

Production of Ligninases and Degradation of Lignin in Agitated Submerged Cultures of *Phanerochaete chrysosporium*

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Research on the extracellular heme protein ligninases of *Phanerochaete chrysosporium* has been hampered by the necessity to produce them in stationary culture. This investigation examined the effects of detergents on development of ligninase activity in agitated submerged cultures. Results show that addition of Tween 80, Tween 20, or 3-[(3-colamidopropyl)dimethylammonio]1-propanesulfonate to the cultures permits development of ligninase activity comparable to that routinely obtained in stationary cultures. The detergent-amended cultures express the entire ligninolytic system, assayed as the complete oxidation of [¹⁴C]lignin to ¹⁴CO₂. The detergent effect is evidently not merely in facilitating release of extant enzyme. Development of ligninolytic activity in the agitated cultures, as in stationary cultures, is idiophasic. Ion-exchange fast protein-liquid chromatography indicated that the heme protein profiles in agitated and stationary cultures are very similar. These findings should make it possible to scale up production of ligninolytic enzymes in stirred tank fermentors.

The lignin-degrading basidiomycete *Phanerochaete chrysosporium* secretes several heme peroxidases that degrade lignin and lignin substructure model compounds (3, 14; T. K. Kirk, S. Croan, M. Tien, K. E. Murtagh, and R. Farrell, *Enzyme Microb. Technol.*, in press; A. Paszczynski, V.-B. Huynh, and R. L. Crawford, *FEMS Microbiol. Lett.*, in press). Both basic and applied studies of these ligninases have been hampered by the difficulty of producing them in sufficient quantity. The enzymes were first isolated from shallow stationary cultures grown in 125-ml Erlenmeyer flasks. Those cultures had been optimized for complete oxidation of lignin to CO₂ (5). Attempts to scale up to larger flasks or to shallow trays resulted in lower activity per milliliter (Kirk et al., in press). Agitated submerged cultures in Erlenmeyer flasks or in stirred tank fermentors permitted good growth as mycelial pellets, but only traces of or no ligninase activity (2; Kirk et al., in press). These results with agitated cultures parallel earlier findings in our laboratory that lignin is not degraded to CO₂ in agitated cultures (5). Paszczynski et al. (in press) recently scaled up production of ligninase by *P. chrysosporium* by using a rotating drum apparatus in which the mycelium was immobilized on the interior wall. Similarly, we have scaled up ligninase production by using a rotating disk fermentor and a mutant strain (Kirk et al., in press). These mycelial film reactors, however, are awkward to use compared with ordinary stirred tank fermentors, and equipment for scale-up and control is not readily available. A recent report by Gold et al. (3) of a strain of *P. chrysosporium* that produces ligninase in agitated culture encouraged us to continue investigations into the use of agitated cultures and eventually stirred tank fermentors. Gold et al. (3) described neither the origin of their special strain nor its properties.

In this paper we describe the production of the ligninase—and of the complete lignin-degrading enzyme system—by

wild-type strains of *P. chrysosporium* in agitated submerged cultures exhibiting only pellet growth. The enzyme activity is equal to or higher than that obtained in stationary cultures or in the disk fermentor. Obtaining activity in the agitated cultures is made possible by the simple expedient of adding detergent to the cultures. In the absence of detergent, no ligninase activity is detected. Our results suggest that the effect of the detergent is not due simply to release of preformed enzyme.

MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* Burds strains ME-446 (ATCC 34541) and BKM-F-1767 (ATCC 24725) were grown in the following sizes of submerged agitated cultures: 30 ml per 125-ml Erlenmeyer flask, 300 ml per 1-liter Erlenmeyer flask, and 600 ml per 2-liter Erlenmeyer flask. Nonagitated shallow cultures (10 ml per 125-ml Erlenmeyer flask) were also used for comparison. All cultures were grown at 39°C. Agitated cultures were grown on a rotary shaker (200 rpm, 2.5-cm-diameter cycle). All cultures were flushed at the time of inoculation and every third day thereafter with 100% O₂ and maintained closed.

