APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1833–1838 Vol. 61, No. 5
0099-2240/95/$04.00+0
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Ligninolytic System Formation by *Phanerochaete chrysosporium* in Air

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Received 14 September 1994/Accepted 23 February 1995

This study characterizes the effect of oxygen concentration on the synthesis of ligninolytic enzymes by *Phanerochaete chrysosporium* immobilized on polyurethane foam cubes in a nonimmersed liquid culture system and maintained under different carbon-to-nitrogen (C/N) ratios and levels. Lignin peroxidase (LIP) activity was obtained in cultures exposed to air when the C/N ratio was low (7.47), i.e., when nitrogen levels were high (C/N = 56/45 mM) or carbon levels were low (C/N = 5.6/4.5 mM). At the low C/N ratio, the fungus was carbon starved and did not produce extracellular polysaccharides. At a high C/N ratio (153), i.e., under conditions of excess carbon (nitrogen limitation) (C/N = 56/2.2 mM), cultures exposed to air produced large amounts of polysaccharide, and LIP activity was detected only in cultures exposed to pure oxygen. Under high-nitrogen conditions, LIP production was 1,800 U/liter in cultures exposed to pure oxygen and 1,300 U/liter in cultures exposed to air, with H1 and H2 being the main isoenzymes. The oxygen level did not significantly alter the isoenzyme profile, nor did low-carbon conditions. The formation of manganese peroxidase was generally less affected by the oxygen level than that of LIP but was considerably reduced by a low C/N ratio. The effects of oxygen level and C/N ratio on the synthesis of glyoxal oxidase paralleled their effects on LIP synthesis except in the case of high nitrogen, which totally suppressed glyoxal oxidase activity.

The white rot fungus *Phanerochaete chrysosporium* efficiently degrades lignin as well as a broad spectrum of recalcitrant aromatic pollutants. It has therefore been considered for various applications in environmental biotechnology (10). The fungus produces an extracellular lignin-degrading enzyme system, consisting of two families of glycosylated heme peroxidases, designated lignin peroxidase (LIP) and manganese peroxidase (MNP), and glyoxal oxidase (GLOX), which produces extracellular H_2O_2 (17, 21, 30). The ligninolytic enzymes are synthesized in response to nitrogen, carbon, or sulfur limitation, and synthesis is particularly active at high oxygen tension (4, 7, 13).

The role of the high oxygen level required for the synthesis of LIP in liquid culture is thought to overcome problems of oxygen availability arising from culture conditions. An undetectable oxygen level has been reported in the mycelial mat at depths below 2 mm in nonagitated liquid cultures of *P. chrysosporium*, even in 100% oxygen (23). A similar result was obtained with a submerged liquid culture (25). It was shown that the addition of Tween 80 or Tween 20 to the growth medium results in higher permeability of oxygen through the cell membranes and in enhanced production of LIP (24).

Most laboratory studies on the formation of ligninolytic enzymes in *P. chrysosporium* have been done with submerged or shallow cultures and have been conducted in pure oxygen or in an oxygen-enriched environment, because these growth conditions are thought to enhance lignin degradation and are critical for the production of LIP (3, 7, 19). On the other hand, there is evidence that the degradation of lignin in solid-state cultures by *P. chrysosporium* and other white rot fungi proceeds at virtually the same rate in air as in an oxygen-enriched atmosphere (11, 15).

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Recent experiments in our laboratories, in which *P. chrysosporium* was immobilized on polyurethane foam in a nonimmersed liquid culture, have demonstrated overproduction of LIP under nonlimiting nitrogen conditions as a result of increased oxygen availability (5). This cultivation system provides a high surface area and thereby resembles the growth conditions on woody tissue. It therefore becomes possible to determine whether the differences in oxygen levels required to degrade lignin in nature versus those required to degrade lignin and produce LIP in the laboratory are due to differences in oxygen availability to the mycelia.

