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Physiological Aspects of Biosynthesis of Lignin Peroxidases by *Phanerochaete chrysosporium*

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Methods based on UV-visible diffuse reflectance spectroscopy were used to study the physiological aspects of lignin-peroxidase biosynthesis by *Phanerochaete chrysosporium*. Here we introduce the use of cytochrome aa_3 as an indicator of active fungal biomass and of its redox state to calculate the oxygen mass transport coefficient between the growth medium and the fungal cell interior. When lignin peroxidase biosynthesis was enhanced by the addition of Tween 80 or Tween 20 to the growth medium, a higher proportion of reduced cytochrome aa_3 and a higher oxygen diffusion barrier were observed compared with control cultures. In cultures supplemented with Tween 80 or Tween 20, a higher oxygen mass transport coefficient between the growth medium and the interior of the fungal cell was also found. The beginning of the lignin peroxidase activity in these cultures was found to coincide with a temporary cessation in the dry biomass increase and a reduction in the relative active-biomass concentration. During the lignin peroxidase activity, a decrease in the intracellular pH and an increase in the growth medium pH were determined in cultures supplemented with Tween 80.

The white rot fungus *Phanerochaete chrysosporium* produces a variety of extracellular lignin peroxidases, which are potentially important in future technologies in the paper industry (11) and for degradation of various hazardous aromatic compounds in wastewaters and materials (7).

In screening for potential stimulators of the *P. chrysosporium* lignin peroxidase system, some surfactants and related compounds such as Tween 80, Tween 20, the alcoholic moiety of Tween 80 hydrolysate, and polyoxyethylene oleate were found to markedly enhance lignin peroxidase production (1, 13, 21). Reese and Maguire (29) studied the effect of surfactants on enzyme production by various fungi and postulated that the surfactants act at the cell membrane to release the enzymes. Stimulators of lignin peroxidase activity influenced the fatty acid composition of *P. chrysosporium* phospholipids, particularly an increase of palmitoleic acid and the subsequent increase of the unsaturation index of polar lipids (21). However, the stimulative effect of those compounds cannot be attributed to the release of lignin peroxidases from the mycelium to the external medium. High lignin peroxidase activities in the growth medium were observed only in cultures in which there was high intracellular lignin peroxidase activity (20). Capdevila et al. (9) and Asther et al. (2) reported that lignin peroxidase synthesis was stimulated by some exogenous phospholipids. They observed that these phospholipids also enhanced energy metabolism of fungi and markedly modified fungal physiology.

The recognition of the actual physiological state of the microorganisms can offer the key to understanding and regulating metabolite production. Furthermore, the intracellular metabolic activities and changes of physiological state are far more intensive than the changes in the cellular environment, yet the latter are usually determined as parameters of bioprocess control. For filamentous fungi the methods of applied physiological research are poorly developed.

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Several methods based on diffuse reflectance spectroscopy were recently adapted and developed in our laboratory for the determination of important physiological parameters in pellets of filamentous fungi. These methods were used to determine the intracellular pH, cytochrome content, and intracellular lignin peroxidase activity of *P. chrysosporium*. A measurement system was also designed for quantitative, continuous, and in vivo determination of the redox state of respiratory cytochromes in fungal pellets (22).

In the present paper, these methods are introduced for the study of the physiological aspects of lignin peroxidase biosynthesis in *P. chrysosporium* based on the comparison of the physiological state of cultures in which lignin peroxidase activity was enhanced by addition of the surfactant Tween 80 or Tween 20 and control cultures with no added surfactant, in which very little such activity is usually measured (21).

MATERIALS AND METHODS

Organism. The organism used in this study was *P. chrysosporium* MZKIBK B-223 (ATCC 24725). The freeze-dried culture was obtained from the American Type Culture Collection and transferred to the Culture Collection of the Institute of Chemistry, Ljubljana, Slovenia (MZKIBK), where it was maintained as an active agar culture. Subcultures were kept on malt agar slants at room temperature until use.

