Effects of Homologous Expression of 1,4-Benzoquinone Reductase and Homogentisate 1,2-Dioxygenase Genes on Wood Decay in Hyper-Lignin-Degrading Fungus Phanerochaete sordida YK-624

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Effects of homologous expression of 1,4-benzoquinone reductase and homogentisate 1,2-dioxygenase genes on wood decay in hyper-lignin-degrading fungus *Phanerochaete sordida* YK-624

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Abstract

We investigated the function of 1,4-benzoquinone reductase (BQR)- and homogentisate 1,2-dioxygenase (HGD)-like genes in wood degradation by *Phanerochaete sordida* YK-624, which exhibits high ligninolytic activity and selectivity. We determined homologous expression in the genomic and cDNA sequences of *BQR*- and *HGD*-like genes in *P. sordida* YK-624 (*PsBQR* and *PsHGD*). Both genes shared high homology (≥90% amino acid sequence similarity) with the corresponding genes in *P. chrysosporium*. These genes were co-transformed with a reporter gene into an uracil auxotrophic mutant of *P. sordida* YK-624. The *PsBQR* and *PsHGD* co-transformants exhibited lower holocellulolytic activity and higher ligninolytic selectivity than the control transformants. In liquid culture with vanillin, both co-transformants significantly accelerated vanillin degradation. Thus, we suggest that the rapid metabolism of low-molecular weight lignin fragments, due to the homologous expression of BQR- and HGD-like genes, affects quinone redox cycling to produce hydroxyl radicals, thereby decreasing holocellulose degradation and increasing ligninolytic selectivity.

Introduction

Lignocellulosic materials, especially wood materials, are considered to have high potential for use as a biorefinery feedstock due to their abundance and renewability. However, lignin is one of the major components of lignocellulosic biomass, and it is resistant to biological and chemical treatments because it forms a complex structure with cellulose and hemicellulose, thereby hindering the biological/chemical degradation of whole lignocellulosic biomass. In particular, lignin that covers the surface of cellulose prevents the activity of cellulase. Therefore, yeast produces lesser ethanol from native wood using the simultaneous saccharification and fermentation method than from pretreated wood. Thus, delignification is a very important pretreatment process to facilitate wood biorefining by fermentation.

Several pretreatment methods are available for delignification, which can be roughly classified as biological and physical/chemical processes. Most physical and chemical pretreatment methods require special instruments and large amounts of energy, but they generate inhibitors such as vanillin and furfurals that affect enzymatic hydrolysis and fermentation [11, 13]. Among the biological pretreatments, white-rot fungi have been most widely used for degrading lignin in the lignocellulosic biomass. The advantages of biological delignification (eco-friendly and no generation of inhibitors) were summarized by Sindu et al. [14]. However, this process has a major problem, i.e., it is too slow for industrial applications. Therefore, the delignification efficiency of white-rot fungi needs to be improved.

It is well-known that white-rot fungi secrete ligninolytic enzymes such as lignin peroxidase, manganese peroxidase, and laccase and that these enzymes facilitate the degradation of lignin [12]. First, ligninolytic enzymes oxidize and depolymerize lignin. Then, the low-molecular weight lignin fragments obtained (e.g., vanillic acid) are taken up by the fungal cells. Finally, the fragments are decomposed into water and carbon dioxide [1]. In *Phanerochaete chrysosporium*, the gene expression levels of 1,4-benzoquinone reductase- (BQR) and homogentisate 1,2-dioxygenase (HGD)-like enzymes are increased by exogenous vanillin [13]. Thus, it has been suggested that these enzymes are strongly involved in the degradation of low-molecular weight lignin fragments. However, the roles of these enzymes in the degradation of lignin remain unclear.

P. sordida YK-624 produces manganese peroxidase and lignin peroxidase, and this fungus exhibits high ligninolytic activity/selectively [15]. Therefore, in this study, we investigated the effects of the homologous expression of BQR- and HGD-like genes on wood degradation in *P. sordida* YK-624, which is closely related to *P. chrysosporium*.

