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Wheat bran biodegradation by *Pleurotus ostreatus*: A solid-state Carbon-13 NMR study

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Abstract

Solid-state ^{13}C nuclear magnetic resonance (NMR) and elemental analysis techniques were used to monitor the degradation of wheat bran by the white-rot fungus *Pleurotus ostreatus* during a 62-day cultivation period. The weight loss and *in vitro* organic matter digestibility of the substrate were also evaluated after fungal treatment. The ^{13}C NMR spectra of degraded wheat bran samples showed a lower content in carbohydrates and a higher content in aliphatic and carboxylic groups than the untreated control sample. In parallel, changes in the wheat bran elemental composition evidenced a decrease in carbon content and a concomitant increase in nitrogen and oxygen content during mycelium growth. These results clearly indicate the occurrence of progressive changes in the composition of wheat bran during fungal treatment and are interpreted in terms of preferential degradation of amorphous vs. crystalline polysaccharides by the fungal mycelium and accumulation of proteins in the substrate. At the end of the cultivation period, the treated samples experienced an average weight loss of 20% and an increase in organic matter digestibility of 17%.

Keywords: Wheat bran; *Pleurotus ostreatus*; Fungal degradation; Solid-state ^{13}C NMR

1. Introduction

The white-rot fungus *Pleurotus (Pl.) ostreatus* is an edible basidiomycete known for its ability to degrade agro-industrial lignocellulosic wastes, which are mainly composed by cellulose, hemicelluloses, and lignin. It is generally cultivated on wheat straw, but other lignocellulosic substrates, such as cotton stalks, have proved adequate for its growth (Hadar et al., 1993; Kerem et al., 1992; Tsang et al., 1987; Valmaseda et al., 1991; Vane et al., 2001; Yildiz et al., 2002). The fungal colonisation of these substrates has far-reaching economical implications (Cohen et al., 2002). Indeed, besides the traditional production of fruit bodies of high nutritional value (Wang et al., 2001; Yildiz et al., 2002), the biodegradation of lignocellulose makes the spent mushroom compost exploitable as material with an improved rumen digestibility (Agosin et al., 1985a; Akin et al., 1993; Soto-Cruz et al., 1999). In fact, as a consequence of the selective degradation of lignin and hemicelluloses, cellulose in the substrate is more exposed and can be utilised by ruminants. Interestingly, the enzymatic activity of *Pl. ostreatus* is greatly affected by the cultivation media: for instance, when grown on cotton stalks, it mainly degrades lignin (Hadar et al., 1993; Kerem et al., 1992), while, during the colonization of wheat straw, it also reduces the hemicellulose content significantly (Tsang et al., 1987; Valmaseda et al., 1991; Vane et al., 2001).

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Like many lignocellulosic agro-residues, wheat bran is produced in considerable amounts as a by-product of the wheat milling industry and is generally used as is in animal feeds. It is made up of hemicelluloses (notably arabinoxylans, ca. 30%), cellulose (10–15%), starch (10–20%), proteins (15–22%), lignin (4–8%), and other minor components such as cutin and lipids (Beaugrand et al., 2004; Maes and Delcour, 2001). Despite its high carbohydrate content, part of the energy reserve of wheat bran (mainly the hemicelluloses content) is unexploited by animals.

In the light of the above considerations, we decided to test whether the treatment of wheat bran with *Pl. ostreatus* could be a valuable upgrading method for this substrate. To our knowledge, wheat bran has never been tested for growing of *Pl. ostreatus*, but it has been shown to be good as an additive for fungal growth on beer grain (Wang et al., 2001). We used solid-state ^{13}C NMR spectroscopy and elemental analysis to investigate the ability of *Pl. ostreatus* to degrade wheat bran during a 62-day cultivation period. The solid-state NMR technique has been proved to be a powerful non-invasive tool for the structural investigation at a molecular level of insoluble lignocellulosic materials (Maunu, 2002). Indeed, it provides exhaustive information directly on the polymeric components without their isolation or fractionation and it is well suited for studying the changes in the chemical structure induced by fungal treatment (Vane et al., 2001, 2003). *In vitro* organic matter digestibility of degraded wheat bran was also evaluated.

2. Methods

Industrial wheat bran was obtained from F.lli Cellino (Oristano, Italy) and used without modification.

2.1. Strain and culture conditions

Mycelia from *Pl. ostreatus* ATCC 36044 were grown for 5 days at room temperature in Petri dishes with the Sabouraud dextrose agar medium (OXOID).

