



Research article

The effect of *Ralstonia pickettii* bacterium addition on methylene blue dye biodecolorization by brown-rot fungus *Daedalea dickinsii*Badzlin Nabilah, Adi Setyo Purnomo^{*}, Hamdan Dwi Rizqi, Herdayanto Sulisty Putro, Refdinal Nawfa

Department of Chemistry, Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember (ITS), Kampus ITS Sukolilo, Surabaya, 60111, Indonesia

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ABSTRACT

Methylene blue (MB) is one of synthetic dyes that is used in the textile industry which is difficult to degrade in nature. Previously, the brown-rot fungus (BRF) *Daedalea dickinsii* had shown a good ability to degrade MB, however, the decolorization ability was relatively still low and had a long period of incubation. Therefore, improvement of process is needed to increase the ability of *D. dickinsii* to decolorize MB. In this study, the effect of *Ralstonia pickettii* bacterium addition on MB biodecolorization by the BRF *D. dickinsii* in potato dextrose broth (PDB) medium was investigated. The amount of *R. pickettii* that was added to the culture of *D. dickinsii* were 2, 4, 6, 8, and 10 mL (1 mL $\approx 1.39 \times 10^8$ CFU). The cultures had ability to decolorize MB (100 mg/L) at 30 °C after 7 days incubation. The highest percentage of MB biodecolorization was obtained at addition of 10 mL of *R. pickettii* approximately 89%, while biodecolorization process by particularly *D. dickinsii* was approximately 17%. The MB degradation metabolites by mixed cultures of *D. dickinsii* and 10 mL of *R. pickettii* were Azure A, thionine, glucose-MB, C₁₂H₁₁N₃SO₆ and C₁₂H₁₃N₃O₆. This study indicated that the addition of *R. pickettii* could enhance MB biodecolorization by fungus *D. dickinsii*. Besides that, this study also indicated that mixed cultures of *D. dickinsii* and *R. pickettii* has great potential for high efficiency, fast and cheap dye wastewater treatment.

1. Introduction

Industrial sector is one of economic indicator of a country which can provide great employment opportunities for many people (Rusastira, 2018). Textile, leather, and footwear industries become one of the mainstays industries. In the production process, those industries cannot be separated from the coloring process, where the dyes that are often used are synthetic dyes. Methylene blue (MB) is one of manufactured dye among dyes that often used in the fabric industries. Besides being used in the fabric industries, MB dye is also used in the paper coloring industry, office equipment and also disinfectant. In the coloring process, only 5% of MB is used, while 95% of the rest is discarded as waste (Machiril and Kusumastuti, 2017). This compound is a stable compound which is difficult to degrade in nature. Waters with high concentration of MB cause the death of aquatic organism due to the high concentration of MB can increase the COD and BOD numbers in the contaminated waters (Lellis et al., 2019). In addition, high concentration of MB also can be harmful to human. It can cause eye burns, if inhaled it can cause rapid problems breathing, while ingestion can

cause nausea, vomiting, mental confusion and methemoglobinemia (Muchanyereyi et al., 2014).

Some remediation processes have been studied including chemical, physical and biological treatments (Azam et al., 2020; Forgacs et al., 2004; Rizqi and Purnomo, 2017). Even though chemical and physical remediation process are more quickly than biological remediation process, they are commonly harmful and causing new problem, more energy-intensive, and often more expensive than bioremediation. Among the several methods, biodegradation is relatively low-cost, more secure and efficient (Foght et al., 2001).

Fungi are one of degradation agents that have high tolerance to chemical pollutants. Brown-rot fungi (BRF) is one of fungi that can degrade some pollutants including dyes (Purnomo et al., 2010; Rizqi and Purnomo, 2017; Wetzstein et al., 1997). The ability of BRF to degrade MB dye had been reported previously, which *Daedalea dickinsii* have shown a good ability to decolorize and degrade MB through hydroxyl radical system. *D. dickinsii* could decolorized MB up to 54% in potato dextrose broth (PDB) medium for 14 days incubation period (Rizqi and Purnomo, 2017). The decolorization percentage of MB through *D. dickinsii* was

^{*} Corresponding author.

