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SACCHARIFICATION OF LIGNOCELLULOSIC RESIDUES BY BROWN-ROT FUNGI: MECHANISMS AND POTENTIAL APPLICATIONS

Project No 936-5542
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Rec'd in SCI MAY 19 1989

1./SELECTION OF THE MOST PROMISING BROWN-ROT FUNGI

This part of the work was done before the beginning of the grant. A paper was already written with the results obtained and submitted to Enzyme and Microbial Technology. The paper is currently in press. I enclosed you a copy of it.

From this work, it appeared that the brown-rot fungi Gloeophyllum trabeum and Wolfiporia coccos are able to grow relatively quick on pine sawdust, allowing a very important increase of rumen digestibility of structural polysaccharides during the early stages of decay. This seems to be related to the rapid depolymerization of these polymers during wood colonization.

Therefore, all our efforts since June 1988 were directed towards the identification of the agent responsible of the oxidative depolymerization of cellulose, as well as of its regulation, as it was proposed in "Physiology of cellulose depolymerization by brown-rot fungi" (see pp. 8-12 of the grant).

II.IDENTIFICATION OF THE AGENT RESPONSIBLE OF CELLULOSE DEPOLYMERIZATION

During these experiments, the strains Poria placenta and Coniophora puteana were also included because they are characterized by the absence of secretion of aromatic metabolic compounds and for possessing an hydrolytic and oxidative cellulolytic system, respectively.

Part of this work has been done in collaboration with Dr. T.K. Kirk, during the stay of the principal investigator at the Inscitute of Enzyme and Microbial Technology, Forest Products Laboratory, USDA, Madison, Wisconsin,

The following experiments were conducted:

1. Depolymerization of cotton cellulose by Coniophora puteana, Gloeophyllum trabeum and Poria placenta under solid-state conditions

Three different carbon sources (mannose, glucose and cellobiose at 0.2, 0.5 and 1%) and two oxygen levels (21% and 50% oxygen) were tested. No significant growth could be obtained for any of these cultures after 20 days of incubation.

2. Efect of an "inducer" on cellulose depolymerization by Poria placenta

The same conditions as above were employed, except that after ten days of fungal growth, a water extract and the residue from a highly depolymerized culture were added at 0.1 and 1% (w/w) level. Lactose was also assayed at the same levels. No

significant growth could be obtained under these conditions, too. Therefore, it was concluded that under these conditions, inhibition by end-products of cellulose decay generated during the first steps of degradation could have been occurred.

3. Effect of dry matter content on cellulose depolymerization by *Poria placenta*

Colonized feeder strips were incubated in Petri dishes with 200mg of cotton cellulose containing 2, 10, 20, 30 and 40% final dry matter (w/v). After three weeks incubation, depolymerization of cellulose was significantly higher for cellulose with 30% dry matter content and more (Figure 1). No difference was determined in the degree of depolymerization (DP) of cotton cellulose between dry matter contents superior to 30% as well as for those that contained less than 30% dry matter.

These results imply that the examination of cultural parameters important for cellulose depolymerization, as indicated in page 9 of the grant, will have to be reassessed.

4. Kinetics of cellulose depolymerization by *Poria placenta*

As a consequence of the results obtained above, the kinetics of cellulose depolymerization was determined using the soil block procedure (Figure 2). Two phases of cellulose depolymerization were observed. The first phase was very rapid (5 days); cotton cellulose was depolymerized to a DP of 800. Then, a slower phase took place, during which cellulose was linearly depolymerized to 230 after 21 days. Finally, a constant DP of 200-250 was determined until 42 days of incubation. 1% NaOH solubility increased linearly till 21 days, after which time, although increase still linear, the rate was slowered. A maximum of 32% alkali-soluble compounds was reached after 42 days of decay. MW distribution of the different period times of depolymerization (currently under determination) should allow us to explain the reasons of the occurence of two different rates for cellulose depolymerization.

5. Pulse-labelling of ^{14}C-glucose during depolymerization of cellulose by *Poria placenta*

In an effort to identify if there was any low molecular weight compound(s) which were specifically secreted during the active phase of cellulose depolymerization, a pulse-labelling of 14C-glucose was set up. 15ul of 14C-glucose (7,5x106 dpm/culture) were added to decayed feeder strips containing 4x100mg of cotton cellulose at time when the second phase of depolymerization was developping (i.e. 10 days). Two "hot" cultures and one cold culture (a total of 1.2 gr of cellulose) were harvested after 1, 3 and 7 days of incubation with radiolabeled glucose. Cotton cellulose was harvested and 5ml of 500mM NaCl were added. After 2 hours with occasional stirring, extracellular water-soluble

compounds were squeezed, centrifuged, and low molecular compounds were separated in a 50x1 cm Sephadex G-25 column (eluent: distilled water). 1 ml fractions were collected and assayed for ninhydrine, absorbance at 280nm and 14C. Results indicated that two new low MW compounds were synthesized under these conditions, which have a MW of aproximately 1100 and 2000 D, respectively (Figures 3, 4 and 5). Only the second one was ninhydrine positive, pointing to the presence of primary amines or that it could be a peptide. Currently, transition metals analysis of the different fractions collected is underway to determine if one (or both) peaks carry Fe or Mn, which have been recently shown to be the only transition metals that accumulate in brown-rotted cellulose (T.K.Kirk, personnal communication). It is interesting to point out that both elements have been shown to participate in lignin degradation by white-rot fungi, a closely related fungi.

FUTURE WORK

- MW distribution of different period times of cellulose depolymerization.

- Transition metals analyses for samples extracted and fractionnated by gel permeation chromatography. The solid residue remaining after extraction for days 13, 17 and 50 will also be analyzed in order to determine the relative importance of extracellular transition metals (see exp. 5).

- In the case that a chelator for Mn, Cu or Fe was discovered, and important amount (50-100gr) of cellulose will be decayed under the same conditions for 20-30 days, extracted and fractionated as above (a bigger column of Sephadex G-25 will then be needed). The main peaks collected will be concentrated, filter-sterilized and added to similar cultures as above. Depolymerization will be followed and compared to non-inoculated cultures. If one of these peaks represent the limiting step in cellulose depolymerization of inoculated cultures, as compared to controls.

- The high MW fraction will be fractionated in a column of Sephadex G-50. Collected peaks will be characterized for general oxidase activity, endo and exoglucanase, heme proteins, etc...

