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Analysis of Fungal Pellets by UV-Visible Spectrum Diffuse Reflectance Spectroscopy

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The application of the UV-visible spectrum diffuse reflectance spectroscopy for the determination of intracellular pH in vivo, for determination of cytochrome content, and for the noninvasive in vivo detection of the redox state of fungal mitochondrial cytochromes in filamentous fungi is introduced. The time course of the intracellular pH values, mitochondrial cytochromes, and CO-binding pigments content and the correlations between the actual redox state of cytochrome aa_3 and saturation of growth medium with oxygen in pellets of the basidiomycete *Phanerochaete chrysosporium* were determined. As the test microorganism, the yeast *Saccharomyces cerevisiae* was used. UV-visible spectrum diffuse reflectance spectroscopy proved to be a promising method for the quick and simple analysis of light-impermeable biological structures for which the classical transmittance spectrophotometric methods are difficult to implement.

For the determination of intracellular pH in bacteria, yeasts, and animal and plant cells, various methods are in use: the distribution of weak acids or bases, measurements with a microelectrode, ^{31}P nuclear magnetic resonance colorimetry, and fluorometry. With improved instrumentation and the use of new spectroscopic probes, UV-visible spectrum and fluorescence spectroscopy offer certain advantages. They are highly sensitive, can be applied to very small cells, have a faster response, and are not destructive as the method of weak acid or base distribution is; they require instruments that are less expensive and are far less complicated than the ^{31}P nuclear magnetic resonance method (25, 26).

UV-visible spectrum (VIS) spectrometry is used also for a determination of mitochondrial cytochromes and related pigments in light-permeable suspensions and whole-cell pastes (4, 11, 20–23). Yet after an extensive survey, no such technique was found for determination of cytochromes or intracellular pH in pellets of filamentous fungi.

In this paper, UV-VIS diffuse reflectance spectroscopy is introduced to determine the intracellular pH, the cytochrome content, and the physiological redox state of mitochondrial cytochromes in the light-impermeable pellets of filamentous fungi.

Reflectance spectroscopy is the investigation of the spectral composition of surface-reflected optical radiation with respect to its angularly dependent intensity and the composition of the incident radiation. It is further divided into specular and diffuse reflectance. Specular reflectance is a mirrorlike reflection of optical radiation from a glossy surface, while diffuse reflectance occurs when optical radiation is partly absorbed and partly reflected in different directions from a matte surface (31). In diffuse reflectance spectroscopy, two conceptions are possible for the occurrence of this reflected, directionally independent, optical radiation. For particles with a diameter much greater than the wavelength, the radiation is partly reflected from the surfaces and partly penetrates into the inside of the sample, where it then undergoes numerous reflections, refractions, and diffractions and finally emerges diffusely from the surface. For particles with diameters in the order of the wavelength, scattering occurs. Measurements of diffuse reflectance may be performed by using an integrating sphere which is designed to permit the collection of diffusely reflected light on a high-performance photomultiplier. The interior of the integrating sphere is, therefore, coated with highly reflective MgO, $MgCO_3$, or $BaSO_4$ paint (17, 31).

Diffuse reflectance spectroscopy is used today for quantitative and qualitative analysis of various materials, such as paper and thin-layer chromatograms; color matching of building materials; measurement of color, whiteness, brightness, and gloss of paper and textiles; determination of surface properties of plastics; as well as for basic research in solid-state reactions, absorptions, surface phenomena, etc. (31). Since the introduction of reflectance spectroscopy in biological research, the continuous, noninvasive study of respiratory enzymes in vivo has become possible. This technique in particular has been widely tested with tissues of living animals (8, 14–16). It was also successfully applied to the determination of the intracellular activity of ligninase in pellets of *Phanerochaete chrysosporium* (18).

The subject of our investigation was pellets of the basidiomycete *Phanerochaete chrysosporium*, the well-known producer of ligninases, enzymes potentially important in future technologies of lignin modification in the pulp and paper industry.

MATERIALS AND METHODS

Organism. *P. chrysosporium* MZKIBK B-223 (ATCC 24725) was obtained from the American Type Culture Collection and transferred to the Culture Collection of the Institute of Chemistry in Ljubljana, Slovenia (MZKIBK). Subcultures were prepared every 3 to 4 weeks on malt agar slants which were kept at room temperature until use.

