Effect on growth, sugar consumption, and aerobic ethanol fermentation of homologous expression of the sugar transporter gene Pshxt1 in the white rot fungus Phanerochaete sordida YK-624

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short title: **Homologous expression of *Pshxt1* in *P. sordida***

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Abstract

Major facilitator superfamily (MFS) transporters are found in all organisms. Although numerous studies have examined the functions of yeast and mold MFS transporters in terms of sugar affinity and metabolic regulation, no functional analyses of MFS sugar transporters in white rot fungi have been reported. This study identified an MFS sugar transporter gene (*Pshxt1*) of the white rot fungus *Phanerochaete sordida* YK-624 expressed in liquid culture containing low concentrations of nitrogen source. Homologous expression of *Pshxt1* dramatically increased the rates of glucose, fructose, mannose, and xylose consumption. Galactose consumption increased slightly but significantly. These data suggest that Pshxt1 has broad affinity for monosaccharides. In contrast, a transformant homologously expressing *Pshxt1* consumed glucose in preference to xylose in wood enzymatic-digestion liquor and liquid culture. Additionally, homologous expression of *Pshxt1* improved mycelial growth, aerobic ethanol production, and simultaneous aerobic saccharification and fermentation efficiency, whereas secretion of the ligninolytic enzyme manganese peroxidase was clearly decreased in the presence of glucose by *Pshxt1* expression. These results suggest that *Pshxt1* is involved in the repression of ligninolytic enzyme activity via carbon catabolite repression at sufficiently high glucose concentrations for activation of primary metabolism.

Introduction

Lignocellulosic biomass is the most abundant and renewable bioresource on earth. Recently, many researchers have demonstrated the potential of using lignocellulosic biomass for sustainable production of chemicals and fuels (1). Therefore, it could be said that lignocellulose materials are critically important raw materials for development of a sustainable society with zero carbon emissions. Biofuels such as ethanol and butanol are produced by fermentation of monosaccharides generated by the hydrolysis of polysaccharides, which are the primary components of lignocellulosic materials. However, polysaccharides in lignocellulosic materials are protected from physical/chemical and microbial attack by the recalcitrant aromatic polymer lignin; lignin removal is thus required for effective saccharification.

White rot fungi are wood-rotting basidiomycetous fungi that have the unique ability to generate polysaccharides by degrading lignin in lignocellulosic materials. Several white rot fungi are capable of fermenting ethanol from lignocellulosic materials (2-4). By exploiting these capabilities, Kamei and co-authors developed an integrated fungal fermentation (IFF) process using the white rot fungus *Phlebia* sp. MG-60 (5). The IFF process is a sequential biological reaction involving solid-state aerobic delignification and subsequent direct ethanol fermentation from delignified wood in supplemented liquid medium under semi-aerobic conditions using a single microorganism. However, low polysaccharide decomposition activity of white rot fungi often causes a bottleneck in ethanol production.

Phanerochaete sordida YK-624 is a white rot fungus that exhibits excellent delignification activity during wood decay and high lignin degradation selectivity (6). In addition, *P. sordida* YK-624 can produce ethanol from glucose under semi-aerobic (7) and aerobic conditions (8). A previous study indicated that this aerobic fermentation by *P. sordida* YK-624 is the result of an overflow in glucose metabolism and that the rate of glucose uptake by the fungus is limited (8). These data suggest that *P. sordida* YK-624 could be useful in aerobic IFF if its polysaccharide degradation activity and rate of monosaccharide uptake were markedly enhanced. Therefore, in this study, we identified a candidate

major facilitator superfamily (MFS) sugar transporter gene (*Pshxt1*) in *P. sordida* YK-624 and elucidated the function of Pshxt1 by homologous gene expression. We also investigated the effect of improved monosaccharide uptake on aerobic ethanol fermentation by this fungus.

Materials and Methods

Fungal strain and primers

Phanerochaete sordida YK-624 (ATCC 90872) and its uracil-auxotrophic strain UV-64 (9) were maintained on potato dextrose agar (PDA) at 4°C. All primers used in this study are listed in Table S1.

Cloning of the *P. sordida* hexose transporter–encoding gene (*Pshxt1*)

Two PDA discs (i.d. 10 mm) on which the surface was covered with *P. sordida* YK-624 mycelia were inoculated into 10 ml of Kirk's low nitrogen (LN) medium (containing 1.0% D-glucose, 1.2 mM ammonium tartrate, and 20 mM 2,2-dimethyl succinate) (10) and incubated for 3 days at 30°C. Grown mycelia were recovered by filtration, and total RNA was extracted using an RNeasy Mini kit (Qiagen). Reverse transcription–polymerase chain reaction (RT-PCR) assays were performed using a PrimeScript RT-PCR kit (TaKaRa Bio Inc.) to obtain a partial *Pshxt1* cDNA sequence. The oligo dT primer included with the RT-PCR kit and degenerate primers (dgHxt-F and -R) were used for the RT reaction and RT-PCR, respectively. The degenerate primers were designed from amino acid sequences of putative MFS sugar transporters from several basidiomycetous fungi archived in the Joint Genome Institute (JGI) and National Center for Biotechnology Information databases (Fig. S1). Residual 3'- and 5'-cDNA sequences of *Pshxt1* were determined using a GeneRacer kit (Invitrogen). The gene-specific and nested primers (GR3spe-F, GR3nest-F, GR5spe-R, and GR5nest-R) for 3'- and 5'-RACE reactions were designed from the partial cDNA sequence. To obtain the *Pshxt1* full-length genomic DNA sequence, gene-specific 3'- and 5'-end primers were designed (gPCR-F and -R). Genomic DNA of *P. sordida* was extracted using an ISOPLANT II kit (Nippon Gene), and the *Pshxt1*

coding sequence (CDS) was amplified by PCR in reactions containing 2% DMSO using TKs Gflex DNA polymerase (TaKaRa Bio Inc.).

Construction of the *Pshxt1* recombinant strain

Kpn I sites were incorporated in the 3'- and 5'-ends of the *Pshxt1* CDS by PCR using TKs Gflex DNA polymerase with the primers *Kpn*Iadd-F and -R. The PCR product was then appended with dA at the 3'-terminal ends using 10× A-attachment mix (Toyobo Co., Ltd.) and cloned into T-vector. The vector was digested, and *Kpn* I-containing *Pshxt1* was purified by agarose gel electrophoresis. The purified product was ligated into *Kpn* I-digested p*GPDpro* (11) to construct the *Pshxt1* expression plasmid (designated p*GPDpro-Pshxt*). The p*GPDpro-Pshxt* expression plasmid was co-transformed with p*PsURA5* into UV-64 protoplasts, as described previously (9). A total of 78 regenerated prototrophic (Ura^+) transformants (designated Hxt1 to Hxt78) were recovered as previously reported (9), and the resulting colonies were screened for the presence of the p*GPDpro-Pshxt* sequence by genomic PCR with specific primers (GR3nest-F and *Psgpd*ter-R) using an approach similar to that reported previously (12).

