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Transformation of wheat straw in the course of solid-state fermentation by four ligninolytic basidiomycetes

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Abstract

Biological upgrading of wheat straw by the white-rot fungi *Phanerochaete chrysosporium*, *Pleurotus eryngii*, *Phlebia radiata*, and *Ceriporiopsis subvermispora* was monitored during 60-day solid-state fermentation. Analysis of straw included determination of weight loss and lignin content, color analysis, and infrared spectroscopy, whereas the studies on the water-soluble fractions were carried out by infrared spectroscopy, elementary analyses and quantification of the total phenols and reducing sugars. The most selective degradation of lignin was produced by *P. eryngii* and especially by *C. subvermispora*, the former species releasing the greatest amount of colored water-soluble products, whereas an increase in straw brightness was caused by *C. subvermispora*. In general, the composition of the water-soluble fraction correlated with the extent of straw transformation. The initial fermentation stage (0–15 days) was characterized by the accumulation of water-soluble products from lignin degradation and fungal metabolism, the concentration of which tended to stabilize in the second stage (16–60 days). The degree of delignification at the second stage tended to coincide with the decrease of the water-soluble nitrogen.

Keywords: Ceriporiopsis subvermispora; Pleurotus eryngii; Phanerochaete chrysosporium; Phlebia radiata; Solid-state fermentation; Straw biopulping

1. Introduction

Cereal straw is a major agricultural byproduct that can be upgraded successfully by microbial treatment. Investigations on straw biodelignification mostly have implied treatments with white-rot fungi to obtain carbohydrate-enriched substrates for animal feeding or, subsequently, sugars and/or ethanol by using further enzymatic transformations [1,2]. However, application of lignin-degrading fungi in the pulp and paper industry has the greatest economic importance, and much attention has currently been drawn to the development of new environmentally friendly technologies for paper and paperboard manufacture [3–7]. The high degree of esterification of the aromatic domain in straw lignocellulose, as compared with wood, favors defibrillation by low-energy reactions [7–9]. Ligninolytic basidiomycetes are able to cause specific changes in lignin content and structure, which lead to fiber individualization and decolorization of substrate, both intended in the pulp and paper industry [10–14].

The concept of pulp quality is complex and can be defined by a series of independent factors [8,15]. Whereas papermaking properties of refined pulps largely depend on the beating degree (i.e. energy applied during mechanical defibrillation), in the case of nonrefined lignocellulosic materials (such as fungal-treated substrates), the quality tests are frequently focused on chemical and optical characteristics (such as lignin removal and substrate decolorization). However, in both cases, the selection of the most suitable fungal species for biopulping requires research on the changes produced in the lignocellulosic substrates during fungal treatment. Such studies include monitoring of several parameters that reflect substrate degradation patterns characteristic of the different microbial strains, with special emphasis on the counterbalance between weight loss and lignin removal in the upgrading substrate [15,16].

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In this study, a series of analyses was carried out to monitor the degree of transformation of small amounts of straw treated with the ligninolytic fungi *Phanerochaete chrysosporium*, *Pleurotus eryngii*, *Phlebia radiata*, and *Ceriporiopsis subvermispora* during a 60-day incubation period.

2. Materials and methods

2.1. Fungal species

The strains of ligninolytic basidiomycetes used in the present study as well as their potential for lignin degradation have been described elsewhere [3,13,17–24]. *Phlebia radiata* 79 (ATCC 64658) was obtained from the culture collection of the Department of Applied Chemistry and Microbiology, University of Helsinki, Finland. *Phanerochaete chrysosporium* VKM F1767 (ATCC 24725) and *C. subvermispora* FP 90031-sp were obtained through the courtesy of M. Leisola (presently at Helsinki University of Technology, Espoo, Finland) and R.A. Blanchette (Department of Plant Pathology, University of Minnesota, St. Paul, MN, USA), respectively. *Pleurotus eryngii* IJFM A1869 was obtained from the culture collection of the Center of Biological Research (CIB, CSIC, Spain).