The present paper reports on the effects of the oxygen level on the formation of the ligninolytic enzyme system in a nonimmersed liquid culture under different carbon-to-nitrogen (C/N) ratios and demonstrates the synthesis of LIP, MNP, and GLOX by *P. chrysosporium* grown in air.

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 (31) but with acetate buffer (20 mM, pH 4.5) instead of dimethyl succinate buffer, as previously reported (3). Veratryl alcohol was added at inoculation (0.4 mM) and again after 48 h of incubation (2 mM), as previously reported (5). The initial glucose and ammonium concentrations for the three different nutrient conditions were, respectively, 5.6 mM and 4.5 mM for low carbon (C/N = 7.47), 56 mM and 2.2 mM for nitrogen limitation (C/N = 153), and 56 and 45 mM for high nitrogen (C/N ratio = 7.47).

Culture system. The experiments were conducted in the nonimmersed liquid culture system reported by Dosoretz et al. (5). Flasks containing cultures were sealed with rubber stoppers and continuously flushed with water-saturated O_2 by means of two hypodermic needles (3 cm, 23 gauge), at a flow rate of 10 ml/min. Cultures grown under free air exchange were stoppered with paper plugs. Oxygen gas was of medical grade.

Enzymatic activities. LIP activity was measured by the method of Tien and Kirk (31), with 1 U representing 1 μmol of veratryl alcohol oxidized to veratraldehyde per min. MNP activity was measured as described by Kuwahara et al. (21), with phenol red as the substrate. One unit of activity was calculated as 1 μmol of phenol red oxidized per min per ml of reaction mix, using the extinction coefficient described by Michel et al. (25). GLOX activity was measured as

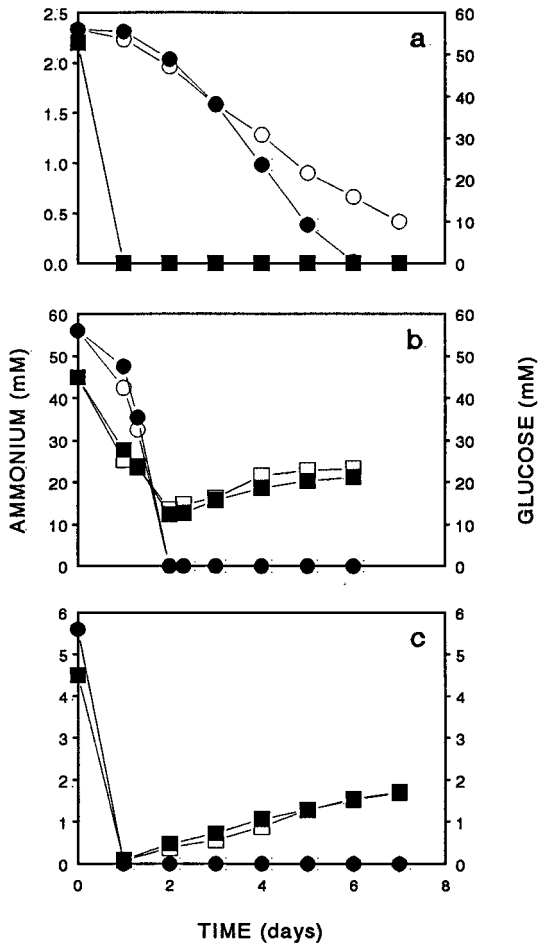

FIG. 1. Time course of nutrient consumption as a function of nutrient concentration and oxygen levels. (a) Nitrogen limitation; (b) high nitrogen; (c) low carbon. Symbols: glucose concentration for cultures exposed to air (○) or oxygen (●); NH_4 concentration for cultures exposed to air (□) or oxygen (■).

described by Kersten and Kirk (17), with methyl glyoxal as the substrate. One unit of activity was calculated as 1 nmol of peroxide produced per min.

Heme protein analysis. Equal volumes of defrosted extracellular fluid were concentrated 25-fold by ultrafiltration, using a 10-kDa-cutoff 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-pressure liquid chromatography (HPLC) with a MonoQ column (Pharmacia, Piscataway, N.J.) at a flow rate of 1 ml/min and monitoring at 409 nm (18). Heme protein nomenclature (H1 to H10) was based on elution properties and activity tests, as in previous reports (2).

