Full Length Research Paper

Isolation, identification and application in lignin degradation of an ascomycete GHJ-4

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This study was undertaken to isolate an ascomycete producing ligninolytic enzyme and characterize its lignin degradation capability. Among 20 isolates, GHJ-4 was isolated from decayed wood of *Salix matsudana Koidz* in Mount Tai, China, by different indicator compounds assay. The taxonomy of the fungi was *Paraconiothyrium variabile* Damm, Verkley and Crous, which had been confirmed by both morphological and 5.8S rDNA/ITS analyses. The capability of utilizing several lignin model compounds and decoloration of aromatic dyes by GHJ-4 strain revealed its ligninolytic potentiality. After incubation for 40 days, the weight loss of the wood was 20.91% and lignin loss was 22.99%, which indicated that, GHJ-4 strain had higher degradation ability for lignin. To our best of knowledge, this study represented the first report that *P. variabile* could produce ligninolytic enzyme and degrade lignin.

Key words: Ligninolytic enzyme, ascomycete, identification, lignin degradation.

INTRODUCTION

Biofuel plays an essential role in replacing petroleum-based fuels in current worldwide energy situation (Yuan et al., 2008). Among all the feedstocks, lignocellulosic biomass, which could be acquired from biomass crop and agricultural residuals, might be the best choice in the long term (Smeets and Faaij, 2007). However, the key obstacle for transitioning lignocellulosic feedstock was the complicated structure of the plant cell wall, particularly the presence of lignin. It was highlighted that, microbial degradation of lignin had potential advantages over the prevailing chemical degradation, due to energy and environmental concerns (Keller et al., 2003). However, degrading lignocellulosic biomass by current available microorganism is still far to meet the industrial demands (Shary et al., 2007; Afrida et al., 2009). Thereby, isolation of new strains for degradation of lignin is still essential.

Extracellular oxidoreductases, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase produced by wood-decomposing microorganism, are directly involved in the degradation of lignin in their natural lignocellulosic substrates and various xenobiotic compounds including dyes (Abrahão et al., 2008; Thurston, 1994; Call and Mücke, 1997; Leonowicz et al., 2001). Some wood-degrading fungi contain all three classes of lignin-modifying enzymes, while the others contain only one or two of these enzymes (Hatakka, 1994; Dhouib et al., 2005). The production of ligninolytic enzymes is observed as a colourless halo around microbial growth (Dhouib et al., 2005). So, the lignin degradation capability of microbes is initially screened indirectly on solid media containing different indicator compounds.

Although, wood decay fungi are primarily basidiomycetes, other microorganisms are also involved in the lignocellulosic decaying processes (Nilsson et al., 1989). Wood biodegradation by certain ascomycetes was first described in detail and designated as "soft rot" by Savory (1954). Nilsson et al. (1989) demonstrated that, some higher ascomycetes, particularly *Daldinia concentrica,*

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Abbreviations: **LiP,** Lignin peroxidise; **MnP,** manganese peroxidase; **PDA,** potato dextrose agar; **SNA,** synthetic nutrient agar medium; **OA,** oatmeal; **CMA,** cornmeal; **MEA,** malt extract; **PDA,** potato dextrose; **ITS,** internal transcribed spacer; **PCR,** polymerase chain reaction; **BLAST,** Basic Local Alignment Search Tool; **NJ,** Neighbors joining; **RBBR,** Remazol Brilliant Blue R; **LiP,** lignin peroxidise; **MnP,** manganese peroxidise.

degraded Aspen wood with the same intensity as *Trametes versicolor,* a basidiomycete typically classified as white-rot fungus. The ascomycete, *Chrysonilia sitophila* could degrade rice hull and *Pinus radiata* bark products and also produce ligninolytic and cellulolytic enzymes (Ferraz et al., 1991). Whereafter, different *Penicillium* strains was described as potential degraders or was able to degrade compounds with related lignin structures (Rodriguez et al., 1994). The family *Xylariaceae* had been reported to cause a special type of extensive wood-rot, which could produce laccase and low amounts of peroxidase as potential ligninolytic enzymes along with different polysaccharide-cleaving hydrolases as well as esterase (Liers et al., 2006).

Ascomycetous fungi with coniothyrium-like anamorphs are common colonizers of woody host plants (Damm et al., 2008). Nilsson (1973) found that, *Coniothyrium cerealis* could degrade wood. A new woody plants hosted genus *Paraconiothyrium* was established by Verkley et al. (2004), which included the old *Coniothyrium minitans* and *Coniothyrium sporulosum*. Later species were frequently isolated from wood and leaves of *Prunus*, *Actinidia*, *Laurus* and *Dendrobium*. Recently, two additional new species, *Paraconiothyrium africanum* and *Paraconiothyrium variabile,* were identified based on their DNA sequence data and unique morphological characteristics (Damm et al., 2008).

In this study, an ascomycete producing ligninolytic enzyme, forming coniothyrium-like anamorphs, was isolated from decayed wood of *Salix matsudana Koidz* in Mount Tai. It was a new strain with lignin-transformation capability. The fungus was morphologically and phylogenetically identified and its ability for lignin degradation was characterized.

