The addition effect of *Pseudomonas aeruginosa* on biodegradation of methyl orange dye by brown-rot fungus *Fomitopsis pinicola*

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**Abstract.** The addition effect of *Pseudomonas aeruginosa* on methyl orange (MO) biodecolorization by *Fomitopsis pinicola* had been investigated. *P. aeruginosa* was added into *F. pinicola* culture at 2, 4, 6, 8, 10 mL (1 mL = 5.05 × 10^{12} CFU). The addition of 4 ml of *P. aeruginosa* showed the highest MO biodecolorization approximately 99.53%, while by *F. pinicola* only was 77.22% in potato dextrose broth (PDB) medium for 7 days incubation. Four metabolites were detected from MO degradation by mixed cultures such as 4-(4-(dimethylimino) hydroxy-cyclohexa-2,5-dien-1-ylidine) hydrazinil) phenolate; 4-(hydroxy-4-iminio-cyclohexa-2,5-dien-1-ylidine) hydrazinil) benzene sulfonate; 4-(4-(dimethylimino) methoxy-cyclohexa-2,5-dien-1-ylidine) hydrazinil) hydroxy-benzene sulfonate; and 4-(hydroxy-4-iminio-cyclohexa-2,5-dien-1-ylidine) hydrazinil) hidroxy-benzene sulfonate. The mixed cultures transformed MO via three pathways: (1) desulfonation, (2) demethylation, and (3) hydroxylation. These results indicated that *P. aeruginosa* can enhance MO biodecolorization by *F. pinicola*.

1. **Introduction**

The synthetic dye industry is currently showing fairly rapid development. One of the major uses of synthetic dyes is in the textile industry. In 2012, textile production increased rapidly as a result of the increasing demand for imported textile and textile products. Along with the development of the textile industry that continues to increase, textile industry waste production also increased. One type of waste generated from the textile industry is textile dye waste [1]. In textile dye waste, many synthetic dyes are dissolved in water, potentially causing pollution of the aquatic environment [2]. Azo dye is a dye that is often used in the textile industry which is 70% of the total dye [3]. Methyl orange (MO) is one of the most commonly used azo dyes. In the process of textile dyeing, 10–15% of MO dye is unused and thrown as waste [4]. Water-soluble MO dye as a textile industry waste disrupts the balance of the water ecosystem. In addition, the MO dye is quite stable because it is composed of aromatic compounds that cause MO difficult to be degraded in nature and harmful to the environment due to its carcinogenic.

Chemical-physical methods can be done to solve synthetic dye waste problems in industrial environments such as adsorption, precipitation, chemical degradation, and so on [5]. However, these methods generally relatively expensive and sometimes still produce toxic sludge. Therefore, another method is needed that is relatively cheaper and environmentally friendly method, that is through bioremediation [6–10]. In the bioremediation method, waste handling is done by utilizing the biological...
activity of microorganisms both fungi and bacteria in degrading textile dye waste. One of the fungi that can degrade dyes is the brown-rot fungi [1, 2, 11, 12].

Brown-rot fungi can degrade cellulose and hemicelluloses by using Fenton reactions by involving Fe(II) and $\text{H}_2\text{O}_2$ producing OH radicals [13]. *Fomitopsis pinicola* is one of brown-rot fungi that can degrade some organic pollutants such as DDT [14-21] and dyes [11]. *F. pinicola* degraded approximately 97% on liquid medium Potato Dextrose Broth (PDB) incubated for 14 days [11]. Although *F. pinicola* can degrade MO, but it is still required relatively long incubation times, thus, culture modification is needed to optimize *F. pinicola* ability to degrade MO.

In addition to using fungi, synthetic dye waste can also be degraded by bacteria [12, 22-24]. Besides, *P. aeruginosa* also degraded DDT in particular as well as in mixed cultures [19, 25, 26]. Therefore, the purpose of this study is to investigate the effect of addition of bacterium *P. aeruginosa* on the MO degradation process by brown-rot fungus *F. pinicola* including identification of metabolites and degradation pathway.

