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ORIGINAL ARTICLE

Storage of maitake mushroom (*Grifola frondosa*) culture medium after harvesting fruit bodies is an effective pretreatment for ethanol conversion

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Abstract The storage of spent maitake culture medium (SMCM) under various conditions was investigated as a potential pretreatment of SMCM for increased ethanol conversion. When SMCM was stored at 25°C for 12 weeks, the glucose yield by enzymatic saccharification increased from 22 to 52%. Selective degradation of lignin and hemicellulose occurred in SMCM during storage. The optimal storage temperature of SMCM was at the active growing temperature of maitake mycelium, which is between 25 and 30°C. Storage of SMCM under anaerobic conditions did not increase the glucose yield compared to non-stored conditions. The glucose yield from the SMCM stored for 4 weeks at 25°C was increased by about 30% with either NaOH or vibrating ball milling pretreatment. After 12 weeks of storage, the glucose yield from SMCM without any other pretreatment was higher than that of non-stored SMCM with additional pretreatments. An ethanol yield of 42.1% was obtained from SMCM stored for 12 weeks by simultaneous saccharification and fermentation, which was comparable to yields after NaOH (41.3%) or vibrating ball milling (44.1%) pretreatments. Therefore, storage of SMCM is a very useful pretreatment for bioethanol production.

Keywords Lignocellulosic biomass · Biological pretreatment · Simultaneous saccharification and fermentation · Spent culture medium · *Grifola frondosa*

Introduction

Production of bioethanol is expected to solve the problem of fossil fuel depletion and prevent global warming. Thus, bioethanol production is now increasing in many countries. Since bioethanol is chiefly produced from food crops such as corn and sugarcane, an increase in bioethanol production can cause a global food crisis. Therefore, it is necessary to develop ethanol conversion technologies from non-food lignocellulosic biomass sources [1, 2].

The maitake mushroom (*Grifola frondosa*) is one of the most well-known sources for the commercial mass production of mushrooms in Japan. Maitake is cultured on a solid medium consisting of hardwood sawdust and agriculture products such as corn bran. Therefore, large amounts of spent maitake culture medium (SMCM) including sawdust after harvesting fruit bodies are available at maitake factories. SMCM is a non-food lignocellulosic biomass that is available in large amounts at one area. In addition, SMCM can be constantly obtained throughout the year. Therefore, SMCM could potentially

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be an excellent lignocellulosic biomass resource. Some maitake factories are equipped with a boiler for the combustion of SMCM to generate the heat and steam utilized in the factories, but the high water content (approximately 60%) of SMCM decreases the thermal efficiency of the boiler over time. Thus, we have investigated the application of SMCM as a resource of clean fuel or chemicals by measuring its ethanol conversion rate [3–6].

When converting a lignocellulosic biomass to ethanol, enzymatic hydrolysis of cellulose is a key step. However, cellulose is surrounded by lignin and hemicellulose, which prevents enzymatic hydrolysis. Therefore, lignocellulosic biomasses require pretreatment. Many pretreatment techniques have been studied, including chemical (acid or alkaline), physical (e.g., milling or steam explosion), and biological pretreatments. Of these pretreatments, biological pretreatments are regarded as the most environmentally friendly with low energy consumption [7, 8]. In biological pretreatments, white rot fungi, mainly class *Basidiomycetes*, have been the most extensively studied class, because they release several ligninolytic enzymes such as laccase, lignin peroxidase, and manganese peroxidase [9, 10]. Many edible fungi are white rot fungi, and therefore, several edible fungi have been studied for the biological pretreatment of lignocellulosic biomasses. *Pleurotus ostreatus* was used to pretreat wheat straw [11] and rice straw [12]. *Flammulina velutipes* was investigated for consolidated bioprocessing for bioethanol production from sorghum [13]. Maitake was also reported to degrade lignin and hemicellulose more than the cellulose of beech wood; the cellulose of beech wood becomes susceptible to enzymatic hydrolysis after 6 months of cultivation [14]. However, in that test, maitake mycelium was cultured without the development of a fruit body in the test medium, which differs from industrial maitake production.

The spent culture medium (SCM) of mushrooms can be regarded as the medium in which mushroom mycelium has already been grown. Since the lignin of SCM has been attacked by mushroom mycelium during cultivation, SCM is considered to have undergone biological pretreatment. For example, Lee et al. [15] evaluated waste logs of *Lentinus edodes* as a better material for bioethanol production compared to native logs. However, our previous study indicated that the influence of mycelial growth in the SCM was limited, i.e. only $\sim$25% of glucose yield was obtained from SMCM by enzymatic saccharification after cultivation. Therefore, we have studied several pretreatments for ethanol production from SMCM, and found that NaOH pretreatment [3, 4, 6] or vibrating ball milling [4] were suitable pretreatments of SMCM. In addition, SMCM, which is the waste after maitake cultivation, was more susceptible to these pretreatments than beech wood [3, 6]. Similar results have been reported by Hideno et al. [16].

In our studies, we found that stored SMCM was saccharified and converted to ethanol more easily than non-stored SMCM. Thus, we call this phenomenon the "storage effect" of SMCM. We investigated the storage of SMCM as a pretreatment method for ethanol conversion. To evaluate the storage effect in this study, we investigated the influence of environmental conditions on the glucose yield and ethanol conversion of SMCM, and compared it with that of NaOH or vibrating ball milling pretreatments.

