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12 TEXTILE DYES DECOLORIZATION BY COPPER-RESISTANT-BACTERIA KLEBSIELLA GRIMONTII, SHIGELLA FLEXNERI, ENTEROBACTER CLOACAE ISOLATED FROM CISADANE RIVER TANGERANG

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Abstract

Dyes and copper are dangerous contaminants because they are toxic. Bioremediation using indigenous bacteria is the best solution to overcome water pollution. Copper resistant bacteria usually have resistance to dyes thereby helping the bioremediation of dye and copper wastes. This study aims to examine the ability of indigenous bacteria isolated from the Cisadane River, namely Klebsiella grimontii IrCis3, Shigella flexneri IrCis5, Enterobacter cloacae IrCis6, and Enterobacter cloacae IrCis9 in terms of resistance and ability to decolorize 12 textile dyes namely methylene blue, malachite green, congo red, mordant orange, reactive black, direct yellow, basic fuchsin, reactive orange, dispersed orange, remasol red, wantex yellow and wantex red. The results showed that Shigella flexneri IrCis5, Enterobacter cloacae IrCis6, and Enterobacter cloacae IrCis9 were resistant to all dye concentrations of 200 and 500 ppm except Klebsiella grimontii IrCis3 did not grow on malachite green and basic fuchsin at concentrations of 200 ppm and methylene blue, malachite green and basic fuchsin concentration of 500 ppm. Only Shigella flexneri IrCis5 has the ability to decolorize 200 ppm basic fuchsin up to 87.23% after 3 days of incubation. The addition of 3 mM CuSO4 reduced the ability to decolorize Shigella flexneri IrCis5 to 0.57%.

Keywords: Copper-resistant-bacteria; Bioremediation; Decolorization; Dyes

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INTRODUCTION

Environmental pollution has long been one of Indonesia's major problems. Many cases of environmental pollution, especially water pollution, have greatly disadvantaged humans and other organisms. Water pollution usually occurs in rivers, due to the high frequency of human activities that involve rivers. According to Zuraya (2019), around 82% of Indonesia's 550 rivers are heavily polluted. A major contributor to the water pollution is the wastewater or discharge from textile industry. For example, 39 industrial plants that line the Citarum River are textile plants. In another example, textile plants have caused rivers and creeks in the city of Solo, West Java to have an overall water quality that is lower than the minimum water quality required. Other rivers, like the Bengawan Solo River, Brojo River, Jenes River, and the Bhayangkara River have also experienced declining water qualities (Zunariyah, 2018).

Some types of dyes used in the textile industry are azo dyes, direct dyes, reactive dyes, triphenylmethane, mordant dyes, indanthrene, disperse dyes, acid dyes, base dyes, naphthol dyes, metallic dyes, and sulfur dyes. More than 10% of all dyes used in Indonesia are disposed of incorrectly in sewage. Several dyes are also found to contain metals, particularly copper (Komarawidjaja, 2017). Aside from metals, improperly disposed dye can also harm the environment due to its organic compound, metal cation, and microorganism content. Hence, these untreated dyes in the water body are the non-biodegradable organic compounds that can cause high levels of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Wang, 2016; Bhatia, 2017).

Additionally, there are some notable disadvantages with using conventional wastewater treatment, namely the economic cost and difficulty involved when using standard physical and chemical treatment processes. These physical and chemical processes, such as adsorption, flocculation, and coagulation, tend to be rather difficult to carry out and require high capital costs. There is also the possibility that large amounts of toxic sludge are produced (Bayoumi *et al.,* 2014). As an alternative, bioremediation can be used.

Bioremediation is a method that is frequently used to solve problems regarding environmental pollution, due to the economic cost of using bacteria. Several species of bacteria can accumulate xenobiotics or convert them

into more environmentally friendly substances (Igiri *et al.* 2018; Xochitl and Rabaey, 2018). The types of bacteria that have potential to be used as bioremediation agents are usually the strains of bacteria that are found at the site of pollution, otherwise known as indigenous bacteria (Fidiastuti, 2017; Irawati, 2020; Putri *et al.,* 2021). According to Velan *et al*. (2012) and Lade *et al*. (2015), these bacteria are able to utilize organic nutrition from pollutants to thrive in toxic conditions. This process of breaking down pollutant dye compounds using a biological agent's ability to utilize the pollutants as nutrition, is called decolorization.

