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Cho, Nam-Seok
Wood and Paper Science, Chungbuk National University

Hatakka, Annele I.
Department of Applied Chemistry and Microbiology, University of Helsinki

Rogalski, Jerzy
Department of Biochemistry, Maria Curie-Sklodowska University

Cho, Hee-Yeon
Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California

他

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Directional Degradation of Lignocellulose by *Phlebia radiata*

Nam–Seok CHO[1], Annele I. HATAKKA[2], Jerzy ROGALSKI[3], Hee–Yeon CHO[4] and Shoji OHGA*

Laboratory of Forest Resources Management, Division of Forest Ecosphere Management,
Department of Forest and Forest Products Sciences, Kyushu University,
Sasaguri, Fukuoka 811–2415, Japan
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The white–rot fungus *Phlebia radiata* preferably degrades lignin and is thus a potential fungus for biopulping and other applications in the pulp and paper industry. To elucidate important factors involved in the degradation of lignin carbohydrate complex (LCC) by this fungus, the metabolism of [U–^{14}C]–labelled wheat straw, [^{14}C]–labelled cellulose and [^{14}C]–labelled wheat straw hemicellulose was studied. The degradation of hemicellulose and lignin were apparently linked together and controlled by some common factors (*e.g.* oxygen, the effect of supplemented aromatic compounds) whereas the degradation of cellulose usually occurred under different conditions. In most cases the amount of nutrient nitrogen did not influence the evolution of $^{14}CO_2$ from carbohydrates. Addition of a small amount of glucose (0.05%, 2.8 mM) strongly decreased the degradation of cellulose but enhanced lignin degradation. Under oxygen atmosphere the degradation of hemicellulose was not influenced by added glucose (low nutrient nitrogen), which implied that low amounts of glucose could be used to specifically promote lignin degradation by *P. radiata*. The accelerating effect was most prominent under 100% oxygen atmosphere. Aromatic compounds strongly repressed the degradation of cellulose. In contrast, the degradation of hemicellulose was not influenced by aromatic compounds in air but under 100% oxygen the fungus degraded 35–70% more hemicellulose (to $^{14}CO_2$) when aromatic compounds were present than without them. The degradation of lignin was either stimulated or repressed by aromatic compounds, depending on the type of the compound used.

INTRODUCTION

Suggested applications of bio–ligninolytic systems in pulp and paper industry are usually based on selective removal or *in situ* modification of lignin. For example, it is possible using lignin–degrading white–rot fungi to decrease the amount of energy needed for mechanical pulping, *i.e.* biopulping, or to facilitate pulp bleaching (Kirk and Chang, 1990; Messner and Srebotnik, 1994; Ragauskas, 2002, Hatakka *et al*., 2002). Besides wood, annual plants, *e.g.* cereal straw and grass species contain substantial amounts of cellulose, which could be used as feed for ruminants, as a growth substrate for microorganisms and also as raw material for pulp and paper industry. However, due to the close association between cellulose and other plant cell wall polymers, *i.e.* lignin and hemicellulose (Eriksson *et al*., 1990; Daniel, 1994) cellulose is not easily available as a carbon or fiber source unless the structure of lignin is chemically or biologically modified or lignin is partially removed. Microbial delignification may be considered as a noteworthy alternative (Kirk and Farrell, 1987; Hatakka *et al*., 1989; Lewis and Yamamoto, 1990; Sarikaya and Ladish, 1997; Scott *et al*., 2002). In these cases a key question is how to break down the connection between lignin and carbohydrates, especially hemicellulose. The knowledge of the critical factors that are involved in the biodegradation of lignin carbohydrate complex (LCC) would help to understand and regulate selective delignification.

The white–rot fungi that belong to Basidiomycetes are the most efficient of all known lignin degraders (Eriksson *et al*., 1990, Hatakka, 2001) but they also degrade cellulose and hemicellulose. Among these fungi Phlebia radiata is a very efficient degrader of lignin (Hatakka and Uusi–Rauva, 1983), it preferentially degrades lignin (Ander and Eriksson, 1977; Hatakka, 1983, 1994) but also was characterized as cellulase (Rogalski *et al*., 1993; Longa, 1997) and hemicellulase producer (Rogalski *et al*., 1993a; Tokarzewska–Zadora, 2001). *Phlebia. radiata* produces directly lignin–modifying enzymes, including lignin peroxidase (ligninase, LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), laccase (EC 1.10.3.2) and dioxygenase (EC 1.13.11), which have been characterized (Niku–Paavola *et al*., 1988; 1990; Hatakka *et al*., 1989; Karhunen *et al*., 1990; Lundell, 1993; Vares *et al*., 1995; Rogalski *et al*., 1996). The second group of enzymes involved in lignin degradation does not attack wood components directly. They include aryl alcohol oxidase and glyoxal oxidase providing H_2O_2 for the peroxidases, and also the feed–back type enzymes as glucose oxidase and cellobiose:quinone oxidoreductase (cellobiose dehydrogenase) that play an important role in joining the metabolic chains during the bioconversion of high–molecular mass wood constituents. All these enzymes

[1] Wood and Paper Science, Chungbuk National University, Cheongju 361–763, Korea
[2] Department of Applied Chemistry and Microbiology, University of Helsinki, FIN–00014, Finland
[3] Department of Biochemistry, Maria Curie–Sklodowska University, Lublin, Poland
[4] Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA
* Corresponding author (E–mail: ohga@forest.kyushu–u.ac.jp)

were found in this fungus (Leonowicz *et al*., 1999; Rogalski *et al*., 2001).

The ability of a particular fungus to selectively remove lignin from natural lignocellulosic materials depends in part on the wood species (Kirk and Moore, 1972). Several cultural parameters regulate the attack on the lignin component (Kirk *et al*., 1978; Bar–Lev and Kirk, 1981; Jeffries *et al*., 1981), and may also affect the selectivity of the attack. We have studied the effect of atmosphere, nutrient nitrogen, and simple aromatic compounds, on the degradation of lignin, cellulose and hemicellulose by *P. radiata*. In these studies the evolution of $^{14}CO_2$ from specifically ^{14}C–labelled components of wheat straw was followed. In addition, the influence of glucose known to repress cellulolytic enzymes in low concentrations on these biodegradation processes was studied. Our results indicate that factors controlling degradation of lignin and hemicellulose showed common features whereas degradation of cellulose was differently regulated in this fungus.

