JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Lignin Degradation by *Agaricus bisporus* Accounts for a 30% Increase in Bioavailable Holocellulose during Cultivation on Compost

RIMKO TEN HAVE,*,† HILDE WIJNGAARD,† NICOLE A. E. ARIËS-KRONENBURG,† GERBEN STRAATSMA,‡ AND PETER J. SCHAAP†

Wageningen University and Research Centre, Laboratory for Microbiology, Section Fungal Genomics, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands and Applied Plant Research, Mushroom Research Unit, P.O. Box 6042, 5960 AA Horst, The Netherlands

The common mushroom *Agaricus bisporus* is a non-white rot saphrophytic fungus that can degrade lignin to free and utilize holocellulose embedded in fermented straw as present in compost. A new method is described to estimate the actual amount of bioavailable holocellulose in 3.8 kg compost cultures spawned with *A. bisporus* Horst U1 prior to and during a cultivation with two cycles of mushroom harvesting. The method shows that the initial amount of bioavailable holocellulose per culture, accounting for 130 ± 22 g, is lower than the total holocellulose consumption by *A. bisporus* accounting for 182 ± 15 g. This difference is explained by a 30% increase in bioavailable holocellulose. The increase is caused by the degradation of 95 ± 3 g of holocellulose-shielding lignin. The results are discussed within the scope of the *A. bisporus* mushroom yield and lignin degradation by white rot fungi during growth on lignocellulose-containing materials.

KEYWORDS: ***Agaricus bisporus*; *Scytalidium thermophilum*; bioconversion; holocellulose; lignin; white rot fungi**

INTRODUCTION

Agaricus bisporus is cultivated commercially on a fermented mixture of wheat straw, straw-bedded horse manure, chicken manure, and gypsum. This compost contains a rich microflora of which the thermophilic fungus *Scytalidium thermophilum* is an example (*1*, *2*). The presence of *S. thermophilum* in the compost is beneficial for *A. bisporus* cultivation as it has been shown that the presence of *S. thermophilum* is positively correlated with *A. bisporus* mushroom yield (*3*). *S. thermophilum* also stimulates the hyphal extension rate of *A. bisporus* (*1*, *3*), thereby minimizing the chance for pathogen outbreaks.

Previous research has shown that *A. bisporus* degrades holocellulose (hemicellulose and cellulose) and lignin during the commercial cultivation (*4*−*6*). Lignin is a polyaromatic polymer that provides rigidity to woody tissues. Since most microorganisms are not able to degrade lignin, it prevents these tissues from a microbial attack by shielding of the underlying holocellulose (*7*). Hence, this shielding minimizes the bioavailability of holocellulose present in compost which is evidenced by the finding that the biodegradability of lignocellulose fibers is negatively correlated with the lignin content (*8*).

A. bisporus is a litter degrading fungus and not a typical wood degrading fungus and is therefore not classified as a brown rot (*9*) or white rot fungus (*7*). However, like white rot fungi *A. bisporus* can degrade lignin in lignocellulose-containing materials such as compost. Degradation of lignin by white rot fungi has been studied in detail. Those studies suggest that the extracellular enzyme system involved is induced by starvation. Therefore, it has been suggested that the main purpose of degrading lignin is to increase the bioavailability of the underlying holocellulose (*7*). A novelty of this research is that it provides experimental evidence for this hypothesis. By using relatively simple techniques it is even possible to quantify the ratio between lignin degradation and the increase in bioavailable holocellulose during *A. bisporus* cultivation on compost. This is done by using a calculation method in which data obtained from a chemical method, yielding total holocellulose, are compared with bioavailable holocellulose data from a newly developed method in which *S. thermophilum* is used. The latter method may also be of use for applied mushroom research as the data may contribute to the further understanding and optimization of the commercial *A. bisporus* cultivation.

MATERIALS AND METHODS

Cultivation of *S. thermophilum*. *S. thermophilum* was maintained on agar slants containing (L^{-1}): 10 g of glucose, 4 g of yeast extract and 12 g of agar. Growth occurred in the dark at 43 °C.

