Short Communication:

Decolorization and Transformation of Synthetic Dye Methylene Blue by Brown-Rot Fungus *Fomitopsis pinicola*

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Abstract: *Methylene blue (MB) is a synthetic dye widely used in industries that is difficult to degrade in the environment due to its stability. Brown-rot fungus* Fomitopsis pinicola *is an organism that is known to be able to degrade some organic pollutants such as DDT, aldrin, dieldrin, and methyl orange dye. This study aimed to explore the ability of* F. pinicola *on MB biodecolorization, to identify metabolites and propose a biodecolorization pathway.* F. pinicola *was evaluated for MB biodecolorization on PDA agar and PDB liquid media. The metabolites were determined by using LCTOF/MS. The PDA agar medium's largest index decolorization (ID) was 0.915%. The MB decolorization in liquid PDB medium showed the highest percentage of decolorization of 92.56% at MB concentration of 100 mg/L after 14-days incubation. The analysis using LC-TOF/MS showed metabolites from MB biodecolorization, namely 3-amino-7-(methylamino) phenothiazin-5-ium (Azure C), 3-(dimethylamino)-7-(methylamino) phenothiazin-5-ium (Azure B), and 3,7-bis(dimethylamino)-4aH-phenothiazin-5-one. The MB degradation pathway was proposed through demethylation and oxidation reactions based on the detected product metabolites. These results indicated that* F. pinicola *is a suitable agent for biodecolorization of MB, and can potentially be used for bioremediation of MB waste in the environment.*

Keywords: *biodegradation; biodecolorization;* Fomitopsis pinicola*; Brown-rot fungi; methylene blue*

■ INTRODUCTION

The advancement of industry and technology opens vast opportunities for synthetic dyes, widely applied in the textile, food, cosmetic, and pharmaceutical industries. However, the artificial dyes derived from aromatic hydrocarbons, such as benzene, toluene, and naphthalene, are toxic, mutagenic, and carcinogenic [1]. One synthetic dye in liquid waste that can pollute the environment is methylene blue (MB). MB is a heterocyclic aromatic compound discovered by Heinrich Caro in 1876 and was first used as a synthetic drug in the medical field. MB is widely used as a dye for cellulose fibers, silk, leather, wool, and paper [2]. However, only 5% of its solution is used in staining, while the remaining 95% is disposed of as waste [3].

MB is a relatively stable compound and very difficult to degrade in nature. MB is dangerous when the concentration exceeds 2 mg/kg because it causes several problems, including increasing COD (Chemical Oxygen Demand) and the disruption of the ecosystem balance, which is marked by the death of aquatic organisms around the waste disposal site, and indirectly in the human food chain. Furthermore, some of the MB dye's effects on humans include nausea, vomiting, hemolytic anemia, hyperpyrexia, hypotension, and bluish skin [4]. Therefore, it is necessary to develop a remediation method in cleaning dye waste for a safe environment.

Various physical, chemical, and biological methods have been used to treat waste-containing dyes, including phytochemical techniques, such as

adsorption, chemical deposition, flocculation, oxidation by chlorine, hydrogen peroxide and ozone, electrolysis, reduction, electrochemical treatment, ion-pair extraction, and nanotechnology [5-6]. Although the physical and chemical methods are technically feasible for use as dye wastewater treatment, they have many disadvantages, including high operational costs, the formation of dangerous byproducts, large energy consumption, and producing a large amount of residue in the form of sludge [7]. Meanwhile, biological remediation offers a safe, efficient, and low-cost method. For example, Biodecolorization, a method used to reduce color density or the level of contaminants to less or non-toxic materials in soil, water, and the environment by using organisms (fungi or bacteria) as decolorizing agents. Fungi are robust organisms with more tolerance to chemical pollutants at high concentrations than bacteria [8].