MATERIALS AND METHODS

^{14}C–labelled preparates

[^{14}C–U]–cellulose from *Nicotina tabacum* L. (185–740 kBq/mg) (NEN Research Products, DUPONT, Dreiech, Germany) was obtained from Dr. P. Ander (present address Department of Forest Products, Swedish University of Agricultural Sciences, Uppsala, Sweden). Uniformly [^{14}C–U]–labelled wheat straw (250 Bq/mg) and [^{14}C–LIGNIN]–labelled wheat straw (125 Bq/mg) were from Dr. E. Odier (Laboratorie de Microbiologie, Centre de Biotechnologies Agro–Industries, I.N.R.A., France). [^{14}C–U]–hemicellulose (320 Bq/mg) was prepared from [^{14}C–U]–labelled wheat straw by the method of Adams (1965). In this method the [^{14}C]–labelled sample was hydrolyzed with 2 N trifluoroacetic acid in 95 °C for 20 hours (Abe *et al*., 1984). The monosaccharide composition of the hydrolyzate was determined by Shimadzu HPLC *vp* system composed of LC 10AD *vp* pump, RID–10A refractive index detector, SCL–10A *vp* system controller, CTO–10A *vp* column oven and the model 7725i sampling valve (Rheodyne, Berkeley, CA, USA) with 20 l loop on REZEX RSM–oligosaccharyde column (Phenomenex, Torrance CA, USA). The mobile phase Mili–Q water was run at the flow rate of 0.4 ml/min at 75 °C. Uronic acids were analyzed by the method of Blumenkrantz and Asboe–Hansen (1973).

Approximate monosaccharide composition of [^{14}C]–labelled hemicellulose was D–xylose, 67%, uronic acid, 11.5%, L–arabinose, 10.5%, D–glucose, 5%, D–mannose, 3% and D–galactose, 1%. Combustion of [^{14}C]–labelled samples for specific activity determination was performed as described by Hatakka and Uusi–Rauva (1983).

Fungus and culture conditions

Phlebia radiata Fr. 79 (ATCC 64658) (Hatakka and Uusi–Rauva, 1983) was maintained on 2% (w/v) malt agar slants. Broken mycelia was prepared for inoculum as previously described (Lundell *et al*., 1990). Basal medium was asparagine ammonium nitrate dimethylsuccinate (ADMS) medium (Hatakka and Uusi–Rauva, 1983). After 6–day of growth at 28 °C the mycelial mats were collected and broken in a Waring Blender to give an inoculum suspension. After inoculation with 4% (v/v) of the homogenate, 100 ml conical flasks, each containing 10 ml of ADMS–LN (2 mM–N) or ADMS–HN (20 mM–N) medium supplemented with 1% (w/v) microcrystalline cellulose (Avicel, Serva Feinbiochemica, Germany), milled wheat straw or

Table 1. Distribution of ^{14}C–labelled compound by *Phlebia radiata* during 25–day cultivation in air condition

Medium, isotope	Nitrogen concentration [mM]	$^{14}CO_2$ evolved [%]	^{14}C residue/mycelium [%]	^{14}C growth liquid [%]	Total ^{14}C [%]
1% cellulose	2	68.19 ± 4.30	5.63 ± 0.50	15.89 ± 1.71	89.72 ± 4.63
[^{14}C–U] cellulose	20	66.02 ± 3.94	10.41 ± 0.79	15.97 ± 2.22	92.44 ± 2.76
1% cellulose + 0.05% glucose	2	19.65 ± 0.94	64.24 ± 3.11	9.77 ± 1.54	93.66 ± 4.77
[^{14}C–U] cellulose	20	10.35 ± 0.87	75.49 ± 4.14	9.30 ± 0.99	95.14 ± 5.11
1% wheat straw	2	49.11 ± 7.20	44.19 ± 1.33	3.00 ± 1.11	96.32 ± 1.66
[^{14}C–U] cellulose	20	78.52 ± 10.07	22.36 ± 1.27	1.22 ± 0.68	102.11 ± 1.95
1% wheat straw + 0.05% glucose	2	17.41 ± 0.59	71.75 ± 3.68	1.78 ± 0.38	90.94 ± 3.92
[^{14}C–U] cellulose	20	16.24 ± 2.08	75.70 ± 4.04	2.68 ± 1.11	94.62 ± 4.67
1% wheat straw	2	10.22 ± 2.37	88.60 ± 2.53	2.78 ± 0.91	101.62 ± 3.74
[^{14}C–U] wheat straw	20	–	57.99 ± 3.00	34.31 ± 1.59	92.32 ± 4.21
1% wheat straw + 0.05% glucose	2	31.23 ± 2.22	49.17 ± 3.14	13.05 ± 0.87	93.45 ± 5.17
[^{14}C–U] wheat straw	20	12.79 ± 1.43	55.26 ± 3.63	27.11 ± 1.54	95.16 ± 4.76
1% wheat straw	2	–	99.26 ± 4.21	2.44 ± 1.06	101.72 ± 5.66
[^{14}C–lignin] wheat straw	20	2.69 ± 0.51	84.18 ± 3.23	9.83 ± 1.50	96.72 ± 3.67
1% wheat straw + 0.05% glucose	2	34.12 ± 3.15	66.60 ± 7.27	2.48 ± 0.14	103.22 ± 6.64
[^{14}C–lignin] wheat straw	20	10.42 ± 0.82	79.84 ± 6.69	8.64 ± 0.51	98.89 ± 7.07
1% wheat hemicellulose	2	16.43 ± 1.39	56.43 ± 2.76	23.56 ± 1.08	96.42 ± 3.86
[^{14}C–U] hemicellulose	20	13.74 ± 0.93	52.43 ± 3.04	31.69 ± 1.77	97.86 ± 4.23
1% wheat hemicellulose + 0.05%	2	4.28 ± 0.31	50.43 ± 3.04	41.13 ± 1.81	95.84 ± 3.21
glucose [^{14}C–U] hemicellulose	20	11.39 ± 0.75	37.54 ± 3.30	45.99 ± 3.12	94.92 ± 6.39

wheat straw hemicellulose as a carbon source, were incubated stationary at 28 °C. In some experiments, the flasks contained in addition to cellulose, wheat straw or wheat hemicellulose, also 0.05% (w/v) of glucose. To each inoculated flasks about 1 kBq of [^{14}C–U]–labelled cellulose, uniformly [^{14}C–U]–labelled wheat straw, [^{14}C–lignin]–labelled wheat straw, or [^{14}C–U]–labelled hemicellulose from wheat straw, was added in the beginning of cultivation. In some cases non–labelled aromatic compounds, vanillic (4–hydroxy–3–methoxybenzoic) acid, veratric (3,4–dimethoxybenzoic) acid, veratraldehyde (3,4–dimethoxy– benzaldehyde), veratryl (3,4–dimethoxybenzyl) alcohol, or ferulic (4–hydroxy–3–methoxycinnamic) acid were added 3 days from inoculation to make a final concentration of 1 mM. All aromatic compounds were reagent grade. Veratryl alcohol was vacuum distilled prior to use. At least three parallel flasks were used in every cultivation.

