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Octyl Glucoside Inhibits [^{14}C]DHP Mineralization Whereas Peroxidase Activity Is Stimulated in *Phanerochaete Chrysosporium*

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ABSTRACT

Octyl glucoside stimulated peroxidase formation in *Phanerochaete chrysosporium* ME-446 cultivated in cellulose-based media. Addition of 0.1% of the nonionic surfactant resulted in a ninefold (143 U/L) and sixfold (119 U/L) increase in LiP formation under conditions of N limitation and N excess, respectively. Octyl glucoside also stimulated MnP formation, but to a lesser extent than observed with LiP. The cellobiose-oxidizing enzymes (cellobiose dehydrogenase and cellobiose:quinone oxidoreductase) were stimulated by octyl glucoside when used at a concentration of up to 0.05%, but higher concentrations gave values similar to those for the controls. Little proteolytic activity was detected in the presence of the surfactant. In general, activities of the enzymes studied were of the same order as those seen using Tween-80. In contrast with Tween-80, octyl glucoside markedly inhibited [^{14}C]DHP mineralization. Attempts to account for the observed inhibition of synthetic lignin degradation by *P. chrysosporium* in the presence of octyl glucoside are discussed.

Index Entries: [^{14}C]DHP mineralization; *P. chrysosporium;* Tween-80; octyl glucoside; lignin peroxidase; Mn peroxidase; cellobiose dehydrogenase; cellobiose:quinone oxidoreductase.

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Abbreviations: CBQ, cellobiose:quinone oxidoreductase; CDH, cellobiose dehydrogenase; DHP, dehydrogenative polymerizate (synthetic lignin); LiP, lignin peroxidase; MnP, manganese peroxidase.

INTRODUCTION

About 10^{11} to 10^{12} tons of lignocellulosic material are produced annually via photosynthesis. The many biotechnical applications that are implicit in the exploitation of this abundant polymer account for the vast amount of research carried out on the biodegradation of this biomass (*1,2*). The key to the effective utilization of lignocellulosic biomass is the removal of lignin, which limits the accessibility of cellulose and hemicellulose to enzymatic hydrolysis. White-rot fungi have the potential to selectively biodegrade lignin, and, among these, *P. chrysosporium* has been extensively studied as a model system.

We have previously shown that synthetic lignin can be degraded by *P. chrysosporium* cultivated in cellulose-based media (*3–5*). Apart from the advantage of being the C source available *in situ*, the cellulose-based media used made it possible to study the simultaneous formation of lignin peroxidase (LiP), manganase peroxidase (MnP) and the cellobiose-oxidizing enzymes. The latter enzymes, namely, cellobiose dehydrogenase (CDH, formerly known as cellobiose oxidase [*6*]) and cellobiose:quinone oxidoreductase (CBQ), were discovered in the 1970s (*7,8*). Many years later, Ander et al. (*9*) showed that CBQ was involved in the in vitro depolymerization of Kraft lignin. Although data reinforcing the probable role of these cellobiose-oxidizing enzymes as an interface between lignin and cellulose degradation has steadily increased (*10–12*), much remains to be known about the role of these enzymes in nature.

In our studies with cellulose-grown cultures of *P. chrysosporium*, high levels of synthetic lignin (DHP) mineralization were only observed when media was supplemented with the nonionic surfactant, Tween-80 (*5*). The use of surfactants in studies on lignin degradation can be traced back to the work of Jäger et al. (*13*). These workers studied the effect of methylbenzethonium chloride, Tween-20, Tween-80 and 3-[(3-cholamidopropyl) dimethylammonio]1-propanesulfonate (CHAPS) on LiP production in agitated submerged cultures of *P. chrysosporium*. Out of the various surfactants tested, it was reported that 0.05–0.1% Tween was optimal in enhancing LiP activity. These authors also reported that when 0.1% Tween-80 was added to cultures containing synthetic [^{14}C]lignin, the rate of mineralization was similar to that in stationary cultures lacking detergent. Asther and co-workers studied LiP production and conversion of [^{14}C]DHP to $^{14}CO_2$ by *P. chrysosporium* INA-12 in a medium containing Tween-80 in the presence of oleic acid and other oils (*14,15*). These authors confirmed the earlier work of Jäger et al. (*13*) in concluding that the stimulatory effect of Tween 80-on LiP production could be explained, in part, by the fungus's

