The effect of addition of bacterium Pseudomonas aeruginosa on biodegradation of methyl orange dye by brown-rot fungus Gloeophyllum trabeum

A S Purnomo*, F D Rahmadini, R Nawfa, and S R Putra
Department of Chemistry, Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember, ITS Sukolilo Campus, Surabaya, 60111, Indonesia

Email: adi_setyo@chem.its.ac.id; adi.spurnomo@yahoo.com

Abstract. The methyl orange (MO), one of common textile dyes from azo groups, has negative impact in human life and the environment. Therefore, many attempts have been devoted to find the most effective method for MO degradation. Brown-rot fungus Gloeophyllum trabeum has identified as the biodegradable agent of MO, but its efficiency is still low, and it requires a long incubation time. In this work, the biodegradable performance of brown-rot fungus Gloeophyllum trabeum was investigated for MO degradation in the presence of bacterium Pseudomonas aeruginosa with various volumes (2-10 mL, 1 mL = 5.05 x 10^12 Colony Forming Unit (CFU)). The addition of 10 mL of bacteria into G. trabeum culture showed the maximum degradation of 88.6% in potato dextrose broth (PDB) medium for the 7-day incubation. The identified metabolites were 4-[(4-dimethylimino) cyclohexa-2,5-dien-1-ylidene] hydrazinyl] phenolate (C_9H_6N_2O, compound 1), 4-[(4-imino-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] benzenesulfonate (C_9H_6N_2O_3S, compound 2), 4-[(hydroxy-4-imino-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] benzenesulfonate (C_10H_8N_2O_3S, compound 3), 4-[(4-dimethylimino)-hydroxy-cyclohexa-2,5-dien-1-ylidene] hydrazinyl] methoxy benzenesulfonate (C_10H_8N_2O_3S, compound 4), and 4-[(4-dimethylimino)-dihydroxy-cyclohexa-2,5-dien-1-ylidene] hydrazinyl] dimethoxy benzenesulfonate (C_10H_8N_2O_5S, compound 5). Based on the identification of metabolic products, the mixed cultures transformed MO via three pathways: (1) desulfonfylation, (2) demethylation, and (3) hydroxylation. These results indicate that P. aeruginosa can enhance MO biodecolorization by G. trabeum.

1. Introduction
The development and progress of the textile industry in addition to providing many benefits to the community also has a negative impact on the environment because this industry always produces textile dye liquid waste in the production process. A large number of dyes in the textile industry cannot fully be absorbed into the colored fiber fabric so that it will be released as waste [1]. Wastewater that contains dyestuff is very dangerous because most dyes are difficult to biodegrade (non-biodegradable), resistant, and toxic. Thus, if dyes are released into the aquatic environment without prior treatment, it can cause serious pollution to water sources which result in damage to the ecosystem environment and threats to human health [2]. Methyl Orange (MO) is a type of azo dyes that is found in textile industry waste and is a toxic compound and harms the environment [3]. To prevent the risk posed by such pollution, efforts are needed to reduce the entry of dyes into the aquatic environment. A variety of methods have been developed for handling dyestuff liquid waste from the textile industry [4]. Bioremediation is considered a quite cheap and efficient method for handling dyes waste [5-10]. Brown-rot fungi are one of the
microorganisms that can degrade some pollutants including dyes [11-14] by involving the Fenton reaction [15-18].

Other research has been carried out to degrade MO dye by utilizing the brown-rot fungus *Gloeophyllum trabeum* which can produce radical hydroxides through the Fenton reaction [13]. Purnomo et al. [13] reported that *G. trabeum* was able to degrade MO in liquid PDB media by 47.53% for 14 days of incubation. The yield of MO degradation by *G. trabeum* was still relatively low and requires a long incubation time, hence a culture modification is needed to improve its ability.

