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Degradation of Eucalypt Waste Components by *Lentinula edodes* Strains Detected by Chemical and Near-Infrared Spectroscopy Methods

M. Brienzo, E. M. Silva, and A. M. F. Milagres*

[1]*Dept. of Biotechnology, Escola de Engenharia de Lorena–USP, Lorena–SP, CP 116 CEP 12 602 810- Brazil, E-mail: adriane@debiq.eel.br*

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Abstract

There are many changes, both qualitative and quantitative, in eucalypt waste during growth and fructification of *Lentinula edodes*. Wet chemical analysis and near-infrared (NIR) spectroscopy were used in conjunction with multivariate regression and principal components analysis to monitor biodegradation of eucalyptus waste during growth of several *L. edodes* strains. Weight and component losses of eucalypt residue after biodegradation by *L. edodes* strains were compared for periods of 1 to 5 mo. Decrease in cellulose, hemicellulose, and lignin contents occurred, however it was not concomitant. Measurement of lignin degradation by NIR and wet chemical analysis indicated its attack in the early stages of biodegradation. Selective lignin degradation by *L. edodes* was observed up to 2 mo of biodegradation for strains DEBIQ and FEB-14. One group of degraded substrate was identified based on the principal component analysis (PCA) of the data on their biodegradation time. Samples treated for 5 months by *L. edodes* strains (DEBIQ, UFV or FEB-14) differed from other, but no discrimination was observed among them. By the end of 5 mo, NIR analyses showed decrease of about 18–47% cellulose, 35–47% polyose and 39–60% lignin. These data were used for comparison with those obtained by wet chemical

*Author to whom all correspondence and reprint requests should be addressed.

method for the degradation of the substrate by other five *L. edodes* strains cultivated at the same conditions. NIR calibration developed in this study was proven to be perfectly suitable as an analytical method to predict the changes in lignocellulose composition during biodegradation.

Index Entries: *Lentinula edodes*; biodegradation; Near infrared; lignin selectivity.

Introduction

The exploitation of eucalypt forest by cellulosic pulp mill and timber industries generate a great volume of lignocellulosic wastes, mainly composed of leaves, branches and barks. These wastes are commonly used as a substitute for sawdust in commercial production of shiitake *(1,2)*, an edible mushroom. Mushroom cultivation represents one of the efficient ways by which these residues can be recycled. Spent mushroom compost is made up of fungal mycelium, extracellular enzymes produced during growth and unutilized lignocellulosic substrate. In addition, this residue could be used for production of bulk enzymes and as organic fertilizer.

One of the most important edible mushrooms traditionally cultivated in the world is the basydiomycete *Lentinula edodes*, which degrades woody substrates and has been considered a nonselective in its atack on the cell wall components *(3)*. However, studies have revealed the presence of wide variations in polysaccharides and lignin degradation ability among the strains of *L. edodes (4,5)*. Because the rate of lignin degradation appears to be the limiting step in substrate colonization by wood-utilizing fungi, strains with highest rates in the early stages of the vegetative growth period would be interesting to improve the utilization of lignocellulosic wastes *(6,7)*.

From the standpoint of chemical analyses of the plant cell wall, numerous studies have been developed, however, the methods are very time-consuming and laborious *(8)*. Using diffuse reflectance infrared spectroscopy (DRIFT), biodegradation of plant cell-wall components, especially lignin degradation by *L. edodes* has also been investigated (*5*). Recently, several reports have demonstrated the use of near-infrared spectroscopy (NIR) technique for rapid measurement of chemical composition of different lignocellulosic residues *(9,10)*. NIR in reflectance mode is a fast, sensitive, and nondestructive technique to estimate the plant cell wall components.

In this work, we provided information on quantitative variations in lignin and carbohydrate degrading ability among strains of *L. edodes* to obtain a correlation coefficient between band absorbances in second derivative NIR spectra of eucalypt waste and wet chemical analyses. Our main objectives were to find out whether wet chemical analyses and NIR-predicted values show any correlation on quantification of cellulose, polyose, and lignin content of the biodegraded residue and also how much difference in chemical composition exists among the biodegraded residues by *L. edodes* strains.

