Direct lactic acid production from beech wood by transgenic white-rot fungus Phanerochaete sordida YK-624

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Abstract

A lactic acid (LA)-producing strain of the hyper-lignin-degrading fungus *Phanerochaete sordida* YK-624 with the lactate dehydrogenase-encoding gene from *Bifidobacterium longum* (*Blldh*) was constructed. When the endogenous pyruvate decarboxylase gene-knocked down and *Blldh*-expressing transformant was cultured with beech wood meal, the transformant was able to successively delignify and ferment the substrate. Supplementation of calcium carbonate into the culture medium, significantly increased the level of LA accumulation. Direct LA production (at 0.29 g/l) from wood was confirmed, and additional inclusion of exogenous cellulase in this fermentation yielded significant further improvement in LA accumulation (up to 1.44 g/l). This study provides the first report of direct production of LA by fermentation from woody biomass by a single microorganism, and indicates that transgenic white-rot fungi have a potential use for development of simple/easy applications for wood biorefinery.

Highlights

· *Phanerochaete sordida* YK-624 acquired lactic acid (LA) fermentation ability by transformation with bacterial *ldh*.

· Knock-down of the endogenous pyruvate decarboxylase-encoding gene improved LA productivity of the *ldh* transformant.

· Consecutive biological delignification and fermentation using a transgenic white-rot fungus provided direct LA production from wood.

· Inclusion of exogenous cellulase in the medium significantly improved LA production by the transgenic fungus.

Keyword

biorefinery, lactic acid, white-rot fungi, woody biomass

1. Introduction

Lactic acid (LA) is a naturally occurring α-hydroxy acid with an asymmetrical carbon atom. This compound is widely used as an acidulant, flavoring agent, and preservative in food; as a moisturizer in cosmetics; and as a starting material for the synthesis of other substances in the pharmaceutical and chemical industries (Wee et al., 2006). There is also increasing interest in the use of this compound in the production of polylactic acid (PLA), a renewable and biodegradable material. LA can be produced by either chemical synthesis or fermentation. Chemical synthesis from petrochemical resources produces racemic DL-LA. On the other hand, microbial fermentation using an appropriate microorganism can produce optically pure L(+)- or D(-)-LA from renewable resources (Wang et al., 2015). Fermentation resulting in optically pure LA has a major advantage in that enantiomeric PLA has higher crystallinity, higher melting point, and more favorable mechanical properties than racemic PLA, which is amorphous. Additionally, enantiomeric PLA is believed to have superior biocompatibility (Södergård and Stolt, 2002).

LA is obtained by fermentation from both bacteria and fungi. Generally, LA bacteria such as *Lactobacillus* species are used in bacterial fermentation, in which LA is produced from pyruvate by an NAD-dependent lactate dehydrogenase (LDH), a process that permits LA production at levels approaching the theoretical maximum (Wang et al., 2015). *Rhizopus oryzae* is the best-known LA-producing fungus. This fungus naturally produces LA at high yield from glucose and other carbohydrates. Moreover, *R. oryzae* is able to utilize starch and hydrolysates of hemicellulosic and cellulosic materials (Wang et al., 2015). There are several reports of LA fermentation by genetically modified yeasts (Abdel-Rahman et al., 2013). Separate work has demonstrated that *Aspergillus brasiliensis* transformed with the LDHA-encoding gene from *R. oryzae* is able to produce LA from monosaccharides, starch, and hemicellulose (Liaud et al., 2015). These results show that filamentous fungi are extremely useful microorganisms for LA fermentation. On the other hand, to our knowledge, there have been no reports of LA fermentation from woody biomass by filamentous fungi. Indeed, lignin, a major component of wood, is known to exhibit strong negative effects on LA fermentation, an effect believed to reflect lignin-mediated inhibition of the degradation of polysaccharides (Gomes et al., 2015). Thus, delignification is an important step for LA fermentation from woody biomass.

Several wood-degrading and filamentous fungi are known to harbor enzymes that facilitate the breakdown of woody biomass and growth on various woody biomass-derived polysaccharides. However, woody biomass contains 15-35% lignin, a branched, heterogeneous, and amorphous aromatic polymer, and the lignin prevents the breakdown of holocellulose (Gomes et al., 2015). Therefore, white-rot fungi, which are able to degrade lignin, would be suitable candidates for LA fermentation from woody biomass. However, to our knowledge, there are no reports of LA fermentation using white-rot fungi, presumably due to lack of or weak LDH activity in these

organisms. Our goal in the present work was to construct a fungal strain that is able to produce LA from woody biomass by one-step cultivation. Therefore, we tested the effect on LA production of introducing a bacterial LDH-encoding gene (*ldh* from *Bifidobacterium longum*, *Blldh*) into the white-rot fungus *Phanerochaete sordida* YK-624, a strain that shows superior lignin degrading ability. In further work, we aimed to enhance the efficiency of direct LA fermentation from woody biomass by knock-down (using RNA interference, RNAi) of the endogenous pyruvate decarboxylase-encoding gene of *P. sordida* YK-624 (*Pspdc*).

2. Materials and Methods

2.1. Strains and materials

P. sordida YK-624 (ATCC 90872) (Hirai et al., 1994) and the isogenic uracil auxotrophic strain UV-64 (Yamagishi et al., 2007) were used in this study. All strains used in this study were maintained on potato dextrose agar (PDA) at 4°C. Primers used in this study are listed in Table S1. Extract-free beech wood meal was prepared by soxhlet extraction with ethanol-benzene (1 : 2) for 48 hours; analysis showed that this beech wood meal was composed of 74.2% holocellulose as well as 25.8% lignin.

2.2. Construction of Blldh expression vector and RNAi vector, and co-transformation of UV-64

The native sequence of *B. longum ldh* was obtained from Genbank as accession number M33585. The *Blldh* sequence then was redesigned based on *P. chrysosporium* high-frequency codon usage, and the resulting optimized gene was synthesized (LC106309) by GeneScript Japan, Inc. The synthetic *Blldh* (*Blldh* hereafter) expression plasmid (designated p*GPDpro-blldh*; Fig. 1) was constructed as shown in Fig. S1. The silencing vector p*PDC*i (Fig. 1; details in Fig. S2) was designed to express an intron-containing hairpin RNA with complementarity to *Pspdc* (GenBank LC057149).

