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Title	Decolorization of a Melanoidin-Containing Wastewater, Waste Sludge Heat Treatment Liquor, by Coriolus hirsutus
Author(s)	Miyata, Naoyuki
Citation	大阪大学, 1998, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3143956
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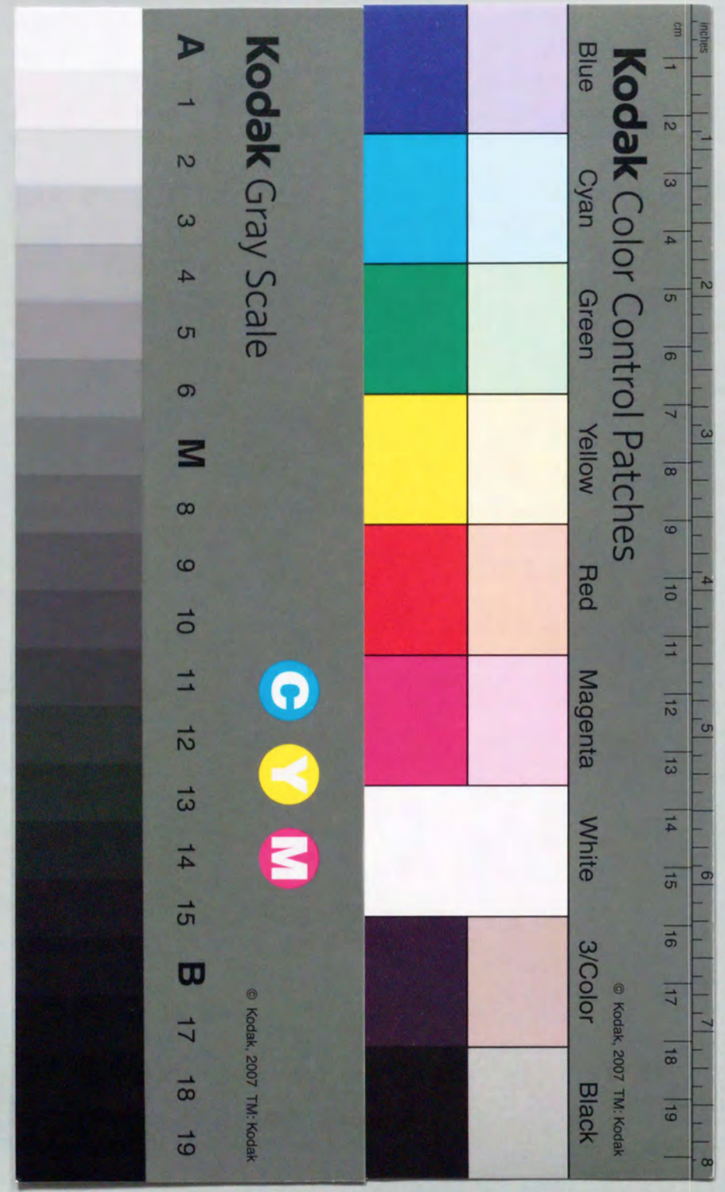

Decolorization of a Melanoidin-Containing Wastewater, Waste Sludge Heat Treatment Liquor, by *Coriolus hirsutus*

（*Coriolus hirsutus* によるメラノイジン含有汚泥熱処理分離液の脱色に関する研究）

1998

Naoyuki Miyata

宮田直幸

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Contents

General introduction

Recently, much attention has focused on the application of white rot fungi to remove recalcitrant compounds from industrial wastewaters [1,2]. The wood-rotting basidiomycetous fungi are unique microorganisms which cause substantial degradation of the recalcitrant polymer lignin in vascular plants. Interestingly, the fungal ligninolytic activity also functions in the degradation of various chemicals such as polychlorinated biphenyls, chlorinated phenolic compounds, polycyclic aromatic hydrocarbons, pesticides, dyes, *etc* [3,4]. This extremely nonspecific activity of the fungi is closely related to the ligninolytic enzyme system. The ligninolytic enzymes are the heme proteins lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13) and the copper-containing protein laccase (EC 1.10.3.2) [5-7]. LiP catalyzes one-electron oxidation of phenolic and nonphenolic compounds by H_2O_2 and the corresponding production of free radicals. Besides various aromatic compounds are utilized as substrates, the radical products react with other compounds as strong oxidants [4]. MnP catalyzes oxidize the H_2O_2-dependent oxidation of Mn(II) to Mn(III); the Mn(III) is then used to oxidize phenolic compounds in a similar manner as LiP [8,9]. Laccase also oxidizes phenolic and nonphenolic compounds to the radicals in the presence of O_2 [10,11]. In addition, the ligninolytic system includes a variety of H_2O_2-producing oxidases [7,12]. Noting the nonspecific nature of the enzyme system, the fungi have been used for treatment of various wastewaters, including removals of color, lignin-related compounds and polychlorinated phenolic compounds from pulp and paper mill effluents [1,2,13], phenolic compounds from olive mill effluents [14,15], and synthetic dyes from textile and dyestuff effluents [16-19]. In practice, the fungal treatment of pulp and paper mill effluents has been conducted with pilot-scale reactors so far [1,13].

Melanoidins, which are well-known as natural browning polymers in various foods, often cause a coloration problem of wastewaters, *e. g.*, molasses wastewaters from distillery and fermentation industries [20]. They are generated through the

Maillard (amino-carbonyl) reaction of aldoses and amines [21] and are closely related to humic substances in the natural environment [22]. Since they are resistant to microbial attack, the conventional biological treatments such as activated sludge processes are inapplicable to color removal from melanoidin-containing wastewaters. At present, the color removal of these wastewaters has to rely on chemical and physico-chemical treatments such as ozonation [23,24] and flocculation treatment [20,25]. The ozonation techniques, however, have not become popular because of the high operational costs. Although the flocculation treatment is more effective, it results in the production of a large volume of waste sludge. Since the colored wastewaters are usually released into water environment without decolorization treatment, development of effective and cost competitive decolorization processes is strongly desired.

The ligninolytic activity of white rot fungi appears to also function in the decolorization of melanoidins and humic substances [26-29]. Therefore, development of wastewater decolorization processes employing these fungi looks promising. In practice the fungi have been used for decolorization treatment of molasses wastewaters [30] and sugar refinery effluents already [31]. Some results from those investigations should be noticed from the viewpoint of the practical use as an alternative to the chemical and physico-chemical treatments. First, the rate of the decolorization seems relatively slow, although it is unclear whether that is caused by the slow rate of fungal growth or the decolorizing ability itself. In general, the time needed to reach sufficient or maximum decolorization ranges from several days to a week [26,27,32]. Furthermore, the fungi appear to require a considerably high concentration of a carbon source (5 to 20 g/l) such as glucose for sufficient decolorization. Addition of a large amount of carbon to wastewater is undesirable for practical processes. Therefore, enhancement of the efficiency of the fungi in melanoidin decolorization is essential to develop useful treatment processes for melanoidin-containing wastewaters. Nevertheless, little attempt to solve those problems has been carried out. This may be caused largely by insufficient

understanding of the melanoidins decolorization. Only a few reports have mentioned the decolorizing system [26,32-34].

In this study, application of the fungi to decolorize heat treatment liquor of waste sludge (HTL) was attempted. It is assumed that HTL is one of the melanoidin-containing wastewaters, though the colored components have not been characterized yet. HTL shows a dark brown color based on heating of the sludge organic matters: HTL is produced by a conditioning process, heat treatment, of waste sludge at sewage treatment plants (Figs. 1 and 2). Activated sludge treatment of sewage wastewater produces a lot of sludge. The sludge produced is usually conditioned to remove the moisture effectively in the sludge-dewatering processes [35]. In the conditioning, the sludge concentrated in the gravity thickener is heated with the heat exchanger unit and maintained at 150 to 200°C for about 1 hr in the heat treatment reactor [35]. HTL consists of the supernatant and filtrate from the mixture of the heated sludge. Ozonation treatment is used for color removal of HTL at a few plants in Japan [M. Sakagami, Takuma Co.; personal communication]. In most cases, HTL is returned to the aeration tank, treating sewage wastewater, or treated in an extended aeration tank. These biological processes, however, hardly remove the color.

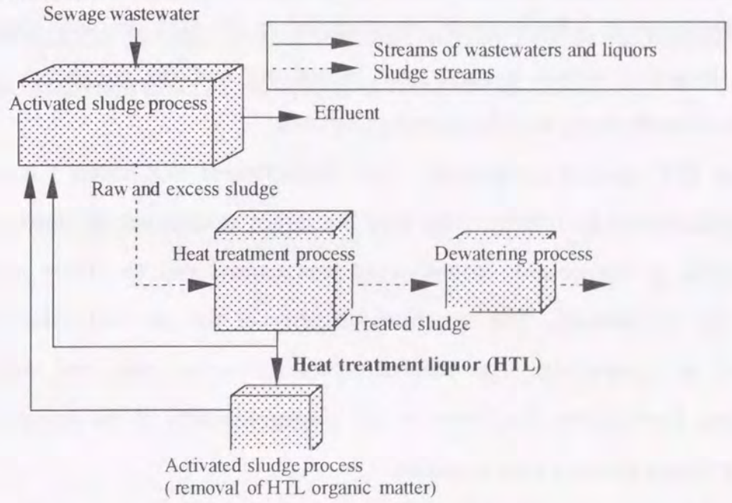

Fig. 1. Heat treatment process in sewage wastewater treatment plant.

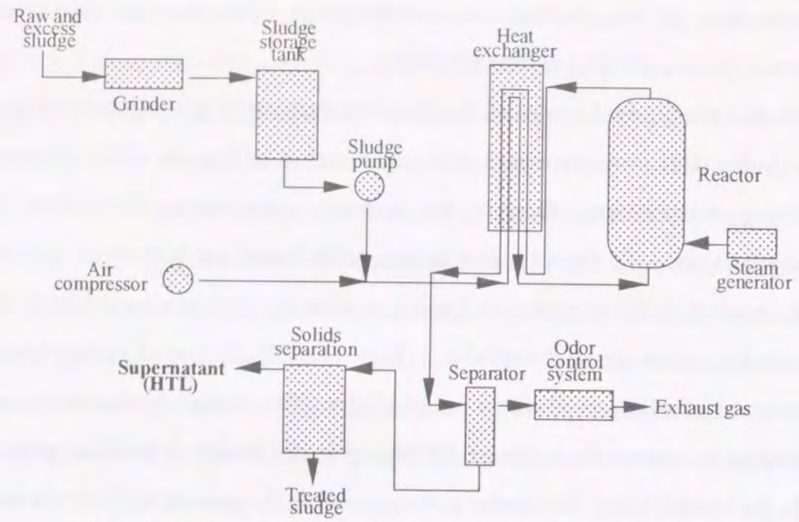

Fig. 2. Schematic diagram for heat treatment process (Ref. 35).

The purpose of this study is to show the possibility of the development of microbial HTL decolorization processes employing white rot fungi as the effective microorganism. It is expected that the fungi are useful microorganisms for the HTL decolorization treatments, since they presumably have a potential ability to decolorize HTL owing to the melanoidin-decolorizing activity described above. To achieve the purpose, a fundamental investigation on the HTL decolorization by the selected fungus *Coriolus hirsutus* was carried out and the enhancement of its decolorization efficiency was discussed as follows:

First, HTL colored components were characterized in Chapter I in order to obtain fundamental knowledge regarding the target substances of decolorization. Fractionation of the colored components was carried out to clarify major and noteworthy components. The structural property of the clarified components is discussed in comparison with that of synthetic melanoidins and soil humic substances. Furthermore, the behavior and biodegradability of the components in activated sludge process were examined.

In Chapters II to IV, HTL decolorization of a white rot fungus was characterized in order to obtain knowledge relating to enhancement of the HTL

decolorization. *Coriolus hirsutus* was selected as a useful fungus because of its high HTL-decolorizing ability, and effects of carbon addition and HTL dilution degree on the HTL decolorization were examined (Chapter II). It was suggested that the decolorizing activity is depressed in concentrated HTL. Also, the fungus required larger amounts of added carbon in concentrated HTL. In Chapter III, the enzyme system involved in the HTL decolorization of *C. hirsutus* was specified using synthetic melanoidin. Extracellular H_2O_2 and two kinds of peroxidases, MnP and manganese-independent peroxidase (MIP), functioned in melanoidin decolorization by the fungus. In Chapter IV, the HTL component that has an inhibitory effect on the HTL decolorization was specified using artificial liquor prepared with synthetic melanoidin. It was concluded that HTL organic nitrogen components lower the decolorization efficiency.

Finally, the approach for enhancement of HTL decolorization by *C. hirsutus* is mentioned in Chapter V. Activated sludge treatment of HTL was introduced as a pretreatment for the fungal HTL decolorization, since it is useful for decomposition of the organic nitrogen component inhibiting the decolorization. Combined use of the sludge and fungus raised the fungal decolorization efficiency. Furthermore, stimulation of the HTL-decolorizing enzymatic reaction by adding Mn(II) and H_2O_2 was confirmed. On the basis of that knowledge, perspectives on the development of a HTL decolorization process are described in the last section.

Chapter I Fractionation and characterization of colored components of waste sludge heat treatment liquor (HTL)

I-1. Introduction

HTL contains complex colored components, since they originate from sludge organic matter. Therefore, an effective technique for separation of the colored components is needed to enable their characterization.

HTL colored components are closely related to colored polymers, melanoidins, which are generated by heating of amino and carbonyl compounds [21]. Furthermore, the colored components are presumably related to natural humic substances. Because humic substances are at least partially generated through the Maillard reaction, and they have a common structure as synthetic melanoidins prepared from simple sugars and amino acids [36,37]. Melanoidins and humic substances are groups of complex polymers that do not have a definite structure, and the individual molecules can not be isolated from those groups [22]. Therefore, fractionation of these two groups into some subgroups, which consist of components with a certain definite nature, has been attempted. Melanoidins have been fractionated by gel filtration chromatography and electrofocusing [38,39]. Fractionation techniques for humic substances have been well established. They are usually fractionated into humus (dilute alkali-soluble fraction) and humin (dilute alkali-insoluble fraction), and the former is fractionated into humic acid (acid-insoluble fraction) and fulvic acid (acid-soluble fraction) [40]. In general, aquatic humic and fulvic acids are concentrated by adsorption chromatography with XAD resin [41]. Furthermore, these two fractions from humus have been fractionated by gel filtration chromatography and anion exchange chromatography [40,42,43].

In this chapter, fractionation of HTL colored components by the method described above for humic substances fractionation was investigated. The major fraction that had an abundance of colored components was characterized on molecular weight distribution and partial structure. Furthermore, the behavior of the major colored components in the activated sludge process was investigated.

I-2. Materials and methods

HTL sample

HTLs (samples A, B and C) were collected from heat treatment facilities at sewage wastewater treatment plants. They were taken from different plants: Sample A was the dewatering-filtrate from heated sludge, and samples B and C were supernatants of sludge mixed liquor before dewatering. An effluent sample (D) from extended aeration tank treating HTL was also collected. It was collected at the same plant as sample A. All samples were filtered with filter paper (No. 131, ADVANTEC Toyo), and were stored at 4°C. The filtrates were centrifuged at 1,800 x *g* for 15 min to remove suspended solids before use. General characteristics of samples A, B and C are listed in Table I-1.

Table I-1. General characteristics of HTL samples

Sample	A	B	C
Treatment conditions			
Temperature (°C)	175	200	175
Retention time (min)	60	45	60
pH	5.7	5.2	5.4
TOC (mg/l)	4,500	3,300	2,300
BOD (mg/l)	7,400	4,300	3,400
T-N (mg/l)	1,300	770	530
NH_4^+-N (mg/l)	770	340	310
NO_3^--N (mg/l)	<1.0	<1.0	<1.0
NO_2^--N (mg/l)	<1.0	<1.0	<1.0
T-P (mg/l)	92	48	57
Color (CU)	7,300	5,700	2,700

Procedure for fractionation of HTL colored components

HTL samples (30 to 100 ml) were acidified to pH 1 with concentrated HCl and stirred moderately for 3 hr. The HTL suspensions were centrifuged at 1,800 x *g* for 15 min to separate acid-insoluble precipitate and supernatant. The former was rinsed with deionized water, and dissolved in 50 ml of 0.05 M NaOH. Then, this solution was adjusted to pH 7 and used as fraction I. The supernatant was further fractionated with a XAD-7 resin column (Rohm and Haas; column size, 3 x 8 cm),

previously equilibrated with 0.1 M HCl. The column was washed with 100 ml of 0.1 M HCl, and the eluate was adjusted to pH 7 and used as fraction II. The components adsorbed on the resin were eluted with, successively, 100 ml of methanol and 100 ml of 0.1 M NaOH. Both eluates were evaporated and dissolved in 50 ml of 0.1 M NaOH. The solutions prepared from the eluates obtained with methanol and alkali were adjusted to pH 7 and used as fractions III and IV, respectively.

To collect colored components of fractions I and III, 500 ml of HTL sample A was acidified and centrifuged. Five hundred grams of XAD-7 resin was added to the supernatant, and the mixture was stirred moderately for 30 min. The resin was washed with a sufficient volume of 0.1 M HCl on a Büchner funnel. The components adsorbed on the resin were eluted with 400 ml of methanol. Fractions I and III were brought to a final volume of 50 ml for both the precipitate and the eluate with methanol.