Materials and methods

Maitake cultivation

Maitake (*Grifola frondosa*) strain M51 maintained in our laboratory was used in this study. Beech (*Fagus crenata*) sawdust was crushed by 80% to a size between 0.5 and 2.0 mm, and was purchased from a sawdust maker for mushroom cultivations (Yukiguni Aguri Co., Ltd, Japan). Maitake was cultured on maitake culture medium consisting of beech sawdust and corn bran (mixed rate, beech:corn bran = 9:1 by volume) with 65% moisture content. After 2.5 kg of maitake culture medium was placed in maitake cultivation plastic bag with a gas-exchange filter (Plabag; Santomi Sangyo Co., Ltd, Japan), it was shaped into a block-shaped form (a 20-cm long, 12-cm wide, and 13-cm high rectangular prism). It was autoclaved at 105°C for 2 h. After cooling, the maitake mycelia were inoculated with previously cultivated maitake mycelia in the same medium as above, and the plastic bag was tightly closed. After the maitake mycelia were cultured at 25°C for 2.5 months, the top of the plastic bag was opened. The fruit body was developed at 16°C and a relative humidity of >90%. After harvesting of fruit bodies, the remaining culture medium (i.e., SMCM) was used in the following tests.

Storage

SMCM was put into a new maitake cultivation plastic bag, and the upper side of the bag was sealed with heat. When SMCM was stored under anaerobic conditions, the plastic bag was sealed under the filter to prevent gas exchange. The prepared SMCM was stored at different temperatures (4, 15, 20, 25, 30, 33, or 37°C) for different times (2, 4, 8, or 12 weeks); SMCM was then collected, weighed, and deformed to ensure proper mixing. The water content was determined using an infrared moisture determining balance (FD-620 or FD-600; Kett Electric Laboratory, Japan). The mixed SMCM was dried at 105°C overnight and weighed to determine its dry weight.

For one test (Fig. 7), SMCM was stored in a plastic bottle; 60 g of deformed SMCM was put into a sterilized

850-ml plastic bottle appropriate for mushroom cultivation. The bottles were closed with a cap equipped with an air filter. The bottles were stored at 4 or 25°C for 4 or 11 weeks. After storing, SMCM was collected and dried as described above.

Enzymatic saccharification

Stored or non-stored SMCM (0.1 g dry weight) was mixed with 10 ml of the cellulase solution that contained 50 mM sodium acetate buffer (pH 4.8), 50 FPU/g-SMCM cellulase, and 1 mM sodium azide. Cellulase "ONOZUKA" RS (Yakult Pharmaceutical Ind. Co., Ltd, Japan) was used for enzymatic saccharification. The reaction mixture was incubated at 50°C for 3 days with reciprocating at 200 rpm. The reaction mixture was boiled for 5 min and centrifuged at 12,000×*g* for 15 min to obtain the supernatant. The glucose concentration in the supernatant was analyzed using a biosensor (BF-4; Oji Scientific Instruments, Japan) equipped with an electrode for glucose determination. The glucose yield was calculated using following formulae.

For calculation on the basis of stored SMCM:

$$\text{Glucose yeild}\ (\%) = \frac{\text{The amount of glucose released from stored SMCM}}{\text{The amount of cellulose in stored SMCM} \times 1.1} \times 100. \quad (1)$$

For calculation on the basis of non-stored SMCM:

$$\text{Glucose yeild}\ (\%) = \frac{\text{The amount of glucose released from stored SMCM}}{\text{The amount of cellulose in non-stored SMCM} \times 1.1} \times 100. \quad (2)$$

Wood chemical composition analysis

Dried SMCM was milled and screened to a size between 180 and 250 μm. Approximately 2 g of milled sample was subjected to Soxhlet extraction with ethanol/benzene (1:2 v/v) solvent. Soxhlet extraction was carried out with a solvent extractor (SER 148; Velp Scientifica, Italy) at 180°C for 1 h followed by 1-h washing. After additional washing with ethanol and water, the solid was dried at 105°C until a constant weight was achieved. The extractive (EXT) was determined by subtracting the weight after extraction from the weight before extraction. Then, 0.5 g of dried solid was dissolved with 7.5 ml of 72% sulfuric acid, and the mixture was incubated at 20°C for 4 h. The mixture was diluted with 280 ml of distilled water and autoclaved at 121°C for 1 h. The cooled liquor was filtered through a 1G4 glass filter (AGC Techno Glass Co., Ltd, Japan). The insoluble residue was washed with hot water and dried, and the constant weight as the acid-insoluble lignin (AIL) was measured. The absorbance of the filtrate sample was measured at 240 nm to calculate the amount of acid-soluble lignin (ASL), following the method of the NREL Biomass Program [17]. Another sample of the filtrate was neutralized with $Ca(OH)_2$, and the supernatant was subjected to sugar analysis by HPLC (Prominence; Shimadzu Corporation, Japan). The HPLC was equipped with an Aminex HPX-87P column (Bio-Rad Laboratories, USA) and an evaporative light scattering detector (ELSD-LTs; Shimadzu Corporation, Japan). The analysis conditions were as follows: distilled water was the mobile phase (0.6 ml/min), the column temperature was set at 85°C, and the detector temperature was set at 50°C. The cellulose (CEL) and hemicellulose (HMC) contents were determined from the glucose concentration and the sum of xylose, arabinose, and mannose concentrations, respectively.