E-mail addresses: adi_setyo@chem.its.ac.id, adi.spurnomo@yahoo.com (A.S. Purnomo).

relatively low and the incubation period was long. Thus, the process has to be improved by culture modification to increase the capability of *D. dickinsii* to decolorize MB.

Several studies regarding the use of bacteria as MB decolorizing agents had been reported. *Sphingomonas paucimobilis* had been reported could decolorize MB by 85%. The process was done aerobically for 5 days with the initial concentration of MB was 1000 mg/L (Noraini et al., 2012). *Bacillus thuringiensis* also had been reported could remove MB up to 95%. The process was conducted aerobically on Luria Bertani medium with the initial concentration of MB was 25 mg/L (Chen et al., 2015). Eslami et al. (2017) reported that *Pseudomonas aeruginosa* that was isolated from MB contaminated soil could remove MB with removal efficiency up to 97.82%. Other bacteria that can be used as MB degradation agent are *Ralstonia mannitolilytica*, *Comamonas aquatic* and *Ralstonia eutropha*. Bacterium *R. eutropha* showed good MB removal ability with removal efficiency up to 82% in the presence of supplementary carbon and nitrogen source (Habibi and Mehrabi, 2017). *R. mannitolilytica* and *C. aquatic* had been reported could decolorize MB by 67.9% and 60.3% respectively (Michelle et al., 2020). These two findings show that genus *Ralstonia* might have capability to decolorize MB.

Ralstonia spp. are non-fermentative rods, Gram-negative and aerobic bacteria that are found in water and soil. *Ralstonia pickettii* is one of organism species that had been shown to have good biodegradative abilities. It had been reported that *R. pickettii* showed a good ability to degrade aromatic compound, such as: benzene, cresol, phenol, and toluene (Ryan et al., 2006, 2007). Mahendra and Alvarez-Cohen (2006) reported that *R. pickettii* PK01 could degrade 50 mg/L 1,4 dioxane by co-metabolic dioxane transformation. Recent study conducted by Purnomo et al. (2021) reported that *R. pickettii* was capable to decolorize MB dye. The bacterium could decolorize 100 mg/L MB for about 98.11% after 18h of incubation time. The use of *R. pickettii* as MB decolorization agent is more beneficial than other bacteria. It was reported as non-pathogenic bacterium in nature and also could survive on extreme environments, such as low nutrient aquatic and soil environment (Ryan et al., 2007). That kind of ability might help it to survive in polluted environments and use some pollutants as its carbon and energy source. Besides, *R. pickettii* showed a better potential to decolorized MB than any other bacteria (Chen et al., 2015; Eslami et al., 2017; Michelle et al., 2020; Noraini et al., 2012; Purnomo et al., 2021).

Modified methods to increase biodecolorization process had been developed. Mixed cultures of fungi and bacteria can be a good potential for biodegradation process. Fungi play more significant role in biosorbition, transport agent and settleability, while bacteria play more significant role in biodegradation. The advanced biosorbition and settleability of fungi, mixed with the excellent biodegradation capability of bacteria resulting a synergistic effect of the co-cultures microorganism (Zhou et al., 2014). Bacterial-fungal co-culture had been reported about its synergistic effect and its capability regarding pollutants degradation. It was capable to degrade some pollutants, such as herbicide diuron (Ellegaard-Jensen et al., 2014), pesticide dichlorodiphenyltrichloroethane (DDT) (Purnomo et al., 2018), petroleum contaminated sediment (Y. Li and Li, 2011), and some azo dyes (Mani and Hameed, 2019; Mawad et al., 2020). Therefore, the co-culture of *D. dickinsii* and *R. pickettii* might also have synergistic effect to decolorize MB. In this study, the effect of *R. pickettii* addition on MB decolorization by *D. dickinsii* was investigated. The addition of *R. pickettii* itself is expected to improve *D. dickinsii* ability to decolorize MB. In addition, the product metabolites and the MB biotransformation pathway by the mixed cultures was also investigated. This report was the first report on proposing MB biodecolorization pathway by mixed cultures of brown-rot fungus *D. dickinsii* and bacterium *R. pickettii*.

