Applied and Environmental Microbiology, June 1993, p. 1919–1926 Vol. 59, No. 6
0099-2240/93/061919-08$02.00/0
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# Overproduction of Lignin Peroxidase by *Phanerochaete chrysosporium* (BKM-F-1767) under Nonlimiting Nutrient Conditions

CARLOS G. DOSORETZ,[1,2]* NATHAN ROTHSCHILD,[2] AND YITZHAK HADAR[3]

*Department of Environmental Engineering and Water Resources, Technion-Israel Institute of Technology, Haifa 32000,[1] MIGAL-Galilee Technological Center, Kiryat Shmona 10200,[2] and Department of Plant Pathology and Microbiology, Faculty of Agriculture, Hebrew University, Rehovot 76200,[3] Israel*

Received 11 January 1993/Accepted 31 March 1993

**The ligninolytic enzymes synthesized by *Phanerochaete chrysosporium* BKM-F-1767 immobilized on polyurethane foam were characterized under limiting, sufficient, and excess nutrient conditions. The fungus was grown in a nonimmersed liquid culture system under conditions close to those occurring in nature, with nitrogen concentrations ranging from 2.4 to 60 mM. This nonimmersed liquid culture system consisted of fungal mycelium immobilized on porous pieces of polyurethane foam saturated with liquid medium and highly exposed to gaseous oxygen. Lignin peroxidase (LIP) activity decreased to almost undetectable levels as the initial $NH_4^+$ levels were increased over the range from 2.4 to 14 mM and then increased with additional increases in initial $NH_4^+$ concentration. At 45 mM $NH_4^+$, LIP was overproduced, reaching levels of 800 U/liter. In addition, almost simultaneous secretion of LIP and secretion of manganese-dependent lignin peroxidase were observed on the third day of incubation. Manganese-dependent lignin peroxidase activity was maximal under nitrogen limitation conditions (2.4 mM $NH_4^+$) and then decreased to 40 to 50% of the maximal level in the presence of sufficient or excess initial $NH_4^+$ concentrations. Overproduction of LIP in the presence of a sufficient nitrogen level (24 mM $NH_4^+$) and excess nitrogen levels (45 to 60 mM $NH_4^+$) seemed to occur as a response to carbon starvation after rapid glucose depletion. The $NH_4^+$ in the extracellular fluid reappeared as soon as glucose was depleted, and an almost complete loss of $CO_2$ was observed, suggesting that an alternative energy source was generated by self-proteolysis of cell proteins. The peak level of $CO_2$ concentration in the cultures increased with increasing $NH_4^+$ concentrations, reaching an almost asymptotic value at 24 mM $NH_4^+$ and paralleling glucose consumption. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and anion-exchange high-performance liquid chromatography analyses of heme protein composition in the extracellular fluid revealed that LIP synthesis in the presence of sufficient and even excess nitrogen concentrations was characterized not only by increasing activity but also by isoenzyme distribution. The H2 heme protein was the predominant LIP isoenzyme (around 70% of the total heme content) under nonlimiting conditions, in contrast to the substantial levels of all of the ligninolytic isoenzymes under N-limiting conditions.**

White rot fungi are able to biodegrade lignin as well as a broad spectrum of organic chemicals containing carbon skeletons similar to those found within the lignin polymer (5, 6, 12). Consequently, they and their extracellular ligninolytic enzymes have been considered for various applications in environmental biotechnology. However, significant improvements in the enzyme productivity and physiological conditions of the white rot fungi in liquid culture still need to be made. The basidiomycete *Phanerochaete chrysosporium* is one of the best-characterized white rot fungi. It produces no phenol oxidase activity; the major components of its extracellular lignin-degrading enzyme system are two families of extracellular glycosylated heme peroxidases and an $H_2O_2$-generating system. The two families of extracellular glycosylated heme peroxidases comprise 10 to 15 different isoenzymes and are designated lignin peroxidase (LIP) and manganese-dependent peroxidase (MNP) (15, 36, 38, 40). The expression of ligninolytic enzymes by *P. chrysosporium*, an idiophasic event triggered by nutrient limitation, is particularly active at a high $O_2$ tension and is highly dependent on culture conditions and medium composition (8, 11, 25, 32). The expression of the multiple genes encoding LIP and MNP isoenzymes has been reported to be differentially regulated at the mRNA level, depending on the conditions of nutrient limitation (5, 16).

