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Studies on the Ligninolytic Enzyme Activities During Biological Bleaching of Kraft Pulp with Newly Isolated Lignin-Degrading Fungi

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ABSTRACT

A screening has been performed to find hyper-ligninolytic fungi, which degrade beech and pine lignin extensively in order to broaden the understanding of the ligninolytic enzymes elaborated by various white-rot fungi. One hundred and twenty two ligninolytic strains were selected from decayed woods with a selective medium for screening ligninolytic wood-rotting fungi. Two of them, *Phanerochaete sordida* YK-624 and YK-472, showed much higher ligninolytic activity and selectivity in beech-wood degradation than typical lignin-degrading fungi, *Phanerochaete chrysosporium* and *Coriolus versicolor*. They also degraded birch dioxane lignin and residual lignin in unbleached kraft pulp(UKP) much more extensively than *P. chrysosporium* and *C. versicolor*. During fungal treatment of beech wood-powder, the fungus strain *P. sordida* YK-624 showed higher activity of extracellular manganese peroxidase(MnP) in the medium than *P. chrysosporium*. It also showed MnP activity, which would not be lignin peroxidase during treatment of oxygen-bleached kraft pulp(OKP) and under enzyme-inducing condition.

1. Introduction

Although lignin is resistant to degradation by microorganisms, lignin-degrading activity has been recognized by certain microorganisms. White-rot fungi(basidiomycetes), of which *P. chrysosporium* and *C. versicolor* are presently well characterized, are responsible for the initiation of lignin degradation in wood. When cultured under ligninolytic conditions, *P. chrysosporium* produces two extracellular peroxidases, lignin peroxidase(LiP) and manganese peroxidase (MnP), and *C. versicolor* produces laccase in addition to LiP and MnP. It was reported that LiP and MnP can catalyze the partial depolymerization of DHP in vitro when DHP is employed as a diluted colloidal dispersion.[1-3] However, it is still uncertain what roles LiP and MnP have in lignin degradation.

White-rot fungi are the most prominent candidates for the biological removal of residual lignin from kraft pulp. Kirk and Yang were the first to recognize that white-rot fungi could partially delignify unbleached kraft pulp.[4] Fujita *et al.* developed a combined lignin-degrading fungal (IZU-154) and chemical bleaching process which could reduce the use of chlorine-based chemicals in hardwood and softwood unbleached kraft pulp bleaching system.[5,6] Murata *et al.* established a chlorine-free bleaching process with a

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combination of fungal treatment of oxygen-bleached hardwood kraft pulp.[7] Paice *et al.* reported that MnP was produced by bleaching cultures of *C. versicolor*, and that the peak production of enzyme occurred at the same time as the rate of fungal bleaching was the maximum.[8] However, the extensive increase in brightness observed in fungal-treated pulp was not found with MnP alone.

In this work, we isolated some lignin-degrading fungi which showed higher ligninolytic activity and selectivity than those reported to date. We investigated their lignin-degrading characteristics and extracellular enzyme activities, which may involve in lignin-degrading stage.

2. Materials and Methods

2.1 Microorganisms

One hundred and twenty-two ligninolytic strains isolated from decayed woods were used. *Phanerochaete chrysosporium*(ME 446), *C. versicolor* (IFO-6482) and *Tyromyces palustris*(FES-0507) were obtained from Dr. Gold at the Oregon Graduate Institute of Science and Technology, Oregon, U.S.A., from Institute for Fermentation, Osaka, Japan and from the Forestry and Forest Products Research Institute, Tsukuba, Ibaragi Prefecture, Japan, respectively.

2.2 Isolation and Screening of Lignin-Degrading Fungi

Each piece of decayed wood was placed on a screening medium containing 0.02% guaiacol and 2.0% beech wood powder(100 mesh pass) and 1.6% agar with the pH of the medium adjusted to 5.0. After incubation at 30℃ for 7 days, the strains that showed red-colored zones were isolated.

2.3 Ligninolytic Enzyme Activity and Selection Factor

One gram of extractive free wood powder(wood: beech and pine, size: 60-80 mesh) and 2.5ml distilled water in a 100ml Erlenmeyer flask were inoculated with each strain of fungi and incubated at 30℃. After incubation, weight loss, Klason lignin content and acid-soluble lignin content[9] of the incubated wood powder were determined. The selection factor(S.F) of fungal degradation of wood was calculated as follows: S.F = lignin loss/holocellulose loss, and the holocellulose loss was calculated as follows: holocellulose loss = weight loss - lignin loss.