2. Materials and methods

2.1. Materials

BRF *D. dickinsii* NBRC 31163 and bacterium *R. pickettii* NBRC 102503 (NITE Biological Resources Center, NBRC; Chiba, Japan) were obtained

from the collection of laboratories of Microbial Chemistry, Department of Chemistry, ITS. MB, aqua distillation, and technical alcohol were acquired from SAP Chemicals, Indonesia. Bacteria growing medium nutrient agar (NA) and nutrient broth (NB) were acquired from Merck, Germany. Fungi Growth Medium Potato dextrose agar (PDA) was also acquired from Merck, Germany, and Potato Dextrose Broth (PDB) was acquired from Himedia, India.

2.2. Fungus culture

The stock culture of *D. dickinsii* was inoculated on 90-mm diameter of PDA plates and incubated at 30 °C for 7 days. The mycelia obtained from the incubation process were transferred into an aseptic blender jar containing 25 mL of aseptic aqua distillation. Then the mycelia were homogenized for 3 min. A milliliter of this homogenate was added into 8 mL of PDB medium in 100-mL Erlenmeyer flasks. The cultures were incubated statically at 30 °C for 7 days (Purnomo et al., 2010).

2.3. Bacterium culture

The bacteria culture stock of *R. pickettii* was inoculated on 90-mm diameter of NA plates and incubated at 37 °C. The colony of the bacterium was inoculated into 250 mL of sterile NB medium in a 500 mL Erlenmeyer flask. The culture was pre-incubated on a shaker incubator with shaker condition 180 rpm for 44 h (Wahyuni et al., 2016, 2017).

2.4. MB biodecolorization by *D. dickinsii* culture

Pre-incubated culture of *D. dickinsii* was added into 10 mL PDB medium (total volume 20 mL). MB was injected into the culture with final concentration 100 mgL⁻¹. The incubation process of the cultures was conducted statically for 7 days at 30 °C (Rizqi and Purnomo, 2017).

2.5. MB biodecolorization by *R. pickettii* culture

The pre-incubated *R. pickettii* culture was added into sterile PDB with variation of 2, 4, 6, 8, 10 mL volume (1 mL of bacterium culture ≈ 1.39 × 10⁸ CFU). MB was injected into each culture with final concentration of the culture was 100 mgL⁻¹ (total volume 20 mL). The incubation process of the cultures was conducted statically for 7 days at 30 °C (Purnomo et al., 2021).

2.6. MB biodecolorization by mixed culture of *D. dickinsii* and *R. pickettii*

Pre-incubated *R. pickettii* with variation 2, 4, 6, 8, 10 mL (1 mL of bacterium culture ≈ 1.39 × 10⁸ CFU) was added separately toward pre-incubated *D. dickinsii* cultures. Then, PDB medium was added toward the culture (total volume 20 mL). MB was injected into each culture with final concentration of the culture was 100 mg/L. The incubation process of the cultures was conducted statically at 30 °C for 7 days (Purnomo and Mawaddah, 2020).

2.7. MB recovery and identification of metabolites

After incubation process, the biomasses obtained were removed by centrifugation process at 3000 rpm for 10 min. The supernatant obtained was measured its absorbance by UV-Vis's spectrophotometer. For abiotic control, MB was added into PDB with the final concentration was 100 mg/L without any microbial addition. The percentage of MB decolorization was calculated by Eq. (1).

$$\% \text{decolorization} = \frac{A_k - A_t}{A_k} \times 100\% \quad (1)$$

where A_k is control absorbance and A_t is treatment absorbance.

