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Physiological Aspects of the Regulation of Extracellular Enzymes of *Phanerochaete chrysosporium*

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ABSTRACT

The formation and decay of lignolytic enzymes, along with the generation of other extracellular metabolites in submerged cultures of *Phanerochaete chrysosporium,* were studied under different physiological conditions. Whereas lignin peroxidase (LiP) was detectable only in a narrow range of O_2 tension and nitrogen concentration, manganese peroxidase (MnP) reached considerable levels over a broad range. The decay of LiP and MnP activities under lignolytic conditions paralleled that of heme and total proteins. The conditions that decrease or suppress LiP or MnP activities resulted in high levels of extracellular protease activity and/or polysaccharides.

Index Entries: *Phanerochaete chrysosporium;* fungal physiology; ligninases; heme protein; peroxidases; protease; microbial exopolysaccharides.

INTRODUCTION

Lignin is the second most abundant polymer in nature and constitutes an enormous source of renewable raw material for use in new technologies (*1*). Several industrial applications of lignolytic enzymes have been proposed: in pulping, bleaching, production of valuable chemicals, and waste treatment (*2*). The lignolytic system of the white rot basidiomycete

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Phanerochaete chrysosporium is the most widely studied (*3*). Its major components consist of two families of extracellular glycosylated heme peroxidases, designated lignin peroxidase (LiP) (*4,5*) and manganese-dependent lignin peroxidase (MnP) (*6,7*), along with the H_2O_2-generation system (*8*). Expression of lignolytic enzymes by *P. chrysosporium* is idiophasic; occurs under nitrogen, carbon, or sulfur limitation, and is particularly active at high O_2 tension (*1,3*). Cultivation parameters are very critical to the lignolytic expression system (*1,9*). Various nutritional supplements (*10*), veratryl alcohol (*11,12*) and other inducers (*13*), MnO_2 (*14*), and fatty acids and temperature shifts (*15*) have been employed to improve their production. The composition of the isoenzyme mixture and its specific activity are also affected by the medium composition (*10*), culture age and carbon source (*15–17*), Mn(II) levels (*18*), and the type of buffer (*19*). Different isoenzyme patterns were found under carbon or nitrogen limitation (*17,20*).

Production of lignolytic enzymes is sensitive to shear forces; however, their production in submerged cultures was made possible under mild agitation with the addition of detergents (*21,22*). Different low-shear-scale-up approaches were reported, including the rotating disk reactor (*10*), air-lift reactor (*23*), and a variety of immobilization systems (*13,24*). Enzyme production was also reported in a 42-L tank reactor (*13*). However, LiP and MnP produced in static, submerged, or immobilized liquid cultures displayed, in most cases, relatively sharp transient peaks of activity, regardless of the strain or carbon source used. The causes of this sudden decrease is not known yet. Although partial inactivation of LiP has been attributed to endogenous H_2O_2 (*25*), other factors may interfere with LiP production. When grown in liquid culture, *P. chrysosporium* secretes other metabolites, depending on the cultivation conditions; some of them seem to be indirectly involved in the regulation of the lignolytic system, such as a β-glucan degrading enzyme (*26*), and some of them seem to interfere with lignin degradation, such as polysaccharides (*27*). *P. chrysosporium* grown on cellulose in static cultures has been shown to produced extracellular proteases involved in the activation or release of endoglucanases (*28,29*). Recently we have shown the presence of protease activity during the idiophase of lignolytic submerged cultures of *P. chrysosporium* grown on glucose, which is inversely related to LiP activity (*30*). This work reports on the simultaneous effect of culturing conditions on formation and decay of LiP and MnP, substrate consumption, and generation of extracellular protease and polysaccharides.

MATERIALS AND METHODS

Chemicals

All chemicals used were of reagent grade. Gases were of medical-grade purity.

