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NOTE

Ozone treatment of spent medium from *Auricularia polytricha* cultivation for enzymatic saccharification and subsequent ethanol production

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Abstract We examined the enzymatic saccharification and ethanol fermentation of the spent media (SMs) from *Auricularia polytricha* cultivation using wood meals of *Falcataria moluccana*, *Shorea* sp., and *Tectona grandis*. Although the hydrolysis weight decrease and reducing sugar yield were higher in SM of *F. moluccana*, the ethanol yield was higher in SM of *Shorea* sp. Ozone treatment of SM further increased the hydrolysis weight decrease, reducing sugar, and ethanol yields in *Shorea* sp. These results indicate that SM of *A. polytricha* is a suitable biomass material to produce fermentable sugars for ethanol production, and that ozone treatment is a suitable method for increasing the ethanol yield.

Keywords Spent media from mushroom cultivation · Ozone treatment · Enzymatic saccharification · Ethanol fermentation

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Introduction

Auricularia polytricha is one of edible mushrooms known as black jelly. *A. polytricha* can be cultivated in a wide range of regions in the world, such as Indonesia which is one of the tropical countries. Some Indonesian wood species are also suitable for *A. polytricha* cultivation [1]. In Indonesia, this species has been cultivated by farmers using a simple technology. After several months of cultivation period, farmers replace the mushroom media with the new ones, and the spent media (SMs) are usually just dumped away near mushroom farms or burnt out. In fact, these treatments have bad effects on the environment, mainly air pollution. Thus, SM after cultivation of *A. polytricha* should be utilized for other applications.

A. polytricha is a member of the class Basidiomycetes. Some of the Basidiomycetes degrade lignin during their growth [2]. Thus, SM after cultivation of *A. polytricha* is regarded as a potential material for the production of fermentable sugars for subsequent ethanol production. In fact, we have succeeded in producing high yield of fermentable sugars from the *A. polytricha* SMs originally made of 5 different Japanese wood species [3]. However, the residual lignin content of the SM from *A. polytricha* cultivation was still rather high [3]. A pretreatment process, therefore, is needed to reduce the residual lignin content from SM [4].

Ozone is known to be a powerful oxidizing reagent and has a potential to degrade lignin without producing harmful compounds [5, 6]. Ozone is highly reactive toward compounds with double bonds and functional groups [7, 8]. So far, ozone has been applied mainly to pulp bleaching process [9, 10], delignification of wood for animal feed [11], treatment of ground and industrial wastewaters [12, 13], and pretreatment on wood liquefaction [14]. In terms of pre-treating lignocellulose for subsequent enzymatic

saccharification, the advantage of ozone treatment is highly selective to removal of lignin with slight degradation of hemicelluloses, whereas it hardly affects cellulose [15]. Moreover, ozone reaction can be performed at room temperature under atmospheric pressure, leading to low pretreatment cost.

The objective of this study is to examine the effects of ozone pretreatment of *A. polytricha* SMs originally made of Indonesian wood species on the production of fermentable sugars and subsequent ethanol. To evaluate the effects of ozone treatment on the SMs, the contents of wood chemical components in SMs were determined after ozone treatment. Furthermore, the ozone-treated spent medium (SMO) was enzymatically hydrolyzed and then fermented. Based on the results obtained, the effects of ozone treatment on enzymatic saccharification and ethanol production were discussed.

Materials and methods

Biomass materials

Wood meals (9–80 mesh) of three Indonesian wood species (*Falcataria moluccana, Shorea* sp., and *Tectona grandis*) with 8.3–12.1 % moisture content (MC) were used for cultivation experiment of *A. polytricha*.