Incubation of HTL with activated sludge

The activated sludge used in this study had been acclimated to synthetic organic wastewater for a long period by a fill and draw cultivation method. The synthetic wastewater had the following composition (/l-tap water): 100 mg meat extract, 160 mg peptone, 70 mg $Na_2HPO_4 \cdot 12H_2O$, 15 mg NaCl, 7 mg KCl, 7 mg $CaCl_2$, and 5 mg $MgSO_4$. The sludge was centrifuged at 800 x *g* for 5 min and then, added to the sample A. The mixture contained 40 ml of sample A, 100 mg sludge (dry weight) and 50 mM Na phosphate buffer (pH 5.3) in a total volume of 50 ml. It was incubated for four days at 25 °C in a reciprocal shaker at 120 rpm and then, filtered with filter paper.

Analytical methods

To measure the color of HTL and its fractions, they were diluted with 0.1 M Na phosphate (pH 7.0) to give an absorbance at 465 nm (A_{465}) of less than 1.0. the color was calculated from the A_{465} value on basis of the platinum-cobalt method [44,45] as follows:

$$\text{Color (CU)} = \frac{1{,}000 \times A2}{A1}$$

where A1 is the absorbance of the 1,000 CU Pt-Co standard solution (A_{465} = 0.272), and A2 is A_{465} of the sample. Total organic carbon (TOC) was measured with a Shimadzu TOC-analyzer, TOC-500. Biochemical oxygen demand (BOD), total nitrogen (T-N), ammonium, nitrate and nitrite nitrogen, and total phosphorus (T-P) were determined according to the sewage analytical methods [46].

Molecular weight distributions of the colored components of fractions I and III were analyzed by gel filtration and dialysis. Fractions I and III were diluted with deionized water to give an A_{465} of 1.0 (3,700 CU). The diluted solutions of fractions I and III contained 250 and 2000 mg/l TOC, respectively. Four milliliters of the diluted fractions were applied to Sephadex G-50 and G-25 columns (each 3 x 25 cm) and then, eluted with deionized water at a flow rate of 60 ml/h. Besides the deionized water, 10 mM Na phosphate (pH 7.0) and the same buffer containing 0.1 M NaCl were used, as higher-ionic-strength eluents. All of the eluents contained 0.5 mM NaN_3. Elution of colored components was monitored at 465 nm. Exclusion limits of G-50 and G-25 gels were taken as molecular weight of dextran, 10,000 and 5,000, respectively [40]. Void volumes (V_Os) of the columns were measured with Blue Dextran 2,000 (Pharmacia Biotech). In addition, the diluted fractions I and III (each 10 ml) were dialyzed with Spectra/Por 6 membrane (cutoff, < Mw 1,000; Spectrum) against 1 l of deionized water or 10 mM Na phosphate (pH 7.0) containing 0.1 M NaCl. The dialyses were maintained for three days at room temperature with two changes of the outer solution, before color and TOC of the dialyzed fractions were determined.

Identification of degradation products from HTL colored components

Components in fractions I and III were degraded with alkaline H_2O_2 in order to elucidate the partial structures of the HTL colored components. The two fractions (each 50 ml) were dialyzed against 3 l of deionized water containing 0.5 mM NaN_3 The dialyses were maintained for six days with five changes of the outer solution. The reaction mixture (160 ml), containing 200 mg-TOC of fraction I or III

nondialyzable component and 10% (v/v) H_2O_2, was adjusted to pH 9 with NaOH and incubated for 24 hr at 28°C. The nondialyzable components in the dilute alkali solution (pH 9.0) were also incubated and used as controls. After the reaction mixture was adjusted to pH 2.0, a small part was used for determination of acetic acid content. The greater part of the mixture was used for separation of acidic and ampho-ionic degradation products (Fig. I-1). The acidic fraction was prepared by extraction with ether, and the ampho-ionic fraction was prepared using two ion exchange columns, Dowex-50 x 8 (H^+ form) and Dowex-2 x 8 (OH^- form) (Dow Chemical, each 0.8 x 5 cm). Acids in the acidic fraction were methylated with trimethylsilyldiazomethane (TMSDM) [47]. Amino acids in the ampho-ionic fraction were trimethylsilylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [48].

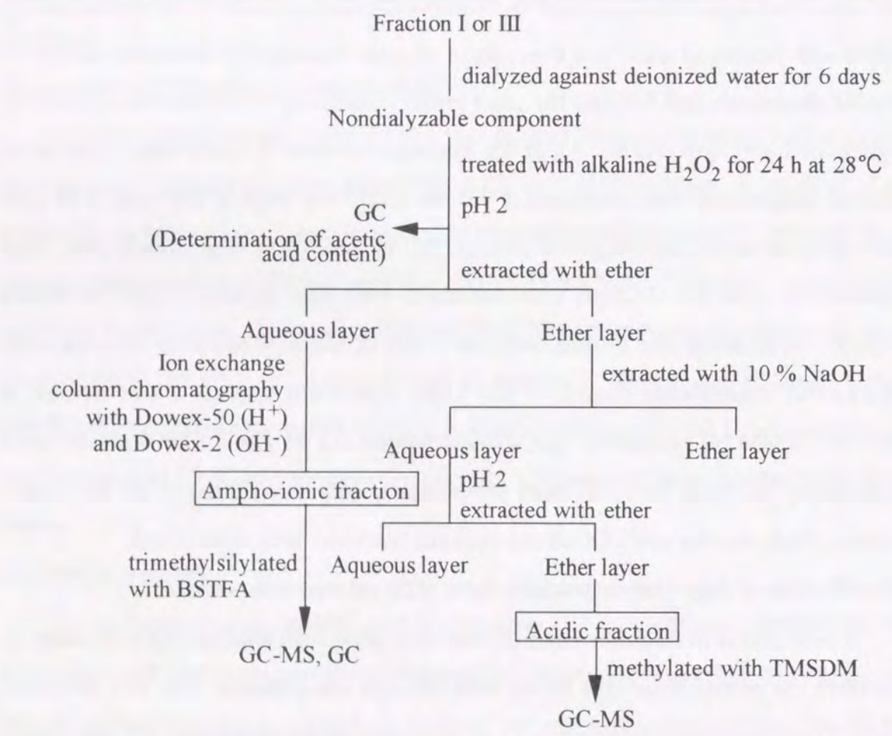

Fig. I-1. Preparation of acidic and ampho-ionic fractions from degradation mixtures of fractions I and III

Acetic acid content was determined by gas chromatography (GC) with a Hitachi, model 263-50 equipped a flame ionization detector (FID). The analytical conditions were as follows: a Shincarbon A packing 5% Thermon 3,000 column (3.2 mm x 1.6 m, Shinwa Chemical Industries) was used. The temperatures of column oven, injector and detector were 130, 260 and 260°C, respectively. The derivatives of acids and amino acids were analyzed by gas chromatography-mass spectrometry (GC-MS) with a Shimadzu GC-17A/QP-5000. For acid derivatives, a CBP-20-M column (0.22 mm x 25 m, Shimadzu) was used. The temperature of the column oven was raised from 60 to 210°C at a rate of 3°C/min after injecting a sample. The injector and detector temperatures were maintained at 230°C. For amino acid derivatives, a CBP-1-M column (0.22 mm x 25 m, Shimadzu) was used. The temperature of the column oven was kept at 80°C for 8 min after injecting a sample and then, raised from 80 to 240°C at the rate of 4°C/min. The temperatures of injector and detector were maintained at 240°C. The derivatives were identified by computer comparison of the mass spectra with the NIST library. Mass spectra of fatty acids (C_{8-17}), dicarboxylic acids (C_{2-10}), 2-furancarboxylic acid, and all of the amino acids were confirmed with the authentic compounds. Furthermore, the amino acids identified were determined with a Shimadzu GC-14A equipped a FID. The analytical conditions were the same as those of the GC-MS analysis.

I-3. Results and discussion

I-3.1. Fractionation of colored components

The methods for fractionation of soil and aquatic humic substances [41] were introduced to characterize HTL colored components. Color and TOC contents of HTL fractions I to IV are listed in Table I-2. In all of the samples, fractions III had the most color, 55, 77 and 63% of samples A's, B's and C's total color, respectively. Therefore, the colored component of fraction III contributed most to the HTL color. The fraction I of sample A also had a high level of color (22% of the total color), though those of B and C had low levels (less than 7% of the total color). This shows that the contribution of the fraction I component to HTL color is also important in

some HTLs. Fraction II contained a large amount of non-colored component, but little colored component. Both color and TOC contents of Fraction IV were at considerably low levels. These results show that the major colored components of HTL are fractionated into fractions I and III.

Table I-2. Color and TOC contents of HTL fractions.

		Fraction				
	Total[a]	I	II	III	IV	Yield (%)
Sample A						
Color (CU)	7,300	1,600	720	4,000	20	88
TOC (mg/l)	4,500	140	2,200	1,800	89	93
Sample B						
Color (CU)	5,700	200	240	4,400	32	86
TOC (mg/l)	3,300	33	1,400	1,500	40	89
Sample C						
Color (CU)	2,700	183	240	700	18	78
TOC (mg/l)	2,300	22	880	1,100	13	85

[a], color and TOC of original HTL samples.

I-3.2. Molecular weight of major colored components

The greater part of fraction I colored component (from sample A) was eluted in the void volume of the G-50 column when it was eluted with deionized water (Fig. I-2A). In contrast, the elution of fraction III component was relatively slow (Fig. I-2B). As the ionic strength of the eluent increased, both elution patterns shifted to right. As a result, they were superposed under the highest-ionic-strength condition. The apparent decreases in their molecular weights by high ionic strength were also obtained, when fractions I and III were dialyzed against high-ionic-strength buffer (Table I-3). Fraction I lost about 60% of the initial color during the dialysis with the buffer, while it lost only 20% during the dialysis with deionized water. The use of the buffer likewise increased the loss of fraction III color, and the loss of color was 10 points greater than that of fraction I color. These results show that both colored components of the fractions changed in their apparent molecular weight owing to ionic strength of the solvent. This is consistent with the behavior of aquatic humic substances: De Haan *et al.* showed the ionic strength-induced changes in the configuration of aquatic humic substances [49]. In the report, they concluded that high ionic strength decreases their molecular size and/or shape.

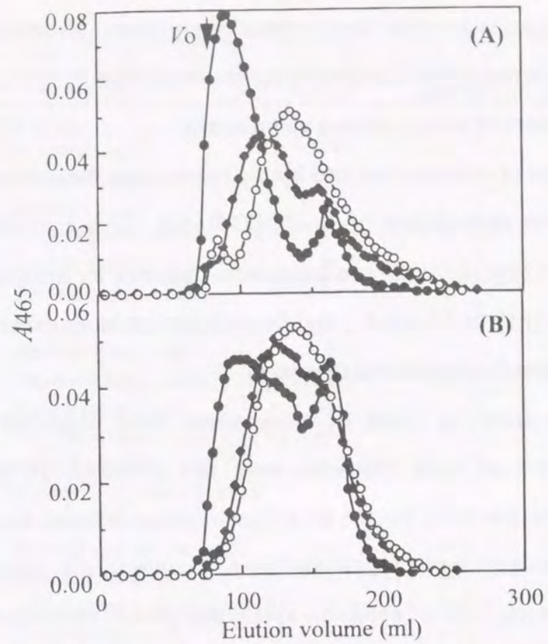

Fig. I-2. Sephadex G-50 gel filtration of colored components in fractions I (A) and III (B).
Eluent: —●— , deionized water; —◆— , 10 mM phosphate buffer; —○— , 10 mM phosphate buffer containing 0.1 M NaCl.

Table I-3. Remaining color and TOC in fractions I and III after dialysis with different-ionic-strength solvents

	Fraction I		Fraction III	
	-i [a]	*+i* [b]	*-i*	*+i*
Color (%)	81	39	69	30
TOC (%)	82	45	31	9

[a], dialyzed against deionized water.
[b], dialyzed against 10 mM phosphate buffer containing 0.1 M NaCl.

Both I and III colored components have apparent molecular weights of less than 10,000 as shown in Fig. I-2. Furthermore, the G-25 gel filtration of the components showed that some of the components have molecular weights of less than 5,000. The elution peak of the fraction III component was broader than that of the fraction I component. As shown in Table I-3, the fraction III component may have a lower-

molecular-weight distribution than the fraction I component. However, more details of their characteristics were not found in these investigations.

I-3.3. Partial structure of major colored components

Structural units of melanoidins and humic substances have been specified by identification of their degradation products [23,50-53]. Hayase *et al.* attempted to use alkaline H_2O_2 to degrade synthetic melanoidin obtained by heating of a glucose and glycine mixture [51]. In this study, that degradation technique was applied in the analysis of HTL colored components structure.

Nondialyzable fractions I and III components were degraded with alkaline H_2O_2. TOC contents of both fractions were not changed by this treatment. Decolorization of the fractions by the treatment reached 90% of their initial color. The degradation products from the fraction I and III components included 42 and 48 μg of acetic acid per mg-TOC of nondialyzable components, respectively. No acetic acid was detected in the controls of the components. The acidic products identified by GC-MS are listed in Table I-4. It was unclear whether some of fatty acids and butanedioic acid were degradation products, since they were also detected in the controls. Many kinds of acids including fatty acids, dicarboxylic acids, furans and aromatic acids were detected in both of the fraction I and III acidic fractions. This shows that the two colored components have a considerable amount of common structural units. Furthermore, 10 amino acids were detected in the ampho-ionic fraction of the fraction III component (Fig. I-3). Calculated total amount of amino acids was 1.6 μg/mg-TOC of the nondialyzable fraction III component.

In the report of Hayase *et al.*, acetic acid as a main product and some kinds of dicarboxylic and furancarboxylic acids, including methylpropanedioic, propanedioic, butanedioic and 2-furancarboxylic acids as minor products, were identified after degradation of glucose-glycine melanoidin [51]. They postulated the partial structural units of melanoidin as follows: **CH_3-CO-R**, **R-CO-CO-R''**, **R-CO-R'-CO-R''**, *etc*. HTL major colored components must also include those units. On the other hand, the higher dicarboxylic hydrocarbons and the aromatic acids have not been

Table I-4. Acidic compounds identified in degradation mixtures of fractions I and III.

		Fraction I		Fraction III	
Retention time	Compound[a]	H_2O_2[b]	Control[c]	H_2O_2	Control
(min)	Fatty acid				
9.0	Octanoic acid	+[d]	–[e]	+	–
12.3	Nonanoic acid	+	–	+	–
15.9	Decanoic acid	+	–	+	–
17.9	Undecanoic acid	+	–	+	–
23.4	Dodecanoic acid	+	+	+	+
30.7	Tetradecanoic acid	+	+	+	+
34.1	Pentadecanoic acid	+	+	+	+
37.4	Hexadecanoic acid	+	+	+	+
40.5	Heptadecanoic acid	+	–	+	–
43.6	Octadecanoic acid	+	+	+	+
44.0	9-Octadecenoic acid	+	+	–	–
	Dicarboxylic acid				
11.7	Methylpropanedioic acid	–	–	+	–
13.2	Propanedioic acid	+	–	+	–
15.1	Methylbutanedioic acid	+	–	+	–
16.1	Butanedioic acid	+	+	+	+
19.5	2-Methylpentanedioic acid	+	–	+	–
19.9	Pentanedioic acid	+	–	+	–
23.0	2,3-Dimethyl-2-butenedioic acid	–	–	+	–
24.1	Hexanedioic acid	+	–	+	–
27.8	Heptanedioic acid	+	–	+	–
31.4	Octanedioic acid	+	–	+	–
34.8	Nonanedioic acid	+	–	+	–
38.1	Decanedioic acid	+	–	+	–
	Furancarboxylic acid				
15.4	2-Furancarboxylic acid	+	–	+	–
18.6	2-Methyl-3-furancarboxylic acid	+	–	+	–
	Pyrrolecarboxylic acid				
32.1	2-Pyrrolecarboxylic acid	+	–	–	–
	Aromatic acid				
16.7	Benzoic acid	+	–	+	–
21.8	Benzeneacetic acid	+	–	+	–
22.0	2-Hydroxybenzoic acid	–	–	+	–
24.8	Benzenepropanoic acid	–	–	+	–
33.0	3- or 4-Methoxybenzoic acid	–	–	+	–
37.8	2-Aminobenzoic acid	+	–	+	–
38.0	1,4- or 1,3-Benzenedicarboxylic acid	–	–	+	–
39.6	1,2-Benzenedicarboxylic acid	+	–	+	–
42.0	Nitrobenzoic acid	+	–	–	–

[a], identified as methylated derivatives; [b], the degradation mixture (with alkaline H_2O_2); [c], the control mixture (with deionized water, see materials and methods); [d], detected in the mixture; [e], not detected.

found as degradation products of simple sugar-amino acid melanoidin. Presumably, this depends on the generation of HTL colored components from complex sludge organic matter such as microorganism constituents. Benzenecarboxylic acids have been considered to comprise a significant part of humic substances [52]. Recently, Saiz-Jimenez detected higher fatty acids and dicarboxylic acids in the pyrolysates of soil fulvic acids [53]. It is interesting that the HTL colored components and humic substances have those compounds as common structural units, because the former was generated from sludge organic matter by heating within a short period, whereas the latter was generated from soil organic matter by natural processes through long period [22].