2.4 Fungal Treatment of Birch Dioxane Lignin

Birch dioxane lignin(10mg) impregnated into glass fiber filter papers were set on the agar plate. The agar plate consisted of high-carbon, low-nitrogen(HCLN) medium.[10] Membrane filter(pore size 0.45μm, Toyo Roshi Kaisha, Ltd.) was set on the lignin sample. The membrane filter was inoculated with pieces of mycelial mat (4mm×4mm) which had been incubated on PDA medium. After 30 days of incubation at 30℃, the dioxane lignin was recovered from the glass filter paper with 50ml aliquot of 0.1 M NaOH. The extract was measured for the absorbance at 280nm.

2.5 Fungal Treatment of Kraft Pulp

Mycelial mats were prepared as previously described.[5] The mycelia were separated from liquid medium, and fragmented with water for 30 seconds in a sterile Waring blender. For pulp treatments with fungi, 4g of unbleached hardwood kraft pulp(UKP) was sterilized(121℃, 10 min) together with 8ml of water in a 200ml Erlenmeyer flask. The pulp, to which 8ml of

mycelium suspension was added(3% on pulp), was incubated statically at 30℃.

In order to prepare crude enzymes, which were secreted during fungal treatment of oxygen-bleached hardwood kraft pulp(OKP), 10g of sterilized OKP was incubated with 18 ml of mycelium suspension statically at 30℃ in a 500 ml Erlenmeyer flask. After incubation, the pulp was defiberized with water or 50mM malonate buffer(pH 4.5) containing 0.05% Tween 80 in a Waring blender. Here, the buffer solution was used in order to recover proteins. Kappa number of pulp was determined according to Tappi useful method 246.

2.6 Fungal Treatment of Beech Wood-Powder

Mycelial mat and mycelium suspension were prepared as above. For wood-powder treatment with fungi, beech wood medium[200ml of 10mM dimethylsuccinate(pH 4.5) containing 1.0% beech wood-powder] was incubated with mycelium suspention(2% on wood-powder) under shaking condition(150 rpm) at 30℃ for 10 days in a 500ml Erlenmeyer flask. After incubation, the culture was filtered, and the filtrate was concentrated by ultrafiltration(ADVANTEC UK-10, 10kDa cut off) and stocked at 4℃. Beech wood-powder after fungal treatment was dried(room temperature), and Klason lignin and acid-soluble lignin were determined.

2.7 Incubation of the Fungus *P. sordida* YK-624 in Liquid Cultures

Five hundred-ml Erlenmeyer flasks containing 200ml of the culture medium was inoculated with a suspension of mycelial fragments of *P. sordida* YK-624. The culture medium contained the following compounds per liter of distilled water; glucose, 20g; ammonium tartrate, 0.22g; sodium acetate, 1.64g; KH_2PO_4, 2.0g; $MgSO_4 \cdot 7H_2O$, 1.6g; $CaCl_2 \cdot 2H_2O$, 0.17g; thiamine-HCl, 1.0mg; nitrilotriacetate, 0.55g; $MnSO_4 \cdot 4\text{-}5H_2O$, 0.26g; NaCl, 0.37g; $FeSO_4 \cdot 7H_2O$, 37mg; $CoSO_4 \cdot 7H_2O$, 68mg; $ZnSO_4 \cdot 7H_2O$, 68mg, $CuSO_4 \cdot 5H_2O$, 3.7mg; $AlK(SO_4)_2 \cdot 12H_2O$, 6.8mg; H_3BO_3, 3.7mg; $Na_2MoO_4 \cdot 2H_2O$, 4.3mg; Tween 80, 1.0g. The cultures(adjusted to pH 4.5) were incubated at 30℃ on a rotary shaker(150rpm). The fungus was grown under air for first 3 days, then the cultures were cultivated under the following conditions; control: further incubation for 4 days, condition A: on fourth day, addition of veratryl alcohol(0.252g) and purging with O_2 at 24-hour intervals.

After 7 days of incubation, the cultures were filtered through glass fiber filter paper and the resultant filtrates were stocked at 4℃.

