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Research Article

Ethanol Production from Various Sugars and Cellulosic Biomass by White Rot Fungus *Lenzites betulinus*

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Abstract *Lenzites betulinus*, known as gilled polypore belongs to Basidiomycota was isolated from fruiting body on broadleaf dead trees. It was found that the mycelia of white rot fungus *Lenzites betulinus* IUM 5468 produced ethanol from various sugars, including glucose, mannose, galactose, and cellobiose with a yield of 0.38, 0.26, 0.07, and 0.26 g of ethanol per gram of sugar consumed, respectively. This fungus relatively exhibited a good ethanol production from xylose at 0.26 g of ethanol per gram of sugar consumed. However, the ethanol conversion rate of arabinose was relatively low (at 0.07 g of ethanol per gram sugar). *L. betulinus* was capable of producing ethanol directly from rice straw and corn stalks at 0.22 g and 0.16 g of ethanol per gram of substrates, respectively, when this fungus was cultured in a basal medium containing 20 g/L rice straw or corn stalks. These results indicate that *L. betulinus* can produce ethanol efficiently from glucose, mannose, and cellobiose and produce ethanol very poorly from galactose and arabinose. Therefore, it is suggested that this fungus can ferment ethanol from various sugars and hydrolyze cellulosic materials to sugars and convert them to ethanol simultaneously.

Keywords Cellulosic biomass, Ethanol, *Lenzites betulinus*, White rot fungus

The first generation bioethanol has been produced mainly from crops such as sugarcane, sugar beet and corn starch, which were used for human food and cattle feed. One of the main debate on the bioethanol was that increasing production of first generation bioethanol was related to raise the food prices in the world and it threaten to the survival of poverty-stricken peoples in the third world countries. Therefore, in recent years, utilization of lignocellulosic biomass as sustainable alternative substrates for bioethanol production has attracted more attentions [1]. To produce ethanol from lignocellulosic materials, physiochemical pretreatment such as acid, alkali, and enzymatic process and fermentation by *Saccharomyces cerevisiae* are necessary.

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However, sugars released from degradation of lignocellulosic biomass by various pretreatments are composed of not only hexose sugars including glucose, mannose, and galactose, but also of pentose sugars such as xylose and arabinose [2]. Although *S. cerevisiae* has been used for ethanol fermentation for thousand years, *S. cerevisiae* could not assimilate and convert pentose sugars to ethanol. The pentose sugars are main components of hemicellulose, which is originated from lignocellulosic biomass [3]. Thus, to improve the microbial bioethanol fermentation from various hexose, pentose, and lignocellulosic materials, screening for suitable microbial strains with efficient ethanol production are necessary. Among the fungal species found in the nature, several species of yeast strains including *Pichia stipitis*, *Candida shehatae*, *Candida tropicalis* [4-6], and filamentous fungi such as *Fusarium oxysporum*, *Paecilomyces lilacinus*, and *Neurospora crassa* exhibited the ability to convert xylose to ethanol [7-9]. However, these yeasts cannot ferment lignocellulosic materials directly to ethanol, and the ethanol converting efficiency from lignocellulosic biomass by the fungi were slow and inefficient. Therefore, it is necessary to find suitable microbial strains for producing bioethanol directly from various sugars and lignocellulosic biomass.

White rot fungi belonging to basidiomycetes are able to degrade lignin, cellulose, and hemicellulose and some of them have potentials to produce ethanol directly from various sugars and lignocelluosic materials [10-12]. In preliminary experiments, we screened wood rotting fungi

which produced ethanol directly from various hexose, pentose sugars and lignocellulosic biomass. Among them, *L. betulinus* IUM 5468 strain showed bioethanol production potential from monosaccharide, disaccharides, and lignocellulosic biomass. *L. betulinus*, known as gilled polypore, is a white rot fungus found in live or dead wood of broadleaf trees, and distributed worldwide [13]. The purpose of this study was to investigate the ability of *L. betulinus* IUM 5468 to produce ethanol from various sugars including pentose, hexose, disaccharide, and lignocellulosic biomass including rice straw and corn stalks.

MATERIALS AND METHODS

Microorganism. Mycelia of *L. betulinus* IUM 5468 was isolated from fruiting body of fallen broad leaf tree in July 2014 from Hwaseong City, Gyeonggi Province, Korea (coordinates 37°12′32.34″ N~126°59′27.32″ E). The fungal isolate was incubated on potato dextrose agar (PDA) medium (Difco, Detroit, MI, USA) at 25°C and preserved at 4°C. The culture of *L. betulinus* IUM 5468 was deposited in Culture Collection and DNA Bank at the Division of Life Sciences, Incheon National University.