UV-64 protoplasts were prepared and then co-transformed with marker plasmid p*PsURA5* (Yamagishi et al., 2007) and p*GPDpro-blldh* with or without p*PDC*i using a polyethylene glycol method (Yamagishi et al., 2007). Prototrophic (Ura^+) transformants were selected on the regeneration medium (CYM medium with 1% SeaPlaque Agarose, Takara Bio) without uracil, and the resulting colonies were screened for the presence of the co-transformed sequences by genomic PCR, as described previously (Sugiura et al., 2009). Screening for p*GPDpro-blldh* and p*PDC*i sequences was performed using primer pairs (ldh-rtF/ldh-rtR and rnai-cF/rnai-cR, respectively) designed to specifically amplify the respective introduced genes. A clone transformed with p*PsURA5* alone, denoted strain U, was used as the negative control. The co-transformants were further screened based on LA production in 4-day semi-aerobic fermentation cultures without $CaCO_3$, as described below. The resulting transformants were subcultured 5 times on PDA, and fermentation ability then was

re-assessed for evaluation of the stability of the transformants.

2.3. Measurement of LA and ethanol productivity

A basal liquid culture medium (10 ml; 10 g/l yeast extract, 10 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, pH 4.5) (Okamoto et al., 2010) containing 2% glucose with or without 0.2% $CaCO_3$ was used for the fermentation experiments. As described in the previous report (Wang et al., 2016), cultivation was performed under aerobic and semi-aerobic conditions, and the resulting spent media were subjected to HPLC analysis for quantification of LA, ethanol, and glucose concentrations. HPLC was performed using a JASCO PU-2089 pump with a JASCO RI-2031 detector, fitted Shodex SH 1821 column (8.0 mm × 300 mm, 75 ºC). The eluent was 0.5 mM H_2SO_4, at 0.6 ml/min. Oxygen concentration in the headspace was analyzed every 4 days by gas chromatography (GC, GL Science GC-3200) on a packed column (Molecular Sieve 5A 30/60, 3 m × 1.6 mm outer diameter) with a thermal conductivity detector (TCD). Argon was used as the carrier gas at a flow rate 35 ml/min. The operational temperatures of the injector, detector, and column were 100, 100, and 80 °C, respectively.

After cultivation, $CaCO_3$ was removed in 0.02N HCL, and residual mycelium washed with distilled water. Then, dried fungal weight was measured. For enzymatic quantification of L(+)-LA, D-lactic-/L-lactic acid test kit (Roche Diagnostics) was used according to an instruction manual.

2.4. Transcriptional analysis of strain PigBL64

Mycelia of co-transformant PigBL64 were harvested from fermentation cultures and stored at -80 °C. RNA extraction and reverse transcription (RT) -PCR were performed as described previously (Wang et al., 2016). RT-PCR employed the gene-specific primer pairs act0-rtF/act0-rtR (for actin-encoding gene), ldh-rtF/ldh-rtR (for *Blldh*), and rnai-rtF/rnai-rtR (for *PDC*i) (Table S1). The PCR conditions were as follows: 25 cycles at 94 °C for 30 s, 58 °C (*actin* and *Blldh*) or 56 °C (*PDC*i) for 30 s, and 72 °C for 30 s.

2.5. Single-pot LA fermentation from beech wood

A mycelial disk of co-transformant PigBL64 was used to inoculate extract-free beech wood meal (solid content 70% weight, dry weight 0.5 g, 80–100 mesh) in a 100-ml Erlenmeyer flask. After incubation for 50 days at 30 °C, an aliquot (10 ml) of glucose-free basal liquid medium with 0.2% $CaCO_3$ was added to the wood culture, and the resulting suspension was mixed well. After incubation for another 50 days at 30 °C, another aliquot (10 ml) of glucose-free basal liquid medium with 0.2% $CaCO_3$, with or without cellulase “Onozuka” RS (30 FPU; Yakult Pharm. Ind. Co., Ltd.), was added to the wood culture. The resulting mixture was re-suspended manually, sealed with a silicone cap, and incubated for another 10 days at 30 °C.

Following this incubation, the concentrations of oxygen, glucose, LA, and ethanol were analyzed as described above. Lignin content and sugar composition of wood were determined as described previously (Kamei et al., 2012).

2.6. Statistical analysis

All culturing was performed as 3 independent experiments. Statistical analyses were performed using two-tailed Student's t-tests. Differences between means at a confidence level of 5% ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Isolation of a LDH-expressing transformant

The expression plasmid p*GPDpro-blldh* was introduced into UV-64 by co-transformation with the marker plasmid p*PsURA5* (Yamagishi et al., 2007). Screening of the regenerated Ura^+ isolates yielded 17 clones with confirmed presence of the heterologous *Blldh* (gBL strains). Fourteen of these 17 gBL isolates produced LA when cultured under semi-aerobic conditions for 4 days. The culture fluids of these strains accumulated LA to concentrations ranging from 0.05–0.5 g/l at 4 days; in contrast, the wild-type strain and control strain U were unable to produce LA at detectable levels (Fig. 2A). After 5-time subcultivation, co-transformant gBL10, which showed the highest LA productivity, was chosen for further experimentation.

Time courses of LA and ethanol accumulation in semi-aerobic cultures of gBL10, wild type, and strain U are shown in Fig. 3. LA accumulation was not observed in the culture fluid of either wild type or strain U during a 12-day cultivation. In contrast, gBL10 produced detectable levels of LA at 4 days of incubation, and the amount of lactate continued to rise, reaching 0.73 g/l at 12 days (Fig. 3A). Similar levels of ethanol production were observed in each of the three strains at 12 days (2.94 g/l in wild type, 2.82 g/l in U, and 2.57 g/l in gBL10); no significant differences in ethanol production were detected among the three strains at any of the examined incubation periods (Fig. 3B). These results indicated that only a small part of the glucose had been used for LA fermentation by gBL10. These results also suggested that ethanol fermentation functions as one of the major pyruvate metabolic pathways of this fungus under semi-aerobic conditions. The L(+)-LA amount in the culture fluid which was enzymatically quantified was equivalent to the value of HPLC analysis.