- Finally, the kinetics of secretion of organic acids and its possible relationship with cellulose depolymerization will be evaluated during this new research period.

FIG. 1

Effect of Dry Matter Content on Cellulose Depolymerization

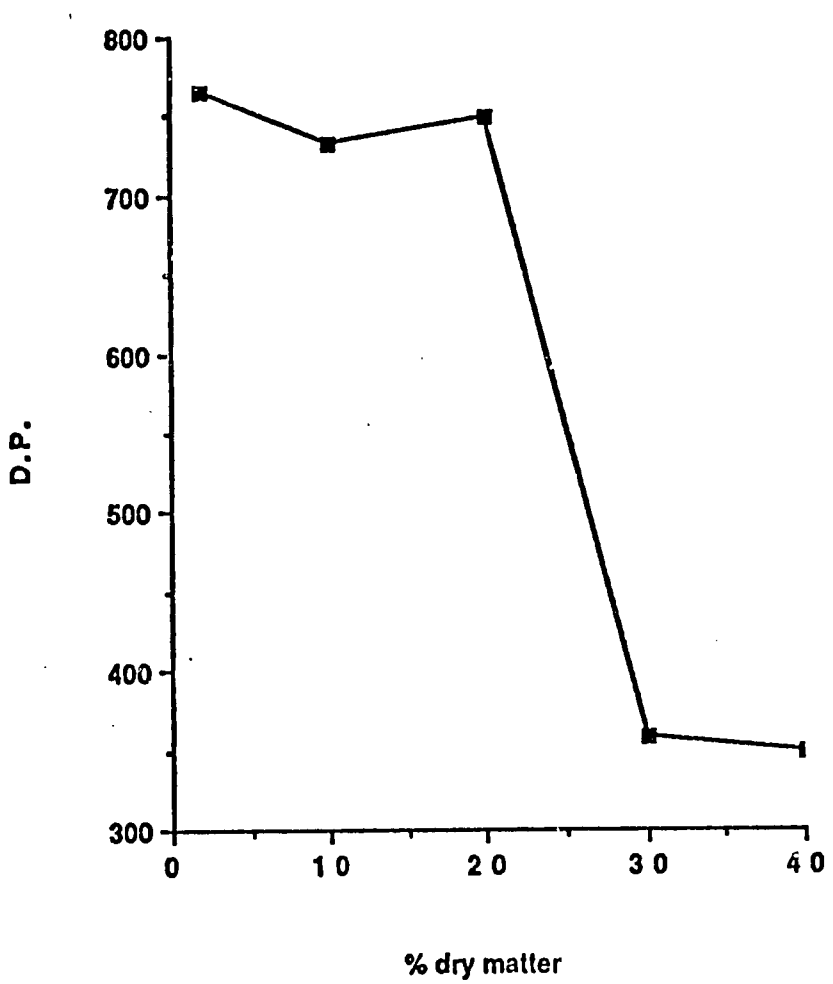

FIG. 2
Kinetics of Cellulose Depolymerization by P. placenta

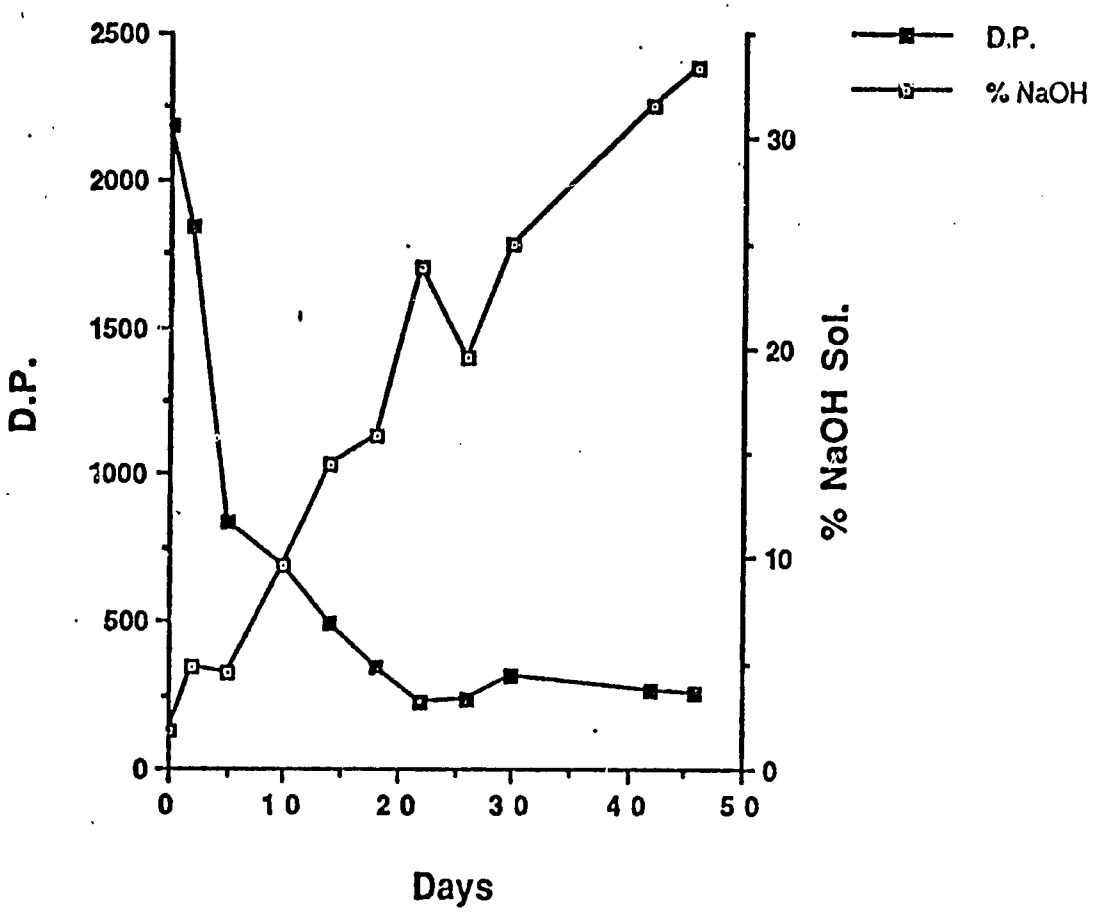

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Fig. 3. Pulse labeled ^{14}C glucose after one day addition to activily depolimerizing cellulolytic cultures of P. placenta

