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Decolourisation and Degradation of Methylene Blue Dye by Brown-rot Fungus *Gloeophyllum trabeum*

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The methylene blue (MB) dye is a compound that is widely applied for textile dyes, which has a negative effect on the environment. MB can cause several effects such as irritation of the skin if touched, gastrointestinal irritation if swallowed and cyanosis if inhaled. Due to the negative effects of MB, the residue of MB in the environment must be removed. One of the effective methods are bioremediation using microorganisms. In this study, the ability of brown-rot fungus *Gloeophyllum trabeum* to decolourise and degrade MB was investigated. *G. trabeum* cultures were added to potato dextrose agar (PDA) medium which contains MB at concentrations of 50, 75, and 100 mg L^{-1}. The result shows that MB was decolourised by *G. trabeum* in PDA medium with decolourisation index (DI) of 0.874, 0.879, and 0.878 at MB concentrations of 50, 75, and 100 mg L^{-1}, respectively. *G. trabeum* degraded MB approximately 47, 62 and 76% of 100 mg L^{-1} after incubated for 0, 7, and 14 days, respectively. Five metabolites were identified by LC-TOF/MS analysis. Based on data from m/z and references, the metabolite products were 2-nitro-5-(*N,N*-dimethyl)amino-benzene sulfonate ($C_8H_{10}N_2SO_5$), 2,5-diaminobenzene sulfonic acid ($C_6H_8N_2SO_3$), 4-(dimethylamino)-2-[m-(dimethylamino) phenylsulfinyl] benzenamine ($C_{16}H_{20}N_3SO$), 2-amino-benzenesulfonic acid ($C_6H_7NSO_3$), and thionine ($C_{12}H_{10}N_3S$). This study indicated that *G. trabeum* can be used to decolourise and degrade MB dye.

Keywords: biodecolourisation; biodegradation; brown-rot fungus; *Gloeophyllum trabeum*; methylene blue

I. INTRODUCTION

Synthetic dyes are widely applied in various industries to dye materials such as in the paper, textile, cosmetics, leather, wool, plastic, and printing industries (Rauf *et al.,* 2010). The dyes waste contains persistent organic residues such as aromatic compounds which give a negative effect to the environment. Some colouring wastes are generally carcinogenic, mutagenic, and teratogenic to humans. Among the synthetic dyes that often used is methylene blue (MB). Besides being used as a textile dye, MB is a heterocyclic aromatic dye that also used in many fields such as agriculture, pharmaceutical, microscopy and bacteriology. MB is stable in water that give negative effects on water ecosystems such as hindering the light penetration which may influence the photosynthetic processes of water ecosystems. MB also can cause several effects such as irritation of the skin if touched, gastrointestinal irritation if swallowed and cyanosis if inhaled (Rauf *et. al.,* 2010; Guo *et. al.,* 2014; González *et al.,* 2014).

Many methods have been studied to treat dye waste such as chemical and physical processes. In the previous study, physical method such as adsorption using activated carbon is given good result, but this method needs more money to get some adsorbent agent to bind the dye compounds (Guo *et. al.,* 2014; Laowansiri *et. al.,* 2008; Rahman *et al.,* 2012). The chemical treatment such as using coagulants also give good result but that method also resulting in some sludge, which causes new environmental problems (Madhu *et al.,* 2009). MB degradation by the chemical Fenton reaction has been reported (Dutta *et. al.,* 2001; Zhou *et. al.,* 2015; Wang *et al.,*

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2014). However, this reaction has some disadvantages such as the generation of iron sludge in large quantity, deactivation by the iron complexation reagents and difficult for recycling catalysts (Zhou *et al.,* 2015). On the other hand, biodegradation method offers more effective and environmentally friendly method. In biodegradation, the use of microorganisms such as using brown-rot fungi capable of providing environmentally friendly degradation methods to degrade some persistent organic pollutant such as pesticide and dye (El-Sersy, 2007; Pant *et. al.,* 2008; Purnomo *et. al.,* 2013; Purnomo *et. al.,* 2014; Purnomo *et. al.,* 2017a; Purnomo *et al.,* 2017b).