utilization of the fatty acids from the surfactant. However, the fact that CHAPS, which lacks utilizable fatty acids, also enhanced LiP production (*13*), indicated that fatty acid utilization alone cannot explain the effect of surfactants on the ligninolytic system of white-rot fungi.

In the present work we have studied the effect of a surfactant, with properties different from those used in earlier studies, on the ligninolytic system of *P. chrysosporium*. Octyl glucoside was chosen because, apart from being nonionic, its physicochemical characteristics are markedly different from those of Tween-80 (*16*). The latter belongs to the polyoxyethylene group of surfactants and the former is a glucosidic surfactant with an alkyl C_8 side chain. The work presented examines the effect of octyl glucoside on the formation of lignocellulolytic enzymes, namely peroxidases and cellobiose-oxidizing enzymes, from cellulose-grown cultures of *P. chrysosporium*. Synthetic lignin degradation in the presence of this surfactant was also determined. In an attempt to understand the basis for the observed inhibition of [^{14}C]DHP mineralization by octyl glucoside, a comparison is made with data obtained using Tween-80.

MATERIALS AND METHODS

Fungus, Medium and Culture Conditions

P. chrysosporium ME-446 (ATCC 34541) was used throughout this work. The carbon source was 1% fibrous cellulose (Whatman CF 11, Maidstone, England). The nitrogen source was asparagine used at a concentration of 2.4 m*M* N in low nitrogen medium and 24 m*M* N in high nitrogen medium. Other components of the medium were as follows: $MgSO_4 \cdot 7H_2O$, 2.15 m*M*; KH_2PO_4, 15 m*M*; $CaCl_2 \cdot 2H_2O$, 0.19 m*M*; thiamine hydrochloride, 1 ppm; nitriloacetate, 78 μM; $MnSO_4 \cdot H_2O$, 5 ppm; NaCl, 10 ppm; $FeSO_4 \cdot 7H_2O$, 1 ppm; $CoCl \cdot 6H_2O$, 1 ppm; $ZnSO_4 \cdot 7H_2O$, 1 ppm; and $CuSO_4 \cdot 5H_2O$, 0.1 ppm, in 20 m*M* sodium tartrate buffer, pH 4.2. The medium was supplemented with 1 m*M* veratryl alcohol (Aldrich, Milwaukee, WI) and octyl glucoside (1-O-N-octyl-β-D-glucopyranoside, Boehringer Mannheim, Germany) or Tween-80 (sorbitan polyoxyethylene monooleate, Merck, Darmstadt, Germany) at concentrations (w/v) specified in the text.

For enzymatic studies, cultures were grown in 250 mL Erlenmeyer flasks containing 20 mL of medium. These were inoculated with 10^7 conidiospores and allowed to incubate under stationary conditions at 30°C. Each cultivation was performed in triplicate, and repeated at least three times. Appropriate controls showed that octyl glucoside did not interfere with any of the assays used for this work.

[^{14}C]DHP Mineralization

Synthetic [ring-U-^{14}C]lignin was a gift of Dr. Paul Ander of the Swedish University of Agricultural Sciences, Uppsala. The specific activity was

277 kBq/mg DHP. Assays were initiated by the addition of about 2 μg of the labeled DHP. These were performed using 5 mL of medium in 125 mL Erlenmeyer flasks, and incubated at 30°C without agitation. Cultures were flushed with humidified 100% oxygen and evolved $^{14}CO_2$ was trapped and counted (*17*).

