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# Bioconversion of ethanol from various sugars and cellulosic materials by brown rot fungus *Phaeolus schweinitzii*

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**ABSTRACT:** A novel brown rot fungus *Phaeolus schweinitzii* IUM 5048 was firstly used for ethanol production. It was found that this fungus produced ethanol with various sugars, such as glucose, mannose, galactose and cellobiose at 0.28, 0.22, 0.06, and 0.22 g of ethanol per g of sugar consumed, respectively. This fungus showed relatively good ethanol production from xylose at 0.23 g of ethanol per g of sugar consumed. However, the ethanol conversion rate of arabinose was relatively low (at 0.08 g of ethanol per g sugar). *P. schweinitzii* was capable of producing ethanol directly from rice straw and corn stalks at 0.11 g and 0.13 g of ethanol per g of substrates, respectively, when the fungus was cultured in a basal medium supplemented with 20 g/L rice straw or corn stalks. These results suggest that *P. schweinitzii* can hydrolyze cellulose or hemicellulose to fermentable sugars and convert them to ethanol simultaneously under oxygen limited condition.

**KEYWORDS:** Brown rot fungus, Cellulosic biomass, Ethanol, *Phaeolus schweinitzii*

## Introduction

The increasing demand and shortage of energy supply has led to a worldwide interest to find alternative energy sources. Among them, bioethanol is considered as one of sustainable and renewable biofuel, which can replace the fossil fuel (Ho *et al*., 2014). Bioethanol is a fuel source produced from lignocellulosic materials consisting of cellulose, hemicellulose, and lignin originated from crop residues and un-used forest resources (Lin *et al*., 2010). Production of bioethanol from cellulosic biomass requires physicochemical pretreatment such as diluted acid, alkali pretreatment, and steam to delignification and saccharification of cellulose and hemicellulose to liberate fermentable sugars (Nakamura *et al*., 2001; Park and Kim, 2012; Jung *et al*., 2013), however, these pretreatments may produce furfural, a strong inhibitor of ethanol fermentation (Brazdausks *et al*., 2014).

Although enzymatic pretreatment is preferable because inhibitors are not generated during saccharification of lignocellulosic biomass, the cost of treatment is higher than any other processes (Sun and Cheng, 2002).

Recently, simultaneous saccharification and fermentation (SSF) process has been used for ethanol fermentation from lignocellulosic materials. The SSF process enables the two-step fermentation process of saccharification and ethanol fermentation to shift a one-step ethanol fermentation process in a single vessel. SSF is considered to be good process because it reduces the cost of ethanol fermentation. However, the efficiency of ethanol fermentation from lignocellulosic raw materials is relatively low because proper and effective microorganisms have not been found (Ohgren *et al*., 2007).

*Saccharomyces cerevisiae* is one of the widely used yeast in brewing process. However, this microbe cannot ferment xylose, one abundant pentose component of hemicellulose (Chu and Lee, 2007). Therefore, it is necessary to develop microbes that can play multiple roles in delignification, saccharification, and ethanol fermentation from various sugars and lignocellulosic materials with high efficiency.

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Brown rot fungi belonging to basidiomycetes are capable of degrading cellulose, and hemicellulose. Several of them have potentials to produce ethanol directly from various sugars and lignocelluosic substances (Okamoto *et al*., 2011; Okamoto *et al*., 2012; Rasmussen *et al*., 2010). *P. schweinitzii*, a brown rot fungus, commonly known as velvet-top fungus or pine dye polypore is a plant pathogen that causes butt rot on spruce, pine, and larch and distributed worldwide (Park and Lee, 2011). In preliminary experiments, we screened brown rot fungi that produced ethanol from various sugars and lignocellulosic biomass. Among them, *P. schweinitzii* IUM 5048 strain exhibited relatively good ethanol production potential from monosaccharide, disaccharides, and lignocelluosic materials. In this study, we investigated the ability of *P. schweinitzii* IUM 5048 to produce ethanol from various sugars, including pentose, hexose, disaccharide, and lignocellulosic biomass such as rice straw and corn stalks.

## MATERIALS AND METHODS

### Microorganism

Mycelia of *P. schweinitzii* IUM 5048 was isolated from fruiting body of old pine tree trunk in Deokyusan National Park, Mujoo-gun District, Korea (Fig. 1). The fungal culture was incubated on potato dextrose agar (PDA) medium (Difco, Detroit, MI, USA) at 25°C and preserved at 4°C until use. The mycelial culture of *P. schweinitzii* IUM 5048 strain was deposited in "Culture Collection of Mushrooms" at Division of Life Sciences, Incheon National University.

