Influence of inducers, Tween 80, and agitation on the enhancing decolorization of black liquor by *Trametes versicolor* F200

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**Abstract.** Black liquor wastewater was generated from bioethanol pre-treatment process. The combination of coagulant-floculants, Poly Aluminium Chloride (PAC) and Anionic Polyacrylamides 3 % has able to reduce COD concentration 99.8 %. After coagulation-flocculation process, this wastewater was treated by *Trametes versicolor* F200. The aim of this study was to determine the decolourisation of coagulated black liquor by *T. versicolor* F200 and the effects of inducers, Tween 80, and agitation to enhance the decolourisation of coagulated black liquor. In addition to the decolourisation rate, COD concentration and enzyme activity were also measured in this study. Further, the characterization of lignin from black liquor was measured by LC-MS to determine the molecular weight of lignin before and after treatment by *T. versicolor* F200. The result showed that the optimum condition for obtaining the highest decolorization of coagulated black liquor were 97.56 % with addition of 2mM CuSO₄, 2mM MnSO₄, 2 % Tween 80 and agitation 150 rpm. The ranges of decolourisation during that condition were (97.56 – 97.89) %. Further, the decreasing molecular weight (m/z) of lignin during degradation process (539 to 325) showed that lignin compound can be degraded into smaller compound by using *T. versicolor* F200.

1. Introduction

Bioethanol pretreatment process from oil palm empty fruit bunches (OPEFB) generated black liquor wastewater. Production of bioethanol in a pilot plant from 600 kg of OPEFB resulted 76.46 kg of bioethanol and 3,000 liters of black liquor wastewater [1]. Black liquor contains lignin amount to 37.5 %, which this compound difficult to be decomposed and has potential to pollute the environment [2–5]. Utilization of white-rot fungi (WRF) can be one alternative for handling this wastewater because it is biodegradable and secretes several ligninolytic enzymes, which can reduce the toxicity of pollutant. One of WRF that commonly used to degrade the pollutant compounds was *Trametes versicolor* F200. This fungus was effective for degrading lignin in black liquor because it has three ligninolytic enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase [6,7].

Degradation of lignin in black liquor occurs color change from dark to light; this phenomenon was expressed as decolourisation of black liquor. However, the biodegradation of black liquor by WRF is less effective if implemented by itself, without combination with physical-biological methods. It was caused by the ability of WRF for alive only in small concentration of black liquor. Before biodegradation
The second treatment after coagulation-flocculation is still necessary to be conducted. Biological method was chosen because the enzymatic systems can metabolize the pollutant compound to be less toxic compound. The aim of this research was to determine the decolourisation rate of coagulated black liquor and the effects of inducers, Tween 80, and agitation to enhance the decolourisation of coagulated black liquor. Several variations of concentrations of inducers, Tween 80, and agitation were made to know the optimum condition for decolorization of coagulated black liquor. In addition to the decolourisation rate, COD concentration and enzyme activity were also measured in this study. Further, the characterization of lignin from black liquor was measured by LC-MS to determine the molecular weight of lignin before and after treatment by *T. versicolor* F200.

## 2. Methods

### 2.1. Chemicals

Black liquor from bioethanol pretreatment process in Research Center for Chemistry LIPI, *Trametes versicolor* F200 from InaCC Research Center for Biology, and other chemicals from Wako Japan, include: Poly Aluminium Chloride (PAC), polyacrylamide, Potato Dextro Agar (PDA), glucose, KHPO₄, KHPO₃, HSO₄, phenol, 2,6-dimetilfenol (2,6-DMP), a buffer solution LiP, H₂O buffer, malonate buffer solution, syringal arudazin, MnSO₄, and sodium acid.

### 2.2. Procedures

*Trametes versicolor* F200 was maintained on potato dextro agar (PDA) medium, incubated at 25 °C for 7 days. The fungus was inoculated into 100 mL of a liquid medium containing: glucose 10 g L⁻¹, KHPO₄, 0.5 g L⁻¹, KHPO₃, 0.6 g L⁻¹, CuSO₄, 0.4 mg L⁻¹, MnCl 0.09 mg L⁻¹, NaNO₃, 0.02 mg L⁻¹, FeCl₃, 1 mg L⁻¹, ZnCl₂, 3.5 mg L⁻¹, and thiamine hydrochloride 0.1 mg L⁻¹. This sample was concentrated at pH 4.5 and pre-incubated at 25 °C for 7 days. The extracellular fluid was blended and added to coagulated black liquor. Several concentrations of inducers (CuSO₄, and MnSO₄ 0.1; 0.5; 1; and 2 mM), Tween 80 (0.5 %, 1 % and 2.5 %), and agitation (50, 100 and 150 rpm) were used. The decolourisation, COD, and enzyme activity of fungus were analyzed at 0, 2, 4, 14, 24, 48, 72, and 96 hours of incubation time.

