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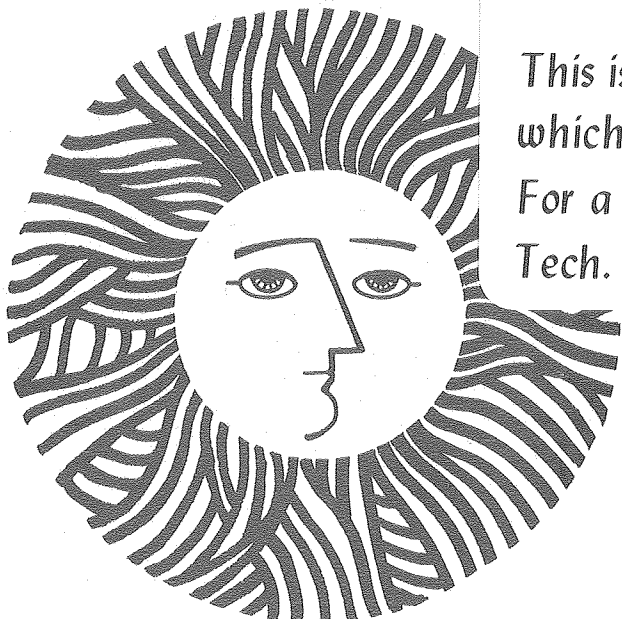
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PATTERNS OF DIFFUSIBILITY OF LIGNIN
AND CARBOHYDRATE DEGRADING SYSTEMS
IN WOOD-ROTTING FUNGI

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Presented at the American Chemical Society Meeting, September 9-14, 1979.
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SUMMARY

In an attempt to identify organisms that produce diffusible lignin-degrading systems, a culturing apparatus was constructed which contained two compartments separated by a bacteriological membrane filter. Lignin-degrading fungi were grown with lignocellulose in one compartment, and diffusion channels were maintained through the membrane to sterile lignocellulose in the adjoining compartment. For the fungi tested both lignin and carbohydrate were degraded when the mycelium and the substrate were in physical contact, but only carbohydrate was degraded significantly in the adjoining compartment containing sterile lignocellulose. Two organisms, Coriolus versicolor and Trichoderma reesii QM 9414 displayed slight diffusible lignin-degrading activity. Some fungi produced more diffusible carbohydrate-degrading activity than others.

INTRODUCTION

Lignin is one of the most abundant organic compounds in nature, yet while a number of fungi (9) and some actinomycetes (5) and bacteria (22,18) are known to be able to degrade lignin, the biochemistry of the process is still only poorly understood (2,1).

The main impediment to understanding lignin metabolism is the absence of a demonstrated cell-free lignin-degrading system. Discovery of such a system would not only allow description of the process but would also, potentially, allow development of schemes for the biodelignification of lignocellulosic materials and, perhaps, the production of useful organic chemicals from lignin. Delignification may be useful or necessary (depending on the process and substrate) in cellulose-based bioconversion processes where the presence of lignin inhibits the action of cellulolytic enzymes and thus the production of soluble sugar (12,23,21)

In a previous report (20) an apparatus termed a diffusion chamber was described which consisted of two aerobic compartments separated by a bacteriological membrane filter. The device allowed continuous passage of enzymes and metabolites between a growing mold culture in one compartment and a moist, sterile lignocellulosic substrate in the other.

It was felt that this apparatus would be useful for a preliminary investigation of cell-free lignin and carbohydrate degradation because more traditional enzyme isolation techniques could involve inactivation of one or more components of the systems (21). In the diffusion chamber there is the potential for continuous synthesis of necessary enzymes and other metabolites.

The organism originally tested in this apparatus, Chrysosporium pruinosum (= Phanerochaete chrysosporium), a thermotolerant lignocellulose-degrading fungus, produced diffusible carbohydrases which hydrolyzed approximately 50% of the carbohydrate in the sterile lignocellulosic substrate. The mold grew at the expense of the hydrolysis products in the chamber on the opposite side of the bacteriological filter separating the two compartments. These facts indicated that diffusion of enzymes and hydrolysis products was occurring between the two compartments. However, little or no lignin was degraded in the sterile substrate. Under conditions where the organism grew in direct contact with the lignocellulosic substrate 50% of the lignin and 80-90% of the carbohydrate were degraded (20).

Among cellulolytic organisms both soluble (diffusible) and cell-bound cellulase systems are encountered (3,17). It was considered possible that the same dichotomy might apply to the enzymes of lignin-degrading species. It was, thus, decided to test a number of known lignin-degrading organisms in the diffusion-culture apparatus to see if any would give evidence of producing a soluble lignin-degrading system. In addition the diffusibility of the carbohydrate-degrading enzymes could be examined.

MATERIALS AND METHODS

Table 1 lists the organisms tested (all fungi) and the approximate temperature and pH optima for growth. These were determined using 5 degrees temperature intervals and 0.5 unit pH intervals as described previously (19). Each organism was grown using the conditions of temperature and pH listed. Cultures were stored at 4°C on slants of the complex media indicated and transferred every 9 months. The composition of these media has been described (19).