2.2. Solid-state fermentation (SSF)

Wheat *(Triticum aestivum)* straw was treated with fungi in 250-cm^3 Erlenmeyer flasks containing 2 g of straw collected in Zaragoza (Spain), chopped to 2–4 cm, and autoclaved for 15 min at 120°C. *Pleurofus eryngii* inoculum was prepared by using static cultures in 500-cm^3 flasks containing 50 cm^3 of the medium described earlier [25]. After development of the fungal mat, the mycelium was homogenized and incubated at 160 rev./min for 4 days in a flask with 100 cm^3 of the above-described medium. The pellets were washed with water, and the straw was inoculated with 6 cm^3 of this fungal suspension.

Cultures of *P. radiata* and *C. subvermispora* were inoculated with mycelial suspension (4%) prepared from the liquid cultures in low nitrogen asparagine ammonium nitrate dimethyl succinate medium [18]. Cultures of *P. chrysosporium* were inoculated with 0.2 cm^3 of spore suspension.

2.3. Color analysis

Several methods were tested to quantify the differences in pulp color at various stages of fungal transformation. Conventional methods based on visual comparisons with reference charts were discarded. On the other hand, methods involving diffuse reflectance spectrophotometry require expensive instrumentation. The method used was based on analysis of the sample images (acquired with a flatbed scanner connected to a personal computer) with a commercial image processor software. Because the determination of color requires homogeneous flat surface, a series of 1.3-cm Ø wafers were prepared from 50 mg of sample milled to <250 μm and compacted in a standard press for sample preparation in infrared (IR) spectroscopy. The samples were compacted to $9 \cdot 10^3$ kg for 3 min. The resulting wafers were placed in a HP Scanjet IIcx and the image files (including the whole set to be analyzed) were acquired at 200% size in the 16.78 Megacolor mode and were stored in JPG format. The images were examined by using the Photostyler 2.0 software [26]. To extract the different color components from the samples, a square area was defined in each wafer image with the color picker tool, and an average color for the selected region was stored as the foreground color. When the foreground color was activated (color editing tool), a pop-up menu showed the color components. The numeric values were taken in the two usual ways to define color components: the RGB (red, green, blue) method for electronic color composition and the CYMK (cyan, yellow, magenta and black) method for color printing. In addition, the HSB components (hue, saturation, brightness) also were extracted automatically. For comparative purposes, the numerical values were normalized by adjusting to 100 the data in the control wafer. Similar calculations also were carried out with the other physical and chemical parameters of the straw to homogenize the values at zero time, reducing to nonsignificant the differences in the original substrate, which included straw as well as fungal biomass. The results of analyses were presented in graphs, where the differences between the species could be recognized more straightforwardly.

2.4. Infrared spectroscopy

A Bruker Fourier-transform IR spectrophotometer and wafers with 200 mg of KBr and 3 mg of sample were used. A total of 100 interpherograms was accumulated in the 4 000–400 cm^{-1} range. The spectra were subjected to baseline subtraction. The following bands were selected as index peaks reflecting chemical changes in the lignocellulosic matrix [27]: 1 510 cm^{-1} and 1 610 cm^{-1} (aromatic bonds), 1 740 cm^{-1} (nonconjugated carbonyl groups), 1 660 cm^{-1} (conjugated carbonyl groups), 1 460 cm^{-1} (alkyl bending vibration), 1 330 cm^{-1} (syringyl ring breathing), 1 270 cm^{-1} (guaiacyl units), and $\approx$1 040 cm^{-1} (pyranoside ring vibrations).

2.5. Chemical analyses

Water-soluble fraction was extracted at room temperature in 40-cm^3 centrifuge tubes (1:30 solid/water ratio) and dried on Petri dishes at 40°C. After weighing, the dried yellowish fraction was scrapped off and used for additional spectroscopic and chemical analyses by using solutions adjusted to 5 mg/cm^3. The lignin was gravimetrically determined from 1 g of sample after Saeman's hydrolysis and expressed as oven-dry, ash-free basis [28] content.

