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Effect of Agitation on Ligninase Activity and Ligninase Production by *Phanerochaete chrysosporium*

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The white rot fungus *Phanerochaete chrysosporium* produces extracellular ligninases as part of its idiophasic ligninolytic system. Agitation has been widely reported to suppress both ligninase production and lignin degradation. Results show that mechanical inactivation of ligninase is possibly the reason why ligninase accumulation is low or absent in agitated shake-flask cultures. Agitation seems to affect the catalytic activity of ligninase and has no apparent effect on either the rate of ligninase production or the physiology of *P. chrysosporium*. The detergents Tween 20, Tween 40, Tween 60, Tween 80, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) are able to protect both purified ligninase and extant ligninase in culture fluids (free of biomass) against mechanical inactivation due to agitation. Addition of Tween 80 at the end of primary growth to agitated shake flasks containing either pelleted or immobilized mycelial cultures results in production and maintenance of high levels of ligninase activity over several days under conditions of high agitation. Possible mechanisms by which the detergents could protect ligninase are discussed.

Phanerochaete chrysosporium, a white rot wood-decaying basidiomycete, secretes a variety of extracellular enzymes involved in lignin degradation (37). Among these enzymes, a class of isoenzymes, identified as peroxidases and termed ligninases, have been discovered (11, 21, 23, 25, 27, 32, 37). These peroxidases are produced as part of the idiophasic ligninolytic system and appear after the completion of growth under carbon, nitrogen, or sulfur limitation (8, 17, 20). Commercial application of ligninase has been proposed because of its ability to depolymerize lignin and lignin substructure model compounds in vitro (11, 37) and its involvement in the degradation of recalcitrant environmental pollutants (12–14).

Research studies with ligninase have been hindered by low productivity levels. Kirk and co-workers (8, 21) have shown that addition of veratryl alcohol, a secondary metabolite of *P. chrysosporium*, and excess trace elements improve ligninase activity levels. Initially workers used stationary shallow cultures in Erlenmeyer flasks for ligninase production (22). Early research had shown that agitation of submerged cultures in flasks led to the formation of mycelial pellets and suppression of both lignin degradation and ligninase activity (8, 22). Shimada et al. (34) had postulated that pellet formation due to agitation might have serious physiological effects in *P. chrysosporium* and could affect secondary metabolism. Thus far, the deleterious effect of agitation has not been explained satisfactorily.

Later, some success was achieved with agitated flask cultures (15, 26, 31). Jager et al. (15) showed that ligninase production in agitated pelleted cultures (30 ml of medium in 125-ml Erlenmeyer flasks shaken on a rotary shaker at 150 rpm [2.5-cm-diameter circle]) was made possible by including detergents Tween 20, Tween 80 or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in the culture medium. Jager et al. (15) and Asther et al. (2) did not explain the mechanism by which these detergents allowed the accumulation of ligninase. Leisola and Fiechter (26) developed only low levels of ligninase (maximum activity, ca. 23 U/liter) in pelleted cultures under milder agitation (50 ml of medium in 200-ml Erlenmeyer flasks shaken on a reciprocating shaker at 100 strokes per min; 2.5 cm per stroke). Reid et al. (31) demonstrated lignin degradation under agitated conditions (10 ml of medium in 125-ml Erlenmeyer flasks shaken on a gyrotory shaker at 136 rpm; 2.4 cm in diameter), but only when a single large mycelial pellet was formed with wood particles being enmeshed in the pellet.

Attempts to produce ligninase in suspended systems such as stirred-tank reactors have met with limited success. Linko (28) was able to produce ligninase in a 10-liter bioreactor in which mixing was provided with an air and/or oxygen supply without an impeller. Janshekar and Fiechter (16) have demonstrated ligninase production in a 42-liter stirred-tank reactor only when polypropylene glycol was present in the culture medium. Attempts by Janshekar and Fiechter to scale up the process to a 300-liter reactor resulted in no observable ligninase production.

The limited success in achieving ligninase production in stirred-tank reactors led to an investigation of the effect of agitation on both ligninase production and inactivation. In this paper, mechanical inactivation of ligninase is shown as the reason why ligninase accumulation is either low or absent in agitated cultures. Studies have demonstrated that agitation affects the catalytic activity of ligninase and has no apparent effect on either the rate of production of ligninase or the physiology of *P. chrysosporium*. Furthermore, the detergents Tween 20, Tween 40, Tween 60, Tween 80, and CHAPS were shown to protect both purified ligninase and extant ligninase in culture fluids (free of biomass) against mechanical inactivation. Addition of Tween 80 to agitated cultures at the end of primary growth resulted in high levels of ligninase activity. Scaling up of ligninase production is probably hampered by the sensitivity of ligninase to mechanical inactivation. Large-scale production of ligninase should be possible in systems which either utilize detergents or minimize intensive shear.

