J Wood Sci (2015) 61:65–69
DOI 10.1007/s10086-014-1441-z

ORIGINAL ARTICLE

Ozone oxidation pretreatment for enzymatic saccharification of spent culture media after *Lentinula edodes* cultivation

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Received: 4 April 2014 / Accepted: 1 October 2014 / Published online: 30 October 2014

Abstract Ozone oxidation pretreatment was carried out on spent culture media (SCMs) after *Lentinula edodes* cultivation to improve fermentable sugar production by enzymatic saccharification. SCM samples treated with ozone for different treatment time under various moisture contents (MCs) were enzymatically saccharified, and hydrolysis weight decrease, reducing sugar yield, and monosaccharide yields were determined. Klason lignin content in SCM samples with 20 and 40 % MC was greatly decreased by ozone pretreatment for all ozone treatment time. In addition, in the ozone-treated sample at 40 % MC, the highest hydrolysis weight decrease after saccharification was obtained in the SCM sample treated for 1 h. After saccharification, glucose yield from the ozone-treated sample for 1 h was higher than that treated for 2 h. These results indicate that ozone treatment is effective to improve fermentable sugar production by enzymatic saccharification for the SCMs of *L. edodes*.

Keywords Enzymatic saccharification · *Lentinula edodes* · Ozone treatment · Spent culture media

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Introduction

In 2010, the total amount of edible mushroom produced in Japan was about 470000 tons, and shiitake mushroom (*Lentinula edodes* (Berk.) Pegler) is one of the main commercially produced edible mushrooms [1]. In addition, 81 % of shiitake production in 2010 was produced by wood meal-based cultivation [2], making this the main shiitake cultivation method in Japan. Disposal of large amounts of spent culture media (SCMs) causes serious problems with the increase in the mushroom production by wood meal-based cultivation. Fresh weight of SCMs after *L. edodes* cultivation is estimated to be more than 50000 t/year [3]. These SCMs have been used as litter for livestock, animal feed, fuel, fertilizer, and raw material for mushroom cultivation [4]; however, alternative utilization methods need to be developed for SCMs after mushroom cultivation.

When ethanol is produced from the SCMs, they need to be saccharified by enzymes or chemicals [5, 6]. It is known that the presence of lignin prohibits the enzyme from accessing polysaccharides during enzymatic saccharification of lignocellulosic materials [7]. Many edible mushroom species, which are cultivated on wood meal-based media, belong to the white-rot fungi. White-rot fungi can degrade wood components such as lignin, cellulose and hemicellulose [8]. It has been reported that the lignin content of SCM after mushroom cultivation is lower than that of fresh media and wood meal [9] and for this reason, SCM is considered a suitable raw material for ethanol production by enzymatic saccharification. Use of SCMs as raw material for ethanol production has been investigated by several researchers [5, 6, 10]. The effect of pretreatment by cultivation with white-rot fungi on the saccharification of SCMs was not significant: only 25 % of the potential glucose yield was obtained from SCM by enzymatic

saccharification after maitake mushroom (*Grifola frondosa*) cultivation [11]. The residual lignin in SCM still inhibits the saccharification of cellulose material by cellulase. Alternative pretreatments to degrade residual lignin are required for enzymatic saccharification to produce ethanol from SCM.

Because ozone has very high oxidizing potential, it has been used in the bleaching of pulp, chemical oxidation of pollutants in the industrial wastewater and other applications [12–14]. Ozone has been applied as a pretreatment method for enzymatic saccharification of lignocellulosic biomass, such as herbaceous plants, wood, and SCM [15–18]. Sugimoto et al. [17] treated Japanese cedar (*Cryptomeria japonica*) sawdust with ozone, resulting in an increase in sugar yield by enzymatic saccharification associated with an increase in lignin degradation by the ozone pretreatment. However, the effects of ozone pretreatment of SCM from *L. edodes* have not been intensively investigated.

In this study, to improve fermentable sugar production by enzymatic saccharification, SCM samples were treated with ozone prior to enzymatic saccharification. The optimal moisture content (MC) of SCMs and ozone treatment duration of the samples were examined for delignification of SCMs. Ozone-treated samples were enzymatically saccharified, and hydrolysis weight decrease, reducing sugar yield, and monosaccharide yields were determined. Based on the results obtained, the effects of ozone treatment on enzymatic saccharification of SCMs were discussed.