Medium and culture conditions. The fungus was grown in a nitrogen-limited medium (23) buffered to pH 4.5 with 20 mM sodium tartrate in agitated 500-ml Erlenmeyer flasks containing 100 ml of growth medium. Flasks were inoculated with 2×10^6 spores from 3- to 4-week-old agar slants (17), and the cultures were incubated at 37°C. A rotary shaker operating at 125 rpm was used.

The surfactant used as a stimulator of lignin peroxidase activity of the fungus was 0.1% (vol/vol) sorbitan polyoxyethylene monooleate (Tween 80) or 0.1% (vol/vol) sorbitan polyoxyethylene monolaureate (Tween 20); the surfactant was added to the growth medium at the start of culture. The experiments were repeated at least twice.

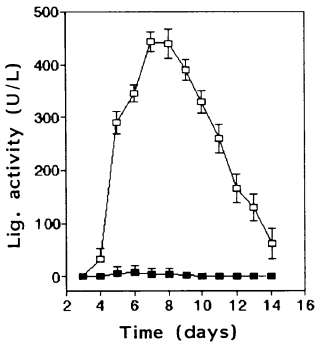

FIG. 1. Lignin peroxidase activity of *P. chrysosporium* control cultures with no added surfactants (■) and cultures supplemented with Tween 80 (□).

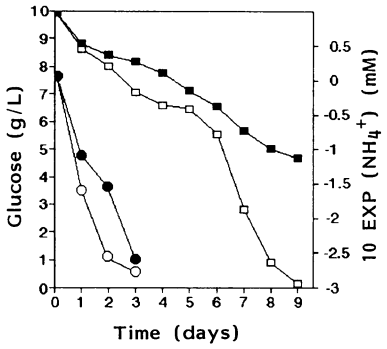

FIG. 2. Glucose (□, ■) and ammonium ion (○, ●) concentrations in the growth medium of *P. chrysosporium* culture without surfactant (solid symbols) and with Tween 80 (open symbols).

Mycelium dry weight. Mycelia were rinsed with distilled water and weighed after being dried at 60°C for 24 h.

Lignin peroxidase assay. Lignin peroxidase activity was measured as described by Tien and Kirk (33). One unit of lignin peroxidase activity was defined as 1 μmol of veratryl alcohol oxidized in 1 min, and the activities are reported as units per liter.

Glucose and ammonium ion determination. Glucose in the growth medium was measured spectrophotometrically by the method of Lever (24). The ammonium ion concentration was measured with an ion-selective electrode (Orion) after alkalinization of the growth medium.

Apparatus. Diffuse reflectance spectra were measured with a Varian DMS 80 spectrophotometer fitted with an integrating sphere accessory (20), and their intensity was evaluated as reflectance.

Measurements of intracellular pH. The intracellular pH of *P. chrysosporium* pellets was determined by the spectrophotometric technique, as described previously (32) and adapted to diffuse reflectance spectrophotometry (22). The dye 6-carboxyfluorescein was used as the intracellular pH indicator.

Mitochondrial cytochrome determination. *P. chrysosporium* mitochondrial cytochrome spectra were obtained as the difference between diffuse reflectance spectra of dithionite-reduced and H_2O_2-oxidized pellets (22).

The dry weight of scanned pellets in the measurement cuvette varied as the density of the pellets decreased during the time of culture. The spectral reflectance intensity (Ref) of pellets from 2- to 8-day-old cultures linearly correlated with the scanned pellet dry weight:

$$\mathrm{Ref}/\mathrm{Ref}_{0.02} = 0.616 + (0.443 \cdot \text{sample dry weight})/0.02, r^2 = 0.88 \quad (1)$$

Equation 1 was used to calculate the reflectance of sample spectra as if the sample dry weight were 0.02 g. The spectra of cytochromes with such a modified reflectance ($\mathrm{Ref}_{0.02}$) was then used in the system of equations proposed by Williams (35) to calculate the relative contents of particular cytochromes.