S. thermophilum was cultured on liquid medium containing (L^{-1}): sodium glutamate·H_2O 0.149 g, KCl 0.20 g,

* To whom correspondence should be addressed. Phone: +31 317−484976. Fax: +31 317−484011. E-mail: Rimko.tenHave@wur.nl.
† Wageningen University.
‡ Applied Plant Research.

10.1021/jf021131h CCC: $25.00
Published on Web 03/14/2003

$MgSO_4 \cdot 7H_2O$ 0.409 g, $CaCl_2 \cdot 2H_2O$, KH_2PO_4 1.0 g, yeast extract 0.1 g (GibcoBRL, Paisley, Scotland) and unless stated otherwise one of the respective carbon sources: microcrystalline cellulose 10 g (ca. 0.019 mm, Serva Feinbiochemica, Heidelberg, Germany), oat spelt xylan 10 g (Sigma, Steinheim, Germany), beech wood xylan 10 g (Sigma, Steinheim, Germany), and glucose$\cdot H_2O$ 11 g. During growth, carbon dioxide was measured in the headspace by a gas chromatograph with a model 427 apparatus (Hewlett-Packard, Palo Alto, California) fitted with a thermal conductivity detector (140 °C). The column (Hayesep Q, Chrompack, Middelburg, The Netherlands) was maintained at 110 °C and the carrier gas was helium (30 mL/min). The injection port was maintained at 110 °C and the injection volume was 100 μL.

For culturing *S. thermophilum* on compost, serum bottles (250 mL) containing 3.0 g of compost and 7.0 mL of H_2O were autoclaved for 30 min. at 121 °C. Two agar plugs (diameter = 0.5 cm) containing *S. thermophilum* were used as an inoculum. The bottles were loosely capped and placed away in a humidified sauerkraut jar at 43 °C. After 3 days, the *S. thermophilum* cultures were homogenized by shaking. For the compost samples, a total incubation time of 20 days was applied. Thereafter, the cultures were dried at 60 °C for 16 h and subsequently stored until further analysis.

Cultivation of *A. bisporus*. A conventional cultivation procedure was used to cultivate *A. bisporus*. This cultivation was performed in boxes (length × width × height = 28.3 × 21.5 × 13.3 cm) filled with 3.8 kg of phase II compost (*10*). As an inoculum, 15 g grains of corn colonized with *A. bisporus* Horst U1 were used. Nylon screen, containing square openings of 9 mm^2, was used to keep the compost separated from the casing soil. This was essential for easy recovery of the compost. The subsequent cultivation occurred according to van Gils (*11*). Three cultures were taken away after inoculation (day 1), at venting (day 24), after the first harvest period (day 37), and after the second harvest period (day 44). The harvested cultures were dried for 16 h at 111 °C, weighed, and milled ($\leq$ 1 mm^2).

Compost Analysis. *Dry Weight and Ash Content.* The dry weight was determined by weighing the samples after drying at 111 °C for 16 h. The ash content was analyzed by weighing the samples before and after burning at 550 °C for 16 h.

Klason Lignin. Klason lignin was determined in the straw part of the compost. An initial hot water extraction was used to prepurify the compost straw. Dry compost samples/cultures were mixed with H_2O (37.5 g/L) and autoclaved for 30 min at 121 °C. The undissolved straw fraction was obtained by filtering (glass microfiber, Whatman, USA) and was used for further analysis after drying at 111 °C for 16 h. After 10 mL of ice cold H_2SO_4 (72%) was added to 1.0 g of the dried straw fraction, the mixture was placed in a water bath at 30 °C for 1 h and the mixture was shaken vigorously at 15 min intervals. After the total incubation period, the mixture of straw and H_2SO_4 was added to 350 mL of H_2O and subsequently autoclaved for 2 h at 121 °C. The brownish solids, a mixture of lignin and ash, were obtained by filtering (glass microfiber filter, Whatman, USA). After drying the sample at 111 °C for 16 h, the ash content was determined in the solids. Klason lignin was calculated according to Klason lignin = total solids − ash in the solids.