Bacteria with resistances to copper and multiple dyes are required to effectively reduce water pollution due to dyes and copper. Previous studies show that several Indonesian indigenous bacteria are resistant to both copper and dyes. The indigenous bacteria *Acinetobacter sp*. strain CN5 isolated from the Cikapundung River in West Java (Irawati *et al.,* 2021[a]), and *Bukholderia* sp. IrV are copper-resistant bacteria and able to decolorize the dyes methylene blue, congo red and basic fuchsin (Irawati *et al.,* 2021[b]). Four bacteria isolates isolated from the Cisadane River, *Klebsiella grimontii* stran IrCis3, *Shigella flexneri* strain IrCis5, *Enterobacter cloacae* strain IrCis6, and *Enterobacter cloacae* strain IrCis9, are also copper-resistant. The four isolates are able to accumulate copper and possess a very high resistance to copper, with a minimum inhibitory concentration (MIC) score of 7-9 mM $CuSO_4$. This aim of this study is to test the copper resistance and decolorizing potential of *Klebsiella grimontii* strain IrCis3, *Shigella flexneri* strain IrCis5, *Enterobacter cloacae* strain IrCis6, and *Enterobacter cloacae* strain IrCis9 towards twelve different dyes. The dyes used are methylene blue, malachite green, congo red, mordant orange, reactive black, direct yellow, basic fuchsin, reactive orange, disperse orange, remasol red, wantex yellow, and wantex red. This study also aims to test the effect of copper towards the copper resistance and decolorizing ability of the bacterial isolates.

RESEARCH METHODS

Growth Medium

The growth medium was prepared by dissolving 20 g of Luria Bertani (LB) broth (Miller) in 1000 ml of distilled water. The solid medium was made by adding 20 g of bacteriological agar to 1L of liquid LB medium. Liquid and solid mediums were sterilized using an

autoclave, with a temperature of 121°C, 15 atm, for 15 minutes. The twelve dyes used were methylene blue, malachite green, congo red, mordant orange, reactive black, direct yellow, basic fuchsin, reactive orange, disperse orange, remasol red, wantex yellow and wantex red. Each dye solution was sourced from a 10.000 ppm stock solution. The type of copper used was copper sulfate ($CuSO_4$), sourced from a stock solution with a concentration of 1 M. The dye and copper stock solutions were sterilized using membrane filters. The concentration of dyes and copper to be used was determined, and then the dyes and copper were added aseptically to the sterilized medium.

Bacterial Growth Test on Different Dyes

A loopful of each strain i.e. *Klebsiella grimontii* strain IrCis3, *Shigella flexneri* strain IrCis5, *Enterobacter cloacae* strain IrCis6, and *Enterobacter cloacae* strain IrCis9 were inoculated to sterile solid medium containing 200 ppm or 500 ppm dye. Inoculation was done using the streak plate method and then incubated at 37℃ for 24 hours. The same conditions were applied to solid medium containing 3 mM $CuSO_4$ to determine the effect of $CuSO_4$ towards bacterial growth. After incubation, the colonies' growth, color changes, and clear zone formations were observed. This method is done to assess the growth of the bacteria in medium containing dye, as well as measure the effects of copper on the bacterial activity.

Decolorization Assay in Various Dyes and $CuSO_4$

A starter culture was produced by transferring one inoculation loop of *Klebsiella grimontii* strain IrCis3, *Shigella flexneri* strain IrCis5, *Enterobacter cloacae* strain IrCis6, and *Enterobacter cloacae* strain IrCis9 each from test tubes of slanted growth agars to 50 ml of liquid LB medium.