SEM. Polyurethane cubes colonized by mycelia were harvested on day 4 except for the carbon-starved cultures, which were harvested on day 3. The colonized cubes were fixed for 2 h at ambient temperature in 4% (vol/vol) glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.1, and analyzed by scanning electron microscopy (SEM) by the method of Flegler and Baker (8).

Analytic techniques. Glucose was determined by the dinitrosalicylic acid method described by Ghose (9). Nitrogen ammonia was determined by the phenol-hypochlorite method of Weatherburn (33).

RESULTS

Nutrient consumption by immobilized cultures. Cultures of *P. chrysosporium* immobilized on polyurethane foam were grown under two different oxygenation regimens by exposing them to a continuous supply of either pure oxygen or air. The effect of these treatments on NH_4 and glucose consumption was examined under three different nutrient conditions: nitrogen limitation, high nitrogen, and low carbon (Fig. 1). In the case of cultures grown under nitrogen limitation, almost identical time-dependent consumption profiles of glucose and nitrogen were observed up to day 3 of incubation, in both air and oxygen. However, an increase in the rate of glucose consumption was evident from day 4 onward in the culture maintained in pure oxygen, resulting in its complete depletion on day 6, while in the culture grown in air, trace amounts of glucose were seen even at day 7. In both cases, ammonia was completely depleted within 24 h (Fig. 1a).

Under both high-nitrogen (Fig. 1b) and low-carbon (Fig. 1c) conditions, glucose was completely depleted, within 48 and 24 h, respectively, regardless of the oxygen level. Under both conditions, the time course of the consumption of NH_4 was not affected by the oxygen level, and nitrogen assimilation ceased after complete depletion of the glucose, followed by a gradual increase in nitrogen level. However, under high nitrogen, 25% of the original NH_4 remained when nitrogen assimilation ceased (Fig. 1b).

Synthesis of ligninolytic enzyme system. (i) LIP. The effect of oxygen level on LIP formation was highly dependent on nutrient composition (C/N ratio) (Fig. 2, LIP). Under nitrogen limitation, LIP activity was totally dependent on the oxygen level (Fig. 2a, LIP). In the high-oxygen cultures, LIP activity peaked at 180 U/liter on day 4, whereas almost no LIP was detected in the presence of air. Under high-nitrogen conditions, high levels of LIP were produced with air and oxygen, reaching a maximum of 1,300 and 1,800 U/liter, respectively, on day 4 (Fig. 2b, LIP). Under low-carbon conditions, the peak level of LIP activity was approximately 300 U/liter on day 3 in both air and oxygen (Fig. 2c, LIP). Under both low-carbon and high-nitrogen conditions, the onset of LIP synthesis coincided with the depletion of glucose from the medium. The level of LIP activity beyond the peak decreased more slowly in air-grown than oxygen-grown cultures.

(ii) MNP. MNP activity was found under all growth conditions tested (Fig. 2, MNP). In general, it appeared that the oxygen level has less influence on MNP activity than the C/N ratio. Under nitrogen limitation, relatively high levels of MNP were produced in both air and oxygen, reaching a maximum of 2.6 and 3.1 U/liter, respectively. In high oxygen, MNP peaked 24 h earlier, but activity was less stable than in air-grown cultures (Fig. 2a, MNP). In contrast, 50% higher activity was observed in oxygen than in air under high-nitrogen conditions (Fig. 2b, MNP), though even this peak was only 45% of the peak under nitrogen limitation. Under low-carbon conditions, slightly higher but less stable MNP activity was observed in oxygen than in air (Fig. 2c, MNP). However, peak activity was only 20% of that under nitrogen limitation. These results indicate that carbon starvation, resulting from a low C/N ratio, strongly repressed MNP activity, an effect that was more marked in air than in oxygen, as can be seen from the comparison of its level with that of LIP under the same conditions.