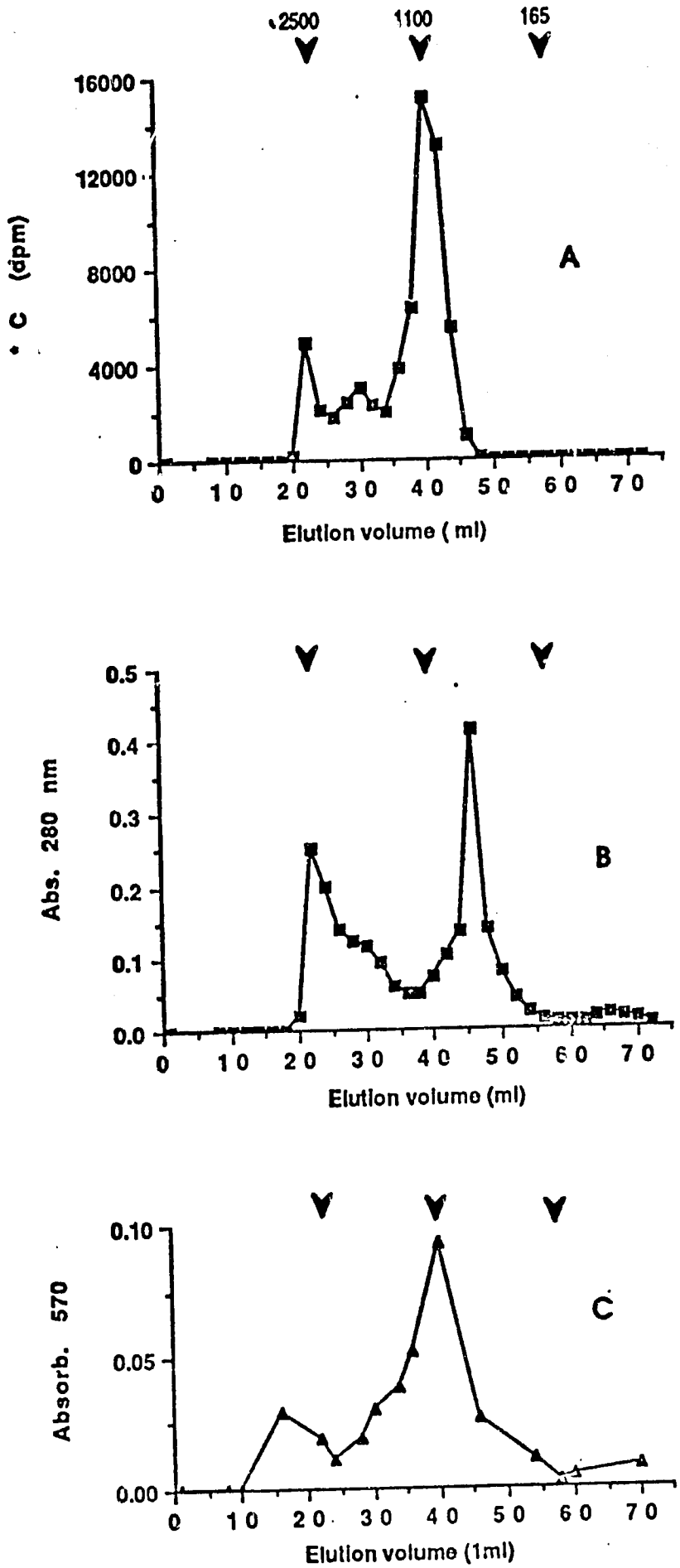


Fig. 4. Pulse labeled ^{14}C glucose after three days addition to activily depolimerizing cellulolytic cultures of P. placenta

