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# Methylene blue decolorizing bacteria isolated from water sewage in Yogyakarta, Indonesia

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**Abstract.** *Michelle, Siregar RAN, Sanjaya A, Jap L, Pinontoan R. 2020. Methylene blue decolorizing bacteria isolated from water sewage in Yogyakarta, Indonesia. Biodiversitas 21: 1136-1141.* The textile industry contributes to water pollution issues all over the world. One of the most commonly applied cationic dye in the textile industry is methylene blue. This study aimed to isolate bacteria with the potential to decolorize methylene blue from dye contaminated sewage water located in Kulon Progo District, Yogyakarta, where several textile industries within the proximity, are located. Characterizations of bacterial candidates were done morphologically and biochemically. Molecular identification was conducted by 16S rRNA sequencing. The ability of isolates to decolorize methylene blue was observed by the reduction of methylene blue's maximum absorption at the wavelength of 665 nm. The results showed that isolates were identified as *Comamonas aquatica* and *Ralstonia mannitolilytica*. *C. aquatica* PMB-1 and *R. mannitolilytica* PMB-2 isolates were able to decolorize methylene blue with decolorization percentage of 67.9% and 60.3%, respectively when incubated for 96 hours at 37°C. These findings present information on the capability of the genus *Ralstonia* and *Comamonas* to decolorize methylene blue cationic dye.

**Keywords:** *Comamonas aquatica*, decolorization, methylene blue, *Ralstonia mannitolilytica*, Yogyakarta

## INTRODUCTION

The textile industries are known as the major source of water pollution all over the world (Ito et al. 2016). Textile industrial wastes contain dyes which are often disposed into the nature aggravating pollution problems due to their toxicity. Most of these dyes are not readily degradable under natural conditions and resistant to conventional wastewater treatment systems (Yaseen and Scholz 2019). The emitted textile effluents impose substantial adverse effects on water quality, soil fertility, and marine life, causing ecosystem disruptions (Croce et al. 2017). For those living adjacent to contaminated riverbanks or other water sources, short-term exposure to textile dyes can lead to skin and eye irritation as well as allergic reactions, while continued exposure could even lead to cancer due to mutagenicity and carcinogenicity of these dyes (Lellis et al. 2019).

One of the most common cationic dyes that are often applied in textile, leather, paper, plastic, and craft industries is methylene blue (Rafatullah et al. 2010). Methylene blue contaminated waste was reported to impose health risks such as nausea, dizziness, and chest pain to the exposed population and reduce the biodiversity of the affected ecosystems (Zhou et al. 2019). Many technologies have been employed to eliminate the contamination of methylene blue in the environment. Recently, physicochemical approaches, including photocatalysis, physiochemical adsorption, and non-thermal plasma technology, have garnered popularity due to its high efficiency and degradation percentage (Das et al. 2019; Myung et al. 2019; Wu et al. 2019). However, high complexity, low economic feasibility, and disposal problems by some of these methods hinder their broad application, especially in a developing country (Zhou et al. 2019). Wastewater treatment by biological approach offers a more cost-efficient and sustainable alternative solution is contrary to the huge expenditure specified by the physicochemical approach. In the biological method, microbes were utilized for the degradation of the toxic textile dyes. Microorganisms that have been studied and demonstrated effective removal of methylene blue include *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* isolated from contaminated soil, *Sphingomonas paucimobilis* isolated from the drainage system, and *Bacillus thuringiensis* (Noraini et al. 2012; Chen et al. 2015; Eslami et al. 2017; Kilany 2017).

Although Indonesia owns high biological diversity, there are only limited bacteria species being reported as a bioremediation agent of a textile dye in contaminated water. Therefore, this study aimed to isolate potential methylene blue-decolorizing bacteria from dye contaminated sewage water located in Kulon Progo District, Yogyakarta, where several textile industries are located within the proximity.

