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PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

DEPARTAMENTO INGENIERIA QUIMICA

FINAL REPORT OF ACTIVITIES

"SACCHARIFICATION OF LIGNOCELLULOSIC MATERIALS BY BROWN ROT FUNGI: MECHANISMS AND POTENTIAL APPLICATIONS"

GRANT NUMBER: DPE-5542-G-55-8076-00

PRINCIPAL INVESTIGATOR: EDUARDO AGOSIN T.

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Executive Summary: Brown-rot fungi destroy wood by selective removal of cellulose and hemicelluloses, without significant depletion of the lignin component. These fungi have received little evaluation for biotechnological purposes, despite their ability to circumvent the lignin barrier in gaining access to polysaccharides. Moreover, its mechanism of wood decay, leaves a highly functionalized lignin. This final report describes the research carried out on the bioconversion of lignocellulosic materials to fermentable sugars, using brown-rot fungi. Specifically, we proposed to study the basic mechanism involved in the depolymerization of the structural polysaccharides of wood. The aim of this project pointed to the optimal utilization of lignocellulosic residues (*Pinus radiata* sawdust) due its high production generated from man made forest exploitation in Chile. As well as in Chile, in Guatemala, high amounts of lignocellulosic materials are produced every year (principally cane bagasse). Hence, joint research efforts were conducted by chilean an guatemalean research teams with the support of PSTC Program, in order to contribute solving wastes disposal by development of bioprocesses that would result in the alternative production of valuable products.

The research was carried out in Chile at the Laboratory of Biotechnology under the direction of the main investigator Dr. Eduardo Agosin at the Institute of Nutrition and Food Technology (INTA), of the Universidad de Chile. In 1990, the main investigator moved to the Department of Chemical Engineering at the P. Universidad Católica de Chile, so the project was prosecuted at the new Laboratory of Industrial Microbiology under the direction of Dr. E. Agosin. Research emphasis was directed towards elucidating cellulose depolymerization underlying brown-rot decay of softwoods, particularly *Pinus radiata* Specifically, investigations like the role of oxalic acid in cellulose depolymerization, the characterization and regulation of endoglucanases secreted by the fungus *Gloeophyllum trabeum*, the role of transition metals and the pattern of cellulose depolymerization by these fungi were conducted.

At the same time, the collaborator Marcela Olivares, a chemical engineer from the Faculty of Engineering of the P. Universidad Católica de Chile, studied the extraction and utilization of the lignins remaining after brown-rot decay as part of crosslinking agents for the manufacture of particle-board panels. Also, part of the program was conducted in Guatemala by M. Sc. Roberto de León, Director of the Fermentation Laboratory at the Central American Research Institute for Industry (ICAITI). This group evaluated the biological pretreatment of tropical lignocellulosic residues to foster microbial degradation and / or animal nutrition.

Results obtained under this grant resulted in the publication of six scientific papers, four already published and two accepted for publication. Several communications were done at International and National Congresses, too. These activities assure the divulgation of the research on an international level with ample credit being given to the generous support by AID's Program. Also the project provided an opportunity for training young scientists as well as the possibility to visit and work at important centers of investigation in the United States (Forest Products Lab., Madison, Dept. of Plant Pathology, U. of Minnesota). Part of the work done under this grant, was conducted in collaboration with Dr. T. Kent Kirk. Three M.Sc. theses were done under the direction of the main investigator and two Ph.D. theses are currently in progress.

Research Objectives: The mechanism of wood decay by brown-rot fungi, is characterized by a very rapid cellulose depolymerization during the early stages of plant tissue invasion. This happens before any significant loss in the weight of wood occurs (**Agosin et al, 1989**). This makes them potential candidates for the direct bioconversion of wood polysaccharides into fermentable sugars. From a biotechnological point of view, the potential to release low cost fermentable carbon sources by fungal pretreatment of wood, give to this project an unexpected potentiality. Moreover, the development of basic research generated new and important information to

understand the chemistry and the biochemistry involved in cellulose depolymerization by brown-rot fungi.

The current working hypothesis on the nature of the depolymerizing system, is that oxygen-derived radical species are responsible for the initial attack of the cellulose polymer. Indeed, Halliwell (1965), and Kirk et al. (1991) have shown that Fenton's reaction [a mixture of Fe(II) and H_2O_2, which is known to generate $^{\bullet}OH$ radicals] causes a degradation of cellulose similar to that resulting from brown-rot decay (**Rojas, 1989**). Koenigs (1974) demonstrated that H_2O_2 is secreted by these fungi; the latter could react with the iron present in wood, making this hypothesis feasible. Oxalic acid is produced by brown-rot fungi in significant amounts in liquid cultures (Takao, 1965), as well as in semi-solid cultures (**Espejo and Agosin, 1991**). Schmidt et al. (1981), demonstrated that Fe(III) rendered Fe(II) (one of the Fenton's reactive) after reduction by oxalic acid. On this view, we found that oxalic acid is secreted by brown-rot fungi and further degraded to CO_2 in semi-solid cultures *in vivo*. Concomitantly, cellulose is strongly depolymerized. Extracellular extracts from these cultures were capable to degrade oxalic acid to CO_2, too. We are currently pursuing the identification of the responsible agent, possibly a novel extracellular oxalate oxydase.

Classical fungal cellulolytic systems, thoroughly investigated in the soft-rot fungus *Trichoderma reesei* and in the white rot fungus *Phanerochaete chrysosporium* (Coughlan et al. 1988), contain three different types of enzymes: cellobiohydrolases (EC 3.2.1.91); endo-1,4-ß-glucanases (EC 3.2.1.4) and 1,4-ß-glucosidase (EC3.2.1.21). Unlike these fungi, most brown-rot fungi do not possess cellobiohydrolase activity (Highley, 1988). Even though endoglucanases activity by brown-rot fungi has been reported in liquid media (Highley, 1973), they have been not thoroughly studied. There is only one report on the partial purification and characterization of an 1,4-ß-endoglucanase produced by the brown-rot fungus *Lenzites trabea* (syn. *Gloeophyllum trabeum*) (Herr et al. 1978). There are striking differences in the regulation of endocellulase production, too. We found that

Gloeophyllum trabeum produced endoglucanases in the presence of monosaccharides such as glucose or mannose as the only carbon source, but the production was higher in the presence of cellulose or cellobiose. We concluded that this kind of enzymes are not subject to catabolite repression in these fungi, but are inducible by cellulose. Furthermore, cellobiose was found to be the best inducer studied so far (**Cotoras and Agosin, 1992a; Cotoras and Agosin, 1992b**). Currently, the purification, characterization and cloning of these enzymes is in progress. Milena Cotoras, a Ph.D. graduate student is doing her thesis in this field.

Brown-rot fungi differ in their mechanism of cellulose depolymerization from white-rot fungi principally in two aspects: in the kind of cellulases produced, as mentioned above, and in the pattern of cellulose degradation. Brown-rot fungi, produce a rapid depolymerization, before a significant weight loss of wood occurs. The kinetics of cellulose depolymerization was studied, comparing modifications of cotton cellulose by a white-rot fungus (*Phanerochaete chrysosporium*) and a brown-rot fungus (*Postia placenta*) under semi-solid culture conditions. The molecular size distribution during the changes of the degree of polymerization (DP) of cellulose during brown-rot attack, showed one major peak centered over a progressively lower DP than the starting substrate. On the other hand, *P. chrysosporium*, showed a continuous consumption of cellulose with a substantially different molecular size distribution pattern. This is the first report, that clearly shows the differences between brown- and white-rot decay of cellulose. From these results we conclude that the brown-rot fungus *P. placenta* cleaved completely through the cellulose microfibrils, whereas the white-rot fungus *P. chrysosporium* attacked the surfaces of the microfibrils (Agosin, E., Report 1988; **Kleman-Leyer et al, 1991**).

Wood decay by brown-rot fungi, results in remotion of polysaccharides without significant depletion of lignin (Kirk and Highley, 1973). However, during wood attack this aromatic polymer suffers several chemical transformations (demethylations, hydroxylations, etc.) (Kirk

et al, 1975), leading to the accumulation of an oxydized polymeric lignin. Experiments with lignin model compounds catabolized by two brown-rot fungi (*G. trabeum* and *W. cocos*), showed the ability of this strains to modified these compounds. Moreover, some of these modifications were similar to that obtained through the generation of oxygen derived radicals generated by water radiolysis (**Espejo, 1989**; **Espejo et al, 1990**). The generation of a functionalized lignin by these fungi, makes it attractive for its utilization as part of modified phenolic resins in plywood adhesives, for example. This study is pertinent assuming that this aromatic polymer is currently subutilized and, according to porfessor H. Bungay, "lignin is the product of most value in biomass refining" (Bungay, 1991). The study of the mechanism of lignin modification by fungal treatment could lend to the obtention of more valuable products from residual wastes. Sound lignin extracted from washing liquors of steam-exploded wood chips, was applied to holocellulose semi-solid cultures inoculated with *G. trabeum*. After 45 days of incubation, the recovered lignin, showed 50% demethylation, witohut significant molecular weight modifications. Functionalized lignin was employed for the formulation of modified phenolic resins (see **Hojas, 1991**).

Finally, in cooperation with the Industrial Fermentation Laboratory at ICAITI, Guatemala, the main agroindustrial wastes in Guatemala, were used as substrates in semi-solid cultures inoculated with *G. trabeum* or *W. cocos* Results from this study, indicated a good correlation between aireation and degradation of cellulose (see Final Report from ICAITI).

References in **bold** correspond to publications generated under this project.

Methods and Results: Methods and results of this project are extensively described in attached papers and reports.

Impact, Relevance and Technology Transfer: Our first goal was to evaluate rumen digestibility *in sacco* of pine sawdust. A very important increase in digestibility of wood was found after only one

week fermentation with several brown-rot fungi, lending support to further studies on the potential of brown-rotted wood as an energetic substitute in animal feeding. New aspects of cellulose depolymerization of wood by brown-rot fungi have been discovered under this grant. Indeed, as mentioned before, the differences between brown-rot and white-rot cellulose depolymerization were for the first time demonstrated and reported, clearly showing the unique pattern of cellulose decay by brown-rot fungi. Also, we had obtained promisory results in the substitution of formaldehyde with functionalized lignin by brown-rot fungi, in order to utilize it as part of crosslinking resins in the manufacture of particle-board panels, a good example of the application of basic discoveries. The prosecution of this study is under progress in order to perform large-scale processes. Correct transfer of knowledge and the know-how of the utilization of brown-rot fungi for the degradation and utilization of tropical raw lignified materials under semi-solid culture conditions, was performed with Guatemalean investigators.

Very good expertise was received by many worldwide reknown researchers, such as Dr. T.K. Kirk (USA) or Professor B. Halliwell (UK) . Furthermore, we were able to send young scientists to train in important laboratories from the USA, as well as to transfer our knowledge to scientists from Guatemala at the ICAITI and from Uruguay. Moreover, the moving from the P.I. to the Faculty of Engineering at the P. Universidad Católica de Chile, one of the major Universities of Chile, potentiate the utilization of our findings in larger scale programmes.

Project Activities/Outputs:

Publications:

Scientific Journals

1. Eduardo Agosin, Sergio Jarpa, Eduardo Rojas and Eduardo Espejo. 1989. Solid-state fermentation of pine sawdust by selected bronw-rot fungi. *Enzyme and Microbial Technology*, **11**: 511-517.

2. Eduardo Espejo, Eduardo Agosin and Rafael Vicuña. 1990. Catabolism of 1,2- diarylethane lignin model compounds by two brown-rot fungi. *Archives of Microbiology*, **154**: 370-374.

3. Eduardo Espejo and Eduardo Agosin. 1991. Production and degradation of oxalic acid by brown-rot fungi. *Applied and Environmental Microbiology*, **57**(7): 1980-1986.

4. Karen Kleman-Leyer, Eduardo Agosin, Anthony H. Conner and T. Kent Kirk. 1992. Changes in molecular size distribution of cellulose during attack by white-rot and brown-rot fungi. *Applied and Environmental Microbiology*, **58**(4): 1266-1270.

5. Milena Cotoras and Eduardo Agosin. 1992. Regulatory aspects of endoglucanase production by the brown-rot fungus *Gioeophyllum trabeum*. *Experimental Mycology*, in press.

Books

1. Milena Cotoras and Eduardo Agosin. 1992. Recent Advances in Wood Decay by Brown-Rot Fungi. *Biotechnology in Pulp and Paper Industry*. Kuwahara and Shimada Ed., Uni Publishers Corp, Japan, in press.

Theses

1. Eduardo Rojas. 1989. Sistema degradativo de residuos lignocelulósicos por hongos de pudrición parda. Universidad de Concepción, Concepción, CHILE.

2. Eduardo Espejo. 1989. Degradación de compuestos modelo tipo lignina por hongos de pudrición parda. Universidad de Concepción, Concepción, CHILE.

3. María S. Hojas. 1991. Bioconversión de eucaliptus explotado-uso en resinas modificadas. P. Universidad Católica de Chile, Santiago, CHILE.

Presentation to Congresses:

International

1. Saccharification of pine sawdust by brown-rot fungi. Poster presented at the IVth International Conference on Biotechnology in the Pulp and Paper Industry, Raleigh, North Carolina, USA, May, 1989.

2. AID workshop on Biomass Utilization, Faisalabad, Pakistan, December, 1989.

3. Wood Decay by Brown-Rot Fungi: Mechanism and Potential for Saccharification of Lignified Biomass. Conference presented at the International Symposium on Biotechnology for Energy, organized by NIBGE & NIAB, Faisalabad, Pakistan, December, 1989.

4. Mecanismos de degradación de la madera por hongos de pudrición parda. Conference held at the II Latin -American Congress of Biotechnology at La Habana, Cuba, July, 1990.

5. Recent advances in cellulose degradation by brown-rot fungi. Conference presented at the 5th International Conference in Biotechnology in the Pulp and Paper Industry in Kyoto, Japan, May, 1992.

National

1. Estudio del mecanismo degradativo de lignocelulosas por hongos de pudrición parda. Poster presented at XXXI Meeting of the Chilean Society of Biology, La Serena, Chile, November, 1988.

2. Sacarificación de aserrín de pino por hongos de pudrición parda. A conference given at the 1st Congress of Biotechnology, Talca, Chile, January, 1989.

3. Degradación biológica de materiales lignocelulósicos. A lecture presented the Conferences on Biotechnology, Valdivia, Chile, October 1989.

4. Catabolismo de compuestos modelo de lignina tipo 1,2-diariletano. A lecture presented by E. Espejo for his incorporation to the Chilean Society of Microbiology was presented at the XIII Chilean Society of Microbiology Congress, Viña del Mar, Chile, April, 1990.

5. Degradación de celulosa por hongos de pudrición parda. A lecture presented by E. Agosin for his incorporation to the Chilean Society of Microbiology was presented at the XIII Chilean Society of Microbiology Congress, Viña del Mar, Chile, April, 1990.

6. Expresión de celulasas por hongos de pudrición parda. Poster presented at the XIV Chilean Society of Microbiology Congress, Santiago, Chile, April, 1991.

7. Depolimerización de celulosa por hongos de pudrición parda: rol del ácido oxálico. A lecture held at XIV Chilean Society of Microbiology Congress, Santiago, Chile, April, 1991.

Training : Eduardo Agosin spent four months at IMBT at the Forest Products Laboratory, Madison, Wisconsin, working with Dr. T.K. Kirk in the chatacterization of low molecular weight compounds secreted by brown-rot fungi (August- November, 1988).

Eduardo Rojas (1988), Eduardo Espejo (1989) and Marisol Hojas (1991) obtained their Master's degree with a thesis entitled: "Degradative System of Lignocellulose by Brown-Rot Fungi", "Degradation of Lignin-Related Model Compounds by Brown-Rot Fungi" and, "Bioconversion of

Steam-Exploded Eucaliptus sp.- Utilization of Modified Phenolic Resins", respectively.

Eduardo Espejo and Verónica Quiñones, both contracted under this grant, followed the International training course sponsored by UNDP, at the Biochemical Engineering Dept. of the Universidad Católica de Valparaíso, Valparaíso, Chile, on "Enzymatic Hidrolysis of Lignocellulosic Materials" (9-16/7/89).

Eduardo Agosin, followed an International training course by UNDP, at the Biochemical Engineering Dept. of the Universidad Católica de Valparaíso, Valparaíso, Chile, entiltled "Solid-State Fermentation" (3-6/10/89).

M. Sc. Roberto de León, Director of the Fermentation Laboratory at ICAITI, Guatemala, spent eight days in the P.I.'s Laboratory. He learned several techniques employed in the cultivation of brown-rot fungi and in the determination of cellulose depolimerization by these fungi (9-16/6/89).

M. Wilkens, M. Cotoras, V. Wilhelm and A. Muñoz, postgraduate students of the Ph. D. programme on Microbiology of the Universidad de Chile, followed a three to six month's research training at the P.I.'s laboratory in relation with this project.

Milena Cotoras, a post-graduate student is currently working on her Ph. D. thesis on the regulation of endoglucanases expression by the brown-rot fungus *Gloeophyllum trabeum* (March, 1990-...).

Eduardo Espejo spent four months at the Institute of Microbial and Biochemical Technology at Forest Products Laboratory, Madison, Wisconsin. During his stay he has learned several techniques and prosecuted his work in the identification of the responsible agent of oxalic acid degradation by brown-rot fungi, under the supervision of Dr. T. K. Kirk. Also, he spent a week at the Dept. of Plant Pathology at

the University of Minnesota, working with Prof. Robert Blanchette on TEM and SEM techniques (Nov.1990-Feb. 1991).

Milena Cotoras spent two months at the Institute of Microbial and Biochemical Technology at Forest Products Laboratory, Madison, Wisconsin. She worked under the supervision of Drs. Dan Cullen and T. K. Kirk on molecular biology techniques in the identification of DNA sequences and synthesis of DNA probes for endoglucanases produced by *G. trabeum* (Dic, 1991- Feb. 1992).

M. Sc. Mary Lopretti, Dept. of Biochemistry, PEDECIBA, Universidad de la República, Montevideo, Uruguay, is currently working on her Ph. D. thesis with the P.I., on the "Mechanisms of Lignin Demethylation by Brown-Rot Fungi" (1991-...).

Future Work: The development of a new research field and training of several investigators involved in the prosecution of this project, lent support to a promising area of basic and applied investigation, as well as, to the constitution of a multidisciplinary research group working in enzymology, molecular biology, semi-solid state fermentation, protein purification, down-stream processing, etc... under the direction of the P.I.. These features, would make feasible further investigations in the same area at the P.I. laboratory as well as in association with other national and international groups. As mentioned before, two Ph. D. theses are currently under way, one of it in cooperation with the Universidad de la República, Montevideo, Uruguay. Both theses are the prosecution of the work initiated during the accomplishment of this project. Particularly, three important aspects of the mechanism of wood decay by brown-rot fungi are currently under study: the expression of endoglucanases, the agent responsible for cellulose depolymerization and the mechanism involved in lignin demethylation. Moreover, a chilean grant in collaboration with the Department of Molecular and Cellular Biology of the P. Universidad Católica de Chile, concerning the characterization and purification of ß-mannanases produced by brown-rot fungi was

recently obtained for these purposes. All these activities should allow to progress in the elucidation of the strategies employed by these fungi during attack to the different wood components.

Litterature Cited:

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FINANCIAL STATEMENT

PROJECT: SACCHARIFICATION OF LIGNOCELULOSIC MATERIALS BY BROWN-ROT FUNGI: Mechanisms and Potential Applications.

Project Number: 936-5542

Grant Number: DPE-5542-G-SS-8076-00

A.I.D. Grant Project Officer: John Kadyszewski

Reception of USAID funds

Date	cash advances.
April/1989	$ 38,885.00
June/1989	$ 15,585.00
Dec./1989	$ 30,790.00
June/1990	$ 23,390.00
July/1991	$ 37,132.00
April/1992	$ 4,200.00
	$ 149,982.00

16A

Period Covered: 08/31/88 To 05/31/92

TOTAL FUNDS ADMINISTERED
EXPENDITURES PER YEAR STATEMENT

	Y1	Y2	Y3	TOTAL
Period	8/31/88 8/31/89	8/31/89 8/31/90	8/31/90 5/31/92	8/31/88 5/31/92
Total	43,679.00	52,676.00	53,618 16	149,973 16

ITEM	TOTAL BUDGET	EXPENDITURES
Salaries	44,600 00	49,598.35
Equipment	22,300.00	23,270 77
Travel	16,000.00	14,142.18
Supplies*	37,300.00	34,113 09
Literature	4,200.00	2,995.72
Overhead	25,582.00	26,062.21
Total	149,982.00	149,982.32

*US$ 4,200 extra funds obtained from USAID, were added to the US$ 33,100 original Supplies item.
Detailed supporting information if AID may require will be furnished on request.

The research was performed by the scientific staff of the Department of Chemical Engineering, Faculty of Engineering, Universidad Católica de Chile, and the Central American Research Institute for Industry (ICAITI) .

INSTITUTION: **P. UNIVERSIDAD CATOLICA DE CHILE** (Santiago, CHILE)

Principal Investigator:	Dr. Eduardo Agosin (*Project Leader*)
Collaborator:	Eng. Marcela Olivares
Co-Investigators:	M. Sc. Milena Cotoras
	M. Sc. Eduardo Espejo
Scientists:	Eduardo Rojas
	Marisol Hojas

INSTITUTION: **INSTITUTO CENTROAMERICANO DE INVESTIGACION Y TECNOLOGIA INDUSTRIAL** (Guatemala, GUATEMALA).

Collaborator: M. Sc. Roberto de León

PUBLICATIONS

PAPER 1

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

Eduardo Agosin, Sergio Jarpa, Eduardo Rojas and Eduardo Espejo

Laboratorio de Biotecnologia, Universidad de Chile, Santiago, Chile

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 of 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.*

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.

Address reprint requests to Dr. E. Agosin, Laboratorio de Biotecnología, Instituto de Nutrición y Tecnología de los Alimentos, INTA, Universidad de Chile, Casilla 15138, Santiago 11, Chile
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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.[13,14]

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,[15] containing 0.1% glucose and 0.02% yeast extract, as well as the ground mycelium.[13] The flasks were stoppered with rubber stoppers 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 orcinol-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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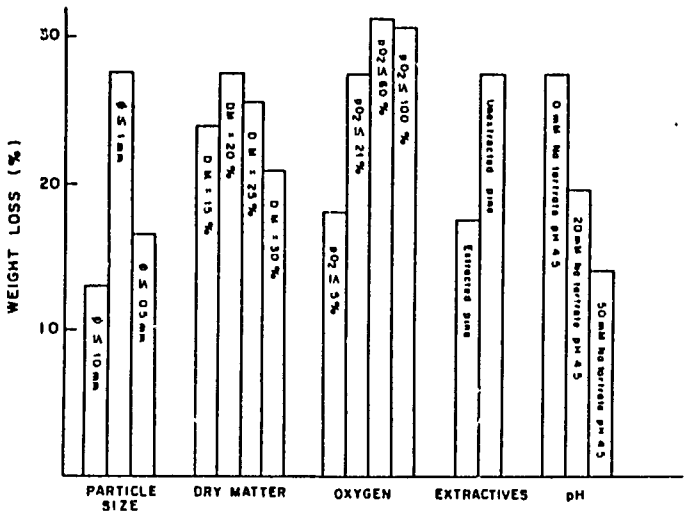

Figure 1 Effect of some cultural parameters on degradation of pine sawdust by the brown-rot fungus *Gloeophyllum trabeum* under semisolid conditions. Each 500-ml conical flask contained 6 g of radiata pine sawdust moistened with a mineral medium (20% final D.M.). Incubation period: 4 weeks at 30°C. Data 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 Koenigs[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%.

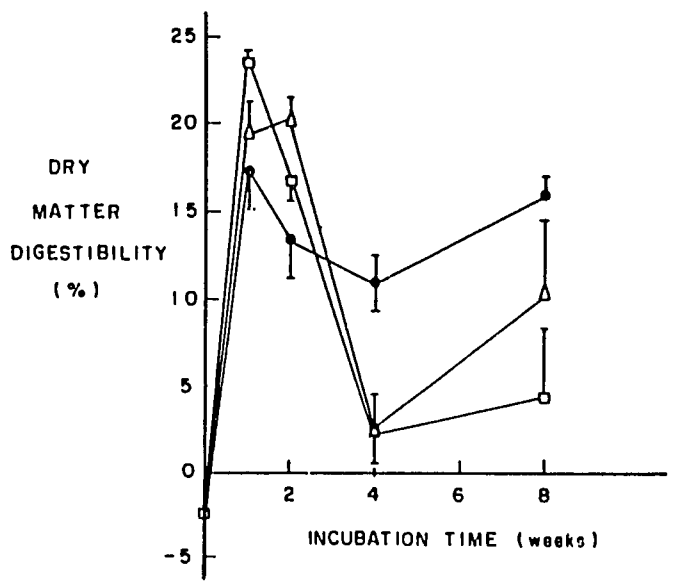

Figure 2 Changes of *in situ* dry matter digestibility by rumen microflora during solid-state fermentation of pine sawdust by the brown-rot fungi *Gloeophyllum trabeum* (●), *Lentinus lepideus* (□), and *Wolfiporia cocos* (△). Each point is the mean ± s.d. of two replicates

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	55.8	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]

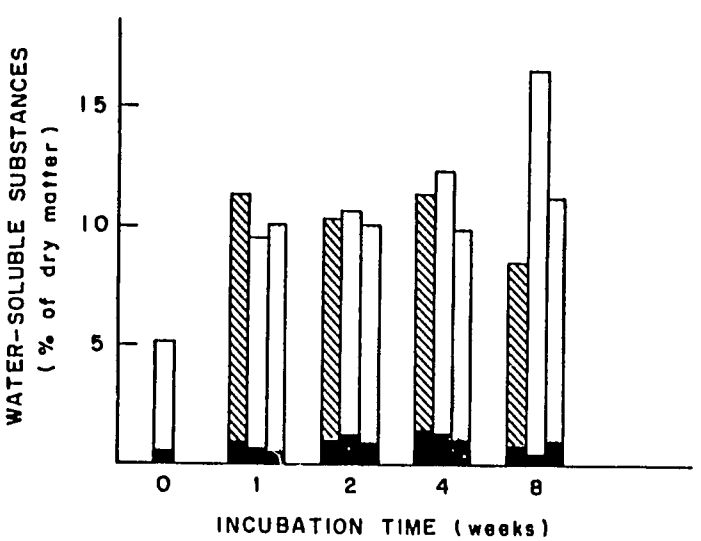

Figure 3 Amount of water-soluble substances and reducing sugars (■) formed during solid-state fermentation of pine sawdust by the brown-rot fungi *Gloeophyllum trabeum* (▨), *Lentinus lepideus* (□), and *Wolfiporia cocos* (▭)

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

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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	28.9	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 5000 (D.P. of 30) after 8 weeks of incubation. In the case of *W. cocos*, significant depolymerization occurred only after 4 weeks of decay.

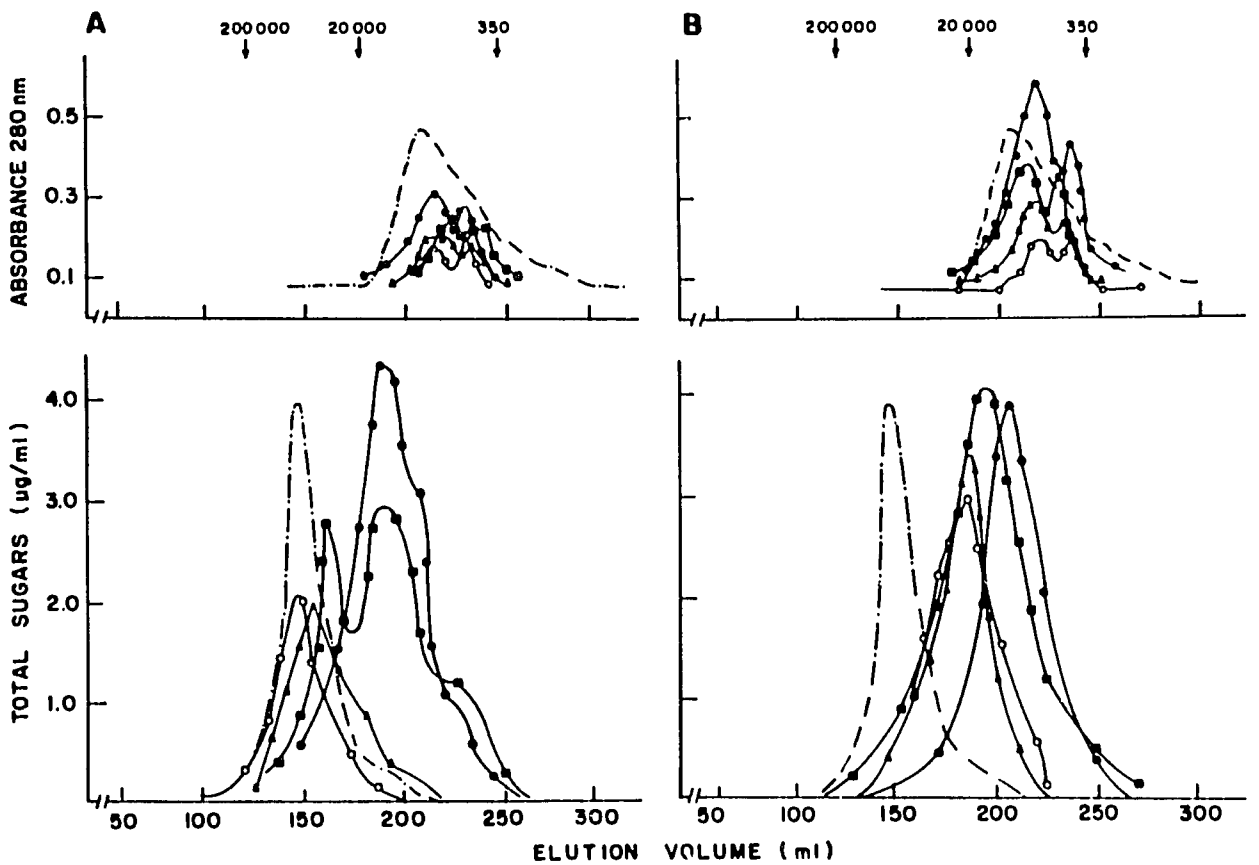

Figure 4 Molecular weight distribution of alkali-soluble polysaccharides and lignin released after 1 (○), 2 (△), 4 (■), and 8 (●) weeks incubation of pine sawdust with the brown-rot fungi *Wolfiporia cocos* (A) and *Gloeophyllum trabeum* (B). Fractions were eluted from a 56 × 2 cm column packed with 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 (—·—·—)

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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 in progress.

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PAPER 2

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Catabolism of 1,2-diarylethane lignin model compounds by two brown-rot fungi

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Abstract. The catabolism of dimethoxybenzil, anisoin and hydroanisoin in nitrogen-limited stationary cultures of the brown-rot fungi *Wolfiporia cocos* and *Gloeophyllum trabeum* was analyzed. These three 1,2-diarylethane lignin model compounds, which differ in the degree of oxidation of the alkylic chain, gave rise to *p*-anisaldehyde in both cultures, suggesting that cleavage between the two aliphatic carbons had occurred. In turn, both strains reduced dimethoxybenzil and anisoin to hydroanisoin, whereas only *Wolfiporia cocos* was able to oxidize hydroanisoin to anisoin. On the other hand, chemically derived hydroxyl radical, but not superoxide radical, produced *p*-anisaldehyde plus other unidentified compounds from anisoin and hydroanisoin. Neither radical modified dimethoxybenzil significantly.

Key words: Brown-rot fungi – Lignin degradation – Ligning model – Compounds – Anisoin – Oxygen derived radical species

Brown-rot fungi attack preferentially softwoods, where decay results in remotion of polysaccharides without a significant depletion of the lignin component (Kirk and Highley 1973). However, during wood decay this aromatic polymer suffers several chemical transformations (demethylations, hydroxylations, etc.) (Kirk 1975) which lead to the progressive accumulation of an oxidized polymeric lignin residue (Agosin et al. 1989).

The current working hypothesis for the mechanism involved in this process is related with the production of a low molecular weight oxidant during fungal attack of wood. This agent would be able to penetrate the cell wall structure, accessing to the structural polysaccharides during the early stages of decay, despite the presence of the lignin barrier (Koenigs 1974; Highley 1977; Highley and Murmanis 1985). In support of this hypothesis, Reitberger has found that hydroxyl radical promotes demethoxylation as well as Cα-Cβ cleavage in the lignin model compound veratrylglycerol-β-guaiacyl ether (Reitberger and Gierer 1986). Furthermore, he assumed that cleavage was only possible if previous *o*-hydroxylation of the aromatic ring had taken place. These modifications have also been found in brown-rotted lignins (Kirk 1975).

The use of lignin-model compounds facilitates the identification of intermediate compounds and products generated by ligninolytic enzymes. This approach has been particularly successful in the elucidation of the ligninolytic system present in the white-rot fungus *Phanerochaete chrysosporium* (Weinstein et al 1980; Glenn et al. 1983; Kirk and Tien 1983; Tien and Kirk 1984; Kirk et al. 1986). Unfortunately, very few studies using this type of approach have been utilized with brown-rot fungi. In one of them, Enoki reported the degradation of β-1 and β-O-4 lignin model compounds by some brown-rot fungi, although the products derived from their catabolism were not identified (Enoki and Takahashi 1983; Enoki et al. 1985). Even though the degradation of these compounds was not as extensive as that reported with white-rot fungi, it was indicative that brown-rot fungi can attack lignin substructures and possibly cleave some intermonomeric bonds.

A suitable lignin model compound of the β-1 type is anisoin. It has been utilized both in fungal (Shimada and Gold 1983) and bacterial (González et al 1986) lignin biodegradation studies. Based on this evidence, we decided to examine the capacity of two brown-rot fungi, *Wolfiporia cocos* and *Gloeophyllum trabeum*, to metabolize anisoin, hydroanisoin and dimethoxybenzil. The last two differ from anisoin in the degree of oxidation of one of the aliphatic carbons. The action of specific oxygen-derived radical species on these lignin model compounds

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Abbreviations: HGLN, high glucose, low nitrogen; HGHN, high glucose, high nitrogen; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; A, anisoin; HA, hydroanisoin; DMB, dimethoxybenzil; ·OH, Hydroxyl radical; O_2^-, superoxide radical

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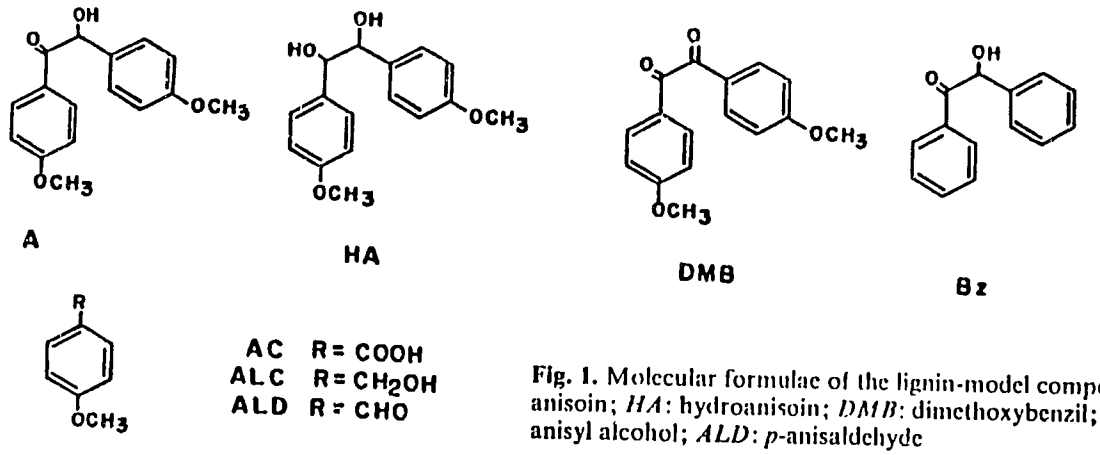

Fig. 1. Molecular formulae of the lignin-model compounds mentioned in this work. *A*: anisoin; *HA*: hydroanisoin; *DMB*: dimethoxybenzil; *Bz*: benzoin; *AC*: anisic acid; *ALC*: anisyl alcohol; *ALD*: *p*-anisaldehyde

was also studied, in order to compare the products obtained with those generated in vivo from these diarylethane structures.