To assess lignocellulose degradation, organisms were grown in moist lignocellulose (washed cattle manure fiber) on the surface of agar plates or in diffusion chambers (20). The mineral medium contained the salts solution described previously (19) plus a final concentration of 1.6 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ added in the trace elements solution, 0.01% (w/v) Difco yeast extracted and, for the plates, 1.5% (w/v) Difco agar. Aliquotes of medium were autoclaved at the pH's indicated in Table 1 except for the pH 4.0 solutions containing agar. These were autoclaved at pH 6.0 and titrated to pH 4.0 with sterile HCl before pouring.

After preparation of the diffusion chambers as described previously (20), 200 mg of dry, sterile manure fiber was added to the top compartments which were then inoculated with 2.0 ml of a culture suspension. All plates and diffusion cultures were incubated in sealed plastic bags ("Clavies," Cole-Parmer Corp., Chicago, Ill, U.S.A.) containing water and an atmosphere of pure oxygen. One ml of medium of the appropriate pH was added weekly to each diffusion culture, and the bags were flushed and re-filled with oxygen and re-incubated. Procedures for analysis of residual lignin and carbohydrate have been described (20).

RESULTS

The organisms tested have been grouped on the basis of the relative amount of lignin degradation seen in the agar plate cultures (Fig. 1). Members of Group I showed the greatest ability to degrade lignin (~ 90% loss in 30-60 days). P. viticola was included in this group on the basis of the results from the inoculated diffusion culture. The P. viticola data are from 60 day cultures whereas the other diffusion cultures were harvested after 30 days. Values for residual lignin and carbohydrate in the 30 day inoculated P. viticola diffusion cultures were: lignin $59.2 \pm 4.3\%$ and carbohydrate

64.0 ± 5.0%. The amounts of residual lignocellulose in the sterile compartments of the 30 day cultures approximated that seen in the uninoculated control.

Members of Group II showed 70-80% lignin loss in the plate cultures and 90% or more carbohydrate loss. Members of Group III showed 35-65% lignin loss and 70-85% carbohydrate loss. Plate data for L. edodes were not available, but it was included with the members of Group III on the basis of results seen in the inoculated diffusion cultures.

The two Trichoderma strains in Group IV are not known to be ligninolytic, nevertheless, they consistently showed the ability to remove slightly more lignin than was lost in the uninoculated controls. Carbohydrate loss was approximately 60%. Members of the last group showed little or no lignin-degrading activity and variable carbohydrate-degrading abilities. Diffusion cultures were not carried out with members of this group.

It was expected that the inoculated compartments of the diffusion chambers would show lignin and carbohydrate losses similar to those seen in the agar plate cultures since in both cases the organism was in intimate contact with the substrate. This is seen to be generally true, but in a number of cases lignocellulose degradation is less complete in the diffusion cultures. One striking example of this is P. chrysosporium, ME-446. Here only a slight amount of lignin was degraded relative to that seen in the agar plate cultures, and carbohydrate degradation was also depressed.

With the possible exceptions of C. versicolor and T. Reesii, QM9414, none of the fungi tested gave evidence of being able to produce diffusible lignin-degrading systems even though in a number of cases lignin degradation in inoculated substrates was excellent

An interesting pattern emerged with respect to the diffusibility of the carbohydrate-degrading enzyme systems. Members of Group I which degraded lignin and carbohydrate best when in contact with the substrate appeared to produce only very small amounts of diffusible carbohydrate-degrading activities. Members of Group II-IV which showed poorer overall lignocellulose-degrading ability displayed, in general, much higher levels of diffusible carbohydrate-degrading activities.

DISCUSSION

Humphrey (7) reported the temperature relations of a number of wood-destroying fungi. Four species used in this work were also studied by him. He found the following growth optima (with my values given in parentheses). S. frustolatum (frustulosum), 28°C (30); C. (Polystictus) versicolor, 28°C (30); F. annosa--two isolates--24° and 34°C (35); P. pini, 24°C (30). Except for the values for P. pini, the numbers agree rather well. It is unusual that different isolates of the same species (F. annosa) would have such different temperature optima. Similar differences were found between strains of T. reesii, (Table 1) but in this case the organisms were the product of specific mutagenesis and selection (15,16).

With the exceptions of Poria placenta (4,11), a brown rot, and the two Trichoderma strains the fungi tested in this work have all been reported to degrade lignin (and cellulose). They were chosen for this study because they appeared to represent a spectrum of physiological types with respect to their lignin and carbohydrate-degrading abilities. It was hoped that by examining organisms with known differences in lignin metabolism the chances of finding one that produced a diffusible lignin-degrading system would be enhanced.