The inoculated liquid mediums were then incubated in an incubator shaker with a speed of 150 rpm and a temperature of 37℃. Starter culture growth was measured using a spectrophotometer, measured at wavelength 600 nm. The starter culture was considered fit to be used once the optical density (OD) of the culture reaches 0.6. About 1% of each starter culture was inoculated to 10 ml of liquid LB medium, each containing twelve different dyes with concentrations of 200 ppm or 500 ppm. The same procedures and conditions were used to inoculate the culture to 10 ml

liquid LB containing a dye and 3 mM $CuSO_4$. Bacterial isolates were incubated for 24 hours in an incubator shaker with a speed of 150 rpm and a temperature of 37^oC (Irawati *et al.,* 2022).

After 24 hours and 48 hours, as much as 1000 uL of each liquid culture were then transferred to sterile microtubes and centrifuged for one minute at 15,000 rpm. The supernatant formed was analyzed using a spectrophotometer, measured at wavelength 300-900 nm, to determine its absorbance value. The medium containing no dye was used as negative control. The percentage decolorization value was measured using the formula shown below.

$$\% \text{ decolorization} = \frac{AbsC - AbsS}{Absc} \times 100\%$$

AbsC = Absorbance of Control
AbsS = Absorbance of Sample

RESULTS AND DISCUSSION

Bacterial Growth on Various Dye Media

Table 1 showed bacterial growth in 12 different dyes media in 500 ppm and Table 2 showed growth in 200 ppm. *Shigella flexneri* strain IrCis5, *Enterobacter cloacae* strain IrCis6, and *Enterobacter cloacae* strain IrCis9 showed growth in all twelve dyes media, as evidenced by the murky suspension in the medium. On the other hand, *Klebsiella grimontii* strain IrCis3 did not grow in the media containing malachite green and basic fuchsin. There was no murky suspension found in the medium. The growth of *Shigella flexneri* strain IrCis5 in 200 ppm twelve dyes media could be seen in Figure 2.

According to Schothorst & Renaud (1985), basic fuchsin inhibited bacterial growth. Basic fuchsin contains pararosaniline, rosaniline, and magenta II hence it acts as bacteriostatic (Kong *et al.,* 2014; Zeyada *et al.,* 2015). Basic fuchsin belongs to the triphenylmethane dye group which are synthetic dyes that contain aromatic xenobiotic compounds and are often used in the textile industry, the cosmetic industry and the paper-printing industry. Triphenylmethane dyes are very dangerous due to their ability to induce mutagenic and carcinogenic effects, especially in humans (Nidadavolu *et al*, 2013). Aside from basic fuchsin, malachite green is also classified as a triphenylmethane dye and has toxic properties, and hence is harmful to both living things and the environment (Hashimoto *et al.,* 2011; Wang *et al.,* 2012).

Table 1. Bacterial growth in 200 ppm dye media

Bacterial Strain	Growth in 200 ppm dye media											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Klebsiella grimontii strain IrCis3	+	-	+	+	+	+	-	+	+	+	+	+
Shigella flexneri strain IrCis5	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae strain IrCis6	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae strain IrCis9	+	+	+	+	+	+	+	+	+	+	+	+

Descriptions: 1. *Methylene blue*, 2. *Malachite green*, 3. *Congo red*, 4. *Mordant orange*, 5. *Reactive black*, 6. *Direct yellow*, 7. *Basic fuchsin*, 8. *Reactive orange*, 9. *Disperse orange*, 10. Remazol red, 11. Yellow wantex, 12. Red wantex. (+) bacterial growth visible, (-) no bacterial growth visible.