The types of fungi that degrade organic pollutants are brown-rot and white-rot fungi. The brown-rot fungi (BRF) use hydroxyl radicals generated from the Fenton reaction to degrade cellulose and hemicellulose by modifying the surrounding lignin. BRF rapidly depolymerize the cellulose in wood before detecting any loss in total wood mass. Furthermore, fungal enzymes such as cellulases and peroxidases are too large to penetrate cell walls during the early stages of wood degradation by brown-rot fungi, which suggests that a brown-rot wood-degrading system component is a component of the brown-rot wood-degrading small enough to diffuse into the sound wood cell wall. The pattern of holocellulose depolymerization in brown rotted wood is similar to that caused by Fenton reagent and natural cellulose depolymerized by hydroxyl radical (·OH) is identical in the chemical structure of brown-rotted cellulose.

Furthermore, some brown-rot fungi degrade lignin-related model compounds to yield relatively large amounts of the same products obtained via ·OH attack on the lignin model compounds. These findings suggest that BRF has a more rapid system to degrade organic pollutants than other fungi [9]. In addition, to produce hydroxyl radicals, these fungi produce several enzymes, such as cellulase, hydrolase, xylanase, glucanase, and glucosidase, which are used to degrade cellulose, a source of carbon and energy [10]. Previously, BRF *Daedalea dickinsii* was found to be able to decolorize and transform MB [11]. Another BRF that can degrade organic pollutants is *Fomitopsis pinicola*. In a previous study, Purnomo et al. [9] reported that the fungus *F. pinicola* could degrade 50 µL of 5 mM DDT (84%) incubated for 28 days in pure culture. Furthermore, Purnomo et al. [10] also reported that *F. pinicola*, *D. dickinsii*, and *Gloeophyllum trabeum* were able to transform methyl orange (MO) dye. Since *F. pinicola* has been proven to degrade some pollutants, including dye, it is necessary to investigate its potential in subverting MB. There has not been any report about the biodecolorization of MB dyes using the BRF *F. pinicola*. Therefore, this research aims to explore the ability of this organism in MB Biodecolorization. In addition, the primary metabolites and the proposed biodecolorization pathway were also determined.

■ EXPERIMENTAL SECTION

Materials

The BRF *F. pinicola* NBRC 8705 was purchased from NITE Biological Resource Center, Japan. This fungus was inoculated in a petri dish containing sterile potato dextrose agar (PDA, Merck, Darmstadt, Germany). Methylene Blue (Merck, C.I. 52015), and methanol (Merck, 99%) were purchased from Sumber Ilmiah Persada (SAP, Indonesia).

Instrumentation

The instruments used in this study included UV-Vis Genesys 10s (Thermo Scientific) and LC-TOF/MS (Bruker), with an ESI mass range of 50–1000 m/z. The column was AcclaimTM RSLC 120 C18, 2.1 × 100 mm with a particle size of 2.2 µm at 33 °C.

Procedure

Culture condition

The BRF *F. pinicola* NBRC 8705 was inoculated in a petri dish containing sterile potato dextrose agar (PDA, Merck, Darmstadt, Germany) and incubated at 30 °C for 14 days until the entire surface of the medium was covered with mycelium.

Biodecolorization of MB on solid medium

F. pinicola culture was inoculated using a loop needle (1 cm in diameter) in a petri dish, which contained PDA and MB dye (purchased from SAP Chemicals, Indonesia) with various final concentrations of 50, 75, and 100 mg/L. The cultures were incubated at 30 °C for 14 days. During this period, the mycelium was measured on the surface of the agar plate (mycelium diameter, MD), while the decolorization was also assessed as the clear zone in the treatment culture (decolorization diameter, DD). The index of decolorization (ID) was obtained from the DD and the MD (ID = DD/MD). As a control, *F. pinicola* was grown on a PDA agar plate medium without the addition of MB, respectively [11].

