J Wood Sci (2011) 57:429–435
DOI 10.1007/s10086-011-1185-y

© The Japan Wood Research Society 2011

ORIGINAL ARTICLE

Ryo Hiyama · Seiki Gisusi · Akira Harada

Evaluation of waste mushroom medium from cultivation of shiitake mushroom (*Lentinula edodes*) as feedstock of enzymic saccharification

Received: October 13, 2010 / Accepted: February 25, 2011 / Published online: June 28, 2011

Abstract The feasibility of using waste mushroom medium (WMM) as a substrate for enzymic saccharification to produce bioethanol or other bioproducts was investigated. WMM was broken up or left unbroken and stored at constant temperatures (5°, 15°, and 25°C) for 1 or 2 month(s) to accelerate lignin degradation by shiitake, which is a white rot fungus, and to increase the saccharification ratio. When the physicochemical properties of WMM and stored WMM were investigated, it was evident that the mushroom medium (MM) lignocellulose had decomposed during shiitake cultivation and its subsequent storage at a constant temperature. WMM and stored WMM were more susceptible to attack by cellulase than MM. When the unbroken WMM that was stored at 25°C for 2 months was saccharized with meicelase at 5 FPU/g substrate, its saccharification ratio was higher than that of unstored WMM. The maximum glucose yield of the stored WMM was 200.5 mg/g substrate, approximately 4.1 times as large as the value for autoclaved MM under the same saccharification condition. The saccharification ratio of the constituent cellulose was approximately 60%.

Key words Waste mushroom medium · *Lentinula edodes* · Enzymic saccharification · Lignocellulosic biomass · Constant temperature storage

Introduction

Shiitake mushroom (*Lentinula edodes*) is one of the most commonly produced edible mushrooms in the world.[1] Total production was more than 1.6 million t in the world in 1997. Shiitake production is currently increasing,[2] and commercially grown shiitake are cultivated on natural bed logs or synthetic media. The use of synthetic media is increasing in several countries such as China,[3] Japan,[4] and the United States.[5] Approximately 79% of the 70,342 t of fresh shiitake that was harvested in Japan in 2008 was cultivated on synthetic media.[6] The fresh weight of waste mushroom medium (WMM) after the cultivation of shiitake is estimated to be in excess of 50,000 t/year.

Mushroom medium (MM) is composed of hardwood meal. It is expected that certain levels of cellulose, hemicelluloses, and lignin remain after cultivation.[7] WMM can be regarded as a lignocellulosic biomass that could be used as a feedstock for enzymic saccharification to produce bioethanol or other bioproducts as an alternative to fossil resources, because lignocellulosic biomass is a renewable resource. However, lignocellulosic biomass has not been extensively used as such a feedstock, the main reason being the high cost for its transportation, storage, and conversion. Transportation for collecting this resource is costly because lignocellulosic biomass is bulky and has a low density.[8,9] Storage cost is incurred because lignocellulosic biomass has seasonal availability.[9] Conversion cost is also high because of the rigid structure. Lignocellulosic biomass has a cellulose-hemicellulose-lignin network that limits the access of enzymes such as cellulase and hemicellulase.[10,11]

WMM is more advantageous than other types of lignocellulosic biomass because of its high density, availability throughout the year, and decreased structural rigidity resulting from the growth process of shiitake fungus. The shift from natural bed log cultivation to synthetic medium cultivation has consolidated scattered mushroom production areas into narrow production zones in mushroom factories.[12] The WMM obtained from these narrow zones therefore can be rapidly utilized. WMM can be supplied throughout the year because the shiitake mushroom is stably produced throughout the year in the mushroom factories.[12] Therefore, WMM does not incur large transportation and storage costs. In addition, because steady amounts of WMM can be obtained, a biomass conversion facility could operate without interruption.

R. Hiyama (✉) · S. Gisusi · A. Harada
Hokkaido Research Organization Forest Products Research Institute, Nishikagura 1-10 Asahikawa, Hokkaido 071-0198, Japan
Tel. +81-166-75-4233; Fax +81-166-75-3621
e-mail: hiyama-ryo@hro.or.jp

A part of this work was presented as a poster at the Symposium of the Society for Biotechnology, Japan, Kitami Institute of Technology, July 23, 2010

Shiitake is a type of white rot fungi that can degrade lignin, cellulose, and hemicellulose.[7] The enzymic saccharification ratio of the lignocellulose of WMM increases after cultivation of some white rot fungi such as maitake[13] and enokitake.[14] However, there are not many reports on the enzymic saccharification ratio of shiitake WMM. The cultivation method of shiitake is significantly different from that of maitake and enokitake; shiitake requires a longer cultivation period and allows multiple harvests, rather than the single harvest of maitake and enokitake. These differences may result in a higher ratio of lignin degradation and enzymic saccharification.

Storing shiitake WMM could possibly enhance the enzymic saccharification ratio. Enzymic saccharification of maitake WMM stored at a constant temperature was higher than that not stored.[15] However, there are few studies on shiitake WMM storage.

The aim of this work was to evaluate WMM of shiitake as a feedstock for enzymic saccharification to produce bioethanol or other biorefined products. Thus, we surveyed changes in the properties of synthetic media before and after cultivation, and the subsequent storage at a constant temperature. Broken and unbroken WMM were stored at three temperatures, and the enzymic saccharification ratios of all treatments were compared.

