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Effect of oxygenation conditions on submerged cultures of *Phanerochaete chrysosporium*

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Summary. The effect of the oxygen supply pattern on the onset and development of the lignolytic enzyme system of *Phanerochaete chrysosporium* was studied in submerged culture employing the serum bottle approach. Periodic or continuous flushing through the head phase, and continuous bubbling through the liquid phase with either oxygen (O_2) or air were applied. The nature of the O_2 supply had a crucial regulatory effect not only on the formation of lignin-degrading peroxidases but also on their decay and on the production of extracellular protease activity and polysaccharides. Continuous oxygenation or aeration increased the glucose consumption rate, extracellular protease activity and polysaccharides. Gassing with air, whether continuous or periodic, sustained Mn-peroxidase activity while ligninase was undetectable. Continuous O_2 supply speeded up ligninase decay, displaying a sharper maximum, while a broader maximum and slower decay of ligninase activity were observed when supplying periodic O_2. Cultures initially grown with free exposure to air displayed a higher but sharper ligninase activity maximum when shifted to continuous rather than periodic O_2 supply. In general, the higher levels of either polysaccharides or protease activity corresponded to the lower levels and faster decay of ligninase and Mn-peroxidase activities.

Introduction

The major components of the lignolytic system of the white-rot fungus *Phanerochaete chrysosporium* consist of two extracellular heme peroxidases, lignin peroxidase (ligninase) (Tien and Kirk 1983; Glenn et al. 1983) and Mn-peroxidase (Kuwahara et al. 1984; Paszczynski et al. 1985). The complete system is idiophasic, triggered by nutrient limitation and particularly active in cultures grown under high oxygen (O_2) tension implying a role for molecular O_2 and its reduced species in the decomposition of lignin (Kirk and Fenn 1982; Faison and Kirk 1983; Kirk and Farrell 1987; Palmer et al. 1987). Degradation of soluble lignin-related compounds by *P. chrysosporium* was reported to be about threefold higher under 100% O_2 than under air (Kirk et al. 1978). High O_2 tension was also reported to stimulate degradation of aspen wood and thermomechanical pulp lignins by *P. chrysosporium* (Reid and Siefert 1980; Yang et al. 1980) as well as wood decay by a series of white rot and brown rot fungi (Reid and Siefert 1982; Highley et al. 1982).

Bar-Lev and Kirk (1981) showed that the concentration of O_2 during tropophase had a marked influence on the late activation of the lignolytic system during idiophase. More recently, Faison and Kirk (1985) reported that both lignolytic and ligninase activities of *P. chrysosporium* were increased in cultures initially supplied air during the growth phase and then shifted to an O_2 atmosphere. Most laboratory-scale studies of ligninase formation in submerged or stationary cultures of *P. chrysosporium* have used periodic pure O_2 (Leisola and Fiechter 1985; Jäger et al. 1985; Faison and Kirk 1985; Buswell and Odier 1987). Most scale-up approaches for the production of lignolytic enzymes in either low shear or immobilized reactor systems have employed continuous O_2-enriched air or pure O_2 (Kirk et al. 1986; Janshekar and Fiechter 1988; Linko 1988).

Remarkably, ligninase activity generally displays sharp transient peaks using different strains, carbon sources, limiting nutrients or cultivation conditions (Kirk et al. 1986; Faison and Kirk 1985; Roch et al. 1989). The reason for this sudden decrease cannot be explained solely by enzyme inactivation due to H_2O_2 (Tonon and Odier 1988). Lignolytic conditions, namely high O_2 tension under nutrient limitation, imply extreme environmental conditions which affect either physiological or metabolic functions (Kirk and Fenn 1982), including formation of other secondary metabolites that may have an adverse effect towards the production of the lignolytic enzymes. Before one can effectively design a process for continuous production of

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lignolytic enzymes, additional knowledge is needed to control the secretion of different metabolites generated by *P. chrysosporium* in liquid culture. In this study we report on the regulatory effect of the oxygenation conditions on both onset and decay of the peroxidative system and the production of extracellular proteases and polysaccharides using a common experimental approach.