Materials and Methods

Fungal Cultures

Eight isolates of *L. edodes* from Botanic Institute mushroom culture collection (CCB)/São Paulo were used in this study. They were identified as SJC, DEBIQ, UFV-52, FEB-14, CCB-514, CCB-515, CCB-581 and CCB-559 and maintained on potato-dextrose-agar (PDA-Neogen Corporation) medium at 4°C *(1)*. Innoculum was prepared by growing these organisms on plates containing the same medium and incubated at 27 ± 2 °C for 15 d in the dark.

Instrumentation

A Perkin Elmer Identicheck NIR spectrometer equipped with a reflectance accessory was used in this study. The elemental analyzer used was a FISONS instruments EA 1108 CHNS-O and moisture was determined with a Mettler-Toledo infrared dryer.

Materials

Eucalyptus waste were collected from a local plantation, in Suzano, São Paulo, Brazil with approx 30% moisture. The general substrate composition (dry weight basis) consisted of 75% bark, 15% leaves, 10% branches and the mixture was previously ground to pass a 7 mesh-screen (size <2.8 mm) using a knife mill (Manesco & Ranieri).

Biodegradation Experiments

The milled eucalyptus waste was submerged in distilled water for 24 h, and the excess of water was drained and moisture was adjusted to about 65–70%. The material (600 ± 5 g) was packed into heat-resistant polipropylene bags, autoclaved at 121°C for 2 h, incubated at room temperature for 24 h, and then autoclaved again. The actively growing cultures were inoculated into the bags by introducing five mycelial PDA plug pieces (Ø = 8 mm). Bags were sealed again and incubated at 27 ± 2°C in the dark for 1, 2, 3, 4 and 5 mo. After each biodegradation period substrates were dried at 85 ± 2°C and weighed for mass-loss determination. Sterilized substrates were used as controls.

Sample Preparation

After mass loss determination, each biodegraded sample were ground (0.5 mm) using a knife mill (Manesco & Ranieri). The sieved samples were dried at 55°C overnight and subdivided for NIR, wet chemical, and elemental analyses determinations.

Wet Chemical Analyses: Lignin, Cellulose, and Polyose Content Determinations

Approximately 3 g of each biodegraded eucalyptus residue was extracted with 95 % ethanol for 6 h in a Soxhlet apparatus. Extracted

samples were hydrolyzed with 72% sulfuric acid (Merck) at 30°C for 1 h (300 mg of sample and 3 mL of sulfuric acid). The acid was diluted to a final concentration of 3% (addition of 79 mL of water). The mixture was heated at 125°C for 1 h. The solids were dried to constant weight at 105°C and determined as insoluble lignin *(11)* . The soluble lignin was determined by measuring the absorbance at 205 nm and the monomeric sugars were determined by high-performance liquid chromatography (HPLC) *(12)*. The acid-insoluble and the acid-soluble lignin were combined and reported as the total lignin content.

Concentrations of monomeric sugars in the soluble fraction were determined by HPLC using a BIORAD HPX87H column at 45°C, eluted at 0.6 mL/min with 0.005 mol/L sulfuric acid. Sugars were detected in a 30°C temperature-controlled RI detector (Waters HPLC pump and detector). Glucose, xylose, arabinose and acetic acid from Merck were used as external calibration standards. No corrections were performed due to sugar degradation reactions during acid hydrolysis. The factors used to convert sugar monomers to anhydromonomers were 0.90 for glucose and 0.88 for xylose and arabinose. Acetyl content was calculated as the acetic acid content multiplied by 0.7. These factors were calculated based on water addition to polysaccharides during acid hydrolysis.

NIR Measurements

An aliquot of about 1 g was scanned with a NIR spectrophotometer which covers a spectral range of 1000–2500 nm. During each scan the instrument takes 50 measurements collected at 2.0-nm intervals which are combined to produce as single spectrum. Raw reflectance data were converted to Kubelka-Munk units (K-M) and the spectra baseline were corrected to the regions near 1320 nm, 1870 nm, 2220 nm, and 2410 nm. Two spectra were recorded from each wood or pulp sample. Duplicate spectra were averaged using the Perkin Elmer software facilities.