2.8 Enzyme Assays

As ligninolytic enzyme activities, peroxidase and phenoloxidase activities were assayed by the oxidation of 2,6-dimethoxyphenol at 37℃. MnP activity was also assayed with addition of 1.2mM $MnSO_4$. The increase of absorbance at 470 nm in the reaction was recorded. LiP activity was assayed by oxidation of veratryl alcohol[11] and Azure B.[12] In oxidation of veratryl alcohol the increase of absorbance at 310nm, and in oxidation of Azure B the decrease of absorbance at 651 nm were recorded.

3. Results and Discussion

3.1 Screening of Lignin-Degrading Fungi and Determination of Ligninolytic Activities and Selection Factors by the Newly Isolated Fungi

A number of decayed wood samples(1212 samples) were collected from Kyushu University Experiment Forests and Yakushima, Japan. Among them, 387 samples showed red-color

zones, and 122 strains could be isolated.

At first, we compared weight losses of beech wood and pine wood between new isolates and typical lignin-degrading fungi, *P. chrysosporium* and *C. versicolor* during fungal treatment for 30 days, as indexes of ligninolytic activities. Many strains showed much lower in weight loss than *P. chrysosporium* and *C. versicolor*. Twenty four strains showed less than 2% in weight loss of beech wood and 42 strains showed less than 2% in weight loss of pine wood. The weight loss of beech wood by P. chrysosporium and *C. versicolor* were 12.2% and 12.7%, respectively, and that of pine wood were 7.6% and 3.0%, respectively. We could isolate 7 strains, which showed higher weight loss of beech wood than *P. chrysosporium* and *C. versicolor*.

Lignin-degrading fungi generally attack angiosperm woods in preference to gymnosperm woods, and they decay the former more rapidly.[13] The lignin-degrading fungi, which degrade gymnosperm lignin more than angiosperm lignin have not so far been isolated. In present works, we compared degradation of angiosperm lignin to gymnosperm lignin with newly isolated fungus. As shown in Fig. 1, most strains showed higher weight loss of beech wood than pine wood. Although only the fungus YK-491 showed weight loss of pine wood as much as that of beech wood, it showed higher ligninolytic

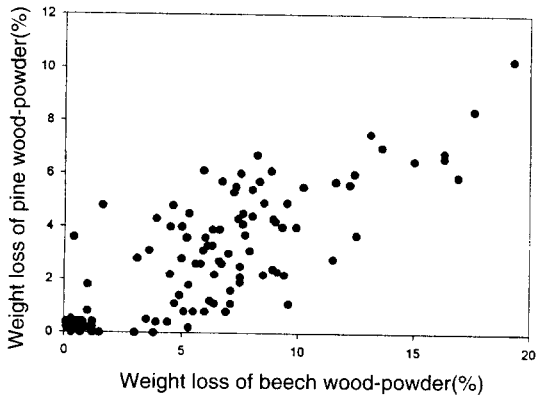

Fig. 1. Weight loss of beech and pine wood-powders by each isolated fungus.

Table 1. Degradation of beech and pine wood lignin by lignin-degrading fungi

Strain	Species	Weight loss(%)	Lignin loss(%)	S.F
YK-491	beech	6.1	7.1	0.4
	pine	6.0	1.0	〈0.1
YK-663	beech	19.5	51.1	2.2
	pine	11.1	23.7	1.5
P. chrysosporium	beech*	12.2	23.7	1.0
	pine	7.6	11.6	0.8
C. versicolor	beech*	12.7	27.2	1.2
	pine	3.0	4.1	0.6

*The strains were incubated for 28 days.

Table 2. Degradation of beech wood lignin by lignin-degrading fungi

Strain	Incubation time(day)	Lignin loss(%)	S.F
KS-62	30	38.5	2.4
MZ-142	30	35.2	2.8
MZ-179	30	51.2	1.7
MZ-210	30	43.3	1.7
MZ-348	30	37.6	2.1
MZ-438	30	42.1	2.6
YK-472	28	40.1	2.5
YK-505	30	36.7	1.6
YK-624	28	40.6	3.5
YK-639	30	35.9	3.7
YK-663	30	51.1	2.2
P. chrysosporium	28	23.7	1.0
C. versicolor	28	27.2	1.2

enzyme activity in beech wood lignin than in pine wood lignin(Table 1). In the present study, no strain degraded gymnosperm lignin more than angiosperm lignin. The fungus YK-663 showed highest ligninolytic activity in pine-wood lignin in all strains employed here(Table 1).