(iii) GLOX. The effects of oxygen level and C/N ratio on the synthesis of GLOX, which produces extracellular H_2O_2 (Fig. 2, GLOX), were found to resemble their effects on LIP production except that GLOX was totally repressed by high nitrogen, regardless of the oxygenation conditions (Fig. 2b, GLOX). In cultures grown under nitrogen limitation, GLOX activity (2.9 U/ml) peaked on day 4 in oxygen, while with air, no activity was observed (Fig. 2a, GLOX). On the other hand, under low carbon, higher but less stable GLOX activity peaked on day 3 with both oxygen (6.1 U/ml) and air (4.3 U/ml) (Fig. 2c, GLOX).

(iv) Isoenzyme analysis. The influence of the oxygen level on LIP and MNP isoenzyme profiles, measured at peak LIP activity, was found to be directly linked to the C/N ratio, as in the

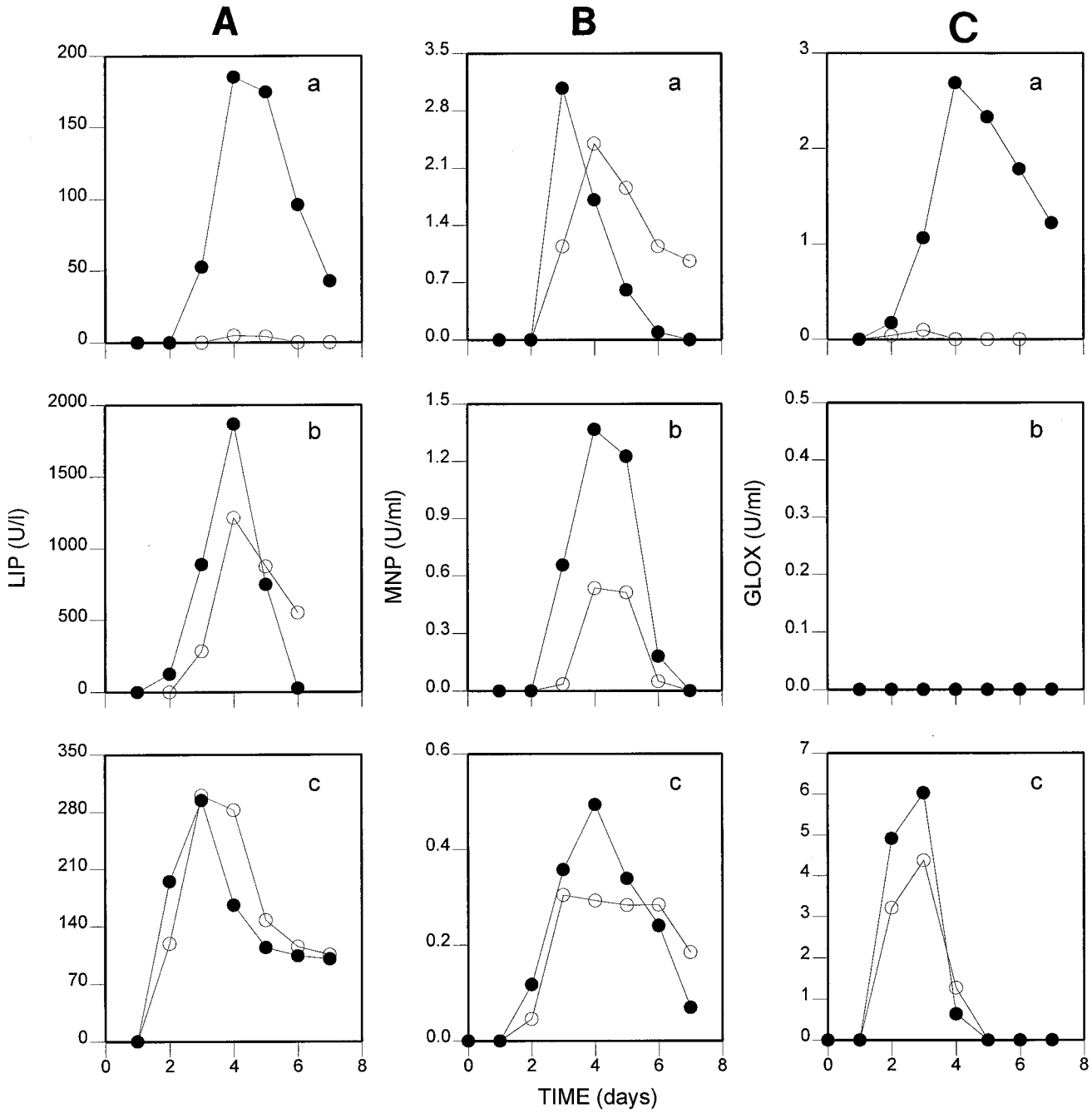

FIG. 2. Effect of O_2 level on (A) LIP, (B) MNP, and (C) GLOX activities in *P. chrysosporium* grown under three nutrient conditions: (a) nitrogen limitation; (b) high nitrogen; and (c) low carbon. Cultures were exposed to air (○) or oxygen (●).

case of enzyme activity (Fig. 3). Under both conditions involving a low C/N ratio, the oxygen level had little effect on the heme protein profile observed in the extracellular fluid on the day of peak LIP activity. H1 and H2 were the dominant isoenzymes in high-nitrogen cultures, and H2 and H6 were the dominant isoenzymes in low-carbon cultures. A substantial level of Ha was found under both conditions, whereas MNP isoenzymes were almost absent. However, the heme profile for the high C/N ratio was markedly affected by the oxygen level. In the culture grown under air, MNP isoenzymes dominated. H3 was the most prominent MNP isoenzyme, with only minor quantities of H8 and H10 LIP isoenzymes. Under oxygen, H2 and H6 were the major LIP isoenzymes, while the MNP isoenzyme H4 appeared at a lower intensity.

Study by SEM. An SEM study of immobilized mycelia on day 4 of growth (corresponding to peak LIP activity) is presented in Fig. 4. Under nitrogen limitation, the mycelia became enveloped in copious quantities of polysaccharides in air-grown cultures (Fig. 4A). The formation of polysaccharides was greatly reduced with 100% oxygen (Fig. 4B). Under high nitrogen, no polysaccharides were found attached to the mycelia in cultures exposed to either air or oxygen (Fig. 4C). A