Materials and methods

Strain and culture conditions. P. chrysosporium Burds strain BKM-F-1767 (ATCC 24725) was maintained on 2% malt agar slants (Kirk et al. 1978). All the cultures were grown at 37° C in N-limited media (Tien and Kirk 1988), but with 20 m*M* acetate buffer instead of dimethylsuccinate. All the experiments were done with agitation (225 rpm) in an orbital shaker (G50, New Brunswick Scientified, N. J., USA) in 120-ml serum bottles containing 50 ml culture. Serum bottles were stoppered with butyl rubber septa (Bellco, Bellco, Glass Inc., Vineland, NJ, USA, no. 2048-11800) and sealed with aluminum seals. Cultures grown with free air exchange were stoppered with foam plugs. For cultures shifted from air to oxygen the foam stopper was replaced by a butyl rubber septum after 2 days incubation. Periodic cultures were flushed for 1 min at 1.2 MPa with O_2 or air, as indicated, at the time of inoculation and every 24 h by means of two hypodermic needles (21 gauge by 3.8 cm) aseptically inserted at the time of flushing. Continuous gassing was done as follows; (a) flushing humidified gas through the head space (64 and 95 µl/min per millilitre of gas phase, of O_2 and air respectively) by means of two hypodermic needles (the same as above); (b) bubbling humidified gas through the liquid phase (88 and 133 µl/min per millilitre of liquid phase, of O_2 and air respectively) by means of a 14 gauge by 8.9 cm hypodermic needle and exhausted from the head space by means of a 21 gauge by 3.8 cm needle.

All the experiments were performed at least four times using three or four replicates for each condition each time. The results from the same conditions were averaged.

Enzymatic assays. Ligninase activity was measured according to Tien and Kirk (1988). One unit (U) represents 1 µmol veratryl alcohol oxidized to the aldehyde per minute. The Mn-peroxidase activity was measured as described by Kuwahara et al. (1984) using phenol red as substrate. Activity was expressed as the absorbance change at 610 nm (A_{610}) in 3 min for 20 µl sample. Protease activity was measured using azocoll (Sigma, St. Louis, Mo, USA) as substrate as described by Dosoretz et al. (1990). Activity in units per liter (U/l) was calculated assuming one unit as the amount of enzyme which 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 dinitrosalicylic acid method using D-glucose as standard (Ghose 1987). Protein was measured with the Bio-Rad (Richmond, Calif., USA) reagent according to the specifications of the manufacturer. CO_2 was measured by a TCD (thermal conductivity detector) gas chromatograph equipped with a 3 mm × 183 cm column packed with Porapak Q (80/100) and using helium as the carrier gas. Samples (0.5 ml) were taken directly from the head space of the serum bottles by means of a pressure lock syringe. Fungal biomass (in mg/ml) is the dry weight at 105° C of the whole content of a serum bottle filtered through a pre-tared GF/C membrane and washed twice with 30 ml distilled water.

Polysaccharide extraction and assay. The whole content of a serum bottle (50 ml) was homogenized for 5 min in a Virtis (Virtis Inc., Gardiner NJ, USA) homogenizer, mixed with ethanol (3/1, v/v) and kept at −20° C for 12 h, and thereafter centrifuged for 15 min at 6000*g*. The supernatant was discarded and the precipitate was redissolved in 50 ml distilled water and centrifuged under the same conditions. The solids were then washed with 15 ml distilled water, centrifuged and discarded. The supernants were combined and washed twice by successive precipitation and redissolving in ethanol and water and freeze dried. All the steps were done in the cold (4° C). Polysaccharides were determined by HPLC using an HPX-87P column (Bio-Rad) and a Refractive Index detector after 4% H_2SO_4 hydrolysis (60 min autoclaving at 1.22 MPa) of the freeze-dried samples, and represent the sum (in mg/ml) of the sugars detected.

Scanning electron microscopy (SEM) and pellet micrographs. Mycelial pellets for SEM 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. The SEM was according to Flegler and Baker (1983). For close-up micrographs 30 ml culture was poured into a 90-mm diameter petri dish and photographed.

Chemicals. All chemicals used were of reagent grade. Oxygen gas was of medical grade purity.

