THE KOREAN JOURNAL OF MYCOLOGY
Vol. 26, No 2, p144-152 June 1998

Printed in S. KOREA

Screening of White Rot Fungi with Selective Delignification Capacity for Biopulping

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백색목재부후균중 Biopulping에 이용가능한 선택적 리그닌분해균의 스크리닝

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ABSTRACT: To obtain white rot fungi which have selective delignification capacity and can be used in biopulping processes, 94 different wood rotting fungi were screened and the capabilities of selected species were evaluated on deciduous and coniferous wood blocks. White rot fungi, first of all, were selected by simple enzyme tests, i.e., cellulase activity test; phenol oxidase activity test; laccase and peroxidase activity test. Most organisms that gave a positive Bavendamm gave a strongly positive laccase test with syringaldazine whereas most of those that gave a negative Bavendamm test also negative test for laccase and peroxidase, even if some exceptions were noted. Wood decay experiement were carried out to select fungal species with selective lignin-degrading ability by inoculating selected fungi to both wood blocks of *Populus tomentiglandulosa* and *Larix leptolepis*. After 12 weeks of incubation, weight losses, lignin losses, and morphological characteristics of the decayed wood were investigated. Almost all fungi tested caused 2 or more times of weight losses in *P. tomentiglandulosa* than in *L. leptolepis*, while no weight losses were detected from the un-inoculated wood blocks. *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* were the best delignifiers for both hardwood and softwood. *P. chrysosporium*, however, was less effective than *C. subvermispora*. *Bjerkandera adusta* and two unidentified spp. caused delignification for only *P. tomentiglandulosa*. *B. adusta* caused simultaneous rot of all cell wall components, resulted in thinning of the secondary cell wall layers. Other fungi caused selective delignification resulting in the removal of lignin from middle lamella and separation of cells from each other.

KEYWORDS: Biopulping, Chemical analysis, Scanning electron microscopy (SEM), Selective delignification, White rot fungi, Wood decay

Although wood can be attacked by many kinds of microorganisms, fungi are the predominant decomposers in forest ecosystems. Wood rotting fungi obtains nutrients by degrading major wood cell wall components, i.e., cellulose, hemicellulose, and lignin. Most of these fungi belongs to Basidiomycota and distributed worldwide. Brown rot fungi cause rapid and extensive depolimerization of cellulose early in the decay progresses. Wood polysaccharides are degraded, lignin modification occurs, and relatively small amounts of lignin are lost as decay progresses. White rot fungi have the capacity to degrade all cell wall components, including lignin. The extent of lignin degradation can vary considerably among species of white rot fungi. Some species deplete lignin, cellulose, and hemicellulose

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in varying ratios; many species attack both nonselectively and selectively in different areas of the same substrate. There are hundreds of white rot fungi with varying capacities to degrade lignin, cellulose, and hemicellulose.

Recently, the biotechnical potential of fungi that delignify wood is enormous (Blanchette, 1991, 1995). The effluents produced during chemical bleaching of kraft pulping are major contributors to water pollution. New methods of bleaching wood pulp with fungi or their enzymes may substitute with the chemicals now used (Farrell, 1987). The recent investigations also showed that fungi with high lignolytic activity are able to degrade environmental pollutants, such as PCB, DDT, dioxins, industrial dyes, and chlorinated phenols (Bumpus and Aust. 1986; Ferry *et al.*, 1995; Kim *et al.*, 1995). Another aspect of white rot fungi that may be of significant use is delignification processes used to modify wood or agricultural byproducts to increase nutritional value for ruminant animal feed (Agosin *et al.*, 1990; Gonzalez *et al.*, 1986; Reade *et al.*, 1983; Zadraril *et al.*, 1982). Of major significance are the pretreatment of wood chips with fungi that selectively remove lignin for pulp and paper production (Akhtar *et al.*, 1992; Blanchette *et al.*, 1988, 1992, 1996).

The fungi that degrade lignin selectively would be the best candidates for biopulping. To obtain the best possible species for use in biopulping processes, numerous species should be screened for rapid growth and selective degradation of lignin from wood. In this study, selection of several white rot fungi was made from screening large numbers of different wood rotting fungi and their capacities were evaluated on deciduous and coniferous wood blocks.

Materials and Methods

Wood rotting fungi used

Ninety four isolates of wood rotting fungi were collected and used. Among these, 38 isolates were obtained by the isolation from fruiting bodies and decayed woods in several forest areas. Twenty isolates were provided by IFO (Japan), 31 isolates from FRI (Korea), and 5 isolates from the University of Minnesota (USA).