MATERIALS AND METHODS

Isolation of ligninolytic enzyme producing fungi GHJ-4 strain

Different indicator compounds were added to potato dextrose agar (PDA) in order to detect ligninolytic enzyme production. These compounds included 0.04% Remazol Brilliant Blue R (RBBR), 0.04% guaiacol and 0.02% tannic acid (Dhouib et al., 2005). The plates were incubated at 28°C for at least a week under dark. Positive strains were subcultured when clear positive reactions were visible.

Morphology

To enhance sporulation, autoclaved wood sawdust was placed onto the surface of synthetic nutrient agar (SNA) medium (Kwaśna and Bateman, 2007) and incubated at 25°C for 1 to 4 weeks under natural light. Measurements, photographs of characteristic structures and vertical sections through conidiomata were made according to Damm et al. (2007). Cultural characteristics and radial growth rates were determined on oatmeal (OA), cornmeal (CMA), 3% malt extract (MEA) and PDA agars (Damm et al., 2008). Plates were incubated in a growth chamber at 25°C under natural light. Growth characteristics were studied on MEA plates incubated in the

dark at temperatures ranging from 5 to 35°C, with 5°C intervals.

DNA isolation, amplification and analyses

Genomic DNA of GHJ-4 strain was extracted from fungal mycelium grown on PDA plates following previously described protocols (Pryor and Gilbertson, 2000). The internal transcribed spacer (ITS) regions of 5.8S rDNA gene was amplified and sequenced using primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Polymerase chain reaction (PCR) was performed by denaturation for 5 min at 95°C followed by 35 cycles of 1 min at 94°C for 1 min at 56°C and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products with the expected size (about 600 bp) were cut from gel and purified by DNA gel extraction kit (TaKaRa, Japan), cloned into pEASY-T3 vector (TransGen, China) and transformed into *Escherichia coli* competent cells DH5α. Sequences were performed with an AB13730XL DNA autosequencer (Applied Biosystems). Sequence homolog analysis was performed by Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov). Fungi identification was based on 5.8S rDNA gene sequence similarity to BLAST hits. Neighbors joining (NJ) phylogenetic trees were generated based on partially sequenced 5.8S rDNA and closely related sequences. In the NJ analyses, the Kimura distance calculation was used. A bootstrap analysis (1000 replicates using NJ option) was performed to determine the confidence levels of the nodes.

Cultivations in liquid media

GHJ-4 strain was cultivated on a basic media (Media V) for production of laccase on 200 rpm at 28°C. Media V contained 15.0 g sucrose and 200.0 g potato extract per liter. Subsequently, it was cultivated in four types of rich media to improve laccase production. Media I contained (g/l): 3.0 soluble starch, 2.0 yeast extract, 0.17 KH_2PO_4, 0.44 $MgSO_4$, 0.37 $CaCl_2$, 0.5 Tween-80 ; media II contained (g/l): 200.0 potato extract, 25.0 glucose, 3.0 yeast extract, 3.0 KH_2PO_4, 2.5 $MgSO_4$, 0.1 vitamin B1, pH 6.0; Media III contained (g/l): 24.0 soluble starch, 24.0 glucose, 10 bran, 3.2 KH_2PO_4, 0.2 $MgSO_4$, 0.006 $CuSO_4$, 3.2 NH_4Cl, pH6.5; Media IV contained (g/l): 30 bran, 2.0 peptone, 3.0 beef extract, 3.0 KH_2PO_4, 0.5 $MgSO_4$, 0.02 Vitamin B1, 0.01 $CaCl_2$, 0.1 NaCl.

LiP and MnP production by GHJ-4 strain was followed for 14 days. The fungus was inoculated into 250 ml Erlenmeyer flasks containing 50 ml medium which involve 30.0 g bran, 58.0 g soluble starch, 3.5 g beef extract, 1.0 g $NaNO_3$, 3.0 g KH_2PO_4 and 0.1 g $MnSO_4$.

Laccase activity assay

Laccase activity was measured spectrophotometrically with guaiacol as substrate. A modified method of Chakraborty et al. (2000) was used. An aliquot of enzyme solution was incubated in 4.0 ml of 50 mM sodium acetate buffer (pH 4.5) containing 1.0 mM guaiacol at 30°C. The changes in absorbance due to oxidation of guaiacol in the reaction mixture was monitored at 465 nm (ε_{465}=12,100 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µmol guaiacol per minute under the aforementioned condition.

Lignin peroxidase activity assay

The lignin peroxidase activity was evaluated by UV spectrometry of the veratraldehyde produced (TM_{310} =9,300 $M^{-1}cm^{-1}$) during veratryl alcohol oxidation (Moldes et al., 2003). The reactive mixture was

composed of 2.5 ml sodium tartrate buffer (100 mM, pH 3.0), 1.0 ml veratryl alcohol (10 mM), 100 µl hydrogen peroxide (10 mM) and 400 µl enzyme extract. The reaction was started by adding hydrogen peroxide and the veratraldehyde was determined at 310 nm. One enzyme unit was defined as 1 µmol product formed per minute under the assay conditions.

