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PROPERTIES OF CELLULASES OF TWO BROWN-ROT FUNGI AND TWO WHITE-ROT FUNGI[1]

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ABSTRACT

Cellulases from two brown-rot fungi (C_x) and from two white-rot fungi (C_x and C_1) were compared. The C_x cellulases of the brown-rot and the white-rot fungi responded differently to pH and temperature effects. C_x activity of the brown-rot fungi was optimum in the low pH region—2 to 3, whereas the activity of the white-rot fungi was considerably lower than optimum. C_x activity of the white-rot fungi was more depressed at low temperatures than was that of the brown-rot fungi. Stability of C_x from the brown-rot fungi was uniform for the range 23 to 60 C, whereas C_x from the white-rot fungi was substantially reduced at temperatures above 50 C. The C_1 cellulases produced by only the white-rot fungi were more unstable than those of the C_x to pH and temperature changes. Sulfhydryl groups apparently are not active sites for any of the cellulases. Degradation of carboxymethylcellulose by C_x was similar for the four fungi and proceeded in a random manner; degradation of microcrystalline cellulose by C_1 was in an endwise manner, and evidently acts as a ß-1-4-glucan cellobiohydrolase. ß-glucosidase produced by the four fungi hydrolyzed the cellulase breakdown products to glucose.

Additional keywords: Coriolus versicolor, Ganoderma applanatum, Poria monticola, Gloeophyllum trabeum, cellulase, enzyme, wood decay, enzyme activity, enzyme stability.

INTRODUCTION

The study of cellulolytic enzymes is essential to understand the decomposition of wood by wood-destroying fungi; the enzymes are necessary to break down native cellulose of wood into easily assimilable products, mainly simple sugars. A previous study (Highley 1973) revealed that carbon source had different effects on cellulase production by typical white-rot and brown-rot fungi. Three white-rot fungi produced cellulase (C_x and C_1) only in the presence of cellulosic substrates, and simple sugars such as glucose inhibited production. Conversely, three brown-rot fungi produced abundant cellulase with only glucose as the carbon source and two of the brown rotters produced only traces of cellulase with cellulose as the sole source of carbon. Cellulase preparations from brown-rot fungi could not significantly degrade a highly ordered form of cellulose, whereas preparations from the white-rot fungi could.

The objectives of this investigation were to determine the effect of pH, temperature, and sulfhydryl inhibitors on activity and stability of cellulases from two white-rot fungi and from two brown-rot fungi and to identify their mechanism of depolymerization cleavage reactions on cellulose. Information on these properties of white-rot and brown-rot cellulases may explain their different behavior during decay as well as lead to new methods to control decay.

METHODS

The following fungi were used: Two white-rot fungi, *Coriolus versicolor* (L. ex Fr.) Quél. (Madison 697) and *Ganoderma applanatum* (Pers. ex Wallr. (Pat.)) (Madison 708); and two brown-rot fungi, *Poria monticola* (Murr.) (Madison 695) and *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. (Madison 617). Culture methods and preparation of crude enzyme have been described (Highley 1973). The carbon source for *C. versicolor* and *G. applanatum* was 0.5% microcrystalline cellulose (American viscose) plus 0.1% aspara-

[1]The author thanks Thomas Straka for technical assistance during the study.

gine. For *P. monticola* and *G. trabeum*, the carbon source was 0.5% microcrystalline cellulose plus 0.1% cellobiose. Cellulase studies were conducted with partially purified enzyme. The crude enzyme preparation was partially purified by precipitation with four volumes of ice-cold ethanol. After 4 h in the ethanol at 0 C, the precipitated proteins were separated by centrifugation, resuspended in 0.1 M acetate buffer, pH 5.0, then dialysed 16 h against distilled water. Toluene (1 ml/liter) was added as a preservative.

Ability of enzyme preparations from the four fungi to degrade soluble cellulose (C_x, ß (1→4) D-glucan 4-glucanohydrolase activity E.C.3.2.1.4) was determined by a previously described viscometric assay with sodium carboxymethylcellulose (Fisher, purified with a degree of substitution 0.65–0.85) as substrate (Highley 1973). Viscometric data are expressed as $10{,}000/t_{50}$ per ml of enzyme solution. where t_{50} is time (sec) for the relative viscosity of the solution to be reduced by 50% at 40 C.

