Determination of the Respiration Kinetics for Mycelial Pellets of *Phanerochaete chrysosporium*

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In mycelial pellet cultures of the white rot basidiomycete *Phanerochaete chrysosporium*, low oxygen concentration negatively affects the production of the extracellular lignin peroxidases and manganese peroxidases which are key components of the lignin-degrading system of this organism. To test the hypothesis that oxygen limitation in the pellets is responsible for this effect, oxygen microelectrodes were used to determine oxygen concentration gradients within the mycelial pellets of *P. chrysosporium*. Pellets were removed from oxygenated cultures, allowed to equilibrate with air, and probed with oxygen microelectrodes. The oxygen profiles were modelled assuming that O_2 uptake follows a Michaelis-Menten relationship. The V_{max} and K_m values for oxygen uptake were 0.76 ± 0.10 g/m^3 of pellet per s and 0.5 ± 0.3 g/m^3, respectively. These kinetic values were used to predict respiration rates in air-flushed cultures, oxygen-flushed cultures, and cultures with large pellets (diameter > 6 mm). The predicted respiration rates were independently validated by experimentally measuring the evolution of carbon dioxide from whole cultures.

Mycelial pellet cultures, which are used for the industrial-scale production of products such as pharmaceuticals, organic acids, and enzymes, offer the key advantages of immobilization, low-medium viscosity, and simplified product isolation without the problems associated with artificial immobilization matrices (6, 17). Previous studies have shown that oxygen can become mass transfer limited within mycelial pellets (5, 16, 18, 19), potentially affecting productivity. In this study, we determined the kinetic parameters of respiration in mycelial pellets of the important lignin-degrading fungus *Phanerochaete chrysosporium*, which produces manganese peroxidases (MNPs) and lignin peroxidases (LIPs) during secondary metabolism (4, 7). These enzymes play key roles in the lignin-degrading system (4), in the decolorization of paper mill bleach plant effluents (7), and in the degradation of xenobiotics (4).

Lignin degradation is primarily an oxidative process. The production of the ligninolytic enzyme system, including the LIPs and MNPs, is greatly enhanced in hyperbaric oxygen (1, 3, 13). The partial pressure of oxygen strongly affects both the rate and extent of lignin degradation and the rate of degradation of lignin model substrates (1, 4). One possible explanation of this effect is that the diffusion of oxygen into the mycelial biofilm is mass transfer limited and that higher oxygen concentrations relieve this limitation.

To determine the nature of oxygen limitation on the production of LIPs and MNPs, the kinetic parameters for respiration were determined by modelling oxygen concentration profiles within the pellets. The results indicate that respiration in mycelial pellets of *P. chrysosporium* can become oxygen limited depending on the pellet radius and external oxygen concentration. To quantify this oxygen limitation, the diffusion and respiration of oxygen into the mycelial pellets was modelled as a function of the pellet size and external oxygen concentration. The results also explain why oxygen enrichment enhances LIP and MNP production and ligninolytic activity in cultures of *P. chrysosporium*.

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MATERIALS AND METHODS

Microorganism. The fungus used was *P. chrysosporium* BKM-F-1767 (ATCC 24725). The strain was maintained on 2% malt extract agar slants, pH 4.5.

Culture conditions. *P. chrysosporium* was cultured at 39°C in a defined low-nitrogen medium with glucose as the carbon source and diammonium tartrate as the nitrogen source (7). The medium contained the following (per liter): 10 g of glucose, 2.0 g of KH$_2$PO$_4$, 1.45 g of MgSO$_4$ · 7H$_2$O, 0.132 g of CaCl$_2$ · 2H$_2$O, 1 mg of thiamine hydrochloride, 0.5 g of Tween 80 (not added in stationary starter cultures), 1.2 mmol of D-diammonium tartrate, 20 mmol of sodium acetate (pH 4.5), and 0.4 mmol of veratryl alcohol. The following trace elements were also added (per liter): 0.14 g of nitrilotriacetate, 0.070 g of NaCl, 0.007 g of FeSO$_4$ · 7H$_2$O, 0.033 g of MnSO$_4$, 0.013 g of CoCl$_2$ · 6H$_2$O, 0.007 g of ZnSO$_4$ · 7H$_2$O, 0.0011 g of CuSO$_4$ · 5H$_2$O, 0.0007 g of AlK(SO$_4$)$_2$ · 12H$_2$O, 0.0007 g of H$_3$BO$_3$, and 0.0007 g of Na$_2$MoO$_4$ · 2H$_2$O.

