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## Studies on the Treatment of Kraft Bleaching Effluents with Lignin-degrading Fungi\*

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### Abstract

Typically, for production of high brightness pulp, the residual lignin in unbleached kraft pulp is commonly removed through a multi-stage bleaching process with the use of chlorine species. The effluent from such a bleaching process is dark brown due to their content of chromophoric and polymeric lignin derivatives. Moreover, the organically bound chlorine [measured as total organically bound chlorine (TOCl) or adsorbable organic halogen (AOX)] is present in a wide range of organic materials which have toxic and mutagenic properties. It has recently been discovered that small quantities of dioxines [polychlorinated dibenzo dioxin (PCDD) and polychlorinated dibenzo furan (PCDF)] can also appear in bleach plant effluents. Consequently, this bleaching effluent is not easily recycled within a mill recovery system because of the potential corrosion problems created by its high chlorine content and finally affects the harmful effects on the environment.

The purpose of the present research is to describe the decolorization of the E<sub>1</sub> effluent with white-rot fungi showing the ligninolytic activity, to accomplish efficient treatment of kraft bleachery effluent with less energy and in relatively short treatment-period.

First, it was attempted to design the effective treatment process of the E<sub>1</sub> effluent with the fungus IZU-154 showing ligninolytic activity. It has revealed that the color of the effluent was effectively removed when glucose was added as an additive. Furthermore, the addition of glucono- $\delta$ -lactone to the E<sub>1</sub> effluent produced a more remarkable degree of decolorization than did that of glucose. All white-rot fungi tested showed high decolorization effect of the E<sub>1</sub> effluent in the presence of glucono- $\delta$ -lactone. It is suggested that the effect of glucono-d-lactone on the high decolorization of E<sub>1</sub> effluent are closely associated with the activity of the surfaces of the mycelium due to the change of pH of the effluent during fungal treatment.

Moreover, to establish a more practical system for the decolorization of the bleaching effluent, a screening have been performed to find fungi having high decolorization activity of the E<sub>1</sub> effluent without any additional nutrients. The

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\* 李 宣鎬・近藤隆一郎・坂井克己：リグニン分解菌によるクラフトパルプ漂白廃液の処理に関する研究

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strains which had the activity of decolorization were isolated from 1,212 samples of rotted wood of forest habitats. Among the isolated fungi, the fungus KS-62 showed the most effective decolorization of the E<sub>1</sub> effluent and degraded the chlorinated lignin in the E<sub>1</sub> effluent without any additional nutrients. Moreover, the treatment with KS-62 significantly reduced the COD and AOX and decreased the high molecular weight of chlorolignins in the E<sub>1</sub> effluent.

To obtain a reasonable basis for the evaluation of an industrial fungal treatment, we have performed the treatment of the E<sub>1</sub> effluent with the immobilized mycelium of the fungus KS-62. The fungal biomass of KS-62 could be maintained for a long-term (at least 40 days) without any appreciable loss of activity in the presence of a critical amount (between 0.050% and 0.075%) of glucose. The COD of the E<sub>1</sub> effluent treated with KS-62 was lower than that of initial E<sub>1</sub> effluent in whole treatment period, which indicated that the fungus KS-62 decomposed the chlorolignin in the E<sub>1</sub> effluent. Based on these experiments, it has become apparent that the biological decolorization of bleaching effluents has been enabled with the lignin-degrading fungi.

**Key words** : white-rot fungi ; IZU-154 ; *Coriolus versicolor* ; bleaching effluent ; decolorization ; nucleic acid constituents ; screening ; adsorbable organic halogens ; manganese peroxidase ; additives ; glucono- $\delta$ -lactone ; immobilized mycelium ; successive treatment.

## Contents

1. Introduction
  - 1.1. Chemical components of wood
  - 1.2. General concept of chemical pulping
  - 1.3. Bleaching of kraft pulp
  - 1.4. Composition of bleaching spent liquor
  - 1.5. Treatment of pulp bleaching effluents
    - 1.5.1. Physical and/or chemical treatment
    - 1.5.2. Biological treatment
    - 1.5.3. Treatment with ligninolytic fungi
  - 1.6. Lignin degradation with white-rot fungi
  - 1.7. Lignin biodegradation with white-rot fungus IZU-154
2. Decolorization of Kraft Bleaching Effluents with the Lignin-degrading Fungus IZU-154
  - 2.1. Introduction
  - 2.2. Materials and methods
    - 2.2.1. Microorganisms
    - 2.2.2. Bleaching effluents
    - 2.2.3. Treatment methods of the E<sub>1</sub> effluents
    - 2.2.4. Determination of color units of the E<sub>1</sub> effluents

- 2.3. Results
  - 2.3.1. Decolorization of the E<sub>1</sub> effluents by the treatment method A
  - 2.3.2. Effects of nutrients on the decolorization of the N-E<sub>1</sub> effluent by the treatment method A
  - 2.3.3. Decolorization of the N-E<sub>1</sub> effluent by the treatment method B
- 2.4. Discussion
- 2.5. Summary
  
3. Detection of Nucleic Acid Constituents in the E<sub>1</sub> Effluent Treated with the Lignin-degrading Fungus IZU-154
  - 3.1. Introduction
  - 3.2. Materials and methods
    - 3.2.1. Microorganisms
    - 3.2.2. Bleaching effluent
    - 3.2.3. Preparation of inoculum
    - 3.2.4. Fungal treatment and analysis of the E<sub>1</sub> effluent
  - 3.3. Results
    - 3.3.1. Analysis of the E<sub>1</sub> effluent treated with IZU-154
    - 3.3.2. Examination of the formations of nucleic acid constituents in the E<sub>1</sub> effluents treated with other lignin-degrading fungi
    - 3.3.3. Effects of preculture conditions of IZU-154 on the formations of nucleic acid constituents and the decolorization of the E<sub>1</sub> effluent
    - 3.3.4. Effects of treatment conditions with IZU-154 on the formations of nucleic acid constituents and decolorization of the E<sub>1</sub> effluent
  - 3.4. Discussion
  - 3.5. Summary
  
4. An Accelerating Effect of Glucono- $\delta$ -lactone on the Decolorization of the E<sub>1</sub> Effluent
  - 4.1. Introduction
  - 4.2. Materials and methods
    - 4.2.1. Microorganisms
    - 4.2.2. Bleaching effluents
    - 4.2.3. Treatment method of the E<sub>1</sub> effluent
    - 4.2.4. Analysis
  - 4.3. Results
    - 4.3.1. Effects of various additives on the decolorization of the E<sub>1</sub> effluent with IZU-154
    - 4.3.2. Effects of glucono- $\delta$ -lactone on the decolorization of the E<sub>1</sub> effluent with the lignin-degrading fungi
    - 4.3.3. Consumption and conversion of the additives with *C. versicolor*
    - 4.3.4. Role of glucono- $\delta$ -lactone in the decolorization of the E<sub>1</sub> effluent
    - 4.3.5. Determination of COD in the E<sub>1</sub> effluent during fungal treatment
  - 4.4. Discussion
  - 4.5. Summary
  
5. Screening of Lignin-degrading Fungi for the Efficient Decolorization and Treatment of the

## E<sub>1</sub> Effluent without Additional Nutrients with a Newly Found KS-62

- 5.1. Introduction
  - 5.2. Materials and methods
    - 5.2.1. Isolation of lignin-degrading fungi from decayed wood
    - 5.2.2. Bleaching effluent
    - 5.2.3. Screening of high-decolorization fungi
    - 5.2.4. Physiological characteristics of lignin-degrading fungi
    - 5.2.5. Decolorization of the E<sub>1</sub> effluent with the fungus KS-62
    - 5.2.6. Effect of catalase addition on the decolorization of the E<sub>1</sub> effluent
    - 5.2.7. Analysis
  - 5.3. Results
    - 5.3.1. Screening of fungi having high-decolorization activity
    - 5.3.2. Decolorization of the E<sub>1</sub> effluent with lignin-degrading fungi
    - 5.3.3. Physiological characteristics of a lignin-degrading fungus KS-62
    - 5.3.4. Dechlorination and degradation of chlorolignin in the E<sub>1</sub> effluent with lignin-degrading fungi
    - 5.3.5. Extracellular enzymes revealed during the treatment of effluent with KS-62
    - 5.3.6. Effect of catalase addition on the decolorization
  - 5.4. Discussion
  - 5.5. Summary
- 
6. Successive Treatment of the E<sub>1</sub> Effluent with Immobilized Mycelium of the Fungus KS-62
    - 6.1. Introduction
    - 6.2. Materials and methods
      - 6.2.1. Microorganisms
      - 6.2.2. Bleaching effluent
      - 6.2.3. Treatment of the E<sub>1</sub> effluent with the immobilized mycelium of white-rot fungi
      - 6.2.4. Analysis
    - 6.3. Results
      - 6.3.1. Treatment of the E<sub>1</sub> effluent with the immobilized mycelium of the white-rot fungi
      - 6.3.2. Effects of glucose concentration on the decolorization with the immobilized mycelium of KS-62
    - 6.4. Discussion
    - 6.5. Summary
  - Acknowledgements
  - References
  - 要 約

## 1. Introduction

### 1.1. Chemical components of wood

Wood is a complex and heterogeneous product of nature made up of interpenetrating components, largely of high molecular weight. It is by far the most important raw material for the production of pulp. Main component groups of wood are cellulose,

hemicelluloses, lignin, and extractives.

Cellulose is a linear polysaccharide consisting of  $\beta$ -D-glucopyranose units, which are linked by (1-4)-glucosidic bonds. Wood cellulose in its native state is composed of at least 10,000 anhydro glucose units. Cellulose molecules are bundled together in wood to form microfibrils. These in turn build up fibrils and, finally, cellulose fibers. Approximately 40-45% of the dry substance in most wood species is cellulose, located predominantly in the secondary cell wall.

Unlike cellulose, hemicelluloses are composed of different carbohydrate units and branched to various extents; their relative molecular masses, with a degree of polymerization on the order of 200, are much lower. Also, the content and types of hemicellulose in softwoods differ considerably from those in hardwoods.

Lignin (Latin *lignum*=wood) is the most abundant and widely distributed renewable aromatic polymer in the biosphere. The lignin component comprises about 20-30% of the dry mass of woody plants (Crawford, 1981) (Higuchi *et al.*, 1983) (Eriksson *et al.*, 1985). It is formed by an enzyme-initiated dehydrogenative polymerization of a mixture of three different 4-hydroxyarylpropenyl alcohols. The proportions of these alcohols vary with different wood species. Thus, softwood lignin is largely a polymerization product of coniferyl alcohol (II in Fig. 1) (Adler, 1977). Figure 2 shows a summary of prominent structures in such lignin, as suggested by Adler (Adler, 1977).

The scheme shown in Fig. 2 contains only 16 monomeric units, however, one scheme cannot explain the entire structure of lignin. The schematic drawing shows that lignin is a complex- heterogeneous and random phenylpropanoid polymer. It does show that softwood lignin is a branched molecule in which the phenylpropane-based units are linked by different types of bonds. These include ether bonds of alkyl-aryl, alkyl-alkyl, and aryl-aryl configurations. In hardwoods, lignin is formed by copolymerization of coniferyl and sinapyl alcohols (II and III in Fig. 1, respectively).

A number of important functional roles have been attributed to lignin (Crawford, 1981). These include conferring strength and rigidity with elasticity to plant tissues and minimization of water permeation across cell walls of xylem tissue. Because of its

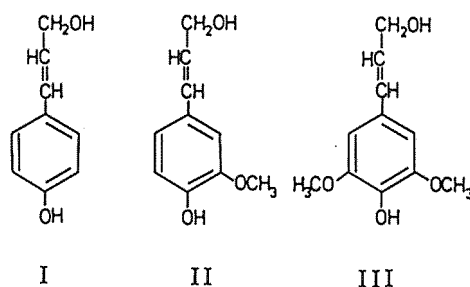


Fig. 1 Lignin precursors.

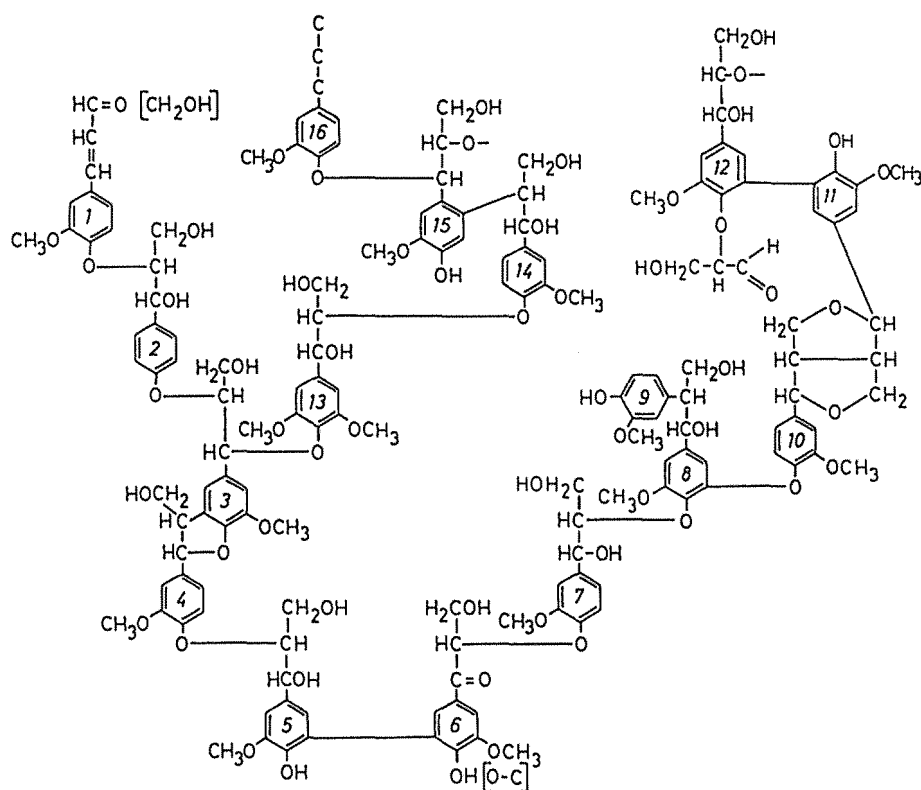


Fig. 2 Prominent structures in softwood lignin

recalcitrance to biodegradation, lignin undoubtedly plays a role in conferring protection against infection by plant pathogenic microbes. About 70-90% of the lignin in wood fiber occurs in the primary and secondary cell wall layers. The middle lamella contains the rest with 10-30% of the lignin. In the plant cell wall, lignin is intimately interspersed with hemicellulose and forms a matrix around the cellulose microfibrils. Lignin also functions as an adhesive cement binding cells together.

The term "extractives" is normally used for those components of wood that can be extracted by organic solvents such as ethanol, acetone, or dichloromethane. They include a variety of compounds that can be divided into aliphatic and phenolic extractives. The total content of extractives in wood varies greatly (1.5-5%), depending on the species, place of growth, and the age of the tree.

## 1.2. General concept of chemical pulping

Chemical pulping is brought about by two types of structural change in lignin which may be connected to each other. The first involves degradation by cleavage of certain interunit linkages and the second entails introduction of hydrophilic groups into the

polymer and its fragments. Most chemical pulping is carried out either according to the kraft (sulfate) process or the sulfite process.

The kraft process (Fig. 3) is presently the most important by far (Kringstad *et al.*, 1984). Depending on pulping conditions, as much as 90-95% of the lignin is removed from wood at this stage. In addition, portions of the wood polysaccharides, especially those of the hemicelluloses, are dissolved during the pulping operation. Wood extractives are also dissolved or dispersed in the kraft pulping liquor. However, the spent liquor (black liquor) is not released from a pulp mill as an effluent, because the liquor is evaporated to a high concentration and then burned to recover energy and inorganic chemicals as shown in Fig. 3.

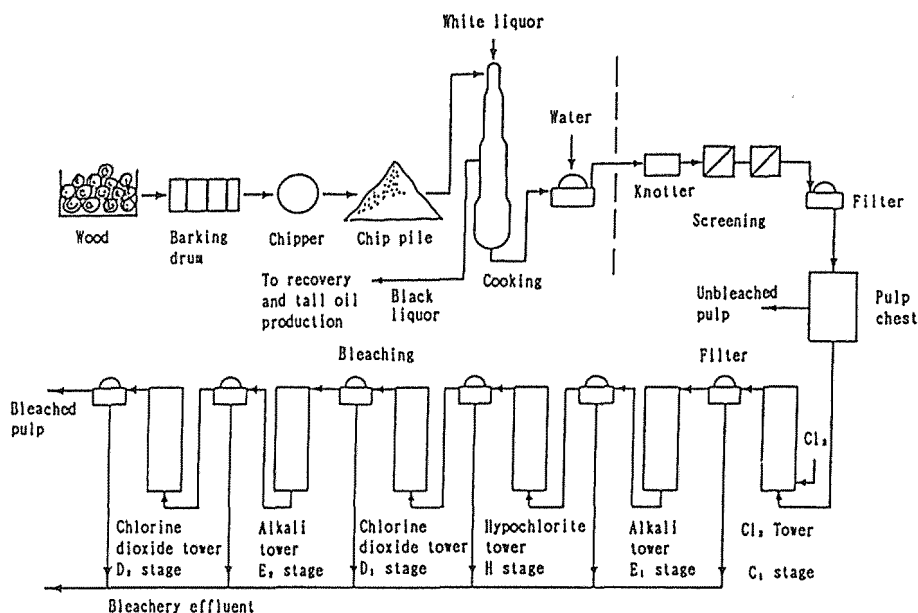


Fig. 3 Kraft pulp mill, including a conventional bleach plant.

### 1.3. Bleaching of kraft pulp

The kraft process can not remove all lignin in the wood. About 5-10% of the original lignin remains in the pulp, since it cannot be removed by extended pulping without seriously degrading the polysaccharide fraction. Typically, for production of high brightness pulp, the residual lignin in unbleached kraft pulp is commonly removed through a multi-stage bleaching process. Multistage bleaching is normally accomplished by successive treatments with chlorine (C<sub>1</sub>), alkali (E<sub>1</sub>), hypochlorite, chlorine dioxide (D<sub>1</sub>), alkali (E<sub>2</sub>) and chlorine dioxide (D<sub>2</sub>) as shown in Fig. 3. Approximately 75% of the dissolved material [chemical oxygen demand (COD) and color] and 90% of the



organically bound chlorine (OCl) are contained in the C1 and E1 bleaching effluent.

#### 1.4. Composition of bleaching spent liquor

There are basically two problems with the use of chlorine species in pulp bleaching. First, bleaching effluent is generally not allowed to enter the recovery system due to the corrosive nature of chloride in the effluent. Therefore, bleaching effluent has to be dealt with separately. Secondly, during bleaching, wood components, mainly lignin, are degraded, heavily modified, chlorinated, and finally dissolved in the effluent. As a result, the effluent from such a bleaching process is dark brown due to their content of chromophoric, polymeric lignin derivatives (Bennett *et al.*, 1971) (Peyton, 1984). Moreover, organically bound chlorine [measured as total organically bound chlorine (TOCl) or adsorbable organic halogen (AOX)] is present in a wide range of organic materials (Pfister *et al.*, 1979) which have toxic and mutagenic properties and may accumulate in aquatic organisms (Eriksson *et al.*, 1979). The AOX test is now preferred (Odendahl *et al.*, 1990).

There have been several studies on chemicals present in pulp mill effluents (Voss *et al.*, 1980) (Suntio *et al.*, 1988). A compilation is presented of some 250 chemicals identified in the effluents from pulp mills (Suntio *et al.*, 1988). In the alkali extraction liquor, biochemical oxygen demand (BOD) and acute toxicity are mainly due to the low molecular weight fraction of chlorinated organics, while the high molecular weight portion of the chlorolignins is responsible for color, COD, and chronic toxicity. High molecular weight chlorolignins are of concern because they are either resistant to biodegradation in nature and, therefore, have a potential to be bioaccumulated in the environment, or they are degraded at a very slow rate resulting in low molecular weight toxic intermediates. It has recently been discovered that small quantities of dioxines [polychlorinated dibenzo dioxin (PCDD) and polychlorinated dibenzo furan (PCDF)] can also appear in bleach plant effluents.

#### 1.5. Treatment of pulp bleaching effluents

The release of chlorinated organic compounds should be reduced or eliminated in order to avoid harmful effects on the environment (Boman *et al.*, 1988). Modifications can be made inside the factory, for example prolonged cooking, oxygen prebleaching and substitution of chlorine by chlorine dioxide. Even after these modifications, to obtain less polluted effluents of bleach plant, an effective external treatment will be needed in many cases. This goal can be achieved in a number of different ways. There are described some possible solutions and results obtained to date with those techniques that have been tested on a larger scale as following statements.

##### 1.5.1. Physical and/or chemical treatment

Many works have been undertaken to study color removal of bleach plant effluents

with physical treatment (such as activated carbon and ultrafiltration), chemical treatment (for example with lime and amines) and combined physical and chemical treatment.

Color removal from waste by physical adsorption is effected by various adsorbents such as an activated carbon, ion-exchange resins (Börjeson *et al.*, 1981), polymeric adsorbents (Rock *et al.*, 1974), and a fly ash (Börjeson *et al.*, 1981). The color removal from caustic extraction effluent of a bleached kraft mill was also performed by ultrafiltration with polymeric membranes (Lundahl *et al.*, 1980).

The chemical treatment included the lime precipitation, lime coagulation, the using high molecular weight amines and an adding certain polyvalent metal ions with the lime after lime treatment (Bennett *et al.*, 1971) (Schmidt *et al.*, 1980) (Börjeson *et al.*, 1981) (Alberti *et al.*, 1981).

The physical-chemical treatment process include a preliminary treatment with lime before activated carbon adsorption, a continuous system of chemical coagulation (using ferric chloride), sedimentation, high-rate filtration and carbon adsorption, and a system comprising a pH adjusting unit, spiral-wound membrane ultrafiltration cell and an incinerator for burning the concentrated colored material (McCuaig *et al.*, 1974). There are many other agents such as metal salts (Almemark *et al.*, 1989) which may be useful for color removal from pulp mill effluents; photochemical decolorization has also been proposed.

These physical and chemical processes appear to be relatively inefficient in removing the chlorinated organic compounds of lower molecular weight (Bennett *et al.*, 1971). Therefore, these methods suffer from the obvious disadvantage, that the pollutants removed must still be degraded. Incineration seems to be the only feasible method to do this, and the problems associated with the burning of chemical sludge or ultrafiltration concentrates containing organochlorine compounds have yet to be solved. These methods also require the high treatment expenses. The main expense of chemical treatment is the price of chemicals, which is consequently sensitive to the total amount of organic compounds discharged with the effluent to be treated. The costs of physical treatment, on the other hand, are sensitive to the volumetric flow of waste water to be treated (Almemark *et al.*, 1989).

### 1.5.2. Biological treatment

Since the conception of the activated sludge adaptation of the bio-oxidation process in 1914, it has become the most widely used type of the treatment of pulp and paper mill wastes. The aerated stabilization basin, or aerated lagoon, is a relatively new form of waste treatment. Because of favorable laboratory and pilot-plant studies, these systems have found widespread application in the pulp and paper industry. In particular, these biological treatments are effective in reducing or eliminating the acute toxicity of pulp mill effluents (Flynn, 1979).