Wild-type (WT) *P. sordida* YK-624 and 30 screened Hxt strains were cultivated on LN medium (1.0% glucose) for 6 days at 30°C, as described above. The culture fluid was then collected, and the amount of residual glucose was measured according to the Somogyi-Nelson method using Somogyi copper solution and Nelson solution (Fujifilm, Wako Pure Chemical Co.). Glucose consumption was followed over time in LN medium (1% D-glucose) for Hxt strains exhibiting higher glucose consumption activity than the WT strain. Five transformants exhibiting higher glucose consumption rates were sub-cultured on PDA medium 5 times, after which the glucose consumption activity was examined again to evaluate the stability of the introduced gene. Transformant Hxt31 exhibited the highest glucose consumption rate and gene stability, and was used for further investigations. The amount of glucose was determined by high-performance liquid chromatography

78 (HPLC) on an instrument equipped with a Shodex SH1821 column and refractive index detector, as
79 previously reported (7).
80
81 **Sugar consumption and ethanol production from saccharides and woody biomass**
82 WT strain and transformant Hxt31 were inoculated into LN medium containing 1% of
83 various monosaccharides (D-glucose, D-fructose, D-mannose, D-galactose, D-arabinose, and
84 D-xylose) and cultured under aerobic conditions at 30°C. The residual sugar concentration, mycelial
85 growth, and ethanol concentration were measured every 2 days. Concentrations of sugar and ethanol
86 in the culture fluid were measured by HPLC, as described above. Mycelia were recovered by filtration,
87 dried at 105°C, and then weighed.
88 Three mycelial discs of WT and Hxt31 were inoculated onto 0.5 g of beech wood meal
89 (extractive free, 80-100 mesh, moisture content 75%, lignin 25.7%, cellulose 54.1% and xylan 20.8%)
90 in a 70-ml vial and incubated for 5 days at 30°C. The discs were then removed, and 3.75 ml of a
91 solution of cellulase (cellulase "Onozuka" RS, Yakult Pharmaceutical Industry Co., Ltd.) dissolved in
92 LN medium (without any carbon source) was added. The cellulase activity was adjusted to 1, 5, and
93 25 filter paper units (FPUs)/g of wood (13). The wood meal culture was then dispersed and incubated
94 aerobically at 30°C for an additional 4 days, after which the culture fluid was recovered, and the
95 amount of glucose and ethanol were determined by HPLC. Un-inoculated beech wood meal was used
96 as control.
97
98 **Manganese peroxidase activity and veratryl alcohol production**
99 The Hxt31 and WT strains were cultured in LN medium containing 1% glucose or 0.5%
100 each of glucose and xylose at 30°C for 2-12 days. Or Hxt31 was cultured in 1% xylose containing LN
101 medium for 4 days, then 0.5% glucose was added to the culture followed further incubation. Mycelia
102 were separated from the culture fluid by filtration (pore size, 0.2 μm) and dried at 105°C for over 12
103 hours following PDA disc removal and then weighed. The resulting filtrate was used for enzymatic

assay of manganese peroxidase (MnP) activity according to a method described previously (14). Protein content in the filtrate was measured using a Bradford protein assay kit (TaKaRa Bio Inc.). Ethanol concentration was analyzed as described above. Sugar concentrations were analyzed by HPLC equipped with NH2P-50 4E column (Shodex) by using 70% acetonitrile as an eluent (1 ml/min). Amount of veratryl alcohol (VA) in the filtrate was measured by absorbance at 276 nm by using HPLC equipped with ODS-3 column (GL Science, 150 mm × 4.6 mm) and to elute with 30% methanol (1 ml/min).

Statistical analysis

Data are presented as the average of three replicates in each experiment. Two-way analysis of variance (ANOVA) was used to compare sugar consumption, mycelial growth, and ethanol production between the WT and transformant strains. The Student's *t*-test was used to assess the significance of differences between the WT and Hxt31 strains. A *P* value <0.05) was considered indicative of statistical significance.

Results

We first determined the full sequence of a putative MFS monosaccharide transporter gene expressed by *P. sordida* YK-624 in LN liquid culture. The gene consisted of 1,821 bp and 4 introns, with a 1,602-bp CDS. A Blast search against the RefSeq_protein database at the amino acid sequence level (15) indicated high similarity between the gene product and MFS monosaccharide transporters of white rot basidiomycetes (~89% identity), especially those of fungi belonging to the order *Polyporales* (Table 1). Therefore, we identified the gene as encoding a MFS monosaccharide transporter, and it was designated *Pshxt1* (accession number: LC438459).

To characterize Pshxt1, we constructed transformants (Hxt strains) expressing *Pshxt1* homologously from the uracil auxotrophic mutant of *P. sordida* YK-624 (9) and compared them to the WT strain. Hxt strains that exhibited higher glucose consumption than the WT strain were screened

130 from among 30 transformants that had a GPDpro-Pshxt gene sequence; the screened strains were then
131 sub-cultured 5 times on PDA medium to assess the recombinant gene's stability. The sub-cultured
132 transformants were then examined with respect to change in glucose consumption over time. Five
133 transformants (Hxt19, 21, 31, 45, and 66) exhibited higher glucose consumption activity than the WT
134 strain and also exhibited increased mycelial growth (Fig. S2). Among all transformant strains, Hxt31
135 exhibited the highest glucose consumption activity and was therefore used for further experiments.
136 To compare monosaccharide consumption, the WT and Hxt31 strains were cultured in
137 ligninolytic medium containing various monosaccharides as a sole carbon source, and then amount of
138 monosaccharide consumed and mycelial growth were measured (Fig. 1). When cultured with glucose,
139 fructose, mannose, or xylose as a sole carbon source, Hxt31 exhibited a significantly higher rate of
140 sugar consumption than the WT strain. In addition, a slight but significance increase in galactose
141 consumption was observed with Hxt31. However, there was no difference in mycelial growth between
142 the WT and Hxt31 strains in cultures containing galactose or pentoses (arabinose and xylose).
143 Moreover, although no ethanol was detected in WT culture fluid regardless of sugar type, Hxt31
144 produced ethanol aerobically in all cultures containing hexoses (Table 2 and Fig. S3). Additionally,
145 xylitol was detected in the Hxt31 culture containing xylose at the maximum 1.2 g/l after 4 days
146 incubation (Fig. S4A). Because Hxt31 was able to ferment hexoses to produce ethanol aerobically in
147 nitrogen-limited culture, as shown in Table 2, simultaneous saccharification and fermentation (SSF)
148 with a low concentration of cellulase was investigated. In this experiment, the fungi were pre-grown in
149 wood meal culture for 5 days, then SSF was started by addition of cellulase solution. The reaction was
150 kept in aerobic, therefore, delignification reaction should proceed during whole cultivation periods.
151 Although no ethanol was produced by the WT strain during 4 days of aerobic SSF with 1 and 5 FPUs
152 of cellulase/g of wood, Hxt31 produced 1.8 ± 0.5 and 3.8 ± 0.3 g ethanol/g of wood, respectively (Fig.
153 2A). After addition of 25 FPUs of cellulase/g of wood, both strains were able to produce ethanol.
154 Although no significant difference was observed in amount of ethanol production between the Hxt31
155 and WT at addition of 25 FPU/g cellulase, the aerobic ethanol productivity of Hxt31 was 1.36 times