A. polytricha cultivation

A strain of *A. polytricha* (Aragekikurage 89, Mori and Company, Ltd., Japan) was used for mushroom cultivation. Commercial rice bran (Satoh Rice, 9–80 mesh size, MC = 11.0 %) (12.5 % w/w), $CaCO_3$ (Kanto Chemical Co. Inc., Japan) (6 % w/w), and distilled water (to adjust the MC to 70 %) were added to wood meal, and 150 g of these mixture was packed in a polypropylene bag (25 × 8 × 4.5 cm) equipped with a porous sterile filter (MilliSeal, 1 cm diameter pore, Millipore). The medium was sterilized at 121 °C for 20 min. After inoculation of *A. polytricha*, the media were cultured for 130 days in a culture room as previously described [3]. The highest yield of fruiting body was obtained in *F. moluccana* (Table 1) [1].

Table 1 Mean yield of fruiting body in each wood species [1]

Wood species	Fresh weight (g)	Dry weight (g)
F. moluccana	65.4 ± 4.5 c	7.6 ± 0.8 c
Shorea sp.	52.6 ± 15.0 b	5.2 ± 1.0 b
T. grandis	32.0 ± 11.3 a	2.6 ± 1.3 a

The same alphabet letter followed by mean and standard deviation shows no significant differences between wood species by Tukey–Kramer test at the 5 % level

Ozone treatment

In ozone treatment, ozone was produced by an ozonizer (ED-OG-R6, EcoDesign, Japan) at a flow rate of 50 mL/min. Before ozone treatment, the samples of the fresh medium (FM) and spent medium were dried in the oven at 45 °C. The dried sample (1 g) was put in a Kjeldahl flask (100 mL) and then MC of the sample was adjusted to 40 % with distilled water. Ozone treatment was done using 6 % concentration of ozone (0.26 g/h flow rate) for 1 h.

Chemical component analysis

Before chemical analysis, the samples were ground by a rotary speed mill (P-14, Fritsch) and then sieved to collect samples in 40–80 mesh size. After that, the samples were dried in an oven at 45 °C. The amounts of α-cellulose, Klason lignin, and acid-soluble lignin of the sample were determined by ordinary methods [16–18]. Although FM and SM contained rice bran, $CaCO_3$, and mycelia, ordinary methods of chemical analysis for wood were applied for determining the amounts of chemical components in these samples. Chemical component analysis was conducted three times for each sample. Values of chemical components in SM and SMO were adjusted to the values based on FM using weight decrease ratio in a bag from FM to SM and FM to SMO, respectively. Decrease ratio from FM to SM or SM to SMO was calculated by dividing dry weight in a bag or chemical components of SM or SMO by these values of FM or SM, respectively.

Enzymatic saccharification

A commercial enzyme, Meiselase (Meiji Seika), was used for the saccharification of each sample. Two hundred mg of oven-dried sample (40–80 mesh size) was saccharified by 50 mg of enzyme at 40 °C for 48 h according to the method previously reported [3]. The hydrolysis weight decrease (HWD) and monosaccharides were quantified by using the methods of our previous study [3]. Reducing sugar yield was determined by the dinitrosalicylic acid (DNS) method [19]. Enzymatic saccharification was conducted three times for each sample.

Ethanol fermentation

Saccharomyces cerevisiae NBRC 0216 was used for ethanol fermentation. The fungus was pre-cultured in agar medium containing 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract (Becton, Dickinson and Company, USA), 3 g/L malt extract (Becton, Dickinson and Company), 15 g/L agar, and 1 L distilled water in a Petri dish (90 mm diameter) at 26 °C for 10 days. Ethanol fermentation was

