitated mode of cultivation. The optimal conditions for lignin bioalteration with this organism have been extensively studied and described by Kirk and co-workers (Kirk et al. 1976, 1978; Bar-Lev and Kirk 1981; Fenn and Kirk 1981; Jeffries et al. 1981).

Recently the ligninolytic phase has also been suggested to have a connection with secondary growth (Janshekar et al. 1982) and production of soluble and cell-bound polysaccharides (Leisola et al. 1982). In this study, further evidence is given to show that when the cells are pregrown in a bioreactor, lignin degradation is preceded by secondary growth rather than by secondary metabolism of the old cells. This secondary growth together with cell-bound and soluble polysaccharides creates cultural conditions where cells probably suffer from oxygen limitation during the non-agitated mode of cultivation and where the lignin is not totally available to the new mycelia. When these facts are taken into consideration, the organism is capable of rapid and complete bioalteration of Kraft lignin.

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Materials and Methods

Media and Culture Conditions. The purified Kraft lignin preparation and media and culture conditions (39° C, 100% O_2) for *Phanerochaete chrysosporium* (ATCC 24725) were as described previously by Leisola et al. (1982). In addition, lignin degradation was studied in non-agitated cultures in 2,000 ml as well as 200 ml Erlenmeyer flasks to study the effect of the surface to volume ratio on degradation. The cultures were buffered with 2,2-dimethylsuccinate at a concentration of 30 mM (Fenn and Kirk 1979).

Analytical Methods. At indicated intervals, the entire contents of each flask was sonicated or blended by a high-frequency homogenizer. Glucose, mycelium dry-weight and total cell carbohydrate were measured as described by Leisola et al. (1982).

Gel Permeation Chromatography. GPC was performed using Sephadex G-75 gel in a 1.6 × 87 cm column with 0.5% NaOH as eluent. The standards used were cytochrome c (MW = 12,500), glucagon (MW = 3,483), and bacitracin (MW = 1,423) (Forss and Fuhrmann 1978).

Lignin Assays. Lignin was measured spectrophotometrically using a simplified modification of the method that was used previously (Janshekar et al. 1982). *Total absorbance* was measured from 0.5 ml homogenized culture by adding 4.5 ml 0.55% NaOH. This mixture was sonicated and centrifuged. The total absorbance was measured from the supernatant after proper dilution at 280 nm. The value obtained with reference cells (no lignin) was subtracted from these absorbances. *Kraft lignin absorbance* was obtained by acidifying (pH ~ 2.5) 2 ml of NaOH extract and allowing lignin to precipitate overnight. After centrifugation, the precipitate was

re-solubilized in 0.5% NaOH and after proper dilution, the absorbance was measured at 280 nm. *Acid soluble absorbance* is the difference between the two above mentioned values.

Results and Discussion

Slow Glucose Feeding

In our earlier work with *Phanerochaete chrysosporium*, the formation of new mycelial structures (Janshekar et al. 1982) and the production of polysaccharides (Leisola et al. 1982) were found to be associated with ligninolytic activity. The excess polysaccharide formation was postulated to be inhibitory to lignin degradation. To determine whether polysaccharide formation does inhibit lignin degradation, an experiment with slow glucose feeding was carried out to prevent polysaccharide formation. The cells for this experiment were taken from the bioreactor after three days batch cultivation. The cells were centrifuged and resuspended in a medium which contained no nitrogen and glucose. Lignin was then added to 25 ml cultures in 200 ml flasks with initial mycelial dry weight of $1.0 \pm 0.05\ g \cdot l^{-1}$ and glucose concentration of $0.5\ g \cdot l^{-1}$. Reference cells had excess glucose so that in the beginning they had $7\ g \cdot l^{-1}$, and on day twelve, another $3.5\ g \cdot l^{-1}$ was added. After three days, various amounts of glucose in 0.04 ml of medium were fed to the cultures daily. Earlier reports of maintenance glucose requirements for *P. chrysosporium:* $0.3\ g \cdot g^{-1} \cdot day^{-1}$ (Jeffries et al. 1981) and $0.2\ g \cdot g^{-1} \cdot day^{-1}$ (Leisola et al. 1982) were used to estimate the feed rates used (Table 1). Results of this experiments are shown in Fig. 1 and Table 1. Only the cultures with excess glucose could substantially degrade lignin. However, an increase in total cell carbohydrate was observed even with the cultures that received only about $0.05\ g \cdot l^{-1} \cdot day^{-1}$, compared to $0.4\ g \cdot l^{-1} \cdot day^{-1}$ which was used by cells that had excess glucose.