Data Analysis

Spectral and chemical composition data were analyzed with the software package QUANT+ available with the Identicheck FT-NIR Perkin Elmer spectrometer. Spectral data were analyzed in digitized form at 2 nm intervals from 1000 to 2500 nm. All spectra were converted to second-derivative (inverted peaks) by a Kubelka-Munk transformation and a standardized normalization procedure by using the SPECTRUM^R software *(13,14)*. Utilizing the Kubelka-Munk theory, the reflectance (R) can be related to the absorption coefficient (K) and scattering coefficient (S) by the following equation *(14)*:

$$\frac{K}{S} = \frac{1 - R^2}{2R}$$

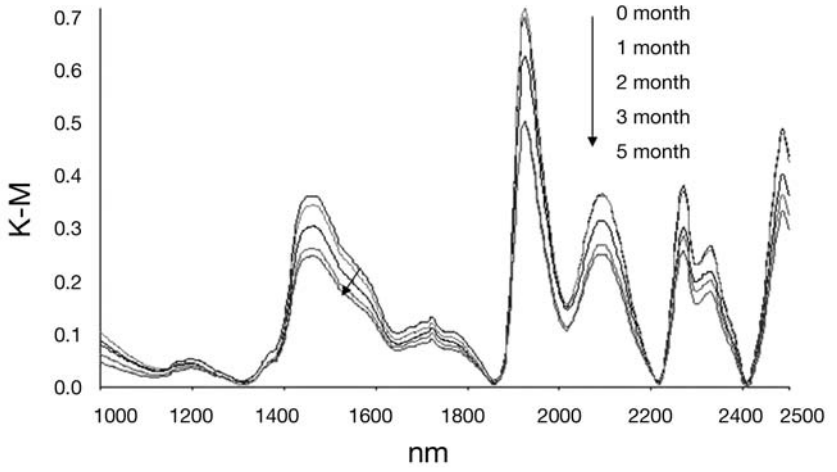

Fig. 1. Near-infrared spectra of eucalyptus residue samples cultivated with *Lentinula edodes* FEB-14 for varying lengths of time.

Standardized normalization procedure was applied in order to remove any systematic variation, reducing the effect of a baseline drift in the original NIR spectra. The area of the peaks at 2119–2150 nm assigned to lignin and the peaks at 2220–2292 nm to carbohydrates *(15)* were used to get a factor between the control and biodegraded samples. Principal component analysis (PCA) of biodegraded samples was performed using the factors obtained in NIR analysis of combined data of three crops of *L. edodes* (PC1) and the effect of biodegradation time (PC2).

Results and Discussion

Spectroscopy Characterization of Biodegraded Residue

Eight isolates of *L. edodes* from our collection were evaluated for the effects of genotypes on consumption of substrate component. The lignin and carbohydrate content were determined by NIR measurement and the correlation factors were obtained in the carbohydrate and lignin regions *(15)*. Figure 1 shows changes in the NIR spectra (range of 1000–2500 nm) as a function of incubation time which was highly correlated to eucalyptus residue weight loss. Curves are given for 1 to 5 mo of incubation by *L. edodes* FEB-14, but the same pattern was observed with the strains DEBIQ and FEB. The signal was constant for the substrate control and for samples of one month of biodegradation, however for the other biodegradation periods there was a decrease in the signal as the degradation time increased. To better resolve the original spectra, the second-derivative spectra was performed to obtain narrow peaks projected downward. The peaks at 2119–2150 nm assigned to lignin and the peaks at 2220–2292 nm to carbohydrates *(15)* were used to get a factor between the control and biodegraded samples

