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Direct bioethanol production from lignocellulosic biomass using white rot fungus *Cerrena unicolor*

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ABSTRACT: White rot fungus *Cerrena unicolor* IUM 5400 produced ethanol from diverse sugars, including glucose, mannose, galactose, and cellobiose at 0.38, 0.28, 0.08, and 0.27 g of ethanol per g of sugar consumed, respectively. The fungus produced relatively high amounts of ethanol from xylose (0.28 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 consumed). When cultured in a basal medium containing 20 g/L rice straw or corn stalks, *C. unicolor* IUM 5400 produced 0.18 g and 0.18 g of ethanol per g of rice straw and corn stalks, respectively. The results suggest that *C. unicolor* IUM 5400 is a white rot fungus that can effectively hydrolyze cellulose or hemicellulose to sugars and simultaneously convert them to ethanol.

KEYWORDS: Bioethanol, *Cerrena unicolor*, Lignocellulosic biomass, White rot fungus

INTRODUCTION

The second generation bioethanol is a fuel source produced from lignocellulosic biomass consisting of cellulose, hemicellulose, and lignin originated from naturally occurring crop residues and un-used forest resources (Busic *et al*., 2018; Guerriero *et al*., 2016). Production of bioethanol from lignocellulosic biomass requires physicochemical pretreatment such as diluted acid, alkali pretreatment, and steam to delignification and saccharification of cellulose and hemicellulose to liberate fermentable sugars (Broda *et al*., 2022). However, these pretreatments may generate furfural, a strong inhibitor of ethanol fermentation (Brazdausks *et al*., 2014). Athough enzymatic pretreatment is preferable because fermentation inhibitors are not produced during saccharification of lignocellulosic biomass, the treatment cost is higher than any other processes (Baruah *et al*., 2018).

Recently, simultaneous saccharification and fermentation (SSF) process has been used for ethanol fermentation from lignocellulosic biomass. The SSF process enables the two-step fermentation process of saccharification and ethanol fermentation to become a one-step ethanol fermentation process. SSF is considered to be a good process because it lessens the cost of ethanol fermentation process. However, the efficiency of ethanol fermentation from lignocellulosic biomass is relatively low because proper and effective microbes have not been found (Huang and Chen, 1988).

Saccharomyces cerevisiae is one of the broadly used fungi in brewing and baker industry. However, this fungus is not able to ferment xylose, one of plentiful pentose sugar in wood and grasses (Chu *et al*., 2007). Therefore, it is essential to establish microbes that can play various roles in delignification, saccharification, and fermentation from diverse pentose and hexose sugars, and lignocellulosic biomass with good efficiency (Sun and Cheng, 2002).

White rot fungi belonging to basidiomycetes can degrade lignin, cellulose, and hemicellulose (Blanchette, 1995). Several of them have potentiality to ferment ethanol directly from diverse sugars and lignocelluosic

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materials (Okamura *et al*., 2001; Kudahettige *et al*., 2012; Horisawa and Nishida, 2014; Horisawa *et al*., 2015). In preliminary experiments, we screened white rot fungi that produced ethanol from diverse sugars and lignocellulosic biomass. Among them, *C. unicolo*r IUM 5400 strain showed fairly good ethanol producing capability from pentose, hexose, disaccharide sugars, and lignocelluosic biomass. *C. unicolor* is a white rot fungus widely distributed worldwide (Park and Lee, 2011). In this study, we investigated the potentiality of *C. unicolor* IUM 5400 to produce ethanol from diverse sugars, including pentose, hexose, disaccharide, and lignocellulosic biomass such as rice straw and corn stalks.

MATERIALS AND METHODS

Microorganism

Mycelia of *C. unicolor* strain IUM 5400 was isolated from fruiting body of old oak tree trunk (Fig. 1) from Incheon City Park, Incheon, Korea. 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 *C. unicolor* strain IUM 5400 was deposited in "Culture Collection of Mushrooms" at the Division of Life Sciences, Incheon National University.

Verification of the fungal strain

C. unicolor IUM 5400 was identified by morphological characteristics of fruiting body and sequencing ITS-5.8S region of ribosomal DNA. Primers ITS 1 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were used to amply ITS and 5.8S rDNA region (White *et al*., 1990).

Fig. 1. *Cerrena unicolor* used in this study.

Amplified ITS and 5.8S sequences of rDNA were searched on NCBI database using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequences of *C. unicolor* IUM 5400 shared 99% similarities with other *C. unicolor* deposited at GenBank with accession number of JQ031127, JX235709 and JN710525, respectively. Based on fruiting body morphology and analysis of ITS region sequence of rDNA, *C. unicolor* IUM 5400 was verified as *C. unicolor*. The ITS region nucleotide sequence of *C. unicolor* IUM 5400 was deposited at GenBank with accession number of KU350754.