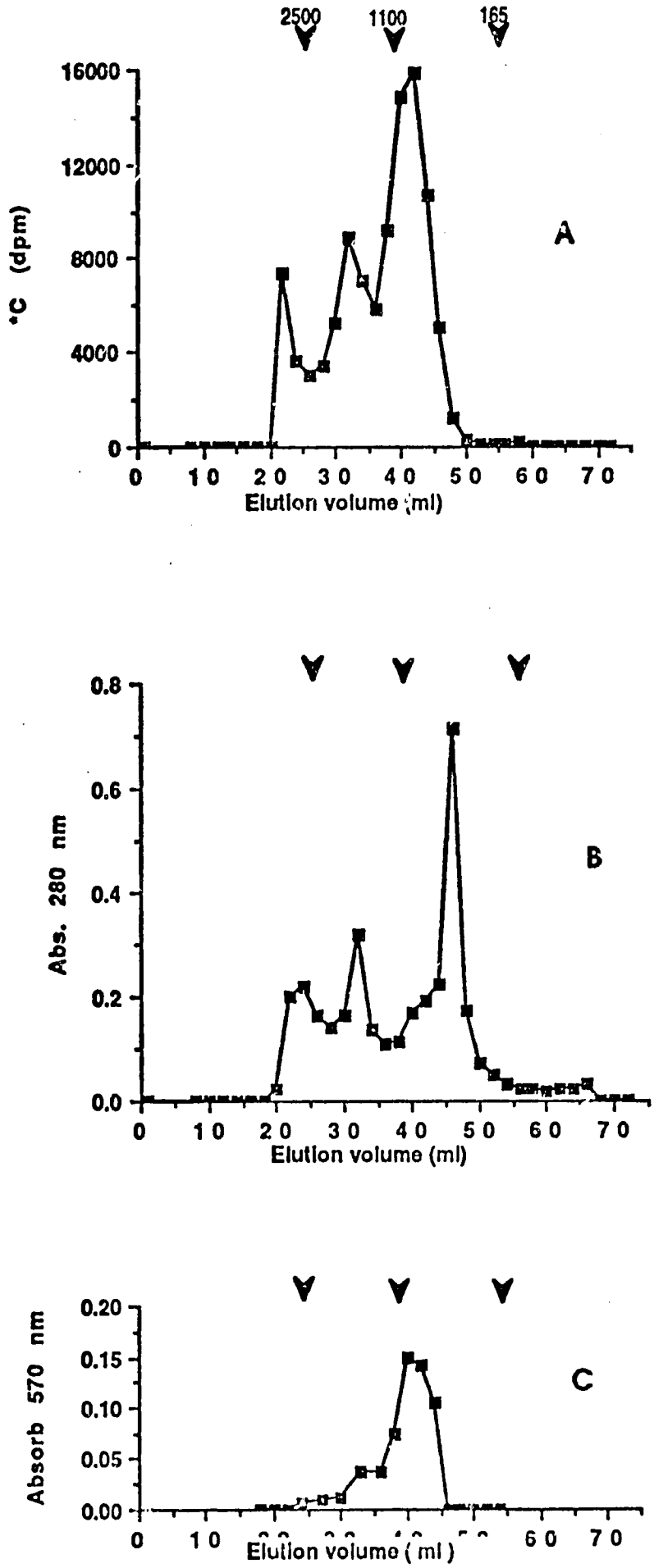
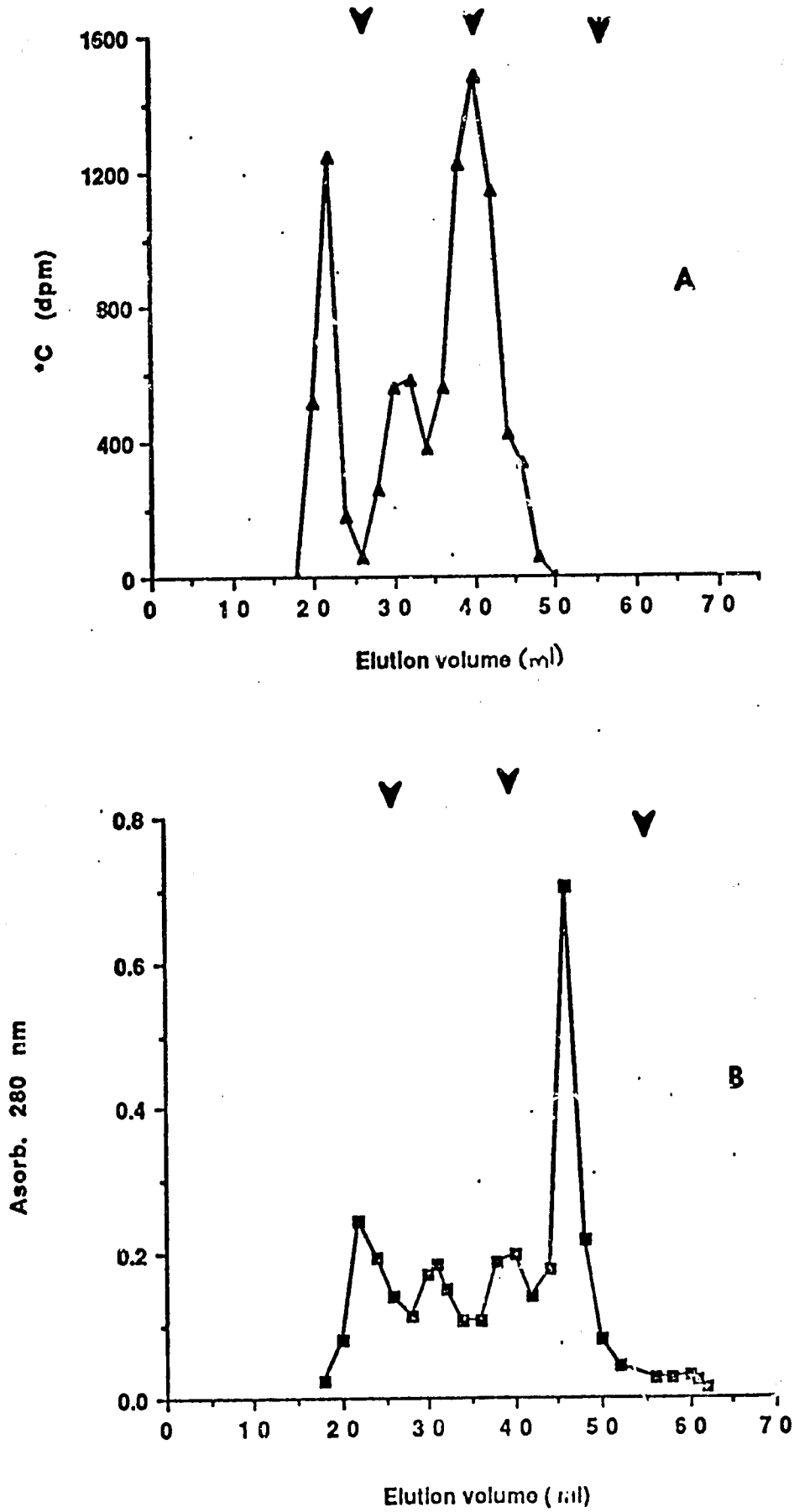


Fig. 5. Pulse labeled ^{14}C glucose after seven days addition to activily depolimerizing cellulolytic cultures of P. placenta



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Solid-state fermentation of pine sawdust by selected brown-rot fungi

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*The effects of selected culture parameters on the solid-state fermentation of radiata pine (*Pinus radiata*) by the brown-rot fungus* Gloeophyllum trabeum *were examined. Oxygen concentration is critical for wood decay; a twofold increase was observed when 21% O_2 was employed, as compared to 5% O_2. The highest yields were obtained with $pO_2 > 50\%$. Although optimum pH for fungal growth was 4.5, pH lower than 4.0 appeared to be essential for wood degradation by these fungi. Culture conditions allowing the highest decay capacities were then employed to follow the chemical changes occurring during decay of radiata pine by* G. trabeum, Lentinus lepideus, *and* Wolfiporia cocos *and their effects on rumen digestibility were assessed. Dry matter digestibility increased from 0 to more than 20% during the first week of wood colonization by the three tested fungi. Then, after 4 weeks incubation, digestibility fell to less than 5% for* W. cocos *and* L. lepideus. *Increase in digestibility could be related to polysaccharide depolymerization, as well as to the alkali-solubilizable fraction of structural polysaccharides and lignin in decayed wood.*

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Keywords: Solid-state fermentation; sawdust; fungus; *Gloeophyllum trabeum*

Introduction

Traditional approaches to the utilization of lignocellulosic materials by microorganisms rely on disruption of the lignin barrier, followed by the conversion of cellulose to glucose. The high cost of these pretreatments and the incomplete utilization of all wood fractions have hampered the economic viability of such processes.[1] Furthermore, most of the studied processes show little response with softwoods.[2,3]

Brown-rot fungi, which occur predominantly on softwoods, have scarcely been studied for biotechnological purposes, despite their unique ability to remove structural polysaccharides in fully lignified native tissues without significant depletion of the lignin fraction.[4] This makes them potential candidates for the direct bioconversion of softwood polysaccharides into fermentable sugars.[5] Furthermore, the residue remaining after decay—mainly lignin—has potential as a source of other valuable products, such as adhesives. Therefore, brown-rot fungi could provide a new saccharification system for lignocellulosic substrates, which may allow the integral use of these abundant, low-cost materials.