Medium and culture conditions. *P. chrysosporium* was grown in a nitrogen-limited medium as described by Leštan et al. (19) and buffered to pH 4.5 with 20 mM sodium tartrate

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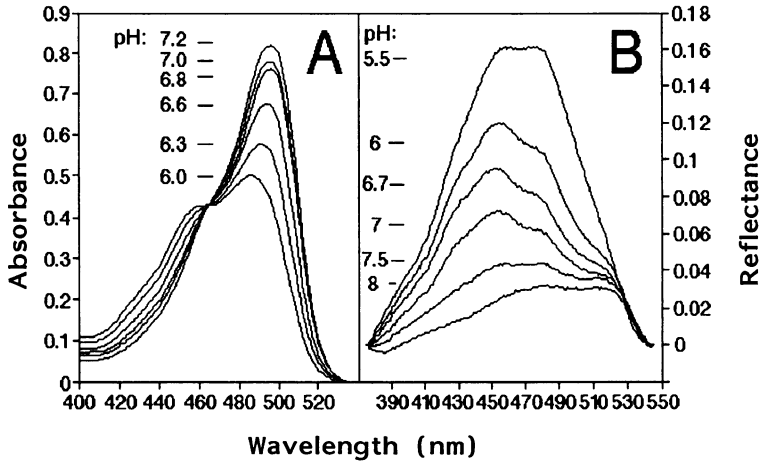

FIG. 1. (A) Transmission spectra of CF in phosphate buffer of different pHs. (B) Diffuse reflectance spectra of CF in phosphate buffer solution of different pHs, imbibed in chromatographic paper.

in agitated (125 rpm) 500-ml Erlenmeyer flasks containing 100 ml of growth medium. Flasks were inoculated with 2×10^6 spores from 2- to 3-week-old agar slants (13), and cultures were incubated at 37°C.

As a blank for the calibration of the spectrophotometer and as a test microorganism, baker's yeast, *Saccharomyces cerevisiae*, was used. It was cultivated aerobically for 6 h at 30°C, pH 4.5, in growth medium containing 0.2% yeast extract, 2% glucose, 0.1% $NH_4H_2PO_4$, and 0.01% K_2SO_4.

Apparatus. Diffuse reflectance spectra were measured with a Varian DMS 80 spectrophotometer fitted with an integrating sphere accessory. The sphere was 73 mm in diameter, coated with $BaSO_4$ paint with a reflectivity greater than 98% over most of the UV-VIS region. Actual measurements of intracellular pH, cytochromes, and pigments were made relative to those for pressed polytetrafluoroethylene (PTFE) powder. Sample cuvettes were made from PTFE with a 12.5-mm diameter and a 3-mm-deep hole. This depth was chosen because the reflectance of *P. chrysosporium* pellets measured with cuvettes with 2-mm-deep and 5-mm-deep samples were approximately 30% lower. Approximately 1 g (wet weight) of blotted fungal pellets with diameters ranging from 1 to 2 mm, approximately 180 of them, were used. Flowthrough sample cuvettes for the determination of the actual redox state of mitochondrial cytochromes were made from PTFE and glass with a hole (3 by 10 by 30 mm [height, width, and length, respectively]) for concentrated, dry biomass approximately 5-g/100 ml, fungal pellet-substrate suspensions. A slit width of 3.0 mm and a scan rate of 100 nm/min were used.

A whole-cell spectrum (see Fig. 7) of mitochondrial cytochromes of aerobically grown *S. cerevisiae* at room temperature was used for calibration of the wavelength scale of the spectrophotometer at the well-known absorption peaks in the α region (603, 560, and 549 nm for the cytochromes aa_3, b, and cc_1, respectively) (5, 29).

The spectrophotometer data were processed by an AT286 personal computer. Instrumental noise was removed by smoothing the data numerically by the method of regression with spline functions (7). A degree of smoothness from 70 to 30 grid points was imposed in order to obtain visually noise-free spectra.

Usually, light-scattering and absorption properties of substances are taken into account by the two constant theories. The well-known Kubelka-Munk theory thus allows evaluation of spectra of finely pulverized substances (17). However, the optical structure of biological substances is much more complicated, and for intracellular pH determination, fluorescent samples were scanned as absorbance and fluorescence were measured. Therefore, the spectra were evaluated simply as reflectance.

Chemicals. 6-Carboxyfluorescein (CF), CF diacetate, and gramicidin were purchased from Sigma Chemical Co. Na dithionite and dimethyl sulfoxide were obtained from Merck-Schuchardt. Other chemicals used in this study were reagent-grade commercial products.

Measurements of intracellular pH. The intracellular pH of *P. chrysosporium* pellets was determined by spectrophotometry (26, 32, 33) adapted to diffuse reflectance. The pH-sensitive dye CF served as the intracellular pH indicator.

To obtain *P. chrysosporium* pellets with CF entrapped within fungal cells, a cell membrane-permeable diacetate ester dye was added to a final concentration of 40 μM from a 10 mM stock solution in dimethyl sulfoxide to 100 ml of culture medium containing approximately 0.6 g (dry biomass weight)/100 ml. Cellular esterases then split the diacetate ester to CF. After 20 min of incubation, the pellets were centrifuged at $200 \times g$, blotted onto thick chromatographic paper, and placed into the PTFE cuvette. Spectra were taken over 375 to 555 nm, with untreated pellets as a blank.

The intracellular pH values were determined from a calibration curve prepared after equilibration of intracellular and extracellular pH with the ionophore technique (32, 33). Pellets were removed from the culture medium, resuspended in 0.01% (wt/wt) gramicidin–40 μM CF diacetate esters–65 mM KCl–solutions of 0.1 M phosphate buffers of various pHs, and equilibrated. The diffuse reflectance spectra of pellets loaded with CF at different intracellular pHs were then taken.