### 2.3. Enzyme activity

Enzyme activity of the fungus was determined by monitoring MnP, LiP and Laccase activity during degradation process. Activity of Laccase was determined by monitoring the oxidation of syringal arudazin and sodium acid buffer at wavelength 525 nm [8]. Activity of LiP was determined by monitoring the formation of 2 mM H₂O and LiP buffer solution at wavelength 310 nm [9]. Activity of MnP was determined by monitoring oxidation of 20 mM 2,6-dimethylphenol (2,6-DMP) in 50 mM malomat buffer solution, 20 mM MnSO₄ and 2 mM H₂O at wavelength 470 nm [10]. Everything was measured after 1 minute, at temperature 20 °C and the activity of enzymes are express in units U/I. This unit describe the amount of enzyme required to oxidize 1 mol of substrate for 1 minute.

| Parameters   | Before       | After coagulation-flocculation |
|--------------|--------------|-------------------------------|
| COD (mg/L)   | 194.458      | 288.44                        |
| pH           | 12.89        | 4.5                           |
| TSS (mg/L)   | 36.550       | 40.72                         |
| Color        | black        | yellow                        |

of black liquor by using WRF, black liquor was treated by coagulation-flocculation method. It is intended to decrease the concentration of black liquor before biodegradation. The black liquor was treated by using coagulant Poly Aluminium Chloride (PAC) 3 % and flocculant Anionic Polyacrilamide 3 %. Table 1 showed the characteristics of black liquor before and after coagulation-flocculation process.

### 2.4. Analysis of black liquor

The concentration of lignin from black liquor was measured by LC-MS to determine the molecular weight of lignin before and after treatment by *T. versicolor* F200.
Figure 1. Decolorization of coagulated black liquor and COD reduction by *T. versicolor* F200.

2.4. *Measurement of chemical oxygen demand (COD)*
COD was measured by UV-Vis spectrophotometer. Sample was added with 1.5 mL of digestive solution and 2.5 mL of H2SO4. After that, the sample was boiled at 150 °C for 2 hours and then sample was measured at wavelength 600 nm [11].

2.5. *Calculation decolourisation*
Concentration of black liquor before and after decolourisation was measured by UV-Vis spectrophotometer at wavelength 393 nm. Percentage of decolourisation was calculated by the following equation:

\[
\text{Decolourisation} \% = \frac{C_c - C_s}{C_c} \times 100 \%
\]

Where \( C_c \) is initial concentration of black liquor (ppm) and \( C_s \) is final concentration of black liquor (ppm).

2.6. *Characterisation by LC-MS*
Sample was centrifuged at 10,000 rpm for 10 minutes. After that, supernatant was added by 6M HCl to pH 1–2, then extracted with ethyl acetate at the same of volume. The liquid phase carried on organic phase was added Na2SO4. Then, sample was filtered and evaporated at 40 °C for evaporation of the organic solvent, so the lignin extractant was obtained. After that, it was analyzed by LC-MS [12].

3. *Results and discussion*

3.1. *Decolorization of coagulated black liquor by T. versicolor F200*
Before black liquor was treated with *T. versicolor* F200, it was treated by coagulation-flocculation method using coagulant PAC 3% and Anionic Polyacrylamide 3% flocculants. PAC was used as coagulant because it has high positive charge with trivalent cation where it is the most effective cation for neutralizing the charge in suspended particles and it forms the floc faster than other coagulants [13]. After large floc was formed, suspended particles will be easily precipitated. Polyacrylamide anionic has able to bring suspended particles and agglomerate microflocs formed by coagulant to form larger flocs [14]. Further, *T. versicolor* F200 was used to degrade coagulated black liquor at the time of incubation 0, 2, 4, 14, 24, 48, 72, and 96 hour. This strain has able to decolourise coagulated black liquor 95.69 % and decrease COD concentration to 243.22 mg/L at 96 h (figure 1).