Brown-rot fungi (BRF) is used for biodegradation due to their ability to produce hydroxyl radicals from the Fenton reaction (Purnomo *et. al.,* 2008; Purnomo *et. al.,* 2010; Purnomo *et. al.,* 2011a; Purnomo *et al.,* 2011b). Hydroxyl radicals are commonly used by BRF as tools to degrade cellulose as well as hemicellulose in wood to obtain carbon source for food (Kaneko *et al.,* 2005). *Gloeophyllum trabeum* is one species of BRF that produces hydroxyl radicals. *G. trabeum* has been reported degrades DDT (aromatic organic pesticide) by involving the Fenton reaction (Purnomo *et. al.,* 2010; Purnomo *et al.,* 2011). Therefore, *G. trabeum* may also can degrade MB as an effective way to clear up the waste textile dye problem. Thus, *G. trabeum* is an appropriate alternative to degrade MB by providing bio-Fenton reaction. This study is the first report on decolourisation and degradation of MB by *G. trabeum*.

II. MATERIALS AND METHOD

A. Materials and Fungus

MB powder was purchased from SAP Chemicals. Analytical grade of methanol and acetone were purchased from Merck. Brown-rot fungus *G. trabeum* NBRC 6509 was purchased from the NBRC (Chiba, Japan). The fungus was cultured on potato dextrose agar (PDA; Merck, Darmstadt, Germany).

B. Decolourisation of MB in Solid Medium

The mycelia of fungus (diameter 1 cm) were put into PDA containing 50, 75, and 100 mg L^{-1} of MB, and was further incubated at 30 °C for 12 days. Decolourisation diameter (DD) and mycelium diameter (MD) were measured based on result on the agar plate. For positive control, *G. trabeum* was cultured in PDA without MB was used, while MB in PDA without fungus was used as a negative control. The decolourisation index (DI) of MB was calculated according to Equation (1) (Jayasinghe *et. al.,* 2008; Purnomo *et al.,* 2019b):

$$DI = \frac{DD}{MD} \quad (1)$$

C. Degradation of MB in the liquid medium

The mycelia of fungus (diameter 1 cm) were inoculated into potato dextrose broth (PDB, 10 mL) medium, and then pre-incubated at 30 °C for 7 days. After the pre-incubation, MB (final concentration, 100 mg L^{-1}) was inoculated to the cultures and incubated for 7 and 14 days. The cultures were centrifuged to separate the biomass, and the absorbance of the supernatant was measured using UV-Vis spectrophotometer (Genesys 10S) at a wavelength of 400-750 nm. The remaining supernatant was further used to identify the metabolites. MB in PDB cultures without fungal was used for control. The percentage of decolourisation of MB was calculated according to Equation (2):

$$\%\, decolorization = \frac{Ac - At}{Ac} x \;\; 100\% \quad (2)$$

where *Ac* is the absorbance of control culture, and *At* is the absorbance of treatment.

D. Identification of Metabolites

Metabolites of MB were identified using LC-TOF/MS (Agilent Technologies) by analysing the supernatants which ionisation source with a mass range of 50-350. The sample was eluted by water and methanol at ratio of 1:99 in 3 min with the flow rate of 0.2 mL min^{-1}, and then 39:61 in 7 min with the flow rate of 0.4 mL min^{-1}. TM RSLC Acclaim 120 C18 with 2.1 × 100 mm size was used as column.

III. RESULT AND DISCUSSION

The ability of brown-rot fungus *G. trabeum* to decolourise MB was investigated in PDA medium as the initial screening to justify the ability of *G. trabeum* to decolourise MB and determine the optimal concentration of MB for degradation

process in liquid medium.

During the incubation period, the decolourisation index (DI) was determined by measuring the decolourisation diameter (DD) and mycelium diameter (MD). DI, DD, and MD were tabulated in Table 1. After 12 days incubation, MD of *G. trabeum* in PDA medium contains a various concentration of MB did not show significantly different comparing with control (without the addition of MB), which indicated the addition of MB into the PDA medium did not inhibit the *G. trabeum* growth. The growth of isolated white-rot fungi (WRF) had no effect on the presence of dyes in the medium, indicated the fungi were able to tolerate the dyes (Vasudev, 2011). Jayasinghe *et al.* (2008) also reported some fungi that have good mycelial growth on medium containing MB, but poor in MB degradation. On the other hand, the PDA medium containing MB did not inhibit the growth of brown-rot fungus *Daedalea dickinsii* (Rizqi & Purnomo, 2017). The growth of some BRF including *G. trabeum* did not show any significant differences in medium contain methyl orange (Purnomo *et al.,* 2019b). Besides, some bacteria were able to growth in medium containing MB and congo red as well as decolourise them (Fulekar *et. al.,* 2013; Li *et. al.,* 2014; Liu *et al.,* 2016). These reports proved that the growth of some microorganisms including *G. trabeum* in medium with the presence of MB could be tolerated.