Results

Metabolism of glucose

With the exception of periodic aeration, no significant differences ($P<0.05$) in the glucose consumption rate were found during the growth phase (first 2 days) regardless of the oxygenation method used (Table 1). During secondary metabolism there was a marked increase in glucose consumption for each method of oxygenation and the increase was largest for the non-periodic ones (see second column in Table 1). Continuous flushing with O_2 increased glucose consumption to a

Table 1. Effect of oxygenation method on the glucose consumption rate[a]

Oxygenation method	Glucose consumption rate (mg · ml^{-1} · day)	
	Growth phase[b]	Idiophase[c]
	Oxygen	
Periodic flushing	1.02 ± 0.08 (b)[e]	1.31 ± 0.03 (ef)
Continuous flushing	1.23 ± 0.03 (a)	1.85 ± 0.10 (a)
Continuous bubbling	1.20 ± 0.05 (a)	1.53 ± 0.03 (b)
To periodic flushing[d]	1.17 ± 0.03 (a)	1.42 ± 0.03 (c)
To continuous flushing[d]	1.17 ± 0.03 (a)	1.83 ± 0.09 (a)
	Air	
Periodic flushing	0.79 ± 0.10 (c)	0.89 ± 0.08 (g)
Continuous flushing	1.19 ± 0.06 (a)	1.37 ± 0.06 (d)
Continuous bubbling	1.17 ± 0.04 (a)	1.31 ± 0.07 (e)
Free exposure	1.16 ± 0.03 (a)	1.29 ± 0.09 (d)

[a] Values express the mean ± standard deviation of four different experiments with four replicates for each condition

[b] Calculated from the slope of the remaining glucose during the first 2 days

[c] Calculated from the slope of the remaining glucose beyond day 2

[d] Cultures were grown with free exposure to air during the first 2 days and then were shifted to pure O_2 as indicated

[e] Different letters indicate statistically different mean ($P<0.05$) calculated separately within each metabolic phase by Student-Newman-Keuls multiple range test

higher level than continuous bubbling, whereas continuous aeration by flushing, bubbling, or free exposure developed nearly the same glucose consumption rate. With periodic aeration the glucose consumption rate was almost 2/3 of the other aeration methods, indicating that O_2 was the limiting factor for metabolic activity. The glucose consumption rate of the cultures that were shifted on the second day from continuous exposure to air to periodic or continuous flushing with O_2 increased to almost same values as the equivalent samples supplied with O_2 from the beginning.

The measurement of the fungal biomass after 8 days incubation (Table 2) showed similar values for all the conditions of O_2 including cultures shifted from air to O_2. However, significantly ($P<0.05$) higher biomass accumulation and greater variability between replicates were found for all the conditions using air due to the large amount of polysaccharides attached to the fungal pellets, even after washing.

The metabolic activity of the cultures measured by the CO_2 evolution rate (Fig. 1) followed fairly well the glucose consumption profile in each case and was highest during idiophase. The CO_2 yields during idiophase, corresponding to the plateau of curves in Fig. 1a, were 4.9 and 2.6 mol CO_2 per mol glucose consumed for periodic oxygenation and aeration respectively.

Extracellular enzymes and protein

The increase in O_2 supply from periodic to continuous showed a regulatory effect on the pattern of extracellular enzyme activities (Fig. 2). Continuous O_2 flushing increased both the formation and decay of ligninase activity (Fig. 2a). Cultures initially grown under air and shifted to O_2 displayed higher activity than those originally grown under O_2 and, moreover, the highest activity but sharpest decay was found on shifting to a continuous rather than periodic regime. Both growth and idiophasic protease activities increased with increasing O_2 supply from periodic to continuous (Fig. 2b). The Mn-peroxidase activity displayed a maximum 24 h earlier for cultures shifted from air to periodic or continuous O_2 and those continuously flushed with O_2 from the beginning (Fig. 2c). In general, the time course of extracellular protein production after day 2 followed that of ligninase activity (Fig. 2d). Note that the high initial protein is from the homogenized inoculum.