Materials and methods

Hokken Industry No. 600 was the shiitake fungal strain (*Lentinula edodes*) used in this study. Commercial wood meal (Watanabe Rinsan, meal size <5 × 5 mm, WM) of *Quercus serrata* was used for mushroom cultivation. The fresh medium (FM) was prepared by mixing the WM (moisture content (MC) = 38.9 %) with commercial wheat bran (Maeda Syokuhin, MC = 22.5 %, WB) (WM:WB = 4:1 (w:w)). The MC of FM was adjusted to 62 %. FM was packed in polyethylene bags (approximately 100 × 130 × 360 mm) to be 1200 g in weight for each bag. The bags filled with FM were autoclaved at 121 °C and 1.2 MPa for 1 h. After cooling the media, 25 g of spawn preincubated for 65 days at 20 ± 1 °C using wood meal was inoculated into the bags. The inoculated media were incubated for 120 days at 20 ± 1 °C and 70 % relative humidity (RH) in the dark. After incubation, the media were removed from the polyethylene bags, soaked in tap water, and then moved to the fruiting room. The fruiting bodies were formed under illumination by the fluorescent lamps for 24 h at 13–20 °C and 60–90 % RH. Spent culture media (SCMs) were collected after the first harvest of the fruiting bodies. Samples of SCMs were ground with a rotary speed mill (P-14, Fritsch, Germany) to obtain powdery samples (42–80 mesh size).

Ozone was generated by an ozone generator (ED-OG-R6, Ecodesign). Oxygen flow rate was adjusted to 50 mL/min, and the electrical power level of the ozone generator was adjusted to 100 %. The concentration of ozone generated was measured according to the conventional iodometric method [19]. The ozone concentration was 6 % under these conditions. MCs of samples were adjusted to 20, 40, 60, and 80 %, respectively. One gram of wet sample was placed into a 100 mL eggplant-shaped flask, and then the flask was connected to a rotary evaporator (N-1110N, EYELA). The ozone oxidation treatment of SCM was performed at 45 rpm with 6.0 % (w/w) ozone at RT for 0.5, 1.0, and 2.0 h. After oxidation, the samples were dried at 45 °C for 1–2 days. Contents of Klason lignin, holocellulose, and α-cellulose were determined according to the standard methods [20].

A commercial enzyme, Meiselase (Meiji Seika), was used for the saccharification of each sample. Dried sample (200 mg, 42–80 mesh size) was placed in an L-shaped test tube (EYELA), and then enzyme (50 mg, 19 filter paper units) dissolved in 10 mL of 0.1 M acetate buffer (pH 5.0) was added to the tube. The L-shaped test tube was sealed with Parafilm (Bemis Flexible Packaging, USA). The mixture was shaken at 60 strokes/min at 40 °C in a water bath shaker (NTS-120, EYELA) for 48 h. Saccharification was performed three times for each sample. Avicel (crystalline cellulose, Funacell II, Funakoshi) was used as a control. After saccharification, the reaction solution was collected in a 15 mL polypropylene centrifuge tube (IWAKI), and centrifuged at 1500×*g* for 15 min (CF15R, Hitachi). After centrifugation, the supernatant was retained and freeze-dried (Flexi-Dry MP, FTS Systems, Germany) to obtain the dried sugar sample. The unreacted residue was resuspended in distilled water, moved to a weighing bottle, and dried at 45 °C and then at 105 °C for 2 days to obtain the hydrolysis weight decrease by saccharification. The hydrolysis weight decrease by saccharification was calculated using the following formula:

$$\text{Hydrolysis weight decrease } (\%) = (W_0 - W_1) / W_0 \times 100$$

where W_0 (g) and W_1 (g) are the oven-dried weight of the each sample before and after saccharification, respectively.

Reducing sugar content in the saccharified product was determined by the 3,5-dinitrosalicylic acid (DNS) method [21]. The measurement was repeated three times for each sample. The calibration curve used glucose (Kanto Chemical Co.) as a standard at concentrations of 0.1, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/mL.

Qualitative and quantitative analyses of the monosaccharides were carried out using a high-performance anion exchange chromatography (HPAE) system (DX500, Dionex, USA) equipped with a pulsed amperometric detector (ED40, Dionex). The analytical column was a CarboPac™ PA-1 column (4 × 250 mm, Dionex) connected with a CarboPac™ PA-1 guard column (4 × 50 mm, Dionex). Temperature of the column oven (Model 502, EYELA) was set at 30 °C. The mobile phase consisted of ultrapure water (A), 100 mM sodium hydroxide (B), and 250 mM sodium hydroxide (C). Eluent flow was set at 1.0 mL/min. Galactose (Wako Pure Chemical Industries, Ltd.), glucose and xylose (Wako Pure Chemical Industries, Ltd.) were used as standard monosaccharides. For monosaccharide analysis, a freeze-dried sample was dissolved in 10 mL distilled water. After that, the sample (1 mL) was filtered through Millex-L (0.20 μm, Millipore, USA), and then 10 μL of filtered sample was injected onto the HPAE system.