In vivo determination of the redox state of mitochondrial cytochromes. On-line measurements of the redox state of mitochondrial cytochromes of *P. chrysosporium*, as a function of the saturation of the growth medium with oxygen, were performed with the measurement system presented elsewhere (22). The growth medium oxygen saturation was detected with a polarographic type of O_2 electrode. Diffuse reflectance spectra of mitochondrial cytochromes in fungal pellets were taken at the different growth medium oxygenations. First the growth medium was saturated with O_2, and then the oxygen saturation was allowed to decrease to 17 to 18% as the oxygen was consumed by the fungus. Below 17 to 18% saturation, N_2 was bubbled through the growth medium to further slowly decrease oxygenation to 0%. The reflectance intensity at 598 nm for cytochrome aa_3 at 100 and 0% oxygenation was then evaluated as 0 and 100% of reduction level, respectively. Reduction levels of cytochrome aa_3 at other oxygenations were calculated accordingly.

RESULTS

The surfactants Tween 80 and Tween 20 are known stimulators of lignin peroxidase biosynthesis by *P. chrysosporium* (1, 13, 21), which usually started at day 4 or 5 of culture. Figure 1 shows that in the control cultures only minor lignin peroxidase activity was produced compared with that in cultures supplemented with Tween 80. Figure 2 presents the glucose and ammonium ion concentration in growth medium of control cultures and cultures containing Tween 80 during the time of culture. The possible effect of Tween 80 and Tween 20 on mitochondrial cytochrome content, relative active biomass, intracellular pH, and oxygen mass transport coefficient between the growth medium and the cell interior of *P. chrysosporium* was therefore investigated.

Figure 3 presents the diffuse reflectance spectra of mito-

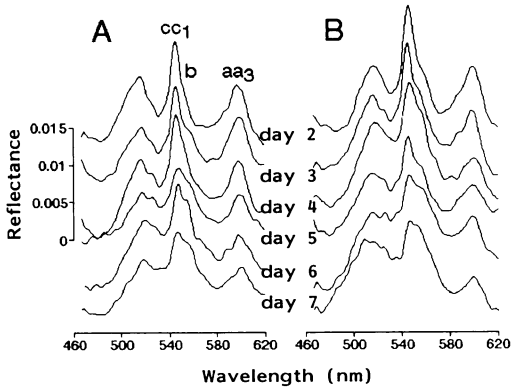

FIG. 3. Diffuse reflectance spectra of reduced minus oxidized mitochondrial cytochromes in whole pellets of *P. chrysosporium*. (A) Control cultures; (B) cultures supplemented with Tween 20.

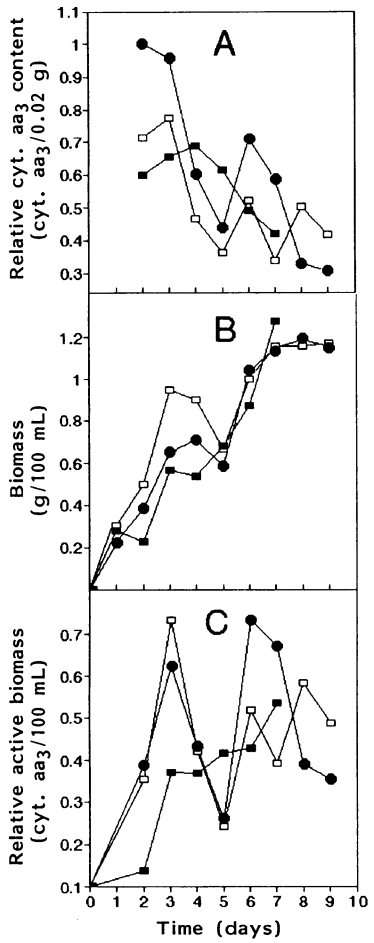

FIG. 4. Relative content of cytochrome aa_3 in 0.02 g (dry weight) (A), biomass dry weight concentration (B), and relative active biomass concentration, expressed as the relative content of cytochrome aa_3 in pellets in 100 ml of growth medium (C). Control cultures (■) and cultures supplemented with Tween 80 (□) and Tween 20 (●) during culture.

