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Improvement of ethanol production by recombinant expression of pyruvate decarboxylase in the white-rot fungus *Phanerochaete sordida* YK-624

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To improve ethanol production by *Phanerochaete sordida* YK-624, the pyruvate decarboxylase (PDC) gene was cloned from and reintroduced into this hyper lignin-degrading fungus; the gene encodes a key enzyme in alcoholic fermentation. We screened 16 transformant *P. sordida* YK-624 strains that each expressed a second, recombinant PDC gene (*pdc*) and then identified the transformant strain (designated GP7) with the highest ethanol production. Direct ethanol production from hardwood was 1.41 higher with GP7 than with wild-type *P. sordida* YK-624. RT-PCR analysis indicated that the increased PDC activity was caused by elevated recombinant *pdc* expression. Taken together, these results suggested that ethanol production by *P. sordida* YK-624 can be improved by the stable expression of an additional, recombinant *pdc*.

[**Key words:** *Phanerochaete sordida* YK-624; White-rot fungi; Pyruvate decarboxylase; Recombinant expression; Ethanol production]

Production of ethanol fuel from biomass has attracted worldwide interest as an alternative to conventional, fossil fuels (1,2). A single microorganism that can hydrolyze cellulose and then ferment the monosaccharides subunits to ethanol could potentially increase ethanol yields and reduce production costs (3). Researchers discovered that white-rot fungi produce enzymes that can be used to pretreat lignocellulosic biomass before downstream enzymatic saccharification (4,5). In addition, Okamoto et al. (6,7) demonstrated that several species of white-rot fungi could produce ethanol directly from hexoses, starch, wheat bran, or rice straw. Since white-rot fungi can hydrolyze cellulose (8,9), white-rot fungi could be used for one-step (direct) alcohol fermentation of lignocellulosic biomass.

Pyruvate decarboxylase (PDC), which catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde with the release of carbon dioxide, is a key enzyme in ethanol fermentation (10). Overexpression of the PDC gene in the yeast *Hansenula polymorpha* was recently shown to increase PDC activity, resulting in elevated ethanol production (11). This finding suggests that overexpression of the PDC gene in white-rot fungi will increase the rate of alcoholic fermentation.

Among white-rot fungi, including the well-characterized species *Phanerochaete chrysosporium* and *Trametes versicolor*, *Phanerochaete sordida* YK-624 is particularly suited for the alcoholic fermentation of lignocellulose due to its high ligninolytic activity and selectivity (12). Here, we generated high *pdc*-expressing transformants of *P. sordida* YK-624 and evaluated their capacity for direct ethanol production from hardwood.

MATERIALS AND METHODS

Fungal strain *P. sordida* YK-624 (ATCC 90872) and uracil auxotrophic strain UV-64 (13) were used in this study. The fungus was maintained on potato dextrose agar (PDA) slants at 4°C.

Cloning of a full-length gene encoding PDC *P. sordida* YK-624 was incubated on PDA plates at 30°C for 3 days, and 10-mm diameter disks then were punched out from the growing edge of the mycelia using a sterile cork borer. Two mycelial disks were placed into a 100-mL Erlenmeyer flask containing 10 mL potato dextrose broth (PDB) medium (Becton, Dickinson and Company). Genomic DNA of *P. sordida* YK-624 was extracted from mycelia using an ISOPLANT II kit (Nippon Gene, Tokyo). All primers used in this process are listed in Table 1. The conserved region of the PDC-encoding gene was amplified using the primer set PcPDCF–PcPDCR, which was designed based on complementary to the *P. chrysosporium* PDC gene archived in the Joint Genome Institute database. The 5′-coding region of the PDC gene from *P. sordida* YK-624 was cloned from genomic DNA using an inverse PCR method (14) with the primer sets InverseF1-InverseR1, InverseF2-InverseR2, InverseF3-InverseR3, InverseF4-InverseR4, InverseF5-InverseR5 and InverseF6-InverseR6. The primer sets InverseF7-InverseR7 and InverseF8-InverseR8 were used to clone the 3′-coding region. The primer set PDCF1-PDCR1, which was designed based on sequences obtained from the inverse PCR, was used to determine the sequence of the full-length gene encoding PDC of *P. sordida* YK-624.

Construction of a PDC gene expression vector, co-transformation of UV-64, and screening of regenerated clones The full-length genomic PDC gene (2124 bp) was amplified using primers PDCF1-PDCR1 (Table 1). The resulting PCR product was ligated into the cloning vector pMD20-T (Takara Bio, Shiga, Japan) and introduced into *Escherichia coli* DH5α for sequencing. Primers PDCF2 and PDCR2 were designed to introduce an *Xba* I site into the *pdc*; these primers were also used to amplify the *pdc* with the *Xba* I from the recombinant plasmid, which was used as a template for sequencing. The plasmid p*PsGPD-pro*, which was generated in our previous study (15), was used to construct a *pdc*-expression

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TABLE 1. Oligonucleotide primers used in this study.