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MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* VKM F-1767 (ATCC 24725) was cultured at 37°C on glucose-yeast extract slants or plates with the medium of Leatham et al. (24), except that 10 g of glucose per liter was substituted for malt extract. After growth and conidiation, slants and plates were maintained at 4°C. Conidia, which were suspended in sterile water and enumerated by using a hemacytometer, formed the inoculum source for liquid cultures (final concentration, 7.5×10^3 spores per ml). Liquid medium was that of Aitken et al. (1), which was patterned after that of Kirk et al. (22), except that glucose-limiting conditions (11.1 mM glucose [2 g/liter] and 5.9 mM ammonium chloride) were used. The medium was buffered with 15 mM (sodium) 2,2-dimethylsuccinic acid (pH 4.5). Cultures were grown in an incubator-shaker (New Brunswick Scientific Co.) at 37°C at the following sizes: 30 ml of medium in a 125-ml Erlenmeyer flask; 50 ml of medium in a 250-ml Erlenmeyer flask; 100 ml of medium in a 500-ml Erlenmeyer flask; and 500 ml of medium in a 2.8-liter Fernback flask. Agitated cultures were shaken at 150 rpm in a rotary shaker with a 2.5-cm-diameter cycle during primary growth. Unless mentioned otherwise, the agitation was reduced to 30 rpm or the culture was transferred to static conditions following glucose consumption, and 1 mM sterile-filtered veratryl alcohol (Aldrich Chemical Co.) was added to induce ligninase production. The detergent polyoxyethylene sorbitan monooleate (Tween 80) (Sigma Chemical Co.) was sterilized by autoclaving 0.2 or 2% (wt/vol; 2 or 20 g/liter, respectively) stock solutions and added to the cultures to give the desired final concentration. Experiments with culture fluids (free of biomass) were conducted with 30-ml volumes in 125-, 250-, and 500-ml Erlenmeyer flasks. The detergents polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene sorbitan monooleate (Tween 80), and CHAPS (all from Sigma) were prepared as 2- or 20-g/liter stock solutions and added to the culture fluids (free of biomass) to give the required final concentration as described in the text.

Immobilization. *P. chrysosporium* was immobilized in agar gel beads by using the procedure outlined by Linko et al. (29). Spore-immobilized agar beads (2.7 g) in 100 ml of culture medium in a 500-ml Erlenmeyer flask were used. Growth was in the form of immobilized mycelia.

Bioreactor. The bioreactor used in this study was a Bioflo II (New Brunswick Scientific Co.) and was run in a batch mode at 37°C with a working volume of 1.5 liters. Agitation was performed by a cell lift impeller (30 rpm) which consisted of a cylindrical tube attached to the impeller shaft with three discharge ports. A ring-shaped sparger placed below the impeller was used for aeration. The combined effect of rotation and air discharge from the sparger holes caused the liquid to move up into the tube and to be expelled from the discharge ports in a continuous loop.

Ligninase assay. Ligninase activity in purified ligninase preparations and culture fluids was determined by measuring the initial rate of veratraldehyde production from veratryl alcohol (36). Reaction mixtures (1 ml) contained 100 mM sodium tartrate buffer (pH 3.0), 1 mM veratryl alcohol, and 305 μl of culture fluid or 10 μl of purified ligninase. Reactions were run at room temperature (23°C) and were started by the addition of 350 μM hydrogen peroxide. One unit of ligninase activity converts 1 μmol of veratryl alcohol to veratraldehyde per min. Reactions were monitored at 310 nm. Activity was expressed in units per liter. The standard deviation of the ligninase assay in culture fluids was 2.4% of the mean. In general, values reported are from single measurements.

Purified ligninase preparation. The basic procedure of Aitken et al. (1) was followed for enzyme purification. Filtered culture fluid was concentrated in an ultrafiltration cell containing a 10,000-molecular-weight-cutoff polysulfone membrane (Millipore Corp.). The concentrate was washed with 5 mM sodium succinate buffer (pH 6.0) and concentrated. The ultrafilter concentrate was applied to a DEAE-Sepharose (Pharmacia, Inc.) anion-exchange column previously calibrated with starting buffer (5 mM sodium succinate [pH 6.0]). The column was then eluted with a linear gradient of starting buffer to final buffer (5 mM sodium succinate [pH 4.5] containing 250 mM NaCl). Fractions exhibiting ligninase activity were split into 500-μl portions and stored at −15°C. A typical ligninase preparation exhibited 5,000 U of ligninase activity per liter and a specific activity of 40 U/mg of protein. Total protein was assayed by the Coomassie blue dye-binding method (5) with bovine serum albumin as the standard.

Glucose. Glucose concentrations were determined by using an enzymatic assay kit (Glucose HK; Sigma).

pH. pH measurements on 50- to 100-μl culture fluid samples were made by using a micro-pH electrode (Microelectrodes, Inc.). The pH within mycelial pellets was determined after squeezing out the culture fluid (ca. 50 μl) within a single mycelial pellet.