## MATERIALS AND METHODS

### Study area

Kulon Progo is one of the regencies located in the Yogyakarta Special Region, Indonesia, named after its location from the Progo River. Being one area that

specialized in the textile industry businesses, the sewers in this area receive a heavy load of textile dyes-contaminated wastewater from the surrounding home textile industries. To isolate bacteria with the ability to decolorize methylene blue, sewage water from a large sewer at Ngentakrejo, Kulon Progo, Special Region of Yogyakarta, Indonesia, was taken as a sample. The sampling location was presented in Figure 1.

**Procedures**

*Isolation of bacteria from Yogyakarta sewage water*

Collected water samples were diluted ($10^{-1}$) with sterile water and plated (200 µl) on GYP (glucose yeast peptone) agar (1% glucose, 0.5% yeast, 0.5% peptone, 2% agar) supplemented with 50 ppm methylene blue (Merck, Germany) and incubated at 37°C for 96 hours. Bacteria isolates with clear zones around their colonies were selected, purified, and maintained on GYP agar supplemented with 50 ppm methylene blue for further analysis.

*Characterization of bacteria from Yogyakarta sewage water*

Cell and colony morphology were observed to characterize potential bacteria. Isolated candidates for methylene blue decolorizer were streak-plated on GYP agar supplemented with 50 ppm methylene blue, incubated at 37°C for 96 hours. Cell appearances observed through Gram-staining examined using a light microscope with a 1000x total magnification. Colony appearances include shape, color, margin, and elevations. Biochemical characterization through Voges-Proskauer (VP) assay, indole assay, catalase assay, and starch hydrolysis were conducted as described by Bergey's Manual of Systematic Bacteriology (Guerrero 2001).

*Molecular identification of bacterial isolates*

The 16S rRNA gene was amplified and sequenced to identify bacteria isolates. Pure isolates were cultured on GYP broth media incubated at 37°C overnight. Three ml of the broth culture of each isolate was centrifuged at 16,000 x g for 3 min and the pellet was used for DNA extraction. The extraction of bacterial genomic DNA was conducted using the Wizard® Genomic DNA Purification Kit according to manufacturer instructions (Promega, USA).

The quality and yield of extracted genomic DNA were assessed by a UV/Vis spectrophotometer (BioDrop, UK) and used as a template for 16S rRNA gene amplification by PCR reactions. All PCR reactions were conducted by employing universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) to amplify 16S rRNA gene using isolated genomic DNA from each isolate. KAPA HiFi™ Taq Polymerase, dNTP and fidelity buffer (Kapa Biosystems, USA) were mixed in 50 µL total volume per reaction under PCR conditions as follow, initial denaturation at 95°C for 3 min, twenty-five cycles of amplification consisted of denaturation at 98°C for 20 sec, annealing at 55°C for 15 sec and extension at 72°C for 60 sec. A final extension phase was performed at 72°C for 2 min.

**Figure 1.** Sewerage in Kulon Progo District, Special Region of Yogyakarta, Indonesia as a water sampling location for bacterial isolation (geographic coordinate: -7.907293,110.264097). Source: Google Inc, 2019

**Table 1**. Cell, morphological colony and biochemical characterization of PMB-1, PMB-2 and PMB-3 isolates

| Characteristics | | Isolates | | |
|---|---|---|---|---|
| | | **PMB-1** | **PMB-2** | **PMB-3** |
| Cellular morphology | Shape | Rod | Rod | Rod |
| | Gram staining | Negative | Negative | Negative |
| Colony morphology | Shape | Circular | Circular | Circular |
| | Color | Blue | Yellow | Blue |
| | Margin | Lobate | Lobate | Lobate |
| | Elevation | Flat | Flat | Flat |
| Biochemical characterization | Voges-Proskauer (VP) | - | - | - |
| | Indole | - | - | - |
| | Catalase | - | - | - |
| | Starch Hydrolysis | - | - | - |

Note: (-) represents negative results

PCR products were visualized on a 0.8% agarose gel stained with ethidium bromide under UV light to confirm the presence of an approximate 1.4 kbp band. The 16S rRNA PCR products were sent to First BASE Laboratories Pte. Ltd., Malaysia, for DNA sequencing.