2. Materials and methods / another section of your paper (for review paper)

2.1. Chemicals
Methyl orange (MO, $\text{C}_14\text{H}_14\text{N}_2\text{NaO}_3\text{S}$), aqua DM, and ethanol 70% were purchased from PT. Sumber Ilmiah Persada Indonesia. Potato dextrose broth (PDB), potato dextrose agar (PDA), nutrient broth (NB), and nutrient agar (NA) were purchased from Merck, Germany.

2.2. Culture conditions
Culture bacterium *P. aeruginosa* NBRC 3080 (NITE Biological Resource Center, was incubated at 37 °C on NA plate. The colony was then inoculated into 60 mL NB, and then pre-incubated for 17 h at 37 °C with a shaker at a speed of 180 rpm [26, 27]. Culture fungus *F. pinicola* NBRC 8705 was maintained on PDA. This fungus was incubated at 30 °C. The mycelia were inoculated into 9 ml PDB then pre-incubated for 7 days at 30 °C [28].

2.3. Biodecolorization MO by *F. pinicola*
After pre-incubation, 10 mL of PDB medium was added into fungal cultures (final volume of 20 mL) and 1 mL MO 2000 ppm (final concentration of 100 ppm) was added to each *F. pinicola* inoculated flask. The cultures were incubated statically for 7 days at 30 °C. As a control, the cultures were killed by autoclave (121 °C, 20 min) after pre-incubation. The experiments were performed in duplicate [11].

2.4. Biodecolorization of MO by bacterium *P. aeruginosa*
After pre-incubation for 17 h, various volume of bacteria cultures at 2, 4, 6, 8, 10 mL (1 mL = 5.05 × 10^{12} \text{CFU}) were inoculated to PDB culture (final volume 20 mL). Each inoculated flask was added 1 mL MO 2000 ppm (final concentration of 100 ppm). The cultures were incubated for 7 days at 30 °C. As a control, the cultures were killed by autoclave (121 °C, 20 min) after pre-incubation. The experiments were performed in duplicate [29].

2.5. Biodecolorization of MO by mixed cultures of *F. pinicola* and *P. aeruginosa*
After pre-incubation (fungus for 7 days and bacteria 17 hours; section 2.2), various concentration of bacteria 2, 4, 6, 8, and 10 mL (1 mL = 5.05 × 10^{12} \text{CFU}) were separately added into *F. pinicola* cultures. PDB medium and MO (final concentration 100 ppm) were then added into the final volume of 20 mL. The headspace of each flask was sealed with a glass stopper. The cultures were incubated statically for 7 days at 30°C. As a control, the cultures were killed by autoclave (121°C, 20 min) after pre-incubation. The experiments were performed in duplicate [30, 31].
2.6. Analytical method and identification of metabolites

The cultures were centrifuged to separate the biomass. The supernatant absorbance was measured with a UV-Vis spectrophotometer at wavelength 350-600 nm, while the rest of supernatants were stored for metabolite product identification. The abiotic control was the cultures without *F. pinicola* and *P. aeruginosa*. The percentage of decolorization of MO was calculated at wavelength 465 nm by using the equation 1.

\[
\% \text{ decolorization} = \frac{A_c - A_t}{A_c} \times 100\%,
\]

(1)

where \( A_c = \) control absorbance and \( A_t = \) treatment absorbance.

Metabolites identification was carried out by analyzing the supernatants using LC-TOF MS, which ionization source with a mass range of 50-350. The sample was eluted by a gradient flow rate of 0.2 mL min\(^{-1}\) in 3 min, and then 0.4 mL min\(^{-1}\) in 7 min. The mobile phases were methanol and water at a ratio of 99:1 in 3 min, and then 61:39 in 7 min remaining. The column was TM RSLC Acclaim 120 C18 with 2.1x100 mm size which column temperature of 33 °C (11, 12, 32).

