Direct Fungal Production of Ethanol from High-Solids Pulps by the Ethanol-fermenting White-rot Fungus *Phlebia* sp. MG-60

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A white-rot fungus, *Phlebia* sp. MG-60, was applied to the fermentation of high-solid loadings of unbleached hardwood kraft pulp (UHKP) without the addition of commercial cellulase. From 4.7% UHKP, 19.6 g L^{-1} ethanol was produced, equivalent to 61.7% of the theoretical maximum. The highest ethanol concentration (25.9 g L^{-1}, or 46.7% of the theoretical maximum) was observed in the culture containing 9.1% UHKP. The highest filter paper activity (FPase) was observed in the culture containing 4.7% UHKP, while the production of FPase in the 16.5% UHKP culture was very low. Temporarily removing the silicone plug from Erlenmeyer flasks, which relieved the pressure and allowed a small amount of aeration, improved the yield of ethanol produced from the 9.1% UHKP, which reached as high as 37.3 g L^{-1}. These results indicated that production of cellulase and ensuing saccharification and fermentation by *Phlebia* sp. MG-60 is affected by water content and benefits from a small amount of aeration.

Keywords: White-rot fungi; Bioethanol; Consolidated bioprocessing; Waste paper fermentation

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INTRODUCTION

Ethanol is the most frequently used liquid biofuel alternative to fossil fuel. Cellulose is the most abundant biomass in nature and has great potential for biofuel production. However, the high cost of enzymatic saccharification step of cellulose has contributed to the high cost of bioethanol production. To reduce the cost of ethanol production from lignocellulosic materials, consolidated bioprocessing (CBP) should be considered (Lynd *et al*. 2005). In CBP, all biological processes (the production of saccharification enzymes, the saccharification of cellulose and hemicelluloses, and the fermentation of hexoses and pentoses) are carried out within one bioreactor. Recently, researchers have focused on modifying non-cellulolytic organisms that are able to produce high amounts of ethanol so that they become cellulolytic (Hasunuma and Kondo 2012; Olson *et al*. 2012).

Lignin-degrading basidiomycetes, referred to as white-rot fungi, are the microbes responsible for degrading all the components of plant cell-wall polymers including polysaccharides (cellulose and hemicellulose) and the recalcitrant aromatic polymer lignin (Kirk and Fenn 1982). White rot fungi have a unique ability to decompose wood lignin *via* the secretion of extracellular lignin-degrading enzymes such as manganese peroxidase, lignin peroxidase, versatile peroxidase, and laccase (Lundell *et al*. 2010). Thus, there are many reports focusing on the biological delignification pretreatment of lignocellulosic biomass for enzymatic saccharification and following fermentation (Bak *et al*. 2009;

Taniguchi *et al*. 2005; Wan and Li 2011; Isroi *et al*. 2011). Therefore, white-rot fungi, which have the ability to saccharify without addition of cellulase and to ferment cellulose simultaneously, may be potentially applicable for one-step fermentation of lignocellulosic biomass.