In order to see whether octyl glucoside influenced the solubility of $^{14}CO_2$, controls were performed in which octyl glucoside (0.1% final) was injected into the sealed flasks that had been cultivated for 6, 9, or 12 d. Following introduction of the surfactant, cultures were allowed to stand for 1 h and subsequently flushed with oxygen. The amount of evolved $^{14}CO_2$ was compared with that evolved from reference flasks to which buffer was introduced, instead of surfactant.

Enzymatic Assays

Determination of Extracellular and Bound CDH and CBQ Activity

The CDH (EC 1.1.3.25) and CBQ (EC 1.1.5.1) activity was determined in the extracellular solution as well as the bound (cellulose + mycelium) fraction. The extracellular solution was obtained after filtration through glass fiber filters (Whatman GF/D). The bound enzymes were determined from the filter retentate after resuspension in the above medium and subsequent homogenization (1 min). CDH activity was determined using 60 μ*M* cytochrome c plus 0.5 m*M* cellobiose (Sigma Type III from horse heart, St. Louis, MO) in 50 m*M* sodium tartrate buffer, pH 4.0, according to our previously described procedure (*5*). The increase in absorbance was determined at 550 nm. For the determination of the bound activity, an aliquot of the homogenized suspension was incubated with the appropriate assay reagent mix for 3 min. The reaction was stopped by the addition of Na_2CO_3 (0.125*M* final) and subsequently centrifuged (13,000*g*, 5 min). The absorbance of the supernatant was determined. A sample containing the boiled enzymatic extract was used as the blank. Appropriate controls showed that the surfactants did not interfere with the assays.

The combined CBQ and CDH activities were determined in the extracellular medium by following the reduction of 3,5-di-tert-butyl-1,2-benzoquinone (DTBB) in 40 m*M* sodium tartrate buffer, pH 4.0, at 420 nm (*18*).The methodology for the determination of the bound activity was as described for CDH, except that the reaction was terminated with trichloroacetic acid (6%).

CBQ activity was defined as the activity using the DTBB assay minus the cytochrome c reducing activity.

Determination of Extracellular Peroxidase Activity

Lignin peroxidase was determined by the method of Tien and Kirk (*19*) by measuring oxidation of 2 m*M* veratryl alcohol (Aldrich) in 50 m*M* tartrate buffer, pH 3.0, at 310 nm.

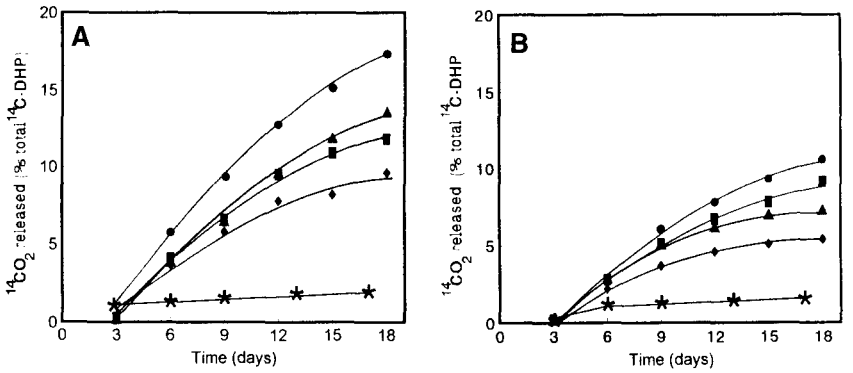

Fig. 1. Effect of octyl glucoside on [^{14}C]DHP mineralization by *P. chrysosporium* in (**A**) cellulose/low N (2.4 m*M* as asparagine) media and (**B**) cellulose/high N (24 m*M* as asparagine) media. The $^{14}CO_2$ evolved was determined in cultures without surfactant (●), 0.025% (▲), 0.05% (■), 0.01% (◆), and 1.34% (*) octyl glucoside.

Mn peroxidase activity was determined at 334 nm using 0.1 m*M* vanillylacetone (ICN Biomedicals) in 50 m*M* tartrate buffer, pH 5.0, containing 0.1 m*M* manganese sulfate, according to Paszczynski et al. (*20*).