Cultures were grown in a chemically defined standard medium (Kirk et al., in press) containing 2.2 mM ammonium tartrate, 1% glucose, and 0.4 mM veratryl-alcohol; a seven-fold excess of trace elements was used as described earlier (Kirk et al., in press). The same buffer, 10 mM (sodium) dimethylsuccinate (pH 4.3) was used in all studies.

For inoculum, cultures of the fungi were grown from conidia (5) in stationary 2.8-liter Fernbach flasks containing 50 ml of the medium described above (without detergent). After 48 h the mycelium was blended for 60 s with a Waring blender. This blended inoculum was added at a concentration of 45 mg (dry weight) of mycelium per liter of medium for both agitated and nonagitated cultures.

Five different detergents were used in the study: methylbenzethonium chloride, Tween 20 (polyoxyethylene-sorbitan monolaureate), and 3-[(3-colamidopropyl)-dimethyl-

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TABLE 1. Effect of concentration of Tween 80 on ligninase activity in *P. chrysosporium* BKM-F-1767 in agitated submerged cultures^a

Tween 80 concn (%)	Ligninase activity (U/liter)	Day of maximum activity ^b
0	0	
0.01	87 ± 23	5
0.05	134 ± 45	5 to 6
0.10	125 ± 34	6
0.20	123 ± 42	7

^a Cultures (30 ml per 125-ml flask) were grown as described in Materials and Methods. Values are means ± standard deviations for four to five replicate cultures.

^b Days after inoculation.

ammonio]1-propanesulfonate (CHAPS) (all from Sigma Chemical Co.); Tween 80 (polyoxyethylene-sorbitan monooleate) (Fisher Scientific Co.); and sodium dodecyl sulfate (Bethesda Research Laboratories). Detergents were solubilized and sterilized by autoclaving 1% aqueous solutions; the solutions were then added to sterile concentrated stock solutions of medium, which were then brought to the correct final volume with sterile water.

Ligninase assay. The ligninase activity in the extracellular fluid was determined as the H₂O₂-dependent oxidation of veratryl alcohol to veratrylaldehyde as described earlier (14; Kirk et al., in press), except that 0.5 M sodium tartrate (pH 2.5) and 100 µl of culture fluid in a total volume of 0.5 ml was used in each assay.

Degradation of lignin. Lignin degradation was assayed as described earlier (4, 5) with synthetic [ring-¹⁴C]lignin (15 kBq/mg). The lignin (833 Bq per culture) was added with the inoculum. Evolved ¹⁴CO₂ was flushed from the cultures, trapped, and counted beginning 3 days after inoculation and then every 24 h.

Mycelium nitrogen determination. The mycelium of a 125-ml Erlenmeyer flask culture was dried overnight at 60°C and then to constant weight over P₂O₅; total Kjeldahl nitrogen was determined.

Reducing sugar determination. Total reducing sugar was determined in the filtered extracellular fluid as described by Somogyi (12).

Fractionation of extracellular proteins. Ten 30-ml cultures of *P. chrysosporium* BKM-F-1767 were grown with agitation for 6 days in standard medium containing 0.1% Tween 80. The mycelium was removed by filtration over glass wool, and the filtrate was concentrated by ultrafiltration from 300 to 30 ml. The proteins in this concentrated extracellular fluid were separated by high-pressure liquid chromatography with a Mono Q column (Pharmacia Fine Chemicals) and a gradient of acetate buffer (pH 6) from 10 mM to 1 M (Kirk et al., in press). For comparison, the 10-fold-concentrated extracellular fluid of stationary 10-ml cultures (5 days old, grown without detergent; Kirk et al., in press) were used.

RESULTS

We first examined the influence of Tween 80 on the formation of ligninase activity in agitated cultures, discovering that the detergent permits development of good activity in both of the examined wild-type strains. Further study revealed that certain other detergents have the same effect, that the effect is apparently not due merely to release of

enzyme, and that the total ligninolytic system is produced in the agitated, detergent-amended cultures.