Production of ethanol from diverse 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 previously (Okamura *et al*., 2001). Glucose, mannose, galactose, xylose, arabinose, and cellobiose were used for ethanol fermentation test. 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 mycelial discs of 0.5-cm^2 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 incubated 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 (Yoon and Lee, 2022).

Production of ethanol from lignocellulosic biomass

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. Powder (1 g) of 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 discs inoculation and ethanol fermentation process were then conducted as described above.

Analytical methods

The cellulose, hemicellulose, and lignin components of rice straw and corn stalks were analyzed using method of Sluiter (Sluiter *et al*., 2008). Total amount of reducing sugars released from rice straw and corn stalks after incubation with *C. unicolor* IUM 5400 were determined using dinitrosalicylic acid (DNS) method (Miller, 1959). Supernatants from ethanol fermenting media (1 mL) were

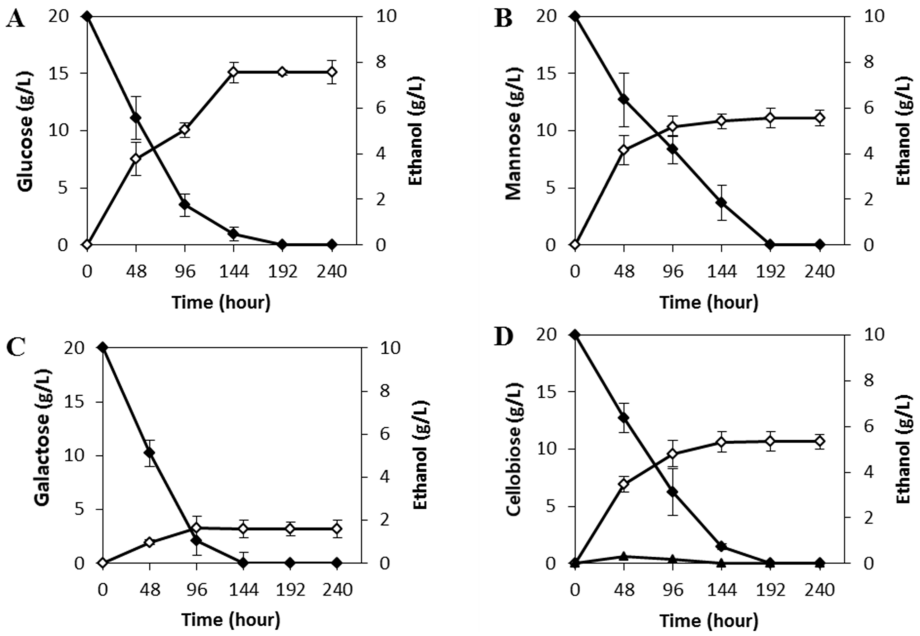

Fig. 2. Time course of ethanol production by *Cerrena unicolor* IUM 5400 with various sugars. The mycelia were cultured in basal medium with 20 g/L glucose (A), mannose (B), galactose (C), and cellobiose (D) under oxygen limited condition. Symbols represent various sugars (filled diamond), ethanol (open diamond) and glucose (filled triangle). Values are expressed as means ± standard deviation (error bars) of three independent experiments.

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 diverse monosaccharides in the ethanol fermenting liquid media were analyzed 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 (Im *et al*., 2016). 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

Production of ethanol from hexose and disaccharide sugars

To determine the ethanol production efficiency of *C. unicolor* IUM 5400 on hexose sugars, glucose, mannose, and galactose were tested as substrates. Hexose consumption and ethanol production by *C. unicolor* IUM 5400 during fermentation period are shown in Fig. 2. All hexose sugars in the media were consumed completely within 192 h of incubation period. The highest ethanol concentration was observed at up to 192 h after incubation. The best hexose sugar that was converted to ethanol was glucose with 7.55 g/L of ethanol from 20 g/L, corresponding to ethanol yield of 0.38 g per g of hexose sugar or theoretical ethanol yield of 74.4% (Fig. 2A). Generally, the theoretical converting efficiency of glucose through fermentation by filamentous fungi such as *Aspergillus awamori A. foetidus*, *A. oryzae*, *A. sojae*, and *Rhizopus javanicus*, and *R. oryzae* is from 27.4 to 99.4% (Skory *et al*., 1997), suggesting that the efficiency of ethanol production by *C. unicolor* IUM 5400 mushroom was moderately good. For the fermentation of mannose, the maximum ethanol concentration was 5.56 g/L, which corresponded to 0.28 g of ethanol per g of hexose or 54.4% of theoretical ethanol yield (Fig. 2B). For the fermentation of galactose, although *C. unicolor* IUM 5400 consumed galactose completely within 192 h of the fermentation period, the fermentation efficiency of galactose was very poor (0.08 g ethanol per g of sugar or