Chlorinated organic chemicals are reported to undergo 30-50% removal during treatment in aerated lagoons (Bryant *et al.*, 1987). Usually only a part (less than 30%) of the load of chlorophenolic compounds is removed (Hakulinen *et al.*, 1982). Dichlorodihydroxybenzoic acid and trichloroguaiacol were poorly biodegradable, whereas tetrachloroguaiacol was biodegradable in systems where the microorganisms were acclimated to this compound. Analyses of untreated and treated mill effluents have shown that chlorophenols and chloroguaiacols are partially degraded, although the level of chlorocatechols was reported to increase. This increase was ascribed to degradation of guaiacols or high-molecular-weight chlorolignin in the aerated lagoon. Newer methods of biological treatment, such as the Enso-Fenox process, an anaerobic-aerobic treatment technique developed in Finland (Hakulinen *et al.*, 1982), with effluents from the first alkaline stage in the bleaching of birch and pine pulps, removed 64-94% of the chlorophenolic load. Recent studies in Scandinavia indicate that aerated lagoon treatment reduces TOC by 25% or so, which is roughly equivalent to the efficiency of chlorophenolic removal. The aerated lagoon treatment, operated for effective BOD removal, was capable of removing approximately one third of the total organic halide (TOX). Using the activated sludge method, AOX was reduced by 48-65%, and chlorophenols decreased by 75-95%, but only 23-32% of AOX was removed by aerated lagoon (Gerkov *et al.*, 1988). Most recently, it has been reported (Collins *et al.*, 1991) that a combination system of aerobic and anaerobic processes of lagoons reduced 80% of BOD at a retention time of ten days.

These biological processes, such as aerated lagoons and activated sludge treatments, have been studied and applied throughout the world. Such treatments reduce the BOD and COD of the effluents but the color of the effluent persists (Collins *et al.*, 1991) (Lankinen *et al.*, 1991). These treatment systems have some disadvantages as follows; the drawback of the aerated lagoon treatment system is the long hydraulic retention time (4-7 days) which requires a large area. Apart from land cost, this means a substantial cooling down of the lagoon in winter, resulting in lower efficiency. A large part of the operation cost is the energy needed for aeration, as in other aerobic processes (Bomam *et al.*, 1988).

### 1.5.3. Treatment with ligninolytic fungi

White-rot fungi are the only known microorganisms which are able to degrade polymeric lignins completely. Industrially modified lignins such as kraft lignin are also degraded by these organisms. In 1977, two reports described the degradation of the extraction-stage effluent from the chlorine bleaching of kraft pulp with ligninolytic fungi (Fukuzumi *et al.*, 1977) (Lundquist *et al.*, 1977). In particular, two species, *Phanerochaete chrysosporium* Burds. and *Coriolus versicolor* (L. ex Fr.) Quel., demonstrating useful levels of native and industrial lignin degradation, lignin chromophore bleaching, and effluent toxicity reduction, have been studied (Royer *et al.*, 1985) (Kirk *et al.*, 1987).

Several investigators demonstrated that kraft bleach plant effluents can be

decolorized by culture of the white-rot fungus, *P. chrysosporium*. Subsequent investigations have optimized culture conditions (Eaton *et al.*, 1980) and demonstrated that the chromophores are both bleached and decomposed (Sundman *et al.*, 1981). Based on these laboratory and bench-scale experiments, a process for efficient color reduction with *P. chrysosporium* has been outlined (Eaton *et al.*, 1982). Several factors are required. At first, the fungi require a growth substrate for high decolorization (Eaton *et al.*, 1980) just as they do for lignin degradation (Ander *et al.*, 1975) (Kirk *et al.*, 1976). Color removal is greatly stimulated by oxygen-enriched atmospheres (Eaton *et al.*, 1980). A growth stage is necessary before decolorization begins (Campbell *et al.*, 1982). Based on these considerations, the biological decolorization of bleaching effluents using the FPL/NCSU mycelial color removal (MyCoR) process (Fig. 4) based on an immobilized white-rot fungus *P. chrysosporium* in a rotating biological contactor (RBC) has been proposed, developed (Eaton *et al.*, 1982) (Joyce *et al.*, 1984) and patented (Chang *et al.*, 1987). An earlier work (Joyce *et al.*, 1983) was focused on decolorization of bleach plant E<sub>1</sub> effluent. Later, the fungus was reported to efficiently degrade chlorolignins and other hazardous organics (Huynh *et al.*, 1985 a). The fungus dechlorinated the bleach plant effluent by

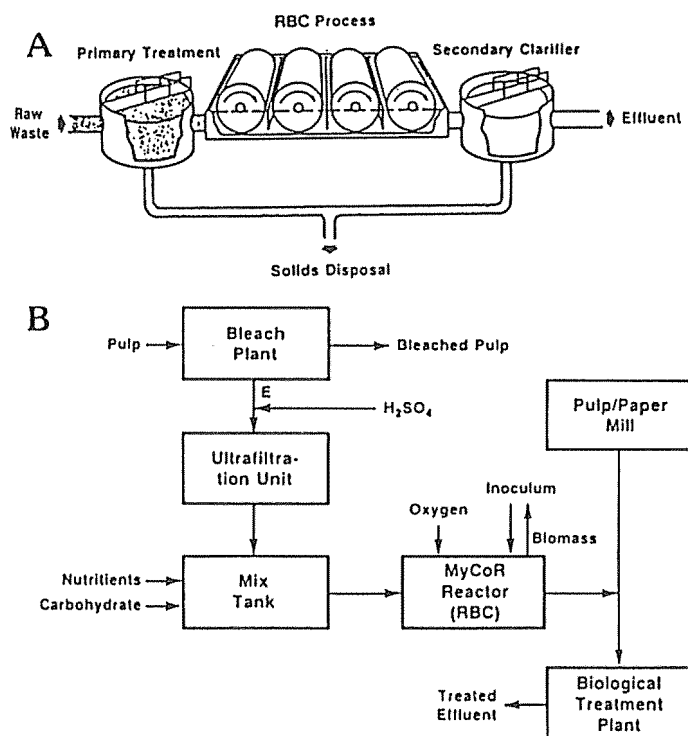


Fig. 4 The rotating biological contractor (A) and the Mycelial Color Removal (MyCoR) process in an integrated pulp and paper mill (B).

converting the organically bound chlorine to inorganic chloride (Matsumoto *et al.*, 1985) (Pellinen *et al.*, 1988), decolorized the effluent color by destroying both chromophoric groups and structures (Sundman *et al.*, 1981), and degraded high and low molecular weight chlorolignins by converting chlorinated organics to CO<sub>2</sub> and chloride (Yin *et al.*, 1989 a). However, the problems with the operation of MyCoR RBC processes were short fungal lifetime (5-7 days), high oxygen concentration required, and biomass wasting (Prouty, 1990) (Cammarota *et al.*, 1992). In particular, this fungus required a high oxygen concentration for treatment of bleach plant effluents and to fulfill this demand, a process was developed which guarantees excellent oxygen supply. This has motivated many authors to study other alternative bioreactors such as packed-bed or fixed-bed reactors (Lankinen *et al.*, 1991). Moreover, the use of trickling filter type bioreactor where the fungus is immobilized on porous carrier material has been adopted in the MYCOPOR system (Fig. 5) (Messner *et al.*, 1989) (Messner *et al.*, 1990). Of all the fermenters tested, this trickling filter system was most effective for the degradation of effluents (Cammarota *et al.*, 1992). In comparison with the MyCoR process, the result achieved with this MYCOPOR process was that the fungal biomass could be maintained for at least 66 days without any appreciable loss of activity (Cammarota *et al.*, 1992). However, many aspects related to its operating conditions should be further investigated and improved (hydraulic retention time, specific air flow rate, etc.). It is one of the disadvantages of these treatment processes that *P. chrysosporium* required the supplement of high concentrations of oxygen and energy sources such as glucose or cellulose as well as various basal nutrients, mineral solution, and Tween 80 (Messner *et al.*, 1990).

One of the other white-rot fungi known to decolorize kraft bleaching effluents is *C.*

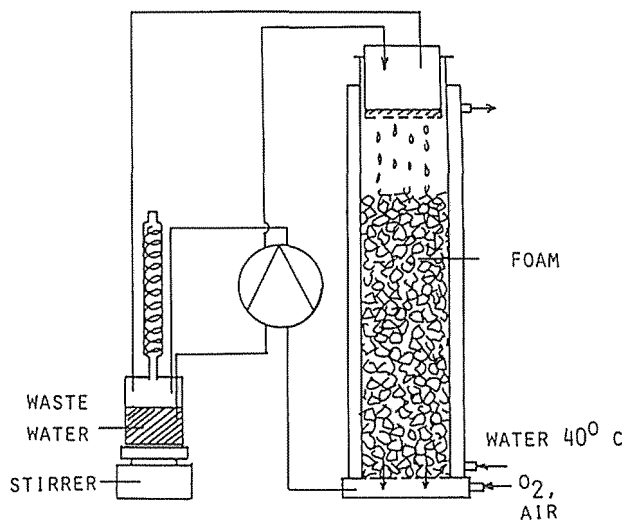


Fig. 5 MYCOPOR trickling filter system.

*versicolor*. In work carried out at PAPRICAN and elsewhere, *C. versicolor* has been shown to produce rapid decolorization of kraft bleaching effluents when applied as mycelium, mycelial pellets, or mycelial fragments immobilized in alginate beads (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983). The presence of malt extract and molasses as complex co-substrates (Bergbauer *et al.*, 1991) or the combination of molasses and brewery cake (Archibald *et al.*, 1990) enhanced the decolorization.

A white-rot fungus *Phlebia radiata* 79 (ATCC 64658) was also investigated in Finland for the efficient lignin degradation (Hatakka *et al.*, 1983) and the dechlorination and decolorization of bleach plant effluents (Lankinen *et al.*, 1991).

### 1.6. Lignin degradation with white-rot fungi

Wood-rotting fungi are classified into three major categories based on the type of wood decay caused by these organisms: white-rot, brown-rot and soft-rot fungi. Of these three groups, white rot fungi are the most important lignin degraders. More than a thousand species of white-rot fungi have been found and almost all belong to Basidiomycota (Kirk, 1971). There are also a few ascomycetes causing a white-rot type of wood decay (Kirk, 1971).

Despite the fact that lignin is rich in carbon, it is not a growth substrate for lignin degrading fungi. They metabolize various lignin model compounds only in the presence of an alternate energy/carbon source (Buewell *et al.*, 1987). Thus, lignin degradation can be truly considered "cometabolism" along with utilization of energy/carbon substrates such as cellulose, hemicellulose, simple carbohydrates, and glycerol. Apparently, the oxidative degradation of lignin with white-rot fungi such as *P. chrysosporium* does not provide sufficient energy for growth (Crawford 1981). Reports of growth of white rot fungi on lignin have not been substantiated (Kirk *et al.*, 1976) (Leisola *et al.*, 1983) (Ulmer *et al.*, 1983). Furthermore, degradation of only lignin during wood decay has not been observed except in a single study with *P. chrysosporium* in which glucose was provided as a cosubstrate (Yang *et al.*, 1980). In another study, a cellulase-negative mutant of *Sporotrichum pulverulentum* (an anamorph of *P. chrysosporium*) degraded lignin in wood without attacking the cellulose, but it simultaneously degraded hemicelluloses (Ander *et al.*, 1976) (Ander *et al.*, 1985). Clearly, lignin does not serve as a growth substrate and strains degrading lignin require an easily metabolizable growth substrate.

Lignin is degraded only during secondary metabolism (Boominathan *et al.*, 1991). The shift to secondary metabolism follows the cessation of growth due to the depletion of certain nutrients. Several ligninolytic fungi, if not all, apparently exhibit this feature (Crawford, 1981) (Jeffries *et al.*, 1981) (Freer *et al.*, 1982) (Leatham *et al.*, 1983) (Buswell *et al.*, 1987). Secondary metabolism in *P. chrysosporium* is triggered by nitrogen, carbon, or sulfur limitation but not by phosphorus limitation. Lignin degradation stops when primary growth is restored by adding excess carbon or nitrogen to cultures limited for those nutrients (Leatham *et al.*, 1983). In nitrogen-limited cultures, ligninolytic activity is

associated with the formation of new secondary mycelia (Leisola *et al.*, 1983). Since nitrogen is limited in wood which is the natural substrate for wood-rotting fungi (Cowling *et al.*, 1966), expression of ligninolytic capability under nitrogen-limited conditions is advantageous for the organism so that sustained lignin degradation occurs. However, lignin mineralization ( $[^{14}\text{C}]$ -lignin to  $^{14}\text{CO}_2$ ) by some white-rot fungi, e.g. *Pleurotus ostreatus* (Freer *et al.*, 1982), *Lentinus edodes* (Leatham *et al.*, 1983) and *Bjerkandera* sp. (Kaal *et al.*, 1993), is not repressed by high nitrogen concentrations. With some mutants of *P. chrysosporium*, e.g. INA-12 (Buswell *et al.*, 1984) and PSBL-1 (Tien *et al.*, 1990) (Orth *et al.*, 1991), the lignin peroxidase (LiP) production was even stimulated by nitrogen.

Degradation of lignin has been referred to as "enzymatic combustion", consisting of nonspecific enzyme-catalyzed burning of lignin (Blanchette, 1991). Biochemical investigations using *P. chrysosporium* have shown that two heme peroxidases are associated with lignin degradation, LiP (Fig. 6) (Tien *et al.*, 1983) (Kirk, 1987) (Tien, 1987) (Farrell *et al.*, 1989) and manganese-dependent peroxidase (MnP) (Fig. 7) (Kuwahara *et al.*, 1984) (Glenn *et al.*, 1985) (Huynh *et al.*, 1985 b) (Gold *et al.*, 1989) (Farrell *et al.*, 1989). LiP is a kind of  $\text{H}_2\text{O}_2$ -dependent enzymes that degrade lignin by a simple initiating reaction (Figs. 8, 9) (Tien and Kirk, 1983) (Umezawa *et al.*, 1985) (Odier *et al.*, 1988): removal of a single electron from aromatic nuclei, producing aryl cation radical species, which undergo non-enzymatic reactions as radicals and as cations. These reactions have been claimed to result in polymer cleavages (Hammel *et al.*, 1985) (Kersten *et al.*, 1985). MnP functions to oxidize Mn(II) to Mn(III), which can oxidize phenolic substructures in lignin (Glenn *et al.*, 1985). Although MnP can oxidize phenolic compounds, it has not been demonstrated to mediate degradation of nonphenolic units that dominate in lignin. During recent years, both LiP and MnP have been found in extracellular filtrates of many different white-rot fungi (Waldner *et al.*, 1988). The molecular weight of these enzymes suggests that they do not readily penetrate into sound wood, since the pores of the cell wall would not be large enough for penetration of the enzymes (Strebotnik *et al.*, 1990). An enzymatically generated oxidized manganese species Mn(III) would be able to diffuse into wood, and recent studies suggest that Mn(III) under certain conditions is capable of ligninolytic activity (Forrester *et al.*, 1988).

However, there are many different avenues that future research can pursue to increase our understanding of the enzymes and interactions associated with lignin breakdown. Although it is clear that our current knowledge of delignification processes is incomplete, the potential for using these fungi, or their enzymes, in biotechnological applications is so great that they are already being suggested for industrial applications (Reid, 1983) (Kirk *et al.*, 1983) (Bumpus *et al.*, 1986) (Farrell, 1987) (Farrell *et al.*, 1987).

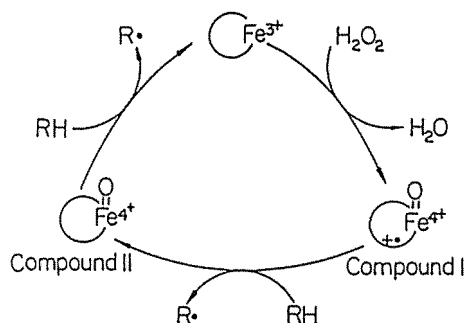


Fig. 6 Catalytic cycle of lignin peroxidase indicating various reduction states of the heme. Resting enzyme reacts with  $\text{H}_2\text{O}_2$  to produce the two-electron oxidized intermediate, compound I, which in turn oxidizes lignin substrate ( $\text{RH}$ ) to yield the one-electron oxidized intermediate, compound II, and a substrate radical. Compound II returns to resting enzyme by oxidizing a second substrate molecule. The free radical ( $\cdot\text{R}$ ) can undergo a variety of reactions.

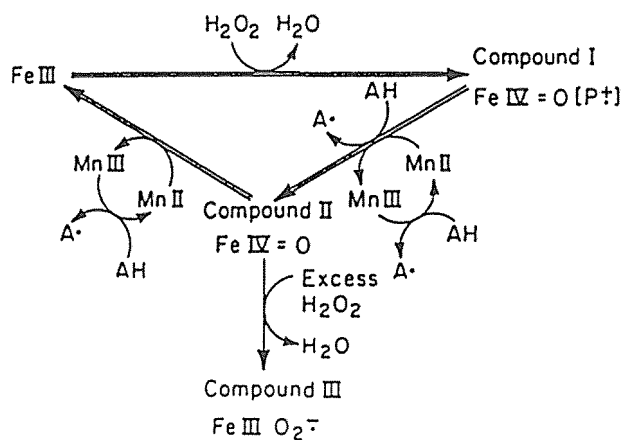


Fig. 7 Catalytic cycle of manganese-dependent peroxidase of *P. chrysosporium*.



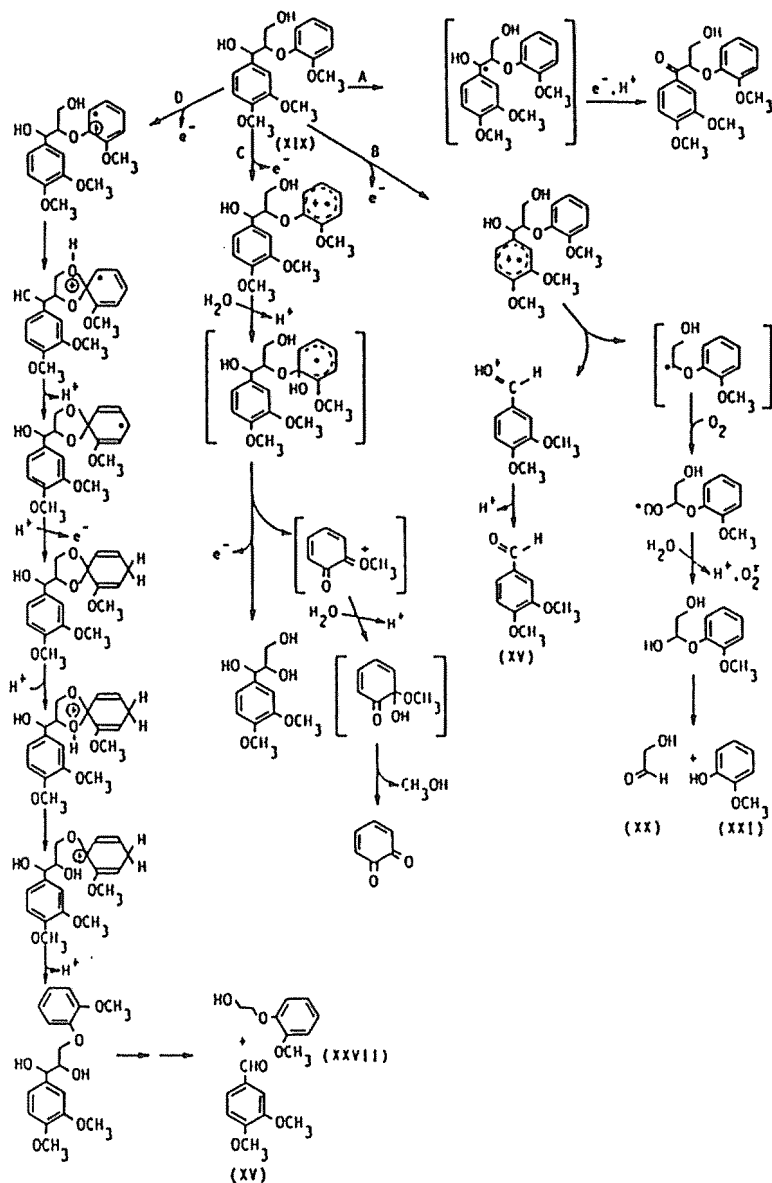


Fig. 8 Degradation pathways for nonphenolic arylglycerol- $\beta$ -aryl ethers by lignin peroxidase; A: ketol formation; B:  $\alpha$ ,  $\beta$ -cleavage; C:  $\beta$ -O-4 cleavage; D: migration of the phenoxy moiety from  $C_\alpha$  to  $C_\beta$  position and  $\alpha$ ,  $\beta$ -cleavage.

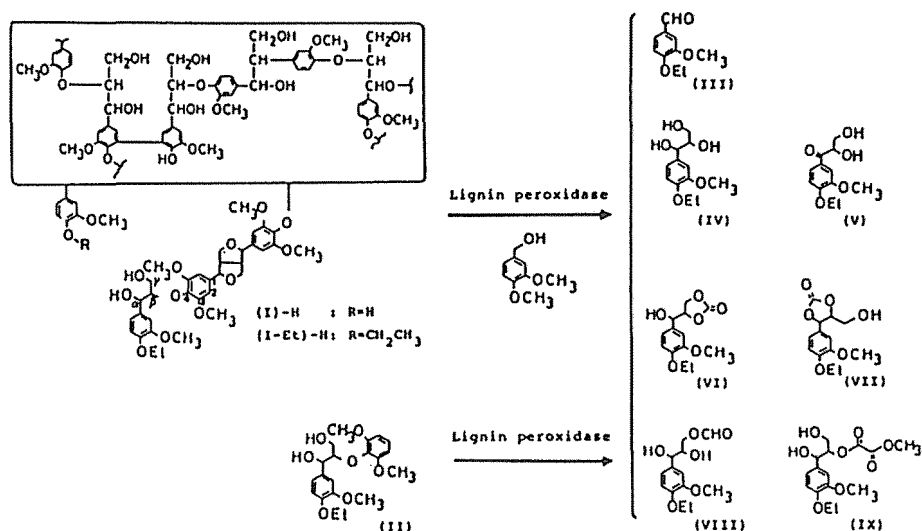


Fig. 9 Degradation of DHP (I)-H, (I-Et)-H, and  $\beta$ -O-4 lignin substructure model dimer (II) by lignin peroxidase in the presence of veratryl alcohol. Note: (III):  $\beta$ -O-4 cleavage product; (IV), (V):  $\beta$ -O-4 bond cleavage products; (VI)-(IX): aromatic ring cleavage products. In the degradation of (I)-H and (I-Et)-H, products formation was dependent on the presence of veratryl alcohol. While the alcohol was not required for the degradation of (II), it accelerated the degradation of (II). In (II)-H and (I-Et)-H, the rectangular enclosure represents an assumed structure of the moiety derived from coniferl alcohol.

