

# Performance of *Ceriporiopsis* sp. in the Treatment of Black Liquor Wastewater

## Unjuk Kerja *Ceriporiopsis* sp. dalam Pengolahan Air Limbah Lindi Hitam

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### ABSTRAK

*Produksi bioetanol dari tandan kosong kelapa sawit menghasilkan air limbah lindi hitam dalam jumlah yang banyak. Lindi hitam ini mengandung NaOH sehingga dengan demikian bersifat toksik pada ekosistem perairan jika dibuang langsung ke lingkungan. Saat ini air limbah lindi hitam telah diolah dengan menggunakan metode koagulasi, akan tetapi tetap membutuhkan pengolahan lanjutan. Penelitian ini bertujuan untuk menentukan kemampuan jamur pelapuk putih dalam mendegradasi air limbah lindi hitam. Lima jamur pelapuk putih diuji kemampuannya untuk mendekolorisasi lindi hitam di media agar dan cair. Dua jamur, *Ceriporiopsis* sp. dan *Phanerochaete chrysosporium*, mampu tumbuh lebih dari 50% di media agar. Lindi hitam dengan dan tanpa koagulasi pada konsentrasi 15000 ppm mampu disisihkan sebesar 70-89% pada hari ke-30i. Unjuk kerja *Ceriporiopsis* sp. dievaluasi dalam kaitannya dengan mendekolorisasi lindi hitam, menyisihkan COD, dan berat kering miselia pada air limbah lindi hitam dengan dan tanpa koagulasi. Warna lindi hitam dengan dan tanpa koagulasi dapat disisihkan masing-masing hingga 90,13 dan 86,65%. COD dapat disisihkan hingga 70,17 dan 40,09% masing-masing dalam lindi hitam dengan dan tanpa koagulasi. Koagulan PAC menghambat degradasi lindi hitam oleh jamur pelapuk putih. Hasil penelitian ini menunjukkan bahwa *Ceriporiopsis* sp. berpotensi untuk digunakan sebagai pendegradasi air limbah lindi hitam.*

**Kata Kunci:** Bioetanol generasi dua; lindi hitam; dekolorisasi; koagulasi; *Ceriporiopsis* sp.; pengolahan air limbah

### ABSTRACT

High amount of black liquor wastewater is generated from bioethanol production by using oil palm empty fruit bunches. It contains an alkaline solution (NaOH), so it is quite toxic for aquatic ecosystems if discharged directly into waters. Actually, black liquor has been treated by coagulation method, but it still needs additional treatment. The aim of this study is to determine the degradation of black liquor wastewater by selected white-rot fungi (WRF). Five different strains of WRF have been tested for their ability to decolorize black liquor on agar and liquid media. Two of five fungi studied, *Ceriporiopsis* sp. and *Phanerochaete chrysosporium*, showed their capacity to grow more than 50% on agar medium. In liquid medium, the percentage of decolorization of 15,000 ppm coagulated and diluted black liquor varied from 70 to 89% at day 30 depending on the fungal strain. *Ceriporiopsis* sp. showed the better ability to decolorize black liquor than *P. chrysosporium*. The performance of *Ceriporiopsis* sp was evaluated in terms of decolorization of black liquor as well as reduction of chemical oxygen demand (COD) and mycelial dry weight, both in original and coagulated black liquor. The color of original and coagulated black liquor can be decolorized up to 90.13 and 86.85%, respectively. Whereas, COD was reduced up to 70.17 and 40.09% in original and coagulated black liquor, respectively. The presence of Poly Aluminum Chloride (PAC) coagulant inhibited degradation of black liquor by fungus. The result demonstrated that *Ceriporiopsis* sp has a potential alternative to treat black liquor wastewater.