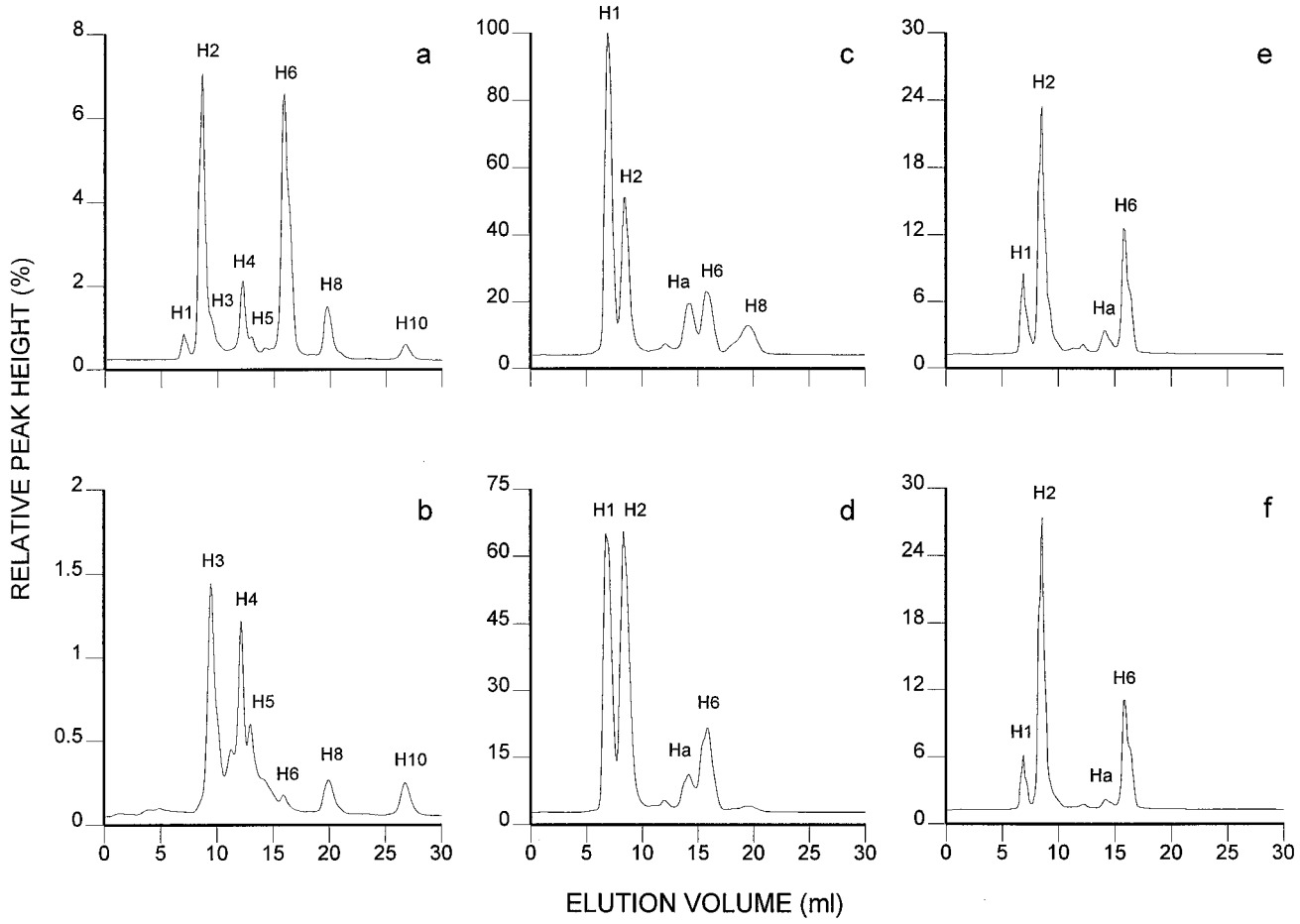

FIG. 3. Typical HPLC profile of heme proteins in the extracellular fluid of *P. chrysosporium* grown under different C/N ratios and oxygen levels: (a) nitrogen limitation and oxygen; (b) nitrogen limitation and air; (c) high nitrogen and oxygen; (d) high nitrogen and air; (e) low carbon and oxygen; and (f) low carbon and air. Equal volumes of extracellular fluid from day 3 in carbon-limited cultures and from day 4 in all others were analyzed by strong anion-exchange HPLC at 409 nm. Peak height for H1 in panel c was set to 100%.

similar situation was found under low-carbon conditions (data not shown).

The effect of immobilization on the morphology of the fungus is also visible in Fig. 4. The micrographs show a spaced and linear filamentous mycelium at low density under all culture conditions, even in the high-nitrogen culture, which gave rise to a high biomass. This is in contrast to the mycelial structure in pellet (nonimmobilized) cultures, especially those grown in high nitrogen, in which a very dense and branched mycelium is found.

DISCUSSION

This study characterizes the effect of oxygen level on the synthesis of ligninolytic enzymes by *P. chrysosporium* BKM-F-1767 immobilized on polyurethane foam cubes in nonimmersed liquid culture under different C/N ratios. This is the first study to report on LIP and GLOX synthesis in the presence of air by the fungus. The nonimmersed liquid culture system used in this work is suggested to increase the availability of oxygen to the mycelia by preventing the formation of a dense mycelial structure and increasing their surface area, thus enabling LIP to be synthesized under nitrogen-rich conditions (5) and even in the presence of air. The results of the present study may explain differences observed with regard to the oxygen level required for the degradation of lignin under natural conditions (woody tissue) versus that in liquid cultures under laboratory conditions.