Manganese peroxidase activity assay

Manganese peroxidase activity was assayed following the method of Moldes et al. (2003). The reaction mixture contained 3.4 ml sodium malonate (50 mM pH 4.5), 0.1 ml $MnSO_4$ (15 mM) and 400 µl enzyme extract. The reaction was started by adding 0.1 ml H_2O_2 (10 mM) and the produced Mn^{3+} complexes was determined at 240 nm (ε_{240}=6,500 M^{-1} cm^{-1}). One activity unit was defined as the amount of enzyme that oxidized 1 µmol $MnSO4$ per minute and the activities were expressed in U ml^{-1}.

Characterization of ligninolytic potential

GHJ-4 strain was incubated in 250 ml Erlenmeyer flasks containing 25 ml minimal salts medium, which involves 2.0 g NH_4Cl, 0.5 g $MgSO_4$, 1.0 g KH_2PO_4, 0.007 g $CuSO_4$, 0.2 g Na_2HPO_4, 0.035 g $MnSO_4$, 0.007 g $FeSO_4$ per liter and lignin (Sigma-Aldrich) and guaiacol as sole carbon source. The flasks were inoculated with 2 pieces (1.0 cm diameter) of 7-day precultured agar plates and the cultures maintained under stationary at 28°C for 15 days. Growth of the fungus and indication of the lignin degradation capability was measured based on the dry weight of the mycelia after filtration, followed by drying at 80°C to constant weight.

Detection of decolourization ability

For dye decolourization studies, GHJ-4 strain was incubated on PDA plate supplemented with 0.02% RBBR, aniline blue and phenol red at 28°C (Rodriguez et al., 1996). Decolourization was observed after 15 days.

Wood biodegradation by GHJ-4 strain

Wood chips (2.0×2.0×0.1 cm) were obtained from *Populus bonatii* Levl (10-year old). Wood biodegradation was performed under solid-state fermentation at 28°C for 40 days. Inoculum was prepared as follows: 3 pieces (1.0 cm diameter) of 7-day precul-tured agar plates were inoculated into a 250 ml Erlenmeyer flask containing 5% Wood chips, 15% bran and 80% nutrient solution that was composed of 2.0 g NH_4Cl, 0.5 g $MgSO_4$, 1.0 g KH_2PO_4, 0.007 g $CuSO_4$, 0.2 g Na_2HPO_4, 0.035 g $MnSO_4$, 0.007 g $FeSO_4$ and 1,000 ml of water. After biodegradation, bio-treated wood chips were washed to remove the mycelium grown on wood surfaces and then dried to constant weight at 105°C. Initial and final dry weights were used to determine weight losses. A set of uninoculated sterilized wood chips served as a control wood sample.

Decayed and non-decayed wood samples were milled in a knife mill to pass through a 0.5 mm screen. Klason insoluble and soluble lignins in benzene/ethanol extracted milled wood samples were determined by acid hydrolysis as described by Ferraz et al. (2000).

RESULTS

Isolation of GHJ-4 strain

Reactions with three different indicators, guaiacol,

Remazol Brilliant Blue R (RBBR) and tannic acid, were tested with collected samples. The positive reactions with guaiacol, RBBR and tannic acid correlated well with each other. Altogether, 20 fungal strains showing positive reactions on indicator plates were isolated from decayed wood of *S. matsudana Koidz* in Mount Tai. Fungal strain GHJ-4 was selected for further studies, as the oxidative polymerization of guaiacol to form reddish brown zones in the medium was higher than other isolated strains. Its lignin degradation capability was further confirmed by other indicators (Figure 1).

Phylogenetic analysis

An expected 589 bp DNA fragment of the 5.8S rDNA/ITS region was obtained from the GHJ-4 strain by PCR ampli-fication. The sequence was deposited in the GenBank database (Accession no. GQ331986). BLAST analysis of the amplificons indicated that, it had the highest similarities (over 99%) sequences generated from *Paraconiothyrium* or *Microdiplodia* strains. To clarify the phylogenetic position of GHJ-4, a phylogenetic tree was constructed based on the 5.8S rDNA sequences (Figure 2). The result revealed that GHJ-4 strain, *P. variabile* and *Paraconiothyrium brasiliense* formed one cluster, which implied they were closely related.

Taxonomy

The strain GHJ-4, obtained from wood of *S. matsudana Koidz*, could be assigned to *P. variabile* based on the DNA sequence data and its morphology (Damm et al., 2008). Its conidiomata were pycnidia and the shape of the conidiogenous cells was more variable. Conidia were smaller than those of most other *Paraconiothyrium* species and grew more slowly than *P. brasiliense*.