**Keyword:** Bioethanol second generation; black liquor; decolorization; coagulation; *Ceriporiopsis* sp; wastewater treatment

## 1. INTRODUCTION

Bioethanol has been produced since the 1970's and then there has been a dramatic increase in bioethanol production based on sugar and vegetable. Unfortunately, this development has attracted criticism because of competition with food availability, actual net energy output, limitation land-use, and the high production cost<sup>(1)</sup>. A solution to this problem is to explore alternative feedstocks such as lignocellulosic biomass, known as second-generation bioethanol.

One lignocellulosic waste that is available in abundance in Indonesia is oil palm empty fruit bunches (OPEFB). OPEFB consists of cellulose (30%), hemicellulose (13%), lignin (37%), and others (20%)<sup>(2)</sup>. Cellulose is converted for bioethanol production via the action of cellulase enzymes into glucose. The breakdown of OPEFB to glucose monomers is the basis for second-generation bioethanol production (G2). Mainly, cellulose is the main part of bioethanol production. Lignin polymer is highly resistant to breakdown because it is a complex aromatic heteropolymer, composed of phenylpropanoid aryl-C3 units, linked together via a variety of ether and C-C bonds<sup>(3)</sup>.

Bioethanol production from OPEFB consists of three primary processes, which are pretreatment, hydrolysis, and fermentation. It requires large quantities of water especially in pretreatment process using alkali chemical NaOH. This production required 600 kg of OPEFB and resulted in 76.46 kg of bioethanol and 3,000 liters of wastewater for pretreatment process, called black liquor. Lignin is the main compound in black liquor<sup>(4)</sup>.

During the production of bioethanol, a wastewater with a high chemical oxygen demand (COD) concentration and also may consist of potentially toxic chlorinated compounds, suspended solids, phenolics, and resin along with lignins is produced<sup>(5)</sup>. The presence of NaOH impacts high pH (10–13) to the wastewater, and it causes poorly biodegradable, and only a few microorganisms can degrade it. The discharge of black liquor with base condition into nature without any treatment is difficult to be degraded, harmful to aquatic ecosystems and constitutes a threat to human health through the contamination of drinking water supplies, recreational water or edible organic species<sup>(6)</sup>.

A wastewater treatment is required for black liquor degradation. Coagulation method by using Poly Aluminum Chloride (PAC) has been developed to treat black liquor. The major advantage of chemical treatment is that high removal of color, COD, and TSS before applying

biological treatment (aerobic treatment, anaerobic treatment, and fungal treatment)<sup>(7,8)</sup>. Because of its chemical complexity contents, a technology that can treat black liquor wastewater simultaneously is needed. The non-biodegradable organic compound fraction and high molecular weight organic compounds need physical and chemical methods. The soluble biodegradable organic compounds are efficiently removed by biological methods<sup>(9)</sup>. Biological methods especially fungal treatment are important ways to depolymerize lignin.

White-rot fungi (WRF) are the most various wood-rotting organisms, because of their ability to degrade lignin, hemicelluloses, and cellulose. WRF can be one option to degrade black liquor wastewater because these fungi secrete one or more enzymes such as lignin peroxidase, manganese peroxidase, and laccase<sup>(10)</sup>. Since the late 1970s, several studies have demonstrated that black liquor as pulp mill effluents could be decolorized by cultures of WRF. Several WRF such as *Phanerochaete chrysosporium*, *Trametes versicolor*, and *T. elegans* are recognized for their ability to degrade lignin and black liquor wastewater<sup>(11,12)</sup>. Decolorization of black liquor from kraft pulping process as 60-90% was needed by several white rot fungi at 60 days<sup>(13)</sup>. The degradation by using WRF is a promising method for efficient, does not require high cost, minimize sludge formed and applicable<sup>(14)</sup>. Adding coagulant is not only effective to remove black liquor, but also create a more complex structures of black liquor. There is a little information about the ability of WRF to degrade coagulated black liquor from bioethanol process.