*Measurement of decolorization activity*

Bacteria isolates were inoculated in GYP broth media supplemented with 50 ppm methylene blue and statically incubated at 37°C for 96 hours under aerobic conditions. Dye degradation was indicated by the reduction of the intensity of the blue-colored media at the end of the incubation period.

The sample was centrifuged at 9,600 x g for 10 minutes to yield pellets of bacterial cells. The decolorization activity was measured in terms of decolorization percentage of supernatant by measuring color intensity reduction using UV/Vis spectrophotometer (BioDrop, UK) with a wavelength range of 400 – 800 nm. The decrease in the absorbance at wavelength of 665 nm indicated methylene blue decolorization. Percentage of decolorization was measured using the equation as follow:

Percentage of decolorization (%) = (A-B)/A×100%

Where,
A : initial absorbance
B : sample absorbance after treatment

**Data analysis**

The 16S rRNA partial sequences of selected isolates were processed using Sequence Scanner 2 (Applied Biosystems, USA) and BioEdit software, and then compared to previously deposited 16S rRNA gene sequences from NCBI GenBank database to identify the bacteria isolate using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The nucleotide sequences of the 16S rRNA gene of the PMB-1 and PMB-2 isolates have been deposited in Genbank under the accession number MH890458 and MH890459, respectively. The phylogenetic tree was constructed using the maximum likelihood, and genetic distances were generated using the Tamura-Nei model with a 1000 bootstrap value, after alignment through MUSCLE, as implemented in MEGA.X software (Pavlopoulos et al. 2010). Statistical analysis of the decolorization activity was done using one-way ANOVA with posthoc Tukey HSD test in GraphPad Prism software to determine the statistical differences between treated samples and control.

## RESULTS AND DISCUSSION

**Isolation and identification of methylene blue decolorizing bacteria**

Three isolates, PMB-1, PMB-2, and PMB-3, collected from sewage water in Kulon Progo-Yogyakarta, showed the ability to decolorize methylene blue in agar media. Cell and the morphological colony, as well as biochemical characteristics of PMB-1 and PMB-3, were indistinguishable when grown on methylene blue supplemented media, indicating that both isolates were most likely to be identical. PMB-2 isolate has a yellow colony which demonstrated different colony color from PMB-1 and PMB-3 (Table 1). The ability of PMB-1 and PMB-3 to accumulate methylene blue inside the cell might contribute to the blue color appearance of their colonies. Based on the characterization of isolates that refer to Bergey's manual showed that PMB-1, PMB-2, and PMB-3 isolates belong to the genus of *Pseudomonas*. (Guerrero 2001).

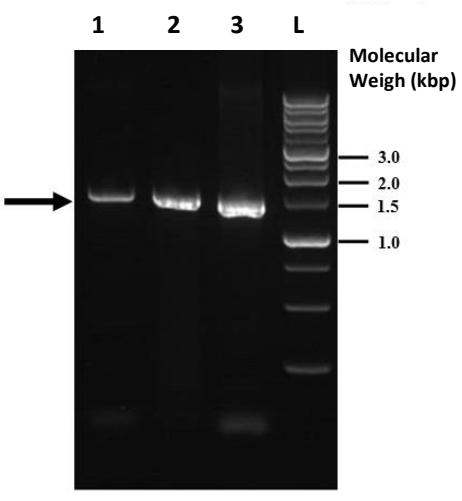

**Figure 2.** The 16S rRNA genes amplification of PMB-1, PMB-2, and PMB-3. Agarose gel electrophoresis was run using 0.8% agarose gel for 20 minutes at 100V. Line 1-3: PMB-1, PMB-2, PMB-3; L: 1 kb DNA ladder (Geneaid, Taiwan). The arrow indicates PCR products of the three isolates.