### 1.7. Lignin biodegradation with white-rot fungus IZU-154

White-rot fungi, *C. versicolor* and *P. chrysosporium*, are the best known lignin-degrading microorganisms (Kirk *et al.*, 1975) (Lundquist *et al.*, 1977) (Ander *et al.*, 1977). However, their ligninolytic activities are insufficient for industrial application because they degrade the lignin portion of wood quite slowly and also degrade the carbohydrate portion simultaneously (Kirk *et al.*, 1973). In other words, lignin-degradation by these fungi is not sufficiently fast and selective enough for it to be applied commercially to pulping and wood saccharification (Nishida *et al.*, 1988). Therefore, an investigation was performed to find the white-rot fungus showing great ligninolytic activity and selectivity towards wood lignin (Nishida *et al.*, 1988).

A rapid and reliable method was designed for screening ligninolytic wood-rotting fungi by observation of a colored zone which is indicative of extracellular phenoloxidases around the mycelium grown on wood powder and agar medium containing guaiacol (Nishida *et al.*, 1988). By this screening method, fungus IZU-154 has been isolated and characterized to be greatly active and selective in lignin degradation.

This fungus was found to metabolize some phenolic and nonphenolic  $\beta$ -O-4-type lignin model compounds under both low and high nitrogen culture conditions (Nishida *et al.*, 1989 a). Phenolic model compounds (Fig. 10), syringylglycerol-b-syringyl ether, and

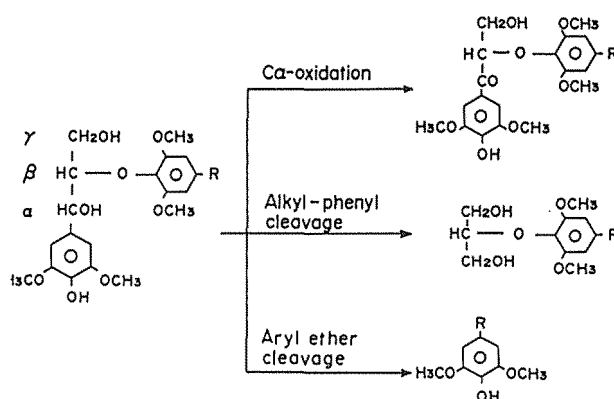


Fig. 10 Degradation products identified from phenolic  $\beta$ -O-4 substructure model compounds by the culture of IZU-154.

syringylglycerol- $\beta$ -(4-methylsyringyl) ether were degraded with this fungus to yield 2-*O*-syringylglycerol and 2,6-dimethoxyphenol from the former and  $\alpha$ -(4-methylsyringyloxy)- $\beta$ -hydroxypropiosyringone, 2-*O*-(4-methylsyringyl) glycerol, and 2,6-dimethoxy-4-methylphenol from the latter. Structures of the degradation products indicated that C $\alpha$ -oxidation, alkyl-phenyl, and aryl ether cleavages could occur with IZU-154. On the other hand, it was suggested that the C-C linkage between C $\alpha$  and C $\beta$  of the propyl side-chain of a nonphenolic model (Fig. 11), 4-*O*-ethylsyringylglycerol- $\beta$ -syringyl ether, was cleaved with this fungus because 4-*O*-ethylsyringyl alcohol was detected in its degradation product.

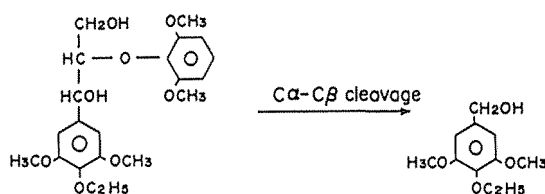


Fig. 11 Degradation products identified from a non phenolic  $\beta$ -O-4 substructure model compound by the culture of IZU-154.

A phenolic  $\beta$ -1 lignin substructure model compound, 1-guaiacyl-2-veratrylpropane-1,3-diol, and a biphenyl (condensed guaiacyl) substructure model compound, dehydrodivanillic acid, were also degraded with IZU-154 under both low and high nitrogen culture conditions (Nishida *et al.*, 1989 b). The former model was degraded to yield 2-veratryl-1,3-propanediol and 2-veratryl-ethanol. The structure of the degradation products indicated that alkyl-phenyl and C $\alpha$ -C $\beta$  cleavages could be brought about by using IZU-154. Dehydrodivanillic acid was also degraded and readily metabolized with IZU-154.

The effect of lignin biodegradation with this fungus on the subsequent enzymatic hydrolysis of beech wood was investigated (Nishida, 1989). The glucose yield of wood pretreated with IZU-154 was about 50 and 60% of the original-wood cellulose after 60 and 80% Klason lignin losses, respectively. This result suggests that IZU-154 preferentially degrades lignin compared with holocellulose, indicating the saccharification of wood by enzymatic hydrolysis is facilitated greatly by the biological pretreatment.

Because the fungus IZU-154 degraded lignin selectively compared with holocellulose and hence had large lignin-degradation selection indexes (Nishida, 1989). A bio-mechanical pulping process with this fungus was performed to take advantages of the fungal treatment as short period as possible (Kashino *et al.*, 1991). It was shown that treatment of hardwood (beech) coarse mechanical pulp with the fungus IZU-154 for only 1 week saved the second refining energy significantly, and the strength properties were increased to almost the same level as a conventional softwood mechanical pulp (Fujita *et al.*, 1991). Also, a biomechanical pulping study of softwood has demonstrated that this fungus achieved significant energy reduction (by one third) and increase in the strength properties after 14 to 10-day fungal treatment of coarse mechanical pulp by inoculating with only the mycelia (Kashino *et al.*, 1991). Moreover, the same effects (energy saving and the improving paper strength) were observed by only one-week treatment with the mycelium suspension which mycelial mat was fragmented without separating from medium.

Because the white-rot fungus IZU-154 extensively and selectively degrades wood lignin (Nishida *et al.*, 1988), this fungus was used to delignify and brighten unbleached kraft pulp. Four white-rot fungi (IZU-154, *C. versicolor*, *P. chrysosporium*, and *Coriolus hirsutus*) were tested for their ability to directly bleach hardwood kraft pulp (Fujita *et al.*, 1991). The greatest increase in pulp brightness was obtained using the fungus IZU-154. The combined fungal (F) and chemical (CED) bleaching process could significantly reduce the use of chlorine-based chemicals and the pollution load of waste liquors compared with the CEDED process. Softwood kraft pulp was also biobleached using this fungus (Fujita *et al.*, 1993). Bleaching of the pulp with IZU-154 and with a successive short CED sequence resulted in the same pulp properties (brightness, strength and pulp yield) as a conventionally bleached (CEDED) pulp while consuming significantly less chlorine. A treatment with this fungus was also applied to bleaching of oxygen bleached hardwood kraft pulp, with aiming to establish a chlorine-free bleaching process with the fungus (Murata *et al.*, 1992). It was revealed that the oxygen-delignified pulp can be fully bleached by a bleaching sequence composed of fungal treatment (F), alkaline extraction (E), and peroxide bleaching (P). However, relatively long periods of F stage and relatively large charge of  $H_2O_2$  were necessary to obtain properties of the bleached pulp that were comparable to those of conventional OCED-bleached pulp.

Since an absolutely chlorine-free chemical bleaching process has not been put to practical use so far, the residual lignin in unbleached kraft pulp is commonly removed

through a multi-stage bleaching process with the use of chlorine species. Therefore, studies are still needed to accomplish efficient treatment of kraft bleachery effluent with less energy and in relatively short treatment-period.

The present research was first attempted the decolorization of the E<sub>1</sub> effluents with the fungus IZU-154. Moreover, for a reality of the E<sub>1</sub> effluent treatment on a commercial scale, the screening of fungi that show the efficient decolorization of the E<sub>1</sub> effluent without any additional nutrients have performed. Finally, the treatment of E<sub>1</sub> effluent with a newly found fungus KS-62 was investigated in more detail. The experiments are consisted of 5 Chapters as follows:

- 2: Decolorization of kraft bleaching effluents with the lignin-degrading fungus IZU-154
- 3: Detection of nucleic acid constituents in the E<sub>1</sub> effluent treated with the lignin-degrading fungus IZU-154
- 4: An accelerating effect of glucono- $\delta$ -lactone on the fungal decolorization of the E<sub>1</sub> effluent
- 5: Screening of lignin-degrading fungi for the efficient decolorization and treatment of the E<sub>1</sub> effluent without additional nutrients with a newly found KS-62
- 6: Successive treatment of the E<sub>1</sub> effluent with immobilized mycelium of the fungus KS-62

## **2. Decolorization of Kraft Bleaching Effluents with the Lignin-degrading Fungus IZU-154**

### **2.1. Introduction**

White-rot fungi have active ligninolytic systems that degrade even heavily modified lignin such as kraft lignin and chlorinated lignin. Several investigators demonstrated that kraft bleaching effluent can be decolorized by the cultures of white-rot fungi, in particular, *C. versicolor* and *P. chrysosporium*. The color removal of kraft bleaching effluents with *C. versicolor* resulted from mycelial pellets or calcium-alginate-immobilized mycelium in the batch cultures (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983). Moreover, the presence of malt extract and molasses as complex co-substrates (Bergbauer *et al.*, 1991) or the combination of molasses and brewery cake (Archibald *et al.*, 1990) enhanced the decolorization. With *P. chrysosporium*, about 2,100 color-unit (CU) reductions in kraft bleaching effluents were achieved in four-day of incubation at 39°C under 80% oxygen with agitation (Eaton *et al.*, 1980) and about 3,000 CU of color was removed after five-day of incubation without agitation under a 100% oxygen atmosphere (Sundman *et al.*, 1981). The addition of a co-substrate, such as glucose or cellulose, was necessary for effective decolorization (Eaton *et al.*, 1980).

The white-rot fungus IZU-154 showed great ligninolytic activity and selectivity towards wood lignin (Nishida *et al.*, 1988), and biomechanical pulping (Kashino *et al.*, 1991) and biobleaching (Fujita *et al.*, 1991) (Murata *et al.*, 1992) (Fujita *et al.*, 1993) also were successfully performed with the fungus IZU-154.

In this chapter, the authors summarize the results of the decolorization of the first alkaline extraction stage ( $E_1$ ) effluents with the fungus IZU-154, compared with *P. chrysosporium* and *C. versicolor*.

## 2.2. Materials and methods

### 2.2.1. Microorganisms

The fungus IZU-154 (Nishida *et al.*, 1988), *C. versicolor* (IFO-6482) and *P. chrysosporium* (ME 446) were used.

### 2.2.2. Bleaching effluents

The  $E_1$  effluent from softwood kraft pulp bleaching (N- $E_1$  effluent) was obtained from a kraft pulp mill.

From unbleached hardwood kraft pulp, L- $E_1$  effluent was prepared under the following conditions: for the C (chlorine) stage, the pulp (kappa number 18.5) was suspended at 4% consistency at room temperature, with 4.44% of active chlorine. After 40 min, the pulp was filtered and washed with water. The  $E_1$  stage was performed at 10% consistency with 2.7% NaOH at 70°C. After 40 min, the pulp was filtered off, and the filtrate (L- $E_1$  effluent) was collected and stored in a refrigerator until required.

To remove suspended particles, the N- $E_1$  and L- $E_1$  effluents were filtered through a glass fiber filter paper (Advantec GB140) before use. The  $E_1$  effluent with or without additive was adjusted to pH 4.5 with 1.0 N HCl or 1.0 N NaOH and sterilized by autoclaving (110°C, 10 min) before fungal treatment. The L- $E_1$  and N- $E_1$  effluents contained 7000 and 7000 to 10000 CU, respectively.

### 2.2.3. Treatment methods of the $E_1$ effluents

In order to decolorize the  $E_1$  effluents with white-rot fungi, two treatment methods were used.

Treatment method A was as follows: the three white-rot fungi were maintained on potato dextrose agar (Difco labs) slants. Potato dextrose agar plates were inoculated with each fungus and incubated for 3-7 days at 30°C. Three disks punched from the grown edge of the mycelium were aseptically added to each 100 ml flask containing 10 ml of the  $E_1$  effluent which was supplemented with nutrients A consisting of 5.0 g of glucose, 0.2 g of ammonium tartrate, 1.0 g of  $KH_2PO_4$ , 0.2 g of  $NaH_2PO_4$ , 0.5 g of  $MgSO_4 \cdot 7H_2O$ , 1.46 g of 2,2-dimethylsuccinate, 100  $\mu g$  of thiamine-HCl, 100  $\mu g$  of  $CaCl_2$ , 100  $\mu g$  of  $FeSO_4 \cdot 7H_2O$ , 10  $\mu g$  of  $ZnSO_4 \cdot 7H_2O$  and 20  $\mu g$  of  $CuSO_4 \cdot 5H_2O$  in one liter of the  $E_1$  effluent. The  $E_1$  effluent was incubated statically with each white-rot fungus for 10 days at 30°C.

Treatment method B was as follows: each white-rot fungus inoculated with an potato dextrose agar plate was homogenized for 10 sec with 50 ml of PMY (glucose 3.0%, pepton 1.0%, malt extract 1.0%, yeast extract 0.4%) medium using a sterile Waring Blender to give a mycelium suspension culture. The culture was then put in a 500 ml Erlenmyer flask with 150 ml of PMY medium and shaken at 150 rpm. After three days, 50 ml of the culture was taken, homogenized once again and then further precultured in a 500 ml Erlenmyer flask with 200 ml of PMY medium for three days on a rotary shaker (150 rpm). The precultured mycelium of white-rot fungus was separated from the PMY medium and then aseptically added to the N-E<sub>1</sub> effluent with or without additives. For decolorization, 1.2 g dry weight of mycelium was added to 60 ml of N-E<sub>1</sub> effluent in a 300 ml flask.

#### 2.2.4. Determination of color units of the E<sub>1</sub> effluents

After incubation for a designated period, the mycelium was removed by filtration through a 0.45  $\mu$ m pore size membrane filter (Advantec). Absorbance readings at 465 nm of both the original and the decolorized E<sub>1</sub> effluents were converted to color units (CU) by the equation:  $CU = 500 \times A_{465} / 0.132$  where 0.132 is the absorbance of 500 CU platinum-cobalt standard solution (Davis *et al.*, 1990).

### 2.3. Results

#### 2.3.1. Decolorization of the E<sub>1</sub> effluents by treatment method A

The L-E<sub>1</sub> effluent treatment by the method A was performed with three white-rot fungi (IZU-154, *C. versicolor* and *P. chrysosporium*) at 30°C for 10 days. As shown in Fig. 12, the fungus IZU-154 started on the third day abruptly to decolorize the L-E<sub>1</sub> effluent at a prominent color removal rate. Sixty-one % (4270 CU) and 86% (6020 CU) of the color were removed with IZU-154 after five and seven days of incubations, respectively. On the other hand, the color removals with *C. versicolor* and *P. chrysosporium* started on the fifth day, and partial color removal of about 3700 CU (53%) took place after seven days of incubations.

The color removals of N-E<sub>1</sub> effluent with the three white-rot fungi proceeded in a way similar to that of the L-E<sub>1</sub> effluent as can be seen in Fig. 13. The fungus IZU-154 also achieved high decolorization degrees of 4550 CU (65%) and 5530 CU (79%) within five and seven days of incubations, respectively, but the color removals with *C. versicolor* and *P. chrysosporium* started slowly on the third and fifth days, respectively, and slight decolorization (about 2800 CU-40%) occurred in seven days of incubations. As no apparent adsorptions of chromophores on the surfaces of the mycelia of the three white-rot fungi were observed, the fungus IZU-154 was selected to further optimize the extent of decolorization in more detail because it showed the high activity for decolorization of the E<sub>1</sub> effluents.

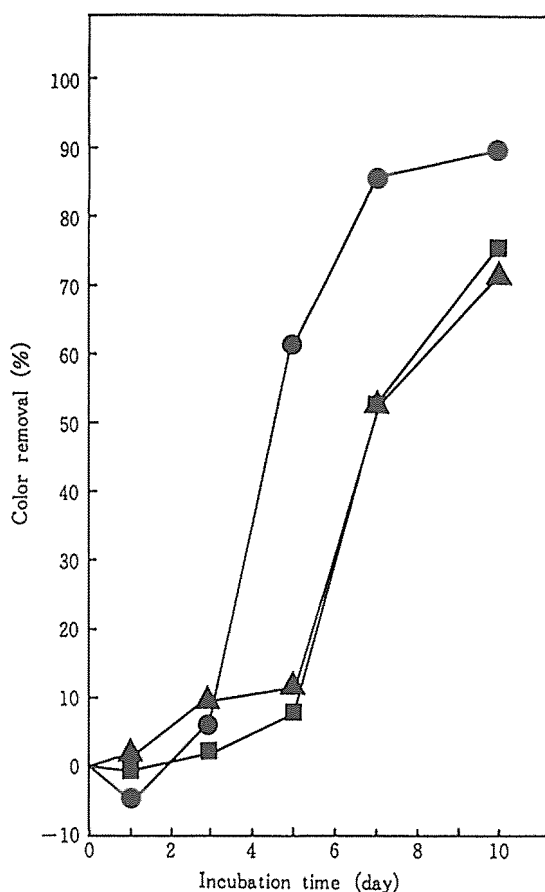


Fig. 12 Decolorization of L-E<sub>1</sub> effluent with white-rot fungi (initial color unit ; 7000 CU, treatment method A).

Legend : ● : IZU-154, ▲ : *C. versicolor*, ■ : *P. chrysosporium*.

### 2.3.2. Effects of nutrients on the decolorization of the N-E<sub>1</sub> effluent by the treatment method A

To elucidate the effects of glucose concentrations on the decolorization, the fungus IZU-154 was incubated with the N-E<sub>1</sub> effluent including nutrients A, but glucose concentration was changed from 0 to 4.0%. As shown in Fig. 14, the addition of glucose significantly enhanced the color removal degree, but in the absence of glucose, a decrease of color was not detected. For the N-E<sub>1</sub> effluent containing 0.5% and 0.1% glucose, 4620 CU (66%) and 3220 CU (46%) of the color, respectively, were removed within five days of incubation. An increase of the glucose concentration of more than 0.5% did not indicate any more increase of decolorization of the N-E<sub>1</sub> effluent than did the result of the 0.5% glucose.

Figure 15 shows the effects of available nitrogen on the color removal of the N-E<sub>1</sub>



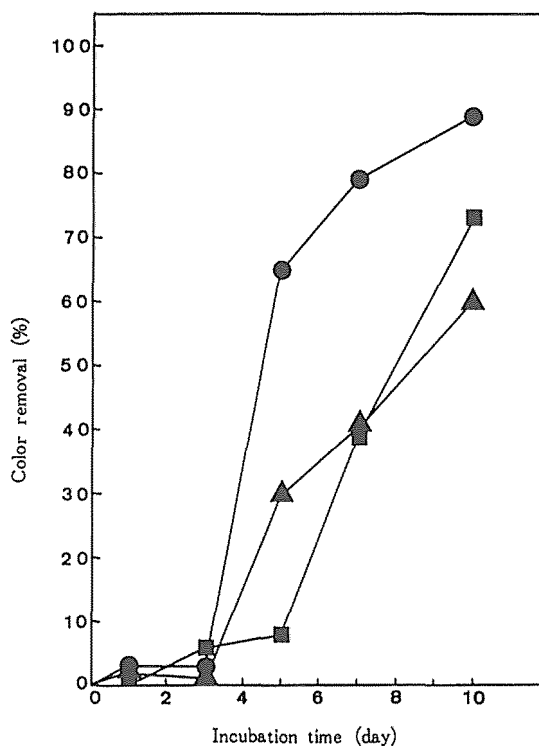


Fig. 13 Decolorization of N-E<sub>1</sub> effluent with white-rot fungi (initial color unit ; 7000 CU, treatment method A).  
 Legend : ● : IZU-154, ▲ : *C. versicolor*, ■ : *P. chrysosporium*.

effluent including nutrients A when ammonium tartrate was added at either 0, 0.005%, 0.01% or 0.02%. Both N-limited (0.005%) and N-excess (0.02%) conditions showed significant effects on the decolorization rates to afford a 6300 CU (90%) color reduction, respectively, within a 10-day incubation under the conditions tested here. Unlike the case of the addition of glucose, even without additional nitrogen a great decolorization (5250 CU-75%) of the N-E<sub>1</sub> effluent was observed within 10 days of incubation.

As the fungal decolorization of the E<sub>1</sub> effluents in the treatment method A exhibited a lag-period of 3-5 days before the rapid decolorization started, it was suggested that the decolorization was accompanied by the fungal growth in the E<sub>1</sub> effluents. Therefore, in order to shorten the lag-period for fungal growth, the E<sub>1</sub> effluents were treated with precultivated fungi in PMY medium without the E<sub>1</sub> effluents.

### 2.3.3. Decolorization of the N-E<sub>1</sub> effluent by the treatment method B

At first, the decolorization of the N-E<sub>1</sub> effluent was done by the treatment method B using the fungus IZU-154 under four kinds of medium conditions, that is, the N-E<sub>1</sub> effluent with nutrients A, with nutrients A without ammonium tartrate, with 0.5% glucose and

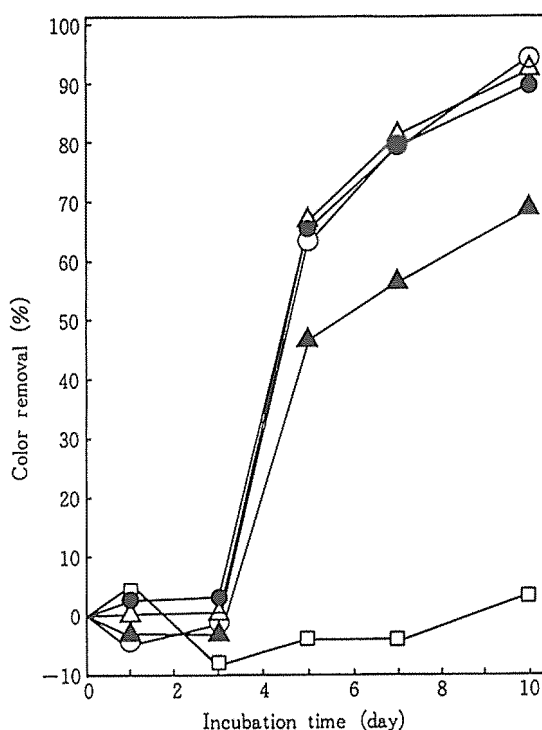


Fig. 14 The effect of glucose concentration on decolorization of N-E<sub>1</sub> effluent with IZU-154 (initial color unit ; 7000 CU, treatment method A).

Legend : ○ : 4.0%, ● : 2.0%, △ : 0.5%, ▲ : 0.1%, □ : without glucose.

without any additives. The results are shown in Fig. 16. In all cases with the additions of nutrients, the rates and extents of the E<sub>1</sub> effluent decolorizations were enhanced so that 83-86% of color removal obtained for one day of incubation, although partial color removal (2940 CU-42%) took place in the absence of additives. This result showed that only a small amount of glucose (0.5%) was necessary, but other additives such as ammonium tartrate as the N-source and trace elements were scarcely required for decolorization of the E<sub>1</sub> effluent with IZU-154.

