ETHANOL PRODUCTION FROM UNPRETREATED WASTE MEDIUM OF SHIITAKE MUSHROOM (*Lentinula edodes*) BY SEMI-SIMULTANEOUS SACCHARIFICATION AND FERMENTATION UNDER HIGH SUBSTRATE CONCENTRATION CONDITIONS

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Waste mushroom medium (WM) from cultivation of shiitake mushroom (*Lentinula edodes*),using meals of willow species *Salix sachalinensis* (WMS) or *Salix pet-susu* (WMP), was air-dried, roughly pulverized, and used to produce ethanol by semi-simultaneous saccharification and fermentation (SSSF) with commercial cellulase (Meicelase) and dry yeast (Ethanol Red). Glucan content of WMS and WMP was 31.5% and 29.6%, respectively. SSSF of WMS at a final concentration of 72% (w/v), introduced over 4 substrate additions, with 5 FPU Meicelase/g of final substrate processed for 232 h, produced an ethanol concentration of 56 g/L and an ethanol yield of 46%. No significant difference was detected in the yield of glucose and ethanol between SSSF performed using air-dried and pulverized WMS (<10 mm) and that using raw and roughly broken WMS (>40 mm). These results indicate that shiitake WM can be used for SSSF without pretreatment and pulverization.

Keywords: waste mushroom medium, *Lentinula edodes*, willow, bioethanol production, simultaneous saccharification and fermentation

INTRODUCTION

The production of bioethanol from lignocellulosic agricultural residues provides an alternative to the use of fossil fuels and represents an important countermeasure against global warming and the depletion of fossil fuels.[1]

Shiitake mushroom (*Lentinula edodes*) is one of the most commonly cultivated edible mushrooms with an annual production of 1.5 million tons, representing approximately 25% of the total world mushroom supply in 1997.[2] Worldwide shiitake production has increased since 1997, and it continues to increase notably in China.[3] In Japan, fresh shiitake production was 66,476 tons in 2012, 87% of which was produced using synthetic media composed of hardwood meal, nutrients, and water.[4] The wet weight of shiitake waste mushroom medium (WM) produced is estimated to be >50,000 tons/year in Japan. Shiitake is cultivated throughout the year in mushroom growing facilities, and collecting and storing costs of WM are extremely low, thus making WM a readily available and low cost biomass resource for saccharification and fermentation.[5–11]

Shiitake, like other white rot fungi, degrades the lignin component of wood, allowing for the 40-50% of cellulose in shiitake WM to be saccharified using cellulase without pretreatment.[5,6,11] Ethanol was obtained from WM by consolidated bioprocessing fermentation using *Phlebia* sp. MG-60.[11]When WM is steamed at 210-235°C as a pretreatment, the saccharification ratio reaches >80%.[7–9] The number of fruiting body harvests affects the saccharification ratio of WM; the ratio is improved when the number of harvests is increased from the standard 3 harvests to 5 harvests.[10] However, our previous study found that the amount of fruiting bodies in the 4th and 5th harvests was lower,[10] and therefore, increasing harvest times was

considered to be economically disadvantageous. However, we are currently developing a new shiitake cultivation method in which the amount of fruiting bodies from the 4th and 5th harvests is not greatly diminished.[12]

To realize the commercial production of bioethanol from lignocellulosic biomass, it is necessary to reduce the cost of pretreatment, enzymes and distillation.[13–15] Although WM has potential for use in bioethanol production, there are few reports on high concentration WM saccharification and fermentation using low levels of cellulase without pretreatment.

One of the objectives of this study was to examine the saccharification ratio of shiitake WM after 5 harvests by using a new cultivation method with willow (*Salix* spp.) wood meal as substrate and a low concentration of cellulase, without pretreatment. Another objective was to evaluate semi-simultaneous saccharification and fermentation (SSSF) by using a high concentration of WM substrate.

EXPERIMENTAL

Cellulase and yeast

A commercial cellulase preparation of Meicelase (Meiji Co. Ltd., Tokyo, Japan) derived from *Trichoderma viride* was used in this study. Cellulase assays were performed in 0.1 M sodium citrate buffer (pH 4.8) and the activity of Meicelase was expressed as filter paper units (FPU) based on the established method.[16] A commercial preparation of dry *Saccharomyces cerevisiae* yeast (Ethanol Red; Lesaffre Yeast Co. Nord, France) was used for SSSF.

Saccharification and SSSF

WM was saccharified on a small scale (fluid volume of 12 mL) in 15-mL polypropylene tubes with triple seal caps (ACG Techno Glass Co. Ltd., Shizuoka, Japan). Tubes were placed on a linearly reciprocating shaker (Multi Shaker MMS-310; Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 80 rpm at 40°C.

A 3-L jar fermentor (BJ30-N, Tokyo Rikakikai) with a stirrer (NZ-1200, Tokyo Rikakikai) and a Teflon stirring propeller (T-12, Tokyo Rikakikai) was used for a scale-up of the saccharification and SSSF processes. WM saccharification solutions were stirred at 200 ± 5 rpm and the 3 jar fermentors were connected in series to a low temperature circulator (NCB-2500; Tokyo Rikakikai) and maintained at 30°C or 50°C ± 1°C.