Table 2. Effect of oxygenation conditions on the fungal biomass[a]

Oxygenation method	Dry biomass[b] (mg/ml)	
	Oxygen	Air[c]
Periodic flushing	1.17 ± 0.04 (b)[e]	2.66 ± 0.21 (c)
Continuous flushing	1.02 ± 0.02 (d)	3.46 ± 0.32 (a)
Continuous bubbling	1.53 ± 0.14 (a)	2.90 ± 0.18 (b)
To periodic flushing[d]	1.01 ± 0.05 (d)	—
To continuous flushing[d]	1.09 ± 0.02 (c)	—

[a] Values express the mean ± standard deviation of seven replicates for each condition

[b] Measured after 8 days growth

[c] Dry biomass may contain mycelium-bound unwashable polysaccharides

[d] Cultures were grown with free exposure to air during the first 2 days and then were shifted to pure O_2 as indicated

[e] Different letters indicate statistically different means ($P<0.05$) calculated separately within each oxygenation group by Student-Newman-Keuls multiple range test

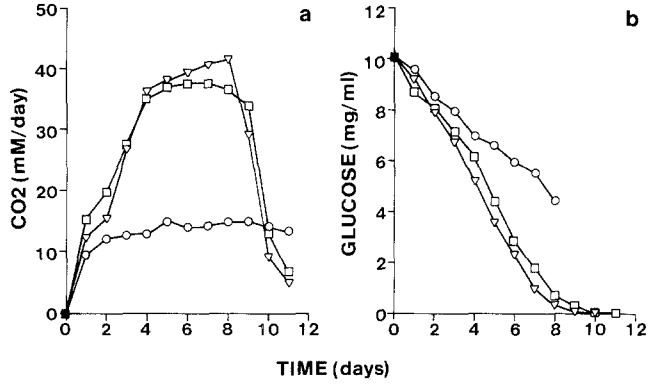

Fig. 1a, b. Effect of the oxygenation conditions on glucose metabolism. **a** The CO_2 production rate. **b** Glucose consumption: □, periodic oxygen; ○, periodic air; ▽, free exposure to air shifted to periodic O_2

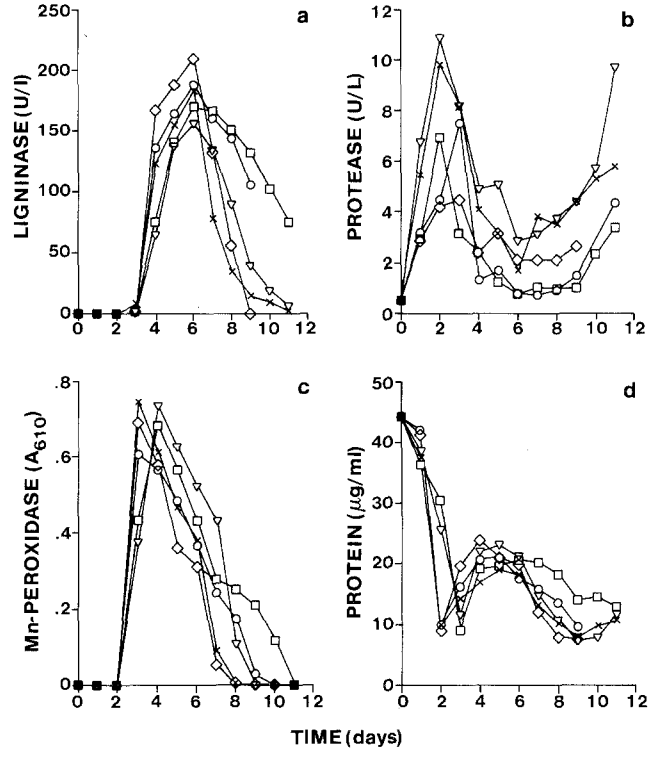

Fig. 2a–d. Effect of oxygenation conditions on extracellular enzyme activity. **a** Ligninase. **b** Protease. **c** Mn-Peroxidase. **d.** Extracellular protein: □, periodic flushing; ×, continuous flushing; ▽, continuous bubling; ○, air shifted to periodic flushing; ◇, air shifted to continuous flushing. U, units; A_{610}, absorbance at 610 nm