Ligninolytic enzyme assay

A mixture of 7.5 g (wet weight) of SMCM with 50 ml of 50 mM sodium malonate buffer (pH 4.5) was stirred on ice for 15 min and filtered through No. 2 filter paper (Advantec Toyo Kaisya, Ltd, Japan). The filtrate was centrifuged at 12,000×*g* for 15 min at 4°C. The supernatant was used for the ligninolytic enzyme assay. The modified method of Hirai et al. [18] was used for the ligninolytic enzyme assay. In our previous study, the optimum temperature of both laccase and MnP was found at 60°C (unpublished data). Therefore, the enzyme assay was done at 60°C.

For the laccase assay, 1 ml of the supernatant was mixed with 1 ml of 50 mM sodium malonate buffer (pH 4.5) in a cuvette. After the reaction mixture was incubated at 60°C for 5 min, 1 ml of 3 mM 2,6-dimethoxyphenol (2,6-DMP) substrate in 50 mM sodium malonate buffer was added. An increase in absorbance at 470 nm was monitored at 60°C for 15 min by a spectrophotometer (U-2000; Hitachi High-Technologies Corporation, Japan) equipped with a thermoelectric cell holder. The laccase activity was defined as the increase in the absorbance per minute.

For the manganese peroxidase (MnP) assay, 1 ml of the supernatant was mixed with 0.5 ml of 6 mM $MnSO_4$ in 50 mM sodium malonate buffer in a cuvette. After the reaction mixture was incubated at 60°C for 5 min, 1 ml of 3 mM 2,6-DMP and 0.5 ml of 1.2 mM H_2O_2 was added (each reagent was dissolved in 50 mM sodium malonate buffer). An increase in absorbance at 470 nm was monitored as described for the laccase assay at 60°C for 1 min. MnP activity was determined by subtracting the laccase activity from the increase in the absorbance per minute.

Other pretreatments

The NaOH treatment was performed as follows: 10 g of dried SMCM was mixed with 50 ml of 5% NaOH solution in an 850-ml plastic bottle, and then, the mixture was heated at 100°C for 1 h. The NaOH-treated SMCM sample was filtered with a stainless sieve (opening, 180 μm) and washed with water until the pH of the wash water was neutral. The NaOH-treated SMCM was dried at 105°C overnight and weighed to calculate the weight loss due to the NaOH treatment. This weight loss value was used to calculate the net glucose yield on the basis of the amount of cellulose in SMCM without any pretreatment.

The vibrating ball milling was performed as follows: dried SMCM (0.2 kg) was placed into a plastic milling jar (4.5 l volume) with 1.2 kg of zirconium balls (1 cm diameter). The jar was set on a rocking mill (RM-40; Seiwa Giken Co., Ltd, Japan), and the sample was milled at 50 Hz for 30 min, followed by sample cooling for 1 h. The milling and cooling cycle was repeated 4 times, corresponding to a total milling time of 2 h, which was the optimum milling time for SMCM in our previous study [4].

Large-scale ethanol conversion

For large-scale fermentation, 5 blocks of SMCM (3.1 kg dry weight) were subjected to storage, NaOH pretreatment, or vibrating ball milling.

The storage was performed as follows: 5 blocks of SMCM were put in a basket and covered with a large plastic bag with a loose closing, and stored at 25°C for 12 weeks. Stored SMCM was subjected to SSF after deforming. The dry weight of SMCM was 2.3 kg after storing, which was used for the SSF process.

NaOH pretreatment was performed in a reactor (50 l volume). Five blocks of SMCM were deformed and soaked in 20 l of 5% NaOH solution. The mixed solution was reacted at 100°C for 60 min with stirring at 120 rpm. The reacted mixture was transferred to a centrifugal dewatering device (YS-7; Iwatsuki Kikai Seisakusho Co., Ltd) with pouring water to wash and neutralize the pretreated SMCM. After NaOH pretreatment, the dry weight of SMCM was 1.7 kg, which was used for the SSF process.

Vibrating ball milling was performed as follows: 5 blocks of SMCM were deformed, and vacuum dried overnight at 80°C. Then, 0.45 or 0.54 kg of dried SMCM was placed into a 4.5- or 5.4-l plastic milling jar, respectively, with 7.2 times the SMCM weight of zirconium balls (1 cm diameter). Both milling jars were set on a rocking mill, and SMCM was milled for 2 h, as described above. This milling operation was performed 3 times to mill all dried SMCM. After milling, 2.9 kg of milled SMCM was collected, which was used for the SSF process.