Table 2. Bacterial growth in 500 ppm dye media

Bacterial Strain	Growth in 500 ppm dye media											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Klebsiella grimontii strain IrCis3	-	-	+	+	+	+	-	+	+	+	+	+
Shigella flexneri strain IrCis5	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae strain IrCis6	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter cloacae strain IrCis9	+	+	+	+	+	+	+	+	+	+	+	+

Descriptions: 1. *Methylene blue*, 2. *Malachite green*, 3. *Congo red*, 4. *Mordant orange*, 5. *Reactive black*, 6. *Direct yellow*, 7. *Basic fuchsin*, 8. *Reactive orange*, 9. *Disperse orange*, 10. Remazol red, 11. Yellow wantex, 12. Red wantex. (+) bacterial growth visible, (-) no bacterial growth visible.

CIS Isolates Decolorization Test

Table 3 and Table 4 showed that only *Shigella flexneri* strain IrCis5 had the ability to decolorize 200 ppm basic fuchsin, but none of the isolates were able to decolorize the dyes at 500 ppm. The growth of *Shigella flexneri* strain IrCis5 at 200 ppm concentration can be seen in Figure 1.

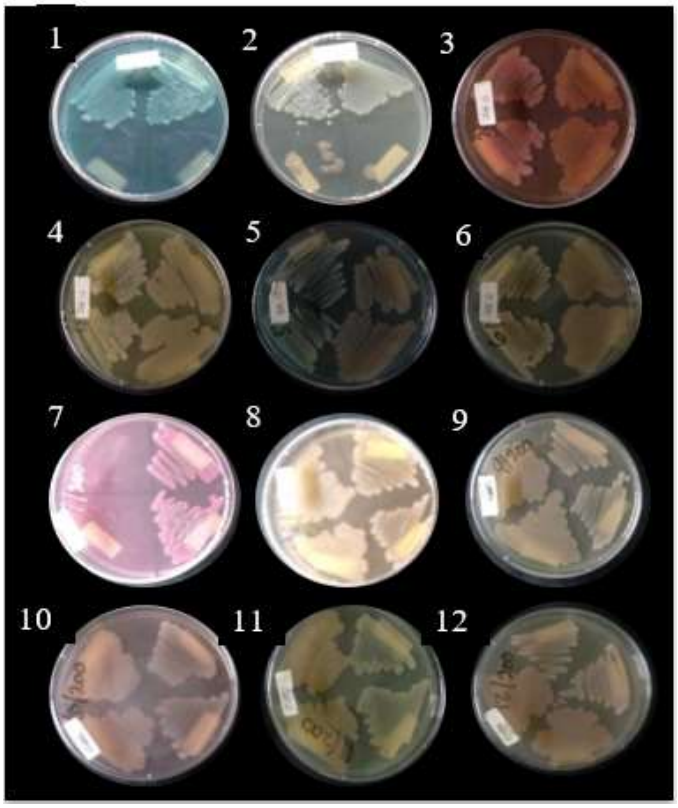

Figure 1. *Shigella flexneri* strain IrCis5 growth in 200 ppm dye media
Descriptions: 1. Methylene blue, 2. Malachite green, 3. Congo red, 4. Mordant orange, 5. Reactive black, 6. Direct yellow, 7. Basic fuchsin, 8. Reactive orange, 9. Disperse orange, 10. Remazol red, 11. Yellow wantex, 12. Red wantex.

Table 3. Decolorization test result of four bacteria isolates on 200 ppm dye concentration

Bacterial Strain	**Growth in 200 ppm dye media**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Klebsiella grimontii strain IrCis3	-	-	-	-	-	-	-	-	-	-	-	-
Shigella flexneri strain IrCis5	-	-	-	-	-	-	+	-	-	-	-	-
Enterobacter cloacae strain IrCis6	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter cloacae strain IrCis9	-	-	-	-	-	-	-	-	-	-	-	-

Descriptions: 1. *Methylene blue*, 2. *Malachite green*, 3. *Congo red*, 4. *Mordant orange*, 5. *Reactive black*, 6. *Direct yellow*, 7. *Basic fuchsin*, 8. *Reactive orange*, 9. *Disperse orange*, 10. Remazol red, 11. Yellow wantex, 12. Red wantex. (+) decolorization occurs, (-) no decolorization occurs.