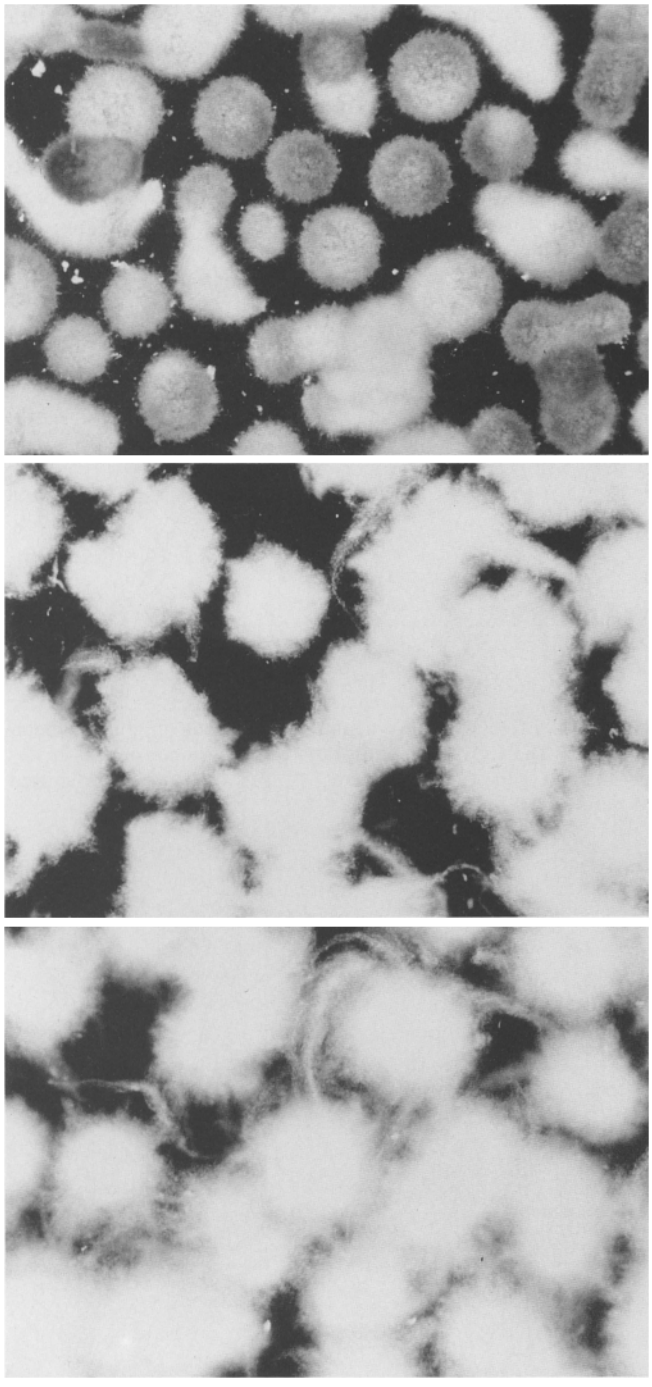

Fig. 3. Close-up micrograph (×5) of pellets formed by *Phanerochaete chrysosporium* grown in shaken cultures: *top,* periodic flushing of O_2; *middle,* continuous flushing of air; *bottom,* free exposure to air. Note the dark appearance of lignolytic pellets in contrast to the white-hairy aspect of non-lignolytic ones. Pellets were collected in day 6