Strain and Culture Conditions

P. chrysosporium Burds wild-type strain BKM-F-1767 (ATCC 24715) was maintained on 2% malt-agar slants (*9*). All the cultures were grown at 37°C in a nitrogen-limited media (*31*), but with 20 m*M* buffer acetate (pH 4.5). For studies on the effect of nitrogen, the concentration was modified as indicated, whereas other medium components remained the same. Oxygenation studies were done in 120-mL serum bottles (50 mL, 225 rpm) sealed with butyl rubber septa and covered with aluminum seals. Humidified gas was flushed through the head space by penetrated needles (21 gage, 2.25 cm) as follows: periodic air or O_2, 1 min at 1.2 MPa at the time of inoculation and every 24 h; continuous air, 0.13 mL/mL/min; continuous O_2, 0.09 mL/mL/min. For free exposure to air, bottles were stoppered with foam plus. All other studies were done in 250-mL Erlenmeyer flasks (90 mL, 175 rpm), sealed with rubber stoppers and periodically flushed with O_2 as above.

Air-Lift Reactor

A 2.2-L-working-volume air-lift reactor (LH Fermentation, Stoke Poges, UK) was maintained at 0.13 mL/mL/min humidified O_2 at 37°C. Cultures were started as above in three 2-L Erlenmeyer flasks (750 mL, 125 rpm). After 24 h of incubation, the cultures were aseptically transferred to the reactor, and 0.3 mL of silicon antifoam was added. The initial glucose concentration was 10 g/L, and daily additions of 1.5 g/L/day were started after 140 h.

Enzymatic Assays

LiP activity was measured according to the method of Tien and Kirk (*31*). One unit (U) represents 1 μmol veratryl alcohol oxidized to the aldehyde per minute. MnP activity was measured as described by Kuwahara et al. (*6*) with phenol red as substrate. Unless otherwise indicated, MnP activity was expressed as the absorbance change at 610 nm (ΔA_{610}) in 3 min for a 20-μL sample. Protease activity was measured as previously described (*30*) using azocoll (Sigma Chemical Co.) as substrate. Activity (U/L) was defined assuming 1 U as the amount of enzyme that catalyzes the release of azo dye causing an absorbance change (at 520 nm) of 0.001/min.

Analytical Techniques

Reducing sugar was determined by the dinitrosalysilic acid method using D-glucose as the standard (*32*). Protein was measured according to the method of Bradford (*33*). Polysaccharides were precipitated with ethanol (3:1 v/v) at −20°C for 12 h, washed twice by successive redissolution and precipitation in water and ethanol, and freeze-dried. Polysaccharides (in mg/mL) represent the sum of the sugars detected on 4% H_2SO_4

hydrolysis (60 min autoclave) by HPLC (HPX-87P column, Bio-Rad, Richmond, CA) and RI detector.

FPLC Analysis

Equal volumes of extracellular fluid were concentrated 25-fold by ultrafiltration (10 kdalton cutoff YM-10 membrane; Amicon, Danvers, MA), subsequently dialyzed against 10 m*M* sodium acetate, pH 6.0; and analyzed for heme protein by anion exchange FPLC (MonoQ, Pharmacia, Piscataway, NJ), as described by Kirk et al. (*10*), at a flow rate of 1 mL/min, monitoring at 409 nm. Heme proteins nomenclature (H1–H10) were assigned according to elution properties and activity test, and based on previous reports (*10,16,19*).

Electrophoresis

Equal volumes of extracellular fluid of the different culture conditions were concentrated 25-fold (Centricon-10 unit, Amicon) and analyzed for protein composition (100 μL/lane) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (*34*). The stacking gel was 4%, and the running gel was 10%. Proteins were seen by Cosmassie-brilliant-blue staining.

