Selective Production of Extracellular Peroxidases from *Phanerochaete chrysosporium* in an Airlift Bioreactor

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Mn(II) concentrations had little effect on substrate uptake and growth rates, but they profoundly changed the composition of extracellular proteins. At low Mn(II) (0.3 ppm), *Phanerochaete chrysosporium* produced lignin peroxidase (LiP) isozymes (total activity= 760 nmol ml$^{-1}$ min$^{-1}$), and the levels of Mn(II) peroxidase (MnP) isozymes were very low (130 nmol ml$^{-1}$ min$^{-1}$), whereas, with high Mn(II) (40 ppm), *P. chrysosporium* preferentially produced MnP isozymes (950 nmol ml$^{-1}$ min$^{-1}$), and LiPs were essentially absent. The airlift reactor provided a suitable low-shear environment for enzyme production by free mycelial pellets. This is a simple method to selectively produce LiP and MnP isozymes.

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Culture parameters are critical to the expression of ligininolytic activity in *Phanerochaete chrysosporium*. Nitrogen and carbon both repress lignin mineralization (1-3), and even brief agitation can disrupt ligininolytic activity (4). Inhibition has been attributed to shear forces, because ligininolytic activity is known to increase with O$_2$ concentration (4, 5).

*P. chrysosporium* produces extracellular liginin peroxidases (LiPs) (6, 7) and Mn(II) peroxidases (MnPs) (8, 9) that are implicated in the biodegradation of lignin. Attention has focused on the regulation of these enzymes so that their production might be optimized. Various nutritional supplements, detergents, and inducers have been employed to enhance enzyme production (10-12). LiP production has been achieved under nitrogen sufficiency with the nitrogen derepressed mutant *P. chrysosporium* INA-12, using glycerol as a carbon source (13). Ligininolytic enzymes have been produced in shake flask cultures by adding detergents or veratrityl alcohol to the culture medium (14, 15) or by supplementing the medium with oleic acid (16). Temperature shifts can also enhance enzyme production (17).

Recently, we discovered that Mn(II) is a regulatory effector in the production of LiPs and MnPs (18). At low Mn(II) levels, LiP is produced with nearly no MnP, whereas at high Mn(II) levels, MnP is formed without LIp. Thus, varying a single nutritional factor can shift the enzyme profile. We were interested in determining whether this nutritional factor could be used to regulate enzyme production in large scale.

Scale-up in submerged cultures requires agitation, but early reports showed that LiP production is inhibited by agitation (5, 15). A rotating-disk contactor provides a suitable low-shear environment for lignin biodegradation (19), and it can be applied to the production of LiPs (10). Immobilization on various supports such as polyurethane foam (20) or nylon web (21, 22) can also allow production of LiPs in agitated bioreactors.

LiPs have been produced by freely suspended mycelial pellets in a 42-l stirred tank reactor (23). In such cultures, the pellet size is critical, but difficult to control (24 and Leisola, M.S.A. et al., Third Intl. Conf., Stockholm, June 16-19, p. 46-48, 1986). Another difficulty is that *P. chrysosporium* adheres readily to impellers, baffles, and other protrusions. Airlift designs do not employ baffles and impellers, and they achieve O$_2$ transfer with little accompanying shear. We therefore decided to try an airlift bioreactor to achieve enzyme production in a low-shear environment.

This paper reports on the selective production of LiPs and MnPs in a 7-l airlift bioreactor by *P. chrysosporium* BKM-1767 and the regulation of their production by varying Mn(II) concentrations in the culture medium.

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**MATERIALS AND METHODS**

**Organism and culture conditions** *P. chrysosporium* BKM 1767 (ATCC 24725) was used for all experiments. It was maintained on yeast malt peptone glucose (YMGP) slants grown at 30°C (18). All experiments used the Mn-free basal medium previously described (18). This was supplemented with 0.83 mg/l or 110 mg/l MnSO$_4$ in order to obtain Mn(II) final concentrations (as the free ion) of either 0.3 ppm (low) or 40 ppm (high).

Scale-up was carried out at 34°C in a 7-l (5 l working volume) airlift bioreactor (L.H. Fermentation, Ltd., Bells Hill, Stoke Poges, England). The bioreactor was equipped with a polarographic dissolved O$_2$ (DO) probe and a pH sensor (Ingold Electrodes, Inc., Wilmington, MA). The airlift bioreactor was sterilized empty and aseptically filled with liquid medium. Spore density was 3.2 x 10$^4$/ml for cultures with low Mn(II) and 6.7 x 10$^5$/ml for cultures with high concentration of Mn(II). In our experiment, pH was allowed to vary freely; pH remained between 3.9 and 4.7 at the low concentration of Mn(II) and between 4.2 and 5.2.
at the high concentration of Mn(II). During the whole cultivation the overall flow of gas (pure air or air enriched with O₂) was kept constant at 1.3 to 1.4 l/min (0.26 to 0.28 l·l⁻¹·min⁻¹). This produced small pellets (1 to 3 mm dia) suitable for the production of these enzymes. Enrichment with pure O₂ was started after 24 to 48 h and adjusted as described. At the same time O₂ enrichment was initiated, veratral alcohol (2.5 mM) and Mn(II) were added. Initial attempts at scale-up were carried out in a 2-l (1.5 l working volume) stirred tank reactor (Braun Biostat M, Melsungen, GDR) at 34°C. Aeration was 0.3 l·l⁻¹·min⁻¹ for the first two days then pure O₂ after that. Agitation was varied between 150 to 600 rpm. In some experiments, polyurethane foam (1.5 mm pore size, 1 to 2 cm cubes) was used as a support. The stirred tank reactor was operated in both batch and continuous modes. With the stirred tank reactor, the foam was loosely packed into the tank (2/3 of working vol.). In the airlift reactor, the foam was loosely packed into the space between the riser tube and the vessel wall.