Materials and methods

Materials

MM was prepared according to the instructions of Kinokoseisan Sougoukenkyusho, Hokkaido, Japan. MM was composed of lignocellulose powder: 244 g (oven dry matter, ODM) of *Quercus mongolica* var. *grosserrata*, 105 g ODM of *Betula platyphylla* var. *japonica*, and 22 g ODM of buckwheat husk. The mixture was supplemented with 141 g ODM of a mixture of rice bran, wheat bran, hominy feed (Sunny Maize, Shizuoka, Japan), and Derutoppu (Mori, Gunma, Japan). Water was added to the mixture to obtain a moisture content of 61% and weight of 1,300 g. MM (fresh weight, 1,300 g) was packed in a plastic bag (approximately 10 cm W × 9 cm D × 35 cm H; Miki-pack 1.3 × 380BF, Miki-sangyo, Aichi, Japan) and then autoclaved at 121°C for 1 h (AMM).

WMM produced from the commercial cultivation of shiitake Mori XR-1 (Mori) was supplied by Kinokoseisan Sougoukenkyusho. The mushrooms were cultured for 85 days (20°–25°C, ≈70% RH), after which the shiitake fruiting bodies were harvested. After the first harvest, the medium was soaked in water, and subsequently cultured for 20 days (15°–20°C, > 80% RH), which was followed by fruiting body formation. This procedure was repeated twice. The fruiting bodies were harvested three times in total.

WMM (cylinder-shaped, approximate height 15 cm, φ 10 cm) was stored under the following conditions. A total of 15 samples of WMM were manually broken into pieces less than 4 × 4 × 4 cm (BWMM), and the other 15 samples were left unbroken (UWMM). Both samples were packed in Miki-packs with two filters (MT-S25B; approximate 19 cm W × 11 cm D × 40 cm H) and stored at a constant temperature. Room humidity was maintained at 60–80% RH. During storage, light was excluded from WMM, except once a week for observations. BWMM and UWMM were stored at 5°C for 2 months and at 15° or 25°C for 1 month or 2 months, respectively. BWMM stored at 15°C for 2 months was designated as B15-2, UWMM stored at 25°C for 1 month was designated as U25-1, and so forth. All samples, except for MM, were prepared in triplicate. However, chemical analysis and enzymic saccharification in MM was performed in triplicate.

Pulverization and sieving

All substrates were manually broken into pieces less than 4 × 4 × 4 cm, then oven-dried at 50°–60°C until the moisture content of the sample became less than 10%. The pieces were milled with a Wiley mill (type 1029-08; Yoshida Seisakusho, Tokyo, Japan) at 980 rpm using a 2-mm mesh grating. The substrates were screened with five sieves with mesh sizes of 0.125, 0.25, 0.5, 1.0, and 2.0 mm, respectively. The percentage of the substrate remaining between the screens was determined with dry sieving apparatus according to JIS Z 8815.

Chemical component analysis

Lignin and constituent polysaccharide (glucan, xylan, galactan, arabinan, and mannan) content was determined using the method of Sluiter et al.[16] with the following minor modifications. Extraction with ethanol–benzene was omitted from the method because it had very little effect on determining the contents of lignin and sugars in preliminary analyses. The substrates were hydrolyzed with 72% sulfuric acid; the residue was used to determine the content of acid-insoluble lignin and the supernatant was used to determine the content of acid-soluble lignin and the constituent sugars. Constituent sugar monosaccharides (D-glucose, D-xylose, D-galactose, L-arabinose, and D-mannose) were analyzed using an HPLC system (L2000 series; Hitachi High-Technologies, Tokyo, Japan) equipped with an RI detector and a tandemly arranged Aminex HPX-87P × 2 (Bio-Rad Laboratories, Hercules, CA, USA). Column temperature was set at 80°C. The eluent was deionized water with a flow rate of 1 ml/min. Because the content of galactan, arabinan, and mannan was relatively small, they were combined and termed "minor sugars."

The acid-soluble lignin content was estimated spectrophotometrically at a maximum absorbance of 199–206 nm according to JISP 8008-1961 using a 228A spectrophotometer (Hitachi, Tokyo, Japan).

The total content of α-glucan (starch and glycogen) in the substrate was measured according to the method of Hideno et al.[13] α-Amylase (>1.5 U/mg, from *Bacillus subtilis*; Wako Pure Chemicals, Osaka, Japan) and glucoamylase (0.1 U/mg, from *Rhizopus* sp.; Wako Pure Chemicals) were used to hydrolyze the α-glucan. The hydrolysate was assayed using a

colorimetric kit (Glucose C2; Wako Pure Chemical) with a 228 A spectrophotometer (Hitachi). The content of β-glucan was calculated by subtracting the content of α-glucan from that of total glucan. The content of "other components" was calculated by subtracting the sum of the amounts of acid-insoluble lignin, acid-soluble lignin, β-glucan, xylan, minor sugars, and α-glucan from the dry weight of the substrate. The other component possibly included ash and extracts.

All lignin and sugars were calculated based on the dry weight of the substrate and expressed in weight percentages. Dry weight was calculated by drying a subsample of the substrate in an oven at 105°C for 24 h and weighing it to obtain an accuracy of ±0.1 mg.

Enzymic saccharification

A commercial cellulase preparation (Meicelase; Meiji Seika, Tokyo, Japan) derived from *Trichoderma viride* was used to saccharize the substrates. Meicelase activity was expressed as filter paper units (FPU), based on the method of Adney and Baker.[17] The substrate (240 mg ODM) was placed into a test tube, and meicelase (4.8 FPU) in 12 ml 0.1 M sodium acetate buffer (pH 4.8) was added. The tubes were incubated at 50°C for 24 h on a shaker (Multi Shaker MMS-310; Tokyo Rikakikai, Tokyo, Japan) at 80 rpm.