Selection of white rot fungi by simple enzyme test

Cellulase activity and phenol oxidase activity tests were carried out to select white rot fungi from wood rotting fungi, based on the fact that extracellular enzymes produced by wood rotting fungi and subsequent decomposition processes differ between white rot and brown rot fungi, resulting in different types of wood degradation.

Cellulase activity test

Dye diffusion method was chosen by using dyed cellulose substrates such as cellulose azure (Smith, 1977). Dispensed a basal medium into screw-capped tubes. Final composition of a basal medium (per liter) was as follows: $NH_4H_2PO_4$ 1g, KCl 200 mg, $MgSO_4 \cdot 7H_2O$ 200 mg, Agar 12g. After sterilization by autoclaving at 121°C for 20 minutes, the medium was allowed to solidify vertically. Overlay this with medium containing the dyed cellulose. A second batch of basal medium(A) was prepared by two-thirds the specified volume of water. The remaining one-third volume of water(B) was mixed with cellulose azure to provide a 1% suspension when A and B were combined. A and B were autoclaved separately, and then mixed while still hot, and the mixture was layered on the top of the basal medium. The medium was inoculated with an agar disc of inoculum and incubated at 27°C for up to 10 days.

Phenol oxidase activity test

A modified Bavendamm medium (Rigling

et al., 1989) has been used in a preliminary and diagnostic test for detecting secretion of phenol oxidases by wood rotting fungi. If secretion of phenol oxidases was once detected, then syringaldazine [*N,N'-bis*-(3,5-dimethoxy-4-hydroxybenzylidene) hydrazine] and hydrogen peroxide were used for easy and rapid differentiation of laccase, or in its absence, of peroxidase (Harkin and Obst, 1973). To test fungal cultures, 2 or 3 drops of 0.1% solution of syringaldazine in ethanol were added to 7 days old mycelium growing on agar plates. The intensity of pink to purple color indicated the amount of laccase present. If no colors developed, dilute hydrogen peroxide was added to test for the presence of peroxidase.

Wood block preparation and culture inoculation

Fungi (Table 2) were grown for 10 days in 60 m*l* glass square tablet bottles (Qorpak®) containing 4 m*l* malt yeast broth (15g malt extract, 2g yeast extract, 1 *l* distilled water). Wood blocks (1.5×1.5×4 cm) were cut from freshly harvested Suwon poplar (*Populus tomentiglandulosa*) and Japanese larch (*Larix leptolepis*), dried for 72 hrs at 60°C and measured dry wight. Blocks were then placed in 237 m*l* glass square wide mouth bottles containing 60 m*l* vermiculite and 20 m*l* distilled water. Bottles were loosely capped, autoclaved, cooled, and inoculated. Each wood block was inoculated with a mat of mycelium. Nine poplar and nine Japanese larch blocks were inoculated with each isolate and nine uninoculated blocks of each served as controls. All bottles were incubated in the dark at 27°C and 90% RH for 12 weeks. After 4, 8, and 12 weeks of incubation, wood blocks were washed out to remove mycelium, then dried for 72 hrs at 60°C and weight loss was calculated.

Scanning electron microscopy and chemical analysis

Blocks with weight losses approximately equal to the mean of three replications were chosen from blocks of 12 weeks incubation for both scanning electron microscopy and chemical analysis. Blocks to be observed with the scanning electron microscope were saturated with water, frozen to −20°C, and sectioned with a cryostat microtome (HM505E, MICROM®) in transverse planes. Specimens were observed with JSM-5410 scanning electron microscope (JEOL Co. Ltd. Japan). For the chemical analysis, the wood blocks should be ground in a Wiley mill to pass at least a 20-mesh screen and extracted in Soxhlet extracting tubes with ethyl alcohol-benzene (1 : 2 v/v) for 8 hrs in 100°C water bath. Holocellulose was obtained by sodium chlorite method. Dried samples were treated with distilled water, glacial acetic acid (CH_3COOH), and sodium chlorite ($NaClO_2$) in Erlenmeyer flasks at 80°C water bath for 1 hour. This procedure was repeated 3 times and 4 times for Suwon poplar and Japanese larch, respectively. Treated samples were filtered through glass filter (1G2) with cold distilled water and finally with acetone until the color and smell of ClO_2 are completely removed. After drying of samples in dry oven at 105°C for 2.5 hrs, the percentage of holocellulose from wood samples was calculated.

