Ethanol fermentation by saprotrophic white-rot fungus Phanerochaete sordida YK-624 during wood decay as a system for short-term resistance to hypoxic conditions

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Ethanol fermentation by saprotrophic white-rot fungus *Phanerochaete sordida* YK-624 during wood decay as a system for short-term resistance to hypoxic conditions

Short-title

Function of ethanol fermentation by *P. sordida*

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ABSTRACT

In this study, major factors involved in regulating ethanol production from wood by the saprotrophic white-rot fungus *Phanerochaete sordida* YK-624 were investigated. *P. sordida* YK-624 produced ethanol from wood meal culture without the addition of any nutrients, and ethanol was produced from wood culture only when the oxygen concentration in headspace was reduced to ≤5%; thereafter, ethanol production ceased within a few days. Analyses of gene expression during aerobic incubation indicated that *P. sordida* simultaneously upregulates the glycolytic pathway from sugar uptake to pyruvate conversion during ethanol fermentation and suppresses pyruvate influx into the TCA cycle. Upon termination of ethanol fermentation, the expression of all tested genes was repressed, and the fungus ceased to grow. In contrast, the fungus could utilize ethanol for aerobic growth. These results suggest that ethanol fermentation by *P. sordida* functions as a short-term stress response system under anaerobic conditions during wood decay, enabling the fungus to rapidly resume growing when oxygen is supplied (e.g., following breakdown of plant cell walls or removal of the fungus from water immersion). This is the first report to describe the physiologic significance of ethanol fermentation in saprotrophic white-rot fungi.

INTRODUCTION

White-rot fungi possess the ability to decompose lignocellulosic materials such as plants and degrade all major plant components. Ligninolytic activity, a characteristic unique to white-rot fungi, refers to the enzymatic degradation of lignin, a major plant component. Lignin, an aromatic polymer that is recalcitrant to biological and chemical degradation, protects the plant structures against various environmental stresses. Degradation of lignin via the activity of white-rot fungi improves the access of cellulolytic enzymes to cellulose fibers (1, 2). Therefore, despite consumption of polysaccharides during treatment with white-rot fungi, the application of white-rot fungi for delignification pretreatment in lignocellulosic biorefinery processes is anticipated.

A number of other studies have reported that white-rot fungi can ferment ethanol directly from lignocellulosic materials (3-7). In these studies, white-rot fungi were found to produce ethanol from lignocellulosic materials supplemented with a nutrient medium under oxygen-limiting conditions and without any additional supplements, such as cellulases or yeast. A number of previous studies also reported ethanol production by several white-rot fungi of differing taxa, such as *Ceriporia* (Irpicaceae), *Peniophora* (Peniophoraceae), *Phlebia* (Meruliaceae), *Punctularia* (Corticiaceae), and *Schizphyllum* (Schizophyllaceae), in addition to *Phanerochaete* (Phanerochaeteceae) and *Trametes* (Polyporaceae). Two studies showed that a majority of white-rot fungi can produce ethanol from glucose, but only few strains could convert lignocellulosic materials into ethanol (5, 7). Phlebioid fungi, particularly *Phlebia* sp. MG-60 and *Phlebia radiata*, can efficiently produce ethanol from lignocellulosic materials (7, 8). However, no studies have discussed the significance of ethanol fermentation by white-rot fungi with

regard to their lifecycle or how fermentation contributes to wood decay. Therefore, in the present study, we investigated the major factors involved in regulating ethanol fermentation by a typical saprotrophic white-rot fungus, *Phanerochaete sordida* YK-624, which exhibits superior ligninolytic activity (9). In addition, we attempted to elucidate the biological significance of ethanol fermentation by *P. sordida* YK-624.

MATERIALS AND METHODS

Evaluation of ethanol production in liquid medium and wood culture

Phanerochaete sordida YK-624 (ATCC 90872) was grown on PDA medium at 30°C. Three mycelial discs (1.0 cm diameter) were placed in vials containing beech wood meal culture (0.5 g of dry wood meal [80-100 mesh] and 1.25 mL of water). The culture vials were incubated for 20 days under aerobic conditions at 30°C to allow for delignification. After aerobic cultivation, the culture vials were sealed to create semi-aerobic conditions. Before sealing, the vials were supplemented with 5 mL of glucose-free basal liquid medium (gf-BLM; 10 g/L yeast extract, 10 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ [pH 4.5]) (3). For nutrient supplementation-free conditions, the PDA pellets were removed from the wood culture vials before initiating semi-aerobic cultivation to exclude effect of the PDA medium. After 10 days of semi-aerobic cultivation, the culture broth was recovered from the vials containing gf-BLM and centrifuged (16,800 × *g*, 4°C, 10 min). For un-supplemented cultures, 3.75 mL of 20 mM sodium phosphate (pH 6.8) was added, and the fungi were homogenized (on ice) using a Polytron homogenizer (PT1200E, Kinematica AG). The homogenate was then centrifuged (16,800 × *g*,

4°C, 10 min) and the supernatant collected. The supernatants obtained from both vials were filtered through a membrane filter (0.2 μm pore size) and adjusted to 0.5 mM H_2SO_4, after which the samples were analyzed by high-performance liquid chromatography (HPLC). HPLC analyses were performed using a JASCO PU-2089 pump equipped with a JASCO RI-2031 detector fitted with a Shodex SH 1821 column (8.0 mm × 300 mm, Showa Denko K.K., Japan) maintained at 75°C. The eluent was 0.5 mM H_2SO_4 at a flow rate of 0.6 mL/min, and the concentration of ethanol was measured using a refractive index detector, as reported previously (10).