New mycelial growth was visually observed in all of the cultures. When the glucose feeding rate was less than $0.144\ g \cdot l^{-1} \cdot day^{-1}$, the dry weight of the cultures actually decreased, although mycelial carbohydrate increased (Fig. 2). The decrease in dry weight is probably a result of old mycelium dying, and the increase in mycelial carbohydrate indicates that new cell walls are being synthesized, supporting the visual observations. The overall process was limited by the rate of glucose feeding so that the mycelium dry weight decreased in spite of the new growth.

The lignin degradation thus seems to be associated with the secondary growth phase and occurs only when sufficient energy is available. Kirk et al. (1978) have also observed an increase in cell biomass when lignin is degraded under similar conditions.

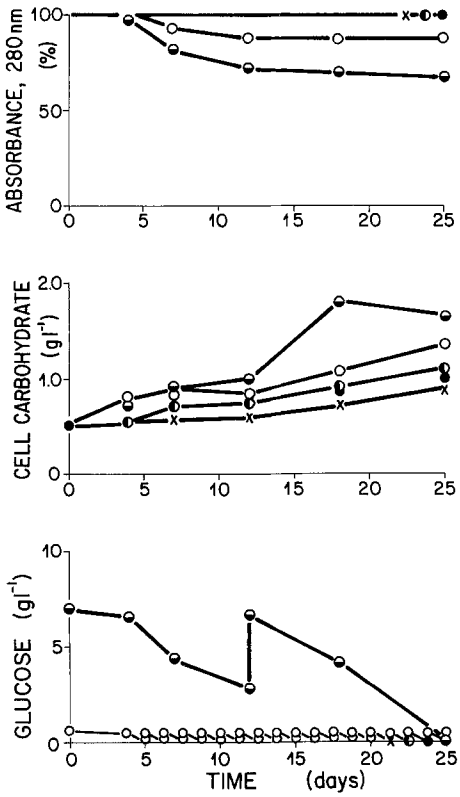

Fig. 1. Total lignin absorbance, cell carbohydrate and glucose concentration as a function of time during slow feeding of glucose in 25 ml cultures. Feed rates as $g \cdot l^{-1} \cdot day^{-1}$: −◒− excess, −○− 0.304, −◐− 0.192, −●− 0.094, −×− 0.047

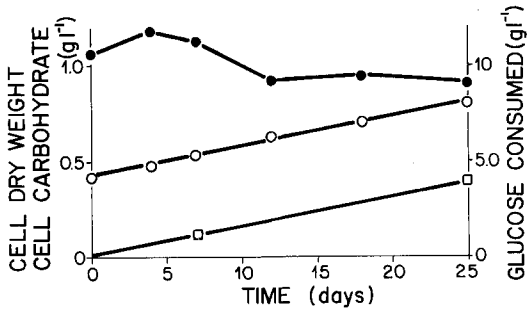

Fig. 2. Changes in mycelium dry weight (−●−) and cell carbohydrate (−○−) during slow ($0.144\ g \cdot l^{-1} \cdot day^{-1}$) glucose feeding. −□− total glucose consumed

They have also noticed a rapid protein turnover during the transition period from inactive to active cultures (Fenn and Kirk 1981). This seems to point to active new growth where the proteins of old cells are degraded and used as substrate for new growth. Part of the approximately 3-fold increase in total cell carbohydrate during the ligninolytic phase (Leisola et al. 1982) is probably due to formation of the new cell wall. The role of excess cell and soluble polysaccharide remains unclear.

Table 1. Decrease in Kraft lignin absorbance after 25 days of incubation using daily glucose feeding in 25 ml cultures

Glucose feed rate $g \cdot l^{-1} \cdot day^{-1}$	Kraft lignin % from original
Excess	51
0.304	80
0.240	100
0.192	100
0.144	100
0.094	100
0.047	100

Depth of the Culture

When the cells are taken from the bioreactor after the primary growth phase is completed, a homogeneous mat is quickly formed during the nonagitated lignin-olytic phase. Lignin is absorbed by this mat before the new mycelial growth occurs. Part of this new growth in 50 ml and 25 ml cultures always occurred in medium where no lignin was present.