In this study, an investigation was conducted to explore the lignin-degrading capacity of attached-growth white-rot fungi. In the screening method, the ability of several WRF to degrade black liquor wastewater was tested on agar medium. In the future, the selected fungi were used to degrade black liquor wastewater in a liquid medium. The ability of WRF to degrade the original black liquor wastewater and the remained black liquor wastewater after coagulation was compared. The removal efficiency of the treatments was determined by monitoring the decrease of color, COD, and dry mycelial weights.

## 2. MATERIAL AND METHODS

### 2.1 Organisms

Five white-rot fungi (WRF) consisted of *Volvariella volvacea*, *Pycnoporus sp.*,

*Ceriporiopsis sp.*, *Pleurotus ostreatus*, and *Bjerkandera sp.* were collected from Research Center for Chemistry, Indonesian Institute of Sciences. The history of their origin came from various areas such as Southeast Asia, East Asia, or northern temperate areas. They were cultured on a malt extract agar medium (malt extract 20 g/l, glucose 20 g/l, agar 20 g/l, and polypeptone 1 g/l) at 25° C for seven days and maintained at low temperature 4°C before being used.

## 2.2 Black liquor wastewater

Black liquor wastewater was obtained from bioethanol process in pilot plant Research Center for Chemistry, Indonesian Institute of Sciences. The waste liquor formed in the pretreatment process consists of an alkaline aqueous solution that contains organic and inorganic solids and has a distinctive dark coloration. pH wastewater is 13, and the color is black. The sample was diluted with distilled water to obtain the concentration of black liquor was 15,000 ppm. Coagulation method to degrade black liquor wastewater was used in our previous experiment<sup>(4)</sup>. Black liquor 1% was mixed with poly-aluminum chloride (PAC) at 200 rpm for one minute and then mixed at 50 rpm for two minutes. Next, the effluent sample was left steady for 30 minutes for sedimentation. In this experiment, residual coagulation wastewater was used as a medium for fungal growth and as a pollutant which should be degraded by WRF. To study the effects of PAC for fungal growth and its degradation, diluted black liquor wastewater in the same concentration with coagulated wastewater was also used.

## 2.3 Decolorization on agar medium

Growth rates of strains were assessed in micronutrient medium prepared with black liquor wastewater 15,000 ppm. Chemicals used for agar media can be found in the previous study<sup>(5)</sup>. Petri dishes were inoculated in the center with one agar plug (7 mm diam) cut from the cultured fungus. A control plate with no black liquor added was also inoculated with each strain. The inoculated plates were incubated at 25°C for five days, and the level of growth was assessed by measuring the radial extension of mycelium. The growth percentage was calculated by measuring the increase disc diameter of each fungus, according to the formula (1):

$$\% \text{ growth} = \left( \frac{\phi_c - \phi_s}{\phi_c} \right) \times 100\% \quad (1)$$

where:

$\phi_s$  = fungal diameter in treated sample with black liquor (cm)

$\phi_c$  = fungal diameter in control without black liquor (cm)

The fungi chosen for next experiment were fungal with growth more than 50%.

## 2.4 Decolorization in liquid medium

Three agar plugs of each fungus were inoculated in 20 mL of micronutrient liquid medium (without agar) in 100-mL Erlenmeyer flasks. Inoculated flasks were pre-incubated for seven days under static conditions at 25°C. After pre-incubation, each inoculated flask was supplemented with 15,000 ppm coagulation residual black liquor or diluted black liquor wastewater. Furthermore, the best strain in fungi screening was used to decolorize original coagulation residual black liquor or black liquor wastewater. This wastewater was also used as a nutrient source. For determination adsorption ability, after growing in a liquid medium, the best strain was autoclaved and then each inoculated flask was also supplemented with black liquor wastewater. The samples were collected periodically after 3, 7 and 15, 21, and 30 days for measuring color, COD, and mycelial dry weight. After degradation time, samples were centrifuged to get supernatant that measured in the next experiment.