Aerobic wood culture (for 5, 20, or 40 days) of *P. sordida* YK-624 was performed as described above. The mycelial discs were removed from the wood culture vials, and the vials were sealed. The concentration of oxygen in the headspace was analyzed every 4 days via gas chromatography with a thermal conductivity detector (GC-TCD; GL Science GC-3200) equipped with a packed column (Molecular Sieve 5A 30/60, 3 m × 1.6 mm outer diameter), as described in a previous study (11). The operational injector, detector, and column oven temperatures were 80, 80, and 50°C, respectively. Argon was used as the carrier gas at a flow rate of 35 mL/min. After incubation, 3.75 mL of 20 mM sodium phosphate (pH 6.8) was added to each vial, and the cultures were homogenized. The resulting homogenate was centrifuged (16,800 × *g*, 4°C, 10 min), and the supernatant was filtered through a membrane filter. For simultaneous analysis of glucose and ethanol concentration, HPLC analysis fitted Shodex KS-801 column were performed (eluent: Milli-Q water, flow rate: 1.0 mL/min, column temp. 80°C).

To evaluate lignin degradation, wood meal cultures were dried overnight at 105°C after aerobic

incubation, and the weight loss and lignin content (sum of acid-soluble and -insoluble lignin) were determined (9).

To investigate the relationship between ethanol fermentation and oxygen/glucose concentrations, two mycelial discs were inoculated into 10 mL of gf-BLM and incubated for 3 days at 30°C under ambient atmospheric (aerobic) conditions. The mycelial mats were recovered and the discs discarded. After washing with distilled water, the mycelial mats were homogenized with 10 mL of distilled water, and 200 µL of mycelial homogenate (1.5 mg dry weight) was added to a vial containing 5 mL of fresh gf-BLM. This process ensured the elimination of any pre-cultivation medium. After 5 days of aerobic incubation, the vials were tightly sealed with butyl rubber plugs. The headspace gas of the cultures was replaced with a mixture of oxygen and nitrogen (1.5%, 5%, 7.5%, 12%, or 21% oxygen gas in nitrogen). After an additional 24 h of incubation, 100 µL of glucose solution was added to the vials using a micro syringe to adjust the final concentration to 0.05, 0.1, 0.3, 0.6, or 1.0 g/L. After 24 h, the oxygen concentration in the headspace was determined using a GC-TCD, whereas ethanol production and residual glucose in the culture medium were measured using HPLC.

Transcriptional analysis

Phanerochaete sordida YK-624 was cultivated on wood medium for 0, 4, and 10 days under semi-aerobic conditions following 5 days of aerobic culture. The wood culture was mixed uniformly, and 200 mg of wet wood culture was collected and stored at −80°C until further use. Total RNA was extracted from the culture sample by bead beating in 1.0 mL of Plant RNA Purification Reagent (Invitrogen) and

then purified using an RNeasy Mini kit (Qiagen). cDNA was synthesized from 100 ng of total RNA by reverse transcription (RT) using a PrimeScript RT-PCR kit (TaKaRa). Quantitative PCR (qPCR) was performed on a LightCycler Nano system (Roche) using SYBR Premix ExTaq II (TaKaRa). Gene-specific primers for actin (LC377777, *act*; 5'-ccctcaagcatgaaggtcaag-3'/5'-tagaagcacttgcggtggac-3'), hexose transporter (LC438459, *hxt*; 5'-ttcttcatctggggcacgac-3'/5'-gcgtcgagttctggtagagg-3'), glyceraldehyde-3-phosphate dehydrogenase (AB285023, *gpd*; 5'-gcaggaatcaacggtttcgg-3'/5'-tcgatgaaggggtcgttgac-3'), pyruvate dehydrogenase E3 subunit (LC377778, *pdhE3*; 5'-gtctcaagaccgcctgcattg-3'/5'-gacgccctcgacttcgatac-3'), pyruvate dehydrogenase kinase (LC377779, *pdhk*; 5'-gaaacgaagctgcaaagcca-3'/5'-gagagccacatgttgaccga-3'), pyruvate decarboxylase (LC057149, *pdc*; 5'-ggtcacggaaatctttggcg-3'/5'-cgttcagctcgttgcagttt-3'), and manganese peroxidase (AB585997, *mnp*; 5'-cgacccttaccactgatcc-3'/5'-gctagctgttgactaagcgaag-3') were designed previously (12-15). Primers for β-glucosidase (*bgl*; 5'-attcgcaacatcactggggt-3'/5'-cgcaacatagggatgggtgt-3'), endoglucanase (*egl*; 5'-gtccccgacatctacaacgg-3'/5'-tagggagaggcgagcgatat-3'), and exoglucanase (*exg*; 5'-gatcgggcaggtcgtagatg-3'/5'-actttcacgtggctggactc-3') were designed from the sequences LC557126–LC557128 obtained from total RNA extracted from a wood culture of *P. sordida* YK-624. These DNA sequences were determined by thermal asymmetric interlaced (TAIL)–PCR using degenerate primers (16, 17), then cDNA sequences were determined. Fold-change in gene expression was calculated by determining the difference between the Cq values for the expression of each gene after 4 or 10 days of semi-aerobic cultivation and on day 0. Relative expression was calculated according to the ΔΔCq method using *gpd* as the reference gene. Because *P. sordida* YK-624 expresses several alcohol