Determination of cytochromes and CO-binding pigments. Pellets from 30 ml of culture (approximately 0.2 g [dry biomass]), were separated by centrifugation ($200 \times g$), washed three times with 0.05 M potassium phosphate buffer (pH 7), and suspended in 20 ml of the same buffer. The pellets were then either reduced by the addition of 0.04 g of Na dithionite and incubated in an N_2 atmosphere for 10 min or oxidized with 0.8 ml of 30% H_2O_2 for 10 min. For determination of CO-binding pigment, CO was prepared from formic acid and concentrated sulfuric acid (34), purified by bubbling through a KOH solution, and then bubbled through the Na dithionite-reduced pellet suspension for 20

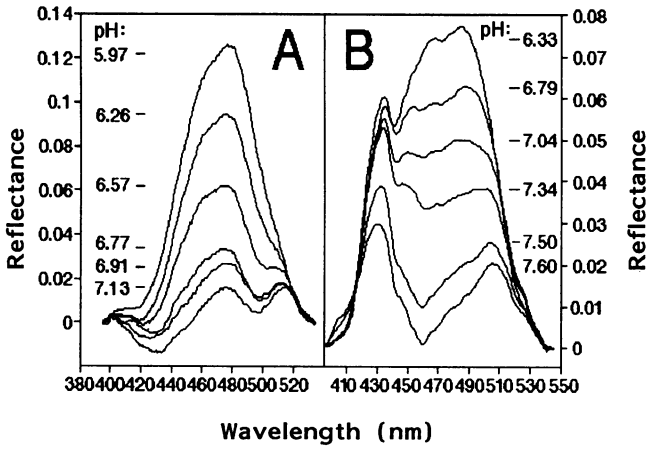

FIG. 2. Diffuse reflectance spectra of *P. chrysosporium* pellets (A) and *S. cerevisiae* cells (B) loaded with CF. Spectra were taken after equilibration of internal and external pHs.

min. Pellets were then separated by blotting on thick chromatographic paper and placed into the PTFE cuvette. Spectra of reduced, oxidized, and reduced and CO-bubbled pellets were taken at room temperature over 650 to 350 and 500 to 380 nm, with PTFE powder as a blank. Cytochromes were determined from dithionite-reduced minus H_2O_2-oxidized difference spectra, whereas CO-binding pigments were estimated from the CO-plus-dithionite-reduced versus dithionite-reduced difference spectra (35).

S. cerevisiae was concentrated by centrifugation at 600 × *g*. Yeast suspensions of approximately 50 g (wet weight)/100 ml were then reduced or oxidized, and cytochromes were determined as described for *P. chrysosporium*.

Determination of the redox state of mitochondrial cytochromes. Actual measurements were performed with the apparatus presented in Fig. 10.

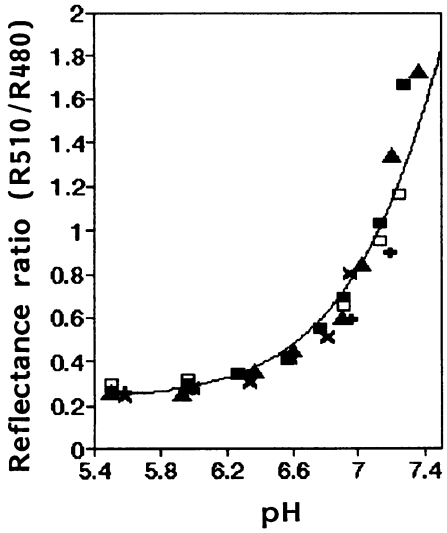

FIG. 3. Calibration curve for determination of intracellular pH in *P. chrysosporium* pellets with CF. Fungal pellets from five different batches, either 3-day-old (+, ×, and ▲)- or 4-day-old (□ and ■) cultures, were used. The ratio of reflectances at 510 and 480 nm was utilized.

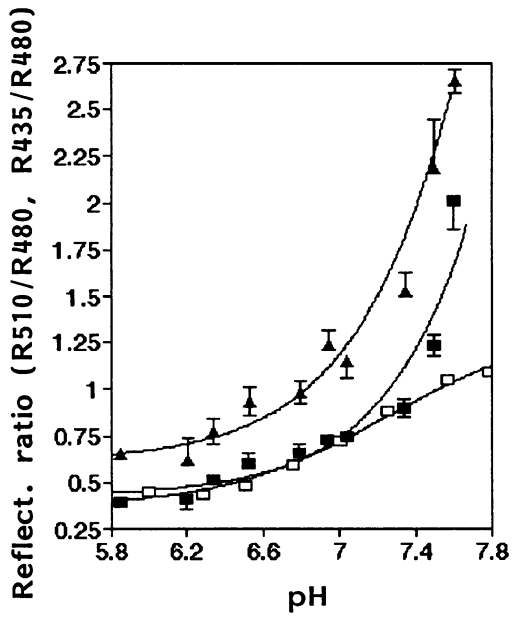

FIG. 4. In vitro calibration curve constructed for ratios of reflectances at 510 and 480 nm of CF spectra imbibed in chromatographic paper (□) and calibration curves for determination of intracellular pH in *S. cerevisiae* cells with CF. The ratios of reflectances at 510 and 480 nm (■) and 435 and 480 nm (▲) were used.