The enzyme preparations from only the two white-rot fungi were tested for ability to degrade insoluble cellulose (C_1 activity 1 since filtrates from the two brown-rot fungi were unable to significantly degrade insoluble cellulose (Highley 1973). To determine C_1 activity, increase in reducing groups from microcrystalline cellulose was measured by Nelson's modification of the Somogyi method (Nelson 1944). Details of the assay have been described (Highley 1973); reducing group data are expressed as micrograms of glucose released in 24 h/ml of culture filtrate at 40 C.

ß-Glucosidase (ß-D-glucoside glucohydrolase; E.C.3.2.1.21) was detected by incubating 1 ml of 1% salicin with 1 ml of enzyme solution for 16 h, then assaying for glucose by Nelson's method (1944).

The effect of temperature on cellulase activity was determined by measuring cellulase activity at 23, 40, 50, 60, and 70 C. Temperature stability was determined by holding enzyme solutions at 23, 40, 50, 60, and 70 C for 1 h. After treatment the cellulase activity was determined. The effect of pH on cellulase activity was determined by measuring enzyme activity in various buffers at pH's ranging from 2 to 9. The buffers were the following: pH 2 to 6, 0.05 M McIlvaine; pH 7, 0.05 M phosphate; and pH 8 and 9, 0.05 M tris-HCl. The effect of pH on stability was determined by incubating 1 ml of filtrate with 1 ml of the buffers for 24 h at 4 C, and cellulase activity was determined as described.

Four thiol inhibitors were tested against the cellulases to determine if sulfhydryl groups are essential to enzyme activity. Parachlormercuribenzoate, iodacetate, indole, and n-methyl maleimide were incubated at different conc (0.05 mM to 20 mM) with the enzyme preparation for 1 h at 23 C before assaying for activity.

The Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.) was used to determine glucose concentrations.

The products of hydrolysis were determined by descending paper chromatography. Substrate in 0.1 M acetate buffer, pH 5, was incubated with the enzyme solution for varying periods. After incubation, the mixture was boiled for 10 min, cooled, and desalted with Amberlite IR-20 and IR-45 ion exchange resins (Sison et al. 19581, then concentrated under vacuum. Ethyl acetate-acetic acid-water (6:3:3, v/v) and isopropanol-water (160:40, v/v) (2:1) were used as developing solvents. The separated sugars were detected by aniline spray reagent (Smith 1960) or alkaline $AgNO_3$.

RESULTS AND DISCUSSION

The curves for the relationship of pH and temperature to activity and stability of C_x for the two brown-rot fungi, *Poria monticola* and *Gloeophyllum trabeum*, were generally similar as were the curves for the two white-rot fungi, *Coriolus versicolor* and *Ganoderma applanatum* (Figs. 1 and 2). There were, however, pronounced differences between the C_x cellulases of the brown rotters and those of the white rotters in their response to pH and temperature. This suggests structural dif-