2.2. Fungal treatment

Wheat bran biodegradation experiments were performed in 1 L flasks containing 25 g of bran moistened with 150 ml of distilled water, which were sterilized by autoclaving. The flasks were sealed with a cotton plug to facilitate aeration. Four replicates were inoculated with *Pl. ostreatus* at room temperature by adding 10 colonies from Sabouraud plates (dish diameter 10 cm) and incubated at room temperature for 62 days up to the end of the stationary phase of the culture. Wheat bran samples were removed from each flask at 15, 30, 54, and 62 days, then freeze-dried, powdered, and used for NMR experiments. Elemental analyses were performed with a FISONS EA-1108 CHNS-O instrument.

2.3. Analytical methods

Dry weight loss of wheat bran was determined from the weight difference between the control and the 62-day treated dried substrates (48 h at 60 °C) (Agosin and Odier, 1985b).

The number of fungal colony forming units (CFU) at the end of the cultivation period was evaluated as described by Pinholt et al. (1979).

The digestibility of organic matter was measured before and after the fungal treatment using *in vitro* technique (Aufrère and Demaquilly, 1989).

2.4. NMR spectroscopy

Solid-state ^{13}C NMR spectra were acquired at room temperature on a Varian 400 Unity Inova spectrometer operating at 100.57 MHz and equipped with a 7 mm probe-head. The samples were packed into cylindrical zirconia rotors sealed with Kel-F caps. All ^{13}C spectra were recorded under magic angle spinning (MAS) conditions at a spinning speed of 6 kHz.

Conventional Bloch decay (BD) experiments were carried out with a 7.5 μs (90°) pulse and a 100 s recycle delay. Improvements in the signal-to-noise ratio were gained by using cross-polarisation (CP) to transfer magnetization from proton to carbon. This technique enhances the intensities of carbons in rigid structures with attached protons more efficiently than those of carbons remote from protons or carbons with high mobility, such as methyl (CH_3), methoxy (OCH_3), and methylene carbons (CH_2) in long alkyl chains. CP experiments were performed using a 5 s recycle delay, a 600 μs contact time and 2048 scans. A matched Hartmann–Hahn condition was established at the spin-lock field of 33 kHz.

CP spectra with dipolar-dephasing (DD) were recorded to improve the detection of quaternary carbon signals at the expense of signals from protonated carbons in rigid environments. Indeed, by inserting a short delay period, without decoupling, between CP and signal acquisition (dephasing delay), the signal is rapidly lost from carbons with attached hydrogens in rigid domains, while it is retained for quaternary carbons and protonated carbons in mobile structures, such as methyl carbons and long CH_2 chains. DD experiments were performed using a delay period of 35 μs.

The chemical shifts of all spectra were referenced to the methylene resonance of solid hexamethylbenzene (17.3 ppm).

3. Results and discussion

3.1. Undegraded wheat bran

Solid-state ^{13}C BD-MAS, CP-MAS, and DD-MAS NMR experiments were carried out on undegraded wheat bran to gain information on its chemical composition.

The spectra are shown in Fig. 1. The NMR resonances were assigned according to data from the literature (Garbow and Schaefert, 1991; Gauthier et al., 2002; Ha et al., 1997; Matulova et al., 2005; Pacchiano et al., 1993; Vane et al., 2001; Wallace et al., 1995; Zlotnik-Mazori and Stark, 1988).

The spectra in Fig. 1 are all dominated by the set of resonances of oxygenated aliphatic carbons in carbohydrates, the most abundant components of wheat bran: the region between 65 and 90 ppm includes peaks from *O*-alkyl carbons, while di-*O*-alkyl carbons resonate in the 90–120 ppm range. In particular, the resonance at 62 ppm and the shoulder at 83 ppm are due to the C-6 and C-4 carbons of amorphous cellulose, starch, and hemicelluloses. The shoulders at 65 and 89 ppm correspond to the C-6 and C-4 carbons of crystalline cellulose, respectively; these signals are clearly visible in the CP spectrum due to the efficient cross-polarisation of carbons in rigid domains. The intense peaks at 73 and 75 ppm are overlapping signals due to the C-2, C-3, and C-5 carbons of all polysaccharides. The resonance at 105 ppm is assigned to the anomeric carbon (C-1) of cellulose and the shoulder at 103 ppm to C-1 of hemicelluloses and starch. Finally, the signal at 56 ppm is associated with the methoxyl groups of hemicelluloses and lignin.

