Role of Fe²⁺-dependent Reaction in Biodecolorization of Methyl Orange by Brown-rot Fungus *Fomitopsis pinicola*

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1. Introduction

Synthetic dyes are widely used in industries because they have a complex and stable structure, therefore, they are difficult to degrade naturally (Ali 2010; Lade et al. 2015; Das and Mishra 2017; Shah 2019). In the last few decades, various techniques of decolorization and degradation of colored wastes have been studied, such as membrane filtration (Yuan and He 2015), sorption (Zhou et al. 2019), electrochemical, and oxidative degradation (Zhou and He 2007). However, biological methods were mostly focused on as alternative methods that are environmentally friendly and cost-effective for chemical decomposition processes (Purnomo 2017; Boelan and Purnomo 2018; Nadaroglu et al. 2019; Wu et al. 2019; Purnomo et al. 2010a, 2011a, 2014, 2017a, 2020a). Methyl orange (MO) is an azo group dye (-N=N-), which has a very wide spectrum of applications including in the textile, leather, paper, cosmetics, and foodstuff industries. Therefore, the potential for its contamination is very high (Dawkar et al. 2008; Purnomo and Mawaddah 2020).

Generally, studies on biological decolorization and degradation of azo compounds focus on bacteria and fungi. Meanwhile, fungi with no history of toxicity to the environment and living organisms are considered very efficient and safe (Shah et al. 2018; Zahid et al. 2020). Brown-rot fungi (BRF) degrade lignocellulose through the Fenton reaction mechanism (Eq. 1, Contreras et al. 2007). Furthermore, these fungi can produce hydrogen peroxide (H₂O₂) and utilize minerals (Fe/Cu ions) in the substrate or media as a catalyst for the decomposition of H₂O₂ to produce hydroxyl radicals (OH, Purnomo and Mawaddah 2020).

Fe²⁺ + H₂O₂ → Fe³⁺ + OH + OH⁻ ..........................(1)
Fomitopsis pinicola is a brown-rot fungus (BRF) that can produce metabolites to support the Fenton reaction (Contreras et al. 2007; Purnomo and Mawaddah 2020; Purnomo et al. 2010b, 2011b, 2020b; Rizqi et al. 2021). Furthermore, several previous studies have reported that BRF can degrade toxic pollutants, namely (1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane, DDT; (Sariwati et al. 2017; Sariwati and Purnomo 2018; Setyo et al. 2018; Purnomo et al. 2020b), polyvinyl alcohol (PVA; Tsujiyama and Okada 2013), as well as some dyes (Rizqi and Purnomo 2017; Purnomo et al. 2019a, 2020a, 2020c; Purnomo and Mawaddah 2020), both as a monoculture and a consortium with bacteria. This degradation ability correlates with the hydroxyl radical (•OH) produced in the DDT degradation media by the BRF (Purnomo et al. 2010b, 2020c). The involvement of Fenton reaction was confirmed by treating pollutants in media containing Fe²⁺, which showed higher degradation rather than that in media without Fe²⁺ (Purnomo et al. 2010b, 2011a, 2020c). Therefore, in this study, it is possible that F. pinicola can degrade and decolorize methyl orange (MO) in a media conditioned by the Fenton reaction by adding Fe²⁺ (Contreras et al. 2007).

Previous studies have reported that F. pinicola can degrade 1,1,1 trichloro 2,2 bis (4 chlorophenyl) ethane (DDT) (Purnomo et al. 2010b) and polyvinyl alcohol (Tsujimaya and Okada 2013) by involving the Fenton mechanism. Besides, F. pinicola is also able to degrade azo methyl orange (MO) dye (Purnomo et al. 2019a), however, the involvement of Fenton reaction in MO degradation by F. pinicola is needed to further investigation. Therefore, this study investigated the involvement of Fenton reaction including the quantification of MO degradation in the Fe²⁺-dependent media, identification of metabolites, and the MO degradation pathway.

2. Materials and Methods

2.1. Materials

The Brown-rot fungus (BRF) F. pinicola NBRC 8705 (NITE Biological Resource Center, Japan) was obtained from the Microbial Chemistry Laboratory, Department of Chemistry, ITS, Indonesia). The MO (C.I. 13025, Merck), potato dextrose broth (PDB, Merck), magnesium sulfate (MgSO₄, Merck), calcium chloride (CaCl₂, Merck), boric acid (H₃BO₃, Merck), cobalt sulfate (CoSO₄, Merck), copper sulfate (CuSO₄, Merck), ammonium molybdate [(NH₄)₆MoO₂₄, Merck], manganese sulfate (MnSO₄, Merck), zinc sulfate (ZnSO₄, Merck), and Ferro sulfate (FeSO₄, Merck) were used in analytical grade.