G. dichrous, M. taxicola, and S. frustolatum were reported by Kirk and Kelman (11) to give atypical phenol oxidase reactions in tests used to identify white rot (lignocellulose-degrading) fungi. C. yamanoi and F. ulmaria were reported by Kirk and Moore (12) to degrade lignin initially more rapidly than carbohydrate in wood samples, compared to other fungi. P. chrysosporium strains 104297-Sp and ME 446 and L. edodes were also reported to have this ability in varying degrees (10) as was P. cremea (2). P. viticola was reported by Eriksson (6) to be able to degrade the middle lamellar regions of woody tissue selectively while leaving the cell walls largely intact. The middle lamella contains mainly lignin, while the cell wall contains mostly cellulose and hemicellulose. P. pini, F. annosa and T. amarus have been classified by Liese (14) as true white rots. These organisms degrade lignin and hemicellulose initially while cellulose is degraded later. C. versicolor is classified as a simultaneous rot with all three major wood components being degraded at approximately the same rate (14,12,4).

In all cases cited above where disproportionate rates of lignin degradation were claimed, the rates were based on the amount of material originally present in the wood substrate. Thus, percentage losses were compared. Since lignin is normally a minority (15-30%) constituent of wood, comparison of lignin and carbohydrate losses on an absolute basis in terms of grams of each material lost often obliterates or even reverses the claimed disproportionalities. Nevertheless, the relative degradation rates reported are still useful for establishing phenotypic differences between organisms.

Except for the two Trichoderma strains and P. placenta, the organisms used in this study do not fall into any obvious groupings based on the selection criteria stated earlier. Indeed, none of the organisms which were

described as initially "preferential" lignin degraders displayed this character under my cultivation conditions. The most likely explanation for these differences is the nature of the substrate. Manure fiber is unlike the woody tissue upon which most of these organisms are adapted to grow. Not only is the fiber derived from straw, but it has undergone fermentation in the rumen where much of the carbohydrate has been removed. The residual fiber is thus depleted in enzymatically accessible carbohydrate and enriched in lignin. On a weight basis the amounts of lignin and carbohydrate are equal. The composition of this material as reported previously (20) is: 37% lignin, 37% carbohydrate, 14% ash and 10% crude protein.

If lignin degradation is necessarily linked to carbohydrate degradation as suggested by Ander and Eriksson (2) and Kirk, et al. (13), the absence of lignin degradation shown by C. yamanoi and T. amarus may be due to a lack of enzymatically accessible carbohydrate. P. placenta, which does not degrade lignin, presumably, is unable to degrade the carbohydrate in manure fiber for the same reason as C. yamanoi and T. amarus. However, F. ulmaria, S. frustolatum and P. pini which are known lignin-degraders are able to degrade some of the carbohydrate but still show little or no lignin degradation. The explanation for this is not clear. It is possible that the presence of a rather high concentration of silica (the major ash component) or the composition or structure of the lignin itself may inhibit degradation.

The two T. reesii strains examined were selected because they were both known to product high levels of cellulase, and it was of interest to see how much of this relatively refractile substrate was susceptible to cellulase attack. The strains behaved similarly except that QM 9414 appeared to be able to cause a small loss in lignin content in the sterile substrate. Upon

cessation of growth 40% of the substrate carbohydrate remained in the plate cultures. The same degree of carbohydrate degradation was seen by Kaneshiro, et al. (8) for another strain of T. reesii (viride) grown in submerged shake-flask cultures with washed manure fiber. The balance of the carbohydrate is probably protected from enzyme attack by lignin (12). A similar situation was seen with the lignin-degrading mold Chrysosporium pruinosum (= P. Chrysosporium) growing with manure fiber in shake flask cultures (20). Under these conditions lignin degradation was inhibited, and carbohydrate degradation was reduced from a normal 90% to 50%. In diffusion cultures the same picture was seen. In the absence of lignin degradation in the sterile fiber carbohydrate loss reached a maximum of only 50% (20).

Members of Groups II and III showed good to moderate lignin-degradating ability on plates and in the inoculated diffusion cultures except for P. chrysosporium ME 446. For Group II strains carbohydrate degradation on plates was extensive, however, lignin and carbohydrate degradation in the inoculated diffusion cultures was sometimes not as extensive as seen on the plates. This may be attributable to the fact that the diffusion cultures received nutrients and buffer only at weekly intervals while a continuous supply of these was available in the plate cultures.

P. chrysosporium ME 466 appeared not to be able to degrade lignin in the inoculated diffusion cultures. It was thought that soluble sugars produced in the sterile fiber might be repressing the synthesis or activity of lignin-degrading enzymes in the inoculated compartment. To test this hypothesis, cultures were incubated for 60 days to insure that all free sugars would be depleted. The same degradation pattern was seen. At the

present time, I can only suggest that the poorer control of environmental parameters in the diffusion cultures vis à vis the agar plate cultures is responsible for the absence of lignin loss in the former.

In earlier work with this system using C. pruinatum no substrate was included in the inoculated portion of the chamber; nevertheless growth occurred, and carbohydrate was degraded in the sterile substrate (20). It was recognized at the time that the absence of lignin degradation in the sterile compartment might be due to the lack of a lignin-related inducer of lignin-degrading enzymes in the growth chamber. To test this hypothesis, the experiments were repeated with lignocellulose fiber included in the inoculated chamber. The same results were obtained (S. Rosenberg, unpublished data). Carbohydrate, but not lignin, was degraded in the sterile chamber. In the inoculated chamber both lignin and carbohydrate were lost. The pattern was similar to that seen in this study for P. chrysosporium HHB.