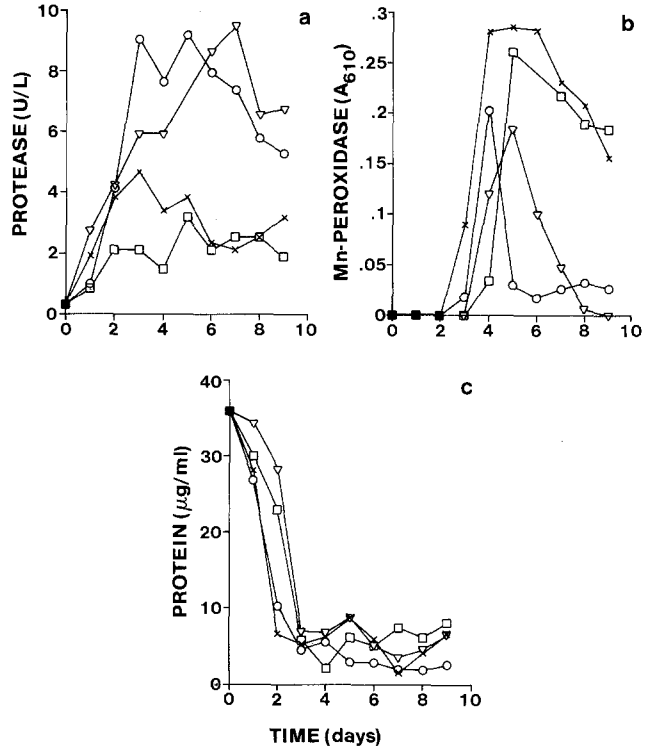

Fig. 4a–c. Effect of aeration conditions on extracellular enzyme activity. **a** Protease. **b** Mn-Peroxidase. **c** Extracellular protein: □, periodic flushing; ×, continuous flushing; ▽, continuous bubbling; ○, free exposure. Note that ligninase activity was always undetectable

Cultures grown under air displayed no detectable ligninase activity regardless of the aeration method employed and, as shown in Fig. 3, those pellets never changed to the brown-black color characteristic of lignolytic pellets.

The protease activity of cultures grown under continuous bubbling or free exposure to air remained at high levels whereas cultures grown under periodic or continuous flushing of air displayed lower protease activity (Fig. 4a). The Mn-peroxidase activity (Fig. 4b) was found in all conditions of aeration, even though these values were about 40% lower than those found with the respective conditions of O_2 (Fig. 2c). Continuous flushing with air stimulated Mn-peroxidase activity. In all the cases where air was supplied the level of extracellular protein (Fig. 4c) was 50% or less of that obtained with O_2.

Extracellular polysaccharide formation

A marked increase in free (Fig. 5) and mycelial-attached (Fig. 6) extracellular polysaccharide formation, accompanied with increasing medium viscosity, were found using air rather than O_2. The exterior appearance of lignolytic pellets are small, smooth and brown while the non-lignolytic pellets appeared white and hairy with an accumulation of polysaccharides (see Fig. 3). The exposure of the cultures to free air exchange led to the highest levels of extracellular polysaccharides. Periodic oxygenation, which gave a broader maximum for ligninase activity (see Fig. 2) and the lowest glucose consumption rate (see Table 1), resulted in the lowest polysaccharide accumulation (see Fig. 5). Upon acid hydrolysis these polysaccharides yielded glucose, xylose, galactose and mannose in the molar ratios of

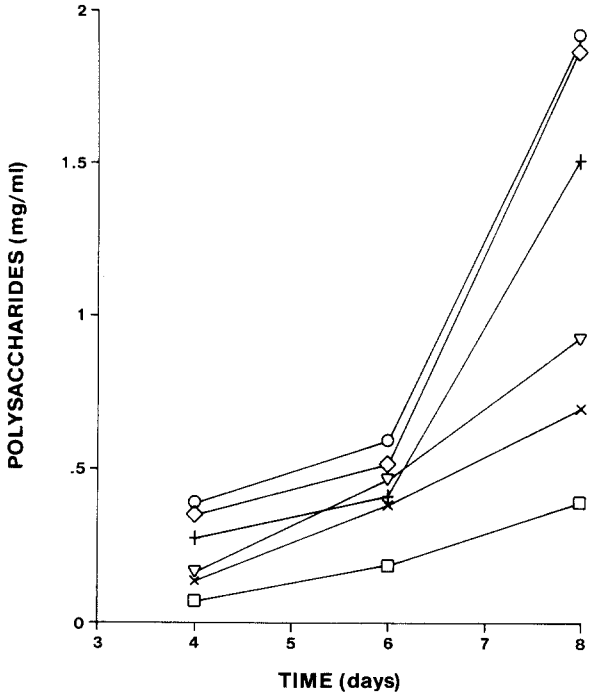

Fig. 5. Extracellular polysaccharide formation. In oxygen: □, periodic flushing; ×, continuous flushing; ▽, continuous bubbling. In air: ○, free exposure; ◇, continuous flushing; +, continuous bubbling

1.00:0.01:0.03:0.02 for O_2 and 1.00:0.02:0.03:0.02 for air. The chemical composition found here corresponded to that of a β-D-glucan identified in stationary and submerged cultures of *P. chrysosporium* (Bes et al. 1987; Buchala and Leisola 1987).