The decolorization abilities of the three white-rot fungi were determined using the E<sub>1</sub> effluent containing 0.5% glucose. As shown in Fig. 17, for the E<sub>1</sub> effluent containing 10000 CU, treatment with IZU-154 resulted in 7800 CU (78%) and 8900 CU (89%) reductions of color after one- and two-day incubations, respectively. On the other hand, when the E<sub>1</sub> effluent was treated under the same conditions, only 3200 CU (32%) and 3600 CU (36%) of decolorizations with *C. versicolor* and 4900 CU (49%) and 7200 CU (72%) of decolorizations with *P. chrysosporium* were observed within one- and two-day incubations, respectively.

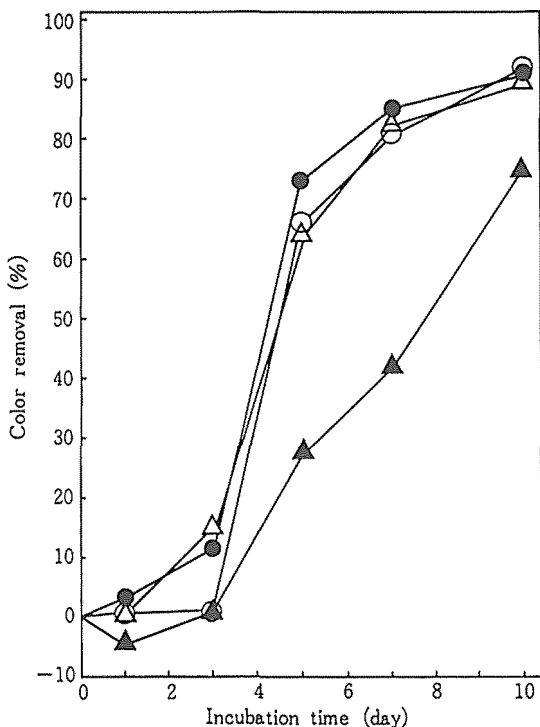


Fig. 15 The effect of ammonium tartrate concentration on decolorization of N-E<sub>1</sub> effluent with IZU-154 (initial color unit ; 7000 CU, treatment method A).

Legend : ○ : 0.02%, ● : 0.01%, △ : 0.005%, ▲ : without ammonium tartrate.

#### 2.4. Discussion

The white-rot fungus IZU-154 showed great ligninolytic activity and selectivity towards wood lignin (Nishida *et al.*, 1988), compared with typical lignin-degrading fungi, *C. versicolor* and *P. chrysosporium*. The fungus IZU-154 also showed successful results on the biobleaching of kraft pulp (Fujita *et al.*, 1991) (Murata *et al.*, 1992) (Fujita *et al.*, 1993). Therefore, we investigated the possibility of the application of the fungus IZU-154 for the decolorization of the E<sub>1</sub> effluents. It can be concluded that IZU-154 showed great decolorization effects on the E<sub>1</sub> effluents, compared with *C. versicolor* and *P. chrysosporium*.

No white-rot fungus has been convincingly shown to use lignin as its principal metabolic carbon or energy source (Ander *et al.*, 1975) (Kirk *et al.*, 1976). Therefore, the addition of easy metabolizable nutrients such as pure glucose or glucose-containing complex microbiological media were required during the decolorization of chlorolignins with *C. versicolor* (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983). Yin *et al.* also have indicated that to degrade lignin and lignin-derived chromophoric structures using *P. chrysosporium*, a co-substrate such as glucose must be added (Yin *et al.*, 1989 b). In order

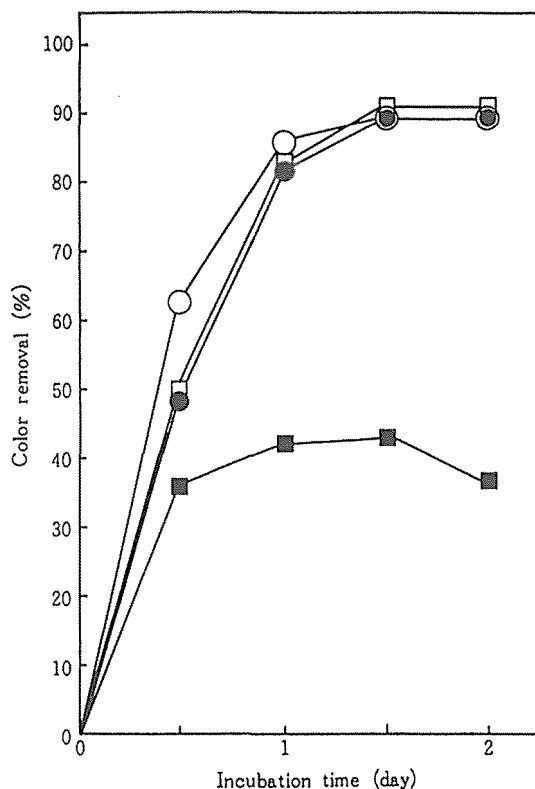


Fig. 16 The effect of nutrients on decolorization of N-E<sub>1</sub> effluent with IZU-154 (initial color unit ; 7000 CU, treatment method B).  
 Legend : ○ : E<sub>1</sub> effluent containing nutrients A, ● : E<sub>1</sub> effluent containing nutrients A without ammonium tartrate, □ : E<sub>1</sub> effluent with 0.5% of glucose, ■ : E<sub>1</sub> effluent without additives.

to obtain high degrees of decolorization of the E<sub>1</sub> effluents with IZU-154, a small amount of a suitable additive such as glucose was required.

The decolorization with this fungus, IZU-154, was hereinafter investigated in further detail, because the fungus IZU-154 showed the most effective decolorization of the E<sub>1</sub> effluent.

## 2.5. Summary

Three white-rot fungi (IZU-154, *P. chrysosporium*, *C. versicolor*) were tested for their abilities to decolorize kraft bleaching effluents from the first alkaline extraction stage. By the method A, the fungus IZU-154 exhibited the effective decolorization of not only hardwood but also softwood kraft pulp bleaching effluent which were supplemented with the nutrients A, compared with *C. versicolor* and *P. chrysosporium*.

For the softwood bleach plant effluent containing 10000 color units (CU), treatment

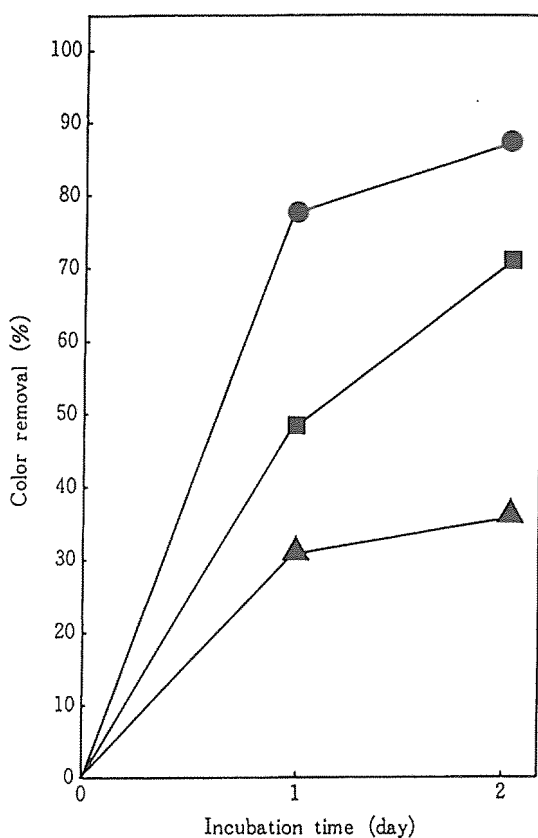


Fig. 17 Decolorization of N-E<sub>1</sub> effluent with white-rot fungi (initial color unit ; 10000 CU, treatment method B).  
 Legend : ● : IZU-154, ▲ : *C. versicolor*, ■ : *P. chrysosporium*.

with IZU-154 by the method B, in the presence of a small amount of glucose (0.5%), resulted in 78% and 89% reductions of the color after one- and two-day incubations, respectively. On the other hand, when the effluent was treated under the same conditions, only 32% and 36% of the decolorization with *C. versicolor* and 49% and 72% of the decolorization with *P. chrysosporium* were observed within one- and two-day incubations, respectively.

### 3. Detection of Nucleic Acid Constituents in the E<sub>1</sub> Effluent Treated with the Lignin-degrading Fungus IZU-154

#### 3.1. Introduction

In Chapter 2, the fungus IZU-154 having great ligninolytic activity and selectivity towards wood lignin showed high decolorization of the first alkaline extraction stage (E<sub>1</sub>) effluents within a short treatment period, compared with *C. versicolor* and *P.*

*chrysosporium*.

When the E<sub>1</sub> effluent was treated with IZU-154 for different periods of time, the author found the phenomenon that an absorbance of the E<sub>1</sub> effluent at 280 nm decreased once, and then increased with the fungal treatment time, although color units of the E<sub>1</sub> effluent were decreasing. Although many papers have demonstrated that kraft mill effluents can be decolorized with the white-rot fungi *C. versicolor* (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983) (Royer *et al.*, 1983) (Archibald *et al.*, 1990) (Bergbauer *et al.*, 1991) and *P. chrysosporium* (Eaton *et al.*, 1980) (Sundman *et al.*, 1981) (Campbell *et al.*, 1982) (Yin *et al.*, 1989 a) (Yin *et al.*, 1989 b), no report has commented on this phenomenon. In this Chapter, the authors have attempted to clarify this phenomenon.

## 3.2. Materials and methods

### 3.2.1. Microorganisms

Three white-rot fungi used were IZU-154, *C. versicolor* and *P. chrysosporium* as described in Chapter 2.

### 3.2.2. Bleaching effluent

E<sub>1</sub> effluent from softwood kraft pulp bleaching (N-E<sub>1</sub> effluent, 10000 CU) was obtained from a pulp mill.

### 3.2.3. Preparation of inoculum

To prepare the mycelium of the white-rot fungus IZU-154, preincubation was performed with the following five medium conditions:

- (1) PMY medium (pH 6.3); 3.0% of glucose, 1.0% of peptone (Kyokuto), 1.0% of malt extract (Difco) and 0.4% of yeast extract (Difco).
- (2) PM medium (pH 6.3); 3.0% of glucose, 1.0% of peptone and 1.0% of malt extract.
- (3) PY medium (pH 6.4); 3.0% of glucose, 1.0% of peptone and 0.4% of yeast extract.
- (4) MY medium (pH 6.0); 3.0% of glucose, 1.0% of malt extract and 0.4% of yeast extract.
- (5) Synthetic medium containing the following components (per liter); 20 g of glucose, 0.2 g of ammonium tartrate, 1.0 g of KH<sub>2</sub>PO<sub>4</sub>, 0.26 g of NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O, 0.5 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.46 g of 2,2-dimethylsuccinate, 100 µg of thiamine-HCl, 100 µg of CaCl<sub>2</sub>, 100 µg of FeSO<sub>4</sub> · 7H<sub>2</sub>O, 10 µg of ZnSO<sub>4</sub> · 7H<sub>2</sub>O and 20 µg of CuSO<sub>4</sub> · 5H<sub>2</sub>O. This medium was adjusted to pH 4.5 with 1.0 N HCl.

The fungus IZU-154, inoculated with a potato dextrose agar plate, was homogenized for 10 sec with 50 ml of the medium described above using a sterile Waring Blender to give a mycelium suspension culture. The culture was then put in a 500 ml Erlenmeyer flask with 150 ml of the medium and shaken at 150 rpm at 30°C. After three days, 50 ml of the culture was homogenized once again and then further precultured in a 500 ml Erlenmeyer flask with 200 ml of the medium for three days on a rotary shaker (150 rpm). The

precultured mycelium of the fungus IZU-154 was separated from the medium and then aseptically added to the E<sub>1</sub> effluent. *C. versicolor* and *P. chrysosporium* were preincubated with PMY medium.

### 3.2.4. Fungal treatment and analysis of the E<sub>1</sub> effluent

The E<sub>1</sub> effluent containing 0.5% of glucose was adjusted to pH 4.5 and sterilized by autoclaving (110°C, 10 min) before fungal treatment. Sixty ml of the E<sub>1</sub> effluent was incubated statically with 1.2 g dry weight of mycelium in a 300 ml flask at 30°C. After incubation for a designated period, the mycelium was removed by filtration through a 0.45  $\mu$ m pore size membrane filter (Advantec).

Color units (CU) of both the original and the decolorized E<sub>1</sub> effluent were obtained as described in Chapter 2. Both qualitative and quantitative analyses of the nucleic acid constituents in the treated E<sub>1</sub> effluent were performed by high-performance liquid chromatography (HPLC) with a TSK gel-G 3000 PW column (Tosoh) using 0.1 mol/l lithium chloride as an eluent (flow rate: 0.4 ml/min). The eluate was monitored by absorbance at 260 nm using a Tosoh UV-8011 detector. The analysis of the treated E<sub>1</sub> effluent also was performed with a Waters Model 510 Liquid Chromatograph. The analysis was conducted at 25°C with a m Bondasphere C-18 column (Waters) using distilled water adjusted to pH 3.0 with trifluoroacetic acid as an eluent (flow rate: 0.8 ml/min). The eluent was monitored with a Waters Model 481 absorbance detector at 260 nm.

## 3.3. Results

### 3.3.1. Analysis of the E<sub>1</sub> effluent treated with IZU-154

Figure 18 shows the color removal and the change of UV absorbance at 280 nm of the E<sub>1</sub> effluent treated with IZU-154 precultivated in PMY medium as a function of treatment time. The color of the E<sub>1</sub> effluent decreased by 80% within one day of incubation, afterwards the decolorization degrees of 89% and 91% were attained within two- and three-day incubations, respectively. On the other hand, the reduction of the UV absorbance at 280 nm was 55% for one day of incubation, then the absorbance increased with the fungal treatment time. The degree of increase of the mycelial weight during the treatment of the E<sub>1</sub> effluent was negligibly small: it was less than 5%.

The change of the UV spectrum of the E<sub>1</sub> effluent treated with IZU-154 is shown in Fig. 19. After one day of incubation, the UV absorbance of the effluent decreased, which indicates that portions of the lignin components of the effluent were degraded. When the fungal treatment period was extended, however, the UV spectrum of the E<sub>1</sub> effluent had a maximum absorption at about 260 nm. The absorbance of this maximum band increased with the fungal treatment time. Components responsible for the band at about 260 nm were supposed to be nucleic acid constituents.

The HPLC profiles for the E<sub>1</sub> effluent before and after treatment with IZU-154 for

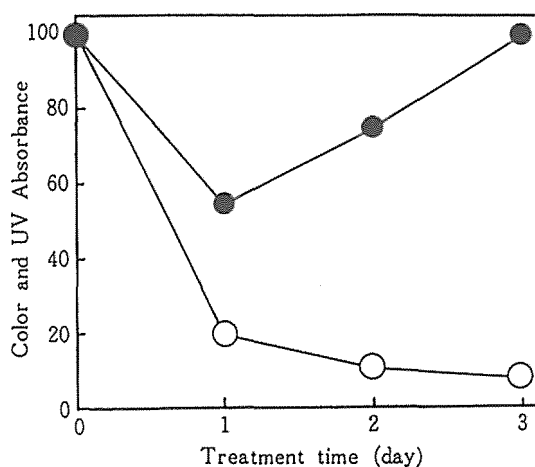


Fig. 18 Decolorization and reduction of UV absorbance of N-E<sub>1</sub> effluent with IZU-154 (initial color unit ; 10000 CU, treatment method B).

Legend : ○ : Color, ● : UV absorbance.

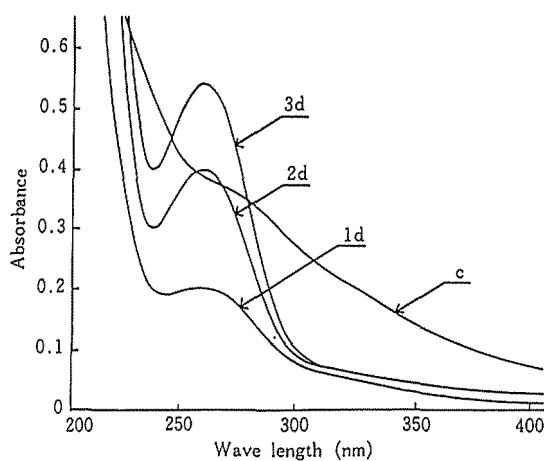


Fig. 19 Change of UV spectrum of E<sub>1</sub> effluent treated with IZU-154.

Notes : C : Control, 1d : After treatment for 1 day, 2d : After treatment for 2 days, 3d : After treatment for 3 days.

two days are shown in Fig. 20. Five main components (A-E) being not regarded as effluent components were detected. Table 1 shows the elution volumes of authentic samples of three nucleosides and two nucleobases analyzed with two kinds of columns using the chromatographic conditions described in the paragraph of Materials and Methods of this Chapter. By direct comparison with these authentic samples, the peaks A, B, C, D and E in Fig. 20 were regarded as uridine, uracil, guanosine, adenosine and adenine, respectively. Therefore, it is assumed that two nucleobases (uracil and adenine) and three nucleosides (uridine, guanosine and adenosine) were present in the effluent



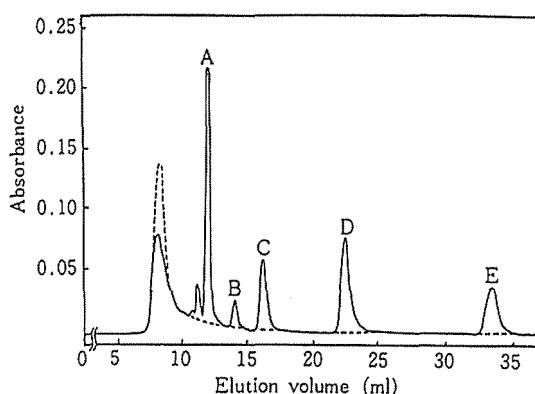


Fig. 20 HPLC profile for E<sub>1</sub> effluent treated with IZU-154.

Notes : Column : TSK gel-G3000PW, Eluenting solvent : 0.1 mol/l lithium chloride, Flow rate : 0.4 ml/min, Detector : 260nm.

Legend : - - : Before fungal treatment, — : After fungal treatment.

Table 1 Elution volumes of nucleosides and nucleobases by HPLC.

Compounds	Elution volumes (ml)		Components <sup>a)</sup>
	TSK gel-G3000PW	μ Bondasphere C-18	
Uridine	12.3	10.5	A
Uracil	14.1	3.5	B
Guanosine	16.2	37.0	C
Adenosine	22.6	41.1	D
Adenine	33.5	6.7	E

a) The components correspond to the peaks in Fig. 20.

treated with IZU-154.

### 3.3.2. Examination of the formations of nucleic acid constituents in the E<sub>1</sub> effluents treated with other lignin-degrading fungi

To determine whether or not this phenomenon is characteristic of IZU-154 or a general tendency of white-rot fungi, other white-rot fungi, *C. versicolor* and *P. chrysosporium* were incubated with the E<sub>1</sub> effluent under the same conditions as with IZU-154. The mycelium of each white-rot fungus was preincubated with PMY medium. The HPLC profiles of the effluent treated with two white-rot fungi are shown in Fig. 21. For the E<sub>1</sub> effluent containing 0.5% glucose, 47% and 71% of decolorizations with *C. versicolor* and *P. chrysosporium*, respectively, were observed, when the treatment with IZU-154 resulted in a 83% reduction after two days of incubation. In contrast to the results obtained with the fungus IZU-154, the nucleic acid constituents were hardly detected in the E<sub>1</sub> effluents treated with *C. versicolor* and *P. chrysosporium*. The phenomenon, for which no report has mentioned to date although many researchers have demonstrated

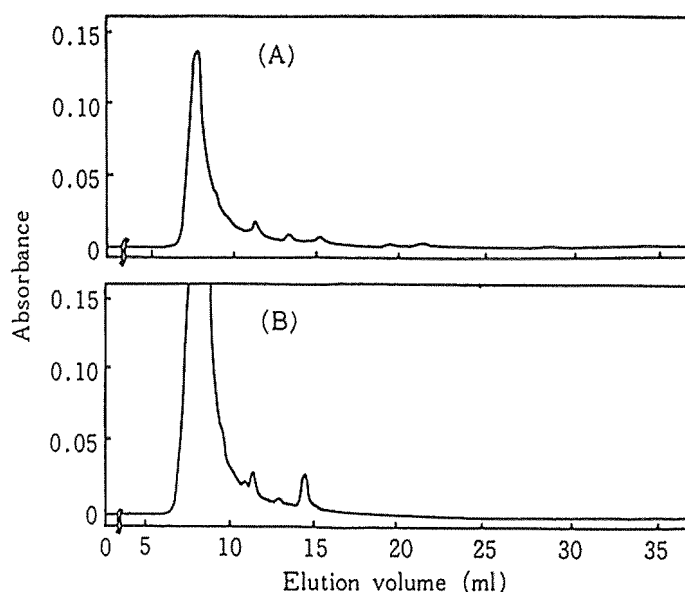


Fig. 21 HPLC profile for  $E_1$  effluent treated with white-rot fungi.  
 Note : Chromatographic conditions are described in the notes of Fig. 20.  
 Legend : (A) : *P. chrysosporium*, (B) : *C. versicolor*.

that kraft mill effluents can be decolorized with white-rot fungi, is a characteristic of IZU-154 having great ligninolytic activity and selectivity towards wood lignin.

### 3.3.3. Effects of preculture conditions of IZU-154 on the formations of nucleic acid constituents and the decolorization of the $E_1$ effluent

To explain the effects of preculture conditions of IZU-154 on the formations of nucleic acid constituents in the treatment media and the decolorization of the  $E_1$  effluent, the effluent was treated for two days with the fungus IZU-154 prepared under various precultural media conditions. The decolorization of the effluent and the amounts of nucleic acid constituents formed are shown in Table 2. The nucleic acid constituents were considerable in the  $E_1$  effluent treated with the mycelium prepared with PMY, PY and MY media. On the other hand, the quantitative yields of these compounds were quite small in the treatment with the mycelium prepared with PM medium. None of the nucleic acid constituents were detected, when the effluent was treated with the mycelium prepared with synthetic medium. That is to say, the nucleic acid constituents in the  $E_1$  effluent treated with IZU-154 were thought to be derived from the yeast extract contained in the precultivation media.

Figure 22 shows the HPLC profile for the yeast extract aqueous solution analyzed by a TSK gel G-3000 PW column. The peaks A, B, C, D and E correspond to the peaks in Fig. 20. By quantitative investigation, however, the HPLC profile for the yeast extract

Table 2 Effect of preculture medium condition on the decolorization of E<sub>1</sub> effluent and the formation of nucleic acid constituents in the culture medium treated with IZU-154.

Preculture medium	Decolorization (%)	Nuclei acid constituents (ppm)				
		Uridine	Uracile	Guanosine	Adenosine	Adenine
PMY	83	80	4	33	50	22
MY	87	88	6	41	69	23
PY	85	125	7	51	108	27
PM	52	2	9	3	3	0
Synthetic medium	28	0	0	0	0	0

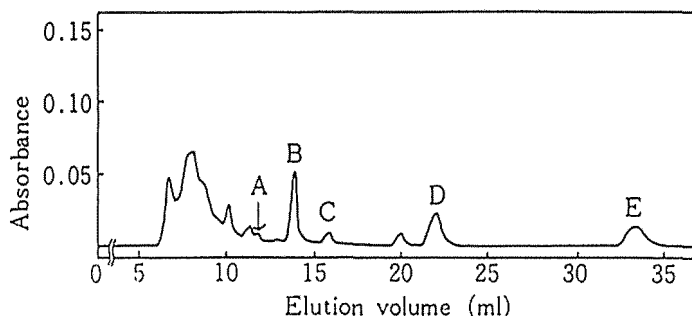


Fig. 22 HPLC profile for 0.4% yeast extract solution.