The three members of Group I showed a pattern of cellulose degradation in diffusion cultures different from the other organisms tested. While lignocellulose degradation was excellent when hyphae were in contact with the substrate, both lignin and carbohydrate degradation were poor in the sterile substrate. This suggests that both the lignin and carbohydrate-degrading systems or one or more key components of these systems are closely associated with the mycelium in these organisms.

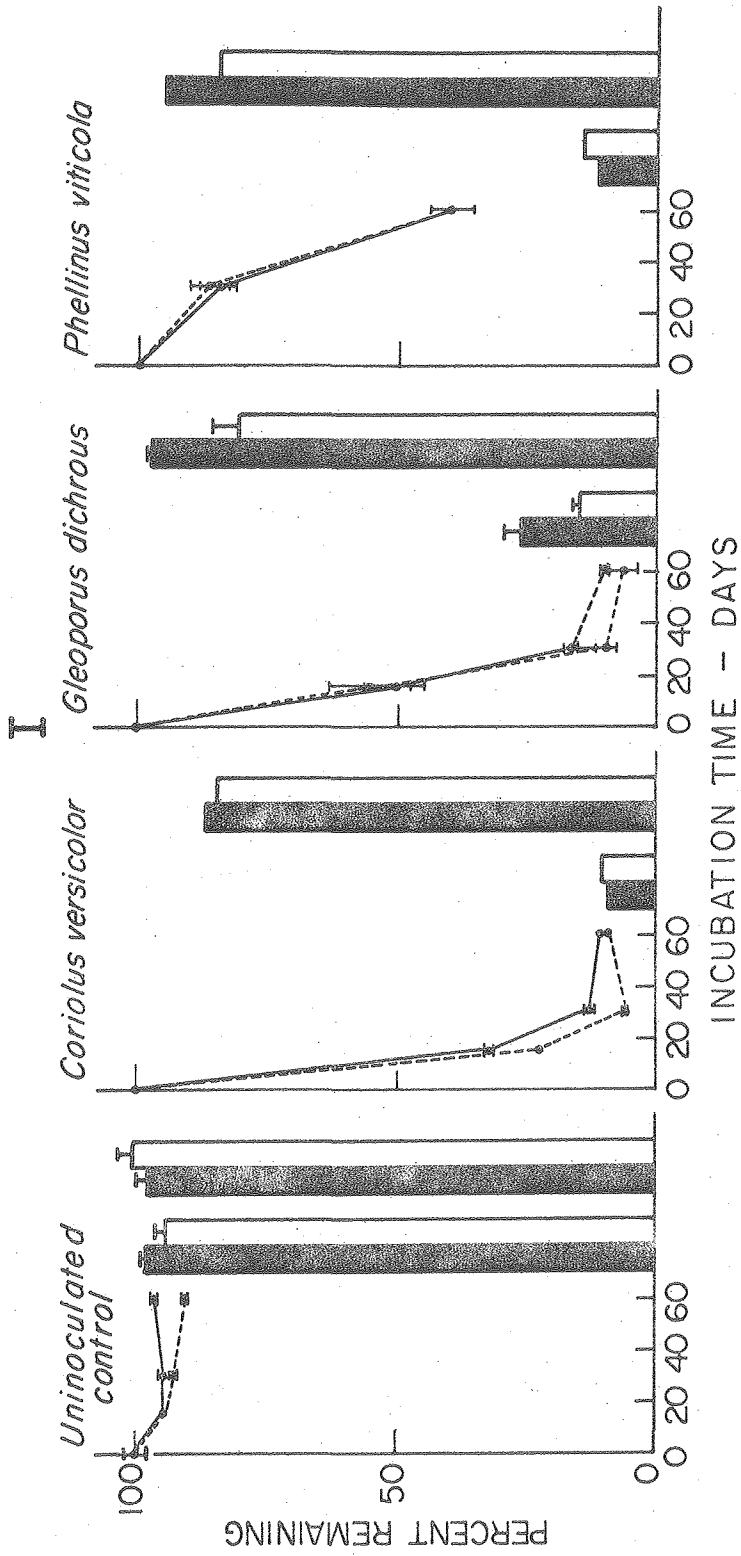
Data from previous studies indicate that as much as 10% of the lignin can be lost from uninoculated controls using these growth systems (20). Lignin losses of 10% or less in inoculated cultures are, thus, not considered to be significant. The significance of the small lignin losses (ca 14%) seen in the sterile fiber of the C. versicolor and T. reesei QM9414 cultures

is not clear. The losses appear to be real, but they are limited. None of the other organisms tested gave evidence of being able to produce a diffusible lignin-degrading system.