RESULTS

Intracellular pH. Figure 1A presents the transmission spectra of CF at several pH values in the aqueous 0.05 M phosphate buffer solution. Figure 1B presents the diffuse reflectance spectra of CF aqueous solution imbibed onto pieces of thick chromatographic paper with clean chromatographic paper used as a blank. Compared with the transmission spectra, the diffuse reflectance spectra showed different characteristics. The spectra of CF in 0.05 M phosphate buffer exhibited a maximum near 495, the absorbance increasing with pH. Spectra of CF imbibed in chromatographic paper had maxima at approximately 460 and 480 nm, and reflectance increased as the pH was lowered.

Figure 2 shows diffuse reflectance spectra of *P. chrysosporium* pellets and *S. cerevisiae* cells entrapped with CF and treated with the ionophore gramicidin to equilibrate intracellular pH and external pH. The spectra of CF imbibed in chromatographic paper (Fig. 1B) and in *P. chrysosporium* pellets (Fig. 2A) show shoulders or maxima at 460 and 480 nm, while the spectra of CF in *S. cerevisiae* cells (Fig. 2B) have maxima at approximately 435, 465, and 480 nm. The spectra of CF imbibed in chromatographic paper, *P. chrysosporium* pellets, and *S. cerevisiae* cells also had a shoulder at approximately 510 nm, which converted to a peak at higher pHs.

Calibration curves (Fig. 3 and 4) were constructed after the ratio (*R*) of the reflectance at 510 and 480 nm was calculated by a nonlinear least-squares fit of ratios to a standard pH titration curve (1, 3). These wavelengths were chosen because the reflectances of CF imbibed in chromatographic paper, in *P. chrysosporium* pellets, and in *S. cerevisiae* cells here were visually most pH insensitive and pH sensitive. A similar approach was used by Graber et al. (9). The best-fit pK_a values for CF in chromatographic paper, *P. chrysosporium* pellets, and *S. cerevisiae* cells were at 7.24 ±

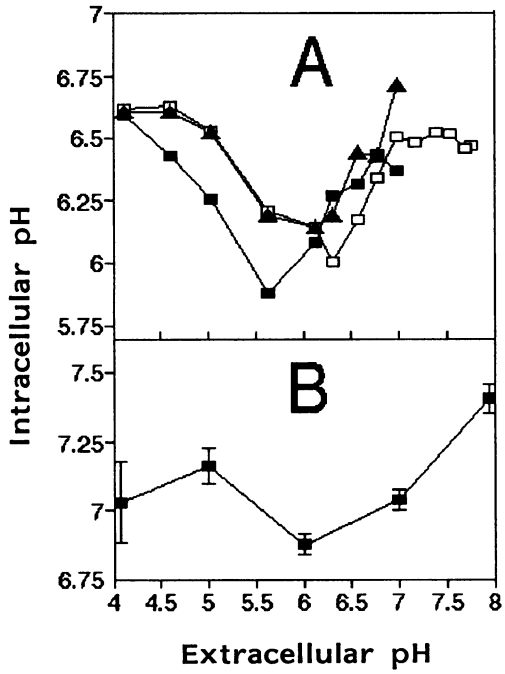

FIG. 5. Dependence of intracellular pH on extracellular pH in pellets of *P. chrysosporium* (A) and in *S. cerevisiae* cells (B) measured with CF. The pellets of *P. chrysosporium* from three different batches of 2 (□)-, 3 (■)-, and 4 (▲)-day-old cultures were used.

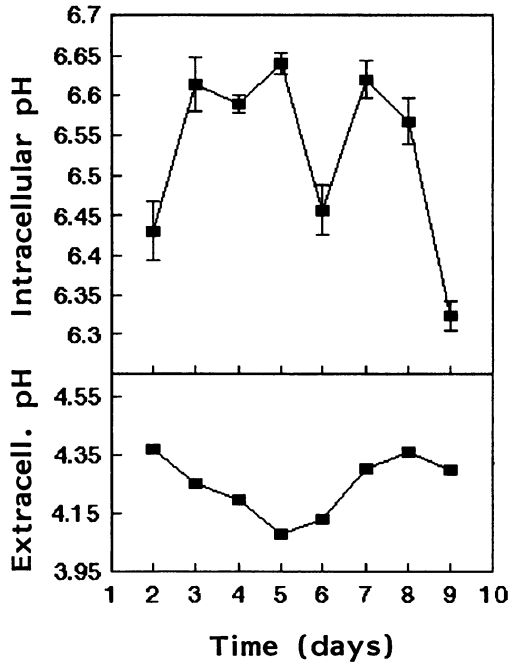

FIG. 6. Time profiles of pH in the growth medium and intracellular pH in *P. chrysosporium* pellets during the time of cultivation.