Note : Chromatographic conditions are described in the notes of Fig. 20.

solution differed widely from that for the E<sub>1</sub> effluent treated with IZU-154. In the treated effluent, uridine and adenosine were present in fairly large quantities, and uracil was detected in small amounts. The yeast extract solution, on the other hand, contained uracil in a large amount, and uridine in a small amount. The incorporation of nucleic acid constituents of the yeast extract into the mycelium and the liberation of these compounds to the treated effluent seemed to be selectively performed by the fungus.

The decolorization of the E<sub>1</sub> effluent was determined using IZU-154 mycelium prepared by various preincubation media conditions. As shown in Table 2, using either mycelium prepared by PMY, PY or MY media conditions, high color removal of over 80% was obtained. However, when the effluent was treated with the mycelium prepared by synthetic or PM media, slight decolorizations of 28% and 52%, respectively, were obtained. That is to say, the greater decolorization of the E<sub>1</sub> effluent was observed when nucleic acid constituents were abundantly detected in the treated effluent.

#### 3.3.4. Effects of treatment conditions with IZU-154 on the formations of nucleic acid constituents and the decolorization of the E<sub>1</sub> effluent

In Chapter 2, the rate and extent of E<sub>1</sub> effluent decolorization in the presence of 0.5% glucose were enhanced so that 82% of the color removal was obtained within one day of incubation, although only partial color removal (41%) took place in the absence of glucose (Fig. 16). Therefore, the relationship between the decolorization of the E<sub>1</sub> effluent and the formation of nucleic acid constituents was studied with the fungus IZU-154 using the effluent with and without glucose. After two days of incubation, 25% of partial color removal took place in the absence of glucose, whereas 83% of decolorization was observed with the addition of glucose. None of the nucleic acid constituents were detected in the treated effluent without glucose.

Furthermore, the E<sub>1</sub> effluent containing 0.5% glucose was treated for two days at 4 °C. The decolorization of this effluent was scarcely 1%, and no formation of the nucleic acid constituents were observed in the treated effluent.

That is to say, the nucleic acid constituents in the treatment medium were not supposed to be formed by either a desorption of these components adsorbed on the surface of the mycelium or a leakage from the fungus, but by the fungal activity with the participation of glucose.

### 3.4. Discussion

In Chapter 2, the fungus IZU-154 showed high decolorization of the E<sub>1</sub> effluents within a short treatment period, compared with *C. versicolor* and *P. chrysosporium*.

When the E<sub>1</sub> effluent was treated with IZU-154 for different periods of time, an absorbance of the E<sub>1</sub> effluent at 280 nm decreased once, and then increased with the fungal treatment time, although color units of the E<sub>1</sub> effluent were decreasing. The UV spectrum of the E<sub>1</sub> effluent treated with IZU-154 had a maximum absorption at about 260 nm. It was assumed that two nucleobases (uracil and adenine) and three nucleosides (uridine, guanosine and adenosine) were present in the effluent treated with IZU-154.

Nucleic acid constituents are of fundamental importance in controlling the reproduction, growth and metabolism of living systems. The fungus IZU-154 showed incorporation of nucleic acid constituents in the culture medium into the fungus during the precultivation period and the liberation of these incorporated constituents to the treated effluent.

Although many papers have demonstrated that kraft mill effluents can be decolorized with the white-rot fungi *C. versicolor* (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983) (Royer *et al.*, 1983) (Archibald *et al.*, 1990) (Bergbauer *et al.*, 1991) and *P. chrysosporium* (Eaton *et al.*, 1980) (Sundman *et al.*, 1981) (Campbell *et al.*, 1982) (Yin *et al.*, 1989 a) (Yin *et al.*, 1989 b), no report has commented on this phenomenon. The phenomenon is a characteristic of IZU-154 having great ligninolytic activity and selectivity towards wood lignin. At the present stage, it would be difficult to explain the relationship between the decolorization of the E<sub>1</sub> effluent and the incorporation and liberation of nucleic acid components during fungal treatment, because the physiological

function-mechanism of the components in the mycelium is still not completely elucidated.

After two days of incubation, 83% of decolorization was observed with the addition of glucose and the nucleic acid constituents were present in fairly large quantities in the treated effluent, whereas 25% of partial color removal took place in the absence of glucose and none of the nucleic acid constituents were detected in the treated effluent. In order to obtain high degrees of decolorization of the E<sub>1</sub> effluents with IZU-154, a small amount of glucose was required.

### 3.5. Summary

When a softwood pulp bleaching effluent from the first alkaline extraction stage was treated with the fungus IZU-154, some nucleic acid constituents, that could not be regarded as being derived from the effluent, were detected. These nucleic acid constituents were estimated to be two nucleobases (uracil and adenine) and three nucleosides (uridine, guanosine and adenosine) with HPLC. These nucleic acid constituents in the E<sub>1</sub> effluent treated with IZU-154 were thought to be derived from yeast extract contained in a precultivation medium. A great decolorization of the E<sub>1</sub> effluent was observed when nucleic acid constituents were detected in the treated E<sub>1</sub> effluent. In contrast to the results obtained with the fungus IZU-154, the nucleic acid constituents were hardly detected in the E<sub>1</sub> effluents treated with *C. versicolor* and *P. chrysosporium*. The phenomenon that the decolorization of the E<sub>1</sub> effluent was well accompanied with the incorporation of the nucleic acid constituents into the fungus and the liberation of them to the treatment medium, was one of the characteristics of IZU-154.

## 4. An Accelerating Effect of Glucono- $\delta$ -lactone on the Fungal Decolorization of the E<sub>1</sub> Effluent

### 4.1. Introduction

Many white-rot fungi have active ligninolytic systems that degrade even heavily modified lignin such as kraft lignin and chlorinated lignin as well as lignin in wood. However, an addition of a co-substrate proved to be essential for effective degradation in all cases. Kirk *et al.* have already reported that *C. versicolor* and *P. chrysosporium* require a growth substrate such as glucose or cellulose in order to metabolize lignin to CO<sub>2</sub> (Kirk *et al.*, 1976).

Several investigators demonstrated that the E<sub>1</sub> effluents can be decolorized with the white-rot fungi, in particular, *C. versicolor* and *P. chrysosporium*. To extensively decolorize the E<sub>1</sub> effluent, the white-rot fungi required a suitable co-substrate such as glucose. When the E<sub>1</sub> effluent with addition of 2.0% glucose was treated with *C. versicolor*, 593 CU (78%) of color removal was obtained for 6-day incubation, but partial color removal (122 CU, 16%) took place in the absence of glucose (Bergbauer *et al.*, 1991). Similar effects of adding glucose on the decolorization of the E<sub>1</sub> effluent have been reported by other

authors for this fungal species (Livernoche *et al.*, 1983) (Archibald *et al.*, 1990). With *P. chrysosporium*, Yin *et al.* and Eaton *et al.* showed that addition of easily metabolizable nutrients such as glucose or cellulose are required during fungal decolorization (Yin *et al.*, 1989 b) (Eaton *et al.*, 1982).

In the previous Chapters 2 and 3, it was demonstrated that the fungus IZU-154 showed high decolorization of the E<sub>1</sub> effluents within a short treatment period, compared with *C. versicolor* and *P. chrysosporium*. In order to obtain high degrees of decolorization of the E<sub>1</sub> effluents with IZU-154, a small amount of a suitable additive such as glucose was required.

In the present Chapter, the authors investigated the effects of various additives including glucose derivatives on the decolorization of the E<sub>1</sub> effluent with IZU-154. It was proved that the addition of glucono- $\delta$ -lactone to the E<sub>1</sub> effluent produces a more remarkable degree of decolorization than did that of glucose. Consequently, attempted have been the clarification of the mechanism of the high color removal obtained with the addition of glucono- $\delta$ -lactone during fungal treatment.

## **4.2. Materials and methods**

### **4.2.1. Microorganisms**

The white-rot fungi used were IZU-154, *C. versicolor* (IFO 6482) and *P. chrysosporium* (ME 446).

### **4.2.2. Bleaching effluents**

E<sub>1</sub> effluents from softwood kraft pulp bleaching (N-E<sub>1</sub> effluents, 5200, 7500 and 10000 CU) were obtained from a kraft pulp mill.

### **4.2.3. Treatment method of the E<sub>1</sub> effluent**

Each white-rot fungus inoculated with a potato dextrose agar plate was homogenized for 10 sec with 50 ml of PMY (glucose 3.0%, pepton 1.0%, malt extract 1.0%, yeast extract 0.4%) medium using a sterile Waring Blender to give a mycelium suspension culture. The culture was then put in a 500 ml Erlenmyer flask with 150 ml of PMY medium and shaken at 150 rpm. After three days, 50 ml of the culture was taken, homogenized once again and then further precultured in a 500 ml Erlenmyer flask with 200 ml of PMY medium for three days on a rotary shaker (150 rpm). The precultured mycelium of white-rot fungus was separated from PMY medium and then added to the E<sub>1</sub> effluent with additives (28 mM). For decolorization, 0.6 g or 1.2 g dry weight of mycelium were added to 60 ml of the E<sub>1</sub> effluent in a 300 ml flask.

### **4.2.4. Analysis**

After incubation for a designated period, the mycelium was removed by filtration through a 0.45  $\mu$ m pore size membrane filter (Advantec).

#### 4.2.4.1. Analysis of the E<sub>1</sub> effluent treated with white-rot fungi

Color units (CU) of both the original and the decolorized E<sub>1</sub> effluent were determined as described in Chapter 2. The change of pH was determined during the fungal treatment period. COD was measured by the Japan Industrial Standard (JIS) method, K 0102.

#### 4.2.4.2. Analysis of additive solution treated with white-rot fungi

The analysis of consumption and conversion of the additives in the treated aqueous solutions was performed by HPLC with a TSK gel Amide-80 column (Tosoh) using 55% CH<sub>3</sub>CN/45% 20 mM KH<sub>2</sub>PO<sub>4</sub> as an eluent (flow rate: 1.0 ml/min, column temperature: 80°C). The eluate was monitored using a Millipore RI-R401 detector.

### 4.3. Results

#### 4.3.1. Effects of various additives on the decolorization of the E<sub>1</sub> effluent with IZU-154

As been mentioned in previous Chapters 2 and 3, adding a small amount of glucose (0.5%) to the E<sub>1</sub> effluent was shown to be essential. Therefore, various carbon sources including mannose, xylose and glycerol were tested as possible substitutes for glucose as can be seen in Fig. 23. After one-day incubation, there were considerable differences in their efficiencies for decolorization of the E<sub>1</sub> effluent containing 7500 CU. In the presence of mannose, 6540 CU (86%) of the color was removed by one-day incubation, but both xylose (4350 CU-58%) and glycerol (5400 CU-72%) were noticeably less efficient than glucose (7125 CU-95%).

Moreover, the same concentration (28 mM, that is equivalent to 0.5% glucose) of various glucose derivatives such as glucono- $\delta$ -lactone, glucuronolactone, sodium gluconate, sodium glucuronate and glucitol were added to the E<sub>1</sub> effluent for testing their efficiencies as additives for decolorization. As shown in Table 3, in comparison with 5300 CU (53%) and 7800 CU (78%) of color reductions achieved with the E<sub>1</sub> effluent including glucose, the addition of glucono- $\delta$ -lactone to the E<sub>1</sub> effluent showed 83% and 94% of decolorizations within 12- and 24-hour incubations, respectively. With glucuronolactone instead of glucose, the reduction of the color was at almost the same efficiency as with glucose. In contrast, sodium gluconate, sodium glucuronate and glucitol had practically no effects on the extents of color removals. Furthermore, organic acids such as glyceric acid, glycolic acid and acetic acid were tested as additives for the decolorization of the E<sub>1</sub> effluent. The addition of acetic acid enhanced effectively the removal of the color of the E<sub>1</sub> effluent, although glyceric and glycolic acids showed no effects on the decolorizations.

The color removal efficiencies of the E<sub>1</sub> effluent with various concentrations of acetic acid as an additive are shown in Fig. 24. The initial rates of decolorization of the E<sub>1</sub> effluent with 0.5% and 0.2% of acetic acid were more than that with 0.5% of glucose within a 12-hour incubation, although decolorization of the E<sub>1</sub> effluent with glucose was more effective than that obtained with acetic acid after a 24-hour incubation.

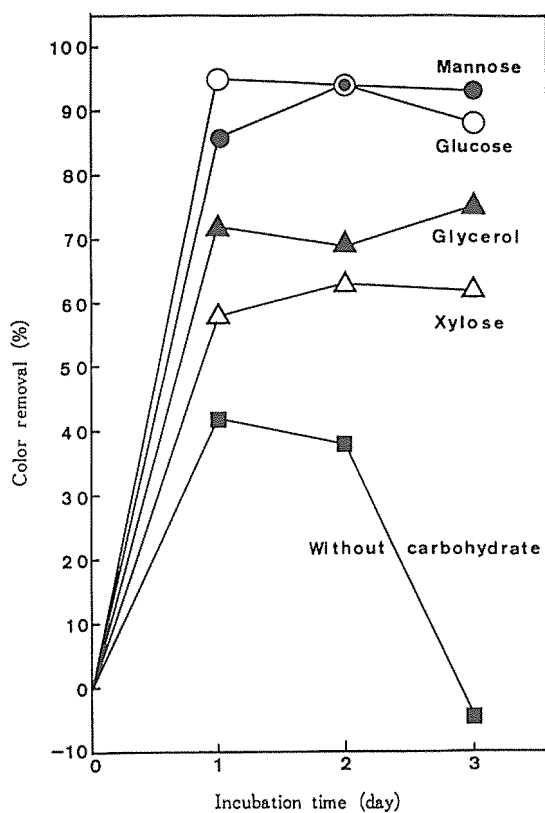


Fig. 23 The effect of various carbohydrates on decolorization of N-E<sub>1</sub> effluent with IZU-154 (initial color unit; 7500 CU, treatment method B).

Table 3 The effect of various additives on decolorization of N-E<sub>1</sub> effluent by IZU-154 (initial color unit; 10000 CU).

Additive	Decolorization (%)	
	12 hr-incubation	24 hr-incubation
Without additive	21	28
Glucose	53	79
Glucono- $\delta$ -lactone	83	94
Glucuronolactone	60	63
Sodium gluconate	32	36
Sodium glucuronate	17	25
Glucitol	21	29
Glyceric acid	n.d.	22
Glycolic acid	n.d.	32
Acetic acid	48	59

n.d. = not determined



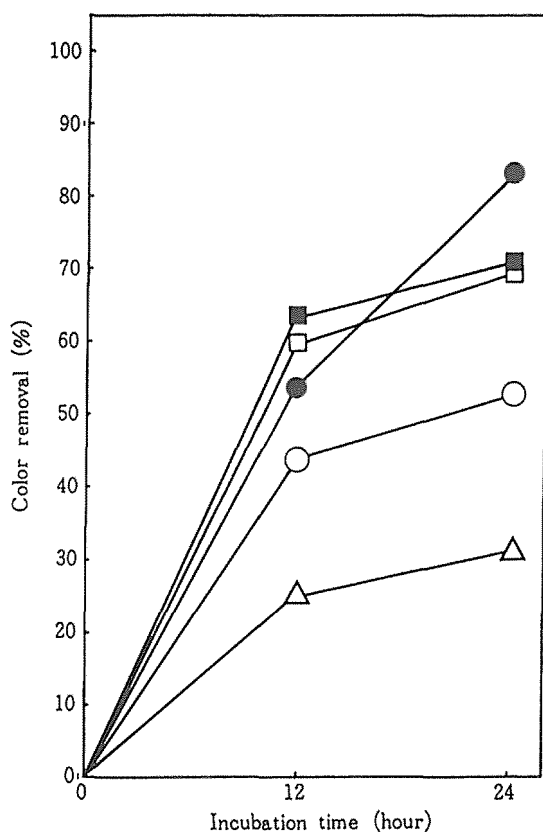


Fig. 24 The effect of acetic acid concentration on decolorization of N-E<sub>1</sub> effluent with IZU-154 (initial color unit; 10000 CU, treatment method B).

Legend : ● : glucose 0.5%, ■ : acetic acid 0.5%, □ : acetic acid 0.2%, ○ : acetic acid 0.1%, △ : without additive.

Because glucono- $\delta$ -lactone was very effective for the decolorization of the E<sub>1</sub> effluent with IZU-154, the effect of concentration of glucono- $\delta$ -lactone on decolorization was determined. As shown in Fig. 25, the extent of color removal of the E<sub>1</sub> effluent increased with an increase of the concentration of glucono- $\delta$ -lactone and the greatest decolorization degree (8300 CU-83%) was observed with the addition of 0.5% glucono- $\delta$ -lactone (28 mM) within a 12-hour incubation. The degree of decolorization by adding 0.2% of glucono- $\delta$ -lactone (11 mM) corresponded to that by adding 0.5% of glucose (28 mM).

#### 4.3.2. Effect of glucono- $\delta$ -lactone on the decolorization of the E<sub>1</sub> effluent with the lignin-degrading fungi

We demonstrated that the addition of glucono- $\delta$ -lactone produced a more remarkable degree of decolorization than did that of glucose. To determine if the high effect of an addition of glucono- $\delta$ -lactone on the decolorization of the E<sub>1</sub> effluent is a general

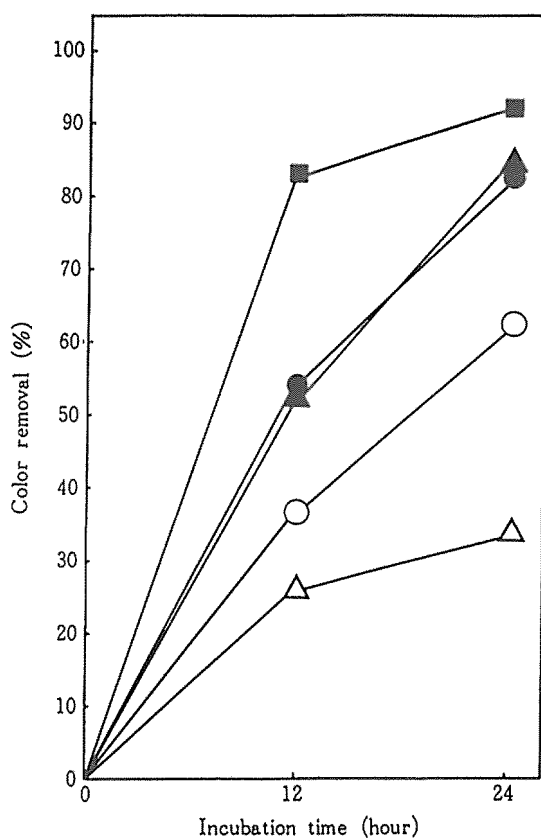


Fig. 25 The effect of glucono- $\delta$ -lactone concentration on decolorization of N-E<sub>1</sub> effluent with IZU-154 (initial color unit; 10000 CU, treatment method B).

Legend : ● : glucose 0.5%, ■ : glucono- $\delta$ -lactone 0.5%, ▲ : glucono- $\delta$ -lactone 0.2%, ○ : glucono- $\delta$ -lactone 0.1%, △ : without additive.

tendency of white-rot fungi, other white-rot fungi, *C. versicolor* and *P. chrysosporium*, were incubated with the E<sub>1</sub> effluent under the same conditions as with IZU-154. The effect of glucono- $\delta$ -lactone on the decolorization of the E<sub>1</sub> effluent with two white-rot fungi were shown in Fig. 26. In the same manner as the results for the fungus IZU-154, the color of the effluents was effectively removed by the two fungi when glucono- $\delta$ -lactone was added as an additive.

It was observed that glucono- $\delta$ -lactone was a very effective additive for the decolorization of the E<sub>1</sub> effluent with the three white-rot fungi. Among them, *C. versicolor* showed the most pronounced effect of glucono- $\delta$ -lactone on the decolorization, compared with that of glucose. Using *C. versicolor*, the mechanism of the high color removal obtained by the addition of glucono- $\delta$ -lactone was hereinafter investigated in further detail.

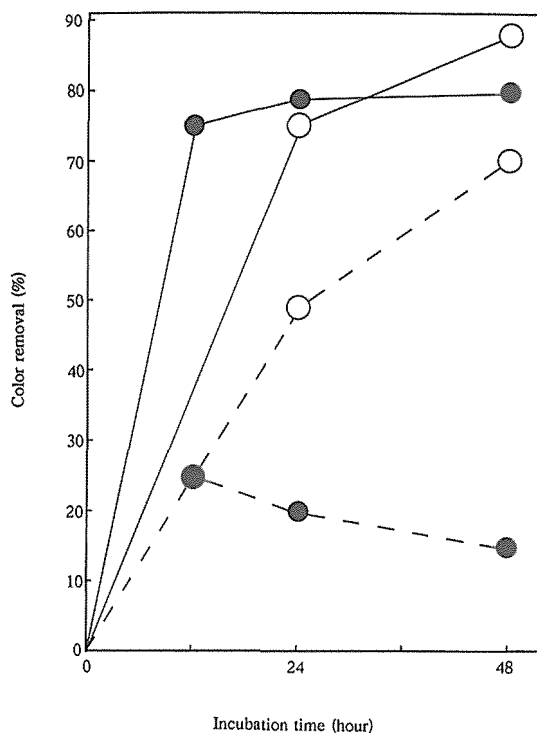


Fig. 26 The effect of additives on decolorization of N-E<sub>1</sub> effluent with white-rot fungi (initial color unit; 7500 CU, treatment method B).

Legend : ● : *C. versicolor*, ○ : *P. chrysosporium*, - - : glucose 0.5%, — : glucono- $\delta$ -lactone 0.5%.

#### 4.3.3. Consumption and conversion of the additives with *C. versicolor*

To investigate the mechanism of the high color removal obtained by the addition of glucono- $\delta$ -lactone, at first the consumption and conversion of the additives added during fungal treatment were determined.

The HPLC profiles for the aqueous solutions of additives before and after treatment with *C. versicolor* for 12-hour incubation are shown in Fig. 27-A and B. In Fig. 27-A the peak (elution volume 9.8 ml) of glucose almost disappeared within a 12-hour incubation. This indicates that almost all glucose added were consumed during fungal treatment by *C. versicolor*. The conversion of the added glucono- $\delta$ -lactone during fungal treatment are shown in Fig. 27-B. In contrast to glucose, glucono- $\delta$ -lactone (elution volume 8.4 ml) was converted abundantly into gluconic acid (elution volume 8.0 and 10.4 ml) after a 12-hour incubation. In other words, almost all glucose added was metabolized by *C. versicolor*, but the glucono- $\delta$ -lactone was hydrolyzed abundantly to gluconic acid within short treatment period, and remained unmetabolized in the culture.