Because of the dependence of the ligninolytic system on nutrient limitation, most studies on the production of lignin-degrading enzymes by *P. chrysosporium* have been performed in unbalanced media, usually with nitrogen limitation, resulting in low productivity of both biomass and biocatalyst. LIP production has never been found under conditions of nitrogen sufficiency or in balanced media, whether the fungus was grown as free pellets in shaken cultures or as a filamentous mat in shallow stationary cultures (11, 13, 18). Several strategies have been employed to enhance LIP productivity, including the use of lignin model-amino acid adducts, the use of veratrylamine, the addition of manganese oxide or phospholipids, and the use of several immobilization systems (2, 18, 19, 23, 33, 41). However, these studies were conducted with limiting nutrient concentrations or by using glycerol, which is a slowly metabolizable substrate (44). Heterologous expression of *P. chrysosporium* LIP in bacteria, yeasts, and even fungi has not been obtained to date (16). However, expression of LIP gene clones, in their active form, in the host insect *Spodoptera frugiperda* (SF-9 cells) after addition to the growth medium of exogenous heme was reported recently (21, 37). A few N-deregu-

* Corresponding author.

lated mutants have been described which are able to synthesize part of the ligninolytic system under nonlimiting nutrient conditions in shallow stationary cultures, albeit in relatively low amounts and with expression of multiple isoenzymes (4, 7, 28). More recently, a lysine auxotrophic mutant capable of producing about 700 to 900 U of LIP activity per liter has been described (35, 43). Although there was a predominance of H1 and H2 isoenzymes, almost all of the ligninolytic isoenzymes were formed. These results notwithstanding, no LIP production has been obtained with the wild-type strain under nonlimiting nutrient conditions by using glucose as the substrate. However, a recent paper has reported the production of LIP activity under nitrogen-sufficient conditions (24 mM $NH_4^+$) by cultures of wild-type strain BKM-F-1767 immobilized on polyurethane foam in the presence of 56 mM glucose (9).

In this study we characterized the synthesis and isoenzyme composition of the ligninolytic system under nonlimiting nutrient conditions in *P. chrysosporium* BKM-F-1767. To do this, we used a nonimmersed liquid culture system and conditions close to those occurring in nature; this system consisted of fungal mycelium immobilized on porous pieces of polyurethane foam saturated with liquid medium and highly exposed to gaseous oxygen. Six- to eight-times-higher LIP activity and two- to three-times-lower MNP activity were obtained under excess nutrient conditions compared with limiting nutrient conditions.

## MATERIALS AND METHODS

**Strain and medium composition.** *P. chrysosporium* Burds BKM-F-1767 (= ATCC 24725) was maintained at 37°C on 2% malt extract agar slants. The growth medium was based on that described by Tien and Kirk (42), but contained 20 mM acetate buffer (pH 4.5) instead of dimethyl succinate buffer, as previously reported (10). Veratryl alcohol was added at the time of inoculation (0.4 mM) and again after 48 h of incubation (2 mM). The initial glucose concentration was 56 mM (10 g/liter), and the nitrogen concentration (nitrogen was supplied as diammonium tartrate) was modified as indicated below.

**Immobilization system.** The immobilization system consisted of cubes of polyurethane foam, approximately 0.5 cm per side, which were rinsed and autoclaved in double-distilled water and dried before use. Cultures were incubated with agitation at 140 rpm in 250-ml shaking flasks containing 90 ml of culture and 1.8 g of polyurethane foam cubes embedded in liquid medium. The cubes were arranged such that the level of the liquid was about one-fourth of the total height of the bed. The inoculum used was 10% (vol/vol) homogenized mycelium, as described previously (42). Flasks were sealed with rubber stoppers and flushed with pure $O_2$ for 3 min at the time of inoculation and then twice a day for the first 4 days and once a day thereafter.

**Enzymatic activities.** LIP activity was measured as described by Tien and Kirk (42), with 1 U defined as 1 μmol of veratryl alcohol oxidized to veratraldehyde per min. MNP activity was measured as described by Kuwahara et al. (29) with phenol red as the substrate; 1 U of activity per ml was defined as 1 μmol of phenol red oxidized per min per ml of reaction mixture when the extinction coefficient described by Michel et al. (34) was used.

**Heme protein analysis.** Equal volumes of defrosted extracellular fluid were concentrated 25-fold by ultrafiltration by using a 10-kDa cutoff type YM-10 membrane (Amicon, Danvers, Mass.), centrifuged for 10 min at 25,000 × $g$, and then dialyzed against 10 mM sodium acetate (pH 6.0). Samples were analyzed for heme protein by anion-exchange high-performance liquid chromatography (HPLC) by using a MonoQ column (Pharmacia, Piscataway, N.J.) and a flow rate of 1 ml/min and monitoring the preparation at 409 nm (5, 11). The heme protein nomenclature used (isoenzymes H1 through H10) was based on elution properties and activity tests, as reported previously (5).

**Electrophoresis.** Equal volumes of extracellular fluid were dialyzed against double-distilled water, concentrated 25-fold by freeze-drying in a SpeedVac centrifuge, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 μl/lane) as described by Laemmli (30), using a 4% stacking gel and a 12.5% running gel. Proteins were visualized by Coomassie brilliant blue staining.

**Analytical techniques.** Glucose content was determined by the dinitrosalicylic acid method described by Ghose (14). Nitrogen ammonia content was determined by the phenol-hypochlorite method described by Weatherburn (45). $CO_2$ content was measured by using a gas chromatograph equipped with a thermal conductivity detector, as previously reported (10). Samples were taken directly from the headspaces of the culture flasks by using a pressure lock syringe. The $CO_2$ values reported below represent micromoles of gas accumulated in the headspace between oxygenation periods per milliliter of liquid medium.