Enzymic saccharification of AMM, WMM, U15-1, and U25-2 was examined with different amounts of meicelase and for different incubation times. The combinations of enzyme activity (FPU/g ODM) for the substrate and incubation time (FPU/h) were 5-48, 20-48, 50-48, 20-24, and 20-72, respectively. These experiments were performed at 40°C because no incubator able to keep the substrate at 50°C was available. However, Teratani et al.[18] showed that meicelase activity had little difference between 40°C and 50°C of incubation temperature. In addition, the differences observed in the result of preliminary saccharification analyses between 40°C and 50°C were also small. Therefore, this change of cultivation temperature was considered to have little influence on the saccharification.

Meicelase saccharification of starch (starch, soluble; Kanto Chemical, Tokyo, Japan) was also examined in the same way as just described. The monosaccharides in the hydrolysate were analyzed using the HPLC system as described above.

The saccharification ratio of β-glucan (S_1) was calculated using Eq. 1:

$$S_1(\%) = [\{B - (C \times D/100)\}/A] \times 100 \quad (1)$$

where A is the content of β-glucan (mg), B is the amount of glucan hydrolyzed by meicelase (mg), C is the α-glucan content (mg), and D is the percentage rate in meicelase saccharification of starch (%).

The saccharification ratio of xylan or minor sugars (S_2) was calculated using Eq. 2:

$$S_2(\%) = (E/F) \times 100 \quad (2)$$

where E is the amount of xylan or minor sugars hydrolyzed by meicelase (mg) and F is the content of xylan or minor sugars (mg).

The β-glucan saccharification yield (Y) was calculated using Eq. 3:

$$Y(\%) = [\{B - (C \times D/100)\}/G] \times 100 \quad (3)$$

where G is the content of β-glucan of MM (mg).

Statistical analysis

The homoscedasticity of particle size, chemical components, and saccharification ratio of each substrate were assessed using Bartlett's test. The difference among the substrates whose homoscedasticity was assumable was examined by one-way analysis of variance (ANOVA) and the Tukey–Kramer test. The difference among the substrates whose homoscedasticity was not assumable was examined using the Games–Howell test. $P < 0.05$ was considered significant.

Results and discussion

Distribution of particle size

When lignocellulose is saccharized with enzymes, its particle size significantly influences saccharification efficiency.[11,13,14] As shown in Table 1, there are almost no differences in the size-class ratios between MM and AMM. The ratio of size-class 1.0–2.0 mm of WMM and stored WMM was lower than that of MM and AMM. The ratio of size-class 0.5–1.0 mm of WMM and stored WMM, except for U15-1, was lower than that of MM and AMM. In contrast, the ratio of size-class 0–0.125 mm of WMM and stored WMM was higher than that of MM and AMM. In addition, the ratio of size-class 0.125–0.25 mm of WMM and stored WMM was higher than AMM. The ratio of size-class 0.125–0.25 mm of six stored WMM substrates (B5-2, B25-1, U5-2, U15-2, U25-1, U25-2) was higher than that of MM. Although the ratio of size-class 0.25–0.5 mm of WMM and six stored WMM substrates was higher than that of MM or AMM, the difference in their averages was smaller than those of size-classes 1.0–2.0 mm and 0–0.125 mm. Thus, autoclaving had relatively little influence on the particle size whereas cultivating shiitake reduced the particle size. Thus, increase in the ratio of finer substrate powder indicated that WMM and stored WMM was ground more easily than MM and AMM for use for subsequent enzymic saccharification.

The ratios of size-class 0.5–1.0 and 0.25–0.5 mm of U15-1 were higher than those of U15-2. In contrast, the ratio of size-class 0–0.125 mm of U15-1 was lower than that of U15-2. In addition, the ratios of size-classes 0.5–1.0 and 0.25–0.5 mm of U25-1 were higher than those of U25-2. Thus, crushability may be improved by lengthening the storage period in UWMM.

Changes in components

The contents of lignin and sugars obtained from the substrates and dry weight changes are shown in Table 2. The

Table 1. Changes in size-class ratios of the shiitake substrate after autoclaving, cultivation, and storage

