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# Accumulation of sugar from pulp and xylitol from xylose by pyruvate decarboxylase-negative white-rot fungus *Phlebia* sp. MG-60

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## HIGHLIGHTS

- Regenerated strains from *Phlebia* sp. MG-60-P2 show the uniform phenotypes.
- Homologous recombination achieved a stable *pdc*-knockout line (KO77).
- Production of ethanol was inhibited successfully at strain KO77.
- KO77 accumulated xylitol from xylose or glucose from cellulose.
- Metabolic engineering of *Phlebia* sp. MG-60 can directly convert lignocellulose.

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## ABSTRACT

*Phlebia* sp. MG-60 is a white-rot fungus that produces ethanol with high efficiency from lignocellulosic biomass without additional enzymes. Through engineering of this powerful metabolic pathway for fermentation in *Phlebia* sp. MG-60, chemical compounds other than ethanol could be produced. Here, we demonstrate sugar accumulation from unbleached hardwood kraft pulp and conversion of xylose to xylitol by pyruvate decarboxylase (*pdc*)-negative *Phlebia* sp. MG-60. We isolated *Phlebia* sp. strain MG-60-P2 from protoplasts to unify the protoplast phenotypes of the regenerated strains. Homologous recombination achieved a stable *pdc*-knockout line, designated KO77. The KO77 line produced traces of ethanol, but accumulated xylitol from xylose or glucose from unbleached hardwood kraft pulp. These metabolic changes in the *pdc*-knockout strain reflect the potential of metabolic engineering in *Phlebia* sp. MG-60 for direct production of chemical compounds from lignocellulosic biomass.



## 1. Introduction

Lignocellulosic biomass is the most abundant renewable and sustainable material, and is therefore expected to be exploited as a substitute for fossil fuels. In particular, bioethanol is becoming a widespread renewable energy alternative to fossil fuels, although edible materials are generally used as the fermentation substrates for ethanol production (Hayes, 2009). Fermentation of lignocellulose is an effective method to convert non-edible materials to low-molecular-weight compounds that can be utilized as biofuels as well as raw materials. A number of researchers have investigated metabolically-engineered bacteria or yeasts that can ferment lignocellulose or xylose, a typical hemicellulose in lignocellulosic biomass (Hasunuma et al., 2014; Kim et al., 2014; Smith et al., 2014; Wang et al., 2013). However, almost of all these genetically-engineered microorganisms could barely exploit lignocellulosic biomass without additional enzymes or pretreatments for lignin degradation.

Lignin-degrading basidiomycetes, white-rot fungi, are microorganisms with innate capability to degrade all components of lignocellulosic biomass, namely cellulose, hemicellulose, and lignin (Kirk and Fenn, 1982). Three major classes of oxidases, manganese peroxidases, lignin peroxidases, and laccases, are recognized to be important for fungal degradation of lignin (Lundell et al., 2010). Biological pretreatment with white-rot fungi for lignin degradation is an effective way to utilize lignocellulosic biomass (Bak et al., 2009; Taniguchi et al., 2005; Wan and Li, 2011). Recently, we established an integrated fungal fermentation process with a white-rot fungus, *Phlebia* sp. strain MG-60, that unites aerobic delignification and semi-aerobic consolidated biological processing using a single microorganism capable of both lignin degradation and saccharide fermentation (Kamei et al., 2012a,b). *Phlebia* sp. MG-60 shows fermentability not only for glucose, but for mannose,

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galactose, fructose, xylose, and disaccharides. Furthermore, it also ferments lignin-containing cellulosic materials, resulting in ethanol production (Kamei et al., 2012a).

Metabolic engineering of white-rot fungi would be an efficient method to produce low-molecular-weight compounds from lignocellulosic biomass as an alternative to fossil fuels. Several previous studies have investigated gene recombination in white-rot fungi (Yamagishi et al., 2007; Kück and Hoff, 2010; Nakazawa et al., 2011; Salame et al., 2012). Yamasaki et al. (2014) reported successful co-transformation of *Phlebia* sp. MG-60. Overexpression of manganese peroxidase MG*mnp2* induced by a *gpd* promotor from *Phlebia brevispora* enhanced both manganese peroxidase activity and lignin degradation. In the same study, wild-type *Phlebia* sp. MG-60 showed various manganese peroxidase activities. Furthermore, there were variations in sizes of protoplasts and numbers of nuclei. Thus, unification of protoplast characteristics appears important to obtain stable phenotypes in metabolically-engineered white-rot fungi.

To produce chemical compounds from lignocellulose biomass by metabolic engineering of *Phlebia* sp. MG-60, inhibition of ethanol fermentation is essential. Here, we achieved metabolic engineering of *Phlebia* sp. strain MG-60-P2 to downregulate pyruvate decarboxylase (*pdc*) gene expression. We isolated a protoplast-regenerated strain, MG-60-P2, that showed stable delignification and fermentation ability of hardwood powder. Although the efficiency of homologous recombination was very low, a stable knockout line, KO77, of *Phlebia* sp. MG-60-P2 was obtained. The KO77 line showed *pdc* suppression, resulting in only traces of ethanol production. Remarkably, the KO77 line accumulated xylitol from xylose fermentation and glucose from unbleached hardwood kraft pulp (UHKP). These findings reflect the first step in metabolic engineering toward an integrated fungal fermentation process.