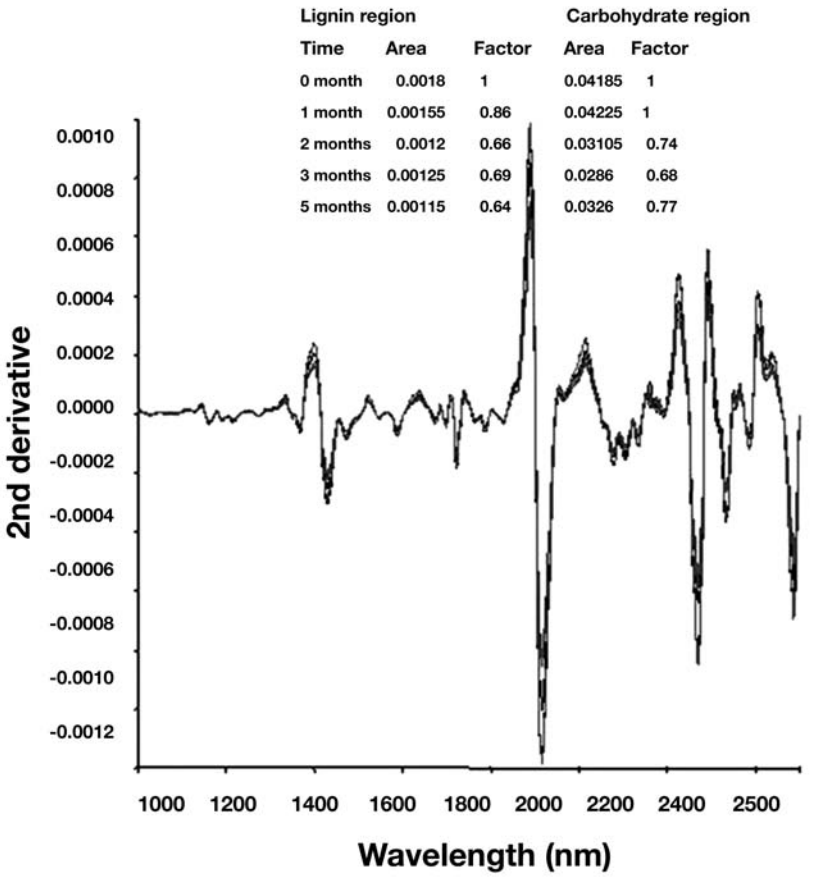

Fig. 2. Second-derivative near-infrared spectra of different period of biodegradation for eucalyptus residue by *Lentinula edodes*-FEB-14. The peaks at 2119–2150 nm assigned to lignin and the peaks at 2220–2292 nm to carbohydrates were used to get a factor between the control and biodegraded samples.

(Fig. 2) and these data are shown in Table 1. The three *L. edodes* strains promoted the highest changes in the lignin region mainly after the first month of biodegradation. After 2 mo, the rate of carbohydrate degradation increased in comparison to lignin, which rates remained relatively constant over a period of five months. Thus, the NIR measurement of lignin degradation indicates its attack in the in early stages of cultivation. The data from Fig. 2 were also analyzed by PCA to recognize the effects of the period of biodegradation and *L. edodes* strains for eucalyptus residue degradation. Samples biotreated for 5 mo differed from the others, but no discrimination was observed among the strains (Fig. 3). The first and second principal components (first and second eigenvectors) explain 90.65% and 4.44% of the data variance, respectively, indicating that the variables time of degradation is more significant that the strain. The higher PC2-score value the higher the degradative activity of the *L. edodes* strains.

Table 1
Change in the Eucalyptus Residue by *Lentinula edodes* Expressed by Correlation Coefficients of Band Absorbances in Second-Derivative Near-Infrared Spectra and by Residual Components Obtained From Wet Chemical Analysis

	NIR[a]		Chemical analysis[b]		
L. edodes isolates	Biodegradation time (month)	2220/2292-nm carbohydrate region	2119/2150-nm lignin region	Carbohydrate	Lignin
DEBIQ	0	1.00	1.00	1.00	1.00
	1	0.90	0.81	0.83	0.75
	2	0.73	0.72	0.75	0.64
	3	0.61	0.66	0.65	0.64
	5	0.63	0.58	0.55	0.46
UFV	0	1.00	1.00	1.00	1.00
	1	0.88	0.92	0.84	0.8
	2	0.71	0.67	0.83	0.74
	3	0.76	0.81	0.73	0.65
	5	0.77	0.5	0.68	0.58
FEB	0	1.00	1.00	1.00	1.00
	1	1.01	0.86	0.93	0.87
	2	0.74	0.66	0.85	0.73
	3	0.68	0.69	0.72	0.72
	5	0.77	0.64	0.62	0.57

[a]Factors determined by the difference between the peak produced at each time of biodegradation and the control sample

[b]Factors obtained by the residual content of lignin or carbohydrate of each sample.