Hydrogen peroxide. H_2O_2 concentrations were determined from the horseradish peroxidase-mediated oxidation of *o*-dianisidine by the method of Faison and Kirk (7). The procedure of Tonon and Odier (38) was followed, except that 50 to 860 μl was used in a final volume of 1 ml. H_2O_2 within mycelial pellets was determined after squeezing out the culture fluid (ca. 50 μl) within a single mycelial pellet.

RESULTS

Effect of agitation on ligninase in shake-flask cultures and culture fluids (free of biomass). In agreement with the reports of the deleterious effect of agitation on ligninase production, agitation of growing shake-flask cultures on a rotary shaker at 150 rpm resulted in the formation of mycelial pellets and no observed ligninase activity. Also, no activity was detected in agitated cultures in which a mycelial mat had been allowed to develop before agitation. Therefore, cultures were routinely agitated during primary growth only and then transferred to a gently agitated state (30 rpm) during the secondary metabolic phase (enzyme production phase).

The effect of agitation in shake flasks during the enzyme production phase by pelleted cultures was examined first. Figure 1 shows the results obtained from two cultures grown in 50 ml of medium in 250-ml Erlenmeyer flasks shaken at 150 rpm. Ligninase activity developed following consumption of glucose (at a culture age of 7 days), at which time the shaker speed was reduced to 30 rpm and 1 mM veratryl alcohol was added. At 15 days after glucose consumption, when the ligninase activity had reached about 75 and 40 U/liter in the two flasks, the shaker speed was increased back to 150 rpm for 24 h and then decreased to 30 rpm. There was a severe loss of activity (100% in one case and 87% in the other) during the period of high agitation (150 rpm). Similar results were obtained with agar gel-immobilized mycelial cultures of *P. chrysosporium* and culture fluids

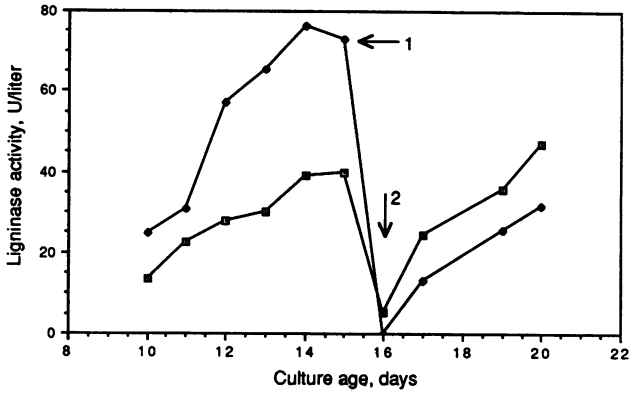

FIG. 1. Effect of agitation on ligninase activity in two replicate *P. chrysosporium* cultures (50 ml of medium in 250-ml flasks) grown as described in Materials and Methods. Arrows indicate events. Event 1, agitation of both cultures was increased to 150 rpm from 30 rpm; event 2, agitation of both cultures was decreased to 30 rpm.

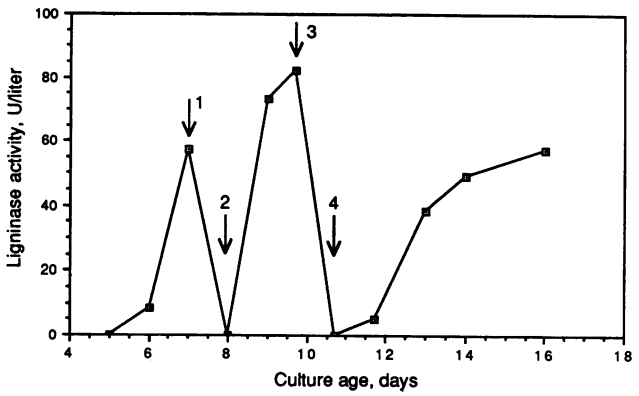

FIG. 2. Effect of repeated agitation on ligninase activity in a *P. chrysosporium* culture (500 ml of medium in 2.8-liter flask) grown as described in Materials and Methods. Arrows indicate events. Events 1 and 3, agitation was increased to 60 rpm from static conditions; events 2 and 4, culture was transferred to static conditions.

from pelleted cultures (after removal of the cells) showing ligninase activity. There was a complete loss of ligninase activity in culture fluids (free of biomass) shaken at 150 rpm and 37°C (30 ml of culture fluid in a 125-ml Erlenmeyer flask). The control culture fluids, shaken at 30 rpm, lost only approximately 10% of their activity during 24 h (data not shown). Reduction of the shaker speed back to 30 rpm in the cell-free system resulted in no increase in ligninase activity. In general the rate of loss of ligninase activity was dependent on the medium volume, the flask volume, and the shaker speed. The rate of loss of ligninase activity in culture fluids (free of biomass) was higher in larger flasks and at higher agitation speeds (Table 1). Purified ligninase tested under similar conditions (30 ml of diluted ligninase in a 125-ml Erlenmeyer flask; 150 rpm agitation) also lost activity as a result of shaking at 150 rpm (data not shown).