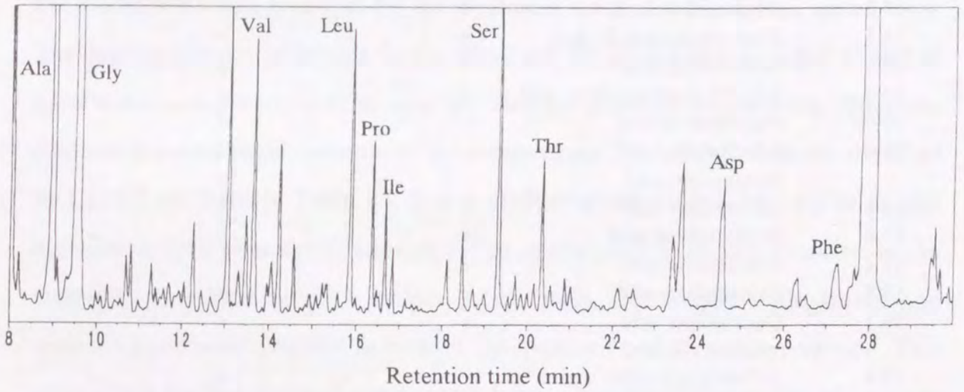

Fig. I-3. GC-MS of amino acids in degradation mixture of fraction III.
All amino acids were identified as trimethylsilylated derivatives.

I-3.4. Behavior of major colored components during activated sludge treatment

In sewage treatment plants, HTL is treated in an extended aeration tank or an aeration tank for sewage treatment. It is important, therefore, to investigate the behavior of the major colored components in the biological processes involved. HTL sample D, which was collected from an aeration tank, was fractionated into fractions I to IV (Fig. I-4). The total color was considerably low, when compared with the reference sample A (non-treated HTL) (Table I-2). It is unclear whether the decrease in color was dependent on activated sludge activity because of dilution of

the HTL in the aeration tank. The result was that fraction III color comprised 90% of the total color and the other fractions color were at low levels. Compared with sample A, the marked decrease in the level of fraction I color in sample D was especially noted. Sample A was incubated with activated sludge and fractionated (Fig. I-4). The fraction I lost 90% of its initial color during the incubation. On the other hand, an increase in the fraction III color was observed. The decrease in the fraction I color appeared to be closely related to the increase in that of fraction III, suggesting that the acid-insoluble colored component is not removed from HTL but remains as an acid-soluble component in the activated sludge processes. Fisher and Swanwick reported that more than 30% of the chemical oxygen demand (COD)-component in HTL is non-biodegradable [54]. The major colored components ought to overlap with such non-biodegradable components.

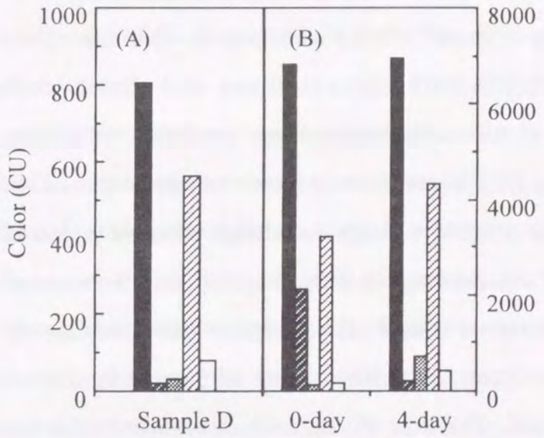

Fig. I-4. Changes of color distribution during HTL activated sludge treatment.
(A) Sample D from extended aeration tank for HTL treatment was fractionated. (B) Sample A was incubated for four days with activated sludge and then, fractionated.
■, total; ▨, fraction I; ▩, fraction II;
▨, fraction III; □, fraction IV

I-3.5. Conclusions

Fractions I and III contained HTL major colored components. They were characterized by comparison with humic substances and sugar-amino acid melanoidin. It can be considered that fraction I colored component corresponds to humic acid, since it is recovered as acid-insoluble precipitate from soil and aquatic organic matters. Fraction III colored component presumably corresponds to hymatomelanic acid and/or fulvic acid [40]. The former is alcohol-soluble and partially acid-insoluble, and the latter is acid, alcohol and water-soluble.

However, this classification of humic substances has been adopted for convenience, and it is difficult to separate those substances clearly [22]. The distinction between the two colored components was not obtained from the viewpoints of their molecular weight distributions or structural unit compositions. Actually, complete removal of the acid-soluble component from the fraction I component was unsuccessful even though the reprecipitation and resolubilization procedures using acid and alkali was repeated. Therefore, they are presumably separated into the two fractions on the basis of a slightly distinct nature, *e. g.*, molecular weight distribution, electric charge, and/or hydrophilicity, as explained for humic substances [40]. These characteristics may be responsible for their behavior in activated sludge processes. Activated sludge was able to decrease the amount of fraction I colored component, but did not appear to remove it substantially. If the difference of their nature is small, the sludge, which insufficiently able to degrade the colored components, may be capable of increasing the solubility of the I component in acid. Fraction III colored component consequently increases contribution to almost all of the HTL color during the treatment. Therefore, the microorganisms that degrade the fraction III component effectively were needed for investigation of microbial HTL color removal.

In the study of the microbial color removal, preparation of simple artificial liquor is needed. Because actual HTL composition is very complex and not steady. Use of an artificial liquor may make the study more efficient and reproducible. HTL colored components are and behave considerably similar to natural humic

substances and synthetic melanoidin, which are readily available. Therefore, it is possible to use these compounds for preparation of the artificial liquor.

I-4. Summary

HTL colored components were fractionated by precipitation with acid and by adsorption chromatography with XAD resin. The acid-insoluble fraction (I) and acid-soluble fraction (III) eluted from the resin with methanol showed high color levels, because these two fractions contained major colored components. These components were further characterized and compared with the related compounds, natural humic substances and synthetic melanoidins. The profiles of gel filtration and dialysis of the two colored components greatly varied as a result of using eluents and solvents with different ionic strength, respectively, *i. e.*, high ionic strength induced apparent reduction of their molecular weights. Therefore, it is suggested that each component exists in aggregation form under low-ionic-strength condition and/or in contracted form under high-ionic-strength conditions. Fractions I and III nondialyzable components including colored components were degraded with alkaline H_2O_2 to identify their partial structure. The colored components had the same structural units that have been identified in humic substances and synthetic melanoidin. However, the detailed differences between the I and III components could not be found by these investigations. Finally, the behavior of the major colored components during HTL activated sludge treatment was investigated. Incubation of HTL with the sludge resulted in the loss of most fraction I color and the increase in fraction III color. This suggests that the fraction I component is converted into acid-soluble component, which can be fractionated into fraction III, in the biological treatment.

Chapter II Decolorization of HTL by the selected fungus *Coriolus hirsutus*

II-1. Introduction

White rot fungi are unique microorganisms which can cause degradation of natural recalcitrant polymer, lignin. Besides lignin degradation, these fungi can oxidatively degrade a wide variety of organic compounds owing to the extremely non-specific nature of the ligninolytic enzymes [3,4]. Therefore, much attention has focused on the removal of pollutants from industrial wastewaters, including removal of lignin-related compounds [44, 55-57] and polychlorinated phenolic compounds [2] from pulp and paper mill effluents, phenolic compounds from olive mill effluents [14,15], and synthetic dyes from textile and dyestuff effluents [16-19].

The fungal activity has also been applied in attempts on microbial color removal of melanoidin-containing wastewaters. *Phanerochaete chrysosporium* showed a decolorization efficiency of about 80% for sugar refinery effluents [31]. Molasses wastewaters were strongly decolorized by *Coriolus versicolor* [27]. Furthermore, continuous decolorization of molasses wastewater and melanoidin medium were successfully conducted using immobilized *C. versicolor* [30] and *C. hirsutus* cells [58] , respectively.

HTL colored components are closely related to the melanoidins as shown in Chapter I. Therefore, the studies on wastewater decolorization using white rot fungi imply that HTL can be decolorized by the fungi. In this session, application of the fungi to HTL decolorization was attempted. *C. hirsutus* was selected on the basis of its high HTL-decolorizing ability. HTL major colored components were degraded during incubation with fungal pellets. The decolorization was greatly affected by HTL dilution degree and amount of carbon source.

II-2. Materials and methods

Preparations of HTL colored components and synthetic melanoidin

The HTL sample used in this chapter was the same liquor as sample A used in Chapter I. It was filtered, stored at 4°C and centrifuged to remove suspended solids before use. HTL major colored components were prepared from the liquor by the fractionation method described in Chapter I. The fraction I and III components were used as the major colored components. The first was the acid-insoluble fraction obtained by precipitation with HCl, and the second was the acid-soluble fraction eluted from resin XAD-7 with methanol.

Melanoidin was prepared by autoclaving a mixture of 1 M D-glucose, 1 M glycine and 0.2 M Na bicarbonate, pH 6.8, for 3 h at 121°C [51].

White rot fungi

C. hirsutus IFO 4917, *C. versicolor* IFO 30340, *Lenzites betulina* IFO 6266, *Pleurotus ostreatus* IFO 30879 and *P. chrysosporium* IFO 31249 were used. *C. hirsutus*, *C. versicolor* and *L. betulina* were the same strains as the melanoidin-decolorizing and/or -degrading strains previously reported [27,28]. All of the strains were maintained at 4°C on malt extract agar, pH 6.0, containing 20 g malt extract, 20 g glucose, 1.0 g peptone, and 16 g agar per liter.

Preparations of mycelium suspension and pellets

The fungi were grown on GPY agar plates [59], containing 20 g glucose, 3.0 g peptone, 2.0 g yeast extract, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 16 g agar (per liter, pH 6.0), at 28°C for a week. The homogeneous mycelium suspension and pellets were prepared by the method of Archibald *et al* [56]. Four 1-cm diameter plugs punched from the periphery of the 7-day GPY agar plate were agitated in 100 ml of GPY liquid medium in a 500-ml polypropylene flask with four 1.8-cm diameter glass marbles for one day at 28°C in a rotary shaker (120 rpm). Ten milliliters of mycerial suspension was transferred into 100 ml of fresh GPY in a 300-ml Erlenmeyer flask, and cultured for four days at 28°C in the rotary shaker (120 rpm). This cultivation produced 200 mg dry weight of 6 to 8-mm diameter pellets per flask.

Decolorization of HTL by *C. hirsutus*

C. hirsutus pellets (dry weight of 200 mg) were washed twice with 100 ml of sterile deionized water on a stainless steel mesh, and transferred into 100 ml of the diluted HTL with deionized water. The HTL contents in the diluted solution were 40 to 100% (v/v). Glucose was added to the liquors as a concentration of up to 20 g/l. Prior to this, the HTL and glucose solutions were autoclaved separately for 20 min at 121°C. Incubation was carried out for eight days at 28°C in a rotary shaker at 120 rpm. HTL fraction I or III component was added to 100 ml of GM medium, referred to as GM-I and GM-III, respectively, and autoclaved for 20 min at 121°C. GM medium contained (per liter) 5.0 g glucose, 1.5 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5g KCl, 0.5g $MgSO_4 \cdot 7H_2O$, and 10 ml trace metal solution, pH 6.0. The trace metal solution was composed of 1.0 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot 5H_2O$, and 0.1 g $CuSO_4 \cdot 5H_2O$ per liter. Addition of the fractions I and III components resulted in media colors of 2200 and 1500 color units (CU), respectively. These color levels correspond to those of fractions I and III from the HTL, diluted to two-times volume with deionized water (*i. e.*, 50% HTL).

Analytical methods

The HTL and melanoidin-containing media incubated with the fungal pellets were filtered with filter paper. After the culture filtrates were diluted to four-times volume with 0.5 M Na acetate, pH 5.0, decolorization was evaluated from the decrease in A_{465} of the filtrates. Color (CU) was determined by combination of the Pt-Co method and measurement of A_{465} as described in section I-2 [44,45]. To recover the colored component adsorbed on mycelium surface, the pellets separated by filtration were washed with deionized water and then, immersed in 0.1M NaOH for 20 min [60]. The alkaline filtrate was adjusted to pH 5.0 and initial culture volume (100 ml), and the color was measured. Dry cell weight of the pellets was measured after drying at 110°C. Reducing sugar contents in the culture filtrates were determined by the method of Somogi-Nelson [61].

Four milliliters of the culture filtrates were applied to a Sephadex G-50 column (3.0 cm x 25 cm) and then, eluted with 20 mM Na phosphate (pH 7.3) containing 0.1 M NaCl. Elution of the colored components was monitored at 465 nm.

II-3. Results and discussion

II-3.1. Selection of HTL-decolorizing fungi

HTL-decolorizing abilities of five fungi were examined (Table II-1). To confirm their decolorizing ability for melanoidin, mycelium suspensions were inoculated in GPY medium supplemented with 0.4% (v/v) synthetic melanoidin solution. All five fungi grew well in the medium. *C. hirsutus* and *L. betulina* removed about 60% of the initial color. In the 50% HTL supplemented with 20 g/l glucose, *C. hirsutus*, *C. versicolor* and *L. betulina* grew well, whereas the other fungi showed a slight growth. Incubation with *P. ostreatus* increased the HTL color. Of the tested fungi, *C. hirsutus* was the most effective fungus removing about 80% of the initial color. Therefore, *C. hirsutus* was selected for further investigations of fungal HTL decolorization.

Table II-1. HTL-decolorizing activity of white rot fungi

Organism	Decolorization (%) A	B
C. hirsutus	57.8	75.6
C. versicolor	37.7	51.3
L. betulina	62.9	28.4
P. ostreatus	33.5	-10.4
P. chrysosporium	19.9	7.2

A, GPY medium supplemented with 0.4% synthetic melanoidin; B, 50% HTL supplemented with 20 g/l glucose.

II-3.2. Behavior of HTL colored components in the fungal HTL decolorization

C. hirsutus pellets were inoculated in 50% HTL with 20 g/l glucose. As shown in Fig. II-1, the decolorization proceeded with the fungal growth, and the efficiency reached 80% after eight days. This strain was reported to be the fungus that effectively decolorized molasses melanoidin [28,58]. The result of the HTL decolorization corresponds to those of the molasses decolorization: the decolorization appeared to be relative to the fungal growth. Fig. II-2 shows gel filtration profiles of HTL colored components before and after the incubation for eight days. *C. hirsutus* was able to decolorize components with a wide-range molecular weight, though low-molecular-weight components tended to be decolorized in preference to higher-molecular-weight ones. In order to clarify the

changes in constitution of the colored components, the 8-day liquor was fractionated into fractions I to IV (Fig. II-3). The fungal incubation of HTL resulted in a disappearance of fraction I color and a considerable decrease in fraction III color. Fractions II and IV, which contained HTL minor colored components, increased in color.

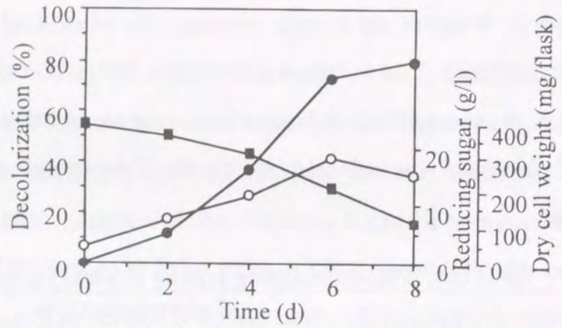

Fig. II-1. HTL decolorization and growth of *C. hirsutus* in 50% HTL supplemented with 20 g/l glucose.
●, decolorization of HTL; ○, dry cell weight; ■, amount of reducing sugar.

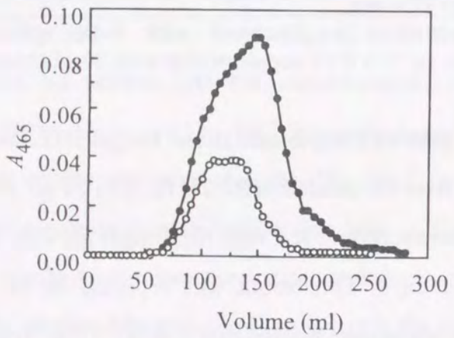

Fig. II-2. Sephadex G-50 gel filtration of colored components of HTL before (●) and after (○) incubation with *C. hirsutus*.
HTL (50%) supplemented with 20 g/l glucose was incubated with the fungal pellets for 8 days. The void volume of the column was 90 ml.

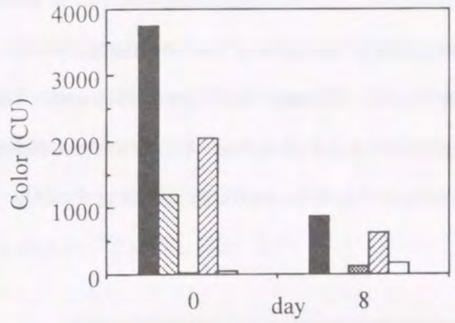

Fig. II-3. Color change in fractions of HTL before (A) and after (B) incubation with *C. hirsutus*. HTL (50%) supplemented with 20 g/l glucose was incubated with fungal pellets for 8 days, and fractionated into fractions I to IV. Symbols: ■, total; ▨, fraction I; ▩, fraction II; ▨, fraction III; □, fraction IV.