The large-scale SSF was performed in an 80-l stainless fermentation tank. The fermentation mixture contained 8.6–15.9% SMCM (depending on the pretreatment), 25 FPU/g-SMCM cellulase, and 1% dry yeast. For this fermentation, cellulase GODO TCD-H3 (Godo Shusei Co., Ltd, Japan) was used. SMCM (with or without pretreatment) was mixed with 18 l of deionized water in the fermentation tank. The mixture was heated at 120°C for 30 min. After cooling to 37°C, 2 l of the cellulase solution (114–209 g/l depending on the amount of SMCM) and 20 g of dry yeast (Nisshin Super Camellia dry yeast; Nisshin Foods Inc., Japan) were added to the fermentation tank. The fermentation mixture was incubated at 37°C for 72 h with stirring at 150 rpm. Fermented samples were removed after 24, 48, and 72 h. The supernatant was obtained by centrifugation at 12,000×g for 15 min and stored at −80°C until ethanol analysis. Ethanol yield was calculated on the basis of the amount of cellulose in SMCM without any pretreatment.

Ethanol concentration was determined by gas-chromatography (GC-14B; Shimadzu Corporation, Japan) equipped with Porapack Q column (Shimadzu Corporation, Japan) and a TCD detector. The analytical conditions were as follows: the injection, column oven, and detector temperatures were 150°C, the mobile phase was helium gas, and the flow rate was 40 ml/min. The ethanol yield was calculated using the following formula:

$$\text{Ethonal yeild}\ (\%) = \frac{\text{Amount of ethanol converted from stored SMCM}}{\text{Amount of cellulose in SMCM without pretreatment} \times 1.1 \times 0.51} \times 100. \quad (3)$$

Results and discussion

Enzymatic saccharification of stored SMCM

After SMCM (average dry weight 710 g, approximate moisture 60%) was stored at 25°C (similar to solid-state cultivation), it was subjected to enzymatic saccharification. As shown in Fig. 1, the amount of glucose released from SMCM increased with the increasing storage time. The amount of glucose increased from 90 to 284 mg/g (based on the dry weight of the stored SMCM) during the

12 weeks of storage. This result indicates that the storage of SMCM enhanced its enzymatic saccharification rate, which was termed as the "storage effect".

Change of the chemical composition of wood in SMCM during storage and the glucose yield

Since it is well known that the biological treatment of the woody biomass for ethanol conversion involves a change in its chemical composition, the change in the chemical composition of wood in the SMCM during storage was investigated and the glucose yield of SMCM was calculated using the cellulose content in SMCM. The results are shown in Table 1.

The total dry weight loss of SMCM reached 23% after 12 weeks of storage. The degradation rate of SMCM during the first 4 weeks was 1.9 times as fast as the rate during the following 8 weeks. The glucose yield from SMCM increased from 22 to 63% (based on cellulose in stored SMCM) after 12 weeks of storage. Since SMCM was degraded during storage, the net glucose yield was calculated based on cellulose content in the non-stored SMCM. The calculated net glucose yield was 52% at 12 weeks of storage, which was 2.4 times higher than the yield in the non-stored SMCM.

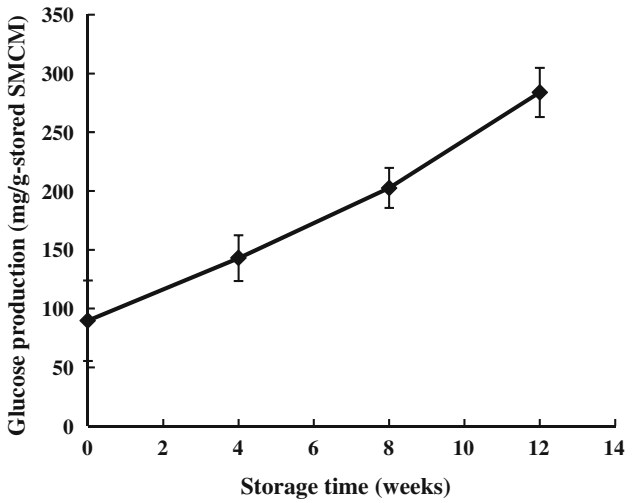

Fig. 1 Enzymatic saccharification-based glucose production from the SMCM stored at 25°C. Average ± SD values from the triplicate tests are shown. The glucose yield was calculated on the basis of the dry weight of the stored SMCM

To compare the degradation pattern of each chemical component of wood in SMCM, weight losses were calculated using the total dry weight loss, and the chemical composition of SMCM is shown in Table 1, and plotted against storage time (Fig. 2). Among the chemical components of wood in SMCM, the most degraded component during 12 weeks of storage was AIL (37%), followed by others (33%), HMC (30%), ASL (27%), CEL (18%), and EXT (−12%). The weight losses of AIL and ASL increased in a linear manner during the storage time. On the other hand, CEL was degraded by 17% in the first 4 weeks, but only by 1.4% in last 8 weeks of storage. The weight loss of HMC was similar to that of CEL in the first 4 weeks, but increased to 30% at 12 weeks. Thus, after 12 weeks of storage, the weight losses of both AIL and HMC were higher than that of CEL. The true weight loss of CEL seems to be lower than that of the calculated loss, because a small amount of glucose derived from some hemicelluloses such as glucomannan [19] was included in the glucose amount that was used to determine the CEL composition. These results indicate that the woody components of SMCM were non-selectively degraded in the first 4 weeks, followed by selective degradation of lignin and hemicellulose in the next 8 weeks of storage. It was reported that maitake degraded lignin and xylan rather than cellulose of beech wood [14, 20]. Therefore, the selective degradation of lignin and hemicellulose observed in SMCM during storage is attributed to degradation by maitake mycelia in SMCM. Removal of lignin and hemicellulose leads to effective saccharification of SMCM by enzymes.