Effect of detergent on development of ligninase activity. Ligninase activity was assayed in agitated submerged cultures of strain BKM-F-1767 (30 ml per 125-ml flask) containing 0 to 0.2% Tween 80 (Table 1). All cultures grew comparably and well, forming 50 to 70 pellets of diameter 0.2 to 0.4 mm per flask. In the absence of detergent, pellet formation was similar, but no significant ligninase activity was detected. In contrast, good ligninase activity was found at all concentrations of Tween 80. The time course of activity development and the maximum reached varied with Tween 80 concentration. At concentrations of 0.01, 0.05, and 0.1% detergent, cultures developed maximum activity on days 5 and 6; at 0.2% Tween 80, maximum activity was seen on day 6. Activity fell off sharply at the lowest concentration (0.01%), but persisted at the higher concentrations. The best results were obtained with 0.05 to 0.1% Tween 80; activity was higher than we routinely obtain in stationary cultures without detergent (Kirk et al., in press). The volume of the culture and flask size had no influence on the development of ligninase activity (0.1% Tween 80) when they were varied proportionately. Thus, activity was the same in 125-ml flasks with 30 ml of medium, in 1-liter flasks with 300 ml of medium, and in 2-liter flasks with 600 ml of medium. The substantial variation in ligninase activity among replicate cultures (Table 1) is caused by asynchrony in culture development. Use of conidia instead of fragmented mycelium for inoculum did not synchronize the cultures; instead it delayed the time required to reach maximum activity by 4 to 5 days. The cultures looked the same, however, and the maximum ligninase activity was comparable.

We also examined a second wild-type strain, ME-446. When grown in submerged culture, this strain, like BKM-F-1767, exhibited good growth as small pellets. Also like BKM-F-1767, ME-446 developed activity in the presence of Tween 80, but not in its absence. In cultures with 0.1% Tween 80, ligninolytic activity appeared after 7 days and peaked on day 8. Maximum activity (~40 U/liter) was higher than we have observed in stationary cultures (~7 U/liter) (2; T. K. Kirk, M. Tien, S. C. Johnsrud, and K.-E. Erikson, *Enzyme Microb. Technol.*, in press), but substantially lower than with strain BKM-F-1767, as is also observed in stationary cultures (Kirk et al., in press). Considerable variation among replicate cultures was also seen with ME-446.

Addition of 0.1% Tween 80 to replicate 6-day-old cultures of strain BKM-F-1767 containing no detergent did not result in activity development; those cultures were assayed 1, 2, 4, 24, 48, and 144 h after detergent addition on day 6.

Effect of other detergents on development of ligninase activity. Four other detergents were examined with strain BKM-F-1767. Both Tween 20 and CHAPS gave results comparable to those with Tween 80; growth in all three cases was in the form of small pellets. The highest activities with Tween 20 and CHAPS were obtained at 0.05%; with both, activity peaked on days 5 and 6, one day earlier than with Tween 80.

Maximum activities with Tween 20, CHAPS, and Tween 80 were not significantly different (Fig. 1). Methylbenzethonium chloride suppressed growth even at a concentration of 0.001%, as did sodium dodecyl sulfate at a concentration of 0.01%. Growth (as pellets) did occur at 0.001% sodium dodecyl sulfate, but ligninase activity did not develop.

Growth and ligninase activity in agitated cultures. The time course of ligninase activity was followed together with mycelial weight, total mycelial nitrogen, and residual glu-