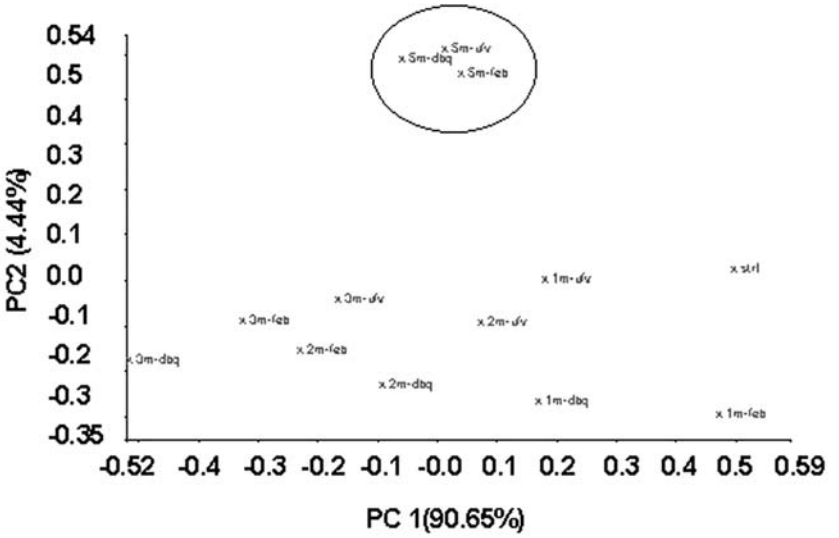

Fig. 3. Principal component analysis plot from data of second-derivative to recognize the effects of *Lentinula edodes* strains (PC1) and the period of biodegradation (PC2) for eucalyptus residue.

Wet Chemical Characterization of Biodegraded Eucalyptus

Chemical composition of biodegraded eucalyptus residue determined by wet chemical analysis revealed few chemical differences among the *L. edodes*-biodegraded residues (Table 2). Average weight loss ranged from 20 to 44% after 5 mo of degradation and glucan, polyoses and total lignin concentrations ranged between 33 and 41%, 11 and 15%, and 27 and 32%, respectively. The remaining percentage should be accounted for as components which were not analyzed such as arabinose, uronic acids and ash and also to sugar losses owing to side degradation reactions occured during hot acid hydrolysis of wood samples *(12)*. *L. edodes* strains rapidly colonized the substrate and the strain DEBIQ promoted highest weight losses after about the same decay period.

Monitoring weight loss, carbohydrates and lignin of the *L. edodes*-biodegraded residues over the biodegradation period indicated that lignin was preferentially degraded. Average weight loss ranged from 1.7 to 44% and the glucan, polyoses and total lignin ranged between 0.79 and 47%, 2.95 and 53%, and 11 and 60%, respectively (Table 2). After 1 mo of treatment with *L. edodes*, the decrease of lignin content was greater than carbohydrates. Selectivity, was expressed by the loss of each component per total weight loss. This ratio decreased after 1 mo and remained practically unchanged after 3 mo. A comparison between glucan losses after 5 mo indicates that DEBIQ was the most efficient degrader (Table 2). At this period, approximately the same amount of polyoses was consumed from the substrates by the strains DEBIQ and FEB, although somewhat more

polyose was removed by the strain DEBIQ during the first month of biodegradation. The strains DEBIQ and FEB showed selective remotion of lignin up to 2 mo of degradation. Among all strains, total lignin loss at the end of the cultivation period ranged between 39 and 60%. The strain FEB promoted only 13.3% of lignin loss in the first month of cultivation, however higher reduction in the lignin component was observed in comparison with the other components.

Almost all the strains used in this work showed selective lignin removal over biodegradation periods no longer than 2 mo. Usually, signifficant differences in lignin removal can be visualized only if substrate is extensively degraded *(4,14)*. Table 1 shows the correlation factors obtained for carbohydrate and lignin between the control and the biodegraded samples. With NIR results, these factors were determined by the difference between the peak produced at each biodegradation period and the control sample, while the results obtained by wet chemical analysis these factors were determined by the residual content of lignin or carbohydrate. Comparison of the results obtained through NIR with those obtained by wet chemical analysis shows that there is a very good correlation between both data sets, lignin ($R^2 = 0.9858$) and carbohydrate ($R^2 = 0.9503$), determined independently by chemical or spectroscopy methods.