Lignin degradation is an oxidative process, and replacing air with atmospheric oxygen stimulates the degradation of lignin by white-rot fungi. As such, there are few reports on white-rot fungal traits under the conditions where aeration is limited (Zadražil *et al*. 1991). A study has recently revealed that the white-rot fungus *Phlebia* sp. MG-60 can convert lignocellulose to ethanol under semi-aerobic conditions (Kamei *et al*. 2012a). When this fungus was cultured with 20 g L^{-1} of unbleached hardwood kraft pulp (UHKP), 8.4 g L^{-1} ethanol was produced after 168 h of incubation, corresponding to an ethanol yield of 0.42 g g^{-1} UHKP, or 71.8% of the theoretical maximum. When this fungus was cultured with waste newspaper, 4.2 g L^{-1} of ethanol was produced after 216 h of incubation, an ethanol yield of 0.20 g g^{-1} of newspaper, or 51.5% of the theoretical maximum (Kamei *et al*. 2012a). Glucose, mannose, galactose, fructose, and xylose were completely assimilated by *Phlebia* sp. MG-60, to give ethanol yields of 0.44, 0.41, 0.40, 0.41, and 0.33 g g^{-1} of sugar, respectively (Kamei *et al*. 2012a). These results indicated that *Phlebia* sp. MG-60 is a good candidate for the production of bioethanol from cellulosic materials. Additionally, this fungus has significant lignin-degrading abilities under aerobic conditions (Li *et al*. 2002; Kamei *et al*. 2008). Thus, a new process (Integrated Fungal Fermentation: IFF) of unified aerobic delignification and anaerobic saccharification and fermentation of wood by *Phlebia* sp. MG-60 was proposed (Kamei *et al*. 2012b). This fungus is able to selectively degrade lignin under aerobic solid state fermentation conditions and to produce ethanol directly from delignified oak wood under semi-aerobic liquid culture conditions. The transition from aerobic conditions (biological delignification pretreatment) to semi-aerobic conditions (saccharification and fermentation) enabled the fermentation of wood by solely biological processes (Kamei *et al*. 2012b). However, a low solids concentration of cellulosic substrates (2% w/w) was used in these studies. Therefore, additional experiments using higher concentrations of cellulosic substrates are needed to evaluate the effects of initial concentration of cellulosic material on ethanol production.

In the present study, the direct fermentation of UHKP was carried out at several high concentrations (2.0, 4.7, 9.1, and 16.5% w/w). Under these conditions, the substrates were essentially solids. The aim of this study was to evaluate the effects of initial concentration of UHKP on ethanol production by *Phlebia* sp. MG-60.

EXPERIMENTAL

Materials

Fungal strains

Phlebia sp. MG-60 (Fungus/Mushroom Resource and Research Center, Tottori University; Japan.) was used in these experiments. The fungus was maintained on potato-dextrose-agar medium. The fungus *Phlebia* sp. MG-60 was screened as a hypersaline-tolerant lignin-degrading fungus from 28 strains isolated from mushrooms and driftwood sampled from mangrove stands in Okinawa, Japan (Li *et al*. 2002). Its phylogenetic position among the *Phlebia* genera based on the internal transcribed spacer (ITS) region (containing 5.8S ribosomal DNA, ITS1, and ITS2) was also previously reported (Kamei *et al*. 2005).

Substrates

The unbleached hardwood kraft pulp (UHKP) was kindly provided by Oji Paper Co., Ltd. (Tokyo, Japan). Alkaline UHKP was washed with water until it acquired a neutral pH (approximately 6.5) and was subsequently air-dried. Before use, the dried UHKP was defibrated using a mixer with mixing blades rotating at 20,000 rpm for 20 s. Japanese waste newspaper (mostly containing mechanically pulped wood) was also used as a substrate for the tests of optimal inoculant amount. One hundred grams of newspaper was added into a mill mixer containing 1 L of deionized water. Then, the contents were mixed vigorously by mixing blades rotating at 20,000 rpm for 20 s, and the wet solid material was separated and air-dried. Before use, dried newspaper was defibrated with the same mixer for 20 s. The compositions of UHKP and newspaper (glucan, 82.0% and 58.5%; xylan, 17.0% and 7.8%; mannan, 0.2% and 4.8%; and lignin, 2.5% and 15.2%, respectively) were described in an earlier paper (Kamei *et al*. 2012a).