3.2. RNAi knockdown of Pspdc in ldh-expressing transformant

We tried to improve the efficiency of LA fermentation by knocking down *Pspdc* expression using *RNA*i. Sixty-four regenerated clones were obtained from uracil-free HN medium. Genomic PCR was used to demonstrate that 23 of these regenerated clones had additionally been transformed with both *Blldh* and *PDC*i; these isolates were designated as PigBL strains and cultured for 4 days. Screening for LA accumulation revealed that strain PigBL64 was the best LA producer at this time point,

with PigBL64 culture medium accumulating products to levels that were higher (LA) and lower (ethanol) than the levels observed with the wild type or control strain U (Fig. 2B). Time courses of LA and ethanol accumulation and of glucose and oxygen consumption in semi-aerobically grown cultures of PigBL64 are shown in Fig. 4A. Notably, PigBL64 accumulated LA to 1.47 g/l by 12 days, a level 2.0-fold that of the production by gBL10 at the same time point (compare to Fig. 3A). Additionally, although ethanol continued to accumulate in the culture medium during the 12-day incubation, the amount of ethanol in the culture fluid of PigBL64 (1.53 g/L) was 0.55-fold that produced by gBL10 (compare to Fig. 3B). RT-PCR confirmed differences in transcript accumulation of recombinant *Blldh* and *PCD*i (Fig. 4B). *Blldh* transcript levels remained similar at sequential time points throughout the incubation period. On the other hand, *PDC*i transcript accumulated over the course of the cultivation period. These results suggested knock-down of *Pspdc* by *PDC*i; this knock-down was expected to decrease endogenous PDC levels, leading to increased flux away from ethanol production and towards lactate production under semi-aerobic conditions. Although the transcripts levels of recombinant *Blldh* remained similar throughout the incubation period (Fig. 4B), LA production plateaued after the fourth day of incubation (Fig. 4A). A complementary trend was observed for oxygen: the remaining oxygen concentration in the headspace fell to 4% on day 4 but did not subsequently change. In long-term (4-week) semi-aerobic culture, LA levels fell during the extended incubation period (reaching 0.49 g/l at 4 weeks), even as ethanol continued to accumulate (reaching 2.87 g/l at 4 weeks). Under aerobic conditions, neither LA or ethanol accumulated at 2 weeks (data not shown). When culturing was repeated in medium supplemented with $CaCO_3$, PigBL64 produced 2.20 g/l of LA at 12 days (Fig. 4C), a level 1.5-fold that observed without $CaCO_3$ (Fig. 4A). However, supplementation with $CaCO_3$ did not yield a significant difference in ethanol productivity, although inclusion of $CaCO_3$ did provide significant increase in glucose consumption (28.2 and 23.4% increase at 8 and 12 day, respectively). And also, no significant difference in oxygen consumption was observed. The mycelial weight of PigBL64 increased during 4-day incubation period in both culture. after that, mycelial weight did not almost change (Fig. 4D). And there was slight difference in fungal weight in the cultures with and without $CaCO_3$ (44 mg and 36 mg, respectively). In either case, the mycelial weight of PigBL64 was lower than wild type strain (58 mg, after 12 days incubation with $CaCO_3$).

3.3. Single-pot LA fermentation from wood

We next tested the ability of our PigBL64 strain to perform successive single-microorganism, single-container delignification and LA fermentation steps. For this purpose, PigBL64 was first cultured aerobically for 50 days in beech wood meal (delignification); liquid medium (containing $CaCO_3$ with or without cellulose) then was added to the culture, which was allowed to ferment for a further 10 days under

semi-aerobic conditions (LA production). After aerobic incubation, 51.3 ± 3.2 % and 50.0 ± 3.0 % of lignin were degraded by wild-type strain and PigBL64, respectively. Because there was no significant difference, the transformation has not affected the ligninolytic activity. However, glucan and xylan decomposition after 50-day incubation with PigBL64 has been significantly decreased (11.0 ± 1.8 % and 22.3 ±4.8%, respectively), while the decomposition by wild-type strain were 34.7 ± 7.0 % and 47.0 ± 9.3 % respectively. In Fig. 5A, we illustrated the scheme of the method of direct LA fermentation from wood meal by using co-transformant PigBL64. Following fermentation without cellulase, the production of LA and ethanol was observed, although the glucose concentration remained quite low (0.02 ± 0.02 g/L). Following fermentation with exogenous cellulase (i.e., simultaneous saccharification and fermentation, SSF), a certain amount of glucose accumulated in the culture fluid, although the values were varied widely among different experiments (2.33 ± 1.41 g/L). Consistent with the differences in glucose levels with and without cellulase, LA and ethanol accumulation were 5.0-fold higher with cellulase supplementation (Fig. 5B). Indeed, the level of LA in the SSF culture reached 64% that obtained in the glucose liquid medium. On the other hand, ethanol production in the SSF culture was 2.1-fold that obtained in the glucose liquid medium. Additionally, note that the lactate:ethanol ratios (0.44 vs. 0.40) in the presence and absence of cellulase (respectively) were similar.