Table 1. MB decolourisation by *G. trabeum* in PDA medium

MB Concentration ($mg\ L^{-1}$)	**MD (cm)**	**DD (cm)**	**DI**
50	8.20	7.17	0.87
75	8.00	7.03	0.88
100	7.67	6.73	0.88
(-) control (Without MB)	8.20		

Data are presented as the mean (n = 3).

However, the DI and DD decrease with the increase of MB concentration. DD values showed significant different which the highest DD value (7.17 cm) was obtained at MB concentration of 50 mg L^{-1} while the lowest ones were that at 100 mg L^{-1} about 6.73 cm. It indicated that the decrease of DD was caused by the increase of MB concentration. Furthermore, the DI values did not show significant differences among various concentrations of MB (Table 1), which the highest DI was obtained at MB concentration of 100 mg L^{-1}. Zeng *et al.* (2015) reported that slower decolourisation rate was caused by high concentrations of dye. Rizqi and Purnomo (2017) reported *D. dickinsii* decolourised MB with the highest ID value (92%) at 50 mg L^{-1}. Jayasinghe (2008) and Vasudev (2011) were also used initial concentration of 100 mg L^{-1} in MB decolourisation by some WRF. However, MB initial concentration of 20 mg L^{-1} was used in MB decolourisation by some bacteria (Fulekar *et. al.,* 2013; Li *et. al.,* 2014; Liu *et al.,* 2016). It indicated that fungi more tolerate high concentration of MB rather than bacteria. Based on screening results in Table 1, 100 mg L^{-1} was selected as the initial concentration for degradation experiment in the liquid medium.

Biodecolourisation of MB in PDB medium was measured using UV-VIS spectrophotometer, which monitored at 0-, 7-, and 14-days incubation period. The profile of MB absorbance was shown in Figure 1. Maximum absorbance at 665 nm was detected in all absorbance profiles which indicated the maximum absorbance of MB, in line with previous report (Rahman *et al.,* 2012). At 0 days incubation, the absorbance was lower than controls, which assumed that MB was degraded by the presence of secondary metabolites or enzymes which is produced by fungus during pre-incubation. Hydrogen peroxide (H_2O_2) was one of the secondary metabolites that could be produced and formed hydroxyl radical via Fenton reaction (Kaneko *et. al.,* 2005; Arantes *et al.,* 2012), which used for MB degradation. Furthermore, at 7 days incubation, the absorbance of MB was slightly decreased compared that at 0 days, due to the fungus might adapt to the addition of MB. However, at 14 days incubation, the MB absorbance was lower than that at 7 days incubation, which assumed that *G. trabeum* might successfully adapt as well as produce more hydroxyl radical to degrade MB intensively.

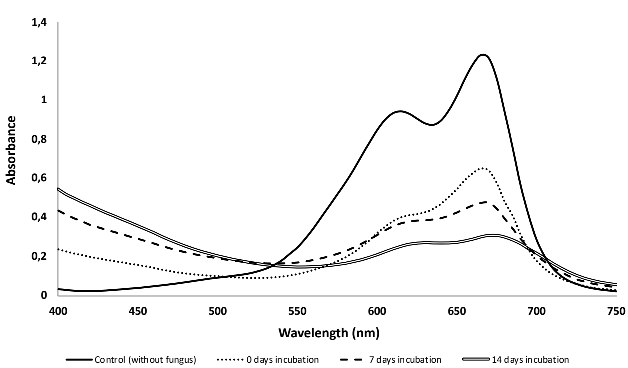

Figure 1. The absorbance profiles of MB degradation by *G. trabeum*

G. trabeum degraded MB in PDB medium was determined quantitatively by determining the decolourisation percentage. The MB decolourisation was approximately 47, 62 and 76% during 0-, 7-, and 14-days incubation periods, respectively, which showed significant differences among them (Figure 2). It indicated that the incubation periods have effect on the decolourisation of MB by *G. trabeum*. During incubation, *G. trabeum* may produce hydroxyl radicals from Fenton reaction as degradative tools. Rizqi and Purnomo (2017) reported *D. dickinsii* decolourised 54% of MB. Some WRF degraded MB (initial concentration 100 mg L^{-1}) in PDB within 20 days incubation (Fulekar *et al.,* 2013). Besides, among some bacteria, *Pseudomonas putida* showed the highest ability to degrade MB by 69% (initial concentration 20 mg L^{-1}) in minimal salt medium (MSM) for 5 days incubation (Li *et. al.,* 2014; Liu *et al.,* 2016). Compared with other microorganisms, *G. trabeum* has high ability to decolourise MB.