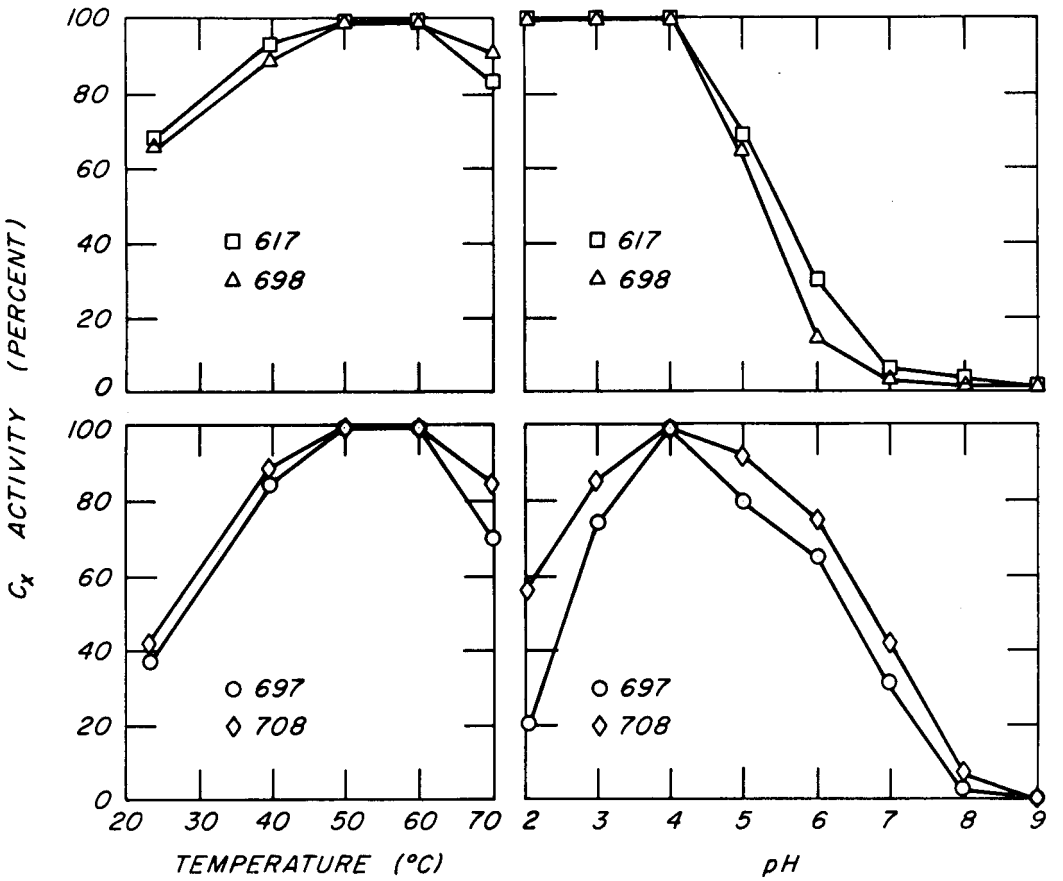

FIG. 1. Effect of temperature and pH on C_x activity of two brown-rot fungi (*Gloeophyllum trabeum,* 617, and *Poria monticola,* 698) and two white-rot fungi (*Coriolus versicolor,* 697, and *Ganoderma applanatum,* 708).

ferences in the C_x's from the brown-rot and the white-rot fungi used in this study.

The pH-curves of the brown-rot fungi and the white-rot fungi differed particularly in the low pH region—2to 3; the brown-rot fungi had high, or optimum, C, activities in this region, whereas the activity of the white-rot fungi was considerably lower than optimum. Also the low pH's affected the stability of C, from the white-rot fungi but not from the brown-rot. The high activity of C_x's of the brown rotters at low pH is unusual because most cellulases lose considerable activity at pH 2-3 (Gascoigne and Gascoigne 1960). The differences in pH stability and activity of the C_x's probably are related to brown-rotted wood being usually more acidic than white-rotted wood.

Temperature affected C, of the four fungi similarly to that reported for other fungal cellulases (Gascoigne and Gascoigne 1960; Keilich et al. 1970). The C_x activity of the fungi increased with temperature to 50 C, leveled off, then decreased. The C, activity (Fig. l) of the two white-rot fungi, however, was depressed more at the low temperatures than was that of the brown-rot fungi. Stability of C, from the two brown-rot fungi was uniform over the range 23-60 C (Fig. 2), whereas from the two white-rot fungi it was substantially reduced at temperatures above 50 C.