Materials and Methods

Strains

P. sordida YK-624 [9] and a uracil auxotrophic strain UV-64 [19] were used in this study.

Cloning procedure for the full-length genes encoding BQR and HGD

The sequences of BQR and HGD in *P. chrysosporium* were obtained from the genome database of the United States Department of Energy Joint Genome Institute (http://genome.jgi.doe.gov/) and their homologous sequences from fungi were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Degenerate primers (degBQRf and degBQRr for BQR, and degHGDf and degHGDr for HGD) were designed based on the high homology regions in these sequences (Table 1).

P. sordida YK-624 was cultured for 3 days , at 30 °C in high-nitrogen basal III medium (HN medium) containing 1.0% glucose, 12 mM ammonium tartrate, and 20 mM 2,2-dimethylsuccinic acid (pH 4.5) [18]. After incubation, 100 µl of 0.2 M vanillin (in N,N-dimethylformamide (DMF) solution) was added and the culture was incubated for another 3 days. Total RNA was extracted from the mycelium using an RNeasy Plant Mini Kit (Qiagen) following incubation. Reverse transcription was performed using a PrimeScript RT-PCR Kit. The partial cDNA sequences of *bqr* and *hgd* were determined by PCR amplification with TaKaRa ExTaq (TaKaRa Bio) and each degenerate primer set. A 3′- and 5′-full RACE core kit (TaKaRa Bio) was used to determine the full length cDNA sequences. Genomic DNA was extracted from *P. sordida* YK-624 using Isoplant II (Nippon Gene). The full length genomic DNA sequences of *P. sordida* YK-624 BQR and HGD (*PsBQR* and *PsHGD*) were amplified by PCR with the primer pairs psBQRf/psBQRr and psHGDf/psHGDr (Table 1), respectively, before determining the DNA sequences.

Construction of BQR and HGD expression vectors, co-transformation of UV-64, and screening of regenerated clones

The primers ndeiBQRsta and xbaiBQRterm, which contained *Nde*I and *Xba*I cut sites, respectively, were designed to amplify the *PsBQR* fragment. The amplicon, which was digested with *Nde*I and *Xba*I, was inserted into the *Nde*I and *Xba*I sites of p*BUNAIIpro* to construct p*BUNA2-BQR*. The *PsHGD* fragment was amplified with kpniHGDsta and xbaiHGDterm primers to construct p*BUNA2-HGD* in a similar manner.

UV-64 protoplasts were prepared before transforming with p*PsURA5* and p*BUNA2pro-BQR* or p*BUNA2pro-HGD* using standard techniques [19]. The co-transformed clones were selected by PCR. The primers confBQR/GPDterm or confHGD/GPDterm, which was fused with a GPD terminator, was designed to amplify *PsBQR* or *PsHGD*, respectively. The clone transformed with p*PsURA5*, which was denoted as strain U, was used as a negative control.

Evaluation of ligninolytic properties

The selected clones with p*PsURA5* and p*BUNA2pro-BQR* (transformant B-line), p*PsURA5* and p*BUNA2pro-HGD* (D-line), strain U, and wildtype were cultured on potato dextrose agar (PDA) medium. A mycelium disk was punched out using a cork borer (i.d. = 10 mm) and used to inoculate beech wood meal (solid

content 28.5% weight, dry weight 0.5 g, 80–100 mesh) in a 100 ml Erlenmeyer flask. After incubating for 4 weeks at 30°C, the weight loss (WL), Klason lignin (KL) content, and acid-soluble lignin contents of the fungal-treated wood meal were determined, as described previously [9]. Transformants were selected based on their ligninolytic properties. The selected transformants were subcultured five times on PDA before measuring their ligninolytic properties again to evaluate the stability of the transformants.

Vanillin degradation and Transcriptional analysis, Enzyme activities.

To evaluate the vanillin degradation capacity of the transformants, two mycelium disks (10 mm) were placed in 10 ml of potato dextrose broth (PDB; Difco) and the culture was pre-incubated for 5 days at 30°C. After pre-incubation, 100 µl of 10 M vanillin (in DMF solution) was added to the culture before incubating at 30°C. After incubation, 20 ml acetone was added to the culture, which was homogenized using a Polytron homogenizer PT1200E. The homogenate was filtered through a membrane filter (pore size = 0.22 µm) before high performance liquid chromatography (HPLC) analysis. HPLC was conducted using a Jasco PU-2089plus quaternary gradient pump with a Jasco MD-2018plus photodiode array detector, which had an Inertsil ODS-3 column (GL Science) with an internal diameter of 4.6 × 250 mm. The analytical conditions for HPLC were as previously described [4].