Radiorespirometry

Cultivation flasks were fitted with polypropene stoppers, and the gas distribution system described by Hatakka and Uusi–Rauva (1983) was used. Radiorespirometric methods to collect evolving $^{14}CO_2$ and the assay of radioactivity were carried out according to Hatakka and Uusi–Rauva (1983). Sterile synthetic air (20% oxygen) or pure oxygen was used for aeration and $^{14}CO_2$ collection purposes. Culture liquors were filtered through Whatman No. 4 filter paper on a glass filter (Shot No. 4, Duran, FRG) and filter paper plus mycelium and residue was combusted as described by Hatakka and Uusi–Rauva (1983) to determine the residual ^{14}C–activity. The radioactivity in the growth liquid plus washings was determined according to Hatakka and Uusi–Rauva (1983). The radioactivity was counted in a liquid scintillation counter (LKB–Wallac Oy, Finland).

The results are presented as per cent of original ^{14}C recovered as $^{14}CO_2$ per hour as a function of incubation time. After every cultivation the distribution of ^{14}C accumulated as $^{14}CO_2$, found in the growth liquor and in the residue, and the total recovery of ^{14}C was calculated and expressed as per cent of applied ^{14}C. The values were means of three parallel flasks standard deviation (SD).

RESULTS AND DISCUSSION

The effect of atmosphere, nutrient nitrogen and glucose

One of the most important factors controlling degradation and mineralization of lignin to CO_2 is oxygen but the effect of oxygen on the degradation of cellulose or hemicellulose has not been studied. Oxygen is known to strongly enhance lignin degradation by *Phanerochaete chrysosporium* (Kirk *et al*., 1978; Reid and Seifert, 1980) and by *Phlebia radiata* (Hatakka and Uusi–Rauva, 1983). The ligninolytic activity of *P. chrysosporium* appears only after the primary growth has ceased, *i.e.* when carbon, nitrogen or sulphur limitation occurs (Keyser *et al*., 1978; Jeffries *et al*., 1981). Results from the degradation of [^{14}C]–labelled lignins by white–rot fungi Hatakka *et al*. (1983) show that the release of carbon dioxide from specifically labelled wood lignins is higher under oxygen aeration than under air. Tables 1 and 2 show that the degradation of [^{14}C]–cellulose was repressed in an oxygen atmosphere in both low (low–N, 2 mM–N) and high (high–N, 20 mM–N) nitrogen concentrations. In contrast, the degradation of [^{14}C–lignin]–labelled wheat straw and [^{14}C–U]–labelled hemicellulose was strongly stimulated by oxygen in both nitrogen concentrations (Table 2).

In this study we mainly determined the final prod-

Table 2. Distribution of ^{14}C–labelled compound by *Phlebia radiata* during 25–day cultivation in oxygen condition

Medium, isotope	Nitrogen concentration [mM]	$^{14}CO_2$ evolved [%]	^{14}C residue/ mycelium [%]	^{14}C growth liquid [%]	Total ^{14}C [%]
1% cellulose	2	50.53 ± 2.89	33.37 ± 1.22	8.82 ± 0.91	92.73 ± 3.00
[^{14}C–U] cellulose	20	49.75 ± 3.60	21.77 ± 0.93	17.78 ± 1.33	89.22 ± 4.25
1% cellulose + 0.05% glucose	2	22.11 ± 1.64	23.83 ± 1.88	44.76 ± 2.03	90.71 ± 4.89
[^{14}C–U] cellulose	20	15.03 ± 0.92	31.60 ± 2.33	45.97 ± 1.94	92.66 ± 3.16
1% wheat straw	2	43.07 ± 1.91	21.09 ± 0.85	19.14 ± 2.00	83.33 ± 3.83
[^{14}C–U] cellulose	20	39.35 ± 3.22	8.07 ± 0.62	38.88 ± 1.56	86.35 ± 3.14
1% wheat straw + 0.05% glucose	2	33.04 ± 1.69	28.02 ± 2.11	30.54 ± 1.47	91.66 ± 4.12
[^{14}C–U] cellulose	20	32.11 ± 2.44	32.64 ± 1.64	25.24 ± 0.96	89.99 ± 3.45
1% wheat straw	2	29.93 ± 5.00	23.55 ± 1.01	32.49 ± 1.62	88.76 ± 4.77
[^{14}C–U] wheat straw	20	38.35 ± 3.54	15.29 ± 0.66	24.26 ± 2.02	77.94 ± 5.99
1% wheat straw + 0.05% glucose	2	54.95 ± 1.89	24.13 ± 1.72	11.02 ± 1.20	90.10 ± 3.61
[^{14}C–U] wheat straw	20	20.69 ± 1.61	43.73 ± 2.37	28.98 ± 1.31	93.42 ± 4.26
1% wheat straw	2	22.65 ± 1.47	38.71 ± 2.79	30.38 ± 2.25	91.74 ± 4.64
[^{14}C–lignin] wheat straw	20	19.39 ± 1.72	39.58 ± 1.94	33.95 ± 4.75	92.92 ± 5.23
1% wheat straw + 0.05% glucose	2	43.98 ± 3.25	47.95 ± 1.68	0.57 ± 0.1	93.43 ± 3.92
[^{14}C–lignin] wheat straw	20	35.13 ± 1.54	44.74 ± 3.80	12.95 ± 1.42	92.82 ± 4.37
1% wheat hemicellulose	2	28.81 ± 0.92	47.87 ± 5.09	19.43 ± 0.64	96.11 ± 5.31
[^{14}C–U] hemicellulose	20	29.46 ± 1.15	49.56 ± 2.47	18.27 ± 0.92	97.23 ± 3.33
1% wheat hemicellulose + 0.05%	2	27.34 ± 1.89	24.47 ± 2.13	45.35 ± 2.67	97.16 ± 4.32
glucose [14C–U] hemicellulose	20	15.90 ± 1.11	12.97 ± 0.99	67.23 ± 4.76	96.13 ± 5.11