Table 1 Changes in the chemical composition of the wood and glucose yield in the SMCM during storage at 25°C

Storage time (weeks)	Total dry weight loss (%)	Chemical composition of the wood (%)						Glucose yield (%)[a]	
		EXT	AIL	ASL	CEL	HMC	Others	Stored SMCM basis	Non-stored SMCM basis
0	0.0	8.5 ± 0.5	15 ± 1.7	6.3 ± 0.3	38 ± 1.8	19 ± 0.2	13 ± 0.2	22 ± 8.9	22 ± 8.9
4	15 ± 6.5	9.5 ± 1.7	16 ± 1.2	6.6 ± 0.3	38 ± 0.7	19 ± 0.6	11 ± 1.2	34 ± 4.2	29 ± 5.7
8	20 ± 5.5	11 ± 0.5	14 ± 1.0	6.6 ± 0.2	40 ± 1.5	18 ± 0.7	11 ± 1.9	48 ± 5.9	39 ± 7.3
12	23 ± 8.7	12 ± 1.6	13 ± 1.2	6.0 ± 0.6	41 ± 0.8	17 ± 1.3	11 ± 2.1	63 ± 7.0	52 ± 6.6

Average is shown from triplicate tests with SD

EXT extractives, *AIL* acid-insoluble lignin, *ASL* acid-soluble lignin, *CEL* cellulose, *HMC* hemicellulose

[a] Glucose yield from stored or non-stored SMCM was calculated using Eq. 1 or 2, respectively

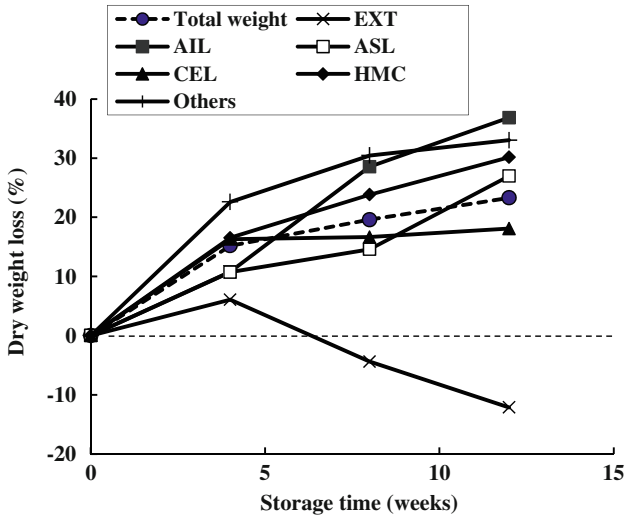

Fig. 2 Changes in the loss of dry weight of woody chemical components in SMCM during storage at 25°C. Abbreviations are shown in Table 1. Dry weight loss of each woody chemical component was calculated from each value shown in Table 1

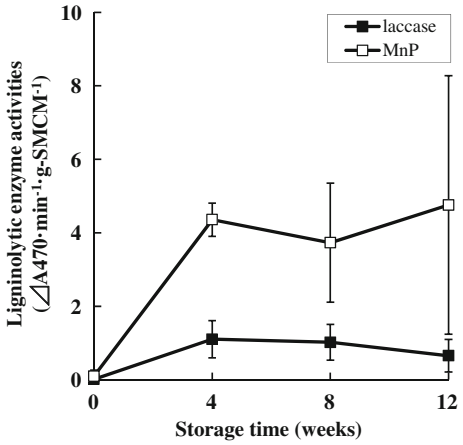

Fig. 3 Changes in the laccase and MnP activities during storage of SMCM at 25°C for 12 weeks. Average ± SD values from the triplicate tests are shown

Only the weight of EXT increased to 112% during the 12 weeks of storage. It is known that EXT contains resin and low-molecular-weight substances [19]. Therefore, the increase in EXT may be due to the decomposed lignin and hemicellulose and some metabolites by the maitake mycelia growing in SMCM during storage.

Hiroi and Tamai [14] investigated the degradation and enzymatic saccharification rates of beech wood during maitake cultivation for 6 months without the development of fruit bodies. The rate of saccharification, lignin degradation and hemicellulose degradation in SMCM was lower (10, 30, and 40%, respectively) than their observations. These differences are probably due to the difference in the contents of the media used by Hiroi and Tamai and the present study. Our medium was nutrient-rich with corn bran (about 25%, based on wood dry weight) to obtain a high-quality fruit body. In addition, maitake prefers to consume glucans derived from corn bran rather than from wood during cultivation. By the time the fruit body was harvested, all the corn bran glucan was consumed by the maitake [16, 21]. As such, the effect of the maitake cultivation on beech wood in SMCM was less than that reported by Hiroi and Tamai. The consumption of corn bran renders the SMCM poor in nutrients, which possibly facilitates the maitake's attack of the beech wood of SMCM during storage.

As shown in Fig. 2, AIL was the most degraded component of SMCM during storage. We have already found laccase and MnP activities in the maitake culture medium during cultivation [22]. To determine if ligninolytic enzymes are involved in lignin degradation, the activities of laccase and MnP during the storage of SMCM were investigated (Fig. 3).