As shown in Chapter I, the color level of fraction I decreases during activated sludge treatment. On the other hand, that of fraction III increases during the same treatment. As a results, fraction III colored component remains predominately in the effluents from activated sludge processes. Therefore, in biological HTL decolorization, much attention should be given to treatment of the fraction III component. Fig. II-4 shows decolorization and adsorption of the fractions I and III components by the fungal pellets. The loss in color of GM-III reached 66% after four days of incubation. The loss in color calculated on the basis of the adsorption was 18% of the medium color loss. The adsorption efficiency might not be underestimated much, since the pellets color after the alkaline treatment seemed to be similar to that of pellets grown in GPY medium. This result indicates that a large part (82%) of the color loss was due to, at least, partial degradation of the fraction III colored component, *i. e.*, degradation of the chromophores. In the case of GM-I, the color loss on basis of the adsorption comprised about 30% of the medium color loss after four days (Fig. II-4). Therefore, the fraction I component must also be degraded during the incubation. Incidentally, the incubation of HTL with the fungus resulted in complete disappearance of fraction I color (Fig. II-3). Despite that result, 42% (600 CU) of the initial color remained after the incubation of GM-I (Fig. II-4). The day 4

culture filtrate was acidified (pH 1), but precipitant of the I component was not obtained. The color of the filtrate consisted out of fractions II, III and IV color accounting for approximately 100, 450 and 50 CU, respectively. This suggests that decolorization and/or degradation of fraction I colored component occurs by conversion into the other colored components as observed in the activated sludge treatment (Chapter I).

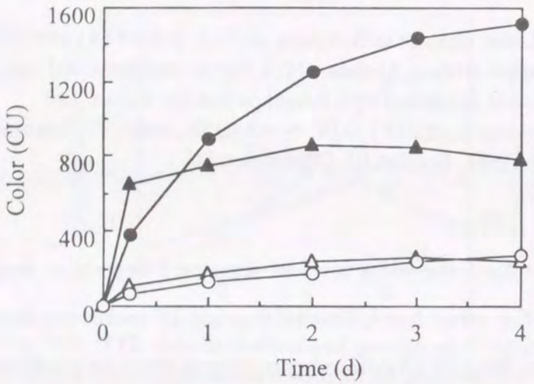

Fig. II-4. Decolorization and adsorption of fractions I and III colored components by *C. hirsutus*. Fractions I and III colored components were incubated with the fungal pellets in media GM-I (▲, △) and-III (●, ○), respectively.
▲, ●: amount of decrease in color of each medium.
△, ○: amount of color recovered from the pellets in each medium.

II-3.3. Effects of HTL dilution degree and carbon source on the fungal HTL decolorization

The HTL media with different degrees of dilution were incubated with fungal pellets (Fig. II-5). Glucose was added to a final concentration of 5 g/l. Hardly any lag time in decolorization was observed in 40% (v/v) HTL. However, it was observed in the HTL media that had a content over 50%. Also, the decolorization level at day 5 of incubation decreased with increasing HTL concentrations. Fig. II-6 shows the influence of the amount of glucose added on the decolorization of 40% HTL. Reduction of the glucose concentration from 5 to 1 g/l caused a considerable decrease in the decolorization rate in the early stage of incubation. Also, the

decolorization level at day 5 decreased. These results show that the fungus requires both an appropriate dilution of HTL and an appropriate amount of glucose for sufficient decolorization. The minimum amount of glucose required for sufficient decolorization could not be determined, since it was closely correlated with the HTL dilution degree: a glucose concentration of 5 g/l appeared to be sufficient in 40% HTL, but insufficient in 70% HTL (Fig. II-5).

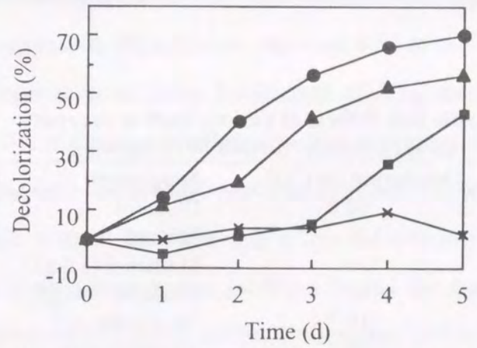

Fig. II-5. Effects of HTL dilution degree on HTL decolorization by *C. hirsutus*.
Glucose (5 g/l) was added to 100% (×), 70% (■), 50% (▲) and 40% (v/v) (●) HTL dilutions.

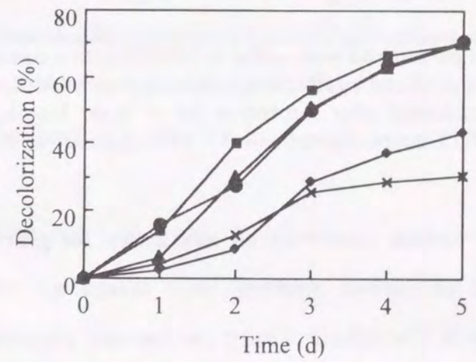

Fig. II-6. Effects of glucose addition on HTL decolorization by *C. hirsutus*.
Glucose was added to 40% HTL to concentrations of 0 (×), 1 (◆), 5 (▲), 10 (●) and 20 (■) g/l.

High concentrations of glucose (5 to 20 g/l) are always used for decolorization of melanoidins by white rot fungi. Ohmomo *et al.* reported that more than 5 g/l of glucose is required for sufficient decolorization of molasses wastewaters by *Coriolus versicolor* [30], though the influence of the wastewater content was not tested. Glucose-rich conditions have also been employed in the fungal treatment of other wastewaters (*e. g.*, see ref. 14 and 18). On the other hand, no attempt has been made to reduce the amount of glucose required. This is considered to be an unavoidable subject in the investigation of a practical fungal treatment of HTL and other wastewaters.

Table II-2. Effects of various carbon sources on HTL decolorization by *C. hirsutus*

Supplement[a]	Decolorization (%)[b]	Supplement	Decolorization (%)
None	18.0	Glycerol	113
D-Glucose	100	Ethanol	118
D-Xylose	100	Methanol (5.0 g/l)	-5.1
D-Fructose	162	Methanol (10 g/l)	-6.9
D-Arabinose	10.7	Na acetate	6.7
L-Arabinose	28.6	Na propionate	7.6
D-Sorbitol	83.7	Molasses (5.0 g/l)	54.3
Sucrose	102	Molasses (10 g/l)	81.4
Cellobiose	71.8	Corn steep liquor (10 g/l)	32.6
Maltose	51.9	Peptone (10 g/l)	40.9
Soluble starch	55.2		
Cellulose	27.2		

[a] Carbon sources without parentheses were added to 50% HTL to a concentration of 5.0 g/l. All of the substrates were sterilized by filtration before they were added to HTL.
[b] Decolorization was evaluated after incubation for 5 days. Decolorization efficiency (56.5%) obtained in the HTL supplemented with 5.0 g/l D-glucose was expressed as 100%.

To find a useful carbon source as an alternative for glucose may also be necessary. A variety of carbon sources were tested as substrates of the decolorization (Table II-2). The effects of most saccharides, glycerol and ethanol on decolorization were comparable to that of glucose. However, using them in a practical decolorization process is difficult because of the high costs. Molasses, which contains abundant monosaccharides such as sucrose and glucose, looks promising in this respect. However, molasses itself contains a considerable amount of melanoidin, and shows a dark brown color. In this experiment, addition of 10 g/l

molasses to 50% HTL raised the liquor color to a two-fold level of the initial color. The color level of the decolorized liquor was equivalent to the original 50% HTL. Therefore, it is very important to find an approach to minimize the amount of carbon giving sufficient decolorization.

II-4. Summary

Five white rot fungi that can decolorize melanoidin were examined on their ability of HTL decolorization. *C. hirsutus* showed the highest HTL-decolorizing activity of the fungi tested. This fungus removed 80% of the HTL color within eight days under appropriate conditions. Incubation of HTL major colored components (fractions I and III) with fungal pellets resulted in a considerable decrease in the color. A large part of the color reduction was due to the degradation of the colored components, while a small part was due to the adsorption onto the pellets. The efficiency of the decolorization was greatly affected by both the degree of HTL dilution and the amount of glucose added: Decreasing of the degree of HTL dilution from 2.5 to one time caused a decrease in the decolorization rate and level. Also, the efficiency decreased with a decrease in the amount of glucose from 5 to 0 g/l. Sufficient fungal decolorization was obtained, when 5 g/l glucose was added to 40% HTL (diluted 2.5 times). The requirement of a large amount of glucose for sufficient decolorization was considered to be one of the most important subjects in the practical use of the fungi.

Chapter III Specification of the enzyme system involved in melanoidin decolorization by *C. hirsutus*

III-1. Introduction

C. hirsutus required a high concentration of a carbon source such as glucose for the decolorization (Chapter II). This is considered to be a main disadvantage of the use of this fungus for HTL treatment. Also, HTL needed to be diluted appropriately to enable sufficient decolorization. In concentrated HTL, the decolorization rate was very slow. To make the HTL decolorization more efficient, an elucidation of the fungal melanoidins decolorization system is important.

Decolorization of melanoidins and/or degradation by white rot fungi is poorly understood, though some works have identified the lignin degradation-related enzymes participating in the decolorization as follows. The intracellular H_2O_2-producing enzymes L-sorbose oxidase [26] and other sugar oxidase [33] were isolated as the melanoidin-decolorizing enzymes in *Coriolus* strains: The produced H_2O_2 secondarily oxidizes melanoidin [51]. Also, *C. versicolor* appears to produce an enzyme which attacks melanoidin directly, without requiring any additions such as sugar and O_2 [33]. The group of Blondeau *et al.* reported the degradation of melanoidin by a Mn-dependent oxidase in cultures of *Trametes* (*Coriolus*) *versicolor* [32,34].

In this chapter, the enzyme system involved in the HTL decolorization of *C. hirsutus* was researched using synthetic melanoidin prepared from glucose and glycine. First, production of extracellular H_2O_2 and its participation in the decolorization were investigated. Since the activities of manganese peroxidase (MnP) and manganese-independent peroxidase (MIP) were detected in the cultures decolorizing melanoidin, participation of these peroxidases in melanoidin decolorization was also investigated.

III-2. Materials and methods

Chemicals

2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), phenol red, Azure B, horseradish peroxidase (HRP, type II) and catalase (from bovine liver, thymol-free) were purchased from Sigma Chemical. Bovine serum albumin (BSA) was purchased from Wako Pure Chemical.

Culture conditions

C. hirsutus IFO 4917 was grown at 28°C on a GPY agar plate [59] containing (per liter) 10 g glucose, 3.0 g peptone, 2.0 g yeast extract, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 16 g agar, pH 6.0. The mycerial suspension was prepared by the procedure described in section II-2. Five milliliters of suspension was transferred into 20 ml of fresh GPY in a 100-ml Erlenmeyer flask, and the fungus was cultured for three days in a reciprocal shaker (110 rpm, 28°C). The fungal mass was obtained in 3 mm-diameter-pellet form. The pellets were washed with sterile deionized water before they were used as inocula.

Decolorization of melanoidin by fungal pellets

Melanoidin was synthesized from a mixture of 1 M D-glucose, 1M glycine, and 0.2 M $NaHCO_3$ (see section II-2). This solution contained 100 mg-organic carbon (C) of melanoidin per milliliter. Medium GPYM was the same as GPY but contained 5.0 g/l glucose and 400 mg-C/l melanoidin. The medium was autoclaved at 121°C for 20 min. The fungal pellets (1.2 g as wet weight) were inoculated in 20 ml of GPYM in a 100-ml Erlenmeyer flask and cultured in the reciprocal shaker (110 rpm, 28°C). The culture supernatant fluid was obtained by centrifugation at 10,000 x *g* for 10 min.

Analytical methods

Decolorization of melanoidin was calculated from the decrease in A_{465} of the culture fluid as described in section II-2.

H_2O_2-producing activity (HPA) of the pellets was measured by HRP-mediated oxidation of phenol red [62]. The pellets (0.5 to 1.0 g as wet weight) collected from GPYM culture were washed twice with 20-ml phenol red solution (PRS) containing 20 mM Na phosphate, pH 6.0, 0.5% (w/v) glucose and 0.1 mg/ml phenol red. The

washed pellets were transferred to 20-ml PRS supplemented with 50 μg/ml HRP and then, incubated for 20 min at 28°C while shaken at 110 rpm. The total mixture was filtered, and the pellets weight was measured after drying at 110°C. A_{610} of the filtrate was measured after adding 0.3 ml of 2 M NaOH. The pellets were also incubated with PRS without HRP as the control. HPA was expressed as amount of H_2O_2 produced by the pellets during the incubation period of 20 min.

To determine the glucose oxidase activity, pellets were mixed with 100 mM Na phosphate buffer (pH 6.0) and blended with sea sand in a mortar. Sea sand and cell debris were removed by centrifugation at 10,000 x *g* for 15 min. The reaction was started by adding 30 μl of 10 mM glucose to the mixture containing 50 mM Na phosphate (pH 6.0), the cell free extract, 50 μg/ml HRP and 0.1 mg/ml phenol red in a total volume of 3.0 ml [63]. The reaction ran for 5 min at 28°C and was then stopped by adding 0.3 ml of 2M NaOH. One unit of glucose oxidase activity was defined as the enzyme amount required to produce 1 μmol of H_2O_2 per minute in the presence of glucose. Protein content of the cell free extract was determined by the method of Bradford [64].

H_2O_2 content of the supernatant fluid of culture was determined by the method of Graf and Penniston [65]. To 0.6 ml of the fluid, 1.8 ml of 80 mM HCl, 0.2 ml of 1 M KI, 0.2 ml of 1.0 mM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 0.5 M H_2SO_4 and 0.2 ml of 5% (w/v) starch were added in that order. A_{570} of the reaction mixture was measured at 20 min after addition of KI solution. Fluid in which H_2O_2 was removed by preincubating with 30 μg/ml catalase for 5 min at 28°C was used as the control.

Laccase (phenol oxidase) activity was determined by the oxidation of ABTS at 420 nm [10]. The assay mixture contained 50 mM Na acetate (pH 5.0), 0.5 mM EDTA, and 0.5 mM ABTS in a total volume of 3.0 ml. MIP activity was calculated by subtracting the amount of H_2O_2-independent ABTS oxidation (owing to laccase activity) from that of ABTS oxidation in the presence of 0.1 mM H_2O_2. One unit of laccase and MIP was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute. MnP activity was measured by the oxidation of phenol red [8] in a mixture containing 20 mM Na succinate (pH 4.5), 25 mM lactate, 1 mg/ml BSA, 0.1

mg/ml phenol red, 0.1 mM Mn(II) as $MnSO_4$ and 0.1 mM H_2O_2 in a total volume of 3.0 ml. The same mixture but with 0.5 mM EDTA instead of 0.1 mM Mn(II) was used to obtain the Mn-independent oxidation. The reaction was stopped by addition of 0.3 ml of 2M NaOH, and the activity was expressed as Mn(II)-dependent increase in A_{610} per minute. Detection of lignin peroxidase (LiP) activity was performed by two different assays using substrates, veratryl alcohol and Azure B, in 50 mM Na tartrate (pH 4.5) [66].

Preparation of crude enzyme

A crude enzyme solution which contained both MIP and MnP was prepared from the 18 to 24-hr cultures. The supernatant fluid from 28 cultures (about 500 ml) was collected and frozen at -80°C until further preparations, and after thawing the precipitate of polysaccharides in the fluid was removed by centrifugation at 10,000 x *g* for 10 min. The protein in the supernatant was salted out with ammonium sulfate (90% saturation) and collected by centrifugation. The protein precipitate was dissolved in 20 ml of 20 mM Na acetate (pH 5.0) and then, dialyzed against 4 l of 20 mM Na acetate for 12 hr. This solution was applied to a DEAE-Toyopearl 650M column (2.0 x 11 cm; Toso Co.) previously equilibrated with 20 mM Na acetate (pH 5.0). The proteins were eluted using a linear gradient of 0 to 0.3 M NaCl in 200 ml of the same buffer. The elution was monitored at 280 and 405 nm to detect protein and heme protein, respectively. Both peroxidases were eluted at a concentration of 0.13 M NaCl as shown in Fig. III-1. Therefore, the active fractions containing both enzymes were collected and used as crude enzyme solution. The residual melanoidin was mostly eluted after elution of MIP and MnP. The activities of MIP and MnP in the enzyme solution were 0.2 U/ml and 0.6 ΔA_{610}-unit/min/ml, respectively, but no laccase activity was detected.

Incubation of melanoidin with MIP and MnP

Decolorization of melanoidin by MIP and MnP was evaluated using Mn-independent reaction mixture (MIRM) made of the MIP assay mixture and using Mn-

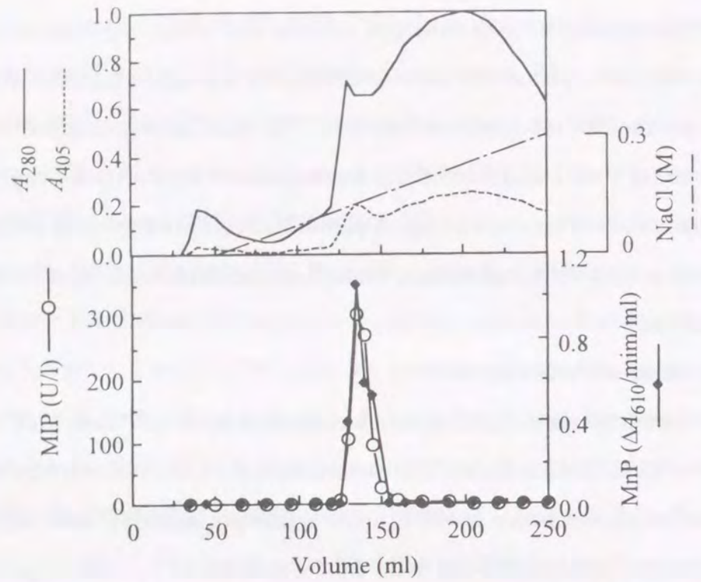

Fig. III-1. DEAE-Toyopearl column chromatography of peroxidases.