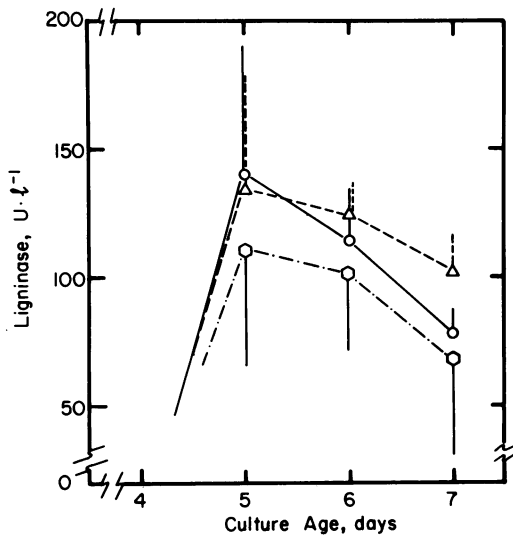


FIG. 1. Ligninase activity in agitated submerged cultures of *P. chrysosporium* BKM-F-1767 amended with Tween 80 (Δ), Tween 20 (\circ), or CHAPS (\diamond), all at 0.05%. Cultures (30 ml) were in 125-ml flasks. Data points are means \pm 1 standard deviation for four to five replicate cultures.

cose in 30-ml agitated cultures containing 0.1% Tween 80 (Fig. 2). The medium is deliberately growth limiting for nutrient nitrogen (5). As a result, growth, measured as total mycelial nitrogen per culture, reached a maximum at about day 2. In accord, the rate of gain in mycelial weight was maximum to day 2 and then slowed considerably. The gain after day 2 can be attributed to accumulation of a polysaccharide sheath (6, 7; Kirk et al., in press), which is not nearly as pronounced in this strain as in others, including ME-446 (Kirk et al., in press). Glucose, supplied in excess, de-

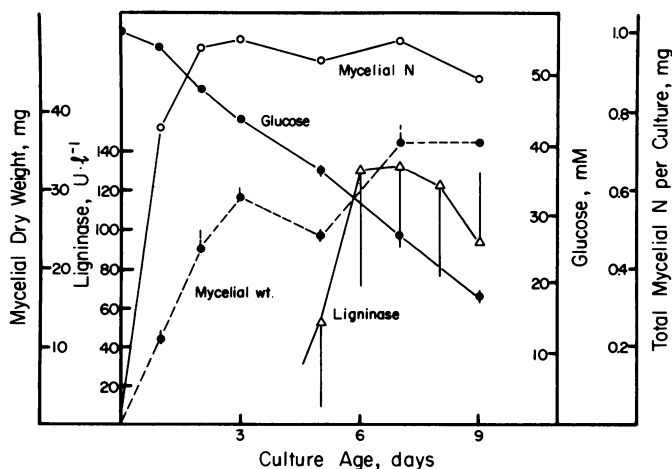


FIG. 2. Relationship between culture growth (mycelial weight, mycelial nitrogen), glucose depletion, and development of ligninase activity in agitated submerged cultures of *P. chrysosporium* BKM-F-1767 amended with 0.1% Tween 80. Values for residual glucose and mycelial dry weight are averages for duplicate cultures. Mycelial nitrogen was determined on pooled duplicate samples. Ligninase values are means \pm standard deviations for five replicate cultures.

TABLE 2. Effect of concentration of nutrient nitrogen on ligninase activity in *P. chrysosporium* BKM-F-1767 grown in agitated submerged cultures with 0.1% Tween 80^a

Nitrogen concn (mM)	Ligninase activity (U/liter)	Day of maximum activity ^b
2.2 (standard)	131, 141	5
4.4	142, 144	6
8.8	51, 58	7
13.2	29, 47	9
22.0	0, 0	

^a Cultures (30 ml per 125-ml flask) were grown as described in Materials and Methods. Values are given for duplicate cultures.

^b Day after inoculation.

creased linearly through the 9-day incubation; glucose was measured as total extracellular reducing sugar. Ligninase activity appeared in the culture fluid between days 4 and 5, more than 1 day after depletion of medium nitrogen; activity reached a maximum on day 6 and persisted.

Development of ligninase activity in the agitated cultures with Tween 80 was affected by nutrient nitrogen concentration. Doubling the concentration to 4.4 mM, however, affected neither the maximum ligninase activity obtained nor the timing of its appearance. At 8.8 mM, maximum activity was only 50% of that at the lower nitrogen concentration and was reached 1 to 2 days later, between days 7 and 8. Higher nitrogen concentrations delayed or completely suppressed development of ligninase activity or both (Table 2).