Materials and methods

Chemicals

p-Anisoin [2-hydroxy-1,2-bis(4′-methoxyphenyl)-ethanone], dimethoxybenzil [1,2-bis(4′-methoxybenzil)-ethanedione], benzoin [2-hydroxy-1,2-diphenyl-ethanone], anisic acid, anisyl alcohol and *p*-anisaldehyde were from Aldrich. Hydroanisoin [1,2-bis(4′-methoxyphenyl)-1,2-ethanediol] was prepared from anisoin according to a previously described procedure (González et al. 1988). The structures of these compounds are shown in Fig. 1.

Fungal strains

The following strains of 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) and *Gloeophyllum trabeum*, obtained by courtesy of Prof. K. E. Eriksson (STFI, Sweden). Mycelial inocula were prepared as described elsewhere (Agosin and Odier 1985).

Culture media

The liquid culture media employed in the present work have been previously described (Espejo 1989). They contained 0.11 M glucose plus either 2.16 mM ammonium tartrate (high glucose, low nitrogen medium, HGLN) or 21.6 mM amonium tartrate (high glucose, high nitrogen medium, HGHN), as carbon and nitrogen sources, respectively. The buffer used was 50 mM disodium tartrate, pH 4.50. Mineral salts were added as reported by Kirk (Kirk et al. 1986).

Degradation of β-1 lignin model compounds

50 µl of a 2.0% solution of the lignin related compound in *N*-*N*-dimethyl formamide were added to 100 ml Erlenmeyer flasks containing 10 ml of culture medium. After inoculation, static cultures were incubated at 25°C in the dark and flushed every 3 days with a mixture of oxygen/nitrogen 1:1. Mycelial mats were separated by vacuum filtration through filter paper (Whatman N° 3). Supernatants were acidified with 2 M HCl to pH 1.0–2.0 and extracted three times with an equal volume of ethyl acetate. The extracts were evaporated under nitrogen to dryness and resuspended in methanol for high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) analyses.

Table 1. Metabolic transformation of anisoin by *Wolfiporia cocos* and *Gloeophyllum trabeum* after 20 days of incubation in stationary liquid cultures

Fungal strain	Culture conditions	Percentage[a]	
		Transformed anisoin	Transformed anisoin/mg mycelium
Gloeophyllum trabeum	HGLN	25	1.1
	HGHN	36	1.0
Wolfiporia cocos	HGLN	28	1.8
	HGHN	41	1.4

[a] Anisoin was quantified by HPLC following the procedure indicated in Materials and methods

Chemical analyses

TLC analyses were performed in 4.0 × 4.0 cm silica gel plates (F254, Merck, Darmstadt, FRG), which were run in a solvent system consisting of dichloromethane:methanol 95:5 v/v. The spots were visualized with a 254 nm UV lamp. When required for spectrophotometric analyses, spots were scrapped from the TLC plates and resuspended in methanol. After centrifugation to remove the silica, spectra were taken directly from the supernatants and compared to those obtained with methanol solutions of authentic standards.

HPLC analyses were carried out in a Waters apparatus equipped with a 25 × 0.39 cm i.d. column packed with a C-18 LiChrospher (Merck) support, 10 µm particle size. The solvent employed was methanol:water (60:40 v/v) at a flow rate of 1.0 ml/min. The eluent was monitored with a UV-detector, set at 280 nm.

Mycelial mat weight was determined gravimetrically after dryness (Espejo 1989).

Generation of radical species

Specific oxygen-derived radical species, i.e. OH and $O_2^{\cdot-}$, were generated by γ-radiolysis of oxygen-saturated water as described by Baker (Baker and Gebicki 1986) and Davies (Davies 1987). A dosis of 940 rad/min was applied to 0.10 mM solutions (7.0 ml) of the lignin model compound during 30 min.

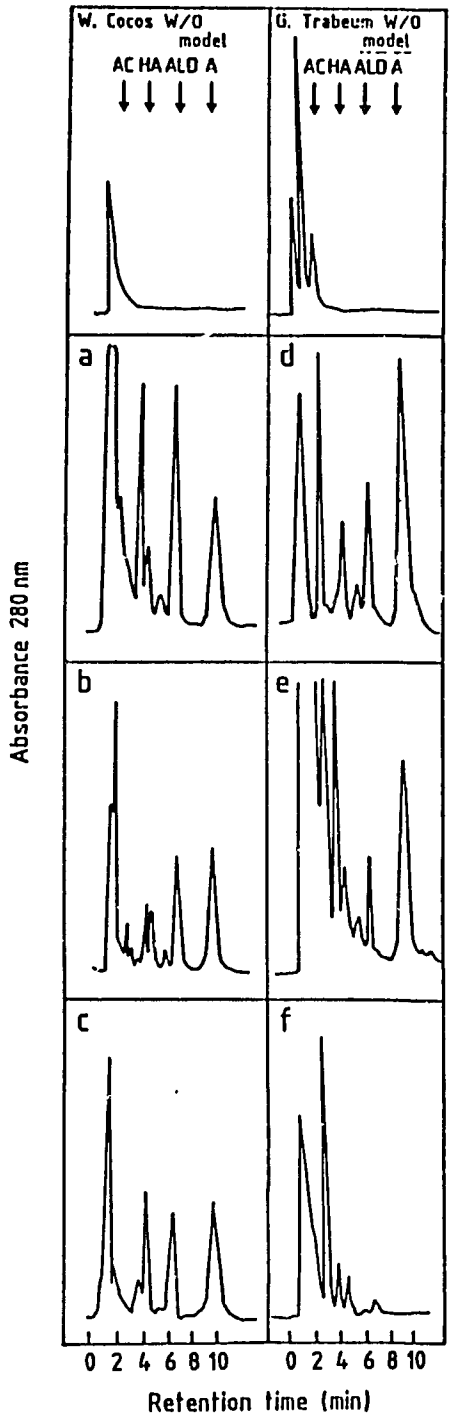

Fig. 2 A – F. HPLC chromatograms of supernatants of HGLN cultures of *Wolfiporia cocos* (A – C) and *Gloeophyllum trabeum* (D – F) containing diarylethane lignin-model compounds. A and C: with DMB; B and E: A; C and F: HA. *Arrows* show peaks identified by coelution with corresponding standards

Results

Degradation of 1,2-diarylethane model compounds and identification of catabolic intermediate in cultures of Wolfiporia cocos and Gloeophyllum trabeum

Table 1 shows the extent of anisoin transformation by both strains after 20 days of incubation under different culture conditions. Limited catabolism of the dimer was obtained in both media, being lower under the nitrogen limited conditions. However, calculation of the specific catabolic activities, i.e. percentage of transformed anisoin/mg mycelium, revealed that the relation of fungal biomass to transformed anisoin is more or less constant, regardless the composition of the medium.

Catabolic intermediates were detected with both strains after two days of incubation in the HGLN cultures and at the fourth day of incubation in the HGHN cultures. In order to identify them, supernatants were analyzed as indicated in Methods. TLC analyses could only be done with samples withdrawn from *Wolfiporia cocos* cultures, since *Gloeophyllum trabeum* secretes several aromatic compounds that interfere with the visualisation of the bands corresponding to 1,2-diarylethane catabolic intermediates (results not shown).

Preliminary identification performed by TLC followed by spectrophotometry showed the formation of *p*-anisaldehyde from the three double ring structures. In addition, anisoin and hydroanisoin were found in cultures containing dimethoxybenzil, indicating the capacity of *Wolfiporia cocos* to reduce carbonylic groups. The opposite reaction was also observed, namely the oxidation of hydroanisoin to anisoin. Similar modifications of anisoin were also found in HGHN cultures of *Wolfiporia cocos* (data not shown).

These results were confirmed by HPLC analyses (Fig. 2). Both strains showed peaks corresponding to anisoin, hydroanisoin and P-anisaldehyde from the catabolism of dimethoxybenzil (Fig. 2A, D). It is noteworthy that only *Wolfiporia cocos* showed the capacity to oxidize hydroanisoin to anisoin (Fig. 2C).

Catabolic activity against anisoin appeared to be inducible: anisoin reduction and cleavage begun only 18 h after this compound was incorporated to the medium in HGLN conditions. Furthermore, degradation started only after both fungi had reached secondary metabolism (data not shown).

Effect of oxygen-derived radical species on 1,2-diarylethane compounds

Hydroxyl radicals generated by the Fenton reaction have been suggested to be involved in the first steps of wood decay by brown-rot fungi (Koenigs 1974). Furthermore, Illman (Illman et al. 1988) and Enoki (Enoki et al. 1989), have shown that these radicals are generated in liquid cultures. Based on these observations, the effect of the specific oxygen radical species $^{\cdot}OH$ and $O_2^{\cdot-}$ on 1,2-diarylethane compounds was investigated. The results showed that only those β-1 dimeric compounds containing one or both alkylic carbons in a reduced form were affected by these radicals (Table 2). Hydroanisoin showed the highest extent of breakdown, whereas the more effective agent was that generated by γ-radiolysis of formiate free solution, i.e. OH. Dimethoxybenzil remained almost unchanged. P-Anisaldehyde was identified by HPLC (data not shown). Several other minor peaks with lower retention times also appeared, although they were not identified.

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Table 2. Effect of ·OH and O_2^- on different β-1 lignin model compounds

Model	Sample	Percentage*	
		Substrate	p-Anisaldehyde
A	Control	96.0	–
	·OH + O_2^-	73.0	2.9
	O_2^-	93.0	–
HA	Control	87.0	–
	·OH + O_2^-	43.1	13.7
	O_2^-	81.7	–
DMB	Control	99.0	–
	·OH + O_2^-	94.0	–
	O_2^-	98.0	–

* Substrates and p-anisaldehyde were quantified as mentioned in Table 1

Discussion

The results obtained in this work indicate that the brown-rot fungi *Wolfiporia cocos* and *Gloeophyllum trabeum* are able to metabolize 1,2-diarylethane lignin model compounds.

Based on the formation of hydroanisoin from anisoin and dimethoxybenzil, reduction of carbonylic groups seems to be a prominent reaction catalyzed by these strains, although *Wolfiporia cocos* can also oxidize hydroanisoin to anisoin. On the other hand, white-rot fungi tend to oxidize both lignin and model compounds at the Cα position (Fenn and Kirk 1984). The transient accumulation of hydroanisoin (or anisoin), together with the results obtained with radical species, suggest that the presence of at least one hydroxyl group in the aliphatic chain is a requirement for Cα-Cβ cleavage. Once produced, anisaldehyde was the only single-ring aromatic compound identified, confirming the observation of Enoki (Enoki et al. 1985). In contrast, anisyl alcohol has been found in culture supernatants during degradation of β-1 model compounds by white-rot fungi (Shimada and Gold 1983; Enoki et al. 1985). Phenolic compounds, either monomeric or dimeric, could not be detected in *Wolfiporia cocos* cultures. Being demethylation one of the most characteristic effects of brown-rot fungi on lignin (Kirk 1975), this was unexpected. One explanation could be that the demethylating system operates only under low water activity levels, as it has been recently shown for the cellulose depolymerizing system of these fungi (Highley personal communication). Another possibility is that single methoxyl group in the *para* position does not constitute a suitable substrate for demethylation (Haider and Trojanowski 1980).

The initial reactions involved in the catabolism of anisoin by these fungi, as well as the location of the agents involved in it are presently unknown. Hydroanisoin is cleaved extracellularly by lignin peroxidase from *Phanerochaete chrysosporium* yielding two molecules of anisaldehyde (Shimada and Gold 1983), whereas anisoin can be cleaved by a specific thiamine-pyrophosphate requiring intracellular enzyme from *Pseudomonas fluorescens* yielding the same products (González and Vicuña 1989). Whether or not this reaction is enzymatic in brown-rot fungi remains to be established. Attempts to reproduce it in vitro with concentrated extracellular fluid and with crude extracts prepared from mycelia have been unsuccessful so far. On the other hand, the effect obtained with ·OH, besides confirming earlier results reported by Kirk (Kirk et al. 1985), points to the possible participation of this radical species in the production of *p*-anisaldehyde from anisoin. Moreover, when the same dosis of radiation was applied in the presence of galactoglucomannan or carboxymethylcellulose instead of a lignin model compound, extended depolymerization took place (Rojas et al. 1988), which reflects the fate of cell wall structural polysaccharides as compared to lignin during wood decay by brown-rotters.

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PAPER 3

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Production and Degradation of Oxalic Acid by Brown Rot Fungi

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Our results show that all of the brown rot fungi tested produce oxalic acid in liquid as well as in semisolid cultures. *Gloeophyllum trabeum*, which accumulates the lowest amount of oxalic acid during decay of pine holocellulose, showed the highest polysaccharide-depolymerizing activity. Semisolid cultures inoculated with this fungus rapidly converted ^{14}C-labeled oxalic acid to CO_2 during cellulose depolymerization. The other brown rot fungi also oxidized ^{14}C-labeled oxalic acid, although less rapidly. In contrast, semisolid cultures inoculated with the white rot fungus *Coriolus versicolor* did not significantly catabolize the acid and did not depolymerize the holocellulose during decay. Semisolid cultures of *G. trabeum* amended with desferrioxamine, a specific iron-chelating agent, were unable to lower the degree of polymerization of cellulose or to oxidize ^{14}C-labeled oxalic acid to the extent or at the rate that control cultures did. These results suggest that both iron and oxalic acid are involved in cellulose depolymerization by brown rot fungi.

One of the most destructive forms of wood decay is caused by fungi that cause brown rots. During the early stages of colonization, fungal hyphae invade wood cells and secrete agents that bring about a rapid decrease in the number of glucosyl residues per cellulose chain, from 10,000 to 15,000 to about 200 (6, 7). The latter corresponds to the average size of cellulose crystallites (11).

Highley (17) has reported that an oxidative system is involved in cellulose depolymerization by brown rot fungi, showing that there was a significant increase of carbonyl and carboxyl groups in the cellulose during decay. Since most of the pore sizes in sound wood are too small to allow cellulolytic enzymes to penetrate the wood, only low-molecular-weight molecules could be responsible for this depolymerization (7, 12). The current working hypothesis on the nature of the depolymerizing agent is that oxygen-derived radical species are responsible for the initial attack of the cellulose polymer. Indeed, Halliwell (16), and Kirk et al. (21) have shown that Fenton's reaction [a mixture of Fe(II) and H_2O_2, which is known to generate ·OH radicals] causes a degradation of cellulose similar to that resulting from brown rot decay. Koenigs (23) demonstrated that H_2O_2 is secreted by brown rot fungi and that wood contains enough iron ions [as Fe(III)] to make this hypothesis feasible. Moreover, Gutteridge (13) found that iron(III) salts in the presence of a chelator (EDTA) and a superoxide-generating system could oxidize several neutral sugars in reactions dependent on the reduction of Fe(III) to Fe(II) by superoxide.

Oxalic acid also has been implicated in the wood-decaying system of these fungi. Takao (34) demonstrated that brown rot fungi secrete significant amounts of oxalic acid in liquid cultures, whereas white rot fungi apparently do not. The latter was attributed to the presence of an intracellular oxalate decarboxylase in white rot fungi which decomposes oxalate to CO_2 and formate. This enzyme could not be detected in cultures of brown rot fungi (32). Later, Schmidt et al. (31) demonstrated in vitro that oxalic acid could reduce Fe(III), normally present in wood, to Fe(II), with oxalic acid simultaneously oxidized to CO_2. Furthermore, this iron reduction significantly increased the cellulose-depolymerizing activity of an iron-H_2O_2 system.

However, the role of oxalic acid in wood decay by brown rot fungi in vivo is not fully understood. Koenigs (23) demonstrated that isolates of *Gloeophyllum trabeum* that caused extensive decay lowered the pH of wood, whereas those that resulted in low amounts of decay did not. In this regard, Agosin et al. (1) showed that the acidic conditions developed by brown rot fungi are essential for wood decay. However, Micales and Highley (26) could not establish a correlation between the wood-decaying ability of various isolates of *Poria placenta* (*Postia placenta*) and their oxalic acid production.

The importance of oxalic acid during the invasion of host tissue by phytopathogenic fungi has been reported previously (5, 24, 25, 33). It has been suggested that the oxalic acid produced by *Sclerotium rolfsii* during pathogenesis acts synergistically with endopolygalacturonase, lowering the pH of the infected tissues to a level optimal for the activity of this enzyme. Furthermore, the calcium present in structural pectates can be strongly chelated by oxalic acid. As a consequence, plant tissues are rendered more susceptible to invasion by this fungus (29).

The aim of the present work, therefore, was to investigate further the possible role of oxalic acid in the cellulose depolymerizing system of brown rot fungi. First, the production of oxalic acid by various brown rot fungi under liquid and semisolid culture conditions was studied, and its relationship with cellulose depolymerization was assessed. Then, ^{14}C-oxalic acid was added at different stages of wood decay to determine the relationship between CO_2 production and cellulose depolymerization. Finally, to assess the participation of iron salts in the depolymerization of cellulose by these fungi, semisolid fungal cultures were incubated in the presence of the siderophore desferrioxamine (14) and the fate of ^{14}C-oxalic acid was followed.

MATERIALS AND METHODS

Fungi. The brown rot fungi *P. placenta* (MAD-698), *G. trabeum* (MAD 617-R), and *Wolfiporia cocos* (FP 90850-R) and the white rot fungus *Coriolus versicolor* (*Trametes versicolor*) (INTA 9A) were used in these experiments.

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These fungi were maintained in malt-yeast extract agar slants at 28°C. Inocula were grown in agitated liquid cultures, and the resulting mycelial pellets were centrifuged, washed, and ground before inoculation, as described elsewhere (2).

Liquid cultures. Static liquid cultures were conducted in 100-ml Erlenmeyer flasks with 10 ml of mineral medium (20) containing 55 mM glucose and 2.2 mM ammonium tartrate as carbon and nitrogen sources, respectively. The medium was buffered with 50 mM sodium 2,2-dimethylsuccinate, pH 4.50.

Semisolid cultures. (i) Cultivation methods. All decay studies were performed in specially designed glass columns (30) which were filled with 1.2 g of pine (*Pinus radiata*) holocellulose, prepared by the method of Timell (35), and humidified to a final moisture content of 75% (wt/wt) with a mineral medium containing the inoculum (20). During incubation at 28°C, the columns were constantly oxygenated through the bottom with humidified air at a flow rate of 3.2 ml/min.

(ii) Radiolabeling studies. ^{14}C-oxalic acid, 5.55×10^5 dpm, (50 μl), was fed to semisolid cultures after 0, 7, and 30 days of decay by applying it with a 50-μl Hamilton syringe to the batch at several sites. Decarboxylation of ^{14}C-oxalic acid was followed after trapping $^{14}CO_2$ from the outlet air flow column in 10 ml of 2-phenylethylamine scintillation solution, as described by Kadam and Drew (19). All experiments were conducted in at least four replicates.

To study the role of iron in the breakdown of oxalic acid, desferrioxamine (Desferal; Ciba-Geigy, Basel, Switzerland), a specific iron-chelating agent, was added to semisolid cultures at a concentration of 15 mg/g of holocellulose. After 7 days of decay, 50 μl of ^{14}C-oxalic acid was fed, and the evolution of $^{14}CO_2$ was followed. The amount of desferrioxamine added to the cultures was calculated considering the average iron content of pine wood (11).

Miscellaneous methods. The degree of polymerization (DP) of cellulose was determined viscosimetrically (6) after solubilization of holocellulose with cupriethylenediamine hydroxide solvent. Cupriethylenediamine was prepared by a standard procedure of the American Society for Testing and Materials (4).

The amount of free oxalic acid present in holocellulose-decayed samples was enzymatically determined on supernatants of thoroughly water-extracted material, using an oxalate kit (Sigma Chemical Co., St. Louis, Mo.). Calcium oxalate was solubilized by 0.2 N HCl from the water-extracted solid residue and determined as free oxalic acid as described above. For liquid cultures, oxalic acid was directly determined from an aliquot of the cell-free culture medium, using the same procedure.

The capacity of brown and white rot fungi to solubilize calcium oxalate was assayed in a semisolid agar culture medium containing 0.5% (wt/vol) calcium oxalate, 0.1% (wt/vol) glucose, and basal mineral salts. The pH of the medium was buffered as described above. Oxalate degraders were detected in this medium by the formation of clear zones around the fungal colonies (8).

The pH of the samples was determined by using an Orion pH recorder, model SA 720.

Mycelial growth in liquid cultures was determined gravimetrically (11).

Weight loss was followed by determining the amount of carbon lost as CO_2. For this purpose, CO_2 was continuously trapped during cultivation in 10 ml of 0.5 N NaOH and quantified every 2 days by titration of the residual NaOH with 0.2 N HCl (2).

Crude extracts were obtained from 1.2 g of holocellulose semisolid cultures decayed by *G. trabeum* for 7 days. The extraction process was done with 2.5 ml of water (three times), squeezing and soaking through a 0.2-μm-pore-size membrane filter. ^{14}C-oxalic acid, 10,000 dpm, was added to 1 ml of the water extracts, and the mixture was incubated for 24 h at 25°C. Oxalic acid oxidation was measured after trapping and counting the $^{14}CO_2$ produced.

RESULTS

Liquid cultures. (i) Production of oxalic acid by brown and white rot fungi. Oxalic acid was secreted into the culture medium from the first day of incubation and increased with growth (Fig. 1). *W. cocos* and *P. placenta* accumulated the highest amounts of oxalic acid, reaching 2.00 and 0.95 mg/ml (standard deviation, $<\pm 0.05$), respectively, after 4 days of cultivation. As a consequence, the pH of these fungal cultures rapidly dropped to around pH 3.0, even though the culture medium was initially buffered at pH 4.5 with 50 mM 2,2-sodium dimethylsuccinate. In contrast, the cultures inoculated with *G. trabeum* exhibited only a limited production of oxalic acid, reaching a maximum of 0.20 ± 0.04 mg/ml after 5 days of incubation. Finally, only trace amounts of the acid were detected in the cultures of the white rot fungus *C. versicolor*.

(ii) Oxalic acid and growth. Liquid cultures containing oxalic acid as the only carbon source showed limited, if any, growth, indicating that oxalate is not utilized after its secretion (data not shown). *C. versicolor* was the only species studied that was able to metabolize calcium oxalate crystals, as shown by its ability to produce clear zones in a calcium oxalate agar medium.

Semisolid cultures. (i) Oxalic acid production and cellulose depolymerization by brown and white rot fungi. Changes in the DP of holocellulose incubated with three brown rot fungi under semisolid culture conditions are illustrated in Fig. 2. *G. trabeum* showed the highest rate of depolymerization, reaching a leveling DP after 15 days of cultivation. In contrast, *C. versicolor*, a white rot fungus, caused only a very limited decrease in the DP of the holocellulose during decay, although an appreciable degradation of the cellulose occurred ($22.0\% \pm 0.5\%$ weight loss after 25 days of incubation). The holocellulose cultures of all three brown rot fungi rapidly dropped to pH values of around 3.0 after 3 to 5 days of cultivation and then remained constant. The DP decrease observed during the early phases of decay is inversely correlated with the increase in oxalic acid content of the semisolid cultures of *P. placenta* and *W. cocos*. Oxalic acid content decreased after the onset of cellulose depolymerization, however, reaching a plateau after 15 days of decay. Surprisingly, in cultures of *G. trabeum* oxalic acid content as well as calcium oxalate were low throughout the experiment (see Fig. 6, closed symbols), although this fungus showed the highest cellulose depolymerizing activity (Fig. 2). The lowering of pH by *G. trabeum* could be due to the production of other unidentified acidic compounds from the metabolism or catabolism of this fungus, as we detected by high-pressure liquid chromatography with an anionic exchange column (data not shown).

Finally, as expected, the white rot fungus *C. versicolor* did not lower the pH of the semisolid cultures, nor did it accumulate oxalic acid during decay.

(ii) Fate of ^{14}C-oxalic acid at different stages of cellulose depolymerization by brown and white rot fungi. The fate of radiolabeled oxalic acid during holocellulose decay is illus-

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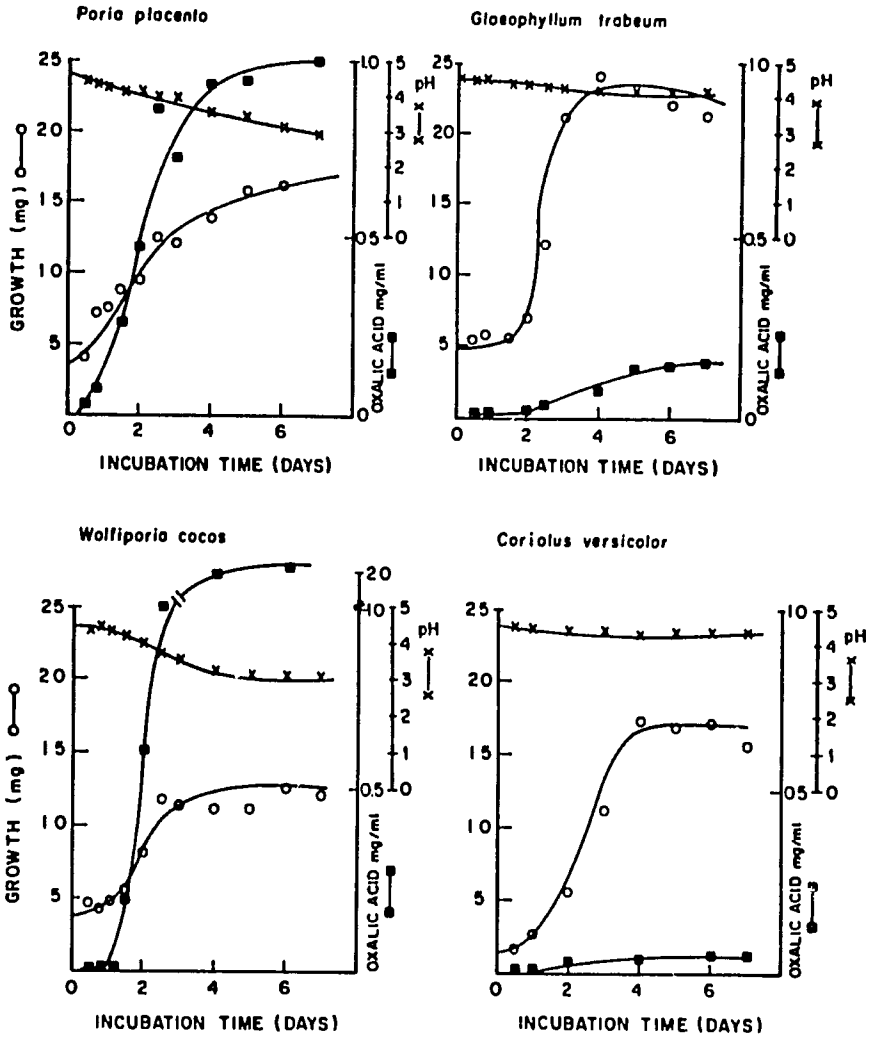

FIG. 1. Oxalic acid production in liquid cultures by the brown rot fungi *P. placenta*, *G. trabeum*, and *W. cocos*. The white rot fungus *C. versicolor* was used as a control. ×, pH; ■, oxalic acid; O, growth.

trated in Table 1 and Fig. 3. When ^{14}C-oxalic acid was added at the beginning of incubation, *G. trabeum* and *P. placenta* generated $^{14}CO_2$ after lags of 2 and 4 days, reaching maxima of 85% ± 4% and 55% ± 6% mineralization of the oxalic acid added after 14 and 16 days of incubation, respectively.

When 7-day-old brown-rotted cultures were fed with the ^{14}C-oxalic acid, a very high mineralization rate was obtained, especially by *G. trabeum* (Table 1 and Fig. 3). Furthermore, no lag in the onset of $^{14}CO_2$ release was observed for either fungus. In contrast, *C. versicolor* showed only a limited ability for degradation of the radiolabeled substrate (Fig. 3). Although both brown rot fungi rapidly reached a maximum for $^{14}CO_2$ production, complete oxidation of the ^{14}C-oxalic acid added was not achieved.

Finally, when cellulose had already reached its lowest DP (about 200), i.e., after 30 days of degradation, the ability to oxidize ^{14}C-oxalic acid to $^{14}CO_2$ was still present, although to a reduced extent (Table 1).

(iii) Effect of desferrioxamine addition on the cellulose depolymerizing system of *G. trabeum*. Our assays showed that *G. trabeum* accumulates low quantities of oxalic acid but has a high cellulose depolymerizing activity and a high ability to oxidize oxalic acid to CO_2. The involvement of iron in the degradation of oxalic acid was examined by testing the action of the Fe(III) chelator desferrioxamine. The effect of desferrioxamine on cellulose depolymerization is shown in Fig. 4. A substantial inhibition of cellulose depolymerization was obtained in the presence of the siderophore. Desferrioxamine also caused a marked inhibition of $^{14}CO_2$ release from ^{14}C-oxalic acid (Fig. 5). Even though a lower weight loss (determined as carbon loss) was found for cultures containing desferrioxamine, a similar extent of decay in cultures without the iron chelator produced a leveling off of the DP of the cellulose component to about 200 (Fig. 4).

Figure 6 shows a significant increase in oxalate and oxalic acid content in cultures containing the iron chelator, suggesting that the low levels of oxalic acid observed in cultures of *G. trabeum* result from its consumption during cellulose depolymerization rather than from low production.

(iv) In vitro mineralization of ^{14}C-oxalic acid. We found that water extracts of 7-day-old pine holocellulose semisolid cultures, decayed by *G. trabeum*, could oxidize ^{14}C-oxalic acid up to 9%. This oxalic acid-oxidizing activity was destroyed when the extract was boiled for 30 min at 100°C.

DISCUSSION

The mechanism implicated in the depolymerization of cellulose during wood decay by brown rot fungi suggests the participation of an oxidative agent of low molecular weight. The current working hypothesis is that highly reactive oxygen-derived radicals are involved in this process. Hy-

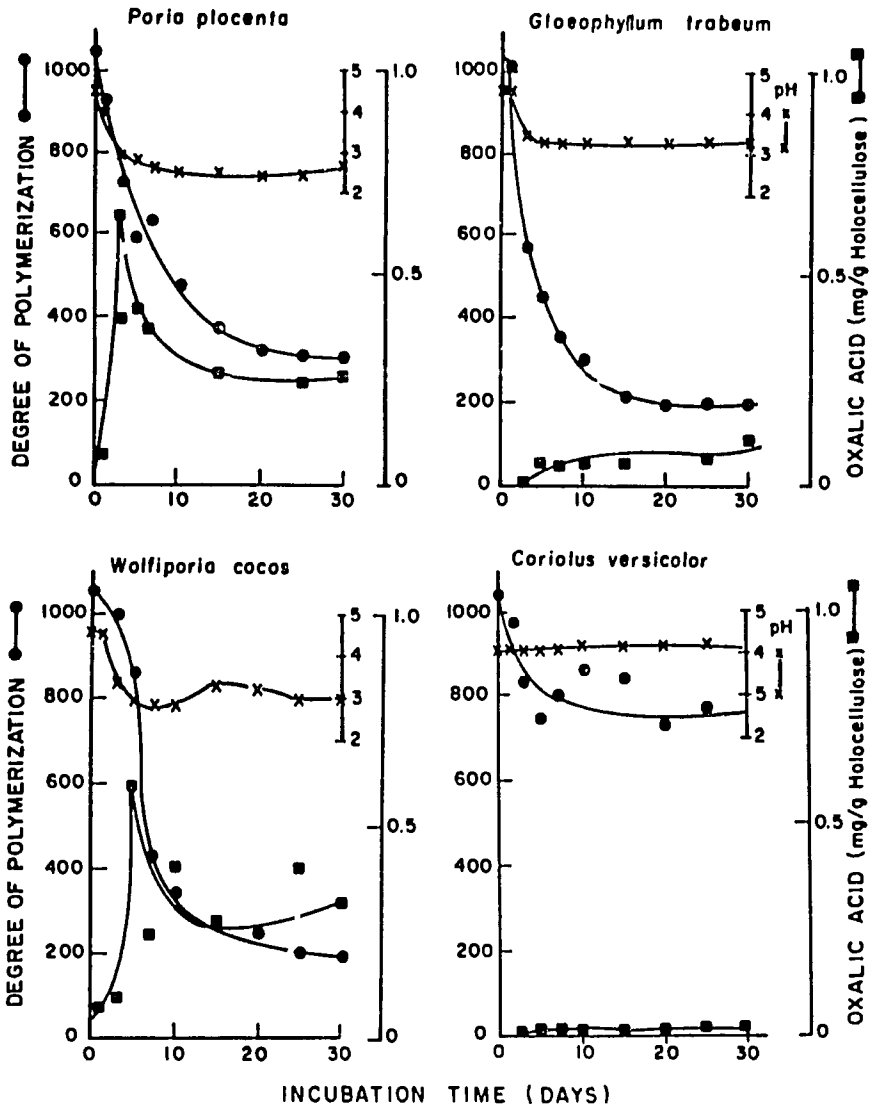

FIG. 2. Evolution of pH (×), oxalic acid (■), and cellulose depolymerization (O) during decay of pine holocellulose by wood-decaying fungi under semisolid culture conditions.

TABLE 1. Characteristics of mineralization rate of ^{14}C-oxalic acid during the decay of pine holocellulose by two brown rot fungi

Strain	Incubation time (days)[a]	Wt loss (%)[b]	Initial DP	$^{14}CO_2$ production		
				Lag (days)	Initial rate (% $^{14}CO_2$ h^{-1})[c]	Total ^{14}C (% total ^{14}C)[d]
G. trabeum	0		1,000	2	0.34	85
	7	3.2	390		28.00	80
	30	29.0	200		5.42	40
P. placenta	0		1,000	4	0.16	55
	7	3.2	500		1.91	65
	30	20.2	298		1.88	20

[a] Incubation time at which the ^{14}C-oxalic acid (50,000 dpm) was added to the cultures.
[b] Determined as CO_2 produced from the oxidation of holocellulose by the action of these fungi (as percentage of initial carbon substrate).
[c] $^{14}CO_2$ produced by the fungus from ^{14}C-oxalic acid (as percentage of total ^{14}C added).
[d] $^{14}CO_2$ recovered after 35 days of incubation.

droxyl radicals could be generated in wood by the Fenton reaction, $Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^-$. The resulting Fe(III) might be converted back to Fe(II) for further $\cdot OH$ generation by the oxalic acid secreted by this type of fungus (23).

Our results confirm previous work by Takao (34) concerning the production of oxalic acid by brown rot fungi during growth in liquid culture, and only trace amounts of oxalic acid could be found in white rot fungal cultures, presumably as a consequence of the presence of an intracellular oxalate decarboxylase produced by these fungi (32) or due to the involvement of oxalic acid in the lignin peroxidase system of *Phanerochaete chrysosporium*, as described recently by Akamatsu et al. (3) and Popp et al. (27).