**Enzymatic assays** Enzymatic activities were determined spectrophotometrically at room temperature as previously described (18). LiP assays used veratral alcohol as a substrate (25), MnP used vanillylacetone (26).

**Analyses** Glucose analyses were performed enzymatically with a glucose analyzer (Beckman Instruments, Inc., Fullerton, CA). Protein concentration in the extracellular fluid was determined by the QuantiGlo method (Diversified Biotech, Newton Center, MA). Culture fluid was concentrated 20 to 30 fold by tangential-flow ultrafiltration (Amicon YM10, 10 kDal cut off; W. R. Grace & Co. Danvers, MA). The concentrated preparation was desalted by anion exchange (Waters Accel Plus QMA anion exchange medium; Millipore Corporation, Bedford, MA). The resulting concentrate was then analyzed by FPLC (MonoQ; Pharmacia, Inc., Piscataway, NJ) (10).

**Isoelectric focusing** Isoenzymes in the concentrate and FPLC fractions were separated by analytical isoelectric focusing. FPLC fractions were concentrated using microconcentrators (Centricon-10, 10 kDal cutoff; W. R. Grace & Co. Danvers, MA). Analytical isoelectric focusing was performed with a pH gradient of 3 to 6 using a thin layer polyacrylamide gel (Servalyt Precotes 3-6; Serva Fine Biochemical, Inc., Westbury, NY). Before loading the samples, the gel was first focused for 5 min at 4 kV and 5 min at 8 kV. The pI points were determined by focusing 5 min at 4 kV and 5 min at 8 kV. Analytical PI standards (Protein Test Mix 9; Serva Fine Biochemical, Inc., Westbury, NY) were run with each sample. Gels were stained with Coomassie Blue (Phast Gel Blue; Pharmacia, Inc., Piscataway, NJ). Isoenzymes were designated following an earlier convention (27).

**Molecular weight determination** Culture concentrates were denatured for 5 min at 99°C by mixing 1:1 with denaturing buffer (20 mM Tris/HCl pH 8—2 mM EDTA—5% SDS—10% β-mercaptoethanol). Relative molecular masses (M) were determined using a continuous polyacrylamide gradient gel from 10 to 15% (Phastgel). Molecular weight markers were obtained from Sigma (St. Louis, MO). The proteins and mol wts (kDa) were as follows: β-galactosidase, *Escherichia coli*, (116); phosphorylase b, rabbit (97.4); Albumin, bovine (66); albumin, egg (45); trypsinogen, PMSF treated (24). Gels were stained with Coomassie Blue.

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**RESULTS AND DISCUSSION**

Initial attempts at scale-up were unsuccessful. Good growth was obtained, and MnP was evident, but only very low titers of LiP were produced (data not shown). We attributed these results to the high shear environment of the stirred tank reactor. Polyurethane foam reduced the shear, but led to the formation of large mycelial masses. Initial attempts using polyurethane foam in the airlift reactor were also unsuccessful. The organism clogged the pores of the foam and prevented good liquid circulation.

LiPs and MnPs were selectively produced in an airlift bioreactor by varying the concentration of Mn(II) in the culture medium. A low concentration of Mn(II) (0.3 ppm) was used to produce LiPs, and a high concentration (40 ppm) was used to produce MnPs. Enzymatic activities and culture parameters were measured during the course of the reaction. Formation of the pellets appeared to be very critical. The volumetric flow rate of gas during the first 12 h affected pellet size. A low flow rate led to the formation of a few large clumps of mycelium and little enzyme production. A high flow rate resulted in excessive foaming. The gas flow rate chosen for the airlift reactor (0.26 to 0.28 l·l⁻¹·min⁻¹) corresponded to an optimum value (data not shown).

**Production of lignin peroxidases** After 50 h of cultivation, MnP activity was detected in the low Mn(II) cultures (Fig. 1a), but it never exceeded 130 nmol/min·ml.