The mycelium was obtained from the culture incubated with vanillin for 6 h. Then, total RNA extraction, reverse transcription and PCR reaction were performed as previous report [15] . Primer pairs targeting *PsBQR* (rtBQRf/rtBQRr), *PsHGD* (rtHGDf/rtHGDr), and *actin* (rtACTf/rtACTr) were used (Table 1).

After 6 h incubation with vanillin, the culture fluid was eliminated by filtration. Residual mycelium was washed with distilled water. Frozen mycelium (1g) was homogenized and suspended in extraction buffer consisted of 50 mM potassium phosphate (pH 6.8) and 0.004% phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 13,800 × *g*, for 15 min. The supernatant was used for enzyme activities. BQR activity was determined by following the oxidation of NADPH at 340 nm (ε_{340} = 6,500 $M^{-1}cm^{-1}$). Reaction mixture (1 ml) was contained 50 mM potassium phosphate (pH 6.8), 100 µM 1,4-benzoquinone, and enzyme. Reaction was initiated by the addition of 100 µM NADPH [3]. HGD was assayed following the formation of maleylacetoacetate at 330 nm (ε_{330} = 13,500 $M^{-1}cm^{-1}$). The HGD assay mixture (1.0 ml) consisted of 50 mM potassium phosphate (pH 6.8), 2 mM ascorbate, 50 µM $FeSO_4$, 250 µM homogentisate, and enzyme [6]. The reaction was carried out at 25ºC with an Ultrospec 2100pro (GE healthcare).

Results and Discussion

In this study, the roles of PsBQR and PsHGD in the degradation of woody components (lignin and holocellulose) by *P. sordida* YK-624 were investigated. First, two genes induced by the ligninolytic fragment exogenous vanillin were obtained from *P. sordida* YK-624: *PsBQR* and *PsHGD*.

The open reading frame (ORF) of *PsBQR* (accession number (AN): AB621802) comprises of 983 base pairs (bp) and four introns, with a coding sequence (CDS) of 609 bp; the CDS of *PsBQR* shares high homology with the gene (79%), cDNA (86%) and amino acid (93%) sequences of the BQR isozyme of *P. chrysosporium* (JGI protein ID: 2979457). The activity of this BQR isozyme of *P. chrysosporium* is not clear, but it has been suggested that it is involved with the degradation of lignin because BQR enzymes are induced by vanillin, vanillate, and quinones [3]. White-rot fungi degrade lignin and the low-molecular weight lignin fragments generated include monomeric quinones. The quinones are taken up into the cells and mineralized to CO_2 and H_2O. The semi-quinones, produced by the reduction of extracellular quinones, can oxidize oxygen to obtain reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). The reduction reactions of quinones are believed to be catalyzed by BQRs [7].

The ORF of *PsHGD* (AN: LC137813) comprises of 2229 bp and 12 introns. The CDS (1410 bp) of *PsHGD* shares high homology with the gene (77%), cDNA (81%) and amino acid (91%) of the HGD isozyme in *P. chrysosporium* (JGI protein ID: 2506060). No previous reports have described the enzymatic activity of this HGD isozyme, but HGD generally catalyzes the oxidative cleavage of the aromatic ring in homogentisate to produce maleylacetoacetate. This isozyme is induced by exogenous vanillin (Shimizu et al. 2005) and thus, it is related to vanillin metabolism.