Whereas lignin degradation in solid-state cultures of *P. chrysosporium* and other white rot fungi is almost identical in air and in an oxygen-enriched atmosphere (11, 15), the synthesis of LIP and the degradation of lignin in liquid cultures have been reported to be highly dependent on the oxygen level (1, 3, 7, 19). The results of the present study are consistent with those of Leisola et al. (23), who, by reducing the depth of the mycelial mat, showed that the diffusion of oxygen into the culture, rather than its concentration in the atmosphere, is the major limiting factor in lignin degradation by *P. chrysosporium*. However, in addition to differences in O_2 level, a lack of CO_2 may also play a role when replacing air with pure O_2, insofar as air contains ca. 0.03% CO_2, whereas O_2 does not.

The lack of LIP activity in cultures grown in air under nitrogen limitation can also be explained in terms of oxygen availability, as a result of the copious formation of polysaccharides attached to the mycelia. Dosoretz et al. (3) showed that extracellular polysaccharides are the major secondary metabolite produced by this fungus when grown under nitrogen-limited conditions in air and suggested that the free and mycelium-attached polysaccharides offer resistance to oxygen and nutrient diffusion. The same authors also reported higher glu-

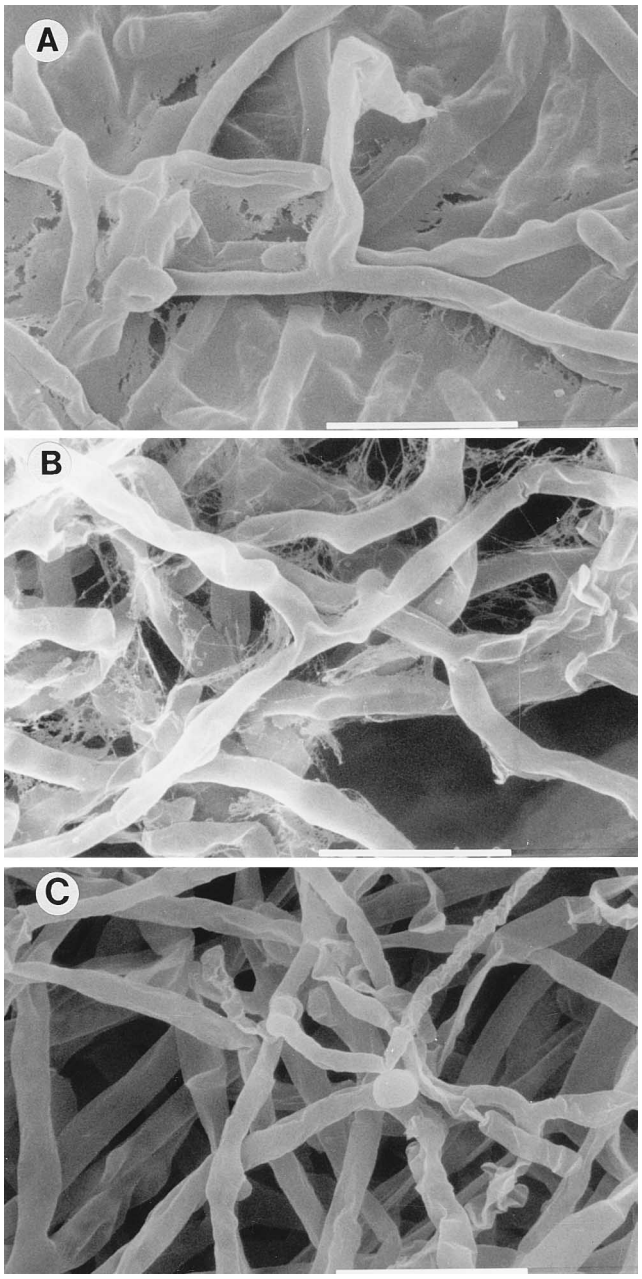

FIG. 4. Typical SEM micrographs of *P. chrysosporium* mycelia immobilized on polyurethane in nonimmersed liquid culture, sampled on day 4. (A) Nitrogen limitation in air; (B) nitrogen limitation in oxygen; (C) high nitrogen in air. Bars, 10 μm.

cose consumption and CO_2 evolution rates in cultures flushed with oxygen instead of air, suggesting that oxygen is a limiting factor. It is therefore likely that the lower rate of glucose consumption obtained under air, as opposed to oxygen, from the fourth day of growth onward reflects lower availability of oxygen to the mycelium, once significant formation of polysaccharides has occurred.