0.18, 8.01 ± 0.17, and 8.05 ± 0.31, with R_{min} and R_{max} at 0.36 and 1.33, 0.22 and 7.0, and 0.35 and 5.72, respectively. R_{min} and R_{max} represent limiting values of R at extremes of acid and alkaline pH. For *S. cerevisiae*, the calibration curve was also constructed for R at the reflectances at 435 and 480 nm (Fig. 4). This calibration curve was then used for intracellular pH determination in *S. cerevisiae* cells (Fig. 5B).

The effects of various concentrations of the dye, length of incubation of *P. chrysosporium* pellets in the growth medium with CF diacetate, and the time elapsed between blotting the pellets on filter paper and scanning for the intracellular pH were statistically insignificant. The mean values and standard deviations of intracellular pH for five concentrations of the dye from 10 to 80 μM, five different times of incubation from 20 to 100 min, and four different elapsed times after blotting of the pellets from 1 to 60 min were 6.638 ± 0.060, 6.696 ± 0.040, and 6.714 ± 0.060, respectively.

As indicated in Fig. 5, the intracellular pH in *P. chrysosporium* pellets and *S. cerevisiae* cells depended on extracellular pH, as also noted by others (27, 28, 30). Therefore, the intracellular pH in *P. chrysosporium* pellets (Fig. 6) was measured after the culture had been incubated in a medium with a set pH of 4.5. Figure 6 presents the changes in intracellular pH of *P. chrysosporium* pellets and the pH of growth medium during a typical fermentation.

Cytochrome determination. Figure 7 shows the diffuse reflectance spectra of cytochromes of aerobically grown *S. cerevisiae*. The reduced minus oxidized difference spectrum served as a biological blank for calibrating the spectrophotometer.

Figure 8 shows the diffuse reflectance difference spectra of *P. chrysosporium* cytochromes, recorded after reduction and oxidation of pellets with dithionite and H_2O_2. Peaks in the α region were observed at 546 nm, corresponding to cytochromes cc_1, at 555 nm, attributed to reduced cytochrome *b*; and at 598 nm, corresponding to aa_3 cytochromes.

Figure 9A indicates the presence of a CO-binding pigment in the pellets of *P. chrysosporium*, during culture. The

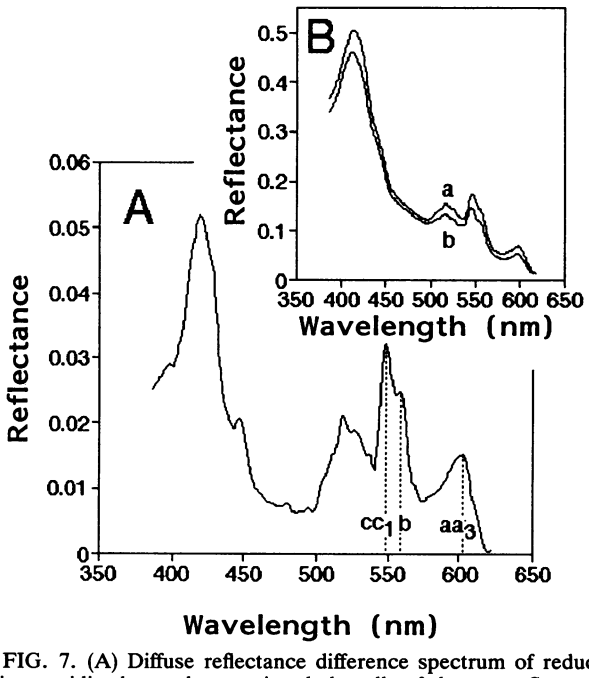

FIG. 7. (A) Diffuse reflectance difference spectrum of reduced minus oxidized cytochromes in whole cells of the yeast *S. cerevisiae*. (B) The diffuse reflectance spectra of reduced (a) and oxidized (b) yeast cells.

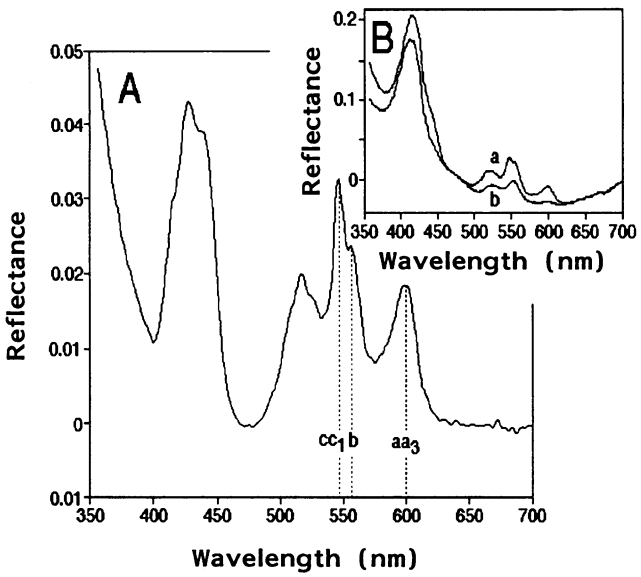

FIG. 8. (A) Diffuse reflectance difference spectrum of reduced minus oxidized cytochromes in whole pellets of 2-day-old *P. chrysosporium* culture. (B) Diffuse reflectance spectra of reduced (a) and oxidized (b) fungal pellets.