Glucose and ethanol analyses

When substrate concentration was 2% (w/v), 0.5 ml of supernatant fluid from the saccharified solution was taken to measure the amount of monosaccharides. In an aliquot of the solution, *meso*-erythritol (>99.0%, Tokyo Chemical Industry Co., LTD., Tokyo, Japan), as an internal standard, and 1.5 ml of deionized water were added for HPLC analysis. The diluted solution was immediately filtrated using a filter with 0.2 μm pore size. When substrate concentration was >30% (w/v), 0.1-0.4 g of slurry was taken to measure the amount of monosaccharides and ethanol. The slurry was weighed to obtain an accuracy of ± 0.01 mg and *meso*-erythritol and 2-8 ml of deionized water were added for HPLC analysis. Monosaccharides and ethanol were extracted from the diluted slurry by 30s of ultrasonic treatment (UT-304; Sharp Co., Osaka, Japan) and 20 s of vortex mixing treatment (TUBE MIXER TRIO HM-1N;AS ONE Co., Osaka, Japan). The solution was immediately filtrated using a filter with 0.2 μm pore size.

Monosaccharides and ethanol were analyzed using an HPLC system (L2000 series; Hitachi High-Technologies Corp., Tokyo, Japan) equipped with an RI detector. Monosaccharides were analyzed using a tandemly arranged Aminex HPX-87P × 2 column (Bio-Rad Laboratories Inc., CA, USA) at 85°C with deionized water as eluent at a flow rate of 0.6 mL/min. Ethanol was analyzed using an Aminex HPX-87H column (Bio-Rad Laboratories) at 60°C with 0.005 M H_2SO_4 as eluent at a flow rate of 0.7 mL/min.

The amount of water in the slurry was calculated by following Eq. 1:

$$A = B - (C + D + E + F + G) \tag{1}$$

where A is the whole weight of water in the slurry (g), B is the whole wet weight of the slurry (g), C is oven-dry matter (ODM) of the substrate (g), D is salt in the buffer (g), E is sodium hydrate for pH adjustment (g), F is Meicelase (g), and G is Ethanol Red (g). The concentration of glucose and ethanol in the slurry was expressed as glucose or ethanol g/L of water in the slurry.

Preparation of WM as substrate

Shiitake mushroom was cultivated in two kinds of synthetic media, containing 390 g oven-dry matter (ODM) of either *Salix sachalinensis* or *Salix pet-susu*in powdered form, 130 g ODM of mushroom nutrient (Derutoppu, Mori& Co., Ltd, Gunma, Japan), and 780 g drinkable tap water. The synthetic media packed in plastic bags (Miki-pack 1.3 × 380BF; Miki-Sangyo, Aichi, Japan) were autoclaved at 121°C for 30 min. Shiitake strain XR-1 (Mori) was inoculated into autoclaved media and cultured for 90 days under dark conditions at 22°C ± 1°C and 70% ± 10% RH. After 90 days, shiitake fruiting bodies were spawned from mushroom media in a spawning room at 16°C ± 1°C and 85% ± 10% RH under lighting conditions (white fluorescent lamp, 350 lx, 12 h/day). After the first harvesting, mushroom media were soaked in water

overnight and fruiting bodies were harvested again in the spawning room after approximately 20 days (second harvest). In this manner, the 3rd-5th harvests were repeated. Fifty blocks were prepared for each medium.[12]

After the final mushroom harvest, all blocks were collected and air-dried at 50-60°C until the moisture content became less than 10%. The air-dried blocks were pulverized through a 10 mm mesh grating using a cutter mill (Rotoplex, Type: R-16/8; Fuji-Sangyo, Osaka, Japan) at 1500 rpm. The pulverized product, designated WMS (WM of *Salix sachalinensis*) and WMP (WM of *Salix pet-susu*), was mixed and used for subsequent analysis and experimentation.

Twenty additional WMS blocks were cultivated as above, and after cultivation, they were removed and stored in a loosely closed plastic bag in a temperature-controlled room at 5°C for 4 weeks. It has been previously shown that storing WM at 5°C for up to 2 months has a minimal effect on the chemical composition and the saccharification ratio.[6] After storage, the raw WMS blocks were roughly broken by hand to a size of >4 × 4 × 4 cm (rWMS). A portion of the rWMS was air-dried at 50-60°C until the moisture content was less than 10% (drWMS). A portion of the drWMS was pulverized in a Rotoplex mill at 1500 rpm equipped with a 10 mm mesh grating (pdrWMS).