Protease Activity

Protease activity was measured using 1% Azocoll (Sigma) in 50 m*M* sodium acetate buffer, pH 4.5 (*21*). Activity was defined assuming 1 U as the amount of enzyme that catalyzes the release of azo dye, causing an absorbance change of 0.001/min.

RESULTS

Effect of Octyl Glucoside on Mineralization of [^{14}C]DHP by *P. chrysosporium*

Mineralization of [^{14}C]DHP by *P. chrysosporium* cultivated in cellulose/low N medium in the absence and presence of octyl glucoside is shown in Fig. 1A. Highest levels of mineralization occurred in control cultures lacking surfactant. Addition of 0.025% octyl glucoside inhibited mineralization and increasing the amount of surfactant inhibited mineralization in a concentration-dependent manner. [^{14}C]DHP mineralization was lower when high N media was used (Fig. 1B), but nevertheless the inhibitory effect of octyl glucoside was notable. In both low and high N media, 0.1% octyl glucoside inhibited DHP mineralization by about 50% relative to the controls. The highest specific rate of [^{14}C]DHP mineralization occurred about day 6, both in the absence and presence of surfactant.

Table 1
Lignin Peroxidase Production by *P. chrysosporium* in the Presence of Octyl Glucoside and Tween-80[a]

Conditions		Lignin peroxidase activity, U/L			
		Octyl glucoside		Tween-80	
Surfactant, %	N, m*M*	day 6	day 12	day 6[b]	day 12
0	2.4	16.2	91.5	1.0	53.9
0.025	2.4	63.0	98.6	nd[c]	nd
0.05	2.4	115.4	102.5	9.1	108.2
0.1	2.4	142.9	114.9	0	104.3
0	24.0	21.0	151.1	9.3	36.1
0.025	24.0	56.6	88.9	nd	nd
0.05	24.0	99.4	76.0	67.8	17.2
0.1	24.0	119.1	50.1	103.5	35.6

[a]Cultivations were carried out without agitation in cellulose-based media containing asparagine as N source, as described under Materials and Methods.
[b]From Costa-Ferreira et al. (1994).
[c]nd = not determined.

Octyl glucoside was also employed at 1.34% (46 m*M*), being about twofold higher than the critical micellar concentration (CMC, see Discussion) in an attempt to compare use of surfactant in micellar form with that in monomeric form. However, at this high concentration, octyl glucoside suppressed fungal growth so that negligible amounts of [^{14}C]DHP were degraded. It is noteworthy that use of up to 0.1% octyl glucoside in the medium did not affect growth of *P. chrysosporium* (data not shown).

Increased Peroxidase Activity in the Presence of Octyl Glucoside

The effect of octyl glucoside on LiP production by *P. chrysosporium* was investigated at various concentrations and compared with enzyme formation in the presence of Tween-80 (Table 1). Following 6 d of cultivation, a clear trend was observed in which the amount of LiP produced was proportional to the amount of octyl glucoside added. Addition of 0.1% octyl glucoside stimulated LiP under conditions of N limitation and N excess by about nine- and sixfold, respectively. In general, octyl glucoside was more effective than Tween-80 in enhancing LiP production. This was particularly evident in low N media following 6 d of cultivation.

MnP accumulation was slightly stimulated by the presence of surfactants (Table 2). In the absence of octyl glucoside, after 6 d of cultivation the MnP activity was not detectable in the extracellular media. Although levels of this enzyme remained low despite the addition of surfactants to the cultivation media, the stimulatory effect was clearly noticeable on day 6.

Table 2
Manganese Peroxidase Production by *P. chrysosporium* in the Presence of Octyl Glucoside and Tween-80[a]

Conditions		Manganese peroxidase activity, U/L			
		Octyl glucoside		Tween-80	
Surfactant, %	N, m*M*	day 6	day 12	day 6[b]	day 12
0	2.4	0	1.5	0.4	2.9
0.025	2.4	0	1.9	nd[c]	nd
0.05	2.4	1.1	1.7	1.1	2.4
0.1	2.4	1.7	1.8	1.2	2.0
0	24.0	0	3.1	0	1.4
0.025	24.0	0	2.6	nd	nd
0.05	24.0	2.1	2.1	0.4	0.8
0.1	24.0	2.1	1.9	0.6	1.1

[a]Cultivations were carried out without agitation in cellulose-based media containing asparagine as N source, as described under Materials and Methods.
[b]From Costa-Ferreira et al. (1994).
[c]nd = not determined.