2.2. Culture Medium

F. pinicola was inoculated into a PDA agar medium, followed incubated at 30°C for 7 days. Furthermore, the mycelium F. pinicola was homogenated for 30 seconds in a sterile blender containing 50 ml of sterile aqua DM. The fungal culture (1 ml) was then inoculated into PDB media and pre-incubated for 7 days at 30°C in static conditions. After pre-incubation, the PDB medium from the culture was removed, and the mycelium was washed with 30 ml of sterile water (Purnomo et al. 2010b, 2020c).

2.3. Batch Bio-decolorization of Methyl Orange

Biodegradation assays were conducted in mineral salt medium (MSM), which contained MgSO₄ 0.8 mM, CaCl₂ 0.2 mM, H₃BO₃ 12 µM, CoSO₄ 0.4 µM, CuSO₄ 0.2 µM, (NH₄)₆MoO₂₄ 0.04 µM, MnSO₄ 2 µM, ZnSO₄ 0.4 µM and FeSO₄ 20 µM in 1 L media. For treatment cultures, the washed F. pinicola mycelium was transferred into Erlenmeyer flasks containing 9 ml of MSM with Fe²⁺ and without Fe²⁺ (Purnomo et al. 2010b, 2020c). Furthermore, the MO (final concentration: 100 mg/l) was inserted into the cultures, incubated at 30°C, and examined after 0, 7, 14, 21, and 28 d of treatments. This was followed by the separation of the F. pinicola mycelium by centrifugation at 3000 rpm for 5 mins (Purnomo et al. 2019a, 2020a; Purnomo and Mawaddah 2020). For control cultures, the washed F. pinicola mycelium was autoclaved for killing the fungus before transferred into MSM.

The MO decolorization was measured using a UV-vis spectrophotometer at a wavelength of 200 to 700 nm, and the rest of the decolorized supernatant was stored for metabolites identification. The decolorization was calculated using the Eq. 2:

\[
\text{% Decolorization} = \frac{\text{Abs}_0 - \text{Abs}_t}{\text{Abs}_0} \times 100\% \quad \text{(2)}
\]
Where Abs was the absorbance control, while Abs was the absorbance treatment (Purnomo et al. 2019a, 2020a; Purnomo and Mawaddah 2020).

2.4. Metabolites Identification

The MO metabolites were identified using LC-TOF/MS analysis with electrospray ionization (ESI) ranging from 50-500 m/z. Furthermore, Elution was carried out using methanol: water with a flow rate of 0.2 ml/min (99:1) in the first 3 minutes and 0.4 ml/min (61:39) in the next 7 minutes. The type of column used in the study was Acclaim TM RSLC 120 C18 (2.1 x 100 mm; Purnomo et al. 2010c, 2017b; Boelan and Purnomo 2019).

2.5. Statistical Analysis

The results were presented as an average of the triplicate measurements. Furthermore, significant differences between or within groups during the decolorization process were determined using a t-test and a confidence level of 5% (Purnomo et al. 2013, 2019b).

3. Results

3.1. Involvement of Fenton Reaction in MO Biodecolorization

Decolorization evaluation was carried out during the incubation phase, following the biomass separation phase using a centrifuge (3,000 rpm, 10 mins), while the absorbance of the filtrate was measured using a spectrophotometer at a wavelength of 400-800 nm. The negative control was a mixture of MO and mineral salt media with and without Fe2+. Figure 1 showed the MO biodecolorization by F. pinicola in the mineral salt media with and without Fe2+. Furthermore, it shows the absorbance profile of MO during degradation by the fungus for 28 days. The decolorization analysis was performed every 7 days. The MO decolorization began to be significant after 7 days of incubation in both conditions, namely 38% (with Fe2+) and 30% (without Fe2+). At the 28 days of decolorization, it was observed that the presence of Fe2+ in the media could cause the optimal degradation of MO, namely 89.47% (with Fe2+) and 80.08% (without Fe2+) (Table 1). Meanwhile, the higher degree of decolorization in the medium containing Fe2+ indicated that F. pinicola involved the Fe2+-dependent reaction in the degradation of MO. Furthermore, the decrease in absorbance during the first 7 days indicated that a metabolite of F. pinicola has been produced to decolorize the MO.