**Chemicals.** All chemicals used were reagent grade or higher. The oxygen gas used was medical grade.

## RESULTS

**Nutrient consumption by the immobilized cultures.** Cultures of *P. chrysosporium* immobilized on polyurethane foam were incubated in the presence of $NH_4^+$ concentrations ranging from 2.4 to 60 mM, with a fixed initial glucose concentration of 56 mM (10 mg/ml). Under the incubation conditions employed, the mycelia attached to the external surfaces and interstices of the porous pieces of polyurethane foam saturated with liquid media and were directly exposed to the oxygen gas supplied to the headspace of each shaking flask. Copious sporulation on the upper layer of the polyurethane bed was observed for cultures containing intermediate $NH_4^+$ concentrations (7 and 14 mM), coinciding with the end of the ligninolytic phase. No spores were evident in the presence of $NH_4^+$ concentrations of 24 mM and above.

Glucose was completely depleted in the presence of any initial $NH_4^+$ concentration (Fig. 1a). Increasing the initial $NH_4^+$ concentration from 2.4 to 14 mM resulted in increasing rates of glucose consumption. At $NH_4^+$ concentrations of 24 mM and above, the same rate of glucose consumption was observed, and complete depletion occurred within about 40 h. Nearly opposite patterns were found for the time course profiles of $NH_4^+$ consumption; 24 mM was the highest concentration above nitrogen-limiting conditions at which all of the $NH_4^+$ was completely consumed, coinciding with complete consumption of glucose (Fig. 1b). The rates and extents of $NH_4^+$ consumption at concentrations above 24 mM were roughly the same (about 30 mM in 40 h), regardless of the initial $NH_4^+$ concentration, indicating that nitrogen assimilation ceased after complete depletion of glucose. $NH_4^+$ reappeared in the medium following glucose depletion, and moreover, the increasing initial levels of $NH_4^+$ followed an almost identical time course for all concentrations (parallel curves in Fig. 1b), observed from the point of complete glucose disappearance. This occurred

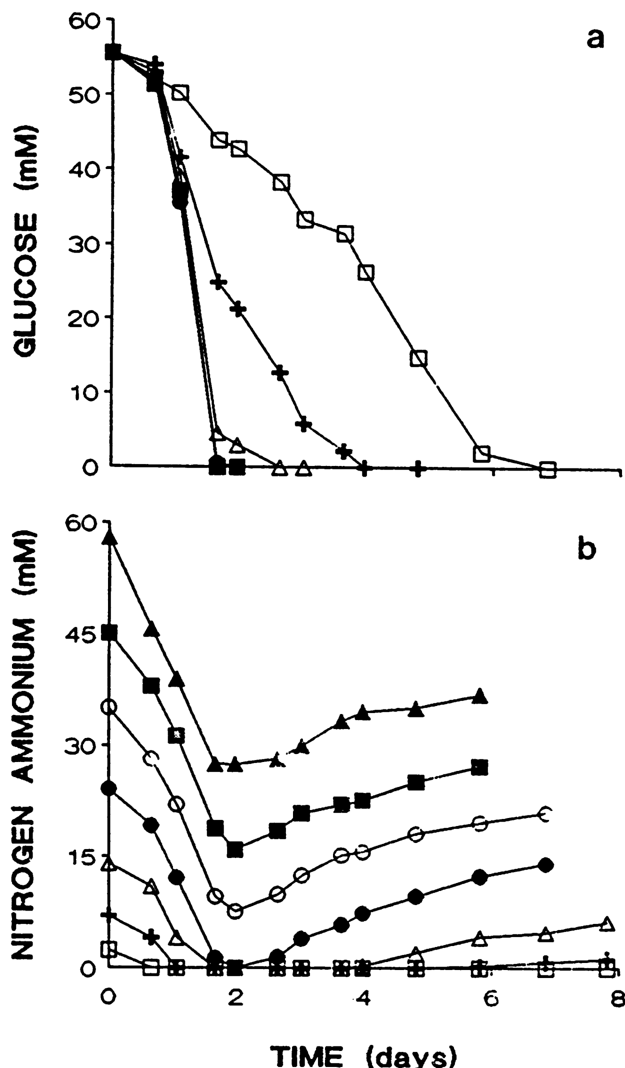


FIG. 1. Effect of initial $NH_4^+$ concentration of *P. chrysosporium* nutrient consumption. (a) Glucose. (b) $NH_4^+$. The initial $NH_4^+$ concentrations were 2.4 mM (□), 7 mM (+), 14 mM (△), 24 mM (●), 35 mM (○), 45 mM (■), and 60 mM (▲). The $NH_4^+$ source was diammonium tartrate. The initial glucose concentration was 56 mM in all cases.