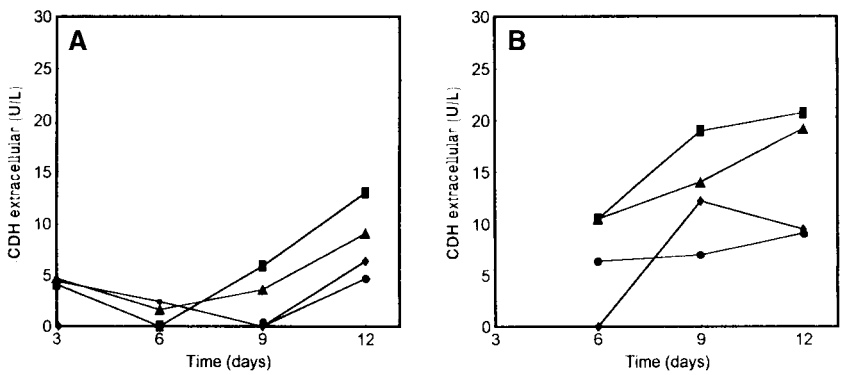

Fig. 2. Effect of octyl glucoside concentration on formation of extracellular CDH by *P. chrysosporium* grown in (**A**) low N and (**B**) high N media. The concentrations of surfactant used were 0% (●), 0.025% (▲), 0.05% (■), and 0.1% (◆).

In general, the activity of this enzyme was of the same order whether octyl glucoside or Tween-80 was employed. This suggests that the inhibitory effect of octyl glucoside on DHP mineralization may not be due to insufficient amounts of MnP being formed.

Effect of Octyl Glucoside on the Cellobiose-Oxidizing Enzymes

The time-course for the formation of extracellular cellobiose dehydrogenase by *P. chrysosporium* in both low (Fig. 2A) and high N (Fig. 2B)

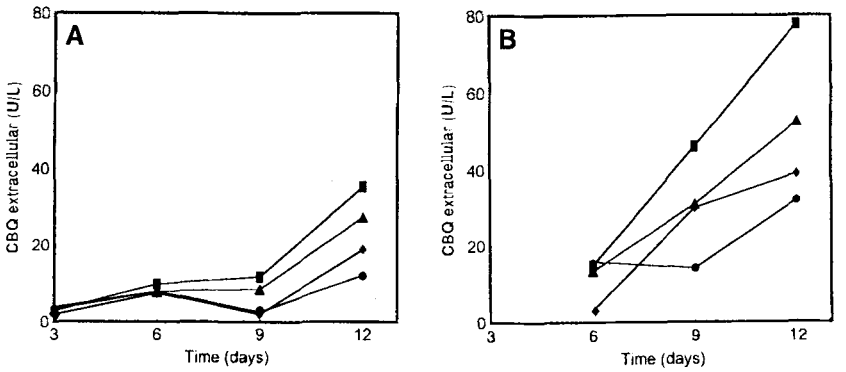

Fig. 3. Effect of octyl glucoside concentration on formation of extracellular CBQ by *P. chrysosporium* grown in (**A**) low N and (**B**) high N media. The concentrations of surfactant used were 0% (●), 0.025% (▲), 0.05% (■), and 0.1% (◆).

showed a similar trend. A twofold enhancement in the CDH activity was observed when 0.05% was used. Not unexpectedly, about twice as much was produced in the high N media (22 U/L) compared with the amount produced under conditions of N limitation (about 14 U/L). When 0.1% octyl glucoside was added to the cultivation media, the amount formed was of the same order as that obtained for the respective controls.