uct of the degradation, carbon dioxide. The rate–limiting reactions cannot be deduced from this. Keeping this in mind, our data suggest that the degradation of hemicellulose and lignin were controlled by many common factors. With both substrates the degradation was strongly stimulated by an oxygen atmosphere. The effect of nutrient nitrogen was not so evident. In our experiments lignin was readily degraded also under 20 mM–N. *P. radiata* degraded rather poorly [^{14}C–lignin]–labelled wheat straw which has been observed also earlier (Hatakka *et al.*, 1983). This fungus efficiently degrades hardwood and especially softwood–type lignins (Hatakka *et al.*, 1983), and manganese peroxidase produced by this fungus may attack milled pine wood (Hofrichter *et al.*, 2001) and mineralizes ^{14}C–labelled guaiacyl type (softwood type) synthetic lignin (Hofrichter *et al.*, 1999).

The highest cellulose degradation (78.5% of the applied activity) was obtained under high nutrient nitrogen (high–N) conditions and under air atmosphere. Degradation of ^{14}C–labelled cellulose in the medium supplemented with 1% wheat straw instead of pure microcrystalline cellulose showed the effect of other lignocellulosic components on cellulose degradation. The effects may be due to difficulties in breaking down lignocellulose, or differences in the induction of cellulolytic enzymes by cellulose vs. lignocellulose. Under low nutrient nitrogen the degradation of cellulose was slightly suppressed in the presence of wheat straw compared with supplementation of microcrystalline cellulose as a carbon source. However, under high nutrient nitrogen a relatively high amount of carbon dioxide was released from the medium supplemented with wheat straw, *i.e.* 78% of applied ^{14}C activity evolved as $^{14}CO_2$. The effect of extra carbon source (0.05% glucose) was not so apparent with hemicellulose as it was in the case of cellulose with which it repressed degradation. In contrast, the addition of glucose stimulated the degradation of uniformly [^{14}C]–labelled wheat straw and [^{14}C–lignin]–labelled wheat straw both in an air and oxygen atmosphere.

This agrees well with our earlier results which showed that an extra carbon source such as cellulose or glucose remarkably increase the degradation of [^{14}C–ring)–labelled poplar wood lignin by *P. radiata* (but not so by *Phanerochaete chrysosporium* (or its anamorph *Sporotrichum pulverulentum*) (Hatakka and Uusi–Rauva, 1983). The degradation of [^{14}C–U]–cellulose under air atmosphere in the presence of either 1% microcrystalline cellulose (Fig. 1: air) or 1% wheat straw were rather similar. Addition of a small amount of glucose decreased the degradation of [^{14}C]–cellulose to 1/3 – 1/2 of the control under oxygen and even more strongly under air. As reported earlier (Hatakka and Uusi–Rauva, 1983), also in the present work glucose strongly stimulated the degradation of lignin by *P. radiata* – the evolution of $^{14}CO_2$ was doubled in an oxygen atmosphere and under air glucose even more clearly stimulated the degradation of lignin. Under oxygen atmosphere the degradation of hemicellulose was not influenced by added glucose (low nutrient nitrogen), which implied that low amounts of glucose could be used to specifically promote lignin degradation and prevent cellulose degradation by *P. radiata*.

In both cases two maxima of $^{14}CO_2$ evolution were observed, but the first maximum was observed a little earlier in the case when cellulose was used as a carbon source. Similar results were obtained in oxygen atmosphere with cellulose as a carbon source under low–N concentration (Fig. l; oxygen) but in the case of oxygen atmosphere and the medium with wheat straw only one peak of $^{14}CO_2$ release was observed. The degradation of [^{14}C–U]–hemicellulose resembled cellulose degradation. The maximum $^{14}CO_2$ release was observed at the same time as in the case of cellulose. Under air atmosphere (Fig. 2: air) only a single maximum could be observed on for 3–4 days from inoculation. Under oxygen atmosphere (Fig. 2: oxygen) the activities were higher and showed two maxima, on the second and fourth day of growth. The fungus did not decompose wheat straw lignin when it was cultivated under air in low–N medium (Fig. 3: air). Replacement of air with oxygen strongly stimulated the evolution of $^{14}CO_2$ from lignin (Fig. 3: oxygen).

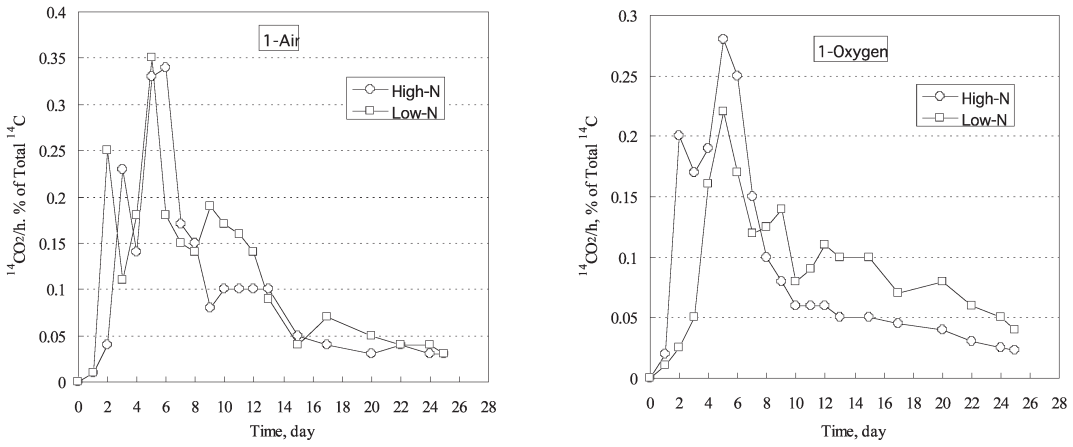

Fig. 1. Evolution of $^{14}CO_2$ from [^{14}C–U] cellulose by *Phlebia radiata* cultivated under air and oxygen on low–N or high–N media.