Figure 1. Absorbance profile of MO by F. pinicola in medium (A) With Fe2+, and (B) Without Fe2+

Thank you for the comment. The further analysis is the identification of metabolites using LC-TOF/MS. The results showed that there was a new peak in the sample chromatogram at retention times of 1.28 and 2.70 mins, indicating metabolites with m/z of 258 and 391, respectively (Figure 2). Based on the TOF-MS data, the metabolite with m/z of 258 and 391 were
Table 1. Percentage of biodegradation of MO by *F. pinicola* in mineral salt media

| Incubation time (day) | Decolorization (%) with Fe^{2+} | Decolorization (%) without Fe^{2+} |
|-----------------------|----------------------------------|------------------------------------|
| 0                     | 4.96±0.14a                       | 4.72±0.16a                         |
| 7                     | 38.24±0.47b                      | 30.13±0.41b                        |
| 14                    | 47.42±0.09c                      | 36.72±0.49b                        |
| 21                    | 68.84±0.46d                      | 64.08±0.36c                        |
| 28                    | 89.47±0.14e                      | 80.08±0.18d                        |

The data were determined by LC-TOF/MS. Data are presented as the mean ± standard deviations (n = 3). Data followed by the different lower letter on each column indicated significant different (P <0.05)

3.2. Metabolites Identification

The metabolites were identified after the MO degradation by *F. pinicola*, using LCMS. The main peak of the chromatogram at a retention time of 7.27 min showed a major peak in the MO (m/z 306) control and

![Figure 2. Chromatograms of MO degradation by F. pinicola in mineral salt media with Fe^{2+}](image)
samples. After 28 days of incubation, the MO showed a very significant decrease in the intensity of the sample chromatogram, and this indicated that the dye was completely decolorized. New peaks on the sample chromatogram as metabolites appeared at the retention time of 1.28 and 2.70 minutes with m/z of 258 and 391, respectively. Furthermore, based on the analysis, the m/z 258 was identified as 4-(2-(4-(dimethyliminio)-2-hydroxycyclohexa-2,5-dien-1-ylidene)hydrazinyl)phenolate, while m/z 391 was 4-(2-(4-(dimethyliminio)cyclohexa-2,5-dien-1-ylidene)hydrazinyl)benzene sulfonate. These two compounds were the metabolites produced during the MO decolorization process.

The metabolite structures identified after incubation of MO with *F. pinicola* are shown in Table 2. Furthermore, based on these structures, the original structure of MO underwent hydroxylation and methylation, and 4-(2-(4-(dimethyliminio)-cyclohexa-2,5-dien-1-ylidene)hydrazinyl)benzene sulfonate was a metabolite obtained from the reactions. In addition, the hydroxylation and desulfonation lead to the production of 4-(2-(4-(dimethyliminio)-2-hydroxycyclohexa-2,5-dien-1-ylidene)hydrazinyl)phenolate. The identified metabolites were the residue from the transformation which would be degraded into simpler compounds.

![Diagram of MO degradation pathway by F. pinicola](https://example.com/diagram.png)

**Figure 3. Proposed MO degradation pathway by F. pinicola**

| Retention time (mins) | m/z   | Molecular formula | Name                                                                 | Molecular structure |
|-----------------------|-------|-------------------|----------------------------------------------------------------------|--------------------|
| 1.28                  | 258   | C\textsubscript{14}H\textsubscript{15}N\textsubscript{3}O\textsubscript{2} | 4-(2-(4-(dimethyliminio)-2-hydroxycyclohexa-2,5-dien-1-ylidene)hydrazinyl)phenolate | ![Molecular structure](https://example.com/structure1.png) |
| 2.70                  | 319   | C\textsubscript{15}H\textsubscript{17}N\textsubscript{3}O\textsubscript{6}S | 4-(2-(4-(dimethyliminio)cyclohexa-2,5-dien-1-ylidene)hydrazinyl)benzenesulfonate | ![Molecular structure](https://example.com/structure2.png) |

Table 2. Metabolites of MO degradation by *F. pinicola* in medium with Fe\textsuperscript{2+}
4. Discussion

BRF is known to carry out the decomposition process of xenobiotic compounds using hydroxyl radicals (OH) produced through the Fenton reaction (Singh 2021). Meanwhile, hydroxyl radicals are the most reactive chemical species after active starch atoms with a relative oxidation power of 2.06 (Yap et al. 2011). The Fe^{2+} in the Fenton reaction is retained in the redox-inactive complex to prevent oxidative deterioration through Fenton's chemistry (Contreras et al. 2007).

