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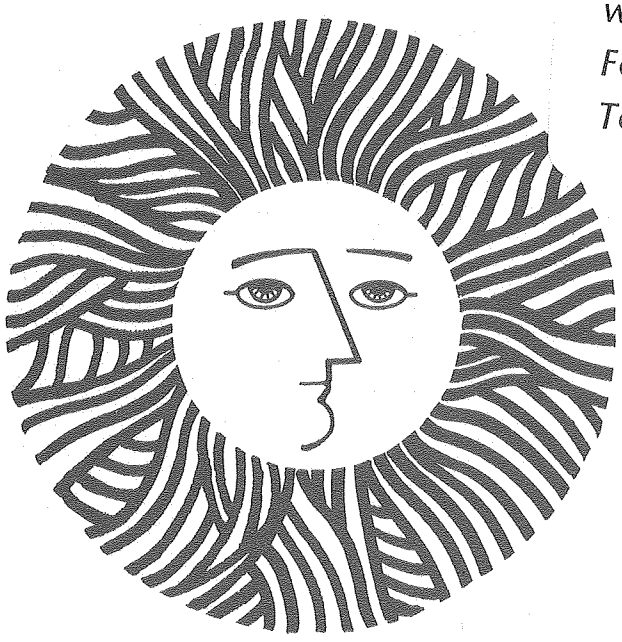

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S.L. Rosenberg and C.R. Wilke

Energy and Environment Division
Lawrence Berkeley Laboratory
University of California
Berkeley, CA 94720

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Chapter 13

LIGNIN BIODEGRADATION AND THE PRODUCTION OF ETHYL ALCOHOL FROM CELLULOSE

S. L. Rosenberg and C. R. Wilke

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I. INTRODUCTION

A. Present Process

During the last several years, our group has been engaged in developing a biochemical process for the conversion of lignocellulosic materials to ethyl alcohol.[1-3] A diagram of the process as presently conceived is shown in Figure 1.[4] Improvements are still being made, however, so this diagram does not represent the final configuration.

Briefly, the process operates as follows. Lignocellulosic material (in this case, corn stover) is milled and then extracted with a hot solution of dilute sulfuric acid. This treatment solubilizes most of the hemicellulose sugars, the primary component of which is xylose. We are currently working on a process for the conversion of xylose to ethanol, but this is not shown in the flow sheet and is not considered in the economic analysis of the process.

The treated solids, composed mainly of lignin and cellulose, are contacted with a cellulase-containing supernatant from a culture of the fungus *Trichoderma reesii (viride)*, strain QM 9414, in the hydrolysis step, and soluble sugars are produced. Cellulase enzymes are initially adsorbed on the cellulosic substrate but are released in the course of cellulose digestion. These enzymes are recovered and reused by contacting fresh acid-extracted substrate. The enzyme-free, glucose-containing hydrolyzate is then concentrated and fermented to ethanol and CO_2 by a continuously growing culture of the yeast *Saccharomyces cerevisiae*, ATCC 4126. The effluent from this fermentation is centrifuged, and part of the cells are recyled to the fermenter to increase the cell density and, thus, the productivity of the fermenter. The rest of the cells are dried for use as an animal feed supplement. Ethanol is recovered from the culture fluid by distillation. Residual unfermented sugars are removed from the waste stream by anaerobic digestion and converted to methane and CO_2. The methane is used to generate steam and electricity for the process. The unhydrolyzed residue from the hydrolysis step which consists mainly of lignin and some cellulose is also recovered and used to generate steam and power for the process.

In the enzyme production step, a continuous culture of *T. reesii* is grown with pure cellulose as the substrate, and cellulase and β-glucosidase enzymes are induced. In the separation step, the mycelium is removed from the enzyme-containing culture fluid as a slurry. Part of the mycelium is recycled to the fermenter. The rest of the mycelium plus residual cellulose is dewatered and recovered for use as an animal feed supplement. It has been calculated[4] that 23 gal of 95% ethanol can be obtained per ton of corn stover at a processing cost of about $1.80 per gallon exclusive of by-product credits.

B. Importance of Delignification

Lignin is recognized as a barrier to cellulose degradation by cellulases.[5] In the present scheme, chemically delignified cellulose is used only as a substrate for mold growth and enzyme induction. The substrate used for saccharification is not delignified because of the cost of such a chemical treatment. A cheap delignification step would benefit the process in two ways. It would increase the amount of cellulose accessible to cellulase hydrolysis and thus increase the overall yield of the process, and it would reduce the amount of inert material carried through the process, reducing the size of the plant and thus the capital cost.

C. Biodelignification

Two processes have been demonstrated for the partial delignification of wood using living cultures of white-rot fungi. The first involves the use of cellulase-less mutants which metabolize lignin and hemicellulose but spare cellulose.[6,7] The second involves