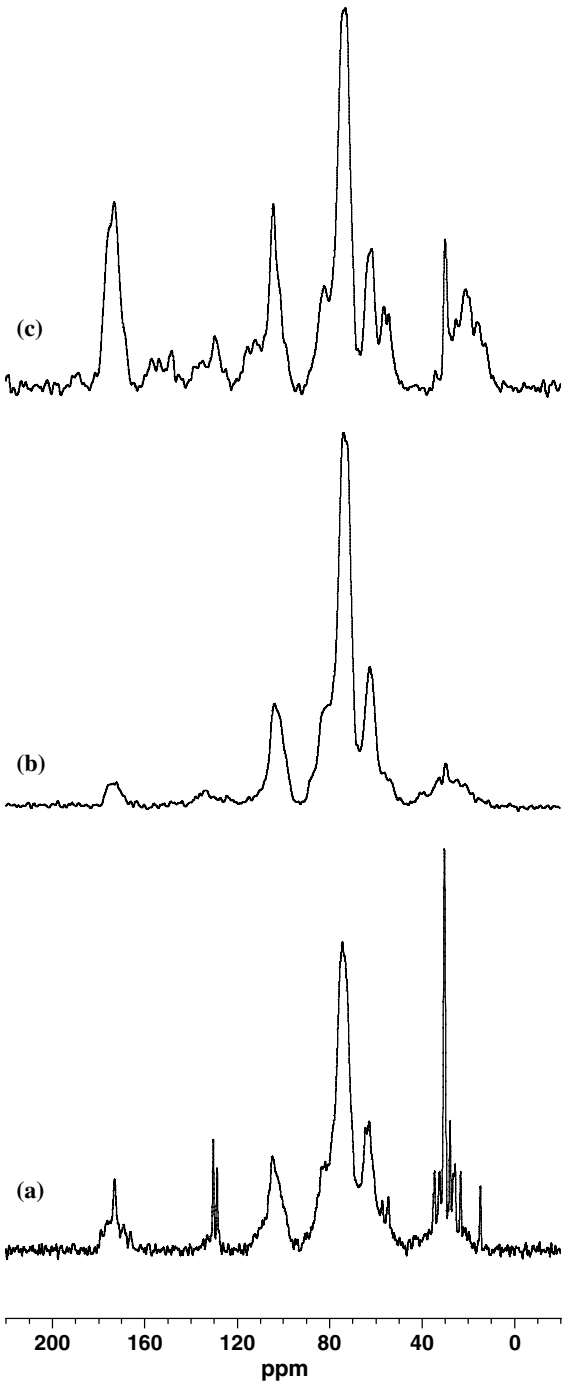

Fig. 1. ^{13}C BD-MAS (a), CP-MAS (b), and DD-MAS (c) spectra of undegraded wheat bran.

The aromatic-olefinic region (120–160 ppm) in both the BD- and CP-MAS spectra lacks the characteristic peaks of oxygen substituted aromatic carbons in lignin at 148 and 153 ppm (Hatfield et al., 1987). Considering the low lignin content in wheat bran (Lequart et al., 1999), detection of these resonances is likely prevented by the low signal-to-noise ratio in the BD spectrum and the low cross-polarisation efficiency of non-protonated aromatic carbons in the CP spectrum. Presence of very little lignin in the sample is revealed by the DD-MAS spectrum of wheat bran (Fig. 1c), where weak signals near the detection threshold of the NMR spectrometer are observed at around 150 ppm. In the light of these findings, we can rule out the contribution of lignin to the methoxyl peak at 56 ppm and attribute this resonance solely to hemicelluloses. As to the 120–140 ppm region, narrow signals are clearly visible at 129 and 130 ppm only in the BD spectrum. These resonances are attributed to unsubstituted aromatic carbons of the phenolic moieties in cutin and/or olefinic carbons in lipids.

Intense alkyl carbon peaks are present in the BD-MAS spectrum of wheat bran (0–45 ppm). These resonances are partially retained in the DD and significantly reduced in the CP spectrum, indicating that they derive from domains characterized by a high molecular mobility. These peaks are mainly ascribable to bulk methylenes in cutin and lipids (29–32 ppm), alkyl groups of hemicelluloses methyl ester (22 ppm), and protein side-chain carbons (41 ppm). Finally, the broad band at 174 ppm in the carboxyl region is assigned to acetate groups of glucuronic acid in hemicelluloses, carboxyl groups of cutin and lipids, and amide carbons of proteins.