Effect of agitation on physiology. Shimada et al. (34) had reported that the biosynthesis of veratryl alcohol, a secondary metabolite of *P. chrysosporium*, was inhibited by agitated pellet cultures and suggested that agitation might "cause deeper physiological effects in *P. chrysosporium* perhaps affecting secondary metabolism in general."

Evidence supporting the theory that agitation affects the physiology of the organism and hence the synthesis of ligninase was not found. Results show that the effect of agitation on ligninase production is reversible (Fig. 1 and 2). In the experiment illustrated in Fig. 1, ligninase activity redeveloped in the culture fluids following transfer of the culture to gently agitating conditions (30 rpm). Repeated recovery of ligninase activity was possible after the culture was subjected to alternating periods of high and no agitation (Fig. 2).

To investigate further the effect on physiology, we considered the possibility that the microenvironment within the mycelial pellets changes as a function of agitation. The pH of the culture medium was noticeably different from the pH within the mycelial pellets (Fig. 3). Figure 3 shows the pH

TABLE 1. Effect of flask volumes and agitation speeds on loss of ligninase activity in culture fluids (free of biomass) of *P. chrysosporium*

Time of agitation[a] (h)	Ligninase activity (U/liter)[b] in:		
	125-ml flask	250-ml flask	500-ml flask
Agitation at 100 rpm			
0 (initial)	70	68	71
5.5	69	54	23
10.5	67	43	2
24	68	19	0
48	63	0	0
Agitation at 200 rpm			
0	78	76	71
1	ND[c]	33	21
2.5	28	0	0
4	8	0	0
6	0	0	0

[a] Agitation was performed with a rotary shaker (2.5-cm-diameter cycle).
[b] Control culture fluids (kept under stationary conditions) did not show any detectable loss in activity over the period tested. All flasks used were Erlenmeyer flasks and contained 30 ml of culture fluid.
[c] ND, Not determined.

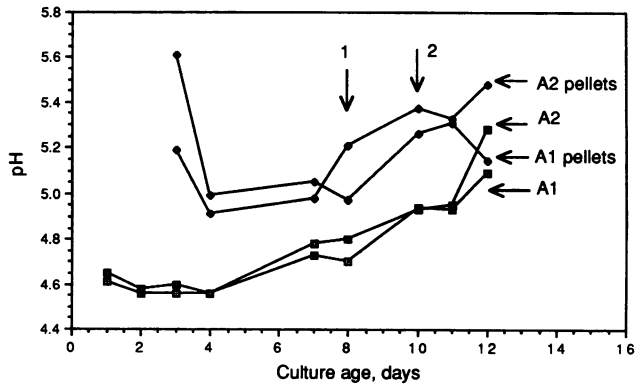

FIG. 3. pH profiles of culture fluids (A1 and A2) and culture fluids within mycelial pellets (A1 pellets and A2 pellets) of *P. chrysosporium* cultures (100 ml of medium in 500-ml flasks) grown as described in Materials and Methods. Arrows indicate events. Event 1, agitation of culture A1 was increased to 150 rpm from 30 rpm; event 2, agitation of culture A1 was decreased to 30 rpm. The shake speed of the control (culture A2) was maintained at 30 rpm after day 5.

profiles of two cultures, A1 and A2. Both cultures were agitated at 150 rpm during primary growth. On day 4 the shaker speed for both cultures was reduced to 30 rpm. On day 8 the shaker speed for culture A1 was increased to 150 rpm for 2 days and then decreased to 30 rpm. The pH of the culture medium increased during the secondary metabolic phase from 4.56 to about 5.0. The pH within the A1 mycelial pellets was always above 4.9; there was a difference of 0.2 to 1.0 pH unit between the pH of the culture medium and the pH within the mycelial pellets. However, agitation seemed to have no obvious impact on the change in pH for the mycelial cultures in culture A1 between days 8 and 10. The pH of the culture fluid and the pH within the mycelial pellets continued to increase as in the control (culture A2). Furthermore, we observed no effect on the pH when the culture was continuously shaken during the growth and the secondary metabolic phases.

Extracellular hydrogen peroxide is produced by *P. chrysosporium* as part of its ligninolytic system (9, 10). Tonon and Odier (38) have shown hydrogen peroxide production (maximum concentration, ca. 23 μM) by nitrogen-limited cultures growing on glucose. In general, hydrogen peroxide could not be detected in the studies reported herein. However, hydrogen peroxide at 20 to 25 μM was detected in culture fluids within mycelial pellets of 7- or 8-day-old cultures shaken during primary growth only at 150 rpm. Cultures agitated at 150 rpm showed no hydrogen peroxide within their mycelial pellets. This absence of hydrogen peroxide did not seem to affect ligninase production once the agitated cultures were transferred to static conditions.