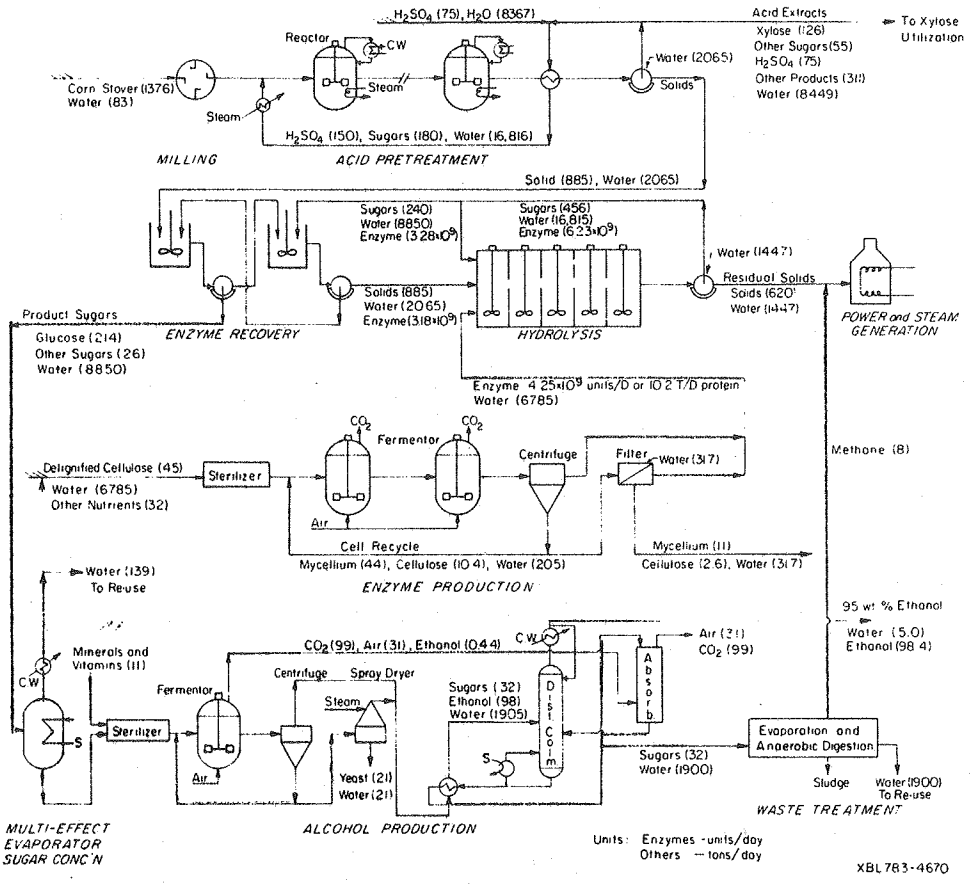

FIGURE 1. Process for the biochemical conversion of corn stover to ethyl alcohol. (From Cysewski, G. R. and Wilke, C. R., *Biotechnol, Bioeng.*, 18, 1297, 1976. With permission.)

the use of natural strains which preferentially ("selectively") degrade lignin in the early stages of their attack on wood.[5,8] In the first approach, significant amounts of hemicellulose sugars are removed during lignin degradation. In the second approach, only small amounts of lignin are degraded before carbohydrate loss begins. In both cases, the degradation products are consumed by the culture and are unavailable for other uses.

C. Potential of Enzymatic Delignification

While at the present time only chemical processes are used to delignify lignocellulosic materials, an enzymatic process might be attractive if a cell-free enzyme system could be developed. Use of an enzyme system should significantly reduce energy requirements for delignification. Such a process would also allow recovery and reuse of the enzymes and possible production of useful chemicals from the dissolved lignin.

The enzymology of lignin degradation has recently been reviewed.[9] No cell-free system for the solubilization of lignin has been demonstrated, although both fungal laccase[10] and peroxidase[11] preparations have been shown to carry out a simultaneous depolymerization and repolymerization of lignin (see Volume II, Chapter 2).

II. WORK WITH THERMOPHILIC AND THERMOTOLERANT FUNGI

A. Basic Attractiveness

In our effort to demonstrate a cell-free enzyme system capable of rapid lignin degradation, we focused our attention on thermophilic organisms because previous work[12,13] suggested that they would be able to degrade lignin and cellulose faster than their mesophilic counterparts. We further restricted our studies to the fungi, because convincing demonstrations of lignin degradation only occurred within this group.

While the thermophilic and thermotolerant[14] fungi had been fairly well characterized with respect to their ability to degrade pure cellulose,[15-18] little was known about their ability to degrade lignocellulose except for two strains, *Chrysosporium pruinosum* and *Sporotrichum pulverulentum*, which had been reported to degrade both lignin and cellulose by Nilsson[19] and Ander and Eriksson.[7] These strains are now characterized as imperfect forms of the mold *Phanerochaete chrysosporium*.[20]

B. Cellulose and Lignocellulose Degradation

Table 1 shows the results of experiments in which growth on and degradation of hanging filter paper (cellulose) or newsprint (lignocellulose) strips were noted for a large assemblage of taxonomically characterized thermophilic and thermotolerant fungi.[21] The paper strips were suspended from hooks in Erlemeyer flasks with the lower 5 to 10 mm of each strip submerged in a mineral medium supplemented with 0.01% yeast extract. Each culture was adjusted to the optimum pH and incubated at the optimum growth temperature as determined previously.[22]

The results obtained allowed the organisms tested to be divided into four groups based on their ability to grow with and degrade cellulose and lignocellulose. Group I fungi neither degraded nor grew on cellulose or lignocellulose. Group II fungi grew on and degraded cellulose but not lignocellulose. An apparent exception is *Torula thermophila* which showed growth on cellulose but no obvious degradation. The degradation which must have occurred was probably too diffuse to be noted. Group III fungi showed good growth on both cellulose and lignocellulose, but obvious degradation was restricted to the cellulose. The growth on lignocellulose may have actually been supported by the supplementary cellulose fibers incorporated in the newsprint (see below). Group IV fungi were able to grow on and degrade both cellulose and lignocellulose.