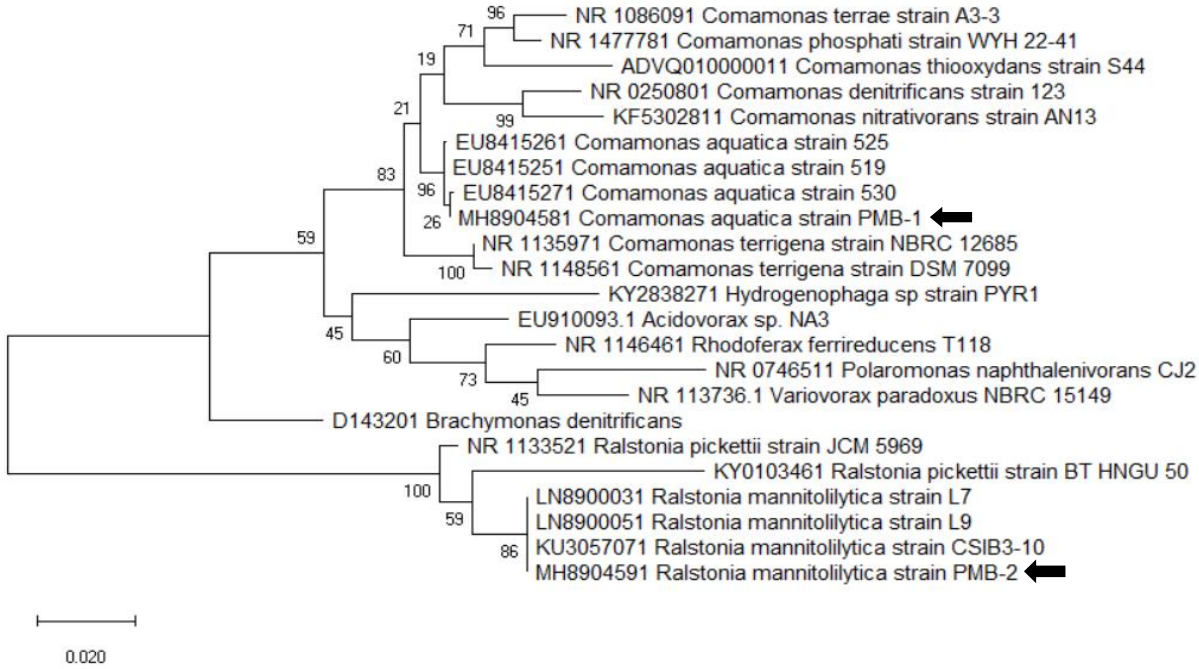


**Figure 3.** Phylogenetic tree of *Comamonas* sp. and *Ralstonia* sp. constructed by MEGA.X software through maximum likelihood tree derived from a distance matrix of 16S rDNA sequences. Arrows indicate *C. aquatica* PMB-1 and *R. mannitolilytica* PMB-2

Molecular identification was conducted to verify the identity of three bacterial isolates that capable of decolorizing methylene blue. The 16S rRNA genes of the three bacterial isolates were successfully amplified through PCR, as indicated by the presence of a single DNA band of approximately 1.4 kbp on agarose gel electrophoresis (Figure 2).

Basic Local Alignment Search Tool (BLAST) showed that PMB-1 and PMB-3 isolates were 100% identical with *Comamonas aquatica*; however, only PMB-1 with 1,378 bp nucleotide length was selected for further analysis in this study. The genus *Comamonas* was previously classified within the *Pseudomonas* genus of γ-proteobacteria and belonged to *Comamonadaceae* lineage in the group of β-proteobacteria. The genus *Comamonas* has been extensively studied for various bioremediations application and was reported to be a natural pollutant degrading microbe. Both of *C. nitrativorans* and *C. denitrificans* isolated from denitrification of activated sludge were reported to convert nitrate to nitrogen (Xing et al. 2010; Maintinguer et al. 2013), while *C.tertosteroni* can reduce heavy metals selenite (Zheng et al. 2014). *C. terrae* strain A3-3 isolated from tannery wastes and agricultural soils have shown to potentially oxidize arsenite to arsenate (Chitpirom et al. 2009). *C. jiangduensis* SZZ 10 has the potential to degrade the diuron herbicide (Hanapiah et al. 2018). Despite the genus *Comamonas* involvement in several bioremediations, there is currently no study on the ability of *C. aquatica* to decolorize dyes.