Substrate[a]	Granulometry (%)				
	Size-class				
	1.0–2.0 mm	0.5–1.0 mm	0.25–0.5 mm	0.125–0.25 mm	0–0.125 mm
MM	6.0 ± 0.9 a	49.0 ± 2.3 a	28.3 ± 1.0 f	9.4 ± 1.2 de	7.3 ± 1.1 d
AMM	5.4 ± 0.9 a	49.9 ± 0.2 a	29.3 ± 0.6 ef	9.5 ± 0.2 e	6.0 ± 0.4 d
WMM	1.5 ± 0.1 b	32.2 ± 1.5 bcd	33.9 ± 1.1 abc	16.4 ± 0.6 cd	16.0 ± 1.7 abc
B5-2	1.3 ± 0.4 b	28.5 ± 1.2 bcd	33.4 ± 1.7 abc	17.8 ± 0.4 abc	19.1 ± 2.3 ab
B15-1	1.4 ± 0.3 b	30.1 ± 1.8 cd	33.5 ± 0.3 abc	17.1 ± 0.5 cd	18.0 ± 2.0 abc
B15-2	1.5 ± 0.3 b	28.9 ± 2.2 cd	31.1 ± 1.0 cdef	18.2 ± 1.7 abcd	20.3 ± 0.8 a
B25-1	1.1 ± 0.2 b	28.2 ± 0.9 cd	32.8 ± 0.3 bcd	18.0 ± 0.5 abc	20.0 ± 0.7 a
B25-2	1.3 ± 0.0 b	27.6 ± 3.4 cd	32.0 ± 2.0 cde	19.2 ± 2.5 abcd	19.8 ± 2.9 a
U5-2	0.9 ± 0.1 b	29.0 ± 1.9 cd	34.4 ± 0.2 abc	18.2 ± 0.4 ab	17.6 ± 1.6 abc
U15-1	1.0 ± 0.1 b	35.0 ± 3.0 ab	35.6 ± 1.5 ab	15.3 ± 0.9 abcd	13.2 ± 2.2 c
U15-2	1.4 ± 0.4 b	28.4 ± 0.5 cd	32.0 ± 0.8 cde	17.9 ± 0.3 abc	20.3 ± 1.1 a
U25-1	0.9 ± 0.2 b	32.6 ± 1.7 bc	36.3 ± 1.5 a	15.9 ± 0.7 bc	14.2 ± 2.3 bc
U25-2	1.3 ± 0.2 b	26.7 ± 1.0 d	32.8 ± 0.7 bcd	18.6 ± 0.4 a	20.7 ± 1.9 a

Values represent means of three repetitions ± SD
Numbers with different letters indicate significant difference among substrates ($P < 0.05$)
Numbers after B and U are temperature (°C) and months of storage
Significance in size-classes except for 0.25–0.125 mm according to Tukey–Kramer test, and in size-class 0.125–0.25 mm according to Games–Howell test
[a] MM, mushroom medium of shiitake mushroom (*Lentinula edodes*); AMM, autoclaved MM; WMM, waste mushroom medium after cultivation of shiitake; B, WMM stored after it was fragmented; U, unbroken and stored WMM

Table 2. Content of lignin and sugars obtained from the substrates and dry weight changes

Substrate[a]	Component [% (w/w)][b]							Changes in dry weight [%(w/w-MM)]
	Acid-insoluble lignin	Acid-soluble lignin	β-Glucan	Xylan	Minor sugars[c]	α-Glucan	Other components[d]	
MM	24.3 ± 0.5 abc	3.4 ± 0.3 b	28.5 ± 1.6 abc	13.3 ± 1.2 a	6.7 ± 0.5 a	5.7 ± 0.3 a	18.1 ± 3.2	100
AMM	24.5 ± 0.8 ab	3.2 ± 0.4 b	28.2 ± 2.3 abc	13.8 ± 0.3 a	8.3 ± 3.8 abc	5.0 ± 0.5 a	17.0 ± 0.7	98.3 ± 1.1 a
WMM	17.8 ± 1.7 cdef	7.4 ± 1.1 a	27.1 ± 3.4 abcd	11.1 ± 1.3 b	7.1 ± 5.0 abc	1.2 ± 0.3 bc	28.4 ± 5.4	46.8 ± 2.5 bc
B5-2	18.9 ± 3.3 bcdef	7.6 ± 0.8 a	23.7 ± 3.4 bcde	9.1 ± 1.1 bcd	4.9 ± 1.4 abc	1.5 ± 0.8 bcd	34.3 ± 1.2	44.4 ± 5.4 bcd
B15-1	19.1 ± 0.7 bcdef	8.5 ± 1.1 a	24.3 ± 0.5 abcde	9.9 ± 1.2 bc	6.2 ± 2.3 abc	1.3 ± 0.3 b	30.8 ± 5.2	41.1 ± 3.3 bcd
B15-2	22.4 ± 3.5 abcde	8.1 ± 1.2 a	19.7 ± 1.7 e	8.0 ± 0.7 cd	3.5 ± 0.7 bc	0.9 ± 0.3 bc	37.5 ± 1.7	33.7 ± 1.4 d
B25-1	23.1 ± 2.6 abcd	7.6 ± 0.1 a	21.1 ± 1.3 de	8.4 ± 0.6 cd	3.1 ± 0.6 bc	0.8 ± 0.7 bcd	35.9 ± 0.8	34.9 ± 2.3 cd
B25-2	27.6 ± 4.7 a	7.2 ± 0.5 a	17.9 ± 2.0 e	7.3 ± 0.5 d	3.0 ± 1.0 c	0.8 ± 0.1 c	36.2 ± 0.9	26.9 ± 1.5 e
U5-2	17.1 ± 1.6 def	7.3 ± 0.6 a	30.4 ± 0.3 a	9.8 ± 0.4 bc	3.1 ± 0.1 abc	N.D. d	32.2 ± 1.2	43.8 ± 5.1 bcd
U15-1	15.9 ± 1.0 f	7.6 ± 0.2 a	29.4 ± 2.4 abc	9.5 ± 0.4 bcd	4.2 ± 0.3 b	N.D. d	33.5 ± 1.3	47.5 ± 5.1 b
U15-2	19.7 ± 0.9 bcdef	7.7 ± 0.1 a	23.5 ± 1.3 cde	8.3 ± 0.2 cd	3.2 ± 0.7 bc	0.4 ± 0.5 bcd	37.2 ± 1.3	33.4 ± 0.3 d
U25-1	16.7 ± 2.4 ef	7.4 ± 0.3 a	30.0 ± 2.9 ab	9.5 ± 0.3 bcd	3.2 ± 0.3 c	0.1 ± 0.2 d	33.1 ± 0.3	41.1 ± 8.4 bcd
U25-2	16.5 ± 0.3 ef	6.3 ± 1.6 a	29.9 ± 1.1 ab	7.9 ± 0.4 cd	3.5 ± 0.4 c	N.D. d	35.9 ± 1.1	34.3 ± 1.6 cd