Biodecolorization of MB on liquid medium

F. pinicola culture was inoculated using a loop needle (1 cm in diameter) into an Erlenmeyer containing 10 mL potato dextrose broth (PDB, Merck, Darmstadt, Germany) medium then pre-incubated for seven days at 30 °C. One milliliter of MB (final concentration of 100 mg/L) was added to the pre-incubated culture and incubated again for 0, 7, and 14 days, and then centrifuged for 10 min at 4000 rpm. The supernatant was taken, and then the absorbance was measured using a UV-Vis spectrophotometer at 400–700 nm for the absorbance profile of MB decolorization. As a positive and negative control, *F. pinicola* was grown on the PDB medium without and with dye, respectively. The percentage of biodecolorization was calculated based on Eq. (1), in which Abs_0 was absorbance control, while Abs_t was absorbance treatment.

$$\%\text{Decolorization} = \frac{Abs_0 - Abs_t}{Abs_0} \times 100\% \quad (1)$$

Analysis of metabolites

The supernatant resulting from centrifugation on day 14 was analyzed using LC-TOF/MS (Bruker), with an ESI mass range of 50–1000 m/z. The column was AcclaimTM RSLC 120 C18, 2.1 × 100 mm with a particle size of 2.2 μm at 33 °C. The mobile phase used was methanol with a ratio of 99:1 in the initial 3 mins, with a flow rate of 0.2 mL/min and 61:39 for 7 min with a flow rate of 0.4 mL/min [11].

Statistical analysis

The data were calculated as the average of three replicate experiments. The student's t-test was used to detect any significant differences within or between the groups during MB decolorization. The confidence level of 5% ($P < 0.05$) was statistically significant between the mean values [11].

■ RESULTS AND DISCUSSION

Biodecolorization of MB in Solid Media

In this study, the MB biodecolorization was begun by determining the MB dye concentration suitable for the process on the PDA agar plate medium with various MB concentrations. This stage aimed to determine the effect of MB concentrations on the growth of the *F. pinicola* and as an initial screening in determining the optimal concentration of MB that *F. pinicola* can decolorize. The varied MB concentrations of 50, 75, and 100 mg/L were chosen because the PDA agar plate media with an MB below 50 mg/L produced a faint color. In comparison, MB concentrations above 100 mg/L yielded a thick color, making it challenging to observe the decolorization process. The results of the mycelium diameter (MD), decolorization diameter (DD), and index of decolorization (ID) are shown in Table 1.

In addition, to measure mycelium growth, the color change that occurred was calculated to indicate that the fungus could decolorize MB dye. Based on Table 1, the *F. pinicola* mycelium growth diameter (MD) in MB solid medium with variations in the final concentrations of 50, 75, and 100 mg/L was approximately 7.1, 6.6, and 7.4 cm, respectively, while the control without MB was 8.1 cm. The results showed that there was no significant difference in mycelium growth among the three varied concentrations and control, indicating that the growth of *F. pinicola* was not affected by the presence of MB dye. These results corresponded with a previous study that showed that dye concentration has no significant effect on BRF growth [10-11]. The MB biodecolorization capability of *F. pinicola* was determined by measuring the ID, approximately 0.90, 0.91, and 0.84 at MB concentrations

Table 1. MB biodecolorization by *F. pinicola* in PDA medium for 14 days incubation period

MB concentrations ($mg.L^{-1}$)	MD (cm)	DD (cm)	DI
50	7.13 ± 0.60a	6.43 ± 0.55a	0.90 ± 0.03a
75	6.60 ± 0.60a	6.03 ± 0.40a	0.91 ± 0.02a
100	7.43 ± 0.92a	6.23 ± 0.50a	0.83 ± 0.04a
Control (without MB)	8.10 ± 0.51a		

Data are presented as the mean ± standard deviations (n = 3). Data followed by the different lower letters on each column indicated significant differences ($P < 0.05$)

of 50, 75, and 100 mg/L, respectively. The results showed no significant difference among the varied MB concentrations. Rizqi and Purnomo [11] reported that *D. dickinsii* was able to degrade MB in various concentrations of 50, 75, and 100 mg/L with ID values of 0.92, 0.90, and 0.88 respectively. In another study, Purnomo et al. [10] also reported that BRF *F. pinicola*, *D. dickinsii*, and *G. trabeum* were able to decolorize MO dye in PDA medium with various concentrations of 50, 75, and 100 mg/L, in which the highest ID value for *F. pinicola* was 0.91 at MO concentration of 50 mg/L. Due to no significant difference between the ID in MB concentrations of 50, 75, and 100 mg/L, the highest MB concentration (100 mg/L) was selected for MB decolorization in the liquid PDB medium.