In the variation of incubation time, the decolourisation rate of coagulated black liquor has similar result. On the other hand, COD reduction in coagulated black liquor was increased during incubation.
Table 2. Effect of inducers, Tween 80 and agitation to decolourise black liquor

| Effect        | Concentration | Decolourisation (%) |
|---------------|---------------|---------------------|
| CuSO$_4$ (mM) | 0.1           | 97.54               |
|               | 0.5           | 97.66               |
|               | 1             | 97.66               |
|               | 2             | 97.71               |
| MnSO$_4$ (mM) | 0.1           | 97.23               |
|               | 0.5           | 97.27               |
|               | 1             | 97.35               |
|               | 2             | 97.59               |
| Tween 80 (%)  | 0.5           | 97.30               |
|               | 1             | 97.32               |
|               | 2             | 97.89               |
| Agitation (rpm) | 50          | 97.32               |
|                | 100           | 97.35               |
|                | 150           | 97.56               |

This result describes that black liquor can be degraded by *T. versicolor* F200 and hardness of black liquor was getting lower.

Enzyme activity of *T. versicolor* F200 was measured to determine the most effective enzyme to degrade lignin in black liquor. During degradation process, it secreted three ligninolytic enzymes: LiP, MnP, and laccase. The result shows that LiP (571.21 U/l) was higher than MnP (84.83 U/l) and laccase (120.86 U/l) (figure 2).

During COD reduction, LiP activity was increased. It means that secondary metabolism was occurred during degradation process and LiP plays a role during degradation of coagulated black liquor. LiP was specific to lignin substrate, however, MnP and laccase also have bound inductively into lignin substrate.

3.2. Effects of inducers, Tween 80, and agitation to decolourisation of black liquor

Inducers used in this research were CuSO$_4$ and MnSO$_4$ in variation of concentration 0.1; 0.5; 1; and 2 mM to know the optimum condition for decolourisation of coagulated black liquor by *T. versicolor* F200. Inducers act as cofactor Cu$^{2+}$ and Mn$^{2+}$ between the enzyme and lignin substrate [15]. Tween 80 in variation concentration 0.5%, 1% and 2.5% plays a role as a surfactant between organic compound of enzyme and inorganic compound of lignin [16]. The agitation with variation 50, 100, and 150 rpm were used to increase the amount of oxygen in liquid medium of fungus [17].

Figure 2. Enzyme activity of coagulated black liquor by *T. versicolor* F200.
Figure 3. Effects of optimum condition to LiP activity and COD reduction.

Figure 4. Chromatogram (a) lignin before and (b) after process.

The optimum condition for decolorizing black liquor was 2mM CuSO₄, 97.71 %, 2mM MnSO₄, 97.59 %, 2 % Tween 80 97.89 %, and agitation 150 rpm 97.56 % (table 2). The highest decolorization was obtained during addition of 2% Tween 80 because enzyme was more active during presence of Tween 80 in the degradation process. LiP activity and COD concentration at optimum condition is shown in figure 3.

The highest decolourisation of coagulated black liquor during addition of Tween 80 was coincided with the lowest COD concentration and the highest LiP activity. It means the hardness of black liquor was getting lower after addition of Tween 80. Tween 80 is useful in enhancing decolourisation by increasing the bioavailability of coagulated black liquor, activating production of ligninolytic enzymes, and having a carbon source for growth of fungi [18,19].

The characterization of lignin using LC-MS was analysed to determine the molecular weight of lignin before and after treatment with the fungus. The decreasing molecular weight (m/z) of lignin during process (539 to 325) showed that lignin compound could be degraded into smaller compound (figure 4).
The result of chromatogram of coagulated black liquor before decolourisation appears two peaks with the retention times are 1.459 and 2.075 minutes. The chromatogram of coagulated black liquor after decolourisation appears three peaks with the retention times are 1.657; 2.353 and 3.473 minutes. The different peaks and retention times indicates that the lignin in coagulated black liquor has been successfully degraded by *T. versicolor* F200. However, the identification of metabolite products still need further investigation.

4. Conclusions
In this study, black liquor was previously treated by coagulation-flocculation method to decrease concentration of black liquor. After that, *T. versicolor* F200 was used to decolorize black liquor. In this study, this strain produced the high LiP during incubation. *T. versicolor* F200 was able to decolorize coagulated black liquor is 95.69 % and reduce COD to 243.22 mg/L at 96 hours. After addition of inducers (CuSO4 and MnSO4, 2mM), Tween 80 2%, and agitation 150 rpm, the decolourisation was improved 97.71%; 97.59%; 97.89%, and 97.56%, respectively. The highest decolourisation of coagulated black liquor during addition of Tween 80 was coincided with the lowest COD concentration and the highest LiP activity. Tween 80 plays a role as a surfactant between enzyme as organic compound and lignin as inorganic compound. Furthermore, the decreasing molecular weight (m/z) of lignin during the process (539 to 325) showed that lignin compound has been successfully degraded into different compounds.

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