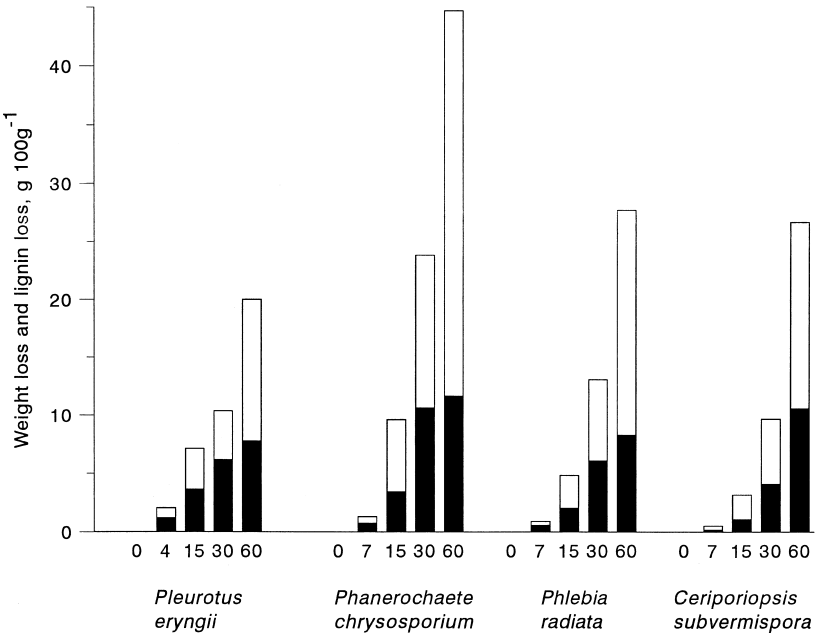

Fig. 1. Weight loss (total bar height) and lignin loss (solid bar) of wheat straw in the course of a 60-day biopulping experiment with four ligninolytic basidiomycetes.

The water-soluble fraction (4 mg in 200 mg of KBr) was used for Fourier-transform IR spectroscopy as indicated above. Elementary composition (C, H, and N) analyses were carried out with a Carlo-Erba EA 1108 microanalyzer. The 0% was obtained by difference to 100 (ash-free percentages) and the H/C and O/C atomic ratios were calculated [29] because these atomic ratios may provide an insight in the nature of the major chemical constituents accumulated in the water-soluble fraction such as aromatic structures and carbohydrates.

Other parameters that could be affected by fungal treatment are related to the color intensity of the extract. Specific extinction at 465 nm (E_4) was determined by using the solution adjusted to a constant concentration. It is considered that E_4 of colloidal humic-like substances reflects the relative amount of aromatic structures [30]. Moreover, the extinction ratio at 465 and 665 nm (E_4/E_6) is considered to depend mainly on the polydispersity [31], decreasing with increasing molecular size.

Additional analyses carried out in the water-soluble extract were estimation of (i) total phenolic compounds (Folin–Ciocalteau reagent) [32], and (ii) total reducing power (Somogyi–Nelson method) [33] providing information on free sugar in the extract.

3. Results

3.1. Weight loss and lignin degradation

The greatest weight loss was caused by *P. chrysosporium* (up to 45% after 60 days), which degraded the straw substrate much faster than the other fungal species, particularly after 15 days of growth (Fig. 1). Substrate weight loss in cultures of *P. eryngii*, *P. radiata*, and *C. subvermispora* was between 20% and 30% at the end of incubation period. For these fungi, the optimal fermentation period was 15–30 days, when the loss of dry matter was relatively low compared to the extent of lignin removal. By 30 days of incubation, *P. eryngii* caused very selective lignin degradation (Fig. 1). On the contrary, *P. radiata* produced higher weight loss than the other two species. Results obtained with *P. eryngii* and *C. subvermispora* coincided with previous reports on these species as selective lignin degraders suitable for biopulping [3,6–9,13,19,21–24]. The low selectivity of *P. chrysosporium* for lignin degradation at prolonged fermentation stages confirmed data reported earlier [17]. In general, the original lignin content in the straw (19%) was reduced by fungal treatments to values between 12% and 15% after the whole fermentation period.