Biodecolorization MB in Liquid Media

This study also carried out MB biodecolorization by *F. pinicola* on liquid media because MB waste is dominant in liquid form in the environment. In addition, the biodecolorization of MB in liquid media also aims to determine the optimal incubation period for MB biodecolorization by *F. pinicola*. For MB decolorization in a liquid medium, the MB profile absorbance during biodecolorization by *F. pinicola* is shown in Fig. 1. Fig. 1 indicates that the absorbance profile of MB was decreased during the various incubation times. The decrease of the MB absorbance profile indicated the reduction of its concentrations.

Meanwhile, *F. pinicola* degraded MB during the incubation period of 0-, 7-, and 14-days. When MB was added to the pre-incubated culture of *F. pinicola* (0 days), the absorbance profile decreased, indicating that *F. pinicola* already produced some extracellular enzymes and the Fenton during the pre-incubation process reactions as the decolorizing agents [12]. In Fig. 1, the maximum absorbance of MB was detected at a wavelength of 665 nm. Therefore, it was used to determine the percentage of decolorization. The MB biodecolorization ability of *F. pinicola* was quantitatively determined by measuring their rate, as shown in Table 2. The percent decolorization of MB at 0-, 7-, and 14-days incubation were 17.14, 25.85, and 92.56%, respectively. The most significant decolorization percentage was found at the 14-day incubation period, at about 92.56%. This result indicated that the incubation period greatly influenced the degradation of MB by *F. pinicola*.

The ability of *F. pinicola* to degrade MB is associated with their potential to produce extracellular enzymes and Fenton reactions. Joo et al. [13] reported

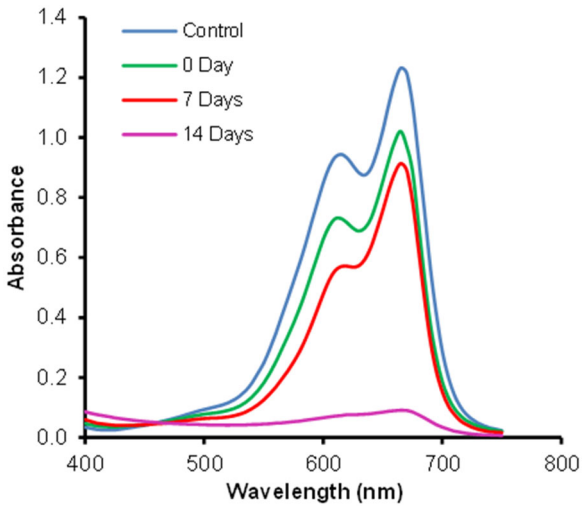

Fig 1. MB absorbance profiles during biodecolorization by *F. pinicola* in various incubation times. Blue lines = Control, green lines = 0-day incubation, red lines = 7-days incubation and pink lines = 14-days incubation

Table 2. Biodecolorization of MB by *F. pinicola* in PDB medium during the various incubation times

Incubation times (day)	MB biodecolorization (%)
0	17.14 ± 0.34a
7	28.85 ± 0.28b
14	92.56 ± 0.72c

Data followed by the different lower letters on each column indicated significant differences among various concentrations of MB ($P < 0.05$). Data are presented as the mean ± standard deviations (n = 3)

That *F. pinicola* secretes an extracellular enzyme, namely β-1,4-glucosidase and endo-β-1,4-glucanase, the enzymes of BRF used to convert cellulose into glucose. Park and Park [14] reported that *F. pinicola* has a novel laccase enzyme. In another research, Shah et al. [15] reported that *F. pinicola* showed extracellular enzyme activity to degrade lignocellulose polysaccharides and proteolytic substrates, with the potential for oxidative decay via Fenton chemistry. *F. pinicola* has also been reported to have the capability to degrade organic pollutants, such as DDT and MO dye, by the involvement of Fenton reactions and extracellular enzymes, such as peroxidase and oxidoreductase.