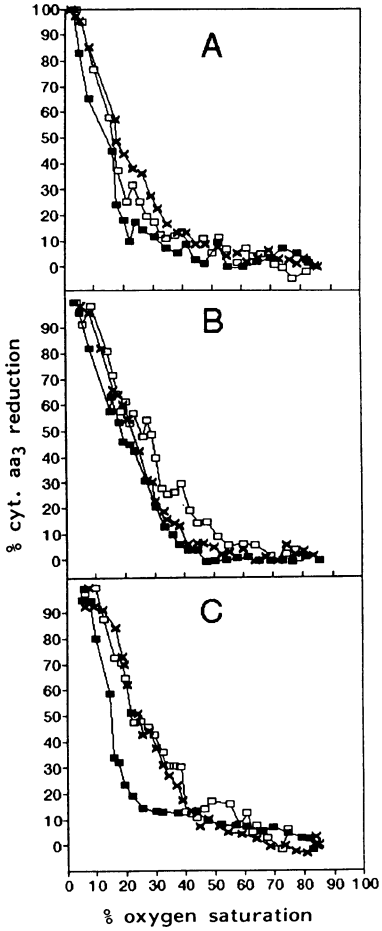

FIG. 5. Dependence of reduction of cytochrome aa_3 on the oxygen saturation of the growth medium of 1-day-old (A), 2-day-old (B), and 3-day-old (C) *P. chrysosporium* control cultures (■) and cultures supplemented with Tween 80 (□) and Tween 20 (×).

chondrial cytochromes of control cultures and cultures containing Tween 20 during cultivation. A peak in the α region was observed at 546 nm, corresponding to cytochrome cc_1; a shoulder at 555 nm was attributed to reduced cytochrome *b*, and a peak at 598 nm corresponded to cytochrome aa_3.

The reflectance of sample spectra was calculated as if the sample dry weight were 0.02 g, as described in Materials and Methods. The system of equations proposed by Williams (35) and the differences in reflectance at 545 and 530 nm, 546 and 534 nm, 555 and 576 nm, and 598 and 621 nm for cytochromes *c*, c_1, *b*, and aa_3, respectively, were then used to calculate the relative contents of particular cytochromes from the mitochondrial cytochrome spectra. The value of 1 was assigned to the relative cytochrome aa_3 content in the sample with the highest calculated content. The relative contents in other samples were normalized accordingly (Fig. 4A). The relative content of cytochrome aa_3 in 0.02 g (dry weight) (Fig. 4A) was used as an indicator of active fungal biomass. We have defined the relative active biomass (Fig. 4C) as the relative content of cytochrome aa_3 in pellets in 100 ml of the growth medium. It was calculated by multiplying the relative content of cytochrome aa_3 in pellets (Fig. 4A) with the biomass dry weight (Fig. 4B).

Leštan et al. (22) described the measurement system based on diffuse reflectance spectroscopy for in vivo monitoring of the redox state of mitochondrial cytochromes in pellets of *P. chrysosporium* suspended in growth medium. The reflectance of cytochrome spectra increases as the growth medium oxygenation decreases (22). Since mitochondrial cytochromes absorb light only in the reduced state, the increased reflectance indicates the higher reduction level.

Figure 5 shows the dependence of the redox state of cytochrome aa_3, measured at 598 nm, in pellets of the control culture and cultures supplemented with Tween 80 or Tween 20 on growth medium oxygenation. Table 1 summarizes the main characteristics of that dependence: the percent oxygen saturation at a 50% reduction of cytochrome aa_3 and the diffusion barrier of oxygen. The latter indicates the percent oxygen saturation of the growth medium when reduction of cytochrome aa_3 appears. The growth medium oxygenation in agitated Erlenmeyer flask cultures and the expected cytochrome aa_3 reduction level at that oxygen saturation, defined from curves in Fig. 5, are also shown in Table 1.