Results and Discussions

Selection of white rot fungi by simple enzyme tests

Fifteen isolates out of 16 isolates which have already been known as brown rot fungi produced cellulase, but phenol oxidases were not produced at all. *Fomitopsis pinicola*, however, produced both cellulase and phenol oxidases even though it has been known as a brown rot fungus. There are a definite relationship between the production of phenol-oxidizing enzymes by wood rotting fungi and

the ability of these fungi to attack and modify or destroy lignin. Among 64 isolates known as white rot fungi, 44 isolates produced both cellulase and lignin degrading enzymes; 7 produced only lignin degrading enzymes; 11 produced only cellulase; 2 isolates produced neither. Thirteen isolates out of 14 unidentified isolates seemed to be white rot fungi since they produced both cellulase and lignin-degrading enzymes (Table 1).

Most organisms that gave a positive Bavendamm reaction gave a strongly positive laccase test with syringaldazine, whereas most of those that gave a negative Bavendamm test also gave negative tests for laccase and peroxidase. However, some exceptions were noted. Based on the results of enzyme tests, 7 isolates showing rapid growth and different results in simple enzyme tests were selected and inoculated to the wood blocks.

Table 1. Fungal cultures of wood rotting fungi grown on culture plates or test tubes for the test of cellulase, phenol oxidase, laccase, and peroxidase

No.	Wood rotting fungi	B/W[1]	CL[2]	BVD[3]	LC[4]	PO[5]
1	*Bjerkandera adusta*	W	++	-	-	-
2	*Coniophora puteana*	B	+	-	-	-
3	*Coriolus pubescens*	W	+	+	++	
4	*Coriolus brevis*	W	+	++	+	
5	*Coriolus consors*	W	+	-	-	-
6	*Coriolus elongatus*	W	+	+	++	
7	*Coriolus pargamenus*	W	-	+	++	
8	*Daedalea dickinsii*	B	++	-	-	-
9	*Daedaleopsis styracina*	W	-	++	+	
10	*Daedaleopsis tricolor*	W	++	++	-	-
11	*Coriolus unicolor*	W	++	-	+++	
12	*Fomes fomentarius*	W	+	+	+	
13	*Fomitopsis insularius*	B	+++	-	-	-
14	*Ganoderma neo-japonicum*	W	+	+	-	-
15	*Lentinus lepideus*	B	++	-	-	-
16	*Panellus serotinus*	W	++	+	-	-
17	*Phanerochaete chrysosporium*	W	++	-	-	-
18	*Pholiota aurivella*	B	++	-	-	-
19	*Poria cocos*	W	+	-	-	-
20	*Tyromyces palustris*	W	+	-	-	-
21	*Bjerkandera adusta*	W	++	+	-	-
22	*Flammulina velutipes*	W	+	+	-	-
23	*Laetiporus sulphureus* var. *miniatus*	B	+	-	-	-
24	*Pholiota squarrosa*		++	+	-	-
25	*Pholiota adiposa*		++	-	-	-
26	*Cryptoderma pini*	W	+	++	+	
27	*Fomitopsis annosa*	W	++	+	+	
28	*Fomitopsis officinalis*	B	++	-	-	-
29	*Coriolellus laricinus*	B	+	-	-	-
30	*Sparassis crispa*	B	+	-	-	-
31	*Fomitopsis cytisina*	W	+	+	+++	
32	*Laetiporus sulphureus* var. *miniatus*	B	+	-	-	-
33	*Phaeolus schweinitzii*	B	+	-	-	-
34	*Sparassis crispa*	B	+	-	-	-
35	*Coriolus hirsutus*	W	-	+	+	