Results and discussion

Effect of MC of the sample and treatment time in ozone pretreatment

As shown in Fig. 1, Klason lignin content in SCM samples with 20 and 40 % MC was greatly decreased by ozonization. On the other hand, the 80 % MC sample showed almost no changes in Klason lignin after ozone treatment. It is known that the MC of the sample affects the ozonolysis of lignocellulose [16, 17, 22]. The optimal MC of sample for ozone treatment is different by type and condition of sample [16, 17]. It is considered, therefore, that the optimal MC condition of sample for degrading the lignin by ozone treatment depends on the type of material. Moreover, it was reported that MC affects lignin degradation by ozone [16]. In the present study, the same tendency was found. The previous study reported that moisture content affected the consumption of charged ozone in Japanese cedar and ozone consumption decreased when moisture content was higher than 40 % [22]. In general, when water surrounds a sample, ozone needs to approach the sample through the water. In addition, during the ozonization of wood material, it is necessary to minimize the distance between ozone and the material for conducting diffusion-controlled, rapid and homogeneous reactions of ozone with wood material. Moreover, when woody material is adequately saturated with water, ozone gas can reach the material directly [23]. In this study, when the MC of samples was adjusted to be more than 60 %, lignin was not degraded efficiently by ozone, suggesting insufficient access of ozone to the sample. Hence, it is considered that the sample could not react

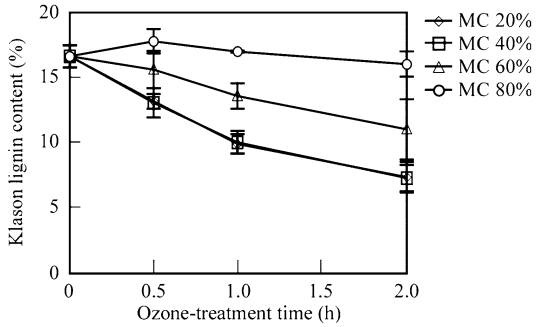

Fig. 1 The relationship between ozone treatment time and Klason lignin content. *Bars* indicate standard deviations. *MC* moisture content

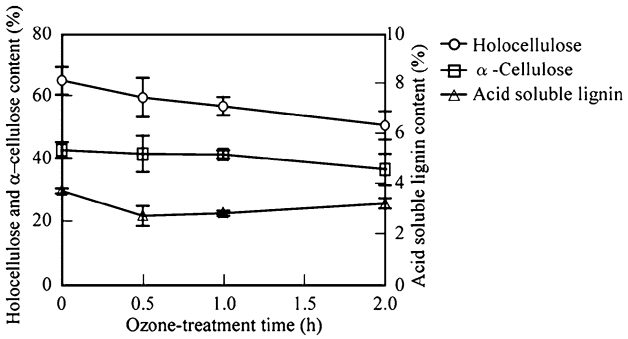

Fig. 2 The relationship between ozone treatment time and holocellulose, α-cellulose, or acid-soluble lignin content. The moisture content of spent culture medium samples was adjusted to 40 %. *Bars* indicate standard deviations

with ozone enough because the sample was surrounded with a thick water layer under high MC, and the distance between the sample and ozone was large. The non-reacted ozone with the sample was considered to be degraded with air and/or water. Based on these results, in the present study, the optimal MC was found between 20 and 40 % MC for lignin degradation by ozone treatment in the SCM of *L. edodes*.

Changes in holocellulose, α-cellulose, and acid-soluble lignin contents during ozone treatment with different treatment times are shown in Fig. 2. In this study, the Klason lignin content in SCM with MC of 20–60 % decreased with an increase in ozone treatment time (Fig. 1). In addition, holocellulose content also decreased gradually over 2 h of ozone treatment, while α-cellulose did not change significantly throughout the treatment (Fig. 2). These results suggest that ozone treatment resulted in the degradation of lignin and hemicellulose in the SCM sample. In general, ozone treatment mainly degrades lignin but also hemicellulose in lignocellulosic biomass, whereas cellulose is only slightly affected [7, 24]. Acid-

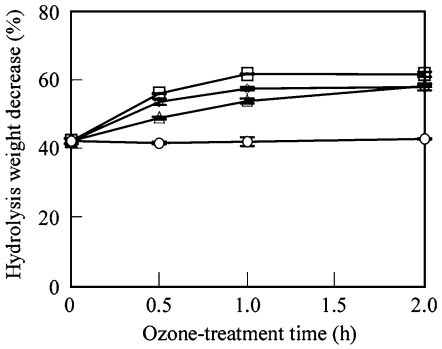

Fig. 3 The relationship between ozone treatment time and hydrolysis weight decrease after saccharification of the spent culture medium treated with ozone. *Bars* indicate standard deviations. Refer to Fig. 1 for symbols