Values represent means of three repetitions ± SD
Numbers with different letters indicate significant difference among substrates ($P < 0.05$)
Significance in the components except for minor sugars and α-glucan according to Tukey-Kramer test, and in minor sugars and α-glucan according to Games–Howell test
N.D., not detected
[a] See Table 1 for identification of substrates
[b] Percentage of weight of lignin and sugars on the basis of dry weight of each substrate
[c] Total content of galactan, arabinan, and mannan
[d] Dry weight minus the amount sum of acid-insoluble lignin, acid-soluble lignin, β-glucan, xylan, minor sugars, and α-glucan

dry weight of AMM and WMM was 489.8 ± 5.3 g and 233.0 ± 12.5 g (mean ± SD), respectively. When the fruiting bodies were harvested, 52% of the raw substrate had been consumed. The dry weight of B15-2, B25-2, and U15-2 was lower than that of WMM; the dry weight of B25-2 was the lowest of all stored WMM substrate. Thus, decrease in dry weight depends on temperature and the period of storage.

The acid-insoluble lignin content of WMM was lower than that of AMM, which indicates that the weight decrease of acid-insoluble lignin was more than the entire weight decrease of AMM from shiitake cultivation. The acid-insoluble lignin of U15-1, U25-1, and U25-2 was lower than that of B15-2, B25-1, and B25-2, respectively. This result showed that acid-insoluble lignin content differed according to the storage method. The acid-soluble lignin content of WMM and stored WMM was greater than that of MM and AMM. In addition, lignin degradation by shiitake enzymes might contribute to the increase in acid-soluble lignin.

β-Glucan content in WMM and stored WMM, except for B15-2, B25-1, and B25-2, did not significantly decrease

compared to that in MM and AMM. Xylan content in WMM was less than that in MM and AMM. Xylan content in B15-2, B25-2, U15-2, and U25-2 was less than that in WMM. Thus, longer storage periods at high temperatures decreased xylan content. Minor sugars in B15-2, B25-1, B25-2, U15-1, U15-2, U25-1, and U25-2 were less than those in MM, indicating that storage at high temperatures decreased the content of these minor sugars. α-Glucan content in WMM and stored WMM was much less than that in MM and AMM. The decrease in the ratio of α-glucan was larger than that of acid-insoluble lignin, β-glucan, and xylan from WMM. Thus, in the substrates where mycelia or fruiting bodies had grown, α-glucan was the first component to be consumed.

Enzymic saccharification

The saccharification ratio of MM, AMM, WMM, and stored WMM is shown in Fig. 1A. The saccharification ratio of xylan and minor sugars (S_2) of each substrate was rather low. Meicelase has a relatively low xylanase activity,[14,19] explaining the low saccharification ratio of xylan.

The saccharification ratio of β-glucan in WMM was approximately 2.8 times higher than that of AMM, indicating that shiitake cultivation increases the saccharification ratio of the substrate. There were significant differences in saccharification ratio between the substrates stored under different conditions. The saccharification ratio of U25-1 and U25-2 was higher than that of B25-1 and B25-2. Especially, the saccharification ratio of U25-2 was 6.4 times as high as that of B25-2. The saccharification ratios of B15-2, B25-1, and B25-2 were lower than those of B5-2 and UWMM. The residual ratio of β-glucan of B15-2, B25-1, and B25-2 was low (see Table 2). In addition, the acid-insoluble lignin content in B25-1 and B25-2 was higher than that of U25-1 and U25-2 (Table 2). It has been reported that the large content of residual lignin limits enzymic saccharification.[20] Thus, low content of β-glucan and high content of lignin are considered to have caused lower saccharification ratios of β-glucan in B25-1 and B25-2 compared to U25-1 and U25-2. Mold was observed in BWMM, especially under high temperature and long storage conditions, which suggests that mold caused the low saccharification yield. Negligible mold was observed in UWMM. WMM of shiitake had a layer composed of mature mycelia on the substrate surface; this layer appeared to protect the substrate from mold growth. In this study, the interiors of the plastic storage bags were under humid, unsterilized, and slowly permeable conditions during storage. The moisture content of WMM was 50–70%, and that of stored WMM was maintained at 50–70%. Such a storage method was adopted for practical use. In terms of practical storage, WMM, which would be not packed in plastic bags or be unsterilized, could be stacked in stockyards. When WMM is piled up, most of this pile of WMM, except for its surface, will be stored in conditions such that the permeability of air will be low and the humidity will be high.