Table 1. Continued

No.	Wood rotting fungi	B/W[1]	CL[2]	BVD[3]	LC[4]	PO[5]
36	*Fomitopsis cytisina*	W	++	-	+	
37	*Armillaria tabescens*	W	-	+	-	-
38	*Fomitopsis pinicola*	B	++	-	++	
39	*Corticium argenteum*		+	++	-	-
40	*Heterobasidion annosum*	W	++	-	++	
41	*Inonotus mikadoi*	W	++	+	-	-
42	*Inonotus obliquus*	W	++	±	-	+
43	*Laetiporus sulphureus*	B	+	-	-	-
44	*Laetiporus versiporus*	B	+	-	-	-
45	*Perenniporia fraxinea*	W	+	-	++	
46	*Phellinus chrusolooma*	W	++	+	-	
47	*Phellinus hartigii*	W	+	+	++	
48	*Phellinus pini*	W	++	+	-	-
49	*Phellinus punctatus*	W	-	++	++	
50	*Phellinus weirii*	W	++	++	-	+
51	*Phellinus linteus*	W	+	-	-	-
52	*Laetiporus sulphureus*	B	+	-	-	-
53	*Phellinus hartigii*	W	+	+	-	+
54	*Armillaria mellea*	W	++	+		
55	*Armillaria mellea*	W	-	++		
56	*Armillaria mellea*	W	-	-	-	+
57	*Armillaria mellea*	W	++	+++		
58	*Armillaria mellea*	W	+	+	-	-
59	*Ganoderma tsugae*	W	+	++	+++	
60	*Poria cocos*	W	+	-	-	-
61	*Poria cocos*	W	+	+	-	-
62	*Poria cocos*	W	-	-	-	-
63	*Poria cocos*	W	+	-	-	-
64	*Phellinus linteus*	W	++	+	-	-
65	*Phellinus linteus*	W	+++	-	-	-
66	*Hericium erinacium*	W	-	-	-	-
67	*Lentinus edodes*	W	+	+++	+	
68	*Lentinus edodes*	W	+	+++	++	
69	*Agrocybe cylindraceae*		++	-	-	+
70	*Phellinus linteus*	W	+++	-	-	+
71	I (unidentified)		+	-	+	
72	II (unidentified)		-	++	+	
73	III (unidentified)		-	+	-	-
74	IV (unidentified)		++	+	++	
75	V (unidentified)		++	++	-	-
76	VI (unidentified)		-	-	+	
77	VII (unidentified)		++	+	-	-
78	VIII (unidentified)		++	+	+	
79	X (unidentified)		±	++	+	
80	XI (unidentified)		++	++	-	-
81	*Microporus affinis*	W	+	+	++	
82	*Fomitopsis cytisina*	W	+	-	++	
83	*Elfvingia applanata*	W	++	+	+	
84	*Tryomyces albellus*	W	+	+	++	

Table 1. Continued

No.	Wood rotting fungi	B/W[1]	CL[2]	BVD[3]	LC[4]	PO[5]
85	*Tryomyces albellus*	W	++	+	+	
86	*Tyromyces borealis*	W	++	-	-	-
87	*Daedaleopsis* sp.	W	++	++	-	-
88	*Coriolus* sp.	W	+	+	++	
89	*Coriolus* sp.	W	+	+	++	
90	*Phlebia subvermispora*	W	++	+	-	-
91	*P. chrysosporium* BKM F-1767	W	+	-	-	-
92	*Ceriporiopsis subvermispora*	W	+	+	+	
93	*C. subvermispora* FP-90031-sp	W	++	+	+	
94	*C. subvermispora* L-14807 ss-10	W	++	++	+	

[1] B/W: Types of rot, B: Brown rot, W: White rot.
[2] CL test: Cellulase activity test, +++: dark blue, ++: blue, +: light blue, −: no reaction.
[3] BVD test: Bavendamm test, +++: dark brown, ++: brown, +: light brown, −: no reaction.
[4] LC test: Laccase activity test using syringaldazine, ++: dark pink, +: light pink, −: no reaction.
[5] PO test: Peroxidase activity test using syringaldazine and H_2O_2, +: pink, −: no reaction.

Wood decay experiment, structural and chemical analysis

Several methods have been developed to select fungal species with selective lignin-degrading ability. However, one of the most appropriate methods appeared to be an assessment of decay using wood blocks in decay chambers.

The fungi tested caused a wide range of weight losses from wood blocks with differences occurring between substrates. Most fungi caused 2 or more times of weight losses in *Populus tomentiglandulosa* than in *Larix leptolepis*. A mean percent weight loss by *Bjerkandera adusta,* however, was 1.66 in Japanese larch, but was 1.09 in Suwon poplar. In general, weight losses in both substrates were increased by the incubation periods. *B. adusta* and *Poria cocos* caused equal or less weight losses in both substrates when the incubation periods were increased from 8 to 12 weeks. No weight losses were detected from the wood blocks incubated without fungal inoculation. The percent weight

Table 2. Dry weight loss (%) after 4, 8, and 12 weeks incubation of wood blocks from *Larix leptolepis* and *Populus tomentiglandulosa* inoculated with wood decay fungi dry weight loss (%)