4. Discussion

The goal of this study was to create (by molecular breeding) a transgenic microorganism that was by itself capable of producing LA from raw lignocellulosic material, especially wood biomass. Multiple studies have described the combined use of bacteria and fungi to ferment lignocellulosic materials to LA (Hasunuma et al., 2013); however, these processes typically require the removal of lignin from lignocellulose by physico-chemical pretreatment as well as supplementation with large amounts of saccharification enzymes. Some fungi secrete their own extracellular polysaccharide hydrolyzing enzymes (e.g. cellulase), a situation that is preferable to the use of bacteria for this purpose. Nonetheless, it is necessary to introduce the ligninolytic ability into the fungi to permit the production of LA from lignocellulose, because the lignin in the substrate materials resists and inhibits saccharification. In our previous study, we attempted to improve the ethanol productivity and ligninolytic activity of *Gloeophyllum trabeum*, a brown-rot fungus that does not secrete a ligninolytic enzyme system, by heterologous expression of a laccase-encoding gene (Arimoto et al., 2015). Ligninolytic activity and ethanol productivity were enhanced in the laccase-overexpressing transformant, but yields remained quite small. These results suggested that providing effective (white-rot fungus-like) ligninolytic activity to non-ligninolytic fungi would require not only the introduction of lignin-degrading enzymes (e.g., laccases and peroxidases) but also the incorporation of accessory

enzymatic systems (such as those required for hydrogen peroxide production and for quinone-redox cycling systems) (Dashtban et al., 2010). Additionally, ligninolytic mechanisms in white-rot fungi remain incompletely understood. Therefore, it has proven difficult to endow non-ligninolytic filamentous fungi with ligninolytic functions, given the need to co-transform and co-express multiple genes encoding lignin-degradation-related enzymes. On the other hand, white-rot fungi already encode the lignin- and polysaccharide-degrading functions needed for wood decay, albeit with some differences among organisms. Thus, we decided to introduce an LDH-encoding gene into *P. sordida* YK-624, a white-rot fungus capable of ethanol fermentation but lacking LA fermentation. In the first part of this study, we investigated whether heterologous expression of an LDH-encoding gene in this white-rot fungus was sufficient to permit LA production. Then, it was demonstrated that the transformed white-rot fungus has produced L(+)-LA by the enzymatic analysis. This experiment employed a bacterial *ldh* that was relatively easy to handle, specifically using a synthetic version of the *B. longum ldh* previously used for heterologous LDH expression in yeast (Ishida et al., 2005). Therefore, in the present study, we examined the influence of ethanol fermentation on LA production by a *Blldh*-transformed white-rot fungus.

According to the JGI fungal genome portal MycoCosm (http://genome.jgi.doe.gov/), two species (*P. chrysosporium* and *P. carnosa*) related to *P. sordida* YK-624 do not harbor an *ldh* homolog (Suzuki et al., 2012; Ohm et al., 2014). We therefore presumed that *P. sordida* YK-624 would have little or no endogenous LA fermentation capability. Indeed, we could not detect LA accumulation in the culture medium of strains lacking the heterologous *ldh* gene. Introduction of the heterologous *Blldh* gene was sufficient to endow the white-rot fungal host with the ability to ferment LA.

A previous study reported that inactivation of *Saccharomyces cerevisiae PDC1* enhanced the efficiency of LA production by a yeast expressing a heterologous *ldh* (Ishida et al., 2005). Therefore, we attempted knockdown of *Pspdc* (by *RNA*i) simultaneously with the introduction of *Blldh* into *P. sordida*. This experiment yielded LA-producing transformants with impaired ethanol production. One such transformant, designated PigBL64, was chosen for further evaluation based on its increased LA productivity and decreased ethanol productivity. Under semi-aerobic conditions, this isolate exhibited lower glucose consumption and lower ethanol yield than isogenic strains lacking the heterologous *ldh* gene. LA yield from this strain effectively peaked and plateaued from day 4 to 12 of cultivation. Although the RNAi method was successful in enhancing LA productivity while decreasing ethanol accumulation, the accumulation of LA did not increase greatly after 4 days of incubation. This observation suggested that the transformant began to metabolize LA under anaerobic conditions. Given that individual calcium ions chelate pairs of LA molecules, thereby preventing LA metabolism by polymerization or degradation, we tested the effect of

adding $CaCO_3$ to the fermentation culture. Supplementation with 0.2% $CaCO_3$ provided a significant ($p < 0.01$) increase in the level of LA accumulation at day 12 of incubation, yielding a LA concentration 1.5-fold that obtained in the absence of $CaCO_3$. However, $CaCO_3$ supplementation did not provide a significant change in the level of ethanol accumulation ($p = 0.46$). In LA fermentation, the addition of $CaCO_3$ usually has been used to buffer against LA accumulation by forming calcium lactate, and has been shown to enhance the accumulation of LA and other organic acids in microbial cultures (e.g. Liaud et al., 2015). Additionally, Takao (Takao, 1965) reported that some white-rot fungi accumulate several organic acids in the presence of $CaCO_3$; that work also indicated that white-rot fungi could decompose organic acids when cultured under acidic conditions.

LA accumulation in the fluid obtained from the fermented wood with PigBL64, was observed both with and without the inclusion of cellulase, although LA levels were significantly elevated with SSF compared to those obtained without cellulase saccharification (Fig. 5B). Indeed, LA accumulation with SSF was similar to that obtained by fermentation in glucose medium. These results indicated that the co-transformant PigBL64 is unable to secrete sufficient cellulase activity for wood saccharification during this LA fermentation process. In this experiment, we chose 50-days aerobic cultivation for delignification. Kamei et al. (2012) used a same cultivation method for direct ethanol fermentation from wood using white-rot fungus, *Phlebia sp*. And they have described that *Phlebia* sp. produced more ethanol if the delignification had progressed. Accordingly, the LA fermentation reaction was started after sufficient delignification, even in this present study. The transgenic white-rot fungus, PigBL64 has obtained a LA-production ability, while maintaining the delignification ability after transformation. However, polysaccharide degradation and mycelial growth was markedly lowered. It was suggested that the introduction of *blldh* and/or *PDC*i has given some harmful effects to the fungus. Although we achieved direct LA production from wood by using transgenic white-rot fungus in this study, the productivity has been too low for industrial applications. It is likely that the low fermentation ability of *P. soridida* YK-624 is a major factor (Wang et al., 2016). Further, there is a possibility that the bacterial LDH is not suitable for white-rot fungi. Therefore, in order to improve the production level of this technology for industrial use, it is necessary to select of the white-rot fungus having the higher wood-degrading and fermentation abilities as host, and to find an ideal LDH gene which is able to express the high activity in the host fungus.