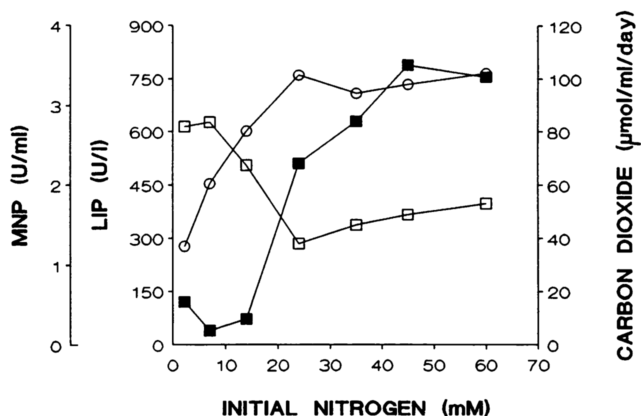


FIG. 2. Changing peak values of ligninolytic enzyme activities and $CO_2$ concentration with increasing initial $NH_4^+$ concentrations. Symbols: ■, LIP activity; □, MNP activity; ○, amount of $CO_2$ accumulated in the headspace. The values represent the peaks of activity or concentration of each parameter for each $NH_4^+$ concentration.

even in the $NH_4^+$ concentration range from 7 to 24 mM, when all of the nitrogen had been utilized.

**Synthesis of the ligninolytic enzymes.** Synthesis of the ligninolytic enzymes by immobilized *P. chrysosporium* was studied as a function of $NH_4^+$ concentration. Figure 2 summarizes the changes in the peak values of each component and of $CO_2$ concentration observed with increasing $NH_4^+$ concentrations (2.4 to 60 mM). The $CO_2$ concentrations in the cultures increased with increasing $NH_4^+$ concentrations, reaching an almost asymptotic value at an $NH_4^+$ concentration of 24 mM. This increase paralleled glucose consumption (Fig. 1a). LIP activity decreased as the initial $NH_4^+$ levels were increased over the range from 2.4 to 14 mM and then increased with additional increases in initial $NH_4^+$ concentration. MNP activity was maximal under nitrogen limitation conditions and decreased 40 to 50% of the maximal level in the presence of sufficient or excess initial nitrogen concentrations. The results of a detailed time course study of the ligninolytic enzyme activities and nutrient consumption in the presence of three $NH_4^+$ concentrations, representing nitrogen limitation, sufficiency, and excess, are described below and are shown in Fig. 3.

**(i) Limiting nitrogen conditions (2.4 mM $NH_4^+$).** The ligninolytic system became active progressively in direct response to nitrogen starvation; first MNP activity appeared, and then LIP activity appeared (Fig. 3a). The $NH_4^+$ was consumed within 24 h, the glucose was progressively consumed at a moderate rate within 6 days of inoculation, and the amount of $CO_2$ produced reached a constant, high level around day 3, indicating that the growth phase had ended (Fig. 3b).

**(ii) Sufficient nitrogen conditions (24 mM $NH_4^+$).** The ligninolytic system became active about 24 h after complete utilization of the carbon and nitrogen sources (Fig. 3c). The appearance of the ligninolytic enzymes coincided with a sudden decrease in $CO_2$ formation and reaccumulation of $NH_4^+$ in the extracellular fluid, following the complete utilization of $NH_4^+$ (Fig. 3d). LIP activity rose to about 500 U/liter, a fourfold increase compared with the level observed under nitrogen limitation conditions, while MNP activity decreased 2.5-fold (Fig. 3a and c). Both glucose and ammonia were completely depleted within 40 h, indicating that there was rapid metabolic activity. This corresponded to a sharp peak of $CO_2$ concentration, which was followed by a rapid decrease in $CO_2$ concentration (Fig. 3d).

**(iii) Excess nitrogen conditions (45 mM $NH_4^+$).** Under excess nitrogen conditions behavior similar to that found under N-sufficient conditions was observed; LIP and MNP appeared almost simultaneously, within 24 h following complete glucose utilization (Fig. 3e). This coincided with a sharp decrease in $CO_2$ formation and reaccumulation of $NH_4^+$ in the extracellular medium. At this time, 37% of the initial $NH_4^+$ remained unconsumed (Fig. 3f). LIP activity reached 800 U/liter (almost seven times the activity observed under N limitation conditions). The MNP activity remained similar to that found under N-sufficient conditions (namely, one-half of the activity under N limitation conditions).