Electron Microscopy

Scanning (SEM) and transmission (TEM) electron microscopy were done essentially as described by Flegler and Baker (*35*). Mycelial pellets were washed twice with saline and fixed for 2 h at 4°C in 5% glutaraldehyde in 0.1*M* phosphate buffer, pH 7.5, for SEM, and overnight for TEM. Pellets for TEM were stained with periodic acid–thiocarbohydrazide–silver proteinate before sectioning.

RESULTS

Exogenous Substrate Addition

Daily additions of glucose during the idiophase, which were based on the average consumption rate of the controls (1.5 mg/mL/d), were started at 140 h, which is the time corresponding to maximum LiP activity (Fig. 1). Exogenous substrate simultaneously maintained LiP at its maximum activity (10–12 U/mg protein), sustained MnP at the level remaining when addition was initiated, and completely repressed extracelluar protease activity. During the period of addition, the glucose concentration remained virtually constant, which indicates that the rate of glucose oxidation was equal to the rate of addition. Thus, the cultures maintained a practically constant metabolic activity, provided that an energy source was supplied.

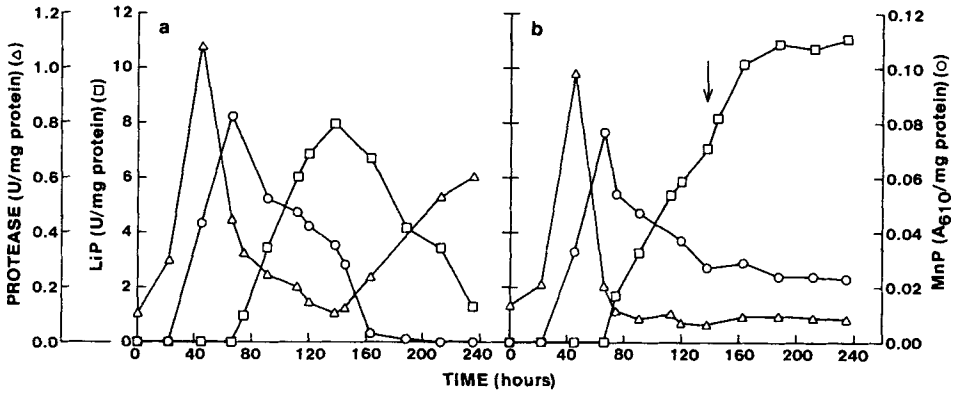

Fig. 1. Effect of daily glucose addition on extracellular enzymes produced in air lift reactor. a. Control, no addition; b. glucose addition. Arrow indicates beginning of addition (1.5 g/L/day). Conditions: 2.2 L working volume, 10 g/L initial glucose and 0.13 mL/mL/min pure O_2.

However, the supply of glucose was restricted in time (5–8 d) because it increased medium viscosity and subsequently decreased LiP activity.

Isoenzyme Pattern

The use of acetate-buffered medium yielded a balanced generation of both types of peroxidases. MnP isoenzymes H4 and H5 were the predominant heme proteins in the early idiophase (day 3) and LiP isoenzymes were predominant later, and day 6 (Fig. 2). At the peak of LiP activity, H2 and H6 constituted the major isoenzymes, and H8 and H10 were found as minor components. The results in Figs. 1 and 2 show that, although LiP and MnP activities, as well as the overall amount of hemeprotein, decreased as a function of the incubation time, the addition of glucose simultaneously retarded this decay and repressed protease activity. The addition of glucose also maintained a practically constant isoenzyme ratio and, consequently, a constant specific activity (*see* Fig. 1). A similar decrease with culture age was observed for total protein comparatively measured by SDS-PAGE (Fig. 3, left panel). The two major bands detected corresponded to the H2 and H6 LiP isoenzymes, and a third major band (P_{43}) included predominantly H4 and H5 MnP isoenzymes and small amounts of the high-mol-wt LiP isoenzymes H8 and H10.

Effect of Nitrogen

The gradual increase of nitrogen concentration resulted in the decrease of both LiP and MnP and in their complete disappearance at conditions of nitrogen sufficiency (Table 1); however, LiP was more repressed than MnP.