Cowling[6] and Cowling and Brown[7] suggested that the basic mechanism employed by these fungi to decay wood is related to their capacity to depolymerize cellulose during the initial stages of decay. It has been proposed[8,9] that cellulose depolymerization opens up the wood cell wall, allowing cellulolytic and hemicellulolytic enzymes of the brown-rot fungi to reach their substrates despite the presence of lignin. The use of this particular mechanism to improve enzymic access to softwood polysaccharides—by rumen microorganisms, for example—has not been evaluated.

Among structural polysaccharides, hemicelluloses, and particularly glucomannans, are preferentially degraded during colonization of softwood by brown-rot fungi.[4] However, the mechanism employed by these fungi to remove the hemicellulose fraction and its role in the opening of the wood cell wall have received very little attention.[10-12]

The present study has the following specific objectives: (i) to determine cultural parameters important for semisolid fermentation of pine sawdust by brown-rot fungi; (ii) to evaluate the potential of these fungi as wood saccharifying agents; and (iii) to provide information on the alkali-soluble substances generated during the process.

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Materials and methods

Fungi and inocula

The following brown-rot fungi were used in this study: *Wolfiporia cocos* FP 90850-R, kindly provided by the Center for Forest Mycology Research, CFMR (Forest Products Laboratory, Madison, USA), *Gloeophyllum trabeum*, obtained by courtesy of Prof. K. E. Eriksson (STFI, Sweden), and *Lentinus lepideus* CTB 67-03-A, donated by Dr. C. Delatour (CNRF, France).

Mycelial inocula were prepared as described elsewhere.[13]

Selection of optimal culture parameters for brown-rot decay

The effects of the following parameters on wood decay have been tested: removal of extractive substances (extracted in a Soxhlet apparatus with ethanol/toluene 1/1), dry matter content (in grams of dry wood/100 g of humidified wood) (15, 20, 25, and 30%), oxygen partial pressure (5, 21, 50, and 100% O_2), and buffer concentration (20 and 50 mM sodium L-tartrate, pH 4.5). The strain *Gloeophyllum trabeum* was employed in these experiments. Cultures were grown for 4 weeks under the general conditions described below, except that flasks were periodically flushed with humidified air.

Cultivation methods

Culture experiments were conducted with *Pinus radiata* sawdust (5.4% H_2O content) under the optimal culture conditions determined above: 6.0 g of pine sawdust (particle size <1 mm) were added to a 500-ml conical flask and autoclaved with 12 ml of distilled water. Twenty replicate flasks per fungal strain were then inoculated with 12 ml of a filter-sterilized double-strength mineral medium,[14] containing 0.1% glucose and 0.02% yeast extract, as well as the ground mycelium.[13] The flasks were stoppered with rubber steppers and incubated at the optimal growth temperature of each fungal strain (26°C for *Lentinus lepideus* and 28–30°C for the others). The flasks were flushed every 2–3 days with a humidified mixture of nitrogen and oxygen in a 1/1 ratio.

After 0, 1, 2, 4, 6, 8, and 10 weeks of cultivation, three cultures per fungus were harvested, the pH of the fermented samples was measured directly, and weight losses were determined after drying the residues at 60°C for 72 h.

Extraction of water-soluble substances

Sawdust samples (3.0 g) were extracted at 80°C with 3 × 40 ml of distilled water, dried at 60°C for 72 h, and weighed. Reducing sugars were determined as described elsewhere.[16]

Chemical analyses

Cellulose, hemicelluloses, and lignin were determined on extracted samples, essentially as described by Effland[17] and Monties.[18]

Alkali solubility of decayed samples was determined after incubating 1.0 g of unextracted wood with 25 ml of 2 M NaOH (30°C, 4 h under nitrogen). The residue was thoroughly washed with distilled water, dried at 60°C for 72 h, and weighed. Cell wall polysaccharides were determined in the residue, as above. The molecular weight distribution of alkali-soluble polysaccharides and lignin was determined by applying 1 ml of the alkali-soluble substances extracted from decayed samples to a column (110 × 2.0 cm) of Fraktogel TSK HW-55/F (Merck), equilibrated in 0.1 M NaOH. Polysaccharides were eluted from the column with the alkali eluent according to their molecular weight. Two-milliliter fractions were collected and total sugars were determined in each tube using the oricinol-sulfuric acid method.[19] Lignin was followed at 280 nm. The column was calibrated using pullulans (Shodex, Japan) with known molecular weights.

Alkali-soluble galactoglucomannan of sound radiata pine was isolated according to Timell.[20]

Digestibility studies

Replicate samples of fungus-treated wood (2.0 g) were weighed into nylon bags of 50-μm pore size (Silk Bolting Cloth MFG Co. Ltd. Switzerland) and suspended in the rumen of three sheep (6 bags/sheep) fitted with a rumen-cannula, and maintained on alfalfa hay. One replicate of sound wood was incubated simultaneously with each series to allow comparisons between the three sheep and between the different series. After 48 h incubation, the bags were removed from the rumen, extensively rinsed under cold water, pressed, dried, and weighed.

Results

Influence of culture parameters on brown-rot decay of pine

The effects of different culture parameters on solid-state fermentation of pine sawdust by *G. trabeum* are illustrated in *Figure 1*. Particle size was found to have a pronounced effect on pine decay. Maximum degradation was observed for pine with a 1-mm particle size, with substantial decrease in wood decomposition for higher or lower sizes.

The dry matter content of fermented wood appeared to have little influence on decay between 15 and 25% dry matter, but higher contents were inhibitory.

The effect of oxygen concentration on pine decomposition was also examined. Results showed that O_2 substantially stimulates pine decay: almost 60% increase in weight loss was reached when the gaseous concentration of oxygen in the cultures increased from 5 to 21%. The highest decomposition was observed when oxygen partial pressure was 50%.

Ethanol/toluene (1/1) extractable pine components were found to have a significant effect on decay.