Some studies suggest that the use of mixed cultures of fungi and bacteria can increase the ability of culture degradation [11, 14, 19-23]. One type of bacterium that can degrade some organic pollutants is *Pseudomonas aeruginosa* because it can produce degrading enzymes such as azoreductase [23-27]. *P. aeruginosa* had been reported to be able to enhance DDT degradation in mixed culture with some wood-rot fungi [28, 29]. Therefore, in this study, the effect of the addition of *P. aeruginosa* on the ability of biodegradation of MO by *G. trabeum* was investigated as an innovation in the effort to deal with the problem of textile dye waste.

2. Materials and methods

2.1. Chemicals

A number of chemicals needed are as follows. Methyl Orange (MO) was purchased from SAP Chemicals, potato dextrose agar (PDA), nutrient agar (NA), and nutrient broth (NB) were purchased from Merck, Germany, while potato dextrose broth (PDB) was purchased from Himedia, India.

2.2 Culture condition

2.2.1 Regeneration of *G. trabeum*. The brown-rot fungus *G. trabeum* NBRC 6509 species was used as the first degradation agent. The fungal mycelium was taken with a sterile ose needle and inoculated into a petri dish containing the sterile PDA medium then incubated at 30±0.5 ºC for 7 days until the mycelium covered the entire surface of the PDA medium [30].

2.2.2 Regeneration of *P. aeruginosa*. The bacterium *P. aeruginosa* NBRC 3080 was used as a second degradation agent. One bacterial colony of *P. aeruginosa* was taken with a sterile ose needle and inoculated into a petri dish containing the sterile NA medium then incubated at 37±0.5 ºC for 24 hours [31, 32].

2.2.3 Preparation of Liquid Culture of *G. trabeum*. One petri dish containing *G. trabeum* in the PDA medium (2.2.1) was homogenized by using a sterile blender that contained 25 mL of sterile distilled water. One milliliter of homogenate was inoculated into 8 mL of sterile PDB medium in 100 mL of Erlenmeyer and then pre-incubated at 30±0.5 ºC for 7 days under a static condition [33].

2.2.4 Preparation of liquid culture of *P. aeruginosa*. One colony of *P. aeruginosa* (2.2.2) was inoculated into 10 mL of sterile NB medium. The culture was incubated at 37±0.5 ºC for 24 hours with shaking at a speed of 180 rpm as a starter. After 24 hours, one milliliter of *P. aeruginosa* bacterium starter was inoculated into 500 mL of sterile NB medium and then pre-incubated at 37±0.5 ºC under a shaking condition with a shaker at a speed of 180 rpm until it reached a maximum OD of the stationary phase of *P. aeruginosa* bacterium [34, 35].

2.3 Biodegradation of MO

*G. trabeum* culture (biotic control), after pre-incubation (2.2.3), was added with 1 mL of 2000 mg/L MO (final concentration 100 mg/L), followed by the addition of 10 mL of PDB medium (the final volume was 20 mL) and then incubated for 7 days at 30 ºC. The bio-decolorization of MO by the mixed cultures was performed by mixing the pre-incubated *G. trabeum* culture (2.2.3) and the pre-incubated *P. aeruginosa* culture (2.2.4) as much as 2, 4, 6, 8, and 10 mL (1 mL = 5.05 × 10¹² CFU), followed by
the addition of MB (the final concentration was 100 mg/L) and PDB to the total volume of 20 mL. As an abiotic control, 1 mL of 2000 mg/L MO (the final concentration was 100 mg/L) was added into 19 mL of PDB medium (the final volume was 20 mL) without an addition of fungal and/or bacterial cultures. All cultures and abiotic control were incubated under a static condition for 7 days at 30±0.5 °C. After 7 days, the cultures were separated by using a centrifuge at 3000 rpm for 15 minutes. The supernatant was taken and filtered by using Whatman filter paper. The supernatant absorbance was measured with a UV-Vis spectrophotometer. The remaining supernatant was stored in a refrigerator for the characterization of metabolites. The calculation of the percentage of MO dye decolorization was according to equation 1 at a wavelength of 465 nm [14].