3.2. Alterations of straw color caused by fungal treatments

There were no highly significant increases of substrate brightness. The fungal treatment did not result in improved substrate bleaching with respect to the control. *Ceriporiopsis subvermispora* was the only species causing an increase in brightness at the end of the fermentation, whereas treatment with *P. eryngii* led to the accumulation of colored products (Fig. 2a). However, with all fungi the color of the straw changed qualitatively, i.e. there was a significant decrease in the intensity of the blue and green components, whereas modification of the red one depended on the fungal

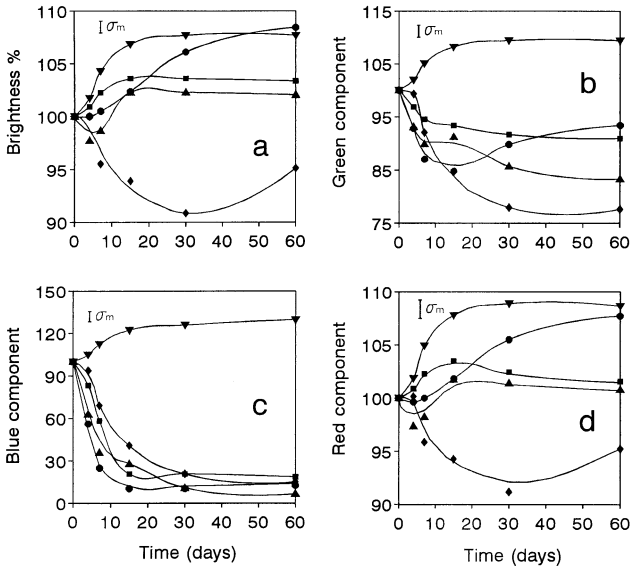

Fig. 2. Color analysis in wheat straw treated with fungi (relative to the values obtained at zero time). Control (▼), *P. chrysosporium* (■), *P. radiata* (▲), *P. eryngii* (♦), *C. subvermispora* (●). Error bars indicate average SD between duplicated runs.

species (Fig. 2b–d). The result was the hue shift in the straw toward the yellowish color.

3.3. Alterations in lignocellulose as determined by IR spectroscopy

Selective removal of lignin could be reflected indirectly by the intensity of the bands in the region centered around 1040 cm^{-1}, in which pyranoside rings of carbohydrate make large contribution (Figs. 3 and 4). The relative intensity of these bands increased during treatment with *C. subvermispora*, whereas with the other fungal species intensities of carbohydrate bands did not change so significantly or even decreased during the first 15 days of cultivation (Fig. 4a). Similar tendency also was reflected by the aliphatic/aromatic ratio (in terms of the 1460 : 1510 cm^{-1} intensity ratio) which increased with *C. subvermispora* although in the case of *P. eryngii*, *P. chrysosporium*, and *P. radiata* the most important increase was observed in the second stage of incubation (Fig. 4b). The increase with cultivation time in the intensities of the 1 740 cm^{-1} and the 1 660 cm^{-1} bands (for unconjugated and conjugated carboxyl groups, respectively) pointed to oxidative alteration of aromatic lignin moieties, which was comparatively more rapid in the cultures of *C. subvermispora* and *P. radiata* (Fig. 4c, d).

3.4. Changes in the water-soluble fraction caused by fungal treatments

It is well known that, in straw compost, the water-soluble fraction indirectly reflects maturity in terms of the concentration and degree of alteration of the insoluble straw macromolecular fractions [34].