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**FIG. 1.** Production of lignin peroxidase and Mn(II) peroxidase in a 7-l airlift bioreactor under low and high Mn(II). (a) Low Mn(II); (b) high Mn(II); lignin peroxidase, ■; Mn(II) peroxidase, □.
LiP activity was produced after 70 h of cultivation and increased steadily to 760 nmol/min·ml at 140 h (Fig. 1a). As the mycelia grew, the DO dropped from 20% to 13% of saturation (where 100% saturation ~ 35 mg/l) (Fig. 2a). When the gas flow was enriched with pure O2, the DO increased to 30%. Then it dropped slowly from 30% to 25% after 150 h of cultivation. No significant variation of the glucose uptake (Fig. 2a) was noticed during the course of the reaction. Nitrogen depletion was followed by a rapid increase in extracellular protein secretion (Fig. 2b).

Production of Mn(II) peroxidases After 50 h of cultivation, MnP activity was produced in the high-Mn(II) culture, but no LiP activity was detected. MnP activity increased to 950 nmol/min·ml after 94 h of culture (Fig. 1b). The DO value dropped from 18% to 11% during the first 25 h of cultivation when air was sparged (Fig. 3a). The flow was then enriched with pure O2. The DO increased to 30%. Then it dropped slowly from 30% to 25% after 150 h of cultivation. No significant variation of the glucose uptake (Fig. 2a) was noticed during the course of the reaction. Nitrogen depletion was followed by a rapid increase in extracellular protein secretion (Fig. 2b).

FPLC analyses The cultures were harvested after 150 h (low Mn(II) cultures) or after 125 h of cultivation (high Mn(II) cultures). The spectrophotometric isoenzyme profiles were then examined. With low Mn(II) (Fig. 4a), we detected five major protein and heme peaks by absorption at 280 and 409 nm, respectively. Each of these had LiP activity (LiP1 to LiP5), but all showed weak MnP activity. The major peak with LiP activity was LiP2. With a high concentration of Mn(II), 11 peaks were detected by absorption at 280 nm. Eight peaks with MnP activity (MnP1 to MnP8) were detected by absorption at 409 nm (Fig. 4b). Some of these did not fully resolve by the methods used. The major peak with MnP activity was MnP4.

pI and molecular weight determination SDS gel electrophoresis was not able to distinguish among the MnP

![Graph A](image1.png)

**FIG. 2.** Cultivation parameters during the production of lignin peroxidase and Mn(II) peroxidase in a 7-l airlift bioreactor at low Mn(II) levels. The reactor was initially sparged with 1.3 l/min of pure air (point 1); at 48 h, the gas mixture was changed to 1.0 l/min air and 0.3 l/min O2 (point 2). Parameters were as follows: (a) glucose, ○ dissolved oxygen, ■; (b) residual nitrogen, □; extracellular protein, ■.

![Graph B](image2.png)

**FIG. 3.** Cultivation parameters during the production of lignin peroxidase and Mn(II) peroxidase in a 7-l airlift bioreactor at high Mn(II) levels. The reactor was initially sparged with 1.3 l/min of pure air (point 1); at 24 h, the gas mixture was changed to 1.0 l/min air and 0.3 l/min O2 (point 2); at 70 h, the gas mixture was changed to 0.5 l/min air and 0.8 l/min O2. Parameters were as follows: (a) glucose, ○ dissolved oxygen, ■; (b) residual nitrogen, □; extracellular protein, ■.
and LiP isoenzymes produced under high and low Mn(II), respectively. At low Mn(II), one major band with a M₉ of 42 kDa was detected, and at high Mn(II) one major band with a M₉ of 45 kDa was detected (Table 1).

Isoelectric focusing was much more effective at resolving differences. Under the low Mn(II) concentration, one major band (Fig. 5b) was found for pI 4.1-4.2. When the individual fractions from the FPLC Mono Q separation were concentrated and run on the same isoelectric focusing gel, the major pI 4.1-4.2 band was found to consist of LiP2 and LiP3. Under high Mn(II), one major band (Fig. 5b) with MnP activity was found with a pI value of 5.1-5.2. Isoelectric focusing of the FPLC fractions showed that it consisted of at least two proteins that corresponded to MnP4 and MnP5. A summary is given in Table 1.

The Mn(II) level did not appear to significantly affect the glucose and nitrogen uptake rates, so one can assume that regulation does not proceed through the broad nutritional effect of nutrient starvation. The effect on O₂ uptake was
more apparent. With high Mn(II), the onset of MnP production correlated with a rapid increase in the O$_2$ uptake rate. With low Mn(III), O$_2$ uptake appeared to remain stable during LiP secretion. One might attribute an increased O$_2$ uptake to increased metabolic activity, but sugar uptake was relatively unaffected, and total protein secretion was actually lower with high Mn(II) than with low Mn(II). It is possible that the secreted MnP activity itself reduced the DO level.

Our results demonstrate that we can selectively produce LiPs and MnPs in an airlift bioreactor, and that Mn(II) is an effective regulator of LiP and MnP production by *P. chrysosporium* BKM during scale up. The use of Mn(II) as a specific nutritional effector gives a new dimension of control over enzyme production by this organism. The effect of Mn(II) is not limited to *P. chrysosporium*. Several species of *Phanerochaete* and other genera of white-rot fungi respond in a similar manner (18). As scale-up conditions are mastered for these fungi, Mn(II) could become an effective regulator of lignin-degradation enzymes with them as well.

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