Table III-1. Compositions of MIRM and MDRM

MIRM	MDRM
Total volume: 3.0 ml	Total volume: 3.0 ml
Crude enzyme: 0.3 ml	Crude enzyme: 0.3 ml
10 mM Na acetate (pH 5.0)	50 mM Na succinate (pH 4.5)
0.5 mM EDTA	0.3 mg-C/ml Melanoidin
0.3 mg-C/ml Melanoidin	25 mM Na lactate (pH 4.5)
H_2O_2 (up to 0.1 mM)	1 mg/ml BSA
	0.1 mM Mn(II)
	0.1 mM H_2O_2

dependent reaction mixture (MDRM) made of the MnP assay mixture, respectively. Compositions of MIRM and MDRM are listed in Table III-1. The mixtures were put in a glass cell set in a spectrophotometer. Melanoidin decolorization was started by adding H_2O_2 and monitored at 465 nm.

H_2O_2 consumption by the peroxidases was also determined with MIRM and MDRM. In those cases, MIRM included 0.1 ml of the crude enzyme and up to 0.8 mg-C/ml melanoidin in a total volume of 0.6 ml. MDRM included 0.1 ml of the crude enzyme and 0 to 0.3 mg-C/ml melanoidin. Residual H_2O_2 content after 5 min of

incubation at 28°C was determined by the method of Graf and Penniston as described above. Mn-independent enzymatic consumption of H_2O_2 was calculated by subtracting the H_2O_2 consumption in MIRM without the crude enzyme from that in MIRM containing the enzyme. The Mn-dependent enzymatic consumption was calculated by subtracting the Mn-independent consumption in MDRM without 0.1 mM Mn(II) from that in MDRM with Mn(II).

III-3. Results and discussion

III-3.1. Production of H_2O_2 by the fungal pellets

Intracellular H_2O_2-producing sugar oxidases have been isolated as melanoidin-decolorizing enzymes of *Coriolus* strains [26,33]. Taking into account the requirement of a high concentration of glucose for HTL decolorization (Chapter II), it is probable that those enzymes function in the HTL and melanoidin decolorization of *C. hirsutus*. However, actual H_2O_2 secretion by the fungi and melanoidin decolorization by the secreted H_2O_2 have not been demonstrated yet.

First, the H_2O_2-producing activity (HPA) of *C. hirsutus* pellets was examined. When the pellets from 1-day culture were incubated with PRS supplemented with HRP, the increase in A_{610} was 0.78/20 min/g-wet cell weight. The increase in A_{610} in PRS without HRP was 0.09/20 min/g. The HPA was estimated by subtracting the oxidation amount obtained without HRP from that obtained with HRP. No HRP-independent phenol red oxidation was found in the pellets from 3- and 5-day cultures. The 1-day pellets had glucose oxidase activity of 0.16 U/mg-protein, while the 3- and 5-day pellets had the activity of less than 0.01 U/mg-protein. Fig. III-2 shows time courses of the fungal HPA and melanoidin decolorization. High HPA was observed during the growth phase, and it decreased rapidly after the growth ceased. Since melanoidin decolorization was also observed during the growth phase, it appeared to be related to expression of the HPA.

Recently, it has been reported that white rot fungi, including *Coriolus* strains, produce extracellular glucose oxidase [12,67]. When glucose, phenol red and HRP were added to the supernatant of 1-, 3- and 5-day cultures, no oxidation of phenol

red was observed. Therefore, in the fungal cultures, major amount of H_2O_2 is presumably produced by the oxidase localized in the intracellular and/or periplasmic space [67].

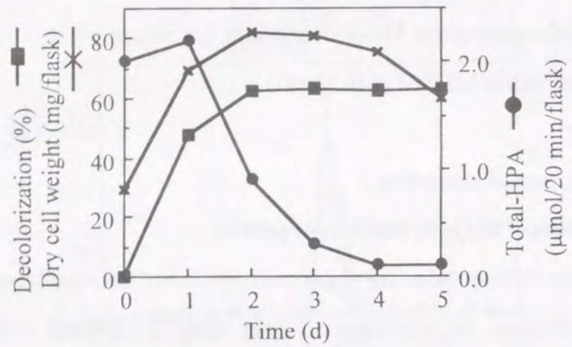

Fig. III-2. H_2O_2-producing and melanoidin decolorizing activities of the fungal pellets.

III-3.2. Participation of H_2O_2 in the fungal melanoidin decolorization

Addition of 30 and 90 μg/ml catalase to the fungal cultures resulted in a significant decrease in the level of decolorization (Table III-2). On the other hand, neither the denatured catalase nor BSA depressed decolorization. The fungal growth was nearly constant, regardless of the addition of proteins. These results show the participation of the secreted H_2O_2 in melanoidin decolorization in fungal cultures. Production of H_2O_2 in the cultures was confirmed as shown in Fig. III-3. H_2O_2 content in supernatant fluid of the culture increased up to 43 μM after start of the incubation. However, it rapidly decreased after 1 hr.

H_2O_2 (98 μM) added to 1-day culture supernatant disappeared within the incubation period of 40 min (Fig. III-4A). When H_2O_2 was added to fresh GPYM medium, the amount was immediately reduced to 85% of the initial concentration. However, the residual H_2O_2 was fairly stable in the medium for 40 min. This is consistent with the observation that the amount of H_2O_2 produced by lactobacilli strains in organic medium remains at acidic pH (< 6.0) for one month at 5°C, while it disappears completely at pH 7.0 to 8.0 within 5 days [68]. Hayase *et al.* reported

Table III-2. Effect of catalase addition on fungal melanoidin decolorization[a]

Addition	Decolorization (%)	Dry cell weight (%)
None	100[b]	100[b]
Catalase		
5 μg/ml	85.9	91.0
30 μg/ml	52.3	96.3
90 μg/ml	46.3	101
Denatured catalase[c]	108	97.1
BSA[c]	102	105

[a] Decolorization of melanoidin (decrease in A_{465} of culture fluid) and dry cell weight at 8 h of incubation after adding protein were measured in triplicate. The results are expressed as the mean percentage of the values obtained from the culture without catalase. [b] The mean values ± SDs of that culture for the decrease in A_{465} and cell weight (mg/flask) were 0.149 ± 0.026 and 37.6 ± 4.5, respectively. [c] Catalase heated at 95°C for 5 min or BSA instead of native catalase was added to a concentration of 90 μg/ml.

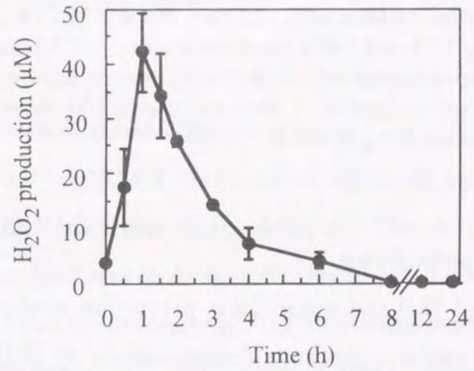

Fig. III-3. Production of extracellular H_2O_2 in the fungal culture. The results are expressed as mean values and SDs (error bars) of the experiments in duplicate.

that the rate of melanoidin decolorization by H_2O_2 at pH 3 to 7 is much slower than at more alkaline pH [51]. In this study, the pH value of the fungal cultures after 24 hr was about 5. Therefore, it is considered that the reacting potential of H_2O_2 *per se* for melanoidin is relatively low in *C. hirsutus* culture. Dilution of the culture fluid with buffer lowered the rate of the H_2O_2 decomposition (Fig. III-4B). Therefore, the rapid decrease in the H_2O_2 as shown in Figs III-3 and -4 was strongly dependent on the

presence of the fungal culture component. Furthermore, addition of melanoidin to the fluid stimulated the decomposition of H_2O_2 (Fig. III-4C).

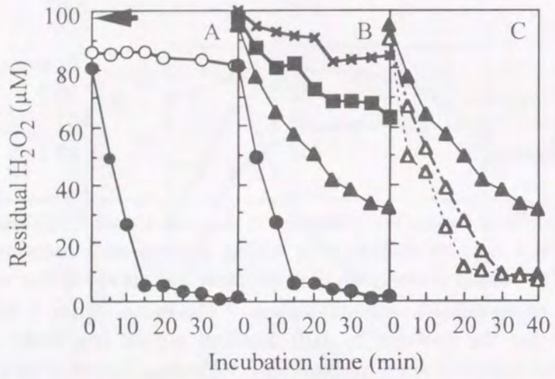

Fig. III-4. Decomposition of H_2O_2 added to supernatant fluid from the 24-hr fungal culture. (A) Addition to fresh GPYM medium (○) and culture fluid (●). (B) Addition to the diluted culture fluid: the contents were 12.5 (×), 25 (■), 50% (▲) in 5 mM Na acetate, pH 5.5, and 100% (no dilution) (●). (C) Addition to the 50% fluid supplemented with 0 (▲) (in the same as the line in B), 0.05 (–Δ–), and 0.1 μg-C/ml (---Δ---) melanoidin. The arrow shows the concentration of H_2O_2 added.

III-3.3. Production of peroxidases

It was postulated that the extracellular peroxidase catalyzes the melanoidin decolorization in the cultures, because decomposition of H_2O_2 occurred in the presence of the culture fluid, and it was stimulated by addition of melanoidin. Activities of MIP and MnP were detected in the cultures exhibiting melanoidin decolorization (Fig. III-5). During the incubation for 72 hr, the culture fluid did not show oxidizing activities of veratryl alcohol and Azure B in the presence of H_2O_2. Therefore, production of LiP was improbable in this experiment. Laccase activity was detected at a low level, less than 10% of that of MIP throughout the period of incubation.

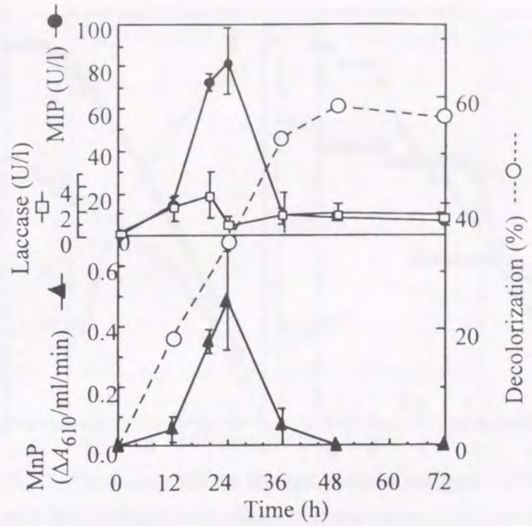

Fig. III-5. Production of extracellular peroxidases and melanoidin decolorization in the fungal cultures. The results are expressed as mean values and SDs of the experiments in triplicate.

III-3.4. Decolorization of melanoidin by peroxidases

Separation of MIP and MnP could not be achieved by the anion exchange chromatography (see materials and methods). Therefore, decolorization of melanoidin by MIP or MnP was investigated using the crude extract containing both enzymes. MIRMs showed a decrease in A_{465}, indicating melanoidin decolorization (Fig. III-6A). Decolorization was not observed when either H_2O_2 or the crude enzyme was omitted from MIRM. This strongly suggests that MIP has melanoidin-decolorizing activity. Also, H_2O_2 and the enzyme were essential for the decolorization in MDRM (Fig. III-6B). The mixture without Mn(II) showed an initial decolorization rate that was equivalent to that of MIRM, and complete MDRM showed a higher decolorization rate than MDRM without Mn(II). MDRM is the solution in which both MIP and MnP can act. The difference in rates between the two mixtures must be due to the decolorizing activity of MnP.

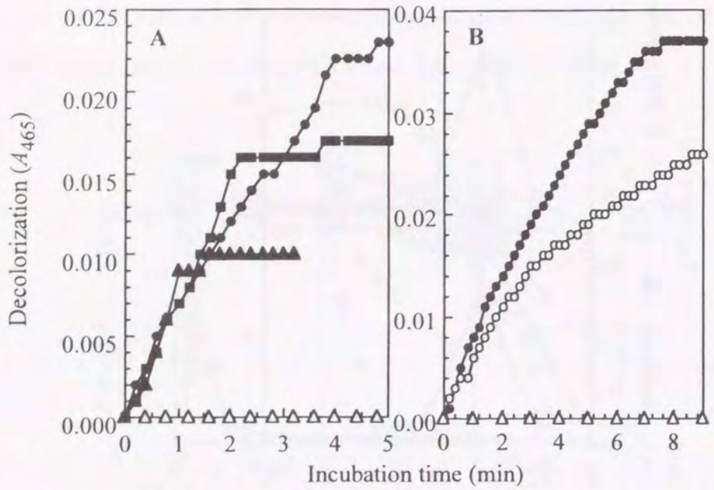

Fig. III-6. Decolorization of melanoidin by crude enzyme in MIRM (A) and MDRM (B). (A): MIRMs containing 50 (●), 33 (■), and 17 μM (▲) H_2O_2 and MIRM without H_2O_2 (△); (B): complete MDRM (●), MDRM without 0.1 mM Mn(II) (○), and MDRM without 0.1 mM H_2O_2 (△).

To determine if the decolorization in the reaction mixtures was catalyzed by peroxidases, the enzymatic H_2O_2 consumptions in the presence of melanoidin were investigated. Fig. III-7A shows the Mn-independent enzymatic H_2O_2 consumption in MIRM. An apparent increase in the rate of the consumption owing to the melanoidin concentration was found. The Mn-dependent enzymatic consumption of H_2O_2 was also calculated by subtracting Mn-independent consumption in MDRM without Mn(II) from that in complete MDRM (Fig. III-7B). It reached its maximum rate at a low content of melanoidin (0.15 mg-C/ml). Consequently, it was concluded that MIP and MnP function in the melanoidin decolorization of the fungal cultures.

Besides the sugar oxidases, a few melanoidin-decolorizing enzymes have been identified in the genus of *Coriolus*. Ohmomo *et al.* reported a *C. versicolor* enzyme which attacks melanoidin directly without any additions such as sugar and O_2 [33]. Dehorter and Blondeau isolated a MnP-like enzyme from *C. versicolor* [32,34].

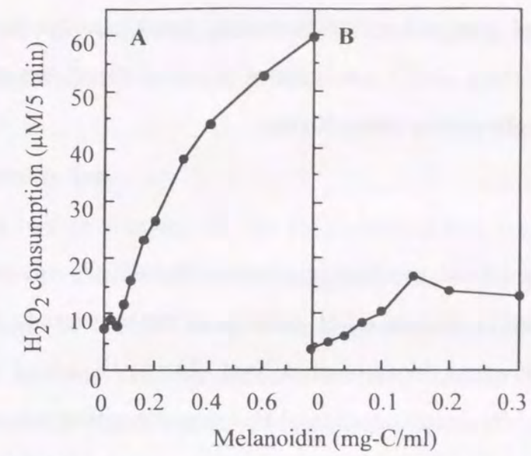

Fig. III-7. Consumption of H_2O_2 by crude enzyme in MIRM (A) and MDRM (B) including various concentrations of melanoidin added. The results show the corrected Mn-independent (A) and dependent (B) enzymatic consumptions (see materials and methods).

That was a hem protein, and it mineralized melanoidin in the presence of Mn(II). Expression of its activity required O_2 rather than H_2O_2. It is still unclear whether the MnP dealt with in this study is a different protein from the Mn-dependent enzyme. Several white rot fungi produce MIP along with the ligninolytic enzymes LiP, MnP, and laccase [69-73]. The ligninolytic activity of MIP was reported using a phenolic lignin model compound as the substrate [74]. Regarding the melanoidin-decolorizing activity of MIP, it has been suggested only for bacterial strains [75,76]. The fungus also produced laccase during melanoidin decolorization (Fig. III-5). Laccase catalyzes one-electron oxidation of phenolic and nonphenolic compounds [10,11] with O_2, and the function is similar to that of the Mn-mediated MnP reaction [8,9]. Although the level of laccase activity in the *C. hirsutus* cultures was very low, it appears rational that the laccase partially participates in the melanoidin decolorization.

In conclusion, it was elucidated that extracellular H_2O_2 and the peroxidases produced participate in the melanoidin decolorization by *C. hirsutus*. Both MIP and MnP are considered to be key enzymes in the decolorization. The use of the fungus

for microbial color removal from melanoidin-containing wastewaters is an attractive subject. Enhancement of H_2O_2 and/or peroxidases production appears to be very important to make the decolorization more efficient.

III-4. Summary

In *Coriolus hirsutus* culture, production of extracellular H_2O_2 was involved in the melanoidin decolorization system, since addition of an appropriate amount of catalase to the culture lowered decolorization level. The H_2O_2 added to culture supernatant fluid was rapidly decomposed, and that decomposition was stimulated by adding melanoidin to the fluid. However, nonenzymatic decolorization of melanoidin by H_2O_2 as previously reported seemed at a low level in the culture. The culture fluid contained two extracellular peroxidases, MIP and MnP. The participation of MIP and MnP in melanoidin decolorization was evaluated using the crude enzyme extract. The reaction mixtures including the enzymes, H_2O_2 and melanoidin showed Mn-independent and dependent decreases in the absorbance at 465 nm, indicating melanoidin-decolorizing activities. The mixtures also showed Mn-independent and dependent H_2O_2-consuming activities. Therefore, it was concluded that production of extracellular H_2O_2 and the activity of peroxidases are the principal systems involved in the melanoidin decolorization by *C. hirsutus*.