5. Conclusions

Lignocellulosic materials can be used as a substrate for LA fermentation, and previous reports have described methods for LA production from wood that has been pretreated by delignification or by lignin modification using physico/chemical treatment (Wee et al., 2006; Zhang et al., 2007). However, there are, to our knowledge,

no previous reports of microorganisms capable of LA fermentation directly from raw wood. In pure culture, white-rot fungi are capable of mineralizing the lignin in wood, and these fungi are the most efficient lignin degraders in nature. Therefore, we evaluated the potential use of *P. sordida* YK-624—one of the best lignin-degrading organisms—for LA fermentation from woody biomass. In a first step, delignification was performed by cultivating PigBL64 (our YK-624 derivative) under aerobic conditions in woody culture containing only beech wood and water. In a second step, fermentation was performed by adding liquid medium (with or without cellulase) to the woody culture and incubating the mixture under semi-aerobic conditions. In the third step, LA production in the spent medium was assessed by HPLC. Finally, we demonstrated the direct one-pot LA production from wood by consecutive biological delignification and fermentation using a single transgenic microorganism. And it has been indicated that transgenic white-rot fungi have a potential use for development of simple/easy applications for wood biorefinery.

Authors's contribution

Conceived and designed the experiments: TM, HKak, HH. Performed the experiments: TM, HKak, TS. Analyze the data: TM, HKaw, HH. Wrote the paper: TM, HH. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

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Figure captions

Figure 1. Restriction maps of p*GPDpro-blldh* and p*PDC*i. The procedures used to construct both plasmids are described in the Materials and Methods section, and also are respectively illustrated in Fig. S1 and S2.

Figure 2. Lactate production at 4 days by (A) gBL and (B) PigBL strains under semi-aerobic conditions. Data points represent means and standard deviations (n=3).

Figure 3. Time courses of substrate and product during LA fermentation by *P. sordida* YK-624 (triangle), transformant strain U (circle), and gBL10 (rhombus) under semi-aerobic conditions. (A) LA production, and (B) ethanol production. Data points represent means and standard deviations (n=3).

Figure 4. Time course of fermentation products and gene expression by co-transformant PigBL64 during cultivation under semi-aerobic conditions. Production of LA (black rhombus) and ethanol (black circle), and consumption of glucose (gray square) and oxygen (white triangle) in the culture of co-transformant PigBL64 (A) without or (C) with the addition of $CaCO_3$ under semi-aerobic conditions. (B) RT-PCR analysis of *actin*, *Blldh*, and *PDC*i expression. Data points represent means and standard deviations (n=3). (D) Fungal weight during cultivation in the culture of PigBL64 with (white) or without (black) $CaCO_3$.

Figure 5. Direct lactate production from wood meal by using co-transformant PigBL64. (A) Scheme of direct lactate fermentation method from wood meal by using transgenic white-rot fungus. (B) Production of lactate and ethanol from wood meal. Data points represent means and standard deviations (n=3).

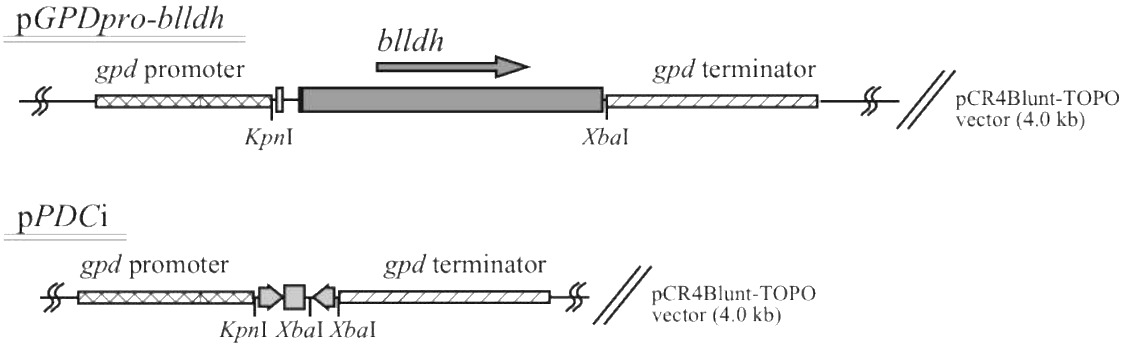
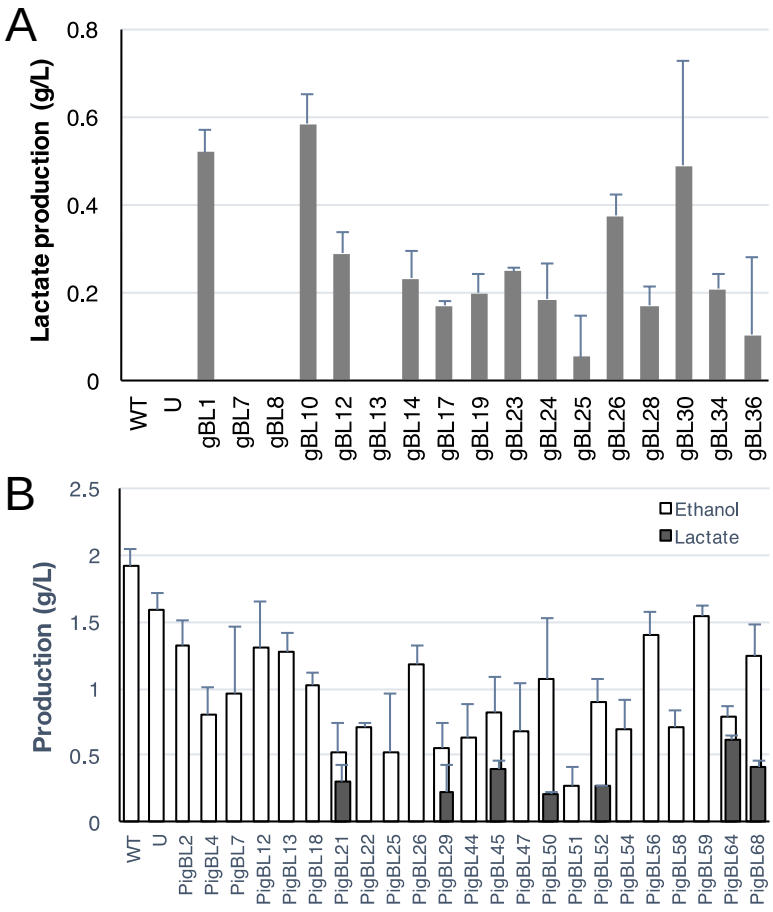

pGPDpro-blldh
blldh
gpd promoter
gpd terminator
KpnI
XbaI
pCR4Blunt-TOPO
vector (4.0 kb)
pPDCi
gpd promoter
gpd terminator
KpnI XbaI XbaI
pCR4Blunt-TOPO
vector (4.0 kb)