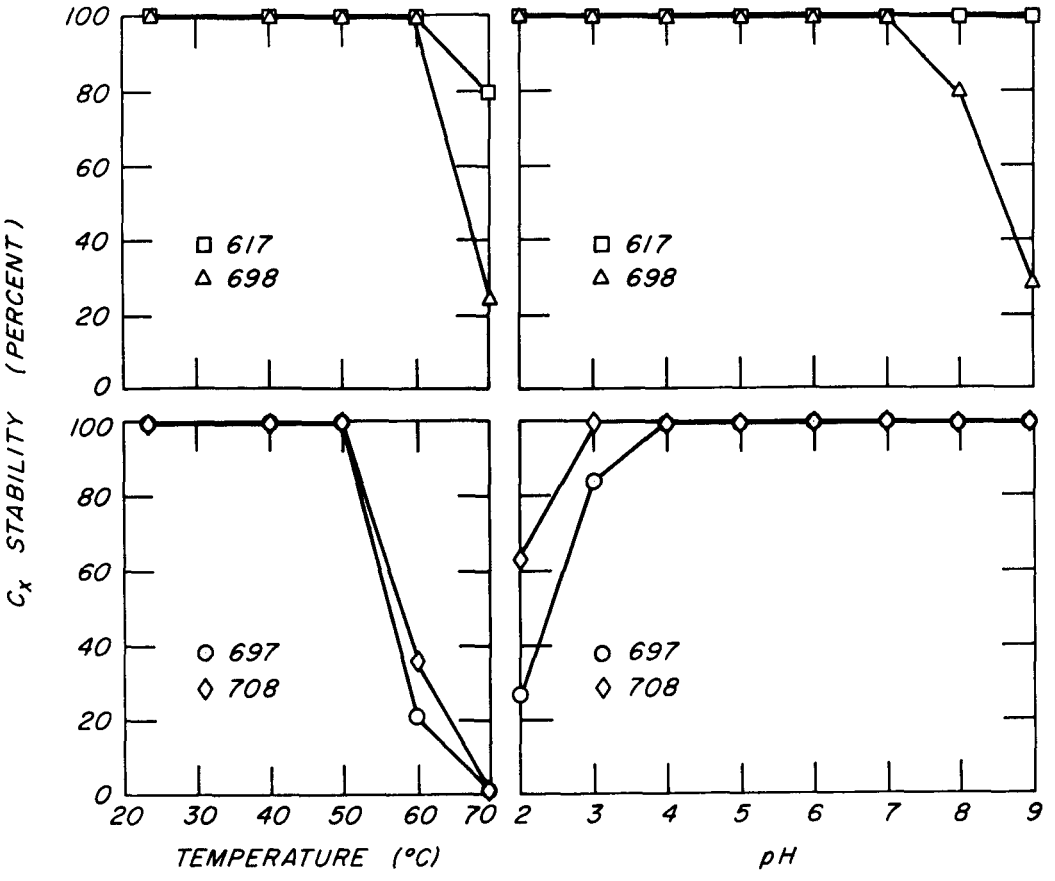

FIG. 2. Effect of temperature and pH on C_x stability of two brown-rot fungi (Gloeophyllum trabeum, 617, and *Poria monticola,* 698) and two white-rot fungi (*Coriolus versicolor,* 697, and *Ganoderma applanatum,* 708).

The curves for the relationship of pH and temperature to activity and stability of C_1 for the white-rot fungi *C. versicolor* and *G. applanatum* were generally similar (Fig. 3). However, the curves for C_1 differed from those for C,. Maximum C_1 activity of both *C. versicolor* and *G. applanatum* occurred at pH 5. C_1 was more sensitive to pH changes than C_x because C_1 activity was markedly reduced at pH values either above or below 5. C_1 stability was also affected more by pH changes than was C, stability. Halliwell and Griffin (1973) report that activity of purified C_1 from *Trichoderma koningii* is also optimum at pH 5, and activity and stability are markedly reduced at low and high pH's.

C_1 activity of both white-rot fungi increased with temperature to 40 C, then decreased substantially. There was also substantial loss in stability of C_1 above 40 C. A similar temperature response was found for C_1 of *Trichoderma koningii* (Halliwell and Griffin 1973).

Sulfhydryl groups are often involved in catalysis by enzymes. Cellulases from fungi vary whether or not sulfhydryl groups are essential for enzyme activity (Gascoigne and Gascoigne 1960). Because thiol inhibitors did not affect C_x or C_1 activity of any cellulase preparations from fungi in this study, sulfhydryl groups do not appear to be part of the active sites of the cellulases.