The MB metabolite products obtained from the decolorization process were identified by analyzing the supernatants. The analysis was conducted using Liquid Chromatography Time of Flight Mass Spectrometry (LC-TOF/MS). The ionization source was ionization electrospray (ESI) with a mass range 100–500. The elution method was gradient method with rate flow 0.2 mL min⁻¹ on the first 3 min. The rate flow for the next 7 min was 0.4 mL min⁻¹. Methanol and water, with ratio 99:1, was used for the mobile phase for the first 3 min and ratio 61:39 was for the rest 7 min. Acclaim TM RSC 120C18 was used for the column. The size of the column was 2.1 × 100 mm and the column temperature was 33 °C (Rizqi and Purnomo, 2017).

3. Results and discussion

3.1. MB biodecolorization

D. dickinsii is one of BRF that has capability to be a bioremediation agent. In this study, 100 mg/L MB was degraded by *D. dickinsii* up to 17% after the 7th day incubation period in PDB medium (Table 1). The result was different from previous report by Rizqi and Purnomo (2017), which obtained approximately 25% of MB decolorization for 7 days of incubation. This might be because of the different method on separating the supernatant. In previous study, filter paper was used to separate supernatant, while in this study, syringe decantation was used for separation. The use of filter paper allowed MB absorption process and make previous study had larger percentage of decolorization. The supernatant was scanned by UV-Vis Spectrophotometer on wavelength range 400–800 nm, where the highest MB absorbance absorbed on 665 nm (Melgoza et al., 2009). Figure 1 showed the biodecolorization profile by *D. dickinsii*, which the maximum absorbance of MB was appeared in wavelength 665 nm. The maximum absorbance of MB in the treatment was smaller than the maximum absorbance of the abiotic control, which indicated MB was decolorized. The capability of *D. dickinsii* to degrade MB could have a correlation with the capability of the fungi to produce hydroxyl radicals resulting from Fenton Reaction and extracellular enzyme (Purnomo et al., 2020; Rizqi and Purnomo, 2017).

MB decolorization by *R. pickettii* only was also carried out with different initial cell density at 2, 4, 6, 8, 10 mL (1 mL ≈ 1.39 × 10⁸ CFU). MB (100 mg/L) was decolorized by approximately 17, 24, 68, 81, and 59% at 2, 4, 6, 8, and 10 mL of bacterial cultures, respectively, within 7 days of incubation in PDB medium (Table 1). Based on the result, the highest percentage of MB decolorization was obtained at the volume 8 mL of *R. pickettii* cultures. The 10 mL *R. pickettii* culture resulted in decreasing of MB decolorization percentage due to the excessed number of bacteria on the culture, thus allowing competition between bacteria to survive (Mairer et al., 2009). The optimum result in this study was different from the previous study conducted by Purnomo et al. (2021). On the previous study, the decolorization process reached higher decolorization percentage (98.11%) within 18 h incubation period. This was due to the aeration process was done in previous study (Purnomo et al., 2021). The aeration on the previous study provided oxygen to the microorganism and fulfilled the Biochemical Oxygen Demand (BOD) (Mohd Tajuddin et al., 2004). Thus, the previous study would have

higher decolorization percentage as aeration is one of abiotic factor that affecting degradation process by microorganism (Joutey et al., 2013). The ability of *R. pickettii* on MB decolorization is dependent on its ability to produce extracellular degradation enzyme on its metabolite process (Purnomo et al., 2021; Ryan et al., 2007).