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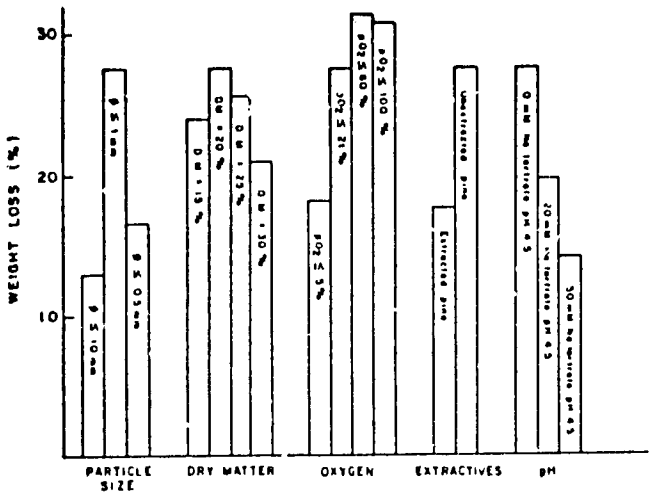

Figure 1 Effect of some cultural parameters on degradation of pine sawdust by the brown-rot fungus *Gloeophyllum trabeum* under semisolid conditions. Each 500-ml conical flask contained 6 g of radiata pine sawdust moistened with a mineral medium (20% final D.M.). Incubation period: 4 weeks at 30°C. Data represent the mean value for 3 replicates. All coefficients of variation were lower than 5%

Extracted wood showed substantially less decomposition, compared to sound pine sawdust.

Finally, the effect of pH on pine decay was assessed. Because the optimum pH for growth was 4.5, and substantial inhibition was observed for values below pH 3.5 (*Table 1*), we tested the effect of sodium tartrate buffer, pH 4.5, on pine decay. Our results (*Figure 1*) confirm those obtained *in vitro* by Koenig,[8] and Schmidt *et al.*[21] in that acidic pH is critical for polysaccharide degradation of pine sawdust by brown-rot fungi.

The culture conditions allowing the highest decay capacity of pine by *G. trabeum* were used in all subsequent experiments. These conditions are described in Materials and Methods under Cultivation Methods.

In situ *dry matter digestibility of fermented wood*

The pattern of development of *in situ* dry matter digestibility of pine sawdust decayed by *G. trabeum*, *W. cocos*, and *L. lepideus* is illustrated in *Figure 2*. All three fungi produced a very rapid increase in dry matter digestibility during the first 2 weeks of incubation from 0 to 17–23%. These results were obtained before weight losses higher than 15% were achieved (*Table 2*). Thereafter, substantial decreases in digestibility occurred, particularly in the cases of *W. cocos* and *L. lepideus*, followed by slight increases.

Polysaccharides present in sound pine sawdust appeared to be inaccessible to rumen microorganisms, as shown by the negative values of dry matter digestibility obtained after 48 h incubation of this substrate in the rumen. The higher weight obtained after rumen incubation of the undecayed wood presumably reflects incomplete removal of adhering rumen microorganisms during the washing step.

Table 1 Effect of medium pH on growth of *Gloeophyllum trabeum*[a]

pH		Growth[b]
Initial	Final	(mg of dry mycelium)
3.0	2.87	38.6 ± 0.7
3.5	3.30	48.4 ± 1.5
4.0	3.86	55.4 ± 2.0
4.5	4.31	62.3 ± 5.6
5.0	4.65	53.1 ± 3.3

[a] Seven day static liquid cultures in 250-ml conical flasks containing 30 ml of mineral salts medium with 2% glucose, 2.6 mM ammonium tartrate, and 50 mM sodium tartrate buffer
[b] Mean ± s.d. of 2 liquid cultures

Water-soluble substances

All the fungi caused a rapid increase in the water-soluble substances from 5% to nearly 10% of total dry matter during the first days of incubation (*Figure 3*). After this time, changes in water-soluble substances were considerably less, although *G. trabeum* solubilized almost 18% of decayed pine after 8 weeks of fermentation.

Reducing sugars increased constantly during the first stages of decay, reaching a maximum of 12% of total water-soluble substances after 4 weeks of cultivation. After 4 weeks, reducing sugars decreased to less than 3%.

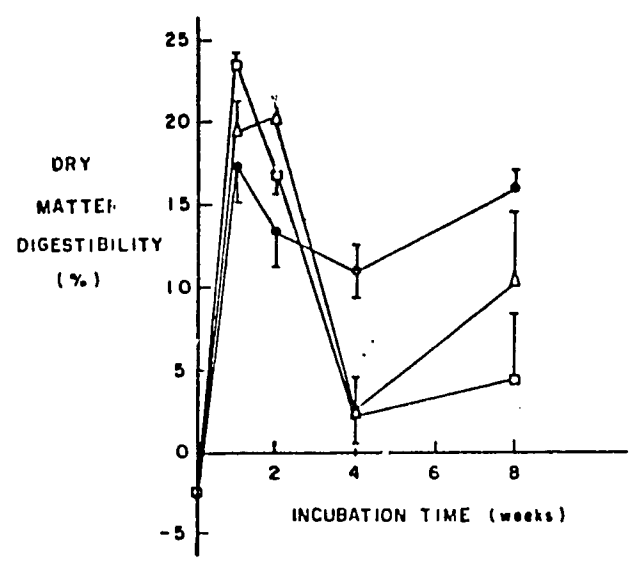

Figure 2 Changes of *in situ* dry matter digestibility by rumen microflora during solid-state fermentation of pine sawdust by the brown-rot fungi *Gloeophyllum trabeum* (●), *Lentinus lepideus* (□), and *Wolfiporia cocos* (△). Each point is the mean ± s.d. of two replicates

Table 2 Evolution of pH, total weight, and structural components during solid-state fermentation of pine sawdust by three selected brown-rot fungi

Strain	Incubation Time (weeks)	pH	Loss[a,c] in Weight	Cellulose	Hemicellulose	Lignin
Gloeophyllum trabeum	0[b]	5.0	0	0 (45.4)	0 (28.7)	0 (25.7)
	1	3.6	11.9	10.8	19.2	3.3
	2	3.5	15.2	11.7	27.9	4.3
	4	3.4	23.3	20.5	40.4	6.6
	8	3.3	39.4	42.8	58.9	8.7
	10	3.3	47.1	55.6	64.3	12.2
Wolfiporia cocos	1	2.9	9.2	9.0	13.3	4.1
	2	3.6	11.8	9.2	21.3	5.0
	4	3.3	13.6	11.1	22.0	6.2
	8	3.2	17.8	14.9	34.7	3.8
	10	3.2	21.6	15.6	41.8	6.6
Lentinus lepideus	1	4.0	9.7	6.2	17.0	5.0
	2	3.9	12.8	12.6	19.9	6.3
	4	3.6	13.6	12.8	21.6	7.0
	8	3.5	21.9	19.7	34.3	9.2

[a] The average coefficient of variation was lower than 6%
[b] Values in parentheses indicate the content of each structural component in sound wood
[c] Results are expressed in % of original component

Evolution of pH, dry matter, and structural components

Table 2 gives the temporal changes in pH, dry matter, and structural components during pine decay by the three selected brown-rot fungi. *G. trabeum* possess the highest decay capability, reaching almost 50% weight loss after 10 weeks of cultivation. *W. cocos* and *L. lepideus* showed a very similar pattern of decay, degrading only 22% of dry matter after 10 weeks of incubation.