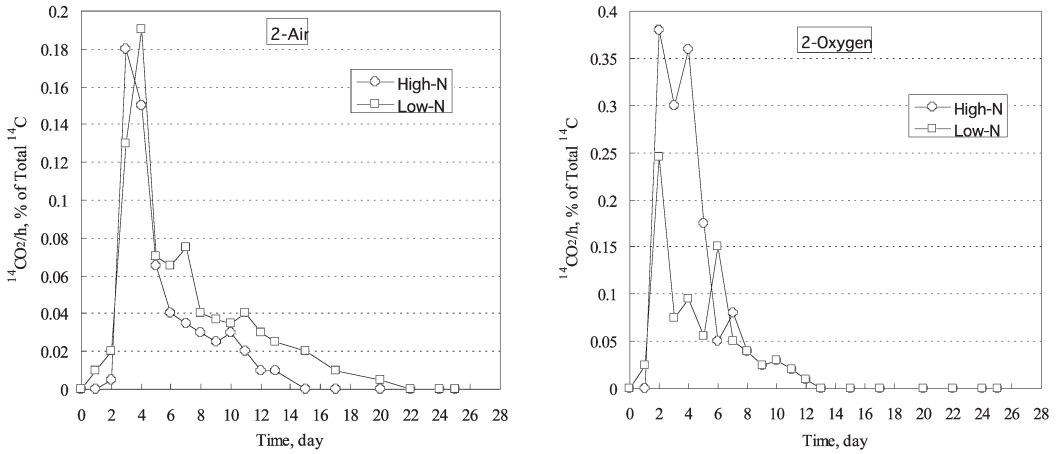

Fig. 2. Evolution of $^{14}CO_2$ from [^{14}C–U] hemicellulose by *Phlebia radiata* cultivated under air and oxygen on low–N or high–N media.

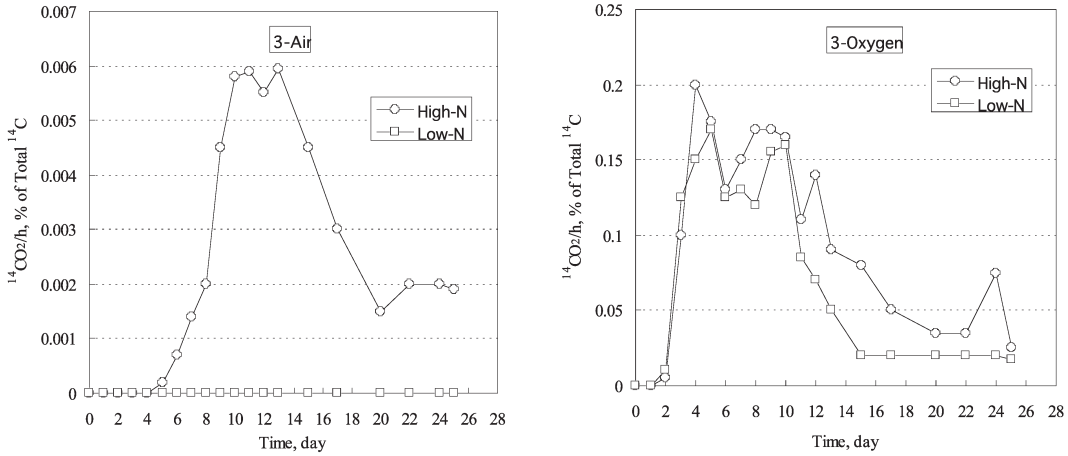

Fig. 3. Evolution of $^{14}CO_2$ from [^{14}C–lignin] wheat straw by *Phlebia radiata* cultivated under air and oxygen on low–N or high–N media.

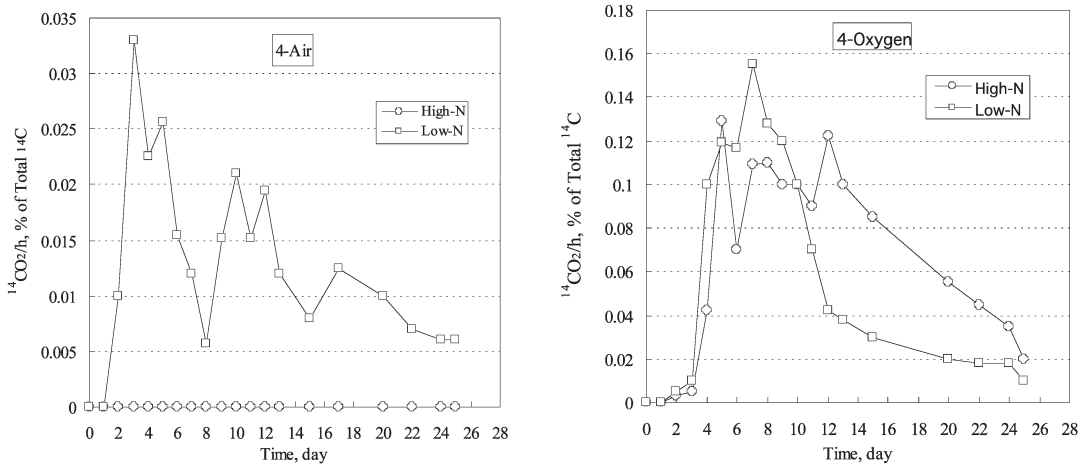

Fig. 4. Evolution of $^{14}CO_2$ from [^{14}C–U] wheat straw by *Phlebia radiata* cultivated under air and oxygen on low–N or high–N media.

Uniformly labelled wheat straw was not degraded by *P. radiata* when the fungus was cultivated in high–N medium under air (Fig. 4: air). However, under oxygen atmosphere the degradation of this preparate was also strongly stimulated, and three maxima of $^{14}CO_2$ evolution were observed (Fig. 4: oxygen).

The effect of aromatic compounds

Cultures were supplemented with aromatic compounds which are known to affect either production of lignin–modifying enzymes or to be lignin degradation products. Degradation of lignin and production of lignin peroxidase is enhanced by veratryl alcohol addition. Vanillic acid has been detected in wood degraded by white–rot fungi. Ferulic acid forms cross–linkages in grass (straw) lignins. The aromatic compounds were added for 3 days from inoculation. The effect of these aromatic compounds on the evolution of $^{14}CO_2$ from

[^{14}C–U]–labelled cellulose hemicellulose and lignin by *P. radiata* are shown in Tables 3, 4 and 5, respectively.