Verification of the fungal strain. *L. betulinus* IUM 5468 was identified by characteristics of fruiting body and sequencing region of ITS-5.8S of ribosomal DNA. Nuclear rDNA was extracted from the mycelia cultured on PDA for 7 days using an Accuprep GMO DNA Extraction Kit (Bioneer Co., Daejeon, Korea) according to the manufacturer's recommendation. To amplify the internal transcribed spacer (ITS) region of ribosomal DNA, primers ITS1 (5′-TCCG-TAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCT-TATTGATATGC-3′) were used. Amplification was performed by the method described by White *et al.* [14]. The mixtures contained 0.5 pmol of each primer, 0.25 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, and 15 ng of template DNA. Conditions for polymerase chain reaction (PCR) were as follows: an initial denaturation step at 95°C for 1 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension cycle at 72°C for 5 min. PCR product were purified and sequenced by Solgent Co. (Daejeon, Korea). The fungal isolate was identified based on sequence homology with fungal sequences retrieved from GenBank database using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

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 [15]. 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 hr. The oxygen limited condition was prepared by sealing the flask tightly with parafilm.

Ethanol production from lignocellulosic biomass. Rice straw and corn stalks were obtained from agricultural area of Ganghwa-gun District, Incheon, Korea in November 2014. They were dried at 45°C for 48 hr 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 procedures. The cellulose, hemicellulose, and lignin components of rice straw and corn stalks were analyzed using method of Sluiter *et al.* [16]. The amount of reducing sugars released from rice straw and corn stalks after incubation with *L. betulinus* IUM 5468 were determined using dinitrosalicylic acid method [17]. Supernatants from ethanol fermenting media (1 mL) were collected at every 48-hr 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, Santa Clara, CA, 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 gram of sugar consumed 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 gram of sugar and that of cellobiose was 0.538 g ethanol per gram of sugar [10].

RESULTS AND DISCUSSION

Verification of the fungal strain. Phylogenetic tree (Fig. 1) showed that the strain of *L. betulinus* IUM 5468 was clustered together with the other same species from GenBank database. The sequences of *L. betulinus* IUM 5468 shared 99%~100% similarities with other *L. betulinus* deposited at GenBank with accession number of KJ668506, KP004982, and KF573021, respectively. Based on morphology of fruiting body and phylogenetic analysis of ITS region sequence of rDNA, *L. betulinus* IUM 5468 was verified as *L. betulinus*. The ITS region nucleotide sequence of *L. betulinus* IUM 5468 was deposited at GenBank with accession number of

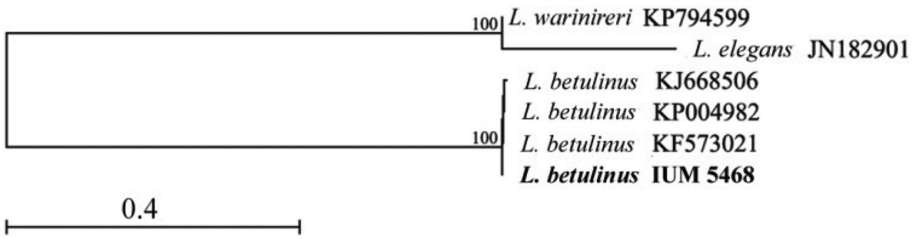

Fig. 1. Phylogenetic analysis using neighbor-joining method comparing the sequence of the internal transcribed spacer ribosomal DNA (rDNA) region from *Lenzites betulinus* IUM 5468 with other *Lenzites* spp. retrieved from GenBank database. The numbers above the nodes are supporting percentages obtained from 1,000 bootstrap replicates. The fungal strain identified in this study is shown in boldface.

KU350750.