Several interesting findings emerged from this work. With respect to cellulose, three organisms in Group II appeared to show regional preferences for growth on and degradation of the paper strip. *Humicola insolens*, *Malbranchea pulchella* var. *sulfurea* and *Myriococcum albomyces* grew best on nonsubmerged regions of the cellulose, while the other organisms which grew with cellulose showed growth or degradation above and below the liquid surface.

With respect to newsprint, which is a mixture of 85% ground wood fibers (lignocellulose) and 15% cellulose fibers, a number of organisms appeared to grow on all regions of the strip (Group III). But only *Chrysosporium pruinosum* and *Sporotrichum pulverulentum* showed the ability to cause significant degradation (thinning) of the substrate, and this degradation occurred characteristically only in regions of the paper strip above the liquid meniscus.

For those organisms that showed it, restriction of growth or degradation to nonsubmerged regions of the substrate is probably related to the local concentration of one or more factors. Important factors may include degradative enzymes, cofactors, metabolic intermediates, minerals, and oxygen.

TABLE I

Growth on and Degradation of Cellulose (Filter Paper) and Lignocellulose (Newsprint) by Thermophilic and Thermotolerant Fungi

Organism	Cellulose		Lignocellulose	
	Growth[a]	Degradation[b]	Growth[a]	Degradation[b]
		I		
Humicola lanuginosa	− (5)[c]	− (5)	− (5)	− (5)
Mucor miehei	− (5)	− (5)	− (5)	− (5)
M. pusillus	− (5)	− (5)	− (5)	− (5)
Stilbella thermophila	− (5)	− (5)	− (5)	− (5)
Talaromyces thermophilus	− (5)	− (5)	− (5)	− (5)
Thermoascus aurantiacus	− (5)	− (5)	− (5)	− (5)
Thermomyces stellatus	− (5)	− (5)	− (5)	− (5)
		II		
Chaetomium thermophile v. *coprophile*	+ a[d] (32)	+ b (32)	− (5)	− (5)
C. thermophile v. *dissitum*	+ a (19)	+ b (19)	− (5)	− (5)
Humicola grisea v. *thermoidea*	+ ab (32)	+ b (32)	− (5)	− (5)
H. insolens	+ b (32)	+ b (32)	− (5)	− (5)
Malbranchea pulchella v. *sulfurea*	+ + bc (32)	+ c (32)	− (5)	− (5)
Myricoccum albomyces	+ b (32)	+ b (32)	− (5)	− (5)
Torula thermophila	+ ab (19)	− (5)	− (5)	− (5)
		III		
Allescheria terrestris	+ + abc (32)	+ + abc (32)	+ abc (8)	− (5)
Aspergillus fumigatus	+ + abc (12)	+ abc (12)	+ abc (5)	− (5)
Sporotrichum thermophile	+ + abc (32)	+ + abc (32)	+ abc (5)	− (5)
Talaromyces emersonii	+ + abc (12)	+ + abc (12)	+ abc (5)	− (5)
Thielavia thermophila	+ + abc (12)	+ + abc (12)	+ abc (5)	− (5)
		IV		
Chrysosporium pruinosum	+ + abc (32)	+ + abc (32)	+ + abc (32)	+ + bc (32)
Sporotrichum pulverulentum	+ + ab (12)	+ + ab (12)	+ + ab (12)	+ + b (12)

[a] (−) Indicates no growth in excess of that seen in control flask. (+) and (+ +) indicate relative amounts of growth in excess of that seen in control flask.

[b] (−) Indicates no obvious degradation compared to uninoculated control. (+) and (+ +) indicate relative degree of thinning or rotting of paper strip.

[c] Number in parentheses indicates day after which no further growth or degradation was observed. Observations were made on days 5, 8, 12, 19, and 32.

[d] a,b, and c indicate regions of the paper strip displaying most growth or degradation. a = submerged, b = 5 mm band above liquid meniscus, c = all area above b.

From Rosenberg, S. L., *Mycologia*, 70, 1, 1978, With permission.

III. PREFERRED CONDITIONS FOR RAPID LIGNOCELLULOSE DEGRADATION

A. Culture Design

Since our qualitative experiments indicated that rapid and extensive lignocellulose degradation could only be expected in nonsubmerged substrates, we set about devel-

oping a culturing system which would allow us to produce large amounts of mycelium and degraded substrate both for quantitative measurement of lignin and carbohydrate degradation and for the isolation of lignin-degrading enzymes.

The system which we developed utilizes a 25 mm high × 150 mm diameter Petri dish half-filled with mineral-agar medium supplemented with thiamin. Up to four sterile 47 mm diameter Nuclepore membrane filters (5 μm pore size) can be laid on the agar surface; 200 mg of dry, sterile lignocellulose fiber are spread on each of the filters, a drop of inoculum is added, and the cultures are incubated.

The lignocellulose fiber used was prepared by washing cattle manure obtained from a feed lot. The procedure was designed to remove both sand and water-soluble materials and yield a particulate residue which was rich in lignin. Much of the carbohydrate present in the original feed grain and straw was solubilized during fermentation in the rumen. The dry, washed manure fiber contained 37 ± 1.5% lignin, 37 ± 2% reducing sugar (cellulose plus hemicellulose), 10 ± 0.5% crude protein and 14 ± 1% ash. Lignin and carbohydrate were analyzed by modifications of the 72% sulfuric acid method of Moore and Johnson[24] and the anthrone method of Fairbairn.[26]. Details of the techniques and analytical methods used will be reported in a forthcoming publication.[23]

Figure 2 shows how various fractions of the lignocellulosic substrate are lost with time in a series of replicate agar plate cultures inoculated with *C. pruinosum*, ATCC 24782.[23] Organics are defined as that fraction of the dry residue which volatilize at 550°C.