Oxalic acid was also secreted by brown rot fungi during the decay of pine holocellulose under semisolid culture conditions. Our data indicate that oxalic acid production is associated with the drop in culture pH during the first stages of decay; moreover, oxalic acid concentration decreases in parallel to cellulose depolymerization. *G. trabeum*, which exhibited the highest rate of cellulose depolymerization, showed the lowest oxalic acid accumulation. Therefore, the drop of culture pH should be associated with other com-

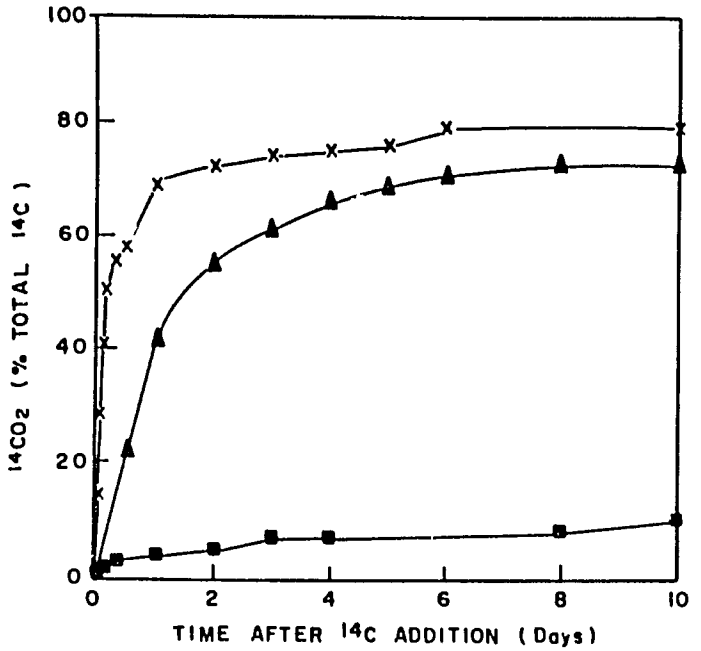

FIG. 3. Mineralization of ^{14}C-oxalic acid in semisolid cultures of pine holocellulose by the wood-decaying fungi *G. trabeum* (×), *P. placenta* (▲), and *C. versicolor* (■). ^{14}C label was added after 7 days of incubation.

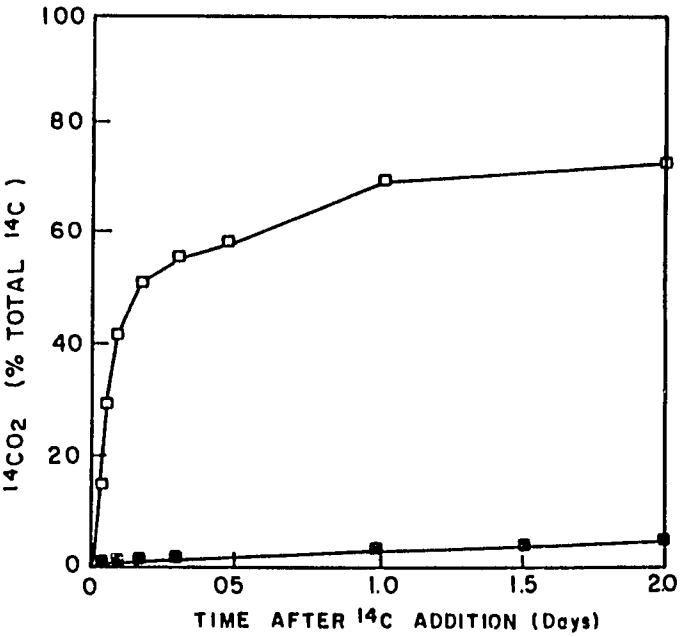

FIG. 5. Mineralization of ^{14}C-oxalic acid in semisolid cultures of pine holocellulose decayed by *G. trabeum* in the presence (■) or absence (□) of desferrioxamine. ^{14}C-oxalic acid was added after 7 days of incubation.

pounds, other organic acids produced by this fungus, and/or sugar acids from the catabolism of polysaccharides (21). Moreover, the onset of cellulose depolymerization in semisolid cultures inoculated with this fungus was found to occur at the beginning of cultivation. Our experiments with ^{14}C-oxalic acid clearly demonstrate that brown rot fungi possess the ability to oxidize oxalic acid at a very high rate, in contrast to previous reports by Takao (34), although the latter reports concerned liquid rather than semisolid cultures. *G. trabeum* showed the highest $^{14}CO_2$ release rate from ^{14}C-oxalic acid. This high oxidation rate may well explain the low amounts of oxalic acid detected in semisolid cultures as a result of the participation of oxalic acid in cellulose depolymerization. *P. placenta* showed a similar pattern of degradation. Finally, as expected, the white rot fungus *C. versicolor* showed a very limited ability to release $^{14}CO_2$ from ^{14}C-oxalic acid or to depolymerize holocellulose during decay.

Experiments conducted in the presence of the siderophore desferrioxamine indicated that sequestration of iron significantly inhibited cellulose depolymerization. At the same

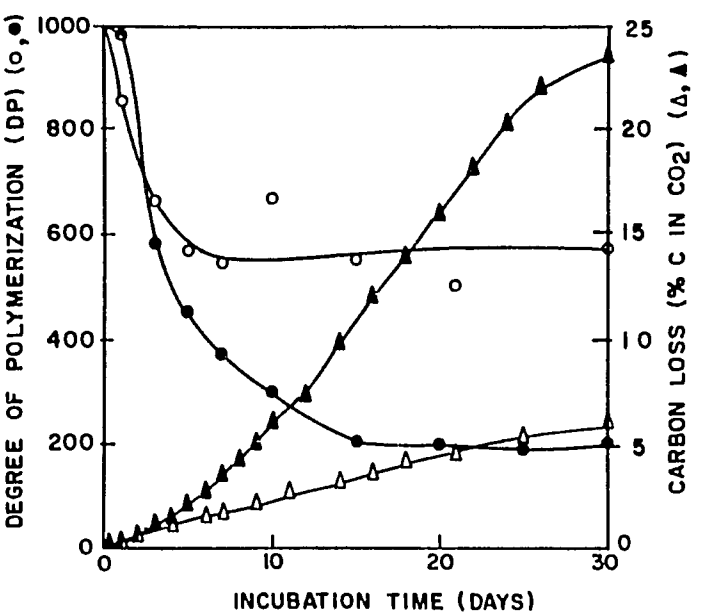

FIG. 4. Evolution of carbon loss (Δ, ▲), and DP (O, ●) during pine holocellulose degradation by the brown rot fungus *G. trabeum* in the presence (open symbols) or absence (closed symbols) of desferrioxamine.

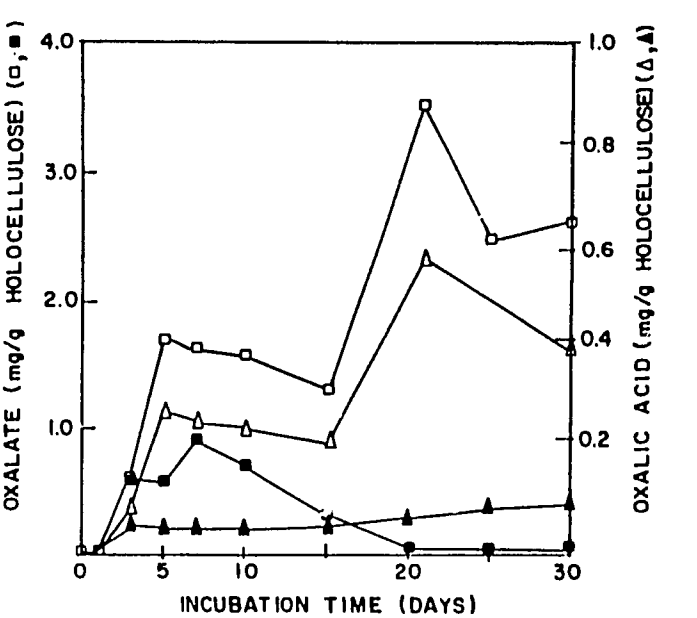

FIG. 6. Evolution of oxalate (□) and oxalic acid (Δ) during pine holocellulose degradation by the brown rot fungus *G. trabeum* in the presence (open symbols) or absence (closed symbols) of desferrioxamine. $P > 0.05$ by t test.

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time, oxalic acid and oxalate concentrations were higher in the presence of desferrioxamine than in the control, and the production of $^{14}CO_2$ from ^{14}C-oxalic acid was decreased. Transition metals (including iron) accumulate during brown rot decay of cotton cellulose (22).

Overall, these results support the proposal that oxalic acid oxidation is iron dependent, presumably causing reduction of Fe(III) to Fe(II), and that the latter is effectively involved in cellulose depolymerization, perhaps by generating ·OH radicals in a Fenton reaction.

The Fenton reaction requires H_2O_2 (16, 21, 31). Detection of H_2O_2 in brown rot cultures has been difficult (18, 23, 36), perhaps because of its high consumption rate. In this respect, another possible explanation for the role of oxalic acid could be that there is an extracellular oxalate oxidase capable of both oxidizing oxalic acid and generating H_2O_2. Indeed, Enoki et al. (9) recently reported the presence of an extracellular one-electron oxidizing activity in wood-decaying cultures of brown rot fungi, but the ability of this enzymatic system to oxidize oxalic acid was not reported.

Hydroxyl radicals are highly reactive and combine with the molecules present at or very close to their site of formation (15, 28). If they are responsible for cellulose depolymerization, then they must be generated adjacent to the cellulose chains. The mechanism by which the required iron for this reaction is translocated to the sites where ·OH generation is required remains to be determined.

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PAPER 4

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Changes in Molecular Size Distribution of Cellulose during Attack by White Rot and Brown Rot Fungi

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The kinetics of cotton cellulose depolymerization by the brown rot fungus *Postia placenta* and the white rot fungus *Phanerochaete chrysosporium* were investigated with solid-state cultures. The degree of polymerization (DP; the average number of glucosyl residues per cellulose molecule) of cellulose removed from soil-block cultures during degradation by *P. placenta* was first determined viscosimetrically. Changes in molecular size distribution of cellulose attacked by either fungus were then determined by size exclusion chromatography as the tricarbanilate derivative. The first study with *P. placenta* revealed two phases of depolymerization: a rapid decrease to a DP of approximately 800 and then a slower decrease to a DP of approximately 250. Almost all depolymerization occurred before weight loss. Determination of the molecular size distribution of cellulose during attack by the brown rot fungus revealed single major peaks centered over progressively lower DPs. Cellulose attacked by *P. chrysosporium* was continuously consumed and showed a different pattern of change in molecular size distribution than cellulose attacked by *P. placenta*. At first, a broad peak which shifted at a slightly lower average DP appeared, but as attack progressed the peak narrowed and the average DP increased slightly. From these results, it is apparent that the mechanism of cellulose degradation differs fundamentally between brown and white rot fungi, as represented by the species studied here. We conclude that the brown rot fungus cleaved completely through the amorphous regions of the cellulose microfibrils, whereas the white rot fungus attacked the surfaces of the microfibrils, resulting in a progressive erosion.

Brown rot fungi comprise a limited number of wood-decaying basidiomycetes that damage wood by rapidly depolymerizing the cellulose component. Generally, depolymerization occurs before significant loss in the weight of wood. At advanced stages of decay, structural polysaccharides are quantitatively removed, and a modified lignin residue remains (4, 14).

The biochemistry of the cellulolytic system of brown rot fungi has received little attention, despite the apparent uniqueness of the mechanism involved and the economic importance of brown rot. Because pores in sound wood are too small to allow cellulolytic enzymes to penetrate, it seems probable that the agent responsible for initial cellulose depolymerization is not a classical cellulase (5). Flournoy et al. (8) recently studied changes in the pore size of wood during decay by the brown rot fungus *Postia placenta*. They concluded that the agent responsible for cellulose depolymerization has a molecular radius of less than 38 Å (3.8 nm) and probably not greater than 15 Å (1.5 nm).

In discussing the nature of the cellulose-depolymerizing agent, Cowling and Brown (5) noted that G. Halliwell (10) had described the depolymerization of cellulose by Fenton's reagent (Fe^{2+} + H_2O_2, which generates a hydroxyl radical or similar oxidant [9]). Subsequently, Koenigs (16, 17) demonstrated that cellulose in wood is depolymerized by Fenton's reagent, that brown rot fungi produce extracellular H_2O_2, and that wood contains enough Fe (Fe^{3+}) to make the hypothesis reasonable. Support for the hypothesis of an oxidative system was later provided by Highley (11) and Kirk et al. (15), who obtained evidence that cellulose subjected to brown rot fungi is oxidized. Schmidt et al. (19) demonstrated that oxalic acid, which is secreted by brown rot fungi in liquid cultures (21), reduces Fe^{3+} to Fe^{2+} under certain conditions.

Recently, Shimada et al. (20) proposed an alternate role for oxalic acid in cellulose depolymerization by brown rot fungi. They reported that oxalic acid (1% [pH 1.3], 35°C, 4 weeks) alone reduces the viscosity of wood pulp to 60% of the original and therefore may be directly involved in the cellulose-depolymerizing process.

Enoki et al. (6) suggested that iron-containing glycopeptides which are able to oxidize 2-keto-4-thiomethylbutyric acid to ethylene in the presence of H_2O_2 are somehow involved in wood degradation by brown rot fungi. However it has yet to be established whether these glycoproteins are involved in cellulose depolymerization.

Although brown rot fungi grow well in chemically defined media, liquid culture conditions in which the depolymerizing system is demonstrable have not been found. However Highley has shown that the system is demonstrable in solid-state (soil-block) cultures (11) and in cultures over an agar medium (12).

Research on the cellulolytic system of brown rot fungi lags behind research on that of the wood-degrading white rot fungi. The cellulolytic system of the latter group is a classical one comprised of endo- and exoglucanases and β glucosidases. Several cellulase components of white rot fungi have been isolated and characterized, and the respective genes have been cloned and sequenced previously (7). Most brown rot fungi possess endoglucanase and β glucosidase activities but not exoglucanase activity (7, 12, 18).

The purpose of our study was to compare the kinetics of cellulose depolymerization by brown and white rot fungi in the solid-state system. Our ultimate goal is to understand the

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chemistry and biochemistry involved in cellulose depolymerization by brown rot fungi.

MATERIALS AND METHODS

Fungal strains, maintenance, and inoculum preparation. *P. placenta* (Fr.) M. Lars. et Lomb. (MAD-698; ATCC no. 11538) was maintained at 27°C on malt agar slants. Bits of mycelium-covered agar from 3 to 4-week-old slants were used to seed petri plates of the same medium. *Phanerochaete chrysosporium* (BKM-F-1767; ATCC no. 11538) was maintained at 30°C on yeast-malt-peptone-glucose agar. Mycelium-covered agar from 7- to 10-day-old slants was used to seed petri plates of malt agar. Ten-millimeter-square sections from the petri dish cultures (<3 weeks old) were used to start the experimental soil-block cultures with both fungi.

Cellulose. Cotton cellulose was purified Type A-600 from Holden Vale Manufacturing Co., Ltd. (Lancaster, England). After incubation with the fungus, cellulose samples were ground in a Wiley mill to pass a 30-mesh screen.

Cellulose depolymerization studies. Cellulose was exposed to the fungi in soil-block bottles (1) that were incubated at 27°C with 70% relative humidity. Weighed balls of cellulose (approximately 100 mg [dry weight]) were placed directly on mycelium-covered wood feeder strips, which in turn rested on sterile, moist soil in French square bottles. The cotton balls were rapidly invaded by the hyphae. Triplicate balls were removed after various incubation times, the surface mycelia were removed, and the residual cellulose was dried at 60°C for 48 h before weighing.

Cellulose analyses. Solubility in 1% sodium hydroxide was determined as described in Technical Association of the Pulp and Paper Industry standard T212 om-83 (22). In the initial experiment, the average degree of polymerization (DP; the average number of glucosyl residues per cellulose molecule) was measured viscosimetrically (2) after cellulose solubilization in cuprietheylenediamine (GFS Chemicals, Columbus, Ohio). In subsequent experiments, molecular size distributions of the samples were determined by size exclusion chromatography (SEC) of the tricarbanilate derivatives dissolved in tetrahydrofuran (24). Increased amounts of derivatizing reagent [(5.0 ml of pyridine plus 1.5 ml of phenylisocyanate) per 5.0 mg of cotton] were used to derivatize cellulose exposed to *P. chrysosporium* because of the excess amount of fungal material present in the samples. The number-, weight-, and viscosity-average degrees of polymerization (DP_n, DP_w, and DP_v, respectively) were calculated from the SEC data. (The SEC analysis of the cellulose sample most excessively attacked by the white rot fungus was complicated because the sample did not completely dissolve during the workup. This was most likely because of the presence of fungal mycelia, which were partially interdispersed with the cellulose and could not be removed completely. The presence of an insoluble residue was also observed when the mycelia alone were analyzed as a control.)

RESULTS

Initial experiments determined the depolymerization of cellulose by the brown rot fungus *P. placenta* in the soil-block cultures as a function of time. The DP was measured by the viscosimetric procedure. Residual cellulose was further characterized by its solubility in alkali. In subsequent experiments with both *P. placenta* and *P. chrysosporium*, changes in the molecular size distribution of cellulose were determined.

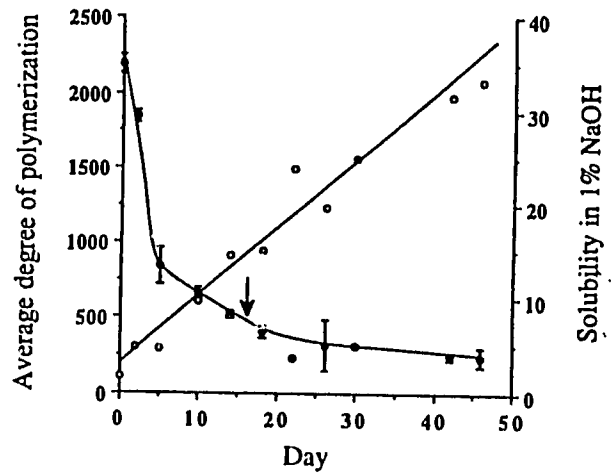

FIG. 1. Time course study of cellulose depolymerization by *P. placenta* in soil-block cultures. Three samples were removed every 4 days, and the DP (●) was determined viscosimetrically. The solubility of the remaining cellulose in 1% NaOH (○) was also evaluated. The arrow indicates the onset of weight loss in the cellulose, and the vertical lines are the standard deviations.

Kinetics of cellulose depolymerization. Depolymerization of cellulose by *P. placenta* (Fig. 1) occurred in two phases as seen by the viscosimetric assay: a phase in which a rapid decrease in the DP from 2,200 to approximately 800 (0 to 5 days) occurred and a slower phase lasting approximately 15 days during which the DP decreased to about 250. Further decrease in the DP was negligible. The solubility of the residual cellulose in 1% sodium hydroxide was not biphasic; rather, it increased in a roughly linear manner throughout the incubation period and reached approximately 33% by the termination of the experiment. Almost all depolymerization occurred before any weight loss occurred. The first loss in weight was observed at day 16, and only 20% of the cellulose was consumed after 42 days.

In a similar experiment with *P. chrysosporium*, the DP of cellulose determined by SEC remained relatively high (DP_w in Table 1). Also, in contrast to results with the brown rot fungus, a continuous weight loss of the cellulose was observed; a maximum of 50% loss was seen after 24 days (Table 1). Solubility in 1% NaOH was not determined but

TABLE 1. Changes in molecular size distribution of cotton cellulose degraded by brown and white rot fungi

Incubation time (days)	Weight loss (%)	DP_n	DP_w	DP_v	Polydispersity (DP_w/DP_n)
Brown rot fungus					
0	0	1,150	2,111	2,044	1.84
5	0	676	1,717	1,642	2.34
14	0	244	754	710	3.09
22	6	86	454	422	5.27
42	20	65	293	273	4.49
White rot fungus					
0	0	1,057	2,009	1,925	1.90
8	9	293	1,134	1,054	3.87
12	21	745	1,433	1,367	1.92
24	50	984	1,570	1,511	1.60

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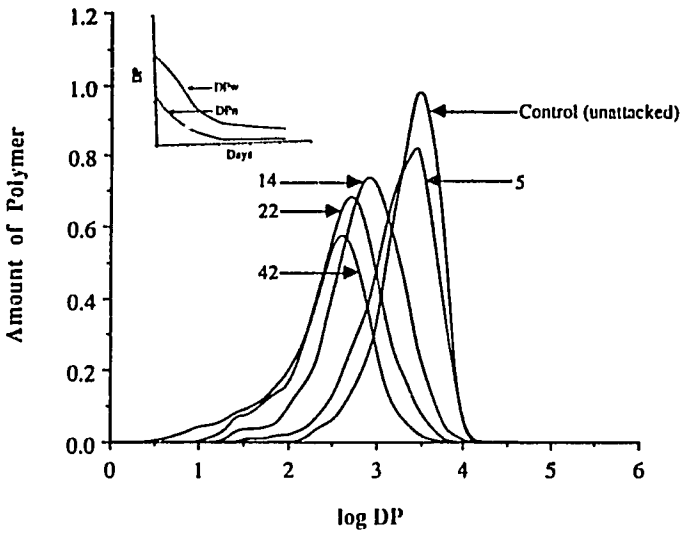

FIG. 2. Changes in molecular size distribution of cotton cellulose during degradation by the brown rot fungus *P. placenta*. The areas under the curves have been adjusted to reflect weight loss due to fungal activity. The numbers next to the arrows are the numbers of days during which cellulose was exposed to the fungus.

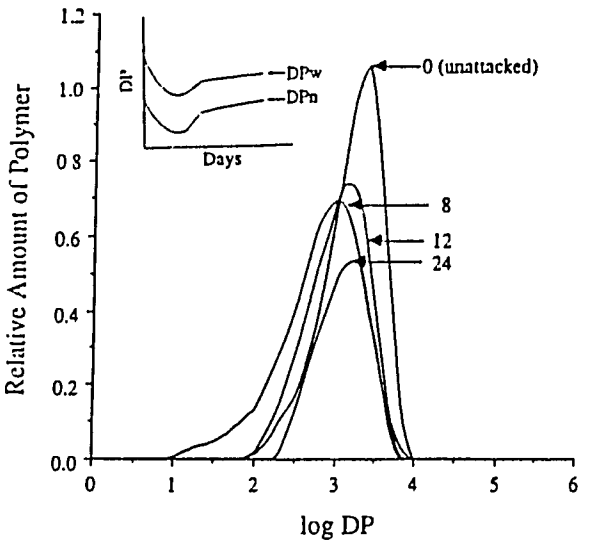

FIG. 3. Changes in the molecular size distribution of cotton cellulose during degradation by the white rot fungus *P. chrysosporium*. The areas under the curves have been adjusted to reflect weight loss due to fungal activity. The numbers next to the arrows are the numbers of days during which cellulose was exposed to the fungus.

had been shown earlier by Highley (12) to increase very slightly during decay.

Molecular size distribution of cellulose decayed by *P. placenta*. Cellulose depolymerization by the brown rot fungus was investigated in detail by examining the change in molecular size distributions during the course of degradation (Fig. 2). Single major peaks were resolved for each sample, irrespective of the length of time of incubation. With time, the peaks became broader and were centered at progressively lower DP values. In addition, by day 14 a shoulder representing low-molecular-size components (DP, <60) appeared; its contribution increased with time, although it was never a major component. A gradual increase in the polydispersity of the samples (DP_w/DP_n, which is an indication of the range of molecular sizes) was observed and is explained by the increase in the proportion of lower-molecular-size components (Table 1). Both DP_w (=DP_v) and DP_n decreased progressively with time (Table 1; Fig. 2).

Molecular size distribution of cellulose decayed by *P. chrysosporium*. The changes in the patterns of molecular size distribution during attack by *P. chrysosporium* (Fig. 3) differed greatly from those observed for *P. placenta*. The molecular-size-distribution curve broadened initially but narrowed as the cellulose was attacked. Single major peaks were resolved for each sample and were centered at a slightly lower average DP than that of starting material. Unlike the observations with the brown rot fungus, both DP_w (= DP_v) and DP_n decreased by day 8 but then gradually increased to near control values thereafter (Table 1; Fig. 2). The polydispersity of the residual cellulose changed in a similar manner.

DISCUSSION

Our study of cellulose depolymerization by *P. placenta* shows that the DP decreased substantially before any weight loss (utilization) occurred. Cowling (4) also observed this phenomenon in experiments with wood blocks from which holocellulose was isolated during decay by this fungus (which was classified at the time as *Poria monticola* Murr.). In addition, Highley (12) reported that a number of brown rot fungi depolymerized cotton before any weight loss occurred. Depolymerization before utilization suggests that the system responsible for utilization (i) acts only on low-molecular-size fragments produced late during depolymerization, (ii) is inhibited during initial depolymerization, (iii) is induced by late-depolymerization products, or (iv) is simply inefficient. The fact that the solubility of the cellulose in 1% NaOH increased in an approximately linear manner during attack by the brown rot fungus indicates that small (soluble) fragments were produced and accumulated during depolymerization; thus, possibilities (ii) and (iv) are more likely than (i) or (iii). Cowling (4) showed that 1% NaOH solubility of holocellulose isolated from wood attacked by brown rot fungi also increased during degradation. Likewise, Highley observed an increase in alkali solubility of cotton cellulose attacked by several brown rot fungi (12). However, our results show that alkali solubility of cellulose decayed by *P. placenta* does not directly correlate with depolymerization and consequently cannot be used as a simple assay for depolymerization.

The kinetic curve of cellulose depolymerization by the brown rot fungus revealed two phases of depolymerization, resulting in an eventual drop in the DP to approximately 250. This may be interpreted as a biphasic mode of depolymerization, but a linear relationship is observed when the log (DP − 250) is plotted against time, suggesting that depolymerization is simply first order.

Cessation at a DP of 250 suggests that extensive cleavages occurred within the noncrystalline (amorphous) regions, producing crystallites. This is analogous to the "leveling off" DP of cellulose observed upon acid hydrolysis in which crystallites are released (3). Highley et al. (13) showed that the overall crystallinity of residual cellulose increases during depolymerization by brown rot fungi. Apparently, the depolymerizing agent is able to cleave through the amorphous regions of the cellulose but not through the cellulose crystallites (4, 15). (Although the DP value of approximately 250 was near the detection limit of our viscosimetric assay [DP =

4C

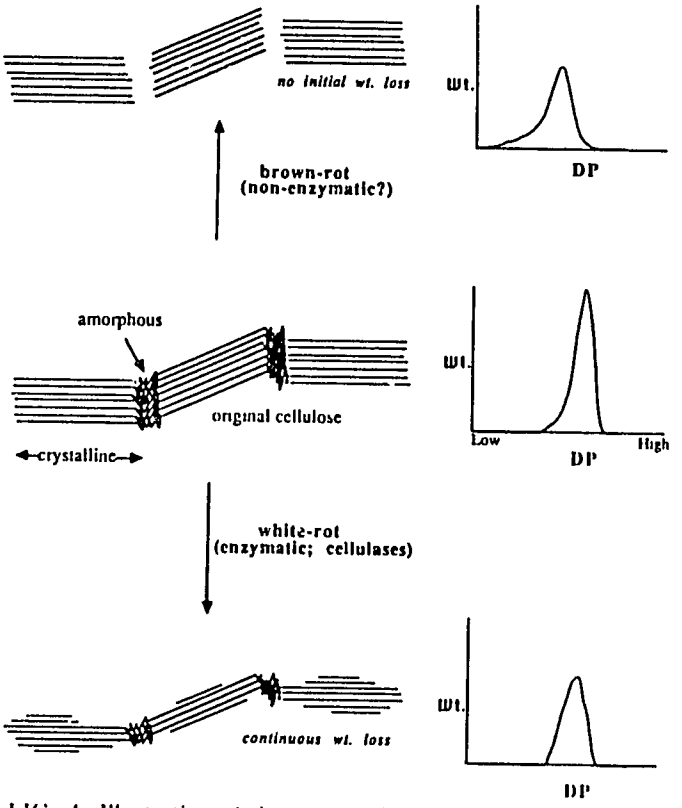

FIG. 4. Illustration of the apparently different modes of degradation of cellulose by *P. placenta* and *P. chrysosporium*. Wt., weight.

196], that it was reached was verified by the molecular size distribution of the depolymerized cellulose [Fig. 2; Table 1]).

The molecular size distribution of the depolymerized cellulose indicated that the increase in polydispersity (DP_w/DP_n) during attack by the brown rot fungus was due to an increasing contribution of fragments of low molecular size. The appearance and accumulation of these fragments indicated that *P. placenta* did not consume the fragments as efficiently as it produced them. The polydispersity calculations gave values higher than those previously reported (13, 15, 24), which is explained by the fact that the cellulose samples prepared in the previous studies were washed with NaOH before analysis, resulting in the removal of low-molecular-size molecules and therefore leading to lower polydispersity. Peak shifts in the molecular size distributions of cellulose during decay by *P. placenta* signify random cleavage completely through the microfibrils at a given site (presumably an amorphous region). That is, the agent worked all the way through the microfibril once it began its attack. Thompson (23) concluded that the same pattern occurs during depolymerization of cellulose by superoxide anion in dimethyl sulfoxide, but we are not aware of any biological precedent.

The time course study of cellulose depolymerization by the white rot fungus *P. chrysosporium* gave markedly different results from those obtained with *P. placenta*, indicating a different mechanism of cellulose degradation, as first recognized by Cowling (4). Unlike *P. placenta*, *P. chrysosporium* continually consumed the cellulose without substantially decreasing the average chain length of the residual cellulose. In accordance with this, Highley (11) reported a decrease in the DP from 2,200 to approximately 1,300 (determined viscosimetrically) in a similar cellulose depolymerization study with several white rot fungi in solid-state cultures. Changes in the molecular size distribution of cellulose in our study during decay by *P. chrysosporium* suggest that initial attack involved the generation of fragments several hundred glucose units long. With time, these were consumed without accumulation of a substantial proportion of fragments of lower DP values. Removal of low-molecular-weight material resulted in low polydispersity values as decay occurred. This suggests that the white rot fungus preferentially consumed the small molecules before generating more.

From these results, it is apparent that the mechanism of cellulose degradation differs markedly between *P. placenta* and *P. chrysosporium*. The brown rot fungus cleaves entirely through the cellulose microfibrils, presumably in the amorphous regions, before utilizing the cellulose, whereas the white rot fungus attacks the surfaces of the microfibrils, consuming the cellulose as it is degraded. These two modes of degradation are illustrated in Fig. 4.

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REGULATORY ASPECTS OF ENDOGLUCANASE PRODUCTION BY THE BROWN-ROT FUNGUS *Gloeophyllum trabeum* *

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Regulation of endoglucanase formation by the brown-rot fungus *Gloeophyllum trabeum* was investigated. This fungus produced endoglucanases in the presence of monosaccharides such as glucose or mannose as the sole carbon source, but the expression of these enzymes was four to five times higher in the presence of cellulose or cellobiose. In a lactose or glucose-containing medium, endoglucanase production was induced by cellobiose. Glucose and glycerol did not repress enzyme production. We concluded that endoglucanase production by brown-rot fungi is inducible by cellulose and not subject to catabolite repression. Cellobiose is the most effective inducer of the system.

Index Descriptors: *Gloeophyllum trabeum* ; brown-rot fungi; cellulases; endoglucanase expression; endoglucanase inducers; cellobiose.

Cellulose is degraded by an extracellular cellulase system, that catalyzes the hydrolysis of this polymer to glucose. The cellulolytic systems of the soft-rot fungus *Trichoderma reesei* and white-rot fungus *Phanerochaete chrysosporium* have been thoroughly investigated (Coughlan and Ljungdahl, 1988). They consist of three basic classes of enzymes: 1,4-ß-D-cellobiohydrolases (EC 3.2.1.91), which release cellobiosyl units from the nonreducing end of cellulose chains; endo-1,4-ß-D-glucanases (EC 3.2.1.4), which cleave internal glycosidic bonds; and 1,4-ß-D-glucosidases (EC 3.2.1.21), which hydrolyze cellobiose and cellooligosaccharides into glucose.

The expression of the cellulolytic system in *T. reesei* is subject to control by both induction and catabolite repression (Merivouri *et al*, 1984). Cellulose is the inducer of the expression of cellulases. Small amounts of constitutive cellulases are secreted releasing the "true" inducer, a low molecular weight compound (El-Gogary *et al*, 1989). The identity of this true inducer is still in dispute: it has been postulated that sophorose is the inducer (Mandels *et al*, 1962; Sternberg and Mandels, 1979), but this disaccharide does not induce all the cellulolytic system components (Messner *et al*, 1988). Other cellulose metabolizing products have also been shown to promote *T. reesei* cellulase formation like cellobiono-1,5-lactone (Iyayi *et al*, 1989) or oxidized cellulose (Kubicek-Pranz *et al*, 1990). Also, under special physiological conditions, cellobiose has been shown to be an inducer (Fritscher *et al*, 1990).

Brown-rotted wood is characterized by extensive degradation of cellulose and hemicellulose but limited degradation of lignin (Eriksson *et al*, 1990). It has been suggested that brown-rot fungi employ a different mechanism for cellulose degradation than that operating in white-rot or soft-rot fungi. Indeed, unique features of cellulose degradation by these fungi have been reported, such as a rapid depolymerization of cellulose during the early stages of wood decay (Kleman-Leyer *et al*, 1992). Highley (1977) has reported that an oxidative system is involved in the initial attack on cellulose, showing that there was a significant increase of carbonyl and carboxyl groups in the cellulose during decay. Because pores in sound wood are too small to allow conventional cellulolytic enzymes to penetrate, it seems probable that the agent responsible for initial cellulose depolymerization is non enzymatic. In this respect, oxygen-derived radical species have been suggested to be implicated in the depolymerizing process. The latter could be produced by the Fenton' s reaction (Fe (II) + H_2O_2) which generates hydroxyl radicals (Halliwell, 1965; Koenigs, 1974; Halliwell and Gutteridge, 1988).

The enzymatic cellulolytic system of brown-rot fungi has not been thoroughly studied, even though some interesting features have been reported. Brown-rot fungi produce endo-1,4-ß-D-glucanases, but no reports exist on the production of exo-1,4-ß-glucanases (Highley, 1973). Thus, these fungi do not seem to degrade crystalline cellulose by the synergistic action between endo- and exo-glucanases, as is

the case in *T. reesei* or *P. chrysosporium*. Endoglucanases produced by the brown-rot fungi *Polyporus schweinitzii* FR and *Lenzites trabea* (syn: *Gloeophyllum trabeum*) have been purified and partially characterized (Bailey *et al*, 1969; Keilich *et al*, 1969; Herr *et al*, 1978). These enzymes have a molecular weight of 45,000 and 29,000 D, respectively.

Striking differences in the regulation of cellulase formation have also been reported; indeed, brown-rot fungi produce cellulases on glucose-grown cultures as the sole carbon source; furthermore, the expression of these enzymes has been claimed to be constitutive (Highley, 1973). In the present work, some regulatory aspects of the synthesis of endoglucanases by the brown-rot fungus *G. trabeum* have been further investigated. The enzymatic production in different carbon sources as well as the effect of glucose, glycerol and cellobiose on the kinetics of cellulase expression in liquid cultures was evaluated.

MATERIALS AND METHODS

Organism

The fungal strain used throughout this study was *G. trabeum* MAD 617-R, kindly provided by the Center for Forest Mycology Research, CFMR (Forest. Products Laboratory, Madison, USA). The fungus was maintained on malt-yeast extract agar slants. Mycelial inocula were prepared, as described elsewhere (Agosin and Odier, 1985).

Conditions of cultivation

G. trabeum was cultivated on 25mM ammonium tartrate, using the carbon sources at initial concentrations of 55 mM, except carboxymethylcellulose (CMC) and galactomannan which were added at 10 g/l. The buffer used was 50mM disodium tartrate, pH 4.5. Mineral salts were added as reported by Kirk *et al* (1986). After inoculation, cultures were incubated on a rotary shaker (150 rpm, 28 °C).

Biomass production was determined as follows: 0.5 ml of a fungal suspension -equivalent to 5 mg dry weight- were added to 100 ml Erlenmeyer flasks containing 50 ml of culture medium. After three days cultivation, cultures were filtered on previously tared filter paper, washed with distilled water, and dried to constant weight at 105 °C.

In order to study the effect of different carbon sources on the expression of endoglucanases by *G. trabeum*, replacement cultivations were used. Pregrown mycelia were obtained inoculating 1 ml of a fungal suspension (corresponding to 10 mg, dry weight) in 250 ml Erlenmeyer flasks containing 100 ml of the same medium supplemented with 1% (w/v) galactomannan (Sigma) and 0.1% (w/v) carbopol. Locust bean galactomannan was employed as carbon source for biomass production, because the highest growth yields were obtained with this polysaccharide (see Table 1). Carbopol, a polymethacrylate derivative, was employed to obtain smaller pellets (Jones *et al* , 1988). After five days, the mycelium was harvested by centrifugation and washed three times with sterile water. Then, mycelium was resuspended in fresh medium without nitrogen and carbon sources and left overnight at 4 °C. Pregrown mycelium was then centrifuged and transferred to the same medium, without nitrogen, using the appropriate carbon source.