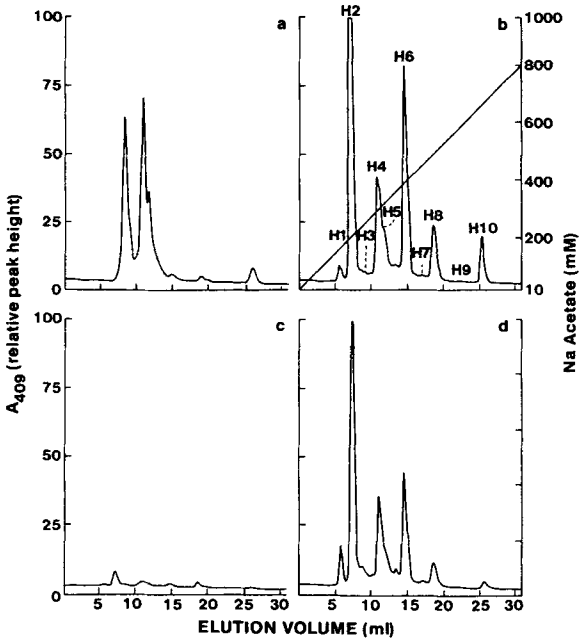

Fig. 2. Change of heme protein profile with culture age and glucose addition. a, b and c are days 3, 6 and 10 of the control, respectively; d. day 10 with glucose addition. Heme proteins were resolved by anion exchange FPLC and monitored at 409 nm. All samples loaded are 25-fold concentrates of equal volumes of culture fluid. H3, H4, H5 and H9 are MnP isoenzymes, others are LiP isoenzymes. Experiments were carried out in shaken flasks.

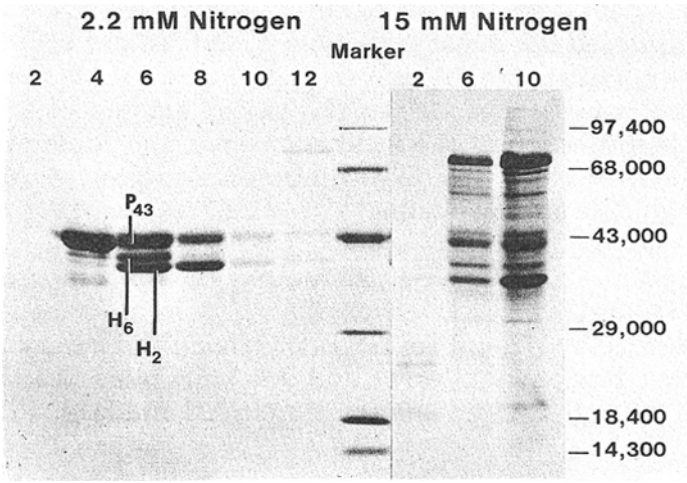

Fig. 3. Protein change with culture age under different initial nitrogen concentrations. Numbers on top of each lane indicate incubation days and numbers on right indicate marker molecullar weight (in Dalton). Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. All samples loaded are 25-fold concentrates of equal volumes of culture fluid. Experiments were done in shaken flasks.

Table 1
Effect of Nitrogen Concentration on Extracellular Enzyme Production[a]

$N-NH_4$ (mM)	C/N[b] ratio	Enzyme activity[c] (U/L)			Glucose[d] consumption rate (mg/mL/day)
		Protease	MnP[e]	LiP	
2.2	130	7.2 ± 1.6	12.4 ± 0.6	196.0 ± 16.5	1.3 ± 0.1
4.4	65	7.8 ± 1.4	12.9 ± 0.9	205.0 ± 28.0	1.7 ± 0.1
11.0	26	12.7 ± 2.1	4.1 ± 0.5	10.2 ± 2.7	2.5 ± 0.2
22.0	13	17.3 ± 1.4	ND	ND	3.3 ± 0.3

a. Values represent the mean ± standard deviations of two independent experiments performed in shaken flasks with 4 replicates for each condition.