higher than that of the WT strain. In un-inoculated beech wood meal, the recovered amounts of free glucose and xylose were increased dependent on the cellulase dosage (Fig. 2B, C). Although it is clear that glucose was consumed by these strains, amounts of free glucose after SSF reaction with WT were almost constant (21.2 ± 9.2 mg/g of wood, this value is corresponded to 2.1 ± 0.9 g/l) regardless to cellulase activity (Fig. 2B). The amount of free glucose in the SSF reaction mixture of the Hxt31 strain was lower than that of the WT strain, and at 1 FPU/g of wood, the difference was significant. During incubation, cellulases and the fungi should continue to release and consume free sugars, respectively. Therefore, total amount of free glucose production and consumption were unable to be estimated in this experiment. However, we have previously reported that *P. sordida* YK-624 changes the rate of glucose uptake dependent on glucose concentration in the case of the glucose concentration is less than 6 g/l (8). Additionally, there is a report described about ethanol production from lignocellulosic materials without exogenous cellulase by some *Phlebia* fungi (16). In that report, the concentrations of released sugars from spruce wood in liquid phase at 7-21 days semi-aerobic fermentation showed almost constant values respectively in every fungus. From these facts, it is speculated that WT has maintained the balance between the glucose release and consumption in SSF reaction, on the other hand, this release/consumption balance in Hxt31 would be inclined because of higher glucose consumption activity than WT. In contrast, the amount of free xylose was consistently and significantly higher in the SSF reaction mixture of Hxt31 than that of the WT strain (Fig. 2C). As this result suggested that Pshxt1 catalyzes glucose transport preferentially to xylose, so we investigated the change in the behavior of sugar consumption in Hxt31 culture by addition of glucose in the middle of cultivation with xylose as a sole carbon source. Although xylose was consumed average 9.9 ± 6.5 % every 2 days at first 4 days, the consumption rate was dropped to average 5.3 ± 3.8 % per 2day after glucose addition (Fig. 3). And ethanol production was observed after glucose addition. This result indicates that xylose uptake of Hxt31 was inhibited by glucose addition. And then, we investigated sugar uptake by the WT and Hxt31 strains in medium containing the same amount of glucose and xylose (0.5 % each). As shown in Fig. 4A, the WT strain consumed glucose at a steady

rate, whereas xylose consumption was slower in comparison. However, Hxt31 consumed almost all of the glucose by day 8, and after then xylose consumption rate has increased. Hxt31 showed better growth during whole cultivation period than WT. Although amounts of extracellular protein of Hxt31 was slightly lower than WT at early stage of culture, it increased after glucose was consumed (Fig S5A). In Hxt31, ethanol was detected in the culture fluid until glucose was completely consumed, and it was observed that Hxt31 tended to always show higher ethanol concentration than WT (Fig. S5B). The time courses of MnP activity and VA concentration of both strains were shown in Fig. 4B. In the present study, MnP and VA were employed as represents of secondary metabolic enzymes and metabolites. In WT, MnP activity was increased at day 4, then decreased at later stage of culture. Contrary, VA concentration was largely increased at later stage. On the other hand, MnP activity of Hxt31 was expressed at day 2 which was earlier than WT, however, the activity decay was also started in earlier. And the maximum activity was much lower than WT. Even though, the activity was started to rise again after glucose was consumed. And also, time course of VA concentration in culture fluid of Hxt31 showed completely different tendency of that of WT. When the WT and Hxt31 strains were cultured with 1% glucose as a sole carbon source for 5 days, 1.2 ± 0.3 and 2.2 ± 0.4 mg/ml of dry mycelia were obtained, respectively. However, the MnP activity of Hxt31 (0.11 ± 0.07 nkat/ml) was significantly lower than that of the WT strain (0.61 ± 0.21 nkat/ml). The amount of extracellular protein in the Hxt31 culture filtrate also tended to be lower compared with the WT culture filtrate.

Discussion

MFS transporters comprise one of the largest membrane transporter families, and these proteins are ubiquitous in both prokaryotic and eukaryotic organisms (17). MFS transporters mediate the transport of a variety of substrates into and out of the cell as a uniporter, symporter, or antiporter. Both high- and low-affinity MFS sugar transporters that function at different sugar concentrations have been described. Sugar uptake into the cell is mediated by whole-sugar transporters. Therefore, because each transporter can complement the function of others, specific individual transporters are

not essential for basic growth on sugars (18). *Saccharomyces cerevisiae* expresses a particularly large number of MFS sugar transporters, enabling this organism to grow over a wide range of glucose concentrations (19). The affinity of each MFS sugar transporter varies for multiple sugars. For example, transporters Hxt1p-Hxt17p of *S. cerevisiae* transport glucose, fructose, and mannose, whereas Gal1p mediates glucose and galactose uptake (19). MFS sugar transporters in *Pichia stipitis* also exhibit differing transport activity; for instance, Sut1 has high affinity for glucose, fructose, and xylose, whereas Sut3 has high affinity for glucose, fructose, galactose, and xylose (20). Among filamentous fungi, *Aspergillus niger* MSTA transports glucose, fructose, xylose, and mannose (21). And also, it has been indicated that *mstA* is subject to carbon catabolite repression (CCR) and pH regulation (19). Two MFS sugar transporters, Stp1 and Crt1 from cellulolytic filamentous fungus *Trichoderma reesei* were already characterized to their roles in cellulose decomposition. Stp1 is capable of transporting glucose and cellobiose, and it has believed that Stp1 represses induction of major cellulase and hemicellulase genes via CCR (22). And Crt1 is essential in cellulase gene induction, although this transporter is not involved in transport of cellulase-inducing sugar (22). Furthermore, it is reported that has MFS sugar transporters HGT-1/-2 from cellulolytic fungi *Neurospora crassa* are up-regulated under carbon-limited or cellulolytic conditions, and also mediate glucose signaling with internal catabolite repression and metabolism (23). As mentioned in above, each MFS sugar transporter shows different sugar affinity in each other. And MFS sugar transporters frequently involved in CCR. In the present study, we employed homologous expression analysis to investigate the uptake of typical plant monosaccharides by PsHxt1, an MFS sugar transporter of *P. sordida* YK-624. As shown in Figure S2, the transformant strain Hxt31 exhibited the highest rate of glucose consumption of all tested transformants, and Hxt31 showed higher consumption rates for glucose, fructose, mannose, galactose, and xylose compared with the WT strain (Fig. 1). These data suggest that Pshxt1 is involved in uptake of these monosaccharides.