Characterization of WM

One liter of air-dried WM (WMS, WMP, drWMS, or pdrWMS) was sieved as previously described through 40-0.125 mm sieves (Table 1).[6] The contents of glucan, xylan, galactan, arabinan, mannan, acid-soluble lignin, and acid-insoluble lignin of WM were determined (Table 2), as previously described.[10]

Saccharification in 15-mL tubes

WMS and WMP were saccharified at 40°C for 72 h in 15-mL tubes using the following conditions: 12 mL of 0.1 M sodium citrate buffer (pH 4.8) containing 0.24 mg of sodium azide, 2% (w/v) of substrate, and 5 FPU Meicelase/g substrate.[10]

Saccharification in 3-L jar fermentors

WMS was saccharified in 3-L jar fermentors in 0.1 M sodium citrate buffer at 50 °C for 96 h, by the addition of 5 FPU Meicelase/g as substrate and 153 mg sodium azide. WMS was saccharified at substrate concentrations of 2%, 30%, and 35% (w/v), representing substrate/buffer ratios of 15/750, 177/588, and 198/567 (g/mL), respectively. The pH of the saccharification was maintained in the range of 4.3-4.8 by the addition of solid sodium hydroxide at 0, 24, 48, and 72 h.

SSSF in a 3-L jar fermentor

WMS, WMP, rWMS, and pdrWMS were saccharified in 3-L jar fermentors in 0.1 M sodium citrate buffer at 50 °C for 24 or 48 h, and then simultaneously saccharified and fermented at 30 °C for 48-232 h. Solid sodium hydroxide was added at 0, 24, and 48 h to maintain the pH between 4.3 and 4.8. The substrate concentration was 35% (w/v) at the start of SSSF, followed by the addition of more substrate with approximately 9% water content in 1-5 steps. The contents of the jar fermentors were kneaded with a spatula after every substrate addition. Meicelase was added to provide 2.7-5 FPU/g of total substrate added. The amount of the dry yeast added was expressed by the following formula:

Dry yeast addition (%) = $W_y / (W_s + V_b) \times 100$ (2)

where W_y is the amount of additional dry yeast (g), W_s is the amount of the final substrate (g), V_b is the amount of the buffer (ml). Dry yeast was added to provide 0.2-2%.

Saccharification ratio and ethanol yield

The saccharification ratio is defined as the ratio of the amount of glucose obtained by enzymatic saccharification to the theoretical yield of glucose based on the glucan content. In our previous study,[6] we found that there is little glucose or other monosaccharides in WM prior to saccharification with cellulase. Therefore, the glucose quantified was regarded as glucose derived from the saccharification of the WM. Ethanol yield is defined as the ratio of the amount of ethanol obtained by SSSF to the theoretical ethanol yield based on the theoretical yield of glucose based on the glucan content of the substrate. Although Meicelase contains lactose as a stabilizing agent, little ethanol was detected in the blank test in which only Meicelase was fermented with the yeast; therefore, the ethanol from Meicelase was considered to be negligible.

Statistical analysis

The homoscedasticity of the saccharification ratio and glucose yield between WMS and WMP, as well as the yield of glucose and ethanol between rWMS and pdrWMS, was assessed using the F-test, and significant differences were determined by the Student's t-test.

The homoscedasticity of the saccharification ratio between different substrate concentrations was assessed using Bartlett's test. Significant differences between samples were evaluated using the one-way analysis of variance (ANOVA), accompanied by the Tukey-Kramer test, or the Games-Howell test. The level of significance was set to 5%.

RESULTS AND DISCUSSION
Enzymatic saccharification in 15-mL tubes

Figure 1A shows the saccharification ratio of WMS and WMP. The saccharification ratio of WMS and WMP at 72 h was 56% and 61%, respectively; no significant difference in the

saccharification ratios was detected ($P< 0.05$) at any time point.

Table 1

Granulometry of substrates for saccharification and SSSF; standard deviations are shown in square brackets (n=3)

Size class (mm)	Substrate[a] (% w/w)			
	WMS[b]	WMP[c]	drWMS[d]	pdrWMS[e]
>40	-	-	69.3 [2.6]	-
40-31.5	-	-	25.3 [2.3]	-
31.5-20	-	-	2.6 [0.7]	-
20-4.0	3.2 [0.3]	3.9 [0.4]	0.9 [0.8]	4.0 [1.0]
4.0-2.0	14.2 [0.8]	14.0 [0.2]	0.4 [0.3]	17.7 [1.9]
2.0-1.0	16.7 [0.3]	17.3 [0.3]	0.4 [0.1]	18.9 [1.0]
1.0-0.5	23.0 [0.2]	26.4 [0.7]	-	19.5 [0.6]
0.5-0.25	21.5 [0.2]	22.7 [0.4]	-	17.7 [0.9]
0.25-0.125	12.7 [0.6]	11.0 [0.5]	-	12.4 [0.6]
0.12-0	8.6 [0.5]	4.8 [0.1]	1.1[f] [0.3]	9.8 [0.3]

[a] Substrate was dried at 50-60°C and pulverized to <10 mm except for drWMS; [b] Waste mushroom medium using *Salix sachalinensis*; [c] Waste mushroom medium using *Salix pet-susu*; [d] WMS roughly broken by hand and dried at 50-60 °C; [e] drWMS pulverized to <10 mm; [f] This value expresses the % of size classes from 1.0-0 mm

Table 2

Dry weight (n=50), moisture content (n= 50) and chemical composition (n=3) of WM, standard deviations are shown in square brackets