We constructed two plasmids containing *PsBQR* and *PsHGD* under the control of the *bee2* promoter, i.e., pBUNA2pro-PsBQR and pBUNA2pro-PsHGD, respectively. The *bee2* promoter was used to drive gene expression during cultivation on beech wood meal [15]. These plasmids were co-transformed with pPsURA5 into UV-64, a uracil auxotrophic mutant of *P. sordida* YK-624 [19]. We obtained 20 clones of *PsBQR* co-transformants (B-line) and 14 clones of *PsHGD* co-transformants (D-line). In addition, 7 uracil prototrophic clones (U-line) were obtained by pPsURA5 single transformation. These clones were cultured on beech wood meal and their ligninolytic properties were determined after 4 weeks (Fig. 1A). The average KL degradation rate and WL were calculated in each transformant line and their characteristics were compared (Fig. 1B). The ratio of KL degradation relative to WL was also calculated as the ligninolytic selectivity. Compared with the U-line, the B-line had a slightly lower WL (1.2%). The WL (3.2%) of the D-line was significantly lower than that of the U-line. Thus, the ligninolytic selectivity was significantly higher (0.11). However, the KL degradation rate of each transformant line was almost the same. Therefore, we speculated that the homologous transformation of *PsBQR*/*PsHGD* in *P. sordida* YK-624 reduced the holocellulose degradation activity.

In order to clarify the effects of PsBQR and PsHGD gene transfer, we selected clones that exhibited higher ligninolytic selectivity. These clones were subcultured five times on PDA medium and re-analyzed for their ligninolytic properties on beech wood meal. Strains U2, B4, and D14 from each transformed clone line were selected based on their ligninolytic activity and selectivity (Fig. 1A), and the capacity of the selected clones to degrade vanillin was investigated. Because *P. chrysosporium* up-regulated BQR and HGD by exogenous vanillin [13], we investigated on the involvement in vanillin metabolism of PsBQR and PsHGD. To eliminate the

oxidation of vanillin by ligninolytic oxidoreductases, potato dextrose broth medium was used to obtain non-ligninolytic conditions in this experiment. The time course of degradation of 10 mM vanillin in culture is shown for each transformant in Fig. 2A. During the initial stage of incubation (~12 h), the vanillin degradation rates were significantly higher with B4 and D14 than U2. However, the rate of degradation by each transformant decreased with the residual vanillin concentration and there was no significant difference overall. The expression of *PsBQR* in B4, and of *PsHGD* in D14 had increased at 6 h incubation after the addition of vanillin (Fig. 2B). BQR activity in B4 (481 ± 3 mU/mg of protein) was significantly higher than that of U2 (340 ± 7 mU/mg) and D14 (422 ± 26 mU/mg), after incubation with vanillin. And also HGD activity in D14 (40 ± 7 mU/mg) was higher than that of U2 (5 ± 1 mU/mg) and D4 (16 ± 3 mU/mg), significantly. Therefore, it was estimated that a high vanillin concentration activated the *bee2* promoter and improved the activities of PsBQR and PsHGD. However, metabolites transformed from vanillin were not detected on HPLC analytical method used in this study. It was considered that the co-transformants of *P. sordida* YK-624 also expressed several enzymes by exogenous vanillin, because the addition of exogenous vanillin had induced various enzymes in *P. chrysosproium* [13]. Therefore, we estimated that some of induced enzymes quickly transformed vanillin into metabolites having a targeting structure of BQR and HGD. *P. chrysosporium* BQR, a homologue of PsBQR is capable of reducing both substituted *para*- and *ortho*-quinones [3]. Although the information of substrate specificity in fungal HGD is poorly, the HGD gene has been specifically induced by the aromatic azo dye/lignin combine treatment in *Irpex lacteus* [16], in addition of the induction by the addition of vanillin in *P. chrysosporium* [13]. From these facts, it was expected that BQR and HGD catalyzes the metabolism of various aromatic compounds during wood-decay. Therefore, we hypothesized that transformants B4 and D14 had expressed higher activity for metabolizing monomeric lignin fragments when there was a high concentration of these fragments.