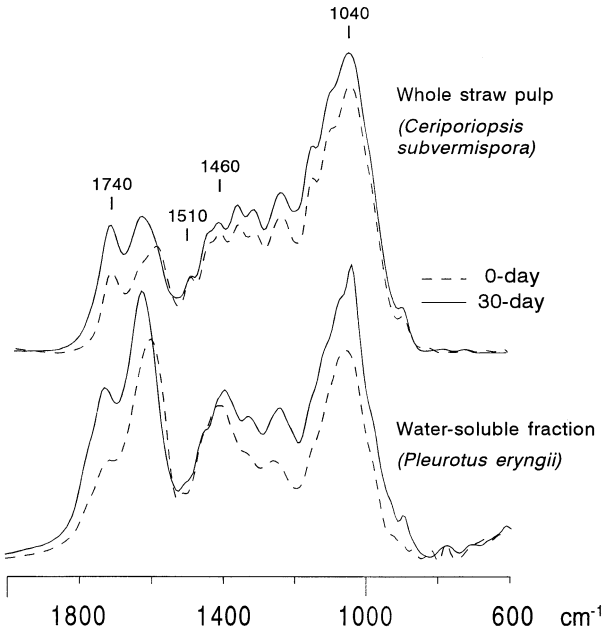

Fig. 3. Infrared spectra of whole wheat straw treated with *C. subvermispora* at 0 days and at 30 days (top) and of the water-soluble fraction obtained after treatment with *PH. eryngii* at 0 days and at 30 days (bottom).

Because the preliminary characterization of the fungal-treated straw indicated rather small degree of chemical transformation of the straw substrate during SSF, at least in comparison with the extent of the changes observed with mild chemical treatments [8], the water-soluble fraction of straw (consisting of a pool of reactive molecules) was studied in more detail. In general, substrate changes did not point to an extensive process of lignin degradation but can

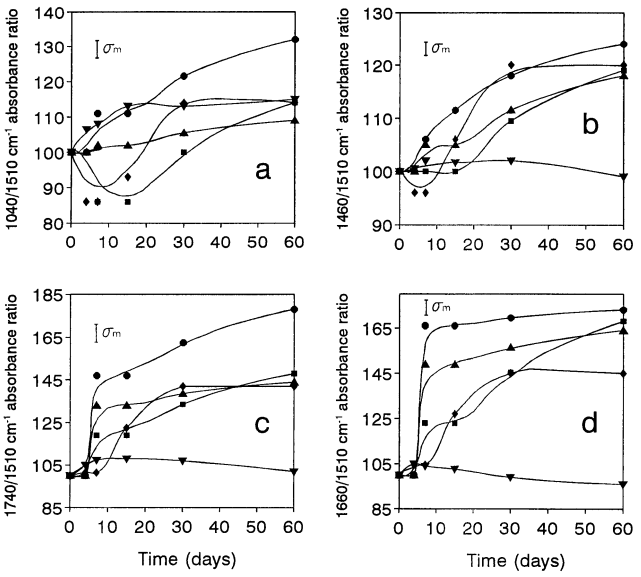

Fig. 4. Changes during incubation in the relative intensity of bands in the IR spectra of wheat straw degraded by fungi. Control (▼), *P. chrysosporium* (■), *P. radiata* (▲), *P. eryngii* (♦), *C. subvermispora* (●).