Although it is held that several MFS sugar transporters are encoded by the genome of white rot fungi (24), to our knowledge, there are no reports of functional analyses of MFS sugar transporters

234 in these organisms. However, several transcriptomic studies conducted under various cultivation
235 conditions indicated the importance of MFS sugar transporters in the life cycle of white rot fungi.
236 Korripally et al. reported that *Phanerochaete chrysosporium* the expression of ligninolytic,
237 polysaccharide-degrading, and cytochrome P450 enzymes along with several putative MFS sugar
238 transporters is upregulated during ligninolysis compared with the fungal growth phase (25). A
239 saprotrophic/necrotrophic wood decaying fungus, *Heterobasidion irregulare*, strongly upregulates the
240 expression of putative high-affinity MFS sugar transporter genes with carbohydrate-metabolizing
241 enzymes during pathogenic growth (26). In addition, Wang et al. reported that the ethanolic white rot
242 fungus *Phlebia* sp. MG-60 upregulates the expression of some MFS sugar transporter–like genes
243 along with glycolytic pathway and ethanol fermentation–related genes under fermentation conditions.
244 These data indicate that white rot fungi express MFS sugar transporter isozymes depending on
245 specific culture conditions, such as during the growth phase and secondary metabolic phase. The fungi
246 are thus thought to regulate sugar uptake based on culture conditions. In the present study, *P. sordida*
247 expressed *Pshxt1* in LN liquid medium containing 1% glucose, suggesting that Pshxt1 functions in the
248 presence of enough high glucose concentrations for making activate the primary metabolic growth.
249 Actually, sugar consumption, mycelial growth, and aerobic ethanol production were promoted in
250 Hxt31, which homologously expresses *Pshxt1* (Figs. 1). Although xylose consumption of Hxt31 was
251 faster than WT as with glucose, it has not been led better mycelial growth (Fig. 1F). Other hand,
252 Hxt31 accumulated xylitol in the culture containing of xylose, but not in the culture of WT (Fig. S4A).
253 Sum of biomass and xylitol yields of Hxt31 from xylose was showed no significance compared with
254 biomass yield of WT (Fig. S4B). So, this result indicates that capacity of xylose utilization for primary
255 metabolism in *P. sordida* YK-624 is much lower than glucose, fructose, and mannose. And also, it
256 was suggested that excessed xylose is converted into xylitol, and temporarily stored at extracellular
257 until depletion of other preferable carbon sources.

258 And then, we investigated whether *Pshxt1* promotes the primary metabolisms during wood
259 decaying, by carrying out the SSF supplemented with low amount of cellulase. Because it is difficult

to quantify the mycelial growth on woody culture, we evaluated the ethanol production as activity of primary metabolism. And ethanol production of Hxt31 in SSF was improved at lower range of cellulase dosage (1 and 5 FPU/g wood) compared with WT. In previous report, it has been suggested that *P. sordida* YK-624 saturates the respiration catabolism in presence of high concentration of glucose, and that resulting overflowed glucose (or its metabolites) is utilized for aerobic ethanol production (8). Therefore, this result suggests that the homologous expression of *Pshxt1* induces the saturation of respiration catabolism at lower sugar concentration due to improvement of sugar uptake. Additionally, Hxt31 left higher amount of xylose in the supernatant of SSF than that of WT (Figs. 2C). And xylose consumption of Hxt31 in liquid culture was inhibited by addition of glucose (Fig. 3). These results suggest that *Pshxt1* plays a role in promotion of primary metabolism by inducing CCR under high-glucose conditions. CCR is a well-known mechanism of carbon source regulation in a variety of microorganisms, including filamentous fungi. If *P. sordida* YK-624 really prefers glucose than xylose, it is predicted that Hxt31 easily occurred CCR then represses xylose uptake because higher expression of *Pshxt1* improves glucose uptake. Of course, xylose consumption wouldn't be suspended in Hxt31, because other sugar transporters are still worked in the transformant. Actually, in the culture containing both xylose and glucose, Hxt31 showed almost same xylose consumption rate as WT, until all glucose was consumed at 8th day as shown in Fig. 4A. In the meantime, WT slightly accelerated xylose consumption after glucose consumed at day 10. And rate of xylose consumption in the Hxt31 culture was decreased by glucose addition (Fig. 3). Therefore, in SSF with 1-25 FPU cellulase addition, it was estimated that residual xylose was increased since xylose uptake of Hxt31 was probably suppressed by CCR and exogenous cellulases released xylose along with glucose (Fig. 2B and C).

Additionally, CCR allows for the repression of certain enzymes necessary for metabolism of less-favored carbon sources when a preferred carbon source is present (27). For example, the expression of enzymes required for the breakdown of lignocellulosic compounds in several filamentous fungi is repressed by the zinc-finger transcription factor CRE1 until glucose is exhausted

(28). CCR also affects secondary metabolism. Various sugars negatively affect β-lactam biosynthesis, which is part of secondary metabolism in *Aspergillus* and *Penicillium* fungi (29). It is reported that *P. sordida* produces high MnP activity during secondary metabolism under LN condition, same as several other white rot fungi, such as closely related strain *P. chrysosporium* (30). And VA is also known a secondary metabolic product of white rot fungus *P. chrysosporium*, the biosynthesis of VA is also suppressed by nitrogenous compounds as with MnP (31). Therefore, we decided to employ these enzyme and metabolite as indicators of secondary metabolism. Lower MnP activity and early drop of the activity were observed during glucose was remaining in the culture of Hxt31. Although MnP activity and VA concentration in WT culture seem to be not unaffected by sugar concentrations, the MnP activity in Hxt31 culture was increased once again after glucose was consumed (Fig. 4B). However, time course of VA concentration in the Hxt31 culture was completely different from that of MnP activity. And Hxt31 showed lower MnP activity than WT, also in the culture containing with glucose as a sole carbon source. From these results, it was suggested that MnP activity was suppressed via CCR induced by *Pshxt1* expression. And production/metabolism of extracellular VA was affected with *Pshxt1* expression, however, it seems to be regulated by the mechanisms different from MnP production. Therefore, it is expected that regulation of VA biosynthesis is not a simple mechanism controlled with sugar concentrations and types.

Finally, *Pshxt1* homologous expression improved aerobic fermentation in *P. sordida*, and the transformant was able to produce ethanol by aerobic SSF even in the presence of low concentrations of cellulase. However, the LN culture conditions used in this study are generally employed for inducing secondary metabolism in white rot fungi (e.g., ligninolytic enzyme production); thus, these culture conditions are probably not suitable for ethanolic fermentation. Therefore, it is possible that the construction of new transformants expressing *Pshxt1* and cellulase genes simultaneously and the establishment of suitable and cost-effective conditions for ethanol production will lead to the development of an IFF process consisting of a single organism, a single batch, and single culture condition.