Fig. 1


A
Lactate production (g/L)
0.8
0.6
0.4
0.2
0
WT
U
gBL1
gBL7
gBL8
gBL10
gBL12
gBL13
gBL14
gBL17
gBL19
gBL23
gBL24
gBL25
gBL26
gBL28
gBL30
gBL34
gBL36
B
Production (g/L)
2.5
2
1.5
1
0.5
0
Ethanol
Lactate
WT
U
PigBL2
PigBL4
PigBL7
PigBL12
PigBL13
PigBL18
PigBL21
PigBL22
PigBL25
PigBL26
PigBL29
PigBL44
PigBL45
PigBL47
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PigBL58
PigBL59
PigBL64
PigBL68

Fig. 2

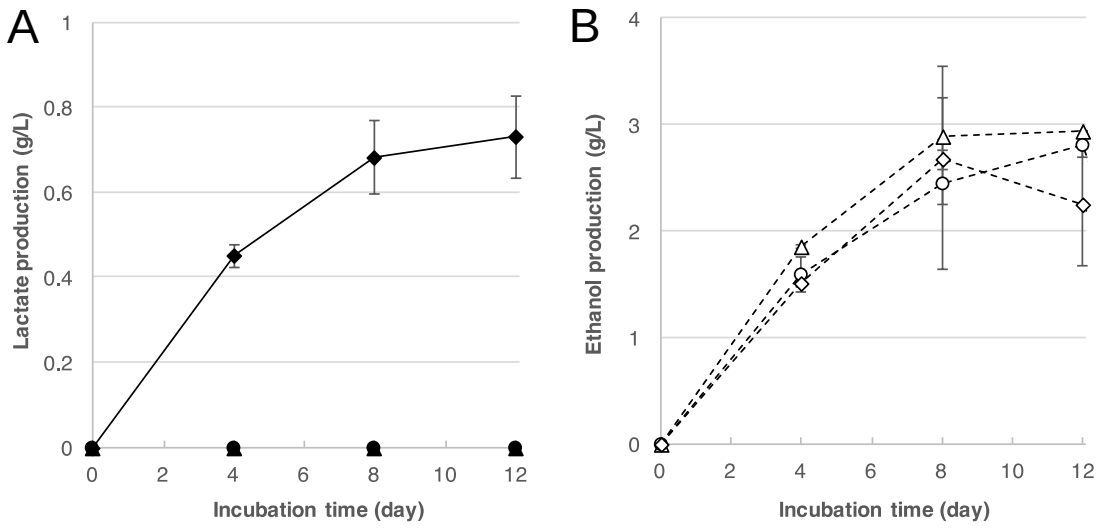
A
Lactate production (g/L)
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
Incubation time (day)
B
Ethanol production (g/L)
4
3
2
1
0
0
2
4
6
8
10
12
Incubation time (day)

Fig. 3

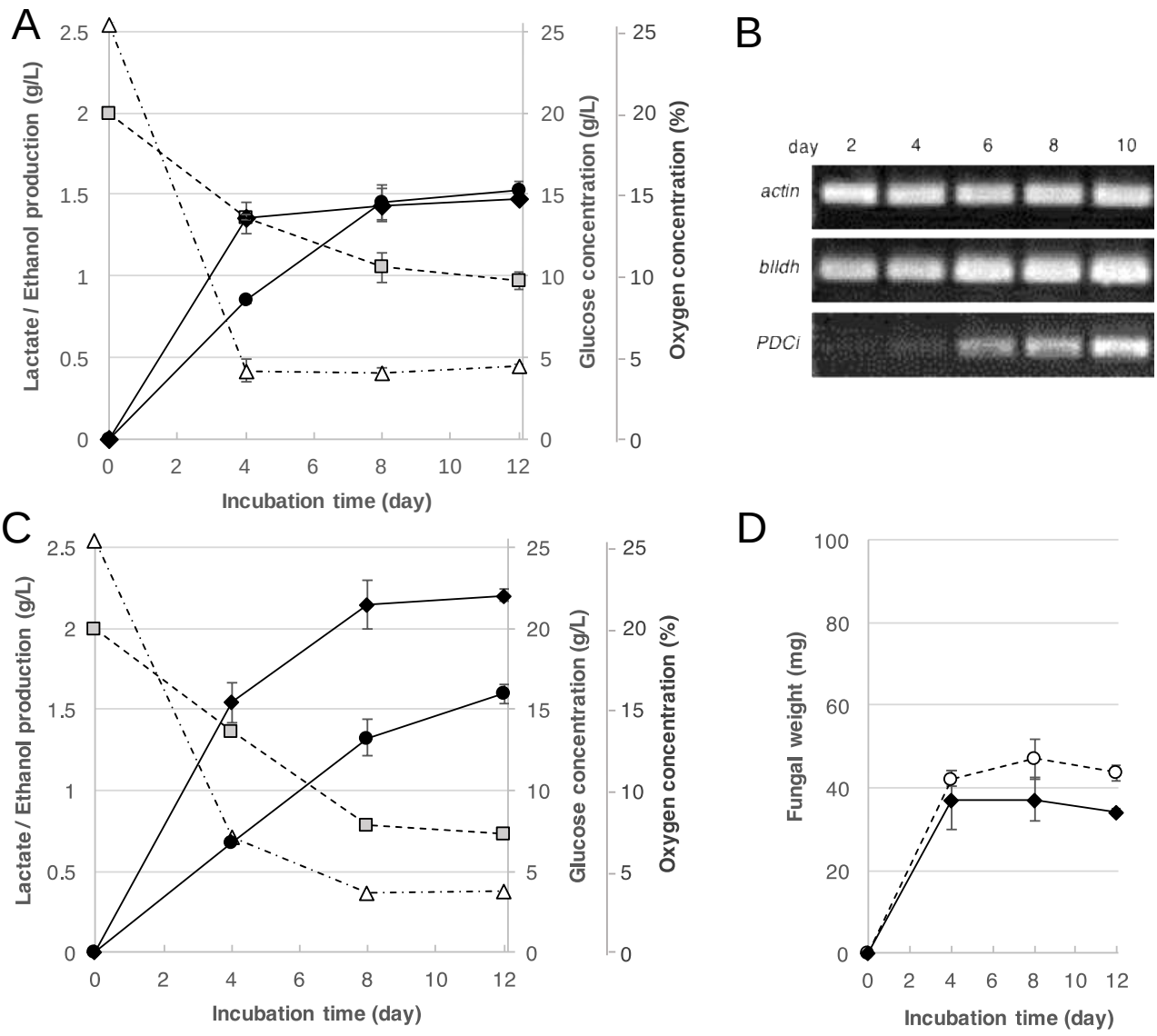
A
Lactate / Ethanol production (g/L)
Glucose concentration (g/L)
Oxygen concentration (%)
Incubation time (day)
B
day 2 4 6 8 10
actin
bldh
PDCi
C
Lactate / Ethanol production (g/L)
Glucose concentration (g/L)
Oxygen concentration (%)
Incubation time (day)
D
Fungal weight (mg)
Incubation time (day)