## 2.5 Analytical methods

The change in absorbance of black liquor was determined at 575 nm with a UV-Vis spectrophotometer (Optizen 2120 UV). Micronutrient medium only containing black liquor was used as a control. The percentage of decolorization was calculated as follows:

$$\text{Decolorization (\%)} = \left( \frac{C_c - C_s}{C_c} \right) \times 100\% \quad (2)$$

where  $C_c$  is the initial dye concentration (ppm) and  $C_s$  is the final dye concentration (ppm).

COD analysis was performed by Standard Methods from APHA/AWWA/WEF<sup>(15)</sup>. The mycelial of fungus was separated from the medium using filter paper, and then dried at 55°C for 24 h. The weight of mycelial on the filter was measured to determine the mycelial mass of fungus. The pH before and after degradation was measured by using the pH meter.

## 2.6 Statistical analysis

The ability comparison of *Ceriporiopsis sp.* and *P. chrysosporium* was conducted by using ANOVA with Tukey Simultaneous Test in the program MINITAB 17. The difference of average

value (mean) was seen from Adjusted P -value in Tukey Test Simultaneous table with the level of confidence of 95%.

### 3. RESULTS AND DISCUSSION

#### 3.1 Screening of fungi on agar and liquid media contaminated black liquor at 15,000 ppm

A preliminary step on plates was carried out to determine the capacity of the fungal strains for black liquor decolorization. Among all the five strains tested, two fungi have the ability to grow more than 50% covering agar medium contaminated black liquor during five days (Figure 1). It means that these fungi have the potential to degrade lignin, and they can adapt to the pollutant. In liquid medium, *Ceriporiopsis sp* demonstrated the highest capabilities for decolorizing black liquor than others. The initial decolorization was fast, reaching  $60.4 \pm 1.43\%$  on day 7. After that, there was a slower rate up to 30 d, probably due to the depletion of glucose in the culture and the production of the enzymes for lignin degradation<sup>(4, 16)</sup>.

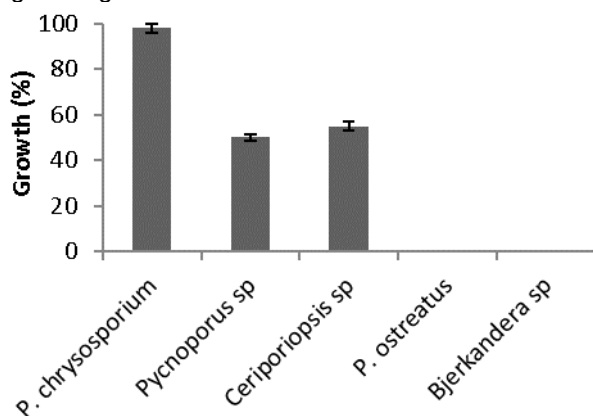


Fig. 1 Growth of fungi on agar medium contaminated black liquor

Fungal cultures were grown on the AG liquid phase and supplemented with black liquor. In the liquid medium, *Ceriporiopsis sp* demonstrated the higher capability for decolorizing black liquor than *Phanerochaeta chrysosporium*. The difference ability to degrade black liquor on agar and in liquid media was caused by the adaptation ability to decolorize black liquor and secretion of ligninolytic enzymes by fungi is different<sup>(17)</sup>.

Both fungi show the similar ability to decolorize coagulated black liquor. *Ceriporiopsis sp* and *P. chrysosporium* started the rapid decolorization of coagulated black liquor, by 74.89 and 72.42%, respectively, at day 3 (Fig.2). After that, from 15 d, it was followed by a slower rate

during the remaining period of incubation. Decolorization of black liquor was mostly affected by the fungal strain, the concentration of black liquor, and the carbon source. The slower changes in the decolorization of black liquor were due to the depletion of glucose as a nutrient source in the culture<sup>(4)</sup>. *Trametes versicolor* has able to detoxify and reduce the color of aromatic compounds and chemical oxygen demand (COD) from black liquors in a soda pulping mill. It reduced the color of aromatic compounds and COD of 70–80% and 60%, respectively<sup>(18)</sup>. *T. versicolor* secreted laccase during degradation of black liquor. In the initial incubation, the decolorization of coagulated black liquor was higher than original black liquor. The small amounts of metals i.e. Aluminum, Cobalt, Copper can enhance the growth and enzyme activities of fungi<sup>(19)</sup>. However, each fungus has difference tolerance level for resistant with these metals. Aluminum can improve  $\beta$ -1,4-N-acetylglucosaminidase enzyme activity of *Trichoderma* fungi<sup>(19)</sup>. Furthermore, several extracellular enzymes remain active under metal concentrations.