The redox state of cytochrome aa_3 can be used as an intracellular oxygen probe (12, 15, 18, 19). The data for

TABLE 1. Dependence of the redox state of cytochrome aa_3 on oxygen saturation of the growth medium for cultures of *P. chrysosporium*, supplemented with Tween 80 and Tween 20 and control cultures

Culture and Time (days)	% Oxygen saturation of growth media at 50% cytochrome aa_3 reduction	Diffusion barrier of oxygen (% oxygen saturation)	% Oxygen saturation of growth media in agitated flasks	% Cytochrome aa_3 reduction at actual oxygenation of growth media in agitated flasks
Control				
1	12	47	16	37
2	15	66	16.5	45
3	17	70	17.5	48
Tween 80				
1	18	50	17	51
2	25	74	18	68
3	22	71	18	63
Tween 20				
1	15	>80	18	31
2	24	>80	18	64
3	24	>80	18	68

cytochrome aa_3 reduction level in fungal pellets and for oxygen saturation of the growth medium can therefore be used for the calculation of the oxygen mass transport coefficient (K) between the growth medium and the cell interior.

The oxygen mass transfer rate (dQ/dt) from the growth medium into the cell, as a result of oxygen consumption in the microorganism, is proportional to the difference of growth medium oxygen saturation ($\%O_{2ext}$) and intracellular oxygen saturation ($\%O_{2int}$); this represents the driving force of oxygen transport:

$$-dQ/dt = K \cdot (\%O_{2ext} - \%O_{2int}) \quad (2)$$

K represents the transport coefficients in the internal and external sites of the cell membrane and its main component, the oxygen diffusion coefficient of the cell membrane. The value of K therefore indicates the permeability of the cell membrane for oxygen. For the correlation between the cytochrome aa_3 reduction ($\%aa_3$) and the intracellular oxygen saturation, the data of Kirman et al. (18) were used and correlated with a linear regression ($r^2 = 0.92$):

$$\%O_{2int} = 5.477 - 0.043\%aa_3 \quad (3)$$

Since the dry weight of pellets (m) and volume (V) of growth medium used in the measurement system are known (Table 2), the specific oxygen consumption rate ($\Delta Q/\Delta t$) can be calculated from equation 4:

$$\Delta Q/\Delta t = V \cdot (\%O_{2ext,t1} - \%O_{2ext,t2}) / [(t_2 - t_1) \cdot m] \quad (4)$$

Equations 3 and 4 can then be used to rearrange equation 2:

$$V \cdot (\%O_{2ext,t1} - \%O_{2ext,t2}) / m = K \left[\int_{\%O_{2ext,t1}}^{\%O_{2ext,t2}} \%O_{2ext}dt - 5.47(t_2 - t_1) + 0.043 \int_{\%aa_{3,t1}}^{\%aa_{3,t2}} \%aa_3 dt \right] \quad (5)$$

The data on the time dependence of the growth medium oxygenation and cytochrome aa_3 reduction level were numerically approximated by orthogonal polynomials (Fig. 6). These data were then used to calculate the coefficient K with equation 5, from the time of 50% oxygenation (t_1) to 20% oxygen saturation (t_2). The results are summarized in Table 2, where $\Delta Q/\Delta t$ and the relative contents of cytochrome aa_3 (also graphically presented in Fig. 4A), as well as their quotients of the relative respiratory activity, are presented for the control cultures and the cultures with Tween 80 or Tween 20.

The redox state of cytochrome aa_3 was measured for the first 3 days of *P. chrysosporium* culture. After that time the fungal pellets became too filamentous and caused clogging in the flowthrough cuvette in the measurement system.

Figure 7 shows the intracellular pH and growth medium pH in control *P. chrysosporium* cultures and cultures supplemented with Tween 80 during the time of culture.

DISCUSSION

In this study, the relative content of cytochrome aa_3 in fungal pellets and its redox state were introduced as an indicator of active fungal biomass and for the calculation of the oxygen mass transport coefficient (K) between the growth medium and the fungal cell interior. These physiological parameters, together with intracellular pH, were used in an attempt to better understand lignin peroxidase biosynthesis by *P. chrysosporium*. We used UV-visible diffuse reflectance spectroscopy to measure these parameters. To estimate which region of the pellets was actually measured by this method, the data of Michel et al. (26) on oxygen concentration in the media within the structure of pellet, determined with an oxygen microelectrode, and the data for cytochrome aa_3 reduction (Fig. 5) can be applied. Michel et al. (26) also proposed a mathematical model describing the oxygen concentration in *P. chrysosporium* pellets with respect to the oxygen concentration in the surrounding medium and to the size of the pellet. Figure 5 shows that above 50% of growth medium oxygenation, the cytochrome aa_3 reduction level is nearly zero, indicating that the cells in the region of the pellets which is actually measured are then excessively supplied with oxygen. For 50% growth medium oxygenation and for 2-mm-diameter pellets, the model of Michel et al. (26) predicts that oxygen is available only to cells in the outer 0.4-mm layer of pellet. This is therefore the broadest possible region of pellets which is actually measured by UV-visible diffuse reflectance spectroscopy.