Table 4. Decolorization test result of four bacteria isolates on 500 ppm dye concentration

Bacterial Strain	Growth in 500 ppm dye media											
	1	2	3	4	5	6	7	8	9	10	11	12
Klebsiella grimontii strain IrCis3	-	-	-	-	-	-	-	-	-	-	-	-
Shigella flexneri strain IrCis5	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter cloacae strain IrCis6	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter cloacae strain IrCis9	-	-	-	-	-	-	-	-	-	-	-	-

Descriptions: 1. *Methylene blue*, 2. *Malachite green*, 3. *Congo red*, 4. *Mordant orange*, 5. *Reactive black*, 6. *Direct yellow*, 7. *Basic fuchsin*, 8. *Reactive orange*, 9. *Disperse orange*, 10. Remazol red, 11. Yellow wantex, 12. Red wantex. (+) decolorization occurs, (-) no decolorization occurs.

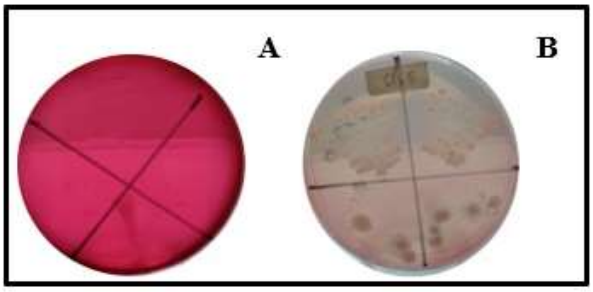

Figure 2. *Shigella flexneri* strain IrCis5 Decolorization results in 200 ppm basic fuchsin media. A. 200 ppm Basic fuchsin medium as control; B. 200 ppm Basic fuchsin medium inoculated with *Shigella flexneri* strain IrCis5.

Figure 2 showed that *Shigella flexneri* strain IrCis5 decolorized basic fuchsin dye medium from purplish red to light pink in three days. Hala *et al.,* (2012) stated that bacteria form proteins on cell membranes, thus toxic dye compounds do not accumulate in the bacterial cells. Triphenylmethane dyes are stable and difficult to degrade. It is suspected that the active metabolic activity of cells can decolorize the dye (Ogugbue *et al.,* 2011).

In addition, it is known that an enzyme called Triphenylmethane Reductase (TMR) can be associated with degradation of dyes from the triphenylmethane group. TMR is a soluble cytosolic NADH/NADPH- dependent and heme-containing oxygenase that catalyzes the reduction of triphenylmethane dyes as a homodimer in solution (Kim *et al.,* 2008). The TMR gene responsible for the expression of TMR enzyme was first found in *Aeromonas hydrophila* strain DN322, another bacterial strain that can degrade dyes (Ren *et al.,* 2006).

***Shigella flexneri* strain IrCis5 Decolorization Percentage and $CuSO_4$ Treatment**

Figure 3 shows the *Shigella flexneri* strain IrCis5 decolorization percentage.

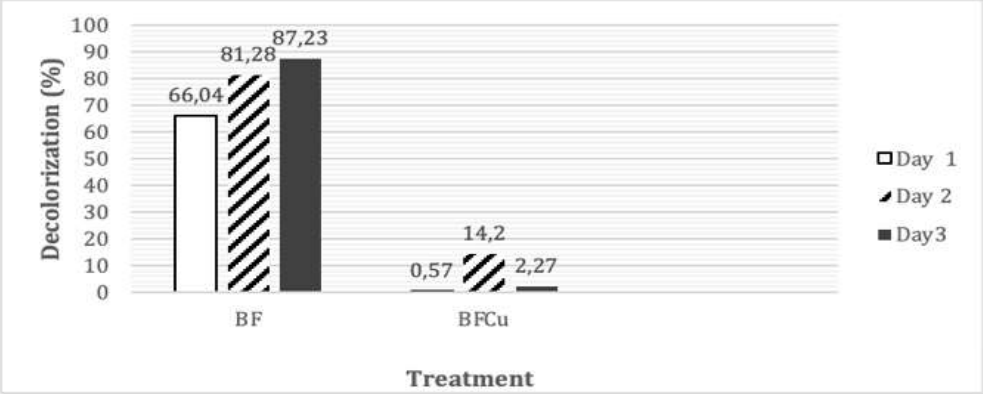

Figure 3. The ability of *Shigella flexneri* strain IrCis5 to decolorize basic fuchsin

The decolorization percentage of the bacterium was 66.04%, 81.28%, and 87.23% in the first, second, and third days of incubation period, respectively.