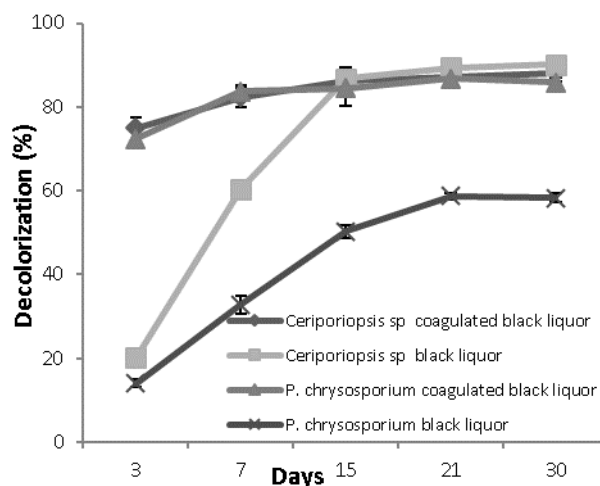


Fig. 2. Decolorization of 15,000 ppm black liquor without and with coagulation by *Ceriporiopsis sp.* and *P. chrysosporium*

*Ceriporiopsis sp.* shows the higher ability to decolorize diluted black liquor than *P. chrysosporium*. A few publications reported the ability of this strain to degrade pollutant compounds. *Ceriporiopsis subvermispora* CZ-3 was able to decolorize black liquor, remove COD, and degrade lignin at glucose at 1 g/l by 90%, 45%, and 62%, respectively<sup>(20)</sup>. However, in the absence of glucose, this fungus only decolorized 62% of black liquor. *Ceriporiopsis sp.* is known as a genus of fungi in the family *Phanerochaetaceae*. This fungus produces manganese peroxidase (MnP) and laccase<sup>(21)</sup>. The ligninolytic enzymes i.e. lignin peroxidase

(LiP), MnP, and laccase in WRF are known having the ability to degrade pollutant compounds. Based on the screening results, *Ceriporiopsis sp.* was selected for degradation of original black liquor wastewater in the liquid medium.

### 3.2 Decolorization of original black liquor by *Ceriporiopsis sp.*

In this experiment, original black liquor both with and without coagulation process was used. In our previous result, PAC 1% has able to decolorize original black liquor 98%. This result was in line with Irfan et al.<sup>(22)</sup> that PAC is excellent for color reduction. Coagulation-flocculation method is one of the best options to treat black liquor wastewater because it is cheaper than other methods such as membrane filtration and ozonation<sup>(22)</sup>. PAC is a pre-polymerized Al(III) chemical, containing a range of hydrolysis and polymeric species. PAC may slow the rate of hydroxide precipitation, so this coagulant is useful for a longer time process, and more strongly adsorb at surfaces<sup>(23)</sup>. However, the high concentration of PAC lead to problems in solid-liquid separation of postprecipitation of Al and the large amount of coagulated sludge<sup>(24)</sup>.

Fig. 3 shows that *Ceriporiopsis sp.* has greater decolorizing of coagulated black liquor than original black liquor. In coagulation method, carboxylic and phenolic groups in black liquor wastewater reacted with metal cations of PAC<sup>(5)</sup>. Resultant compounds from coagulation process were degraded by *Ceriporiopsis sp.* It was assumed that this compound is easier to be degraded than black liquor compound because of its simple structure. WRF seem to be the most likely organisms to detoxify black liquor by degrading low molecular weight phenolic compounds, while increasing biodegradability due to the fractionation of high molecular weight compounds<sup>(18)</sup>.