### Verification of the fungal strain

*P. schweinitzii* IUM 5048 was identified by characteristics of fruiting body morphology and sequencing ITS-5.8S region of ribosomal DNA. Primers ITS 1 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-4 (5'-GGAA-GTAAAAGTCGTAACAAGG-3') were used to amplify ITS and 5.8S rDNA region (White *et al*., 1990). Amplified ITS and 5.5S sequences of rDNA were searched against NCBI database using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequences of *P. schweinitzii* IUM 5048 shared 95% similarities with other *P. schweinitzii* deposited at GenBank with accession number of FJ608591 and LN714583, respectively. Based on morphology of fruiting body and phylogenetic analysis of ITS region sequence of rDNA, *P. schweinitzii* IUM 5048 was verified as *P. schweinitzii*. The ITS region nucleotide sequence of *P. schweinitzii* IUM 5048 was deposited at GenBank with accession number of KU350753.

**Fig. 1**. Fruiting body of *Phaeolus schweinitzii* used in this study.

### Ethanol production from various sugars

Basal medium (pH 6.0) containing 20 g/L sugar source, 10 g/L yeast extract, 10 g/L $KH_2PO_4$, 2 g/L $(NH_4)_2SO_4$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ was prepared as described previously (Okamura *et al*., 2001). Glucose, mannose, galactose, xylose, arabinose, and cellobiose were used for ethanol production tests. Liquid media without sugar was first autoclaved at 121°C for 15 min. After autoclave, each sugar was sterilized by filtration using 0.45-μm membrane filter and added to the basal medium. Five discs of 0.5-$cm^2$ mycelia taken from PDA plates cultured at 25°C for 7 days were then transferred to an Erlenmeyer flask (125 mL) containing 50 mL of the basal medium. Each flask was cultured statically at 28°C under oxygen limited condition up to 240 h. The oxygen limited condition was prepared by sealing the flask tightly with parafilm.

### Ethanol production from lignocellulosic materials

Rice straw and corn stalks were obtained from agricultural field of Ganghwa Island, Incheon, Korea. They were dried at 45°C for 48 h and finely pulverized. One gram of powder from wheat straw or corn stalks was added to flasks containing 50 mL of basal medium. These flasks were autoclaved at 121°C for 60 min. Mycelial disc inoculation and ethanol fermentation process were then conducted as described above.

### Analytical procedures

The cellulose, hemicellulose, and lignin components of rice straw and corn stalks were analyzed using method of Sluiter *et al*. (2008). Total amount of reducing sugars