carried out in an 1.5 mL micro-tube (STF-15, Sansyo, Japan) with a working volume of 1.5 mL. Freeze-dried hydrolysates (three hydrolysate samples were combined) were diluted with 10 mL nutrient solution (1 % (w/v) polypeptone (Becton, Dickinson and Company), 0.5 % (w/v) yeast extract, 0.5 % (w/v) KH_2PO_4, and 0.2 % (w/v) $MgSO_4$), and the pH was adjusted to 6.0 with 0.1 M NaOH [20]. The solution was autoclaved at 121 °C for 20 min. *S. cerevisiae* was inoculated to the medium at 10 % (v/v) mycelium concentration (OD_{660} = 1.63–1.64) and incubated stationarily at 30 °C for 48 h. After fermentation, the culture sample was centrifuged at 5,590 × g at 4 °C for 10 min, and the obtained supernatant was analyzed for ethanol content. Quantitation of ethanol was performed using a gas chromatograph (HP6890 series, Agilent, USA) equipped with a capillary column (DB-ALC1, ID 0.32 mm × 30 m, film thickness 1.8 μm, Agilent) [21]. Other conditions were as follows: column temperature, 100 °C (5 min) → 150 °C (5 °C/min, for 10 min); injection pot temperature, 250 °C; He flow rate, 30 mL/min; FID temperature, 250 °C. Isopropanol (1 %) was used as an internal standard. Ethanol quantitation was conducted three times for each sample. Ethanol yield was defined as obtained ethanol weight (g) after enzymatic saccarification and fermentation processes from 100 g of oven-dried SM or SMO samples.

Results and discussion

Dry weight change in the media

The decrease ratio of dry weight from FM to SM varied between 20.7 and 42.8 % after *A. polytricha* cultivation. The highest decrease ratio was found in *F. moluccana*, suggesting its suitability for *A. polytricha* cultivation. Some of the degraded wood components were incorporated into the fruiting bodies and partly emitted into the atmosphere as carbon dioxide through respiration during mushroom growth [22]. On the other hand, the decrease ratio from SM to SMO was only about 16 % (Table 2).

Table 2 Dry weight and chemical components of fresh medium (*FM*), spent medium (*SM*), and ozone-treated spent medium (*SMO*)

	WM	FM	SM	SMO	Decrease ratio (%)	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	FM to SM	SM to SMO
F. moluccana						
Dry weight in a bag (g)	37.2*	48.4 ± 0.5	27.7 ± 3.8	23.3*	42.8	15.9
Klason lignin (%)	23.3 ± 0.3	19.4 ± 0.5	5.5 ± 0.1	1.3 ± 0.4	71.6	76.4
Acid-soluble lignin (%)	2.8 ± 0.1	2.8 ± 0.1	2.5 ± 0.0	1.4 ± 0.0	10.7	44.0
Holocellulose (%)	80.8 ± 0.6	73.4 ± 2.5	41.0 ± 4.2	33.1 ± 0.8	44.1	19.3
α-Cellulose (%)	45.3 ± 1.1	40.0 ± 0.2	16.9 ± 0.8	15.7 ± 0.5	57.8	7.1
Ethanol–toluene extracts (%)	2.9 ± 0.4	5.2 ± 0.2	2.6 ± 0.0	6.3 ± 0.4	50.0	−142.3
Shorea sp.						
Dry weight in a bag (g)	37.2*	50.3 ± 0.3	34.5 ± 3.5	28.8*	31.4	16.5
Klason lignin (%)	30.9 ± 0.4	27.9 ± 0.3	13.5 ± 1.6	3.0 ± 0.8	51.6	77.8
Acid-soluble lignin (%)	1.5 ± 0.1	1.5 ± 0.1	2.4 ± 0.2	1.7 ± 0.0	−60.0	29.2
Holocellulose (%)	75.7 ± 1.2	78.7 ± 0.3	48.3 ± 0.7	41.8 ± 1.2	38.6	13.5
α-Cellulose (%)	49.2 ± 2.4	54.8 ± 0.8	23.1 ± 1.4	24.0 ± 1.3	57.8	−3.9
Ethanol–toluene extracts (%)	2.9 ± 0.2	3.1 ± 0.1	2.1 ± 0.1	8.3 ± 0.6	32.3	−295.2
T. grandis						
Dry weight in a bag (g)	37.1*	51.2 ± 0.4	40.6 ± 2.4	34.4*	20.7	15.3
Klason lignin (%)	29.9 ± 0.6	27.5 ± 1.1	21.9 ± 1.9	8.7 ± 0.3	20.4	60.3
Acid-soluble lignin (%)	1.4 ± 0.1	1.3 ± 0.0	2.7 ± 0.3	2.0 ± 0.1	−107.7	25.9
Holocellulose (%)	76.0 ± 2.5	73.3 ± 1.0	62.0 ± 1.3	50.5 ± 0.2	15.4	18.5
α-Cellulose (%)	45.7 ± 2.1	45.0 ± 0.9	30.7 ± 0.4	26.6 ± 1.0	31.8	13.4
Ethanol–toluene extracts (%)	6.1 ± 0.3	7.2 ± 0.4	4.7 ± 0.5	12.6 ± 0.6	34.7	−168.1