The biodecolorization absorbance profile of mixed cultures of *D. dickinsii* and *R. pickettii* was shown in Figure 2. The maximum absorbance of treated MB was shown at wavelength 665 nm (Melgoza et al., 2009). The figure also showed that the lowest absorbance was found at the 10 mL bacteria addition. The results of MB decolorization by mixed cultures of *D. dickinsii* and *R. pickettii* were shown in Table 1. After 7 days' incubation period, approximately 16, 30, 37, 55, and 89% of MB was decolorized by mixed cultures of *D. dickinsii* with *R. pickettii* addition at 2, 4, 6, 8, and 10 mL respectively. The addition of 10 mL of *R. pickettii* into *D. dickinsii* could enhance the ability of *D. dickinsii* to decolorize MB with the increase of the percentage about 72%. Thus, it showed that mix culture capability in MB decolorization was higher in comparison with *D. dickinsii* alone. On the previous report, the consortium of *R. pickettii* and *F. pinicola* (Purnomo et al., 2020), *D. dickinsii* (Purnomo et al., 2018), and *P. eryngii* (Purnomo et al., 2019) were discovered to be a better DDT degradation agent than the fungi culture alone. Some study regarding MB biodecolorization by microbial consortium had been reported previously. The consortium of *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger* resulted an optimum MB (150 mg/L) decolorization for about 92% (36 h, 30 °C). The result showed a better decolorization efficiency than the individual culture due to interdependent catabolic activity of the *Aspergillus* sp. consortium (Karaghool, 2021). The mix culture between yeast and bacteria had been reported about its ability in MB degradation. MB (200 mg/L) was degraded by the consortium within 72 h and the process was dependent on the peptone extract addition as the nutrition of the culture. The consortium showed a complete decolorization within 108 h in domestic wastewater, while 57.39% of the dye removed by 144 h in industrial effluent (Eltarahony et al., 2021). These findings result indicating that the microbial consortium might show a better performance in biodecolorization process.

The dyes biodecolorization process by microorganism appear because of various mechanism, through enzymatic and non-enzymatic (via Fenton reaction) mechanisms. Laccase (Sivasakthivelan, 2013), lignin peroxidase (Ollikka et al., 1993), and manganese peroxidase were some enzymes that usually produced by white-rot fungi (WRF) and bacteria. These enzymes were capable to degrade synthetic dyes and numerous aromatic compound due to their nonspecific nature (Sweety, 2018). Some other enzymes that were produced by bacteria, such as azo-reductase (Xu et al., 2021), flavin-reductase (Telke et al., 2015), NADH-DCIP Reductase (Kalyani et al., 2008), polyphenol oxidase etc. were used in dye decolorization process (Telke et al., 2015). In this study, MB was being decolorized and transformed through enzymatic and non-enzymatic mechanism. *D. dickinsii* was playing a role in the non-enzymatic mechanism using hydroxyl radical, as brown-rot fungus (BRF), can produce hydroxyl radical from Fenton reaction (Rizqi and Purnomo, 2017). On the other hand, *R. pickettii* was playing a role in the enzymatic mechanism through reduction and demethylation reactions that were supported by its extracellular enzymes (Purnomo et al., 2021).

3.2. Product metabolites identification and proposed degradation pathway

The metabolites identification of MB degradation was performed by using LC-TOF/MS. Peak at retention time (RT) 16.00 min, both in control and treatment chromatogram (Figure 3), had m/z value of 284. The peaks were identified as MB, where peak on the treatment had much lower intensity than the peak on the control. This result verify the occurrence of MB biodecolorization process and also support the previous decolorization analysis data by UV-Vis spectrophotometer (Cohen et al., 2019). Figure 3 showed that there are 5 peaks of products metabolites that were detected. The peak was detected at RT 2.01, 7.87,

Table 1. The MB decolorization by *D. dickinsii*, *R. pickettii* and mixed cultures after 7 days incubation period.

<i>R. pickettii</i> culture volume (mL)	Methylene Blue Decolorization (%)	
	<i>R. pickettii</i> only	Mixed Cultures
<i>D. dickinsii</i> only	16.51 ± 0,05	
2	16.646 ± 0,001	15.893 ± 0,008
4	23.588 ± 0,002	29.778 ± 0,003
6	67.503 ± 0,001	36.721 ± 0,001
8	80.594 ± 0,001	54.621 ± 0,001
10	59.180 ± 0,001	88.791 ± 0,001