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Figure legends

Fig. 1. Sugar consumption and mycelial growth of WT *P. sordida* YK-624 and Hxt31 transformant in low-nitrogen cultures containing various monosaccharides (1% glucose (A), fructose (B) mannose (C) galactose (D) arabinose (E) and xylose(F)) under aerobic conditions. Symbols (Rhombi and circles) indicate sugar consumption and mycelial growth of the WT (open) and Hxt31 (closed) strains, respectively. Values are mean ± standard deviation of triplicate cultures.

Values for sugar consumption and mycelial growth that differ significantly (P<0.05) from the WT strain in the same incubation period are indicated by † and ‡, respectively. * indicates significant difference between time courses of both strains as determined by two-way ANOVA.

Fig. 2. Amount of ethanol and residual free-sugars produced after 4 days of aerobic SSF with different levels of cellulase activity. Cellulase was added after 5 days of incubation after inoculation with the WT (gray bars) and Hxt31 (black bars) strains. Ethanol (A), D-glucose (B), D-xylose (C) were extracted following additional aerobic incubation for 4 days. The abiotic control (white bars), which received no inoculation, was analyzed in the same way as other samples. Values are the mean ± standard deviation of triplicate cultures. Asterisks indicate significant differences between WT and Hxt31 strains (P<0.05), and n.d. indicates not detectable.

Fig. 3 The time courses of A) xylose (rhombi) and glucose (circles) consumption, and B) mycelial growth (squares) and ethanol production (triangles) of Hxt31, during liquid cultivation initially containing 1.0% xylose and followed 0.5% glucose addition after 4 days incubation (indicated as arrows). Values are mean ± standard deviation of triplicate cultures.

Fig. 4. Time course of A) glucose and xylose consumption and B) MnP and VA concentration from ligninolytic cultures of the WT and Hxt31 strains. A) Medium initially containing 0.5% glucose (circles) and 0.5% xylose (rhombus) was inoculated with the WT (open symbols) and Hxt31 (closed symbols) strains, and the cultures were recovered every 2 days for measurement of residual monosaccharides. And B) shows MnP activity (squares) and VA concentration (triangles) in the extracellular fluid of the WT and Hxt31 culture. Values are the mean ± standard deviation of triplicate cultures. Values that differ significantly (P<0.05) from the WT strain in the same incubation period are indicated by † and ‡, respectively. * indicates significant difference between time courses of both strains as determined by two-way ANOVA.

Table 1. Function and accession number of proteins registered in RefSeq exhibiting high similarity to the translated amino acid sequence of *Pshxt1*.

Function	Organism	Coverage (%)	Identity (%)	Accession no.
putative MFS transporter	[*Phanerochaete carnosa*]	99	89	XP_007400709
MFS monosaccharide transporter	[*Trametes versicolor*]	100	76	XP_008038928
MFS monosaccharide transporter	[*Dichomitus squalens*]	97	75	XP_007362946
MFS sugar transporter	[*Heterobasidion irregulare*]	100	74	XP_009548738
putative sugar transporter	[*Postia placenta*]	96	74	XP_024341010

Table 2. Maximum ethanol concentration and incubation period of WT *P. sordida* YK-624 and Hxt31 transformant cultured in LN medium containing various monosaccharides.

	maximum ethanol concentration g/L (day)	
sugar	wild type*	Hxt31
glucose	n.d.	0.25 ± 0.05 (4)
fructose	n.d.	0.39 ± 0.02 (8)
mannose	n.d.	0.41 ± 0.04 (8)
galactose	n.d.	0.09 ± 0.01 (2)
arabinose	n.d.	n.d.
xylose	n.d.	n.d.

*n.d.: not detectable.

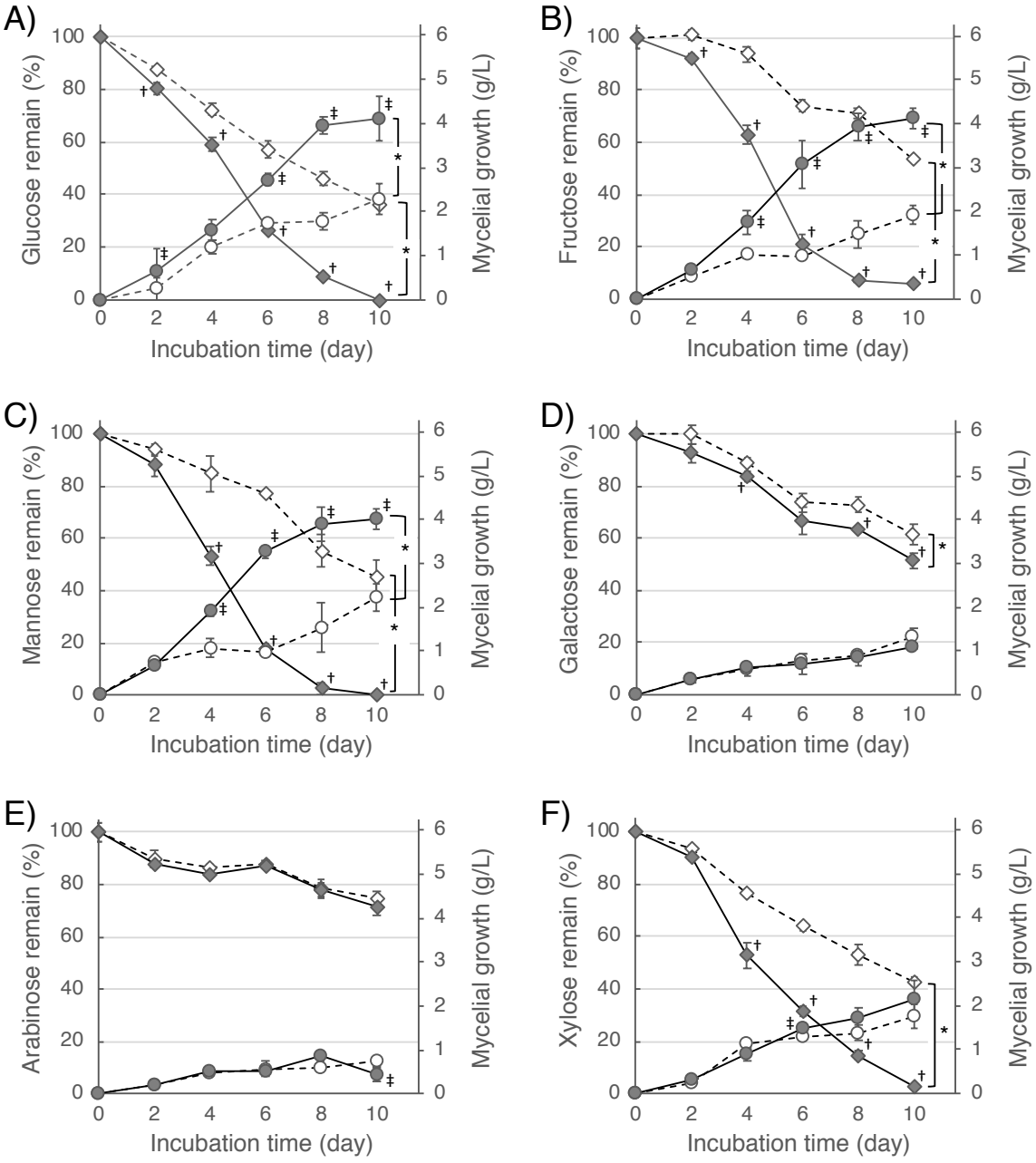
A)
B)
C)
D)
E)
F)
Glucose remain (%)
Fructose remain (%)
Mannose remain (%)
Galactose remain (%)
Arabinose remain (%)
Xylose remain (%)
Mycelial growth (g/L)
Incubation time (day)