Holocellulose. The amount of holocellulose was calculated according to holocellulose = total mass of the straw fraction − total Klason lignin − total ash in the straw fraction.

Statistical Procedure. All experiments were performed in triplicate unless stated otherwise. The data are presented as average values ± standard error of the mean (SEM). Propagation of random errors as used in linear combinations or in multiplicative expressions was calculated according to Miller and Miller (*12*).

Table 1. CO_2 Production (±) by *Scytalidium thermophilum* during Growth on Liquid Medium Supplemented with Several Carbon Sources (10 g/L) at Several Points in Time during the Cultivation[a]

	score based on CO_2 areas		
extra carbon source	day 1	day 5	day 9
none	–	–	–
glucose	+/–	++	++ +/–
xylan from oat spelts	+/–	++++	++
xylan from beech wood	–	++++	++ +/–
microcrystalline cellulose	–	++ +/–	++ +/–
carboxymethyl cellulose	+/–	+	–

[a] The scores are based on average CO_2 GC peak areas from three independent cultures.

RESULTS AND DISCUSSION

This research introduced a new method to estimate bioavailable holocellulose in compost in which the native compost organism *S. thermophilum* was used to utilize all available holocellulose. The use of this method in research on *A. bisporus* cultivation provided a new parameter which was useful in calculating the increase in bioavailable holocellulose during the cultivation of *A. bisporus*.

Assay to Estimate Bioavailable Holocellulose in Compost. *S. thermophilum* was selected for estimating bioavailable holocellulose in compost samples because it is known that it can grow on compost (*1*, *2*), it is suggested to be a nonlignin degrader (*2*), and it was found to colonize the compost quite rapidly near its optimal growth temperature. As shown in **Table 1** *S. thermophilum* produces CO_2 during growth on liquid minimal medium supplemented with all respective carbon sources tested. Cultures without an extra carbon source showed negligible CO_2 production. There was CO_2 production during growth on xylan, which is the major constituent of hemicellulose (*13*), and crystalline cellulose both of which are the constituents of holocellulose.

As shown in **Figure 1** growth of *S. thermophilum* on compost results in a decrease in culture dry weight (panel A) which stabilizes after 10 days of incubation. **Figure 1** also shows that lignin degradation was only marginal and holocellulose is evidently consumed by the fungus (panel B). On the basis of this information, a total incubation time of 20 days was applied for all studied compost samples which proved to be sufficient to utilize all bioavailable holocellulose from the compost samples (data not shown).

On the basis of this information, it was concluded that this assay could be used to estimate bioavailable holocellulose in compost.

Estimating Bioavailable Holocellulose in *A. bisporus* Compost Cultures. Compost samples of small scale *A. bisporus* cultures were analyzed during the cultivation. The dry weight of the cultures decreased proportionally to the increase in ash content (**Figure 2 A**). The dry weight decreased by 254 ± 5 g which was accounted for by the decrease in holocellulose and lignin of 182 ± 11 g, and 95 ± 3 g, respectively (**Figure 2B**). During the analysis, it was noted that the moisture content of the compost decreased from 2623 ± 2 g (n = 3) to 2010 ± 39 g (n = 3) at the end of the studied cultivation. The mushroom wet weights accounted for 903 ± 17 g (n = 9) and 501 ± 7 g (n = 6), respectively.