Substrate[a]	Dry weight of WM (g/block)	Moisture content[b] (%)	Components (% w/w)							
			Acid-insoluble lignin	Acid-soluble lignin	Glucan	Xylan	Galactan	Arabinan	Mannan	Other components[c]
WMS	147.1 [13.8]	47.2 [9.8]	13.5 [1.3]	6.6 [0.2]	31.5 [2.6]	9.3 [0.8]	1.8 [0.4]	3.0 [0.3]	5.3 [0.6]	29.0
WMP	164.4 [9.8]	42.3 [7.4]	14.2 [0.6]	6.7 [0.3]	29.6 [1.2]	9.7 [0.7]	1.7 [0.3]	3.0 [0.3]	5.3 [0.8]	29.7

[a] Substrate was dried at 50-60°C and pulverized to <10 mm; [b] Measurement was performed immediately after the last harvest; [c] Dry weight minus the sum of acid-insoluble lignin, acid-soluble lignin, glucan, xylan,galactan, arabinan, and mannan

Figure 1B shows the amount of glucose obtained from WMS and WMP. After 72 h of saccharification, both WMS and WMP yielded statistically equivalent amounts (176 and 181 mg, respectively) of glucose per gram of substrate, and no significant difference in the amount of glucose obtained was detected at any other time point ($P< 0.05$). Because of the similarities in glucose yield from WMS and WMP, these substrates were considered to be equivalent in subsequent experiments.

WMS and WMP were prepared with a new shiitake cultivation method using the dominant willow species present in the riverbed region of Hokkaido, Japan.[12] This new shiitake cultivation method resulted in numerous large fruiting bodies, and the quantity of mushrooms produced in the 4th and 5th harvests did not diminish appreciably. The saccharification ratio of WMS and WMP was high without the use of an expensive pretreatment, and was also comparable to that of WM produced by the conventional method using birch and oak.[6,9,10] This indicates that WMS and WMP are excellent substrates for enzymatic saccharification.

Enzymatic saccharification in 3-L jar fermentors

When the willow-based WM was saccharified in a 3-L jar fermentor at 40°C, there were cases in which the amount of glucose decreased over the course of the saccharification process, most notably

at lower substrate concentrations (data not shown). Microbial contamination was suspected as the cause of the decrease in glucose; therefore, in subsequent experiments the process was conducted at a temperature of 50°C in order to inhibit microbial growth. As a preliminary study, commercial WM which was made of lignocellulose meal of *Quercus crispula*, *Betula platyphylla*, *Abies sachalinensis*, and buckwheat husk was saccharifiedat 20%, 25%, and 30% (w/v) of substrate concentration. Under these conditions, the saccharification ratios were approximately 45% and the substrate concentration was observed to be higher as well. Therefore, for the purpose of this study, the substrate concentration condition for examining saccharification was set to 30% and 35% (w/v).
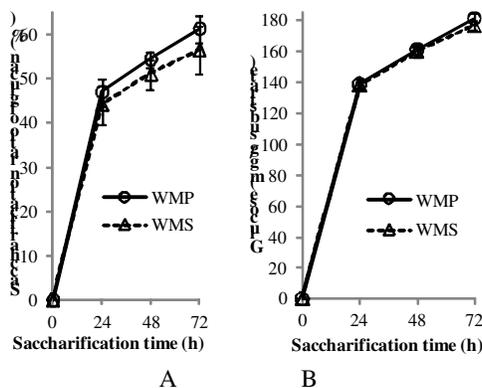

Figure 1: Saccharification ratio of glucan in WMS and WMP (A) and amount of glucose obtained from WMS and WMP by enzymatic saccharification (B)
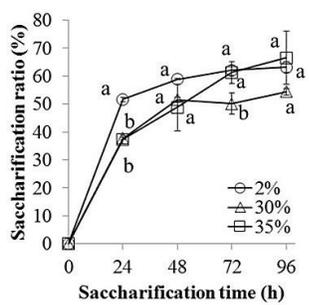

Figure 2: Evolution in time of WMPsaccharification ratio at different substrate concentrations (n = 3; error bar, standard deviation). Letters indicate significant differences among substrates (*P*< 0.05); they were determined using the Tukey-Kramer test, except for those at 48 h, for which the Games and Howell test was used

Figure 3: Evolution in time of the concentration of glucose and ethanol when different amounts of dry yeast were added (n = 1)

Figure 2 shows the saccharification ratio of WMP at substrate concentrations of 2%, 30%, and 35%. At a substrate concentration of 2%, the time course of saccharification in the 3-L jar fermentor was similar to that in the 15-mL tube (Fig. 1), reaching a saccharification ratio of 50% at 24 h, which was earlier obtained at substrate concentrations of 30% and 35% (*P*< 0.05)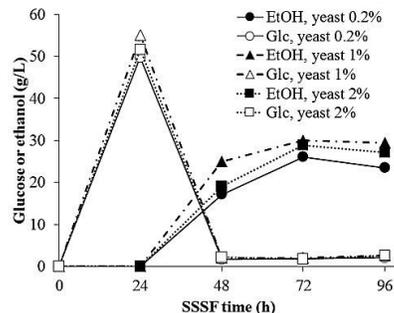. The saccharification ratio at all substrate concentrations was more than 50% at 96 h (P < 0.05), demonstrating that under the conditions of this study, the saccharification ratio at substrate concentrations within the range of 2-35% (w/v) was similar; however, 3- to 4-fold more time was required at the highest concentration. On the basis of these observations, a substrate concentration of 35% (w/v) was chosen as the standard concentration in subsequent experiments.