dehydrogenase (ADH)-like genes during wood decay (18), it is not clear which ones are mainly involved in ethanol fermentation. Therefore, ADHs were not used for transcriptional analysis in the present study.

Viability under semi-aerobic conditions

After 5 days of aerobic culture, decayed wood meal was collected from the 0-, 5-, 10-, and 15-day-old semi-aerobic wood cultures of *P. sordida* YK-624 and inoculated into PDA medium. Mycelial growth was then measured for 2 weeks.

Ethanol utilization test

Mycelial homogenate was prepared using BLM (gf-BLM supplemented with 2% glucose) as described above and then inoculated into 5 mL of fresh gf-BLM. The medium was incubated at 30°C for 5 days under aerobic conditions and then divided into three groups: aerobic, semi-aerobic, and anaerobic. For the aerobic group, ethanol was added to the culture and incubated under the same aerobic conditions. For the semi-aerobic group, ethanol was added to the culture, and the vial was tightly sealed. To create anaerobic conditions, the culture vial was sealed and the headspace was filled with nitrogen gas. Ethanol was then added to the culture using a syringe. The ethanol concentration was adjusted 0.25% for all cultures. All culture groups were incubated for 2, 5, and 7 days at 30°C under static conditions. After incubation, the mycelial mat was separated from the fluid by filtration, dried in an oven, and weighed. The residual volume of ethanol in the filtrate and oxygen in the headspace were measured using HPLC and GC-TCD, respectively. An aerobic culture without ethanol supplementation served as

the control.

Statistical analysis

The Student's *t*-test was used to assess the significance of differences between two samples. For multiple comparisons, one-way analysis of variance (ANOVA) was carried out to determine the significance of differences. Statistical analyses were performed using Tukey's method. Differences between mean values considered statistically significant at a confidence level of 5% ($P<0.05$).

RESULTS

Fermentation of ethanol from wood by P. sordida *YK-624*

Aerobic pre-incubation on wood was carried out for a period of 5, 20, and 40 days, followed by semi-aerobic incubation of sealed cultures for 10 days. Semi-aerobic incubation was carried out in nutrient-free medium or supplemented gf-BLM. As shown in Table 1, *P. sordida* YK-624 produced ethanol from wood regardless of nutrient supplementation in cultures pre-incubated for 20 or 40 days. However, only 4.2 mg of ethanol was produced from 1 g of wood in the 5-day pre-incubated culture, which showed the highest ethanol production of the three pre-incubation periods. The concentration of oxygen remaining after semi-aerobic incubation was <2% in all cultures.

Next, the time (in days) required for ethanol production and oxygen consumption during semi-aerobic incubation of the cultures pre-incubated aerobically for 5 days and 20 days (designated as 5A and 20A, respectively) was investigated (Fig. 1A and B). During semi-aerobic incubation of culture 5A,

oxygen consumption was high, with the concentration falling from 3% on day 2 to <1% on day 4. In contrast, the rate of oxygen consumption of culture 20A during semi-aerobic incubation was lower than that of culture 5A, as the oxygen concentration decreased to <5% after 4 days, and the conditions became anaerobic after 8 days of semi-aerobic incubation. Ethanol production in culture 5A was 0.6 mg/g of wood after 2 days of semi-aerobic incubation, whereas culture 20A required 8 days of semi-aerobic incubation to produce measurable ethanol. Ethanol production continued in both cultures for a short period (4 days or less) before ceasing. Free glucose was detected in both wood cultures throughout the semi-aerobic incubation period. In both cultures, the glucose concentration remained at approximately 1–2 mg/g of wood until all of the oxygen was consumed or the fermentation process stopped, after which the glucose concentration increased. This result indicates that the saccharification enzymes were still active after termination of fermentation while cellular activities such as sugar uptake and fermentation were suspended. The rate of increase in glucose concentration in culture 20A was higher than that in culture 5A, suggesting that cellulolytic activity was higher in culture 20A.

A flock of mycelia was collected from culture 5A every 5 days during the semi-aerobic incubation period and inoculated onto PDA medium. Mycelia collected between days 0 and 5 of the semi-aerobic cultures were able to regrow on PDA, whereas mycelia collected between days 10 and 15 of the semi-aerobic cultures exhibited no re-growth (Fig. S1).