Effect of detergents on ligninase in culture fluids (free of biomass). Jager et al. (15) reported ligninase production and lignin degradation in agitated submerged cultures of *P. chrysosporium* by using a detergent-amended culture medium. Specifically, they used the detergents Tween 20, Tween 80, and CHAPS at concentrations between 0.05 and 0.2% in their culture medium to obtain a maximum activity of ca. 140 U/liter under agitation. Initial findings in this study had indicated that mechanical inactivation of ligninase should have been substantial under the conditions used by Jager et al. (30 ml of medium in a 125-ml Erlenmeyer flask; 150 rpm agitation). This apparent contradiction can be explained if the detergents protect ligninase against mechanical inactivation. To test this possibility, we conducted experiments with culture fluids (free of biomass) exhibiting ligninase activity under the conditions used by Jager et al. (15). It should be noted that the source of culture fluids used in the following experiment for all flasks was the same and was obtained by filtration (to remove biomass) of 7- to 8-day-old cultures of *P. chrysosporium* grown as described in Materials and Methods. Culture fluid B3 contained Tween 80 at 1,000 mg/liter (0.1%, wt/vol) and was agitated at 150 rpm (Fig. 4). Culture fluids B1 and B2 served as the controls with no added detergent. B1 was also shaken at 150 rpm while B2 was kept stationary. As expected, at the end of 1 day B1 lost all of its activity. In complete contrast, B3, which contained the added detergent, retained all of its activity even while being agitated at 150 rpm. In fact, B3 was able to retain its activity over an extended period of high agitation (more than 7 days). Two other controls were also used. One, which consisted of culture fluid kept under static conditions with added detergent, did not show any loss of activity over the period tested. The other control, which consisted of boiled culture fluid with added detergent, tested negative for ligninase activity. The above experiment was performed three more times,

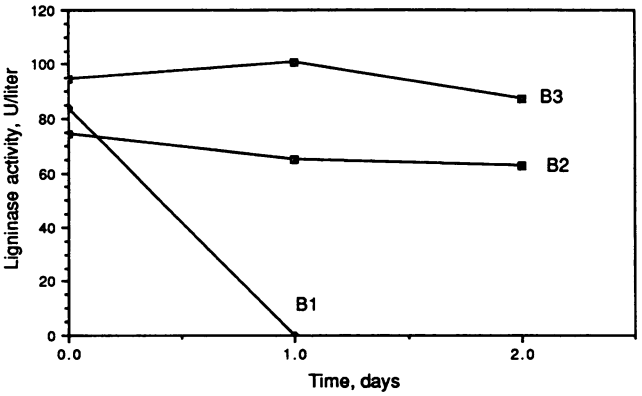

FIG. 4. Effect of Tween 80 on ligninase in agitated culture fluids free of biomass (30 ml in 125-ml flasks). Culture fluid B3 contained Tween 80 at 1,000 mg/liter and was agitated at 150 rpm. Culture fluid B1 was also agitated at 150 rpm, whereas B2 was kept under static conditions. Culture fluids B1 and B2 did not contain Tween 80.

with the same results. An additional effect of Tween 80 at 1,000 mg/liter was a 1.3- to 1.4-fold increase in the observed rate of the ligninase assay in culture fluids (free of biomass) at pH 4.5 under static conditions. Foaming was not observed in culture fluids (free of biomass) with or without detergent addition.

A concentration-dependent effect of Tween 80 on ligninase activity in culture fluids free of biomass (30 ml in 125-ml flasks) agitated at 150 rpm was demonstrated (Fig. 5). At concentrations of 50, 100, and 1,000 mg of Tween 80 per liter, the protective effect was observed with sustained ligninase activity over the entire period tested. At concentrations of 1 and 10 mg of Tween 80 per liter, as in the control without Tween 80, no appreciable activity was detected at the end of 1 day. At 20 mg of Tween 80 per liter, ligninase activity was not sustained over the entire period tested but did, however, persist to some extent over 1 day of high agitation.