3. Results and discussion

3.1. Biodecolorization of MO by *F. pinicola*

In this study, biodecolorization of MO was carried out in the PDB medium due to the most suitable medium for the growth of brown-rot fungi compared to the other liquid media [14]. The ability of *F. pinicola* to degrade MO was quantitatively determined by measuring the percentage of decolorization. Figure 1 showed the profile absorbance of MO decolorization by *F. pinicola*. In the abiotic control (negative control), the maximum wavelength was detected at 465 nm. However, since *F. pinicola* produces some metabolites including organic acids, the pH medium became acid that caused the wavelength shifts to the right and detected at the 510 nm wavelength [12]. Profile absorbance showed that the absorbance of MO decreased when treated with *F. pinicola* (in this case was used as positive control) compared with abiotic control (negative control). Decreasing MO absorbance indicated that MO was degraded by *F. pinicola*. Percentages of decolorization of MO by *F. pinicola* were approximately 77.22%, for 7 days incubation periods. The ability of *F. pinicola* to degrade MO might be correlated with the ability of *F. pinicola* in producing hydroxyl radicals generated by Fenton reaction [14–17].

![Figure 1. Absorbance profile of decolorization of MO by *F. pinicola* (red line) and abiotic control (blue line).](image-url)
3.2. Biodecolorization of MO by *P. aeruginosa*

The absorbance profile of MO decolorization by *P. aeruginosa* was presented in figure 2. The highest peak was detected at 485 nm, due to the culture became acid. *P. aeruginosa* have been reported produces polyuronic acids such as mannanos acid and guluronic acid [33]. The higher volume of bacteria resulted in decreasing of MO peak, indicating MO was further decolorized. The ability of the *P. aeruginosa* to decolorize MO is related to its ability to produce enzymes in its metabolic processes. Besides having the ability to produce degrading enzymes, *P. aeruginosa* is also capable of producing biosurfactant that also plays a role in biodecolorization process [34–36].

![Figure 2. Absorbance profile of decolorization MO by *P. aeruginosa*.](image)

The ability of *P. aeruginosa* to decolorize MO was also determined quantitatively based on the percentage of decolorization (table 1). Percentages of decolorization of MO by *P. aeruginosa* were approximately 49.59%, 90.27%, 91.06%, 91.36%, and 91.59% at 2, 4, 6, 8, and 10 mL of bacteria in PDB medium for 7 days incubation period, respectively. The results showed that the higher amount of *P. aeruginosa*, the higher percentage of MO biodecolorization was obtained, which the highest percentage of biodecolorization of MO was obtained approximately 91.59% at 10 mL of bacteria in PDB medium. However, in variation volume bacterium of 4-10 mL, MO decolorization was not significantly different approximately 91%. Since abundant population of bacteria might produce some toxic metabolites during the stationary phase, the competition of bacteria for surviving might occur rather than to decolorize MO [12].

**Table 1. Percentage of biodecolorization MO by bacterium *P. aeruginosa*.**

| Amount of bacteria culture (mL) | Absorbance control | Absorbance sample | Decolorization (%) |
|---------------------------------|--------------------|-------------------|--------------------|
| 2                               | 3.424              | 1.726             | 49.59±0.09^a       |
| 4                               | 3.424              | 0.333             | 90.27±0.04^b       |
| 6                               | 3.424              | 0.306             | 91.06±0.03^b       |
| 8                               | 3.424              | 0.296             | 91.36±0.03^b       |
| 10                              | 3.424              | 0.288             | 91.59±0.02^b       |

Analyses were conducted by spectrophotometer UV-VIS. Data are mean ± standard deviation (n=3). A 1 mL of bacteria = 5.5 × 10^12 CFU. Data followed by the different minor letter on each column indicates significantly different (P < 0.05).
3.3. Biodecolorization MO by mixed cultures

Figure 3 presented the absorbance profile of MO decolorization by mixed cultures of *F. pinicola* and *P. aeruginosa* in PDB medium for 7 days incubation. The addition of *P. aeruginosa* into *F. pinicola* culture can generally increase the MO decolorization compared to the abiotic control. In addition of 2 mL of *P. aeruginosa*, the absorbance was decreased, which indicated MO decolorization even in small amount due to the process of adaptation between fungus and bacterium. Among the mixed cultures, *F. pinicola* with 4 mL of *P. aeruginosa* showed the highest MO biodecolorization.