The wood samples(13 samples), in which over 10% of weight loss of beech wood was observed by new isolates, were determined lignin content, and S.F was calculated. As shown in Table 2, the fungi, *P. sordida* YK-624, MZ-179, MZ-210, MZ-438, YK-472 and YK-663 showed higher ligninolytic activity in beech wood, and *P. sordida*

YK-624, YK-472 and YK-639 showed higher selectivity.

3.2 Ligninolytic Characteristics of the Fungi, *P. sordida* YK-624 and YK-472

In order to study ligninolytic enzymes, we selected the fungi, *P. sordida* YK-624 and YK-472, which showed much higher ligninolytic activity and selectivity in beech wood lignin than *P. chrysosporium* and *C. versicolor*.

The degradation of beech wood lignin by these fungi was determined as a function of incubation time. In whole incubation time, *P. sordida* YK-624 and TK-472 showed much higher ligninolytic activity than *P. chrysosporium* and *C. versicolor* (Fig. 2).

The degradation of birch dioxane lignin by these fungi under the condition of ligninolytic culture(HCLN medium) was determined. In this study, a membrane filter, which fungi could penetrate the pores, was introduced in order to prevent adsorption of lignin sample to the mycelia. Considering that a part of solid lignin might migrate toward the mycelia through pores or be adsorbed on the membrane filter during incubation, *T. palustris* was inoculated as a control sample. The fungi, *P. sordida* YK-624 and YK-472,

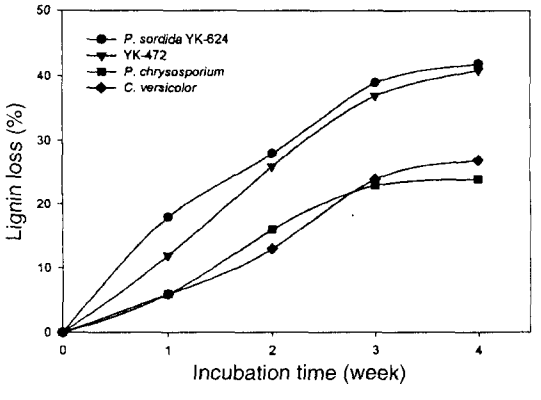

Fig. 2. Degradation of lignin in beech wood powder by lignin fungi as a function of incubation time.

Table 3. Degradation of birch dioxane lignin by lignin-degrading fungi

Strain	Lignin loss(%)
YK-472	22.6
YK-624	28.1
P. chrysosporium	11.7
C. versicolor	8.9
T. palustris	2.2

showed much higher ligninolytic activity in solid lignin than *P. chrysosporium* and *C. versicolor* (Table 3).

The degradation of residual lignin in UKP by these fungi was determined. They showed higher ligninolytic activity than *P. chrysosporium* and *C. versicolor*, as shown in Fig. 3. Although no nutriment was added in the culture, the fungus strain *P. sordida* YK-624 could degrade lignin significantly.

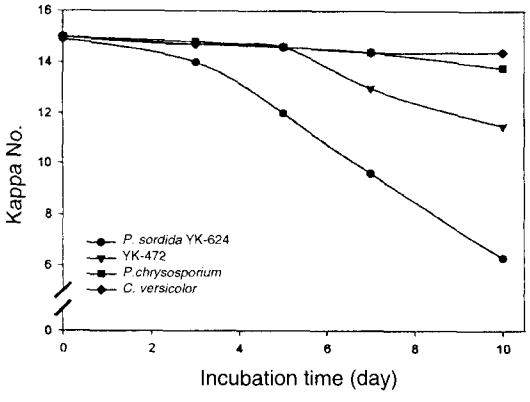

Fig. 3. Pulp kappa number as a function of incubation time for UKP treated with lignin-degarding fungi.

3.3 Enzyme Activities during the Degradation of Beech-Wood Lignin by the Fungus *P. sordida* YK-624

Extracellular enzyme activities were determined during the degradation of beech-wood lignin by the fungus strain *P. sordida* YK-624 and *P. chrysosporium*. After 10 days at incubation, *P. sordida* YK-624 slightly degraded beech-wood

Table 4. Enzyme activities during fungal treatment of beech wood-powder by lignin-degrading fungi

Strain	Lignin content(%)	PO*1 act.(U*2)		PhO*3 act. (U)
		I*4	II*5	
YK-624	24.6	0.53	0.85	0.12
P. chrysosporium	25.3	0	trace	0
control	25.6	-	-	-

*1PO = Peroxidase. *2U(Unit) = ΔA/min. *3PhO = Phenoloxidase.
*4It was assayed without Mn^{2+}.
*5It was assayed with Mn^{2+}.