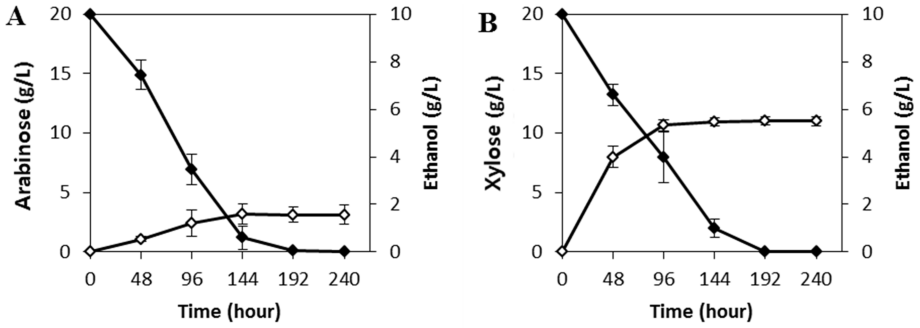

Fig 3. Time course of ethanol production by *Cerrena unicolor* IUM 5400 with pentose sugars. The mycelia were cultured in basal medium with 20 g/L arabinose (A), and xylose (B) under oxygen limited condition. Symbols represent various sugars (filled diamond) and ethanol (open diamond). Values are means ± standard deviation (error bars) of three independent experiments.

15.7% of theoretical ethanol yield). Disaccharide cellobiose consists of two molecules of glucose. It is derived from acidic or enzymatic hydrolysis of cellulose. To investigate the fermenting ability of *C. unicolor* IUM 5400 on cellobiose, the fungus was cultured in media supplemented with 20 g/L of cellobiose. Cellobiose concentration in the medium was decreased gradually while ethanol concentration was increased steadily after 48 h of incubation. The maximum ethanol concentration was observed at 192 h after incubation period with 0.27 g of ethanol per g of cellobiose, which corresponds to 50.2% of theoretical ethanol yield (Fig. 2D). There was only a small amount of glucose detected during fermentation period as degraded glucose from cellobiose was assimilated and converted to ethanol by the fungus quickly. These results suggested that ethanol production from cellobiose by the fungus was occurred by hydrolysis of cellobiose to glucose and conversion of glucose to ethanol simultaneously. Taken together, these results suggest the fungus has good potentials to produce ethanol from wide ranges of hexose sugars and disaccharide.

Production of ethanol from pentose sugars

Pentose sugars including xylose and arabinose are abundant major constituents of hemicellulose originated from biomass of grasses and wood (Plus and Schuseil, 1993). Some brown rot fungi and white rot fungi such as *Gloeophyllum trabeum*, *Phaeolus schweinitzii*, *Neolentinus lepideus*, *Lenzites betulinus*, *Flammulina velutipes* are capable of converting xylose to ethanol efficiently (Rasmussen *et al*., 2010; Yoon and Lee, 2022; Okamoto *et al*., 2012; Im *et al*., 2016; Mizuno *et al*., 2009). In this study, when mycelia of C. *unicolor* IUM 5400 was cultured in 20 g/L of xylose, the fungus consumed xylose completely within 192 h of incubation period with the highest ethanol content at 5.5 g/L (0.28 g of ethanol per g of xylose consumed or theoretical ethanol yield of 53.8%, Fig. 3B). *C. unicolor* IUM 5400 converted xylose to ethanol efficiently under oxygen limited condition. The result was better than that of ethanol produced from xylose by *Hohenbuehelia* sp. ZW-16 (Liang *et al*., 2013). The theoretical ethanol yield of *C. unicolor* IUM 5400 is also higher than those of experimental results (Okamoto *et al*., 2010) from *Peniophora cinerea* (17.6%) or *Trametes suaveolens* (11.4%). The result indicated that *C. unicolor* IUM 5400 may have good ethanol fermenting potential from xylose, which is one of major components of hemicellulose. On the other hand, although arabinose was consumed completely by *C. unicolor* IUM 5400 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 or theoretical ethanol yield of 15.4%, Fig. 3A). In contrast, when wood rot fungus *Phlebia* sp. MG-60 was incubated in 20g/L of arabinose containing liquid medium up to 120 h of incubation period, only a small amount of arabinose was consumed and ethanol production was negligible suggesting that the white rot fungus could not have potential to convert arabinose to ethanol efficiently (Kamei *et al*., 2012).