## 2. Materials and methods

### 2.1. Fungal strain and cultures

*Phlebia* sp. strain MG-60 TUFC40001 (Fungus/Mushroom Resource and Research Center, Tottori, Japan) was maintained on potato-dextrose agar plates. *Phlebia* sp. strain MG-60-P2 was isolated from protoplasts derived from strain MG-60 to unify the protoplast phenotypes of the regenerated strains. Regenerated mycelia from MG-60-P2-derived protoplasts were evaluated by decolorization activity of hardwood powder and fermentability of glucose to confirm their phenotypic stability.

### 2.2. Nucleic acid isolation and cDNA preparation

Mycelia from 10-mL liquid cultures were filtered through Miracloth (Merck Millipore, Darmstadt, Germany), semi-dried with sterile paper towel, rapidly frozen in liquid nitrogen, and stored at −80 °C. The frozen mycelia were ground to a powder by crushing with a hammer. DNA was isolated from the mycelium powder with ISOPLANT (Nippon Gene Co. Ltd., Toyama, Japan). Total RNA was prepared using a combination of Plant RNA Isolation Reagent (Invitrogen Corp., Carlsbad, CA) and TRIzol reagent (Invitrogen Corp.). The obtained RNA was washed with 75% ethanol and dissolved in RNase-free water. The amount and quality of the RNA were calculated by the absorbances at 260 and 280 nm. cDNA was synthesized in a 20-μL reaction mixture containing 1 μg of total RNA, 1 μM oligo (dT)-adapter primer with an M13 primer M4 sequence, 10 U of RNase inhibitor, and 10 U of AMV Reverse Transcriptase (Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. The reaction was carried out for 60 min at 45 °C, and then terminated by heating for 5 min at 95 °C. Finally, the reaction mixture was diluted 1:100 with RNase-free water, and a 1-μL sample was subjected to RT-PCR to amplify the *pdc* partial sequence in *Phlebia* sp. MG-60.

### 2.3. Gene identification and characterization

To isolate the partial sequence of *pdc* in *Phlebia* sp. MG-60, a 1121-bp fragment of MG*pdc1* cDNA was amplified with a pair of primers (Pb-PyruDe-F2 and Pb-PyruDe-R1) designed from the genome sequence of *P. brevispora*. The 3′-downstream region was amplified by PCR using primers M13 and M4. The PCR amplification was performed with the following program: denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 2 min; and final extension at 72 °C for 5 min. To identify the 5′-upstream region of the *pdc* gene, an LA PCR In Vitro Kit (Takara Bio) was utilized with a selection of restriction enzymes. After ligation of a HindIII cassette, the first-round PCR was performed with a cassette primer (C1) and a specific primer (MG-PDC-LA-R1). Annealing was carried out at 55 °C for 30 s and extension was performed at 72 °C for 4 min using LA Taq polymerase (Takara Bio). The second-round PCR was carried out with proper primers C2 and MG-PDC-LA-R2. Approximately 1000 bp of the amplified products including the start codon and 5′-untranslated region was sequenced and used for further primer design. The full-length genomic DNA and open reading frame cDNA of the *pdc* gene were amplified with primers designed on the sequence data obtained from the LA-PCR fragments and 3′-downstream region (MG-PDC-UTR-F1, MG-PDC-UTR-F2, and MG-PDC-UTR-R1). The promoter and terminator regions of *pdc* were obtained using the LA PCR In Vitro Kit, with proper primers and designed specific primers.

Ex-Taq polymerase (Takara Bio) or KOD FX Neo (Toyobo, Osaka, Japan) was used for PCR amplifications. The PCR products were subcloned into the pMD19 or pMD20 vector (Takara Bio), and the ligation products were transformed into *Escherichia coli* strain JM109 according to the manufacturer's protocol. The clones were sequenced by a dideoxy method (BigDye® Terminator v3.1 Cycle Sequencing Kit; Thermo Fisher Scientific Inc., Waltham, MA, USA) with a sequencer (Model 3500xL; Applied Biosystems, Foster City, CA, USA). The obtained sequence of MG*pdc1* gene in addition to both promotor and terminator regions was deposited to GenBank with accession number LC214886.

### 2.4. Preparation of a knockout construct

A knockout construct for MG*pdc1* was prepared as follows (Fig. 1). An *hpt* expression cassette containing *hpt* (hygromycin resistance gene), *gpd* promoter, and terminator of *P. brevispora* (Yamasaki et al., 2014) was inserted into the *pdc* genome sequence. About 5300 bp of the *pdc* gene genome sequence containing the promoter and terminator was amplified with the pMD19 vector using primers MG-PDC-Spe-F1 and MG-PDC-EcoT22-R1 to add restriction sites for SpeI and EcoT22I. The resulting fragment was then ligated with the fragment containing the *hpt* expression cassette with *gpd* promoter and *gpd* terminator, and amplified by PCR with primers PbGPD-EcoT22-F1 and PbGPD-M-R1. The obtained knockout construct containing the *hpt* expression cassette in the *pdc* coding region (total 8998 bp) in pMD19 was amplified in *E. coli* strain JM109.

### 2.5. Transformation

Protoplast isolation and polyethylene glycol (PEG)-mediated co-transformation assays with MG-60-P2 were performed as described previously (Yamasaki et al., 2014). Briefly, MG-60-P2