All aromatic compounds tested strongly repressed the degradation of cellulose irrespective the atmosphere (in medium containing 2 mM–N). The degradation of hemicellulose was not influenced by aromatic compounds in air but under 100% oxygen atmosphere 35–70% more CO_2 was released with added aromatic compounds than in controls. The degradation of lignin was strongly influenced by aromatic compounds so that (under oxygen atmosphere) the degradation was stimulated, in decreasing order, by vanillic acid (59%) > ver-

Table 3. The effect of aromatic compounds on the distribution of the ^{14}C–label from [^{14}C–U] cellulose by *Phlebia radiata* during 25–day cultivation under different aeration condition

Medium, isotope	Aeration condition	$^{14}CO_2$ evolved [%]	^{14}C residue/ mycelium [%]	^{14}C growth liquid [%]	Total ^{14}C [%]
1% cellulose	air	68.19 ± 4.33	5.63 ± 0.51	15.89 ± 1.68	89.74 ± 4.62
[^{14}C–U] cellulose	oxygen	50.53 ± 2.88	33.37 ± 1.19	8.80 ± 0.90	92.73 ± 3.00
1% cellulose + 1 mM vanillic acid	air	8.37 ± 1.57	51.81 ± 1.20	31.52 ± 0.81	91.76 ± 2.77
[^{14}C–U] cellulose	oxygen	15.65 ± 1.88	25.23 ± 1.97	53.72 ± 1.65	94.60 ± 4.11
1% cellulose + 1 mM veratric acid	air	13.36 ± 2.54	54.78 ± 1.59	26.06 ± 1.07	94.22 ± 3.56
[^{14}C–U] cellulose	oxygen	17.48 ± 2.22	25.83 ± 3.06	48.09 ± 2.13	91.40 ± 4.78
1% cellulose + 1 mM veratric aldehyde	air	22.38 ± 2.88	32.36 ± 0.90	34.16 ± 1.79	88.90 ± 3.85
[^{14}C–U] cellulose	oxygen	14.66 ± 1.23	32.39 ± 2.66	43.15 ± 1.11	90.23 ± 3.09
1% cellulose + 1 mM veratric alcohole	air	16.86 ± 1.72	45.27 ± 1.33	29.27 ± 2.02	91.42 ± 4.07
[^{14}C–U] cellulose	oxygen	19.18 ± 2.08	44.31 ± 2.06	29.11 ± 0.93	92.64 ± 3.88
1% cellulose + 1 mM ferulic acid	air	17.62 ± 3.03	45.75 ± 1.05	28.93 ± 1.72	92.33 ± 4.66
[^{14}C–U] cellulose	oxygen	11.12 ± 1.36	30.34 ± 2.56	48.44 ± 1.07	89.90 ± 4.54

Table 4. The effect of aromatic compounds on the distribution of the ^{14}C–label from [^{14}C–U] hemicellulose by *Phlebia radiata* during 25–day cultivation under different aeration condition

Medium, isotope	Aeration condition	$^{14}CO_2$ evolved [%]	^{14}C residue/ mycelium [%]	^{14}C growth liquid [%]	Total ^{14}C [%]
1% wheat hemicellulose	air	16.43 ± 1.39	56.43 ± 2.76	23.56 ± 1.08	96.42 ± 3.86
[^{14}C–U] hemicellulose	oxygen	28.81 ± 0.92	47.87 ± 5.09	19.43 ± 0.64	96.11 ± 5.31
1% wheat hemicellulose + 1 mM vanillic acid	air	19.69 ± 1.81	65.98 ± 3.16	8.67 ± 0.91	94.34 ± 4.34
[^{14}C–U] hemicellulose	oxygen	47.61 ± 2.88	49.02 ± 2.74	2.21 ± 0.31	98.84 ± 5.09
1% wheat hemicellulose + 1 mM veratric acid	air	18.63 ± 0.99	45.86 ± 1.79	32.67 ± 1.31	97.16 ± 2.94
[^{14}C–U] hemicellulose	oxygen	40.47 ± 1.42	35.78 ± 2.47	18.02 ± 0.68	94.27 ± 3.64
1% wheat hemicellulose + 1 mM veratric aldehyde	air	13.81 ± 0.93	48.43 ± 3.03	29.92 ± 2.22	92.16 ± 4.72
[^{14}C–U] hemicellulose	oxygen	36.96 ± 1.77	36.28 ± 3.19	17.62 ± 2.29	90.86 ± 6.22
1% wheat hemicellulose + 1 mM veratric alcohole	air	21.83 ± 1.35	71.58 ± 4.22	2.27 ± 0.12	95.68 ± 5.23
[^{14}C–U] hemicellulose	oxygen	38.37 ± 1.69	45.89 ± 3.58	9.85 ± 0.89	94.11 ± 5.11
1% wheat hemicellulose + 1 mM ferulic acid	air	22.63 ± 1.11	66.17 ± 2.58	4.74 ± 0.30	93.54 ± 3.54
[^{14}C–U] hemicellulose	oxygen	40.38 ± 2.52	50.26 ± 2.46	2.77 ± 0.52	93.41 ± 4.76

Table 5. The effect of aromatic compounds on the distribution of the ^{14}C–label from [^{14}C–lignin] wheat straw by *Phlebia radiata* during 25–day cultivation under different aeration condition

Medium, isotope	Aeration condition	$^{14}CO_2$ evolved [%]	^{14}C residue/ mycelium [%]	^{14}C growth liquid [%]	Total ^{14}C [%]
1% wheat straw	air	–	99.26 ± 4.19	2.44 ± 1.02	101.70 ± 5.57
[^{14}C–lignin] wheat straw	oxygen	22.65 ± 1.47	38.71 ± 2.79	30.38 ± 2.25	91.74 ± 4.64
1% wheat straw + 1 mM vanillic acid	air	24.09 ± 1.81	75.31 ± 6.92	2.21 ± 0.12	101.62 ± 7.44
[^{14}C–lignin] wheat straw	oxygen	36.19 ± 1.81	36.12 ± 2.13	16.41 ± 1.28	88.72 ± 4.36
1% wheat straw + 1 mM veratric acid	air	4.62 ± 0.39	60.24 ± 5.24	33.74 ± 0.72	98.64 ± 6.31
[^{14}C–lignin] wheat straw	oxygen	17.64 ± 1.05	35.00 ± 2.69	33.82 ± 2.87	86.46 ± 5.67
1% wheat straw + 1 mM veratric aldehyde	air	3.03 ± 0.15	56.96 ± 5.01	35.41 ± 3.08	95.44 ± 7.81
[^{14}C–lignin] wheat straw	oxygen	7.29 ± 0.65	47.81 ± 3.54	24.13 ± 1.64	79.23 ± 4.16
1% wheat straw + 1 mM veratric alcohole	air	5.06 ± 0.35	72.01 ± 1.15	19.54 ± 1.54	96.64 ± 2.38
[^{14}C–lignin] wheat straw	oxygen	32.55 ± 1.59	36.77 ± 2.39	17.60 ± 1.02	86.92 ± 4.06
1% wheat straw + 1 mM ferulic acid	air	16.22 ± 1.26	78.93 ± 6.88	7.28 ± 0.42	102.37 ± 7.73
[^{14}C–lignin] wheat straw	oxygen	15.68 ± 1.13	39.58 ± 2.73	22.68 ± 1.18	77.94 ± 4.79

atryl alcohol (44%) but repressed by added veratraldehyde (68%) > ferulic acid (31%) > veratric acid (22%).