Production of ethanol from lignocellulosic biomass

To investigate the fermentation capability of *C. unicolor* IUM 5400 on lignocelluosic biomass, the mycelia was inoculated in the fermentation medium supplemented with powder of rice straw or corn stalks. The compositions of rice straw and corn stalks used in this study were analyzed. Rice straw were composed of 32.82 % of

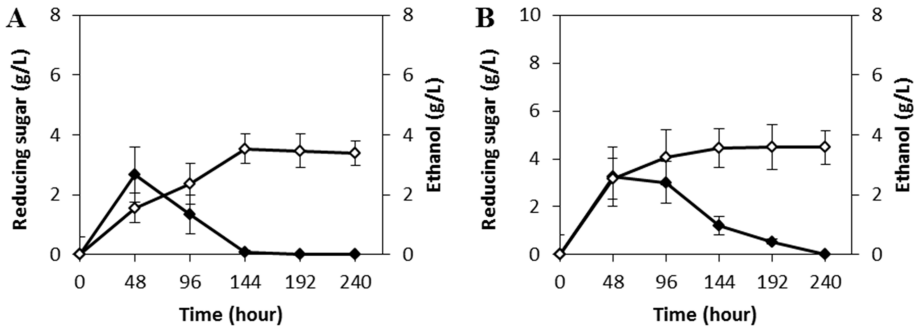

Fig 4. Time course of ethanol production by *Cerrena unicolor* IUM 5400 with lignocelluosic biomass. The mycelia were cultured in basal medium with 20 g/L rice straw (A), and corn stalks (B) under oxygen limited condition. Symbols represent reducing sugars (filled diamond) and ethanol (open diamond). Values are means ± standard deviation (error bars) of three independent experiments.

cellulose, 26.13% of hemicellulose, 16.45% of lignin, 14.21% of ash, and 10.39% of other substances. Corn stalks were consisted of 33.91 % of cellulose, 29.65% of hemicellulose, 19.83% of lignin, 7.25% of ash, and 11.36% of other substances on the basis of dry weight. When *C. unicolor* IUM 5400 was cultured in 20 g/L of rice straw, reducing sugars and ethanol were first detected at 48 h after incubation period (2.67 g/L of reducing sugars and 1.56 g/L of ethanol). The highest ethanol concentration (3.53 g/L) was observed up to 144 h of incubation period (theoretical ethanol yield of 32.8%, Fig. 4A). The concentration of reducing sugar in the media was decreased gradually as the concentration of ethanol in the medium was increased progressively during the incubation period, suggesting that the reducing sugars derived from hydrolysis of the rice straw were converted to ethanol by the fungus simultaneously. With similar attempt, white rot fungus *Irpex consors* was used for direct ethanol production from rice straw. The ethanol yield was 0.12 g ethanol per g rice straw, which was lower than that of rice straw in this experiment (Choi *et al*., 2015).

When mycelia of *C. unicolor* IUM 5400 was cultured with 20 g/L of corn stalks, reducing sugars (3.25 g/L) and ethanol (2.53 g/L) released into the medium were first detected after 48 h of incubation period. The maximum ethanol concentration of 3.59 g/L was observed at 192 h after incubation period (Fig. 4B). Although the concentration of reducing sugar in corn stalks medium was decreased when the time of ethanol fermentation was increased, ethanol concentration was increased gradually up to 192 h of incubation period as the liberated reducing sugars from corn stalks were converted to ethanol by the fungus quickly. Therefore, the fungus showed little higher ethanol conversion rate from corn stalks (3.59 g/L) than that from rice straw (3.53 g/L). The higher ethanol concentration from corn stalks might be due to that the total percentage of cellulose and hemicellulose contents in the corn stalks (63.56%) was higher than that from rice straw (58.95%). When brown rot fungus *Phaeolus schweinizii* was cultured in a medium containing 20 g/L of lignocellulosic biomass such as rice straw and corn stalks, the maximum ethanol concentrations of 2.12 g/L and 2.64 g/L were observed after 192 h of incubation period, respectively (Yoon and Lee, 2022), which were lower than the ethanol yields from rice straw (3.53 g/L) and corn stalks (3.59 g/L) observed in this study.

Therefore, *C. unicolor* IUM 5400 appears to be a good candidate for producing ethanol directly from lignocellulosic biomass.

In conclusion, white rot fungus *C. unicolor* IUM 5400 can assimilate diverse carbon sources and produce ethanol effectively from glucose, mannose, xylose and cellobiose, which are major components of cellulose and hemicellulose originated from grasses and woods. The fungus also converted directly wheat straws and corn stalks to ethanol, suggesting that *C. unicolor* IUM 5400 is a good candidate for producing ethanol from lignocellulosic biomasses through SSF process. Further studies are necessary to improve the fermentation conditions to increase the efficiency of ethanol production.

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