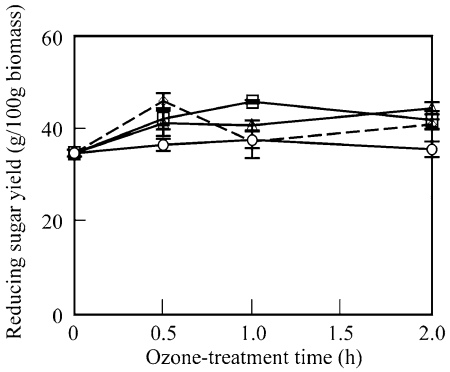

Fig. 4 The relationship between ozone treatment time and reducing sugar yield after saccharification of the spent culture medium (SCM) treated with ozone. *Bars* indicate standard deviations. Reducing sugar yield was expressed as equivalent glucose amount, which was obtained by a calibration curve using glucose as a standard. The yield is indicated as glucose amount (g) per 100 g dry SCM. Refer to Fig. 1 for symbols. *Dotted line* indicates the values of SCM samples with 20 % moisture content

soluble lignin content in the samples of ozone-treated SCM at 40 % MC decreased for the first 0.5 h and then slightly increased between 0.5 and 2.0 h (Fig. 2). Similarly, Garcia-Cubero et al. [16] reported that acid-soluble lignin content increased after ozone treatment in rye straw. In general, acid-soluble lignin is mainly composed of lignin degradation products, with the remainder composed of secondarily formed hydrophilic materials such as lignin–hemicellulose complexes [25]. The slight increase in acid-soluble lignin in the sample of ozone-treated SCM may have been caused by degradation of lignin and lignin–hemicellulose complexes during ozone treatment.

Effect of ozone treatment on enzymatic saccharification

It is known that lignin inhibits enzymatic saccharification by limiting the enzyme accessibility to polysaccharides and by absorbing enzymes [7]. García-Cubero et al. [16] reported a proportional increase in sugar production with lignin degradation by ozone pretreatment. The effects of the duration of ozone treatment on hydrolysis weight decrease after saccharification are shown in Fig. 3. Among the four sample MCs, 40 % MC showed the largest reduction in hydrolysis weight after saccharification for all ozone treatment durations tested. The largest decrease in hydrolysis weight (61.0 %) after saccharification was obtained after ozone treatment for 1 h. Reducing sugar yield from the SCM samples with 20–80 % MC increased up to 0.5 h ozone treatment (Fig. 4). The optimum MC for ozone treatment of shiitake SCM samples to obtain the highest yield of reducing sugars was 40 % (Fig. 4). Furthermore, significant correlation was obtained between reducing sugar yield and Klason lignin content ($r = -0.638$). Table 1 shows the monosaccharide yield after saccharification of the ozone-treated SCM sample (MC 40 %). After saccharification, glucose was the major monosaccharide, followed by xylose and galactose. In addition, glucose and xylose yields from the sample treated with ozone for 1 h were higher than the yields from the sample treated with ozone for 2 h. These results indicate that lignin degradation by ozone pretreatment for 1 h improved effectively the accessibility of the enzyme to cellulose during the saccharification, while prolonged ozone treatment for 2 h caused monosaccharide degradation. From these results, the optimal duration of ozone treatment to obtain the highest glucose yield from the SCM by enzymatic saccharification was 1 h.

Table 1 Monosaccharide yield after saccharification of ozonized spent culture medium samples

Ozone treatment time (h)	Monosaccharide yield (g/100 g dry biomass)			
	Galactose	Glucose	Xylose	Total
0.0	0.8	17.8	4.6	23.2
1.0	0.3	21.8	6.5	28.6
2.0	0.2	16.4	5.2	21.8

Moisture content of spent culture medium sample was adjusted to 40 % before ozone pretreatment. Results were obtained from three replicates. Monosaccharide yield after enzymatic saccharification is indicated in g weight per 100 g of original dried SCM

Conclusion

In this study, the pretreatment conditions for ozone oxidation, i.e., MC and ozone treatment time, were determined for SCMs from *L. edodes*. Ozone oxidation was an efficient

pretreatment for SCMs. Ozone treatment resulted in lignin degradation in the SCMs, resulting in the improvement of subsequent enzymatic saccharification. At the MC of the sample between 20 and 40 %, the lowest lignin content was obtained in the ozone-treated SCM. In addition, in the sample ozonized at 40 % MC, the highest hydrolysis weight decrease and reducing sugar yield after saccharification were obtained in the SCM sample treated for 1 h. Glucose yield after saccharification from the ozone-treated sample for 1 h was the highest among all samples treated. Based on these results, we conclude that ozone is an effective treatment to improve sugar yield by enzymatic saccharification of *L. edodes* SCM.

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