Acknowledgement

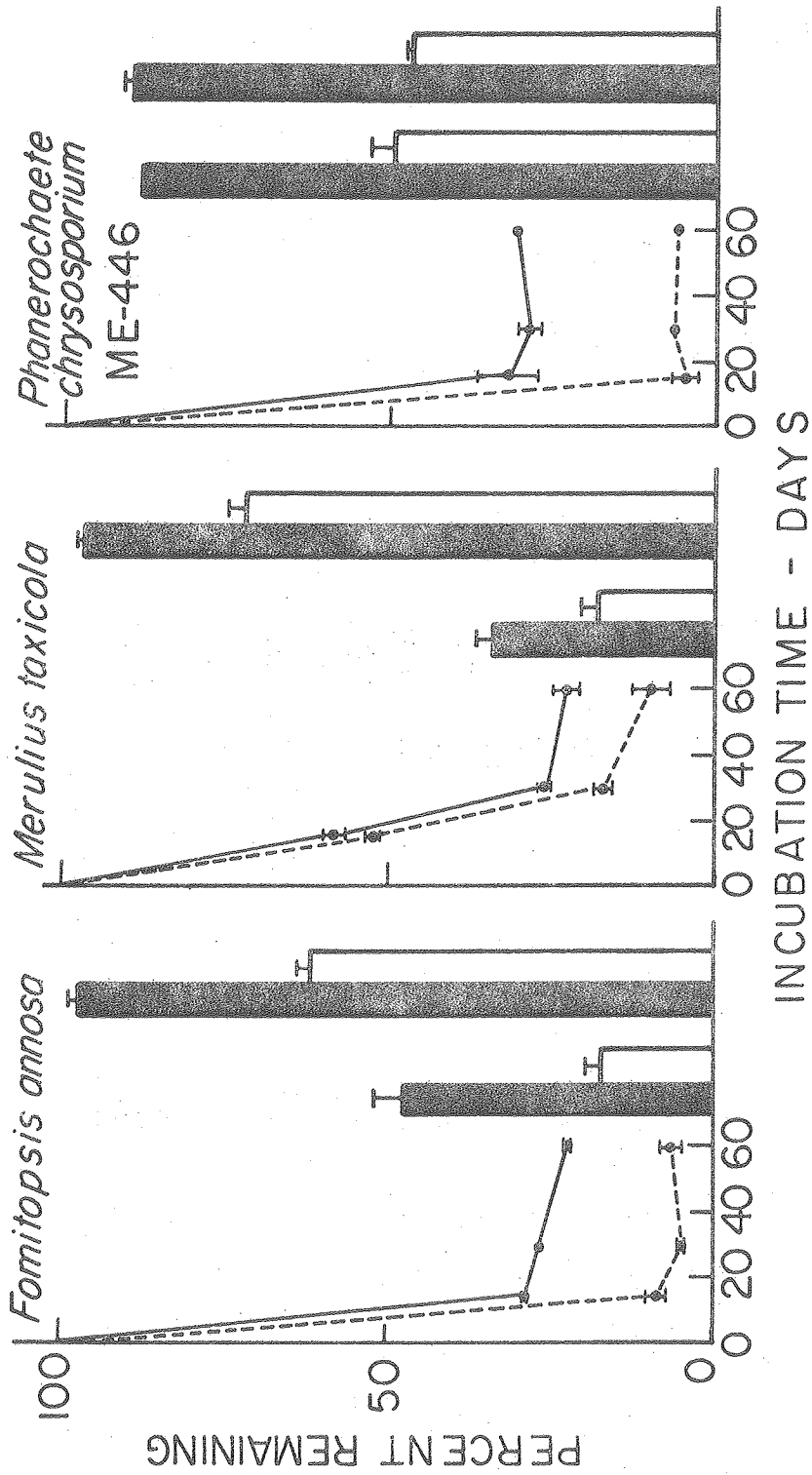
I wish to thank Loretta Baier and Tonya Hill for skillful technical assistance and Frances Lombard of the Forest Products Laboratory, Madison, Wisc. for supplying many of the organisms used in this study. This work was supported by grant # AER75-23686 to C.R. Wilke from the National Science Foundation, RANN program.

Figure 1. Lignin and carbohydrate remaining in washed manure fiber degraded by cellulolytic and ligninolytic fungi. —lignin and ----carbohydrate remaining in agar plate cultures. Dark bar, lignin and light bar, carbohydrate remaining in diffusion cultures. Left-hand bar pair = residual lignocellulose in top (inoculated) chamber. Right-hand bar pair = residual lignocellulose in bottom (sterile) chamber. Error bars indicate range of variability in replicate samples. No error bar means only one sample processed. Diffusion cultures were incubated for 30 days except for the P. viticola culture which was incubated for 60 days.