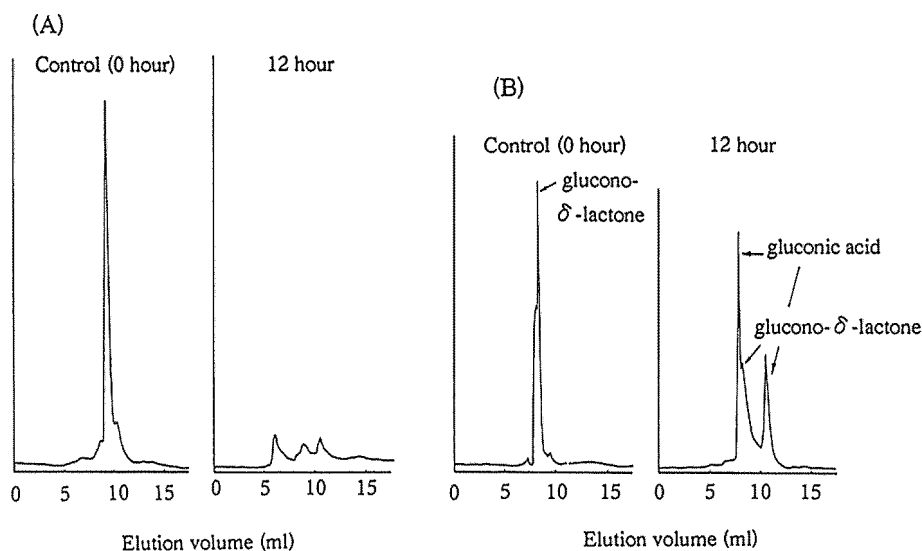


Fig. 27 The HPLC profiles of additives treated with *C. versicolor*.

#### 4.3.4. Role of glucono- $\delta$ -lactone in the decolorization of the $E_1$ effluent

As glucono- $\delta$ -lactone was shown not to be metabolized but only be hydrolyzed to gluconic acid, the change of pH during fungal treatment was determined and shown in Fig. 28. The pH of the  $E_1$  effluent containing glucono- $\delta$ -lactone was decreased from 4.5 to 3.6 with fungal treatment period. On the other hand, the pH of the  $E_1$  effluent without additives was increased gradually to 6.6 within 24-hour incubation.

It was suggested that the effect of glucono- $\delta$ -lactone on the decolorization of the  $E_1$  effluent are closely associated with the change of pH of the  $E_1$  effluent during fungal treatment. Therefore, the pH of the  $E_1$  effluent without additives was exactly adjusted

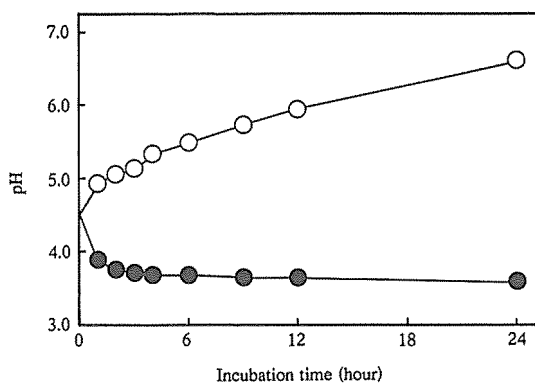


Fig. 28 The change of pH of  $E_1$  effluent during treatment with *C. versicolor*.  
Legend : ● : glucono- $\delta$ -lactone 0.5%, ○ : without additive.

with hydrochloric acid to the change of pH of the E<sub>1</sub> effluent containing glucono- $\delta$ -lactone during treatment with *C. versicolor*. The results of the decolorization of the E<sub>1</sub> effluent are shown in Fig. 29. The addition of glucono- $\delta$ -lactone to the E<sub>1</sub> effluent showed 55% and 64% of high decolorization within 6- and 12-hour incubations, respectively. When the pH of the E<sub>1</sub> effluent without additives was controlled to follow the change of pH of the E<sub>1</sub> effluent containing glucono- $\delta$ -lactone, the reduction of the color exhibited almost the same efficiency as with glucono- $\delta$ -lactone. On the other hand, the decrease of color was not detected in the E<sub>1</sub> effluent, if its pH not being controlled during the fungal treatment.

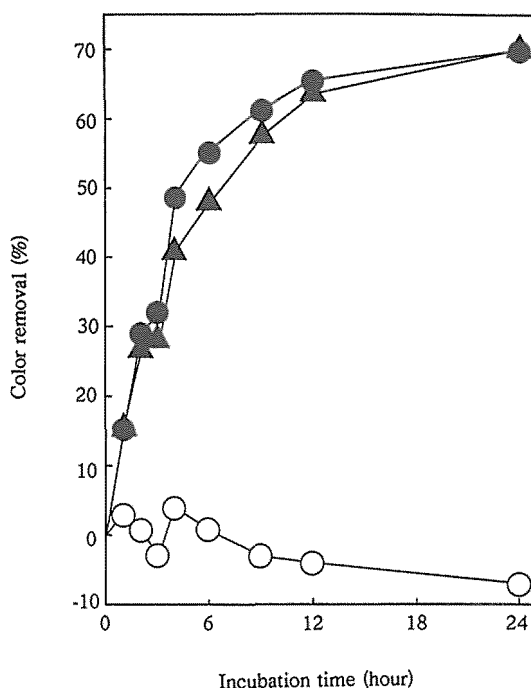


Fig. 29 Decolorization of E<sub>1</sub> effluent with *C. versicolor*.  
 Legend : ● : glucono- $\delta$ -lactone 0.5%, ○ : without additive,  
 ▲ : without additive, adjusting of pH.

When *C. versicolor* showed the effective decolorization of the E<sub>1</sub> effluent by the addition of glucono- $\delta$ -lactone, the adsorption of the color substances on the surfaces of the mycelium of the fungus were also observed. The dark color of the mycelium changed to light color with the extension of the fungal treatment time indicating the decomposition of the color substances adsorbed onto the mycelium.

#### 4.3.5. Determination of COD in the E<sub>1</sub> effluent during fungal treatment

Table 4 shows the COD values and the decolorization of the additive-containing E<sub>1</sub>

effluent before and after fungal treatment with IZU-154 and *C. versicolor*. Eighty % of the color of the E<sub>1</sub> effluent containing 0.5% of glucose was removed with IZU-154 after 24-hour incubation. *C. versicolor* also achieved high decolorization degrees of 75% and 79% within 12- and 24-hour of incubations, in the presence of 0.5% of glucono- $\delta$ -lactone, respectively. The COD value of the E<sub>1</sub> effluent was 1630 ppm, and the values increased to 5750 ppm and 5710 ppm by the addition of glucose and glucono- $\delta$ -lactone, respectively. The COD of the E<sub>1</sub> effluent with 0.5% of glucose was reduced 12% (700 ppm) with IZU 154. With *C. versicolor*, the reductions of COD of the E<sub>1</sub> effluent with 0.5% of glucono- $\delta$ -lactone were obtained 12% (685 ppm) and 19% (1079 ppm) within 12- and 24-hour of incubations, respectively. However, there is a problem that the treated E<sub>1</sub> effluents maintained high levels of COD even after fungal treatment.

Table 4 The COD values and decolorization of the E<sub>1</sub> effluents including additives before and after fungal treatment.

	COD (ppm) (Before fungal treatment)	COD (ppm) (After fungal treatment)		
		IZU-154		<i>C. versicolor</i>
		24 hr. <sup>a)</sup>	12 hr. <sup>a)</sup>	24 hr. <sup>a)</sup>
E <sub>1</sub> effluent with 0.5% of glucose	5750	5050 (80%) <sup>b)</sup>	n.d. <sup>c)</sup>	n.d. <sup>c)</sup>
E <sub>1</sub> effluent with 0.5% of glucono- $\delta$ -lactone	5710	n.d. <sup>c)</sup>	5025 (75%) <sup>b)</sup>	4631 (79%) <sup>b)</sup>

COD of the E<sub>1</sub> effluent ; 1630 ppm.

a) Treatment time. b) ( ) ; decolorization (%). c) n.d. ; not determined.

#### 4.4. Discussion

It was concluded in Chapter 2 that IZU-154 showed great decolorization effects on the E<sub>1</sub> effluents, compared with *C. versicolor* and *P. chrysosporium*.

No white-rot fungus has been convincingly shown to use lignin as its principal metabolic carbon or energy source (Ander *et al.*, 1975) (Kirk *et al.*, 1976). In order to obtain high degrees of decolorization of the E<sub>1</sub> effluents with IZU-154, a small amount of a suitable additive such as glucose was required. Furthermore, the addition of glucono- $\delta$ -lactone to the E<sub>1</sub> effluent produced a more remarkable degree of decolorization than did that of glucose. All white-rot fungi tested showed high decolorization effect of the E<sub>1</sub> effluent in the presence of glucono- $\delta$ -lactone. In particular, *C. versicolor* showed a high degree of decolorization of the E<sub>1</sub> effluent by addition of glucono- $\delta$ -lactone than that of glucose, compared with IZU-154 and *P. chrysosporium*.

When the consumption and conversion of the additives added during fungal treatment were analyzed, almost all glucose added was consumed by *C. versicolor*, but

glucono- $\delta$ -lactone was hydrolyzed abundantly to gluconic acid within short treatment period and remained unmetabolized in the culture. When the change of pH during fungal treatment was determined, the pH of the E<sub>1</sub> effluent containing glucono- $\delta$ -lactone was decreased from 4.5 to 3.6 with fungal treatment period. When the pH of the E<sub>1</sub> effluent without glucono- $\delta$ -lactone was controlled to follow the pH change of the E<sub>1</sub> effluent containing glucono- $\delta$ -lactone, the reduction of the color was almost the same efficiency as with glucono- $\delta$ -lactone. Recently, it have studied the gelation process of soybean milk (Nishinari *et al.*, 1991) and soybean proteins (Kohyama *et al.*, 1992 a) (Kohyama *et al.*, 1992 b) in the presence of glucono- $\delta$ -lactone as coagulant, and they (Kohyama *et al.*, 1993) demonstrated that a decrease in pH by the addition of glucono- $\delta$ -lactone greatly promotes the gelation kinetics. The protein structures of the mycelium surface of *C. versicolor* might be influenced with the addition of glucono- $\delta$ -lactone and as a result, the adsorption of the color substances in the E<sub>1</sub> effluent would be promoted.

The adsorption of the color substances in the E<sub>1</sub> effluent can be assumed to be a first step of the decolorization mechanisms by white-rot fungi. Since in the present study, a large quantity of fungal mass was used for the decolorization of the E<sub>1</sub> effluent, the adsorption of the color substances to the mycelium might be accelerated. It was reported that the mycelium of *Coriolus* sp. No. 20 as well as filamentous fungus, *Rhizoctonia* sp. D-90 adsorbed organic compounds such as melanoidin, and also demonstrated that the degree of melanoidin adsorption activity was influenced by the kinds of sugars used for cultivation (Sirianuntapiboon, 1991).

*C. versicolor* showed the adsorption of the color substances on the surfaces of the mycelium accompanied with the decolorization of the E<sub>1</sub> effluent by the addition of glucono- $\delta$ -lactone. The dark color of the mycelium changed to light color with the extension of the fungal treatment time. From these phenomena, it can be suggested that the color substances of the E<sub>1</sub> effluents, which were adsorbed on the surface of the mycelium of *C. versicolor* by the help of glucono- $\delta$ -lactone, were decomposed by intracellular enzymes as well as cell-bound enzymes related to the lignin degradation.

Although the high decolorization of the E<sub>1</sub> effluent was performed with the supplement of additives including glucono- $\delta$ -lactone, the supplement of additives may increase COD and BOD of the E<sub>1</sub> effluents after fungal treatment and may have harmful environmental effects ultimately, unless the supplied additives would be perfectly consumed and/or mineralized during the decolorization stage. Even though *C. versicolor* and IZU-154 showed the high color removal in the presence of glucose and glucono- $\delta$ -lactone, the COD values remarkably increased by the supplement of the additives. Consequently, there was no favorable effect of fungal treatment on the reduction of COD in the present experiment. It was also demonstrated that the COD of the bioreactor exit stream (4400 ppm) was higher than the COD of the E<sub>1</sub> effluent (700 ppm) that was a component of the induction media fed to the reactor (Camarota *et al.*, 1992). They suggested that the major part of the residual COD might be attributed to Tween 80 and

benzyl alcohol, which might be modified but not metabolized by *P. chrysosporium*.

To overcome a problem that co-substrates such as glucose or glucono- $\delta$ -lactone must be added during fungal treatment for the effective decolorization of the E<sub>1</sub> effluent, the screening of fungi requiring extremely low or, if possible, no additional nutrients is thought to be necessary to find a more advantageous system for the decolorization of the bleaching effluents.

#### 4.5. Summary

The effects of various additives on the decolorization of the E<sub>1</sub> effluent with IZU-154 were investigated. In comparison with 53% and 78% of color reductions of the E<sub>1</sub> effluents containing glucose achieved with IZU-154, the addition of glucono- $\delta$ -lactone to the effluent showed remarkable decolorizations of 83% and 94% within 12- and 24-hour incubations, respectively. Among the fungi tested, *C. versicolor* showed the most pronounced effect of the addition of glucono- $\delta$ -lactone on the decolorization of the E<sub>1</sub> effluent.

When the consumption and conversion of the additives added during the treatment with *C. versicolor* were analyzed by HPLC, glucose added was almost metabolized and glucono- $\delta$ -lactone was hydrolyzed abundantly to gluconic acid, remaining unmetabolized in the culture, within short treatment period. When the pH of the E<sub>1</sub> effluent without additives was controlled from 4.5 to 3.6, that is, to follow the change of pH of the E<sub>1</sub> effluent containing glucono- $\delta$ -lactone the reduction of the color was at almost the same efficiency as with glucono- $\delta$ -lactone. Therefore, it is suggested that the effect of glucono- $\delta$ -lactone on the high decolorization of E<sub>1</sub> effluent are closely associated with the change of pH of the effluent during fungal treatment.

### 5. Screening of Lignin-degrading Fungi for the Efficient Decolorization and Treatment of the E<sub>1</sub> Effluent without Additional Nutrients with a Newly Found KS-62

#### 5.1. Introduction

Several investigators demonstrated that kraft bleaching effluents can be decolorized with white-rot fungi, in particular *C. versicolor* (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983) (Royer *et al.*, 1983) (Archibald *et al.*, 1990) (Bergbauer *et al.*, 1991) and *P. chrysosporium* (Eaton *et al.*, 1980) (Sundman *et al.*, 1981) (Campbell *et al.*, 1982) (Yin *et al.*, 1989 a) (Yin *et al.*, 1989 b). To extensively decolorize the E<sub>1</sub> effluent, however, white-rot fungi required suitable co-substrates such as glucose. As described in previous Chapters white-rot fungi exhibited the effective decolorization of E<sub>1</sub> effluents in the presence of glucose. In particular, the high decolorization of the E<sub>1</sub> effluent was obtained with the addition of an additive, glucono- $\delta$ -lactone. However, COD values of the E<sub>1</sub> effluent remarkably increased by the supplement of the additives, and no favorable



effects on the COD reduction were obtained by fungal treatment. This fact points out a disadvantage associated with the fungal decolorization process with these fungi.

In this Chapter, therefore, the authors summarize the results of screening of fungi that show the efficient decolorization of the E<sub>1</sub> effluent without any additional nutrients and reports the treatment ability of the E<sub>1</sub> effluent with a newly isolated fungus KS-62, compared with *C. versicolor*. Furthermore, we deal with the extracellular enzyme activities exhibited by the fungus KS-62 in the E<sub>1</sub> effluent during the decolorization.

## **5.2. Materials and methods**

### **5.2.1. Isolation of lignin-degrading fungi from decayed wood**

A modification of method of Nishida et al. was used for isolating white-rot fungi (Nishida *et al.*, 1988). The isolation medium was composed of powdered lignocellulosic substrate (100-mesh pass beech wood, 0.2%), guaiacol (0.02%), and agar (1.6%) with the pH of the medium adjusted to 5.5. Each piece of decayed wood was placed on the medium described above. The strains which produced red-colored zones were isolated after incubation at 30°C for 7 days. For decolorization of the E<sub>1</sub> effluent, 124 strains were obtained from 1212 samples of rotted wood of forest habitats in Japan.

### **5.2.2. Bleaching effluent**

The E<sub>1</sub> effluent from softwood kraft pulp bleaching (N-E<sub>1</sub> effluent) was obtained from a kraft pulp mill. To remove suspended particles, the E<sub>1</sub> effluent was filtered through a glass fiber filter paper (Advantec GB140) before use. The E<sub>1</sub> effluent was adjusted to pH 4.5 with 1.0 N HCl and sterilized by autoclaving (110°C, 10 min) before fungal treatment.

### **5.2.3. Screening of high-decolorization fungi**

Potato dextrose agar plates were inoculated with each strain of the isolated wood-rotting fungi and incubate for 2-7 days at 30°C. Three disks punched from the grown edge of the mycelium were aseptically added to each 100 ml flask containing 10 ml of the E<sub>1</sub> effluent with and without the nutrients A medium, as previously mentioned in Chapter 2. The E<sub>1</sub> effluent thus inoculated was incubated statically for 5 days at 30°C. For the isolated fungi showing the high decolorization, furthermore, the color removal activities were examined as a function of treatment time.

### **5.2.4. Physiological characteristics of lignin-degrading fungi**

Fungi were incubated on PDA media at 30°C for a designated period, and mycelial mats of 0.8 cm diameter were transplanted to new PDA media and incubated under different conditions (15-40°C at pH 5.5, and pH 2-10 at 30°C). The diameter of mycelial growth was measured after incubation for 4 days.

### 5.2.5. Decolorization of the E<sub>1</sub> effluent with the fungus KS-62

Potato dextrose agar plates were inoculated with the fungus KS-62 and incubated for 5 days at 30°C. Three disks punched from the grown edge of the mycelium were aseptically added (the dry weight was less than 3.0 mg) to each 100 ml flask containing 10 ml of the E<sub>1</sub> effluents which were supplemented with the nutrients A medium and the Kirk's medium (Tien *et al.*, 1988), respectively. The E<sub>1</sub> effluents were incubated statically for 10 days at 30°C. The original E<sub>1</sub> effluent (incubation time, 0 day) was recovered immediately by filtration after inoculation with the mycelium disks.

### 5.2.6. Effect of catalase addition on the decolorization of the E<sub>1</sub> effluent

After five days of fungal treatment, catalase (Wako Pure Chemicals) was repeatedly added at 12 hours intervals for 2 days. The amount of catalase added to the medium at one time was 3.0 mg (17700 Unit) per 10 ml of the E<sub>1</sub> effluent. The decolorization rate of the catalase-added medium was compared with that of the medium without catalase.

### 5.2.7. Analysis

#### 5.2.7.1. Adsorbable organic halogens (AOX)

The amount of chloro-organic compounds was determined using an AOX analyzer (Mitsubishi Kasei TOX-10).

#### 5.2.7.2. Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was performed with a fast protein liquid chromatograph (FPLC 2249) equipped with a Sephadex G-75 column using 0.01N NaOH containing 0.1 N LiCl as an eluting solvent and monitored at 280 nm.

#### 5.2.7.3. Enzyme assays (Gold *et al.*, 1989)

To determine enzyme activities, the E<sub>1</sub> effluent treated with the fungus KS-62 was concentrated with an ultrafiltration membrane (10 kDa cut-off). Phenoloxidase activity was assayed by the oxidation of 2,6-dimethoxyphenol at 37°C. Manganese peroxidase (MnP) activity was also assayed with addition of 50 mM MnSO<sub>4</sub>. The increase of absorbance at 470 nm in the reaction was recorded. Lignin peroxidase (LiP) activity was assayed by oxidation of 3,4-dimethoxybenzyl alcohol (veratryl alcohol). In oxidation of veratryl alcohol the increase of absorbance at 310 nm was recorded.

## 5.3. Results

### 5.3.1. Screening of fungi having high-decolorization activity

For screening of fungi having high-decolorization activity, 124 strains of white-rot fungi which produced red-colored zones in the medium containing 0.02% guaiacol, were isolated from 1212 samples of rotted wood. Figure 30 shows the distribution of the activities of the isolated strains to decolorize E<sub>1</sub> effluent with and without the nutrients A

medium. Based on the tendency to the color removal of the  $E_1$  effluent, the isolated fungi could be divided into three groups. Group A was that of isolated fungi having high decolorization of the  $E_1$  effluent without additional nutrients. The fungi in group B showed more efficient decolorization of the  $E_1$  effluent with rather than without additional nutrients, and those in group C showed extremely high requirement of the additional nutrients for the decolorization of the  $E_1$  effluent.

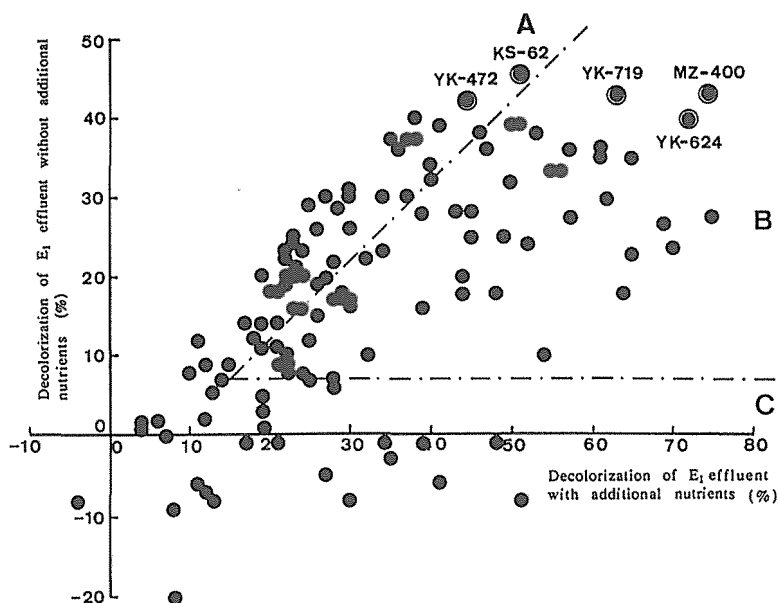


Fig. 30 Decolorization of  $E_1$  effluent with isolated wood-rotting fungi (initial color unit : 4500 CU)

Among the isolated fungi belonging to A and B groups, the treatment of  $E_1$  effluent with five fungi, KS-62, YK-472, YK-719, MZ-400, and YK-624 were hereinafter investigated in further detail, because these fungi showed the effective decolorization of the  $E_1$  effluent without any additional nutrients.