Optimization of the amount of dry yeast for SSSF in 3-L jar fermentor

Figure 3 shows the amount of glucose and ethanol obtained from WMP in SSSF when the amount of additional dry yeast was changed. SSSF was performed under the following conditions: 35% (w/v) of substrate in 0.1 M sodium citrate buffer, 5 FPU Meicelase/g substrate, 24 h of saccharification at 50°C, followed by 72 h of simultaneous saccharification and fermentation at 30°C. The average ethanol yield at 72 h was 48%. Most of the glucose obtained from the slurry of WMP at 35% substrate concentration was converted to ethanol by 48 h after yeast addition. Dry yeast activation, typically used for development of an inoculation culture, was not performed to avoid reduction in the ethanol concentration by water addition; nonetheless, as previously indicated, fermentation proceeded promptly. The lack of the need for yeast activation could provide a small cost advantage as a result of simplified processing, and the elimination of the apparatus and materials required for inoculum development.

Although it seemed that the amount of yeast added (in the range of 0.2-2%) did not greatly influence the amount of ethanol, the amount of yeast to be added in the subsequent examination was set at 1%.

Influence of drying and pulverizing WM to SSSF

Figure 4 shows the amount of glucose and ethanol obtained from rWMS and pdrWMS by SSSF. SSSF was performed under the following conditions: 35% (w/v) substrate in 0.1 M sodium citrate buffer, 5 FPU Meicelase/g substrate, 1% added dry yeast, 24 h of saccharification at 50°C, followed by 48 h of simultaneous saccharification and fermentation at 30°C. Mixing the contents of the jar fermentors was performed under high load conditions for the first 1-2 h, and thereafter, at low load conditions due to reduction in medium viscosity. After the addition of dry yeast, the glucose produced by saccharification was promptly fermented, indicating that fermentation inhibitors were either not produced or produced at very low levels. Significant differences in the amount of glucose produced at 24 h and the amount of ethanol produced at 48 and 72 h were not detected ($P < 0.05$).

WMSblock was originally an aggregation of wood meal of size<9 mm, especially the 80% one was <2 mm. It was observed that the wetter WMS block was more brittle. It was also observed that rWMSin the buffer with Meicelase crumbled by stirring. In this study, pdrWMS was pulverized to be <10 mm. For these reasons, the saccharification ratio and ethanol yield between rWMS and pdrWMS were found to be at the same level.Raw and roughly broken WM was saccharified and fermented almost as efficiently as pulverized WM, demonstrating that the benefits obtained by pulverization for SSSF of WM are low and the pretreatment of WM for SSSF can be greatly simplified.

Increase of precursory saccharification time and substrate concentration

The results of our preliminary examination led us to think that the most suitable temperature for simultaneous saccharification and fermentation (SSF) was 30°C. However, it is known that the activity of Meicelase at 30°C is only 60-65% of that at 40-50°C, which is the optimum temperature range for this enzyme.[17] Therefore, it was considered that precursory saccharification at 50°C before SSF would be important to obtain ethanol earlier in the process.

WMP was saccharified at 50°C for 48 h and then simultaneously saccharified and fermented at 30°C for 112 h. After the start of SSSF, no substrate was added in the jar fermentor. Substrate was added in the other two jar fermentors at 44.6% concentration at 24 and 48 h after the start of SSSF. At the start of SSSF, 5 FPU Meicelase/g of final substrate was added. At 48 h from the start of SSSF, 1% of dry yeast was also added. Figure 5 shows the amount of glucose and ethanol obtained from WMP in this SSSF. It was observed that the difference in the amount of ethanol production in additional substrate timing at 24 and 48 h was not large. By comparing the result of 35% substrate concentration with that of Figures 3 and 4, it seemed that elongating precursory saccharification time had poor efficacy in increasing the amount of ethanol. Though the precursory saccharification time could be shortened, it was decided that the time needed to obtain a certain level of glucose in this study was 24 h.

The ethanol yield of the three jar fermentors whose substrate concentration was 35% and 44.6% with additional substrate at 24 and 48 h was 47, 48
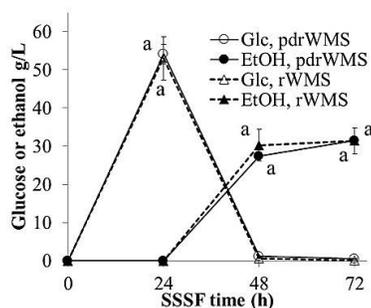
and 50% at 160 h, respectively.