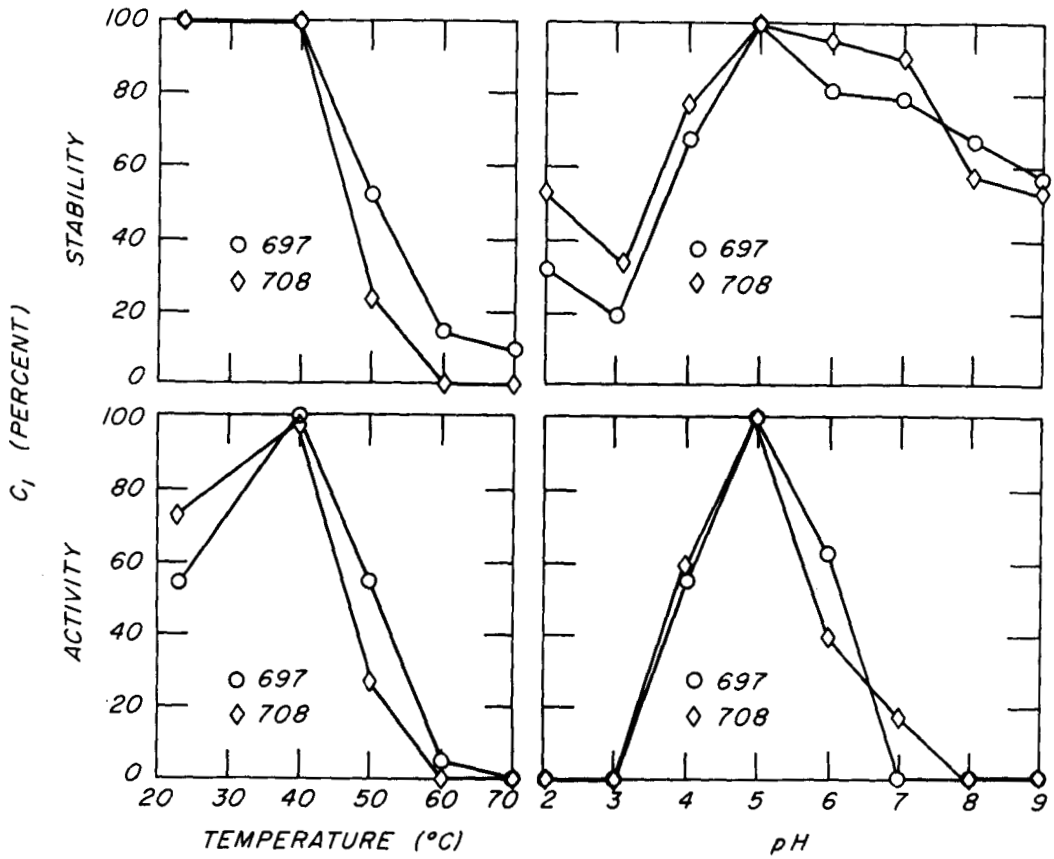

FIG. 3. Effect of temperature and pH on stability and activity of C_1 of two white-rot fungi (*Coriolus versicolor,* 697, and *Ganoderma applanatum,* 708).

Glucose (final product of cellulose hydrolysis) was not detected in filtrates of the two white-rot fungi cultured on cellulose or cellobiose (Table 1); apparently it is immediately assimilated by the fungus cells. With the two brown-rot fungi, however, glucose was abundant in cultures grown on cellobiose, an indication that end products of hydrolysis were accumulated faster than they were assimilated. This corresponds with Cowling's findings in decayed wood (1961); *P. monticola* rapidly depolymerized cellulose, and the end products of hydrolysis accumulated faster than they were used. In contrast, the products of cellulose degradation by *P. versicolor* were metabolized at about the same rate as they were formed. Simple sugars repressed cellulase production (C_x and C_1) by white rotters; utilization of simple sugars as they are formed may prevent cellulase repression (Highley 1973). C_x production by brown rotters was not repressed by simple sugars.

Cellulases from all four fungi rapidly reduced the viscosity of carboxymethylcellulose (CMC) with relatively small amounts of reducing groups formed. This suggests random cleavage (endo-Cx). Also the presence of oligosaccharides and relatively small amounts of glucose or cellobiose on chromatograms of reaction products at 1, 4, and 24 h rules out the presence of exo-C_x. Random cleavage is the most commonly reported type of C, action (Be-Miller 1968; Gascoigne and Gascoigne 1960; Jensen 1971; King 1966; Mandels and Reese 1964).

TABLE 1. ***ß-glucosidase activity and glucose in filtrates of white-rot (WR) and brown-rot (BR) fungi.***

Fungus and type of decay	µg glucose released in 16 hr from salacin[a]	µg/ml glucose in culture filtrate[b] (14 days)	(21 days)
Poria monticola (BR)	210	1,750	730
Gloeophyllum trabeum (BR)	490	2,150	560
Coriolus versicolor (WR)	740	0	0
Ganoderma applanatum (WR)	550	0	0

[a] BR grown on basal salts + 0.1% cellobiose + 0.5% cellulose for 21 days. WR grown on basal salts + 0.1% asparagine + 0.5% cellulose for 21 days.