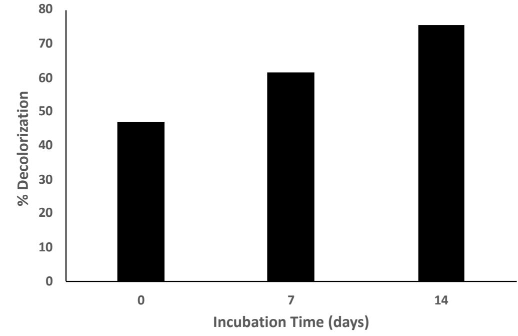

Figure 2. MB Decolourisation by *G. trabeum* in PDB medium during 0-, 7-, and 14-days incubation periods

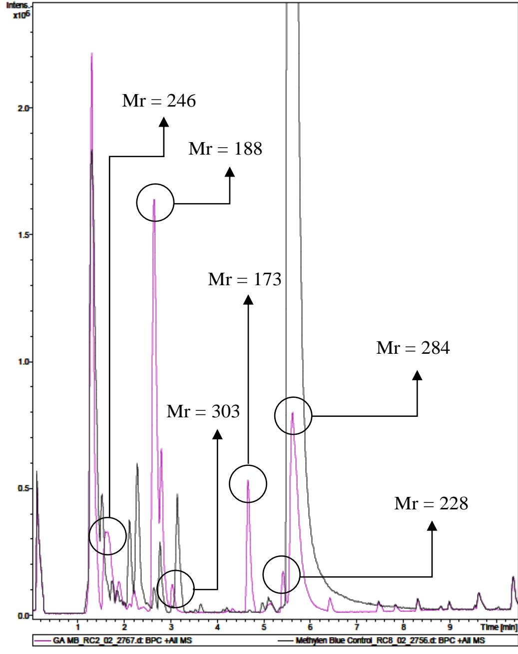

Figure 3. Chromatogram of MB degradation by *G. trabeum* for 14 days incubation. Black line was control, while pink line was treatment

The MB metabolites were identified by using LC-TOF/MS (Figure 3). The MB peaks appeared both in control and treatment samples which had m/z 284 at a retention time of 5.57 min. The MB peak treatment was lower than control, indicated the MB was degraded. Five metabolites were detected at 1.63; 2,64; 3.04; 4.66 and 5.40 min (Figure 3). The peak at a retention time of 1.63 min had m/z 245, which identified as 2-nitro-5-(*N*, *N*-dimethyl) aminobenzene sulfonate with molecular formula $C_8H_{10}N_2SO_5$ (Table 2). It was supported by Zhang *et al.* (2011), who identified this compound from MB degradation by photocatalytic. The peak at retention time 2.64 min had m/z 187, which identified as 2,5-diaminobenzene sulfonic acid with a molecular formula $C_6H_8N_2SO_3$ (Table 2). This compound was also identified from MB degradation by chemical Fenton reaction method (Liu *et al.,* 2016). 4-(dimethylamino)-2-[m-(dimethylamino) phenylsulfinyl] benzenamine was identified from the peak at retention time of 3.04 min, which had m/z 302, with molecular formula $C_{16}H_{20}N_3SO$ (Table 2). Ejhieha and Shamsabadi (2014), also identified this compound from MB degradation by photocatalytic with the present of zeolite X and CuO nanoparticles. The peak at retention time 4.66 min had m/z 172, which identified as 2-amino-benzenesulfonic acid with a molecular formula $C_6H_7NSO_3$ (Table 2). This

compound was also identified from MB degradation by chemical Fenton reaction method (Wang *et al.,* 2014). Thionine was identified from the peak at retention time of 5.40 min, which had m/z 227, with molecular formula $C_{12}H_{10}N_3S$ (Table 2). Rauf *et al.* (2010), also identified this compound from MB degradation by photocatalytic. All identified metabolites that reported previously were produced by involving hydroxyl radical. Since this fungus had been reported degraded DDT by involving hydroxyl radical (Purnomo *et. al.,* 2010; Purnomo *et al.,* 2011), it suggested that *G. trabeum* might also degrade MB by this system. However, further investigation in Fenton reaction involvement on MB degradation is needed.