Values of chemical components in SM and SMO were adjusted to the values based on FM using weight decrease ratio in a bag from FM to SM and FM to SMO, respectively. Decrease ratio from FM to SM and SM to SMO was calculated by dividing dry weight in a bag or chemical components of SM or SMO by these values of FM or SM, respectively. n = 3, *, n = 1

WM wood meal, *FM* fresh medium, *SM* spent medium, *SMO* ozone-treated spent medium, *SD* standard deviation

Chemical characterization

Chemical analysis of the sample was conducted to determine the Klason lignin, acid-soluble lignin, holo-cellulose, and α-cellulose (Table 2). After *A. polytricha* cultivation, the highest decrease ratio (71.6 %) of Klason lignin was obtained in *F. moluccana* and that of α-cellulose (57.8 %) was found in *F. moluccana* and *Shorea* sp. After ozone treatment, the decrease ratio from SM to SMO in Klason lignin was 76.4, 77.8, and 60.3 % for *F. moluccana*, *Shore* sp., and *T. grandis*, respectively. On the other hand, the decrease ratio from SM to SMO in α-cellulose (7.1, −3.9, and 13.4 %, respectively), showed lower values compared to decrease ratio in Klason lignin. These results indicate that ozone treatment is effective for the degradation of lignin, but not for cellulose. Our results are similar to those of other studies focused on lignocellulosic materials [6, 23]. Ozone is highly reactive toward compounds with conjugated double bonds and functional groups with high electron densities. Because lignin has high content of C = C bond, it is easily oxidized by ozone [7, 8].

Acid-soluble lignin increased after *A. polytricha* cultivation in *T. grandis* and *Shorea* sp., while it decreased in *F. moluccana* (Table 2). It seems that *A. polytricha* degraded lignin, hemicelluloses, and cellulose during their mycelial growth, leading to the increase of the acid-soluble lignin content. However, acid-soluble lignin decreased in SMO compared to SM after ozone treatment in all three species (Table 2). In contrast, there is a report that acid-soluble lignin was increased after ozone treatment in rye straw [6]. This discrepancy may be due to the physical and chemical differences of the biomass material used. The chemical compounds with smaller molecular mass derived from degradation by *A. polytricha* cultivation might be further degraded by ozone treatment, and they could be easily extracted with ethanol–toluene solvent. In fact, the ethanol–toluene extract content dramatically increased after ozone treatment (Table 2).

Enzymatic saccharification

Table 3 shows hydrolysis weight decrease, reducing sugar yield, and monosaccharide yield after enzymatic saccharification. Hydrolysis weight decrease, reducing sugar yield, and glucose yield were higher in SM compared to FM, indicating that SM was a potential material for ethanol production. Based on these results, it is considered that cellulases could easily hydrolyze cellulose, because lignin encrusting cellulose in FM was greatly decreased by *A. polytricha* cultivation. In fact, there was negative significant correlation between lignin content and glucose yield (Fig. 1).