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

where \(A_c\) is absorbance abiotic control, and \(A_t\) is absorbance treatment.

2.4 Product metabolite identification

The identification of metabolites was performed by using the LC-TOF/MS (Impact II, Bruker). The source of ionization was the electrospray ionization (ESI) with a mass range of 50-500. The elution method used was the gradient method with a flow rate of 0.2 mL/min in the first 3 minutes and the next 7 minutes using a flow rate of 0.4 mL/min. The mobile phase was methanol and water with a ratio of 99:1 in the initial 3 minutes and 61:39 for the remaining 7 minutes. The column was the Acclaim TM RSLC 120 C18 with a size of 2.1 x 100 mm with a column temperature of 33 °C [12-14].

3. Results and discussions

3.1. The effect of addition of \(P.\) aeruginosa on biodegradation of MO by \(G.\) trabeum

In this study, the addition of \(P.\) aeruginosa on the biodegradation of MO by brown-rot fungus \(G.\) trabeum was investigated. The MO degradation by \(G.\) trabeum fungus was also carried out as the biotic control. The absorbance profile of MO degradation is shown in figure 1. The maximum absorbance of MO for abiotic control is detected at a wavelength of 465 nm. However, in the biotic control and the mixed cultures, the absorbance of peaks decrease which indicates decolorization. Besides, the other peaks appear at the wavelength of 300-390 nm indicating the peaks of metabolites. Since the MO peak appears in the wavelength range of 400-600 nm which is the wavelength region of visible light, the metabolites might be colorless [3]. The absorbance of biotic control of \(G.\) trabeum is lower than that of the abiotic control, indicating \(G.\) trabeum decolorized MO. Besides, the biotic control peak is lower than the mixed cultures with the addition of 2, 4, and 6 mL of bacteria. This suggests that the mixed cultures still adapt to the addition of MO. The MO degradation by the mixed cultures of \(G.\) trabeum and \(P.\) aeruginosa occurs maximally in the additions of 8 and 10 mL of bacteria as evidenced by the lower peaks at 465 nm compared with the other peaks.

The ability of mixed cultures of \(G.\) trabeum and \(P.\) aeruginosa to degrade MO was determined quantitatively by measuring the percent of decolorization (table 1). The percent of MO decolorization by the mixed cultures increased along with the increase in the amount of added bacteria. The more the bacteria added, the greater were the biosurfactants as well as dye degradation enzymes produced by \(P.\) aeruginosa. Thus, it caused an increase in the MO decolorization. Based on table 1, the degradation of MO by mixed culture occurs optimally in the addition of 10 mL of bacteria (1 mL = 5.05 x 10^{32} CFU) approximately 88.67%. However, the percent of decolorization at the additions of 8 mL to 10 mL of bacteria is not significantly different due to the competition of bacteria for surviving that might occur rather than to decolorize MO. Some toxic metabolites might be produced during the stationary phase under an abundant population of bacteria [22].
tyrosinase degrading enzyme, namely the enzyme azoreductase, lignin peroxidase, DCIP reductase, producing oxalic acid and H$_2$O$_2$, which play a role in the Fenton reaction. The ability of producing oxalic acid and H$_2$O$_2$ is related to its ability of degrading MO. The ability of producing oxalic acid and H$_2$O$_2$ is related to its ability of degrading MO. The ability of producing oxalic acid and H$_2$O$_2$ is related to its ability of degrading MO.

It can be seen that each degradator agent can adapt to each other when living in the same medium in degrading MO. The ability of each degradator agent can adapt to each other when living in the same medium in degrading MO.