Table 2. Metabolites of MB degradation by *G. trabeum*

No	Retention Time (min)	Mr	Molecular Formula	Molecular Structure
1	1.63	246	$C_8H_{10}N_2SO_5$	
2	2.64	188	$C_6H_8N_2SO_3$	
3	3.04	303	$C_{16}H_{20}N_3SO$	
4	4.66	173	$C_6H_7NSO_3$	
5	5.40	228	$C_{12}H_{10}N_3S$	

Transformation pathway of MB by *G. trabeum* was proposed based on the identification of metabolic products (Figure 4). MB was degraded initially via three pathways: (1) oxidation to 2-nitro-5-(*N*,*N*-dimethyl)amino-benzenesulfonate. MB was oxidised at the secondary ketimine and sulfide groups. Oxidation of the secondary ketimine group breaks the bonds of the secondary ketimine group with aromatic rings and forms a nitro group. Likes oxidation in the secondary ketimine group, oxidation of the sulfide group also breaks the sulfide group bond with the aromatic ring and forms a sulfo group. (2) Demethylation to thionine, followed oxidation cleavage to 2,5-diaminobenzenesulfonic acid, then undergo to release NH_2 to form *o*-amino benzenesulfonic acid. Demethylation (removal of the methyl group) occurred in the tertiary amine group, which is replaced by hydrogen, so that a primary amine group was formed, followed by cleavage of the bond secondary and sulfide ketimine groups with the aromatic ring. The secondary ketimine group was transformed into a primary amine while the sulfide group was transformed into a sulfo group. The subsequent breaking of the bond occurred by removing another primary amine group. (3) Oxidation of the sulfide group to form 4-(dimethylamino)-2-[m-(dimethylamino) phenylsulfinyl] benzenamine, as reported by Rauf *et al.* (2010), following oxidation on the MB sulfide group to 3,7-bis(dimethylamino)-4OH-phenothiazin-5-one (Huang *et al.,* 2010). Further reduction on the C=N double bond was occurred to yield 4-(dimethylamino)-2-[m-(dimethylamino) phenylsulfinyl] benzenamine (Ejhieha & Shamsabadi, 2014).

Figure 4. Proposed MB degradation pathway by *G. trabeum*

MB degradation pathway was reported by Huang *et al.* (2010) by atmospheric pressure dielectric barrier discharge plasma. Firstly, chloride ion (Cl^-) is ionised and exists in the detached state during dissolution of MB. The radical species bombardment caused the N–CH_3 bond broken, followed by oxidation of the –CH_3 into HCHO or HCOOH, and left the most active parts (C–S and C–N) in MB structure molecule. During the bombardment of •OH, the two bonds are broken more easily. At the same time, phenyl thiophene and other molecular structures were determined. In another research by Li *et al.* (2014) reported Azure B (MB derivate) dye decolourisation mechanism by *Bacillus* sp. MZS10 that the

mechanism of decolourisation mostly involved quinone dehydrogenase. This enzyme reduces the C=N double bond to leuco form, followed by a combined amine group with the other group to form a stable compound. MB degradation pathway by hydroxyl radicals via photocatalytic was also reported by Houas *et al.* (2001). The cleavage N=C double bond at central amino group of MB was induced by the cleavage of the double bond of the $-S^{+}=$ group in *para* position of the central aromatic ring. Hydroxyl radical substituted the amino group to form phenol and release NH_2 radical. Considering above references, MB transformation pathway in this study has similarity, especially in the central aromatic ring by the involvement of N=C double bond cleavage.

IV. CONCLUSION

G. trabeum degraded 76% of MB (initial concentration 100 mg L^{-1}) after incubated for 14 days. Five metabolites of MB degradation were identified: 2-nitro-5-(N,N-dimethyl) amino benzene sulfonate ($C_8H_{10}N_2SO_5$), 2,5-diaminobenzene sulfonic acid ($C_6H_8N_2SO_3$), 4-(dimethylamino)-2-[m-(dimethylamino) phenylsulfinyl] benzenamine ($C_{16}H_{20}N_3SO$), 2-amino-benzenesulfonic acid ($C_6H_7NSO_3$), and thionine ($C_{12}H_{10}N_3S$), which hydroxyl radical might be involved in the degradation pathway. This study proved that *G. trabeum* have ability to decolourise and degrade MB.

V. ACKNOWLEDGEMENT

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