Profile of extracellular proteins. The high-pressure liquid chromatography profiles of extracellular hemeproteins (409 nm-absorbing material) from agitated detergent-amended cultures and stationary cultures were similar (Fig. 3). The major protein in both cases eluted at 23.5 min; we have designated this protein H8 (Kirk et al., in press). In both

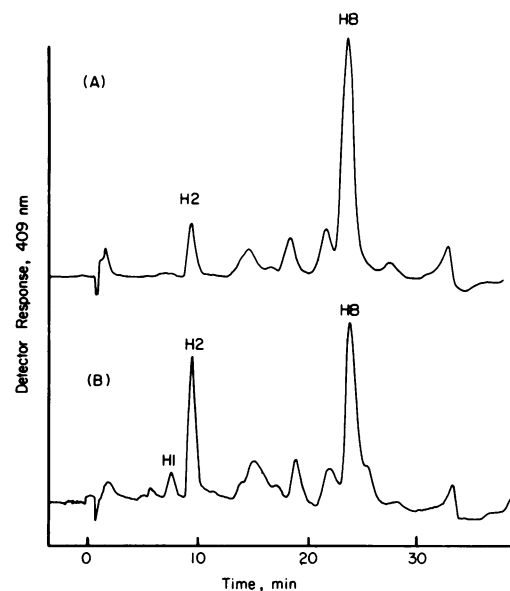


FIG. 3. High-pressure liquid chromatography profiles (absorbance at 409 nm) of the extracellular fluids from 6-day agitated cultures (A) and 5-day stationary cultures (B) of *P. chrysosporium* BKM-F-1767. Peak designations refer to previous work (Kirk et al., in press).

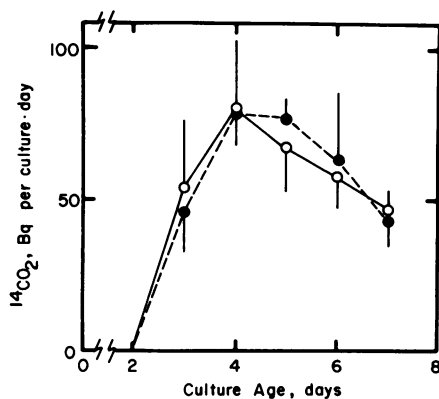


FIG. 4. Oxidation of synthetic [ring- ^{14}C]lignin to $^{14}\text{CO}_2$ by *P. chrysosporium* BKM-F-1767 in agitated submerged cultures (○) and in stationary cultures (●). Agitated cultures had 0.1% Tween 80. Values are means \pm standard deviations for five to six replicate cultures. Submerged cultures were 30 ml per 125-ml flask, and stationary cultures were 10 ml per 125-ml flask.

cases over 80% of the total ligninase activity was associated with this peak and with a second peak (designated H2; Kirk et al., in press) at 9.1 min. Several smaller peaks were evident only in the stationary cultures, most noticeably peaks at 7.4 min (H1) and a peak eluting just after H8, which appeared as a shoulder at about 24 min. The 280-nm profiles of both samples (data not shown) were very similar to the 409-nm profiles, as we have observed earlier with stationary cultures (Kirk et al., in press).

Degradation of synthetic [^{14}C]lignin. Agitated submerged cultures (30 ml) grown with 0.1% Tween 80 oxidized [^{14}C]lignin to $^{14}\text{CO}_2$. The rate of evolution of $^{14}\text{CO}_2$ was virtually the same as in 10-ml stationary cultures, reaching a maximum on days 4 and 5 (Fig. 4).