The influence of aromatic compounds on the degradation of [^{14}C]–hemicellulose differed from the degradation pattern with cellulose (Table 4). In all cases except in one (veratraldehyde, air atmosphere) a slight stimulation of $^{14}CO_2$ release was observed. Decomposition of wheat straw specifically labelled in lignin was highly influenced, either positively or negatively, by aromatic monomers (Table 5). Under air, in all cases a significant increase of $^{14}CO_2$ release was observed. Vanillic acid and veratryl alcohol were the best stimulators both in air and oxygen atmosphere. Veratraldehyde and to some extent ferulic acid strongly repressed lignin degradation to CO_2 under oxygen atmosphere. Earlier we found that these same aromatic compounds affected the production of lignin peroxidase and other ligninolytic as cellulolytic and hemicellulolytic enzymes by *P. radiata*.

All aromatic compounds tested strongly decreased of the release of $^{14}CO_2$ from [^{14}C]–cellulose. However, relatively the amounts of water–soluble compounds derived from [^{14}C]–cellulose were higher in media in which aromatic compounds were added than in control cultures. This may indicate that aromatic compounds did not prevent the initial attack on the cellulose polymer but affected later in fungal oligomer or glucose metabolism. In most cases higher amounts of $^{14}CO_2$ were released in an oxygen atmosphere than in air. Veratraldehyde and ferulic acid most strongly suppressed the degradation of cellulose in an oxygen atmosphere.

As a conclusion, the degradation of hemicellulose and lignin seemed to be regulated by many common factors, but cellulose degradation was regulated separately in *P. radiata*. The reason of the similar regulation of the degradation of lignin and hemicellulose on one hand, and different regulation of the degradation of lignin and cellulose, may be due to the possible chemical linkage between lignin and hemicellulose, but not between lignin and cellulose (Eriksson *et al*., 1990; Hatfield *et al*., 1999; Soulnier and Thibault, 1999). For the enhancement of lignin degradation and the simultaneous protection of cellulose from fungal attack, selection of controlling factors, for example (i) cultivation in an oxygen atmosphere, (ii) addition of a small amount of glucose and (iii) supplementation of the medium with vanillic acid could be suggested.

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REFERENCES

Abe, M., J. E. Sherwood, R. I. Hollingsworth and F. B. Dazzo 1984 Stimulation of clover root hair infection by lectin binding oligosaccharides of *Rhizobium trifolii*. *J. Bacteriol*., **160**: 517–520

Adams, G. A. 1965 Arabinoglucuronoxylan, arabinoxylan and xylan purification using a copper complex and purification by fractional precipitation of acetates upon young wheat plants. *Methods Carbohyd. Chem*., **5**: 170–174

Ander, P. and K.–E. Eriksson 1977 Selective degradation of wood components by white–rot fungi. *Physiol Plant*, **41**: 239–248

Bar–Lev, S. and T. K. Kirk 1981 Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium*. *Biochem Biophys. Res. Commun*., **99**: 373–378

Blumenkrantz, N. J. and G. Asboe–Hansen 1973 New method for quantitative determination of uronic acids. *Anal. Biochem*., **54**: 484–489

Daniel, G. 1994 Use of electron microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiol. Rev*., **13**: 199–233

Eriksson, K.–E., R. A. Blanchette and P. Ander 1990 Microbial and enzymatic degradation of wood and wood components. Springer–Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, Hong Kong, pp. 1–407

Hatakka, A. I. 1983 Pretreatment of wheat straw by white–rot fungi for enzymic saccharification of cellulose. *Eur. J. Appl. Microbiol. Biotechnol*., **18**: 350–357

Hatakka, A. I. 1994 Lignin–modifying enzymes from selected white–rot fungi: production and role in lignin degradation. *FEMS Microbiol. Rev*., **13**: 125–135

Hatakka, A. 2001 Biodegradation of lignin. *In*: Biopolymers. Biology, Chemistry, Biotechnology, Applications. Vol 1. Lignin, Humic Substances and Coal (Hofrichter, M. & Steinbchel, A., eds.), Wiley–VCH, Weinheim, Germany. Chapter 5, pp. 129–180

Hatakka, A. I., J. A. Buswell, T. I. Pirhonen and A. K. Uusi–Rauva 1983 Degradation of ^{14}C–labelled lignins by white–rot fungi. *In*: Higuchi T., Chang H.–m., Kirk T. K. (eds) Recent advances in lignin biodegradation research. UNI Publ Co Ltd, Tokyo, pp. 176–187

Hatakka, A., P. Maijala, A. Mettl, T. Hakala, L. Hauhio and J. Ellmn 2002 Fungi as potential assisting agents in softwood pulping. Biotechnology in the Pulp and Paper Industry: In: Biotechnology in the Pulp and Paper Industry (L. Viikari and R. Lantto, eds.), Elsevier, Amsterdam, London, New York, pp. 81–88

Hatakka, A. I., O. K. Mohammadi and T. K. Lundell 1989 The potential of white rot fungi and their enzymes in the treatment of lignocellulosic feed. *Food Biotechnol*., **3**: 45–58

Hatakka, A. I. and A. K. Uusi–Rauva 1983 Degradation of ^{14}C–labelled poplar wood lignin by selected white–rot fungi. *Eur. J. Appl. Microbiol. Biotechnol*., **17**: 235–242

Hatfield, R. D., J. Ralph and J. H. Grabber 1999 Cell wall cross–linking by ferulates and diferulates in grasses. *J. Sci. Food Agric*., **79**: 403–407

Hofrichter, M., T. Vares, K. Scheibner, S. Galkin, J. Sipil and A. Hatakka 1999 Mineralization and solubilization of synthetic lignin (dehydrogenation polymerizate) by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata*. *J. Biotechnol*., **67**: 217–228