Substrate degradation appears to cease after about 12 days with 75% of the organics, 80% of the carbohydrate, and 50% of the lignin degraded. During the period of active substrate degradation (3 to 12 days), the moisture content of the culture varies between 77 and 82% w/w.[23] Over the first 24 days of the experiment, the protein content (Kjeldahl nitrogen × 6.25) of the original residue doubles from 11.6% to 23.3%.[23]

Based on the amount of time required for equal percentages of degradation, the rate of lignin degradation displayed by *C. pruinosum* growing under these conditions is about four times greater than the rate reported by Kirk and Moore[5] for the mesophilic white rot *Polyporus giganteus* growing on wafers of aspen wood. The rate of lignin degradation displayed by *P. giganteus* is the highest previously reported.

B. Submerged Cultivation-Inhibition of Lignin Degradation

Figure 3 shows how degradation proceeds under conditions of submerged cultivation in a mineral medium using the same organism and substrate in shaking flask cultures.[23] Initial substrate losses are due to solubilization caused by autoclaving the fiber in the mineral medium. Here, little or no lignin was degraded, and losses of carbohydrate and organics were limited to 50 and 40%, respectively. The rate of carbohydrate degradation was also lower under these conditions than in the agar plate cultures. It is possible that shaking the submerged culture prohibits close contact between the mycelium and the substrate, and this may be responsible for the absence of significant lignin degradation (See Volume II, Chapter 4).

Figure 4 shows the results of an experiment in which stationary, submerged fiber cultures were analyzed. A tight mycelial mat formed on the bottom of each flask which encompassed and immobilized almost all of the fiber particles, indicating that close contact between mycelium and substrate was established. The results are similar to those shown in Figure 3 except that somewhat less carbohydrate was degraded. This may be due to the fact that in these cultures the fiber and medium were autoclaved separately and then combined.

These quantitative data confirmed our initial observations (Table 1) that significant lignocellulose degradation by *C. pruinosum* does not occur in a submerged substrate.

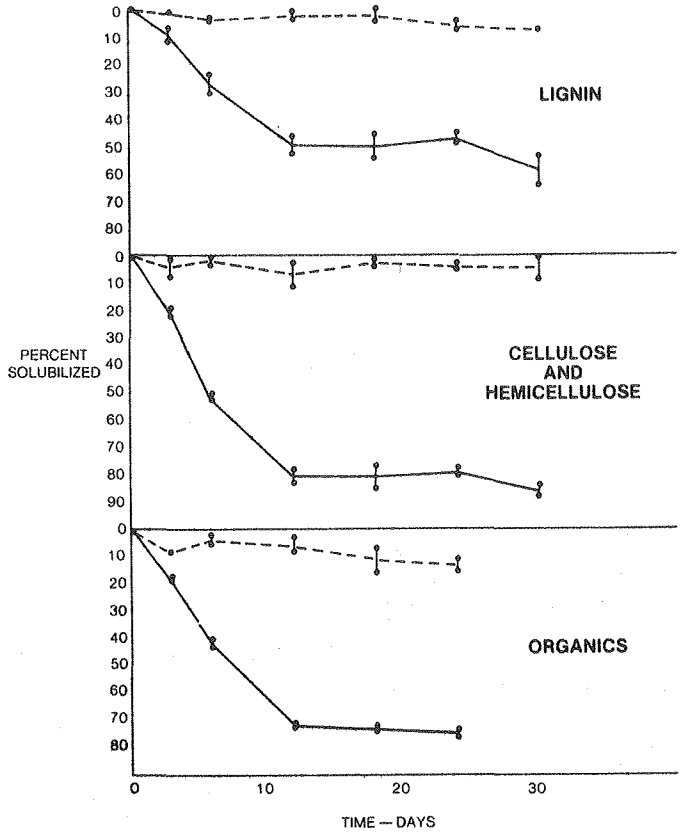

FIGURE 2. Degradation of manure fiber constituents by *Chrysosporium pruinosum* growing in damp fiber on the surface of mineral agar plates. Each data point represents the contents of one filter. All experiments were run in duplicate. Dashed lines indicate uninoculated controls.

C. Effect of Oxygen

It has been suggested that lignin degradation may be inhibited under submerged conditions by a low oxygen tension caused by fungal metabolism. To examine this possibility, a series of submerged standing cultures was prepared and incubated in an atmosphere of pure oxygen. Data in Figure 5 indicate that there was a small but significant increase in lignin degraded under these conditions and a much larger increase in carbohydrate loss (see Figure 4). Degradation appears to be essentially complete after 30 days of incubation (see also Volume II, Chapter 4).

Experiments were also carried out in which shaking submerged cultures were incubated in a pure oxygen atmosphere. Results similar to those shown in Figure 4 were obtained suggesting that both close hyphal-substrate contact and high oxygen tensions are required for significant lignin degradation to occur under conditions of submerged cultivation. We are presently investigating the effects of greater than atmospheric pressures of oxygen on submerged lignin and cellulose degradation.