The effect of different carbon sources on the production of endoglucanase was studied, employing glycerol, monosaccharides (glucose, mannose, galactose, fructose and xylose), disaccharides (lactose and cellobiose) or celluloses (carboxymethylcellulose (CMC) and avicel). All these compounds were added at initial concentrations of 55mM, except celluloses which were added at 10g/l.

For induction studies, the pregrown mycelium was transferred to 1% (w/v) CMC-, 1%(w/v) lactose- or 1%(w/v) glucose-containing cultures. The inducer was added at the time and concentration indicated.

In order to determine the effect of glucose and glycerol on cellulase formation, pregrown mycelia were transferred to media with 1%(w/v) CMC or 1%(w/v) CMC supplemented with 5.5mM of glucose or 5.5mM glycerol.

The white-rot fungus *P. chrysosporium* BKMF 1767 was used as a reference. It was grown under similar conditions to those employed for *G. trabeum*, although the incubation temperature was 39 °C.

Assay of endoglucanases

The endoglucanase activity of the culture supernatants was determined by viscosity at 30 °C, using 6ml of 0.5% (w/v) carboxymethylcellulose (low viscosity CMC, Sigma) in 50mM sodium acetate, pH 4.5 and 1 ml of 50mM sodium acetate containing dilutions of the culture supernatants. The measurements of viscosity were done in an Ostwald viscometer (Water efflux time at 30 °C: 179 sec), submerged in a water bath at 30 °C. One unit (1 U) of enzyme activity is the amount of enzyme that decreases the relative viscosity in 0.01, per minute, in defined conditions of temperature and pH. The relative viscosity is defined as the ratio between the efflux time of the solution and the efflux time of the buffer.

RESULTS

Biomass production on different carbon sources

The effect of different carbon sources on biomass production by *G. trabeum* is shown in Table 1. When glycerol, xylose, cellobiose, maltose, lactose and CMC were used as carbon source, low biomass yields were obtained. Higher biomass yields were found for glucose and mannose, although the highest one was on galactomannan. On the other hand, galactose and fructose were not metabolized, because the fungus did not grow when these monosaccharides were employed as carbon sources.

Production of endoglucanases on different carbon sources.

Replacement experiments were used to study the effect of different carbon sources on the production of endoglucanases by *G. trabeum*. Pregrown mycelia were transferred to media containing different carbon sources. Endoglucanases were produced in all the carbon sources tested (Fig.1); however, the level of enzymatic activity was different. Three different groups of endoglucanase activity can be distinguished. The highest level was reached on cellobiose, CMC and Avicel (between 20 and 48 U/ml). An intermediate level of production was obtained on glucose, mannose and lactose (about 7 U/ml). Finally the lowest yield of cellulases was obtained on fructose,

galactose, xylose and glycerol (1 U/ml). The highest activity was obtained on cellobiose (48.1 U/ml). On glucose, mannose and lactose the endoglucanase activity was significant, although these activities were about five to six times lower than those obtained on cellobiose.

Induction of endoglucanase production.

It was reported that the expression of endoglucanases in brown-rot fungi is constitutive (Highley, 1973). We reinvestigated this asssumption by studying the effect of cellobiose on the production of these enzymes by *G. trabeum*. Pregrown mycelia were transferred to CMC or lactose-containing media. When cellobiose was added to a CMC-containing culture, no further stimulation of endoglucanase production was found (Fig 2a). However, when cellobiose was added to a lactose-containing medium a significant induction of endoglucanase production was observed (Fig. 2b). The induction occurred about one hour after cellobiose was added to the cultures. If glucose was added instead of cellobiose no induction occurred. (Data not shown)

Catabolite repression studies of endoglucanase production.

Since the level of *G. trabeum* endoglucanase production, on glucose and glycerol-containing media was lower than on cellulose, we studied if these compounds repressed the production of the enzymes when the fungus was placed in a CMC-containing medium. (Fig. 3a). Pregrown

mycelia were transferred to media with CMC or CMC supplemented with glucose or glycerol. Both, glycerol and glucose, retarded the production of endoglucanases. Although the retarding effect of glucose is higher, similar levels of enzymatic activity were reached after ten hours of cultivation. Moreover, we verified that glucose and glycerol at a concentration of 5.5 mM did not inhibit the enzymatic activity of *G. trabeum* endoglucanases (data not shown).

As a reference, the same experiments were conducted with the white-rot fungus *P. chrysosporium* (Fig. 3b). In this fungus, the expression of endoglucanases was totally repressed by glucose, but not by glycerol.

To confirm that the endoglucanase production was not subject to catabolite repression, increasing concentrations of glucose (5-40mM, final concentration) were added to five hour old cultures induced by cellobiose (Fig. 4). Addition of glucose to induced cultures did not decrease the expression of endoglucanases. Likewise, when cellobiose was added to a glucose-containing medium (Fig 5), there was induction. Therefore glucose did not suppress the stimulatory effect of cellobiose. However, the induction was slower than that observed on a lactose-containing medium. Indeed, on lactose, the slope of the inductive curve is two times higher than on glucose.

DISCUSSION

The regulation of the cellulase expression has been extensively studied in *T. reesei* . The expression of these enzymes is under catabolite repression, i.e. cellulases are repressed in the presence of easily metabolizable carbon sources, like glucose, glycerol, etc..., and can be induced by growth on cellulose (Merivuori et al, 1984). Glucose also represses the expression of cellulases in *P. chrysosporium* . It is evident from our results that there are striking differences in the regulation of endoglucanase expression in white-rot or soft-rot fungi, as compared to the brown-rot fungus *G. trabeum* .

Early studies postulated that the production of endoglucanases in brown-rot fungi was constitutive (Highley, 1973). In this work, we have showed that endoglucanase production by *G. trabeum* is inducible. Indeed, although this basidiomycete produces endoglucanases in all the carbon sources tested, three different levels of enzyme formation were observed. The lowest level was obtained on galactose, fructose, xylose and glycerol. Galactose and fructose were not employed as carbon sources by the fungus, possibly because the fungus has not an active transport system for these sugars; hence, the expression level observed on the latter would correspond to the constitutive level of expression of endoglucanases in *G. trabeum*, which is many times higher than that reported for *T. reesei*.

Among the sugars that sustain fungal growth, mannose, glucose

and lactose allowed a significantly higher induction level of endoglucanases than xylose and glycerol. Even though the inducer(s) has not been identified yet. we can speculate that some relationship should exist between some of these sugars or their oxidized/transglycosilated products and the induction of endoglucanase expression. Indeed, cellulase production has been shown to be stimulated by a low content of oxidized end groups in the cellulose (Kubicek-Pranz et al, 1990), probably related with the presence of a constitutive cellulose oxidizing enzyme identified by Vaheri (1983). During the initial hydrolysis of this oxidized cellulose, cellobiono-lactone, another inducer of cellulase formation in *T. reesei* , would be released (Iyayi et al, 1989). This could be related with the generation of oxidized low molecular weight compounds during cellulose depolymerization by brown-rot fungi, which occurs before significant loss in the weight of the polymer (Kleman-Leyer et al, 1992). Some of these oxidized compounds could mediate induction of cellulase synthesis in these fungi. In this respect, it is noteworthy that the addition of cellobiose, mannose or glucose - but not xylose - to crystalline cellulose, resulted in a drastic reduction of the degree of polymerization of the cellulose by the brown-rot fungus Postia placenta (Highley, 1977). Thus, the induction or onset of the depolymerizing system of brown-rot fungi by these sugars is extended here to endoglucanase formation.

The most effective induction was obtained with avicel, CMC and cellobiose. Cellobiose is an effective inducer even when the fungus is

grown on lactose or glucose as the sole carbon sources. However, when cellobiose was added to cultures with CMC no further stimulation of endoglucanases production was observed. These results could be explained by a saturation of the inductive system in a CMC-containing medium. Similar effects were observed in *T. reesei* grown in cellulose and induced by sophorose (Fritscher et al, 1990)

On the other hand, in other cellulolytic fungi, as *P. chrysosporium*, it has also been shown that cellobiose is the inducer (Eriksson et al, 1978). In contrast, in *T. reesei*, sophorose is the most potent cellulase-inducing compound identified (Mandels et al., 1962; Sternberg and Mandels, 1979). However, it was recently shown that cellobiose can induce the expression of cellulases, but only when the hydrolysis of this compound is artificially decreased by nojirimycin (Fritscher et al, 1990).

One important difference between the brown-rot fungus *G. trabeum*, and the other systems described, i.e. *T. reesei* and *P. chrysosporium*, is that in the presence of glucose, *G. trabeum* expresses endoglucanases; moreover, glucose added to induced cultures at concentrations of 40mM or higher, did not repress the production of endoglucanases. On the other hand, we were able to show that induction of the endoglucanases was possible with cellobiose, when the mycelia were in the presence of glucose. Indeed, when glucose was added to the cultures from the beginning, a retardation of the stimulatory effect of the inducer was observed. One of the reasons that could explain this retardation is a competition between glucose and the inducer for their transport inside the cell, and not to catabolite

repression. Therefore, we can argue that the production of endoglucanases in *G. trabeum* is not under catabolite repression and that the lower levels of expression observed on glucose or lactose resulted from the absence of a good inducer.

Recently, a similar conclusion was obtained by Messner and Kubicek (1991). They observed that *T. reesei* produced cellobiohydrolase I in the presence of glucose when the mycelia were pregrown on lactose. The level of enzymatic expression was, however, very low and the enzyme could only be detected using monoclonal antibodies . These authors suggested that the expression of cellulases in *T. reesei* is not under catabolite repression and that these enzymes have a low expression on glucose due to the absence of the inducer. However, these authors did not explain the results obtained by EL-Gogary et al, (1989), where a catabolite repression effect is clearly observed. Indeed, EL-Gogary et al (1989) demostrated that *T. reesei* did not produce cellobiohydrolase I gene transcripts when the fungus was grown on Avicel for 21 hours and then exposed to glucose for 1 hour.

In *T. reesei* , cellulose or sophorose induce the expression of cellulases at the transcriptional level (EL-Gogary et al, 1989). In our induction studies by cellobiose, we observed that the inductive effect had a lag of approximately one hour after the inducer was added. The latter suggests de novo RNA and/or protein synthesis. Current experiments are directed towards the study whether the regulation of endoglucanases expression occurs at the transcriptional or translational level.

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Figure 1: Effect of different carbon sources on the production of endoglucanases by *Gloeophyllum trabeum* . 25 ml (corresponding to 170 mg, dry weight) of pregrown mycelia were transferred to media without nitrogen and with different carbon sources. The carbon sources used were Glycerol (Gly), Glucose (Glc), Mannose (Man), Fructose (Fru), Galactose (Gal), Xylose (Xyl) Cellobiose (Cel), Lactose (Lac), Carboxymethylcellulose (CMC) and Avicel (AVI) . These were added at initial concentration of 55mM, except for the celluloses, CMC and Avicel, which were added at 1% (w/v). The endoglucanase activity was measured after 18 hrs of cultivation.

Figure 2: Effect of cellobiose on the production of endoglucanases by *Gloeophyllum trabeum.* (a) Pregrown mycelia were transferred to media containing 1% (w/v) of CMC (□); and 1% (w/v) CMC plus 5.5mM cellobiose (◆) . (b) Pregrown mycelia were transferred to media containing 1% (w/v) lactose (□). At the time indicated by the arrow 5.5mM cellobiose was added (◆). Samples were taken each one hour.

Figure 3: Production of endoglucanases during cultivation of *Gloeophyllum trabeum* (a), or *Phanerochaete chrysosporium* (b); in the presence of 1% (w/v) CMC (■) , 1% (w/v) CMC plus 5.5mM glucose (□) and 1% (w/v) CMC plus 5.5mM glycerol (◆).

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Figure 4: Effect of glucose on endoglucanase production in cellobiose-induced cultures. Pregrown mycelium was transferred to incubation medium containing 1% (w/v) lactose. After three hours of cultivation, cellobiose was added at a concentration of 5.5mM. Then, at the time indicated by the arrow, increasing concentrations of glucose were added. (□) 5mM glucose; (■) 20mM glucose and (◇) 40mM glucose.

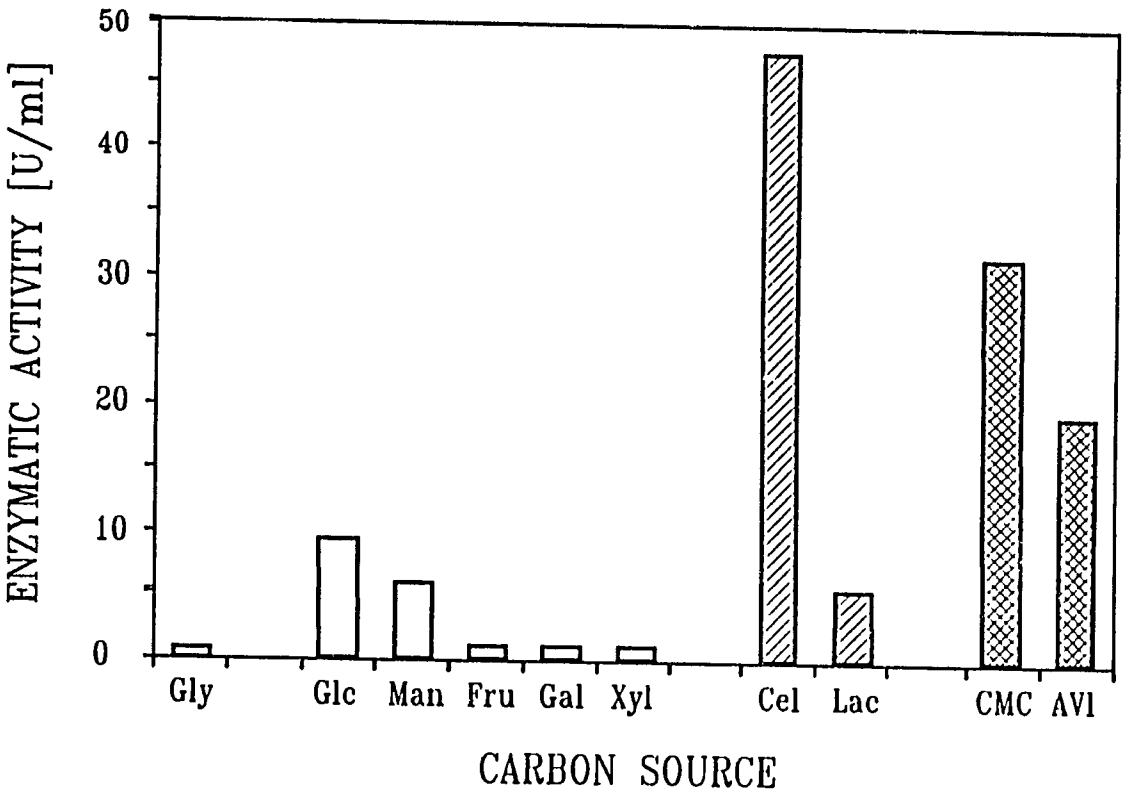
Figure 5: Effect of cellobiose in the endoglucanase production on a glucose-containing medium. Pregrown mycelium was transferred to a medium containing 1% (w/v) glucose (□). Cellobiose (5.5 mM) was added at the time indicated by the arrow (◆).

TABLE 1

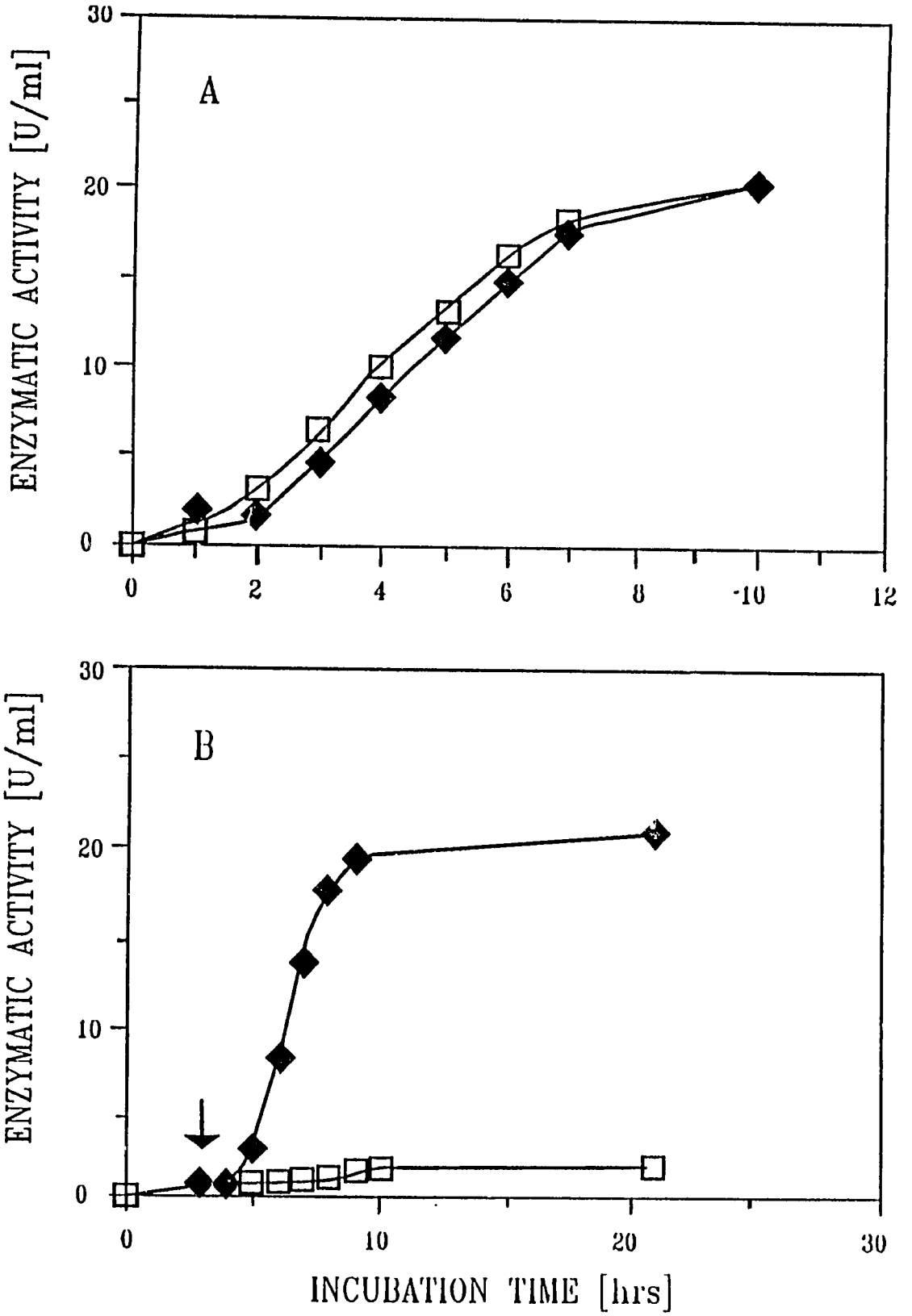
Biomass production by *Gloeophyllum trabeum* on different carbon sources

Carbon source	Mycelial dry weight (mg)
Glycerol	13.9
Fructose	4.3
Galactose	4.7
Xylose	18.9
Glucose	42.4
Mannose	45.6
Cellobiose	18.8
Maltose	25.8
Lactose	22.7
Carboxymethylcellulose	24.3
Galactomannan	164.6

The concentration of all the studied carbon sources was 55mM, except for CMC and galactomannan which were 1% (w/v). In each case, the mycelium was harvested after three days of cultivation.


ENZYMATIC ACTIVITY [U/ml]
50
40
30
20
10
0
Gly
Glc
Man
Fru
Gal
Xyl
Cel
Lac
CMC
AVl
CARBON SOURCE

EFFECT OF CELLOBIOSE ON ENDOGLUCANASE PRODUCTION BY *Gloeophyllum trabeum*

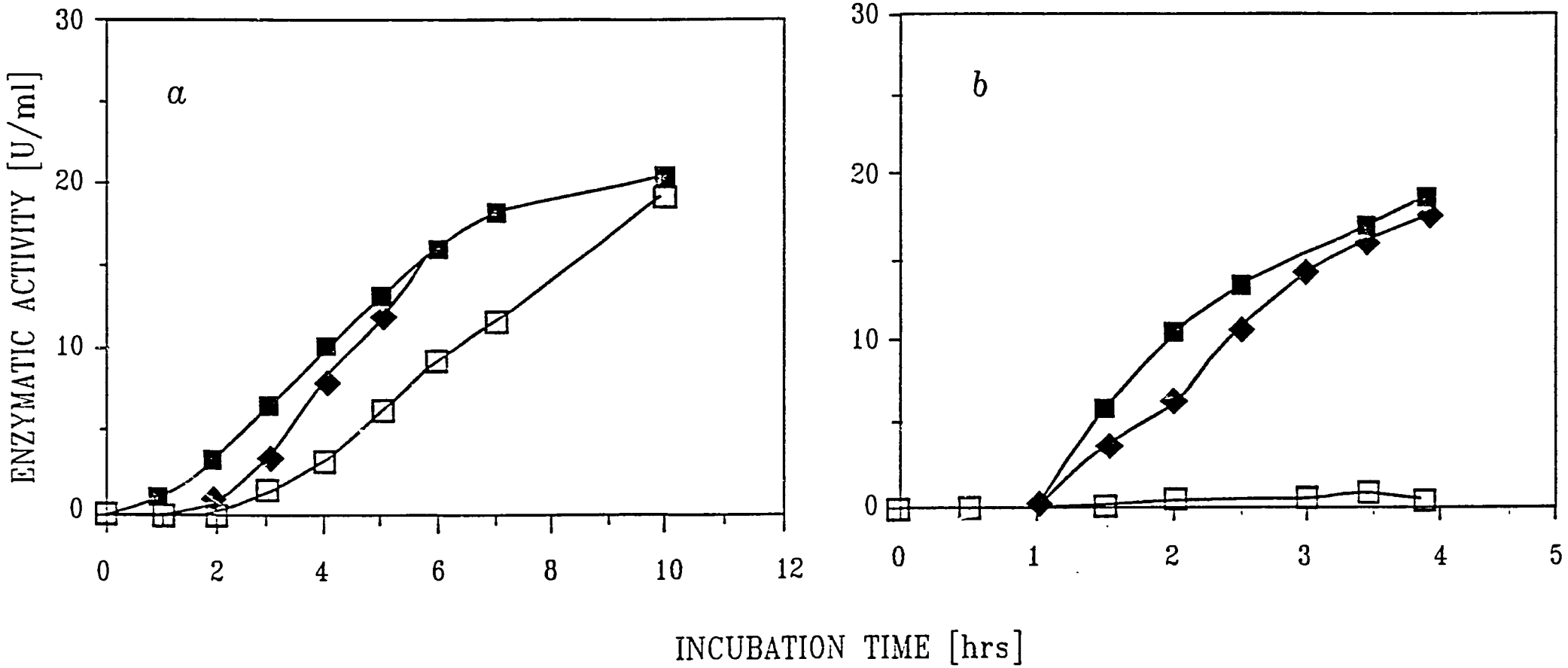


A. CMC-containing medium: 1% CMC (□); 1% CMC + 5.5mM cellobiose (◆)

B. Lactose-containing medium: 1% lactose (□); 1% lactose + 5.5mM cellobiose (◆)

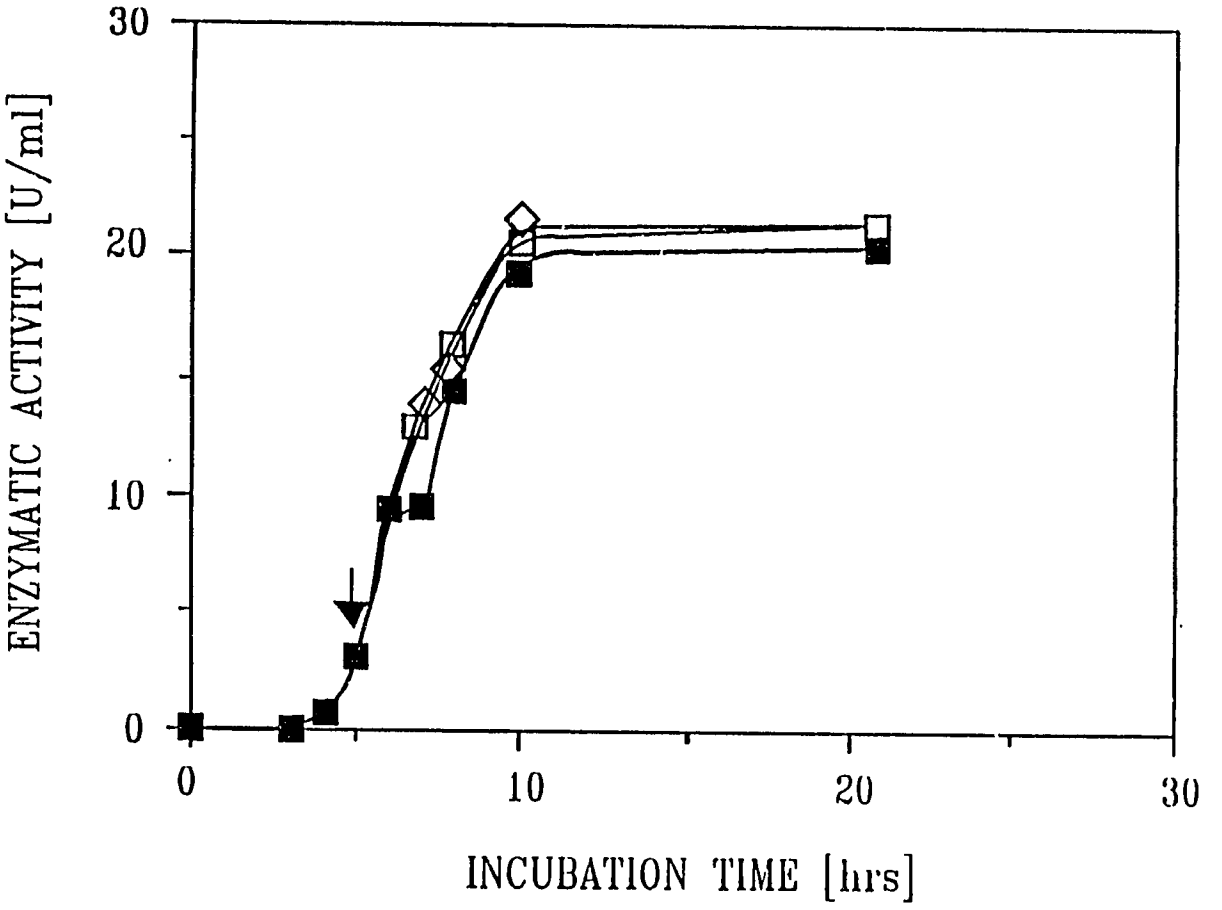
PRODUCTION OF ENDOGLUCANASES DURING CULTIVATION OF

Gloeophyllum trabeum (a) AND *Phanerochaete chrysosporium* (b)



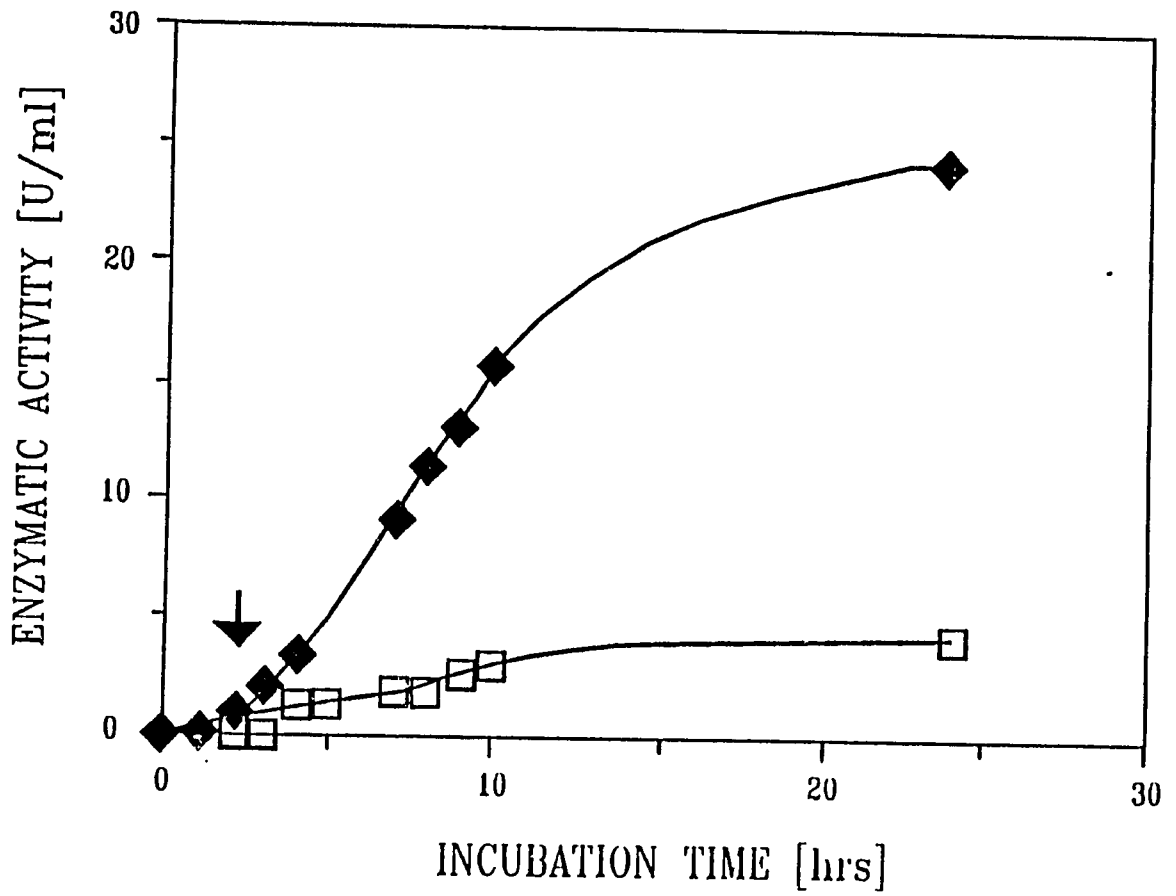
(■) 1% CMC ; (□) 1% CMC + 5.5mM glucose ; (◆) 1% CMC + 5.5mM glycerol

EFFECT OF GLUCOSE ON ENDOGLUCANASE PRODUCTION BY *Gloephyllum trabeum* IN CELLOBIOSE-INDUCED CULTURES



Carbon source was 1% lactose. After 3 hr cultivation, 5.5mM cellobiose was added as inductor. Then,glucose was added at 5mM (□), 20mM (■) or 40mM (◇) concentration at the time indicated by arrow.

EFFECT OF CELLOBIOSE ON ENDOGLUCANASE PRODUCTION BY *Gloeophyllum trabeum* ON A GLUCOSE-CONTAINING MEDIUM



To culture medium containing 1% glucose (□), 5.5mM cellobiose (◆) was added at the time indicated by arrow

PAPER 6

Regulation of endoglucanase formation by the brown-rot fungus *Gloeophyllum trabeum*

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Universidad Católica de Chile, Casilla 306, Santiago, Chile.

Regulation of endo-1,4-ß-D-glucanase formation by the brown-rot fungus *Gloeophyllum trabeum* was investigated. Results showed that the fungus produced several enzymes with endoglucanase activity in the presence of glucose or mannose as the sole carbon source, even though the expression of these enzymes was much higher with cellobiose or cellulose. Moreover, glucose did not repress enzyme production. We concluded that endoglucanase production in this fungus is not subject to catabolite repression, but it is inducible by cellulose. Furthermore, cellobiose is a very effective inducer of the system.

1. INTRODUCTION

The most destructive form of wood decay, brown rot, is caused by a limited number of basidiomycetous fungi that damage wood by rapidly depolymerizing the cellulose component. Generally, polysaccharide depolymerization occurs before significant loss in the weight of wood. At advanced stages of decay, structural polysaccharides are quantitatively removed, and a modified lignin residue remains[1,2,3]. Hence, brown rot fungi might possess a unique cellulolytic system which enable these fungi to circumvent the lignin barrier in gaining access to polysaccharides. Furthermore, the cellulolytic system might remain active towards increasing lignin contents in decaying wood.

The biochemistry and physiology of the cellulolytic system of brown rot fungi has received little attention, despite the uniqueness of cellulose degradation by these fungi. Oxidative depolymerization of cellulose during the early stages of decay has been demonstrated[4]. Considering that pores of sound wood are too small to allow "classical" cellulases to penetrate, a non enzymatic mechanism related with the generation of oxygen-derived radicals has been suggested to be responsible for the depolymerizing process[5,6].

The enzymatic cellulolytic system of brown-rot fungi has not been thoroughly studied, even though some interesting features have been reported. Brown-rot fungi produce endo-1,4-ß-D-glucanases, but no reports are available on the production of exo-1,4-ß-glucanases[7]. Thus, these fungi do not seem to degrade crystalline cellulose by the synergistic action between endo- and exo-glucanases, as is the case in *T. reesei* or *P. chrysosporium*.

Striking differences in the regulation of cellulase formation have also been reported; indeed, brown-rot fungi produce cellulases on glucose-grown cultures as the sole carbon source; furthermore, the expression of these enzymes has been claimed to be constitutive[7]. In the present work, some regulatory aspects of endoglucanase synthesis by the brown-rot fungus *G. trabeum* have been reassessed. N-terminal aminoacid sequence and homology of the major endoglucanases secreted by the fungus in liquid cultures was also determined.

2. MATERIALS AND METHODS

2.1 Organism

Gloeophyllum trabeum MAD 617-R, kindly provided by the Center for Forest Mycology Research (Forest Products Laboratory, Madison, USA) was used throughout this study.

2.2 Conditions of cultivation

G. trabeum was cultivated on 25mM ammonium tartrate, using the carbon sources at initial concentrations of 55 mM, except carboxymethylcellulose (CMC) which was added at 10 g/l. The buffer used was 50mM disodium tartrate, pH 4.5. After inoculation, cultures were incubated on a rotary shaker (150 rpm, 28 °C).

In order to study the effect of different carbon sources on the expression of endoglucanases by *G. trabeum*, replacement cultivations employing pregrown mycelia were used. This inoculum was transferred to the different culture media containing the appropriate carbon source, but without nitrogen.

For induction studies, the pregrown mycelium was transferred to 1% (w/v) CMC-, 1%(w/v) lactose- or 1%(w/v) glucose-containing cultures. The inducer was added at the time and concentration indicated.

2.3 Assay of endoglucanases

Endoglucanase activity was determined viscosimetrically in an Ostwald viscometer at 30°C, using 6ml of 0.5% (w/v) carboxymethylcellulose (low viscosity CMC, Sigma) in 50mM sodium acetate, pH 4.5 .

2.4 Electrophoretic determination of endoglucanases.

Culture supernatants were analyzed by PAGE in non-denaturing conditions. After protein separation (3h at 4°C, 10mA current), polyacrylamide gels were preincubated in 50mM sodium acetate pH 4.5 and laid over an agarose gel containing 0.3% CMC. Endoglucanases were visualized as described by Bertheau et al[8].

For identification of the two major endoglucanases, the bands showing endoglucanase activity were eluted from the non-denaturing gel and precipitated with 10% TCA. Also, extracellular proteins produced on different culture media were concentrated 100x by ultrafiltration, employing 3,000 or 10,000D cut off polysulfonated membranes. Concentrated supernatants, as well as eluted endoglucanases, were separated by SDS-PAGE on gels containing 12% acrylamide and 0.1% SDS, according to Laemmli[9]. Proteins were stained using the Coomassie blue method.

2.5 Determination of N-terminal aminoacid sequence of endoglucanases

The N-terminal amino acid sequence of the two major endoglucanases was determined after protein transfer to a PVDF membrane as described by Matsudaira[10].