An inverse relationship between the level of polysaccharides and the degree of degradation of kraft lignin was reported in stationary cultures of *P. chrysosporium* (22). On the other hand, the absence of polysaccharides under low-carbon, high-nitrogen (low C/N) conditions, which we propose allows the synthesis of LIP to take place in both air and oxygen, is the result of carbon starvation, arising from complete depletion of the glucose and leading to termination of the growth phase. This finding is supported by the predominance of H1 and H2 LIP isoenzymes in the extracellular fluid, which is characteristic of carbon starvation (5, 12). Furthermore, the gradual reaccumulation of NH_4 in the extracellular fluid after complete depletion of glucose may be the result of proteolysis of cell proteins, which may serve as an alternative energy source for the fungus, as previously reported (5). The high level of H1, which appeared under both low-carbon and high-nitrogen conditions, showed that the process of posttranslational dephosphorylation of H2, leading to the formation of H1 (14, 20), is enhanced by carbon starvation. Under the same conditions, a substantial level of Ha isoenzyme appeared, suggesting that its formation may also be the result of dephosphorylation. A previous report (2) ascribes the formation of Ha to the composition of the medium.

GLOX formation, under both carbon and nitrogen limitation, closely paralleled that of LIP. A temporal correlation between the formation of these two enzymes has indeed been reported (17, 29). However, the absence of any GLOX formation under high-nitrogen conditions implies that its synthesis is subject to nitrogen catabolic repression (6), a well-defined regulatory mechanism which has been demonstrated in many fungi (28, 34). It is important to note that the repression of LIP formation in *P. chrysosporium* by nitrogen is also well documented (4, 7, 32), but the fact that this repression can be overcome by immobilizing the fungus on a porous support in a nonimmersed liquid culture may indicate that it results from a decrease in the availability of oxygen rather than from nitrogen catabolic repression. A high level of GLOX activity under nonlimiting nutrient conditions (22 mM NH_4) has been reported (26), but this was achieved only with a lysine auxotrophic mutant. Although it was reported (16) that a high oxygen level is critical for the production of GLOX (as for LIP) in shaken cultures under nitrogen limitation, the present study shows that the process of immobilization enables GLOX to be formed in air, even after the addition of 2 mM veratryl alcohol, which has been reported (26) to decrease extracellular GLOX activity.

Unlike the complete suppression of LIP and GLOX formation, the formation of MNP was much more affected by the C/N ratio than by the oxygen level. Similar results were obtained by Dosoretz and Grethlein (4), who reported a difference between the effects of oxygen on the regulation of LIP and MNP. The synthesis of MNP was partially repressed by the carbon starvation arising from a low C/N ratio, unlike that of LIP. This is consistent with a previous report (27) in which the response to the onset of secondary metabolism is regulated differently in the two enzymes. It should be noted that the level of polysaccharides at the onset of MNP activity (around day 3) may still be low. Evidence for this can be found in the rate of glucose consumption. It is also supported by the time course and level of polysaccharide formation in submerged cultures, reported by Dosoretz et al. (3).

In conclusion, the present study provides evidence that the growth of *P. chrysosporium* under nonimmersed liquid conditions and the suppression of polysaccharide formation by a low C/N ratio enable all the ligninolytic enzymes to form in air. The availability of oxygen in this culture system is suggested to more closely resemble the natural growth conditions under which the fungus utilizes woody material than the more commonly used submerged or stationary culture systems.

ACKNOWLEDGMENT

We are grateful to the Israel Ministry of Science and Arts, whose grant 4786 gave partial support to this work.

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