Proposed vanillin metabolic pathway including with BQR and HGD, and the quinone redox cycling were illustrated in Fig. 3. Various phenolic compounds (such as vanillin) produced in white-rot fungi during the degradation of lignin and these phenolic compounds are readily oxidized to quinones (methoxyquinone; MQ) by ligninolytic enzymes. A part of quinones are rapidly reduced to hydroquinones (methoxyhydroquinone; MHQ) by cellobiose dehydrogenase (CDH) which is an extracellular enzyme produced by various wood-degrading fungi [8], and the increased amount of hydroquinones activates the production of ROS by quinone redox cycling [7]. Semi-quinone radicals are formed from hydroquinones via oxidation by redox enzymes such as manganese peroxidase. Semi-quinone radicals have the ability to reduce oxygen to form the superoxide anion ($O_2^{\bullet-}$), which dismutates to hydrogen peroxide. The hydroxyl radical ($HO^{\bullet}$) produced from H_2O_2 by the Fenton reaction is a likely candidate for wood decay. It can oxidize all of the wood components including lignin and holocellulose [10]. Backa et al. [2] investigated fungal growth and the generation of hydroxyl radicals, and showed that hydroxyl radicals are involved in the initial phase of wood degradation by white-rot fungi. Suzuki et al. have also found that a decrease in holocellulose viscosity was correlated with hydroquinone concentration and that hydroxyl radicals derived from hydroquinone turnover contributed most to major part of the holocellulose

depolymerization during brown rot in wood [17]. In addition, Eriksson et al. have reported that white-rot fungi can metabolize syringic acid, a lignin degradation fragment, much more rapidly than brown-rot fungi [5]. Therefore, it is believed that the hydroxyl radicals produced by quinone redox cycling are responsible for the early phase of holocellulose degradation by wood-rot fungi, and that the different rates of low-molecular weight lignin fragment metabolism in white- and brown-rot fungi are responsible for the variations in holocellulose degradation activity.

Quinones taken into cell are reduced to hydroquinones (MHQ) by BQR, and then the demethylation is followed to give trihydroxylbenzene (THB), which then undergoes ring-cleavage (Fig. 3). It is thought that the demethylation occurs prior to ring-cleavage in *P. chrysosporium* [1], therefore we considered that *P. sordida* YK-624 also probably has similar metabolic system. Thus, it is estimated that the increasing of BQR activity rises the amount of hydroquinones in mycelium, and accelerates the ring-cleavage indirectly. On the other hand, upregulation of HGD which probably works in a minor ring-cleaving pathway is able to increase the velocity of hydroquinones metabolism directly. As a result, the excretion of quinones and hydroquinones from mycelium is decreased, so the extracellular concentration of these compounds that are going to join the quinone redox cycling is lowered. In this study, the homologous expression of *PsBQR* or *PsHGD* in the co-transformant B- and D-lines, respectively, most likely improved the phenol/quinone metabolism in a similar manner to vanillin (Fig. 2). Therefore, we hypothesized that the co-transformants could metabolize phenols and/or quinones more readily under BQR/HGD homologous expression, which reduces the cellulolytic activity due to quinone redox cycling. In contrast, the degradation of lignin was unaffected by the homologous expression of *PsBQR* and *PsHGD* (Fig. 1), but the ligninolytic selectivity was increased.

In this study, we investigated the effects of the homologous expression of BQR and HGD genes on the metabolism of low-molecular weight lignin fragments during wood degradation by *P. sordida* YK-624. The homologous recombination of *PsBQR* and *PsHGD* had quite limited effects on the degradation of lignin. However, we believe that BQR and HGD have roles in the metabolism of low-molecular weight lignin fragments, although they are also involved in holocellulose degradation during white rot in wood, as shown in this study.

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Figure Legends

Fig. 1. Wood degradation properties A) individual co-transformants (B and D) and control transformants (U), or B) averages of each transofrmed lines. Weight loss (WL) and Klason lignin (KL) degradation are shown as white and gray bars, respectively. KL degradation/WL ratios are shown by the black diamond symbol. The asterisks indicate significant difference ($p < 0.05$) from the control (U-line).

Fig. 2. Response of co-transformant (B4, D14) and control strain (U2) to exogenous vanillin. A) Time courses of the degradation of 10 mM vanillin by co-transformants B4 and D14, and control transformant U2 in potato dextrose broth medium. B) RT-PCR analysis of *PsBQR*, *PsHGD* and *actin* expression in the transformants. Asterisks indicate significant difference ($p < 0.05$) from the control.

Fig. 3. Proposed pathway of the metabolism of vanillin including with quinone redox cycling. Dotted lines mean hypothetical pathway or enzymatic activity in *P. sordida* YK-624.