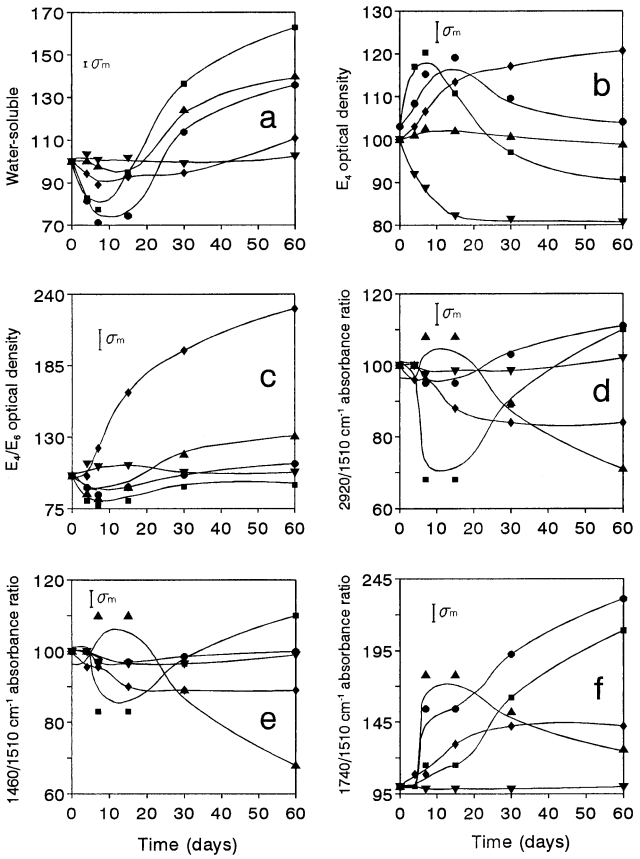

Fig. 5. Straw water-soluble fraction: Total content (a), 460-nm optical density (b), E_4/E_6 ratio (c), and relative intensities of bands in the IR spectra (d–f). Control (▼), *P. chrysosporium* (■), *P. radiata* (▲), *P. eryngii* (♦), *C. subvermispora* (●).

be interpreted as an oxidative alteration of aromatic structures in straw, resulting in the formation of the water or alkali-extractable oligomer fractions.

Among the fungi studied in the present work, *P. eryngii* and *C. subvermispora* produced Mn^{2+}-oxidizing peroxidase (MnP, EC 1.11.1.14) but not lignin peroxidase (LiP, EC 1.11.1.13), and the former has been described as the major ligninolytic enzyme involved in lignin degradation by these fungal species [35,36]. As *P. chrysosporium* and *P. radiata* also produced MnP [37–39] (and LiP), MnP most likely is responsible for solubilization of straw lignin by the fungi studied. Both suitable level of manganese in straw (11.4 mg/kg) and the presence of Mn^{3+} chelators (organic acids), commonly produced by the fungi, favored the catalytic activity of MnP [37–40]. Initial lignin depolymerization by MnP has been suggested earlier in studies on the synthetic lignin (DHP) degradation by *P. chrysosporium*.

In this study, except for *P. radiata*, the total concentration of water-soluble substances decreased during the first 15 days, to increase thereafter (Fig. 5a). Results obtained during the initial 15 days of incubation correspond to the early delignification stage, in which straw soluble products were consumed by the fungi, followed by the advanced stage (days 15–60) when secondary metabolites and/or some products of lignin breakdown accumulate in the substrate. This virtual two-stage process (also coincided with the previous studies on wheat straw degraded by *P. ostreatus*) [17] was confirmed by further analyses on the water-soluble straw fraction discussed below.

3.5. Optical density

The optical density of the water-soluble extract, adjusted to a constant concentration, showed a neat increasing tendency only in the case of *P. eryngii* (Fig. 5b), which pointed to the accumulation of soluble aromatic products upon fermentation time. This observation coincided with the above-described results about decrease of straw brightness caused by *P. eryngii.* With the other fungal species, such tendency was observed only in the initial stage (0–7 days).

Regarding the E_4/E_6 ratio, *P. eryngii* (Fig. 5c) also showed progressive tendency for decrease in molecular weight of water-soluble compounds. Valleys in curves of the other fungi corresponding to 7-day-old cultures indicated consumption of polymer or oligomer substances at the early degradation stage, which were later substituted by other relatively simple products.