Methods

Fermentation of high-concentration UHKP

A basal liquid culture (10 g L^{-1} yeast extract, 10 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4$ $7H_2O$) (pH 6.0) (Okamoto *et al*. 2010) was used for the fermentation experiments. The UHKP substrates (moisture content 5.7%) (0.4, 1.0, 2.0, or 4.0 g of dry weight) were added to 19 mL of liquid medium and autoclaved. A mycelium mat on a PDA plate was transferred to a sterilized blender cup containing 50 mL of sterilized water and was homogenized for 10 s. One milliliter of homogenate was used to inoculate 100-mL Erlenmeyer flasks containing 19 mL of basal liquid medium with 2.0, 4.7, 9.1, or 16.5% (w/w) UHKP as a carbon source, meaning that the final moisture content was 98.0, 95.0, 90.5, or 82.8% (w/w), respectively. After being sealed with a silicon stopper (semi-aerobic conditions), the culture was incubated statically at 28 °C for 312 h. Samples were taken out at every 48 h after an initial 72 h of incubation, in accordance with the method described below (see analytical method section). Three independent experiments were performed.

Testing for the amount of mycelium inoculant needed for newspaper fermentation

Six-millimeter-diameter discs were punched from the edge of the mycelium incubated on PDA plates. Each disc was placed into a 100-mL Erlenmeyer flask containing 30 mL of potato dextrose broth (PDB) medium and then incubated at 28 °C in a shaking incubator (120 rpm, 5 days). After incubation, the mycelium was collected by filtration under sterile conditions and washed twice with 50 mL of sterile water; the collected mycelium was homogenized with 50 mL of basal liquid medium for 10 s. Then, 1, 2, 3, or 4 mL of the mycelial homogenate was inoculated into the autoclaved 100-mL Erlenmeyer flasks containing 16 mL of basal liquid medium and 10 g dry weight of newspaper (moisture content 6.0%), and the total liquid volume was adjusted up to 20 mL by adding distilled water. The final substrate concentration was 9.1%, meaning that the moisture content was 90.9%. After being sealed with a silicon stopper (semi-aerobic conditions), the culture was incubated statically at 28 °C for 360 h.

Analytical method

After the appropriate incubation period, 80 mL of sterile water was added to the flask to extract the ethanol from the substrate. The ethanol was extracted at 4 °C for 15 min on the rotary shaker (150 rpm), before the mycelium and insoluble waste carbon were removed by means of centrifugation at 13,000 ×g for 10 min. The resulting supernatant

was filtered through a PTFE membrane filter (0.45 μm; Millipore, Japan) and analyzed by high-performance liquid chromatography (HPLC) to quantify the amount of ethanol, monosaccharides, and disaccharides. The HPLC used a Shimadzu LC-10A system with a Shimadzu RID-10A differential refractive index detector fitted with a Shodex SUGAR KS-802 column (inner diameter 8.0 mm, length 300 mm) or Shodex SUGAR SP0180 (inner diameter 8.0 mm, length 300 mm) (Showa Denko Inc.; Tokyo, Japan). The supernatant was eluted with water at a flow rate of 1 mL min^{-1} at 80 °C. Student's *t*-test for ethanol production was done to determine any significant difference between the groups of incubation time. Differences between means at the 5% confidence level ($P < 0.05$) were considered to be statistically significant.

Ethanol yield (*Y*, %) was calculated based on the following equation:

$$Y\ (\%) = (\text{ethanol produced, g } L^{-1})/(\text{theoretical ethanol, g } L^{-1}) \times 100 \quad (1)$$

Theoretical ethanol (*E*, g L^{-1}) was calculated based on the following equation,

$$E\ (\text{g } L^{-1}) = \text{glucan in g } L^{-1} \times 1.11 \times 0.51 + \text{xylan in g } L^{-1} \times 1.14 \times 0.46 \quad (2)$$

where 1.11 is the coefficient of glucose obtained from glucan, 1.14 is the coefficient of xylose obtained from xylan, 0.51 is the coefficient of ethanol obtained from glucose, and 0.46 is the coefficient of ethanol obtained from xylose.