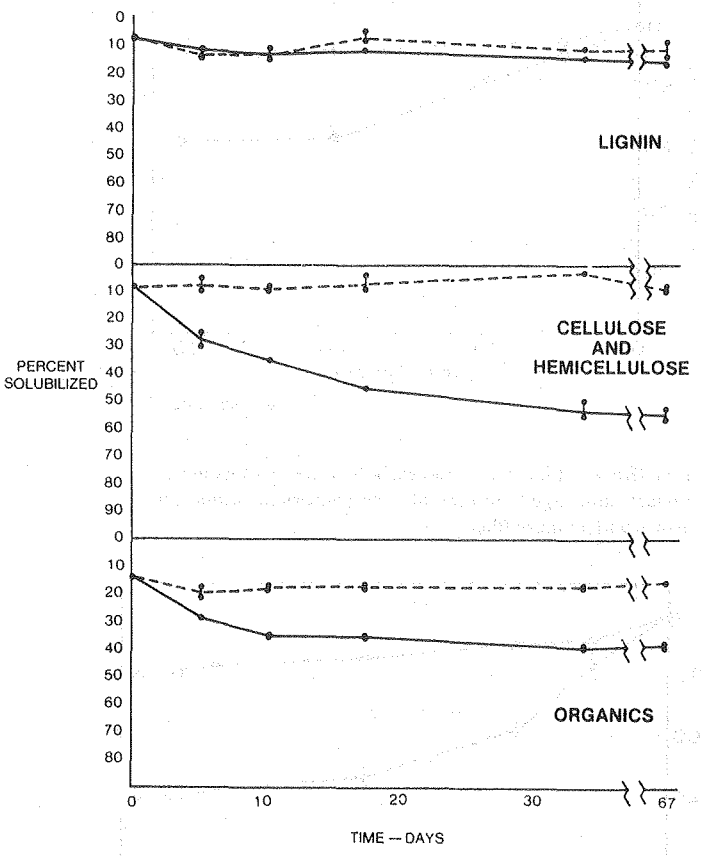

FIGURE 3. Degradation of manure fiber constituents by *Chrysosporium pruinosum* growing in submerged (shake flask) cultures. Each data point represents the combined contents of two shake flasks. Single points indicate that replicates give identical values. Dashed lines indicate uninoculated controls.

IV. ATTEMPTS TO DEMONSTRATE CELL-FREE LIGNIN DEGRADATION

A. Extraction of Cultures

Having developed the plate-culturing technique for *C. pruinosum* which allowed rapid lignin degradation to proceed, we next attempted to derive a cell-free lignin-degrading enzyme system from these cultures.

In our first approach a number of 4-day-old plate cultures were harvested and mixed with half-strength mineral medium[21] at 0°C for 30 min to extract enzymes. The suspension was then centrifuged. The recovered supernate represented a 1:10 dilution of the liquid present in the original fiber cultures. The supernate was concentrated at 4°C for 24 hr in an Amicon pressure cell using a UM-2 membrane (approximate molecular weight cutoff = 1000). A 26-fold concentration of the supernate was achieved. The concentrate was centrifuged, and the supernate was sterilized by vacuum filtration through a 0.4 μm pore size Nucleopore filter. Filtration was very slow, requiring 4 hr at 20°C. Aliquots of the sterile filtrate were added to 200 mg samples of sterile fiber in test tubes, mixed to coat the wall of the tube, and incubated in a humidified incubator at 40°C for 6 days. The aliquot size was chosen to reproduce in the fiber the

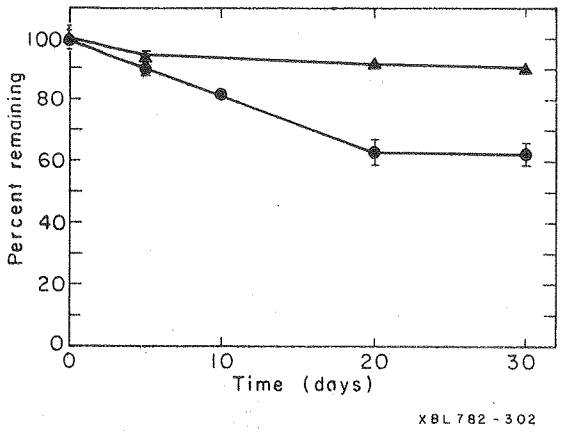

FIGURE 4. Lignin ▲–▲ and carbohydrate ●–● loss in stationary submerged cultures of *Chrysosporium pruinosum* grown with manure fiber.

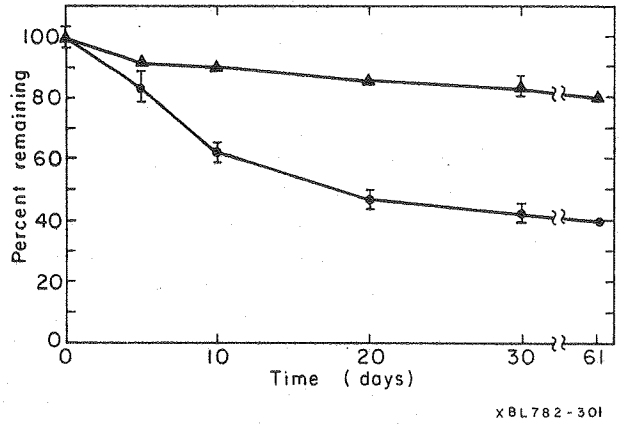

FIGURE 5. Lignin ▲–▲ and carbohydrate ●–● loss in stationary submerged cultures of *Chrysosporium pruinosum* grown with manure fiber in an oxygen atmosphere.