3. RESULTS AND DISCUSSION

3.1 ENDOGLUCANASES PRODUCED ON DIFFERENT CARBON SOURCES.

Non-denaturing PAGE of culture supernatants of *G. trabeum* grown on different carbon sources showed that two major endoglucanases were produced in mono-, di- and polysaccharides, as well (Fig.1). Three other minor bands are also seen in the gel, at least for cellobiose, CMC and Avicel. These results confirm and extend those from Highley[7] concerning endoglucanase formation on glucose-containing media by brown-rot fungi.

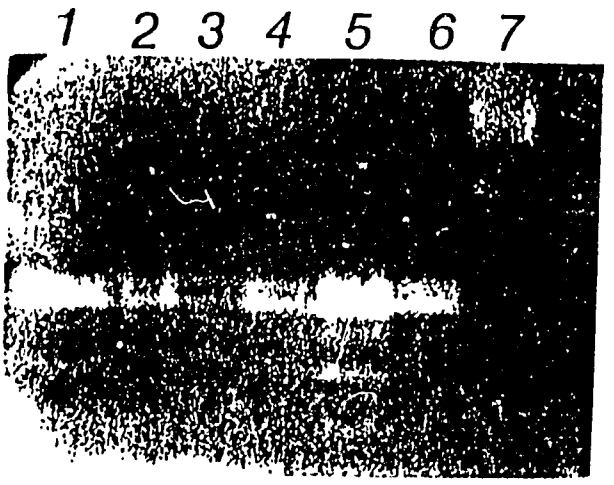

Figure 1. Endoglucanases produced by *Gloeophyllum trabeum* on different carbon sources. Crude culture supernatants (10ul) were applied to each lane. Lanes 1-6, *Gloeophyllum trabeum* grown on CMC (1), glucose (2), fructose (3), lactose (4), cellobiose (5) and Avicel (6). Lane 7, *Phanerochaete chrysosporium* grown on Avicel.

3.2 INDUCTION AND CATABOLITE REPRESSION OF ENDOGLUCANASE FORMATION

The effect of cellobiose on the kinetics of endoglucanase formation by *G. trabeum* was studied. When cellobiose was added to a CMC-containing culture, no further stimulation was found (Fig.2a). However, when cellobiose was added to a lactose-containing medium, a very high induction of endoglucanase production was observed (Fig.2b). One possible explanation for this difference could be that the inductive system is saturated in the CMC-medium, as shown for *T. reesei* grown on cellulose[11]. In all cases the induction occurred about 1 hour after cellobiose addition, suggesting *de novo* RNA and/or protein synthesis.

Cellobiose induction was also found in a glucose-contaning medium (Fig.2c). Thus, glucose did not suppress the stimulatory effect of cellobiose, even though induction was slower than on the lactose-containing medium. Competition for uptake of these sugars inside the cell - and not catabolite repression - can explain this retardation, as recently described for *T. reesei* [12]. Therefore, we can argue that production of endoglucanases in *G. trabeum* is not under catabolite repression. This could be related with the unique mechanism of early decay of wood developed by brown-rot fungi, which results in the release of increasing amounts of low molecular weight carbohydrates during cellulose depolymerization[13].

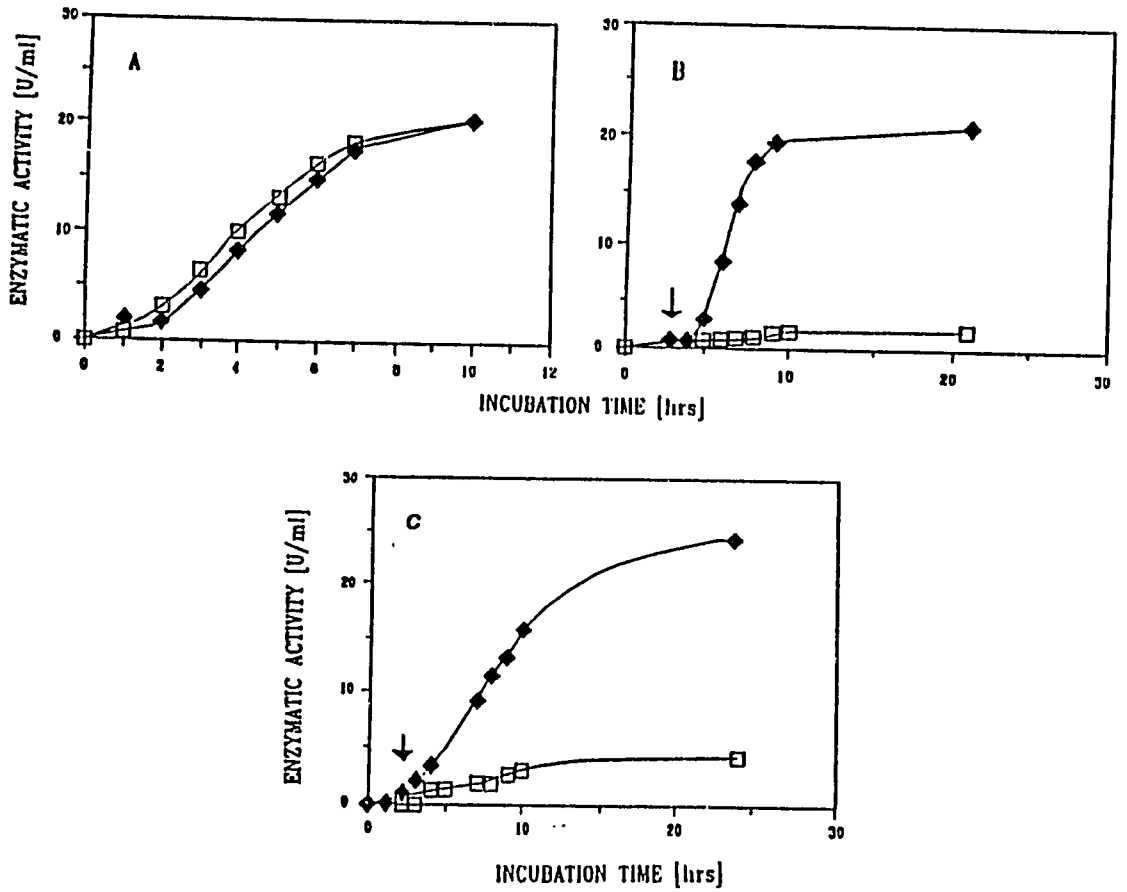

Figure 2. Effect of cellobiose on endoglucanase production by *Gloeophyllum trabeum* on CMC (A), lactose (B) and glucose (C). A.- 1% CMC (□); 1% CMC and 5.5mM cellobiose (♦). Cellobiose and CMC were added at the same time. B.- 1% lactose (□); 1% lactose and 5.5mM cellobiose. C.- 1% glucose (□); 1% glucose and 5.5mM cellobiose (♦). Cellobiose was added at the time indicated by the arrow.

3.3 N-TERMINAL AMINOACID SEQUENCES OF THE TWO MAJOR ENDOGLUCANASES

N-terminal aminoacid sequences of the two major endoglucanases were compared. For this purpose, we took advantage of previous results[14] which showed that only tiny amounts of endoglucanases are expressed when galactose is employed as the culture carbon source. Hence, the proteins secreted in cellobiose and galactose were compared by SDS-PAGE (Fig. 3). Proteins corresponding to bands 1 and 2 are mainly expressed in cellobiose growing cultures, indicating that these proteins might correspond to the major endoglucanases. To confirm this presumption, the two adjoining major endoglucanase activities were eluted separately from non-denaturing gels and run in parallel with concentrated supernatants. Results clearly showed that band 1 corresponds to EG I (Fig. 3). Unfortunately, EGII could never be visualized in the gels. However, SDS/Western blotting/inmunostaining techniques using polyclonal antibodies against EG I of *P. chrysosporium* mainly reacted with band 2 (Cotoras, M., unpublished results). Hence, it is very likely that EG I and EG II correspond to bands 1 and 2, respectively. Their molecular weight, as estimated from SDS-PAGE, was 36,000 and 32,000Da, respectively.

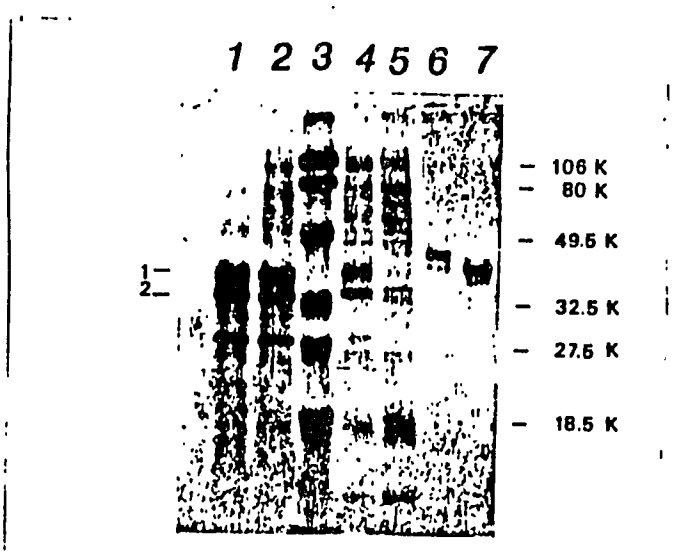

Figure 3. SDS-page on filtrates from cellobiose and galactose culture media of *Gloeophyllum trabeum*. Lane 1, 3,000 D cut off cellobiose supernatant; Lane 2, 10,000 D cut off cellobiose supernatant; Lane 3, marker proteins; Lane 4, cellobiose cultures; Lane 5, glactose; Lane 6, EGI eluted from non-denaturaly gels; Lane 7, idem.

N-terminal aminoacid sequences of the two major endoglucanases secreted by *G. trabeum* are identical (Fig. 4). Thus, it is very likely that EGII arises from EG I by proteolytic modification or partial deglycosylation of the former. This type of events has recently been proved for EG-III of *T. reesei*[15]. Furthermore, it is noteworthy that a region comprising 10 consecutive aminoacids of the N-terminal of EG from *G. trabeum* is homologous to a region close to the O-glycosylated domain of EG III of *T. reesei* [16] (Fig. 5). These results also indicate that the N-terminal region of EG I of *G. trabeum* would not contain a cellulose-binding domain.

EG I:

5 10 15 20 25 30
V-T-G-P-A-P-L-K-F-A-G-V-N-I-A-G-F-D-F-G-X-G-T-D-G-T-X-H-V-S-G-a

EG II:

5 10 15 20 25 30
V-T-G-P-A-P-L-K-F-A-G-V-N-I-A-G-F-D-F-G-G-G-I-d-G-T-X-H-V-S-G-a

Figure 4 N-terminal aminoacid sequence of two major endoglucanases secreted by *Gloeophyllum trabeum*.

```
                                10                  20                  30
EG-I   G. trabeum     V T G P A P L K F A G V N I A G F D F G G G T D G T X H V S G
                        . .   . . X : : : : : : : : : : :   . : : : X
EG-III T. reesei      P P T S S G V R F A G V N I A G F D F G C T T D G T C V T S K
                        90                  100                 110
```

Figure 5 Homology between the sequences of EG-I from *Gloeophyllum trabeum* and EG-III from *Trichoderma reesei*.

CONCLUSIONS

1./ The cellulolytic system of the brown-rot fungus *G. trabeum* is clearly inducible by cellulose and cellobiose. The possibility that more than one inducer could mediate the induction of endoglucanase synthesis is currently under investigation.

2./ The cellulolytic system of *G. trabeum* is not subject to catabolite repression. This "deregulation" could be related with the early non-specific release of low-molecular weight compounds during initial depolymerization of cellulose by these fungi.

3./ Identical N-terminal aminoacid sequences were obtained for the two major endoglucanases secreted by *G. trabeum*, suggesting post-traductional modifications from a single major enzyme.

ACKNOWLEDGEMENTS

The study of aminoacid sequences of endoglucanases was made in collaboration with Drs. D. Cullen and T.K. Kirk of the Institute of Microbial and Biochemical Technology, Forest Products Laboratory, Madison, Wisconsin. We are also indebted to Dr. R. Niece, UW Biotechnology Center, Madison, for running aminoacid analyses. This work was supported by grants AID Nº 76542, FONDECYT Nº 91-0794 and FONDECYT Nº 92-0045.

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THESES

THESIS 1

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INTA
UNIVERSIDAD DE CHILE

SISTEMA DEGRADATIVO DE RESIDUOS LIGNOCELULOSICOS POR HONGOS DE PUDRICION PARDA

TESIS PARA OPTAR AL TÍTULO
DE BIOQUÍMICO.

EDUARDO ARTURO ROJAS CARTES

ENERO 1989

18

SUMMARY

The degradation of lignocellulosic material by brown-rot fungi produce an extensive depolymerization of polysaccharides without removal of lignin. This characteristic make them potential candidates for the direct bioconversion of wood polysaccharides in fermentable sugars. The mechanism employed by these fungi to produce this characteristic degradation is not known yet. The capability to evade the lignin-polysaccharide network, should indicate that the depolymerizing agent could be a low molecular weight chemical compound.

In order to verify this mechanism a solid fermentation of pine sawdust with two strains of brown rotters, Gloeophyllum trabeum and Wolfiporia cocos was made. The results of these experiments showed the characteristic degradation of polysaccharides and the slight removal of lignin. Using pine holocellulose (delignified sawdust) as sustrate, a strong depolymerization of polysaccharides was observed.

The strong decrease of pH observed during the pine sawdust biodegradation is due to organic acids produced by fungi. G. trabeum, in liquid cultures, produced glycolic and /or glyoxalic acid and a non identified acid. However, W. cocos produced large amounts of oxalic acid in addition to glycolic and/or glyoxalic acid.

The oxidatives changes produced by the depolymerizing sistem of brown-rot fungi were not detected using decoloration of Remazol brilliant blue R test. It is due to the low sensitivity of the Remazol test.

The generation oxygen derivated free radicals showed that •OH is highly reactive. Hydroxiethylcellulose and crystalline cellulose were strongly depolymerizated by this specie. HO2• depolymerized crystalline cellulose sligthy and singlet oxygen made no changes in the degree of polymerization of hydroxiiethylcellulose.

THESIS 2

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DEGRADACION DE COMPUESTOS MODELO TIPO LIGNINA POR HONGOS DE PUDRICION PARDA

TESIS DE GRADO PARA OPTAR AL
TÍTULO DE BIOQUÍMICO

EDUARDO ESPEJO DUMENEZ

MARZO 1989

30

SUMMARY

Brown-rot fungi, which occur predominantly in softwoods, possess the unique ability to remove structural polysaccharides in native fully lignified tissues with only a slight depletion of lignin. However, this slight degradation is accompanied by largely oxidative events, principally demethylation of phenolic and non phenolic units. The mechanism employed by these fungi to remove the polysaccharide fraction in spite of the barrier of lignin is unknown. This phenomenon makes them potential agents for the direct bioconversion of softwood polysaccharides into fermentable sugars.

In order to clarify the reactions produced during the process of lignin degradation, the metabolism of lignin-model compounds by two species of brown-rot fungi (Wolliporia cocos and Gloeophyllum trabeum) was investigated using low-nitrogen media. Stationary liquid cultures were employed to study their substrate degrading abilities. Three dimers with a ß-1 linkage and three different stages of oxidation in the alkylic chain were utilized (dimethoxybenzil (VI), anisoin (IV), dihydroanisoin (V)). From the catabolism of these models, p-anisaldehyde and dihydroanisoin were identified. The first compound resulted from the rupture of the Cα-Cß linkage and the second compound came from the anisoin reduction.

The generation of radicals species derived from oxygen, showed the high reactivity of the •OH. This radical produced the rupture of anison and dihydroanisoin with the production of p-anisaldehyde and other unidentified compounds, but it had no action against dimetoxybenzil. Superoxide was not able to produce modifications of these model compounds.

THESIS 3

Pontificia Universidad Católica de Chile
Escuela de Ingeniería
Departamento de Ingeniería Química

BIOCONVERSION DE EUCALIPTUS EXPLOTADO - USO EN RESINAS FENOLICAS MODIFICADAS

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Memoria para optar al Título de
Ingeniero Civil de Industrias con
Mención en Química

Profesor Guía:
EDUARDO AGOSIN T.

Santiago de Chile, 1991

RESUMEN

Los hongos de pudrición parda se caracterizan por la capacidad de degradar los polisacáridos de la madera sin degradar mayormente el polímero de lignina. Sin embargo, se ha determinado que este polímero aromático, sufre principalmente modificaciones de carácter oxidativo, de preferencia demetilación del anillo aromático. Este fenómeno, los convierte en agentes potenciales para la modificación de la lignina y su posterior uso en resinas fenólicas modificadas.

Para estudiar este sistema demetilante y las demás modificaciones de la lignina, ésta se aisló de madera de eucaliptus previamente explotada, para facilitar su extracción, obteniéndose un rendimiento de extracción de lignina del 44,5%. Este sustrato, junto con holocelulosa de pino, fue fermentado bajo distintas condiciones de aireación y contenido de lignina en el medio. Estos estudios mostraron que el sistema demetilante alcanza un grado de demetilación de 50%, a la vez que presenta características oxidativas.

La lignina funcionalizada presentó una extensa demetilación, mientras que conservó su peso molecular, lo cual la hace atractiva para su utilización en resinas fenólicas modificadas. Se formuló una resina con 15% de reemplazo de fenol por lignina funcionalizada en forma natural, la cual se ajustó a las normas requeridas.

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REPORTS

REPORT 1

Low molecular weight compounds secreted by the brown-rot fungus *Poria placenta* during the depolymerization of cotton cellulose

Eduardo Agosin T

(August-November 1988)

REPORT OF ACTIVITIES DONE AT THE IMBT, FOREST PRODUCTS LAB, MADISON WISCONSIN

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INTRODUCTION

Brown-rot fungi are represented by a limited number of basidiomycetes that damage wood structure primarily by causing a rapid depolymerization of the cellulose component. This happens before any substantial loss in the weight of wood occurs. At advanced stages of decay, structural polysaccharides are quantitatively removed, but lignin accumulates (Cowling, 1961).

The biological components of the cellulolytic system of brown-rot fungi have received little attention, despite the uniqueness of the mechanism involved. However, since most of the pore sizes in sound wood are too small to allow cellulolytic enzymes to penetrate inside wood, it is very probable that the agent responsible for initial cellulose depolymerization was non-enzymatic. Furthermore, Flournoy at al (1989) have recently studied the increase in the pore volume available during wood decay by Poria placenta and concluded that the agent responsible for opening up the wood structure during brown-rot decay should have a molecular weight of less than 6,000.

Brown-rotted cellulose has been shown to contain high amounts of carboxyl and carbonyl residues (Highley, 1977) suggesting that the diffusable agent is strongly oxidant. Koenigs (1974) proposed that the latter arises from H_2O_2, which, in the presence of Fe (II), breaks down into hydroxyl radicals (OH.). This oxygen-derived radical species has been shown to duplicate the brown-rot chemistry (Halliwell, 1965, 1978; Kirk et al, 1989). Simetrical molecular weight distribution of a highly decayed cellulose sample, indicating random cleavage of all the cellulose fibers, is also consistent with a possible role of the Fenton reagent in cellulose depolymerization by brown-rot fungi (Highley et al, 1989).

Despite the importance of wood decay by these fungi and the potential of their unique cellulolytic system for efficient saccharification of fully lignified substrates (Agosin et al, 1989), the cultural conditions controlling cellulose depolymerization have not been determined yet. However, it has been shown that liquid cultures do not allow the active depolymerizing system to develop (Highley, 1973). Furthermore, recent experiments where cotton was placed in contact with the agar medium in order to saturate the sample, neither allowed the expression of the depolymerizing system (Highley et al, 1989).

The aim of this work was to identify and partially characterize the low molecular weight compounds secreted during the active phase of cellulose depolymerization by the brown-rot fungus Poria placenta in order to assess their possible role in the mechanism of brown-rot decay of wood. The threshold of water activity necessary for depolymerizing cellulose, as well as the evolution of the molecular size distribution of cellulose during decay were also investigated.

MATERIALS AND METHODS

Fungus. The brown-rot fungus Poria placenta (MAD-698) was employed in all the experiments. The fungus was grown at 28°C in a malt-yeast extract agar medium before inoculation.

Cellulose decay studies. All decay studies of cellulose (purified cotton type A-600, Hercules) were conducted following the soil block procedure of the American Society for Testing and Materials (1971), as described by Highley (1977).

Effect of water content. The effect of dry matter content on cellulose depolymerization was tested in triplicate using the following system: after a 4 weeks decay period, colonized wood blocks were transferred from the culture bottles to Petri dishes and placed in contact with 200mg of cotton cellulose at various dry matter contents (2, 10, 20, 30 and 40% w/v). During the experiment, Petri dishes were maintained in a thermorregulated chamber at a 90% humidity level, in order to avoid sample dessication. After 4 weeks of incubation at 28°C, the cellulose was removed, dried at 80°C and the resulting DP was determined as described below.

Radiolabeling techniques. 4 x 100mg cotton "balls" were equidistantly placed at each corner of a 4 weeks-decayed feeder strip. After 10 days of cellulose colonization, i.e. when the cultures were strongly depolymerizing the cellulose, 15ul of 14C-glucose (ICN; specific activity 10.9mCi/mmol) containing 7.5x106 dpm, were fed at the center of the feeder strip, at 1-1.5 cm distance of each cotton ball. Aerial mycelium was gently removed at this place before feeding. A total of 10 culture bottles were radiolabeled under these conditions. Another 20 cultures without 14C-glucose were also run under the same conditions. Every 2 days, the radioactive cultures were flushed during 15 min with air into a CO_2 trapping fluid ( ) and $^{14}CO_2$ was measured in a scintillation counter.

Separation of low molecular weight (MW) compounds. The secretion of (14C)-low MW compounds during cellulose depolymerization was followed by randomly harvesting 6 cotton balls from the "hot" cultures at 1, 3 and 7 days after the addition of the label, i.e. 11, 13 and 17 days of cellulose decay. The 14C-labeled samples were mixed with 4x100mg cellulose from "cold cultures" (total weight: 1,0g) and extracted twice with 4 and 2 ml of 500mM NaCl solution, respectively. After each extraction (2h, 20°C), the sample was squeezed, centrifuged and 50ul of supernatant were taken for 14C measurement.

The clarified supernatant (1.5 ml) was applied to a 50x1cm Sephadex G-25 column. Distilled water was used as eluent and 1mL

fractions were collected. Each tube was assayed for 14C, absorbance at 280nm and ninhydrin reaction. Transition metals were determined by atomic emission spectrometry on 3ml pooled eluted fractions of supernatants extracted from cellulose decayed for 17 days. Oxydized insulin chain A (MW=2530), ferrichrome A (MW=1100) and veratryl alcohol (MW=165) were employed to calibrate the column.

For comparison, the low molecular weight compounds present in a highly decayed brown-rotted cellulose (50 days incubation) were also separated by gel permeation chromatography following the same procedure.

Mycellaneous procedures

The solubility of cellulose in 1% sodium hydroxyde was tested as described in the TAPPI Standard T 4m-44.

The average degree of polymerization (DP) was measured viscosimetrically (Cowling, 1960) after cellulose solubilization with cupriethylenediamine hydroxide solvent (Ecusta Paper Corp.)

The evolution of the molecular weight distribution of cotton cellulose during brown-rot decay was followed by size exclusion chromatography of cellulose tricarbanilate derivatives, as described by Wood et al (1986).

The presence of primary amines in the column eluent was assayed with the ninhydrin reagent (Moore and Stein , 1947).

RESULTS

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

The effect of dry matter content on cellulose depolymerization is shown in Table 1. Depolymerization was significantly higher for cellulose containing at least 30% dry weight than for lower dry matter contents. Furthermore, no difference was found in the extent of cellulose depolymerization among dry matter contents higher than 30%, as well as for those that contained less than 30% dry matter. This suggests that the threshold for an effective action of the agent responsible for cellulose depolymerization is at least 30% dry matter of the decaying cellulose

2. Kinetics of cellulose depolymerization

The course study of cellulose depolymerization by *Poria placenta* is illustrated in Figure 1. Two phases in the depolymerization of cellulose were observed. The first phase was very rapid: after 5 days of decay, cotton cellulose was depolymerized up to a DP of 800. Then, a slower phase of depolymerization developed, during which cellulose DP decreased linearly. Finally, the levelling off degree of polymerization for cotton cellulose, i.e. a DP of 200-250, was reached after 21 days incubation, and remained constant up to 42 days of decay. Solubility in 1% sodium hydroxide 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 incubation.

The occurence of two different rates in cellulose depolymerization was further investigated by examining the molecular size distribution of the cellulose during decay (Figure 2). Only one main peak was resolved for all assayed samples, irrespectively of the extent of depolymerization acheived. However, with decay time, the peak broadened and evolved to the zone of lower DP. In addition, since day 14, increasing amounts of low molecular weight compounds (DP<60) accumulated. Polydispersity (DPw/DPn) increased gradually with colonization, reaching a maximum after 22 days of decay and then slightly decreased (Table 2).

3. Pulse-labeling of 14C-glucose during cellulose depolymerization

An experiment was performed in which 14C-glucose was fed to to the feeder strip during the most active phase of depolymerization of cotton cellulose, as determined in the previous experiment of this study. The fate of radioactivity was followed after 1, 3 and 7 days of radiolabeling. Almost 70% of the fed 14C-glucose was converted to $^{14}CO_2$ after seven days of incubation. However, 42% mineralization was acheived during the first 20 hours after adding radioactivity (results not shown).

Fungal translocation of 14C-glucose from the feeder strip to cotton cellulose was very efective. 5.3, 7.9 and 1.4% total radioactivity was recovered in cellulose extracts after 1, 3 and 7 days of labeling, respectively. Typical results obtained after separation of the labeled low molecular weight compounds secreted during cellulose decay, as a result of fungal translocation and transformation of 14C-glucose, are shown in Figure 3. Data obtained for 3 days of labeling is shown as an example. The results of this experiment showed that only a minor peak of 14C radioactivity was present at the exclusion volume of the column (MW>4.000). Low, but significant endoglucanase activity was determined on this fraction by mean of viscometric methods (results not shown). However, no exoglucanase activity could be found.

On the contrary, two low MW compounds, with apparent MW of 2,000 and 1,000D, were actively synthesized during this stage of depolymerization. The latter were strongly radiolabeled during the first day of feeding. Essentially the same profile for all 14C compounds shown in Figure 3 was found in labeled samples, whatever was the harvesting time. Furthermore, preliminary experiments where 14C-glucose was added only after 3 days of incubation with cotton cellulose, revealed that the same two low MW compounds were been actively synthetized even at this early stage of decay.

On the opposite, these two compounds could be quantitated by their absorbance at 280nm only after 13 (MW of 2000) and 17 (MW of 1000) days of decay, i.e. when cellulose DP had almost reached its lowest value (Figure 4). Furthermore, in highly depolymerized cellulose (50 days of decay), a new compound absorbing at 280nm, with an approximate MW of 1500 (fraction 35) developed (Figure 4). Recent experiments where 14C-glucose was pulse-labeled to depolymerizing cultures at different incubation times, showed that the onset of the synthesis of the latter begun only after 20 days of decay. only after Finally, the peak showing the lowest molecular weight has strong absorbances at both 280 and 400nm. It was present in all the samples and eluted at the inclusion volume of the column. It is interesting to point out that apparently no 14C-glucose diffused through the feeder strip to the cotton cellulose, as shown by the absence of radioactivity in the fractions eluted at the corresponding molecular weight, i.e. the inclusion volume of the column (Fig. 3).

The presence of primary amine residues in extract components was also determined by assaying all the collected fractions for ninhydrin reaction. As illustrated in Fig. 3, the compound with an apparent MW of 1000 was the only one to give a positive reaction to ninhydrin.

Elemental analysis of a cellulose sample harvested at a stage of active depolymerization showed substantial accumulation of Ca and Mg (Table 3). Among transition metals, only Mn and Fe were found to accumulate. Furthermore, more than 50% of total Mg and Mn, and 30% of total Fe, appeared to be extracellular and water-soluble. Figure 5 shows the results obtained for the elemental analysis of the soluble extract of this sample after separation by gel permeation chromatography. Sodium, calcium, zinc and copper eluted at the inclusion volume of the column, i.e. after fraction 43 (results not shown). A sharp and unique peak of manganese and magnesium was found at fraction 40; therefore, it seems that these cations co-eluted with the main low MW peak radiolabeled during the 14C-pulse (see Fig. 3). Iron was also found in this fraction. The resulting Mg:Mn:Fe ratio was 10:4:1. Finally, it is noteworthy that the second peak that resolved for iron (fraction 31) co-eluted with the second low MW compound which appeared radiolabeled after feeding 14C-glucose to the depolymerizing cultures.

DISCUSSION

The experiments carried out here on the effect of water content on cellulose depolymerization showed that at least a 30% dry matter content is necessary to permit significant depolymerization of cellulose by the brown-rot system. The reasons for this threshold in water content, as well as for the effect of water on the depolymerizing system, are unclear. One possible explanation is that high water concentrations repressed the synthesis of the depolymerizing system. Another possibility is that, although the system is activated, the agent responsible for depolymerization was diluted in the medium and therefore could not rise its target, cellulose; it is also possible that the agent simply reacts preferentially with water than with cellulose or the other structural polymers. Finally, cellulose recrystallization, which has been described to occur in aqueous media (Bertran and Dale, 1985) could also have been partially responsible for the limited depolymerization observed at low dry matter contents. Indeed, while alcohols do not produce recrystallization of amorphous cellulose, water promotes the recrystallization process as a consequence of its high surface tension and hydrogen bonding capacity (Wadhera and Manley, 1965). Therefore, as the oxidative agent attacks cellulose chains within the amorphous regions (Kirk et al, 1989), water could reduce cellulose depolymerization by decreasing the number of areas susceptible for cleavage.

Since the early stages of decay, molecular weight distribution of the cellulose samples resolved in only one main peak (Figure 3). This confirmed previous results of Highley et al (1989), concerning random cleavage within cellulose chains by the brown-rot system. However, polydispersity calculations gave much higher results than those reported by Highley et al (1989), as well as for that obtained on acid prehydrolysis (Wood and Conner, 1988). These differences could be explained by the fact that brown-rotted cellulose samples prepared by these authors were previously washed with NaOH before analysis, resulting in the removal of low molecular weight molecules, leading therefore to lower DPw/DPn values. Furthermore, the method employed by these authors to recover cellulose tricarbanilate derivatives (Schroeder and Haigh, 1979) leads to sample fractionation, and therefore to the loss of the low molecular weight fraction (Wood et al, 1986).

The accumulation of a cellulose-derived low molecular weight fraction, concomitantly to the decrease in cellulose length during decay (Figure 3) could result either from the sequential action of the depolymerizing agent, as well as from endoglucanase activity, which develop in parallel to the reduction in molecular size. Indeed, after the oxidative agent has "opened" the cellulose fibers, endoglucanases should be able to bind and partially hydrolize depolymerized cellulose near the sites where the oxidative cleavage took place. Anyway, this occurs within the

amorphous regions of the polymer, as discussed above, and is consistent with the increase in cristallinity of brown-rotted, as compared to sound, cellulose (Highley et al, 1989).

Fenton reagent has been implicated in cellulose depolymerization by brown-rot fungi (Cowling nad Brown, 1969; Koenigs, 1974). Results of elemental analysis indicated that iron is contained in two low M.W. compounds secreted by P. placenta during cellulose depolymerization. Ninhydrine positive reaction of the lowest MW compound suggests that this compound could be related to siderophore activity. Furthermore, manganese, another transition metal was also found to be associated with iron within the same molecule. To our knowledge, specific manganese bearers have only been described in Bacillus subtilis, although structural characterization and regulation have not been reported. The possible role of this bimetallic compound on cellulose depolymerization has not been assessed, but is currently under investigation.

ACKNOWLEDGEMENTS

We are grateful to Drs. T. Highley and D. Flournoy for valuable suggestions and helpful discussions. We acknowledge also Dr. T. Conner for analysis of the molecular size distribution of brown-rotted cellulose. This work was supported by grants IFS No 0977/2, AID No DPE-5542-G-SS-8076-00, UNDP CHI/87/021 and USDA No 86-FSTY-0212.

Table 1: Effect of dry matter content on the depolymerization of cotton cellulose by *Poria placenta*.