Biomass dry weight measures the total weight of living cells, dead cells, and cell debris. For the determination of kinetic data, yields, and productivity of the microorganism, the metabolically active biomass is therefore a better parameter. To estimate active microbial biomass, methods based on fluorescence measurements of the intracellular reduced NAD levels (36) and on cellular ATP levels (30) have been developed. These methods can be used principally to monitor pure cultures of yeasts and bacteria but are unsuitable for aggregates or filamentous cells. It is also possible to relate the active biomass to the level of some cellular constituents, such as total protein, total RNA, and total DNA (30). These measurements are well established but time-consuming.

The identities of the mitochondrial cytochromes in pellets of filamentous fungi can be easily determined by diffuse reflectance spectroscopy (22). Mitochondrial cytochromes are coupled to oxidative phosphorylation, most important in the energy production of the living, aerobic cell. Therefore, their content seemed to be an appropriate indicator of active

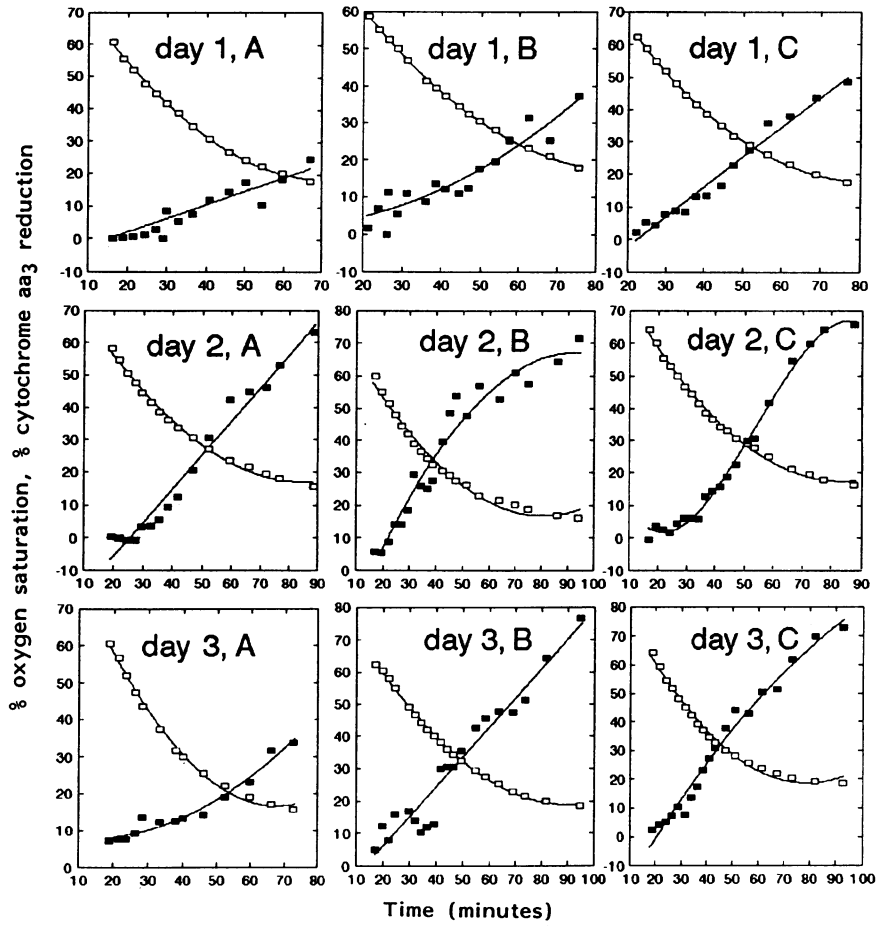

FIG. 6. Time dependence of reduction of cytochrome aa_3 (■) and oxygen saturation of the growth medium (□) measured with the system for cytochrome redox state determination. Pellets of *P. chrysosporium* control culture (A) and cultures supplemented with Tween 80 (B) and Tween 20 (C) were used.