The involvement of the extracellular Fenton BRF reaction was identified in the biodegradation of DDT to (1,1-dichloro-2,2-bis (4-chlorophenyl) ethane [ DDD], 1-dichloro-2,2-bis (4-chloro-phenyl) ethylene (DDE) [24], and DDMU (1-chloro-2,2-bis (4-chlorophenyl) ethylene) (Sariwati et al. 2017). In addition, extracellular mechanisms of the Fenton type have been reported to be involved in the degradation of polycyclic aromatic hydrocarbons (PAHs) (Yap et al. 2011). In other BRF, Gloeophysillum trabeum and Daedalea dickinsii degraded MO through the Fenton reaction in nutrient broth media, at percentages of 46-93% (Purnomo et al. 2019a). Furthermore, though Fenton catalytic is the main mechanism reported in BRF (Purnomo and Mawaddah 2020; Rizqi et al. 2021), the presence of extracellular enzymes is thought to be involved in the MO degradation process. For example, laccase which can oxidize sulfonate groups, and azoreductase which are involved in the reductive cleavage of azo bond (-N = N-) (Ayed et al. 2010).

On the other hand, white-rot fungi (WRF) have also been found to degrade MO, however, with a different degradation mechanism. WRF Ganoderma sp. En3 decolorized MO at 96.7% for 72 hours, and Laccase (120.5±7.92 UI-1) was the enzyme identified in the decolorization process (Zhuo et al. 2011). Apart from BRF and WRF, some groups of bacteria also showed decolorization activity, and an example is Pseudomonas aeruginosa, which showed 91.46% decolorizing activity against the MO dye (Purnomo and Mawaddah 2020). Decolorization of MO occurs in conventional and facultative anaerobic and aerobic conditions, through different groups of bacteria. Meanwhile, reductive attack on azo groups (-N = N-) by azoreductase is the beginning of MO decolorization (Pandey et al. 2007; Wahyuni et al. 2016). The cleavage of the azo bridge produces toxic aromatic amines; however, some microbes can carry out further mineralization such as Klebsiella pneumoniae strain AHM (Kumar et al. 2017). Meanwhile, sulfonated aromatic amines require a consortium of cells for complete mineralization (Barsing et al. 2011; Wahyuni et al. 2017).

BRF such as G. trabeum and D. dickinsii have been reported to be able to degrade MO in Potato Dextro Broth (PDB) media (Purnomo et al. 2019a). Furthermore, these fungi showed similar transformations of the MO structure with F. pinicola in the conditioned medium for the Fenton reaction. G. trabeum transformed the structure of MO through hydroxylation and demethylation reactions, which produce five metabolites with molecular weights of 225, 324, 242, 320, and 276. Besides, D. dickinsii transformed the structure majorly by hydroxylation, and this leads to the production of eleven metabolites with molecular weights of 261, 276, 225, 324, 320, 336, 352, 368, 384, 400, and 432 (Purnomo et al. 2019a). The decolorization of MO using G. trabeum in Fenton media was also reported to produce hydroxylation and methylation metabolites with molecular weights of 351 and 411 (Purnomo et al. 2020c). Meanwhile, these metabolites were reported as photocatalytic metabolites. Photocatalytic degradation in MO using Ag/ZnO resulted in monohydroxylated MO (m/z 320; Chen et al. 2008). The formation of hydroxylation products comes from hydroxyl radicals (OH), where MO radicals are an intermediary in the oxidation of MO by oxidants (Hu et al. 2011). BRF could produce oxalic acid, which would support the oxidation of Fe^{2+} to Fe^{3+}. Meanwhile, the catalysis of H_{2}O_{2} breakdown by Fe^{3+} produces hydroxyl radicals (OH) (Ayed et al. 2010; Tsujiyama and Okada 2013; Purnomo et al. 2010b, 2019a, 2020c; Rizqi et al. 2021).