Table 5. Enzyme activities during fungal treatment of OKP by *P. sordida* YK-624

Incubation time (day)	Kappa number	PO act.(U)		LiP act. (U)	PhO act. (U)
		I*1	II*2		
0	9.6	-	-	-	-
1	9.5	5.6	26.9	0	trace
2	9.2	5.2	22.4	0	trace
3	7.9	12.6	45.0	0	trace

*1 It was assayed without Mn^{2+}.
*2 It was assayed with Mn^{2+}.

lignin in this culture, while *P. chrysosporium* hardly degraded the lignin(Table 4). In the filtrate of *P. sordida* YK-624, peroxidase and phenoloxidase activities were detected. Its peroxidase activity increased when it assayed with Mn^{2+}. In filtrate of *P. chrysosporium* which did not degrade lignin in this culture, we hardly detected peroxidase and phenoloxidase activities(Table 4).

3.4 Enzyme Activities during the Degradation of Residual Lignin in OKP by the Fungus *P. sordida* YK-624

Enzyme activities were determined during the degradation of residual lignin in OKP by *P. sordida* YK-624. Kappa number decreased as a function of incubation time as shown in Table 5. Peroxidase and weak phenoloxidase activities were detected in whole incubation time. The peroxidase activity increased when it assayed with Mn^{2+}. LiP activity was not detected when it was assayed with Azure B method(Table 5).

The discovery in 1983 of fungal lignin peroxidase able to catalyze the oxidation of nonphenolic aromatic lignin model compounds and release some CO_2 from lignin has been seen as a major advance in understanding how fungal degrade lignin. It was reported that MnP performed the initial step of DHP depolymerization but that LiP is necessary for further degradation of the polymer to lower-molecular-weight products and mineralization.[14] The work employing a new lignin peroxidase inhibitor and a new lignin peroxidase assay using the dye Azure B indicated that secreted lignin peroxidases do not play a role in the *C. versicolor* pulp-bleaching system.[15]

3.5 Enzyme Activities of the Liquid Cultures of the Fungus *P. sordida* YK-624

Under the control condition, peroxidase activity was detected, and its activity increased when it was assayed with Mn^{2+}. Under the condition A, peroxidase activity increased greatly, and its activity was stimulated further when it was

Table 6. Enzyme activities of *P. sordida* YK-624 in liquid culture

Condition	PO act.(U)		LiP act.	PhO act.
	I*1	II*2	(U)	(U)
condition A	866	4414	0	trace
control	14	22	0	trace

*1 It was assayed without Mn^{2+}.

*2 It was assayed with Mn^{2+}.

assayed with Mn^{2+}. Under both conditions, LiP activity was not detected, and phenoloxidase activity was trace(Table 6).

4. Conclusions

We isolated some hyper-ligninolytic fungi from decayed woods. The fungi, *P. sordida* YK-624 and YK-472, showed much higher ligninolytic activity and selectivity than other fungi. The fungus YK-663 showed the highest ligninolytic activity in pine-wood lignin among all strains employed here. The fungus strain *P. sordida* YK-624 showed much higher ligninolytic activity in beech-wood lignin, birch dioxane lignin and residual lignin of UKP than *P. chrysosporium* and *C. versicolor*.

During fungal treatment of beech wood and OKP by *P. sordida* YK-624, peroxidase activity was detected in both cultures, and its activity increased when it was assayed with Mn^{2+}. LiP activity was not detected in neither cultures. Under the enzyme-inducing condition(condition A, see Materials and methods), which veratryl alcohol was added to and was purged with O_2, peroxidase activity increased greatly, and its activity was stimulated further when it was assayed with Mn^{2+}. LiP activity was not detected in this condition, either.

In the present report, it was suggested that the fungus strain *P. sordida* YK-624 showed peroxidase and weak phenoloxidase activities in ligninolytic cultures and liquid culture condition.

Acknowledgements

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