Figure 4: Evolution in time of the concentration of glucose and ethanol produced during the saccharification of rWMS and pdrWMS (error bar, standard deviation; n = 3). Letters indicate significant differences among substrates (P < 0.05). Significance among the substrates was determined using Student's t-test
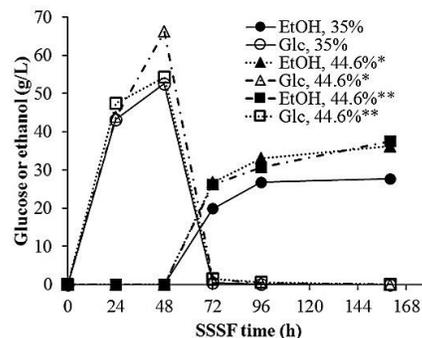

Figure 5: Evolution in time of the concentration of glucose and ethanol when substrate concentration was 35% or 44.6% (n =1). *,** The dry weight of 56.7 g of WMP was added to a fermentor at 24 and 48 h, respectively, to represent 44.6% of final substrate concentration
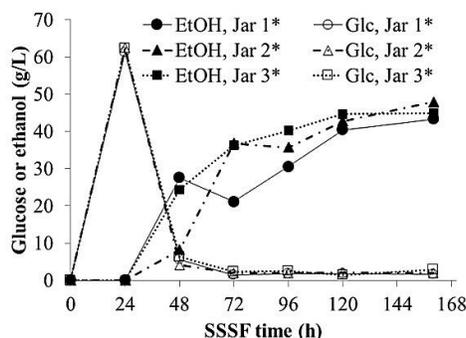

Figure 6: Evolution in time of the concentration of glucose and ethanol when the WM substrate was added to 3-L jar fermentors at different time points; *Table 3 shows the time points for substrate addition

Table 3
Cumulative substrate concentrations (%, w/v) in the 3 jar fermentors

Jar No.	SSSF time (h)						
	0	16	24	40	48	64	72
1	35	44.6	Yeast addition	49.3	53.9	58.6	63.1
2	35	49.3		58.6	63.1	63.1	63.1
3	35	44.6		53.9	53.9	63.1	63.1

SSSF of WM at a substrate concentration of 63.1% (w/v)

WMP was saccharified and fermented at the time points shown in Table 3. At the start of SSSF, 5 FPU Meicelase/g of final substrate was added to each jar fermentor. At 24 h from the start of SSSF, 1% of dry yeast was added. Figure 6 shows the amount of glucose and ethanol produced during SSSF. The concentration of ethanol in the jars 1, 2, and 3 was 43, 48, and 45 g/L, respectively, at 160 h and the ethanol yield was 38%, 42%, and 40%, respectively.

The decrease in ethanol concentration observed at this high concentration of substrate (Fig. 6) may have been due to a non-homogeneous ethanol concentration in the slurry. This led to the

observation that possibly manually powered agitation after substrate addition or before sampling was not sufficient. Thus, to avoid this in the subsequent experiments, the slurry was more thoroughly agitated after substrate addition and before sampling.

Evaluation of the effect of a low cellulase concentration and the gradual addition of cellulase on the SSSF of WMS

Figure 7 shows the effect on glucose and ethanol production and yield using a substrate concentration of 63.1% when the amount of Meicelase and the time of its addition were varied.

At 24 h from the start of SSSF, 1% of dry yeast was added. Reducing the amount of Meicelase from 5 to 2.7 FPU/g of the final substrate concentration resulted in an ethanol concentration of 30 g/L and an ethanol yield of 28% after 160 h. Adding 5 FPU Meicelase/g of final substrate at the start of SSSF and adding Meicelase at every additional injection of the substrate to provide an equivalent amount of enzyme finally produced ethanol at a concentration of 43 and 46 g/L, respectively, and resulted in a corresponding ethanol yield of 40% and 43%, respectively, at 160 h.
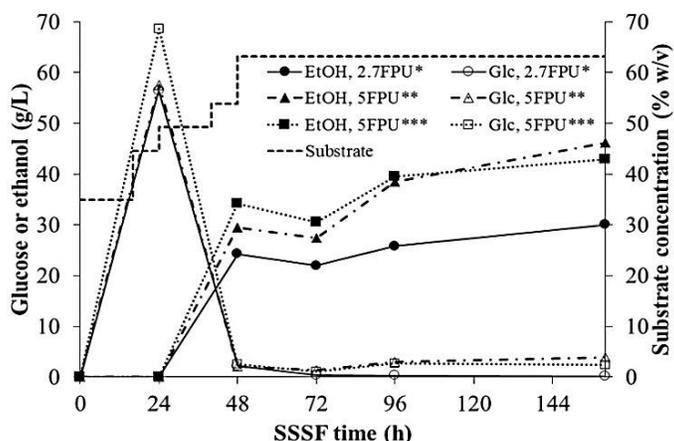