Conidiomata pycnidial, produced on wood sawdust on SNA in 1 to 4 weeks was solitary, subglobose, 1 to 3 ostioles, black, superficial or semi-immersed, 200 to 600 µm diameter, wall consisting of 6 to 8 cell-layers (30 to 45 µm) of thick-walled dark brown textura angularis, be-coming hyaline and thin-walled towards the inside of the pycnidium, that was surrounded by brown hyphal appendages. Conidiophore, lining the inner conidiomatal cavity, was hyaline, branched with conspicuous umbilici, 3 to 10 × 2 to 5 µm. Conidiogenous cells varied in shape, conical to subulate or subcylindrical, broadly or elongated ampulliform, phialidic with periclinical wall thickening or with one or more percurrent proliferations near the apex. Conidia was pale brown or light green, subcylindrical to ellipsoidal, both ends obtuse, 1-celled, smooth walled, 3 to 4.5 × 1.5 to 2.5 µm. Vegetative hyphae were 2 to 5 µm wide, hyaline to pale brown, septate and smooth. On PDA medium, parts of the hyaline vegetative hyphae was transformed to very dark-walled hyphal pieces, which could become locally swollen or accumulate amorphous
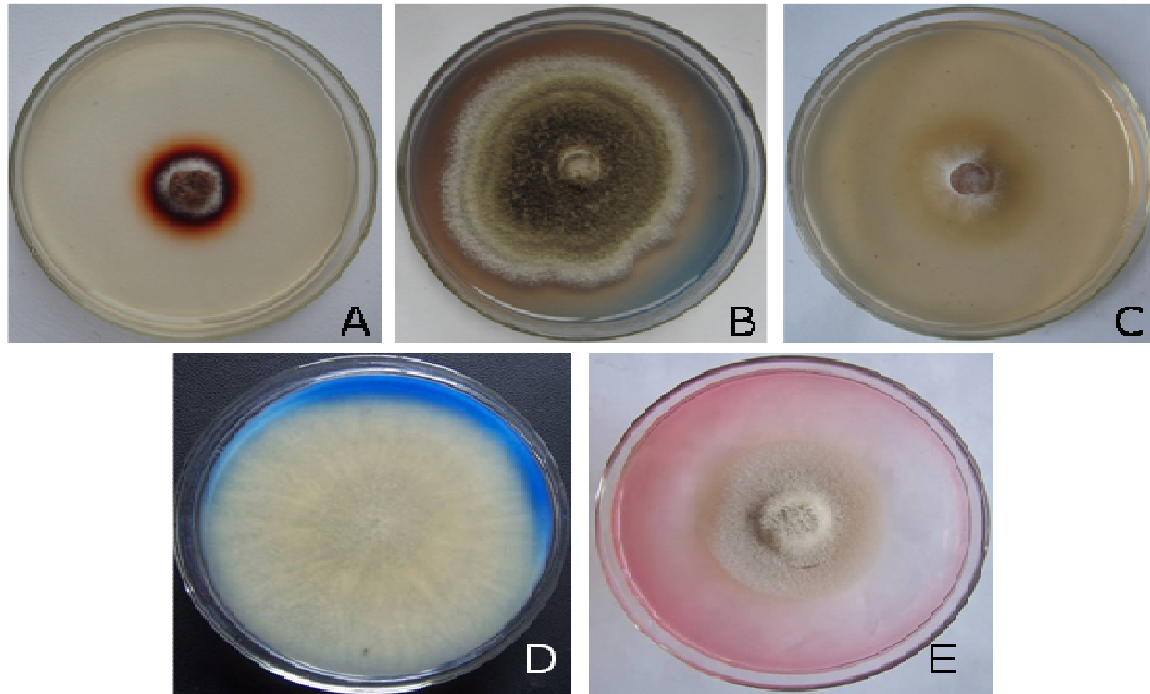
Figure 1. Reactions of GHJ-4 strain with different indicators on PDA plates. A, Reddish brown zones of guaiacol; B, decolourize of RBBR; C, yellow zones of tannic acid; D, decolourize of aniline blue; E, decolourize of phenol red.
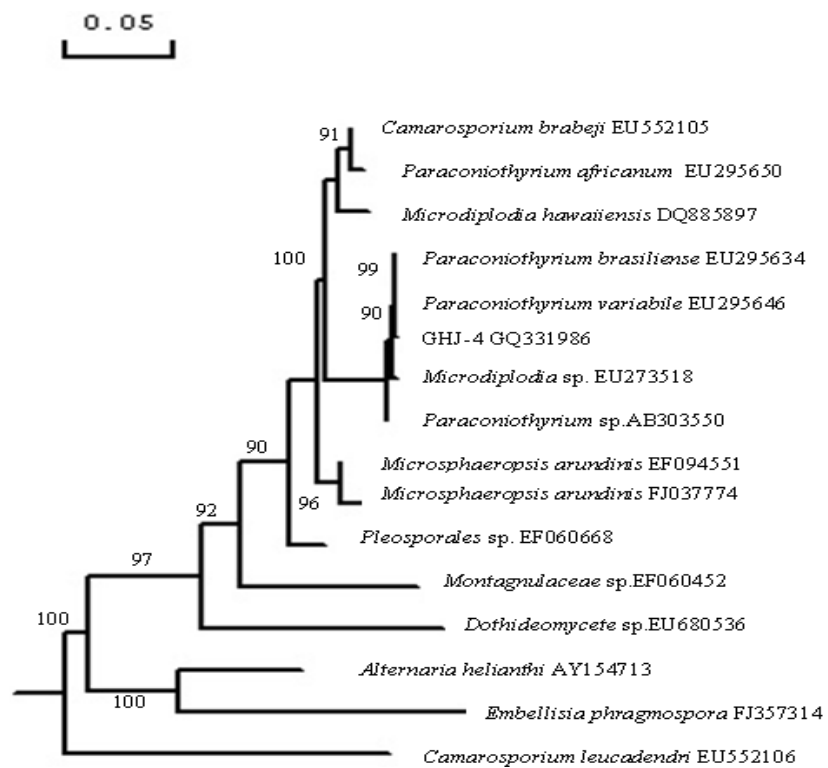
Figure 2. 5.8S rDNA/ITS region phylogenetic tree of GHJ-4 strain obtained by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Numbers at the nodes are the bootstrap confidence values obtained after 1000 replicates. *Camarosporium leucadendri* was used as the out-group.
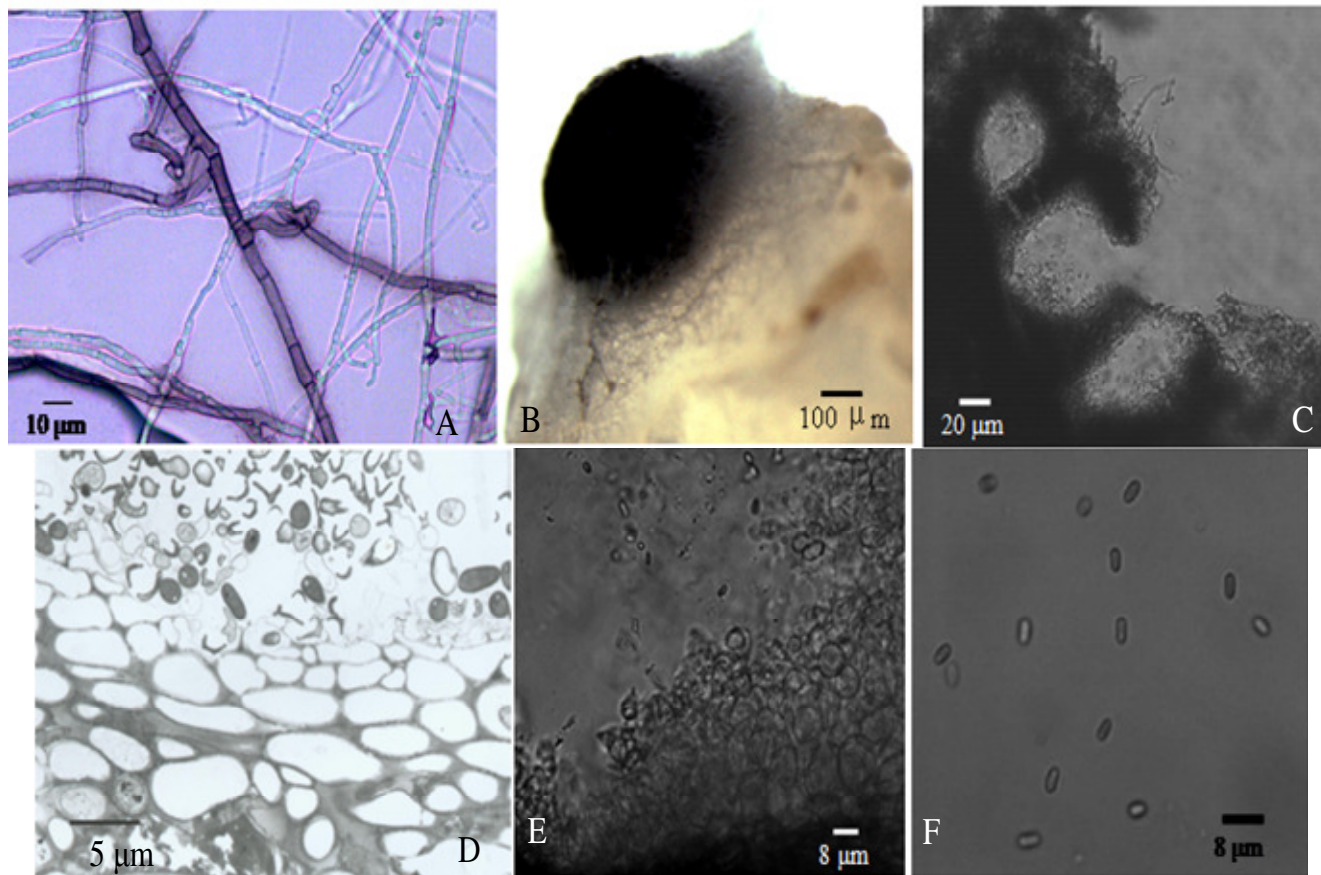
Figure 3. Morphology of GHJ-4 strain. A, Dark walled hyphae; B, conidia oozing from pycnidia; C, longitudinal section through a pycnidium; D, pycnidial wall with conidiophorese; E, conidiophores; F, conidia.