[b] BR and WR grown on basal salts + 0.5% cellobiose.

To determine more closely the nature of the products of CMC degradation by the four fungi, reaction products were assayed simultaneously for reducing end groups and glucose. More reducing end groups were formed than could be accounted for by glucose (Table 2). Since no cellobiose or barely detectable amounts of it were found on chromatograms, it can be assumed that higher molecular weight products are formed from CMC and that a multiple enzyme system including both a ß-1,4-glucanase (ß-1,4-glucan 4-glucanohydrolase) and ß-glucosidase (ß-D-glucoside glucohydrolase) is present. Enzyme preparations from the four fungi were found to contain ß-glucosidase activity (Table 1).

Contrary to C_x, C_1 of *C. versicolor* and *G. applanatum* apparently acts on cellulose from the end of the chain (exo-C_1) because chromatographs of reaction products revealed only glucose and trace amounts of cellobiose. In addition, glucose accounted for almost all of the reducing groups when reaction products of cellulose degradation were assayed simultaneously for reducing groups and glucose (Table 3). Degree of polymerization (DP) measurements also showed very little change in DP. This agrees with Eriksson and Pettersson's (1972) suggestion that the C_1 enzyme is an exo-1,4-ß-glucanase. Since the partially purified filtrates also contain ß-glucosidase activity, C_1 may release terminal cellobiose units as is reported with *Trichoderma koningii* (Halliwell and Griffin 1973; Wood and McCrae 1972) and *Trichoderma viride* (Berghem and Pettersson 1973). The cellobiose units would then be, in turn, hydrolyzed by ß-glucosidase to glucose. Therefore an assay was made in the presence of glucono delta lactone, a specific inhibitor of ß-glucosidase.

Chromatographs of reaction products with glucono delta lactone present showed predominantly cellobiose with only trace amounts of glucose. Glucono delta lactone apparently decreased the amount of reducing end groups formed to about one-half, and the glucose was barely detectable (Table 3). Thus, the C_1 of the two white rotters apparently acts as a ß-1,4-glucan cellobiohydrolase. This action of C_1 from the two white rotters on cellulose agrees with that found for purified C_1 of *Trichoderma koningii* (Halliwell and Griffin

TABLE 2. ***Comparison of reducing groups and glucose formution by cellulase action on carboxymethylcellulose.***

Fungal enzyme preparation	Reducing groups (µg/ml) in: 1 h	4 h	24 h	Glucose (µg/ml) in: 1 h	4 h	24 h
Poria monticola	50	65	130	0	0	30
Gloeophyllum trabeum	115	175	280	5	25	95
Coriolus versicolor	140	175	245	70	135	175
Ganoderma applanatum	35	65	175	0	10	50

TABLE 3. *Comparison of reducing groups and glucose formution by cellulase action on cellulose with and without glucono delta lactone.*

Fungal enzyme preparation	Reducing groups[a]	Glucose[a]
Coriolus versicolor (C.v.)	178	165
C.v. + glucono delta lactone	126	22
Ganoderma applanatum (G.a.)	100	90
G.a. + glucono delta lactone	50	8

[a] µg/ml produced in 24 h .

1973; Wood and McCrae 1972) and of *Trichoderma viride* (Berghem and Pettersson 1973), but is contrary to the earlier concepts of C_1 action (Reese et al. 1950; Mandels and Reese 1964).

The DP of holocellulose in wood rotted by white-rot fungi decreased slowly during decay (Cowling 1961). An exo-C_1 in these culture filtrates of the white-rot fungi would produce this type of effect on DP of cellulose. The exo-C_1 of *Trichoderma koningii* (Halliwell and Griffin 1973) hydrolyzed cellulose without any other component of the cellulase system present. Wood and McCrae (1972), on the other hand, propose that a purveyor of accessible end-groups (endo-1,4-ß-glucanase) is essential for action of C_1. Studies with purified enzyme preparations would be necessary to determine which situation exists with the C_1 from *C. versicolor* and *G. applanatum.*

(Trade or proprietary names are included for identification only and do not imply any endorsement by the Forest Service of the U.S. Department of Agriculture.)

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