b. Theoretical carbon to nitrogen ratio at constant glucose concentration (56 mM). Nitrogen source (variable) was di-ammonium tartarate.

c. Enzyme activities represent their respective maximum values.

d. Calculated from the slope of the curves of the glucose remaining, after 30 hours incubation and beyond.

e. Activity was arbitrarily defined in U/L as ΔA_{610}/min per mL sample, where ΔA_{610} is the absorbance change at 610 nm.

ND. Not detectable.

Concomitantly, a marked increase of both protease activity and glucose consumption rate was observed. Practically undetectable levels of substrate were found for the high-N cultures (11 and 22 m*M*) after 4–5 d. The protease activity shown here corresponded to the primary peak (around day 2) formerly identified under conditions of N limitation (*see* Fig. 1). Beyond day 2 and during the 8 d of the experiments, the protease activity remained at high levels for the 11 and 22 m*M* $N—NH_4$, making it difficult to distinguish between primary and idiophasic activities. In a different set of experiments (Fig. 3, right panel) we also noticed a retardation effect of high N on the appearance of MnP, neglibible LiP levels as well as the presence of a diverse number of proteins. Preliminary tests indicated that the prominent upper band shown with high N contained protease activity and constituted the late idiophasic protease. The band in high-N medium displaying a molecular weight similar to that of P_{43} in N-limited medium appears to be of a similar nature, as indicated by FPLC results (no data shown). The MnP activities in the extracellular fluid were 5.76±0.5 and 5.92±0.5 U/L for days 6 and 10, respectively; no LiP activity was detectable.

Table 2
Effect of the Oxygenation Conditions on Culture Parameters[a]

Oxygenation method	Enzyme activity[b] (U/L)						Polysaccharides (mg/mL)[c]	Glucose consumption rate[d] (mg/mL/day)
	Protease		MnP		LiP			
	Growth	Idioph.	Day 3	Day 6	Day 6	Day 9		
Periodic air	2.1	*	4.6	3.3	ND	ND	-	0.89
Free exposure	8.4	*	3.6	0.9	ND	ND	1.92	1.29
Continuous air	4.6	*	5.1	3.7	ND	ND	1.86	1.37
Periodic O_2	6.9	3.4	12.0	4.7	170.0	131.8	0.39	1.31
Shifted to O_2[e]	7.2	4.1	10.8	4.3	183.0	105.4	0.46	1.42
Continuous O_2	9.8	6.1	13.1	2.1	187.6	14.8	0.69	1.85

a. Values represent the average of 3 independent experiment performed in serum bottles with 3 replicates for each condition.

b. Protease activities represent the value at the maximum, day 2 for growth, and day 11 for idiophasic. MnP and LiP activities are given at their maximum values on days 3 and 6, and three days later, respectively.

c. Polysaccharides were measured on day 8.

d. Calculated from the slope of the curves of the glucose remaining, after 48 hours incubation and beyond.

e. Cultures were grown with free exposure to air for the first two days and then shifted to periodic oxygenation.

*. Activity values remained high during the entire period, and therefore idiophasic proteases were indistinguishable from growth proteases.

Effect of Oxygenation

The increment of the oxygen tension from periodic air to continuous O_2 had a number of effects (Table 2). A practically monotonous increase of the rate of substrate consumption corresponded to a gradual increase of idiophasic protease activity. Extracellular polysaccharides were significantly higher under air than under O_2. The formation of free extracellular polysaccharides resulted in an increase of medium viscosity and coincided