Enzyme assay

The crude enzyme was collected from the basal liquid medium by means of centrifugation at 13,000 ×g, for 20 min at 4 °C, and the supernatant was used for the enzyme assay. All assays were performed in triplicate. The filter paper activity (FPase) activity was determined by the release of reducing sugars produced in 60 min at 50 °C. The reaction mixture for FPase contained 125 μL of crude enzyme solution, 250 μL of 50 mM sodium citrate buffer (pH 4.5), and 20 mg of Watman No. 1 filter paper (1.5 × 1.0 cm), incubated at 50 °C. One filter paper unit (FPU) was defined as the amount of enzyme that released 1 μmol glucose min^{-1} (Montenecourt *et al.* 1978). The glucose equivalents (reducing sugars) generated during the assay were estimated using the 3,5-dinitrosalicylic acid method, with glucose as the standard.

RESULTS AND DISCUSSION

CBP Fermentation of High-concentration UHKP

To evaluate the effects of substrate concentration on the fermentation ability of *Phlebia* sp. MG-60, ethanol fermentations under different concentrations of UHKP, 2.0, 4.7, 9.1, or 16.5% (w/w), were studied. Figure 1 shows the time courses of the ethanol production from cultures with different concentrations of substrate. Both the ethanol concentration produced in each culture (Fig. 1a) and the ethanol yield, which was calculated based on the carbohydrate contents of UHKP (Fig. 1b), are shown. In all conditions, ethanol production reached a maximum value within 240 h of incubation. From the 2.0% UHKP culture, 8.6 g L^{-1} ethanol was produced, which was equivalent to a yield of 72.1% of the theoretical maximum. This result was almost the same as the previous results (Kamei *et al*. 2012a). From the 4.7% UHKP culture, 19.6 g L^{-1} ethanol was

produced, or 61.7% of the theoretical maximum. The highest ethanol concentration (25.9 g L^{-1} ethanol, or 46.7% of the theoretical maximum) was observed in the culture containing 9.1% UHKP. Up to this point, the ethanol concentration produced by *Phlebia* sp. MG-60 increased in a substrate concentration-dependent manner. However, the ethanol concentration produced from the culture containing 16.5% UHKP was only 14.4 g L^{-1}, just 12.9% of the theoretical maximum. Figure 2 shows photographs of the cultures containing 9.1% and 16.5% UHKP at the time of inoculation and after incubation for 168 h. The solubilization of the 9.1% UHKP culture was observed; however, there was no solubilization in the 16.5% UHKP culture, although growth of the fungal mycelium was observed (Fig. 2).

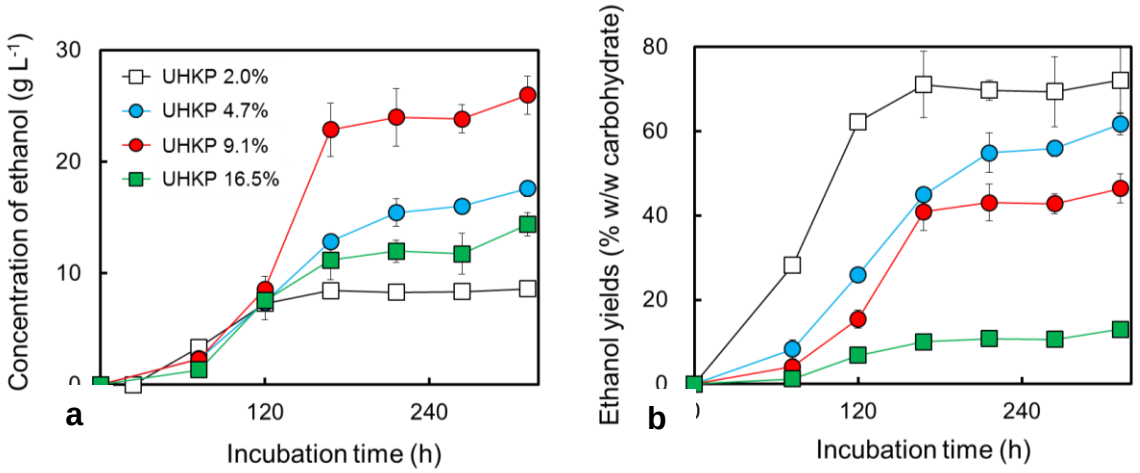

Fig. 1. Time courses of ethanol production from different concentrations of UHKP by wood-rotting fungus *Phlebia* sp. MG-60. (a) Ethanol concentration and (b) ethanol yields based on the carbohydrate are shown. The data represent the average of three independent experiments