Fig. 4

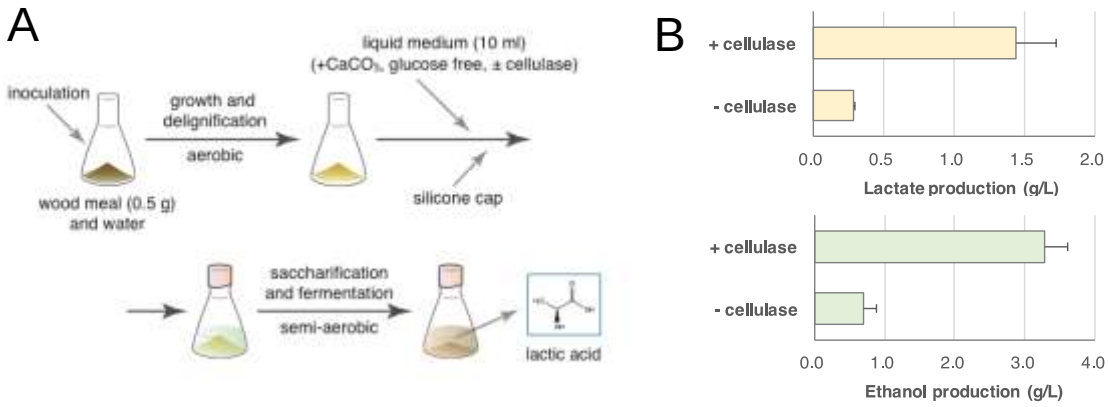
A
liquid medium (10 ml)
(+CaCO3, glucose free, ± cellulase)
inoculation
growth and
delignification
aerobic
silicone cap
wood meal (0.5 g)
and water
saccharification
and fermentation
semi-aerobic
lactic acid
B
+ cellulase
- cellulase
0.0
0.5
1.0
1.5
2.0
Lactate production (g/L)
+ cellulase
- cellulase
0.0
1.0
2.0
3.0
4.0
Ethanol production (g/L)

Fig. 5

Title:

Direct lactic acid production from beech wood by transgenic white-rot fungus *Phanerochaete sordida* YK-624

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Table S1. Oligonucleotides used as primers in this study

Primer name	Sequence (5'-3')[a]	Specificity
gpdpt-F	GGTACCATGCCGGTGAGCACACAG	*Kpn*I + *GPD* (1061-1078)
gpdpt-R	GACGGTGGTCTCCGCGTTGATTCCTGCTTTGACCTG	*blldh* 5'-terminal + *PsGPD* (1119-1139)
ldh-F	AAAGCAGGAATCAACGCGGAGACCACCGTCAAG	*PsGPD* 2nd intron + *blldh* (4-21)
ldh-R	TCTAGATTCAGAAGCCGAACTGGGCAG	*Xba*I + *blldh* (944-963)
sence-F	GGTACCATGCAGGTCGCCGACCAG	*Xba*I + *PsPDC* (1-18)
pdc-F	ATGCAGGTCGCCGACCAG	*PsPDC* 5'-terminal
pdc-R	TCACAGCGTGTCGCTCGC	*PsPDC* 3'-terminal
sence-R	TCTAGACCGAGGTTGAAGTCTCCAG	*Kpn*I + *PsPDC* (160-178)
anti-F	TCTAGACCGTGACCTTGAGCTGGAC	*Xba*I + *PsPDC* (73-91)
anti-R	TCTAGAATGCAGGTCGCCGACCAG	*Xba*I + *PsPDC* (1-18)
ldh-rtF	AGATCGTCCTCGAGGACATC	*blldh* (104-123)
ldh-rtR	GATCGCCTTGAGGATGTTGAC	*blldh* (304-324)
rnai-cF	GGGTACTTGTACAATCCTCC	*PsGPD* (2563-2584) terminator region
rnai-cR	GTGCCTGGAGACTTCAAC	*PDC*i (156-173)
act0-rtF	AGCACGGTATCGTCACCAAC	Misumi et al. (2001)
act0-rtR	AGCGAAACCCTCGTAGATGG	Misumi et al. (2001)
pdc-rtF	CAACGACATCATCAACTGGTG	*PsPDC* (1494-1514)
pdc-rtR	GTCGTGCATCGGCATCATC	*PsPDC* (1659-1677)
rnai-rtF	AAGCAGCGAGGATTGTACC	*PsGPD* (889-907) promoter region
rnai-rtR	GTTGAAGTCTCCAGGCAC	*PDC*i (156-173)

a. Restriction site are underlined.