TABLE 2

Residual Lignin and Carbohydrate (Expressed as Glucose Equivalent) in Manure Fiber Treated with Concentrated Culture Supernate

Addition to fiber	Average % lignin remaining ± average deviation[a]	Average % carbohydrate remaining ± average deviation[a]
Concentrated supernate	98.0 ± 0.7	83.8 ± 3.3
Boiled concentrated supernate	98.7 ± 1.3	97.8 ± 3.5
Mineral medium	97.4 ± 0	100.3 ± 1.5
None	100.0 ± 1.3	100.0 ± 3.4

[a] "No addition" control = 100%; four replicates analyzed.

TABLE 3

Residual Lignin and Carbohydrate (Glucose Equivalent) in Manure Fiber Treated with Expressed Culture Fluid

Addition to fiber	Average % lignin remaining ± average deviation[a]	Average % carbohydrate remaining ± average deviation[a]
Culture fluid	100.7 ± 0	94.4 ± 0.7
Boiled culture fluid	100.0 ± 0.7	100.0 ± 2.1

[a] Boiled culture fluid control = 100%; two replicates analyzed.

moisture content of an agar plate fiber culture (∼80% w/w). Table 2 shows the results obtained. While carbohydrate was degraded, no lignin-solubilizing activity was detected.

There are a number of problems with the above approach. Enzymes may be inactivated during dilution, low molecular weight cofactors may be lost in the concentration step, and significant losses in activity may occur due to the time required for concentration. In order to minimize or eliminate these problems, a different procedure was devised.

B. Use of Expressed Culture Fluid

Agar plate cultures were grown for 5 days and then scraped off the filters directly into the barrel of a plastic syringe. The plunger was inserted and pushed in tightly using a press, and culture fluid was collected. A small disc of polyurethane foam was placed in the bottom of the syringe barrel before loading to prevent clogging of the orifice. In this way, 45% of the liquid content of the cultures could be recovered. The expressed fluid was centrifuged, filter-sterilized, and then added to fiber tubes as before. Table 3 shows the results of this experiment. Again, no lignin loss is seen, and only a small amount of carbohydrate was solubilized.

It is obvious from the results of these experiments that the lignin degradation system produced by this organism does not consist solely of an easily separable set of soluble extracellular enzymes, as is the case for the cellulase system.[25]

C. Use of Diffusion Cultures

Since it was not clear whether the lignin-degrading system produced by this organism was diffusible or required the presence of living mycellium for activity, an apparatus was constructed to try to answer these questions. The apparatus (Figure 6), termed a diffusion chamber, consists of two glass tubes glued to and separated by a 47 mm diameter Nuclepore membrane filter of 0.2 μm pore size. The tubes were 20 to 30 mm long with an O.D. of 46 mm and an I.D. of 44 mm. The ends of the tubes were plugged with well-washed polyurethane sponge plugs. The bottom chamber contained 200 mg of sterile manure fiber moistened with 0.8 mℓ of mineral medium plus thiamin. The top chamber was inoculated with 0.2 mℓ of a culture suspension (inoculated filter). The cultures were incubated in a humidified incubator, and 0.6 mℓ aliquots of mineral medium were added to the top surface weekly to prevent drying and provide minerals for culture growth. These chambers allowed growth of the mold on the top surface of the filter without hyphal invasion of the substrate below the filter. As controls, one series of chambers was prepared, but not inoculated, while another series was prepared with the fiber inoculated. The results of these experiments are shown in Table 4.

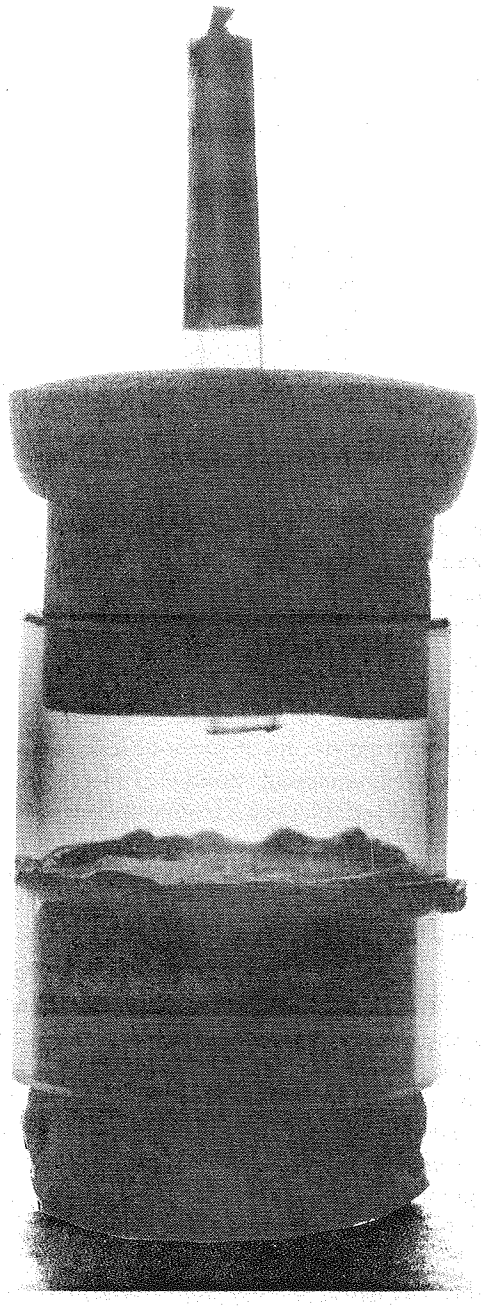

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FIGURE 6. Diffusion chamber. Top plug raised to show details of construction.