The formation of extracellular CBQ in low (Fig. 3A) and high (Fig. 3B) N media was likewise affected by the presence of octyl glucoside. The trend was similar to that observed for CDH. Addition of 0.05% octyl glucoside to the cultivation media stimulated CBQ formation from 11–34 U/L and from 32–78 U/L in low and high N media, respectively. Use of 0.1% of the surfactant was clearly less favorable for the formation of this enzyme.

In addition to the free enzyme present in the extracellular media, bound enzyme was also determined since it is known that these cellobiose-oxidizing enzymes have a high binding affinity for cellulose (*22*). Both of these enzymes can reduce quinones, cation, and phenoxy radicals that may result from the action of peroxidases on lignin (*12*); the combined bound activity is presented in Table 3. In general, the bound enzymes were not affected by the presence of the surfactant. (The values for day 6 cultures grown under conditions of N limitation in the presence of 0.1% were somewhat higher, but the significance of this is not clear). Also, in contrast to the extracellular enzyme, the amount of bound enzyme did not increase as a function of time. A further difference between the extracellular CDH/CBQ and the amount of bound enzymes was that the latter were not affected by the N concentration.

A comparison between the effect of 0.1% Tween-80 and octyl glucoside on the formation of the cellobiose oxidizing enzymes is shown in Table 4. This concentration of surfactants was chosen because it gave optimal stimulation of [^{14}C]DHP mineralization with Tween-80. Octyl glucoside was shown (Fig. 1) to strongly inhibit synthetic lignin degrada-

Table 3
Effect of Octyl Glucoside on the Formation of Bound Cellobiose Oxidizing Enzymes by *P. chrysosporium*

Octyl glucoside, %	N, mM	CDH + CBQ, U/L	
		day 6	day 12
0	2.4	16.9	12.6
0.025	2.4	18.6	17.4
0.05	2.4	16.9	19.7
0.1	2.4	27.0	24.7
0	24.0	20.6	9.1
0.025	24.0	23.0	16.6
0.05	24.0	22.1	14.1
0.1	24.0	21.9	14.2

Table 4
Comparison Between Effect of Octyl Glucoside (0.1%) and Tween-80 (0.1%) on Formation of Cellobiose Oxidizing Enzymes by *P. chrysosporium*[a]

Conditions		Enzymatic activity, U/L			
Surfactant	N, mM	CDH (extracellular)	CDH (bound)	CBQ (extracellular)	CBQ (bound)
Control	2.4	2.3	1.7	7.7	15.2
Octyl glucoside	2.4	0.5	2.3	7.3	24.7
Tween-80[b]	2.4	0	0	2.4	12.8
Control	24	6.4	2.3	15.8	18.3
Octyl glucoside	24	0	2.7	3.0	19.2
Tween 80[b]	24	0.9	1.2	7.5	15.1

[a]Enzymatic activities were determined following 6 d of cultivation in cellulose-based media.
[b]From Costa-Ferreira et al. (1994).

tion. It is noteworthy that, with 0.1% surfactant, the amount of CDH/CBQ in the extracellular and bound fractions were of the same order, regardless of whether Tween-80 or octyl glucoside was present in the cultivation media.

Effect of Octyl Glucoside on Formation of Proteolytic Activity

Protease activity was determined with Azocoll as substrate in cultures of *P. chrysosporium* grown for 6 and 12 d. The results summarized in Table 5 show that for cultures grown in low N medium, octyl glucoside inhibited protease activity in a concentration-dependent fashion. When 0.1% octyl glucoside was added to the cultivation medium, protease activity could

Table 5
Effect of Octyl Glucoside on Protease Activity
Formed in Cellulose Grown Cultures of *P. chrysosporium*

Octyl glucoside, %	N, m*M*	Protease activity, U/L	
		day 6	day 12
0	2.4	0.21	0.07
0.025	2.4	0.13	0.13
0.05	2.4	0	0.20
0.1	2.4	0	0
0	24.0	0.20	0.38
0.025	24.0	nd	nd
0.05	24.0	0.17	0.66
0.1	24.0	0.13	0.76

nd = not determined.

not be detected in the extracellular media. Because in vitro studies have shown that CBQ is a product of the proteolytic cleavage of CDH (23), it is interesting to note that about 10 to 18 U/L of the former enzyme was detected in the absence of the acidic proteases. In high N media, although no trend was discernible, the values obtained are of the same order as those observed using Tween-80 in a similar cellulose-based medium.