Gene expression analysis

All genes examined in this study were expressed in wood cultures of *P. sordida* YK-624 under

aerobic conditions. The expression of genes related to wood decay, respiration, and fermentation in *P. sordida* YK-624 wood cultures incubated for 5 days under aerobic conditions followed by 0, 4, and 10 days of incubation under semi-aerobic conditions (designated 5A0S, 5A4S, and 5A10S, respectively) was analyzed using qPCR. The change in Cq value for each gene with respect to the Cq value on day 0 is shown in Figure 2A. In the day 4 semi-aerobic culture (5A4S), the expression levels of *hxt*, *pdc*, and *pdhk* were significantly increased, whereas the expression levels of *act*, *bgl*, *exg*, *mnp*, and *pdhE3* decreased. No significant differences were observed in the expression levels of *gpd* and *egl* between cultures 5A0S and 5A4S. In contrast, the expression of all tested genes was significantly lower in culture 5A10S as compared with culture 5A0S. The change in the Cq value of *gpd* between cultures 5A0S and 5A4S was <1.0; therefore, the relative expression of the remaining genes was calculated using *gpd* as the reference gene, as shown in Figure 2B. The relative expression of *hxt* and *pdc* was >5-fold higher in culture 5A4S than culture 5A0S, whereas *pdhk* exhibited 2-fold higher relative expression in 5A4S compared with 5A0S. Figure 2B illustrates the dramatic decrease in the expression levels of other genes during the 4-day semi-aerobic incubation period.

Requirement of hypoxic conditions for ethanol fermentation

The relationship between ethanol fermentation and concentrations of glucose and oxygen in *P. sordida* YK-624 cultures was investigated using BLM. After mycelia reached confluence in gf-BLM, the headspace gas of the cultures was replaced with varying concentrations of oxygen. Following an additional 24 h of incubation, glucose solution was added to the cultures, and glucose consumption and

196 ethanol production were analyzed over a 24-h period. Although glucose consumption tended to be lower
197 under conditions of lower headspace oxygen concentration (Fig. 3A), all of the supplemented glucose
198 was consumed within 24 h, regardless of oxygen concentration in the headspace, in the case of lower
199 glucose concentrations (<0.5 g/L). Figure 3B and C shows that the amount and efficiency (yield) of
200 ethanol production increased with decreasing headspace oxygen concentration. In cultures supplemented
201 with a high concentration of glucose (0.5 or 1.0 g/L), ethanol production was observed even under
202 aerobic conditions, and ethanol production in cultures supplemented with a low concentration of glucose
203 (0.05–0.3 g/L) was only observed when the oxygen concentration was ≤5%. The efficiency of ethanol
204 production increased with decreasing oxygen concentration across all tested concentrations of glucose,
205 and the yields clearly increased when the oxygen concentration was ≤5%. Glucose concentration did not
206 have a clearly discernable effect on the efficiency of ethanol production.
207
208 *Ethanol utilization test*
209 The ability of *P. sordida* YK-624 to use ethanol for growth under conditions of nutrient depletion
210 was investigated at different oxygen concentrations (Fig. 4A). Figure 4B shows ethanol and oxygen
211 consumption by *P. sordida* YK-624. The dry weight of mycelia of *P. sordida* YK-624 aerobically
212 incubated in gf-BLM increased over the course of 5 days and thereafter gradually decreased. Therefore,
213 we concluded that the nutrients in gf-BLM were depleted after 5 days of incubation. In the case of
214 ethanol supplementation of gf-BLM cultures on the fifth day followed by aerobic incubation, however
215 some part of ethanol probably volatilized, mycelial weight increased over time and then decreased after

the ethanol was consumed. Semi-aerobic incubation following ethanol supplementation also resulted in an incremental increase in mycelial weight, but mycelial growth stagnated after oxygen declined to ≤5%. In addition, the rate of ethanol consumption in semi-aerobic cultures slowed as the oxygen concentration declined. During anaerobic incubation following ethanol supplementation, no consumption of ethanol or oxygen was observed, and mycelial weight was maintained. These results suggest that *P. sordida* YK-624 is able to utilize ethanol for growth under aerobic conditions.

DISCUSSION

Other studies reported that *Phlebia* sp. MG-60 and *Phlebia radiata* produced >300 mg/g and 100 mg/g of ethanol from oak wood and waste core board, respectively (7, 8). These special ethanol-producing fungi are also known as excellent lignin degraders (19, 20). As *Phlebia* sp. MG-60 was isolated from driftwood in mangrove stands (20), it is thought that this marine fungus can survive on wood immersed in sea water. Although *Phlebia radiata* is generally considered a saprotroph, the fungus is often isolated from areas of heart rot in wood (21). Based on these observations, it is presumed that these fungi can tolerate hypoxia, because they can live under low-oxygen conditions. In contrast, *Phanerochaete* spp. are saprotrophic fungi, and *P. sordida* YK-624 in particular exhibits excellent ligninolytic activity and selectivity under aerobic conditions (9). In the present study, *P. sordida* YK-624 exhibited evident ethanol production from wood alone (without the addition of nutrients), but compared with the productivity of *Phlebia* fungi described above, ethanol production by *P. sordida* YK-624 from woody biomass was quite low, regardless of nutrient supplementation (Table 1). We

hypothesized that this lower productivity level is not sufficient to generate the energy needed for survival under hypoxic conditions. Therefore, we attempted to elucidate the biological significance of ethanol production in *P. sordida* YK-624.