![Absorbance profile of decolorization MO by mixed cultures of *F. pinicola* and *P. aeruginosa*.](image)

**Table 2.** Percentage of biodecolorization MO by mixed culture of *G. trabeum* and *P. aeruginosa*.

| Amount of bacteria culture (mL) | Absorbance control | Absorbance sample | Decolorization (%) |
|---------------------------------|--------------------|-------------------|--------------------|
| 2                               | 3.424              | 0.086             | 97.49±0.02<sup>a</sup> |
| 4                               | 3.424              | 0.016             | 99.53±0.02<sup>b</sup> |
| 6                               | 3.424              | 0.066             | 98.07±0.03<sup>b</sup> |
| 8                               | 3.424              | 0.108             | 96.85±0.05<sup>b</sup> |
| 10                              | 3.424              | 0.105             | 95.18±0.03<sup>b</sup> |

Analyses were conducted by spectrophotometer UV-VIS. Data are mean ± standard deviation (n=3). A 1 mL of bacteria = 5.5 × 10<sup>12</sup> CFU. Data followed by the different minor letter on each column indicates significantly different (P < 0.05).

The ability of mixed cultures of *F. pinicola* and *P. aeruginosa* to decolorize MO was determined quantitatively based on percentage of decolorization (table 2). Percentages of decolorization of MO by mixed culture *F. pinicola* and *P. aeruginosa* were approximately 97.49%, 99.53%, 98.07%, 96.85%, and 95.18% at 2, 4, 6, 8, and 10 mL of bacteria, respectively, in PDB medium for 7 days incubation period. The addition of 2-4 mL of *P. aeruginosa* increased MO decolorization due to the enzyme dehydrogenase produced by *P. aeruginosa* might help to increase the decolorization of MO by *F. pinicola* [35]. However, in addition to 6, 8, and 10 mL, MO decolorization was decreased due to the competition between fungus and bacterium in getting the nutrients contained in the media [12]. The highest MO decolorization was obtained at mixed cultures of *F. pinicola* with the additional 4 mL of *P. aeruginosa*, which enhances the ability of decolorization by 22.31% (from 77.22% by *F. pinicola* culture...
only to 99.53% by mixed cultures). Mixed microbial cultures have more power to degrade pollutants because they produce more metabolites as well as enzymes [24].

3.4. The identification of metabolites

Based on characterization by LC-TOF/MS, four metabolites were detected at time retention 1.31; 2.36; 3.31; and 6.81mins (Figure 4). The identification of metabolites was determined based on the similarity between MS spectrum and time retention from the database.

Based on TOF/MS data (table 3), the peak at retention time 1.31 min has m/z 258 which is identified as 4-(4-(dimethylimino) hydroxy-cyclohexa-2,5-dien-1-ylidine) hydrazinyl) phenolate. The compound was reported previously which had fragments in m/z 118, which is in accordance with previous research on the degradation of azo dyes by laccase enzyme from WRF [37, 38]. At a peak of 2.36 mins, metabolite was identified as 4-(hydroxy-4-imino-cyclohexa-2,5-dien-1-ylidine) hydrazinyl) benzene sulfonate with m/z 294. This compound was supported by the presence of fragments in MS data with m/z 214, fragment m/z 136 in accordance with research on metabolites produced by F. pinicola in MO (11). The peak of 3.31 mins with m/z 348 was identified as 4-(4-(dimethylimino) methoxy-cyclohexa-2,5-dien-1-ylidine) hydrazinyl) hydroxy-benzene sulfonate. This compound was supported by fragments m/z 213 [39]. The peak of 6.81 mins was identified as 4-(hydroxy-4-imino-cyclohexa-2,5-dien-1-ylidine) hydrazinyl) hydroxy-benzene sulfonate with m/z 311. This compound was supported by the presence of fragments in MS data with m/z 214 and m/z 108 in accordance with research on metabolites produced by F. pinicola in MO [11]. Based on the identification of metabolites, the MO decolorization pathway by mixed cultures of F. pinicola and P. aeruginosa cultures were proposed in figure 5.
TABLE 3. Metabolites of MO degradation by mixed cultures of *F. pinicola* and *P. aeruginosa*.