The activities of both laccase and MnP were minimal before storage (immediately after harvesting of the fruit bodies), but increased considerably after storage for 4 weeks. Their activities remained constant for the last 8 weeks of storage. The MnP activity was 3–7 times higher than that of laccase. Therefore, maitake secretes these ligninolytic enzymes to degrade lignin of SMCM during storage.

Effective environmental conditions for storage

If the degradation of lignin and hemicellulose by maitake mycelia could be accelerated by storage conditions, more glucose could be obtained from SMCM by enzymatic saccharification. Therefore, suitable temperature and air conditions for the storage of SMCM were investigated.

Figure 4 shows the effect of storage temperatures of SMCM on its enzymatic saccharification with weight loss. Increasing glucose yields and total dry weight loss was observed with increasing temperature. Storage at 30°C resulted in 22.6% of weight loss, whereas only 3.0% was lost at 4°C. The glucose yield with enzymatic saccharification was 20.3% after storage at 4°C, which increased to 34.1, 41.0, 48.5, and 52.6% after storing at 15, 20, 25, and 30°C, respectively. However, no significant differences in weight loss or glucose yield due to storage at 20, 25, or 30°C were observed by Tukey's test ($P < 0.05$). There was no increase in the glucose yield from the SMCM stored at 33 or 37°C compared to non-stored SMCM (data not shown).

Our laboratory has previously determined that the optimum growth temperature of the maitake mycelia was between 25 and 30°C; growth was not supported at 35°C on potato dextrose agar (unpublished data). Therefore, the high dry weight loss of SMCM noted at 25 and 30°C

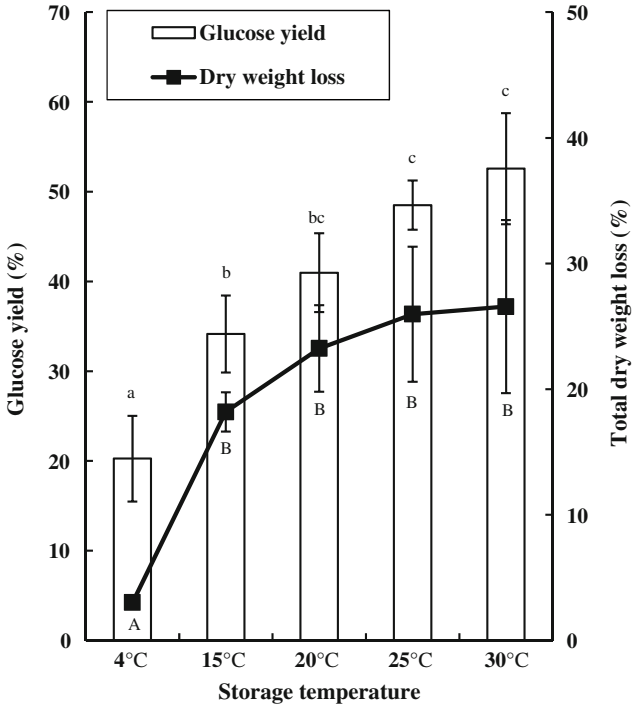

Fig. 4 Effect of storage temperature on enzymatic saccharification and total dry weight loss of the SMCM stored for 12 weeks. The glucose yield was calculated on the basis of the cellulose content of non-stored SMCM. Average ± SD values from the triplicate tests are shown. Values with different *small letters* (*a–c*) or *capital letters* (*A*, *B*) represent significantly different glucose yields or dry weight losses, respectively, at $P < 0.05$, as determined by Tukey's test

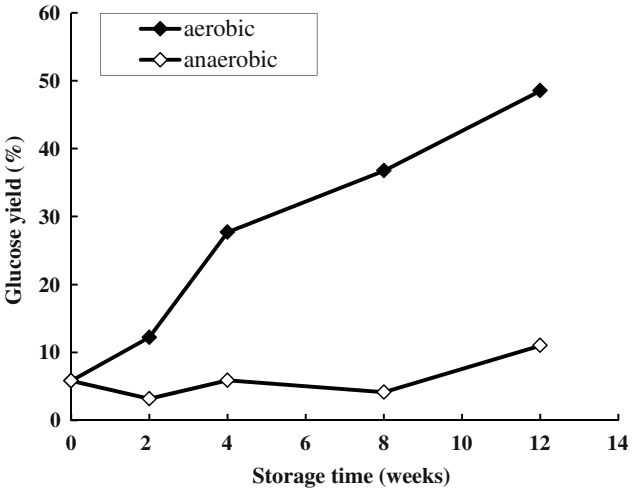

Fig. 5 Yield of glucose produced by enzymatic saccharification from SMCM during storage at 25°C for 12 weeks under aerobic or anaerobic conditions. Anaerobic conditions were established by sealing the plastic bag containing SMCM under the gas-exchange filter. The glucose yield was calculated on the basis of the cellulose content of the non-stored SMCM. Average values from the duplicate (2 and 8 weeks) or triplicate (0, 4, and 12 weeks) tests are shown

reflected the active growing of maitake mycelium with the degradation of lignin and hemicellulose (Table 1; Fig. 2), which led to an increase in the glucose yield. Based on these data, it was identified that temperatures between 25 and 30°C are suitable for storage of SMCM. The storage temperature of SMCM should not exceed 30°C.