It is apparent that organisms growing in direct contact with the lignocellulose fiber (Lines 6 and 7) are able to degrade significant amounts of both lignin and carbohydrate within the first 2 weeks of incubation, although neither lignin nor carbohydrate losses are as complete as in the agar plate cultures (Figure 2).

Cultures where the inoculum is separated from the substrate (Lines 4 and 5) show a mat of mycelial growth and conidia on the top surface of the filter. The fiber below

TABLE 4

Residual Lignin and Carbohydrate (Glucose Equivalent) in Manure Fiber From Diffusion Cultures

Sample	Incubation time-days	Average % lignin remaining ± average deviation[a]	Average % carbohydrate remaining ± average deviation[a]
Uninoculated control	0	100.0 ± 0.8	100.0 ± 0.1
	14	96.7 ± 0.2	94.3 ± 2.8
	31	96.5 ± 0	89.8 ± 0.4
Inoculated filter	14	94.5 ± 1.0	78.1 ± 0.8
	31	92.0 ± 2.5	54.9 ± 4.2
Inoculated fiber	14	77.5 ± 1.0	26.9 ± 7.1
	31	76.0 ± 2.8	27.4 ± 2.5

[a] Relative to average value for zero time uninoculated control. Two replicates analyzed.

remains sterile. Under these conditions an appreciable amount of carbohydrate is solubilized. Growth of the organism above coupled with solubilization of carbohydrate below indicates that both enzymes and soluble products are diffusing through the separating membrane. Lignin loss, while somewhat higher than in the uninoculated controls, is not believed to be significant, since losses of approximately 10% are seen in the uninoculated controls from other experiments (Figures 2, 3).

Taken together, the data from the enzyme-isolation and diffusion experiments suggest that the lignin-degradation system or one or more of its components produced by this organism is either not induced, unstable, nondiffusible (e.g., bound to the cell wall), or inactive, at small distances (about 1 mm) from growing hyphae.

V. PRESENT DIFFUSION CULTURE STUDIES

A. Selection of Mutants of *Chrysosporium pruinosum*

Although we have not yet been able to demonstrate a cell-free lignin-degrading system, the diffusion culture technique may be useful in further attacking this problem. We are presently using two approaches in our attempt to demonstrate a cell-free system. Cultures of *C. pruinosum* are being mutagenized in order to obtain a strain which produces a diffusible system. The selection technique involves the use of a fiber substrate which was previously exhaustively degraded by the wild-type in a diffusion culture. Any organisms able to grow on this partially degraded material in a diffusion culture would presumably possess either a diffusible lignin-degradation system or an altered cellulase activity. Preliminary experiments have shown that the wild type displays only very slight growth in diffusion cultures using previously degraded fiber as a substrate. This suggests that the heavy growth seen using undegraded substrate occurs primarily at the expense of the "diffusate" and not to any significant degree at the expense of nutrients carried in the inoculum.

B. Testing of Mesophilic Lignin-Degrading Fungi

We are also using diffusion chambers to test a number of mesophilic lignin-degrading molds which differ from one another both taxonomically and with respect to the way in which they have been shown to degrade lignin. By analogy with common metabolic pathways described in other microorganisms, it is reasonable to expect that there may be alternative biochemical approaches to the degradation of lignin and that some of these organisms may produce a diffusible system.

VI. SUMMARY

During the last few years our group has been engaged in developing a biochemical process for the conversion of lignocellulosic materials to ethyl alcohol. The present process involves pretreatment of the substrate with hot, dilute acid to remove hemicellulose and increase cellulose reactivity, treatment of the extracted lignocellulose with cellulases from *Trichoderma reesii* to produce glucose, and fermentation of the glucose to ethanol and CO_2 by *Saccharomyces cerevisiae.*

Lignin is a barrier to complete cellulose saccharification in this process, but chemical and physical delignification steps are too expensive to be used at the present time. An enzymatic delignification process might be attractive for several reasons: little energy would be expected to be needed, enzymes could be recovered and reused, and useful chemicals might be produced from dissolved lignin.

We examined a number of thermophilic and thermotolerant fungi for the ability to rapidly degrade lignocellulose in order to find an organism which produced an active lignin-degrading enzyme system. *Chrysosporium pruinosum* and *Sporotrichum pulverulentum* (now recognized as imperfect forms of the mold *Phanerochaete chrysosporium)* were found to be active lignocellulose degraders, and *C. pruinosum* was chosen for further study.

It was found that in shake flask cultures using a lignocellulosic substrate, carbohydrate (cellulose and hemicellulose) was degraded, but lignin was not. Lignin and carbohydrate were degraded when the substrate remained moistened by, but not submerged in, the liquid medium. Carbohydrate degradation was much more extensive in moist cultures than in submerged cultures. In static submerged cultures both lignin and carbohydrate degradation could be stimulated by an atmosphere of pure oxygen.

Attempts were made to demonstrate a cell-free lignin degrading system by both extraction and pressing of cultures grown on moist lignocellulose. Carbohydrate-degrading activity was found but not lignin-degrading activity. This led us to ask whether diffusible lignin-degrading activity could be demonstrated in this organism. Using an apparatus in which the culture was separated from the damp substrate by a bacteriological membrane filter, we found that mycelial growth could oocur on the inoculated side of the membrane at the expense of carbohydrate degraded on the substrate side, but little or no lignin was lost.