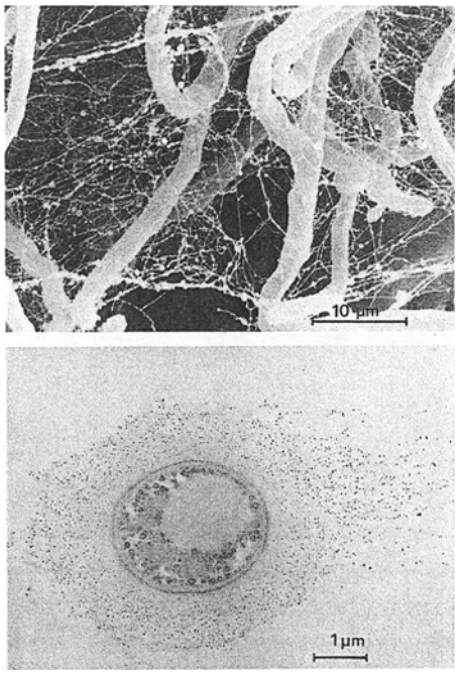

Fig. 4. Typical electron microscopic appearance of pellets formed by *P. chrysosporium* grown in submerged culture under continuous aeration. Top: SEM of whole pellets; Bottom: TEM of pellets stained with periodic acid-thiocarbohydrazide-silver proteinate before sectioning. Black dots show presence of polysaccharides. Pellets were collected on day 4.

with the presence of mycelial-attached polysaccharides (Fig. 4). The material attached to the pellets and interlocked with the mycelial hyphae shown by SEM corresponded to the polysaccharides further identified by specific staining in TEM. Respective controls with or without partial staining were done (no data shown). Intensification of the oxygenation conditions led not only to higher levels, but also to faster decay of the lignolytic enzymes (*see* Table 2). LiP activity, which increased with the increase of O_2 tension, was undetectable under the different conditions of aeration employed. A practically constant MnP production was found under O_2, regardless of the conditions employed. MnP activity was present in the entire range of oxygenation, and the values found under air were 30–40% of those obtained under corresponding conditions with pure O_2. However, a delay in onset of 24–30 h was noticed under air. As shown in Fig. 5, MnP isoenzymes were the predominant heme proteins under air, and the major peaks (P3 and P4) had elution properties similar to those of H4 and H5 isoenzymes (*see* Fig. 2). Whether the other MnP peaks are "new" proteins or are at undetectable amounts under lignolytic conditions is still unknown.

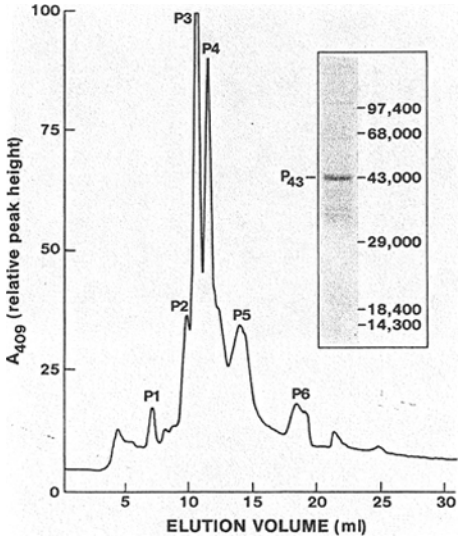

Fig. 5. Heme and total protein produced by *P. chrysosporium* grown in nitrogen limited medium under air. Heme proteins were resolved by anion exchange FPLC and total proteins (insert) by SDS-PAGE. Samples represent 250-fold concentrates of extracellular fluid of cultures harvested on day 5. P1 and P6 display LiP activity, other fractions display MnP activity. Peak number were arbitrarily assigned according to the order of appearance.