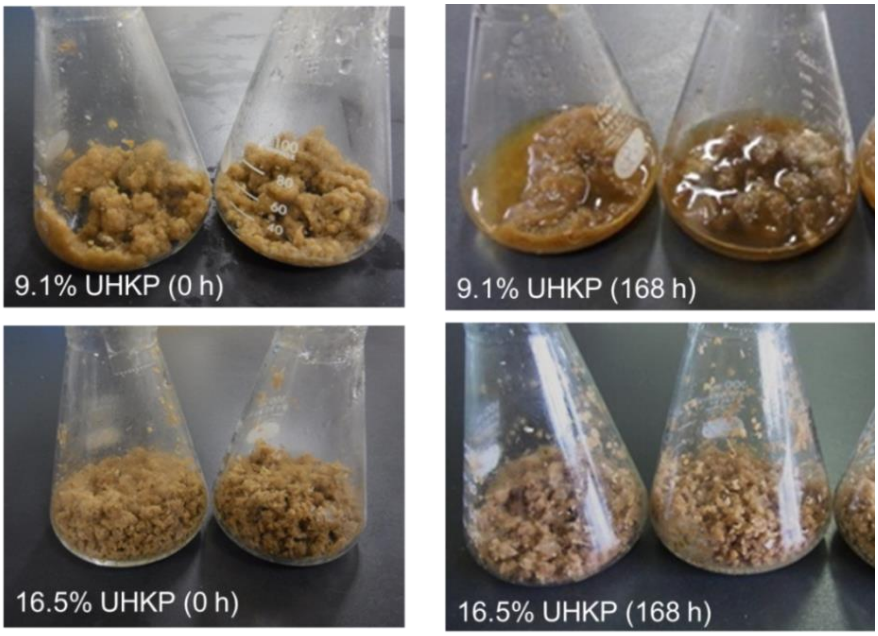

Fig. 2. Photographs of fermenting cultures containing 9.1% (upper) and 16.5% (lower) UHKP

The CBP fermentation is a promising way to reduce the cost of producing ethanol from cellulosic materials. Therefore, the addition of the cellulolytic traits into fermentable yeast has been of interest to scientists. The heterologous expression of cellulases and hemicellulases has been primarily studied in bacterial hosts such as engineered *Escherichia coli* (Ryu and Karim 2011), *Klebsiella oxytoca* (Zhou and Ingram 2001), and *Zymomonas mobilis* (Vasan *et al*. 2011) and in the yeasts *Saccharomyces cerevisiae* (reviewed in Hasunuma and Kondo 2012), *Hansenula polymorpha* (Voronovsky *et al*. 2009), and *Kluyveromyces marxianus* (Yanase *et al*. 2010a). The direct ethanol fermentation from phosphoric acid swollen cellulose (PASC) using cellulase-coexpressing yeast, which produces endoglucanases, cellobiohydrolases, and β-glucosidases, was reported. The ethanol yield was 2.1 g L^{-1} from 10 g L^{-1} of PASC (Yanase *et al*. 2010b). However, many of those reports are limited to the tests at low substrate concentrations or on amorphous cellulose. It was also reported that the display of cellulases on recombinant yeast cell surfaces produced an ethanol concentration of 42.2 g L^{-1} by means of the cell recycle batch fermentation of hydrothermally pretreated high-solid rice straw (200 g L^{-1}). The ethanol yield when using recombinant yeast was higher than that when using wild-type yeast (34.5 g L^{-1}) because of the activity of the cellulase produced by the recombinant yeast strains (Matano *et al*. 2013). However, this system requires the addition of cellulase, so CBP is less likely to be able to improve this process.