Carbon and hydrogen of control and biodegraded substrates determined by elemental analysis shows that the C/H rate had a slight increase over the biodegradation period, confirming that the biodegraded substrates have higher carbohydrate content than lignin *(5)*. Oxygen was determined by difference and nitrogen was below the limit of detection. An important characteristic of compost to be used as organic fertilizer is that because of a low C:N value of approx 25 *(5)*, despite the fact that all *L. edodes* strains have grown well on the eucalyptus residue, the nitrogen content was below limit detection. Although the lignocellulosic material treated with fungi contained residual mycelium, the contribution of fungal biomass was insignificant.

Conclusions

Chemical and spectroscopy analyses used in this study were proven to be suitable as analytical method to follow changes in lignocellulose composition during biodegradation. The results obtained through NIR spectroscopy and chemical analyses showed a good correlation for different *L. edodes* strains. Among the eight isolates of *L. edodes*, significant differences in weight losses were observed after 5 mo of biodegradation, in addition, samples of *L. edodes*-biodegraded residues treated for short periods were not statistically different. Isolates SJC, DEBIQ, and FEB appear to have the greatest potential application for substrate degradation; however, 5 mo of biodegradation by *L. edodes* were not sufficient for chemical transformations that enable their use as organic fertilizer.

Table 2
Chemical Composition of Eucalyptus Residue Biodegraded by *Lentinula edodes* Strains and Calculation of Component Losses and Selectivity

		Chemical composition (%)				Component losses (%)			Selectivity		
L. edodes	Degradation time (month)	Glucan	Polyose	Total Lignin	Weight loss (%)	Glucan	Polyose	Lignin	Glucan	Polyose	Lignin
Control		39.54	13.97	32.31							
	1	36.11	12.43	30.1	4.54	12.82	15.07	11.07	2.82	3.32	2.44
	2	36.8	12.55	28.07	18.01	23.69	26.35	28.77	1.32	1.46	1.60
SJC	3	39.28	12.76	29.14	27.15	27.63	33.46	34.30	1.02	1.23	1.26
	4	39.54	12.29	22.41	34.64	34.64	42.50	54.67	1.00	1.23	1.58
	5	36.84	10.74	21.37	39.67	43.79	53.62	60.09	1.10	1.35	1.52
	1	39.07	13.81	29.32	17.12	18.10	18.07	24.79	1.06	1.06	1.45
	2	41.51	14.63	27.52	24.62	20.86	21.05	35.79	0.85	0.86	1.45
DEBIQ	3	37.51	12.76	31.84	34.46	37.82	40.13	35.41	1.10	1.16	1.03
	4	36.14	12.03	32.23	33.51	39.22	42.74	33.67	1.17	1.28	1.00
	5	39.45	12.5	27.04	44.59	44.71	50.42	53.62	1.00	1.13	1.20
	1	42.11	15.1	30.83	16.10	10.65	9.31	19.94	0.66	0.58	1.24
	2	41.09	14.74	29.03	17.21	13.97	12.65	25.62	0.81	0.73	1.49
UFV-52	3	38.21	12.84	28.95	27.31	29.76	33.19	34.87	1.09	1.22	1.28
	4	40.48	14.22	25.62	24.10	22.30	22.74	39.82	0.93	0.94	1.65
	5	37.83	12.85	27.80	31.14	35.07	40.84	41.61	1.09	1.27	1.29
	1	39.53	13.87	30.11	7.01	7.03	7.67	13.34	1.00	1.09	1.90
	2	38.83	13.85	27.84	15.31	16.83	16.03	27.02	1.10	1.05	1.77
FEB-14	3	35.47	12.16	32.21	28.11	35.51	37.43	28.34	1.26	1.33	1.01
	4	37.27	12.37	31.63	29.52	33.56	37.59	31.00	1.14	1.27	1.05
	5	33.36	11.32	29.57	37.65	47.39	49.47	42.93	1.26	1.31	1.14
	1	37.14	12.82	27.03	10.42	15.85	17.79	25.06	1.52	1.71	2.41
	2	37.14	12.3	19.08	20.0	24.86	29.57	52.76	1.24	1.48	2.64
CCB-514	3	40.27	12.38	19.43	25.02	23.63	33.55	54.91	0.94	1.34	2.19
	4	36.12	12.01	19.89	20.94	27.78	32.03	51.33	1.33	1.53	2.45
	5	40.57	13.02	24.53	20.32	18.25	25.74	39.51	0.90	1.27	1.94
	1	39.63	12.3	27.70	18.61	18.42	28.34	30.00	0.99	1.52	1.61