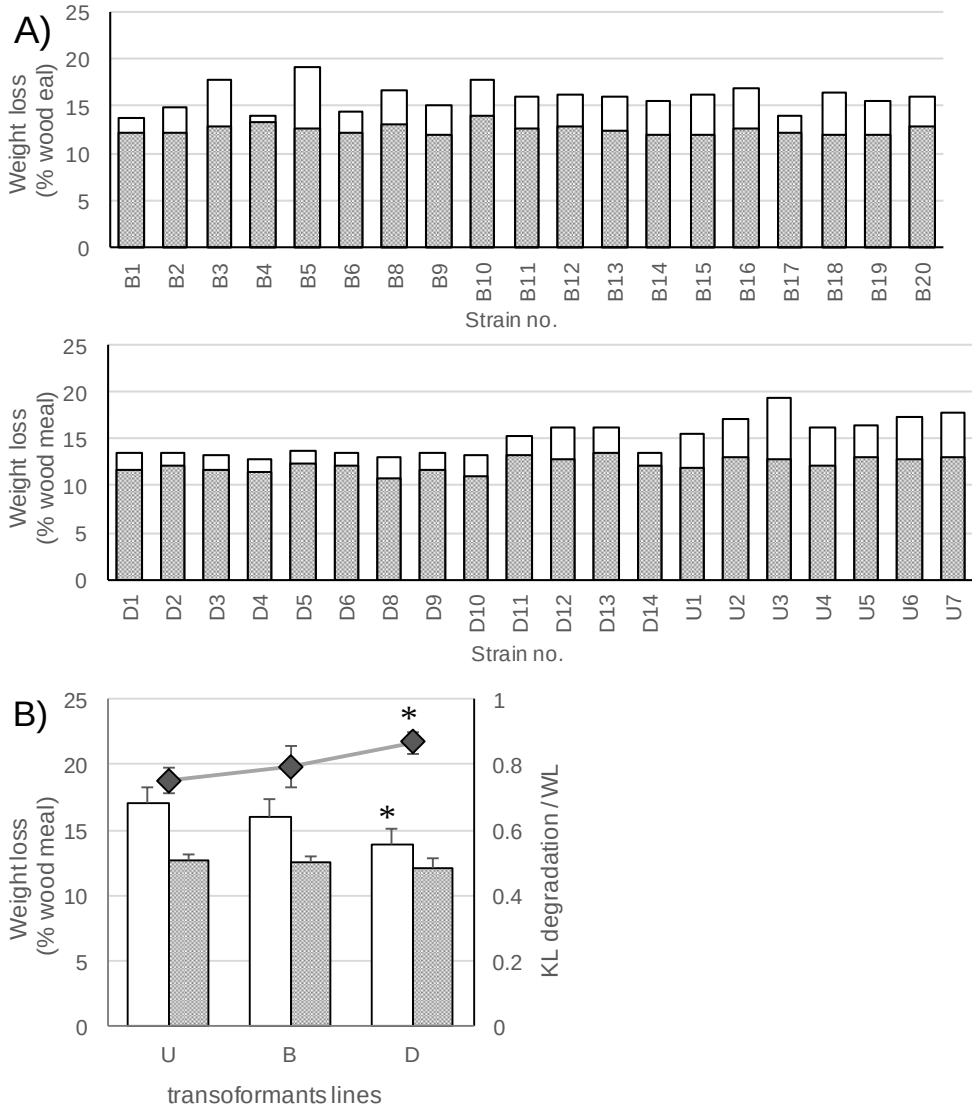

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Fig. 1

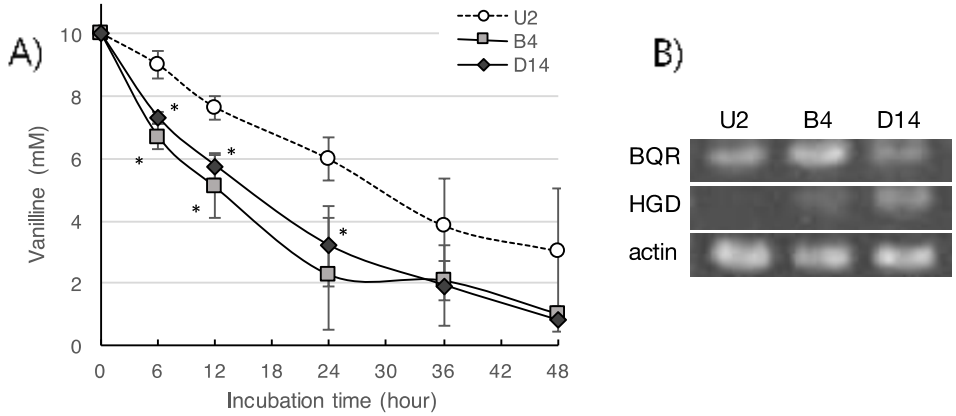

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Fig. 2

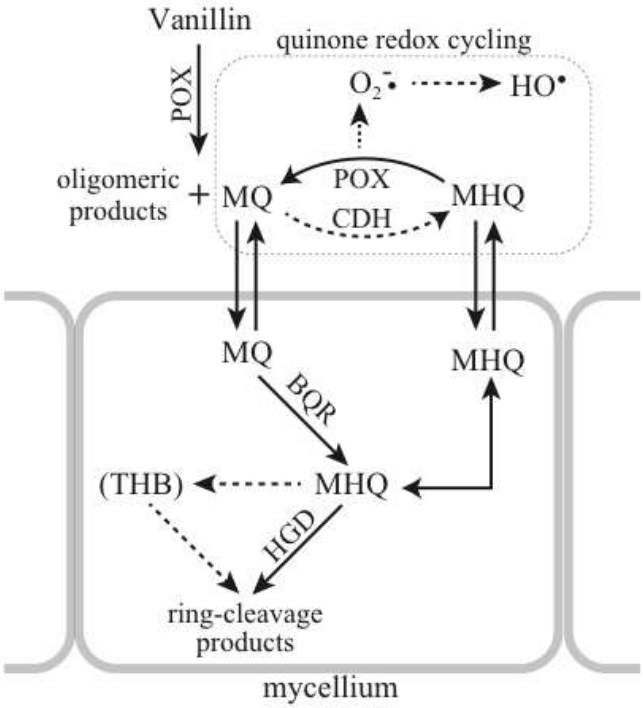

Fig. 3. Proposed pathway of the metabolism of vanillin including with quinone redox cycling. Dotted lines mean hypothetical pathway or enzymatic activity in *P. sordida* YK-624.

Fig. 3

Table 1. Primers used in this study

Primer name	Sequence* (5′ → 3′)
degBQRf	TAYWSIATGTAYGGNCAYRT
degBQRr	TCCCATRAANGCNYKCCAYTG
degHGDf	AYATGGGNAANGARGCNTTY
degHGDr	TGCCANGCNACNAYRTCRAA