The significant differences occur in the mixed cultures with the additions of 6 mL and 8 mL of bacteria, from 75.32% to 87.53%. It suggests that each degradation agent can adapt to each other when living in the same medium in degrading MO. The ability of G. trabeum to degrade MO is related to its ability of producing oxalic acid and H$_2$O$_2$, which play a role in the Fenton reaction. In addition to involving the Fenton reaction, G. trabeum also produces several extracellular enzymes to degrade wood and some pollutants, such as endoglucases and xylanases that are known produced by G. trabeum in the very large quantities. The ability of bacterium P. aeruginosa in degrading MO is also related to its ability to produce a degrading enzyme, i.e. enzyme azoreductase, lignin peroxidase, DCIP reductase, and tyrosinase. Apart from producing degrading enzymes, Scheibenbogen et al. [40] reported that P. aeruginosa was able to produce biosurfactants of rhamnolipid types which could also be applied in increased degradation of hydrocarbon pollutants.

Table 1. % Decolorization of MO.

| Amount of bacteria addition (mL) | decolorization (%) |
|---------------------------------|--------------------|
| G. trabeum (biotic control)     | 84.67 ± 0.14a      |
| 2                               | 69.92 ± 0.02b      |
| 4                               | 71.96 ± 0.04b      |
| 6                               | 75.32 ± 0.01b      |
| 8                               | 87.53 ± 0.02a      |
| 10                              | 88.67 ± 0.02a      |

3.2 Metabolites identification

The profile of chromatogram of MO metabolites is shown in figure 2. It can be seen that the treatment MO peak is lower compared with the control MO peak, identified at the retention time of 7.29 minutes. Besides the MO peak, in the treatment chromatogram, there are 5 new peaks appear that do not yet exist in the control chromatogram. They are identified as the metabolites produced during the process of MO degradation by the mixed cultures of G. trabeum and P. aeruginosa. Identification of metabolites is determined based on the similarity between MS spectrum and time retention from database (table 2). As seen in table 2, the peak at the retention time of 1.29 minutes has m/z 241 which is identified as 4-[(4-
dimethyliminio)-cyclohexa-2,5-dien-1-ylidene) hydrazinyl phenolate (C\textsubscript{14}H\textsubscript{15}N\textsubscript{3}O, compound 1). The peak at the retention time of 2.55 minutes has m/z 276 identified as 4-[(4-iminio-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] benzenesulfonate (C\textsubscript{13}H\textsubscript{10}N\textsubscript{3}O\textsubscript{2}S, compound 2). Meanwhile, the peak at the retention time of 2.72 minutes has m/z 292 identified as 4-[(hydroxy-4-iminio-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] benzenesulfonate (C\textsubscript{13}H\textsubscript{10}N\textsubscript{3}O\textsubscript{2}S, compound 3), and the peak at retention time of 4.38 mins has m/z 350 identified as 4-[(4-dimethyliminio)-hydroxy-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] methoxy benzenesulfonate (C\textsubscript{15}H\textsubscript{16}N\textsubscript{3}O\textsubscript{3}S, compound 4). The peak at the retention time of 5.54 minutes has m/z 396 identified as 4-[(4-dimethyliminio)-dihydroxy-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] dimethoxy-benzenesulfonate (C\textsubscript{16}H\textsubscript{18}N\textsubscript{3}O\textsubscript{5}S, compound 5). Based on this identification of metabolites, the MO degradation pathway by the mixed cultures of G. trabeum and P. aeruginosa were proposed in this study (figure 3).

Figure 2. Chromatogram profiles of MO degradation by the mixed cultures of G. trabeum and P. aeruginosa. Purple chromatogram: control (a); green chromatogram: treatment (b).