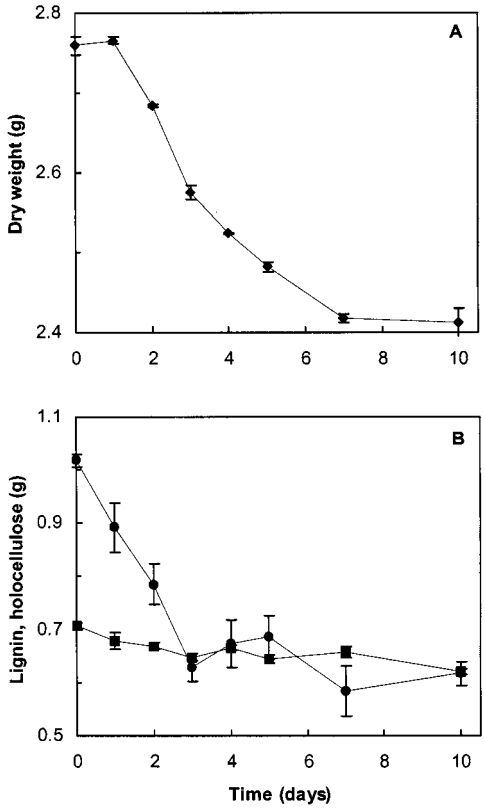

Figure 1. Analysis data obtained from *Scytalidum thermophilum* cultures grown on sterilized compost (3.0 g) at several stages during the cultivation. Panel A depicts the dry weight (g). Panel B depicts the amount of lignin (■, g) and holocellulose (●, g). Average values ± SEM ($n = 2$) are presented.

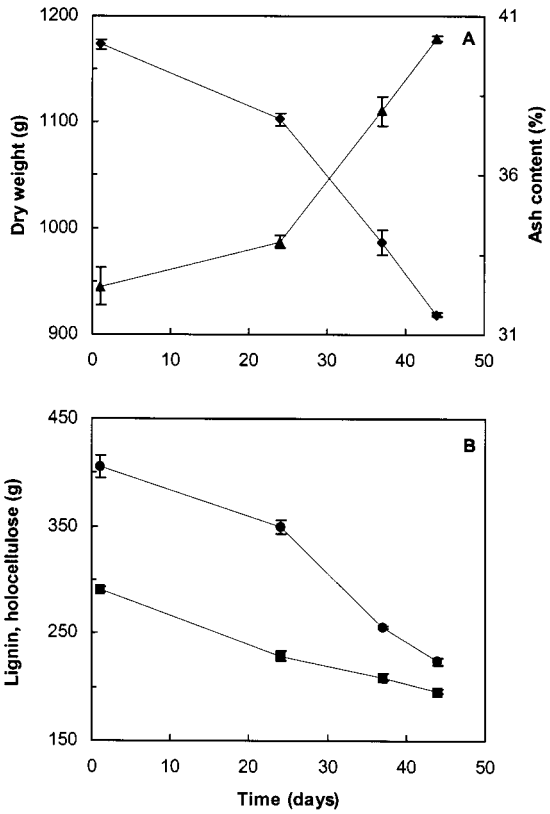

Figure 2. Analysis data obtained from *Agaricus bisporus* cultures grown on compost (3.8 kg) after inoculation (day 1), venting (day 24), the first harvest period (day 37), and the second harvest period (day 44). The experimental setup is representative for commercial cultivation. Panel A depicts the dry weight (◆, g) and the ash content (▲, %). Panel B depicts the amount of lignin (●, g) and holocellulose (■, g). Average values ± SEM ($n = 3$) are presented.

As shown in **Table 2** growth of *S. thermophilum* on the compost samples did not result in lignin degradation and did result in a significant decrease in the total amount of holocellulose during cultivation. From these data, the bioavailable holocellulose percentage was calculated. The resulting percentages were used to calculate the amount of bioavailable holocellulose in the *A. bisporus* cultures. As shown in **Table 3** the amount of bioavailable holocellulose sharply decreased from 166 ± 12 to 36 ± 2 g during the studied *A. bisporus* cultivation. The decrease is especially steep from day 24 to day 37, which corresponded with mushroom initiation and development of mushrooms for the first harvest period. The decrease in total holocellulose and bioavailable holocellulose was 45 ± 5% and 78 ± 9%, respectively.