The medium (85 ml) was dispensed into sterile 250-ml rubber-stoppered Erlenmeyer flasks and inoculated with a 10% (vol/vol) homogenized mycelial inoculum which was grown in shallow stationary culture. The cultures were sealed and agitated at 173 rpm, and the headspace (165 ml) was flushed daily with pure oxygen. The number of mycelial pellets was determined by placing a known volume (15 to 20 ml) of culture fluid in a petri dish and counting the number of pellets. To measure the diameters of the pellets, the dish was photographed and enlarged. Twenty pellets selected at random were measured, and the average diameter and standard deviation were calculated. The average number of pellets was 7,320 ± 1,100 liter^{-1}. For determining mycelial dry weight, cultures were vacuum filtered through tared GF/C grade filter paper, rinsed with 100 ml of distilled deionized water, weighed, dried to a constant weight, and reweighed and the cell mass was calculated by difference. The pellet density (ρ; g dry weight/m^3 of mycelia) was calculated by dividing the culture dry weight (g/m^3 of medium) by the pellet volume fraction (m^3 of mycelia/m^3 of medium) and had an average value of 65,000 ± 10,000 g/m^3. The pellet number and size are inversely related to the rate of agitation;

however, high rates of agitation are known to be detrimental to LIP and MNP production (8).

Enzyme and analytical assays. Enzyme activities were measured by spectrophotometry. MNP activity was measured as previously described (7). The sample volume was 10 to 40 μl, the pH was 4.5, and the reaction time was 4 min. A unit of MNP activity was defined as 1 μmol of phenol red oxidized per liter per min, using an extinction coefficient of 4,460 M^{-1} cm^{-1} as determined in our laboratory. LIP activity was measured at pH 2.5 according to the procedure of Tien and Kirk (15). Glucose was measured as reducing sugar by the dinitrosalicylic acid method, using D-glucose as the standard (3, 7). Ammonium concentration was measured by using an ammonia electrode (Orion model 95-12).

Oxygen profile measurements. The oxygen profile within the mycelial pellets was measured with a hand-made oxygen microelectrode with a tip diameter of approximately 3 μm (14). The electrode was calibrated with pure oxygen and pure nitrogen sparged into distilled water. Pellets were removed and placed onto plates of culture fluid agar (15 ml of extracellular culture fluid + 0.3 g of agar [Difco]). An additional 15 ml of the extracellular culture fluid was added onto the surface of the culture fluid agar, and the system was equilibrated in air. To determine the oxygen concentration profiles in a pellet, a microelectrode was inserted with a micromanipulator (0.01-mm gradations). The procedure was monitored with a stereo dissecting microscope. The micromanipulator was also used to measure the diameter of the pellet being probed. A total of 19 pellets were probed on days 1, 2, 4, and 5 of incubation.

A computer program was used to calculate values of V_{max} and K_m which provided the best fit of the model (represented in equations 3 and 5, below) to experimental oxygen profile data. The value of the diffusivity used for modelling was the published value (11) of the diffusivity of oxygen in water, scaled to 39°C by assuming that $\mathcal{D} \, T_{abs}/\mu$ is a constant ($\mathcal{D} = 2.9 \times 10^{-9}$ m^2/s).

Oxygen concentrations in the headspace and the culture fluid were measured with a polarographic dissolved-oxygen sensor (model IL531; Ingold Electrodes, Inc., Wilmington, Mass).

Respiration rate measurements. Carbon dioxide evolution was monitored as previously described (3) by using gas chromatography (Porapak Q 80/100; helium as carrier gas, column temperature of 90°C). Samples were removed from the headspace with a pressure-lock syringe.