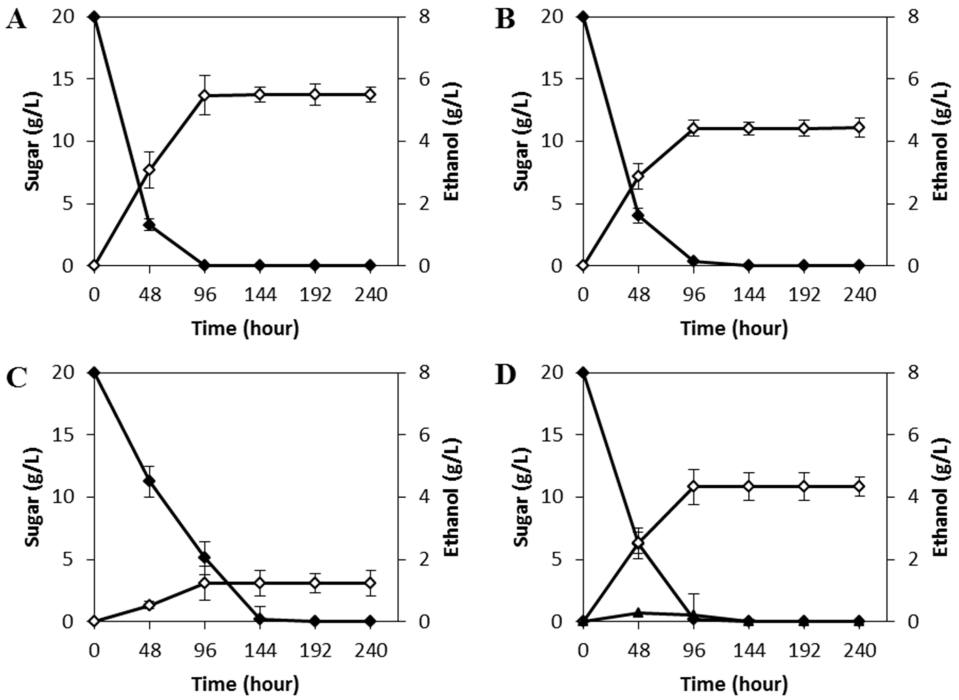


**Fig. 2.** Time course of ethanol production by *Phaeolus schweinitzii* IUM 5048 with hexose and disaccharide sugars. The mycelia were cultured in basal medium with 20 g/L glucose (A), mannose (B), galactose (C), and cellobiose (D) under oxygen limited condition. Symbols represent various sugars (filled diamond), ethanol (open diamond) and glucose (filled triangle). Values are means ± standard deviation (error bars) of three independent experiments.

released from rice straw and corn stalks after incubation with *P. schweinitzii* IUM 5048 were determined using dinitrosalicylic acid (DNS) method (Miller, 1959). Supernatants from ethanol fermenting media (1 mL) were collected at every 48 h interval, centrifuged at 15,000 × g for 10 min and filtered with a 0.22-μm membrane filter. Concentrations of ethanol and various monosaccharides in the ethanol fermenting liquid media were determined with high-performance liquid chromatography (HPLC, Agilent 1200 system; Agilent Technologies, USA) using a refractive index detector and a Shodex KS-801 column. HPLC was operated at 80°C with deionized distilled water as the mobile phase at a flow rate of 0.6 mL/min and an injection volume of 20 μL. Concentrations of ethanol and reducing sugars were calculated using calibration curve obtained from standard solution curve. The percentage of theoretical ethanol yield per g of sugar was determined by dividing the actual ethanol yield per 1 g of sugar consumed. The theoretical ethanol yield of glucose, mannose, galactose, xylose, and arabinose was 0.511 g of ethanol per g of sugar and that of cellobiose was 0.538 g ethanol per g of sugar consumed (Kudahettige *et al*., 2012).

## RESULTS AND DISCUSSION

**Ethanol production from hexose and disaccharide sugars**

To determine the ethanol production efficiency of *P. schweinitzii* IUM 5048 on hexose sugars, glucose, mannose, and galactose were tested as substrates. Hexose consumption and ethanol production by *P. schweinitzii* IUM 5048 during fermentation period are shown in Fig. 2. The highest ethanol concentration was observed at up to 192 h after incubation and all hexose sugars in the media were consumed completely. The best hexose sugar that was converted to ethanol was glucose with 5.5 g/L of ethanol from 20 g/L, corresponding to ethanol yield of 0.28 g per g of hexose sugar or theoretical ethanol yield of 53.8% (Fig. 2A). Generally, the theoretical converting efficiency of glucose through fermentation by filamentous fungi such as *Aspergillus foetidus*, *A. awamori*, *A. oryzae*, *A. sojae*, *Fusarium verticilloides*, and *Rhizopus javanicus* is from 2 to 99.4% (Skory, *et al*., 1997; de Almeida *et al*., 2013), suggesting that efficiency of ethanol production from *P. schweinitzii* IUM 5048 fungus was moderately efficient. For the fermentation from mannose, the maximum ethanol concentration was 4.45 g/L, which

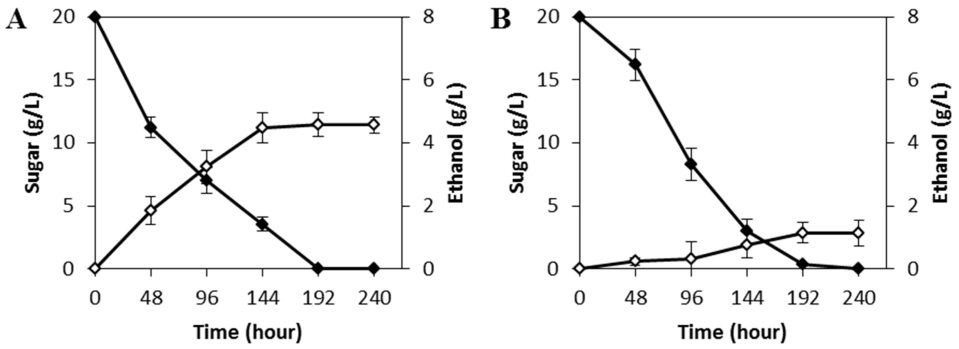


**Fig. 3.** Time course of ethanol production by *Phaeolus schweinitzii* IUM 5048 with pentose monosaccharide. The mycelia were cultured in basal medium with 20 g/L xylose (A), and arabinose (B) under oxygen limited condition. Symbols represent various sugars (filled diamond) and ethanol (open diamond). Values are means ± standard deviation (error bars) of three independent experiments.