3.2. Wheat bran treated with Pl. ostreatus

Fig. 2 shows the ^{13}C CP-MAS NMR spectra of some of the wheat bran samples at different fungal cultivation times. For the sake of comparison, the spectrum of control wheat bran is also reported. While the spectral features of the samples remain unchanged during mycelium growth, variations are observed in the relative intensities of the

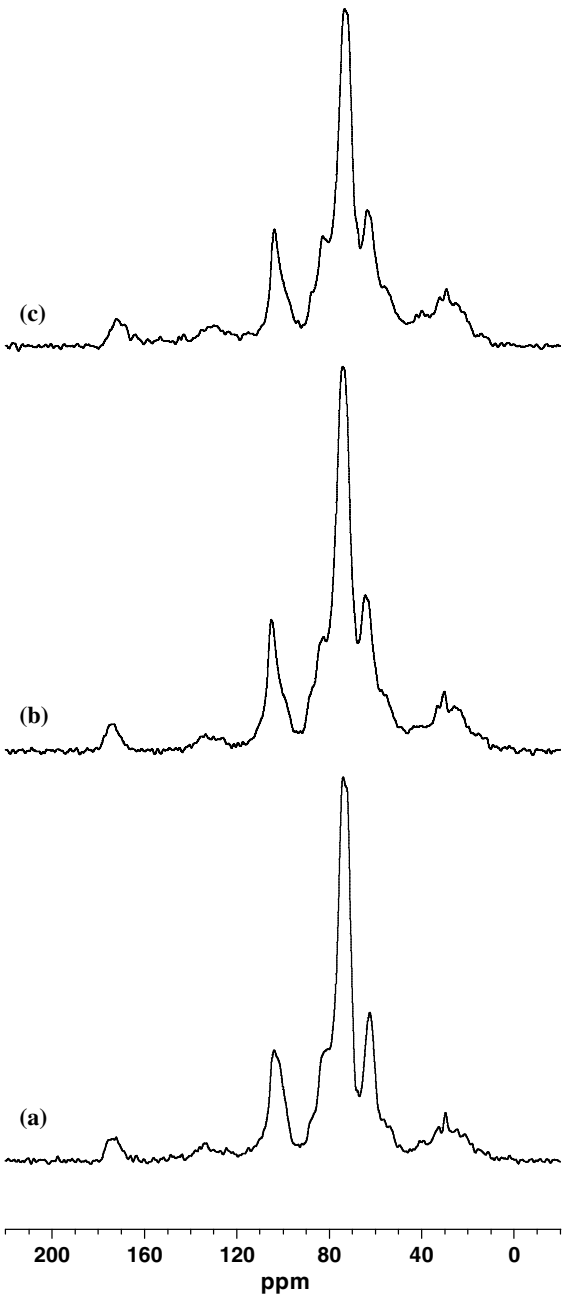

Fig. 2. ^{13}C CP-MAS spectra of control wheat bran (a) and wheat bran treated with *Pl. ostreatus* at 30 days (b) and at 62 days (c).

signals. In order to evaluate the percentages of the different carbon types in the CP-MAS spectra, these were divided into four different chemical shift regions, and the corresponding integrated areas were measured. It is worth noting that, though the CP experiment does not by its nature provide rigorous quantitative information, this approach has been proved to give reliable estimate of the relative variations in carbon distribution in similar samples with similar type of carbons when analysed under identical experimental conditions (Vane et al., 2001, 2003). This is the case with undegraded and degraded wheat bran under investigation.

In Fig. 3 the relative ^{13}C CP-MAS integrated areas are graphically shown as a function of cultivation time. Peaks in the 45–120 ppm range arising from cellulose, hemicelluloses, and starch were accounted together, since their overlap makes it difficult to quantify each carbohydrate component. The data in Fig. 3 show a progressive variation in the relative carbon distribution with increasing fungal cultivation time. In particular, a significant decrease in *O*-alkyl and di-*O*-alkyl carbons takes place together with an increase in alkyl carbons. As to the variation of carboxyl and aromatic-olefinic intensities, they are within the experimental error.