Fig. 1

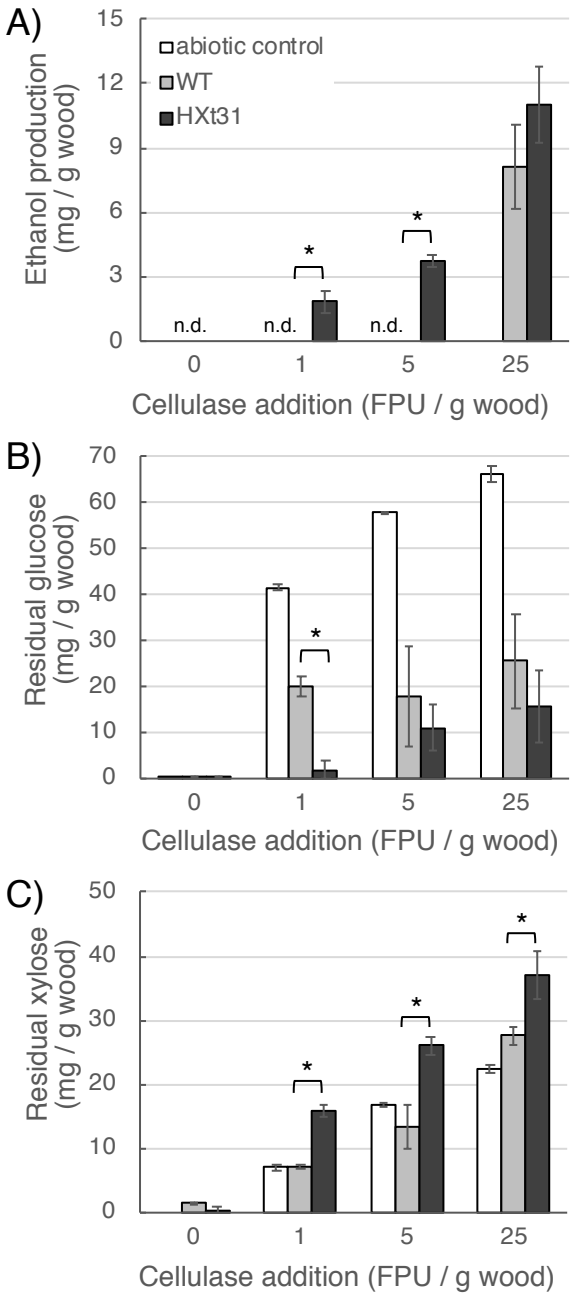
A)
abiotic control
WT
HXt31
Ethanol production (mg / g wood)
15
12
9
6
3
0
n.d.
n.d.
n.d.
*
*
0
1
5
25
Cellulase addition (FPU / g wood)
B)
Residual glucose (mg / g wood)
70
60
50
40
30
20
10
0
*
0
1
5
25
Cellulase addition (FPU / g wood)
C)
Residual xylose (mg / g wood)
50
40
30
20
10
0
*
*
*
0
1
5
25
Cellulase addition (FPU / g wood)

Fig. 2

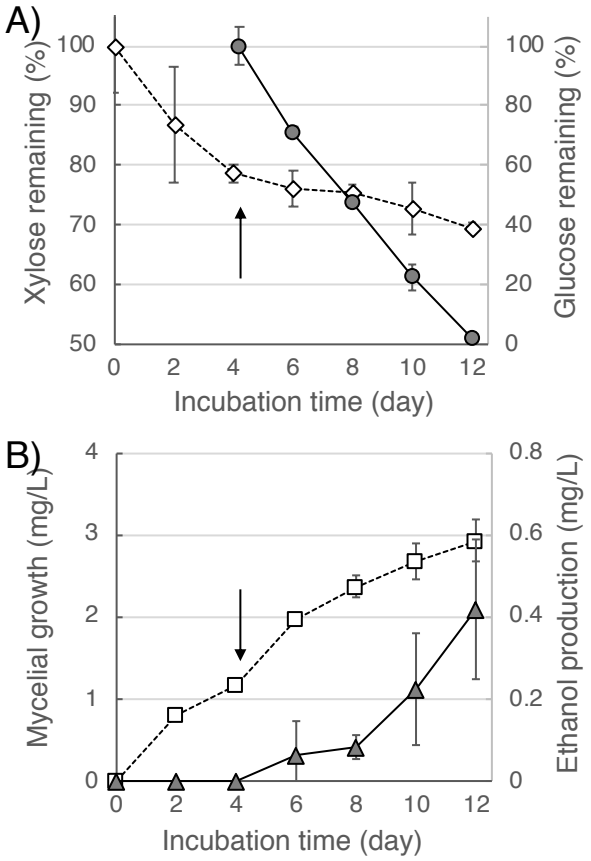
A)
Xylose remaining (%)
Glucose remaining (%)
Incubation time (day)
B)
Mycelial growth (mg/L)
Ethanol production (mg/L)
Incubation time (day)

Fig. 3

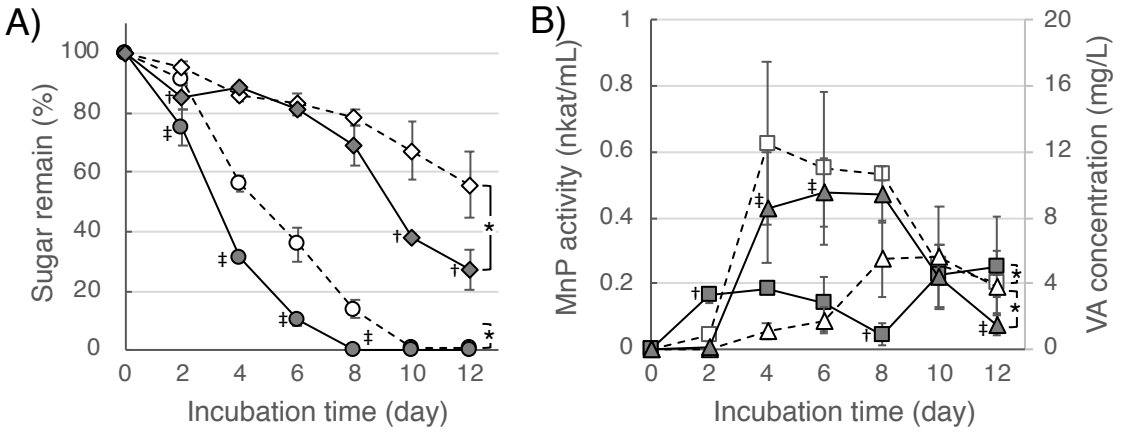
A)
Sugar remain (%)
100
80
60
40
20
0
0 2 4 6 8 10 12
Incubation time (day)
B)
MnP activity (nkat/mL)
1
0.8
0.6
0.4
0.2
0
VA concentration (mg/L)
20
16
12
8
4
0
0 2 4 6 8 10 12
Incubation time (day)

Fig. 4

Table S1. Primers used in this study.