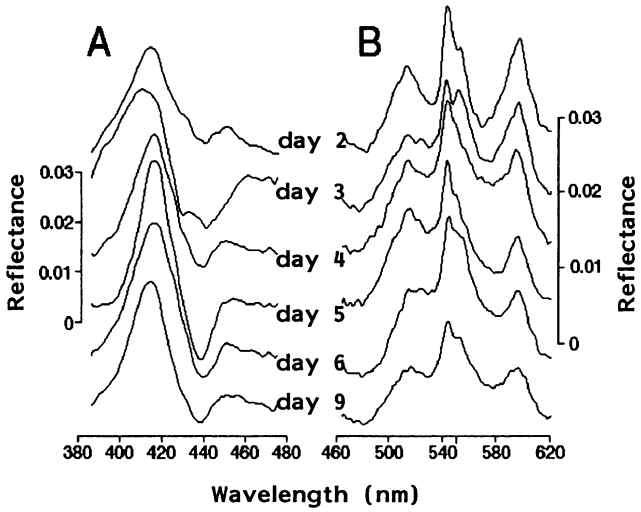

FIG. 9. Diffuse reflectance spectra of CO-binding pigments (A) and mitochondrial cytochromes (B) in the pellets of *P. chrysosporium*, during the time of cultivation.

intensity of absorption peaks of mitochondrial cytochromes decreased with the time of cultivation (Fig. 9B).

Redox state of mitochondrial cytochromes. On-line measurements of the redox state of mitochondrial cytochromes of *P. chrysosporium* were performed with the measurement system as shown in Fig. 10. The saturation of the perfusion substrate with oxygen was detected with a polarographic type of O_2 electrode. Oxygen saturation was controlled with O_2 and N_2 bubbling through the substrate in a mixing cuvette. The 100 and 0% of reduction levels of cytochrome aa_3, with a peak located at 600 nm, were determined by using substrate flow saturated with N_2 or O_2.

Diffuse reflectance spectra of mitochondrial cytochromes were taken at different oxygen saturation levels of the perfusion substrate. As presented in Fig. 11 and 12, the redox states of respiratory cytochromes and particularly cytochrome aa_3 of *P. chrysosporium* were strongly dependent on the oxygen saturation of the perfusion substrate.

The rapid reduction of the terminal member of the respiratory chain, cytochrome aa_3, of 1- to 2-day-old cultures was observed when oxygen saturation of the perfusion substrate was less than 40%. With 3- to 4-day-old cultures, the reduction rate of cytochrome aa_3 was slower and started at about 60% oxygen saturation of the substrate (Fig. 12).

DISCUSSION

In the experiments presented in this paper, the basic principles of intracellular pH and cytochrome UV-VIS spectrophotometric determination usually used for bacteria, yeasts, and animal and plant cells, were also applied to fungal pellets. The differences are in the use of diffuse reflectance instead of transmittance or fluorescence spectroscopy, in the procedure applied for sample preparation, and in the morphological and structural properties of the microorganism.

When we tested, we found that nonfluorescent dyes ($CuSO_4$, $CoCl_2$, and methyl orange in pH 8 phosphate buffer) either in aqueous solution or imbibed onto thick chromatographic paper have similar transmittance and diffuse reflectance spectra. Furthermore, the diffuse reflectance spectrum of intracellular veratryl aldehyde in pellets of *P. chrysosporium*, generated by intracellular enzymatic oxidation of veratryl alcohol, is again similar to transmittance spectra of veratryl aldehyde (18). The diffuse reflectance spectra of cytochromes in *S. cerevisiae* cells and *P. chrysosporium* pellets (Fig. 7 and 8) also have the well-known and expected shape (4). The transmittance and diffuse reflectance spectra of fluorescent dyes such as CF are, however, different (Fig. 1). The reason is in the construction of the integrating sphere which is designed to collect total radiant energy which is reflected and also emitted from the fluorescent sample. When in the integrating sphere the fluorescent sample is exposed to monochromatic radiation, there is a wavelength range where the fluoroprobe is excited and fluorescence occurs. The emitted fluorescence is at higher wavelengths, but the integrating sphere is unable to differentiate between wavelengths, seeing only total radiation energy. Therefore, fluorescence is recorded at the excitation wavelength of the sample together with reflected light at that wavelength (31). The eye sees the fluorescence radiation at its emission wavelengths. Thus, for the experiments for which it is important to obtain data consistent with visual observation, as, for example, in the evolution of fluorescent textile brighteners, other constructions of an integrating sphere are used. Here the sample is illuminated by white light, and the reflected and fluorescent radiation is dispersed by the monochromator before reaching the detector. The fluorescence is then measured at its emission wavelength in addition to the reflectance at that wavelength (31). The differences in CF spectra consequently occur, since transmittance spectra (Fig. 1A) contain information about absorbance properties, while diffuse reflectance spectra (Fig. 1B) contain mixed informations about absorbance and fluorescence properties of the dye. The same results were obtained with fluorescein (data not shown).