Wood Decay Fungi / incubation periods (wks)	*Larix leptolepis*			*Populus tomentiglandulosa*		
	4	8	12	4	8	12
Bjerkandera adusta	0.86	1.86	1.66	0.50	1.65	1.09[a]
Lentinus lepideus	2.42	7.54	9.73	5.07	15.73	19.51
Phanerochaete chrysosporium	2.45	3.20	4.75	6.00	12.00	14.75
Poria cocos	1.90	10.01	6.22	6.01	9.47	9.14
II (unidentified)	1.39	3.67	2.86	5.20	8.99	10.24
X (unidentified)	1.47	1.89	4.31	1.13	5.58	7.18
Ceriporiopsis subvermispora L-14807 ss-10	1.52	3.89	6.10	2.47	8.41	12.20
Control[b]	0.00	0.00	0.00	0.00	0.00	0.00

[a] Each figure is the mean of three replicates and indicates percentage of dry weight loss.
Dry weight loss (%)={(pre-dw − post-dw)/pre-dw}× 100
pre-dw: dry weight of wood block before inoculation of wood decay fungi
post-dw: dry weight of wood block after 3 months of incubation with fungi
[b] Control means wood blocks incubated without fungal inoculation.

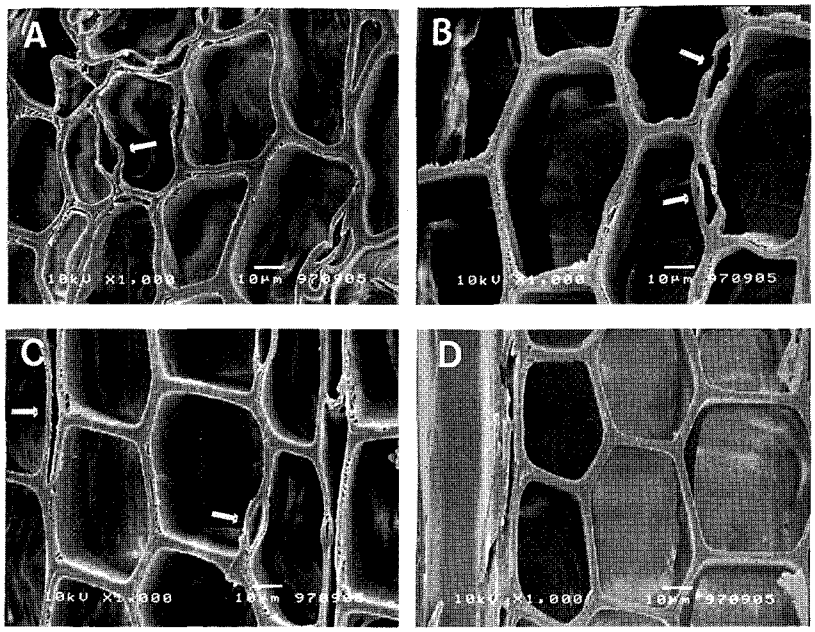

Fig. 1. Scanning electron micrographs of wood from *Larix leptolepis* delignified by white rot fungi. Transverse section of woods with simultaneous removal of all cell wall components (A) by *Bjerkandera adusta* and selective degradation of lignin by X (unidentified) (B) and *Ceriporiopsis subvermispora* (C). Lignin in middle lamella regions between cells has been removed by selective delignification. Arrows indicate that cells separate partially from adjacent cells as a result of the degradation of middle lamella. The cellulose-rich secondary wall remains. The normally rigid cell wall structure is shown in wood incubated without fungal inoculation(D). Bar=10 μm.

losses of each wood blocks by inoculation periods for all the fungi tested are listed in Table 2.

Electron microscopic studies showed that some fungi caused a selective delignification, while others caused a simultaneous rot. Wood blocks decayed by *B. adusta* caused a type of simultaneous rot which resulted in extensive thinning of the secondary cell wall layers (Fig. 1A). Lignin is distributed throughout the wood cell wall layers, but the greatest concentrations are in the compound middle lamella and cell corner regions. *C. subvermispora* and X (unidentified) caused a selective delignification of Japanese larch wood resulting in the removal of lignin from localized areas of wood blocks and separation of cells from each other indicating a loss of middle lamella (Fig. 1B, C).