The data on the total amount of holocellulose from the chemical analysis and the bioavailable holocellulose can also be used to calculate the decrease between two successive time points or time frame. The data presented in **Table 3** indicate that there was no significant difference between the decrease in the total holocellulose (Δ_{total}) and the decrease in bioavailable holocellulose ($\Delta_{bioavailable}$) between the consecutive time points. However, by calculating the decrease over the total period (day 1−44) the decrease in total holocellulose (182 ± 11 g) differed significantly and exceeded the decrease in bioavailable holocellulose (130 ± 12 g). If *A. bisporus* only consumed the available holocellulose, the latter two values would have been the same. However, the data suggest that growth of *A. bisporus* was associated with an increase in bioavailable holocellulose. This increase accounted for 31 ± 10%, roughly 30%, of the total initial bioavailable amount of holocellulose (166 ± 12 g). A factor that was likely to contribute to the apparent increase in bioavailable holocellulose could have been lignin degradation. **Figure 2** shows that *A. bisporus* degraded 95 ± 3 g of lignin in 44 days. The degradation of this amount of lignin resulted in the release of 52 ± 16 g of holocellulose, a ratio of roughly 2:1. Autoclaving, milling, and aging of the compost samples only slightly contributed to the release of additional holocellulose (data not shown).

Table 2. Estimation of the Amount of Bioavailable Holocellulose (%) Based on compost analysis data prior to and after cultivation of *Scytalidium thermophilum* on Compost Samples (3.0 g)[a]

	lignin (g)		holocellulose (g)		
sample	initial	end	initial	end	%
day 1	0.71 ± 0.01	0.73 ± 0.02	1.03 ± 0.01	0.61 ± 0.02	41.0 ± 2.7
day 24	0.59 ± 0.01	0.59 ± 0.01	0.94 ± 0.01	0.59 ± 0.02	38.8 ± 2.2
day 37	0.63 ± 0.01	0.64 ± 0.01	0.75 ± 0.02	0.59 ± 0.02	21.5 ± 3.9
day 44	0.63 ± 0.01	0.65 ± 0.00	0.70 ± 0.01	0.59 ± 0.00	15.9 ± 1.0

[a] For each compost sample the sampling times are indicated. The table also shows the amount of lignin (g) and the amount of total holocellulose (g). Average values ± SEM ($n = 3$) are presented.

A. bisporus is not a white rot fungus, but nonetheless appeared to be a very efficient lignin degrader (see **Figure 2B**) having the extracellular enzymes manganese peroxidase (*14*) and laccase (*15*). White rot fungi also excrete these oxidative enzymes to degrade lignin (*7*) suggesting that *A. bisporus* and white-rot fungi use similar oxidative mechanisms involved in lignin degradation. This also suggests that white rot fungi and *A. bisporus* degrade lignin for the same purpose, which is to

Table 3. Total Amount of Holocellulose (g) and Bioavailable Holocellulose (g) in the Studied *Agaricus Bisporus* Cultures (3.8 kg each) at Different Sampling Times (day)[a]

	holocellulose		time frame	holocellulose	
day	total (g)	bioavailable (g)	(day)	Δ_{total} (g)	$\Delta_{bioavailable}$ (g)
1	406 ± 11	166 ± 12			
24	349 ± 7	132 ± 8	1–24	57 ± 13	34 ± 14
37	256 ± 1	55 ± 10	24–37	93 ± 7	77 ± 13
44	224 ± 3	36 ± 2	37–44	32 ± 3	19 ± 10
			1–44	182 ± 11	130 ± 12

[a] The table also shows the differences in total (Δ_{total}, g) and bioavailable holocellulose ($\Delta_{bioavailable}$, g) for the indicated time frames. Average values ± SEM ($n = 3$) are presented.

gain access to the underlying holocellulose. These results confirm the hypothesis on the significance of lignin degradation by white rot fungi as proposed in the literature (*7*).

From published results, it is known that the *A. bisporus* mushroom yield as obtained during a typical third harvest period is generally significantly lower than the subsequent respective harvest periods (*16*). Our results indicate that the amount of bioavailable holocellulose is sufficient to form all mushrooms as obtained during a typical third harvest period. Therefore, it is conceivable that also other factors determine the mushroom yield as already pointed out elsewhere in the literature (*17*).

The results also indicate that the amount of holocellulose that will be used for mushrooms beyond the second harvest period has been entirely released by lignin degradation during the previous cultivation. This underlines the importance of lignin degradation in view of commercial *A. bisporus* cultivation.