Primer name	Sequence* (5' to 3')
dgHxt-F	TAYCARTGGGCNATHACNAT
dgHxt-R	ACCCANGCDATNGGNCCCCA
GR3spe-F	TTCATGACCCTCCCCGGCATGTG
GR3nest-F	TCGCGCTCGTCTGCATCTACATC
GR5spe-R	GAGGCGACTCAGGGAGATAGAAC
GR5nest-R	GTTGTTGATGACGGAGGCGAGGA
gPCR-F	ATGGCTGGTGGTCCCGCTGCATC
gPCR-R	CTAGACCTTTTCGGACTCGTCC
*Kpn*Iadd-F	GGTACCATGGCTGGTGGTCC
*Kpn*Iadd-R	GGTACCCTAGACCTTTTCGGACTC
*Psgpd*ter-R	CGTTTGTCAGTGCTGCACAC

* Y=C/T, R=A/G, H=A/C/T, N=A/T/C/G
Restriction sites are underlined.

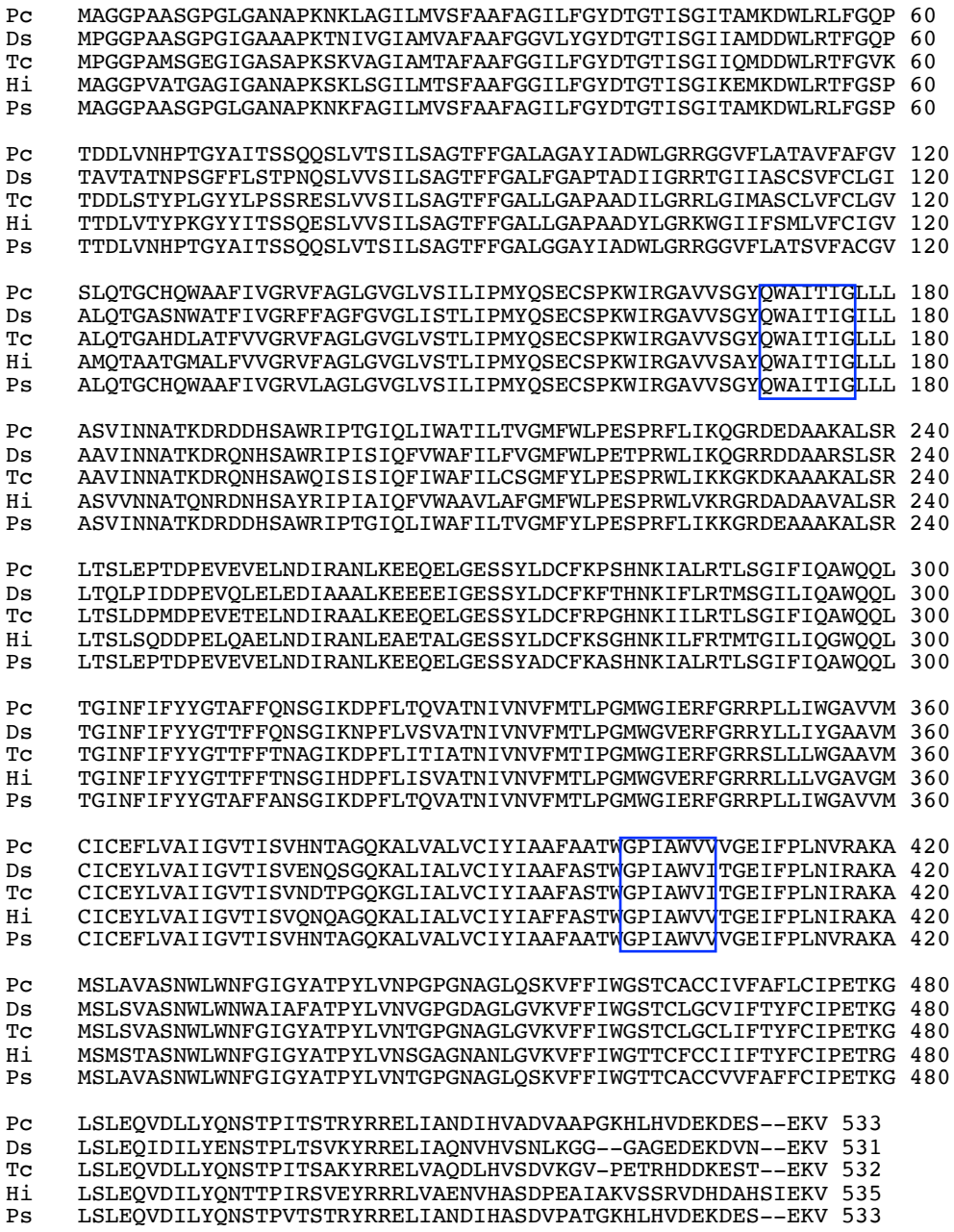

Fig. S1. Multiple sequence alignment (Clustal W) of MFS sugar transporters from *Phanerochaete chrysosporium* (PC; protein ID 3015932, JGI), *Dichomitus squalens* (Ds; XP_007362962, GenBank), *Trametes coccinea* (Tc; OSD02952, GenBank), *Heterobasidion irregulare* (Hi; XP_009548738, GenBank), and *Phanerochaete sordida* (this study). Strictly conserved residues are indicated by red letters. The positions of degenerate primers are indicated with boxes.