% dry matter	DP
2	760
10	720
20	750
30	350
40	320

Table 2: Evolution of average molecular weight and polydispersity of cotton cellulose during its incubation with the brown-rot fungus *Poria placenta*

Incubation time (days)	MW	Polydispersity (DPw/DPn)
0	2044	1.84
5	1642	2.34
14	710	3.09
22	422	5.27
42	273	4.49

TABLE 3. ELEMENTAL CONCENTRATIONS (μG/G COTTON) IN COTTON CELLULOSE AFTER 17 DAYS OF DECAY BY *PORIA PLACENTA*

	ELEMENTS							
	P	CA	MG	ZN	MN	FE	CU	CO
TOTAL	43.0	220.1	67.5	18.0	31.9	32.7	ND	ND
SOLUBLE EXTRACT (%)	18	19	63	36	56	32	-	-

N.D.: not detected

Fig. 1: Kinetics of Cellulose Depolymerization by P. placenta

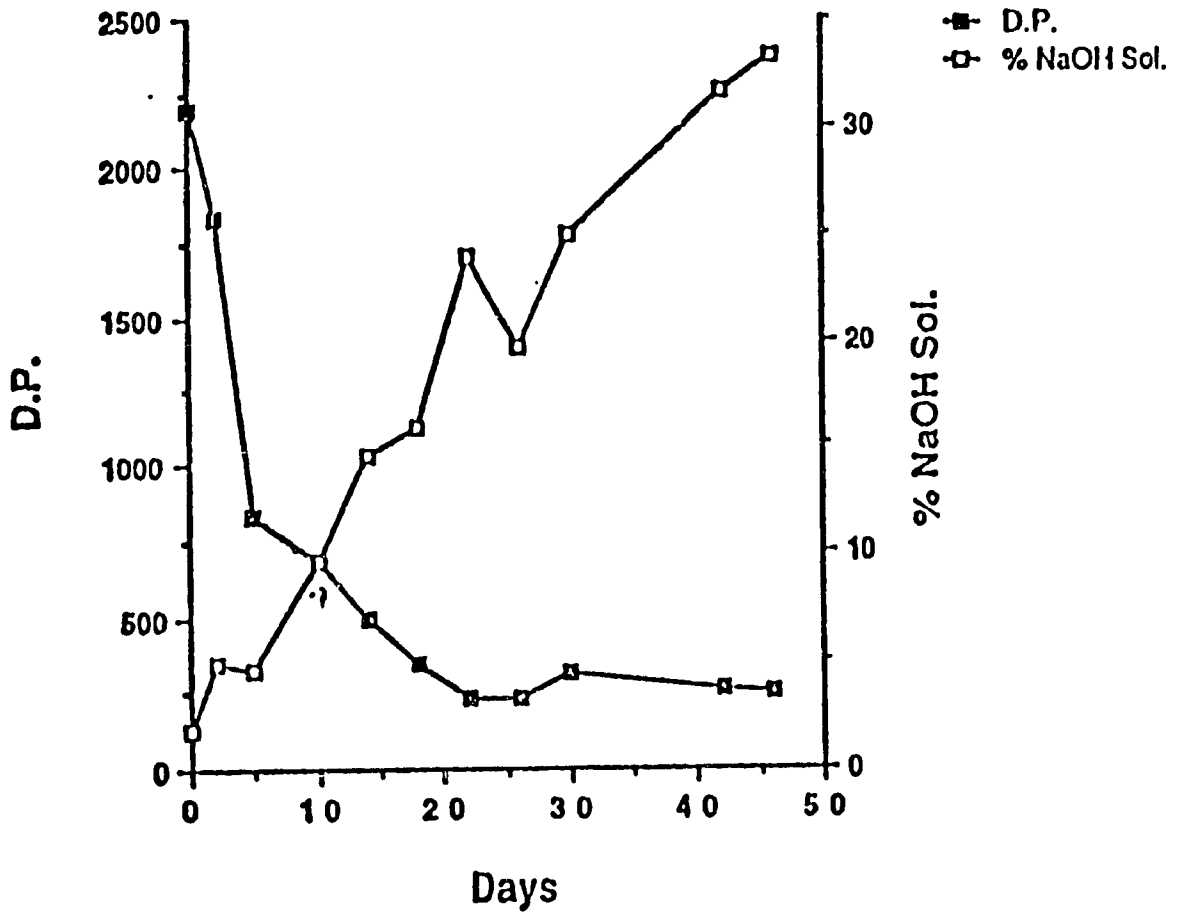


Fig. 2: Molecular weight distribution of depolymerized cotton cellulose

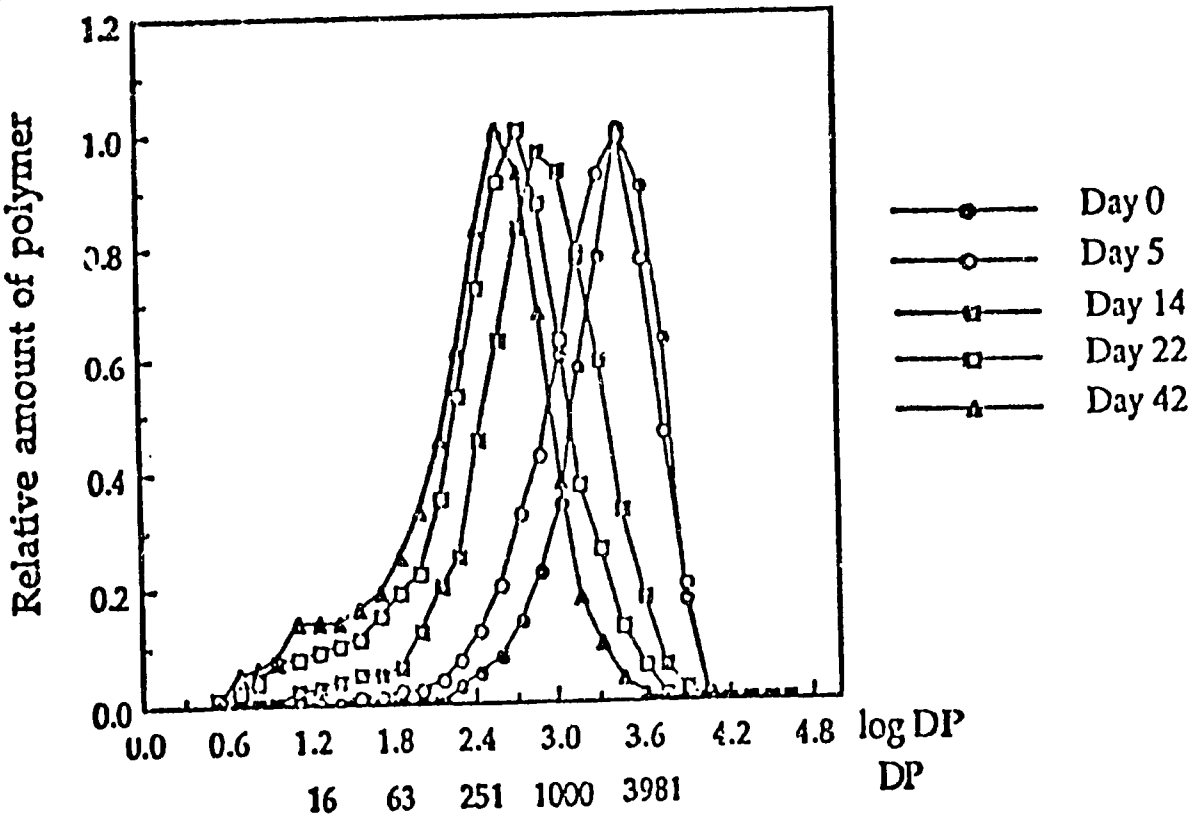


Fig. 3: SEPARATION OF EXTRACELLULAR RADIOLABELLED COMPOUNDS SYNTHETIZED 3 DAYS AFTER ^{14}C-GLUCOSE ADDITION DURING THE ACTIVE PHASE OF CELLULOSE DEPOLYMERIZATION BY *Poria placenta*

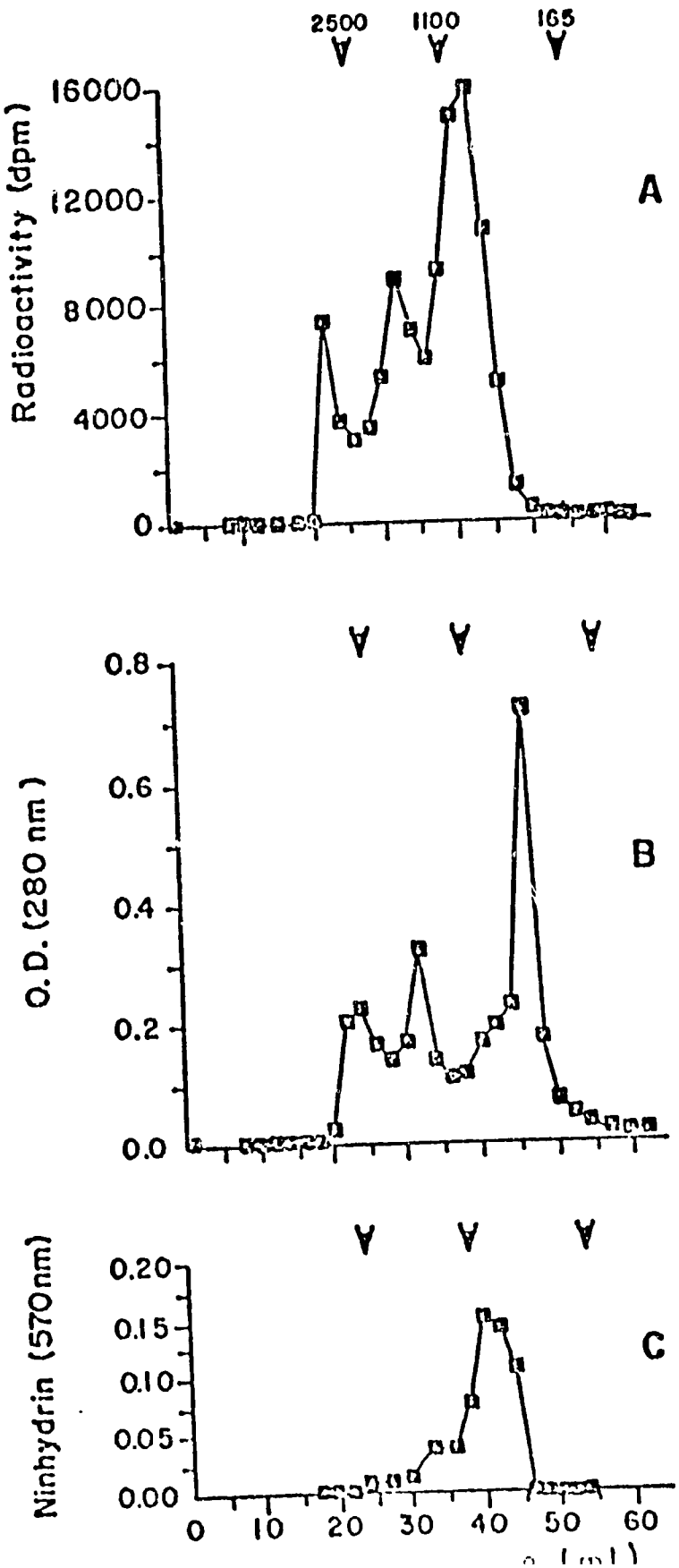
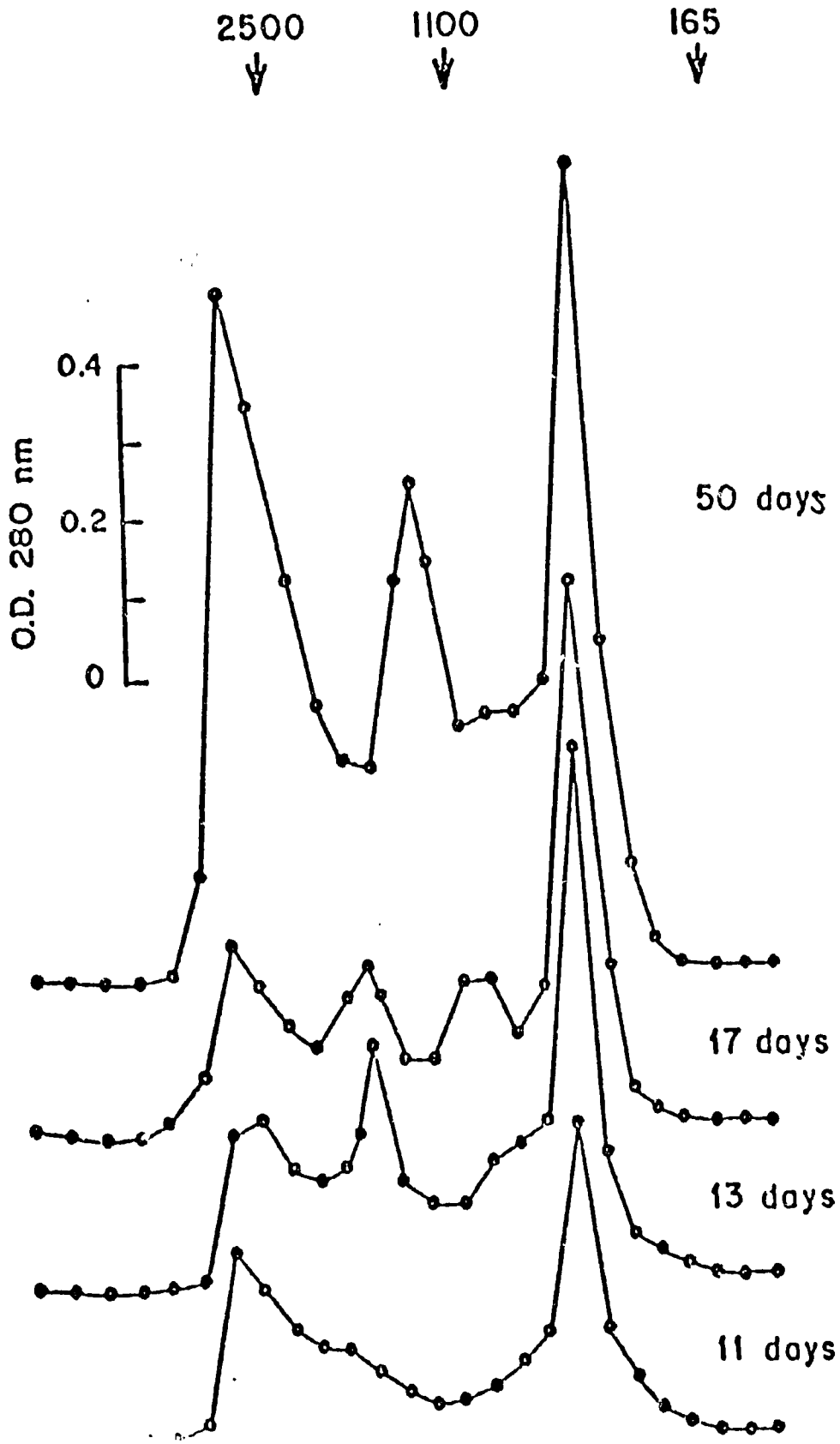


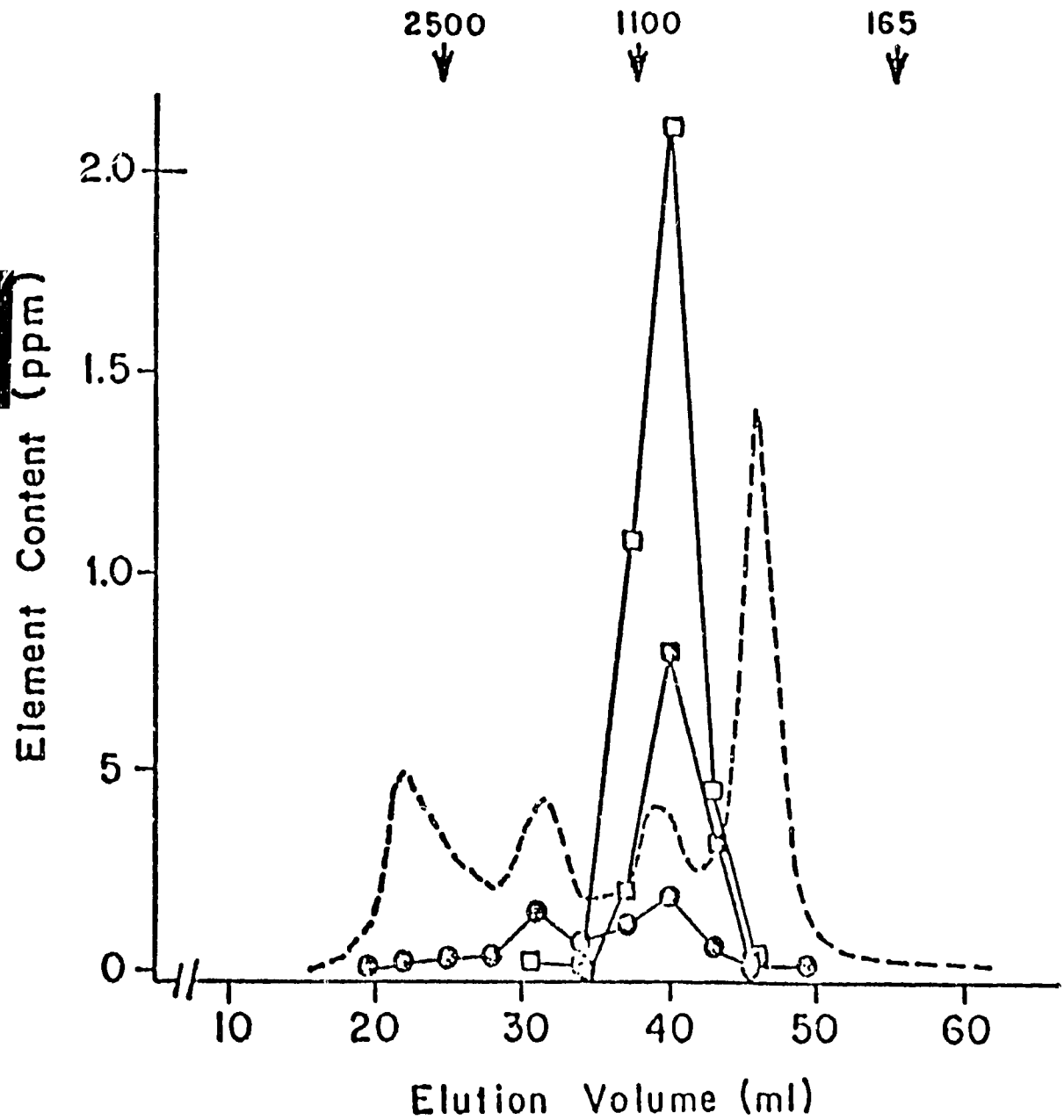
Fig.4: EVOLUTION OF EXTRACELLULAR LOW MOLECULAR WEIGHT MOLECULES DURING CELLULOSE DEPOLYMERIZATION BY *Poria placenta*

280 nm ABSORBING COMP. WERE SEPARATED IN A 50x1 cm SEPHADEX G-25 COLUMN.



MOLECULAR WEIGHT DISTRIBUTION OF TRANSITION METALS PRESENT IN THE SOLUBLE FRACTION OF COTTON CELLULOSE DEPOLYMERIZED BY THE BROWN-ROT FUNGUS *Poria placenta*

Mg (□) ; Fe (○) ; Mn (◘)



REPORT 2

FINAL REPORT OF ACTIVITIES

"Saccharification of Lignocellulosics Materials by Brown Rot Fungi"

Presented by the Industrial Fermentation Laboratory
Applied Research Division
ICAITI

Guatemala, January 1992

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List of Contents

1. Visit of Dr Eduardo Agosín to ICAITI in Guatemala City

2. Visit of M.Sc. Roberto de León to the Biotechnology Unit at INTA in Santiago, Chile

3. Collection Trips for local Strains of Brown Rot Fungi in Guatemala

4. Experiments with agroindustrial residues available in Guatemala

4.1.Experimental set up

4.2.Results

4.2.1 Preliminary Biodegradation of agroindustrial residues

4.2.2 Optimization of conditions

Annex I
Figures 1 - 24

Annex II
Tables 1-29

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1. Visit of Dr Eduardo Agosín to ICAITI in Guatemala

During the week of the 29th of may to the 2nd of June, Dr Eduardo Agosín visited ICAITI to carry on the arrangements for the collaborating study. As it is stated in the work plan of the project, ICAITI was to contribute to the project by attempting brown rot fungi isolation and test local agroindustrial residues in solid state fermentation at laboratory level with strains donated by Dr Agosín.

During his stay Dr. Agosin gave a lecture on the work carried out by the Biotechnology Unit at the Institute of Nutrition and Food Technology (INTA) in Santiago, Chile. Also some highlights of the Congress on Biodegradation of Lignocellulosics that took place in North Carolina, USA, the preceding week.

Also time was devoted to some attempts of isolation of brown rot fungi. Several sites were visited of different clime conditions, however the season is the driest for the year in Guatemala and it was decided to repeat the isolations during the rainy season.

An agreement was written to define the activities to be taken by ICAITI.

2. Visit of Roberto de León to the Biotechnology Unit at INTA

During the week of the 10th to the 14th july, 1989, Roberto de León made a visit to the Biotechnology Unit (BU) at INTA in Santiago, Chile. The main purpose was to get familiar with the procedures of solid state fermentation carried out in samples of wood chips. At the B.U., an initial preparation for wood is made by extraction with ethanol:toluene, followed by a thermal treatment at 65° C with acetic acid and sodium hypochlorite.

Solid State fermentation is carried out by using the glass columns designed years ago in France, with an air inlet inserted in a glass tube containing water, to humidy the incomming air. The whole system is placed in a incubator so the temperature is up to 35° C. Glass wool is used to avoid any enzymic action on cellulose in the case of using cotton. The column system is aerated by using an aquarium pump. The whole system is incubated from 6 to 8 weeks . Afterwards, or during the incubation period, the evaluation of the attack is made. This includes the measurement of the degree of polymerization according to the TAPPI method.

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Other activities during the visit included getting in touch with the Chemical Engineering group working in collaboration with the B.U. for this project and in charge of making tests for the lignin utilization. Another activity was to present a lecture to the B.U. personnel on ICAITI's research activities

Additional training was taken for column preparation, soil block techniques and the method for carboxylic group determination.

From the visit to the B.U. eleven strains of brown rot fungi were brought to Guatemala.

Strain	Culture collection No
-*Poria placenta*	MAD-698-R
-*Glorophyllum trabeum*	STPI-Suecia
-*Lentinus lepideus*	CTB 67-03-A
-*Wolphiporia cocos*	FP-40580-R
-*Glorophyllum trabeum*	6M-R
-*Lactiporus sulphureum*	OKM-8051-S
-*Serpula lacrymans*	CBS-751.79
-*Poria monticola*	HHB 1195-SP
-*Coniophora puteana*	CBS 132.71
-*Inonatus weiri*	CBS 663.85
-*Phaseolus schweinitzii*	14.854-5

3. Collection trips

During the stay of Dr Eduardo Agosin in Guatemala two main attempts of isolation of strains were done. One in the nearby areas of Guatemala city, at the local camp site of the boy scouts association and the other in the mountains surrounding lake Amatitlán, both sites within a range of 15 km of the city. The first site visited was a park were intensive maintenance is given and rests of decaying wood are readily taken away. The second site was a forest near coffee plantations by the edge of a small stream of water, some samples were taken because more evidences of fungal growth were found here.

A second collection trip was done to a University Park located about 120 km to the north. This is a place were the forest is left untouched, actually it was created to preserve a natural habitat for the national bird, the Quetzal. However less evidences of brown rot fungi were found, perhaps because

it is a kind of forest of the "rain type" were plants live shorter lifes and are made of more soft wood.

After the departure of Dr Agosin, collection trips were initiated again until the rainy season appeared. This time the trips were made into forests of 1500 to 1800 m above sea level. On this occasion, a forest with new and young trees was found about 30 km east from the city and were old trees were chopped down for firewood, however the old stumps remained there and they were attacked and presented decaying symptoms. Samples were collected and isolations were attempted from this.

Another collection trip was done to the west on the highlands of Guatemala. A visit made in 1990, to promote a mushroom project, put us in contact with people who harvest mushrooms in the forest and they collected and showed us samples of decaying wood. Again populated areas are difficult for this kind of collection due perhaps to the demand for firewood. It is difficult to find samples of trees old enough to present the type of attack that we were looking for.
From all the isolates obtained in the collection trips, the Bavendam test was performed but only two isolates were considered potential brown rot fungi.

4. Experiments with materials available in Guatemala.

In the agreement between ICAITI and Dr E. Agosin the tests for biodegradation were to performed on agroindustrial wastes such were considered to be: sugar cane bagasse, citronela and lemon tea bagasse, oil palm bagasse, wheat straw , pine and eucaliptus sawdust. These are the main agroindustrial waste of lignocellulosic nature that would be available in the country. Experimentes were to be carried out with the two main strains of brown rot fungi that were brought from INTA Biotechnology Unit in Chile.

4.1. Experimental set up

The experimental design was carried out in columns aireated at the bottom with the air passing through a glass tube filled with water. The set-up was placed in an incubator room with the controlled at 35° C, therefore temperature of the material and the water was equilibrated. Twelve columns were used for each treatment, two were taken every two weeks for analysis.

The columns, plastic tubings and accessories, were

sterilized at 121° C for twenty minutes. Air came from the central system of the institute that uses a compressor that maintains pressures of 70 to 100 psi; with the help of a regulator, the pressure was dropped to five psi and then conducted via a Tygon plastic tube to the incubator room. There it went through a glass wool filter and bubbled in sterile water to saturate it with moisture.

The samples were taken every two weeks and they were analyzed for cellulose, hemicellulose, lignin, degree of polymerization and total sugars. A preliminary run was made with sugar cane bagasse. With sugar cane, three levels of nitrogen were assayed because of previous experiments with white rot fungi showed low activity of the tested strains due to low levels or unavailability of nitrogen; the levesl were no supplementation, and supplementation to the ratio of 12:1, and 25:1, carbon to nitrogen.

Analysis performed on the residues were

-Degree of polymerization (according to the method Viscosity of pulp T-230 om-82) TAPPI, 1988

-Lignin-acid-detergent, according to USDA, 1979

-Neutral detergent, according to TAPPI, 1988

-Lignin permanganate, cellulose, insoluble ash

4.2. Results

4.2.1 Preliminary Degradation of agroindustrial residues

The first trials were made on sugar cane bagasse. This is one of the most important lignocellulosic residues available for many reasons and one of them is their abundance and easiness to collect due to its accumulation in the sugar factories. Because of previous assays with white rot fungi, two levels of nitrogen supplementation were tested against no supplementation.

Results are presented in Figures 1-10 and in Tables 1-5 in Annex I and Annex II respectively. Results after eight weeks of incubation gave some interesting hints, apparently no supplementation was neccesary in the case of G. trabeum and did some effect with W. cocos. For G. trabeum the best results were obtained for decreasing the DP in 43.8 % with no added nitrogen (C:N ratio of 180:1), for C:N, 25:1, 11.8 % in reduction of DP and for 25:1, C:N, a reduction of 17.3%.

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Cellulose digestion for 180:1 C:N, was of 43.77 % from the original whereas for 25:1 C:N, 11.6 % and for a C:N of 12:1, 17.3 % of cellulose digestion when compared to the original concentration. The tendencies in lignin and hemicellulose were much the same.

For **W. cocos**, the change in DP was of 15.3 % when not nitrogen supplemented whereas in 25:1 and 12:1 the change was of 46.16 and 42.79 % respectively. Cellulose degradation was of 15.5 % for no supplementation and 42.8 % for 12:1 and 45.9 % for 25:1 ratios of C:N. Hemicellulose and Lignin were increased as they are fractions not really touched in this kind of biodegradation.

The second part of the assays, based on the results obtained with sugar cane bagasse, were made in lemon grass, oil palm bagasse and wheat straw.

Results are presented in Figures 11- 20 and in Tables 6-25 in Annex I and Annex II respectively.

The obtained results show a similar activity in the three substrata, lemon grass, oil palm and wheat straw when treated with **Glorophyllum trabeum** and **Wolphiporia cocos.**

We will revise the results by components, the first one, cellulose, is presented in Figure 11 for the treatment with **G. trabeum** and in Figure 12 for the treatment with **W. cocos**. In six weeks in all cases of substrates, **G. trabeum** ends up with about 22 % of cellulose as % dw begining with values in the range of 33 to 38 %; action on sugar cane seems more constant than the other substrates where the final two weeks worked better. The results with **W. cocos** are more diverse, lemon grass and wheat straw cellulose seem more degraded, followed by oil palm specially in the last two weeks, whereas in sugar cane the decrease is much smaller; final cellulose concentrations however never got down from 20 % in both strains of brown rot fungi.

In the case of hemicellulose, we can see in Figure 13 that **G. trabeum** had some effect on wheat straw hemicellulose and apparently little effect on the other substrates, sugar cane hemicellulose is not affected and actually increases from an initial value of 26.62 %, to 37.9%, likely from the loss in cellulose. In Figure 14, **W. cocos** has a similar effect either in sugar cane as well as with the other substrates.

In Figure 15 we find the results obtained with **G. trabeum** on the concentration of lignin, **G. trabeum** leaves the lignin at 34.6% from an initial value of 20.77% in the case of sugar cane (a 66.6% increase) the next best substrate is lemon grass with an increase of 54%, oil palm though not having an

effect of more than 29.31% of increase, ends at six weeks with 32 % dw of lignin roughly the same as lemon grass. **W. cocos** as noticed in Figure 16 has a minor effect in sugar cane with only 21 % of increase, whereas for wheat straw the increase is in the order of 60 % to a final concentration of 36.87 %, the highest of the tests.

The highest decrease in the degree of polimerization was obtained in the sugar cane bagasse with 28.56 %, when this substrate was treated with **G. trabeum** and for the other substrate the effect was small.

When **W. cocos** was used, the effect was similar but in the case of sugar cane it was a matter only of 15%

The presence of total sugars was evident only in the case of lemon grass treated with **W. cocos** lemon grass with an increase of 93.85% .

The behavior in the concentration of total sugars was definitively a consequence of its liberation during the incubation time as well as its consumption.

The overall impression is that for these strains of fungi, sugar cane bagasse seems a better substrate, and particularly if it compares well even for the non nitrogen supplemented data.

4.2.2. Optimization of conditions

From the above experiments it was noticed that the most promising substrate was sugar cane bagasse. For the tested strains, no nitrogen supplementation was made at this time.

A first condition was to optimize aeration, which was being provided in the first two sets of experiments by the central air unit of the laboratories and though equipped with a regulator, the flow was extremely variable; by using aquarium pumps and a manifold with keys that could regulate the flow more precisely. Every two days the flow was checked and corrected to the value of one volume of air for volume of substrate per minute (VVM).

Results are presented in Figures 21-24 and Tables 26-29 in Annex I and Annex II respectively. For DP, there was an improvement for **G. trabeum** because we obtained a change of 43.69% in the first assay and with a regulated aeration of 1 VVM, 51.19 % , however, for **W. cocos** the response without

nitrogen supplementation was of 15 % and with 1 VVM, the change was of 21.74%.

Cellulose consumption was originally 43.67% in six weeks for **G. trabeum** and this time the consumptiom was of 47.61%. In the case of **W. cocos**, the first assays gave a consumption of 15.36% and with the air at 1 VVM, 19.35%.

Hemicellulose was concentrated because of its low consumption; in the first trials, for **G. trabeum**, the change was to become 42.41% higher, and with regulated aeration, 49.1% For **W. cocos** the first change was 50.34% higher and with air 52.97%.

About the lignin concentration, in the first part the results obtained gave for **G. trabeum** of 66.59% increase in lignin content and 21.57% for **W. cocos**. The aeration controlled trials gave results of 88.06 % increase in lignin concentration for **G. trabeum** and 52.77 % for **W.cocos**.

The regulation of air at the level of 1 VVM was picked up because at higher rates, there was more drying and cooling effects. With a volume of about 120 cm3 occupied in the glass column, the flow rate was set at 120 cm3 of air per minute. Small changes had to be made about every two days when a rotameter was used to check the flow.

Anyhow we could increase the performance of the strains on the sugar cane bagasse only by supplying the air in a constant flow and at a level high enough to provide oxygen.

Optimization trials were to conducted on several aspects of substrate composition and operation of the solid state fermentation. Levels of minerals besides air flow were to be estimated. Unfortunately time was not enough to cover all aspects

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Annex I

Figures 1 to 24

Figure 1
Gloeophyllum trabeum
Sugar cane bagasse

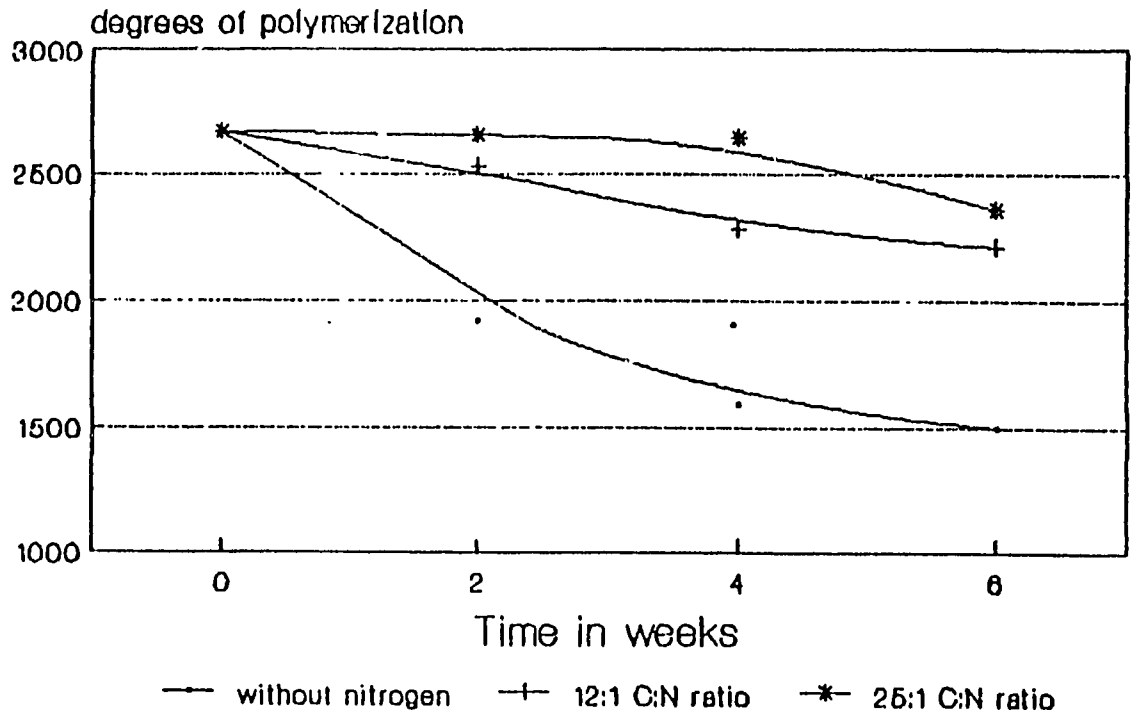

Figure 2
Wolphiporia cocos
Sugar cane bagasse

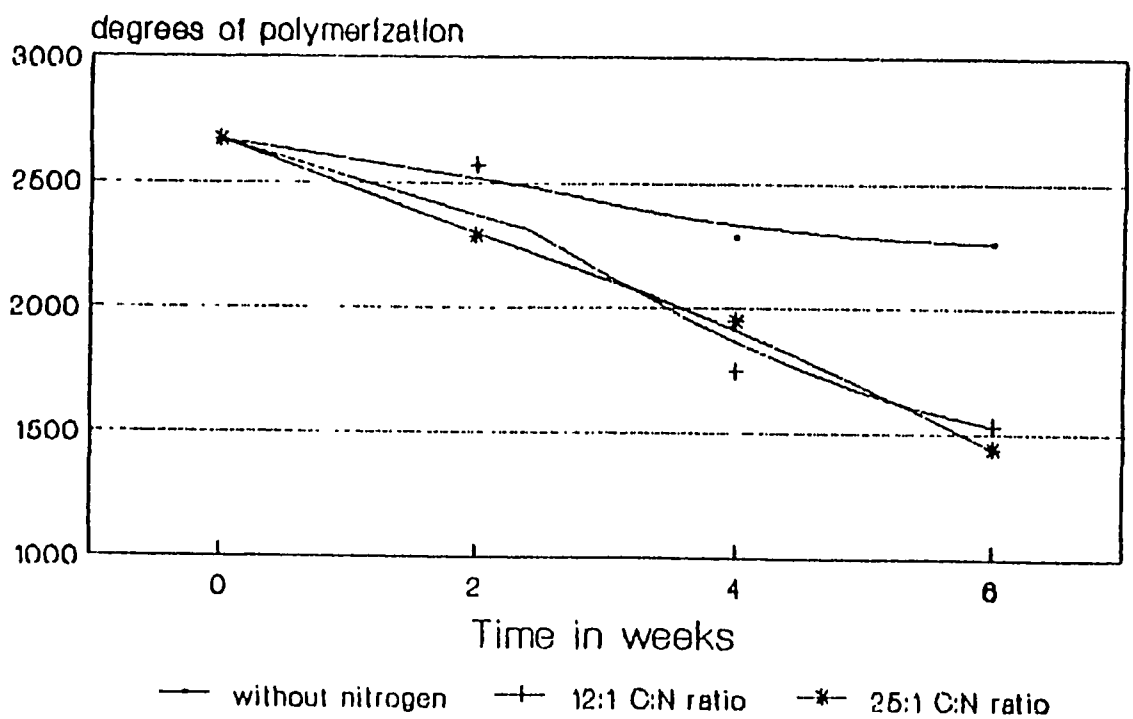

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Figure 3
G. trabeum
Sugar cane bagasse