BLAST sequence analysis for the 16S rRNA gene showed that PMB-2 with 1,398 bp nucleotide length was 100% identical to *Ralstonia mannitolilytica*. Isolate PMB-2 was thought to be the genus *Pseudomonas* based on the biochemical characteristics (Ryan et al. 2011). *Ralstonia mannitolilytica* was previously identified as *Pseudomonas thomasii*, which is commonly found in soil and water and mostly present in industrial and polluted biotopes, and metallurgic wastes (Lucarelli et al. 2017). *R. mannitolilytica* was previously reported as cadmium bioremediating bacteria (Ojuederie and Babalola 2017).

The phylogenetic tree of β-proteobacteria species was constructed by MEGA.X based on 16S rRNA gene sequences (Figure 3) showed that PMB-1 belongs to the clade of *C. aquatica*, whereas isolate PMB-2 belongs to the clade of *R. mannitolilytica*. Other definitive genera in the β-proteobacteria include *Acidovorax*, *Brachymonas*, *Hydrogenophaga*, *Polaromonas*, *Rhodoferax*, *Variovorax*, and *Xylophilus* (Willems 2014). The group of β-proteobacteria was described as an efficient bioremediating agent. *Acidovorax* sp. is capable effectively in the bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (Singleton et al. 2009). *Brachymonas denitrificans* has activity as nitrogen-removal bacteria (Zhao et al. 2017). *Hydrogenophaga* sp. isolated from polycyclic aromatic hydrocarbon-contaminated soil can degrade pyrene under iron-reducing anaerobic conditions (Yan et al. 2017). *Rhodoferax ferrireducens* isolated from subsurface sediments showed the activity as

microbial fuel cells for electricity generation (Risso et al. 2009). Similar to *Acidovorax* sp., *Variovorax* sp. isolated from a coal tar-contaminated aquifer possess putative aromatic ring hydroxylation dioxygenase enzymes that enable bacteria to degrade benzene (Posman et al. 2017). However, there is no information regarding the ability of *Xylophilus* to facilitate bioremediation.

**Decolorization activity of isolates PMB-1 and PMB-2**

Decolorization activity was done by measuring the absorbance of culture supernatants at the maximum wavelength of methylene blue absorption, which is 665 nm (Briggs et al. 2018) by spectrophotometer. The absorbance of the culture supernatant of *C. aquatica* PMB-1 and *R. mannitolilytica* PMB-2 compared to GYP broth as a control showed a similar reduction of the peak at a wavelength of 665 nm with decolorization percentage ± SEM (standard error of the mean) of 67.9 ± 9.94 % and 60.3 ± 12.86%, respectively (Figure 4). The results showed a significant reduction ($P \leq 0.05$) in the absorbance of methylene blue in the treated samples; it showed the ability of both isolated bacteria to reduce the concentration of methylene blue in the sample. Statistical analysis showed that there was no significant difference between the absorbance value of PMB-1 and PMB-2 treated samples at 665 nm ($P > 0.05$).

The exact mechanism of methylene blue decolorization by *C. aquatica* PMB-1 and *R. mannitolilytica* PMB-2 still needs to be studied. However, previous studies on methylene blue-decolorizing bacteria showed that methylene blue removal from aqueous solution mainly achieved by two primary mechanisms, i.e.,: biosorption and biodegradation (Noraini et al. 2012; Eslami et al. 2017; Habibi and Mehrabi 2017; Kilany 2017). Biosorption of cationic dyes which was observed not only on bacterial surface but also on other biomass including fungi (Maas et al. 2018), algae (Guarin et al. 2018) and water plant (Pinontoan et al. 2019), occurs through interaction between negatively-charged phosphate groups on the surface of the biomass and positively-charged dyes. Electrostatic attraction keeps the dyes bound to the biomass, which indicated by a noticeable change of the biomass color (Habibi and Mehrabi 2017; Kilany 2017). Biodegradation occurs because of the presence of enzymes produced by the living cell, which can break down directly the toxic dyes. Many bacteria employ both mechanisms in the decolorization process, in which cationic dyes are first adsorbed to their surface and enzymatically degraded in subsequent reactions (Chen et al. 2015).