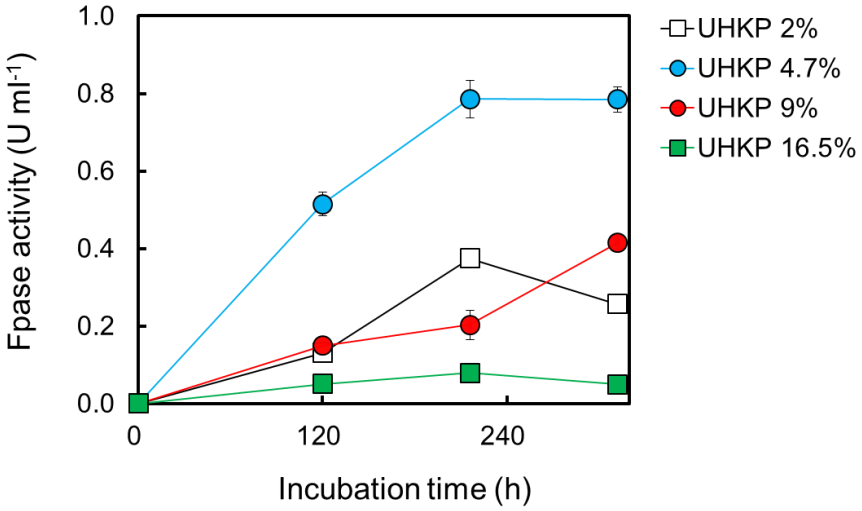

Fig. 3. Time course of FPase activity produced by *Phlebia* sp. MG-60 in cultures with different concentrations of UHKP. The data represent the average of three independent experiments. Values are means ±SD for samples.

In the case of white-rot fungi, *Flammulina velutipes* (Mizuno 2009a,b) and *Trametes hirsuta* (Okamoto *et al*. 2010) were reported as candidates for CBP fermentation. *F. velutipes* could produce ethanol from sorghum. However, the author indicated that the production of cellulases from *F. velutipes* was not sufficient (Mizuno 2009a). Recently, the same group reported ethanol production from cultures containing high concentrations of sugarcane bagasse cellulose (prepared by treatment with 1 N NaOH) by *F. velutipes.* The highest amount of ethanol (an ethanol yield of 69.6%) was produced when 15% (w/w) sugarcane bagasse cellulose was incubated with 9 mg/g biomass of commercial cellulase by *F. velutipes*. However, no ethanol production was observed from the culture without added cellulase (Maehara *et al.* 2013). In our present study, *Phlebia* sp. MG-60 was

observed to produce ethanol from cultures containing 2.0, 4.7, and 9.1% UHKP at ethanol yields of 72.1, 61.7, and 46.7%, respectively, without the addition of any enzymes. Although the highest ethanol production obtained was lower than that of *F. velutipes* with added cellulase, our process has a great advantage because added cellulase is not required, making it more feasible for the CBP fermentation of lignocellulose.