Identification of MB Metabolites

In this study, MB biodecolorization metabolites were also observed in order to determine what compounds are produced from the biodecolorization process of MB by *F. pinicola* and estimate the MB biodecolorization pathway by *F. pinicola*. The MB biodecolorization metabolite was analyzed using LC-TOF/MS. The LC-TOF/MS chromatogram (Fig. 2) show the separated compounds as several peaks with different

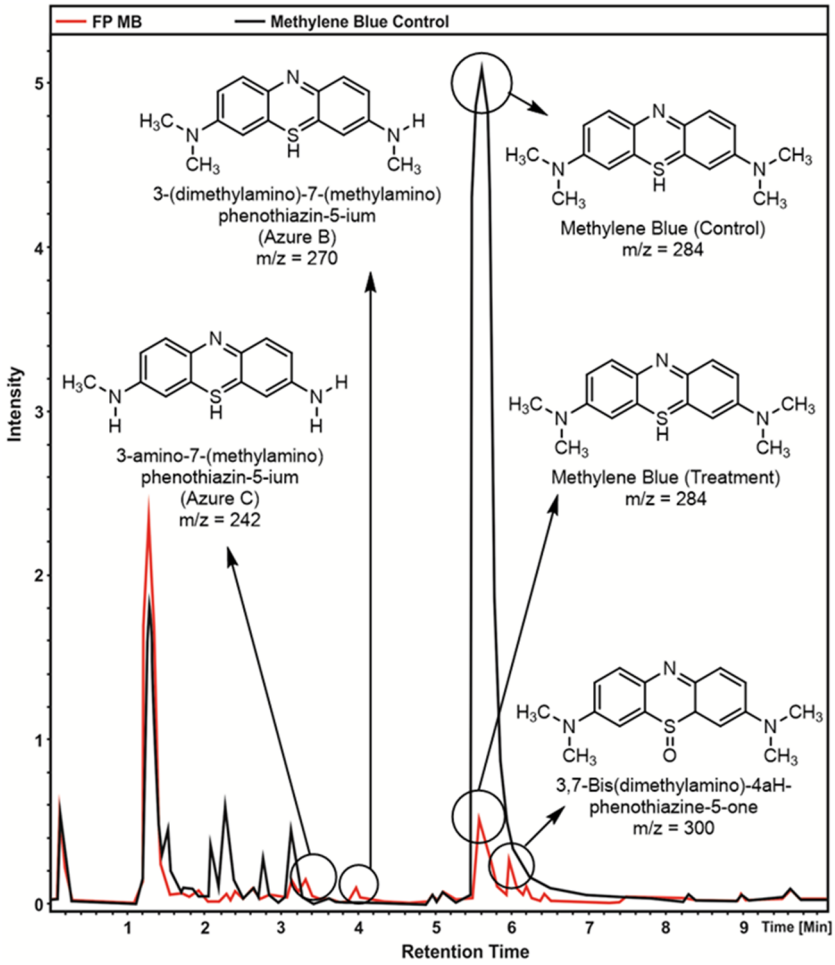

Fig 2. Chromatogram profile of MB biodecolorization by *F. pinicola* during a 14-days incubation