Discussion

The data presented here indicated that oxygenation conditions have a direct regulatory effect on both production and decay of lignolytic enzymes, and on the formation of extracellular protease activity and polysaccharides. The higher O_2 tension (pure O_2) corresponded to higher rates of glucose oxidation (measured by both glucose consumption and CO_2 evolution) and lower levels of polysaccharides, and vice versa, indicating increasing maintenance energy requirements associated with the more severe physiological conditions. The increase in O_2 tension increased the level and rate of formation of ligninase and Mn-peroxidase activities, but also enhanced their decay associated with faster substrate depletion and the temporal increase of idiophasic protease activity. This inverse temporal relationship suggests the involvement of starvation-generated proteases in decay of the lignolytic activity. In fact, we have recently shown the simultaneous stabilization of ligninase activity and repression of protease activity by addition of glucose or a specific inhibitor of protease activity (Dosoretz et al. 1990).

Extracellular polysaccharides were the major secondary metabolites produced by *P. chrysosporium* grown on glucose in N-limited medium under atmospheric O_2, whether periodic or continuous. Bes et al. (1987) suggested that the formation of polysaccharides by *P. chrysosporium* may play a favorable role in lignin degradation by the release of the catabolic substrate repression of the glucose-oxidizing system under low nitrogen concentrations. Leisola et al. (1982) reported an inverse relationship between the level of polysaccharides and the extent of Kraft lignin degradation in standing cultures of *P. chrysosporium*. Kikpatrick and Palmer (1989) reported a polysaccharide-containing fraction isolated from BKM-F cultures which inhibited in vitro purified isoenymes of ligninase.

Our results show that the levels of both extracellular free and mycelial-attached polysaccharides strongly depend on the aeration rate, and their formation coincided with the onset and development of the lignolytic system. Furthermore, our data made a clear distinction between both kinds of polysaccharides. Indeed, the polysaccharides attached to the mycelium form a resistance to internal diffusion of O_2 and nutrients in the pellets and contribute to apparent cell mass. On the other hand, the free polysaccharides contribute to liquid viscosity and offer resistance to O_2 and nutrient diffusion to the pellets, in line with previous reports (Leisola et al. 1983; Buchala and Leisola 1987).

The enrichment of the O_2 supply, from periodic air to continuous O_2, resulted in a different effect on the expression of ligninase and Mn-peroxidase. Ligninase reached undetectable levels under all the conditions of aeration while Mn-peroxidase reached sustantial levels over the entire range of O_2 supply. These observations are in line with recent work (Pease et al. 1989) reporting that, in spite of the constitutive and triggering similarities between both peroxidases, the response to the onset of the secondary metabolism is regulated differently for the two. Furthermore, the data presented here suggested a substantial role for Mn-peroxidase in lignin degradation in liquid culture under air. In fact, earlier work reported a 2–3 fold higher lignin degradation under O_2 than under air (Kirk et al. 1978; Reid and Siefert 1980, 1982; Bar-Lev and Kirk 1981). This assumption is further supported by recent work (Boominathan et al. 1990) that reports a 16% mineralization of a synthetic lignin by a ligninase-negative/Mn-peroxidase positive mutant of ME-446. One should note that the onset of Mn-peroxidase, with a peak on days 3–4, corresponded to low levels of polysaccharides and enough carbon source to support metabolic activity.

In conclusion, the present study shows that the method of oxygenation used in liquid cultures of *P. chrysosporium* grown on N-limited media has a crucial influence not only direct on the production of ligninase and Mn-peroxidase but also by regulating the levels of polysaccharides produced and the extent of the extracellular protease activity. Although both polysaccharides and proteases are triggered by different factors and the way in which both adversely affect production of lignolytic enzymes is distinct, their presence is still a major impediment to the continuous production of the lignolytic enzymes. In contrast to ligninase, Mn-peroxi-