Hofrichter, M., T. Lundell and A. Hatakka 2000 Conversion of milled pine wood by manganese peroxidase from *Phlebia radiata*. *Appl. Environ. Microbiol*., **67**: 4588–4593

Jeffries, T. W., S. Choi and T. K. Kirk 1981 Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol*., **42**: 290–296

Karhunen, E., A. Kantelinen and M.–L. Niku–Paavola 1990 Mn–dependent peroxidase from the lignin–degrading white–rot fungus *Phlebia radiata*. *Arch. Biochem. Biophys*., **279**: 25–31

Keyser, P., T. K. Kirk and J. G. Zeikus 1978 Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. *J. Bacteriol*., **135**: 790–797

Kirk, T. K. and R. L. Farrell 1987 Enzymatic "combustion": the microbial degradation of lignin. *Annu. Rev. Microbiol*., **41**: 465–506

Kirk, T. K. and H.–M. Chang 1990 Biotechnology in pulp and

paper industry. Butterworth–Heinemann, Stoneham MA, pp. 1–666

Kirk, T. K. and W. E. Moore 1972 Removing lignin from wood with white–rot fungi and digestibility of resulting wood. *Wood Fiber*, **4**: 72–79

Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz and J. G. Zeikus 1978 Influence of culture parameters of lignin metabolism by *Phanerochaete chrysosporium. Arch. Microbiol.*, **117**: 277–285

Leonowicz, A., A. Matuszewska, J. Luterek, D. Ziegenhagen, M. Wojtas–Wasilewska, N.–S. Cho, M. Hofrichter and J. Rogalski 1999 Biodegradation of lignin by white–rot fungi. *Fung. Gen. Biol.*, **27**: 175–185

Lewis, N. G. and E. Yamamoto 1990 Lignin: Occurrence, biogenesis and biodegradation. *Ann. Rev. Plant. Physiol. Plant Mol. Biol.*, **41**: 455–496

Longa, B. 1997 The investigation on cellulolytic enzymes in *Phlebia radiata*, Ph.D. Thesis, Maria Curie–Sklodowska University, Lublin, pp. 1–179

Lundell, T. 2003 Ligninolytic system of the white–rot fungus *Phlebia radiata*: Lignin model compound studies, Ph.D. Thesis, University of Helsinki, Helsinki, pp. 1–402

Lundell, T., A. Leonowicz, J. Rogalski and A. Hatakka 1990 Formation and action of lignin modifying enzymes in cultures of *Phlebia radiata* supplemented with veratric acid, *Appl. Environ. Microbiol.*, **56**: 2623–2629

Messner, K. and E. Srebotnik 1994 Biopulping: an overview of developments in an environmentally safe paper making technology. *FEMS Microbiol. Rev.*, **13**: 351–364

Niku–Paavola, M.–L., E. Karhunen, P. Salola and V. Raunio 1988 Ligninolytic enzymes of the white–rot fungus *Phlebia radiata. Biochem. J.*, **254**: 877–884

Niku–Paavola, M.–L., E. Karhunen, A. Kantelinen, L. Viikari, T. Lundell and A. Hatakka 1990 The effect of culture conditions on the production of lignin modifying enzymes by the white–rot fungus *Phlebia radiata. J. Biotechnol.*, **13**: 211–221

Ragauskas, A. J. 2002 Biotechnology in the pulp and paper industry. – A chalange for change. In: Biotechnology in the Pulp and Paper Industry (L. Viikari and R. Lantto, eds.), Elsevier, Amsterdam, London, New York, pp. 7–12

Reid, I. D. and K. A. Seifert 1982 Effect of an atmosphere of oxygen on growth, respiration and lignin degradation by white–rot fungi. *Can. J. Bot.*, **60**: 252–260

Rogalski, J., A. Hatakka, M. Wojtas–Wasilewska and A. Leonowicz 1993 Cellulolytic enzymes of the ligninolytic white–rot fungus *Phlebia radiata. Acta Biotechnol.*, **13**: 41–45

Rogalski, J., A. Hatakka, B. Longa and A. Leonowicz 1993a Hemicellulolytic enzymes of the ligninolytic white–rot fungus *Phlebia radiata*. I. Determination of enzyme activities. *Acta Biotechnol.*, **13**: 47–51

Rogalski, J., M. Wojtas–Wasilewska, B. Bialy, J. Luterek and A. Leonowicz 1996 A study of aromatic ring cleavage enzymes in *Phlebia radiata. In*: 6th Intern. Conf. Biotechnol. Pulp Paper Ind., (E. Srebotnik and K. Messner eds.), Facultas–Universitatverlag, vienna, Austria, pp. 477–450

Rogalski, J., J. Fiedurek and A. Leonowicz 2001 Production of lignolytic and feed–back type enzymes by *Phlebia radiata* on different media. *Acta Biol. Hungarica*, **52**: 149–160

Sarikaya, A. and M. R. Ladish 1997 Mechanisms and potential applications of bio–ligninolytic systems in a CELLS. *Appl. Biochem. Biotechnol.*, **62**: 131–149

Saulinier, L. and J.–F. Thibault 1999 Ferulic acid and diferulic acids as components of sugar–beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.*, **79**: 396–402

Scott, G. M., M. Akhtar, R. E. Swaney and C. J. Houtman 2002 Recent development in biopulping technology at Madison, WI. In: Biotechnology in the Pulp and Paper Industry (L. Viikari and R.Lantto, eds.), Elsevier, Amsterdam, London, New York, pp. 61–68

Tai, D., M. Terasawa, C.–L. Chen and H. M. Chang 1983 Biodegradation of guaiacyl–syringyl lignins in wood by *Phanerochaete chrysosporium. In*: Higuchi T, Chang H–m, Kirk TK (eds) Recent advances in lignin biodegradation research. UNI Publ Co Ltd, Tokyo, Japan, pp. 44–63

Tokarzewska–Zadora, J. 2001 The investigation on xylanolytic enzymes in *Phlebia radiata* Ph.D. Thesis, Maria Curie–Sklodowska University, Lublin, pp. 1–216

Vares, T., M. Kalsi and A. Hatakka 1995 Lignin peroxidases, manganese peroxidases and other ligninolytic enzymes produced by *Phlebia radiata* during solid state fermentation of wheat straw. *Appl. Environ. Microbiol.*, **61**: 3515–3520