3.6. IR spectroscopy

The IR profiles of the water-soluble straw fraction obtained from 30-day-old culture of *P. eryngii* (Fig. 3, bottom) had broad bands, which corresponded to a complex mixture of carbohydrates, amino acids, phenolic acids, and high-molecular-weight polyelectrolytes of undefined structure [41]. The IR spectra (Fig. 5d) showed a decrease in the intensity of the bands related to aliphatic structures (2 920 cm^{-1} and 1 460 cm^{-1}), which indicates accumulation of aromatic products at the second stage of SSF (15–60 days) and coincides with the data inferred from the E_4 values (Fig. 5b) for *P. eryngii.* A similar process occurred, but at more advanced stage of degradation, in cultures of *P. radiata* (Fig. 5d, e). In general, the curves suggested the two-stage transformation, more pronounced in treatment with *P. chrysosporium,* where aromatic products accumulated in the water-soluble fraction only during the first 4 to 7 days. The relative intensity of the 1 740 cm^{-1} peak (Fig. 5f) indicated that, except for *P. radiata* at the final stage, the water-soluble fraction might consist of a pool of oxidized products such as phenolic acids or cinnamyl esters. In addition, the intensity of the 1 040 cm^{-1} peak (Fig. 6a) suggested that soluble carbohydrate might be the aliphatic product accumulated at the final cultivation stages.

The above-described results are complementary to those obtained by the direct determination of the major water-soluble compounds such as phenolics and reducing sugars (Fig. 6, c). Colorimetric determination of phenolic compounds reflected their accumulation in the cultures of *P. eryngii*, whereas the decrease in the total phenols was ob-

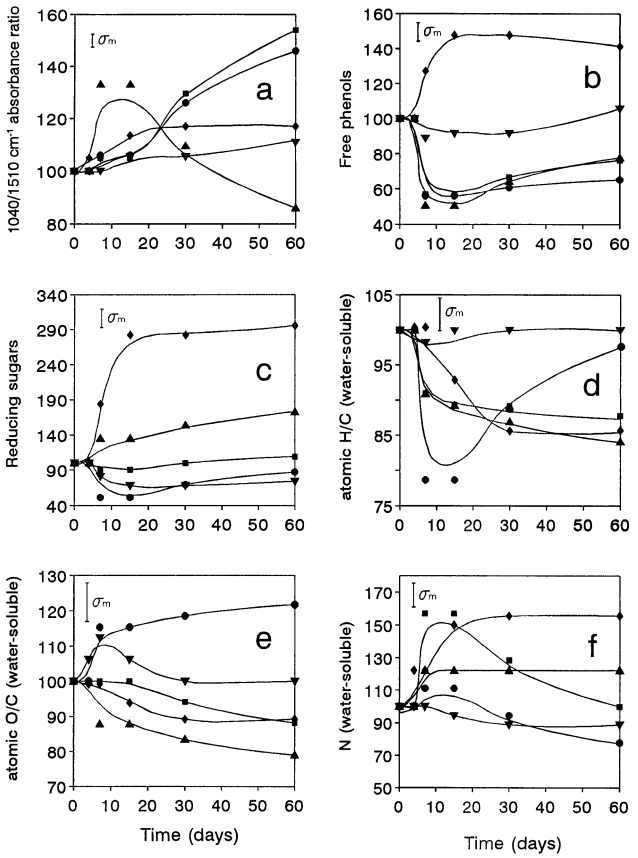

Fig. 6. Straw water-soluble fraction: relative intensity of the 1 040 cm^{-1} IR band (a), concentration of phenols and reducing sugars (b and c), atomic ratios (d and e) and nitrogen concentration (f). Control (▼), *P. chrysosporium* (■), *P. radiata* (▲), *P. eryngii* (♦), *C. subvermispora* (●).

served in the case of the other fungal species (Fig. 6b). The results of reducing sugar determination (Fig. 6c) confirmed conclusions inferred from the IR analyses and showed accumulation of monosaccharides mostly in *P. eryngii* and *P. radiata* cultures.

3.7. Elementary composition

The atomic H/C ratio of the water-soluble straw fraction tended to decrease in all species, especially in the cultures of *C. subvermispora* at the earlier degradation stages (Fig. 6d), which could be due to the concentration of lignin-derived water-soluble compounds. Unlike to the other fungi studied, the H/C ratio increased in the water-soluble straw fraction obtained from *C. subvermispora* cultures at the advanced stages of SSF. Correspondingly, the O/C atomic ratio increased only in *C. subvermispora* cultures (Fig. 6e), which also coincided with the intensity of the IR carbonyl vibrations (Fig. 5f).