Chapter IV Relationship between HTL organic component and HTL-decolorizing activity of *C. hirsutus*.

IV-1. Introduction

As shown in Chapter II, the HTL-decolorizing activity of *C. hirsutus* is depressed in concentrated HTL. This suggests that the fungal HTL decolorization is greatly affected by the HTL component. If the component that shows the inhibitory effect can be removed, the decolorization efficiency may be improved for development of HTL decolorization processes.

In *P. chrysosporium* cultures, production of ligninolytic peroxidases, LiP and MnP, has been found to be triggered by limitation of nutrients such as nitrogen [77-79]. High concentrations of N suppressed ligninolytic activity and production of ligninolytic peroxidases [77, 80-82]. This knowledge has been extensively reported for cultures of other fungi [83-85]. Therefore, it is postulated that melanoidin decolorization by the fungi is greatly affected by the nutrient status. In *P. chrysosporium* cultures, melanoidin decolorization occurred under N-limited conditions [29,31]. Nevertheless, the melanoidin decolorization by *Coriolus* strains including *C. hirsutus* has been investigated using nutrient rich media with high concentrations of C and N [27,33]. Nutrient conditions appear to be not so important for their abilities for melanoidin decolorization, since they can express decolorizing activity in those media. In those reports, however, little attention was given to the clarification of the relationship between melanoidin decolorization and nutrient conditions.

This chapter describes the influences of the HTL component on HTL decolorization by *C. hirsutus*. Artificial HTL (AHTL) with a similar composition to that of HTL was prepared and used in the tests of the fungal decolorization. The AHTL decolorization greatly varied with the content of organic components in the liquor. Furthermore, effects of the components on MIP and MnP production in the fungal cultures were investigated.

IV-2. Materials and methods

Preparation of artificial HTL (AHTL)

AHTL was prepared by mixing a matrix component solution (MCS) and a colored component, melanoidin, solution (CCS). MCS contained (per liter) 180 g peptone, 120 g meat extract and 51.2 g ammonium acetate, pH 5.5, and it was autoclaved for 20 min at 120°C. CCS was prepared by autoclaving a mixture which consists of 75 g glycine, 180 g glucose and 16.8 g $NaHCO_3$ (per liter) for 3 hr at 120°C. These solutions were stored at 4°C. AHTL contained, per liter, the following: 28 ml MCS, 4.6 ml CCS, 25 mg $FeSO_4 \cdot 7H_2O$ [5 mg Fe(II)], 4.4 mg $MnSO_4 \cdot 5H_2O$ [1.0 mg Mn(II)] and 4.4 mg $ZnSO_4 \cdot 7H_2O$ [1.0 mg Zn(II)]. General characteristics of AHTL are listed with those of actual HTLs in Table IV-1.

Table IV-1. General characteristics of actual HTLs and AHTL

	HTL[a]		AHTL
	(min. - max.)	(mean)	
pH	5.1 - 5.7	5.3	5.5
Color (CU)	1,700 - 7,300	4,200	6,400
TOC (mg/l)	1,800 - 7,800	4,500	4,000
BOD (mg/l)	2,400 - 7,400	4,000	5,400
Organic-N (mg/l)	220 - 530	380	1,000
NH_4^+-N (mg/l)	150 - 840	430	290
NO_3^--N (mg/l)	0.0 - 2.5	< 2.5	1.1
Total-P (mg/l)	48 - 170	98	69

[a], the compositions were obtained from seven HTL samples including samples A, B, C, E and F (see Chapters I and V).

To investigate the effects of the matrix organic components on the fungal AHTL decolorization, the initial MCS content of 28 ml/l was varied in a range of 0.35 to 44 ml/l. The total organic carbon (TOC) contents of these liquors ranged from 500 to 6,000 mg/l because of the variation in the MCS contents. Furthermore, organic and inorganic Ns were added to the dilute liquor, containing 0.35 ml/l MCS and 4.6 ml/l CCS (500 mg/l TOC). The N sources, including peptone, meat extract, ammonium tartrate and $NaNO_3$, were sterilized with a membrane filter (pore size of 0.22 μm).

Culture conditions

C. hirsutus pellets with a diameter of 3 mm were cultured in 20 ml GPY medium [12] as described in section III-2. The pellets were washed with sterile deionized water before they were used as inocula. Forty milliliters of AHTL was autoclaved at 121°C for 20 min in a 100-ml Erlenmeyer flask and then, supplemented with 1.0 or 5.0 g/l glucose which was previously sterilized by autoclaving at 121°C for 20 min. Fungal pellets (wet weight 1.8 g) were inoculated in the liquors. Incubation was performed at 28°C in a reciprocal shaker at 120 rpm.

Analytical methods

Supernatant fluid of the incubated liquor was obtained by centrifugation at 10,000 x *g* for 10 min. Color of the fluids was determined by a combination of the Pt-Co method and the A_{465} measurement (see section I-2). Liquor decolorization was expressed as the amount of the decrease in color (CU).

Laccase and MIP activities were determined by the oxidation of ABTS as described in section III-2. MnP activity was determined by the oxidation of 2,6-dimethoxyphenol (2,6-DMP, purchased from Tokyo Kasei Industries) [9]. The reaction mixture contained 50 mM Na malonate (pH 5.0), 1.0 mM 2,6-DMP, 1.0 mM $MnSO_4$ and up to 500 μl of the fluid in a total volume of 3.0 ml. The reaction was initiated by addition of 0.1 mM H_2O_2, and peroxidase activity was corrected for laccase activity present. In the mixture supplemented with 5 mM EDTA instead of Mn(II), oxidation of 2,6-DMP by MIP was observed. Therefore, MnP activity was corrected by subtracting the MIP activity from the peroxidase activity in the mixture with Mn(II) [86]. One unit of MnP was defined as the amount of enzyme required to oxidize 1 μmol 2,6-DMP per minute.

IV-3. Results and discussion

IV-3.1. Decolorization of artificial HTL (AHTL) by *C. hirsutus*

AHTL (4,000 mg/l TOC) including 28 ml/l MCS and the dilute liquors with various concentrations of MCS were supplemented with 5.0 g/l glucose and then, incubated with *C. hirsutus* pellets. Fig. IV-1 shows the decrease in their color during the incubation. Rapid decolorization of AHTL was obtained after 24 hr, and 51% of

the initial color was lost after 72 hr. In the case of the liquor (6,000 mg/l TOC) containing 44 ml/l MCS, both rate and level of decolorization were strongly depressed. On the other hand, decreasing the MCS content caused a gradual increase in decolorization rate and level. Incubation of the liquor (500 mg/l TOC) containing 0.35 ml/l MCS removed 71% of the initial color without a lag time for decolorization, which was observed in more concentrated liquors. These results indicate that the behavior of the fungal AHTL-decolorizing activity is similar to that of the actual HTL-decolorizing activity as shown in Chapter II: Increasing the degree of HTL dilution increases the HTL decolorization efficiency.

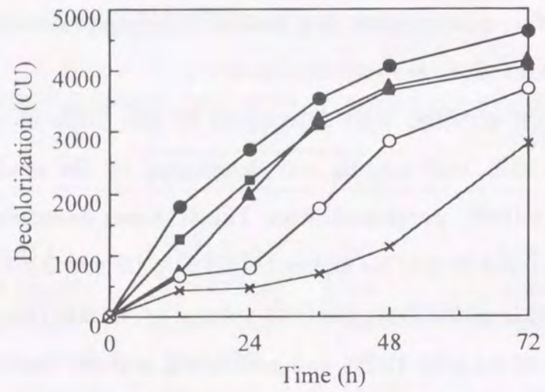

Fig. IV-1. Decolorization of liquors with various concentrations of TOC by *C. hirsutus*.
Glucose (5.0 g/l) was added to the liquors with following TOC concentrations: ● , 500 mg/l; ■ , 1,000 mg/l; ▲ , 2,000 mg/l; ○ , 4,000 mg/l (original AHTL); × , 6,000 mg/l.

Decolorization levels after 48 hr of incubation as shown in Fig. IV-1 are represented in Fig. IV-2 with the levels for the liquors supplemented with 1.0 g/l glucose. In the figure, the data on the decrease in color are plotted against the TOC contents of the liquors. The carbon added as glucose added is not included in the TOC contents. Only a slight color removal (ca. 14% of the initial color) was obtained when 5.0 and 1.0 g/l glucose were added to the 6,000 mg/l-TOC liquor. In the range of 1,000 to 4,000 mg/l TOC, decreasing the amount of glucose added from 5.0 to 1.0

g/l caused a decrease in the amount of color removal. However, addition of 1.0 g/l glucose to 500 mg/l-TOC liquor resulted in a considerably high decolorization level which was nearly equivalent to that of the liquor supplemented with 5.0 g/l glucose. The decolorization profile up to 48 hr of the 500 mg/l-TOC liquor supplemented with 1.0 g/l glucose was also equivalent to that of the liquor supplemented with 5.0 g/l glucose (Fig. IV-1). Therefore, it was suggested that a decreasing TOC content, *i. e.* MCS content, of AHTL caused a decrease in the amount of glucose required for effective decolorization.

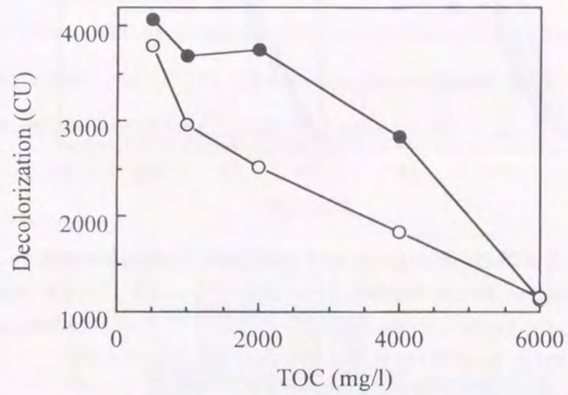

Fig. IV-2. Effects of TOC concentration on glucose amount required for the fungal liquor decolorization.
Glucose was added to a concentration of 1.0 (○) or 5.0 g/l (●).
Decolorization of liquors was determined after 48 hr of incubation.

IV-3.2. Effects of organic components on the fungal AHTL decolorization

MCS contains organic and inorganic nitrogen (N) components, peptone, meat extract and NH_4^+. To specify the component that depresses the AHTL decolorization, one of these N sources was added to dilute AHTL containing 0.35 ml/l MCS and 4.6 ml/l CCS (TOC, 500 mg/l; organic N, 77 mg/l; inorganic N, 4.1 mg/l). Addition of peptone in concentrations from 250 to 1,000 mg/l N increasingly depressed the fungal liquor-decolorizing activity (Fig. IV-3A). In contrast, NH_4^+-N showed only a slight inhibitory effect on the decolorization even if the concentration increased up to 1,000 mg/l (Fig. IV-3B). The inhibitory effect of 500

mg/l-N meat extract was similar to that of 500 mg/l-N peptone. These results demonstrate that the organic N components tested contribute to the depression of the AHTL decolorization by an increase in the MCS content.

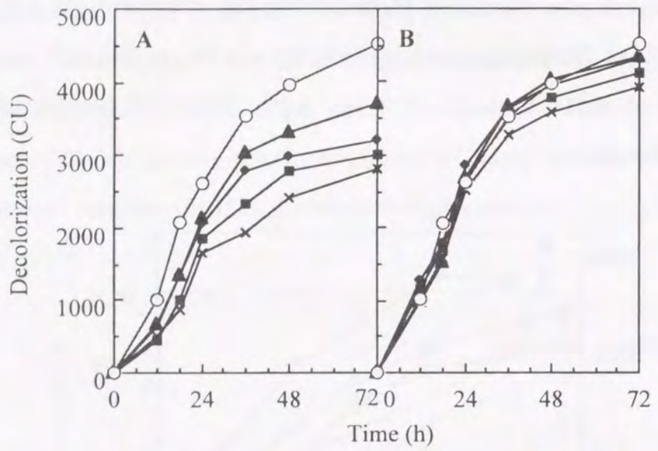

Fig. IV-3. Effects of organic and inorganic N supplements on fungal liquor decolorization. Peptone-N (A) or NH_4^+-N (B) was added to the liquor containing 500 mg/l-TOC: ○ , no supplement; ▲ , 250 mg/l; ◆ , 500 mg/l; ■ , 750 mg/l; × , 1,000 mg/l. Glucose was added to a concentration of 5.0 g/l.

Aoshima *et al.* reported that in *C. versicolor* cultures, addition of 5 g/l organic N source such as casate or peptone is effective for melanoidin decolorization [27]. The N concentration is estimated to have been 500 to 700 mg/l by assuming that their N contents are 10 to 14%. However, in their experiments glucose was added to much higher concentration (30 g/l). As shown in Fig. IV-2, the depression of decolorization by MCS organic components depends on the glucose concentration: Influence of the components on decolorization was more marked in the liquors with 1.0 g/l glucose than in those with 5.0 g/l glucose. Addition of glucose to a high concentration such as 30 g/l may counteract the depression by organic components.

IV-3.3. Effects of addition of different nitrogen sources on the AHTL-decolorizing activity

The extracellular peroxidases activity in the cultures with various concentrations of N sources was measured after 12, 18 and 24 hr of incubation (Fig. IV-4). The level of MnP activity was greatly reduced by additions from 250 to 1,000 mg/l-N peptone. Increasing the NH_4^+ concentration also resulted in a reduction of the MnP level. However, the influence of NH_4^+ addition on the enzyme level was less than that of peptone addition. The cultures supplemented with 250 and 500 mg/l-N NH_4^+ had MnP activities at levels of more than 50% of the control after 18 hr of incubation. The level of MIP activity slightly increased by addition of peptone. In contrast, it decreased to some extent by addition of NH_4^+. Therefore, the extensive decrease in MnP level by peptone addition presumably caused the depression of the decolorization (Fig. IV-3). These results suggest that in the cultures, the contribution of MnP to the liquor decolorization is more important than that of MIP.

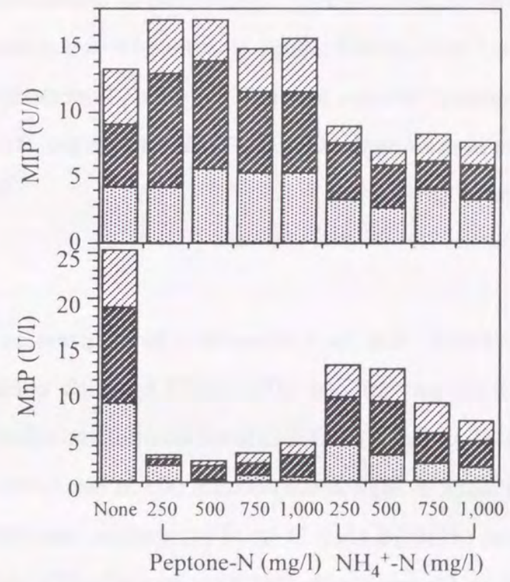

Fig. IV-4. Effects of organic and inorganic N supplements on peroxidase production of *C. hirsutus*. The supplement conditions are described in the legend of Fig. IV-3.The peroxidases activities in the 12- (▨), 18- (▨) and 24-hr (▨) cultures were measured.

In *P. chrysosporium* [87] and an unidentified ligninolytic fungus IZU-154 [88] cultures, limitation of nutrient-N causes MnP production. On the other hand, *Bjerkandera* sp. BOS55 has been found to produce ligninolytic enzymes, including MnP and MIP, under N-rich conditions [86]. In the present study, production of MnP by *C. hirsutus* seemed to be regulated by nutrient N (Fig. IV-4). However, the depression of MnP activity was not so strong when NH_4^+ was added to a concentration of 500 mg/l-N [88]. Although the depression by the organic N sources was stronger, some MnP activity could be detected. At present it is unclear what kind of mechanism controls constituent changes in the enzymes by addition of N sources.

In conclusion, a high concentration of AHTL organic N nutrient depressed the liquor-decolorizing activity of *C. hirsutus*. This implies that the low efficiency in the fungal HTL decolorization is related to the HTL organic N component. Therefore, reduction of the component concentration should lead to enhancement of the HTL decolorization efficiency. In activated sludge treatment of wastewater, organic N is rapidly degraded to inorganic N forms as NH_4^+ and NO_3^-. Pretreatment of HTL with sludge may be effective in proving the fungal HTL decolorization. Its usefulness will be discussed in the next chapter.

IV-4. Summary

To specify the factor that is responsible for depression of the HTL decolorization of *C. hirsutus*, artificial HTL (AHTL), which contained a matrix organic component solution (MCS) and a colored component solution (CCS), was used in decolorization tests. A high decolorization rate in the AHTL supplemented with 5.0 g/l glucose was obtained after 24 hr of incubation, and 51% of the initial color was lost after 72 hr. Decreasing the MCS content of AHTL caused a gradual increase in the decolorization rate and level. In the liquor with a low content of MCS, lowering of the decolorization efficiency did not occur even though the final concentration of glucose was reduced from 5.0 g/l to 1.0 g/l. Since MCS contained organic N components, peptone and meat extract, and an inorganic N component, NH_4^+, effects of these components on the liquor decolorization were investigated. Addition of 500 mg/l-N peptone or meat extract to the liquor with a low content of MCS strongly depressed the decolorization rate and level. On the other hand, addition of the same amount of NH_4^+ did not affect the decolorization. These results indicate that the organic N components of AHTL are responsible for the depression of the fungal activity. Addition of the organic source peptone caused great decrease in the level of MnP activity in the cultures. Therefore, it was suggested that the depression of decolorization by organic N components results from the decrease in MnP activity.