After the first week of incubation, the cultures became increasingly acidic, especially for pine fermented with *W. cocos*. As the incubation progressed, the pH slowly dropped to pH 3.3–3.2.

The highest decay rate (between 0.9% for *W. cocos* and 1.1% per day for *G. trabeum*) was observed for the three strains during the first 2 weeks of decay. Upon further incubation, the decay rate decreased, especially between 2 and 4 weeks of cultivation, after which a new increase in the degradation rate occurred.

Hemicelluloses were degraded in preference to cellulose by the three strains tested. Thus, after a 21–23% loss in total dry matter, twice as much of the hemicelluloses as cellulose had been removed by *G. trabeum* and even more in the case of *W. cocos*. Similar results have been published by Kirk and Highley,[4] although these authors found that loss of cellulose was always greater than total weight loss. This was not the case with radiata pine, at least until 30% dry matter degradation was achieved.

Lignin, as shown also by others,[4,6] was poorly degraded by the brown-rot fungi. The slight depletion determined could have resulted either from solubilization of lignin during extraction of water-soluble substances, or from an increase in the acid-soluble lignin fraction during decay.[22]

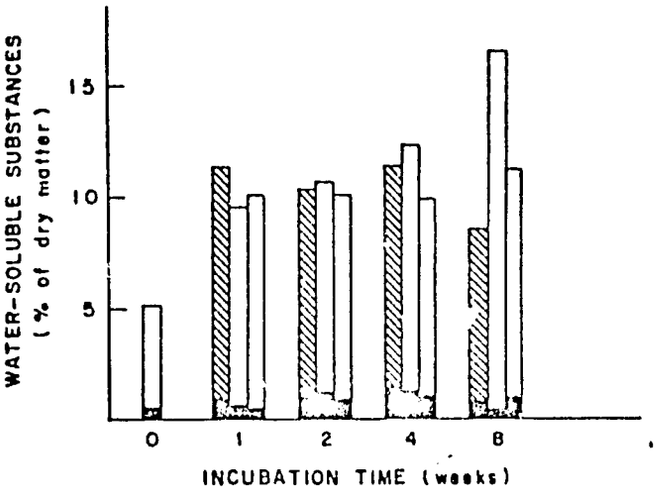

Figure 3 Amount of water-soluble substances and reducing sugars (▨) formed during solid-state fermentation of pine sawdust by the brown-rot fungi *Gloeophyllum trabeum* (□), *Lentinus lepideus* (▨), and *Wolfiporia cocos* (▭)

Alkali-soluble substances

Alkali-soluble substances substantially increased in cultures during decay, reaching almost 60% of the dry matter (decayed weight basis) after 40% weight loss (*Table 3*). Although similar amounts (10%) of each structural component of sound pine sawdust were released by sodium hydroxide, the rate and extent of solubilization of these components during the decay process were rather different depending upon the

Table 3 Evolution of 2 M, sodium hydroxide-soluble structural components during decay of pine sawdust by two brown-rot fungi[1]

Strain	Weight loss (%)	Alkali-soluble: Total substances	Cellulose	Hemicellulose	Lignin
Gloeophyllum trabeum	0	9.8	8.6	9.8	11.6
	11.9	26.5	26.2	21.8	32.7
	15.2	29.9	28.2	34.7	27.8
	23.3	35.0	38.5	38.2	28.0
	39.4	58.1	61.1	67.5	46.0
Wolfiporia cocos	9.2	15.5	11.5	18.6	19.5
	11.8	20.6	14.5	31.2	20.5
	13.4	21.3	23.9	20.4	17.7
	17.8	36.1	38.9	43.1	26.0

[1] Results are expressed as % of each component in decayed wood

fungus. Thus, after 12% weight loss, 22% of the remaining hemicelluloses in pine decayed by *G. trabeum* were soluble in 2 M sodium hydroxide, whereas more than 31% of these carbohydrates were solubilized in pine fermented by *W. cocos*.

The changes in the molecular weight of alkali-soluble polysaccharides released during brown-rot decay are illustrated in *Figure 4*. *G. trabeum* strongly depolymerized structural polysaccharides, as compared to sound pine hemicelluloses, during the first days of incubation. The average molecular weight of alkali-soluble polysaccharides decreased from 25,000 (D.P. of 150) after 1 week of incubation with *G. trabeum* to about 5,000 (D.P. of 30) after 8 weeks of incubation. In the case of *W. cocos*, significant depolymerization occurred only after 4 weeks of decay.