No ethanol was detected in culture 5A0S, which was grown under aerobic conditions, but ethanol production was clearly observed in culture 5A4S, which reached a nearly anaerobic state, and ethanol production ceased on 8 day or later in semi-aerobic cultivation (Fig. 1). However, it is difficult to investigate the status of a fungus based on residual enzymatic activities, because wood-degrading enzymes can retain activity for a long period of time, similar to the glucose production shown in Figure 1A and B. Additionally, quantification of cytosolic NAD(P)H/NAD(P)$^+$ and ATP/ADP for estimation of cellular redox and energy status of the fungus on wood culture is quite difficult, because the methods for exclusion of negative effect on analysis of various contaminants produced during wood decay have not been established and amount of mycelia on decay wood is probably too low. Therefore, a change in fungal metabolic status was suspected based on the relative gene expression levels during aerobic respiration (culture 5A0S), ethanol fermentation (culture 5A4S), and post-fermentation stagnation (culture 5A10S). In culture 5A4S, increased expression of *hxt*, *pdc*, and *pdhk* and decreased expression of the other genes examined was observed when compared with aerobic cultures (Fig. 2). The function of the *P. sordida* YK-624 genes *hxt*, which mediates the uptake of monosaccharides, and *pdc*, which is involved in ethanol production, have been reported (10, 15). It is well known that PDH is a key enzyme at the pyruvate branch point, catalyzing the conversion of pyruvate to acetyl-CoA and carbon dioxide, with PDHK inactivating PDH via dephosphorylation (22). Therefore, lower *pdhE3* and higher *pdc* and

pdhk expression indicate the response of the fungus to low-oxygen conditions, which causes a change in metabolism from aerobic respiration to anaerobic fermentation. Repression of *mnp* and other cellulolytic enzyme genes during the ethanol fermentation period suggests that *P. sordida* YK-624 ceases active wood decay and utilizes the monosaccharides provided by previous enzyme activity. In contrast, repression of *act* in culture 5A4S and repression of all of the tested genes in culture 5A10S indicate that hypoxic conditions induce fungal cell death. In the viability test, mycelia collected from cultures in which ethanol production had terminated showed no viability, whereas mycelia collected during ethanol fermentation regrew under aerobic conditions. These results suggest that *P. sordida* YK-624 carries out ethanol fermentation in order to survive for a few days under hypoxic conditions and that it can regrow if wood decay progresses and breaks down the plant cell walls to create aerobic conditions. Hence, ethanol fermentation by *P. sordida* YK-624 could be a stress response to anaerobic or low-oxygen conditions. Schaffer (23) studied the growth and survival of wood-rot fungi under low-oxygen conditions. In that study, heart rot fungi tolerated low-oxygen conditions and survived for longer periods than saprotrophs. The duration of survival of wood-rot fungi under low-oxygen conditions varied, with some species dying in less than 1 week. Therefore, the sensitivity of *P. sordida* YK-624 to low-oxygen conditions is likely a typical response of saprotrophic fungi.

The amount of ethanol produced by *P. sordida* YK-624 was affected by the concentrations of both glucose and oxygen, whereas the efficiency of ethanol production appeared to be affected by oxygen concentration alone (Fig. 3). Under anaerobic conditions (<5% oxygen), the amount of ethanol produced and the efficiency of ethanol production (yield) increased significantly with a reduction in oxygen

concentration. These results suggest that the fungus responds to low levels of oxygen (≤5%) via a change in metabolism from respiration to fermentation. This hypothesis is supported by data regarding ethanol production and gene expression in wood cultures when the oxygen concentration was decreased to <5%. It is well known that oxygen diffusion from the surface of wood is very limited. Covey et al. (24) reported that the internal oxygen concentration in dead wood from forests is so low as to be nearly anaerobic, regardless of the state of decay. In addition, van Keulen et al. (25) reported that even a thin layer of water (1–2 mm) can easily create hypoxic conditions. Finally, *P. sordida* YK-624 utilized ethanol for growth under oxic conditions, whereas it did not consume ethanol under anaerobic conditions (Fig. 4). There are several fungi utilizing ethanol as a carbon source. For example, Srivastava et al. (26) reported isolation of ethanol-utilizing fungi and they found that ADH and acetaldehyde dehydrogenases (ALD) play crucial role in the utilization of ethanol by fungus. It is also known *Saccharomyces cerevisiae* has a potential of ethanol utilization. In yeast cells, ethanol is metabolized to acetaldehyde by ADHs. Acetaldehyde is oxidized to acetic acid by ALDs and then acetate is conjugated to coenzyme-A by acetyl-CoA synthetases. Acetyl-CoA plays important roles in various metabolic pathway including energy production in TCA cycle (27).