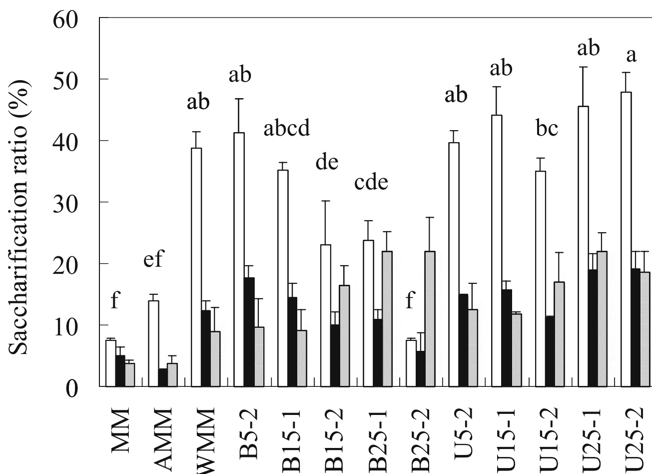

Fig. 1. Saccharification ratio of MM, AMM, WMM, and stored WMM to each constituent polysaccharide by meicelase [20 filter paper units (FPU)/g substrate] at 50°C for 24 h ($n = 3$; *error bar*, standard deviation). *Open bars*, β-glucan; *solid bars*, xylan; *diagonal striped bars*, minor sugars. Substrates: MM, mushroom medium of shiitake mushroom (*Lentinula edodes*); AMM, autoclaved MM; WMM, waste mushroom medium after cultivation of shiitake; B, WMM stored after it was fragmented; U, unbroken and stored WMM. The significance of saccharification ratio of β-glucan among the substrates was examined using the Tukey-Kramer test. Different letters of saccharification ratio of β-glucan indicate significant differences among substrates ($P < 0.05$)

The residual ratio of β-glucan and the saccharification ratio in BWMM were low, indicating that WMM should be stored in the unbroken state.

The amounts of glucose obtained by enzymic saccharification from MM, AMM, WMM, B5-2, B15-1, B15-2, B25-1, B25-2, U5-2, U15-1, U15-2, U25-1, and U25-2 were 23.5 ± 0.6, 43.5 ± 0.1, 116.7 ± 19.1, 108.0 ± 15.0, 88.5 ± 12.3, 51.2 ± 19.3, 55.8 ± 11.1, 14.0 ± 0.8, 134.3 ± 7.7, 145.1 ± 25.7, 91.7 ± 9.9, 153.5 ± 36.6, and 159.2 ± 14.7 g/g substrate ODM (mean ± SD), respectively. There was a significant difference only between MM and AMM examined using the Games–Howell test.

Effects of saccharification conditions

To increase the low saccharification ratios of β-glucan (less than 50%; see Fig. 1A), various enzymic saccharification conditions were examined. Figure 2 shows the effect of processing time on the saccharification ratio of β-glucan in AMM, WMM, U15-1, and U25-2. Saccharification ratios were significantly different between WMM and U25-2 at 24 h. The saccharification ratio of U25-2 at 72 h was 60.3% ± 2.0% (mean ± SD). The glucose obtained from U25-2 under this saccharification condition was 200.5 ± 13.3 mg/g substrate (mean ± SD). Although the saccharification ratio tended to increase when the time was increased to 72 h, the inclination of the graph appeared to tail off. Therefore, even if the saccharification time is extended from 72 h, it is unlikely to have a significant effect on saccharification ratio.

Figure 3A shows the effects of the amount of meicelase on the saccharification ratio of β-glucan of AMM, WMM, U15-1, and U25-2. The saccharification ratio did not significantly increase, even when the amount of meicelase added

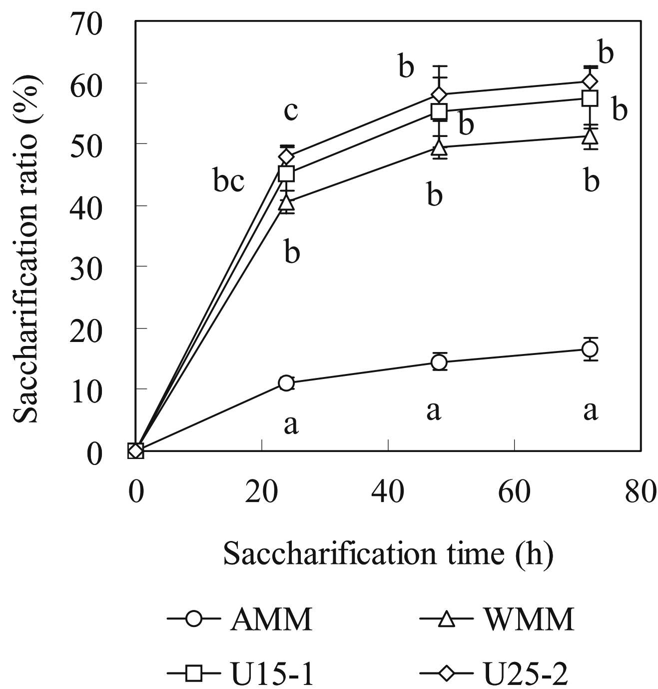

Fig. 2. Time-course change in saccharification ratio of AMM, WMM, U15-1, and U25-2 by meicelase (20 FPU/g substrate) at 40°C when processing time changed ($n = 3$; *error bar*, standard deviation). Significance among the substrates was examined using the Tukey–Kramer test. Different letters indicate significant differences among substrates ($P < 0.05$)