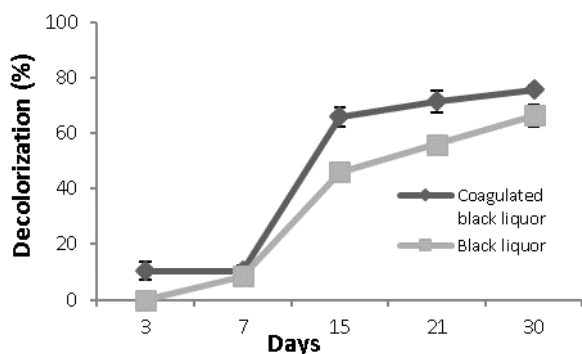


Fig. 3. Decolorization of original black liquor by *Ceriporiopsis sp.*

Based on the results of ANOVA, in all the incubation times, the decolorization of coagulated black liquor by using *P. chrysosporium sp* and *Ceriporiopsis sp.* was not significantly different. On the other hand, the decolorization of original black liquor by using *P. chrysosporium sp* and *Ceriporiopsis sp.* was significantly different. Therefore, from statistical analysis, it can be concluded that *Ceriporiopsis sp.* has better decolorization of original black liquor level than *P. chrysosporium*

Fig. 4 shows that PAC was effective coagulant to remove COD in black liquor wastewater. This removal could be enhanced by the addition of fungal treatment by *Ceriporiopsis sp.* Unfortunately, by only fungal treatment, COD removal was less than 30% at 30 d incubation. Inter-unit bonds in lignin are not hydrolyzable, so they are difficult to be biologically degraded. COD of removal can be increased if WRF secreted the higher enzyme activity. Enhancing enzyme activity could be done by addition of inducers, trace elements or carbon and nutrient source.

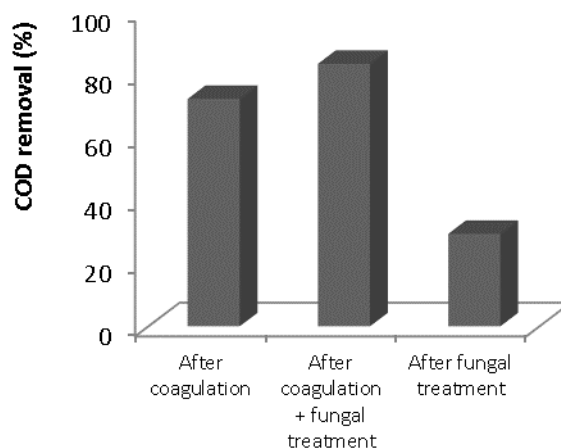


Fig. 4. COD removal in black liquor treatment

Fig. 5 shows that growth of *Ceriporiopsis sp.* was not affected by black liquor. It means that black liquor was not toxic for this strain. Lignin in black liquor was used as carbon source for growth of fungi. Furthermore, in line with the previous result from Kredics et al.<sup>(19)</sup>, aluminum enhanced the growth of fungi and *Ceriporiopsis sp.* has adaptation to heavy metal concentration in black liquor. Some microorganisms have difference tolerable rate to heavy metals depend on their ability to adsorb, bio-accumulate and transform metals<sup>(25)</sup>. Conidia produced by fungal strains growing in different heavy metal concentrations showed different morphological structure caused by resistance mutations<sup>(26)</sup>. The mycelial dry weight of *Ceriporiopsis sp.* without black liquor was similar with the mycelial dry

weight of dead fungus cell contaminated black liquor, means adsorption did not play a role for this decolorization.