psBQRf	TTGAATCGCACTCCTGATCC
psBQRr	ATCTGGAGATAGGTCGTGTC
psHGDf	GCAGCACTCTATTGCTCAAG
psHGDr	ACAAGGCTCCAGGTTTCTTG
ndeiBQRsta	CAGCCCATATGCCCGCGAAGATCGCC
xbaiBQRterm	GACGCTCTAGATTAGAAGGAAACCTTCGCAAGAGTG
kpniHGDsta	GGTACCATGGTGCGTGTTATTCTGAC
xbaiHGDterm	TCTAGACTACAAAGCCAGGGGC
confBQR	GCTACCGGCCAGCTTTG
confHGD	CGACAACGAGGTCTTCACC
GPDterm	GGGTACTTGTACAATCCTCC
rtBQRf	AGTTGGCTGAGTCCGTCAAG
rtBQRr	GTAGAGACGAAGAGAGCGGC
rtHGDf	TTCCAGTGTTCGGCAGACTC
rtHGDr	TGTACGTCACTTCCCACTGC
rtACTf	AGCACGGTATCGTCACCAAC
rtACTr	AGCGAAACCCTCGTAGATGG

* Y = C/T, W = A/T, S = G/C, N = A/T/G/C, R = A/G, K = G/T, I = inosine. Restriction sites are underlined.