In the present study, the ethanol production from the culture containing 16.5% UHKP was much lower than that from the 4.7% and 9.1% UHKP cultures. Figure 3 shows the time courses of the cellulase (FPase) activity in the cultures containing different concentrations of UHKP with *Phlebia* sp. MG-60. The highest FPase activity was observed in the 4.7% UHKP culture, while the cultures containing 2.0 or 9.1% UHKP also showed progressive increases in FPase activity (Fig. 3). It was considered that synthesis of cellulases by fungi is induced by cellulose substrates and/or more specifically by their water soluble short chain depolymerization products such as cellobiose (Hulme and Stranks 1970). The weak production of FPase in the culture with 2.0% might be caused by the rapid depletion of cellulosic substrate, as estimated by the results for ethanol production. However, the production of FPase in the 16.5% culture was much lower (Fig. 3). This phenomenon might have been caused by the water content of the substrate. It is well known that the production of enzymes can be affected by water content and water activity (Krishna 2005). In our past study, both FPase and xylanase activities were barely detected at the time of termination of the aerobic-solid culture conditions, when the moisture content was 77%. However the high activities of both enzymes were detected 120 h after the culture was changed to the semi-aerobic liquid culture conditions (Kamei *et al*. 2012b). These results demonstrated that a clear switch of metabolism from selective lignin degradation to polysaccharide degradation occurred at the transition from aerobic-solid culture conditions to semi-aerobic-liquid culture conditions (Kamei *et al*. 2012b). This phenomenon might also have been affected by the water content. Although additional detailed study is needed to optimize the culture conditions for the production of cellulase and the fermentation by *Phlebia* sp. MG-60, it is evident that MG-60 can produce ethanol from cultures containing 9.1% UHKP and may be a good candidate for the CBP fermentation of cellulosic materials.

Effects of Relief of Pressure and Inoculation Amount of Mycelium

In the fermentation of cultures with high-solid loadings of pulp, the pressure in the flask derived from CO_2 production was not negligible. Therefore, the effects of pressure on fermentation were evaluated by temporarily removing the silicone stopper for 1 min. The effects on the ethanol production from cultures containing 9.1% UHKP by *Phlebia* sp. MG-60 are shown in Fig. 4. When the silicone stopper was removed once after 120 h of incubation, the production of ethanol reached approximately 25 g L^{-1} after 240 h incubation, and then remained constant (Fig. 4; green square). Conversely, temporary removal of the silicone stopper twice at 120 h and 240 h (Fig. 4; red circle) or once at 240 h (Fig. 4; blue circle) increased the production of ethanol from 9.1% UHKP up to 37.3 g L^{-1} or 31.4 g L^{-1}, respectively, after 360 h incubation. This remarkable improvement might have been caused by the release of pressure and by a small amount of gas exchange. A recent study revealed that, while this fungus could also produce ethanol from UHKP under aerobic conditions, the produced ethanol disappeared under the aerobic conditions (Kamei *et al*. 2012a). These results indicated that a suitably small amount of gas exchange would improve the production of saccharification enzymes and/or the activity of fermentation. In the case of *S. cerevisiae*, positive effects of aeration on ethanol fermentability and cell viability were reported (Ryu *et al*. 1984). Although further detailed study is needed to

determine the optimum amount of oxygen, a small amount of added air might improve the CBP fermentability of cellulose by *Phlebia* sp. MG-60, in a similar way to that seen with *S. cerevisiae*.

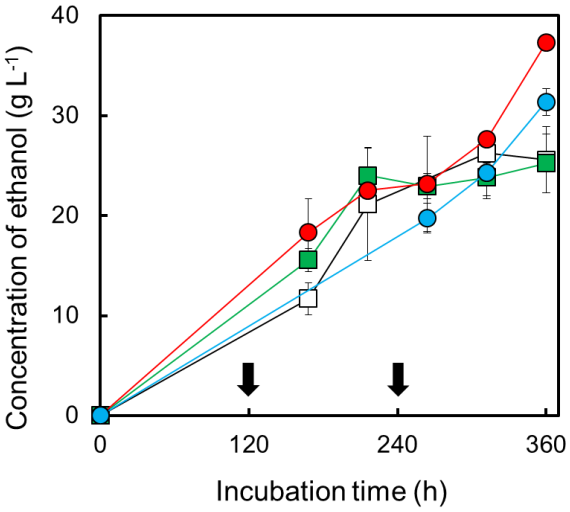

Fig. 4. The effects of the temporary removal of the silicone stopper on ethanol production from 9.1% UHKP by *Phlebia* sp. MG-60. The time points where the stopper was temporarily removed are shown by the arrows. White squares indicate control with silicone stopper. Green squares indicate the ethanol production when the stopper was removed once after 120 h of incubation. Blue circles indicate the ethanol production when the stopper was removed once after 240 h of incubation. Red circles indicate the ethanol production when the stopper was removed twice (after 120 h and 240 h of incubation). The data represent the average of three independent experiments. Values are means ±SD for samples.