The results of the present study suggest that *P. sordida* YK-624 initiates ethanol fermentation from free saccharides when hyphae are placed under oxygen-limiting conditions and that the fungus suspends respiratory metabolism to enhance tolerance to hypoxic stress. For example, when hyphae invade the inner region of a piece of dead wood or when the hyphae are immersed in water, the fungus consumes oxygen by respiration until conditions are hypoxic and then initiates ethanol fermentation.

296 Although *P. sordida* YK-624 can tolerate hypoxic stress without respiration for several days, eventually
297 the fungus will die under these conditions. However, if wood decay progresses from aerobic regions and
298 leads to the breakdown of the cell walls of the wood, resulting in replenishment, or alternatively, if the
299 hyphae are no longer immersed in water, the fungus probably starts to utilize ethanol for re-growth. It is
300 speculated that *P. sordida* YK-624 has evolved to specialize in aerobic sapwood decay, particularly with
301 regard to its ligninolytic properties, instead of developing sufficient tolerance for the hypoxic conditions
302 that would probably be needed for heart wood decay, as with *Phlebia*. This is thus the first report to
303 describe the physiological significance of ethanol fermentation by a saprotrophic white-rot fungus.
304 However, cellular energy and redox status during ethanol fermentation and termination period are still
305 not clear. In the future work, it is necessary to develop a method to investigate the cellular redox and
306 energy status under different oxygen condition during wood decay, in order to better understand the
307 physiological significance of ethanol fermentation of this saprotrophic white-rot fungus.
308
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390

391 **Figure Legends**

392 **Fig. 1** Time courses of ethanol production, oxygen consumption, and change in glucose concentration
393 during semi-aerobic incubation following 5 days (A) and 20 days (B) of aerobic incubation on wood
394 culture inoculated with *P. sordida* YK-624. Black circles and gray rhombi indicate ethanol and
395 glucose concentration in the wood culture (mg/g of wood), respectively. Oxygen concentration in the
396 headspace is represented by white squares. Values are mean ± standard deviation of triplicate cultures.

397 **Fig. 2** Fold-change in the expression of various genes during semi-aerobic incubation. (A) Difference in
398 the Cq value of each gene between wood cultures at the start of semi-aerobic incubation (day 0) and
399 day 4/10. Values are mean ± standard deviation of triplicate cultures. Asterisks indicate significant
400 differences compared with day 0 ($P < 0.05$). (B) Relative gene expression of day 4 cultures compared
401 with day 0 cultures. Relative expression was calculated according to the ΔΔCq method using *gpd* as
402 the reference gene.

403 **Fig. 3** Glucose remaining (A), ethanol concentration (B), and ethanol yield (C) of *P. sordida* YK-624 in
404 the presence of varying concentrations of oxygen and glucose. Each sample was analyzed after 24 h
405 of incubation with glucose supplementation. Values are mean ± standard deviation of triplicate
406 cultures. Open circles, triangles, rhombi, square, and closed circles are indicated the analyzed values
407 of 0.05, 0.1, 0.3, 0.6 and 1.0 g/L glucose supplemented cultures, respectively.

408 **Fig. 4** Mycelial growth and ethanol consumption by *P. sordida* YK-624 after ethanol supplementation
409 of gf-BLM cultures. A) Effect of ethanol supplementation on mycelial growth. Open squares show
410 change in dry mycelial weight of *P. sordida* YK-624 under aerobic culture without ethanol

supplementation (control). Closed circles, rhombi, and triangles indicate change in the dry weight on ethanol-supplemented aerobic, semi-aerobic, and anaerobic cultures, respectively. Five-day old aerobic cultures were supplemented with ethanol. B) Time courses of ethanol concentration (closed symbols) and headspace oxygen concentration (open symbols) in ethanol-supplemented cultures. Circles, rhombi, and triangles indicate aerobic, semi-aerobic, and anaerobic cultures, respectively. Values are mean ± standard deviation of triplicate cultures.