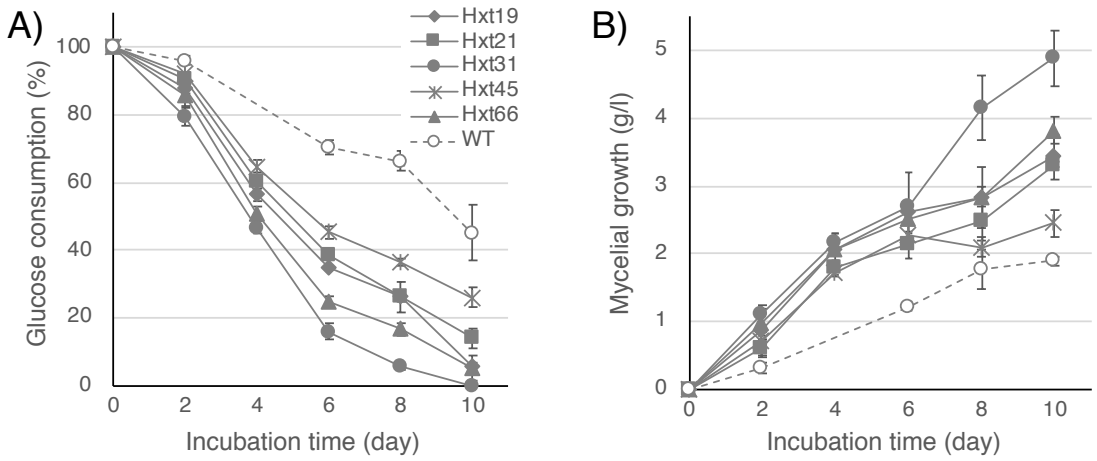

Fig. S2. Time course of glucose consumption (A) and mycelial growth (B) of the WT *P. sordida* YK-624 and Hxt transformant strains in low-nitrogen cultures under aerobic conditions. Values are the mean ± standard deviation of triplicate cultures.

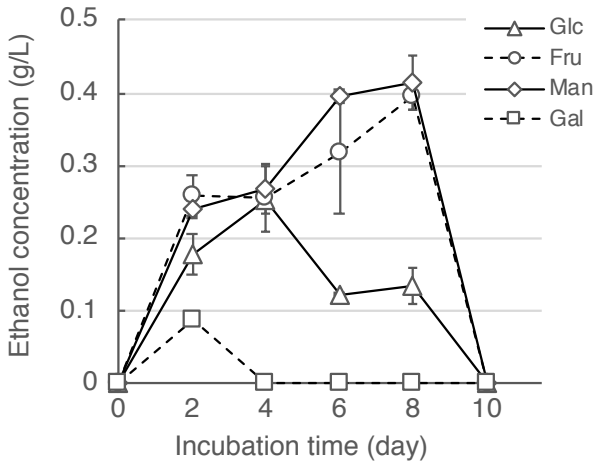

Fig. S3. Time course of ethanol production by the Hxt31 transformant cultured in low-nitrogen medium supplemented with various monosaccharides. Values are the mean ± standard deviation of triplicate cultures.

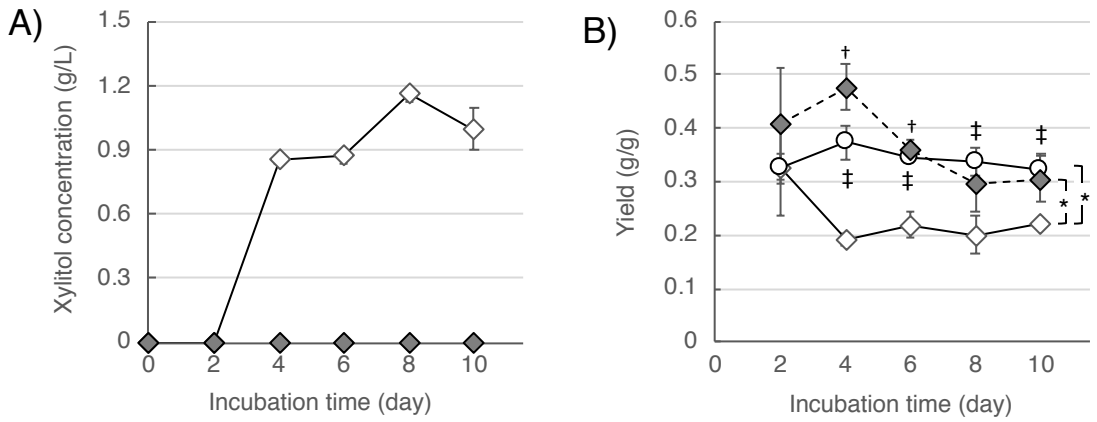

Fig. S4. Xylitol production (A) and biomass yields with or without xylitol (B) of WT and Hxt31 in the LN culture containing with xylitol as a sole carbon source. (A) Closed and open rhombi indicate xylitol production of WT and Hxt31, respectively. (B) Biomass yields of WT and Hxt31 are shown as closed and open rhombi, respectively. And sum yield of biomass and xylitol of Hxt31 are also shown as open circles. Values are the mean ± standard deviation of triplicate cultures. Values for biomass yield of WT or (biomass + xylitol) yield of Hxt31 that differ significantly ($P<0.05$) from the biomass yield of Hxt31 in the same incubation period are indicated by † and ‡, respectively. * indicates significant difference between time courses of both strains as determined by two-way ANOVA.

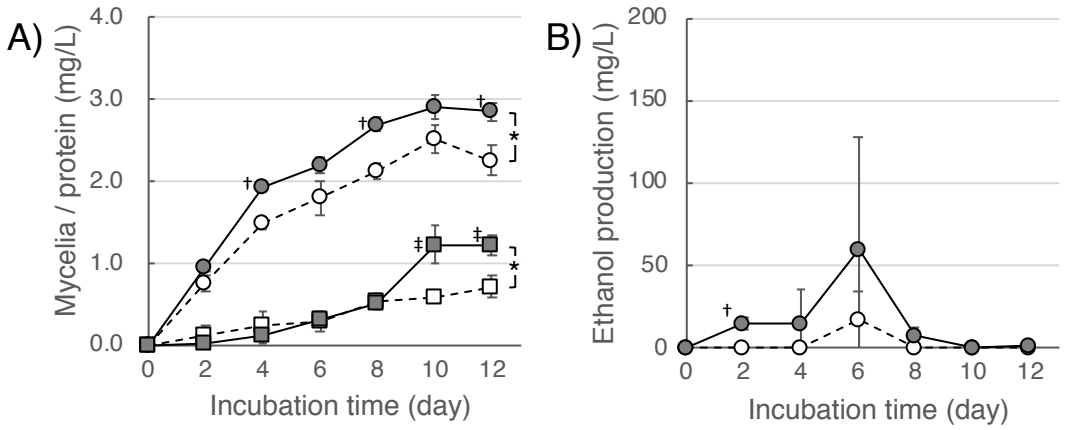

Fig. S5. Time courses of mycelial growth, extracellular protein and ethanol production of WT and Hxt31 in LN cultures containing with 0.5% each of glucose and xylose. Panel A) showed time courses of mycelial growth (circles) and extracellular protein (squares) and panel B) showed ethanol concentration. Open and closed symbols indicated WT and Hxt31, respectively. The values are the mean ± standard deviation of triplicated cultures. Each value that differ significantly ($P<0.05$) from the WT strain in the same incubation period are indicated by † and ‡, respectively. * indicates significant difference between time courses of both strains as determined by two-way ANOVA.