LITERATURE CITED

(1) Straatsma, G.; Samson, R. A.; Olijnsma, W. O.; op den Camp, H. J. M.; Gerrits, J. P. G.; van Griensven, L. J. L. D. Ecology of thermophilic fungi in mushroom compost, with emphasis on *Scytalidium thermophilum* and growth stimulation of *Agaricus bisporus* mycelium. *Appl. Environ. Microbiol.* **1994**, *60*, 454−458.

(2) Tuomela, M.; Vikman, M.; Hatakka, A.; Itävaara, M. Biodegradation of lignin in a compost environment: a review. *Bioresour. Technol.* **2000**, *72*, 169−183.

(3) Straatsma, G.; Gerrits, J. P. G.; Augustijn, M. P. A. M.; op den Camp, H. J. M.; Vogels, G. D.; van Griensven, L. J. L. D. Population dynamics of *Scytalidium thermophilum* in mushroom compost and stimulatory effects on growth rate and yield of *Agaricus bisporus*. *J. Gen. Microbiol.* **1989**, *135*, 751−759.

(4) Wood, D. A.; Leatham, G. F. Lignocellulose degradation during the life cycle of *Agaricus bisporus*. *FEMS Microbiol. Lett.* **1983**, *20*, 421−424.

(5) Gerrits, J. P. G. Organic compost constituents and water utilized by the cultivated mushroom during spawn run and cropping. *Mushroom Sci.* **1969**, *7*, 111−126.

(6) Gerrits, J. P. G.; Bels-Koning, H. C.; Muller, F. M. Changes in compost constituents during composting, pasteurization and cropping. *Mushroom Sci.* **1967**, *6*, 225−243.

(7) ten Have, R.; Teunissen, P. J. M. Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chem. Rev.* **2001**, *101*, 3397−3413.

(8) Vikman, M.; Karjomaa, S.; Kapanen, A.; Wallenius, K.; Itävaara, M. The influence of lignin content and temperature on the biodegradation of lignocellulose in composting conditions. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 591−598.

(9) Xu, G.; Goodell, B. Mechanism of wood degradation by brown-rot fungi: chelator-mediated cellulose degradation and binding of iron by cellulose. *J. Biotechnol.* **2000**, *87*, 43−57.

(10) van Gils, J. J. In *Nutrition and Compost*; Darlington Mushroom Laboratories: Rustington, United Kingdom, 1988; pp 29−72.

(11) van Gils, J. J. In *Nutrition and Compost*; Darlington Mushroom Laboratories: Rustington, United Kingdom, 1988; pp 263−308.

(12) Miller, J. C.; Miller, J. N. In *Statistics for Analytical Chemistry*; Ellis Horwood Limited: Chichester, United Kingdom, 1993; pp 46−47.

(13) Fanta, G. F.; Abbott, T. P.; Herman, A. I.; Burr, R. C.; Doane, W. M. Hydrolysis of wheat straw hemicellulose with trifluoroacetic acid. Fermentation of xylose with *Pachysolen tannophilus*. *Biotechnol. Bioeng.* **1984**, *26*, 1122−1125.

(14) Lankinen, P. V.; Bonnen, A. M.; Anton, L. H.; Wood, D. A.; Kalkinen, N.; Hatakka, A.; Thurston, C. F. Characteristics and N-terminal amino acid sequence of manganese peroxidase from solid substrate cultures of *Agaricus bisporus*. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 170−176.

(15) Wood, D. A. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.* **1980**, *117*, 327−338.

(16) Chen, Y.; Chefetz, B.; Rosario, R.; Heemst, J. D. H.; Romaine, C. P.; Hatcher, P. G. Chemical nature and compostion of compost during mushroom growth. *Compost Sci. Util.* **2000**, *8*, 347−359.

(17) Kües, U.; Liu, Y. Fruiting body production in basidiomycetes. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 141−152.

Received for review November 18, 2002. Revised manuscript received January 21, 2003. Accepted January 27, 2003. This research is supported by the Technology Foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs.

JF021131H