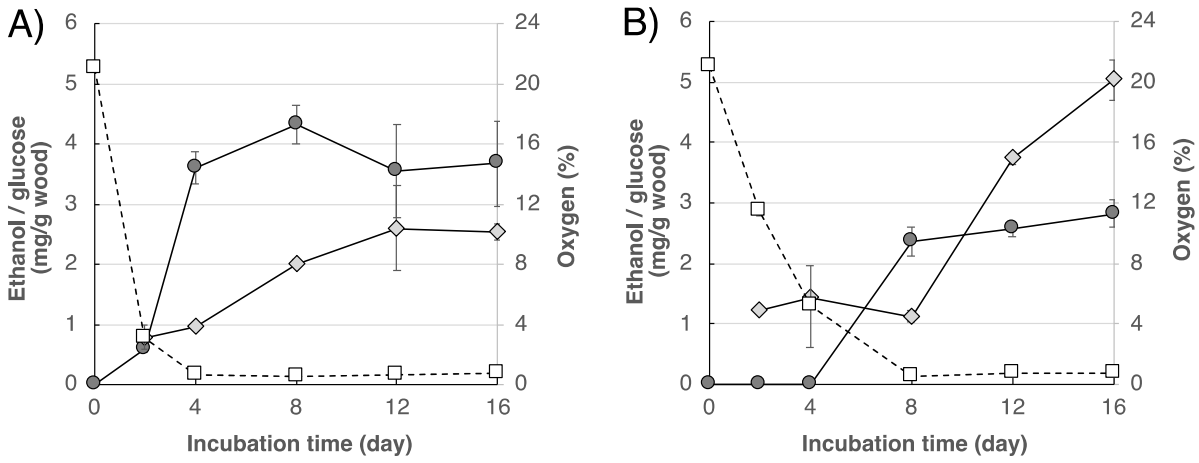
A)
B)
Ethanol / glucose (mg/g wood)
Oxygen (%)
Incubation time (day)
0
1
2
3
4
5
6
0
4
8
12
16
20
24

Fig. 1

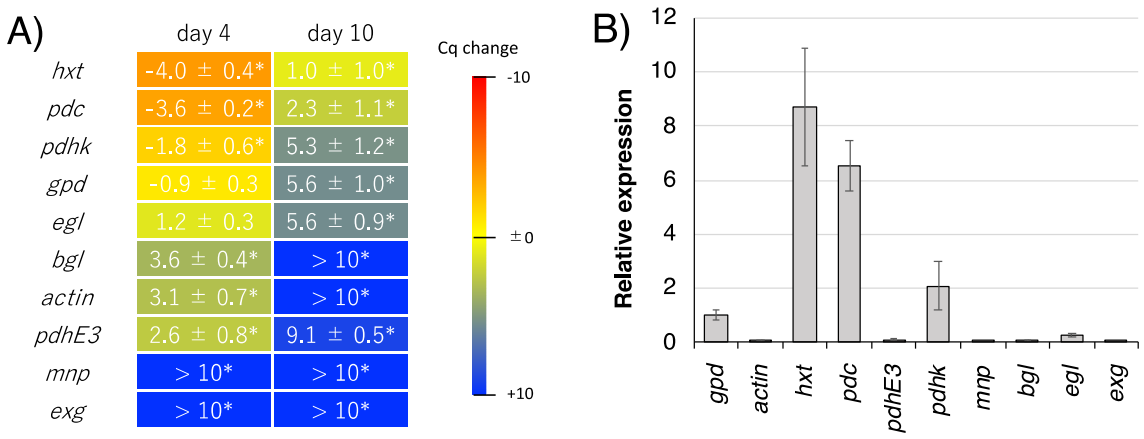
A)
day 4
day 10
hxt
-4.0 ± 0.4*
1.0 ± 1.0*
pdc
-3.6 ± 0.2*
2.3 ± 1.1*
pdhk
-1.8 ± 0.6*
5.3 ± 1.2*
gpd
-0.9 ± 0.3
5.6 ± 1.0*
egl
1.2 ± 0.3
5.6 ± 0.9*
bgl
3.6 ± 0.4*
> 10*
actin
3.1 ± 0.7*
> 10*
pdhE3
2.6 ± 0.8*
9.1 ± 0.5*
mnp
> 10*
> 10*
exg
> 10*
> 10*
Cq change
-10
±0
+10
B)
Relative expression
12
10
8
6
4
2
0
gpd
actin
hxt
pdc
pdhE3
pdhk
mnp
bgl
egl
exg

Fig. 2

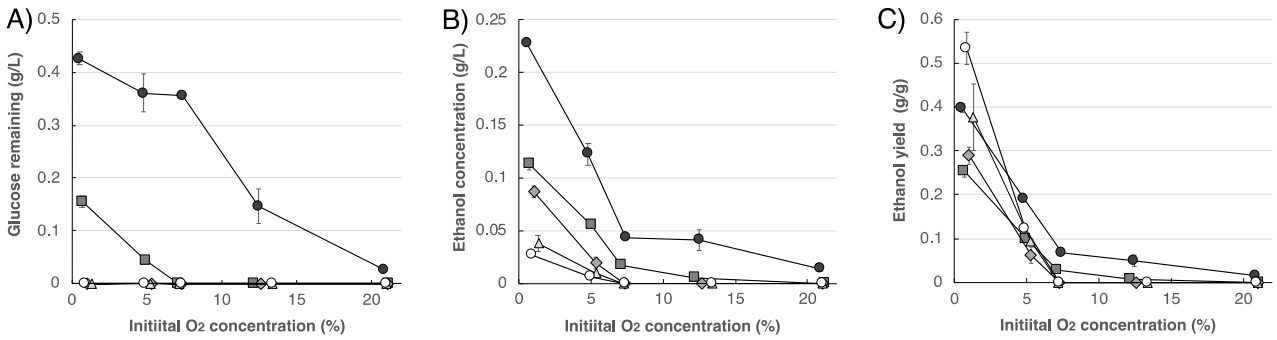
A)
Glucose remaining (g/L)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
Initiital O2 concentration (%)
B)
Ethanol concentration (g/L)
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
Initiital O2 concentration (%)
C)
Ethanol yield (g/g)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
Initiital O2 concentration (%)