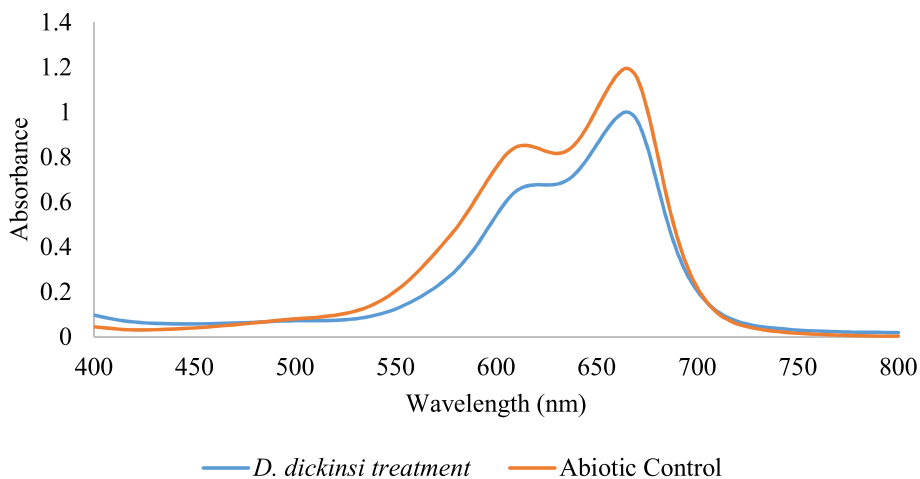


Figure 1. Profile absorbance of MB decolorization by *D. dickinsii*.

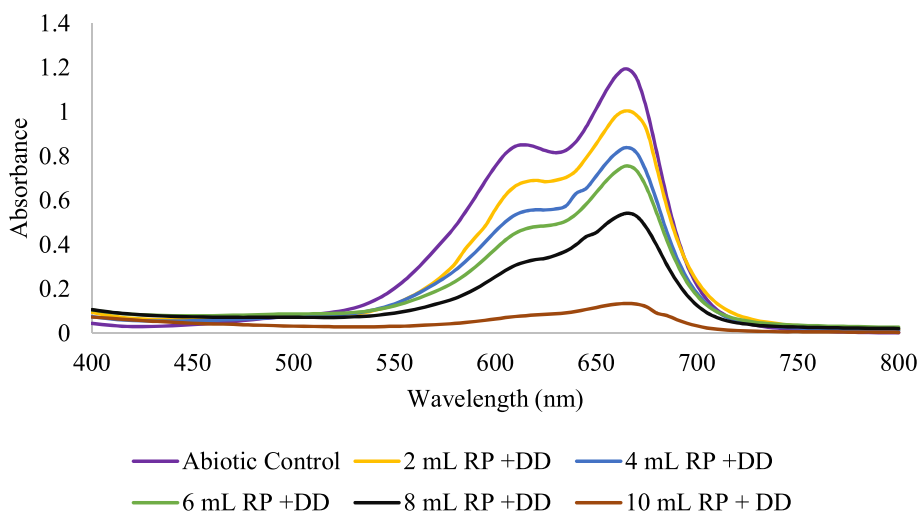


Figure 2. Profile absorbance of MB decolorization by mixed cultures of *D. dickinsii* (DD) and *R. pickettii* (RP).