biomass. In selecting the cytochrome to be used as an indicator, aa_3 seemed to be applicable. It is the terminal part of the respiratory chain; therefore the energy produced in oxidative phosphorylation is equal to the electron transfer through cytochrome aa_3. Cytochrome aa_3 levels can also be easily evaluated from the mitochondrial cytochrome spectrum (Fig. 3).

Respiratory enzymes use most of the O_2 available to the cell; therefore the redox state of mitochondrial cytochromes reflects the ratio of the cellular energy pool (NADPH, NADH, and $FADH_2$) and the intracellular oxygen concentration. The redox state of cytochrome aa_3, the most immediate reductant of O_2, is the least sensitive to other metabolic factors, including substrate delivery or cell activity state (3), and was proposed as an intracellular oxygen probe (15, 18, 19). The affinity of cytochrome aa_3 for oxygen in vivo differs from that reported for isolated mitochondria (18). Because the correlation between the redox state of cytochrome aa_3 and the intracellular oxygen saturation is not available for fungal cells, the data of Kirman et al. (18), obtained with several types of tissue cells, were used for calculation of intracellular oxygen saturation (equation 3).

The stimulative effect of the Tweens on lignin peroxidase synthesis by *P. chrysosporium* was first described by Jäger et al. (13) and Asther et al. (1). They proposed that surfactants modify cell membrane permeability and hence enhance the transport of compounds into and from the cell. They did not explain the actual mechanism. Jäger et al. (13) reported that Tween 80 was effective only if added at the beginning of primary growth. In contradiction to this finding, Venkatadri and Irvine (34) reported that addition of Tween 80 after the end of primary growth also resulted in an enhancement of lignin peroxidase levels. They proposed that the effect of the detergents is to protect lignin peroxidases in liquid culture against mechanical deactivation, presumably through formation of protective detergent micelles around the enzymes. Leštan et al. (21), however, reported that other detergent-related compounds, unable to form micelles in water, also stimulate lignin peroxidase synthesis. They also observed that after 3 days of culture only 2% of the initial concentration of Tween 80 remained in the growth medium. The changes in *P. chrysosporium* culture, related to the addition of Tween 80, which later enable lignin peroxidase activity therefore seem to have occured in the first days of culture. The cytochrome aa_3 redox state at this time is presented in Fig. 5 and 6 and in Tables 1 and 2.

In cultures with Tween 80 or Tween 20, a higher K and a higher $\Delta Q/\Delta t$ were found compared with control cultures (Table 2). No specific trend was observed for the specific respiratory activity (Table 2). The higher K indicates the higher oxygen permeability of the cell membrane and therefore better

TABLE 2. Dry biomass of the pellets and volume of growth medium in the measurement system for cytochrome redox state determination and parameters related to the energy pool of cultures of *P. chrysosporium* supplemented with Tween 80 and Tween 20 and control cultures

Culture and time (days)	m (g)	V (liters)	K (liters/g · min)	$\Delta Q/\Delta t$ (kPa · liters/g · min)	Relative cytochrome aa_3 content	Relative respiratory activity (kPa · liters/g · min · aa_3 content)
Control						
1	0.042	0.059	0.0435	1.187		
2	0.050	0.054	0.028	0.777	0.59	1.32
3	0.052	0.039	0.0277	0.769	0.65	1.18
Tween 80						
1	0.0334	0.059	0.0479	1.283		
2	0.0369	0.059	0.0421	1.204	0.72	1.67
3	0.0340	0.058	0.0376	1.046	0.78	1.34
Tween 20						
1	0.0335	0.041	0.0357	0.995		
2	0.0364	0.0535	0.0391	1.095	1.00	1.09
3	0.0320	0.047	0.0395	1.102	0.95	1.16