DISCUSSION

Culture conditions affect not only the formation, but also the decay of lignolytic enzymes, and also regulate the production of polysaccharides and protease activity in liquid cultures of *P. chrysosporium*. The decay of LiP and MnP activities under strict lignolytic conditions (N limitation and pure O_2) paralleled the decrease of heme and total protein along with increased idiophasic protease activity. Periodic glucose addition suppressed this protease activity and simultaneously delayed the decay of MnP and LiP proteins and stabilized specific activity in either shaken flasks or airlift reactors. In a previous work, we also showed the stabilization of LiP specific activity by the addition of a general protease-activity inhibitor (*30*). Furthermore, intensifying the oxygenation conditions that speeded up substrate depletion and, supposedly, the generation of starvation conditions resulted in an increase of extracellular protease activity and a faster decrease of LiP activity. The synthesis and active release of proteases started at the initial reorganizational phase of starvation is thought to reflect the ability of microbial cells to improve their uptake capacity at low nutrient concentrations (*36*). All these results indicate the involvement of starvation-promoted proteases in the decay of peroxidase activity. In

contrast to the apparently inducible idiophasic protease, the primary protease activity reported here appears to be a constitutive enzyme, since it was present in all the conditions employed and seems to be involved in the growth process.

Extracellular polysaccharides are an intrinsic metabolite of *P. chrysosporium* grown in N-limited culture (*26,37*), and, as appears from our work, their levels strongly depend on the physiological conditions. The inverse relationship between the rate of substrate consumption and the level of polysaccharides produced under air or O_2 may be attributable to the increase of maintenance energy associated with more severe physiological conditions. Bes et al. (*26*) suggested that the formation of polysaccharides by *P. chrysosporium* is involved in the release of the catabolic substrate inhibition of the glucose-oxidizing system under high C/N ratio. Furthermore, our results also inferred the detrimental effect of mycelial-attached polysaccharides on nutrient and O_2 mass transfer inside the pellets, in addition to the increase in meduim viscosity, in line with previous reports (*27,37*).

The gradual change of culture conditions from lignolytic (high-O_2, low-N) to nonlignolytic (low-O_2, high-N) resulted in a differential effect on the regulation of LiP and MnP. Both enzymes were maximized at high-O_2 and N-limitation conditions as previously reported (*1,3*). However, MnP was present at substantial levels over the whole range of oxygenation employed and up to 15 m*M* nitrogen, in contrast to LiP activity, which was detectable at substantial levels only at high O_2 tension and low nitrogen concentration. Pease et al. (*38*) recently reported that the response to the onset of secondary metabolism proceeds differently between the two catalytically distinct families of peroxidases, despite their regulatory and constitutive similarities (*17,39*) A similar differential effect on LiP and MnP was reported for manganese (*18*). The sequential maximum of MnP and LiP isoenzymes and activities found in this work in days 3 and 6, respectively, is in line with previous reports (*17,23,38*). In addition, our experiments were carried out in acetate-buffered medium; therefore, more balanced levels of both families of peroxidases are expected (*19*). Interestingly, the secretion of heme proteins by *P. chrysosporium* (Fig. 2) over time, rather than being a random process, seems to follow a sequence from high to low molecular weight. Differences in heme protein profiles between different reports can be explained on the basis of posttranlational modification or gene transcription as well as by differences in culture conditions and medium composition (*16,17,20*).

In conclusion, extracellular proteases and polysaccharides appear to be intrinsic metabolites produced by *P. chrysosporium* because of the lignolytic conditions, playing a detrimental role in either formation or decay of lignolytic enzymes. Proteases and polysaccharides, as well as the rapid decrease of LiP and MnP activities (regardless of the strain, substrate, or conditions employed), were also reported in static cultures

and low-shear reactor systems, which suggests that our findings are a more general phenomena. Because synthesis of lignolytic enzymes is confined to secondary metabolism, a feasible process for continuous production should have a considerably high number of production cycles per batch of biocatalyst. In spite of the improvement achieved, a major drawback in the scale-up of enzyme production is still the fast decay of enzyme productivity at a low number of reactor cycles. Thus, better understanding and control of the conditions leading to the decay of LiP and MnP in the presence of the organism is still required to improve process productivity.

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