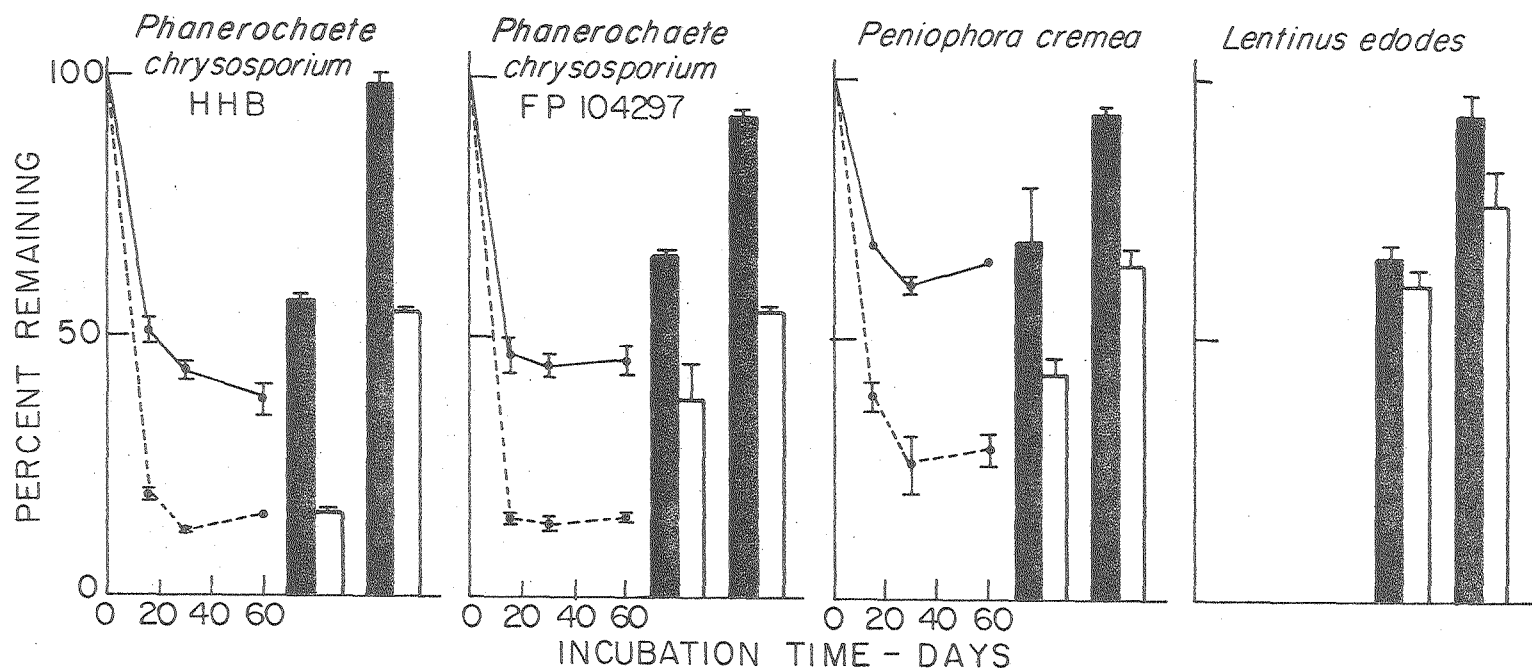
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II



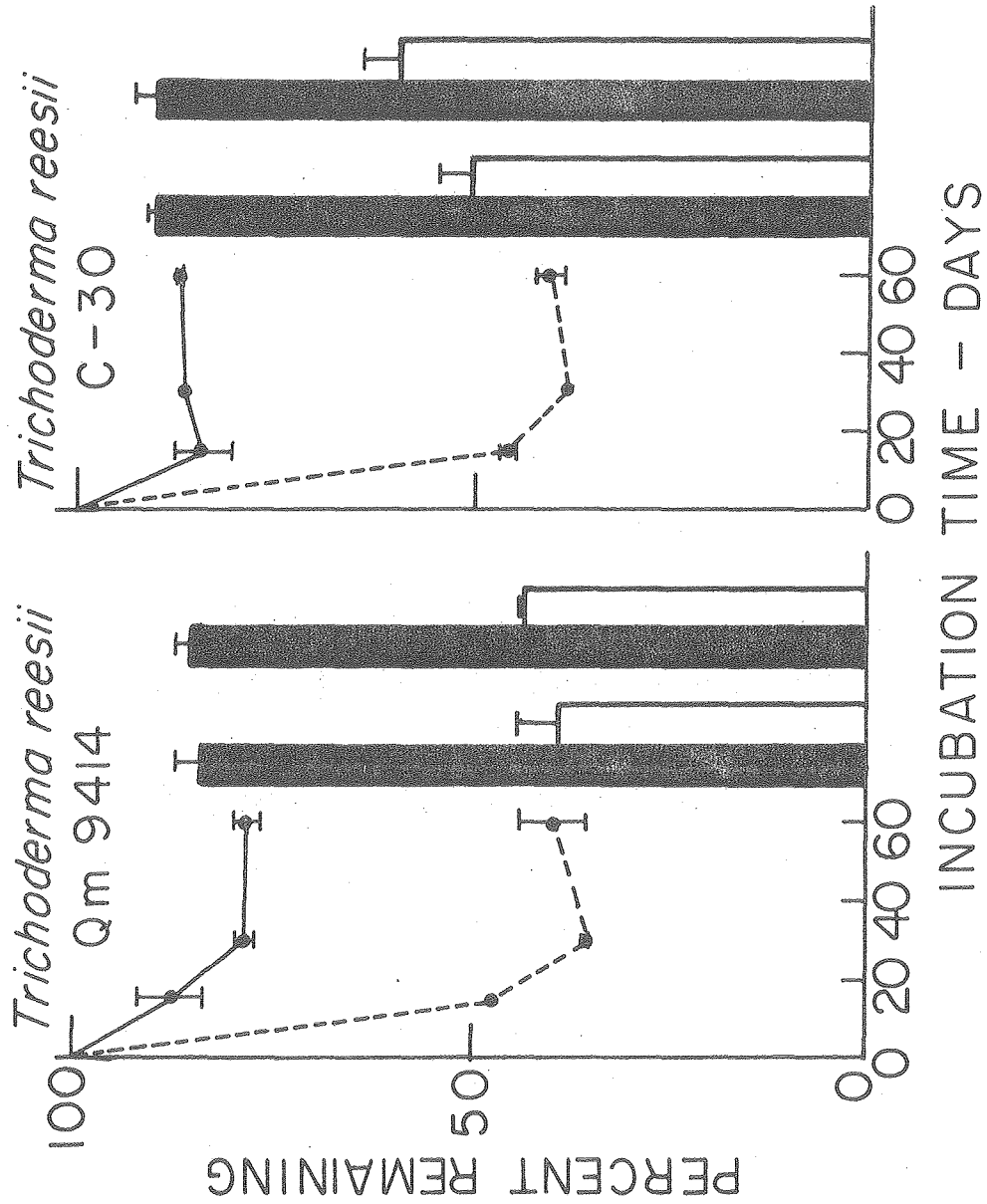
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III