Chemical analysis showed large losses of lignin from wood blocks. Lignin losses from both wood blocks by *Lentinus lepideus* were small as compared to those by other fungi tested, even though weight losses after incubation was large in both hardwood and softwood. Wood blocks decayed by *P. chrysosporium* or *C. subvermispora* caused large losses of lignin from both *P. tomentiglandulosa* and *L. leptolepis*. However, although *C. subvermispora* caused large weight losses for both *P. tomentiglandulosa* and *L. leptolepis*, *P. chrysosporium* caused large weight losses for *P. tomentiglandulosa* only. *B. adusta*, II, and X caused relatively large losses of lignin only in *P. tomentiglandulosa* (Table 3). It has been reported that some species are effective with hardwood only, whereas others are effective on both hardwood and softwood. As a

Table 3. Chemical analyses of decayed wood blocks (%)

Wood Decay Fungi / Chemical components	*Larix leptolepis*		*Populus tomentiglandulosa*	
	Holocellulose	Lignin	Holocellulose	Lignin
Bjerkandera adusta	58.0	42.0	74.4	25.6[a]
Lentinus lepideus	56.8	43.2	59.2	40.8
Phanerochaete chrysosporium	60.4	39.6	74.0	26.0
Poria cocos	55.6	44.4	65.2	34.8
II (unidentified)	58.4	41.6	73.6	26.4
X (unidentified)	57.6	42.4	71.2	28.8
Ceriporiopsis subvermispora L-14807 ss-10	62.4	37.6	75.6	24.4

[a] Each figure is the mean of three replicates and indicates percentage of chemical components of wood blocks.

conclusion, among the fungi tested in this experiment, *C. subvermispora* showed the best selective delignification capacity for both hardwood and softwood, and *P. chrysosporium* did the second capacity. It had been also reported that different strains within the selected species varied in their selectivity towards lignin and there were large differences among the strains in capacity to degrade lignin and in selectivity. Therefore, more strains within the *C. subvermispora* should be screened to obtain the best possible strain for use in biopulping processes.

적 요

선택적 리그닌 분해능을 가지고 생물펄프공정에 사용가능한 백색부후균을 얻기 위하여 94종류의 목재부후균을 검정하였고 선발된 7개 종에 대하여 활엽수와 침엽수의 부후능력을 측정하였다. 우선 백색부후균은 셀룰로오즈 분해효소, 페놀산화효소, laccase, peroxidase 등의 효소활성을 간단한 방법으로 검정하여 선발하였는데, Bavendamm test에서 양성을 나타내는 대부분의 균들은 syringaldazine을 사용한 laccase test에서도 강한 반응을 나타낸 반면, 음성반응을 나타낸 대부분의 균들은 laccase와 peroxidase test에서도 음성반응을 나타내었다. 선택적 리그닌 분해능력을 지닌 부후균을 선발하기 위하여 부후균을 은사시나무와 일본잎갈나무(낙엽송) 목재블럭에 접종하여 12주간 배양한 후에 부후된 목재의 중량감소율, 리그닌 량의 감소, 형태적 변화들을 화학분석과 주사전자현미경을 통하여 분석하는 목재부후 실험을 실시하였다. 이 실험에서 사용한 거의 모든 균주는 목재블럭의 중량감소율이 일본잎갈나무 보다 은사시나무에서 2배이상 높게 나타났으며 균을 접종하지 않은 목재블럭에서는 중량감소가 전혀 나타나지 않았다. *Ceriporiopsis subvermispora*와 *Phanerochaete chrysosporium*이 다른 균주에 비해서 침엽수와 활엽수의 리그닌을 모두 잘 분해시키는 것으로 나타났으나 분해능력은 *Ceriporiopsis subvermispora*가 더욱 우수하였다. *Bjerkandera adusta*와 미동정된 2균주는 은사시나무에서만 상대적으로 높은 리그닌 분해능력을 나타내었다. *B. adusta*는 모든 세포벽 성분을 동시에 분해시켜서 2차세포벽을 얇게 만들었으나 다른 균주들은 선택적 리그닌 분해력을 나타내어 두 세포의 세포벽 사이에 위치하는 중벽에 존재하는 리그닌을 분해시켜서 세포를 분리시키는 것이 관찰되었다.

Acknowledgement

The authors wishes to acknowledge the financial support of the Korea Research Foundation made in the Program Year 1996. We also thank Department of Forest Microbiology, Forset Research Institute, Korea and Department of Plant Pathology, University of Minnesota, USA for providing culture isolates of wood rotting fungi.

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