The data indicate that the lignin degradation system, or one or more of its components, produced by this organism is either unstable, non-diffusible, or inactive at small distances (about 1 mm) from growing hyphae.

At present, studies are being conducted using diffusion cultures to select mutants of *C. pruinosum* that do produce a diffusible lignin degradation system. We are also examining a number of mesophilic lignin-degrading molds for this ability.

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REFERENCES

1. **Cysewski, G. R. and Wilke, C. R.,** Utilization of cellulosic materials through enzymatic hydrolysis. I. Fermentation of hydrolysate to ethanol and single cell protein, *Biotechnol. Bioeng.*, 18, 1297, 1976.
2. **Wilke, C. R., Cysewski, G. R., Yang, R. D., and von Stockar, U.,** Utilization of cellulosic materials through enzymatic hydrolysis. II. Preliminary assessment of an integrated processing scheme, *Biotechnol. Bioeng.*, 18, 1315, 1976.
3. **Wilke, C. R., Yang, R. D., and von Stockar, U.,** Preliminary cost analysis for enzymatic hydrolysis of newsprint, *Biotechnol. Bioeng.*, Symp. No. 6, 155, 1976.
4. **Wilke, C. R., Yang, R. D., Sciamanna, A. S., and Freitas, R.,** Raw materials evaluation and process development studies for conversion of biomass to sugars and ethanol, paper presented at the 2nd Ann. Symp. on Fuels from Biomass, Rensselaer Polytechnic Institute, Troy, N.Y., June 20 to 22, 1978.
5. **Kirk, T. K. and Moore, W. E.,** Removing lignin from wood with white-rot fungi and digestibility of resulting wood, *Wood Fiber*, 4, 72, 1972.
6. **Eriksson, K.-E. and Goodell, E. W.,** Pleiotropic mutants of the wood-rotting fungus *Polyporus adjustus* lacking cellulase, mannanase and xylanase, *Can. J. Microbiol.*, 20, 371, 1974.
7. **Ander, P. and Eriksson, K.-E.,** Influence of carbohydrates on lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*, *Sven. Papperstidn.*, 78, 643, 1975.
8. **Ander, P. and Eriksson, K.-E.,** Selective degradation of wood components by white-rot fungi, *Physiol. Plant*, 41, 239, 1977.
9. **Ander, P. and Eriksson, K.-E.,** Lignin degradation and utilization by microorganisms, *Prog. Ind. Microbiol.*, 14, 2, 1977.
10. **Ishihara, T. and Miyazaki, M.,** Oxidation of milled wood lignin by fungal laccase, *Mokuzai Gakkaishi*, 18, 415, 1972.
11. **Sopko, R. S.,** Degradation of spruce and sweetgum lignins by *Polyporus versicolor* and *Pleurotus ostreatus*, Master's thesis, North Carolina State University at Raleigh, 1972.
12. **Brock, T. D.,** Life at high temperatures, *Science*, 158, 1012, 1967.
13. **Waksman, S. A. and Gerretsen, F. C.,** Influence of temperature and moisture upon the nature and extent of decomposition of plant residues by micro-organisms, *Ecology*, 12, 33, 1931.
14. **Cooney, D. G. and Emerson, R.,** *Thermophilic Fungi*, W. H. Freeman, San Francisco, 1964.
15. **Chang, Y.,** The fungi of wheat straw compost. II. Biochemical and physiological studies, *Trans. Br. Mycol. Soc.*, 50, 667, 1967.
16. **Eriksson, K.-E. and Rzedowski, W.,** Extracellular enzyme system utilized by the fungus *Chrysosporium lignorum* for the breakdown of cellulose. I. Studies on the enzyme production, *Arch. Biochem. Biophys.*, 129, 683, 1969.
17. **Fergus, C. L.,** The cellulolytic activity of thermophilic fungi and actinomycetes, *Mycologia*, 61, 120, 1969.
18. **Tansey, M. R.,** Agar-Diffusion assay of cellulolytic ability of thermophilic fungi, *Arch Mikrobiol.*, 77, 1, 1971.
19. **Nilsson, T.,** Mikroorganismer i Flisstackar, *Sven. Papperstidn.*, 68, 495, 1965.
20. **Burdsall, H. H., Jr. and Eslyn, W. E.,** A new *Phanerochaete* with a *Chrysosporium* imperfect state, *Mycotaxon*, 1, 123, 1974.
21. **Rosenberg, S. L.,** Cellulose and lignocellulose degradation by thermophilic and thermotolerant fungi, *Mycologia*, 70, 1, 1978.
22. **Rosenberg, S. L.,** Temperature and pH optima for 21 species of thermophilic and thermotolerant fungi, *Can. J. Microbiol.*, 21, 1535, 1975.
23. **Rosenberg, S. L.,** Physiological studies of lignocellulose degradation by the thermotolerant mold *Chrysosporium pruinosum*, *Developments in Industrial Microbiology*, 20, 133, 1979.
24. **Moore, W. E. and Johnson, D. P.,** Procedures for the chemical analysis of wood and wood products, Forest Products Laboratory, U.S. Department of Agriculture, 1967.
25. **Streamer, M., Eriksson, K.-E., and Pettersson, B.,** Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum (Chrysosporium lignorum)* for the break down of cellulose, *Eur. J. Biochem.*, 59, 607, 1975.
26. **Fairbarn, N. J.,** A modified anthrone reagent, *Chem. Ind. (London)*, No. 4, 86.