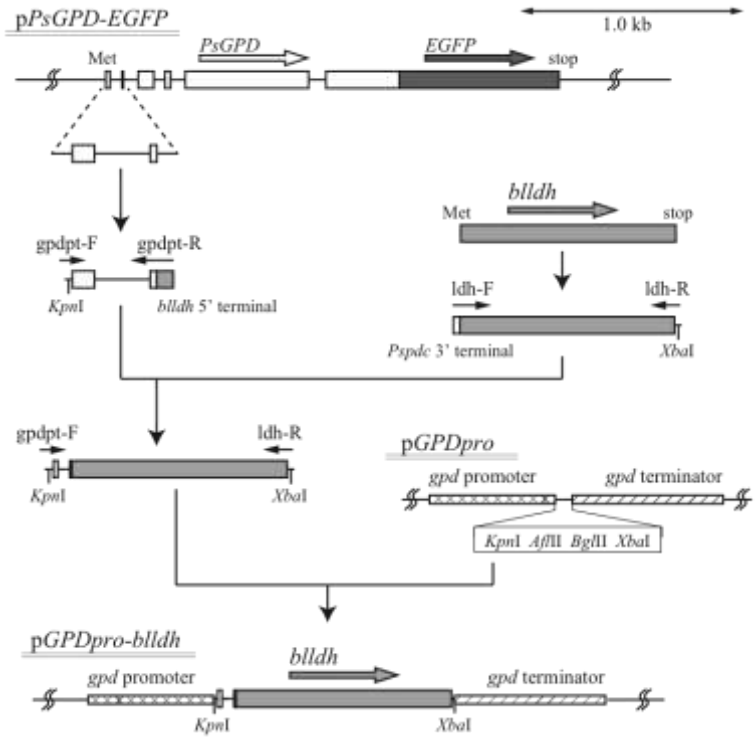

Fig. S1.

Construction of *ldh* expression plasmid p*PsGPD-blldh.* The horizontal arrows indicate the locations and directions of the primers. Plasmids p*PsGPD-EGFP* and p*GPDpro*, generated previously (Yamagishi et al., 2007; Suzuki et al., 2014), were used for the construction of p*GDPpro-blldh.* The *blldh* expression vector was constructed by a series of PCR procedures using the primers listed in Table S1. Primers gpdpt-F and gpdpt-R were designed to amplify a *P. sordida PsGPD* (GenBank: AB285023) fragment containing the 5'-upstream region, the first exon and first intron, and a portion of the second exon. These primers also contained a *Kpn*I site and 15 bases of the *blldh* 5'-terminal sequence. For enhancing gene expression, a *PsGPD* first intron was placed upstream of the *blldh* coding sequence (Jeon et al., 2000; Scholtmeijer et al., 2001). *Blldh* was amplified with primer pair blldh-F and blldh-R, which were engineered to contain 15 bases of the *PsGPD* second exon and a *Xba*I site at the respective 5'-terminus. The engineered amplicons were connected by joint PCR with gpdpart-F and blldh-R. The joined amplicon was digested by *Kpn*I and *Xba*I. After that, the digested fragment was inserted between the KpnI and XbaI sites of p*GPDpro*.

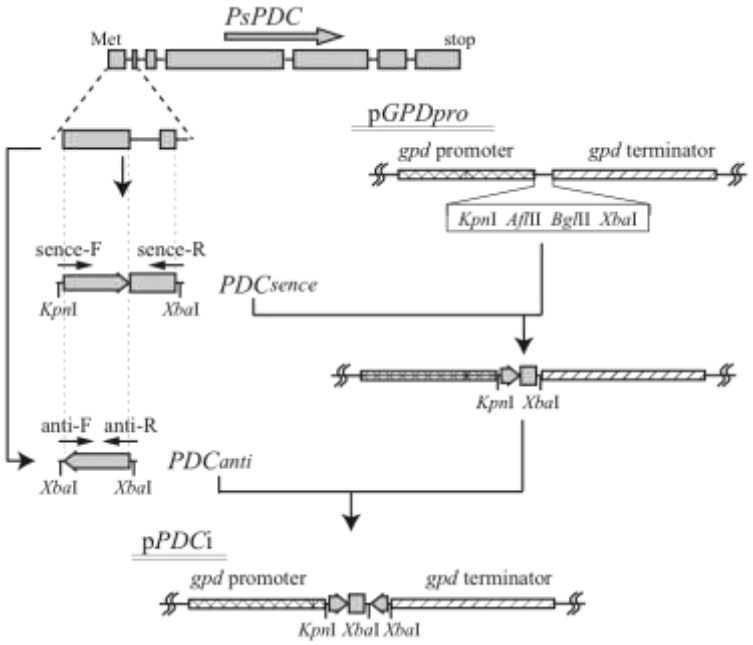

Fig. S2.

Construction of plasmid p*PCD*i. The horizontal arrows indicate the locations and directions of the primers. The *P. sordida* YK-624 *PDC* gene (*PsPDC,* GenBank LC057149) was amplified by inverse PCR using several primers designed to be complementary to the *pdc* of *P. chrysosporium* in the database of the Joint Genome Institute (JGI), and finally primer set pdc-F1 and pdc-R1 (Table S1) was used for determination of the full-length gene encoding *PsPDC*. *PsPDC* silencing vector p*PDCi* directed transcription of an intron-containing hairpin RNA. Two *pdc* fragments, PDC_{sense} and PDC_{anti}, were amplified from *PsPDC* using the primers sense-F/sense-R and anti-F/anti-R, respectively. The PDC_{sense} fragment containing the first and second exon, and the first intron of the *PsPDC* was digested and inserted between the *Kpn*I and *Xba*I sites of the plasmid p*GPDpro.* Then, the PDC_{anti} fragment was inserted into *Xba*I sites of p*GPDpro-*PDC_{sense}.

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