RESULTS

Mycelial pellets of the lignin-degrading white rot fungus *P. chrysosporium* were produced in sealed shake-flask cultures which were flushed daily with pure oxygen. The average diameter of the mycelial pellets increased from 1.6 mm on day 1 of incubation to 2.0 mm on day 10 (Fig. 1). The standard deviation indicated that approximately 68% of the pellets had a diameter between 1.3 and 1.85 mm on day 1 and between 1.4 and 2.3 mm on day 9 (Fig. 1). The depletion of nutrient nitrogen triggered the onset of secondary metabolism on day 2. The culture dry weight increased slowly after day 2 and reached a maximum (2,300 g/m^3) on day 9 (Fig. 1). MNP activity was first detected on day 3 and reached a maximum of 840 U/liter on day 4. LIP activity was first detected on day 4 and reached a maximum of 190 U/liter on day 7 (Fig. 1). The glucose concentration in the medium (initially 10,000 g/m^3) decreased to less than 200 g/m^3 by day 11.
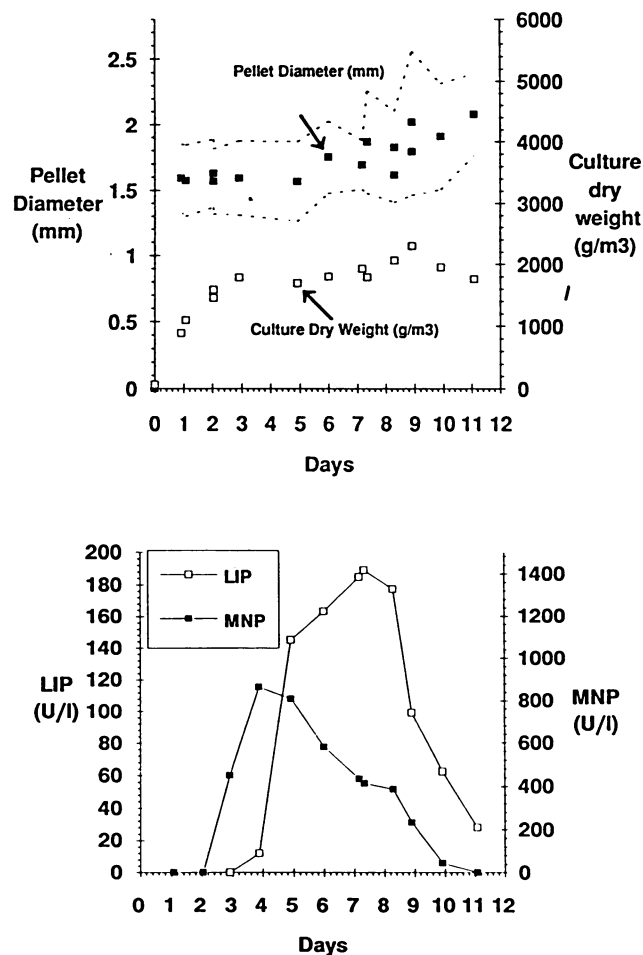

FIG. 1. Characteristics of agitated low-nitrogen mycelial pellet cultures of *P. chrysosporium*. Upper panel: average mycelial pellet diameter and culture dry weight. The dashed lines represent mean pellet diameter ±1 standard deviation. Lower panel: LIP and MNP production (points represent averages for triplicate cultures).

Previous studies have assumed that the main resistance to oxygen mass transfer in mycelial pellet cultures lies within the mycelial pellet itself (12, 16, 19). This is in contrast to what is found with small, nonflocculating bacterial or yeast cultures, where the primary oxygen mass transfer resistance lies at the gas-liquid interface. To validate this assumption for mycelial pellet cultures of *P. chrysosporium*, the oxygen concentrations were measured in the headspace and in the culture fluid with a polarographic oxygen electrode and at the pellet surface and within the pellet interior with an oxygen microelectrode. It should be noted that in contrast to oxygen, which has a low solubility in culture medium (32.9 g/m^3 at 39°C), glucose is present at a relatively high concentration (initially 10,000 g/m^3) in the culture medium; glucose concentration, therefore, is not limiting within the pellet. The results showed that the oxygen in the gas phase was in rapid equilibrium with the liquid phase and that the culture fluid and gas phase oxygen concentrations were the same, indicating that mass transfer resistance at the gas-liquid interface was not limiting in this system.