| RT (min) | Mr  | Name                                                                 | Molecular formula | Structure |
|---------|-----|----------------------------------------------------------------------|-------------------|-----------|
| 1.31    | 258 | 4-(4-(dimethylamino) 2-hydroxy cyclohexa-2,5-dien-1-ylidene) hydrazinyl phenolate | C_{14}H_{13}N_{3}O_{2} | ![Structure](image1) |
| 2.36    | 294 | 4-(2-hydroxy-4-imino cyclohexa-2,5-dien-1-ylidene) hydrazinil) benzenesulfonate | C_{12}H_{12}N_{3}O_{4}S | ![Structure](image2) |
| 3.31    | 348 | 4-(4-(dimethylamino) methoxy cyclohexa-2,5-dien-1-ylidene) hydrazinyl hydroxyl benzene sulfonate | C_{12}H_{11}N_{3}O_{5}S | ![Structure](image3) |
| 6.81    | 311 | 4-(2-hydroxy-4-imino cyclohexa-2,5-dien-1-ylidene) hydrazinil) 2-hydroxy benzene sulfonate | C_{12}H_{11}N_{3}O_{5}S | ![Structure](image4) |

* The data were determined by LC-TOF/MS.

Figure 5. Proposed pathway degradation of MO by mixed cultures of *F. pinicola* and *P. aeruginosa*. 
The MO degradation pathway by mixed cultures of *F. pinicola* and *P. aeruginosa* was proposed to divide into three ways. The first pathway: the MO was ionized by the media solution released Na⁺, and then transformed into compound 2 by demethylation and hydroxylation, followed hydroxylation into compound 4. The second pathway: the ionized MO was transformed into compound 1 by desulfonation, followed hydroxylation. The third pathway: ionized MO was dehydroxylated on both aromatic rings, followed methylated into compound 3. Hydroxylation occurs due to the presence of radical hydroxides that might be produced from Fenton reaction by *F. pinicola* that attack MO [19]. Previously, *F. pinicola* transformed MO via three pathways: (1) demethylation, followed by hydroxylation reactions; (2) hydroxylation, followed by demethylation; and (3) desulfonation [11].

4. Conclusion

The addition of bacterium *P. aeruginosa* enhanced the ability of brown-rot fungus *F. pinicola* to decolorize and degrade MO. The highest MO decolorization was obtained in the addition of 4 mL of *P. aeruginosa* with decolorization percentage by 99.73%. The mixed cultures of *F. pinicola* and *P. aeruginosa* transformed MO into 4-(dimethyliminio) hydroxy-cyclohexa-2,5-dien-1-ylidine)-phenolate; 4-(hydroxy-4-iminio-cyclohexa-2,5-dien-1-ylidine) hydrazinyl) benzene sulfonate; 4-(dimethyliminio) methoxy-cyclohexa-2,5-dien-1-ylidine) hydrazinyl) hydroxy-benzene sulfonate; and 4-(hydroxy-4-iminio-cyclohexa-2,5-dien-1-ylidine) hydrazinil) hidroxy-benzene sulfonate. Based on the identification of metabolic products, the mixed cultures transformed MO via three pathways: (1) demethylation, (2) hydroxylation, and (3) desulfonation. This research indicated that the bacterium *P. aeruginosa* can increase the decolorization of the MO dye by the brown-rot fungus *F. pinicola*.

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