### 5.3.2. Decolorization of the $E_1$ effluent with lignin-degrading fungi

The color removal activities of the five strains showing the high decolorization were examined as a function of treatment time. The results are shown in Fig. 31. The decolorization of the  $E_1$  effluent treated with YK-624, MZ-400, YK-472, and YK-719 increased with time of treatment for 5 days of incubation, but prolonged fungal treatment longer than 5 days did not result in any appreciable increase of decolorization of the  $E_1$  effluent. The fungus KS-62, on the other hand, showed a remarkable decolorization with treatment period and resulted in 70% and 80% reduction of the color after 7- and 10-days

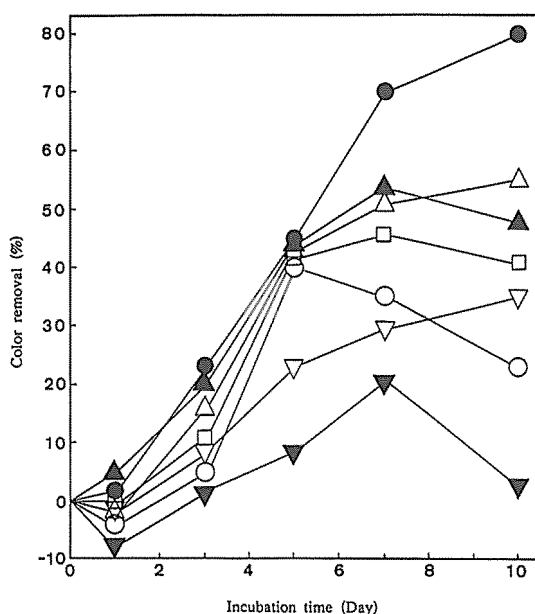


Fig. 31 Decolorization of E<sub>1</sub> effluent without any additional nutrients with white-rot fungi (initial color unit : 4500 CU).

Legend : ● : KS-62, ○ : YK-624, △ : MZ-400, ▲ : YK-472, □ : YK-719, ▽ : *C. versicolor*, ▼ : *P. chrysosporium*.

Notes : Average of five repetitions.

of incubations, respectively. As the precipitation or the adsorption of chromophores on the surfaces of the mycelium disks was not observed, it is thought that the colored materials of the E<sub>1</sub> effluent were degraded by the fungus KS-62. On the other hand, when the E<sub>1</sub> effluent was treated under the same conditions, only 29% and 13% of decolorization were observed with *C. versicolor* and *P. chrysosporium* for 5 days of incubations, respectively. Because the fungus KS-62 showed the most effective decolorization of the E<sub>1</sub> effluent without any additional nutrients, the treatment of E<sub>1</sub> effluent with this fungus was hereinafter investigated in further detail, compared with *C. versicolor* that showed more efficient decolorization than *P. chrysosporium*.

### 5.3.3. Physiological characteristics of a lignin-degrading fungus KS-62

The effect of pH and temperature on the mycelial growth of KS-62 was compared with that of *C. versicolor* and *P. chrysosporium*. As shown in Fig. 32, KS-62 could grow between pH 3-9, and the optimum pH was 6.

Regarding temperature (Fig. 33), KS-62 grew between 15-35°C, and the optimum temperature was 25-30°C. The fungus grew more slowly than *P. chrysosporium*, but faster than *C. versicolor*.

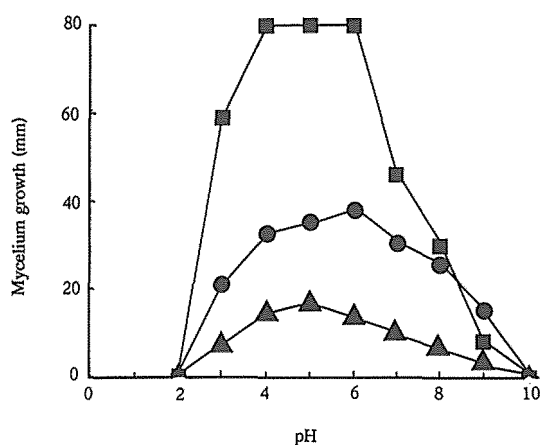


Fig. 32 Effect of pH on mycelium growth (PDA medium, 30°C, three days of incubation).

Legend : ● : KS-62, ▲ : *C. versicolor*, ■ : *P. chrysosporium*.

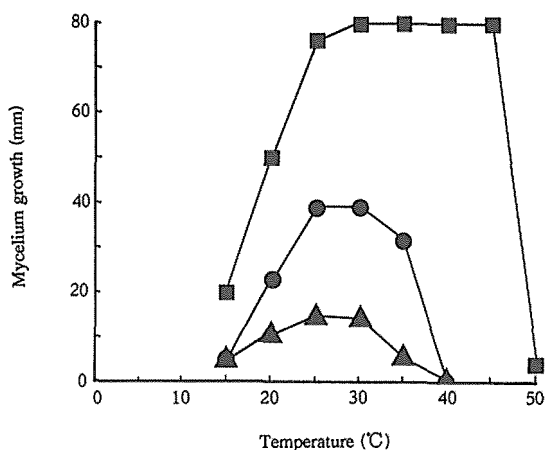


Fig. 33 Effect of temperature on mycelium growth (PDA medium, pH 5.5, three days of incubation).

Legend : ● : KS-62, ▲ : *C. versicolor*, ■ : *P. chrysosporium*.

#### 5.3.4. Dechlorination and degradation of chlorolignin in the E<sub>1</sub> effluent with lignin-degrading fungi

The reduction of COD and AOX is expected to reflect the extent of dechlorination and degradation of the chlorolignins. With KS-62, COD and AOX decreased with the fungal treatment period and resulted in 34% and 40% reductions after 7-day incubation, respectively. Under the same conditions, COD and AOX reductions with *C. versicolor* were only 20% and 25%, respectively, as shown in Fig. 34.

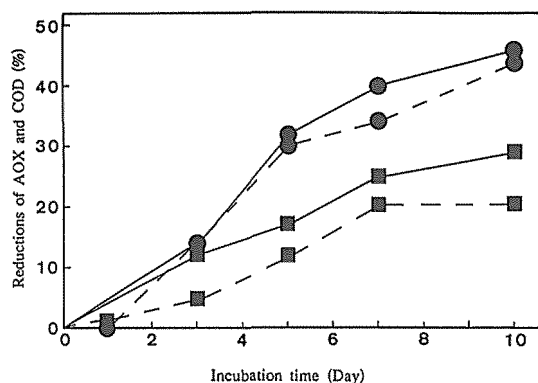


Fig. 34 Reductions of AOX and COD of  $E_1$  effluent without any additional nutrients with white-rot fungi (initial AOX : 102 ppm, initial COD : 1549 ppm).

Legend : — : AOX, -- : COD, ● : KS-62, ■ : *C. versicolor*.

Note : Average of three repetitions.

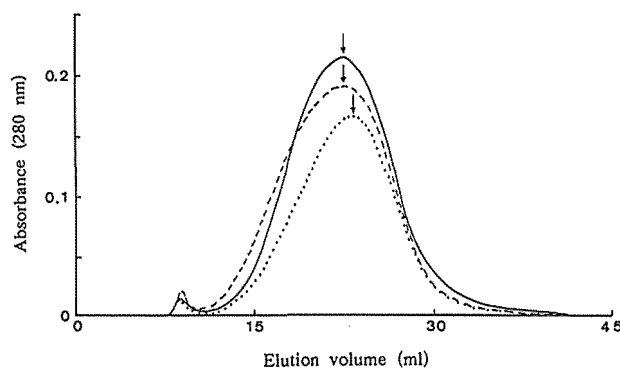


Fig. 35 Gel permeation chromatography profiles of the  $E_1$  effluent treated with white-rot fungi for 7 days.

Legend : — : untreated  $E_1$  effluent, -- : treated  $E_1$  effluent with *C. versicolor*,  
 •• : treated  $E_1$  effluent with KS-62.

Note : The arrows indicate main elution fractions.

The changes of elution profile that indicates the molecular weight distribution of the  $E_1$  effluent during the treatment with two white-rot fungi are illustrated in Fig. 35. When the  $E_1$  effluent was treated with the fungus KS-62, the high molecular weight portion of  $E_1$  effluent was appreciably degraded, however, with *C. versicolor* the molecular weight distributions shifted towards high molecular weights.

These findings clearly indicate that the fungus KS-62 significantly depolymerized and decomposed the chlorolignin in the  $E_1$  effluent without any additional nutrients.

### 5.3.5. Extracellular enzymes revealed during the treatment of effluent with KS-62

The extracellular enzyme activities in the  $E_1$  effluent treated with the fungus KS-62 were determined. As shown in Fig. 36, rapid decolorization of the  $E_1$  effluent without any additional nutrients was observed on days 3 through 5. When rate of decolorization reached a maximum between days 3 and 5, MnP activity reached its highest level, but LiP activity was not detected. Phenoloxidase activity appeared at moderate levels on day 3, then declined with the incubation time.

Moreover, the influence of co-substrate on decolorization and the extracellular enzyme activities were tested with the nutrients A medium and the Kirk's medium. The composition of these media are shown in Table 5. There were considerable differences in

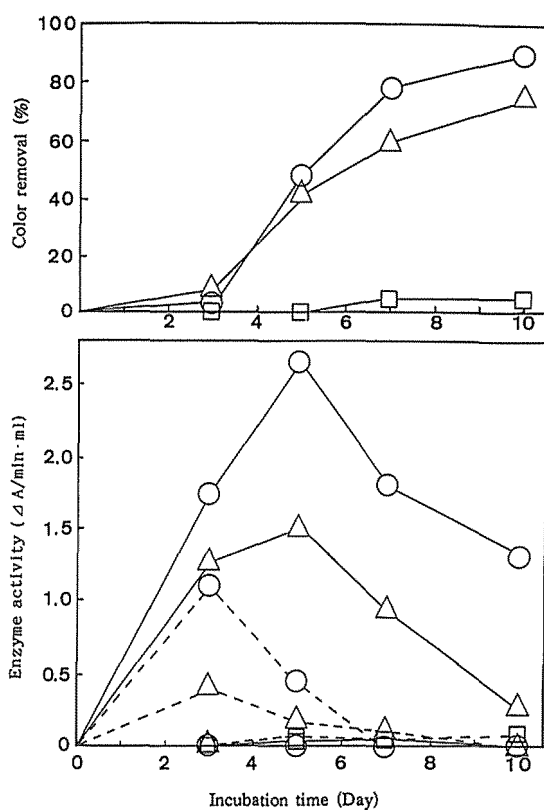


Fig. 36 The relationship between decolorization of  $E_1$  effluent with KS-62 and its extracellular enzyme activities.

Legend : Color removal :  $\circ$  :  $E_1$  effluent with the nutrients A medium ;  
 $\square$  :  $E_1$  effluent with Kirk's medium ;  
 $\square$  :  $E_1$  effluent without any additional nutrients  
 Enzyme activity :  $\circ$ —,  $\triangle$ —,  $\square$ —, MnP,  
 $\circ$ ---,  $\triangle$ ---,  $\square$ ---, Phenoloxidase.

Note : Average of three repetitions.

Table 5 Composition of Kirk's medium and nutrients A medium (per liter).

	Kirk's medium	Nutrients A medium
Glucose	10g	5.0g
Ammonium tartrate	0.2g	0.2g
KH <sub>2</sub> PO <sub>4</sub>	2.0g	1.0g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	1.45g	0.5g
Thiamine-HCl	1.0mg	100 µg
CaCl <sub>2</sub>	0.1g	100 µg
FeSO <sub>4</sub> · 7H <sub>2</sub> O	7mg	100 µg
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	7mg	10 µg
CuSO <sub>4</sub> · 5H <sub>2</sub> O	7mg	20 µg
MnSO <sub>4</sub>	35mg	—
CoCl <sub>2</sub>	7mg	—
NaCl	70mg	—
AlK(SO <sub>4</sub> ) <sub>2</sub> · 12H <sub>2</sub> O	0.7mg	—
H <sub>3</sub> BO <sub>3</sub>	0.7mg	—
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	0.7mg	—
Nitrilotriacetate	0.105g	—
NaH <sub>2</sub> PO <sub>4</sub>	—	0.2g

their abilities for decolorization of the E<sub>1</sub> effluent. In the presence of the nutrients A medium, 48% and 79% of color removals were showed for 5 and 7 days of incubations, respectively, but the decrease of color of the E<sub>1</sub> effluent was scarcely detected in the presence of the Kirk's medium. The time course of the extracellular enzyme activities were determined during the decolorization of the E<sub>1</sub> effluent containing the nutrients A medium. MnP activity was detected at a higher level than other enzyme activities in the culture fluid on day 3 of incubation, then increased to a maximum on day 5, and decreased to low levels by day 10. Phenoloxidase activity was detected to a maximum extent on day 3 of incubation, and then rapidly declined. However, no LiP activity was detectable. In the E<sub>1</sub> effluent containing Kirk's medium, the fungus KS-62 scarcely decolorized the effluent, and these extracellular enzyme activities were hardly detected in whole incubation time; that is to say, they were less than 0.1 (ΔA/min.ml).

### 5.3.6. Effect of catalase addition on the decolorization

Because it is suggested that MnP might play the major role in the decolorization of the E<sub>1</sub> effluent with the fungus KS-62, the contribution of the peroxidases system including MnP to decolorization was examined by adding catalase to the decolorizing medium. The effect of the catalase addition is shown in Fig. 37. The significant reduction was observed for the decolorization of catalase-added culture. This result indicates that the peroxidases system including MnP is likely to play an important role in the E<sub>1</sub> effluent decolorization with the fungus KS-62.



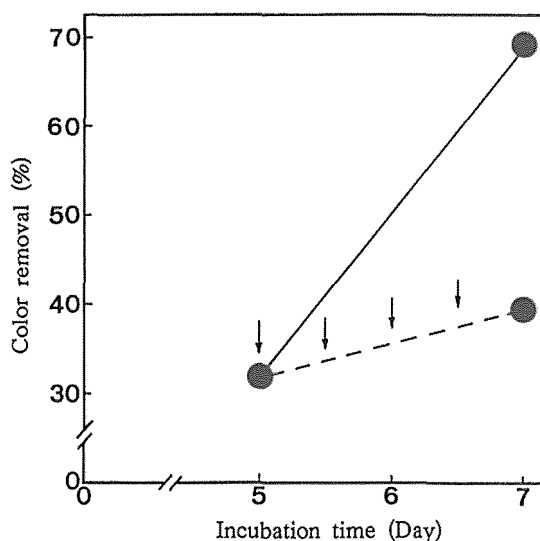


Fig. 37 The effect of catalase addition on decolorization of E<sub>1</sub> effluent with KS-62.

Legend : — : without catalase, -- : with catalase

Note : The arrows indicate the catalase addition. Average of four repetitions.

#### 5.4. Discussion

It has been reported that ligninolytic white-rot fungi such as *P. chrysosporium* and *C. versicolor* can efficiently decolorize and dechlorinate of the pulp bleach plant effluents (Lundquist *et al.*, 1977) (Sundman *et al.*, 1981) (Livernoche *et al.*, 1981) (Eaton *et al.*, 1982) (Huynh *et al.*, 1985 a) (Yin *et al.*, 1989 a) (Archibald *et al.*, 1990). To obtain the high decolorization of the E<sub>1</sub> effluents with white-rot fungi, however, a suitable co-substrate such as glucose was required (Livernoche *et al.*, 1981) (Royer *et al.*, 1985) (Archibald *et al.*, 1990) (Bergbauer *et al.*, 1991).

Some papers dealt with the screening of white-rot fungi for the E<sub>1</sub> effluent treatment. Most of them, however, dealt with the effluent supplemented with nutrients. Among the fungi selected from 25 species of tropical fungi and 10 species of Japanese isolates (Fukuzumi *et al.*, 1977), *Tinctoporia* sp. showed the best results of decolorization of the E<sub>1</sub> effluent under the culture condition of adding saccharides. *Polyporus* sp., *Coriolus* sp. and *Polyporus sanguineus* were evaluated for the decolorization in the presence of 1.0% of glucose for nine days of incubation (Esposito *et al.*, 1991). *Coriolus* sp. showed the greatest efficiency for decolorization (85%). *P. chrysosporium*, *C. versicolor*, *Ramaria* sp., *Poria placenta* (MAD-698), *Gloeophyllum trabeum* (MAD 6M-R) and several unidentified ligninolytic fungi were evaluated for their ability to decolorize the E<sub>1</sub> effluent (Galeno *et al.*, 1990). *Ramaria* sp. showed the highest potential for the biological treatment of the E<sub>1</sub> effluent: 13050 CU (90%) of the color was removed after eight days of incubation in

Kirk's medium (Kirk *et al.*, 1986) under air. According to Esposito *et al.*, among fifty one ligninolytic strains tested, *Lentinus edodes* (UEC-2019) showed a remarkable decolorization of the E<sub>1</sub> effluent without any additional nutrients and resulted in 73% (830 CU) of the color removal and 60% COD-reduction of the effluent after five days of incubation (Esposito *et al.*, 1991).

From decayed wood samples, the authors isolated the fungus KS-62 that showed the most effective decolorization of the E<sub>1</sub> effluent without any additional nutrients. The fungus KS-62 showed 45% (2025 CU) of color removal which was more than twice higher than that of *L. edodes*. reported by Esposito *et al.* (Esposito *et al.*, 1991). The reduction of COD shown by KS-62 was 30% (465 ppm) within five days of incubation. But it was not described the value of COD reduction with *L. edodes* (Esposito *et al.*, 1991).

The treatment with the fungus KS-62 resulted in significant reductions of the COD and AOX of the E<sub>1</sub> effluent, compared with *C. versicolor*. The elution profile on GPC of the E<sub>1</sub> effluent implies a decrease of the high molecular weight fraction of chlorolignins during the treatment with the fungus KS-62. These results indicated that a portion of chlorinated lignin components of the E<sub>1</sub> effluent was degraded by this fungus.

The fungus KS-62 has not yet been identified exactly. However the results of 38.5% of lignin loss of beech wood-powder and 25% of brightness increase of kraft pulp after five-day of incubation (Hirai *et al.*, 1993), the fungus KS-62 would be a white-rot fungus.

Decrease of AOX is becoming the most important criterion for the efficiency of pulp mill effluent treatment in the 1990s (Lankinen *et al.*, 1991). In activated sludge plants, the decrease of AOX is 48-65% for 7-9 days, but only 23-32% in aerated lagoons within 15-17 days (Gerkov *et al.*, 1988). The treatment of bleach plant effluent by MyCoR also markedly decreased total organic chlorine (TOCl) (Matsumoto *et al.*, 1985). With carrier-immobilized *Phlebia radiata* in 2 l bioreactors, AOX decrease was ca. 4 mg/l in one day (Lankinen *et al.*, 1991). These results were obtained under the condition of a biotechnological treatment method containing an adding of various nutrients and a flushing with oxygen. On the other hand, the reduction of AOX with the fungus KS-62 was ca. 6 mg/l in one day.

The extracellular peroxidases of *P. chrysosporium* are widely implicated in lignin degradation and detoxification of a wide variety of toxic aromatic compounds (Lamar *et al.*, 1990) (Valli *et al.*, 1991). Paice *et al.* have shown, on the other hand, that horseradish peroxidase can catalyze the decolorization of the E<sub>1</sub> effluent (Paice *et al.*, 1984). Recently Michel *et al.* and Lackner *et al.* showed that MnP plays a more important role than LiP for decolorization of the E<sub>1</sub> effluent with *P. chrysosporium* (Michel *et al.*, 1991) (Lackner *et al.*, 1991). On the other hand, Momohara *et al.* reported that either peroxidases and hydrogen peroxide itself do not participate directly in the decolorization with *P. chrysosporium* (Momohara *et al.*, 1989).

The authors determined the extracellular enzyme activities in the E<sub>1</sub> effluent, being decolorized with the fungus KS-62. High rates of the decolorization of the E<sub>1</sub> effluent

were seen between days 3 and 5 of incubations, when the cultures exhibit high levels of MnP activity but no LiP activity. Fujii *et al.* reported that the oxidation of veratryl alcohol by LiP is inhibited when some phenolic compounds are present (Harvey and Palmer, 1990) (Fujii *et al.*, 1993). Therefore, we incubated the fungus KS-62 using nutrients A medium and Kirk's medium without the E<sub>1</sub> effluent. No LiP activity was detected when the extracellular enzyme activities were determined (data not shown). The decolorization with KS-62 was significantly suppressed by the catalase-addition to the culture. Therefore, it can be suggested that MnP might play the major role in the decolorization of the E<sub>1</sub> effluent. This result is in agreement with the finding of Michel *et al.* and Lackner *et al.* for *P. chrysosporium* (Michel *et al.*, 1991) (Lackner *et al.*, 1991).

There were considerable differences in the efficiencies of co-substrates for decolorization of the E<sub>1</sub> effluent, when their influence on decolorization was tested with both the nutrients A medium and the Kirk's medium. Higher color removal was showed in the presence of the nutrients A medium, whereas color was scarcely reduced in the presence of the Kirk's medium. For the extracellular enzyme activities, high levels were determined in the culture containing the nutrients A medium, but these enzyme activities were hardly detected in the culture containing the Kirk's medium in the whole incubation time. The Kirk's medium contains more constituents (Mn<sup>2+</sup>, Co<sup>2+</sup>, Al<sup>3+</sup>, MoO<sub>4</sub><sup>2-</sup> etc.) and larger amounts than nutrients A medium. This different decolorization by the Kirk's medium may be attributed to the inhibition of the fungal growth and the various enzyme productions. It is not clear now whether the inactivity of the fungus KS-62 was related to a specific constituent or the excess of the constituents in the Kirk's medium.

### 5.5. Summary

A screening have been performed to find fungi having high treatment activity of the E<sub>1</sub> effluent without any additional nutrients. Among some isolated fungi, particularly, the fungus KS-62 showed the excellence in decolorizing the E<sub>1</sub> effluent and degrading the chlorinated lignin in the E<sub>1</sub> effluent without any additional nutrients.

Treatment with KS-62 for 5 days resulted in 62% reductions of the color of the softwood E<sub>1</sub> effluent containing 4500 color units (CU). This decolorization was much more efficient than conducted with *C. versicolor* for 10 days. The treatment with KS-62 significantly reduced the COD and AOX of the E<sub>1</sub> effluent, compared with *C. versicolor*. The high molecular weight fraction of chlorolignins in the E<sub>1</sub> effluent decreased during the treatment with KS-62, but the chlorolignins treated with *C. versicolor* were mainly polymerized.

During the E<sub>1</sub> effluent treatment with the fungus KS-62, high levels of MnP were produced, but little or no LiP activity was shown. The significant reduction was observed for the decolorization of catalase-added culture. This suggests that MnP may play an important role in decolorization of the E<sub>1</sub> effluent with the fungus KS-62.

## 6. Successive Treatment of the E<sub>1</sub> Effluent with Immobilized Mycelium of the Fungus KS-62

### 6.1. Introduction

Immobilization of biocatalysts such as microbial cells is attracting worldwide attention since they are, in general, stable and easy to handle compared with native counterparts. One of the most important features is that they can be used repeatedly in a long-term series of batch reactions or continuously in flow systems (Fukui *et al.*, 1982) (Fukui *et al.*, 1984).