It is well known that maitake requires significant aeration for cultivation; indeed, many wood-decaying fungi, including maitake, are aerobic organisms [23]. Furthermore, Reid and Seifert [24] reported that lignin degradation by some white rot fungi containing maitake was faster in an oxygen-rich atmosphere compared to air. Therefore, we hypothesized that the storage effect of SMCM would decline under anaerobic storage conditions. As shown in Fig. 5, the glucose yield did not increase after storage for 12 weeks under anaerobic conditions. These data also indicate that the increase in glucose yield from SMCM requires active growth of maitake mycelia during storage.

Effect of NaOH or vibrating ball milling pretreatment on stored SMCM

Our previous studies have shown that both alkaline treatment and vibrating ball milling treatment were effective pretreatments for enzymatic saccharification of SMCM [3, 4, 6]. It was expected that the storage of SMCM would enhance the effect of sequential alkaline or vibrating ball milling treatment, because some lignin and hemicellulose would be removed from SMCM during storage (Table 1).

As shown in Fig. 6a, the glucose yield from the NaOH-pretreated SMCM after 4 weeks of storage increased from 41.7 to 58.0%, parallel to that without NaOH pretreatment (from 15 to 25%). From 4 to 12 weeks of storage, the glucose yield from NaOH-pretreated SMCM did not increase, whereas the yield from SMCM without NaOH pretreatment continued to increase. Consequently, after 12 weeks of storage, the glucose yield from SMCM without NaOH pretreatment (59.4%) was nearly the same as the yield from NaOH-pretreated SMCM (58.2%). In addition, the glucose yield from SMCM after 12 weeks of storage without NaOH pretreatment was higher than the yield from non-stored SMCM after NaOH treatment.

For the vibrating ball milling-treated SMCM after storage (Fig. 6b), similar to NaOH pretreatment, the glucose yield increased from 37.9 to 58.3% after 4 weeks of storage. A slight increase in glucose yield (to 62.3%) was observed during the subsequent 8 weeks of storage, which was higher than the yield without milling (53.6%). However, the glucose yield from SMCM after 12 weeks of storage without milling was considerably higher than the yield from SMCM after milling but without storage (37.9%).

Itoh et al. [25] reported that biological treatment of beech wood with selective lignin-degrading fungi (*Ceriporiopsis subvermispora*) improved the subsequent ethanolysis

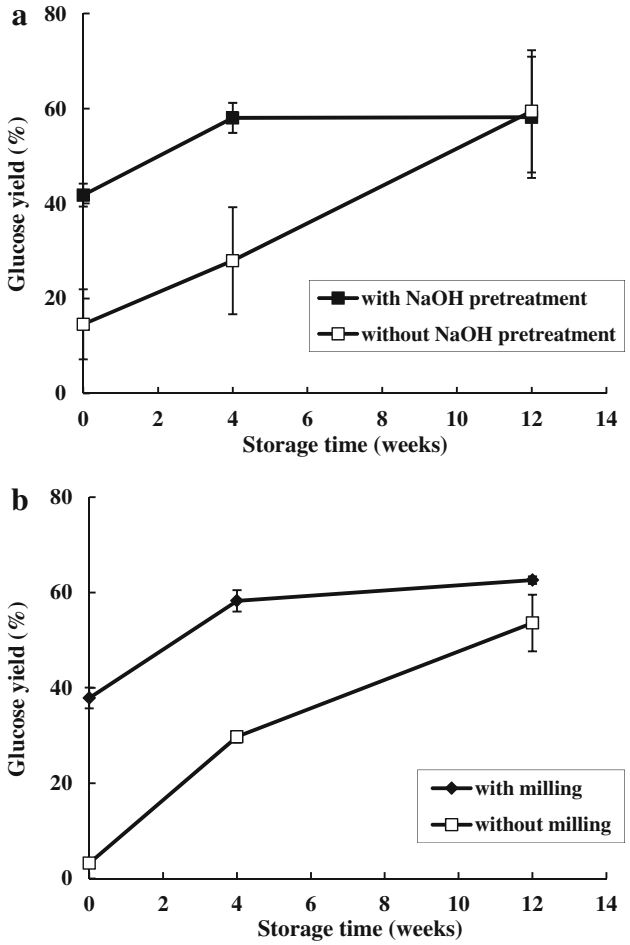

Fig. 6 Effects of NaOH pretreatment (**a**) and vibrating ball milling pretreatment (**b**) on the enzymatic saccharification of SMCM during storage at 25°C. The glucose yield was calculated on the basis of the cellulose content of SMCM without storage and without pretreatment. Average ± SD values from the triplicate tests are shown. The test season differed between **a** and **b**. Therefore, the nature of the beech sawdust used in the maitake culture was slightly different