**Profile of extracellular proteins.** The SDS-PAGE profiles of the extracellular proteins (Fig. 4) were consistent with the

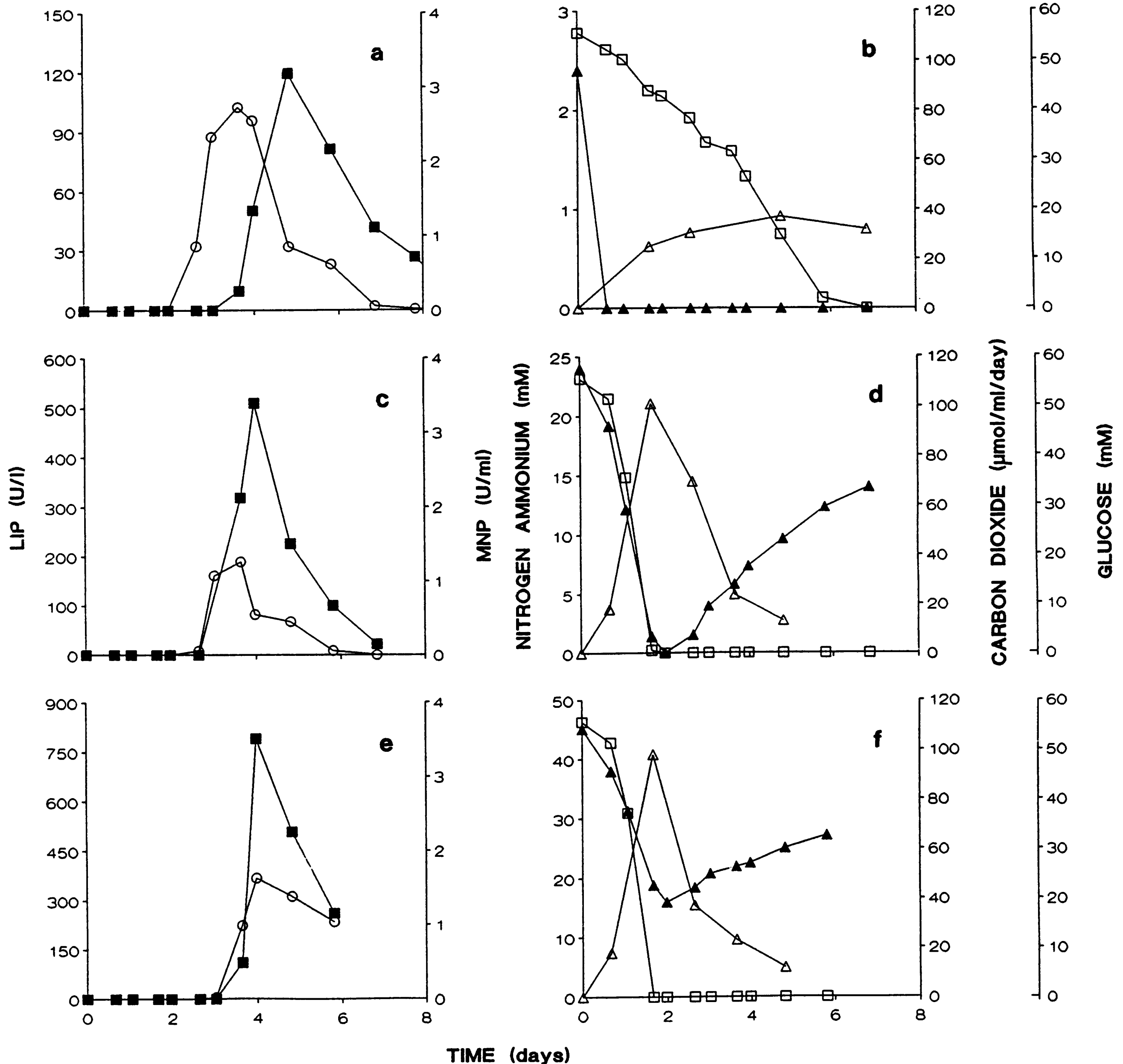


FIG. 3. Time course study of ligninolytic enzyme activities (a, c, and e) and nutrient consumption (b, d, and f). (a and b) N limitation conditions (2.4 mM). (c and d) N sufficiency conditions (24 mM). (e and f) N excess conditions (45 mM). Symbols: ■, LIP activity; ○, MNP activity; □, glucose concentration; ▲, $NH_4^+$ concentration; △, amount of $CO_2$.

peak activity value profiles of the ligninolytic enzymes (Fig. 2). The predominant protein band at $NH_4^+$ concentrations of 24 mM and above corresponded to the H2 isoenzyme, whereas the bands corresponding to the MNP proteins (Fig. 4, band P) and especially the H6 LIP isoenzyme were evident primarily at an $NH_4^+$ concentration of 2.4 mM; the intensity of these bands decreased at concentrations toward sufficient nitrogen conditions, and the bands intensified in the presence of excess nitrogen. At $NH_4^+$ concentrations of 7 and 14 mM, the bands corresponding to the ligninolytic proteins were very weak. However, two other major proteins with molecular masses of 36.7 and 71.5 kDa were present. An analysis of heme protein compositions in the extracellular fluid by using strong anion-exchange HPLC (Fig. 5) revealed that LIP synthesis in the presence of sufficient and even excess nitrogen in the medium was characterized not only by increased activity, but also by a different distribution of the LIP isoenzymes. The level of H1 and H2 isoenzymes increased from about 25 to 90% of the total heme, and the level of H6 decreased from around 45% of the total heme in the presence of limited nitrogen to less than 15% in the presence of excess nitrogen. Typical heme protein levels in the presence of 24 to 60 mM $NH_4^+$ consisted of roughly 20% H1 and 70% H2; the rest of the