The ability of white-rot fungi to degrade chlorolignins and to decolorize kraft bleach effluents has been investigated in recent years. Treatment of phenolic effluents with immobilized white-rot fungi removes color and degrades toxic compounds (Eaton *et al.*, 1982) (Royer *et al.*, 1985). *Coriolus versicolor* (L. ex Fr.) Quel. immobilized in calcium alginate beads (Livernoche *et al.*, 1981) (Livernoche *et al.*, 1983) (Royer *et al.*, 1983) has been used in air-lift reactors. The MyCoR process is based on the decolorization by *Phanerochaete chrysosporium* Burds. immobilized on a rotating biological contactor (RBC) (Eaton *et al.*, 1982). The use of trickling filter type bioreactor where *P. chrysosporium* is immobilized on a porous carrier material (polyurethane) has been adopted in the MYCOPOR system to guarantee excellent oxygen supply (Messner *et al.*, 1990) (Camarota *et al.*, 1992). Recently, *Phlebia radiata* 79 (ATCC 64658) was immobilized onto polypropylene carriers in 2 l glass bioreactors, and aerated with sterile air to remove color from a medium containing effluent. To obtain the effective decolorization with immobilized white-rot fungi, however, the fungi required the supplement of a high oxygen concentration and high concentration of energy sources such as glucose or cellulose as well as various basal nutrient, mineral solution, Tween 80, and/or benzyl alcohols (Royer *et al.*, 1985) (Jaklin-farmer *et al.*, 1992).

In the previous Chapter 5, the authors have reported that a screening have been performed to find fungi having high decolorization activity of the E<sub>1</sub> effluent without any additional nutrients. Among some isolated fungi, particularly, the fungus KS-62 showed the excellence in decolorizing the E<sub>1</sub> effluent and degrading the chlorinated lignin in the E<sub>1</sub> effluent without any additional nutrients under the batch treatment conditions. In this Chapter, to obtain a reasonable basis for the evaluation of an industrial fungal treatment, the authors have performed the treatment of the E<sub>1</sub> effluent with the immobilized mycelium of the fungus KS-62. Effects of the addition of glucose during the decolorization stage on color removal and reduction of COD are also reported.

### 6.2. Materials and methods

#### 6.2.1. Microorganisms

Three white-rot fungi used were the fungus KS-62 and *C. versicolor* and *P. chrysosporium* as described in Chapter 2.

### 6.2.2. Bleaching effluent

The E<sub>1</sub> effluent from softwood kraft pulp bleaching (N-E<sub>1</sub> effluent, 6800 CU) was obtained from a mill and filtered to remove suspended particles as described in Chapter 2. The E<sub>1</sub> effluent with or without additive was adjusted to pH 4.5 with 1.0 N HCl and sterilized by autoclaving (110°C, 10 min) before fungal treatment.

### 6.2.3. Treatment of the E<sub>1</sub> effluent with the immobilized mycelium of white-rot fungi

Potato dextrose agar plates were inoculated with each fungus and incubated for a designated period at 30°C. Three hundred and fifty disks punched from the grown edge of the mycelium were aseptically added to each 100 ml flask containing 30 ml of the E<sub>1</sub> effluent which was supplemented with various concentrations of glucose. The effect of nitrogen source as ammonium tartrate in the cultures on the decolorization activity was also determined. The E<sub>1</sub> effluent was incubated statically or agitationally at 150 rpm at 30°C. After treatment for a designated period, the E<sub>1</sub> effluents treated with white-rot fungi were replaced with fresh E<sub>1</sub> effluent, and the reaction was continued. This operation was repeated several times.

### 6.2.4. Analysis

After incubation for a designated period, the mycelium was removed by filtration through a 0.45  $\mu$ m pore size membrane filter (Advantec). Color units (CU) of both the original and the decolorized E<sub>1</sub> effluent were obtained as described in Chapter 2. The change of pH of the E<sub>1</sub> effluent during fungal treatment was determined. The COD was measured as described in Chapter 4.

## 6.3. Results

### 6.3.1. Treatment of the E<sub>1</sub> effluent with the immobilized mycelium of the white-rot fungi

The treatment of the E<sub>1</sub> effluents was performed with the white-rot fungi immobilized in PDA medium. The dotted lines and the arrows in the Figs. represent the initial pH and the addition of 0.5% of glucose to the E<sub>1</sub> effluent, respectively. As shown in Fig. 38, the fungus KS-62 showed from 70% to 80% of the color removal of the E<sub>1</sub> effluent without any nutrients for four times of replacement within one or two days of incubations under the agitational condition. The color removal started to decrease at the fifth replacement with the fresh E<sub>1</sub> effluent without any nutrients. However, the decolorization activity of the fungus KS-62 was restored by once replacing by the E<sub>1</sub> effluent containing 0.5% of glucose and the great decolorization within one or two days of incubations was continuously observed for three and four times of the replacement in the absence of glucose. With the fungus KS-62, such decolorization activity was repeatedly obtained for seventeen times of replacement within 29 days of total treatment period. However, partial color removal (from 15% to 20%) took place and the decolorization activity was hardly

restored under the static condition, even the addition of 0.5% of glucose to the  $E_1$  effluent.

The change of pH during fungal treatment was determined. As shown in Fig. 38, when a sufficient decolorization more than 70% was observed with the immobilized mycelium of KS-62, the pH of the treated  $E_1$  effluent decreased down to 3.6 which is smaller than the initial pH 4.5. But the pH increased when the partial color removal took place.

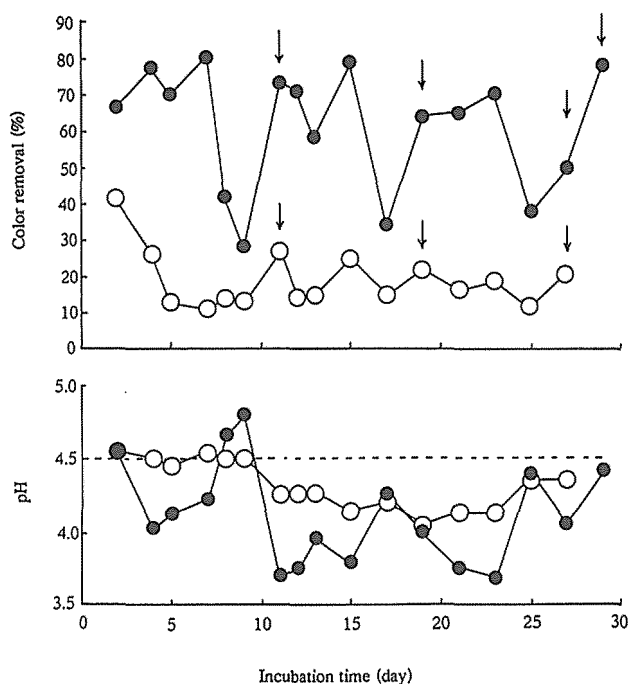


Fig. 38 The decolorization and the change of pH of the  $E_1$  effluent treated with immobilized mycelium of KS-62 (initial color unit ; 6580 CU).

Legend : ● : the agitational treatment (150 rpm), ○ : the static treatment.

Note : Arrows indicate the addition of 0.5% of glucose to the  $E_1$  effluent.

The dotted line represents the initial pH of the  $E_1$  effluent.

The effect of the supplement of nitrogen source in the culture on the decolorization activity during fungal treatment was also determined. Unlike the case of the addition of glucose, distinct increase of the color removal was not shown by the addition of ammonium tartrate as a nitrogen source to the  $E_1$  effluent (data not shown).

The decolorization abilities of the two immobilized white-rot fungi, *C. versicolor* and *P. chrysosporium*, were determined under the same conditions as with KS-62. As shown in Fig. 39, *C. versicolor* hardly showed the color removal of the  $E_1$  effluent without glucose under the agitational condition. When replaced by the  $E_1$  effluent containing 0.5% of glucose, the effective decolorization (60%) was observed. However, the color removal

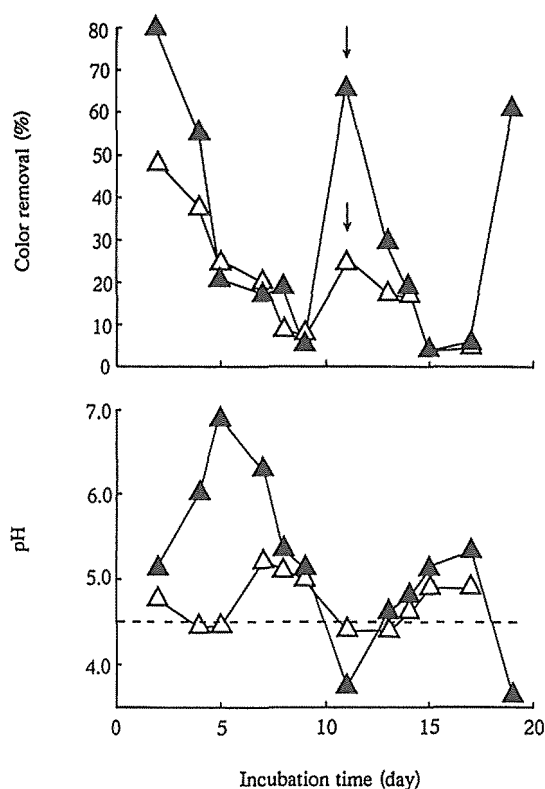


Fig. 39 The decolorization and the change of pH of the E<sub>1</sub> effluent treated with immobilized mycelium of *C. versicolor* (initial color unit ; 6800 CU).

Legend : ▲ : the agitational treatment (150 rpm), △ : the static treatment.

Note : Arrows indicate the addition of 0.5% of glucose to the E<sub>1</sub> effluent.

The dotted line represents the initial pH of the E<sub>1</sub> effluent.

was immediately decreased after the replacement of the E<sub>1</sub> effluent without glucose. Under the static condition, only partial color removal took place, even if 0.5% of glucose was added to the E<sub>1</sub> effluent. The change of the pH during fungal treatment was similar to that the results shown with KS-62. That is to say, when higher decolorization was shown, the pH of the treated E<sub>1</sub> effluent was lower. The relationship between the degrees of decolorization and pH change during fungal treatment with *C. versicolor* was similar to that observed with KS-62. Figure 40 indicates the color removal of the E<sub>1</sub> effluent with *P. chrysosporium* under the agitational and the static conditions. Unlike the results of the decolorization obtained with KS-62 and *C. versicolor*, *P. chrysosporium* showed the partial color removal under the agitational as well as the static conditions, even though 2.0% of glucose was added to the E<sub>1</sub> effluent and the fungal treatment period was prolonged to seven days.

Under the agitational treatment condition, the mycelium of the fungus KS-62 immobilized in the PDA medium showed the most effective decolorization compared with

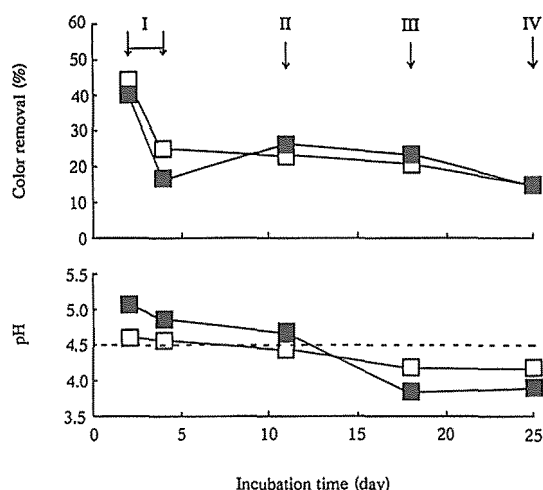


Fig. 40 The decolorization and the change of pH of the E<sub>1</sub> effluent treated with immobilized mycelium of *P. chrysosporium* (initial color unit ; 6580 CU).

Legend : ■ : the agitational treatment (150 rpm), □ : the statical treatment.

Note : I, II, III and IV indicate the addition of 0%, 0.5%, 1.0% and 2.0% of glucose to the E<sub>1</sub> effluent, respectively.

The dotted line represents the initial pH of the E<sub>1</sub> effluent.

*C. versicolor* and *P. chrysosporium*. Consequently, the decolorization of the E<sub>1</sub> effluent with this fungus was hereinafter investigated in further detail.

### 6.3.2. Effects of glucose concentrations on the decolorization with the immobilized mycelium of KS-62

To obtain the effective decolorization continuously with the immobilized mycelium of the fungus KS-62, the addition of a small amount of glucose to the E<sub>1</sub> effluent was required. The efficiencies of the color removal and the reduction of COD during the decolorization of E<sub>1</sub> effluents containing various amounts of glucose are summarized in Fig. 41. In the absence of glucose, the fungus showed the color removal more than 80% for three times of replacement (treatment period 6 days). By the reuse of this mycelium, the high decolorization degrees around 85% for six times of replacement (treatment period 12 days) were achieved in the presence of 0.1% of glucose. When the addition of glucose to the E<sub>1</sub> effluent was reduced to 0.075%, furthermore, a sufficient decolorization more than 70% was maintained for seven times of replacement (treatment period 15 days). Seventy % of the color removal was obtained for three times of replacement (treatment period 7 days) with the E<sub>1</sub> effluent containing 0.05% of glucose. This finding showed that a critical amount (between 0.05% and 0.075%) of glucose was needed to maintain the decolorization activity. When the critical amount of glucose was present in the E<sub>1</sub> effluent, the fungus KS-62 showed high color removal activity during nineteen times of replacement of the E<sub>1</sub> effluent for 40 days of treatment period.



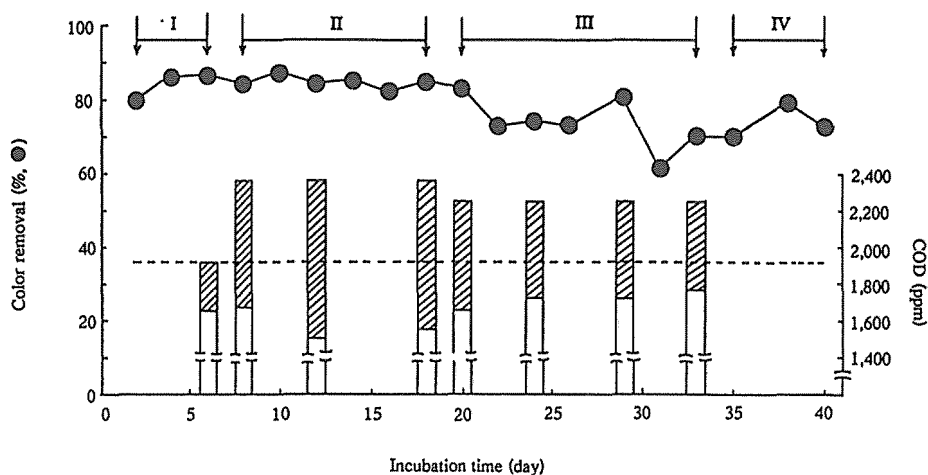


Fig. 41 The decolorization and the reduction of COD of the  $E_1$  effluent treated with immobilized mycelium of KS-62 under the agitational condition (initial color unit : 6800 CU).

Legend :  $\square$  : reduced COD,  $\square$  : remained COD.

Note : I, II, III and IV indicate the addition of 0%, 0.1%, 0.075% and 0.05% of glucose to the  $E_1$  effluent, respectively.

The dotted line represents the COD value of the  $E_1$  effluent.

Figure 41 also showed the reduction of COD of the  $E_1$  effluent treated with KS-62. The COD value of the original  $E_1$  effluent was 1910 ppm, and the values increased to 2370 ppm and 2250 ppm by the addition of 0.1% and 0.075% of glucose, respectively. As shown in Fig. 41, partial COD decrease of 255 ppm (13%) took place in the absence of glucose. With the additions of 0.1% and 0.075% of glucose, 29%-37% and 21%-26% of the COD reductions were observed, respectively. The COD of the treated effluent is the sum of the COD due to the  $E_1$  effluent itself and the glucose added for fungal treatment. In the present experiment, the COD of the treated effluent was lower than the  $E_1$  effluent COD in the whole incubation time. If the reduction of COD is expected to reflect the extent of degradation of the chlorolignins, these findings indicate that the fungus KS-62 decomposed the chlorolignin in the  $E_1$  effluent.

#### 6.4. Discussion

The treatment of the  $E_1$  effluent in bioreactors has been so far studied mainly with *P. chrysosporium* using MyCoR and MYCOPOR processes. With MyCoR process, Prouty reported that 1300 CU (16%) of color removal was obtained in an air atmosphere per day (Prouty, 1990). Campbell *et al.* determined 2000 CU (50%) and 600 CU (15%) of color removals in an oxygen-enriched atmosphere and an air atmosphere per day, respectively (Campbell *et al.*, 1982). Using MYCOPOR process in which *P. chrysosporium* was immobilized on a trickling filter, Messner *et al.* reported that the mean decolorization rate

of the E<sub>1</sub> effluent was 2590 CU (70%) with daily replacement of the medium/effluent solution (Messner *et al.*, 1989). Jaklin-Farcher *et al.* also evaluated a maximum of 2960 CU (80%) decolorization after one passage of the E<sub>1</sub> effluent through a trickling filter (Jaklin-Farcher *et al.*, 1992). Campbell *et al.* described that their bioreactor promoted 2730 CU (70%) decolorization during 5.8 days of treatment period (Campbell *et al.*, 1982).

As mentioned in Chapter 5, the fungus KS-62 is a white-rot fungus requiring low additional nutrients during decolorization. In the present Chapter, the authors determined the decolorization of the E<sub>1</sub> effluent with the immobilized mycelium of the fungus KS-62. In the absence of additives, the color reduction between 4760 CU (70%) and 5440 CU (80%) was obtained within two days of incubation. About 85% (5780 CU) of color reduction with KS-62 was obtained in the presence of 0.1% glucose. The removal of color substances obtained with KS-62 was about twice higher than those of MYCOPOR process. Moreover, the results obtained with KS-62 are remarkable insofar as this value is achieved without any further biotechnological optimization such as the introduction of bioreactor as well as flushing with oxygen.

To obtain the effective decolorization in the MyCoR and MYCOPOR processes, *P. chrysosporium* required the supplement of a high oxygen concentration and high concentration (0.4%-1.0%) of energy sources such as glucose or cellulose as well as various basal nutrient, mineral solution, Tween 80, and/or benzyl alcohols (Campbell *et al.*, 1982) (Messner *et al.*, 1990) (Cammarota *et al.*, 1992). From an economic point of view, however, nutrients added during fungal treatment must be minimized. Adding glucose to the E<sub>1</sub> effluent also will result in the increase of COD and BOD of the E<sub>1</sub> effluent after fungal treatment because it may not be perfectly consumed during the decolorization stage. Cammarota *et al.* actually reported that although 50% of COD was reduced by MYCOPOR treatment, the COD of the exit stream was higher than the COD of the E<sub>1</sub> effluent because Tween 80 and benzyl alcohol were added at the inlet of the reactor (Cammarota *et al.*, 1992). On the other hand, the reductions of COD of 22%-26% and 29%-37% with KS-62 were obtained by the additions of 0.075% and 0.1% of glucose, respectively, and the COD of the treated effluent was lower than the E<sub>1</sub> effluent COD in the whole treatment period.

The change of pH during the treatment with mycelium of immobilized KS-62 was determined. The pH of the treated E<sub>1</sub> effluent decreased down to 3.6 when high decolorization more than 70% was observed. The change of the pH was similar to that the results shown with *C. versicolor* by the addition of glucono- $\delta$ -lactone in Chapter 4. It might be suggested that the glucose added to the E<sub>1</sub> effluent during fungal treatment was converted into gluconic acid.

In the present experiment, *P. chrysosporium* showed the low decolorization activity. This result might be attributed to the medium used during fungal treatment. The treatment of the E<sub>1</sub> effluent with *P. chrysosporium* needs to be investigated in further detail using the E<sub>1</sub> effluent containing various nutrients.

With immobilized mycelium of the fungus KS-62, practically important and favorable result achieved was that the fungal biomass could be maintained for at least 40 days without any appreciable loss of the decolorization activity in the presence between 0.05% and 0.075% of glucose. Using further biotechnological optimization such as the introduction of a bioreactor, the treatment period can be expected to be extended with high decolorization activity for a possible biotechnological application.

### 6.5. Summary

The treatment of the E<sub>1</sub> effluents was performed with the mycelium of white-rot fungi immobilized in PDA medium. The fungus KS-62 showed from 70% to 80% of the color removal of the E<sub>1</sub> effluent within one- or two-day incubation without any nutrients for four times of replacement under the agitational condition. To achieve the effective decolorization continuously, the addition of glucose to the E<sub>1</sub> effluent was required. The fungal biomass of KS-62 could be maintained for at least 40 days without any appreciable loss of activity in the presence of a critical amount (between 0.05% and 0.075%) of glucose. This is much smaller than the glucose amounts which must be added in the MyCoR and MYCOPOR systems using the immobilized mycelium of *P. chrysosporium*. It is noteworthy that KS-62 did not require any enzyme-inducers and stabilizers such as benzyl alcohols and Tween 80 which are necessary for decolorization in the MyCoR and MYCOPOR systems. With *C. versicolor*, the distinct color removal of the E<sub>1</sub> effluent without glucose was not shown under the same condition as with KS-62. By the addition of 0.5% of glucose to the E<sub>1</sub> effluent, 60% of color removal was shown, but the color removal was immediately decreased if followed by the replacement of the E<sub>1</sub> effluent without glucose. Unlike the decolorization with KS-62 and *C. versicolor*, only partial color removal with *P. chrysosporium* was detected even if 2.0% of glucose was added to the E<sub>1</sub> effluent and the fungal treatment period was prolonged to seven days. The COD of the E<sub>1</sub> effluent treated with KS-62 was lower than that of initial E<sub>1</sub> effluent in the whole treatment period, which indicated that the fungus KS-62 decomposed the chlorolignin in the E<sub>1</sub> effluent.

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## リグニン分解菌によるクラフトパルプ漂白廃液の 処理に関する研究

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### 要 約

上質紙の材料となるクラフトパルプには難漂白性のリグニンが残留するため、塩素系の薬品による多段漂白が必要である。この漂白廃液は強く着色しており、また微量ながらダイオキシン類を含むことが明かとなったが、塩素イオンを含むことから回収処理が困難であり、環境汚染源の一つとして問題となっている。本研究は、リグニン分解能を有する白色腐朽菌による漂白廃液処理の効果とその機構を明らかにし、実用化への指針を示したものである。

まず、強力なリグニン分解能を有する IZU-154 株を用いて、効果的な廃液処理プロセスの設定を試み、添加剤として糖類の共存下、ほぼ完全な廃液の脱色が可能であることを明らかにした。添加剤としては、グルコノ- $\delta$ -ラクトンが最も効果的であり、その効果はリグニン分解菌に共通して認められることを示し、その機構は処理系内での pH の微少な変化に基づく菌体表面の活性化によるものと推定した。

さらに、より実際的な漂白廃液処理プロセスを構築するために、栄養成分要求性が極めて低い高活性廃液分解菌のスクリーニングを試みた。自然界より採集した 1212 点の腐朽材より、多数のリグニン分解菌を分離し、その中から、何ら栄養成分を添加しなくとも廃液のリグニンを分解する特異なリグニン分解菌である KS-62 株の単離に成功した。さらに、本菌株は漂白廃液の色度、化学的酸素要求量 (COD) 及び吸着性有機ハロゲン化合物量 (AOX) を大幅に減少させることを明らかにした。この KS-62 株の菌体を固定化し、廃液の連続処理を試みたところ、微量のグルコースの存在下、長期間にわたって顕著な脱色と COD の減少が継続されることを認め、リグニン分解菌によるパルプ漂白廃液処理プロセス構築のための基礎的知見を明示した。

キーワード：白色腐朽菌, IZU-154, カワラタケ, 漂白廃液, 脱色度, 核酸類, スクリーニング, 吸着性有機ハロゲン化合物, Manganese peroxidase, 固定化菌体, 連続処理