brown material on the outer wall surface (Figure 3).

Cultural characteristics

Colonies on OA reached 53.2 mm after 7 days and 85 mm after 14 days (25°C, in diffuse daylight). It was flat, with an even to slightly ruffled colourless and glabrous margin, mycelium whitish, aerial mycelium absent or consisting of sparse, scattered white to grayish tufts. Colonies on MEA reached 42.6 mm diameter in 7 days and 68.5 mm in 14 days, with an even to slightly ruffled, glabrous and colourless margin, colony surface almost entirely covered by a dense mat of woolly aerial mycelium, which was pale olivaceous-grey, in the centre olivaceous-black and near the margin paler to almost pure white. Colonies on PDA reached 54.7 mm diameter in 7 days, 82.8 mm diameter in 14 days. It was flat, with an even, whitish margin, most of the colony surface covered by felty floccose aerial mycelium, in the centre becoming olivaceous-grayish. Colonies on CMA reached 85 mm diameter in 14 days (46 mm in 7 days), as on PDA. It was flat, white, in the centre becoming olivaceous-grayish (Figure 4).
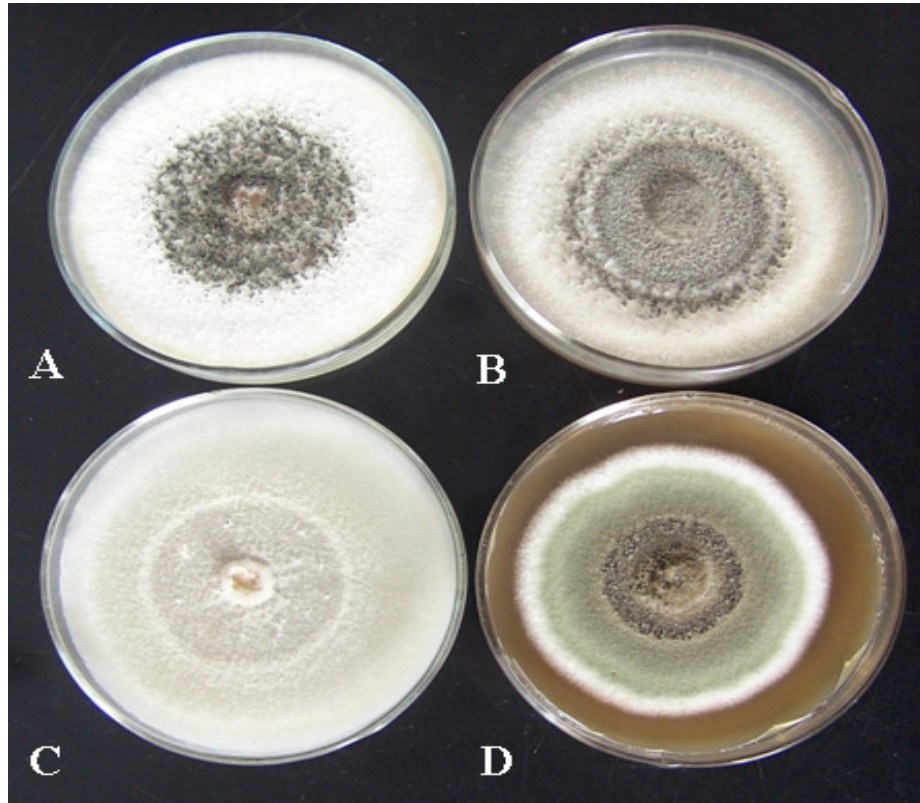
Growth characteristics

The growth temperature range of GHJ-4 was 5 to 35°C and the optimum growth temperature was approximately 25°C.

Production of ligninolytic enzyme by GHJ-4 strain

Laccase production by GHJ-4 strain was studied in liquid cultures and proved to be highly dependent on the medium. The production level of laccase in liquid cultures was quite low in basic media. Laccase production was enhanced by the addition of bran. Among the five media tested in this study, media IV had the highest laccase production (Figure 5). The production of laccase activity occurred on day 2 and reached its maximum (484.72 U/ml) on day 10 and then declined gradually.

Furthermore, GHJ-4 also produced LiP and MnP. LiP activity first appeared on the 2nd day (0.31 U/ml) and reached its highest level of activity (7.28 U/ml) on the 12th day, after which the activity started to decrease (Figure 6). As regards MnP activity, it began on the 4th day (0.73 U/ml) and then it increased peaked on the 8th day of