DISCUSSION

The nonionic surfactant octyl glucoside markedly inhibited mineralization of [^{14}C]DHP by *P. chrysosporium* ME-446. The use of a surfactant which stimulated peroxidase activity, but inhibited [^{14}C]DHP mineralization, has not been previously described. In contrast to the effect of octyl glucoside, in our earlier reports we showed that Tween-80 enhanced mineralization of synthetic lignin in a similar cellulose-based system (*3,4*).

The observed inhibition of [^{14}C]DHP mineralization by octyl glucoside did not result from

1. growth inhibition, because this was not affected by the presence of up to 0.1% octyl glucoside
2. the unique cultivation conditions used (static, cellulose cultures), because the same inhibitory phenomenon was seen, using the classical glucose/low N media (results not shown); or
3. the surfactant's influence on the partitioning of carbon dioxide in an aqueous medium, because appropriate controls for the mineralization assay excluded this possiblilty.

Octyl glucoside was found to be more effective than Tween-80 in stimulating LiP formation by *P. chrysosporium*. The N concentration of the cultivation media was not critical and similar trends for LiP enhancement

by octyl glucoside were seen under both low and high N conditions. This may be due to the use of the recalcitrant C source, cellulose, which could have induced conditions similar to those occurring under secondary metabolism. Although correlation between LiP activity and DHP mineralization in *P. chrysosporium* has been observed (*14,24*), the present work shows that by manipulating cultivation parameters it is possible to get an inverse relationship between LiP accumulation and degradation of synthetic lignin.

Octyl glucoside stimulated the formation of cellobiose-oxidizing enzymes at concentrations up to 0.05%. However, use of 0.1% of this surfactant gave CDH/CBQ activities comparable to those observed using Tween-80. Also, the absense of detectable proteolytic activity in cultures containing low N in the presence of 0.05–0.1% octyl glucoside strongly suggests that ligninolytic enzyme inactivation, or degradation of a protein structure involved in [^{14}C]DHP mineralization, may not account for the observed inhibitory effect. Taken together, the effect of octyl glucoside on the enzymatic activities studied was, in principle, favorable for synthetic lignin degradation to occur, but the opposite effect was seen.

The differences in the physicochemical characteristics between octyl glucoside and Tween-80 may provide an insight into the differential effects of these two surfactants. The HLB (hydrophile-lipophile balance) for octyl glucoside, calculated using an empirical equation, (*25*) gives a value of 24; Tween-80 has a HLB value of 15, indicating that the former is more hydrophilic. Another major difference between the two surfactants is that the CMC for Tween-80 is more than a thousandfold lower than that for octyl glucoside, being 0.02 and 20–25 $\times$ $10^{-3}M$, respectively (*26*). At concentrations up to 0.1%, octyl glucoside was present mainly as monomers. Because of its hydrophilic nature, even at these low concentrations, octyl glucoside could be incorporated into the fungal cell wall (*27*), thereby nonspecifically hindering the binding of the synthetic lignin molecule. That nonsaturable (i.e., nonspecific) binding of lignin precedes degradation was shown in an early study by Chua et al. (*28*). Whether the surfactant must be present in micellar form in order to facilitate degradation of synthetic lignin is being studied, using a homologous series of glucosidic surfactants. Elongation of the alkyl chain by two methylene groups causes a decrease in the CMC by a factor of approximately 10 (*29*). (*29*). By using tetradecyl glucoside, which has a CMC similar to Tween-80's, it will be possible to get further information on the importance of the structure (micellar or otherwise) of the surfactant in relation to its effect on the ligninolytic system.

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