Oxygen concentration profiles were determined in pellets which were removed from the flask cultures described above
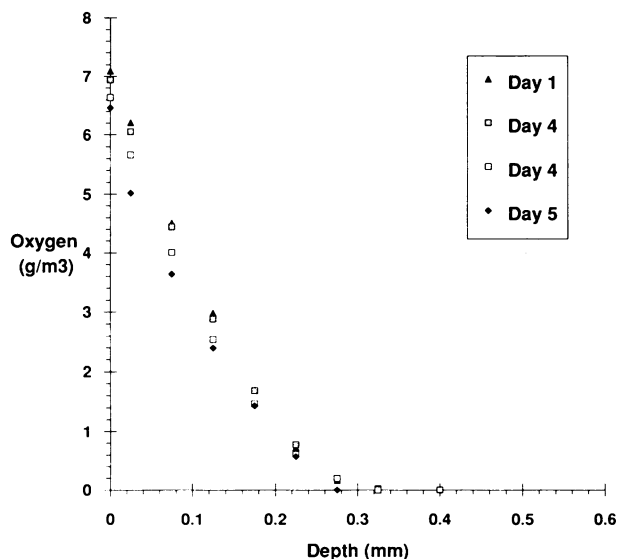
FIG. 2. Oxygen concentration gradients measured with an oxygen microelectrode in mycelial pellets of P. chrysosporium. The profiles were measured by using 1.85-mm-diameter pellets from 1-, 4-, and 5-day-old cultures which were equilibrated to air-saturated culture fluid collected on the same day as the pellets. Two sets of datum points are shown for day 4.

and were allowed to equilibrate in air-saturated culture fluid collected on the same day of incubation as the pellets. Pellets with a diameter of 1.85 mm were selected on days 1, 4, and 5 of incubation, and the oxygen concentration gradient was measured with an oxygen microelectrode (Fig. 2). The oxygen concentrations at the pellet surface and in the culture fluid surrounding the pellet were the same (between 6.4 and 6.9 g/m^3; Fig. 2), indicating that mass transfer to the pellet surface was not rate limiting. As the microelectrode entered the pellets, the oxygen concentration dropped precipitously, reaching undetectable levels at a depth of less then 0.4 mm (Fig. 2). Therefore, the assumption that the oxygen mass transfer within the mycelial pellets is rate limiting is valid for pellets of P. chrysosporium under the conditions tested. The oxygen concentration profiles did not vary significantly with culture age, provided the pellet sizes were the same (Fig. 2).

A mathematical model of the oxygen concentration profile was used to determine the kinetic parameters (V_{max} and K_m) for respiration in mycelial pellets of P. chrysosporium. Once the kinetic parameters V_{max} and K_m were obtained, predictions were made about oxygen gradients and respiration rates in air-flushed cultures, in oxygen-flushed cultures, and in pellets of different sizes.

The kinetic parameters were determined by using the following model. Assuming that oxygen transfer occurs by Fickian diffusion, the respiration of oxygen by mycelial pellets can be represented by the following second-order differential equation (a definition of the notation used is presented in Table 1).

$$\mathfrak{D}\left(\frac{d^2C}{dr^2} + \frac{2}{r}\frac{dC}{dr}\right) = v \qquad (1)$$

Various methods have been used to solve this equation (5, 10, 16, 19). We used a finite-difference approach (2) to solve equation 1 for the oxygen concentration as a function of the depth within a mycelial pellet.

TABLE 1. Notation used for mathematical model of oxygen diffusion and respiration in mycelial pellets of P. chrysosporium

Symbol	Definition	Unit
C	Oxygen concentration in a mycelial pellet	g/m^3
$\mathfrak{D}$	Oxygen diffusivity	m^2/s
E	Oxygen effectiveness factor	
i	Finite element index value	
K_m	Kinetic coefficient for respiration	g/m^3
n	Number of finite elements	
r	Radial distance from pellet center	m
R	Pellet radius	m
X	Culture dry weight	g/m^3
v	Rate of oxygen depletion	g/m^3/s
V_{max}	Kinetic coefficient for respiration	g/m^3/s
Subscripts		
i	Index value	
g	Gas phase	
l	Liquid phase	
n	Number of finite elements	
s	Surface	

$$\frac{C_{i-1} - 2C_i + C_{i+1}}{\Delta r^2} + \frac{C_{i+1} - C_{i-1}}{i\Delta r^2} = \frac{v}{\mathfrak{D}} \qquad (2)$$

$$C_{i+1} = \left(\frac{i}{i+1}\right)\left[2C_i + \left(\frac{1}{i} - 1\right)C_{i-1} + \frac{v|_{C=C_i}\Delta r^2}{\mathfrak{D}}\right] \qquad (3)$$