The similar study was done by Rani *et al.,* (2014), who used two types of fungi, namely *Aspergillus niger* and *Phanerochaete chrysosporium* that isolated from dye effluent soil. The fungi were incubated for 10 days and the decolorization percentage obtained on the last day for *A. niger* was 81.85% and for *P. chrysosporium* was 89.8%. In another study by Shnada *et al*. (2015), they used *Saccharomyces cerevisiae* isolated from salt-water and palm wine that can degrade basic fuchsin. The concentration of basic fuchsin that was used in this study was 20 mg. The decolorization percentage that carried out by salt-water *S. cerevisiae* which decolorized basic fuchsin treated at 250 ml, 500 ml, and 750 ml of mineral salt media for 12 days, was 60.39%, 41.29% and 24.47% respectively. Meanwhile, palm wine *S. cerevisiae* with the same concentration for 12 days respectively was 72.61%, 48.88% and 33.92%. Research that used two types of blue green algae, namely *Hydrocoleum oligotrichum* and *Oscillatoria limnetica*, was conducted by Wagih (2016) with 7 days incubation time. The result of decolorization showed that basic fuchsin was able to be decolorized by *H. oligotrichum* and *O. limnetica* with the decolorization percentage at 92.44% and 90.23% respectively. From the research that has been done, most of the studies used fungi as biological agents, indicating that the *Shigella flexneri* strain IrCis5 has

a higher ability to decolorize basic fuchsin more than microbial previous study.

The addition of $CuSO_4$ with 3 mM concentration resulted in reduction of decolorization ability of *Shigella flexneri* strain IrCis5. The decolorization percentage for the first day was 0.57, the highest percentage achieved on the second day was 14.2%. The percentage went nosedive to 2.27% on the third day. Copper has toxic properties against bacteria and is classified as antibacterial. When bacteria are exposed to an environment that contains copper, it increases stress on bacteria because bacteria will survive to live. In this research, *Shigella flexneri* strain IrCis5 was treated with the addition of dye and copper which caused stress. When bacteria are exposed to copper, bacterial cellular respiration will be affected, and degradation of bacterial DNA occurs (Warnes *et al.,* 2011). The decrease in decolorization ability is also thought to be caused by excessive stress faced by *Shigella flexneri* strain IrCis5 to survive in the toxic conditions of dyes and copper.

CONCLUSION

Shigella flexneri strain IrCis5, *Enterobacter cloacae* strain IrCis6, and *Enterobacter cloacae* strain IrCis9 were resistant to 200 ppm and 500 ppm methylene blue, malachite green, congo red, mordant orange, reactive black, direct yellow, basic fuchsin, reactive orange, disperse orange, remasol red, wantex yellow and wantex red except *Klebsiella grimontii* strain IrCis3 did not grow at 200 ppm malachite green and basic fuchsin and 500 ppm methylene blue, malachite green, and basic fuchsin. Only *Shigella flexneri* strain IrCis5 could decolorize 200 ppm basic fuchsin up to 87.23% after 3 days of incubation. The addition of 3 mM $CuSO_4$ decreased the decolorization ability of *Shigella flexneri* strain IrCis5 to 0.57%. *Shigella flexneri* strain IrCis5 is capable of being the most potential bacterial agent for basic fuchsin decolorization. Molecular analysis for the future experiment is required to obtain improved understanding about the resistance mechanism of *Shigella flexneri* strain IrCis5 to live under a toxic environment of copper and basic fuchsin.

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