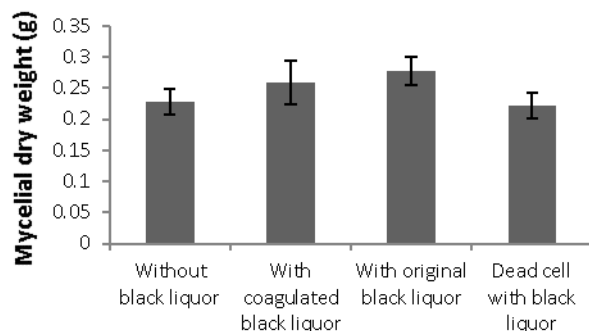


Fig. 5. Mycelial dry weight of *Ceriperiopsis sp.* at 30 days

We assumed two mechanisms might play roles for decolorization of black liquor by *Ceriperiopsis sp.*: pollutant was used as energy source and enzyme produced by WRF was secreted during limitation of glucose. This enzyme was used to attack black liquor<sup>(10)</sup>. The summary of coagulated black liquor removal is shown in Table 1.

Table 1. The removal of coagulated black liquor by *Ceriperiopsis sp.* on day 30

Parameter	Values
Decolorization of black liquor	75.57%
Degradation of lignin	70.4%
COD	83.35%
Mycelial dry weight	0.26 gram
Sludge weight after coagulation	0.5 gram

*Ceriperiopsis sp.* was found competent of reducing COD up to 83.4%, black liquor up to 75.6%, and lignin content up to 70.4% after 30 d. The similar result between decolorization of black liquor and degradation of lignin showed that lignin was broken down into other compounds. WRF are known having the capability to breakdown lignin with their extracellular enzymes i.e. lignin peroxidase, manganese peroxidase, and laccase. *P. chrysosporium* produces LiP and MnP corresponding to lignin degradation<sup>(27)</sup>. The presence of binding sites for oxidation of non-phenolic aromatics in the cases of LiP, and Mn<sup>2+</sup> in the case of MnP, respectively, are used to degrade lignin. The reaction of MnP with b-aryl ether lignin model compounds is proceeded via a phenoxy radical intermediate, followed by Ca–Cb bond cleavage, and also alkyl-phenyl bond cleavage reactions. On the other hand, the reaction of LiP with b-aryl ether lignin model compounds is proceeded via Ca–Cb bond cleavage. Furthermore, due to its high redox

potential, LiP can also oxidize nonphenolic methoxy-substituted lignin subunits. *P. chrysosporium* produced several metabolic products during degradation of lignin such as benzoic acid, benzaldehyde, cinnamic acid, biphenyl-5-5'-dicarboxylic acid, 2,2'-dihydroxy, 3,3'-dimethoxy, and diphenyl ether<sup>(28)</sup>. However, metabolic products of lignin during degradation of black liquor by *Ceriperiopsis sp.* still needs further investigation.

Decolorization by WRF is not much impressive in reducing color but reduction in BOD and COD has been found to be much satisfactory<sup>(29)</sup>. The advantages of the biological treatment are no sludge and less toxic because enzyme activity produced by fungi can break down the structure of black liquor. Unfortunately, a very limited experience is available on the possibility of direct degradation of highly contaminant black liquor by WRF.

#### 4. CONCLUSIONS

*Ceriperiopsis sp.* showed the ability to grow more than 50% on agar medium. In liquid medium, this strain decolorized 15,000 ppm coagulated and diluted black liquor by approximately 90% at 30 days. Further, the color of original and coagulated black liquor can be decolorized up to 90.13 and 86.85%, respectively. COD in original and coagulated black liquor was reduced up to 70.17 and 40.09%, respectively. The result showed *Ceriperiopsis sp.* has a potential alternative to treat black liquor wastewater. By using combination treatment coagulation-fungal treatment, we obtained several advantages such as high decolorization and COD removal, less sludge comparing with only coagulation method by PAC 5%, and less toxic because lignin compound is breakdown. Finally, coagulation–WRF systems are presented in this work as a promising efficient alternative for the treatment of black liquor. Further, to make efficient, this strain can be optimized by immobilization system in an aerated reactor, completed with fluidized, stirred, and air-pulsed reactors.

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