Since the oxygen concentration at the pellet center is lower than that at the surface of the pellet, the specific rate of respiration by a pellet is less than that which would be found for non-oxygen-limited filamentous mycelia of equal mass. The generalized effectiveness factor (E) describes the decrease in the rate of respiration in the pellet due to intrapellet mass transfer resistance. E is defined as the observed reaction rate over the pellet volume divided by the rate without mass transfer limitations and is valid for any rate equation and spherical geometry (equation 4). The effectiveness factor can account for autolysis occurring at the pellet center because of oxygen starvation, since little or no respiration would be predicted in areas of the pellet which are oxygen depleted (provided the pellet density is known).

$$E = \frac{\sum_{i=0}^{n} [(v|_{C=C_i})(r_i^3 - r_{i-1}^3)]}{(v|_{C=C_s})R^3} \qquad (4)$$

For most microbial cultures, the respiration kinetics (v) follows Michaelis-Menten kinetics (equation 5). This equation was used for v in equations 3 and 4.

$$v = \frac{V_{max}C}{(K_m + C)} \qquad (5)$$

Equation 3 was solved by using a shooting algorithm (2). The algorithm was started by using an oxygen concentration at the pellet center (C_o) that is halfway between the pellet surface concentration (C_s) and zero. The oxygen concentration at the pellet surface was then calculated by using equation 3. The oxygen concentration at the pellet center was changed until the calculated surface concentration converged to the known value ($C_n = C_s$). By using a small step
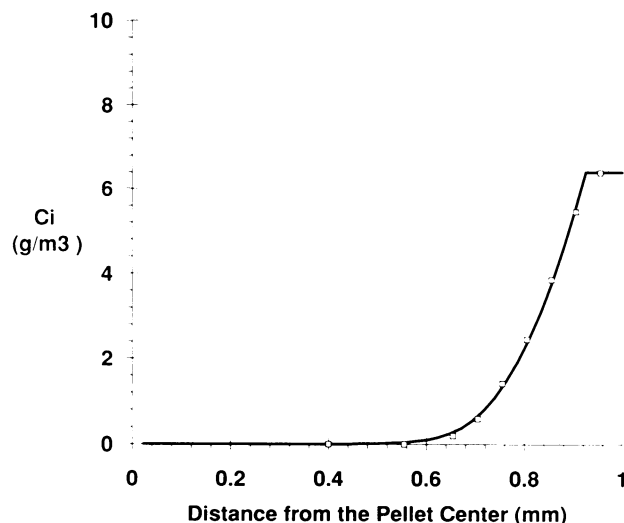
FIG. 3. Oxygen concentration profile for a mycelial pellet of *P. chrysosporium* (diameter 1.85 mm) predicted by a mathematical model for the oxygen profile using V_{max} and K_m values of 0.76 g/m³/s and 0.5 g/m³, respectively (solid line). The open squares represent experimental data as determined by using an oxygen microelectrode.

FIG. 4. The effect of the mycelial pellet radius (R) and the oxygen concentration at the pellet surface (Cs) on the oxygen effectiveness factor (E) for mycelial pellets of *P. chrysosporium*. E is defined as the observed respiration rate divided by the rate without mass transfer limitations.

size (n = 100), the calculated concentration has an error of less than 0.1% of the analytical solution.