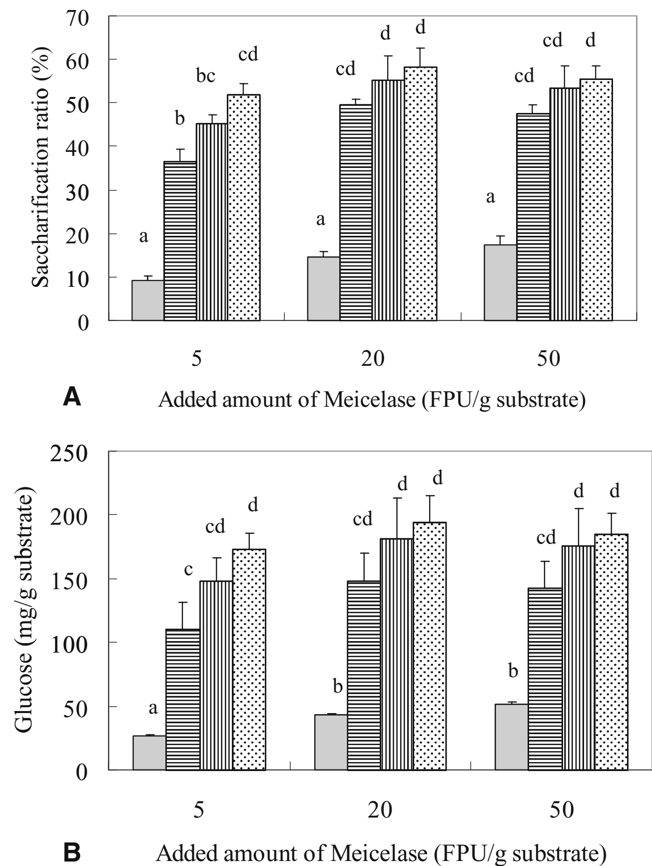

Fig. 3. Hydrolysis of AMM, WMM, U15-1, and U25-2 at 40°C for 48 h by adding different amounts of meicelase ($n = 3$; *error bar*, standard deviation). **A** Saccharification ratio to each constituent polysaccharide. **B** Saccharized glucose (mg) per dry weight of the substrate (g). *Shaded bars*, AMM; *horizontal striped bars*, WMM; *vertical striped bars*, U15-1; *stippled bars*, U25-2. Significance was examined using the Tukey–Kramer test. *Different letters* indicate significant differences among substrates ($P < 0.05$)

was increased from 20 FPU/g substrate to 50 FPU/g substrate. When meicelase was added at 20 and 50 FPU/g substrate, the saccharification ratio only differed significantly between AMM and the other substrates. When meicelase was added at 5 FPU/g substrate, the saccharification ratio differed significantly between WMM and U25-2 in addition to that between AMM and the other substrates. When the amount of meicelase added was decreased from 20 to 5 FPU/g substrate, the decrease in the saccharification ratio of β-glucan differed between stored and unstored WMM: a significant decrease in the saccharification ratio of β-glucan in U25-2 was not detected whereas it was detected in WMM and U15-1. This result suggests that storing WMM at 25°C for 2 months was effective in decreasing the amount of cellulase required.

The β-glucan saccharification yield (Y), which was based on β-glucan content in MM, was calculated for AMM, WMM, U15-1, and U25-2 to compare the saccharification ratios tempered with the weight change caused by cultivation or storage. The β-glucan saccharification yields of AMM, WMM, U15-1, and U25-2 were 9.5% ± 1.0%, 17.2% ± 2.0%, 21.5% ± 3.2%, and 17.6% ± 1.6% (mean ± SD), respectively, when meicelase was added at 5 FPU/g substrate and the substrates were saccharized at 40°C for 48 h. The β-glucan saccharification yield only differed significantly between AMM and the other substrates ($P < 0.05$, Tukey–Kramer test).

Figure 3B shows the amounts of glucose obtained by enzymic saccharification of AMM, WMM, U15-1, and U25-2 when amount of meicelase added was varied. U25-2 yielded 193.5 ± 21.8 g (mean ± SD) glucose under the following saccharification conditions: meicelase added at 20 FPU/g substrate, saccharification for 48 h, and saccharification temperature at 40°C. There were no significant differences between WMM and stored WMM when the added amount of meicelase was 20 and 50 FPU/g substrate. However, when meicelase was added at 5 FPU/g substrate, the amount of glucose obtained from U25-2 was more than that from WMM. Hence, storing WMM is an effective way of reducing the required amount of the enzyme and increasing the amount of glucose obtained from a substrate. It is very important for the practical use of the enzymic saccharification process of lignocelluloses, because the decrease in the amount of the enzyme addition lowers the cost of the process.

The saccharification ratio of β-glucan in shiitake WMM was nearly 50% (see Fig. 2), was similar to that of maitake WMM.[13] When WMM was saccharized for 24 and 48 h with meicelase at 20 FPU/g substrate, the total saccharification ratios of all the constituent polysaccharides of shiitake

WMM were calculated to be 28% and 36%, respectively. These values were higher than the corresponding values of about 18% and 24%, respectively, for enokitake WMM.[14] Hence, shiitake increased the saccharification ratio of the polysaccharides in MM to similar or better levels than other white rot fungi.

In this study, the saccharification ratio of β-glucan from lignocelluloses in shiitake MM, which mainly consists of hardwoods, was 7.4% ± 0.4% (mean ± SD). Some pretreatments are required to obtain a high saccharification ratio from lignocellulosic biomass, including hardwoods.[10,20] Various pretreatments of hardwoods have been examined using acid,[21,22] alkaline,[23] or high-pressure ethanol,[24] and thus, high saccharification ratios (70% to nearly 100%) have been obtained. However, such pretreatment is usually costly in terms of equipment and energy. Because 50–60% glucose was easily obtained from WMM and stored WMM, such as U15-1, U25-1, and U25-2, with only rough pulverizing and no pretreatment, these substrates could be used as cost-effective feedstocks for enzyme saccharification to produce bioethanol or other bioproducts.