Figure 4. Cultural characteristics of GHJ-4 strain incubated on CMA (A), PDA (B), OA (C) and MEA (D) (25°C, natural light).
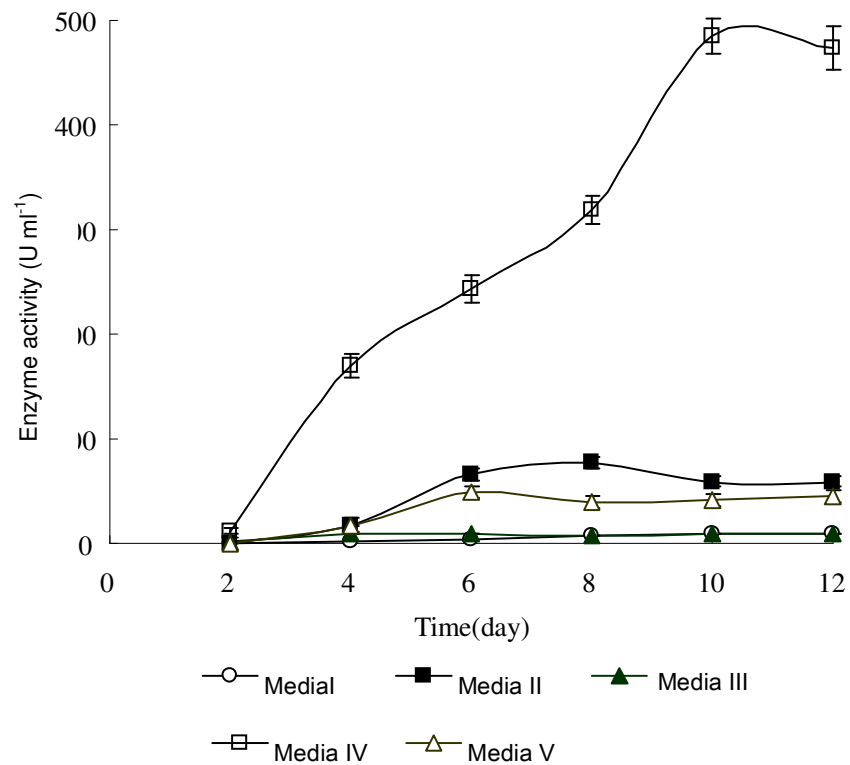
Figure 5. Time course of extracellular laccase activity of GHJ-4 on different media.
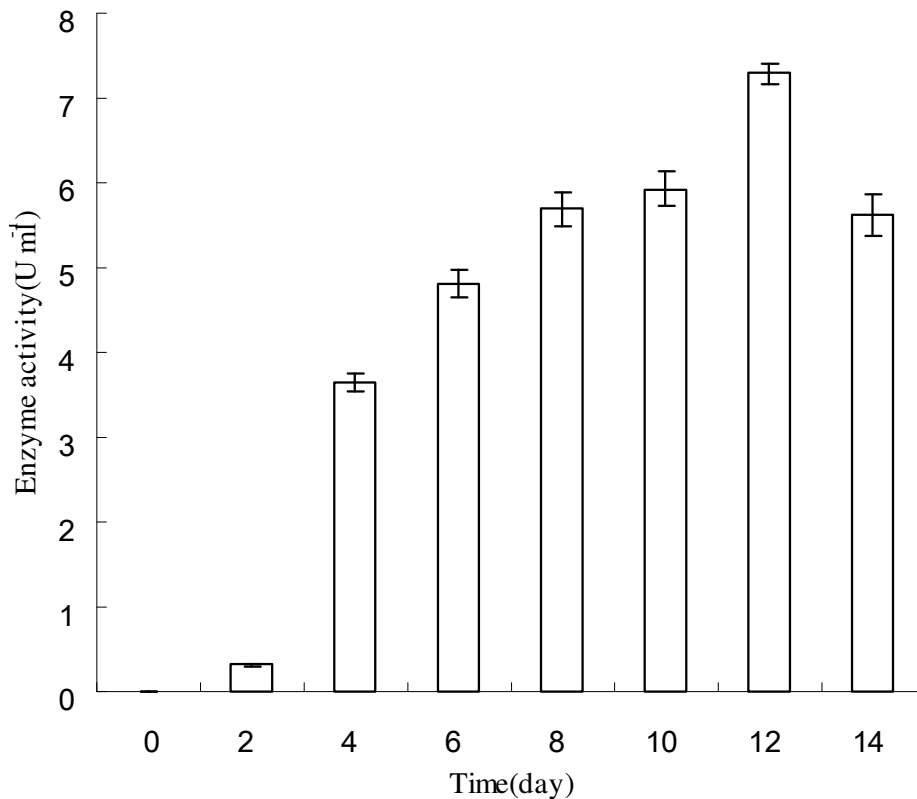
Figure 6. Time course for LiP activity produced by GHJ-4.

cultivation (3.81 U/ml) (Figure 7).

Lignin model compounds utilization capability

The ability of GHJ-4 strain to utilize guaiacol or lignin as the sole carbon and energy source is shown in Table 1. Results showed that lignin and guaiacol, particularly lignin, could be utilized by GHJ-4 strain. So, GHJ-4 was able to utilize some lignin model substrates.

Dye decolourization ability of GHJ-4

The polymeric dye RBBR, an anthracene derivative, was used as a starting material in dye production and represented an important class of often toxic and recalcitrant organopollutants. Results showed that, GHJ-4 could readily decolorize RBBR (Figure 1). We had also found the decolourization of another two polymeric dyes, aniline blue and phenol red, by GHJ-4 in solid culture (Figure 1).

## Biodegradation capacity for wood

The decay capacity of GHJ-4 strain for wood blocks was examined. Results showed that, GHJ-4 had 20.91%

losses of wood weight and approximately 22.99% lignin loss after 40 days incubation period. So, GHJ-4 strain could be considered as an efficient wood degrader.