In general, Fenton is not the only degradation mechanism in BRF, other metabolites also play a role, namely oxalic acid (D’Souza et al. 1996), enzymes such as laccase and the reductase group (Park and Park 2014). Laccase and Azo reductase are the key enzymes most frequently reported in the degradation of azo dyes. Azo reductase showed degradation activity in the azo bridge cleavage. However, in this study, there was no such product (Pandey et al. 2007). Meanwhile, laccase showed the oxidation ability of sulfonate groups (Park and Park 2014) such as the metabolite in this study, namely 4-(2-(4-(dimethyliminio)-2-hydroxycyclohexa-2,5-dien-1-ylidene) phenolate. Laccase activity in BRF has been reported in several research, namely G. trabeum (D’Souza et al. 1996). D. dickinsii
superoxide ion (O$_2^-$) has become an important part of BRF in carrying out the degradation activities of various substrates. A different degradation mechanism is found in WRF, which has different types of extracellular enzymes. Meanwhile, the Fenton-type extracellular mechanism is a mechanism that generates highly reactive hydroxyl radical (OH) species that attack the target substrate. Radicals can be produced in BRF because physiologically these fungi can produce various reactants (oxalic acid) and utilize minerals (ex. Fe$^{2+}$/Cu$^{2+}$), which catalyze the decomposition of H$_2$O$_2$ to hydroxyl radicals (OH). F. pinicola is a BRF species reported to produce up to 50 mm of oxalic acid, which supports various degradation activities (Shah et al. 2018). Increasing the concentration of oxalic acid would increase the production of hydroxyl radicals from the Fenton reaction. However, it should be noted that the reactivity of these radicals has a negative impact on biomolecules that are thought to be involved in the degradation mechanism. Such conditions have been reported in DDT degradation using F. pinicola, where the media with a higher concentration of Fe$^{2+}$ ions correlated with higher radical production. In addition, the increase in radicals inhibited the degradation of DDT and the production of DDD (Purnomo et al. 2011a, 2011b). The impact of increasing hydroxyl radicals can be explained by the metabolites of degradation in this study, which were shown in Figure 3.

Figure 3 showed that the MO transformation/degradation/decolorization mechanism in this study by F. pinicola was dominated by hydroxyl radical oxidation. The first transformation was estimated through hydroxylation and methylation to produce the metabolite, m/z 391. Moreover, further oxidation of the SO$_3$ group resulted in the desulfonation metabolite, m/z 258. Various oxidants such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH), superoxide ion (O$_2^-$), and singlet oxygen ($^1$O$_2$) were involved in the decomposition of wood by BRF and WRF. However, among these oxidants, the hydroxyl radical was the strongest. Therefore, it is possible that the radical was actively involved in the hydroxylation of MO (Singh 2021). Another source reported that F. pinicola is a source of laccase, which is thought to be involved in the oxidation of the MO sulfate group.

In this study, the fungus F. pinicola was pre-incubated in the PDB medium which assumed the starting fungal growth was same. Further, the fungus was transferred into MSM without any carbon source to grow, which the hypothesis was that the fungus utilized MO as a carbon source. Thus, the supplement of Fe$^{2+}$ is used to induce Fe-dependent mechanisms rather than used for the growth of fungus. The supplement of Fe$^{2+}$ did not significant affect the fungal growth directly. Therefore, fungal growth was not discussed in this study. The MO decolorization in this study for 28 days was a hybrid process between Fenton’s mechanism and enzymatic activity. Besides, the use of F. pinicola as a MO biocatalyst was very environmentally friendly, because the fungus did not have a toxic history. F. pinicola has long been used as a medical mushroom in traditional Chinese medicine (Zahid et al. 2020). However, it is important to know the reference to the toxicity of various biocatalysts such as fungi and bacteria, in order to minimize the negative impacts, and monitor the application of the catalysts in waste treatment.

In conclusion, this study showed that F. pinicola could decolorize methyl orange (MO) in mineral salt media containing Fe$^{2+}$ and without Fe$^{2+}$. Furthermore, the decolorization of MO in media with Fe$^{2+}$ and without Fe$^{2+}$ were 90% and 80%, respectively. Based on the LCMS chromatography analysis, MO degradation by F. pinicola produced the metabolites, 4-(2-(4-(dimethyliminio)-cyclohexa-2,5-dien-1-ylidene) hydrazinyl) benzensulfonate and 4-(2-(4-(dimethyliminio)-2-hydroxycyclohexa-2,5-dien-1-ylidene) hydrazinyl) phenolate. This showed that Fe$^{2+}$-dependent as well as enzymatic mechanisms were involved in the biodecolorization of MO by the fungus.

Conflict of Interest

The authors declare that they have no conflict of interest.

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