Ethanol production from hexose and disaccharide sugars. To investigate the ethanol production of *L. betulinus* IUM 5468 on hexose sugars, glucose, mannose, and galactose were tested as substrates. Ethanol production and hexose consumption by *L. betulinus* IUM 5468 during fermentation period are shown in Fig. 2. All hexose sugars in the media were consumed completely at up to 192 hr of fermentation period. The maximum ethanol concentration was observed within 192 hr of incubation. The best hexose sugar that was converted to ethanol was glucose with 7.68 g/L of ethanol from 20 g/L, corresponded to ethanol yield of 0.38 g per gram of hexose sugar or theoretical ethanol yield of 75.1% (Fig. 2A). The theoretical ethanol converting efficiency of glucose by filamentous fungi such as *Aspergillus sojae*, *Mucor corticolous*, *Rhizopus oryzae*, *R. hiemalis*, *A. foetidus*, *A. awamori*, *A. oryzae*, and *R. indicus* is from 20.4% to 84.1% [18, 19], indicating that the ethanol production efficiency of *L. betulinus* IUM 5468 was moderate. In the fermentation from mannose, the maximum concentration of ethanol was 5.11 g/L, which corresponded to 0.26 g of ethanol per g of hexose or 50% of theoretical ethanol yield (Fig. 2B). For the fermentation from galactose, although *L. betulinus* IUM 5468 consumed galactose completely during 192 hr of the fermentation period, ethanol converting efficiency was very low (0.07 g ethanol per gram of sugar or 13.5% of theoretical ethanol yield) (Fig. 2C). Cellobiose, a disaccharide, is composed of two molecules of β-glucose linked by a β-(1 → 4) bond. It can be derived

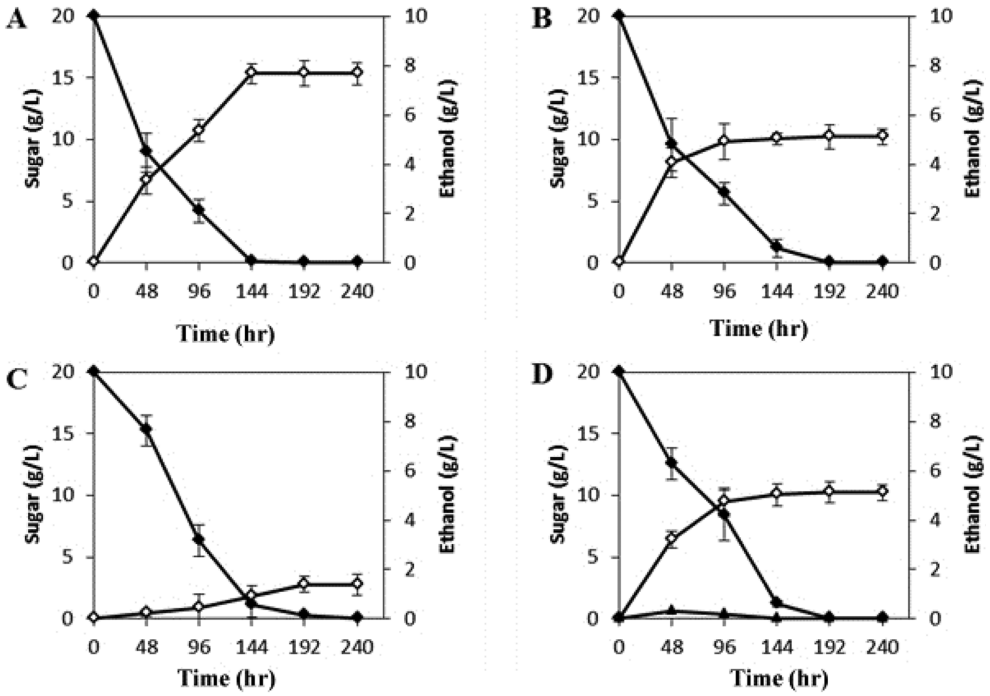

Fig. 2. Time course of ethanol production by *Lenzites betulinus* IUM 5468 with various sugars. The mycelia were cultured in basal medium with 20 g/L glucose (A), mannose (B), galactose (C), and cellobiose (D) under oxygen limited condition. Symbols represent various sugars (filled diamond), ethanol (open diamond), and glucose (filled triangle). Values are expressed as mean ± standard deviation (error bars) of three independent experiments.

from enzymatic hydrolysis of cellulose. To determine the fermenting ability of *L. betulinus* IUM 5468 on cellobiose, this fungus was incubated in medium supplemented with 20 g/L of cellobiose. Cellobiose concentration in the medium was decreased rapidly while ethanol concentration was increased gradually after 48 hr of incubation. The maximum concentration of ethanol was observed at 192 hr after culturing at 0.26 g of ethanol per gram of cellobiose consumed, corresponding to 50.1% of theoretical ethanol yield (Fig. 2D). Only a small amount of glucose was detected during fermentation period as degraded glucose from cellobiose was assimilated and converted to ethanol by the fungus rapidly. These results suggested that most of the fungi possessing ethanol fermenting ability, can produce ethanol efficiently from hexose sugars and disaccharide under oxygen limited condition.