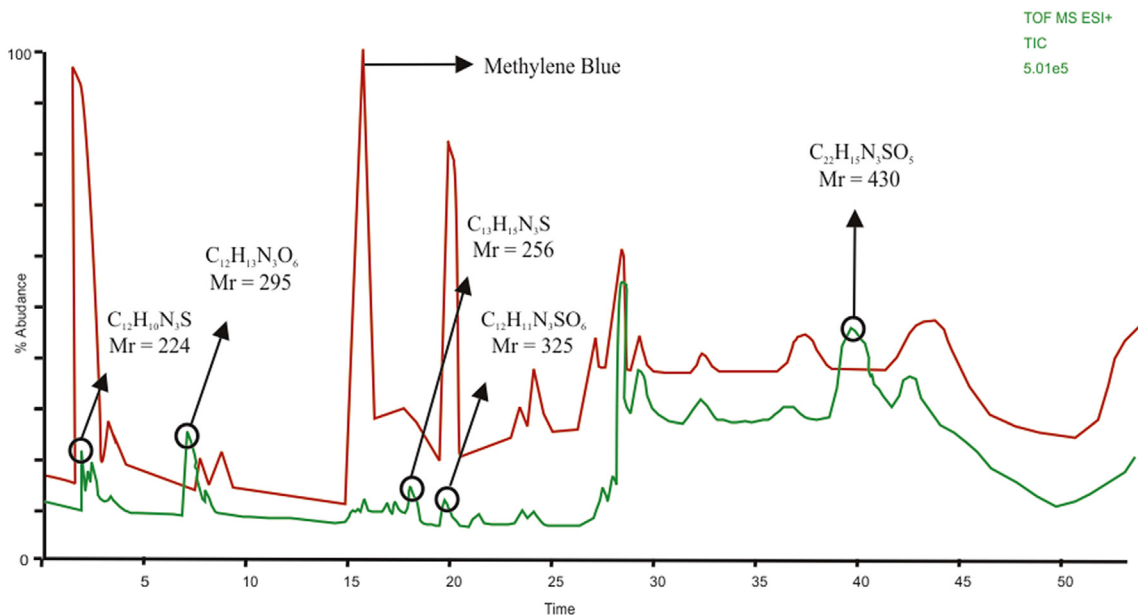


Figure 3. Chromatogram of MB degradation by mixed cultures of *D. dickinsii* and *R. pickettii* after 7 days incubation period (Red chromatogram: MB Control; Green chromatogram: MB Treatment).

18.04, 19.75, and 39.56 min, which identified as $C_{12}H_{10}N_3S$ (thionin, $m/z = 224$), $C_{12}H_{13}N_3O_6$ ($m/z = 295$), $C_{13}H_{15}N_3OS$ (Azure A, $m/z = 256$), $C_{12}H_{11}N_3SO_6$ ($m/z = 325$) and $C_{22}H_{15}N_3SO_5$ (Glucose MB, $m/z = 430$) respectively (Table 2). The product metabolites by microorganisms showed different result due to various enzymes that was produced by the microorganism. However, some microbes showed similar metabolic products. *B. albus* MW407057 and *P. chrysosporium* produced lignin peroxidase (LiP) enzyme to degrade MB via demethylation and deamination resulting Azure A and Thionine (Ferreira et al., 2000; Kishor et al., 2021). *Bacillus* sp. MZS10 produced quinone dehydrogenase enzyme that

could degrade Azure B (MB derivate) via reduction and dehydration reaction resulting glucose MB (H. Li et al., 2014).

Based on identification of metabolites, MB biotransformation pathway was proposed (Figure 4). MB was transformed via three pathways. On the first and second proposed pathway, demethylase enzyme and quinone dehydrogenase enzyme played an important role in the transformation of MB. The first and the second pathway were started with oxidative demethylation transforming MB into Azure A. The first step continued with another oxidative demethylation transforming Azure A into Thionine (Kishor et al., 2021). On the other hand, the second

Table 2. Metabolites of MB degradation by mixed cultures of *D. dickinsii* and *R. pickettii*.

Retention time (mins)	Molecular Weight	Molecular formula	Molecular structures
2.01	224	$C_{12}H_{10}N_3S$	
7.87	295	$C_{12}H_{13}N_3O_6$	
18.04	256	$C_{13}H_{15}N_3S$	
19.75	325	$C_{12}H_{11}N_3SO_6$	
39.56	430	$C_{22}H_{15}N_3SO_5$	