There were three MO degradation pathways by the mixed cultures of G. trabeum and P. aeruginosa proposed. The first pathway: the ionized MO was transformed into compound 1 by desulfonylation. The second pathway: the ionized MO was transformed into compound 2 by demethylation, followed by hydroxylation to compound 3. Last, the third pathway: the ionized MO was transformed into compound 4 by hydroxylation and methylation, followed by hydroxylation and methylation again into compound 5. Hydroxylation occurred due to the presence of radical hydroxides that might be produced from the Fenton reaction by G. trabeum that attacked MO [20]. Previously, G. trabeum transformed MO via three pathways: (1) demethylation, followed by hydroxylation reactions; (2) hydroxylation, followed by demethylation; and (3) desulfonylation [13].
Table 2. Metabolite products of MO degradation by the mixed cultures.

| RT (min) | Mr  | Name                                                                 | Molecular formula | Structure |
|----------|-----|----------------------------------------------------------------------|-------------------|-----------|
| 1.29     | 241 | 4 – [[(4-dimethylimino)cyclohexa-2,5-diene-1-ylidene] hydrazinyl] phenolate | C₁₂H₁₃N₂O       | ![Structure 1](image) |
| 2.55     | 276 | 4 – [[(4-iminio-cyclohexa-2,5-diene-1-ylidene] hydrazinyl] benzenesulfonate | C₁₂H₁₀N₂O₃S     | ![Structure 2](image) |
| 2.72     | 292 | 4 – [[hydroxy-4-iminio-cyclohexa-2,5-diene-1-ylidene] hydrazinyl] benzenesulfonate | C₁₂H₁₀N₂O₃S     | ![Structure 3](image) |
| 4.38     | 350 | 4 – [[(4-dimethylimino)-hydroxy-cyclohexa-2,5-diene-1-ylidene] hydrazinyl] -metoxy-benzenesulfonate | C₁₃H₁₅N₂O₃S     | ![Structure 4](image) |
| 5.54     | 396 | 4 – [[(4-dimethyliminio)-dihydroxy-cyclohexa-2,5-diene-1-ylidene] hydrazinyl] -dimethoxy-benzenesulfonate | C₁₆H₁₈N₂O₃S | ![Structure 5](image) |

![Diagram](image)

**Figure 3.** Proposed MO degradation pathways by mixed cultures of *G. trabeum* and *P. aeruginosa.*
4. Conclusion
The addition of bacterium *P. aeruginosa* increased the ability of brown-rot fungus *G. trabeum* to decolorize MO. The highest MO decolorization was obtained in the addition of 10 mL (1 mL = 5.05 x 10^{12} CFU) of *P. aeruginosa* with decolorization percentage by 88.67% in the PDB liquid medium for the 7-day incubation. The mixed cultures of *G. trabeum* and *P. aeruginosa* transformed MO into 4-[(4-dimethyliminio)-cyclohexa-2,5-dien-1-ylidene] hydrazinyl benzene sulfonate (C_{12}H_{15}N_{2}O, compound 1), 4-[(4-imino-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] benzenesulfonate (C_{12}H_{10}N_{2}O_{3}S, compound 2), 4-[(hydroxy-4-imino-cyclohexa-2,5-dien-1-ylidene) hydrazinyl] benzenesulfonate (C_{12}H_{10}N_{2}O_{4}S, compound 3); 4-[(4-dimethyliminio)-hydroxy-cyclohexa-2,5-dien-1-ylidene] hydrazinyl] methoxy benzenesulfonate (C_{15}H_{16}N_{2}O_{3}S, compound 4); and 4-[(4-dimethyliminio)-di-hydroxy-cyclohexa-2,5-dien-1-ylidene] hydrazinyl] dimethoxy-benzenesulfonate (C_{16}H_{18}N_{2}O_{5}S, compound 5). Based on the identification of metabolic products, the mixed cultures transformed MO via three pathways: (1) desulfonylation; (2) demethylation; and (3) hydroxylation. This research finally indicate that bacterium *P. aeruginosa* can enhance the decolorization of the MO dye by brown-rot fungus *G. trabeum*.