DISCUSSION

Our results demonstrate that *P. chrysosporium* can produce ligninase, and indeed the entire ligninolytic enzyme system, in submerged agitated cultures when certain detergents are added to the growth medium. The basis for the detergents' effectiveness is not yet clear. Two of the effective detergents, Tween 20 and Tween 80, probably can supply fatty acids to the cultures (lauric and oleic acids, respectively). However, the third effective detergent, CHAPS, cannot, which argues against fatty acid supplementation as the basis for detergent effectiveness. The fact that detergent addition to 6-day cultures instead of at the time of inoculation failed to result in development of ligninase activity suggests that the detergents are not functioning simply to foster release of extant enzyme. The effect of the detergents, therefore, has a physiological basis. We were unable to find other examples of the dramatic effect of detergent on production of extracellular enzymes in agitated versus stationary cultures. Indeed, the deleterious effect of agitation in the first place seems to be unusual. Nevertheless, the beneficial effect of detergents in increasing the yields of various extracellular enzymes in filamentous fungi in agitated submerged cultures was reported several years ago by Reese and Maguire (9). They suggested that the effect is on cell membrane permeability, but they also recognized that surfactants have other effects on fungi, and the basis for

the effect was not actually determined. Several other reports since that of Reese and Maguire have confirmed the beneficial effect of detergents on extracellular enzyme production by filamentous fungi (1, 8, 11, 13), but still the basis has apparently not been clarified. In our work, preliminary microscopic examination of the mycelia also failed to provide clues. At the age of maximum ligninase activity, the mycelia of agitated cultures with no detergent and agitated cultures with detergent appeared similar; numerous intercalary chlamydoconidia and a few arthrospores were seen in both. Activity is associated with brown pigmentation, which is also seen when extra trace elements are added, in the absence of detergent in stationary cultures; the brown color, therefore, is not a reflection of detergent. The nature of the brown pigment that is associated with development of ligninase activity is not clear. In preliminary study, we found that the color can be extracted from the pellets with 1 M sodium tartrate buffer (pH 4.5). No ligninase activity was detected in the tartrate buffer extract of the brown mycelia, however, even after overnight dialysis. The brown color passed through the dialysis membrane, indicating that it is of low molecular weight. The pigment did not give a positive reaction for Fe^{3+} with potassium ferrocyanide.

Reid et al. reported recently that agitated cultures of *P. chrysosporium* are able to degrade [^{14}C]lignin to $^{14}\text{CO}_2$ (10). In contrast, our laboratory reported several years ago that culture agitation resulting in growth as submerged mycelial pellets severely suppresses degradation of lignin (5). Such agitated cultures also produce negligible ligninase activity (2), as we have shown again here. The shallow cultures of Reid et al. that were able to degrade lignin grew as single, large rounded colonies in the vortex of the culture medium (10). We reported earlier that such colonies can degrade lignin (5); they apparently are comparable to stationary colonies. Thus it seems that detergent permits development of ligninolytic activity when growth is in the form of numerous small submerged pellets. Interestingly, Reid et al. (10) used Tween 80 in preparing their spore inoculum, but the final detergent concentration in the cultures was probably too low to affect ligninolytic activity.

Degradation of lignin and appearance of ligninase activity are both associated with growth limitation and the resultant secondary (idiophasic) metabolism in the agitated cultures, as they are in stationary cultures (2, 3). In the present study, depletion of nutrient nitrogen triggered the onset of idiophase, and provision of higher levels of nutrient nitrogen delayed or suppressed development of ligninase activity in agitated cultures containing detergent, as it does lignin degradation (5). Use of spore inoculum rather than mycelial fragments also delayed ligninase appearance, evidently reflecting slower initial growth and onset of idiophase.

The extracellular proteins produced by agitated ligninolytic cultures are probably the same as in stationary ligninolytic cultures. We have found that the high-pressure liquid chromatography profile of the proteins changes with culture age in stationary cultures (R. Farrell, K. E. Murtagh, M. Tien, and T. K. Kirk, unpublished data). Based on those results we suspect that the relatively minor differences here between the protein profiles of agitated and stationary cultures simply reflect culture age. We are currently investigating the biochemical basis for the age-dependent profiles; several of the heme proteins are closely related (Kirk et al., in press). The important point in the present work is that all of the extracellular proteins needed to degrade lignin are present in the agitated cultures.

In conclusion, our results provide a simple means—

detergent addition—for obtaining good ligninase titers in agitated submerged cultures of wild-type strains of *P. chrysosporium*. This discovery should make it possible to produce ligninase in ordinary stirred tank fermentors. Investigations aimed at doing so are underway.

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