Fig. 3

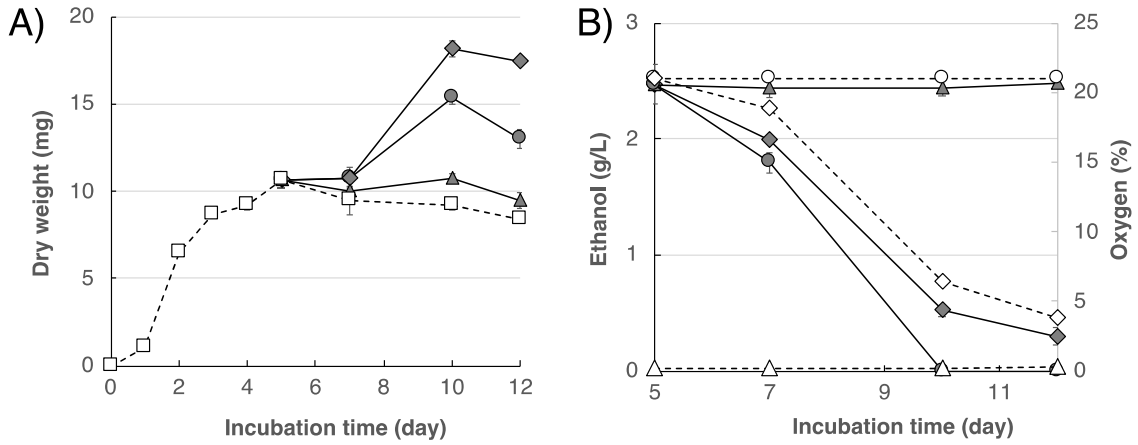
A)
Dry weight (mg)
Incubation time (day)
B)
Ethanol (g/L)
Oxygen (%)
Incubation time (day)

Fig. 4

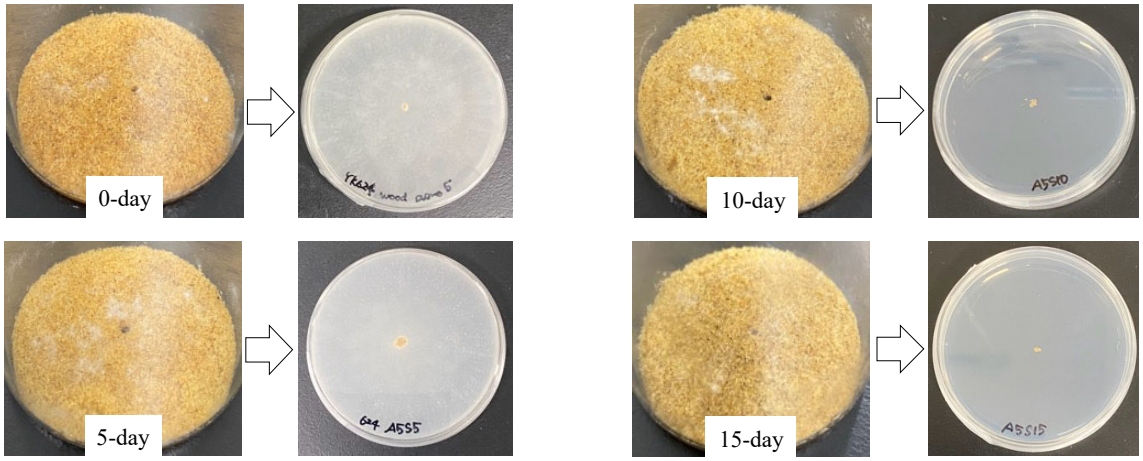

Fig. S1 Photographs of 0, 5, 10 and 15 day-old semi-aerobic wood cultures of *P. sordida* YK-624 and PDA cultures (3 days) inoculated each semi-aerobic cultures.

Table 1. Effect of aerobic pre-incubation duration and nutrient supplementation on ethanol production from wood culture by *P. sordida* YK-624.

pre-incubation (day)	weight loss (%)[a]	lignin degradation (%)[a]	ethanol production (mg/g wood)[bc]	oxygen remaining (%)[b]
without nutrient addition				
5	6.9 ± 1.1	15.9 ± 1.6	4.2 ± 0.6	0.4 ± 0.1
20	16.3 ± 0.4	24.6 ± 1.5	2.7 ± 0.3	1.7 ± 0.6
40	N.D.	N.D.	1.8 ± 0.1	0.4 ± 0.1
gf-BLM supplemented condition				
20			3.4 ± 0.3*	0.7 ± 0.1
40			4.3 ± 0.1*	0.3 ± 0.1

[a] These data were from wood cultures immediately after pre-incubation. N.D. means "not determined".

[b] These data were determined after 10-day semi-aerobic incubation.

[c] Asterisk indicates the significant difference ($P < 0.05$) between the values measured from the cultures with and without supplementation.