By using the microelectrode technique, the range of values determined for V_{max} was 0.76 ± 0.10 g/m³ of mycelia per s. For K_m, the range of values was 0.5 ± 0.3 g/m³. The specific V_{max} for pellets of *P. chrysosporium* was 1.2 × 10⁻⁵ g/g dry weight per s. An oxygen concentration profile for a 1.85-mm-diameter pellet and a computer simulation curve for pellets in equilibrium with air are presented in Fig. 3. The results show that the oxygen concentration decreases to undetectable levels well before the pellet center is reached. This is probably not true for pellets in oxygen-flushed cultures (the oxygen profile measurements were made in air). The value of the effectiveness factor for oxygen-flushed cultures between days 1 and 6 is greater than 0.9. In addition, both the respiration rate and the culture dry weight increased from days 1 to 6 (Fig. 1 and 4). Also, the pellets were solid and showed no sign of autolysis under hyperbaric conditions.

By using the kinetic parameters for respiration, a three-dimensional diagram for the oxygen effectiveness factor (E) was generated with a computer program which calculated the value of E for pellet radii ranging from 0.05 to 2.5 mm and oxygen concentrations ranging from 1 to 33 g/m³ (Fig. 4). This graph quantifies the oxygen limitation in the pellets as a function of the pellet size and external oxygen concentration and is useful in quickly estimating the oxygen effectiveness factor in mycelial pellets of *P. chrysosporium*. The most favorable conditions for aerobic metabolism are represented by the plateau of the surface, where the oxygen effectiveness factor (E) is near one. This region corresponds to small pellet radii and high dissolved oxygen concentrations.

To confirm the prediction of respiration rates in hyperbaric cultures, the carbon dioxide concentration in the culture headspace of hyperbaric cultures was measured daily by gas chromatography immediately before oxygenation. These data were compared with the CO_2 concentration predicted by a kinetic model for submerged cultures of *P. chrysosporium* (9). This kinetic model assumes that all of the

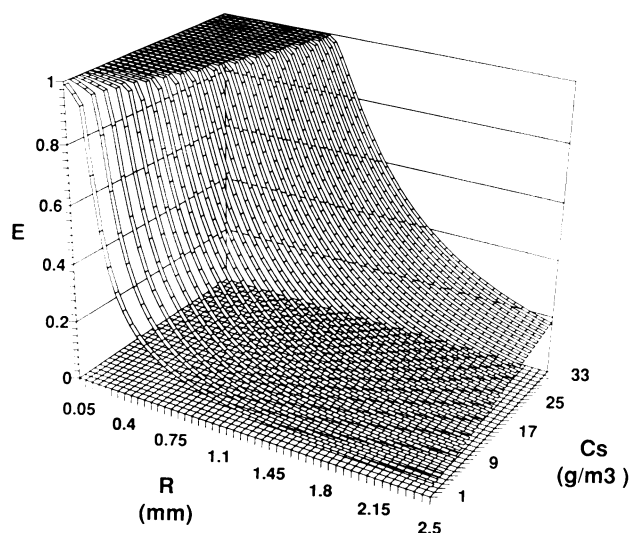oxygen is respired to carbon dioxide. It uses the same kinetic parameters which were determined by modelling oxygen concentration gradients. The evolution of carbon dioxide by the pellets in a sealed shake flask culture can be described as follows (9):

$$CO_2 = 1.38 \, V_l \int_0^t \frac{X}{\rho} E \left(\frac{V_{max} \, C_l}{K_m + C_l} \right) dt \qquad (6)$$

This equation has been previously solved by developing an integrated model describing the biomass, effectiveness factor, and mycelial pellet radius in cultures of *P. chrysosporium* (9) as a function of time and the initial concentration of growth nutrients in the culture medium. The model-predicted carbon dioxide production (Fig. 5) determined by using kinetic parameter values (V_{max} = 0.76 g/m³ of mycelia per s, K_m = 0.5 g/m³, pellet density = 65,000 g of mycelia per m³ of pellet, V_l = 85 ml) closely matched the experimentally determined CO_2 evolution data (Fig. 5). Thus, the kinetic coefficients determined by modelling oxygen concentration profiles in mycelial pellets which are in equilibrium with air can be used to predict respiration behavior in hyperbaric conditions.

Because of the very low K_m for oxygen found for many microbial cultures, a zero-order model for respiration has been used by many investigators (12, 16, 18, 19). If a zero-order model is assumed for pellets of *P. chrysosporium*, then the critical pellet radius at which the center of the pellet first becomes anoxic can be calculated. This radius is 0.86 mm in oxygen-saturated medium (C_s = 32.9 g/m³) and 0.40 mm in air-saturated medium (C_s = 6.9 g/m³) at 39°C.