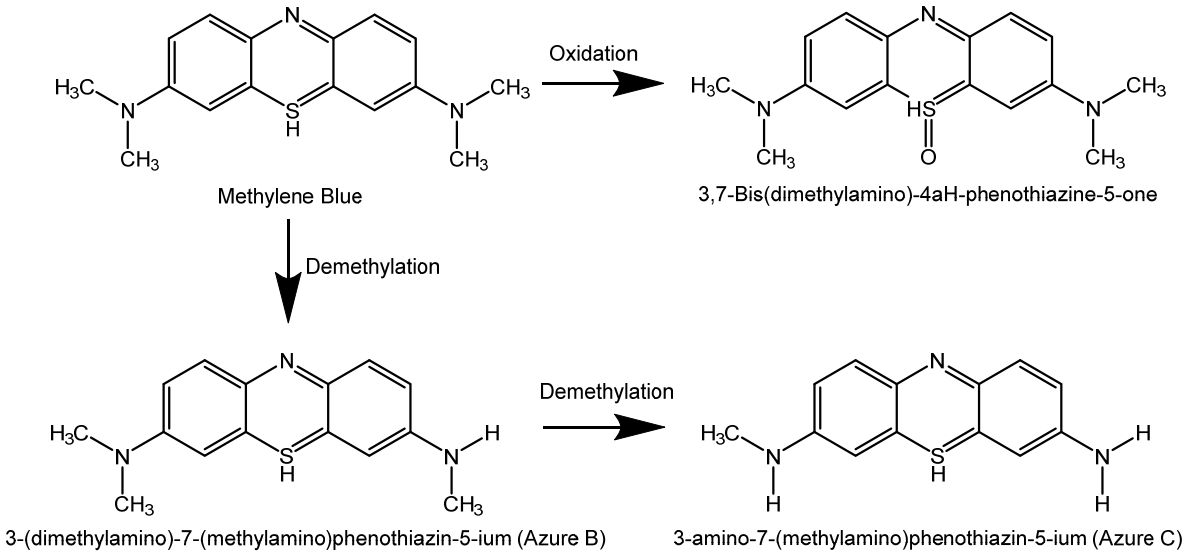

Fig 3. Proposed MB biotransformation pathway by *F. pinicola*

retention times. The black line indicates the control MB peak which exits at a retention time of 5.57 min, while the treated MB peak was shown to be lower than the control, indicating that the MB was degraded by *F. pinicola*. Compared to a standard, the metabolic products of MB biodecolorization by *F. pinicola* were identified by LC-TOF/MS. Three other peaks were detected based on the chromatogram, suggesting the presence of MB biodecolorization metabolites by *F. pinicola.* Azure C (3-amino-7-(methylaminophenothiazin-5-ium) was found in the retention time of 3.50 min, while Azure B (3-(dimethylamino)-7-(methylamino) phenothiazin-5-ium was seen at 3.96 min. Rauf et al. [16] had also found Azure B and C metabolites obtained during MB degradation by a photocatalytic method. Another detected metabolite was 3,7-Bis(dimethylamino)-4aH-phenothiazin-5-one, which was found in the retention time of 6.17 min. Huang et al. [17] found 3,7 bis-(dimethylamino)-4aH-phenothiazine-5-one as the metabolite from MB degradation by atmospheric pressure dielectric barrier discharge plasma method.

Proposed MB Biotransformation Pathway

Based on these results, the MB biotransformation pathway was proposed in Fig. 3. First, the MB underwent an oxidation reaction to form 3,7-bis(dimethylamino)-4aH-phenothiazine-5-one and demethylation to form Azure B. Then, Azure B underwent demethylation again to include Azure C. The capability of *F. pinicola* to carry out an oxidation reaction is due to its ability to produce laccase enzyme. Meanwhile, some studies have reported that laccase activity is involved in the oxidation reaction process [18-19] and the capability of *F. pinicola* to produce O-demethylase enzyme [20]. Venkatesagowda and Dekker [20] reported that *F. pinicola* has O-demethylase activity involved in the lignin demethylation process.

■ CONCLUSION

This study showed that *F. pinicola* successfully biodecolorized MB on PDA agar and PDB liquid media. The largest index decolorization (ID) was 0.915%. The relegated MB in liquid media PDB showed the highest decolorization percentage (92.56%) at a concentration of 100 mg/L after 14-days of incubation. The results of LC-TOF/MS showed metabolites from MB biodecolorization, namely 3-amino-7-(methylamino) phenothiazin-5-ium (Azure C), 3-(dimethylamino)-7-(methylamino) phenothiazin-5-ium (Azure B), and 3,7-bis (dimethylamino)-4aH-phenothiazin-5-one. The MB transformation pathway was proposed through demethylation and oxidation reactions based on the

detected product metabolites. These results indicated that *F. pinicola* is a suitable agent for the biodecolorization of MB, and can potentially be used for bioremediation of MB waste in the environment.

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