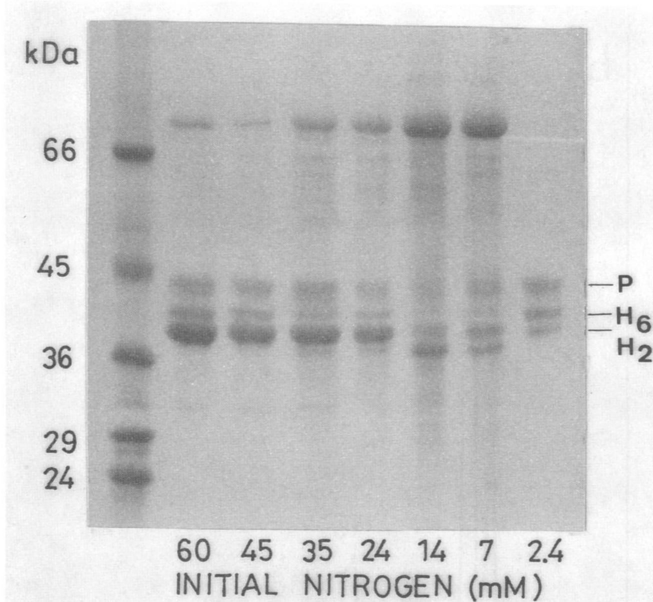


FIG. 4. SDS-PAGE of the extracellular proteins secreted by *P. chrysosporium* at $NH_4^+$ concentrations ranging from 2.4 to 60 mM. Equal volumes of extracellular fluid were sampled on day 4. $H_2$ and $H_6$ indicate the positions of LIP isoenzymes H2 and H6, respectively. Band P is a composite band that includes predominantly H4 and H5 MNP isoenzymes and minor amounts of H8 and H10 LIP isoenzymes.

heme protein was composed mainly of small amounts of H4, H5, H6, and H8 and traces of other minor unidentified peaks. Although the level of H1 and H2 was fairly reproducible, the individual levels of each of these isoenzymes varied, with H1 increasing at the expense of a decrease in H2, depending on the sampling and storage procedures. A typical example of the heme protein distribution, as analyzed by strong anion-exchange HPLC, is shown in Fig. 6. In contrast to a mixture of almost all of the isoenzymes under N limitation conditions, the H2 heme protein was the predominant LIP isoenzyme under excess nitrogen conditions. Interestingly, H10 was detected only in the presence of 2.4 mM $NH_4^+$ and completely disappeared as the $NH_4^+$ concentration increased.

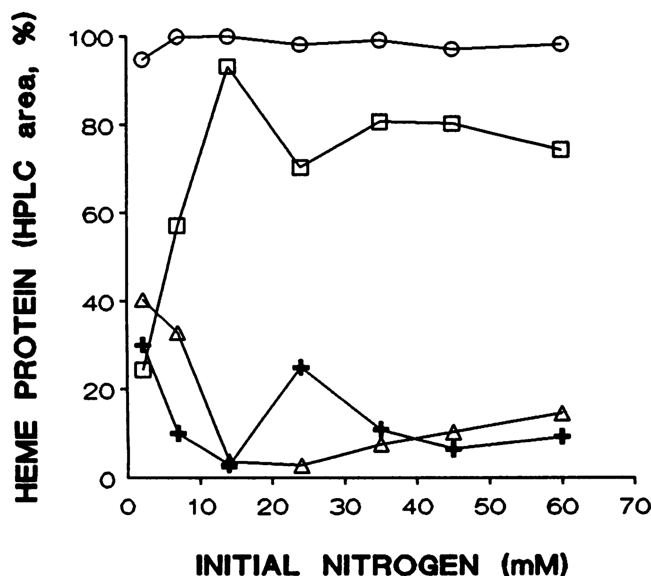


FIG. 5. Area percentages of the heme proteins in extracellular fluids, as analyzed by strong anion-exchange HPLC. Symbols: □, isoenzymes H1 and H2; △, isoenzyme H6; +, isoenzymes H4, H5, and H8; ○, total heme. The proteins were resolved by using a MonoQ column and monitoring the preparation at 409 nm.