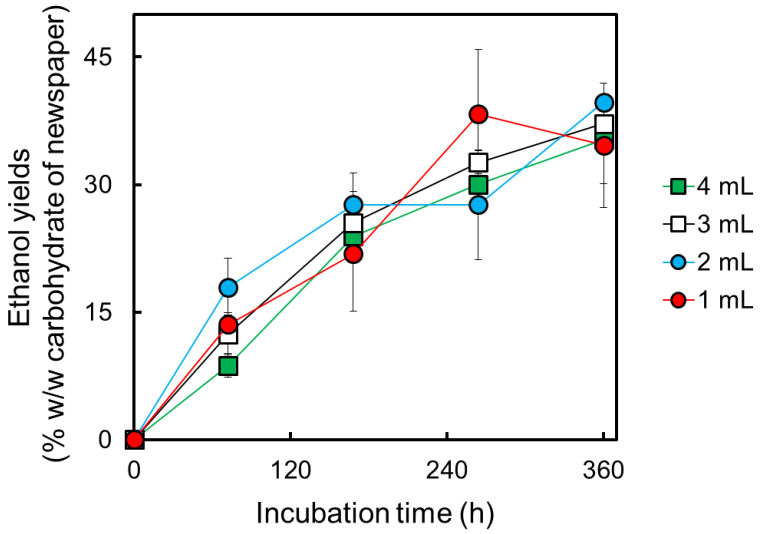

Fig. 5. The effect of the amount of inoculant on the production of ethanol from 9.1% newspaper by *Phlebia* sp. MG-60. The data represent the average of four independent experiments. Values are means ±SD for samples.

The effects of inoculation with different amounts of mycelium were examined. Figure 5 shows the time courses of ethanol production from cultures containing 9.1% newspaper. Although it was difficult to ensure uniformity of the amount of mycelial inoculant, the maximum ethanol yields after 360 h of incubation from cultures starting with

1, 2, 3, or 4 mL of inoculant homogenate reached 38.3, 39.7, 37.1, and 35.3%, respectively, of the theoretical maximum (there was no significant difference). The ethanol production from the cultures with 1 mL of mycelial homogenate from the PDA plate and from those with 3 mL of mycelial homogenate showed almost similar pattern of ethanol production (Fig. 5; red circle and unshaded square). The additional mycelium (2 mL; blue circle) gave rise to a higher production of ethanol than the other volumes of mycelial inoculation after 72 h incubation with significant difference ($P < 0.05$). However, higher volumes of mycelial inoculum (4 mL; green square) showed the lowest yield of ethanol during the initial 72 h of incubation with significant difference. This phenomenon might have been caused by poor dispersion of the mycelial homogenate. The mycelial homogenate in this study formed a high-density gel colloid, and the 4 mL of mycelial homogenate remained close together on the UHKP. Most likely, a good distribution of the fungal inoculant will be more important to the high-concentration substrate used in this experiment than will be the absolute quantity of the fungal inoculant used.

CONCLUSIONS

1. *Phlebia* sp. MG-60 is a good candidate for CBP fermentation of high-solid cellulose.
2. 9.1% UHKP was solubilized without the addition of commercial enzymes and efficiently fermented to yield ethanol by *Phlebia* sp. MG-60.
3. The fermentability of ethanol from UHKP was affected by moisture content and was improved by a small amount of aeration.
4. Lower moisture contents severely inhibited the production of cellulase and the ensuing saccharification and fermentation.

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