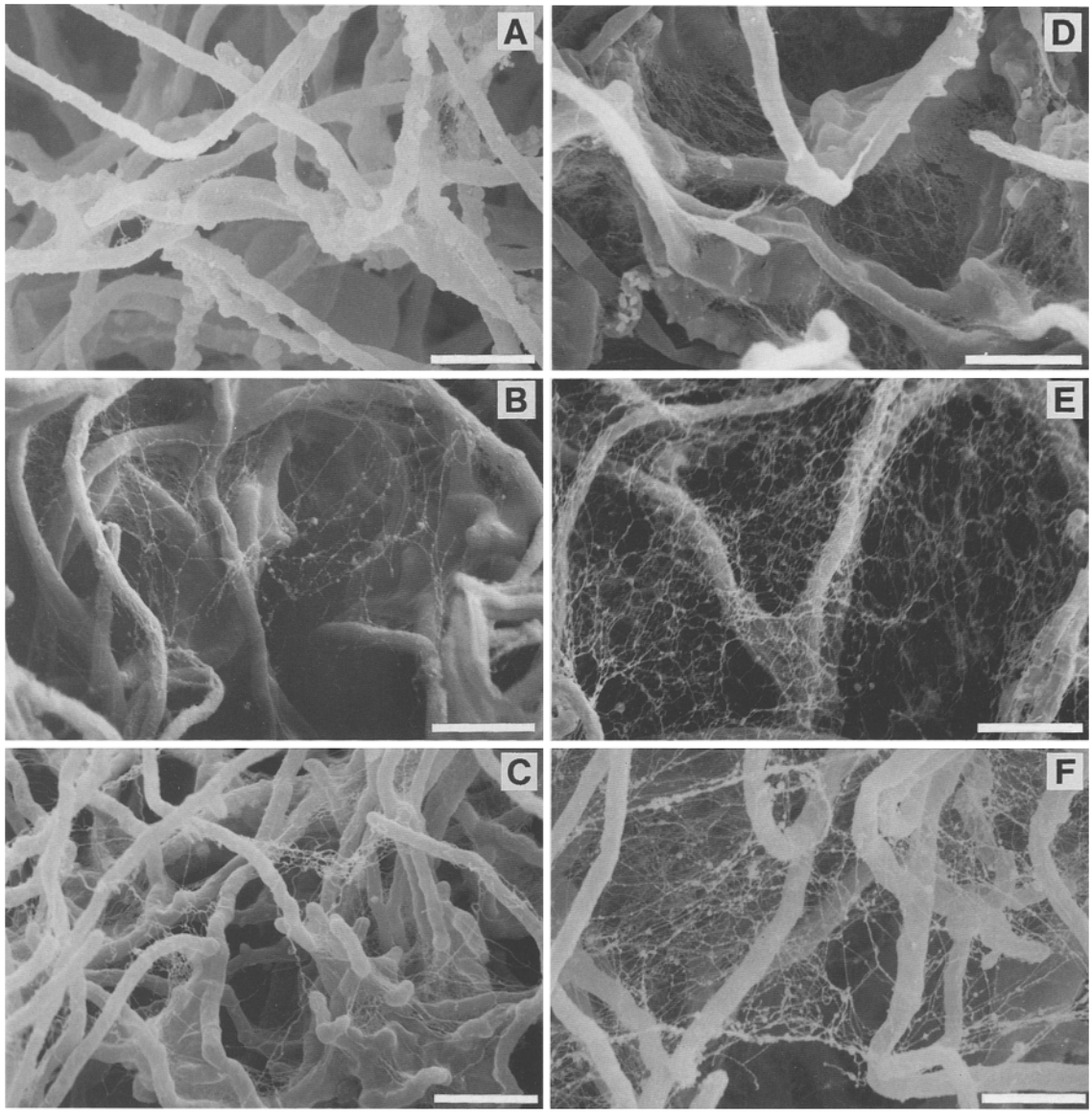

Fig. 6a–f. Scanning electron micrograph of pellets formed by *P. chrysosporium* under different O_2 conditions. Pure O_2: **a** Periodic flushing. **b** Continuous flushing. **c** Continuous bubbling. Air: **d** Free exposure. **e** Continuous flushing. **f** Continuous bubbling. Pellets were collected on day 4, close to the onset of ligninase activity. *Bar* represents 10 μm

dase was shown to be sustained under a very broad range of O_2 tension.

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