The sample preparation applied in our experiments is simpler than that used in transmittance or fluorescence spectroscopy. Scanning of solid samples prevents troubles

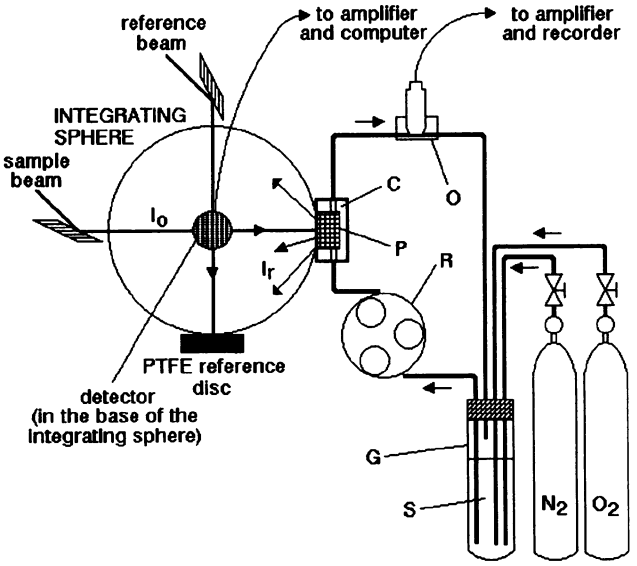

FIG. 10. Schematic diagram of the system for in vivo monitoring of the redox state of the respiratory cytochromes in *P. chrysosporium* pellets. Perfusion substrate (S) (50 ml) was pumped, with a flow rate 10 ml of substrate per min, from a gas mixture cuvette (G) by the roller pump (R) through PTFE-glass sample cuvette filled with a pellet suspension (P). The perfusion substrate then passed the flowthrough cuvette with an O_2 electrode (O) and returned to the gas mixture cuvette. I_0, incident sample beam; I_r, reflection radiation. Arrows indicate the direction of flow.

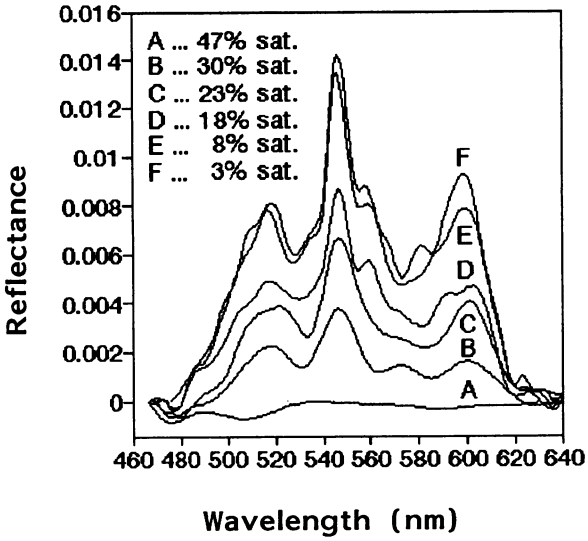

FIG. 11. Diffuse reflectance spectra of respiratory cytochromes in whole pellets of *P. chrysosporium* at the day 2 of cultivation. Spectra were taken at different oxygen saturations of the perfusion substrate.

caused by dye leaking from cells suspended in aqueous solutions.

The yeast *S. cerevisiae* was used as the test microorganism to determine whether the differences in sample preparation and morphological and structural differences of microbial samples have an influence on intracellular pH and cytochrome determination with diffuse reflectance spectroscopy. The reflectance of CF entrapped in *P. chrysosporium* (Fig. 2A) and in test *S. cerevisiae* cells (Fig. 2B) as well as the reflectance of CF imbibed in chromatographic paper (Fig. 1B) decrease similarly with increasing pH. The calibration curves constructed on a base of reflectances ratio at 510 and 480 nm (Fig. 3 and 4) are also similar. Similar results were obtained when fluorescein was used as the intracellular pH indicator and the ionophores nigericin and salinomycin were used for pH equilibration (data not shown).

The possible reason for differences between in vitro (Fig. 1B) and in vivo (Fig. 2) diffuse reflectance spectra and calibration curves (Fig. 3 and 4) are metabolism of the dye, protein-dye binding interactions (1), and differences in structure of fungal pellets, yeast cells, and filter paper which might influence optical processes. The intracellular calibration with ionophores eliminated the need to correct for these differences, and the ratio method minimized the variations due to concentration differences or instrument fluctuation (2, 24).

In Figure 5, there is a valley in the intracellular pH curves of approximately 0.5 and 0.3 at an external pH of 6 for pellets of *P. chrysosporium* and for *S. cerevisiae* cells. The valley of 0.2 pH at an external pH of 5.5 was determined for *P. chrysosporium* (data not shown) when fluorescein was used as the indicator. Fluorescein also measures mitochondrial pH and not just pH in cytosol as CF does (33). In the paper of Slavik (27), in whose experiments CF was used as a pH indicator, the valley in intracellular pH curves of 0.2 to 0.3 at an external pH of 4.5 are presented for the yeasts *Torulopsis versatilis*, *Endomyces magmusii*, and *Lodderomyces elongisporus*. The possible reasons for these phenomena are the adaptation of microorganisms to changes in external pH, but the actual mechanism is not known.