corresponded to 0.22 g of ethanol per g of hexose or 43.5% of theoretical ethanol yield (Fig. 2B). Although *P. schweinitzii* IUM 5048 utilized galactose completely within 192 h of the fermentation period, the efficiency of fermentation was very low (0.06 g ethanol per g of sugar or 11.7% of theoretical ethanol yield) (Fig. 2C). Disaccharide cellobiose is composed of two molecules of β-glucose. It can be obtained from acidic or enzymatic hydrolysis of cellulose. To investigate the fermentation ability of *P. schweinitzii* IUM 5048 on cellobiose, the fungus was incubated in the media supplemented with 20 g/L of cellobiose. Cellobiose concentration in the medium was decreased gradually while ethanol concentration was increased progressively after 48 h of incubation period. The maximum ethanol concentration was observed at 192 h after incubation at 0.22 g of ethanol per g of cellobiose, corresponding to 40.9% of theoretical ethanol yield (Fig. 2D). There was only negligible amount of glucose detected during fermentation period as decomposed glucose from cellobiose converted to ethanol by the fungus immediately. These results indicated that ethanol production from cellobiose by the fungus occurred by hydrolysis of cellobiose to glucose and simultaneous conversion of glucose to ethanol. Taken together, these results suggested this fungus has ethanol fermentation ability using a broad range of sugars such as hexose sugars, and disaccharide.

**Ethanol production from pentose sugars**

Pentose sugars including xylose and arabinose are abundant components of hemicellulose derived from grasses and woody biomass (Puls and Schuseil, 1993). Some brown rot, white rot fungi, and yeasts such as *Gloeophyllum trabeum*, *Flammulina velutipes*, *Candida shehatae*, and *Pichia stipitis* are capable of converting xylose to ethanol efficiently (Rasmussen *et al*., 2010; Mizuno *et al*., 2009; Sanchez *et al*., 2002). In this study, mycelia of *P. schweinitzii* IUM 5048 were cultured in 20 g/L of xylose. This fungus consumed xylose completely within 192 h of incubation period, with the highest ethanol content at 4.57 g/L (0.23 g of ethanol per g of xylose consumed or theoretical ethanol yield of 44.7%, Fig. 3A). *P. schweinitzii* IUM 5048 tended to ferment xylose efficiently under oxygen limited condition. This result was better than those of ethanol production from xylose by white rot fungi including *Hohenbuehelia* sp. ZW-16 (14.7% theoretical ethanol yield), *Peniophora cinerea* (17.6%), or *Trametes suaveolens* (11.4%) (Liang *et al*., 2013; Okamoto *et al*., 2010), indicating that *P. schweinitzii* IUM 5048 may have good ethanol fermenting potential from one component of hemicellulose. On the other hand, arabinose was consumed completely by *P. schweinitzii* IUM 5048 within 192 h of incubation period, the maximum concentration of ethanol produced from 20 g/L arabinose was 1.57 g/L, which was a very poor ethanol yield (0.08 g of ethanol per g of arabinose with theoretical ethanol yield of 15.4%, Fig. 3B). In contrast, when white rot fungus *Phlebia* sp. MG-60 was incubated in arabinose containing liquid medium up to 120 h, only a small amount of arabinose was consumed and ethanol production was not observed (Kamei *et al*., 2012). The result suggested that this white rot fungus almost could not assimilate and convert arabinose to ethanol effectively.

**Ethanol production from lignocellulosic materials**

To investigate the fermentation potential of *P. schweinitzii* IUM 5048 on lignocelluosic materials, the