The effect of Tween 80 addition on purified ligninase being agitated under similar conditions (30 ml of diluted ligninase in a 125-ml Erlenmeyer flask; 150 rpm agitation) was also examined. Here, too, the dramatic effect of the detergent in

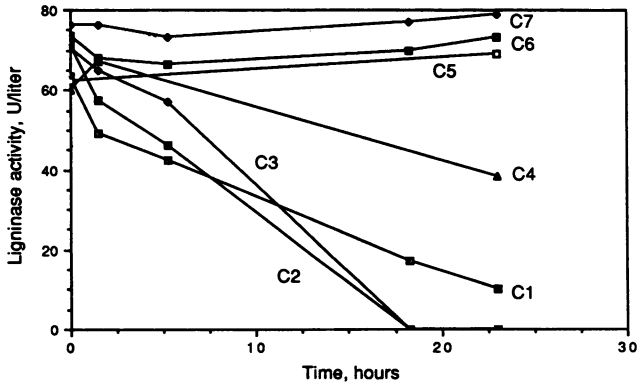

FIG. 5. Effect of Tween 80 concentration on the protection of ligninase in culture fluids free of biomass (30 ml in 125-ml flasks) agitated at 150 rpm. Culture fluids C1, C2, C3, C4, C5, C6, and C7 contained 0, 1, 10, 20, 50, 100, and 1,000 mg of Tween 80 per liter, respectively.

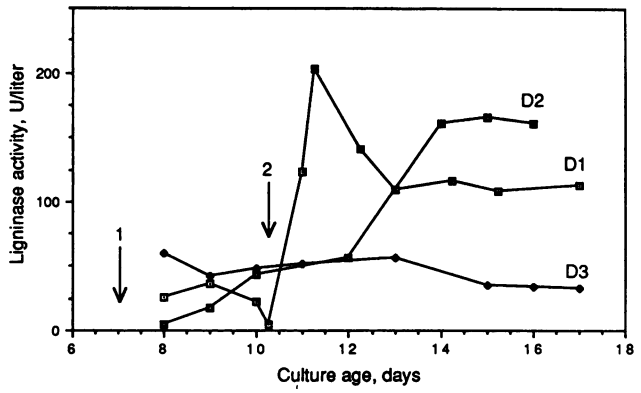

FIG. 6. Effect of Tween 80 addition to agitated cultures of *P. chrysosporium* (100 ml of medium in 500-ml flasks) agitated at 150 rpm. Cultures D1 and D2 were inoculated with spores, whereas culture D3 was inoculated with spore-immobilized agar beads. Cultures were grown as described in Materials and Methods. Arrows indicate events. Event 1, 1 mM veratryl alcohol was added to cultures D1, D2, and D3, and 1,000 mg of Tween 80 per liter was added to cultures D2 and D3; event 2, 1,000 mg of Tween 80 per liter was added to culture D1.

preventing mechanical inactivation was distinctly observed (data not shown).

Four other detergents, Tween 20, Tween 40, Tween 60, and CHAPS (all at 1,000 mg/liter), were also able to prevent mechanical inactivation of ligninase in culture fluids (free of biomass) under extended periods of agitation (data not shown).

Effect of Tween 80 on ligninase production. The effect of Tween 80 on ligninase production was tested by adding it to *P. chrysosporium* cultures at the end of the primary growth period. Other researchers had used detergents as part of their growth medium at the beginning of primary growth (2, 15). In contradiction to the findings of Jager et al. (15), addition of Tween 80 at 1,000 mg/liter to continuously agitated cultures after the end of primary growth resulted in high productivity of ligninase (Fig. 6). Moreover, ligninase was remarkably stable over the period tested under the high agitation conditions. Here again, the results were easily reproducible. This effect was observed with mycelial pellets as well as immobilized *P. chrysosporium* in agar beads (Fig. 6). Addition of Tween 80 at 1,000 mg/liter to an agitated culture 3 days after the end of primary growth also resulted in a remarkable enhancement in ligninase levels (Fig. 6).

Ligninase production in stirred-tank reactors. Mechanical inactivation of ligninase (loss of 25 U of ligninase per liter in 16 h) was significant under agitated conditions in a bioreactor (working volume, 1.5 liters; 30 rpm cell lift impeller).

DISCUSSION

Results indicate that ligninase was inactivated under conditions of high agitation in cultures of *P. chrysosporium* as well as in culture fluids (free of biomass) (Fig. 1 through 3; Table 1) and in preparations with purified ligninase. A likely explanation for the loss of activity caused by agitation seems to be denaturation of ligninase as a result of mechanical forces generated at high agitation speeds (100 to 200 rpm), noting that the loss in activity was well correlated to the degree of agitation (Table 1). The use of larger flasks and higher agitation speeds resulted in greater mechanical forces, leading to a higher rate of mechanical inactivation (Table 1).

The fact that purified ligninase tested under the same conditions as culture fluids (free of biomass) also lost activity as a result of agitation indicates that the loss of activity occurs in the absence of any extraneous factors, such as proteases or other chemical species generated by the organism. Also, there seems to be no indication that loss of activity is not a primary consequence of agitation.

Nevertheless, alternative routes to inactivation of ligninase in culture fluids (free of biomass) were also considered. In activation due to chemical species does not explain the inactivation phenomenon. The control (stationary) culture fluids (free of biomass) were identical to the agitated culture fluids (free of biomass) and demonstrated stable ligninase activity (Fig. 4).