The results indicated that ethanol production from cellobiose by *L. betulinus* IUM 5468 was due to simultaneous hydrolysis of cellobiose to glucose and conversion of glucose to ethanol. The experimental results suggested that ethanol fermenting ability of this fungus has a broad range of sugar utilization from hexose sugars to disaccharide.

Ethanol production from pentose sugars. Pentose sugars such as xylose and arabinose are major components of hemicellulose originated from grasses and wood [20]. Several yeasts and filamentous fungi such as *Candida shehatae*, *Pichia stipitis*, *Pachysolen tannophilus*, *Gloeophyllum trabeum*, and *Flammulina velutipes*, can convert xylose to ethanol efficiently [21-23]. To investigate xylose fermentation ability of this fungus, mycelial discs of *L. betulinus* IUM 5468 were incubated in 20 g/L of xylose containing medium. *L. betulinus* IUM 5468 consumed xylose completely up to 192 hr of incubation, with the maximum ethanol concentration at 5.5 g/L (0.24 g of ethanol per gram of xylose consumed or theoretical ethanol yield of 46.1%) (Fig. 3A). *L. betulinus* IUM 5468 had a good tendency to ferment xylose under oxygen limited condition. This experimental result was better than those of ethanol production from xylose by white rot fungi such as *Peniophora cinerea* (17.6% theoretical ethanol yield), *Trametes suaveolens* (11.4%), and *Hohenbuehelia* sp. ZW-16 (14.7%) [15, 24], indicating that *L. betulinus* IUM 5468 may have ethanol fermenting potential from one major component of hemicellulose. In contrast, arabinose was also consumed completely by *L. betulinus* IUM 5468 up to 192 hr of fermentation period, the maximum concentration of ethanol from 20 g/L arabinose was 1.35 g/L, which was a poor ethanol yield (0.07 g per gram of arabinose with theoretical ethanol yield of 13.2%) (Fig. 3B). When Liang *et al.* [24] cultured *Hohenbuehelia* sp. ZW-16 strain in 20 g/L of arabinose containing medium, the fungus consumed the arabinose completely within 192 hr of ethanol fermentation period with an ethanol yield of 0.3 g, indicating that ethanol converting ability of the fungus from arabinose was very poor and arabinose might be used mainly for energy production. On the other hand, when *Phlebia* sp. MG-60 was incubated in arabinose supplemented liquid medium, only a negligible amount of arabinose was consumed during fermentation period of 120 hr and ethanol production was not observed [25]. Therefore, it seems that most of white rot fungi used in ethanol fermentation, including *L. belutinus* IUM 5468 utilized arabinose for energy source, but not for ethanol production.

Production of ethanol from lignocellulosic biomass. To determine the fermentation potential of *L. betulinus* IUM 5468 on lignocelluosic materials, the mycelial discs were inoculated in 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 consist 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 *L. betulinus* IUM 5468 was cultured in 20 g/L of rice straw, reducing sugars and ethanol were first detected

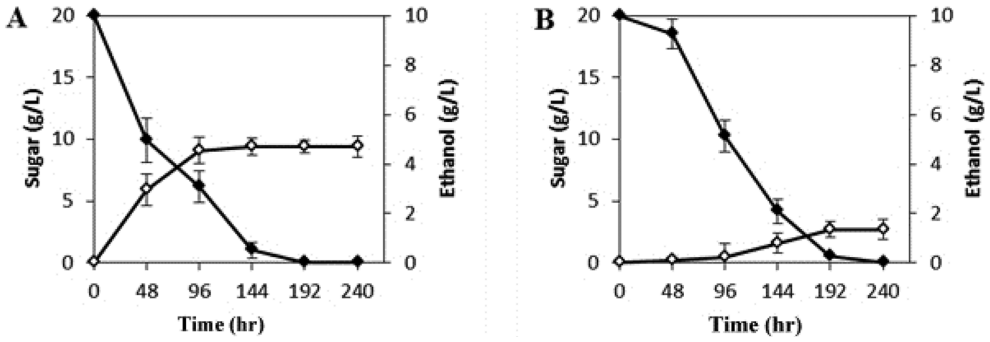

Fig. 3. Time course of ethanol production by *Lenzites betulinus* IUM 5468 with pentose monosaccharide. The mycelia were cultured in basal medium with 20 g/L xylose (A) and arabinose (B) under oxygen limited condition. Symbols represent various sugars (filled diamond) and ethanol (open diamond). Values are expressed as mean ± standard deviation (error bars) of three independent experiments.