References

1. Shang ST (1999) World production of cultivated edible and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* (Berk.) Sing. in China. Int J Med Mushrooms 1:291–300
2. Chen AW (2007) Growing Shiitake mushrooms. (conference & specialty issue) (Report). http://www.entrepreneur.com/tradejournals/article/159865043_2.html. Accessed July 6, 2010
3. Chang ST (2002) Past and present trends in the production of *Lentinula edodes* in Asia. Mushroom Biology and Mushroom Products. http://wsmbmp.org/proceedings/4th%20international%20conference/Documentos%20Word/1%20CM/1%20Shiitake%20Trends%20OK.doc. Accessed July 7, 2010
4. Yamauchi T, Ayusawa S, Eda K, Iizuka K, Yokota S, Ishiguri F, Yoshizawa N (2009) Flushing control of fruit bodies by temperature and moisture content in sawdust-based cultivation of *Lentinula edodes*. Mushroom Sci Biotechnol 17:19–23
5. Pennsylvania State University (2001) Cultivation of Shiitake on Natural and Synthetic Logs. http://pubs.cas.psu.edu/FreePubs/pdfs/ul203.pdf. Accessed July 7, 2010
6. Hokkaido (2009) Hokkaido Tokuyou-rinsan Toukei, pp5, http://www.pref.hokkaido.lg.jp/NR/rdonlyres/625EDB25-ACAC-4170-8FC8-A47F7FFBD443/0/H20tokusantoukei.pdf. Accessed July 8, 2010
7. Okii T, Watanabe H, Ishikawa H (1981) The biodegradation of lignin by shiitake *Lentinus edodes* (Berk.) Sing. (in Japanese). Mokuzai Gakkaishi 27:696–702
8. Thorsell S, Epplin FM, Huhnke RL, Taliaferro CM (2004) Economics of coordinated biorefinery feedstock harvest system: lignocellulosic biomass harvest cost. Biomass Bioenergy 27:327–337
9. Ekşioğlu SD, Acharya A, Leightley LE, Arora S (2009) Analysing the design and management of biomass-to-biorefinery supply chain. Comput Ind Eng 57:1342–1352
10. Octave S, Thomas D (2009) Biorefinery: toward an industrial metabolism. Biochimie 91:659–664
11. Lee S, Teramono Y, Endo T (2010) Enhancement of enzymatic accessibility by fibrillation of woody biomass using batch-type kneader with twin-screw elements. Bioresour Technol 101:769–774
12. Yamauch T, Eda K, Ayusawa S, Nagashima A, Matsumoto K, Iizuka K, Yokota S, Ishiguri F, Yoshizawa N (2010) Cultivation properties of the strain for sugi (*Cryptomeria japonica*) wood in sawdust-based cultivation of *Lentinula edodes* (in Japanese). Mokuzai Gakkaishi 56:113–121
13. Hideno A, Aoyagi H, Isobe S, Tanaka H (2007) Utilization of spent sawdust matrix after cultivation of *Grifola frondosa* as substrate for ethanol production by simultaneous saccharification and fermentation. Food Sci Res 13:111–117
14. Shimokawa T, Nakamura M, Nagasawa N, Tamada M, Ishihara M (2007) Effect of gamma-ray irradiation on enzymatic hydrolysis of spent corncob substrates from edible mushroom, enokitake (*Flammulina velutipes*) cultivation. Bull FFPRI 6:27–34
15. Shimoda T, Baba Y, Nishibori K (2008) Ethanol conversion of spent mushroom culture medium by the ball-vibration simultaneous saccharification and fermentation system (in Japanese). Mokuzai Gakkaishi 54:340–345
16. Sluiter A, Hames B, Ruiz R, Scrarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. Technical Report NREL/TP-510-42618
17. Adney B, Baker J (1996) Measurement of cellulase activities. Technical Report NREL/TP-510-42628
18. Teratani F, Tanzawa S, Ogawa Y, Kai Y, Nishida T, Maezawa E (1997) Hydrolysis of water-soluble cellulose acetate prepared from waste newspapers by immobilized cellulase. Mokuzai Gakkaishi 43:956–964
19. Koreihi M, Imanaka H, Imamura K, Kariyama M, Nakanishi K (2009) Efficient ethanol production from wheat bran by enzymatic saccharification using commercially available enzyme products and fermentation using bakers' yeast (in Japanese). Seibutsu-kogaku Kaishi 87:216–223
20. Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 100:10–18
21. Pristavka A, Kodituvakky PA, Kozlov YP, Zacchi G, Berezin IV, Rabinovich ML (2000) High-solids enzymatic hydrolysis of steam-exploded willow without prior water washing. Appl Biochem Microbiol 36:122–130
22. Jensen JR, Morinelly JE, Gossen KR, Brodeur-Campbell MJ, Shonnard DR (2010) Effect of dilute acid pretreatment conditions on enzymatic hydrolysis monomer and oligomer sugar yields for aspen, balsam, and switchgrass. Bioresour Technol 101:2317–2325
23. Zhang X, Qin W, Paice MG, Saddler JN (2009) High consistency enzymatic hydrolysis of hardwood substrates. Bioresour Technol 100:5890–5897
24. Teramoto Y, Lee SH, Endo T (2008) Pretreatment of woody and herbaceous biomass for enzymatic saccharification using sulfuric acid-free ethanol cooking. Bioresour Technol 99:8856–8863