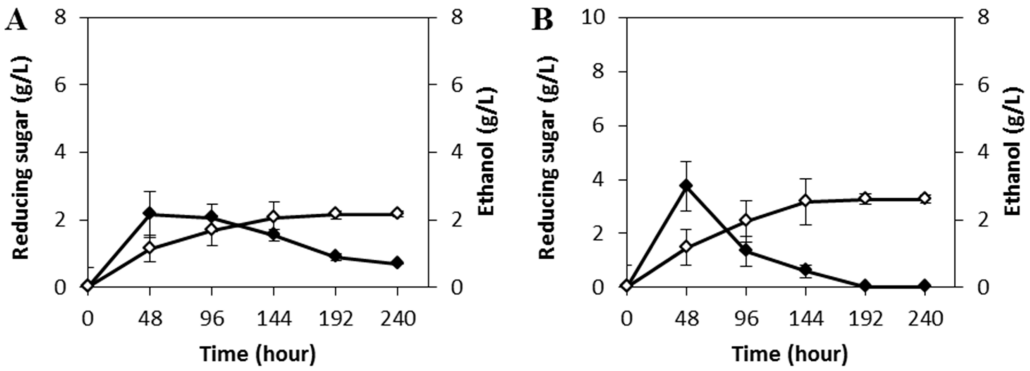


**Fig. 4.** Time course of ethanol production by *Phaeolus schweinitzii* IUM 5048 with lignocelluosic biomass. The mycelia were cultured in basal medium with 20 g/L rice straw (A), and corn stalks (B) under oxygen limited condition. Symbols represent reducing sugars (filled diamond) and ethanol (open diamond). Values are means ± standard deviation (error bars) of three independent experiments.

mycelia was inoculated in the medium supplemented with powder of wheat straw or corn stalks. The compositions of rice straw and corn stalks used in this experiment were analyzed. Rice straw were composed of 32.82% of cellulose, 26.13% of hemicellulose, 16.45% of lignin, 14.21% of ash, and 10.39% of other substances. Corn stalks were composed of 33.91% of cellulose, 29.65% of hemicellulose, 19.83% of lignin, 7.25% of ash, and 11.36% of other substances based on dry weight. When *P. schweinitzii* IUM 5048 was cultured in 20 g/L of rice straw, reducing sugars and ethanol were first detected at 48 h after incubation (2.15 g/L of reducing sugars and 1.15 g/L of ethanol). The highest ethanol concentration of 2.17 g/L was observed up to 144 h of fermentation period (theoretical ethanol yield of 34.0%, Fig. 4A). The concentration of reducing sugar in the media was decreased steadily as the concentration of ethanol in the medium was increased gradually during the fermentation period, indicating that the reducing sugars liberated from the rice straw were converted to ethanol by the fungus simultaneously. With similar experiment, white rot fungus *Trametes hirsuta* were used for direct ethanol production from rice straw. The ethanol yield was 0.15 g ethanol per g rice straw, which was lower that of rice straw in this study (Okamoto *et al*., 2011).

When *P. schweinitzii* IUM 5048 was cultured with 20 g/L of corn stalks, reducing sugars (1.95 g/L) and ethanol (2.12 g/L) released into the medium were also first detected after 48 h of incubation. The maximum ethanol concentration of 2.64 g/L was observed at 192 h after incubation (Fig. 4B). Even though the concentration of reducing sugar in corn stalks medium was decreased when the time of ethanol fermentation was proceeded, ethanol concentration was increased gradually up to 192 h of incubation as the liberated reducing sugars from corn stalks were consumed by the fungus rapidly for ethanol fermentation. This fungus showed higher ethanol conversion rate from corn stalks (2.64 g/L) than that from rice straw (2.17 g/L). The higher ethanol concentration from corn stalks might be due to total percentage of cellulose and hemicellulose contents in the corn stalks (63.56%) was higher than that in the rice straw (58.95%). When brown rot fungus *Neolentinus lepideus* was incubated in a medium containing 20 g/L of lignocellulosic materials such as birch wood xylan and wheat bran, ethanol concentrations were 1.7 g/L and 2.8 g/L, respectively (Okamoto *et al*., 2012), which were similar to those of ethanol concentrations from corn stalks or rice straw observed in this study. Therefore, *P. schweinitzii* IUM 5048 appears to be a good candidate for ethanol production directly from lignocellulosic biomass.

In conclusion, brown rot fungus *P. schweinitzii* IUM 5048 was used to produce ethanol from various sugars and cellulosic materials for the first time. It was found that *P. schweinitzii* IUM 5048 could assimilate various carbon sources and produce ethanol effectively from glucose, mannose, xylose, and cellobiose, the main components of cellulose and hemicellulose. *P. schweinitzii* IUM 5048 also convert rice straw and corn stalks directly to ethanol, indicating that brown rot fungal strain *P. schweinitzii* IUM 5048 has a potential for producing ethanol directly from natural lignocellulosic biomass through SSF.

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