The observed decrease in carbohydrate intensities in degraded wheat bran indicates a progressively lower

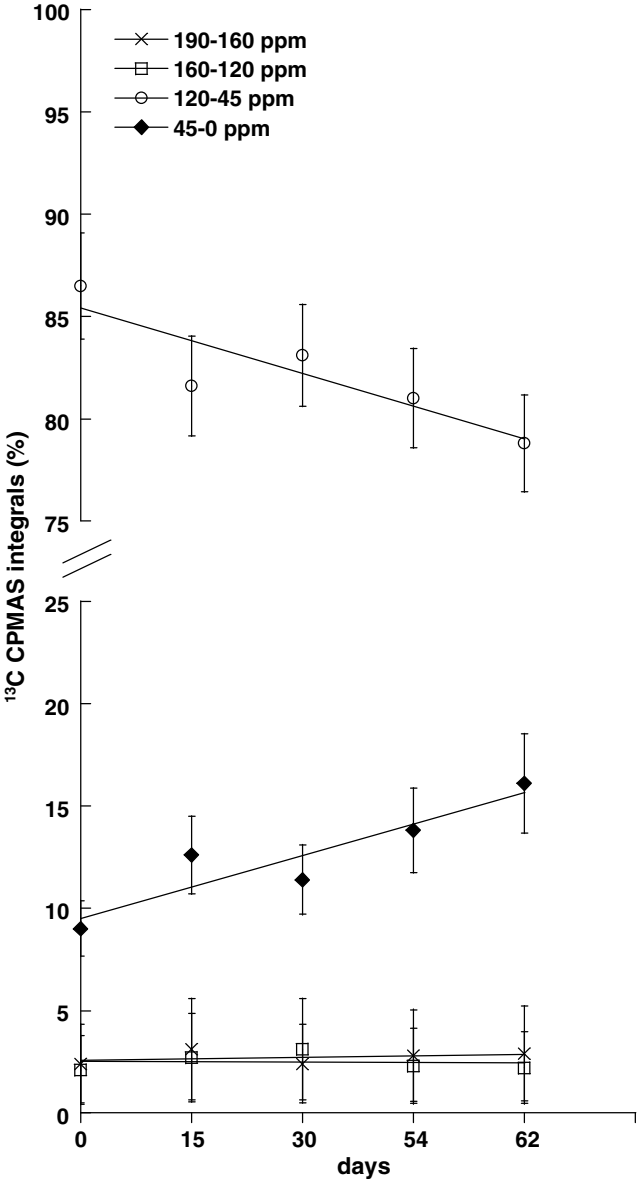

Fig. 3. Integrated areas of the ^{13}C CP-MAS spectra of undegraded and degraded wheat bran (expressed as a percentage of the total carbon area) as a function of cultivation time (days). The lines are a guide for the eye. The error bars represent the experimental error.

amount of polysaccharides during fungal treatment. A possible contribution from fungal polysaccharides to the spectra is excluded (Pizzoferrato et al., 2000), since fungal biomass was estimated to be less than 1% of total biomass at the end of the cultivation period (CFU = $1.8 \pm 0.4 \times 10^9$/ml). Furthermore, from a qualitative comparison among CP-MAS spectra, the decrease in the peak at 83 ppm (C-4 in amorphous carbohydrate moieties) relative to the peak at 89 ppm (C-4 in crystalline cellulose) suggests that a preferential degradation of amorphous versus crystalline polysaccharides occurs. Similar NMR results have been reported for the biodegradation of wheat straw by *Pl. ostreatus*, showing the removal of amorphous arabinoxylans in hemicelluloses (Vane et al., 2001).