## DISCUSSION

This study characterized the overproduction and isoenzyme composition of LIP synthesized by *P. chrysosporium* BKM-F-1767 immobilized on polyurethane foam cubes under nonlimiting nitrogen conditions. The major characteristics of this system under these conditions were found to be: (i) relatively high levels of LIP activity (800 U/liter); (ii) the predominance of the H2 LIP isoenzyme (around 70% of the total heme content), in contrast to the multiplicity of isoenzymes obtained under N limitation conditions; and (iii) nearly simultaneous secretion of both ligninolytic enzymes starting on the third day of incubation. These findings were made possible by the use of a nonimmersed growth system in liquid culture, in which the organism was immobilized on polyurethane foam saturated with liquid medium and highly exposed to gaseous oxygen. The advantage of using polyurethane foam (a solid, hydrophobic, very porous material) over other porous supports and the effects of this material on the thermodynamic adhesion properties and morphology of *P. chrysosporium* have been reported previously (1, 9, 26). Our results indicate that LIP synthesis under sufficient and excess nitrogen conditions occurs in response to carbon starvation (i.e., after complete depletion of the substrate around day 2 and termination of the growth phase). Reappearance of $NH_4^+$ in the extracellular fluid after complete depletion of the substrate and during the ligninolytic phase suggests that an alternative energy source is generated by an autolytic mechanism. This should involve proteolysis of cell proteins, as evidenced by the reaccumulation of $NH_4^+$ in the extracellular fluid as soon as glucose had been completely utilized and $CO_2$ evolution stopped. A phenomenon of disappearance and reappearance of soluble ammonia in nitrogen-limited cultures was also observed by Jeffries et al. (20). On the other hand, the profile of the extracellular ligninolytic enzymes resembled the profile obtained under carbon-limiting conditions by Holzbaur and Tien (17). However, there are significant differences between the carbon-limiting conditions of Holzbaur and Tien and our system. The carbon-limited systems were obtained by decreasing the glucose concentration to 10% (5.6 mM) of the initial concentration, whereas our system employed a glucose concentration of 56 mM. As a result, we achieved eight- to nine-times-higher ligninolytic enzyme activity, on a par with the activity observed in the recently described, improved, overproducing mutants (35, 43). It should be noted that the level of H1 plus H2, which accounted for almost 90% of the extracellular heme proteins, was fairly reproducible; however, the individual levels of each of these isoenzymes varied, with H1 increasing at the expense of a decrease in H2, depending on the sampling and storage procedures. This phenomenon is consistent with the posttranslational dephosphorylation of H2, yielding H1, recently reported (21, 27). The predominance of the H2 isoenzyme under carbon starvation conditions could imply that this isoenzyme has a special role in nature, as well as that a unique regulation mechanism is

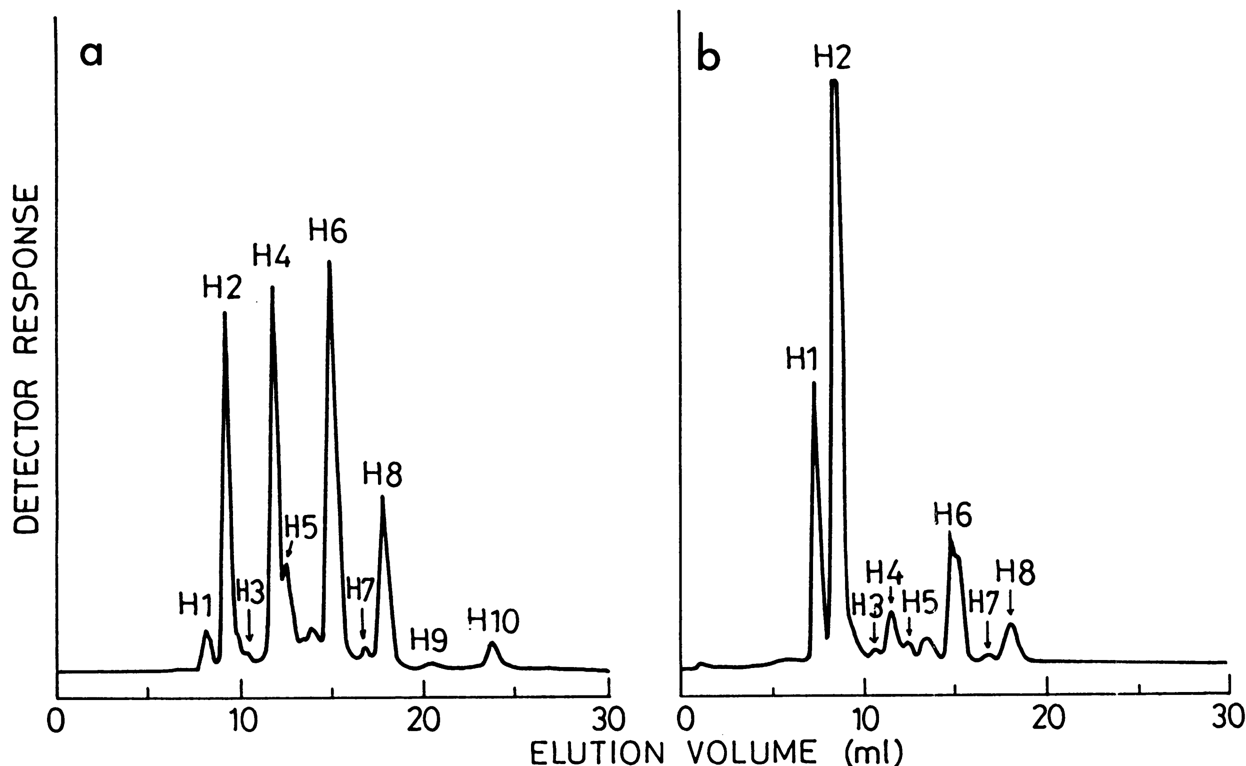


FIG. 6. Typical strong anion-exchange HPLC profiles of the extracellular fluids in cultures containing 2.4 mM $NH_4^+$ (a) and 45 mM $NH_4^+$ (b). Peaks H1, H2, H6, H8, and H10 are LIP isoenzyme peaks; the other labeled peaks are MNP isoenzyme peaks.