	2	41.38	11.94	21.56	26.87	23.47	37.50	51.05	0.87	1.40	1.90
CCB-515	3	41.22	12.50	23.84	26.84	23.73	34.54	45.85	0.88	1.29	1.71
	4	41.20	11.66	23.65	30.80	27.90	42.24	49.19	0.91	1.37	1.60
	5	41.87	11.27	24.37	31.86	27.85	45.03	48.45	0.87	1.41	1.52
	1	39.15	12.85	28.01	6.03	6.95	13.56	18.53	1.15	2.25	3.07
	2	37.79	13.21	26.99	21.99	25.45	26.24	34.84	1.16	1.19	1.58
CCB-581	3	42.86	13.11	26.80	25.12	18.83	29.73	37.89	0.75	1.18	1.51
	4	39.73	12.4	24.47	27.32	26.97	35.49	44.96	0.99	1.30	1.65
	5	41.39	12.2	23.29	30.91	27.67	39.66	50.19	0.90	1.28	1.62
	1	39.90	13.79	27.86	1.69	0.79	2.95	15.23	0.47	1.75	9.03
	2	37.34	12.52	23.96	19.74	24.20	28.07	40.48	1.23	1.42	2.05
CCB-559	3	36.72	12.14	28.81	22.12	27.68	32.33	30.56	1.25	1.46	1.38
	4	35.72	11.33	19.31	23.69	31.07	38.11	54.40	1.31	1.61	2.30
	5	40.11	12.84	23.39	30.39	29.38	36.02	49.60	0.97	1.19	1.63

Component losses [CL= (mci – mcf)/mci] × 100] where, mci: % initial component x initial dry weigth and mcf: % final component × final dry weight Selectivity = (CL/weigth loss) × 100.

Table 3
Time-Dependent Changes in Elemental Compositions in Black Gum Degraded by *Lentinula edodes*

Isolates of *L. edodes*	Biodegradation time (month)	%N	%C	%H	%O	C:H
Control	0	N.D.	47.1 ± 3.8	8.1 ± 2.3	44.8	5.8
	1	N.D.	42.3 ± 1.5	7.1 ± 6.3	50.6	5.9
	2	N.D.	40.7 ± 1.5	6.8 ± 1.2	52.5	6.0
FEB-14	3	N.D.	42.9 ± 4.3	6.1 ± 4.3	51.0	7.0
	5	N.D.	44.8 ± 0.7	5.6 ± 9.7	49.6	8.0
	1	N.D.	45.2 ± 0.6	4.7 ± 12.5	50.1	9.6
	2	N.D.	44.3 ± 0.2	5.8 ± 10.0	49.9	7.6
UFV-52	3	N.D.	44.0 ± 0.4	5.8 ± 3.7	50.2	7.6
	5	N.D.	43.7 ± 0.2	5.8 ± 4.2	50.5	7.4
	1	N.D.	45.1 ± 0.1	7.1 ± 6.8	47.8	6.3
	2	N.D.	45.1 ± 0.1	6.9 ± 2.9	48.0	6.5
DEBIQ	3	N.D.	44.9 ± 0.4	6.1 ± 0.6	49.0	7.4
	5	N.D.	43.6 ± 1.2	6.2 ± 0.3	50.2	7.0

N.D., below of limit detection.

Our results clearly indicate that small discrepancies occur between chemical and NIR data, particularly to carbohydrate moiety. We also found that the main chemical transformation during 5 mo of biodegradation was due to lignin degradation and that NIR method is a powerful analytical procedure for evaluation of lignocellulose biodegradation, but we also suggest that additional component losses be completed with two or more biodegradation periods. This would help minimize the potential independent effect of germoplasm on crop cycle time.

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