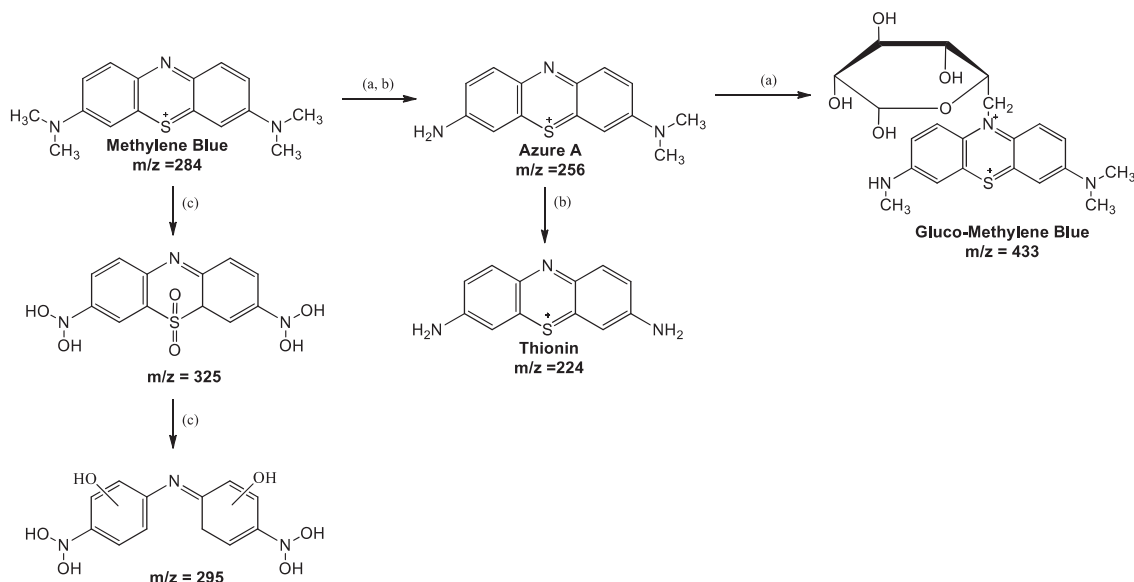


Figure 4. MB biotransformation pathway (a). First transformation pathway, (b). second transformation pathway, and (c). third transformation pathway.

pathway continued with reduction of C=N bond on the thionine ring generating an amino group. The amino group would have a bond with hydroxyl group from glucose assembling stable compound via dehydration reaction (H. Li et al., 2014). On the third pathway, MB would undergo two step oxidation process on its C-S⁺ = S group (thionine ring) producing an intermediate product of C₁₆H₁₉N₃SO₂. The process was continued with hydroxylation reaction on the two symmetrical dimethyl amino groups of the intermediate product. The reaction might be catalyzed by hydroxylase enzyme producing compound C₁₂H₁₁N₃SO₆ with m/z value of 325. The thionine ring would undergo de-sulfonation process resulting metabolite product C₁₂H₁₃N₃O₆ with m/z value of 295 (Su et al., 2019).

The oxidative demethylation of MB was also can be found on MB degradation by *D. dickinsii* and *R. pickettii* alone. Oxidative demethylation of MB by *D. dickinsii* producing Azure B. In addition, two compound, C₁₆H₁₉N₃SO, and C₁₆H₂₁N₃SO, were also produced because of oxidation process on C-S⁺ = S group of the thionine ring and reduction on C=N bond (Rizqi and Purnomo, 2017). On the other hand, oxidative demethylation of MB by *R. pickettii* producing Azure A and Thionine, which showed the same results as the current study. Azure A and Thionine are two MB derivatives that commonly found on the degradation of Methylene Blue. Besides, MB transformation by *R. pickettii* was also resulting glucose MB as a result of reaction that was catalyzed by quinone dehydrogenase enzyme. Yang et al. (2017) reported that P2ABSA-modified TiO₂ nanocomposite could degrade MB via photocatalyst method. The process was producing Azure B, Azure A, Azure C, and thionine as a result of demethylation reaction. Furthermore, Azure A, B, and C were also can be found on the MB degradation by Fenton process based on the use of maghemite/silica microsphere (Cohen et al., 2019).

4. Conclusion

R. pickettii had an ability to enhance MB decolorization by *D. dickinsii*. Approximately 89% of MB was decolorized after 7 days' incubation period. The mixed culture was capable to transform MB into Azure A, thionine, glucose-MB, C₁₂H₁₁N₃SO₆ and C₁₂H₁₃N₃O₆. Thus, it indicated that demethylase, quinone dehydrogenase, and hydroxyl radical played important role on the transformation process.

Declarations

Author contribution statement

Adi Setyo Purnomo: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Badzlin Nabilah, and Hamdan Dwi Rizqi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Herdayanto Sulisty Putro, and Refdinal Nawfa: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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