DISCUSSION

P. variabile was frequently isolated from different plant species worldwide. Riccioni et al. (2007) isolated it from wood of *Actinidia chinensis* and *Actinidia deliciosa* in association with trunk or vine disorders and from necrotic wood under pruning cut surfaces. There was also a report on the isolation of this species from leaves of *Laurus nobilis* (Göre and Bucak, 2007) and branches of *Prunus persica* and *Prunus salicina* with dieback symptoms or pruning debris (Damm et al., 2008). However, there is no isolation of *P. variabile* having been reported in China. Our present study represents the first report of isolation of this species. This indicated its broad host spectrum, including several distantly related host plants and a wide geographical distribution.

The basidiomycetous white-rot fungi and related litter-decomposing fungi are the most efficient lignin-degrading organisms in nature (Fackler et al., 2006; Ohkuma et al., 2001). However, the degradation of lignocellulose by ascomycetes is an important route for carbon cycling in plant litter and soils (Nilsson and Daniel, 1989; Liers et
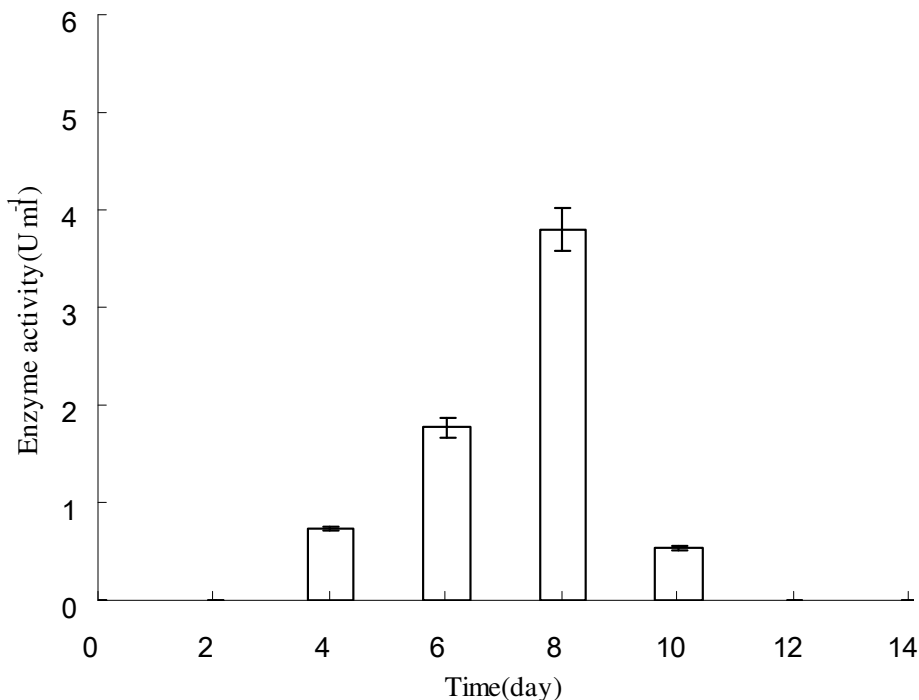
Figure 7. Time course for MnP activity produced by GHJ-4.

Table 1. Growth of GHJ-4 on different medium.

Carbon source	Dry weight of mycelial (mg)				
	3 days	6 days	9 days	12 days	15 days
Lignin	2.2	4.9	10.0	16.9	25.5
Guaiacol	0.5	1.2	6.4	8.8	12.3

al., 2006). Our results showed that, the newly isolated ascomycete GHJ-4 could produce extracellular oxido-reductases including laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP), which is the first record of *P. variabile* producing ligninolytic enzyme. Furthermore, laccase production by GHJ-4 was largely affected by culture conditions, particularly bran, which was in agreement with the reported results (Lee et al., 1999; Schlosser et al., 1997; Dong et al., 2005). Chen et al. (1982) had clearly demonstrated that, part of lignin degradation proceeds via low-molecular-weight aromatic acids and the dye serves as a substrate for fungal enzymes possibly related to lignin degradation (Glenn and Gold, 1983). Our results showed that, the newly isolated *P. variabile* GHJ-4 strain was capable of utilizing several lignin model compounds and decoloration of aromatic dyes, which provided additional evidence of degrading lignin of GHJ-4. In addition, this finding proved that GHJ-4 was able to degrade natural lignin.

In conclusion, our results showed that this new isolated *P. variabile* GHJ-4 had a certain lignin degradation capacity and provided a new microorganism resource for lignin degradation. The ability of this fungi strain could be of interest for lignocellulosic biomass degradation as well as treatments of pulp and paper mill effluent. However, it remains to be studied concerning the role of every ligninolytic enzyme of GHJ-4 strain on lignin biodegradation and how lignin is degraded by it. Furthermore, it would be more informative to compare the ligninolytic capabilities of this fungus with other lignin-degrading ascomycetes and basidiomycetes and further research concerning the role of ascomycetes on lignin biodegradation should be carried out using *P. variabile* GHJ-4 and other microorganisms of this class.

ACKNOWLEDGEMENT

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