Acknowledgments
This study was funded by Deputy for Research and Development Strengthening, Indonesian Ministry of Research and Technology/National Research and Innovation Agency, under the Basic Research Scheme Number: 3/E1/KP.PTNBH/2020 and Amendment Contract Number: 3/AMD/E1/KP.PTNBH/2020.

References
[1] Selvam K, et al. 2003 *Bioresour Technol* **88** 115
[2] Hamdaoui O and Chiha M 2006 *Acta Chim Slov* **54** 407
[3] Alamddine L and El jamal MM 2009 *J Chem Technol Metall* **44** 127
[4] Moreira MT, et al. 2000 *Biotechnol Lett* **22** 1499
[5] Purnomo AS, et al. 2010 *Chemosphere* **80** 619
[6] Purnomo AS, et al. 2010 *Int Biodeterior Biodegrad* **64** 397
[7] Purnomo AS, et al. 2013 *Int Biodeterior Biodegrad* **82** 40
[8] Purnomo AS, et al. 2014 *Environ Sci Pollut Res* **21** 11305
[9] Purnomo AS 2017 1st ed. Springer Nature, Switzerland
[10] Purnomo AS, et al. 2017 *Curr Microbiol* **74** 320
[11] Rizqi HD and Purnomo AS 2017 *World J Microbiol Biotechnol* **33** 92
[12] Purnomo AS, et al. 2020 *AIP Conf Proc* **2237** 020002
[13] Purnomo AS, et al. 2019 *Intl J Environ Sci Tech* **16** 7555
[14] Purnomo AS and Mawaddah MO 2020 *Biodiversitas* **21**(5) 2297
[15] Contreas D, et al. 2005 *Int Biodeterior Biodegrad* **57** 63
[16] Purnomo AS, et al. 2010 *Int Biodeterior Biodegrad* **64** 560
[17] Purnomo AS, et al. 2011 *Int Biodeterior Biodegrad* **65** 691
[18] Purnomo AS, et al. 2011 *Int Biodeterior Biodegrad* **65** 921
[19] Sariwati A, et al. 2017 *Curr Microbiol* **74** 1068
[20] Sariwati A and Purnomo AS 2018 *Indon J Chem* **18** 75
[21] Setyo PA, et al. 2018 *Res J Chem Environ* **22** 151
[22] Griczka BE and Setyo PA 2018 *J Physics: Conf Ser* **1095** 102015
[23] Purnomo AS, et al. 2020 *Heliyon* (**6**), e04027
[24] Asad S, et al. 2007 *Bioresour Technol* **98** 2082
[25] Nawfa R, et al. 2019 *Asian J Chem* **31**(10) 2367
[26] Chen KC, et al. 2003 *Biotechnology* **101** 57
[27] Moosvi S, et al. 2005 *World J Microbiol Biotechnol* **21** 667
[28] Boelan EG and Purnomo AS 2019 *Hayati J Biosci* **26** 90
[29] Purnomo AS, et al. 2017 *J Microbiol Biotechnol* **27**(7) 1306
[30] Purnomo AS, et al. 2008 J Biosci Bioeng 105 614
[31] Purnomo AS, et al. 2020 J Idn Chem Soc 3(1) 53
[32] Purnomo AS, et al. 2019 Mal J Fund Appl Sci 15(3) 377
[33] Purnomo AS, et al. 2019 J Microbiol Biotechnol 29 1424
[34] Wahyuni S, et al. 2016 Asian J Chem 28 2731
[35] Wahyuni S, et al. 2017 Asian J Chem 29 1119
[36] Hatakka A 1994 FEMS Microbiol Rev 13, 125
[37] Maier RM, et al. 2009 Environ Microbiol second Edition. UK: Academic Press Elsevier
[38] Sarayu K and Sandhya S 2010 Appl Biochem Biotech 160(4) 1241
[39] Jadhav JP, et al. 2010 Biodegradation 21(3) 453
[40] Scheibenbogen K, et al. 1994 J Chem Technol Biotechnol 59 53