To bring the new mycelial growth closer to lignin and to avoid possible oxygen limitation by the viscous polysaccharide mat (Leisola et al. 1982), the depth of the cultures was reduced. The first series of experiments were carried out using 50 ml of culture in 200 ml and 2,000 ml flasks (Fig. 3). In larger flasks, the lignin bioalteration started earlier and was almost complete, as compared to lignin bioalteration in the small flasks. Glucose was also consumed at a faster rate in large flasks.

In the second set of experiments, various volumes of culture were incubated in 200 ml flasks for 6 days. Again, a drastic change in rate and extent of lignin bioalteration was observed (Table 2) when the culture volume was reduced. The increased glucose consumption was an indication of oxygen limitation. The large differences between 5 ml and 10 ml cultures were especially surprising. Jeffries et al. (1981) reported maintenance values of $0.3 \cdot g^{-1} \cdot day^{-1}$ for 10 ml cultures in 125 ml Erlenmeyer flasks. Our earlier estimates were $0.2\ g \cdot g^{-1} \cdot day^{-1}$ (Leisola et al. 1982) for 50 ml cultures in 200 ml flasks. Both of these values are probably oxygen limited. Values as high as $0.75\ g \cdot g^{-1} \cdot day^{-1}$ were now measured for homogeneous cell mats that were less than 2 mm thick. The almost complete bioalteration of the original, acid-insoluble, Kraft lignin by 2 ml and 5 ml cultures was probably a result of improved oxygen transfer through the mycelium and increased availability of the lignin to the new mycelium.

Based on these results, a new experiment was carried out using 5 ml cultures in 200 ml flasks to find out the rate of lignin bioalteration. The cells for this experiment were taken from a chemostat which was run at a dilution rate of $0.02\ h^{-1}$. The bioalteration of lignin started after about 2–3 days of non-agitated cultivation (Fig. 4) and was completed in 6 days. During this time, the cell mat turned from dark brown to white. However, simultaneously with the disappearance of the acid-insoluble (< pH 3.0), dark brown lignin, water-soluble (> pH 1.0) products were formed which had an absorbance at 280 nm. After 6 days, the total water- and acid-soluble fraction was equivalent to about 30–35% of the original lignin absorbance, and its amount remained at the same level for the rest of the experiment.

The soluble material was fractionated by gel chromatography and UV-spectra were run for various fractions. The material itself and the various fractions no longer had the typical absorption maximum of lignin at 280 nm (Janshekar et al. 1981). The average molecular weight (Fig. 5) of the soluble material was smaller than that of the original lignin (about 4,000). The average molecular weight was

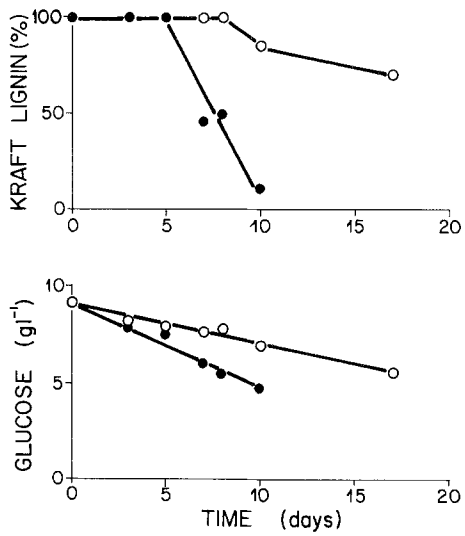

Fig. 3. Kraft lignin absorbance as % from original and glucose consumption in 200 ml (–○–) and 2,000 ml (–●–) flaks using 50 ml of culture

Table 2. Effect of culture volume on lignin degradation and glucose consumption in 200 ml flasks. Cells were taken from bioreactor after 3 days of batch cultivation and incubated for 6 days

Culture		Glucose consumption ($g \cdot g^{-1} \cdot day^{-1}$)	Lignin absorbance % from original	
Volume (ml)	Depth (mm)		Total	Kraft
2	< 1.0	0.75	54	15
5	1.5	0.73	65	15
10	3.0	0.30	100	76
25	7.0	0.20	100	83
50	13.0	0.17	100	88