Chapter V Enhancement of HTL decolorization efficiency of *C. hirsutus*

V-1. Introduction

In recent years, much attention has been given to the use of white rot fungi for treatment of the industrial wastewaters containing recalcitrant pollutants. Many researchers have suggested the possibilities of fungal bioprocesses in the treatment of the wastewaters, including melanoidin-containing ones, as described in section II-1. At present, development of practical processes is nevertheless limited to the treatment of a few wastewaters such as pulp bleachery effluents [13] and phenolic compounds-containing effluents [1] by the fungus *P. chrysosporium*.

Maintenance of the fungal ligninolytic activity at a high and constant level is required to effectively conduct fungal treatment of the wastewaters. As described in section IV-1, expression of the activity takes place under special nutrient conditions, and modes of expression are different in different fungi. Therefore, in certain cases adjustment of the wastewater composition may be needed for expression of ligninolytic activity by a fungus.

In Chapters II and III, the ability of *C. hirsutus* to decolorize HTL and the possible melanoidins-decolorizing system involved in the HTL decolorization were demonstrated. The extracellular peroxidases MIP and MnP were considered to play a role in the HTL decolorization. Chapter II also revealed that the fungus has some property that is disadvantageous to its use for microbial processes for HTL decolorization. The fungus showed a high HTL-decolorizing activity only when a high concentration (5 to 20 g/l) of carbon source such as glucose was added to appropriately diluted HTL. In concentrated HTL, HTL decolorization was at a low level even though 20 g/l glucose was added. This depression appeared to be dependent on the HTL organic nitrogen component, since addition of N nutrient such as peptone or meat extract to artificial HTL caused strong depression of the fungal decolorizing activity (Chapter IV). Therefore, removal of the organic N component is considered to be an important approach to obtain high levels of the HTL decolorization by *C. hirsutus*.

This chapter describes enhancement of the HTL decolorization by *C. hirsutus* by decreasing the HTL organic N concentration. For the removal of the organic N component, activated sludge treatment of HTL was introduced prior to the fungal decolorization treatment. Besides the sludge treatment, effects of Mn(II) and H_2O_2 supplements on the HTL decolorization were investigated to further enhance the decolorization.

V-2. Materials and methods

HTL samples

The HTL samples E and F used in this chapter were collected at the plants where the samples C and A described in section I-2 were collected, respectively, but at a different date. HTL samples were filtered and stored at 4°C. They were centrifuged at 1,800 x *g* for 15 min to remove suspended solid before use.

Activated sludge treatment of HTL

The activated sludge, acclimated with artificial sewage wastewater by the fill and draw cultivation method (see section I-2), was used for pretreatment of HTL. The sludge was collected by centrifuging at 800 x *g* for 5 min and then, added to 100 ml of sample E in a 300-ml Erlenmeyer flask to a concentration of 2,000 mg (dry weight)/l. Also, it was added to 100 ml of the sample F diluted with deionized water to 40% (v/v) content to a concentration of 3,000 mg/l. The sludge treatment was conducted at 28°C for one to four days in a rotary shaker at 120 rpm. The sludge in the liquor was removed with filter paper (No. 1, ADVANTEC Toyo). The filtrate was adjusted to pH 4.5 with a small amount of 5 M HCl and used as pretreated HTL for decolorization tests with *C. hirsutus*. Non-treated samples of E and F (40% content) were also adjusted to pH 4.5 to use them as non-treated HTLs. One hundred milliliters of the HTL was transferred into a 300-ml Erlenmeyer flask and autoclaved at 120°C for 20 min.

Decolorization of HTL by *C. hirsutus*

C. hirsutus pellets were prepared by the method described in section II-2. The pellets (total dry weight of 200 mg) were transferred into 100 ml of the HTL that was

previously autoclaved and supplemented with 1.0 or 5.0 g/l sterile glucose. Incubation of the HTL with the pellets was conducted at 28°C in a rotary shaker (120 rpm). To investigate effects of Mn(II) on the fungal HTL decolorization, up to 40 mg/l $MnSO_4$ was added to the HTLs. The effect of supplementation of H_2O_2 on the HTL decolorization was investigated by addition of 80 U/l glucose oxidase (from *Aspergillus niger*, Wako Pure Chemical Industries) and 1.0 g/l glucose to 24-hr fungal cultures (including the fungal pellets).

Analytical methods

Supernatant fluid of the cultures was obtained by centrifugation at 10,000 x *g* for 10 min. Color was determined by a combination method of the Pt-Co method and the A_{465} measurement (see section I-2). Decolorization of liquors was expressed as amount (CU) of the decrease in color.

Activities of laccase, MIP and MnP in the supernatant fluid were determined by the methods described in section IV-2 [9,10,86].

V-3. Results and discussion

V-3.1. Activated sludge treatment of HTL

Activated sludge treatment reduced the concentration of the organic component in HTL samples, as the decreases in contents of TOC, BOD and organic N indicate (Table V-1). The organic N content of sample E decreased from 282 to 33 mg/l by treatment for four days. Increase in the NH_4^+-N content during treatment shows the progress of mineralization of the organic N component. Although sample F showed a N composition which was greatly different from that of sample E, mineralization of its organic N component likewise progressed during two days of treatment. The sludge treatment was ineffective for decolorization of HTLs, as was already shown in Chapter I.

V-3.2. Decolorization of HTL, pretreated with activated sludge, by *C. hirsutus*

Fig. V-1A shows the fungal decolorization of the HTL sample E that was treated with activated sludge for the indicated periods. In the non-treated HTL, only a low rate of decolorization was obtained. Pretreatment for one and two days caused

Table V-1. Activated sludge treatment of HTL samples

Sample	E				F[a]	
Treatment (d)	None	1	2	4	None	2
pH	5.2	8.0	8.2	8.3	5.4	8.1
Color (CU)	1,710	1,680	1,740	1,700	3,050	2,940
TOC (mg/l)	1,790	650	400	280	2,320	640
BOD (mg/l)	2,500	270	23	29	3,100	-
Organic-N (mg/l)	282	94	91	33	166	93
NH_4^+-N (mg/l)	140	196	224	232	334	366
NO_3^--N (mg/l)	< 0.5	< 0.5	< 0.5	1.1	0.8	< 0.5
T-P (mg/l)	65	51	41	46	67	41

[a], the liquor diluted to 40% content with deionized water. Composition was determined after dilution.

a marked increase in the initial rate of decolorization. A lag time for decolorization was no longer observed in these liquors. Even though the final glucose concentration was reduced from 5.0 to 1.0 g/l, decolorization of the pretreated HTL was not much influenced (Fig. V-1B). These results are consistent with results for fungal decolorization of artificial HTL as shown in Chapter IV: decreasing the content of AHTL organic N component caused enhancement of the decolorization efficiency.

On the other hand, the high rate and level of decolorization were depressed when the period of pretreatment was extended from two to four days. The organic component and/or other components that are required for expression of the fungal ability may be absent in the liquor treated for four days. Therefore, to achieve effective HTL decolorization by *C. hirsutus*, the duration of activated sludge treatment should be noted.

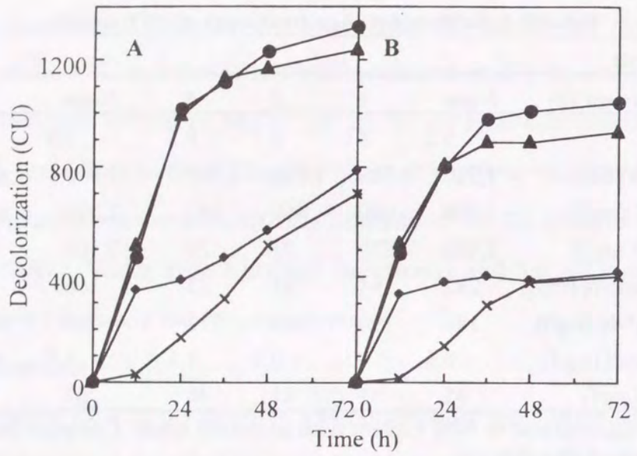

Fig. V-1. Decolorization of pretreated HTL (E) by *C. hirsutus*. Pretreatment period: × , none; ▲ , 1 d; ● , 2 d; ◆ , 4 d. The pretreated liquors were supplemented with glucose to a final concentration of 1.0 (A) or 5.0 g/l (B).

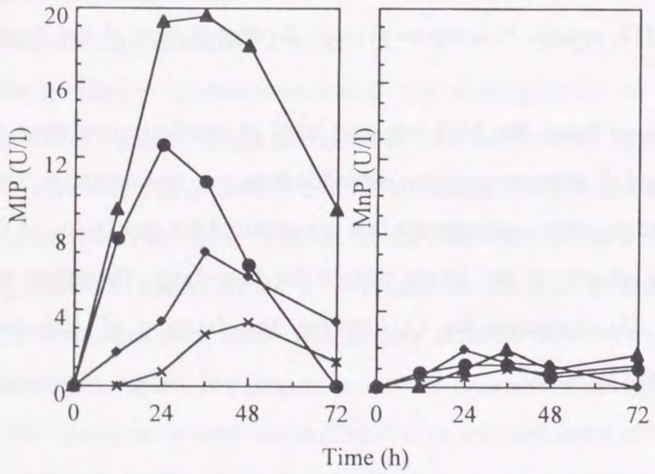

Fig. V-2. Production of peroxidases by *C. hirsutus* in pretreated HTL (E). Symbols are the same as those in Fig. V-1. The liquors were supplemented with glucose to a final concentration of 1.0 g/l.

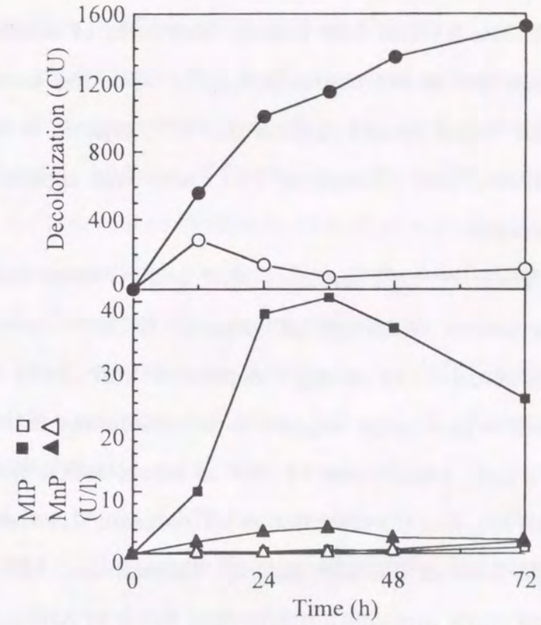

Fig. V-3. Decolorization of pretreated HTL (F) and peroxidases production by *C. hirsutus*. Opened symbols: no pretreatment; closed symbols: pretreatment for 2 days. The liquors were supplemented with glucose to a final concentration of 1.0 g/l.

The stimulatory effect of the pretreatment on the fungal HTL decolorization was analyzed from the viewpoint of the HTL-decolorizing enzymes activity. In the non-treated and treated liquors (sample E) with 1.0 g/l glucose, peaks of MIP and MnP activity were observed at 24 to 48 hr of incubation. No laccase activity was detected in the liquors throughout incubation. In the incubation for 72 hr, the highest activity of MIP was observed in the liquor pretreated for a day (Fig. V-2). Prolonging the treatment caused a significant decrease in MIP activity. In the non-treated liquor, only low activity of MIP was detected. Although the levels of MnP activity increased slightly with increasing treatment times, they were considerably lower than those of MIP activity (Fig. V-2). Therefore, the stimulation of the fungal MIP production by the pretreatment appeared to contribute to its stimulatory effect on the decolorization. Decolorization of the HTL sample F was also stimulated by the pretreatment (Fig. V-3). An marked increase in the level of MIP activity was

served in pretreated HTL. The level of MIP activity after 24 hr of incubation was about 127-fold higher than that of the non-treated HTL, while the level of MnP activity was 18-fold higher. These results suggest that HTL organic N component depresses the fungal MIP and MnP production. The component appears to affect the MIP production, especially.

As described in Chapter IV, high levels of MnP activity were found under organic N-deficient conditions. Addition of organic N to a concentration corresponding to that of actual HTLs strongly depressed MnP levels. However, levels of MIP activity were higher under organic N-rich conditions than under N-deficient ones. In this regard, expressions of MIP in actual HTLs were greatly different from those in AHTLs, *i. e.*, the reduction in HTL organic N content brought about the extremely high level of the MIP activity. Furthermore, MIP activities detected in the pretreated HTLs were much higher than those in AHTLs. It is not clear whether the difference in MIP activity levels in those liquors is dependent on the difference in compositions of HTL and AHTL.

V-3.3. Effects of Mn(II) supplement on the HTL decolorization

In cultures of *P. chrysosporium*, limitation of N nutrient triggers expression of MnP [87,88]. Also, MnP production of white rot fungi is well known to occur in the presence of Mn(II) [89-92]. Frederick *et al.* reported that addition of 12 or 100 mg/l Mn(II) to *P. chrysosporium* cultures containing pulp bleachery effluent results in both high production of MnP and a high rate of effluent decolorization [93]. The increase in the level of MnP activity by the HTL pretreatment was not so great compared with that of MIP activity. Therefore, an acceleration of MnP-mediated HTL decolorization by addition of Mn(II) is expected.

Addition of 40 mg/l Mn(II) to the sample E caused increases in the rate and level of fungal decolorization, though addition of 5 or 20 mg/l Mn(II) did not affect the decolorization (Fig. V-4A). Increasing the Mn(II) concentration caused a gradual increase in the level of MnP activity. The MnP activity detected at 24 hr in the liquor supplemented with 40 mg/l Mn(II) was 48-fold higher than that in the liquor without Mn addition. On the other hand, the level of MIP activity decreased to less than half

by Mn(II) addition. Addition of 20 or 40 mg/l Mn(II) to the sample F also accelerated fungal liquor decolorization (Fig. V-4B). However, addition of 5 mg/l Mn(II) caused a slight depression of liquor decolorization. The MnP production in the liquor F was also stimulated by addition of Mn(II). The effect of 20 mg/l Mn(II) addition on the enzyme production was equivalent to that of 40 mg/l Mn(II) addition. When Mn(II) was added to concentrations of 5 to 20 mg/l, the level of MIP activity decreased to less than 16% of the level in the liquor without Mn.

When Mn(II) was not added to the treated HTLs, high levels of MIP but low levels of MnP were produced (Figs. V-2 and -3). Therefore, in those cultures, therefore, MIP is considered to play a major role in HTL decolorization. High levels of MnP but low levels of MIP were produced in the cultures supplemented with Mn(II) (40 mg/l for the sample E; 20 or 40 mg/l for the sample F), suggesting that MnP plays a important role in the decolorization of those cultures (Fig. V-4). Since addition of Mn(II) to those concentrations caused an acceleration of the decolorization, the production of MnP appears to be sufficient for efficient decolorization. On the other hand, addition of 5 or 20 mg/l and 5 mg/l Mn(II) to the liquors E and F, respectively, did not increase the rates and levels of decolorization. Although the Mn(II) addition results in production of some amount of MnP, simultaneous decrease in the MIP level may counteract the effect derived from the insufficient MnP production.

V-3.4. Effects of H_2O_2 supplement on the HTL decolorization

As shown in Chapter III, the fungal H_2O_2 production appears to be closely related to glucose addition to the culture. Therefore, depression of the fungal HTL decolorization by reduction of glucose amount (Chapter II) may be dependent on depression of the H_2O_2 production. In *Bjerkandera* sp. cultures overproducing ligninolytic peroxidases, the endogenous H_2O_2 production limits oxidation of xenobiotic compounds such as anthracene and polymeric dye [94]. The limited oxidation was promoted by adding glucose oxidase to cultures containing glucose.

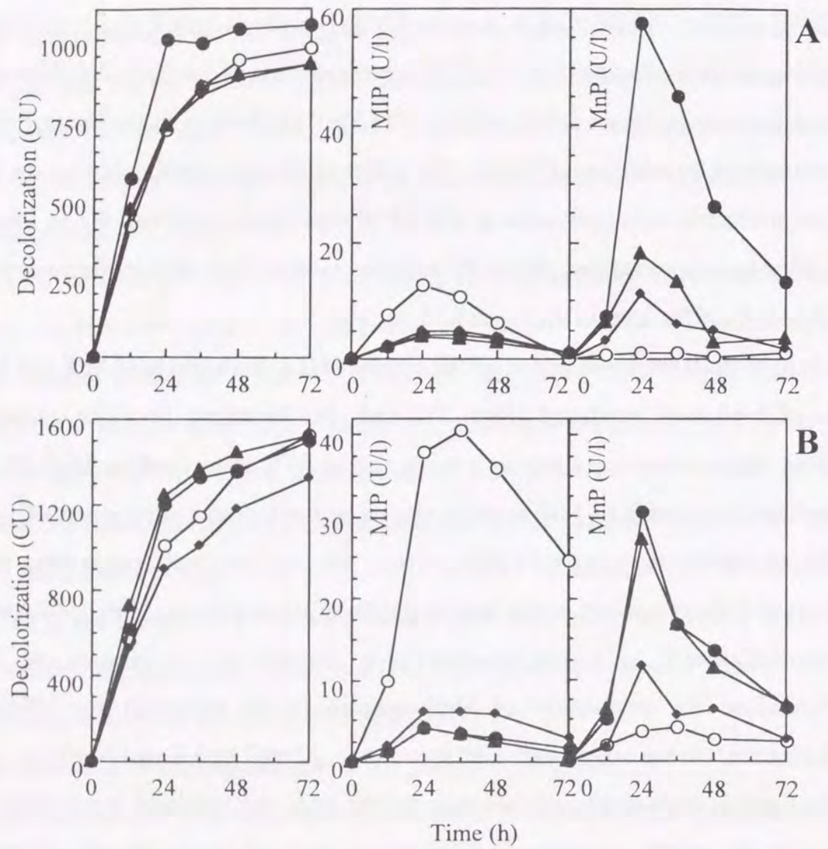

Fig. V-4. Effects of Mn(II) addition on decolorization of the pretreated HTL sapmples E (A) and F (B) by *C. hirsutus*. Mn(II) addition: ○ , none; ◆ ,5mg/l; ▲ , 20 mg/l; ● , 40 mg/l. The liquors were pretreated for 2 days and then, supplemented with glucose to a final concentration of 1.0 g/l.