The possibility that the agitated culture fluids (free of biomass) contained higher levels of dissolved oxygen than the stationary culture fluids (free of biomass), and that this caused inactivation of ligninase, was also considered. However, oxygen has been widely reported to have a beneficial effect on both ligninase production and lignin degradation by *P. chrysosporium* (8, 22, 25, 31). Faison and Kirk (8) have reported that ligninase activity is increased by idiophasic exposure of cultures to 100% O_2. Additionally, from independent results (Venkatadri and Irvine, unpublished data), it was found that very high levels of ligninase activity could be maintained at high dissolved-oxygen levels (greater than 15 mg/liter) during the enzyme production phase. Moreover, ligninase was stable for long periods (more than 4 days) at high dissolved-oxygen concentrations. Our results show that the loss in ligninase activity under agitated conditions was rapid (within 6 h at 200 rpm). Also, ligninase was stable in a culture fluid (free of biomass) with pure oxygen added through a gas-permeable silicone tubing. Therefore, inactivation of ligninase by oxygen was ruled out as an explanation for the loss of ligninase activity.

Involvement of extracellular proteases as a cause of inactivation of ligninase was also ruled out. As mentioned above, the source of the stationary culture fluids (free of biomass) and agitated culture fluids was the same. If inactivation by proteases were involved, ligninase activity would not be expected to be stable in the stationary culture fluids (free of biomass) over the time tested (Fig. 4). Moreover, culture fluids (free of biomass) were obtained from carbon-limited (nitrogen-sufficient) cultures. Nitrogen-sufficient cultures would not favor extracellular protease production.

The fact that the effect of agitation on ligninase production by *P. chrysosporium* is reversible (Fig. 1 and 2) suggests that agitation probably has no short-term effect on the physiology of the organism which controls ligninase synthesis and that its effect is on the activity of extracellular ligninase alone. Therefore, it seems that mechanical inactivation of ligninase is responsible for either low or no ligninase activity detected in cultures agitated continuously during both the primary growth phase and the secondary metabolic phase. It is possible that other extracellular enzymes such as Mn-dependent peroxidases are also inactivated in agitated cultures and result in no lignin degradation.

Reid et al. (31) had reported that cultures of *P. chrysosporium* agitated on a gyrotory shaker at 136 rpm were able, as well as static cultures, to degrade aspen lignin to CO_2 and water-soluble products. The mycelia in their agitated cultures with ligninolytic activity formed a single, large pellet in each of their shake flasks. Lignin degradation was observed

only if the wood particles became enmeshed in a given individual pellet; no degradation occurred in agitated cultures which consisted of separate hyphal fragments, with poor growth and low respiration rates. They suggested that unbound wood particles seemed to have an abrasive effect and prevented good growth, resulting in hyphal fragments and hence no lignin degradation. In contrast, we hypothesize that ligninase, one of the important enzymes in lignin degradation, could be produced by the organism and act on the wood particles within a given single, large pellet without being released into the culture fluid and then mechanically inactivated. In the cultures with separate hyphal fragments and no direct contact of ligninase with the wood particles, ligninase would have to be released into the culture fluid, where it could be mechanically inactivated without noticeable lignin degradation.

Both Jager et al. (15) and Asther et al. (2) have been unable to establish the mechanism by which detergents enhance extracellular enzyme production in agitated cultures. It has been suggested that Tween 80 may modify cell membrane permeability and hence enhance the transport of compounds into and out of the cell (30, 35). In contrast, our results clearly demonstrate that the effect of the detergents is to protect ligninase in culture fluids (free of biomass) and purified ligninase from being inactivated under agitation (Fig. 4 and 5). This surprisingly simple observation has not been reported previously.

The protective effect of Tween 80 on ligninase was dependent on the concentration of Tween 80 in relation to its critical micelle concentration. At concentrations above the critical micelle concentration of Tween 80 (36 mg/liter at 25°C [3]), the protective effect of Tween 80 was clearly seen (Fig. 5). No protection was observed at concentrations below the critical micelle concentration of Tween 80.

The fact that the protective effect of Tween 80 on ligninase was dependent on its critical micelle concentration suggests that micelles are involved in the protection of the enzyme. The structure of a Tween detergent monomer consists of a nonionic polyoxyethylene-sorbitan head group and a hydrophobic linear hydrocarbon chain. The only difference between the different Tween detergents is the hydrophobic end (monolaurate, monopalmitate, monostearate, and monooleate groups in Tween 20, Tween 40, Tween 60, and Tween 80, respectively). It is thought that the polyoxyethylene chains are predominantly in the form of expanded helical coils (3). It is possible for some organic solubilizates to be incorporated in the polyoxyethylene exterior of the micelles of surfactants of the polyoxyethylene type such as the Tweens (3).