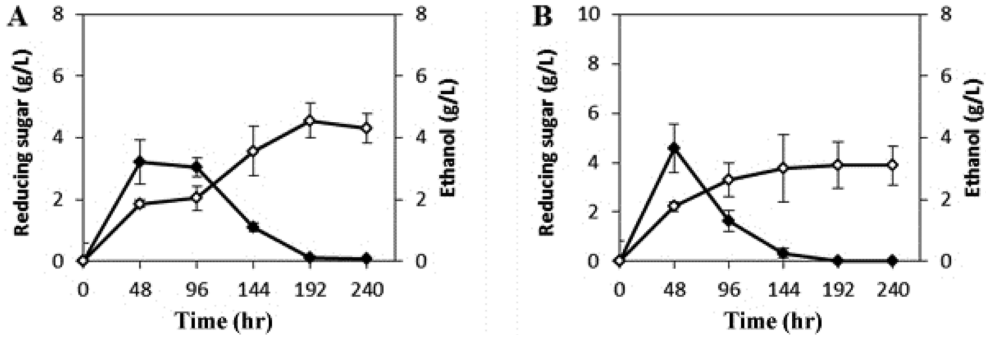

Fig. 4. Time course of ethanol production by *Lenzites betulinus* IUM 5468 with lignocelluosic biomass. The mycelia were cultured in basal medium with 20 g/L rice straw (A) and corn stalks (B) under oxygen limited condition. Symbols represent reducing sugars (filled diamond) and ethanol (open diamond). Values are expressed as means ± standard deviation (error bars) of three independent experiments.

at 48 hr after incubation (3.22 g/L of reducing sugars and 1.86 g/L of ethanol). The highest ethanol concentration of 4.55 g/L was observed up to 144 hr of fermentation period (theoretical ethanol yield of 42.2%) (Fig. 4A). The concentration of reducing sugar in the media was decreased gradually as the ethanol concentration in the medium was increased steadily during the fermentation period, indicating that the reducing sugars decomposed from rice straw were converted to ethanol by the fungus simultaneously. When *L. betulinus* IUM 5468 was cultured with 20 g/L of corn stalks, reducing sugars (4.57 g/L) and ethanol (1.78 g/L) released into the medium were also first observed after 48 hr of cultivation. The maximum ethanol concentration of 3.12 g/L was detected at 192 hr after cultivation (Fig. 4B). Although the concentration of reducing sugars in corn stalks medium was decreased rapidly, ethanol concentration was increased gradually up to 192 hr of incubation as the liberated reducing sugars from corn stalks were taken up by the fungus simultaneously for ethanol production. Even though total cellulose and hemicellulose contents from corn stalks (63.56%) is higher than that of rice straw (58.95%), the ethanol concentration produced from rice straw (4.55 g/L) is higher than that of corn stalks (3.12 g/L). The higher ethanol concentration produced from rice straw might be due to the reducing sugars hydrolyzed by this fungus in the medium were converted to ethanol more efficiently than that from corn stalks. When Okamoto *et al.* [26] cultured white rot fungus *Trametes hirsuta* in a medium supplemented with 20 g/L of lignocellulosic materials such as rice straw and ball-milled rice straw, ethanol concentrations were 3.0 g/L, and 3.4 g/L, respectively. Rasmussen *et al.* [22] observed that brown rot fungus, *Gloeophyllum trabeum* was cultured in a solid medium containing corn fiber for 72 hr, and followed by continuous incubation in buffer containing liquid medium up to 264 hr, the maximum ethanol concentration was detected at 3.3 g of ethanol per 100 g of corn fiber, which were lower than the ethanol concentrations from rice straw (3.47 g/L) or corn stalks (3.58 g/L) observed in this study.

In summary, it is concluded that *L. betulinus* IUM 5468 could assimilate various carbon sources and produce ethanol effectively from glucose, mannose, xylose, and cellobiose, the major components of cellulose and hemicellulose originated from grasses and wood. *L. betulinus* IUM 5468 also could convert wheat straw and corn stalks directly to ethanol, indicating that the white rot fungus *L. betulinus* IUM 5468 has a good potential for producing bioethanol from lignocellulosic biomass through the SSF process. Further studies are needed to increase ethanol fermenting efficiency through improving culture conditions.

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