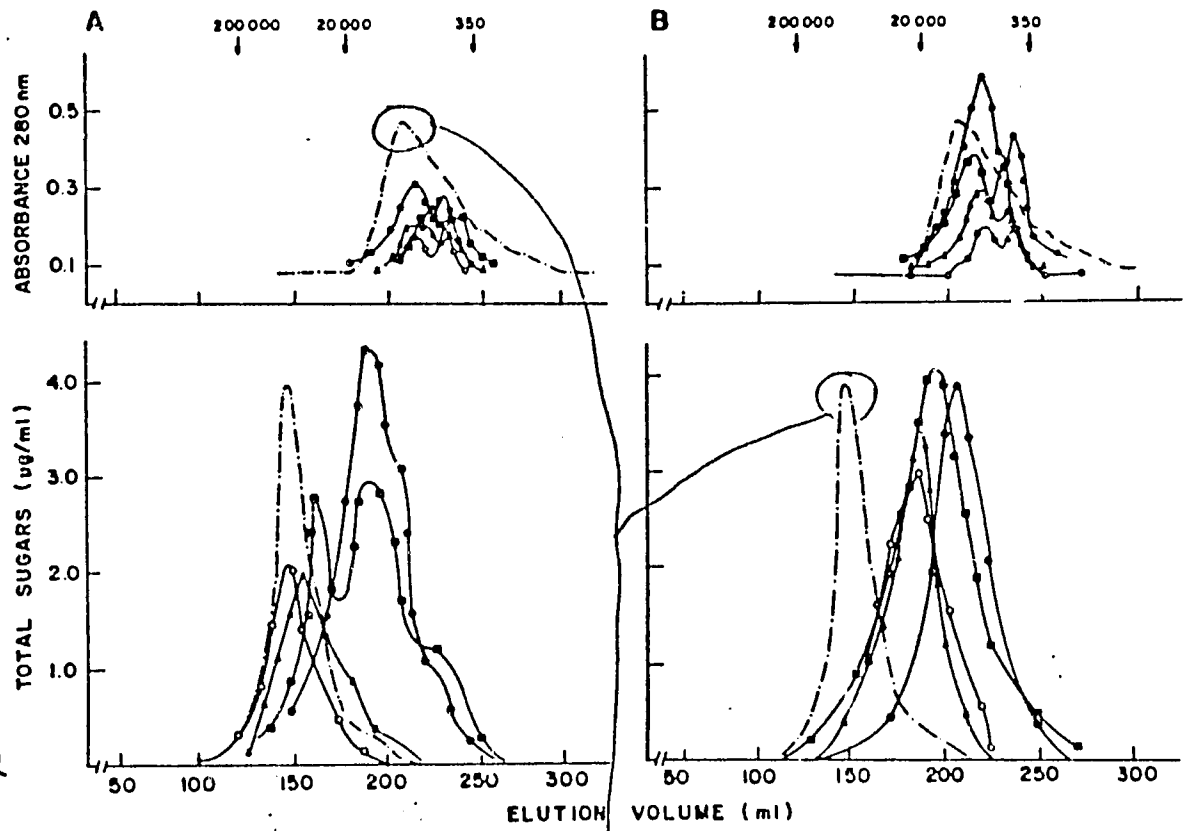

Figure 4 Molecular weight distribution of alkali-soluble polysaccharides and lignin released after 1 (○), 2 (△), 4 (⊠), and 8 (●) weeks incubation of pine sawdust with the brown-rot fungi *Wolfiporia cocos* (A) and *Gloeophyllum trabeum* (B). Fractions were eluted from a 56 × 2 cm column packed with Fraktogel TSK HW 55/F (Merck) using 0.1 M NaOH as eluent. Lignin was monitored as the absorbance at 280 nm and polysaccharides were followed by the orcinol method. Standards: pullulans of known M.W., pine galactoglucomannan (—●—●), and milled pine wood lignin (—○—)

A significant release of alkali-soluble lignin was observed after the first week of incubation, especially for *G. trabeum* (*Table 3*). Soluble lignin increased slightly during the next 3 weeks of decay, after which another substantial increase was found. These soluble fractions are polymeric and show two main elution peaks (*Figure 4*). Upon incubation, a relative increase in the peak for high molecular weight was seen (*Figure 4*).

Discussion

Results obtained in this work indicate that acidic pH and high oxygen concentration are important culture parameters for brown-rot decay of radiata pine under semisolid conditions. Acidic conditions (due to secretion of oxalic acid) developed by brown-rot fungi during wood decay have been related with Fe(III) reduction to Fe(II). The Fe(II) has been suggested to react with H_2O_2 generating hydroxyl radicals, which depolymerize the cellulose.[23] This hypothesis, however, has not been adequately explored. Indeed, H_2O_2 could not be detected in liquid cultures of several brown-rot fungi.[24] Furthermore, several organic acids, and not exclusively oxalate, were detected in cultures of *G. trabeum* and *W. cocos* (unpublished results). In any event, low pH, which could allow the expression of specific depolymerizing enzymes or potentiate oxygen-reduced species, like superoxide, seem to be indispensable for wood degradation by these fungi. More studies are required to better understand the role of secreted organic acids in brown-rot decay of wood.

The rapid depolymerization of cellulose, reported as the initial step of polysaccharide degradation by brown-rot fungi, has been shown to be oxidative.[8,9,21] Furthermore, lignin modifications resulting after brown-rot are also largely oxidative.[22,25] These facts suggest that oxygen concentration might be an important factor for brown-rot decay of wood. The results obtained here confirmed that oxygen concentration is critical for brown-rot decay of pine and are in accord with oxidative reactions being involved in wood decay by brown-rot fungi. Interestingly, the role of cellulose oxidizing enzymes in fungal degradation of this structural polysaccharide was emphasized some years ago by Eriksson and coworkers.[26] These authors suggested that insertion of uronic acid moieties in the cellulose chains leads to cellulose swelling, which could increase the accessibility of hydrolytic enzymes to the crystalline parts. Finally, inhibition of pine decomposition by low oxygen levels, rather than stimulation by high oxygen concentrations, should also be considered to explain our results.

The effect on rumen or polysaccharidase digestibility of wood decayed by brown-rot fungi has apparently previously not been reported. The significant increase in rumen biodegradability caused by these fungi in the first stages of decay is probably related to the rapid early depolymerization of structural carbohydrates. Indeed, as shown by Puri,[27] depolymerization should open up the cell walls, increasing the specific surface area of wood, thus allowing rumen cellulolytic bacteria and their enzymes to gain access to the structural polysaccharides.

The substantial increase in alkali-solubility of both polysaccharides and lignin on the decay of pine is also probably related to this improvement of digestibility. Indeed, the presence of ionizable groups in cellulose and hemicelluloses could increase their susceptibility to polysaccharide hydrolases, as discussed above. Furthermore, Agosin and Odier[13] recently showed the existence of a close relationship between polymeric water-soluble lignins, from white-rotted straw, and improvement of dry matter digestibility. This solubilization probably arose from oxidation of the lignin component, which resulted in the generation of hydrophilic groups such as carboxyl groups.[28] Similar reactions were found in brown-rotted lignins[22,23] and included extensive lignin demethylation[22] and ring hydroxylation,[29] apparently without depolymerization.

All the results presented here suggest that brown-rot fungi, or their polysaccharide degrading system, have potential as saccharifying agents for lignified tissues. Research on the enzymes involved in this process is now under progress.

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