oxygen supply of the cells. The reason for the higher permeability might be the changed fatty acid composition of polar (membrane) lipids of the cultures supplemented with Tween 80, Tween 20, or other stimulative compounds (21). Since it is known that the lignin peroxidase activity of *P. chrysosporium* strongly depends on oxygen availability (4, 17), this better oxygen supply may be the reason for enhanced lignin peroxidase biosynthesis in cultures with Tween 80 (Fig. 1) or Tween 20 (1, 13, 21). In cultures with Tween 80, enhanced metabolic activity was determined as a faster consumption of glucose and nitrogen source (Fig. 2). The higher glucose consumption and CO_2 evolution rate in cultures flushed with oxygen instead of air was reported by Dosoretz (10), indicating that oxygen is a limiting substrate. The physiological evidence that oxygen is a limiting substrate is given in Table 1. It shows the high diffusion barrier of oxygen and a high proportion of reduced cytochrome aa_3 at actual growth medium oxygenation.

In cultures with Tween 80 or Tween 20, a higher energy pool was found during the first 3 days of cultivation compared with control cultures. It was noted as a higher proportion of reduced cytochrome aa_3 at the actual growth medium oxygenation (Table 1). The exhaustion of nitrogen sources (Fig. 2) might have caused a shock to surfactant-added cultures supplemented with an originally high energy pool and metabolic activity. This might be the possible reason for the drop in the cytochrome aa_3 content (Fig. 4A) and the relative active biomass (Fig. 4C) and for the temporary cessation of the increase in dry biomass (Fig. 4B), coincident with the beginning of lignin peroxidase biosynthesis in cultures with Tween 80 (Fig. 1) or Tween 20 (20). The cessation of the increase in dry biomass at the time of the onset of lignin peroxidase activity has also been reported by other authors (13, 14, 16). The energetic state of the cell is indicated by intracellular pH. It has been reported that a low intracellular pH value (intracellular acidification) is associated with decreased cellular activity (28). Therefore the shock mentioned above might be reflected in a reduction of the intracellular pH of 0.35 pH unit (Fig. 7A) and in an increase of the growth medium pH (Fig. 7B), determined in *P. chrysosporium* cultures with Tween 80 during the period of lignin peroxidase activity (Fig. 1). These results compare favorably with those of Bonnarme et al. (5), who measured an increase in the growth medium pH and in the dissolved-oxygen level in the growth medium, a cessation of mycelial dry biomass increase, and a reduction in the mitochondrial content (estimated by an enzyme marker) from the initial high level. All occurred at the onset of lignin peroxidase activity.

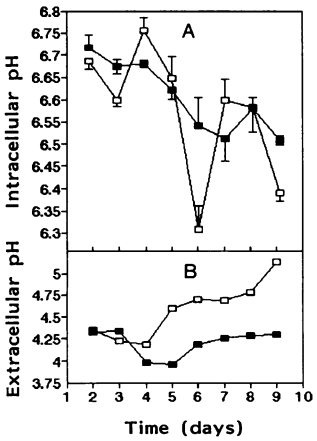

FIG. 7. Intracellular pH (A) and pH of the growth medium (B) of control cultures (■) and cultures supplemented with Tween 80 (□) during cultivation.

Various metabolic events and rates are influenced by intracellular pH levels. For example, a small 0.12 pH unit rise was shown to increase the glycolysis rate by 43% (27). It was also reported that biosynthesis of cyclic AMP (cAMP) is activated at a lower pH as a result of activation of adenylate cyclase and hampered at a higher pH as a result of activation of phosphodiesterase (8, 31). cAMP is presumably connected with the control of biosynthesis of catabolitically repressed lignin peroxidase enzymes (6). MacDonald et al. (25) reported that the intracellular concentration of cAMP in lignin peroxidase active cultures increased 10-fold at the onset of lignin peroxidase activity in the same fungus compared with that in the control cultures. This increase may be connected with the reduction in intracellular pH.

ACKNOWLEDGMENTS

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