The drop of intracellular pH at day 6 during the cultivation of *P. chrysosporium* (Fig. 6) was found to coincide with the onset of *P. chrysosporium* ligninolytic activity and with the temporary stop of the fungal biomass dry weight increase (data not shown). This drop occurred regularly in our experiments and was more intensive in cultures of *P. chrysosporium* in which ligninolytic activity was enhanced by addition of Tween 80.

The difference diffuse reflectance spectra of cytochromes in *P. chrysosporium* pellets (Fig. 8) and in test *S. cerevisiae* cells (Fig. 7) have the expected shape (4): characteristic peaks for cytochromes aa_3, b, and cc_1 in the α region (Fig. 9B), a gap at approximately 465 nm, indicating flavoprotein (all the cytochromes absorb light in the reduced state, but only flavin adenine dinucleotide absorbs in the oxidized state), and peaks at the Soret band. Very similar difference diffuse reflectance spectra of cytochromes were also obtained when pellets of the other filamentous fungi, *Claviceps paspali* and *Rhizopus nigricans*, were scanned (data not shown).

Monitoring the actual redox state of the respiratory enzymes (Fig. 10 to 12) provides an insight into the cellular energy supply and demand. The terminal part of the respiratory chain, cytochrome aa_3, is responsible for more than 90% of the oxygen consumption of a living cell (12, 15). The redox state of cytochrome aa_3 (Fig. 12) reflects the ratio between the cellular energy pool and the oxygen available for respiration, and it can reliably determine the cell oxygen supply (10). Since average substrate oxygen saturations during the time of *P. chrysosporium* culture were 16, 16.5, 17.5, and 20% at days 1 to 4, respectively, about 45% of the reduction of cytochrome aa_3 can be expected for the first 2 days and 65% for the third and fourth day, according to the

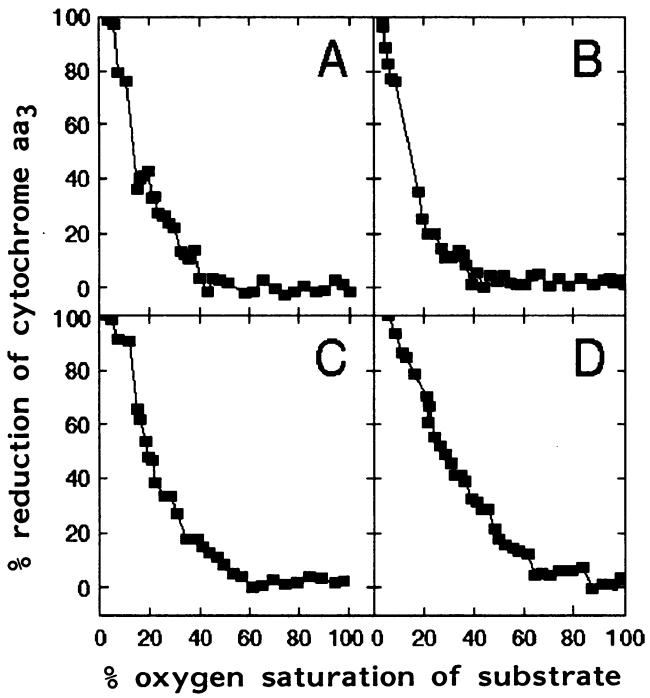

FIG. 12. Redox state of cytochrome aa_3 in *P. chrysosporium* pellets. Percent of reduction versus oxygen saturation of the perfusion substrate was determined after day 1 (A), day 2 (B), day 3 (C), and day 4 (D) of cultivation.

data plotted in Fig. 12. Since at the time of these measurements the concentration of glucose, the main energy source, was not the limiting factor, these data indicate that oxygen is in fact the limiting substrate. Dosoretz et al. (6) studied the effect of oxygenation conditions on submerged cultures of *P. chrysosporium*. They used CO_2 evolution rate to measure metabolic activity of cultures periodically flushed with air or oxygen. In cultures flushed with oxygen, they determined a higher CO_2 evolution rate and faster glucose consumption rate, indicating again that oxygen is a limiting factor for metabolic activity.

The results illustrate that a spectrophotometer coupled with an integrating sphere is a powerful tool in the study of fungal physiology. Besides the fact that diffuse reflectance allows one to apply some spectrophotometric methods to light-impermeable structures, it has other advantages. (i) The analyzed material is in the solid state. Thus, processes external to the pellets can be minimized. (ii) There is minimum sample preparation. In vivo measurements are therefore possible. (iii) The method is rapid enough to study transient states.

The main obstacles for further implementation of this method are expected to be difficulties in the interpretation of diffuse reflectance spectra because of the complexities of microbial structures as well as of the optical processes of light reflectance, absorbance, and emission.

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