Figure 7: Evolution in time of the concentration of glucose and ethanol using different amounts and timing of cellulase addition (n = 1); *2.7 FPU Meicelase/g of final substrate added at the start time of SSSF; **5 FPU Meicelase/g of final substrate added stepwise at the time of each addition of substrate; ***5 FPU Meicelase/g of final substrate at the start of SSSF
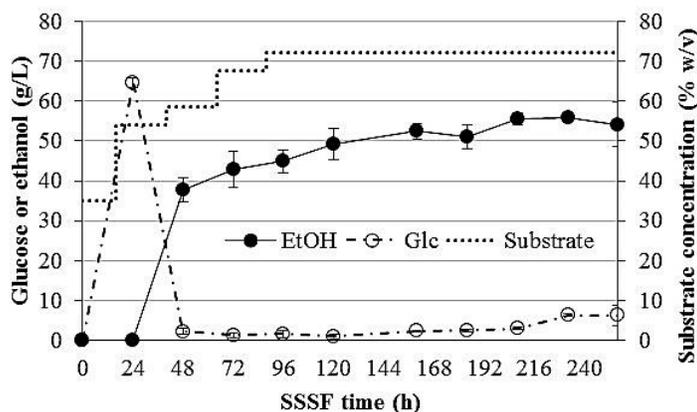

Figure 8: Evolution in time of glucose and ethanol at a substrate concentration of 72.1% (w/v) introduced over the course of 4 additions (error bars, standard deviation; n = 3)

These results show that adding an equivalent amount of Meicelase once at the beginning of SSSF or incrementally throughout the process has little effect on the amount of ethanol produced. In

contrast, reducing the amount of Meicelase from 5 FPU to 2.7 FPU/g markedly reduced the ethanol production and yield. In our previous study[6,10] and as shown in Fig. 1, the saccharification ratio of unpretreated WM was approximately 45-60% at 2% (w/v) substrate concentration and 5-50 FPU/g substrate. Compared with these results, an ethanol yield of 43% in this high concentration SSSF examination (63% w/v) was not considered to be dramatically low. To our knowledge, there are no other studies in which WM was saccharified without pretreatment by using a similarly high substrate concentration and low levels of cellulase (≤5 FPU/g substrate). In this study and our previous study,[6,9,10] the activity of Meicelase was determined to be of 0.39-0.52 FPU/mg Meicelase. Although Asada et al.[7] and Asakawa et al.[8] used steam-exploded, saccharified, and fermented WM at high substrate concentrations (10% and 30% [w/v], respectively), the use of Meicelase was estimated at 39-52 FPU/g substrate.

SSSF of WM at a substrate concentration of 72.1%

WMS was saccharified at 50°C for 24 h and then simultaneous saccharification and fermentation was carried out at 30°C for 232 h. At 0 and 48 h from the start of SSSF, 4.3 and 0.7 FPU Meicelase/g of final substrate, respectively, was added (total 5 FPU Meicelase/g of final substrate). At 24 h from the start of SSSF, 1% of dry yeast was added. Figure 8 shows the concentration of glucose and ethanol over the course of the SSSF. The ethanol concentration and yield at 120 and 232 h was 49 g/L and 41% and 56 g/L and 46%, respectively. Ethanol Red, the commercial yeast used in this study, can convert glucose, sucrose, or fructose to ethanol at approximately 90% fermentation efficiency under favorable conditions.[18] The maximum saccharification ratios obtained from SSSF in this study were estimated to be near 50% even at very high substrate concentrations. This indicates that a high concentration of WM can be saccharified to a similar degree as that of low concentrations of WM. In addition, the variability in the amount of glucose and ethanol produced and the ethanol yield was found to be very low, over a wide range of substrate concentrations. This result demonstrates that the production of ethanol from WM by using *Salix sachalinensis* or *Salix pet-susu* meals is generally

stable and reproducible.

Although an ethanol concentration of >5% (w/v) was achieved in this study, it required a long duration (160h) SSSF. However, there is room for further improvement by optimizing agitation conditions, and the type of cellulase and yeast used for fermentation. When the substrate concentration of the SSSF was high (>49% w/v), the media in the jar fermentors became clayey and agitating the entire content was inefficient when using a stirring propeller (as used in this study), suggesting that an improved agitation system would accelerate saccharification. The activity of Meicelase at 30°C is 60-65% of that at 40-50°C, which is the optimum temperature range for this enzyme.[17] In a recent study, high-temperature (39-45°C) tolerant yeasts were reported.[19–21] If these yeasts were to be used, the temperature of SSSF could be set at 40°C, resulting in near-maximum Meicelase activity. Although Meicelase, a commercial cellulase, was used in this study, various other cellulase enzymes have been developed for hydrolysis of cellulosic biomass.[22] This finding suggests that other cellulase enzymes are also available that may be better suited for hydrolysis of WM, which may accelerate the SSSF process.

It has been reported that using *Phlebia* sp. MG-60 for converting unpretreated WM into ethanol resulted in an ethanol yield of ~40%.[11] However, the ethanol concentration was low (2.4 g/L, estimated) in the study.[11] To our knowledge, there are no other studies reporting on a high concentration of ethanol (>50 g/L) from unpretreated WM with high substrate concentration (72% w/v).