Differences in the concentration of the water-soluble nitrogen forms were comparatively more significant (Fig. 6f). As the total nitrogen concentration in the fermented straw remained constant (degradation was not accompanied by ammonia volatilization), distribution of nitrogen in the substrate during fungal transformation (e.g. its concentration in soluble forms) could reflect different degradation stages. Straw degradation by all the fungi was accompanied by release of soluble nitrogen during the first 15 days, although it tended to decrease at the advanced stage of degradation by *P. chrysosporium* and *C. subvermispora* (Fig. 6f). Nitrogen in straw cell walls mainly is incorporated to plant proteins, which apparently are covalently linked to polysaccharides [42]. Thus, release of nitrogen in the straw water-soluble fraction could be associated with the degradation of carbohydrate components in the substrate. Correspondingly, *P. chrysosporium*, which is an active carbohydrate-degrading species, released the highest amounts of soluble nitrogen during the first 15 days of straw fermentation (Fig. 6f). At final incubation stages, along with increase of fungal biomass, nitrogen is immobilized in mycelia and could be re-used by the fungi upon mycelial autolysis.

4. Discussion

Four ligninolytic basidiomycetes known as efficient lignin degraders were used for treating wheat straw. These fungi possess different systems of ligninolytic and cellulolytic enzymes, and accordingly, they did not cause similar patterns of straw transformation. However, all fungi studied produced Mn^{2+}-oxidizing peroxidase, which probably played a major role in straw lignin modification, causing the formation of water-soluble products from lignin degradation. Solubilization effect on lignin has been reported earlier by other authors [40]. Depolymerization of milled wheat-straw, milled-straw lignin, and synthetic lignin (DHP) by isolated MnP have been described as accompanied by the formation of the water-soluble low-molecular-weight fragments [43,44].

Pleurotus eryngii and *C. subvermispora* caused the minimal substrate weight loss, rapidly degrading lignin during the first 30 days of growth, whereas *P. chrysosporium* was selective only at the very early fermentation stage (7 days). Although *P. eryngii* was highly selective for lignin degradation, it released the greatest amount of low-molecular-weight aromatic compounds, which is unfavorable when the color removal is intended. However, alkaline extraction after biotreatment could improve substrate brightness. The best results in terms of both selective degradation of lignin and straw brightness were obtained with *C. subvermispora.* The IR analyses of straw treated with *C. subvermispora* suggested that noticeable increase in carbohydrates was accompanied by decrease in aromaticity and oxidation of the aromatic lignin structures.

Composition of the straw water-soluble fraction reflected the progress of fungal modification of the whole lignocellulosic substrate. Analyses of water-soluble compounds showed two stages of degradation (0–15 days and 16–60 days) differing in the composition and patterns of water-

soluble fraction. At the early fermentation stage, the amount of water-soluble products decreased, obviously due to their utilization by fungi, whereas by the end of incubation total water-soluble compounds, including soluble carbohydrates and phenols were mainly accumulated in the straw substrate. In this respect, *P. eryngii* differed from the other fungi causing accumulation of low-molecular-weight water-soluble compounds throughout the fermentation period.

Apparently, the composition of the water-soluble fraction depended greatly on the fungal species used. This was reflected in the dynamics of aromatic and aliphatic compounds determined by IR spectroscopy, where *P. radiata* and *P. chrysosporium* showed quite opposite tendencies.

Elementary analyses of the water-soluble compounds pointed out that *C. subvermispora* caused the most intensive oxidation of lignin in straw in comparison with the other fungi studied, which coincided with the other results derived from this study and confirmed earlier reports on this fungus as a selective lignin degrader, able to delignify a wide range of lignocellulosic substrates [3,6,21–24].

Acknowledgments

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