present at the gene expression level among the LIP isoenzymes, in agreement with previous reports (17, 44). Interestingly, H2 is reportedly less related to the other major LIP isoenzymes, on the basis of N-terminal sequences and molecular weight determinations, agreeing with the peptide mapping data and sequences of LIP cDNAs and genes (5, 16, 38).

Jager et al. (18) and Rogalski et al. (39) reported the overproduction of LIP under nitrogen-sufficient conditions by *P. chrysosporium* BKM-F-1767 immobilized on macroporous sintered glass in an upflow fixed-bed bioreactor. These authors concluded that sintered glass mimics the natural environment, providing mechanical support to the mycelium and preventing any damage that may be caused by shear forces. However, in their studies they used glycerol as a sole carbon and energy source. This substrate is known to be slowly metabolized by *P. chrysosporium*, thereby imposing unbalanced nutrient conditions, or more specifically carbon limitation conditions, even during the growth phase (44). Moreover, it has been suggested that, in contrast to glucose, glycerol acts as a nonrepressive substrate in cultures containing sufficient nitrogen (44). In our work, the cultures were grown under rich nutrient conditions with glucose, a rapidly metabolizable substrate, and with high exposure of the fungal mycelium to gaseous oxygen. Therefore, a different metabolism and metabolic rate are to be expected. In addition to the mechanical advantages of the support pointed out by Jager et al. (18), our results may be explained by the larger surface area exposed to gaseous oxygen, which seems to trigger overproduction of the ligninolytic system by speeding up glucose consumption. The importance of oxygen partial pressure on lignin degradation and on the synthesis of the ligninolytic system in *P. chrysosporium* grown in liquid culture has been widely reported (3, 10). Undetectable $O_2$ levels in the mycelial mat at depths lower than 1 mm have been reported in nonagitated as well as submerged liquid cultures of *P. chrysosporium* incubated in the presence of 100% $O_2$ (31, 34). On the other hand, Kerem et al. (22) reported that $O_2$ is not a rate-limiting factor for lignin degradation in solid-state cultures because of the high surface area, even when the fungal mycelium was exposed to atmospheric $O_2$, in agreement with our findings.

Overproduction of the ligninolytic system by *P. chrysosporium* wild-type strain BKM-F-1767 in the presence of sufficient and excess $NH_4^+$ concentrations, obtained by physiological manipulations, resulted in levels similar to those observed with improved mutant strains, such as the lysine auxotrophic mutant PBSL-1 (35, 43). However, it should be noted that the activity level of the ligninolytic system in BKM-F-1767 does not seem to simply follow the level of nutrients present in the medium, as has been reported for the different N-deregulated mutants of *P. chrysosporium* (4, 7, 35). Indeed, in the system described in this paper, LIP levels decreased as initial $NH_4^+$ levels were increased over the range from 2.4 to 14 mM, whereas the fungal physiological activity increased with the increase in $NH_4^+$ concentration from 2.4 to 14 mM, as indicated by the $CO_2$ concentration. Moreover, generally opposite trends were observed for LIP and MNP over the entire range of nitrogen concentrations studied. On the other hand, glyoxal oxidase, which has been reported to be one of the pathways for $H_2O_2$ generation in N-limited cultures of *P. chrysosporium* during the idiophase (24), was found to be repressed by increased $NH_4^+$ levels. In fact, it was present only under

N limitation conditions (data not shown). Orth et al. (35) reported nearly identical trends during overproduction in mutant PBSL-1 of glyoxal oxidase activity and the two ligninolytic peroxidases under nonlimiting nutrient conditions. Thus, the exocellular glyoxal oxidase may not be responsible for $H_2O_2$ production under rich nitrogen conditions by wild-type strain BKM-F-1767.

Taken together, the results described above suggest that whereas a broad regulatory mechanism may control the response of *P. chrysosporium* to any kind of starvation conditions, the factors that trigger overproduction of the individual components of the ligninolytic system may differ. This observation is supported by the data of Zitomer and Lowry (46), who suggested that microbial genes that utilize common oxygen-dependent elements to regulate their expression may also be simultaneously cross-regulated by very different signals, as in the case of the heme and some oxygen-binding proteins. Although greater understanding of the regulation of the ligninolytic system under nonlimiting nutrient conditions is needed, the nonimmersed culture system allows laboratory studies of enzyme regulation by *P. chrysosporium* over a broad range of nutrient conditions and under conditions close to those occurring in nature.

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