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IV



XBL 798-10956

V

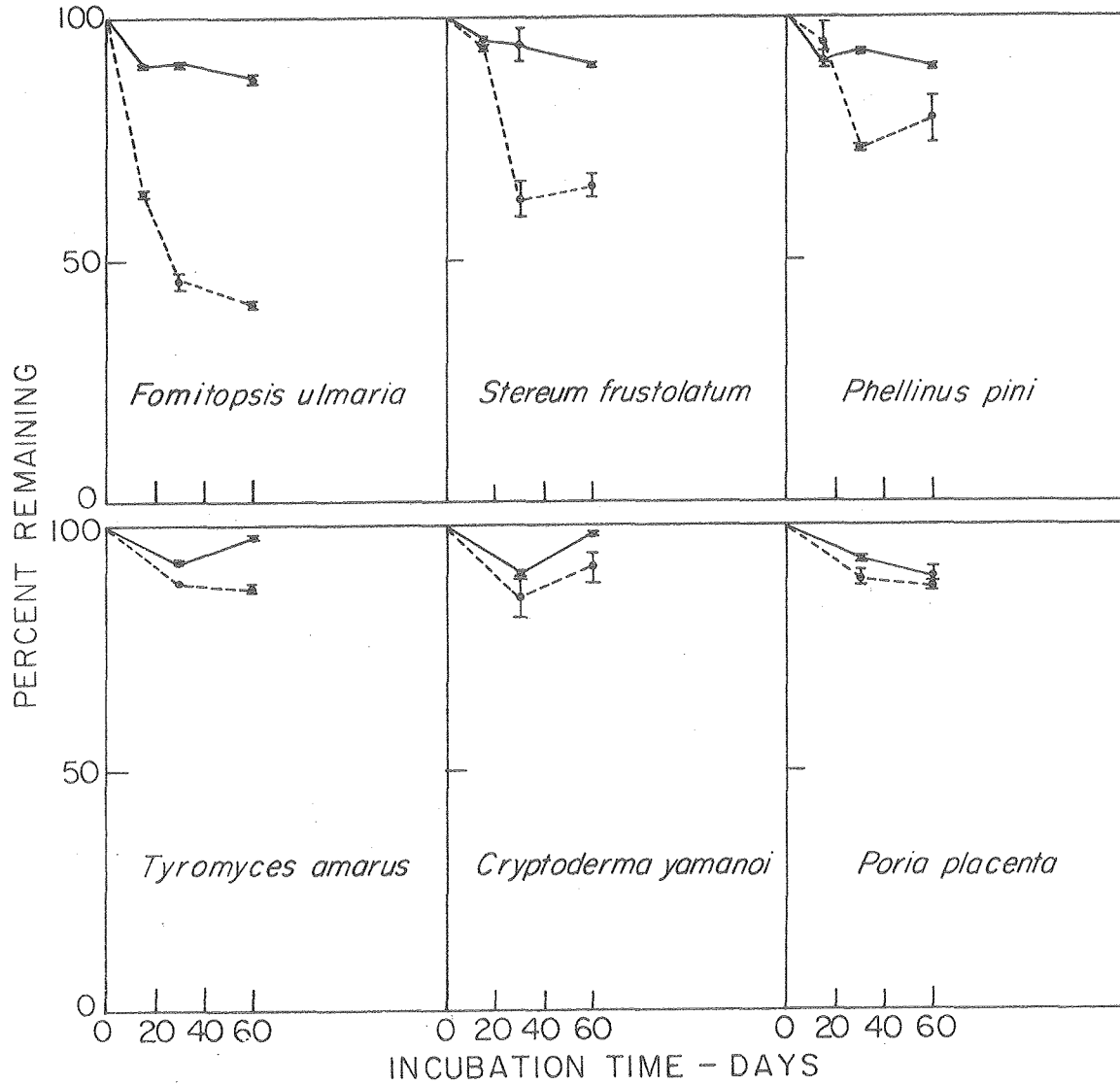


Table 1. FUNGI EXAMINED AND CONDITIONS OF CULTIVATION

ORGANISM	STRAIN	APPROX. TEMP. OPT., °C	APPROX. PH OPTIMUM	STORAGE ^a MEDIUM	SOURCE ^b	REFERENCE
<u>GLEOPORUS DICHROUS</u> (Fr.) BRES. (= <u>POLYPORUS DICHROUS</u>) ATCC 26758	FP-106899-SP	30	5.0	YES	FPL	11
<u>MERULIUS TAXICOLA</u> (PERS.) DUBY (= <u>PORIA TAXICOLA</u>)	L-3356-SP	30	4.0	MX	FPL	11
<u>STEREUM FRUSTOLATUM</u> (PERS. ex Fr.) FCKL.	FP-106073-S	30	4.5	YES	FPL	11
<u>CRYPTODERMA YAMANOI</u> IMAZ	F6P	25	5.0	MX	FPL	12
<u>FOMITOPSIS ULMARIA</u> (Sow. ex Fr.) Bond. and Sing. (= <u>FORMES ULMARIUS</u>)	L-11700-S	30	7.0	YES	FPL	12

Table 1. (Continued)

ORGANISM	STRAIN	APPROX. TEMP OPT. °C	APPROX. PH OPTIMUM	STORAGE ^a MEDIUM	SOURCE ^b	REFERENCE
<u>PHANEROCHAETE CHRYSOSPORIUM</u> BURDSALL	FP-104297-sp	40	4.5	PG	FPL	10
<u>PHANEROCHAETE CHRYSOSPORIUM</u> ATCC 34541	ME-446	40	4.5	MX	FPL	10
<u>PHANEROCHAETE CHRYSOSPORIUM</u> ATCC 34540	HHB-6251-SP	35	4.5	PG	FPL	10
<u>LENTINUS EDODES</u> (BERK.) SING.	117=1+(C Strain)	25	4.0	YES	FPL	6
<u>PELLINUS VITICOLA</u> (Schw. ex Fr.) DONK (= <u>PELLINUS ISABELLINUS</u>)	L-15651-sp	25	4.0	YES	FPL	2
<u>PENIOPHORA CREMEA</u>	P-B1780220	35	4.0	YES	RCF	