To investigate effects of H_2O_2 supplementation on HTL decolorization, a H_2O_2-generating system which consisted of glucose oxidase and glucose was added to 24-hr fungal cultures exhibiting decolorization of the pretreated liquor F. In the cultures without Mn(II) addition, the liquor decolorization was most enhanced in the presence of both glucose oxidase and glucose (Fig. V-5A). Since this culture was not supplemented with Mn(II), a high level of MIP activity (34.0 U/l) but a low level of MnP activity (4.1 U/l) were detected immediately before adding the H_2O_2-generating system (after 24 hr of the preincubation). These enzyme levels decreased

rapidly to less than 50%, while those of the control culture did not (Fig. V-5A). In the presence of excess H_2O_2, ligninolytic peroxidases such as LiP and MnP are converted to an inactivated form of the enzymes, compound III [95-97]. Therefore, addition of glucose oxidase and glucose is considered to lead to a decrease in the levels of the enzymes activity. A relatively high rate of decolorization was observed in the culture supplemented with glucose only (Fig. V-5A). Since fungal pellets can produce extracellular H_2O_2 in the presence of glucose as shown in Chapter III, addition of glucose to the culture also presumably caused the stimulatory effect of H_2O_2 on decolorization. This suggestion is supported by the decrease in the enzyme activities of cultures, as shown for the culture including both of glucose oxidase and glucose. It is considered that the low level of decolorization observed in the heated culture is due to nonenzymatic decolorization by H_2O_2 [26,33,51].

Fig. V-5B shows a stimulatory effect of H_2O_2 supplementation on the decolorization in cultures with 20 mg/l Mn(II). The color loss of liquors during the preincubation for 24 hr was greater than that of the liquors without Mn(II), as shown in Figs. V-4B and -5B. Therefore, the color levels of cultures with Mn(II) at the beginning of post-incubation were lower than those of the cultures without Mn(II). This may be responsible for the low levels of decolorization in the presence of 20 mg/l Mn(II) compared with those in the absence of Mn(II) (Fig. V-5). The liquor decolorization was promoted by addition of glucose oxidase and/or glucose in a similar manner as shown in Fig. V-5A. In these cultures, however, the time courses of enzyme activities were clearly different from those in the cultures without Mn(II). Immediately before adding the H_2O_2-generating system, the cultures had MIP and MnP activities of 5.7 ± 0.4 and 31.2 ± 10.1 U/l, respectively. Addition of glucose oxidase and/or glucose resulted in a 1.5-fold increase in the level of MnP activity of the control culture (Fig. V-5B). Li *et al*. found that addition of Mn(II) and H_2O_2 to Mn-deficient, N-limited cultures of *P. chrysosporium* induces a 1.6-fold higher level of MnP production than addition of Mn(II) alone [98]. Also, in that report, expression of MnP was not stimulated by the presence of H_2O_2 alone. In the present study, additional production of MnP apparently also occurred in the presence of

both Mn(II) and H_2O_2, since it did not occur in the cultures without Mn(II) (Fig. V-5A).

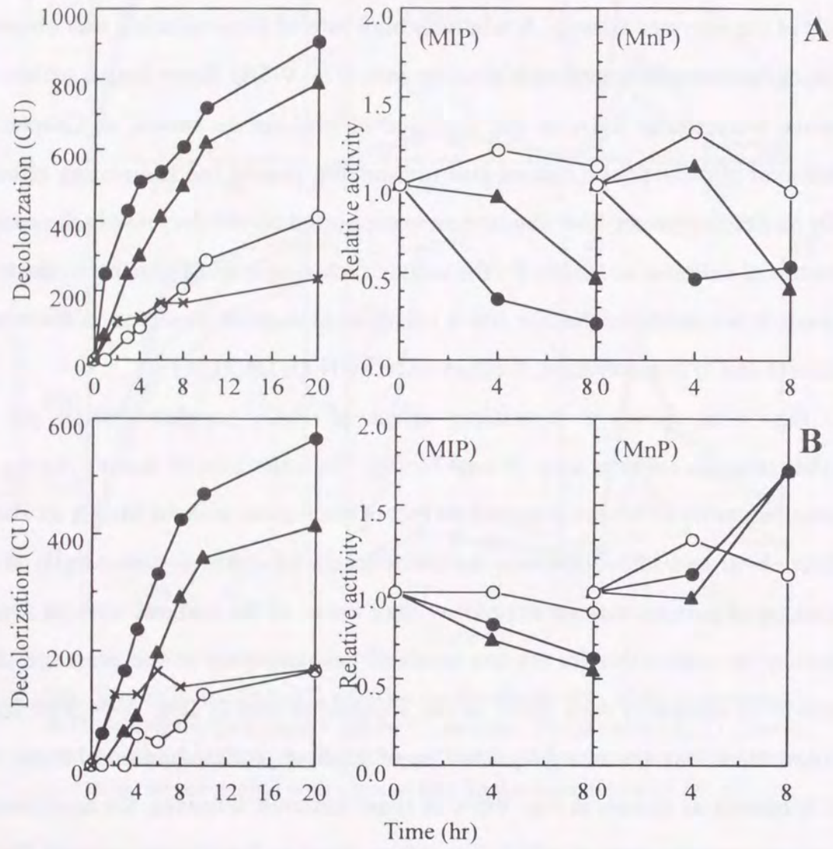

Fig. V-5. Effects of H_2O_2 supplementation on the fungal decolorization of the pretreated HTL sample F without (A) and with (B) Mn(II) addition .
The pretreated liquors containing 1.0 g/l glucose were preincubated with the fungus for 24 hr, and then incubated with the supplements for 20 hr: ● , 80 U/l glucose oxidase and 1.0 g/l glucose; ▲ , 1.0 g/l glucose; ○ , none. The preincubated cultures were also heated at 90°C for 5 min and then, supplemented with glucose oxidase and glucose (control, ×).

V-3.5. Conclusions and perspectives of the development of fungal reactor for HTL decolorization treatment

In conclusion, the use of sludge treatment as pretreatment of HTL enhanced fungal HTL decolorization. Besides pretreatment, the combined supplementation of Mn(II) and H_2O_2 resulted in additional enhancement of decolorization and MnP production. The appropriate conditions regarding the pretreatment time and concentrations of Mn(II) and H_2O_2 are presumably different for different HTLs. Therefore, those conditions should be determined using the artificial liquors prepared in Chapter IV. Particularly, the concentration of H_2O_2 should be considered, since addition of excess H_2O_2 causes inactivation of fungal peroxidases as described above.

Numerous carriers have been tried for immobilization of fungi [99], and application of immobilized fungi to paper and pulp industry effluents has been extensively attempted [15,44,100-102]. Continuous decolorization of melanoidin by *C. hirsutus* cells immobilized to alginate gel beads was also conducted by Tamaki *et al* [58]. Taking into account the relatively slow growth of white rot fungi and the solids separation of treatment processes, introduction of techniques for microorganisms immobilization may be essential. Furthermore, *P. chrysosporium* cells immobilized to a carrier, polyurethane foam, show higher ligninolytic activities than free cells [103-105]. The most important subject may be to prevent microbial contamination in the reactor. Melanoidin-decolorizing activity of *C. hirsutus* is best expressed in a pH range of 4.0 to 4.5 [58]. Controlling pH of the reactor must lead to repression of bacterial growth [4]. In addition, H_2O_2 which is produced by the fungus and/or added exogenously may also repress the bacterial growth. In future, development of a laboratory-scale reactor on the basis of that knowledge will be needed for the investigation of practical processes.

V-4. Summary

To enhance HTL decolorization by *C. hirsutus*, HTLs were treated with activated sludge prior to the fungal treatment. The fungus was able to decolorize the

pretreated HTLs effectively in the presence of 1.0 g/l glucose. The levels of MIP activity increased up to 127-fold by the pretreatment, while the levels of MnP activity increased up to 18-fold. Therefore, it was suggested that the large increases in MIP activity led to enhancement of decolorization. Addition of Mn(II) to the pretreated liquor to a concentration of 20 or 50 mg/l caused a further acceleration of decolorization. Addition of Mn(II) also caused a marked increase in the level of MnP activity and a decrease in the level of MIP activity, respectively, suggesting that MnP plays an important role in the decolorization in the presence of Mn(II). Addition of a H_2O_2-generating system, which consisted of glucose oxidase and glucose, to the culture without Mn(II) resulted in an additional acceleration of decolorization, but simultaneously, in a large decrease in the levels of MIP and MnP activity. On the other hand, the level of MnP activity increased with additional decolorization when the H_2O_2 generating system was added to culture containing 20 mg/l Mn(II). Therefore, it was concluded that simultaneous application of the activated sludge pretreatment and the combined supplementation of Mn(II) and H_2O_2 is very effective in enhancement of HTL decolorization by *C. hirsutus*.

Conclusions

For the development of microbial decolorization processes for HTL, this study was focused at the enhancement of HTL decolorization by the fungus *C. hirsutus* and obtained the knowledge that follows.

First, HTL colored components which are target substances of the decolorization were characterized (Chapter I). The acid-insoluble fraction (I) and acid-soluble fraction (III) eluted from the resin with methanol showed high levels of color, and the two fractions contained HTL major colored components. From the viewpoints of molecular weight distribution and structural unit, these colored components had characteristics which are similar to those of soil humic substances and simple melanoidin prepared from sugar and amino acid. These results suggested that HTL can be decolorized by the white rot fungi that have been reported to decolorize synthetic melanoidins and humic substances. Also, those results showed that simple artificial HTL containing melanoidins or humic substances can be used for investigations of actual HTL decolorization by the fungi.

Since *C. hirsutus* had the highest HTL-decolorizing ability of the fungi tested, it was selected and investigated further for its HTL-decolorizing ability (Chapter II). The fungal pellets removed 80% of the HTL color within eight days under appropriate conditions. The liquor decolorization was mainly due to degradation of the major (I and III) colored components, though some extent of adsorption onto the pellets was observed. To obtain a high rate and level of decolorization, both an appropriate dilution of HTL and an addition of a large amount of glucose were needed, *e. g.*, sufficient decolorization was obtained when 5 g/l glucose was added to 2.5 times diluted HTL. These requirements were considered to be disadvantages in the practical use of the fungus for HTL treatment.

The enzyme system involved in HTL decolorization by *C. hirsutus* was specified using melanoidin that was prepared from glucose and glycine (Chapter III). In *C. hirsutus* cultures, production of extracellular H_2O_2 was involved in the melanoidin decolorization system. However, nonenzymatic decolorization of

melanoidin by H_2O_2 as previously reported seemed slight in the cultures. The fungi produced two kinds of extracellular peroxidases, MIP and MnP, in the cultures exhibiting melanoidin decolorization. Partially purified MIP and MnP showed melanoidin-decolorizing activity. Therefore, it was concluded that production of extracellular H_2O_2 and peroxidases is involved in the principal system of the fungal melanoidin decolorization.

Interestingly, decreasing the organic nitrogen content of artificial HTL (AHTL) resulted in an marked increase in the rate and level of AHTL decolorization (Chapter IV). In the contrast, addition of organic N such as peptone or meat extract caused a decrease in the level of decolorization. Addition of inorganic N NH_4^+ hardly affected the liquor decolorization. Therefore, the low efficiency of the fungal HTL decolorization was considered to result from depression of the fungal decolorizing activity by a high concentration of organic N component. Addition of peptone to dilute AHTL caused a large decrease in the level of MnP activity, while it did not cause a decrease in the MIP level. Therefore, it was suggested that the decrease in a level of MnP activity is mainly responsible for the depression of liquor decolorization.

Finally, based on the knowledge described above, a few approaches to enhance HTL decolorization by *C. hirsutus* were introduced (Chapter V). Since the inhibitory effect of the HTL organic N component on fungal decolorization was shown in Chapter IV, reduction of that component by activated sludge treatment was attempted. The rate of HTL decolorization increased markedly by pretreatment of the liquor with activated sludge. This pretreatment was also very effective for the reduction of amount of the glucose required: addition of 1.0 g/l glucose to the pretreated liquors resulted in decolorization levels which were comparable to levels resulting from addition of 5.0 g/l glucose. In conclusion, the use of the sludge treatment as pretreatment of HTL enhanced the decolorization. Next, enhancement of the enzyme system as shown in Chapter III (Chapter V) was attempted to make the decolorization more efficient. Besides pretreatment, the combined supplementation of Mn(II) and H_2O_2 resulted in enhancement of the decolorization.

Addition of 20 or 40 mg/l Mn(II) to the pretreated liquor caused an acceleration of the decolorization with a marked increase in the level of MnP production. Supplementation of H_2O_2 in the culture expressing high levels of MnP activity gave an additional acceleration of decolorization. Furthermore, it was shown that the level of MnP activity increases in the culture supplemented with Mn(II) and H_2O_2. In conclusion, simultaneous application of the activated sludge pretreatment and the combined supplementation of Mn(II) and H_2O_2 gave good results for the enhancement of HTL decolorization by *C. hirsutus*.

Application of white rot fungi in the treatment of various wastewaters that are not treated effectively by the conventional biological methods has been extensively attempted. At present, the recalcitrant compounds-removal efficiency in the fungal cultures is generally low. Therefore, enhancing the efficiency is one of the most important subjects for the development of wastewater treatment processes. The fungal ligninolytic systems, which are involved in the degradation of the recalcitrant compounds, are different in different fungi. Furthermore, expression manners of the systems are different under different culture conditions. Therefore, it is crucial to specify the degradation system of the selected fungus and to find out the appropriate conditions for expression of the fungal activity. As described above, fundamental investigations relating to decolorization of a melanoidin-containing wastewater, HTL, by *C. hirsutus* have been conducted. This study could consequently identify new approaches for enhancement of HTL decolorization by the fungus. In future, development of laboratory-scale reactor based on this knowledge will be needed for conducting an investigation of the possibilities for practical use of the processes. In addition, noting the nonspecific nature of fungal peroxidases, the fungus can be used for the efficient treatment of various industrial effluents containing lignin, phenolic compounds, dyes, *etc*. Therefore, that knowledge obtained from the present study should likewise prove useful for the development of the fungal treatment processes for various wastewaters.

Acknowledgments

The author wishes to express his sincerest thanks to Professor Dr. Masanori Fujita, Department of Environmental Engineering, Faculty of Engineering, Osaka University, for his kindest guidance and encouragement during the entire course of this work. The author is deeply grateful to Professor Dr. Yoshikatsu Murooka, Department of Biotechnology, Graduate School of Engineering, Osaka University, and Professor Dr. Tatsuji Seki, International Center for Biotechnology, Osaka University, for their valuable suggestions and critical readings of his manuscript.

The author greatly appreciates Associate Professor Dr. Keisuke Iwahori, Institute for Environmental Sciences, University of Shizuoka, for his kindest suggestions and encouragement. The author also wishes to express his thanks to Professor Dr. Kenji Furukawa, Department of Civil Engineering and Architecture, Faculty of Engineering, Kumamoto University, and Instructor Dr. Michihiko Ike and Ms. Yuriko Miyazaki, Department of Environmental Engineering, Faculty of Engineering, Osaka University, for their helpful advice and discussions.

The author greatly appreciates Mr. Masaharu Sakagami and Mr. Tomohiko Hirao, Engineering Division, Takuma Co., LTD., for providing heat treatment liquor samples and for their valuable discussions. Helpful and valuable suggestions by Dr. Shinichi Yoshida, Industrial Research Institute of Tottori Prefecture are also gratefully acknowledged. The author thanks Ms. Masako Yamashita, Nissuikon LTD., Mr. Toru Mori, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, and Mr. Akira Era, Graduate School of Engineering, Osaka University, for the invaluable technical assistance. Thanks are also due to all members of the Laboratory of Water Science and Environmental Biotechnology, Department of Environmental Engineering, Osaka University and the Laboratory of Environmental Engineering, Institute for Environmental Sciences, University of Shizuoka. The author is grateful to Dr. Willem Engelaar, National Agriculture Research Center, Ministry of Agriculture, Forestry and Fisheries and Ms. Bandunee Liyanage, Department of Environmental Engineering, Faculty of Engineering, Osaka University for reading his manuscript and pointing out syntax and grammatical discrepancies.

Last but not least, his gratitude is to all of my family members for their constant support and encouragement during the years of study.

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