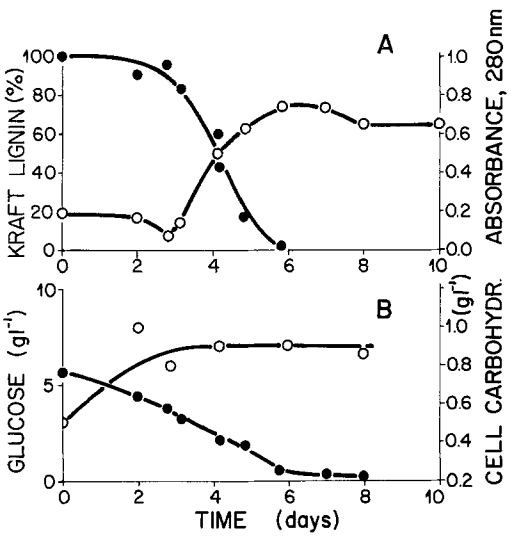

Fig. 4A and B. Changes in **A** Kraft lignin absorbance as % from original (−●−) and water soluble absorbance (−○−), dilution 1 : 10; **B** glucose concentration (−●−) and total cell carbohydrates (−○−). 5 ml cultures in 200 ml flasks

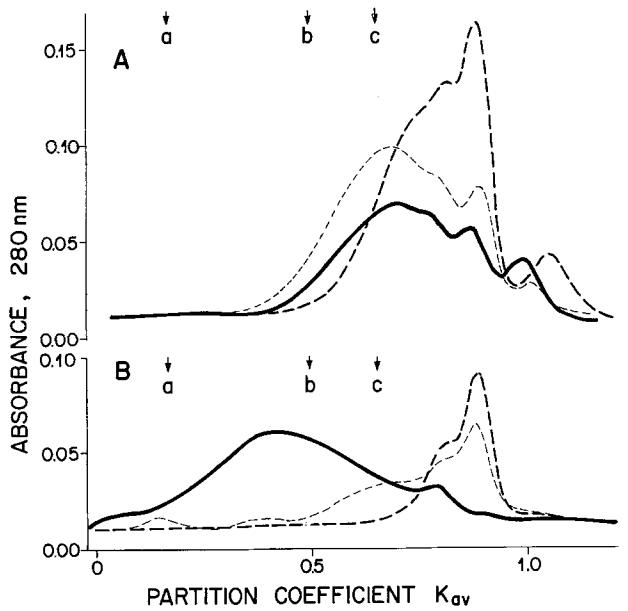

Fig. 5A and B. Elution patterns for various fractions of ligninolytic cultures using Sephadex G-75 gel; **A** Water soluble fraction after 4 (———), 6 (- - - -) and 10 (− −) days of cultivation; **B** Kraft lignin (———), water soluble part of Kraft lignin (- - - -) at pH 4.5 and absorbing material produced by cells without lignin (− −) after 8 days of incubation. 5 ml cultures in 200 ml flasks. Standards used: a) cytochrome c (MW = 12,500), b) glucagon (MW = 3,483) and c) bacitracin (MW = 1,423)

below 1,500 between days 4 and 6, and the material was further depolymerized until day 10. A considerable part of the absorbing material (10–20%) in the small molecular weight region was also produced by cells that had no lignin (Fig. 5).

It can be postulated that the solubilization of Kraft lignin is one step in its degradation. In optimal conditions, this step is quite rapid. One gram of fungal mycelia solubilized and depolymerized 1 g of lignin in about 3–4 days under the described conditions, with the consumption of about 4 g · l^{-1} glucose. This corresponds to a minimum glucose consumption rate of 1 g · g^{-1} · l^{-1} based on the initial mycelial dry weight of about 1 g · l^{-1}. Thus the glucose consumption rate is not only a function of the depth of the culture (Table 2) but is also based on the past history of the cells. This is evident from the the differences observed in the cells obtained from a batch culture (0.73 g · g^{-1} · day^{-1}, Table 2) and cells obtained from a chemostat (1.0 g · g^{-1} · day^{-1}, Fig. 4). The active phase in the small volume cultures was also preceeded by an increase in cell carbohydrates (Fig. 4) and the formation of new mycelia.

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