Primer name	Nucleotide sequence (5′-sequence-3′)
PcPDCF	ACTTCCCGGTATATGCTGC
PcPDCR	AGAGGTGAGTGATCGTCTC
InverseF1	GTCTTTGCGTCTGTCTAACGC
InverseR1	GTAGCGGTCATAGTCTTCGG
InverseF2	GCCTAGATTTACGTCGGAACG
InverseR2	TATCGCCGTCTTGCCCATTG
InverseF3	TCAAGGAAGGAAGCCTCG
InverseR3	ATATGCTGCGTTCAGCTCG
InverseF4	CAGAAGAGCAAGCCCATG
InverseR4	GGAGCTTGATTCTATGTAGTCC
InverseF5	ACTCTGACCACACTCGCATC
InverseR5	AGCTACAAAGCGTGGGTCAG
InverseF6	ATCGGCATGAAGCGTCTGC
InverseR6	AGCGAAAATGCTGGAGCGTAC
InverseF7	CTGACGTTATCCTAGGCTTC
InverseR7	CAGGCACGCCAAAGATTTC
InverseF8	ATACAGCCGTCACTCATGTG
InverseR8	AGTAGGCGTAGATAGAAGTCG
PDCF1	ATGCAGGTCGCCGACCAG
PDCR1	TCACAGCGTGTCGCTCGC
PDCF2	AATCTAGAATGCAGGTCGCCGACCCG
PDCR2	AATCTAGATCACAGCGTGTCGCTCGC
gpdF1	AAGCAGCGAGGATTGTACC
PDCR3	GGAGCTTGATTCTATGTAGTCC
PDCF4	GTCATTCTCTTCGTTGGAGAC
PDCR4	GGGTACTTGTACAATCCTCC
PDCF5	TTGTCAAGCTGATCGAGGAG
PDCR5	CTCCTTCTTGACGTTATGCC
ActinF	AGCACGGTATCGTCACCAAC
ActinR	AGCGAAACCCTCGTAGATGG

plasmid. The DNA fragment amplified with primers PDCF2 and PDCR2 was digested with *Xba* I and cloned into *Xba* I digested-p*PsGPD-pro*, yielding plasmid p*PsGPD*-PDC. The p*PsGPD*-PDC was sequenced to verify the absence of PCR errors. UV-64 protoplasts were prepared via a standard technique involving cellulases and were then co-transformed with p*PsURA5* and p*PsGPD*-PDC using a polyethylene glycol (PEG) method, as previously described (13). The co-transformed strains (GP strains) were selected by PCR using the primers gpdF1 and PDCR3 (Table 1), which were designed to amplify the entire recombinant *pdc* gene.

Assessment of pdc expression transformants based on ethanol production and fermentative capacity for various saccharides The effect of stable *pdc* expression on ethanol production by the *pdc*-expressing transformants (GP strains) was investigated. Briefly, *P. sordida* YK-624 (WT) and 16 GP strains each were incubated on separate PDA plates at 30°C for 3 days; 10-mm diameter disks then were punched out from the growing edge of mycelia using a sterile cork borer. For each strain, two mycelial disks were placed into a 100-mL Erlenmeyer flask containing 10 mL basal liquid medium (20 g L^{-1} glucose, 10 g L^{-1} yeast extract, 10 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$, and 0.5 g L^{-1} $MgSO_4$–$7H_2O$, pH 4.5). After sealing each flask with a silicone plug stopper (semi-aerobic conditions), each culture was statically incubated at 30°C for 4 days (16). Culture samples were separated by high-performance liquid chromatography (HPLC) using a Shodex SH1821 column (8.0 mm × 300 mm, Showa Denko K.K., Tokyo, Japan) at 75°C with 0.5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}, and ethanol and glucose concentrations in the cultures were measured using an online Refractive Index Detector.

The GP strain with the highest ethanol fermentation efficiency (GP7) was also examined for its ability to produce ethanol from each of eight saccharides— D-glucose, D-mannose, D-galactose, D-fructose, D-xylose, L-arabinose, cellobiose, and maltose (Wako Pure Chemical Industries, Osaka, Japan)—using the above-described fermentation method.

Enzyme assay WT and GP7 were each incubated at 30°C for 2, 4, 6, or 8 days in basal liquid medium, as described above. A mycelial sample from each strain was added to 2 mL of 400 mM Tris–HCl buffer (pH 6.0) and was then homogenized on ice using a Polytron PT1200E (Kinematica, Canada). The homogenate was centrifuged (4°C, 10,000 ×*g*, 10 min); 100 μL 10 mM thiamine diphosphate and 100 μL 10 mM $MgCl_2$ were added to each obtained supernatant, which was then incubated at 60°C for 30 min. For each culture, this solution was used as a cell-free extract to measure PDC activity, which was determined by monitoring the oxidation of NADH to NAD^+ ($\varepsilon 340 = 6.22$ mM^{-1} cm^{-1}) as previously described (17). Each reaction mixture (1 mL) consisted of 50 μL cell-free extract, 100 μL 10 mM pyruvate, 150 μL distilled water, 100 μL 6 U mL^{-1} alcohol dehydrogenase solution (Sigma–Aldrich, USA), and 100 μL 0.4 mM NADH and 500 μL 400 mM Tris–HCl buffer (pH 6.0).