DISCUSSION

The enhancement of ligninolytic activity and LIP and MNP production in cultures of *P. chrysosporium* flushed with hyperbaric oxygen as compared with cultures flushed
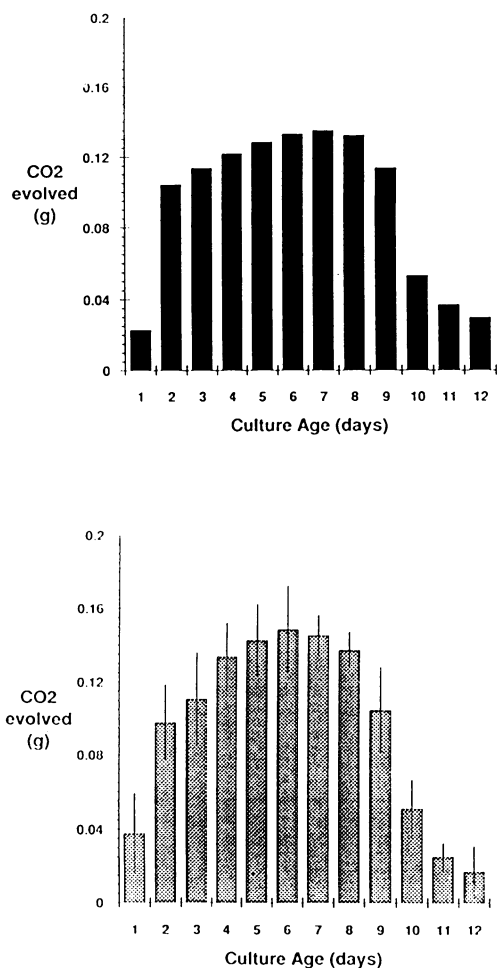
FIG. 5. Daily carbon dioxide evolution in oxygen-flushed mycelial pellet cultures of *P. chrysosporium*. The solid bars represent CO_2 evolution predicted by the kinetic model (equation 6) using parameters determined with an oxygen microelectrode ($V_{max} = 0.76$ $g/m^3/s$, $K_m = 0.5$ g/m^3). The crosshatched bars represent experimental data (mean ± 1 standard deviation for five to seven replicate cultures).

with air has been widely documented (1, 3, 13). However, the mechanism of this oxygen enhancement effect was not clear. In this study, we show that mycelial pellets of *P. chrysosporium* in atmospheric air become oxygen limited even at shallow depths (Fig. 2 and 3) and that hyperbaric oxygen allows deeper oxygen penetration into the mycelia, thereby partially ameliorating the condition of oxygen starvation (Fig. 4). The kinetic parameters for respiration in mycelial pellets are difficult to determine in whole cultures unless models such as the one described here are used to interpret rate data. Oxygen microelectrodes allowed the determination of oxygen profiles from which kinetic parameters (V_{max} and K_m) for respiration could be determined (Fig. 3).

Cultures of *P. chrysosporium* which are oxygen starved at the pellet center produce much lower levels of LIP and MNP activity. For example, Dosoretz et al. (3) showed that in air-flushed cultures (in which the oxygen effectiveness factor ranges from 0.1 to 0.3 on the basis of this model), no LIPs and one-third fewer MNPs were produced. Furthermore,

Michel et al. (8) found that large pellets (6-mm diameter), for which the oxygen effectiveness factor is lower than 0.1, are formed in cultures with low agitation rates and that these cultures fail to produce LIPs. Similarly, Barlev and Kirk (1) found that in stationary cultures of *P. chrysosporium* in which a thick mycelial mat forms, the ligninolytic activity in air-flushed cultures was approximately 20% of that observed with oxygen-flushed cultures. By comparison, cultures of *P. chrysosporium* in which effectiveness factors near unity are predicted by the kinetic model (small pellets and liquid-phase oxygen concentrations near saturation) produce high levels of both LIP and MNP. Therefore, the beneficial effect of hyperbaric oxygen in cultures of *P. chrysosporium* appears to be caused by the amelioration of the oxygen limitations which would occur under atmospheric air.

ACKNOWLEDGMENTS

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