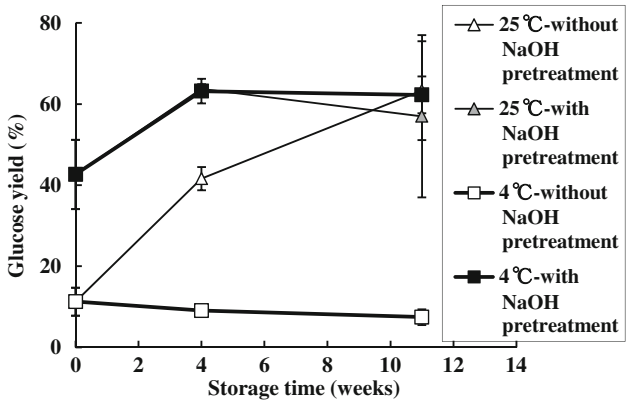

Fig. 7 Effect of storage at cold temperature on NaOH pretreatment of SMCM performed for enzymatic saccharification. SMCM was stored in the mushroom cultivation bottle. The glucose yield was calculated on the basis of the cellulose content of SMCM without storage and without pretreatment. Average ± SD values from the triplicate tests are shown

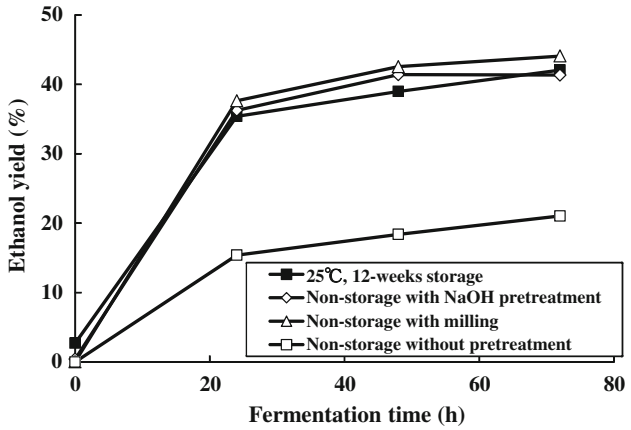

Fig. 8 Large-scale conversion of SMCM into ethanol after storage at 25°C for 12 weeks. The ethanol yield was calculated on the basis of the cellulose content of SMCM without storage and without pretreatment. Average ± SD values from the triplicate tests are shown

pretreatment for ethanol production by SSF. Similarly, our results also indicate that storing SMCM for 4 weeks improves the glucose yield with both NaOH and vibrating ball milling pretreatments. In contrast, storing SMCM for more than 4 weeks after additional pretreatments did not affect saccharification. In addition, storing SMCM at 25°C for 12 weeks was the most effective pretreatment for enzymatic saccharification compared to NaOH pretreatment or vibrating ball milling pretreatment without storage.

Unexpectedly, the glucose yield was high when SMCM was stored at cold temperatures and pretreated with NaOH (Fig. 7). When SMCM was stored at 4°C for 4 weeks, the glucose yield with NaOH pretreatment was 1.5 times higher than the yield before storage. However, the glucose yield from SMCM without NaOH pretreatment did not increase under these conditions. Thus, cold temperature storage of SMCM enhances the glucose yield by enzymatic saccharification after NaOH pretreatment. Since maitake mycelia hardly grow at 4°C, a rationale for the observed increase in the glucose yield following NaOH pretreatment after storage at 4°C is unclear.

Large-scale ethanol conversion

To evaluate the storage effect of SMCM on ethanol conversion, 5 blocks (~3.1 kg dry weight) of SMCM was subjected to storage for 12 weeks, NaOH pretreatment, or vibrating ball milling pretreatment. After each pretreatment, 2.3, 1.7, and 2.9 kg of dry weight were recovered from each SMCM, respectively. Figure 8 shows the ethanol yields from the pretreated SMCMs, which were

subjected to SSF in an 80-l volume fermentation tank. An ethanol yield of 42.1% at 72 h was obtained from stored SMCM, while the yield was 21.0% without any pretreatment. The ethanol yield after NaOH and vibrating ball milling pretreatments was 41.3 and 44.1%, respectively, which is similar to the yield obtained after storage. These results indicate that storage pretreatment of SMCM is comparable to both NaOH and vibrating ball milling pretreatments with regard to ethanol conversion of SMCM. The ethanol yield from stored SMCM (42.1%) was lower than the glucose yield after enzymatic saccharification (48.5–62.3%, mentioned above). These differences may be due to the differences in the nature of the beech wood used in the maitake medium, because maitake is cultivated in different seasons.

In conclusion, we found the storage effect of SMCM which is one of the biological pretreatments by maitake mycelia that have already grown in SMCM. The glucose yield from SMCM became 2.4–16.8 times higher after 12 weeks of storage at 25°C than the yield from the non-stored SMCM. The storage effect of SMCM was comparable pretreatment to NaOH or vibrating ball milling pretreatment for the ethanol conversion. These results suggest that SMCM is a very useful biomass resource; complex pretreatments for sugar and bioethanol production are unnecessary. Furthermore, because the storage does not require any complex equipment or chemicals, it is amenable to scaling up to industrial levels, and ethanol production cost should decrease. However, there are some limitations such as a storage space and storage time, and ethanol yields may still be low for practical applications. To solve the problems, it is necessary to enhance the storage effect of SMCM. Controlling ligninolytic enzyme activities during the storage might be a key to improve saccharification of SMCM. Therefore, our current research is focused on understanding the role of ligninolytic enzymes during the storage.

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