The previous study showed that other Proteobacteria, namely *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* isolated from contaminated soil, were able to decolorize methylene blue effectively through biosorption and biodegradation process (Eslami et al. 2017; Kilany 2017). *Sphingomonas paucimobilis,* isolated from a closed drainage system in Malaysia, can break down methylene blue enzymatically under aerobic conditions (Noraini et al. 2012). Recently, different species of *Ralstonia*, i.e., *R. eutropha*, can adsorb and degrade methylene blue in aqueous solution under aerobic conditions, with biodegradation as its predominant mechanism (Habibi and Mehrabi 2017).

In this study, the culture of *C. aquatica* PMB-1 grown in methylene blue-supplemented medium had blue-colored colonies, suggesting that this bacterium might be able to absorb the dye onto the surface of its cell. However, further experiments are needed to determine the extent of methylene blue biosorption and biodegradation capability of *C. aquatica* PMB-1 and *R. mannitolilytica* PMB-2.

So far, there is no previous report on *C. aqutica* and *R. mannitolilytica* as decolorizer of cationic dyes such as methylene blue. The findings in this study provide additional information on the capability of the *Ralstonia* and *Comamonas* genera to break down cationic dyes. However, toxicity assay and further evaluation of the decolorization potential need to be carried out to optimize the application of both isolates as bioremediation agents.

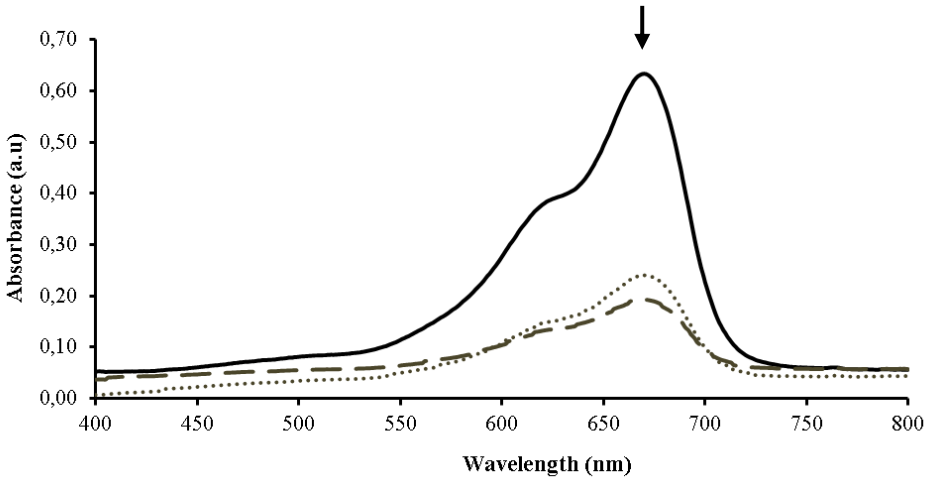


**Figure 4.** The decolorizing activity of *C. aquatica* PMB-1 and *R. mannitolilytica* PMB-2. The isolates were incubated in 50 ppm methylene blue supplemented media incubated at 37°C for 96 hours. The profiles were measured using a UV-Vis spectrophotometer.

Values are expressed as the mean (n=3). Solid line (____): GYP + 50 ppm methylene blue as control; broken line ( - - - - ): GYP + 50 ppm methylene blue + *C. aquatica* PMB-1; Dotted line (…..): GYP + 50 ppm methylene blue + *R. mannitolilytica* PMB-2. The arrow indicates the adsorption peak of methylene blue at 665 nm.

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