Table 1 (Continued)

ORGANISM	STRAIN	APPROX. TEMP. OPT., °C	APPROX. PH OPTIMUM	STORAGE ^a MEDIUM	SOURCE ^b	REFERENCE
<u>PHELLINUS PINI</u> (THORE ex Fr.) A. AMES (= <u>TRAMETES PINI</u>)	FP-53236-S	30	4.5	MX	FPL	14
<u>FOMITOPSIS ANNOSA</u> (Fr.) KARST. (= <u>FOMES ANNOSUS</u>) ATCC 11667	FP-58548-S	35	4.5	MX	FPL	14
<u>TYROMYCES AMARUS</u> (HEDGE) LOWE (= <u>POLYPORUS AMARUS</u>)	FP-94377-S	25	4.0	MX	FPL	14
<u>CORIOLUS VERSICOLOR</u> (L. ex Fr.) QUEL (= <u>POLYPORUS VERSICOLOR</u>) ATCC 11235	R-105	30	4.5	YES	FPL	14,12,4
<u>PORIA PLACENTA</u> (= <u>PORIA MONTICOLA</u>)	704	25	4.0	MX	WW	11,4
<u>TRICHODERMA REESII</u> (= <u>TRICHODERMA VIRIDE</u>)	QM-9414	35	4.0	PG	MM	15
<u>TRICHODERMA REESII</u> (= <u>TRICHODERMA VIRIDE</u>)	C-30	25	4.5	RUT	MX	16

Table 1 (continued)

a) Media Described in Ref. 19.

b) Abbreviations: FPL, U.S. Forest Products Laboratory, Madison, Wisc., USA;
RCF, T. Nilsson, Royal College of Forestry, Ultuna, Uppsala, Sweden;
WW, W.W. Wilcox, Univ. of Calif. Forest Products Laboratory, Richmond, Ca.,
USA; MM, M. Mandels, U.S. Army Natick Laboratories, Natick, Mass., USA.
RUT, B.S. Montenecourt, Dept. of Biochemistry and Microbiology, Rutgers
University, New Brunswick, N.J., USA.

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