Transcriptional analysis of strain GP7 WT and GP7 were cultured as described in the *enzyme assay section*, and were each incubated at 30°C for 2, 4, 6, or 8 days. Mycelia were then collected and stored at −80°C. Under liquid nitrogen and using a mortar and pestle, 100 mg of mycelium mat was ground to a fine powder, and total RNA was then isolated from the mycelial powder using a Qiagen RNeasy Mini Kit (Hilden, Germany). Reverse transcription (RT)-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit and the gene-specific primer sets PDCF4-PDCR4 (recombinant *pdc*), PDCF5-PDCR5 (native *pdc*) and ActinF-ActinR (*actin*, Table 1).

Determination of ligninolytic properties and fermentation with wood meal as a carbon source Mycelial discs of WT and GP7 were added to 50-mL Erlenmeyer flasks containing 0.5 g extractive-free beech wood meal (80–100 mesh) and 1.25 mL distilled water; each resulting culture was incubated at 30°C for 28 days. After the incubation period, weight loss, Klason lignin content, and acid-soluble lignin content in the fungal-treated wood meal were determined, as previously described (12).

After this 28 day-incubation of wood meal culture inoculated with either WT or GP7, 10 mL of basal liquid medium lacking saccharides was added to each culture, which was then homogenized using a Polytron PT1200E. To investigate the effect of saccharification on ethanol fermentation, 36 paper units (FPUs) of Cellulose Onozuka RS/g wood meal were then added to each culture. Each culture flask was sealed with a silicon plug stopper (semi-aerobic conditions) and then further incubated with shaking at 30°C for 3, 6, or 9 days. After fermentation, the wood meal cultures were centrifuged (10,000 ×*g*, 5 min), and the amount of ethanol in the obtained supernatant was analyzed by HPLC, as described in above.

Nucleotide sequence accession number The nucleotide sequence of *pdc* derived from *P. sordida* YK-624 has been deposited in the DDBJ database (http://www.ddbj.nig.ac.jp/) under accession no. LC057149.

RESULTS AND DISCUSSION

Generating pdc-expression transformants and screening for ethanol productivity Full-length genomic *pdc* (2124 bp) was obtained from *P. sordida* YK-624 using PCR, inverse PCR, and genomic DNA template. Amino acid sequence alignment of theoretical translations of *pdc* from *P. chrysosporium* (PcPDC) and *P. sordida* YK-624 (PsPDC) indicated that the sequence identity of the two predicted proteins was 89% (Fig. 1). The procedure for constructing the *pdc* expression plasmid, p*PsGPD*-PDC, is shown in Fig. 2. The p*PsGPD*-PDC expression plasmid was introduced into strain UV-64 together with p*PsURA5* as a marker plasmid. We obtained 16 GP strains co-transformed with p*PsGPD*-PDC and p*PsURA5*.

Ethanol production by each GP transformant strain was then investigated. Ethanol production by all 16 recombinant GP strains was higher than that by the WT strain (data not shown). Transformant GP7 showed the highest ethanol production and glucose consumption among the 16 screened transformants (data not shown) and was therefore selected for further analyses of PDC activity and gene expression.

PDC activity and transcriptional analysis of recombinant pdc in GP7 The intracellular activity of PDC in strain GP7 was compared with that in WT. GP7 showed the highest PDC activity after 4 days of incubation, with 1.3-fold higher PDC activity relative to that of the WT strain (Fig. 3A).

We next used RT-PCR to analyze the temporal transcription profile of *pdc* in GP7 and WT (Fig. 3B). The highest levels of recombinant *pdc* expression were detected after 4 days of incubation, after which the expression levels of *pdc* decreased. Although the native *pdc* was detected in WT by RT-PCR, the expression level was markedly lower than expression of recombinant *pdc* in GP7 (data not shown). Thus, these results suggested that the higher production of PDC was due to the higher expression of recombinant *pdc* in GP7.

Ethanol production from saccharides For WT and GP7, the time course of glucose fermentation is shown in Fig. 4. GP7 produced the maximum ethanol concentration (3.8 g L^{-1}) from glucose after 8 days of incubation, and this maximum was higher than that for WT (3.1 g L^{-1}). The ability of GP7 to ferment each of several monosaccharides and disaccharides as a carbon source was determined (Table 2). In the cultures containing mannose or fructose as the sole carbon source, maximum ethanol