Previous reports have shown that WM can be converted to bioethanol by enzymatic saccharification and fermentation (Table 4). However, in these reports, WM was pretreated, a higher concentration of Meicelase was used, and the ethanol concentration obtained was low. The pretreatment by steam explosion is expensive and requires high-pressure equipment; moreover, fermentation inhibitors produced during the process need to be removed. In the present study, a higher concentration of ethanol (56 g/L) was achieved with a low enzyme dose (5 FPU/g substrate) without pretreatment of the WM. These results demonstrate that the cost of pretreatment, enzymes, and ethanol distillation can be reduced for the production of bioethanol from WM using SSSF.

Table 4
Summary of literature results on the production of bioethanol from shiitake WM

References	Pretreatment	Meicelase (mg/g substrate)	Substrate concentration (g/L)	Ethanol Concentration (g/L)	Yield (%)
Asada *et al.*[7]	Steam explosion and water washing	100	200	38.3	70.5
			300	38.8	47.6
Asakawa *et al.*[8]	Steam explosion and water extraction	100	100	20.0	77.0
This paper	Rough pulverization (<10 mm)	12.6 (5 FPU)	721	56.0	46.2

CONCLUSION

The glucan content and the saccharification ratio of WM produced by a new cultivation method using *Salix* spp. meals were similar to those observed for conventional WM. Unpretreated WM was converted to bioethanol in a high substrate concentration SSSF (72.1% of substrate concentration) with a low concentration of a commercial cellulase (5 FPU Meicelase/g substrate). Under these conditions, a concentration of 56 g/L of ethanol at a yield of 46% of ethanol was achieved. These results suggest that WM, produced using *Salix* spp. as substrate, is a promising feedstock for the production of bioethanol.

REFERENCES

[1] N. Sarkar, S. K. Ghosh, S. Bannerjee and K. Aikat, *Renew. Energ.*, **37**, 19 (2012).

[2] S. T. Chang, *Int. J. Med. Mushrooms*, **1**, 291 (1999).

[3] D. J. Royse, "Cultivation of Shiitake on Natural and Synthetic Logs", Ag. Communications and Marketing, 2009, p. 2.

[4] Hokkaido, *Hokkaido Tokuyou-rinsanToukei* (in Japanese), 2013, pp. 5, http://www.pref.hokkaido.lg.jp/sr/rrm/grp/04/tokusan/tokusantoukei24.pdf;accessed May 13, 2014.

[5] S. Yokota, R. Nakajima, D. Suzuki, F. Ishiguri, K. Iizuka *et al.*, *Cellulose Chem. Technol.*, **41**, 575 (2007).

[6] R. Hiyama, S. Gisusi and A. Harada, *J. Wood Sci.*, **57**, 429 (2011).

[7] C. Asada, A. Asakawa, C. Sasaki and Y. Nakamura, *Bioresour. Technol.*, **102**, 10052 (2011).

[8] A. Asakawa, C. Sasaki, C. Asada and Y. Nakamura, *Int. J. Mod. Phys.*: *Conf. Ser.*, **6**, 745 (2012).

[9] R. Hiyama, S.Gisusi and A. Harada, *J. Wood Sci.*, **58**, 446 (2012).

[10] R. Hiyama, S. Gisusi and A. Harada, *J. Wood Sci.*, **59**, 88 (2013).

[11] I. Kamei, T. Nitta, Y. Nagano, M. Yamaguchi, Y. Yamasaki *et al.*, *Int. Biodeter. Biodegr.*, **94**, 57 (2014).

[12] A. Harada, K.Orihashi, R. Hiyama, S. Gisusi and T. Tanano, *Mushroom Sci.Biotech.*, **22**, 24 (2014) (In Japanese).

[13] Black and Veatch Ltd., Lignocellulosic Ethanol Plant in the UK Feasibility Study –Final Report, NNFCC 08–007, 2008.

[14] P. Alvira, E.Tomás-Pejó, M. Ballesteros and M. J. Negro, *Bioresour. Technol.*, **101**, 4851 (2010).

[15] A. Elliston, S. R. A. Collins, D. R. Wilson, I. N. Roberts and K. W. Waldron, *Bioresour. Technol.*, **134**, 117 (2013).

[16] B. Adney and J. Baker, Technical Report NREL/TP-510-42628,1996.

[17] F. Teratani, S. Tanzawa, Y. Ogawa, Y. Kai, T. Nishida *et al.*,*MokuzaiGakkaishi*,**43**, 956 (1997).

[18] I. Tahminaand C. Sergio, *Int. J. Agr. Boil. Eng.*, **4**, 30 (2011).

[19] B. M. A. Abdel-Banat, H. Hoshida, A. Ano, S. NonklangandR. Akada, *Appl.Microbiol. Biotechnol.*,**85**, 861 (2010).

[20] T. Kitagawa, K. Tokuhiro, H. Sugiyama, K. Kohda, N. Isono *et al.*, *Appl. Microbiol. Biotechnol.*, **87**, 1841 (2010).

[21] H. Shahsavarani, D. Hasegawa, D. Yokota, M. Sugiyama, Y. Kaneko *et al.*, *J. Biosci. Bioeng.*, **115**, 20 (2013).

[22] B. Yang, Z. Dai, S. Dingand C. E. Wyman, *Biofuels*, **2**, 421 (2011).