Interestingly, Tween 80 at 1,000 mg/liter caused a 1.3- to 1.4-fold increase in the observed rate of the ligninase assay in culture fluids (free of biomass) at pH 4.5. This is not unusual, as many detergents have been found to increase enzymatic activity. As an example, five nonionic detergents including Tween 20 and Tween 40 have been reported to increase the activity of L-glutamic acid dehydrogenase toward α-ketoglutaric acid (19). It has been suggested that conformational changes induced by the detergent could lead to an increase in the binding of the substrates required for the reduction of α-ketoglutaric acid (19). This supports the view that ligninase does interact with Tween 80 at a molecular level.

The interaction between surfactants and proteins can be complex. Surfactants can combine with native proteins and cause conformational changes. Interaction of dodecyl sulfate with β-lactoglobulin polymorphs A, B, and C yielded complexes containing 2 mol of dodecyl sulfate per mol of protein (33). There is evidence that the complex formation is abolished when the histidine residues located in the interior of polypeptide chains are destroyed, indicating that the histidine residues are probably the binding sites for dodecyl sulfate (33). In certain cases, surfactants are able to solubilize some insoluble membrane-bound proteins (3). There is some evidence that detergents may protect enzymes against inactivation. Detergents such as dodecyl sulfates, however, irreversibly inhibit the enzyme pancreatic lipase owing to denaturation of the enzyme by unfolding of the structure (4). However, the simultaneous presence of bile salts protects lipase from being irreversibly inactivated, probably owing to the stabilization of the lipase structure (4). Addition of the surfactant Triton X-100 results in the protection of colipase, a small protein which acts as a cofactor of lipase, against proteolytic cleavage in particular at the Arg_5—Gly_6 bond (6). This protection was thought to be due to binding of the detergents to a hydrophobic site on the colipase molecule.

It is possible for ligninase, a soluble protein complex containing hydrophilic surfaces, to favorably interact with the hydrophilic head group of the Tween detergents. This may lend more structural stability to ligninase and hence prevent mechanical denaturation. One possible interaction is the binding of ligninase to the hydrophilic regions in the micelles of the Tween detergents. The fact that all the Tweens were able to protect ligninase, whose common hydrophilic region is the polyoxyethylene-sorbitan group, further supports the theory that the hydrophilic regions of the micelles are involved in the protection. Another possible mechanism by which the detergents could interact with ligninase is the binding of the detergent monomers to ligninase. It is possible for the hydrophobic chain of the Tween monomers to bind with the hydrophobic residues within the ligninase molecule or for the hydrophilic polyoxyethylene head group to bind with the hydrophilic residues of ligninase. Both scenarios could lead to the binding of more detergent monomers to monomers already bound to the protein (3), possibly resulting in a stable complex which is resistant to mechanical denaturation, and hence inactivation, owing to agitation.

The protective effect of Tween 80 on ligninase has been demonstrated in both culture fluids (free of biomass) and cultures of *P. chrysosporium* under agitation (Fig. 6). The protective effect of Tween 80 on ligninase occurs in agitated cultures during the enzyme production phase, which is distinct from the growth period. An additional effect of Tween 80 was demonstrated by Asther et al. (2). They showed that Tween 80 at 500 mg/liter caused a 1.6-fold increase in ligninase production by *P. chrysosporium* even under stationary conditions. They suggested that saturated and unsaturated fatty acids, released by the hydrolysis of Tween 80 and then available for use by the organism, may be involved in the mechanism by which Tween 80 increases ligninase production. It is possible that the hydrolysis of Tween 80 and the disappearance of fatty acids are involved in the activation of ligninase production, either by providing an extracellular energy source for secondary metabolism or by serving as an inducer, as do many aromatic compounds including veratryl alcohol (8). Additionally, the increased ligninase activity demonstrated by Asther et al. (2) could simply be due to the increase in the observable activity in the presence of Tween 80. Thus, the possible effects of Tween 80 seem to be fourfold: (i) it is able to protect ligninase from being mechanically inactivated; (ii) it interacts with ligninase

to cause an increase in the observable activity; (iii) it or its hydrolysis products may serve as an extracellular energy source for use during secondary metabolism; and (iv) it or its hydrolysis products may be involved in the activation (or induction) of ligninase synthesis, resulting in enhanced ligninase production.

The loss of ligninase activity reported above for agitated conditions in both the bioreactor and the shake flasks explains the limited success reported by others for ligninase production in stirred-tank reactors. Kantelinen et al. (18) have recently shown a sixfold enhancement of ligninase production by *Phlebia radiata* in a 1.2-liter (working volume) bioreactor, in which the mixing took place via air bubbles and 500 mg of Tween 80 per liter was added. The facilitated ligninase production in a 42-liter stirred-tank reactor reported by Janshekar and Fiechter (16) involved the use of polypropylene glycol, which probably serves to protect ligninase in a manner similar to that described above for detergents. Both of these studies reinforce the notion that detergents such as Tween 80 can protect ligninase against mechanical inactivation in both bioreactors and shake flasks.

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