After ozone treatment of SM, SMO of *Shorea* sp. showed the highest hydrolysis weight decrease, reducing sugar yield, and glucose yield (Table 3). As shown in Table 2, the highest decrease ratio of Klason lignin from

Table 3 Hydrolysis weight decrease, reducing sugar yield, and monosaccharide yield of fresh medium (*FM*), spent medium (*SM*), and ozone-treated spent medium (*SMO*)

	FM Mean ± SD	SM Mean ± SD	SMO Mean ± SD
F. moluccana			
Hydrolysis weight decrease (%)	10.1 ± 0.5	20.6 ± 2.2	53.1 ± 0.5
Reducing sugar (g)	6.0 ± 0.2	15.8 ± 1.1	24.2 ± 1.0
Glucose (g)	3.1 ± 0.0	8.5 ± 0.3	13.2 ± 1.6
Galactose (g)	0.3 ± 0.0	1.7 ± 0.2	0.3 ± 0.0
Xylose (g)	1.7 ± 0.0	0.7 ± 0.4	3.0 ± 0.3
Shorea sp.			
Hydrolysis weight decrease (%)	8.6 ± 1.8	15.4 ± 2.0	59.1 ± 2.4
Reducing sugar (g)	6.1 ± 0.4	13.9 ± 1.2	27.7 ± 1.3
Glucose (g)	2.4 ± 0.3	9.0 ± 0.7	15.5 ± 0.7
Galactose (g)	0.3 ± 0.0	3.0 ± 0.1	0.3 ± 0.0
Xylose (g)	1.2 ± 0.3	0.9 ± 0.1	1.7 ± 0.1
T. grandis			
Hydrolysis weight decrease (%)	13.6 ± 1.0	10.9 ± 3.0	49.9 ± 3.0
Reducing sugar (g)	6.8 ± 0.1	11.8 ± 1.8	22.5 ± 1.1
Glucose (g)	2.7 ± 0.1	7.3 ± 1.8	11.7 ± 0.7
Galactose (g)	0.3 ± 0.0	3.0 ± 0.3	0.3 ± 0.0
Xylose (g)	1.4 ± 0.1	0.7 ± 0.1	2.2 ± 0.4

Reducing sugar or monosaccharide yield was defined as reducing sugar or monosaccharide weight (g) after enzymatic saccharification process from 100 g oven-dried FM, SM, or SMO samples. *SD* standard deviation, $n = 3$

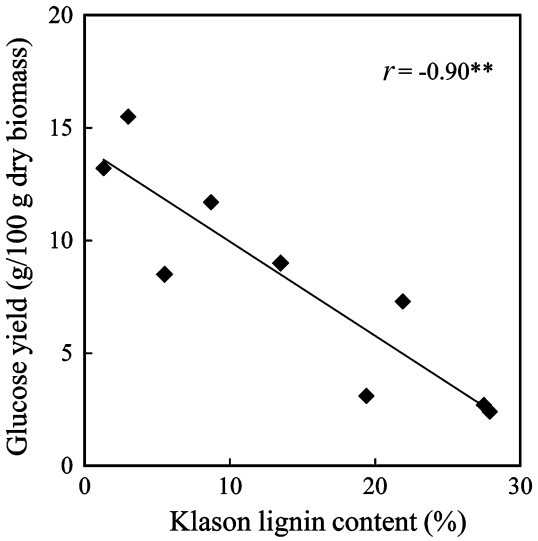

Fig. 1 Correlations between Klason lignin content and glucose yield in the fresh medium, spent medium, and ozone-treated spent medium. ** Significance at the 1 % level, $n = 9$

SM to SMO was observed in *Shorea* sp. On the other hand, the α-cellulose content remaining in SMO of *Shorea* sp. was still high. Thus, it is suggested that a rather higher amount of remaining cellulose was easily hydrolyzed by cellulases, resulting in the higher hydrolysis weight decrease, reducing sugar yield, and glucose yield in SMO of *Shorea* sp.