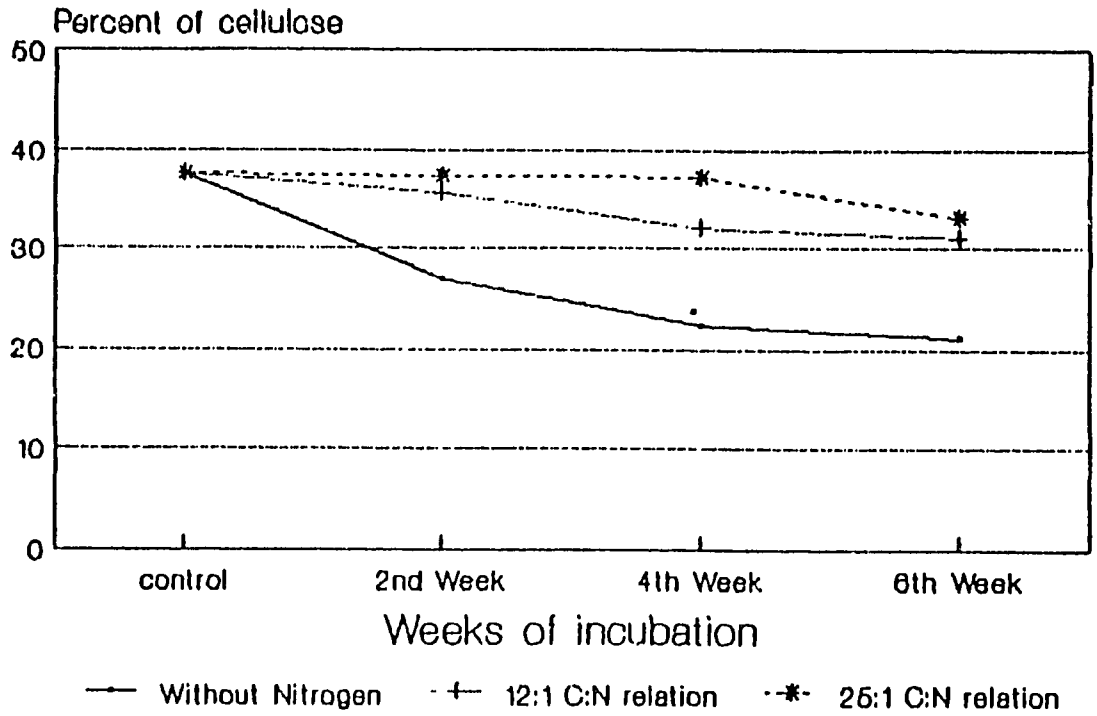


Figure 4
W. cocos
Sugar cane bagasse

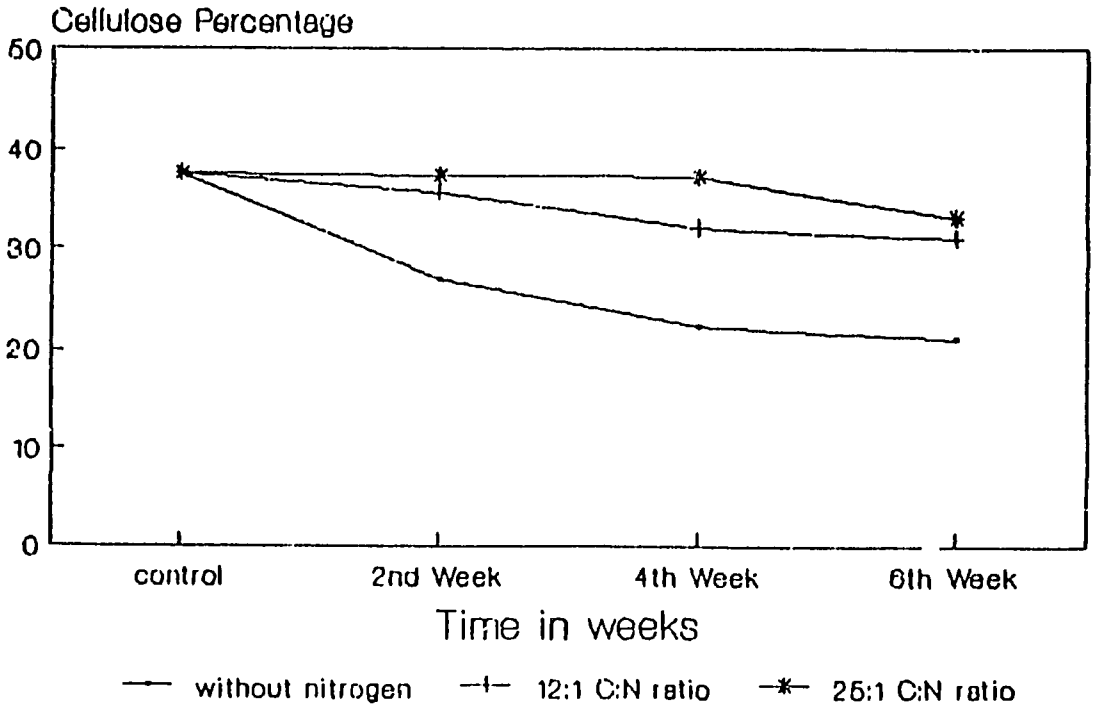


Figure 5
G. trabeum
Sugar cane bagasse

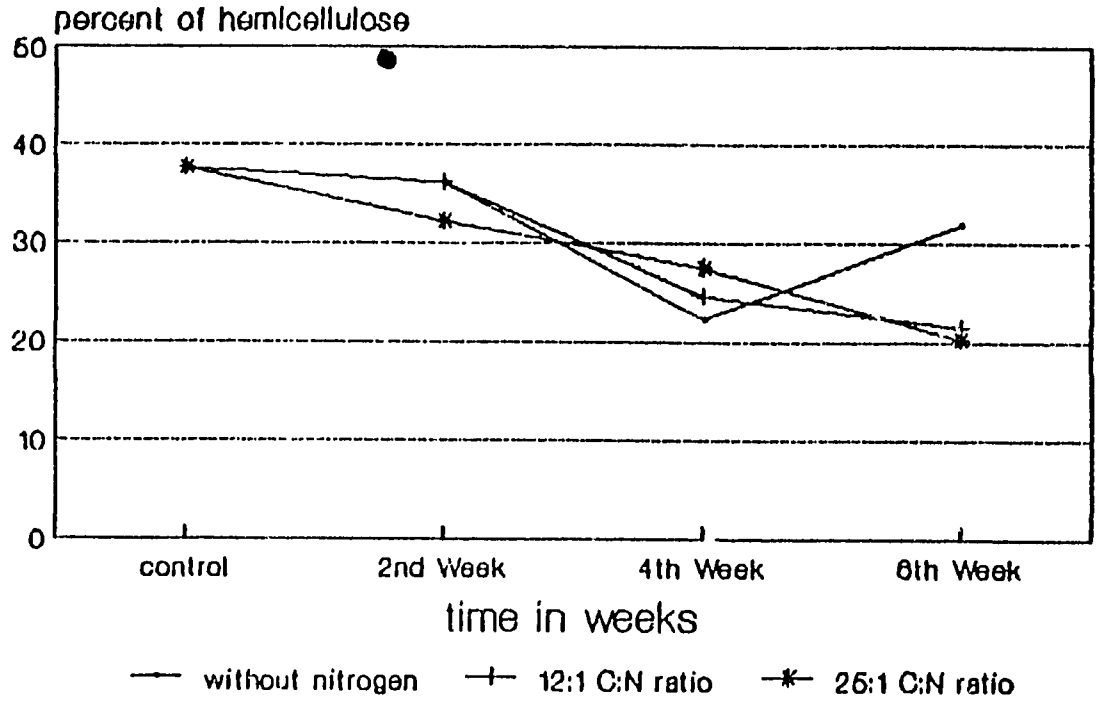


Figure 6
W. cocos
Sugar cane bagasse

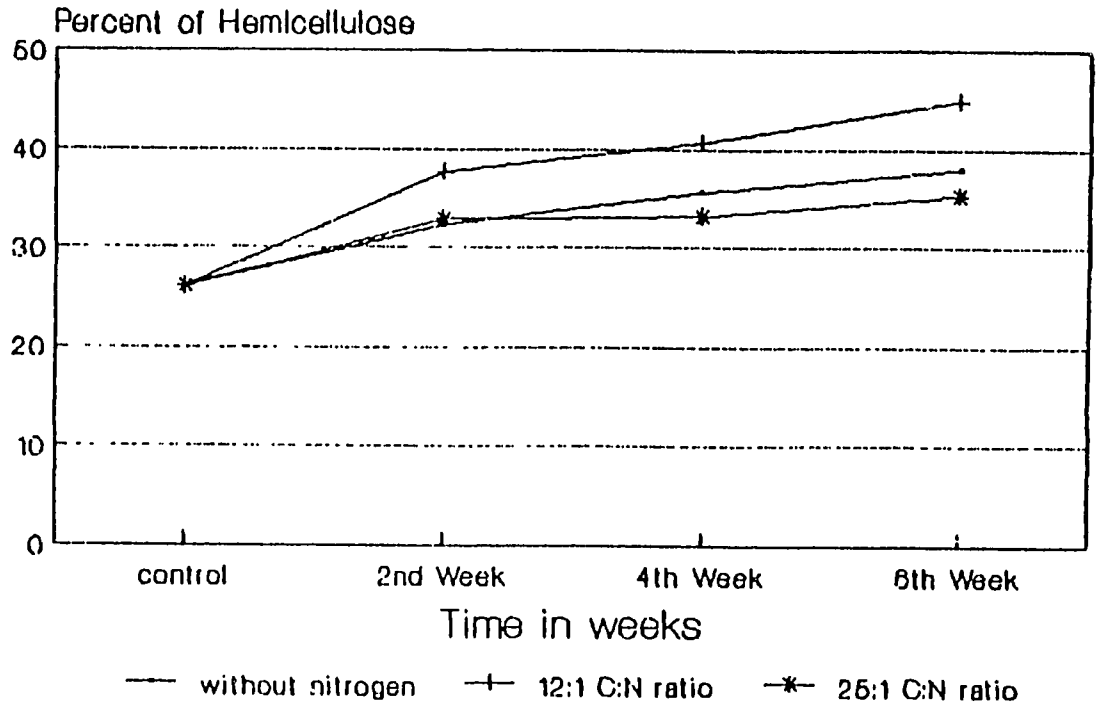


Figure 7
G. trabeum
Sugar cane bagasse

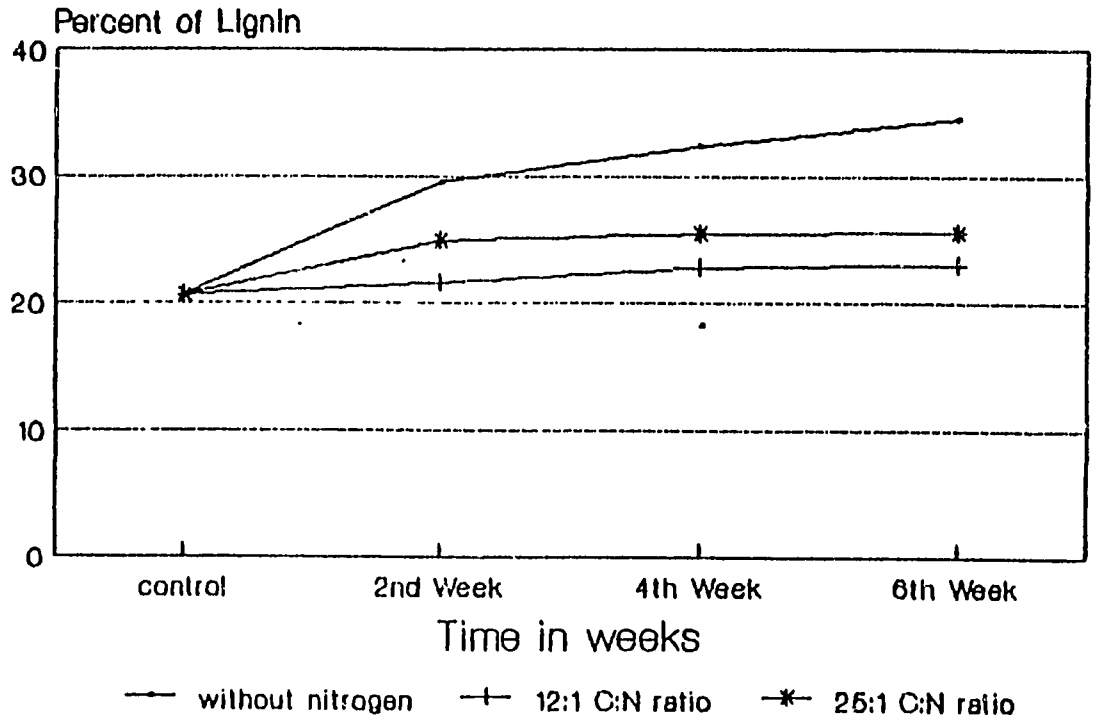


Figure 8
W. cocos
Sugar cane bagasse

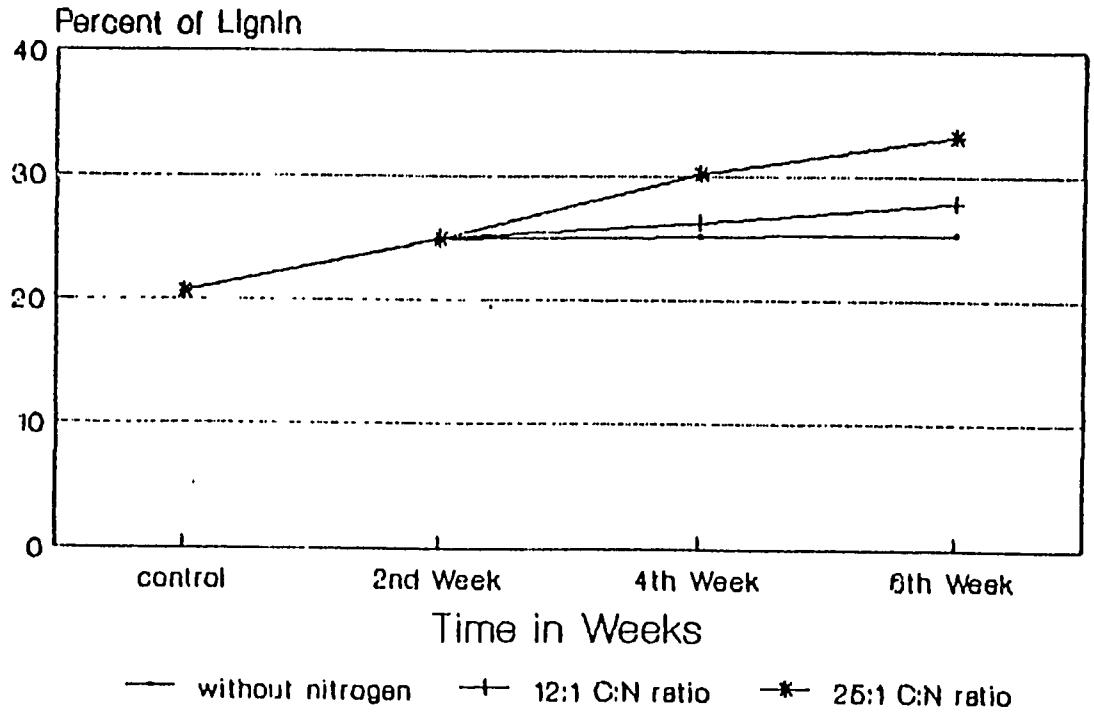


Figure 9
G. trabeum
Sugar cane bagasse

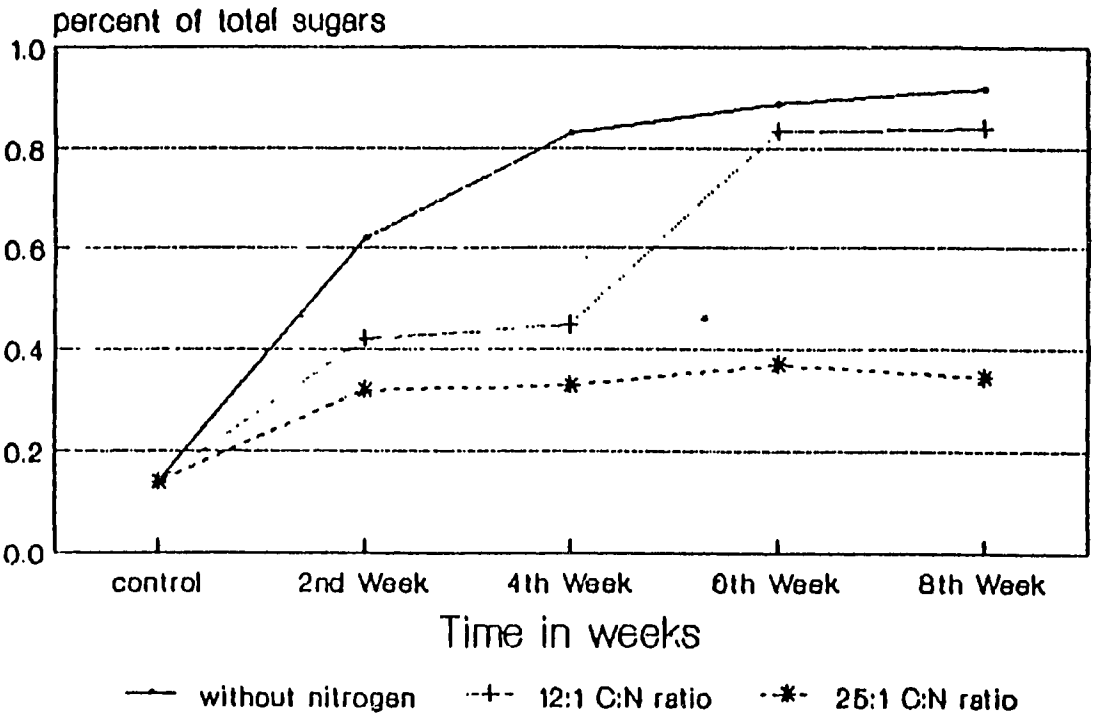


Figure 10
W. cocos
Sugar cane bagasse

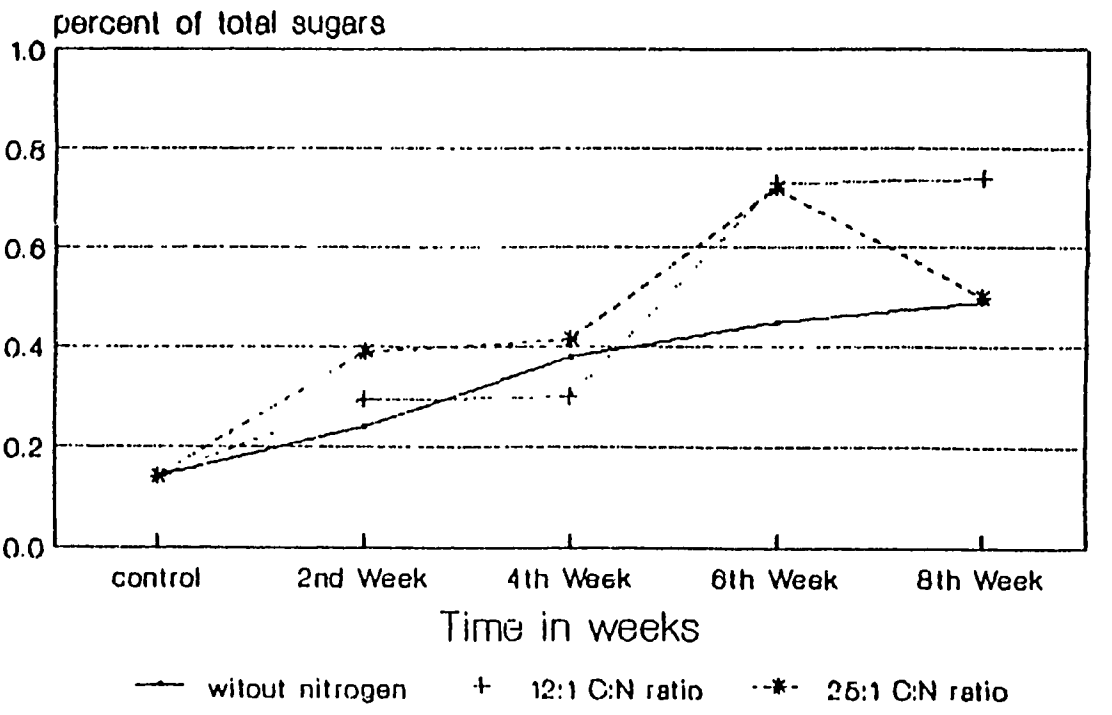
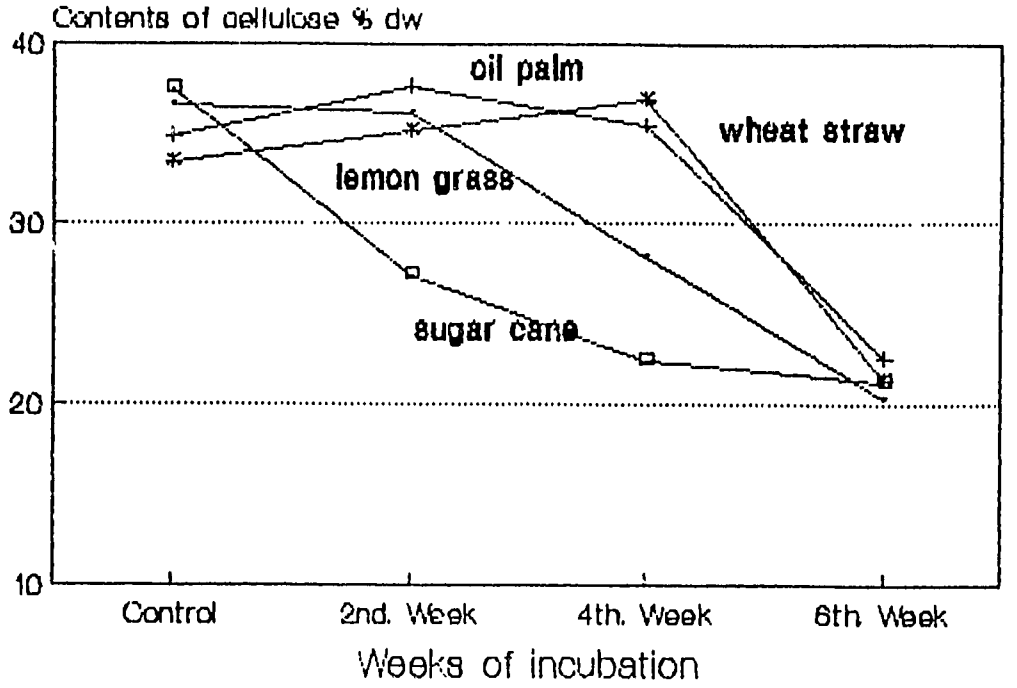
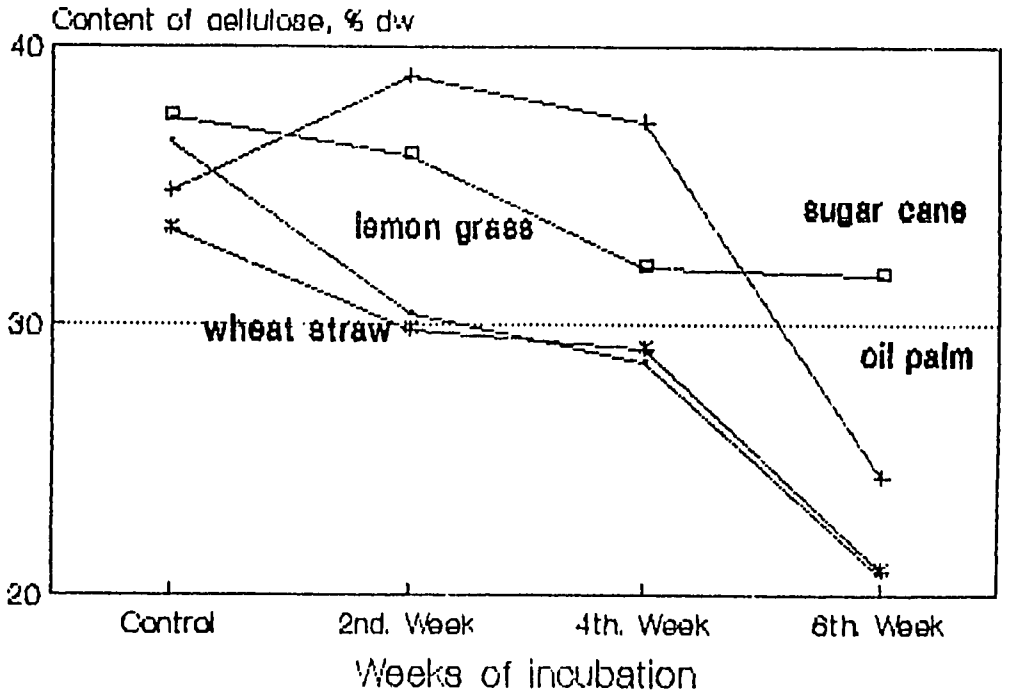