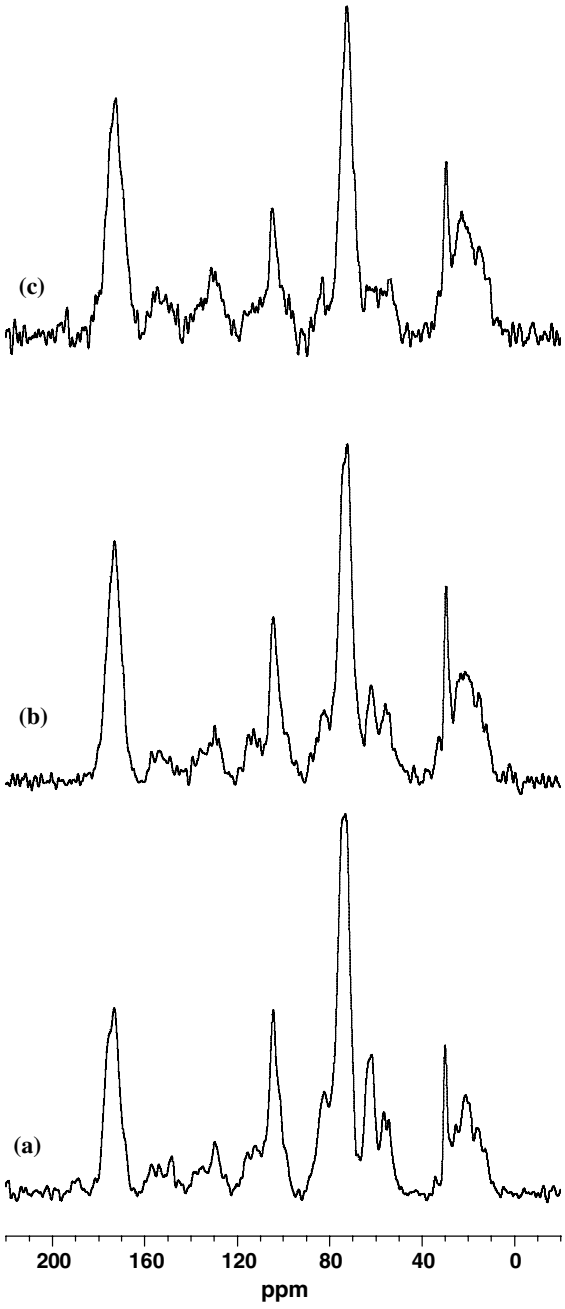

Fig. 4. ^{13}C DD-MAS spectra of control wheat bran (a) and wheat bran treated with *Pl. ostreatus* at 30 days (b) and at 62 days (c).

Table 1
Elemental composition of wheat bran treated with *Pl. ostreatus* at different cultivation times

Time (days)	N (%)	C (%)	H (%)	O (%)
0	2.92	41.33	7.95	47.81
15	3.08	41.60	8.51	46.81
30	3.09	43.00	8.14	53.10
54	3.31	32.41	6.25	58.03
62	4.19	33.47	6.94	55.40

Signals from quaternary and highly mobile carbons were examined in more details in DD-MAS spectra, where the intensities of these resonances are enhanced compared to CP-MAS. The spectra in Fig. 4 show an increase in carboxyl and alkyl intensities in treated wheat bran with respect to the control sample, thus suggesting the accumulation of these structures during *Pl. ostreatus* attack. On the contrary, olefinic and aromatic intensities from cutin and lignin, respectively, seem to be unaffected by mycelium growth. It is worth reminding that an accurate NMR detection of these components is prevented by their very low concentrations. Nevertheless, cutin is likely unaltered due to its high resistance against external agents, while lignin degradation cannot be excluded considering the well established ligninolytic activity of *Pl. ostreatus*. Moreover, the decrease in the methoxyl signal at 56 ppm provides a clear indication of the fungal attack on hemicelluloses and thus of the degradation of this component. This result is accompanied by the marked reduction of the peaks at 62 and 83 ppm arising from amorphous polysaccharides.

The degradation of carbohydrates observed via NMR is supported by changes in the elemental composition of wheat bran samples, which indicate a progressive decrease in carbon content with increasing cultivation time (Table 1). In parallel, the concomitant increase in nitrogen and oxygen contents suggests protein accumulation in the substrate, which is likely to cause the increase in the alkyl and carboxyl groups in the NMR spectra of degraded wheat bran. This finding is consistent with the production of extracellular enzymes by fungal metabolic activity (Wang et al., 2001).

At the end of the cultivation period, the weight loss of the substrate amounted to an average of 20%. In parallel, an improved digestibility of organic matter was observed: from $36 \pm 2\%$ in untreated wheat bran to $53 \pm 2\%$ at the end of the fungal treatment.

4. Conclusions

The overall findings of this investigation clearly indicate that wheat bran is a good substrate for the growth of *Pl. ostreatus* and that the material is efficiently degraded during a 62-day cultivation period. NMR results show that

changes in the molecular composition of the substrate occur during the fungal fermentation, with a preferential loss of hemicelluloses and possibly other amorphous carbohydrates. Although the very low concentration of lignin prevented an accurate analysis of this component via NMR, the fungal attack on lignin can not be ruled out. Finally, the fungal colonisation of the substrate increased its digestibility, thus giving the possibility of improving its use as animal feed.

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