Ozone is effective in disrupting the chemical bonds between the various components so as to produce a substrate with enhanced reactivity to the hydrolytic enzyme [24]. Ozone treatment also can increase the total space volume as well as specific surface area of the lignocellulosic materials [25]. Lee et al. [23] have reported that cellulose conversion into sugars in coastal Bermuda grass by cellulases increased from 30 to 53 %, when materials were pretreated with ozone at concentrations of 4.5 and 26.4 %, respectively.

Glucose was the most abundant monosaccharide among the produced monosaccharides (Table 3). Glucose and galactose yields were higher in SM compared to FM. Although glucose and xylose yields increased, galactose yield decreased after ozone treatment (Table 3). Similarly, Garcia-Cubero et al. [6] have reported that glucose yield from enzymatically hydrolyzed wheat, rye, oat, and barley straws increased after ozone treatment. It is known that the larger amount of glucose is favorable to ethanol fermentation [26]. Therefore, ozone treatment is considered to be effective for increasing the ethanol yield from lignocellulosic materials.

Ethanol fermentation

The obtained hydrolysates from SM and SMO were fermented using *S. cerevisiae* NBRC 0216 for 48 h. The hydrolyzate from FM was not fermented because of its low yield. Table 4 shows ethanol yield from SM and SMO. The ethanol yields from SM of *F. moluccana*, *Shorea* sp., and *T. grandis* were 5.5, 6.8, and 4.2 g/100 g dry biomass, respectively. The ethanol yields from SMO of *F. moluccana*, *Shorea* sp., and *T. grandis* were 7.7, 13.2, and 9.3 g/100 g dry biomass, respectively. The results obtained in the present study were similar to those by Kaida et al. [27], in which they used *Acacia mangium* and *F. moluccana* wood applying ultrasonication as the pretreatment process. Among three species, *Shorea* sp. showed the highest ethanol yield. These results correspond to the higher total hexose yields in SM and SMO of *Shorea* sp. (Table 3), indicating that the higher hexose yield results in a higher yield of ethanol.

The ethanol yield from SMO was higher (40–121.4 %) than that from SM (Table 4). The results indicate that hydrolysates from SMO contain more fermentable sugars than those from SM. Because high fermentation rate was obtained in this study, the hydrolysates from SMO are estimated to have no negative influence on the fermentation process. Ozone oxidation is known not to produce harmful by-products [5, 6]. It is suggested that ozone treatment is a suitable method for increasing fermentable sugar yield to efficiently produce ethanol.

Table 4 Ethanol yield from spent medium (*SM*) and ozone-treated spent medium (*SMO*)

Sample	Ethanol (g/100 g dry biomass)		Increase ratio from SM to SMO (%)
	SM Mean ± SD	SMO Mean ± SD	
F. moluccana	5.5 ± 0.5 b	7.7 ± 0.6 a	40.0
Shorea sp.	6.8 ± 0.5 c	13.2 ± 1.7 c	94.1
T. grandis	4.2 ± 0.6 a	9.3 ± 0.1 b	121.4

The same alphabet letter followed by mean and standard deviation shows no significant differences between wood species by Tukey–Kramer test at the 5 % level. $n = 3$. Ethanol yield was defined as obtained ethanol weight (g) after enzymatic saccharification and fermentation processes from 100 g oven-dried SM or SMO samples. *SD* standard deviation

Conclusion

SM of *A. polytricha* is a promising biomass material for enzymatic saccharification to obtain large amounts of monosaccharides which are fermented to ethanol. In addition, ozone treatment is a suitable method for increasing the ethanol yield. The ethanol yield from SMO was higher (40–121.4 %) than that from SM. Furthermore, SM and SMO of *Shorea* sp. are considered to be the most appropriate biomass material for enzymatic saccharification and subsequent ethanol fermentation.

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