Figure 11
Evolution of cellulose



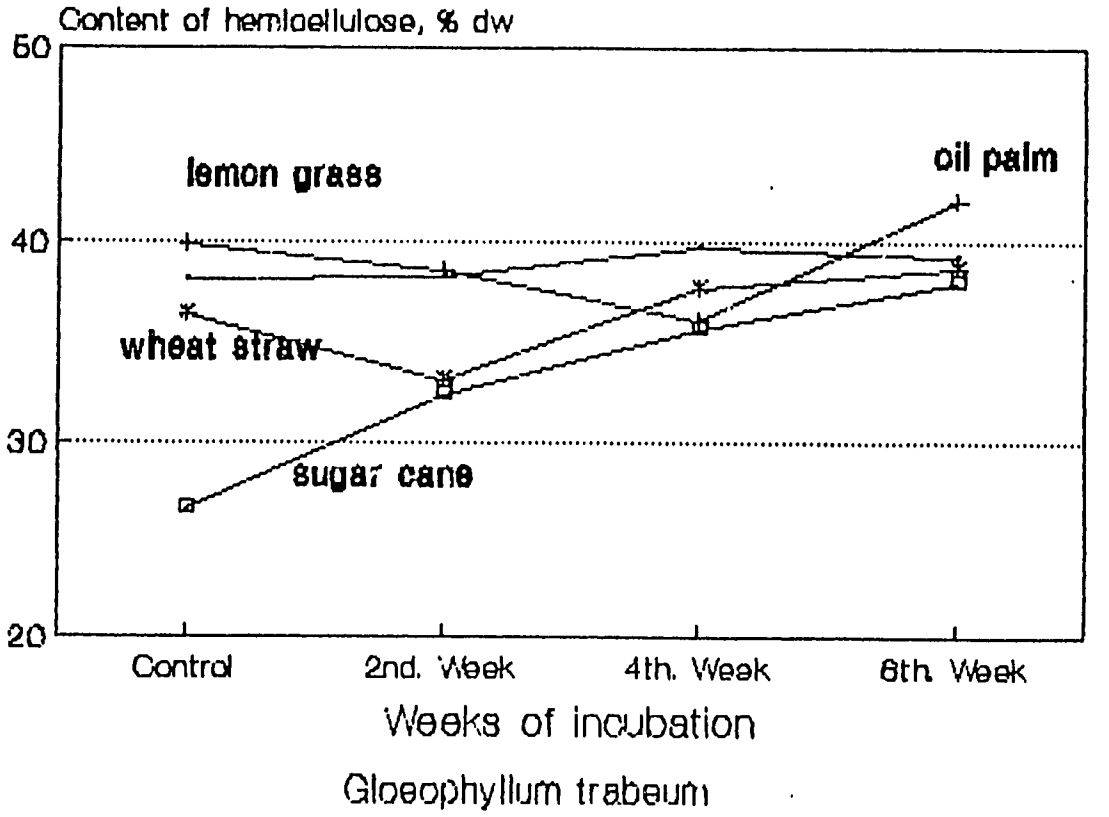
Glorophyllum trabeum

Figure 12
Evolution of cellulose



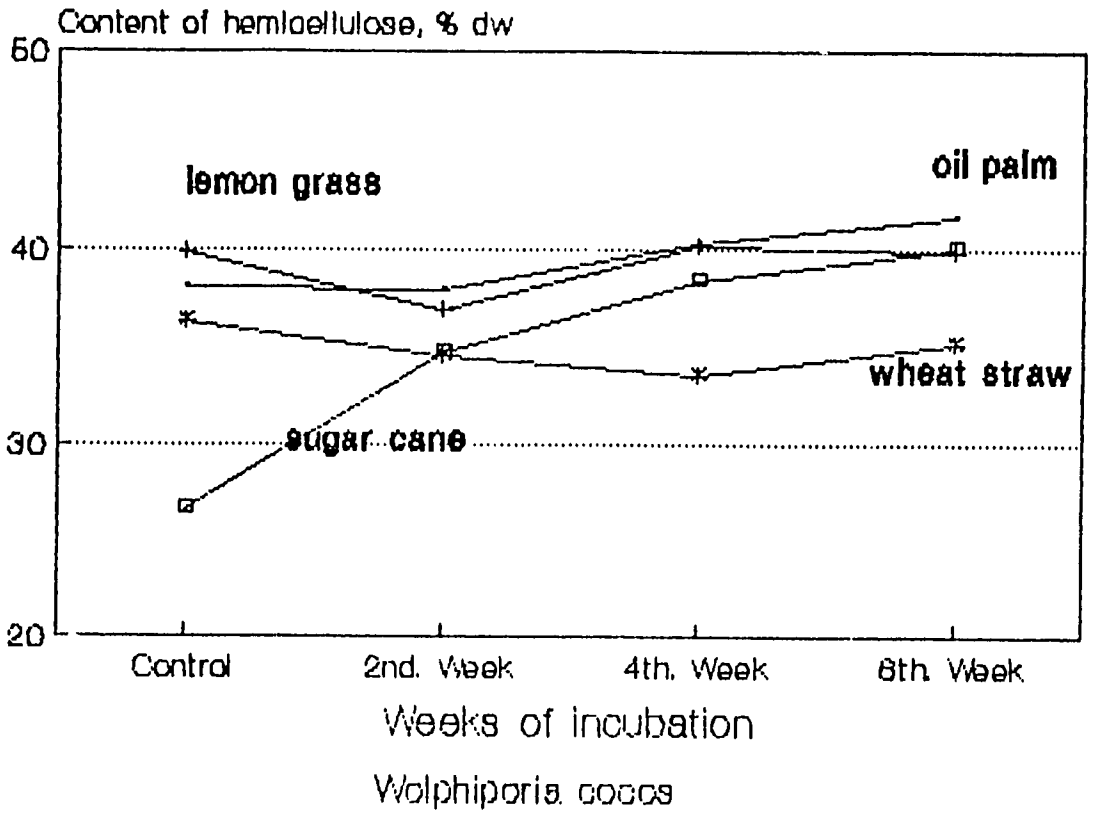
Wolphiporia cocos

Figure 13
Evolution of hemicellulose



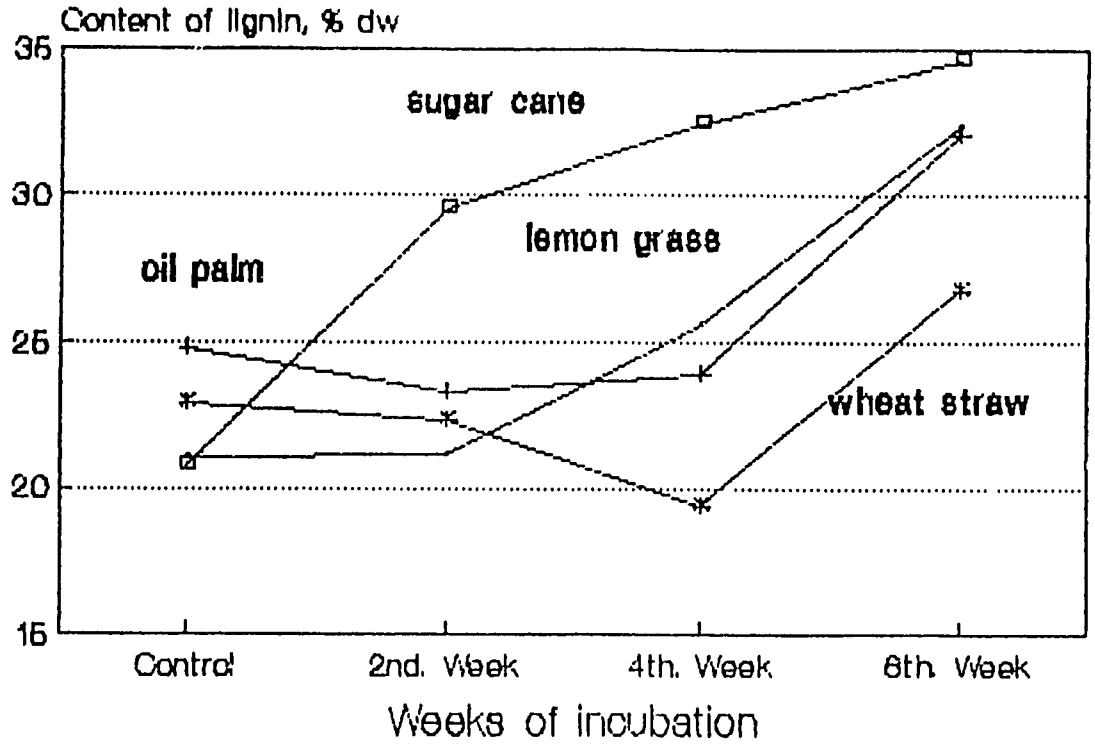
Gloeophyllum trabeum

Figure 14
Evolution of hemicellulose



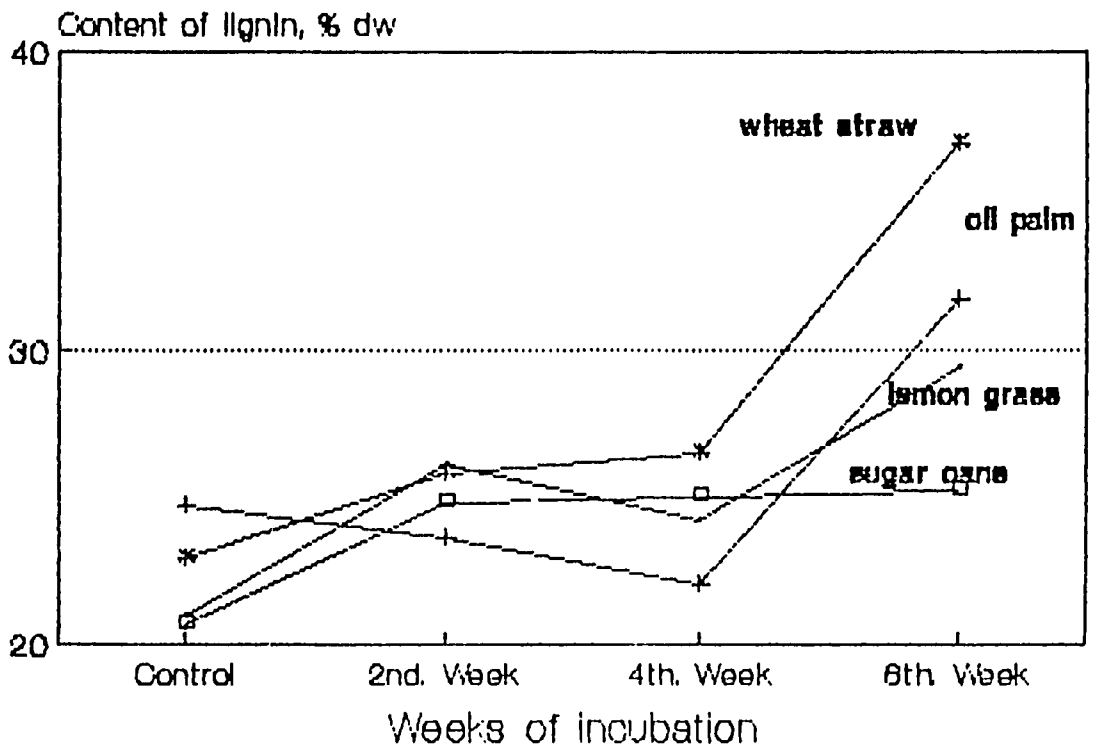
Wolphiporia cocos

Figure 15
Evolution of lignin



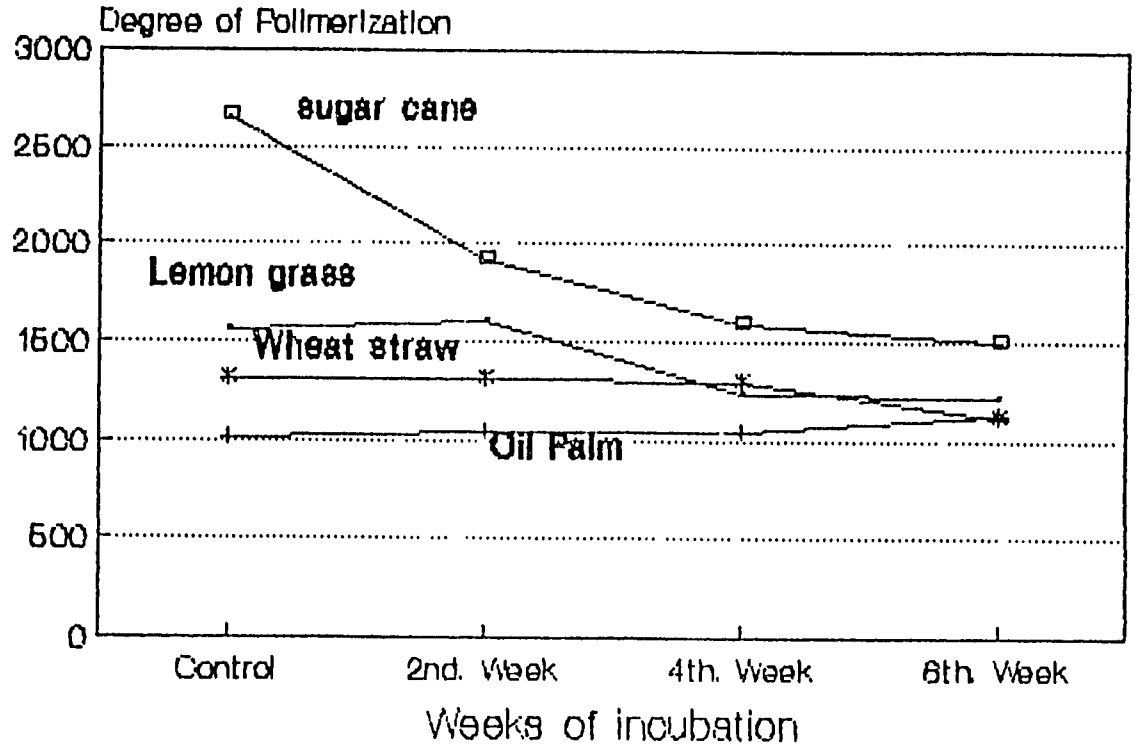
Glorophyllum trabeum

Figure 16
Evolution of lignin



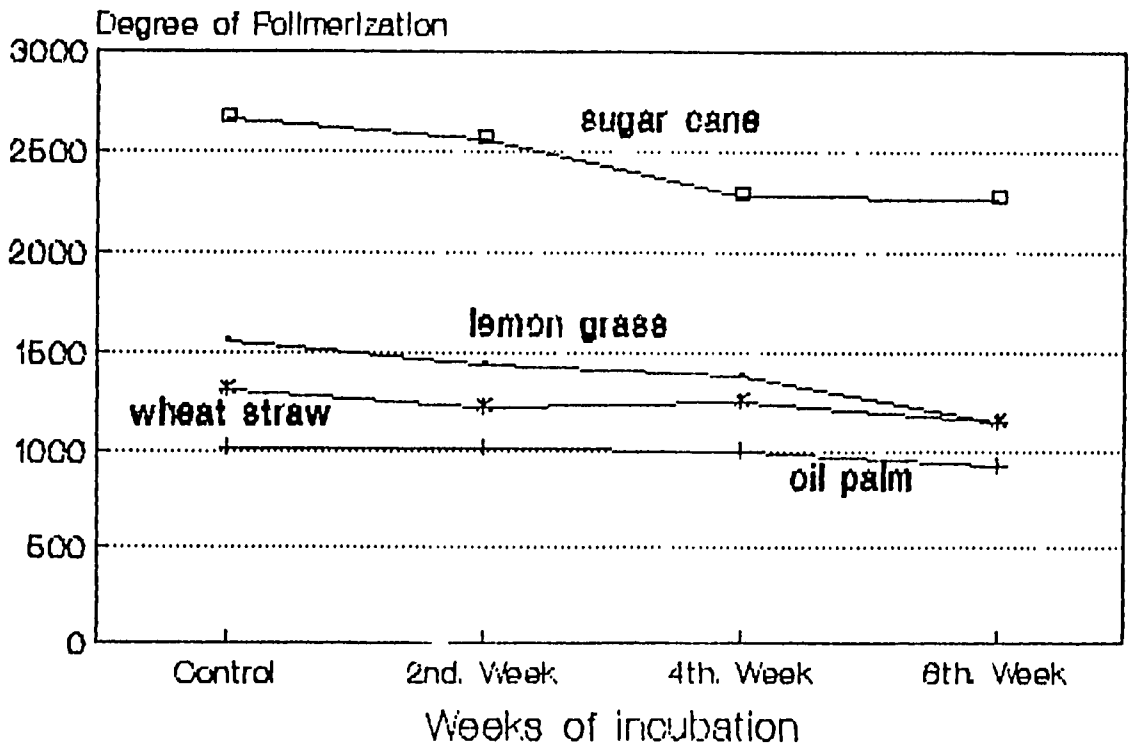
Wolphiporia cocos

Figure 17
Degree of Polimerization



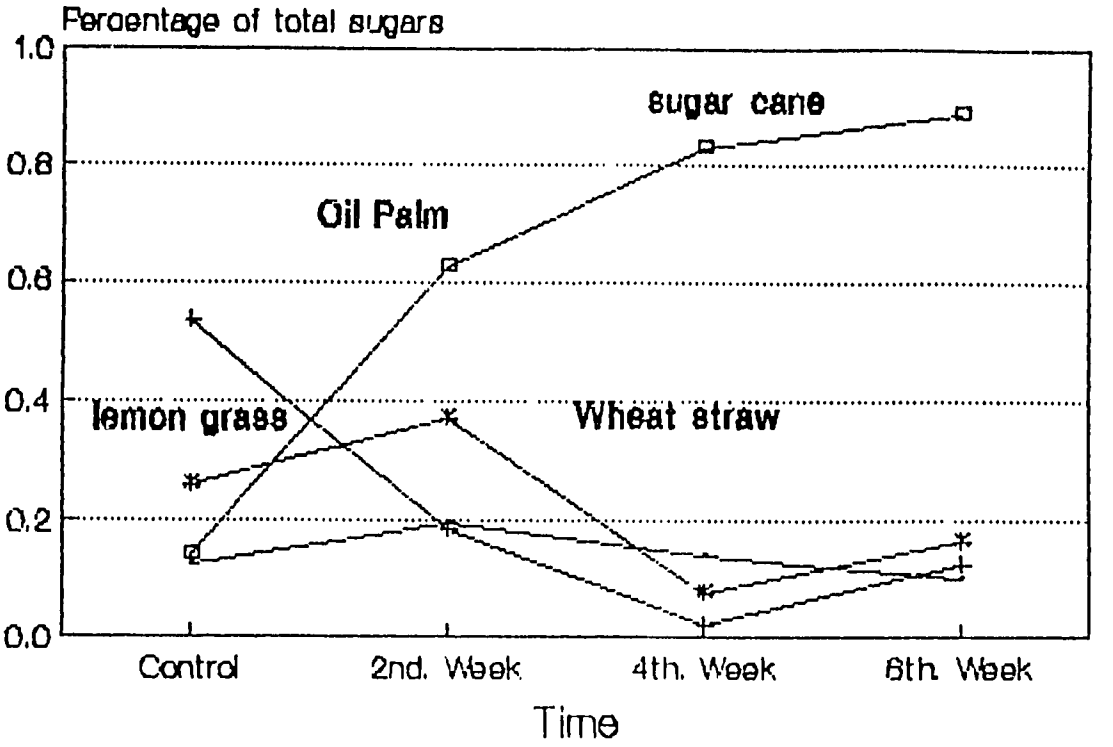
Glorophyllum trabeum

Figure 18
Degree of Polimerization



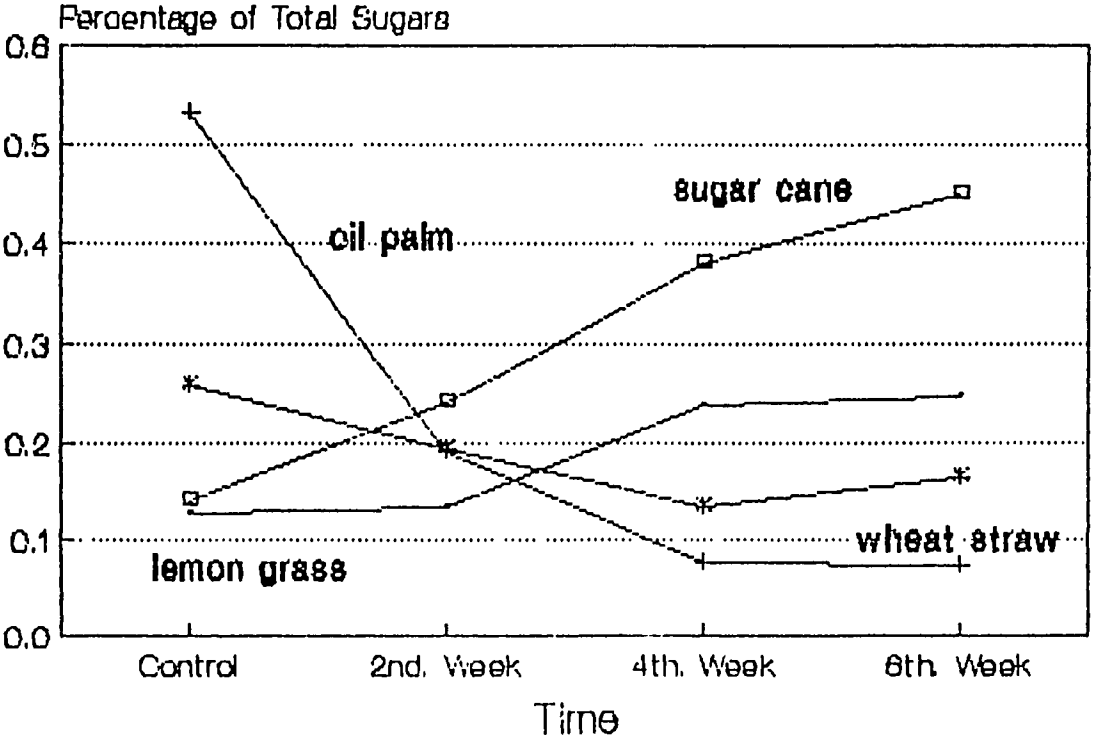
Wolphiporia cocos

Figure 19
Percentage of Total Sugars



Glorophyllum trabeum

Figure 20
Percentage of Total Sugars



Wolphiporia cocos

Figure 21

Sugar cane bagasse. aireation 1 VVM

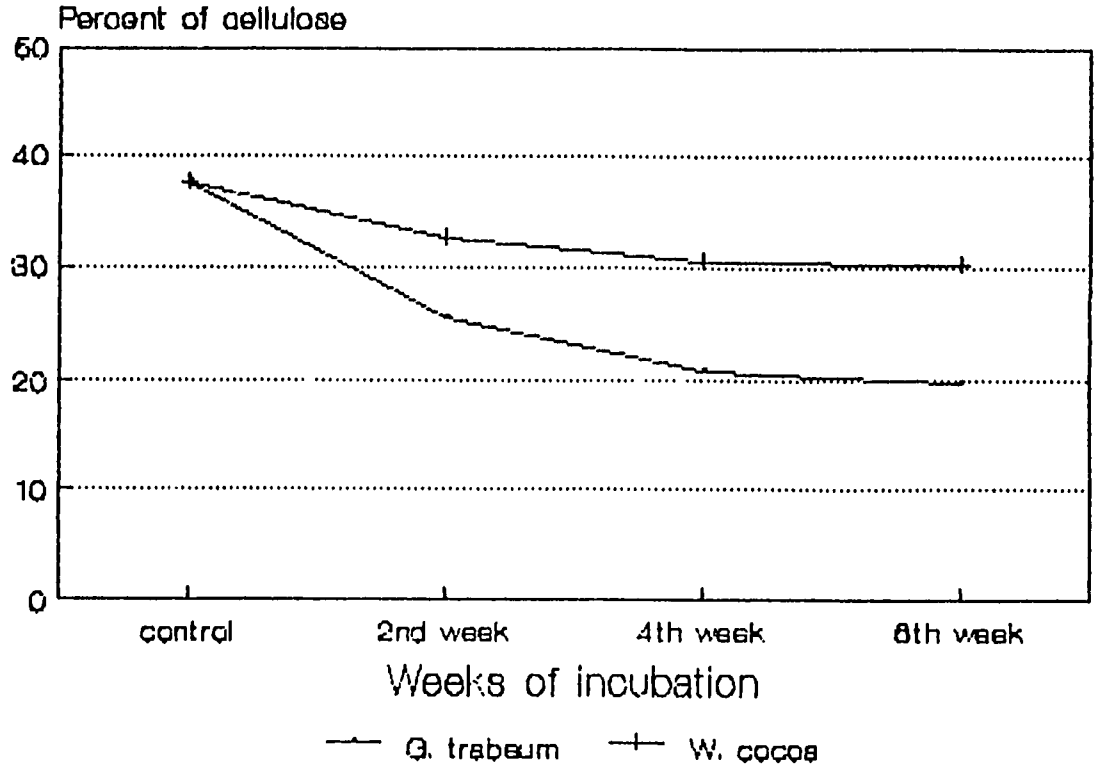


Figure 22

Sugar cane bagasse. Aeration 1 VVM

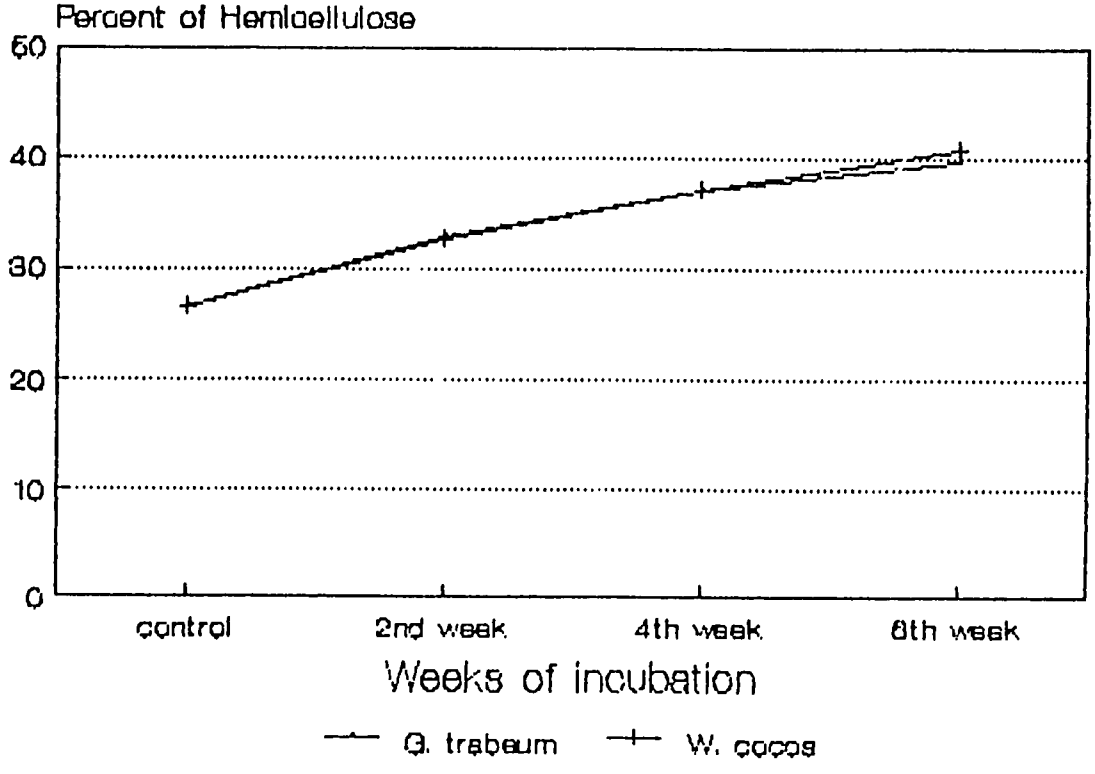


Figure 23

Sugar cane bagasse. Aeration 1 VVM

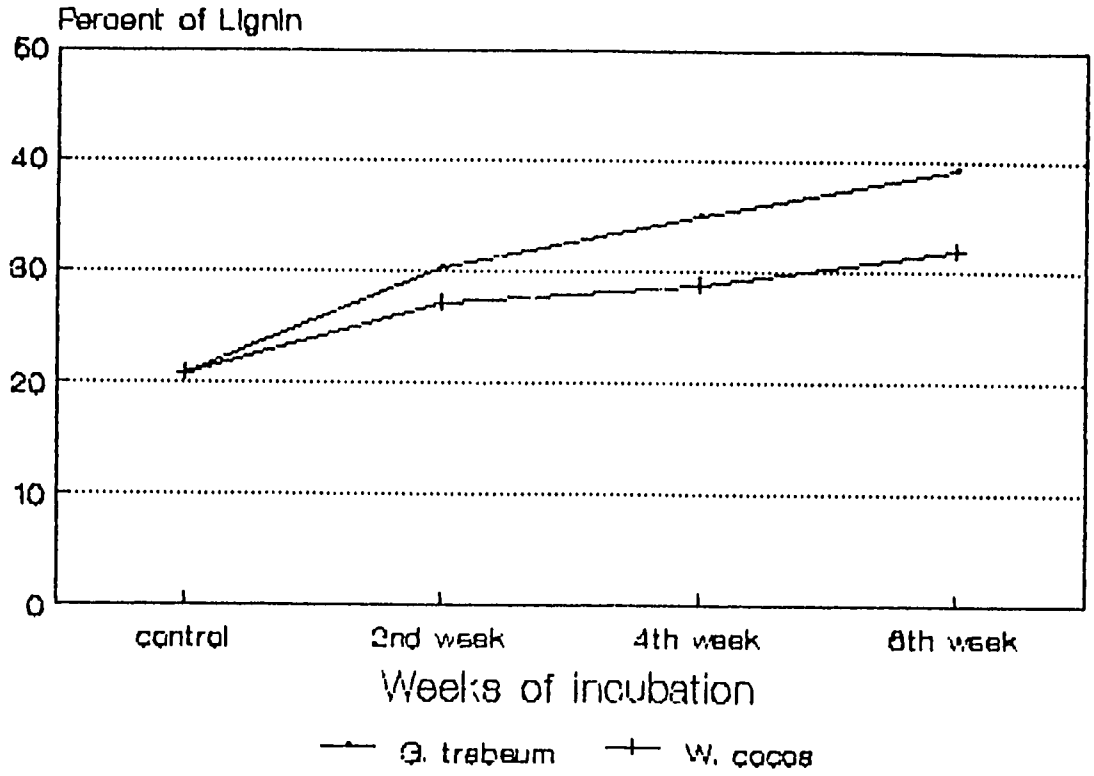
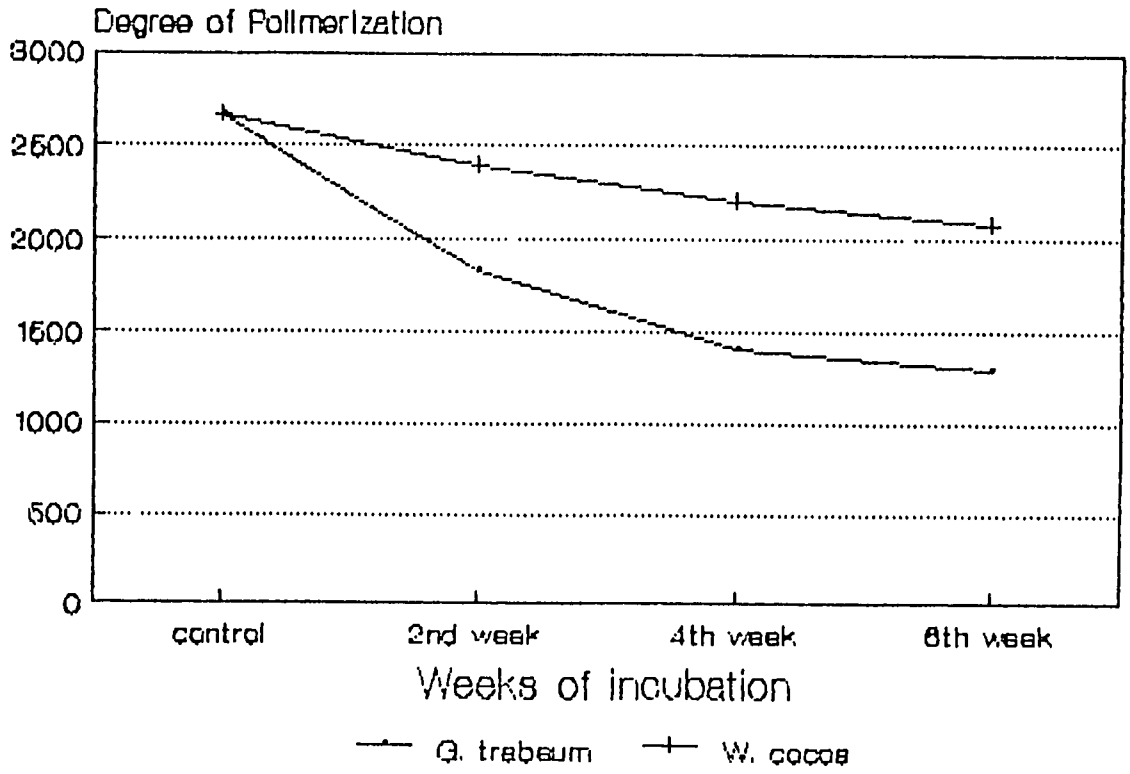


Figure 24

Sugar cane bagasse. Aeration 1 VVM



Annex II

Tables 1 to 29

TABLE 1

Results of degree of polymerization in sugar cane bagasse after growth of G. trabeum and W. cocos.

		Gloeophyllum trabeum			
		Time in weeks			
Control	C:N	Two	Four	Six	Eight
2669.68	180:1	1917.97	1590.94	1500.36	1590.22
	12:1	2529.34	2276.78	2207.72	2413.62
	25:1	2652.04	2643.29	2354.53	2344.50
		Wolphiporia cocos			
	180:1	2566.16	2282.07	2260.53	1966.55
	12:1	2568.76	1748.68	1527.40	1499.05
	25:1	2286.71	1947.57	1437.19	2008.32

TABLE 2

Results of content of % cellulose in sugar cane bagasse after growth of G. trabeum and W. cocos

		Time in weeks			
Control	C:N	Two	Four	Six	Eight
		G. trabeum			
37.58		26.98	22.36	21.13	22.27
	12:1	35.60	32.05	31.05	32.89
	25:1	37.33	37.21	33.15	33.70
		W. cocos			
		36.11	32.06	31.75	27.65
	12:1	36.14	24.59	21.48	21.03
	25:1	32.19	27.54	20.32	21.46

TABLE 3

Results of content of % Hemicellulose in sugar cane bagasse after growth of G. trabeum and W. cocos

Control	C:N	Time in weeks Two	Four	Six	Eight
			G. trabeum		
26.12	180:1	32.38	35.60	37.91	38.11
	12:1	37.68	40.64	44.88	39.38
	25:1	32.88	33.23	35.34	36.18
			W. cocos		
	180:1	34.66	38.35	40.02	45.93
	12:1	38.06	41.76	44.81	44.23
	25:1	29.83	40.22	38.46	38.64

TABLE 4

Results of Lignin content in % in sugar cane bagasse after growth of G. trabeum and W. cocos

Control	C:N	Time in weeks Two	Four	Six	Eight
			G. trabeum		
20.62	180:1	29.54	32.43	34.60	36.15
	12:1	21.53	22.75	22.91	24.61
	25:1	24.81	25.33	25.43	25.70
			W. cocos		
	180:1	24.86	25.08	25.25	23.99
	12:1	24.92	26.18	27.79	31.04
	25:1	24.89	30.11	33.19	26.12

TABLE 5

Results of content of total sugars in % in sugar cane bagasse after growth of G. trabeum and W. cocos

			G. trabeum		
			Time in weeks		
Control	C:N	Two	Four	Six	Eight
0.14	180:1	0.62	0.83	0.89	0.92
	12:1	0.42	0.45	0.84	0.84
	25:1	0.32	0.33	0.37	0.35
			W. cocos		
	180:1	0.24	0.38	0.45	0.49
	12:1	0.30	0.30	0.73	0.74
	25:1	0.39	0.42	0.72	0.50

Table No. 6

PERCENTAGE OF CELLULOSE IN LEMON GRASS BAGASSE

	Time in weeks		
Species	2	4	6
G.t.*			
r1	37.20	29.27	21.53
r2	35.17	26.81	16.95
ø	36.19	28.04	20.24
W.c.*			
r1	29.93	29.83	20.00
r2	30.73	27.45	21.46
ø	30.33	28.64	20.73

Note:
Percentage of cellulose in the original Lemon grass bagasse
r1 = 36.37, r2 = 36.83, ø = 36.60.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 7

PERCENTAGE OF CELLULOSE IN OIL PALM BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	37.33	36.60	23.30
r2	37.90	34.21	21.68
ø	37.62	35.41	22.49
W.c.*			
r1	38.70	38.37	25.83
r2	39.27	36.24	22.96
ø	39.00	37.31	24.40

Note:
Percentage of cellulose in the original Oil Palm bagasse was of: r1 = 34.60, r2 = 35.07, ø = 34.84.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 8

PERCENTAGE OF CELLULOSE IN WHEAT STRAW

Species	Time in weeks		
	2	4	6
G.t.*			
r1	35.30	36.07	22.07
r2	34.84	37.93	20.42
ø	35.17	37.00	21.25
W.c.*			
r1	31.00	30.53	20.63
r2	28.55	27.61	21.19
ø	29.78	29.07	20.91

Note:
Percentage of cellulose in the original wheat straw was of:
r1 = 34.00, r2 = 32.93, ø = 33.47.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 9

PERCENTAGE OF CELLULOSE IN SUGAR CANE BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	26.95	22.41	21.11
r2	27.00	22.31	21.15
ø	26.98	22.36	21.13
W.c.*			
r1	36.21	32.21	31.69
r2	36.01	31.90	31.81
ø	36.11	32.06	31.75

Note:
Percentage of cellulose in original sugar cane bagasse was of: r1 = 37.45 , r2 = 37.56 , ø = 37.51

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 10

PERCENTAGE OF HEMICELLULOSE IN LEMON GRASS BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	39.73	40.17	40.20
r2	36.90	39.13	38.33
ø	38.22	39.65	39.27
W.c.*			
r1	38.43	40.90	43.20
r2	37.37	39.62	40.11
ø	37.90	40.26	41.66

Note:
Percentage of hemicellulose in the original Lemon Grass bagasse was of: r1 = 37.47, r2 = 38.70, ø = 38.09.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 11

PERCENTAGE OF HEMICELLULOSE IN OIL PALM BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	38.97	35.62	41.27
r2	38.00	36.48	42.81
ø	38.49	36.05	42.04
W.c.*			
r1	36.57	39.03	40.90
r2	37.10	41.20	38.69
ø	36.84	40.12	39.80

Note:
Percentage of hemicellulose in original Oil Palm bagasse was of: r1 = 40.67, r2 = 39.06, ø = 39.87.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 12

PERCENTAGE OF HEMICELLULOSE IN WHEAT STRAW

Species	Time in weeks		
	2	4	6
G.t.*			
r1	33.13	37.07	38.57
r2	33.06	38.26	38.91
ø	33.10	37.67	38.74
W.c.*			
r1	35.93	34.77	36.27
r2	33.00	32.26	33.88
ø	34.47	33.52	35.08

Note:
Percentage of hemicellulose in the original Wheat Straw was of: r1 = 35.77, r2 = 36.79, ø = 36.28.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 13

PERCENTAGE OF HEMICELLULOSE IN SUGAR CANE BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	32.34	35.67	37.92
r2	32.42	35.52	37.90
ø	32.38	35.60	37.91
W.c.*			
r1	34.62	38.24	39.99
r2	34.50	38.45	40.05
ø	34.66	38.35	40.02

Note:
Percentage of hemicellulose in original sugar cane bagasse was of: r1 = 26.98 , r2 = 26.25 , ø = 26.62

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 14

PERCENTAGE OF LIGNIN IN LEMON GRASS BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	19.50	25.33	31.40
r2	22.80	25.80	33.26
ø	21.15	25.57	32.33
W.c.*			
r1	25.77	23.60	30.33
r2	26.53	24.92	28.41
ø	26.15	24.26	29.37

Note:
Percentage of lingnin in original Lemon Grass bagasse was of: r1 = 20.30, r2 = 21.73, ø = 21.02.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 15

PERCENTAGE OF LIGNIN IN OIL PALM BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	23.03	23.17	33.90
r2	23.53	24.69	30.15
ø	23.28	23.93	32.03
W.c.*			
r1	24.40	22.40	33.00
r2	22.87	21.86	30.25
ø	23.64	22.13	31.63

Note:
Percentage of lignin in original Oil Palm bagasse was of: r1 = 24.10, r2 = 25.43, ø = 24.77.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 16

PERCENTAGE OF LIGNIN IN WHEAT STRAW

Species	Time in weeks		
	2	4	6
G.t.*			
r1	22.67	19.87	29.90
r2	22.00	18.87	26.68
ø	22.34	19.37	26.79
W.c.*			
r1	24.27	25.10	37.73
r2	27.50	27.89	36.01
ø	25.89	26.50	36.87

Note:
Percentage of lignin in original wheat straw was of: r1 = 22.60, r2 = 23.27, ø = 22.94.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 17

PERCENTAGE OF LIGNIN IN SUGAR CANE BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	29.48	32.51	34.62
r2	29.59	32.35	34.58
ø	29.54	32.43	34.60
W.c.*			
r1	24.80	25.00	25.10
r2	24.92	25.16	25.40
ø	24.86	25.08	25.25

Note:
Percentage of lignin in original sugar cane bagasse was of: r1 = 20.68 , r2 = 20.65 , ø = 20.77

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 18

DEGREE OF POLIMERIZATION IN LEMON GRASS BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	1599.1	1235.9	1215.3
r2	1589.1	1230.1	1218.3
ø	1594.1	1233.0	1216.8
W.c.*			
r1	1435.3	1375.0	1142.1
r2	1431.6	1372.0	1147.7
ø	1433.5	1373.5	1144.9

Note:
The degree of polimerization in the original Lemon Grass bagasse was of: r1 = 1562.0, r2 = 1555.9, ø = 1159.0.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 19

DEGREE OF POLIMERIZATION IN OIL PALM BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	1042.6	1042.6	1004.1
r2	1044.9	1038.2	1002.7
ø	1043.8	1040.4	1003.4
W.c.*			
r1	1002.5	1002.2	918.65
r2	1003.4	1000.2	915.33
ø	1003.0	1001.2	916.99

Note:
Degree of polimerization in the original Oil Palm bagasse was of: r1 = 1126.6, r2 = 1121.5, ø = 1124.1.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 20

DEGREE OF POLIMERIZATION IN WHEAT STRAW

Species	Time in weeks		
	2	4	6
G.t.*			
r1	1304.8	1289.2	1106.8
r2	1298.2	1286.5	1109.8
ø	1301.5	1287.8	1108.3
W.c.*			
r1	1225.7	1245.0	1139.1
r2	1206.8	1236.6	1160.5
ø	1216.3	1240.8	1149.8

Note:
Degree of polimerization in the original wheat straw was of: r1 = 1302.8, r2 = 1304.6, ø = 1303.7.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 21

DEGREE OF POLIMERIZATION IN SUGAR CANE BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	1916.21	1591.62	1500.22
r2	1919.73	1590.26	1500.49
ø	1917.97	1590.94	1500.36
W.c.*			
r1	2573.61	2293.10	2256.64
r2	2558.71	2271.03	2264.41
ø	2566.16	2282.07	2260.53

Note:
Degree of polimerization in original sugar cane bagasse was of: r1 = 2660.45, r2 = 2668.26, ø = 2664.36

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 22

PERCENTAGE OF TOTAL SUGARS IN OF LEMON GRASS BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	0.1901	0.1303	0.09700
r2	0.1980	0.1451	0.09918
ø	0.1941	0.1377	0.09809
W.c.*			
r1	0.1343	0.2335	0.2454
r2	0.1360	0.2401	0.2461
ø	0.1352	0.2368	0.2458

Note:
Percentage of total sugars in original Lemon Grass bagasse was of: r1 = 0.1260, r2 = 0.1275, ø = 0.1268.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 23

PERCENTAGE OF TOTAL SUGARS IN OIL PALM BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	0.1836	0.01893	0.1216
r2	0.1885	0.01942	0.1233
ø	0.1861	0.01918	0.1225
W.c.*			
r1	0.1915	0.07838	0.07600
r2	0.1901	0.07856	0.07551
ø	0.1908	0.07847	0.07576

Note:
Percentage of total sugars in original Oil Palm bagasse was of : r1 = 0.5516, r2 = 0.5134, ø = 0.5325.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 24

PERCENTAGE OF TOTAL SUGARS IN WHEAT STRAW

Species	Time in weeks		
	2	4	6
G.t.*			
r1	0.3763	0.07417	0.1630
r2	0.3649	0.07526	0.1627
ø	0.3706	0.07472	0.1629
W.c.*			
r1	0.1925	0.1303	0.1630
r2	0.1967	0.1390	0.1676
ø	0.1946	0.1347	0.1653

Note:
Percentage of total sugars in wheat straw was of: r1 = 0.2631, r2 = 0.2559, ø = 0.2595.

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 25

PERCENTAGE OF TOTAL SUGARS IN SUGAR CANE BAGASSE

Species	Time in weeks		
	2	4	6
G.t.*			
r1	0.62	0.82	0.87
r2	0.61	0.83	0.90
ø	0.615	0.825	0.885
W.c.*			
r1	0.240	0.380	0.460
r2	0.240	0.380	0.440
ø	0.240	0.380	0.450

Note:
Percentage of total sugars in original sugar cane bagasse was of: r1 = 0.14 , r2 = 0.14 , ø = 0.14

* G.t. = **Glorophyllum trabeum**
* W.c. = **Wolfiporia cocos**
* ø = average
r = repetition

Table No. 26

PERCENTAGE OF CELLULOSE IN SUGAR CANE BAGASSE

AERATION: 1 VVM	Time in weeks		
Species	2	4	6
G.t.*			
r1	25.90	20.40	19.15
r2	25.00	21.00	20.15
ø	25.45	20.70	19.65
W.c.*			
r1	32.21	30.21	29.69
r2	33.01	30.90	30.81
ø	32.61	30.55	30.25

Note:
Percentage of cellulose in original sugar cane bagasse was of: r1 = 37.45 , r2 = 37.56 , ø = 37.51

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 27

PERCENTAGE OF HEMICELLULOSE IN SUGAR CANE BAGASSE

AERATION: 1 VVM	Time in weeks		
Species	2	4	6
G.t.*			
r1	33.43	36.76	40.29
r2	32.24	37.25	39.09
ø	32.84	37.05	39.69
W.c.*			
r1	32.28	37.42	40.29
r2	33.05	36.54	41.15
ø	32.66	36.98	40.72

Note:
Percentage of hemicellulose in original sugar cane bagasse was of: r1 = 26.98 , r2 = 26.25 , ø = 26.62

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 28

PERCENTAGE OF LIGNIN IN SUGAR CANE BAGASSE

AERATION : 1 VVM	Time in weeks		
Species	2	4	6
G.t.*			
r1	30.48	34.15	39.26
r2	29.90	35.53	38.85
ø	30.19	34.84	39.06
W.c.*			
r1	26.81	28.00	31.11
r2	27.29	29.56	32.34
ø	27.05	28.78	31.73

Note:
Percentage of lignin in original sugar cane bagasse was of: r1 = 20.88 , r2 = 20.65 , ø = 20.77

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Table No. 29

DEGREE OF POLIMERIZATION IN SUGAR CANE BAGASSE

AERATION: 1 VVM	Time in weeks		
Species	2	4	6
G.t.*			
r1	1861.12	1419.26	1320.12
r2	1810.37	1390.66	1280.94
ø	1835.75	1404.96	1300.53
W.c.*			
r1	2337.16	2240.10	2166.40
r2	2458.11	2171.43	2004.11
ø	2397.64	2205.75	2085.16

Note:
Degree of polimerization in original sugar cane bagasse was of: r1 = 2660.45, r2 = 2668.26, ø = 2664.36

* G.t. = Glorophyllum trabeum
* W.c. = Wolfiporia cocos
* ø = average
r = repetition

Annex III
Basal Medium Formula

COMPOSITION OF THE BASAL MEDIUM

Formula per Liter of distilled water

2 g NH_4NO_3

2 g KH_2PO_4

0.5 g $MgSO_4.7H_2O$

0.1 g $CaCl_2.2H_2O$

0.57 mg H_3BO_4

0.036 mg $MnCl_2.4H_2O$

0.31 mg $ZnSO_4.7H_2O$

0.039 mg $CuSO_4.5H_2O$

0.018 mg $(NH_4)_6Mo_7O_{24}.4H_2O$

0.015 mg $FeSO_4.7H_2O$

0.001 g Thiamine Hyidrochloride