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The potential of copper-resistant bacteria *Acinetobacter* sp. strain CN5 in decolorizing dyes

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Abstract. *Irawati W, Pinontoan R, Mouretta B, Yuwono T. 2022. The potential of copper-resistant bacteria* Acinetobacter *sp. strain CN5 in decolorizing dyes. Biodiversitas 23: 680-686.* Bacteria with multi-resistance to copper and dyes may be employed in bioremediation of copper and dye waste more effectively than physical or chemical remediation. This study aims at determining the effect of the addition of various textile dyes and copper on the growth of *Acinetobacter* sp. CN5 and its ability to decolorize dyes. The dye resistance test was carried out by inoculating bacterial isolates into Luria Bertani media containing 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 ppm dyes. The twelve dyes used in the test were methylene blue, malachite green, congo red, mordant orange, reactive black, direct yellow, basic fuchsine, reactive orange, dispersion orange, remazol red, wantex yellow, and wantex red. The decolorization activity was analyzed by spectrophotometry at a 300-900 nm wavelength. The study results demonstrated that bacteria thrived in media containing 50 ppm of all dyes, except malachite green dye. *Acinetobacter* sp. CN5 was found to be resistant to up to 500 ppm methylene blue, basic fuchsine, and wantex red and resistant to congo red at 450 ppm. *Acinetobacter* sp. CN5 was also able to decolorize methylene blue, congo red, basic fuchsine, and red wantex by 57.64%, 53.17%, 91.37%, and 67.50%, respectively. The addition of 5 mM $CuSO_4$ to the medium increased the ability of *Acinetobacter* sp. CN5 to decolorize the congo red from 57.64% to 82.58%.

Keywords: Bioremediation, copper-resistant bacteria, decolorization, dyes

INTRODUCTION

Industrial wastes have long been a critical issue to the ecosystem and living organisms, including human existence due to their toxic nature. Textile wastes, particularly dyes, are among the widely known forms of industrial waste. Based on its functional group, dyes can be categorized into azo dyes, triphenylmethane dyes, and phthalein dyes. Based on its use, dyes can be grouped into acid, base, direct, disperse, reactive, or mordant (Kumar et al. 2011). Throughout the process of textile dyeing, approximately 10% of the dyes are discharged into wastewater (O'Mahony et al. 2002). Dye pigments are either almost or completely unable to undergo natural degradation. As a result, the penetration of sunlight into bodies of water is obstructed, disrupting the process of photosynthesis required by deep-water organisms to sustain their lives (Hassan and Carr 2018). Dyes are extremely dangerous toward living organisms due to their carcinogenic effects (Sharma et al. 2018). Dyes can enter the organisms' bodies through the digestive and respiratory systems. Consequently, organisms suffer from skin and eye irritation, nerve disorders, and enzymatic reaction disruptions since dyes can act as a co-factor in causing enzymatic deactivation (Copaciu et al. 2013; Khan and Malik 2018).

Numerous dyes are synthesized using heavy metals. Heavy metals are detrimental to human health and ecosystem and are considered toxic and hazardous elements. Copper is widely used in dye production as found indirect, reactive, acidic, and press dyes. However, due to its naturally recalcitrant properties, the use of copper in dye industry has imposed a new environmental issue as it accumulated in the environment. In addition, the presence of copper in water bodies also reduces the pH and N_2O levels. The condition of a non-optimal suppression of CO_2 and CH_4 levels due to the accumulation of copper can also affect the surrounding environment of the contaminated water bodies (Ahmed et al. 2018). Marine and terrestrial organisms may also bioaccumulate copper which thus creates copper-contaminated food chain. It has been widely believed that elevated copper levels may affect human health and impose physiological disorders such as anemia, liver and kidney diseases, and stomach and intestinal irritation (Wuana and Okieimen 2011).

Emerging environmental issues such as copper and dye contamination necessitate the discovery and development of new technologies and solutions. Bioremediation, defined as the biological process of degrading excess organic matter found in natural surroundings, has been regarded as an eco-friendly and cost-effective solution in cleaning up contaminated environmental sites (Fareed et al. 2017). Bioremediation employs living organisms as remediation agents to degrade pollutants into less toxic forms using the cells' metabolism. Bacteria are known to adapt to new

and/or toxic environments and are thus potential for bioremediation of contaminated sites. The fast reproductive cycle of the bacteria also speeds up bioremediation (Zouboulis et al. 2019).

The capability of bacteria to decolorize dyes is supported by specific enzymes synthesized during the metabolism process, such as oxidative enzymes such as lignin peroxidase (LiP), veratryl alcohol oxidase, laccase, and tyrosinase, as well as reductive enzymes such as azo reductase, riboflavin reductase, dichlorophenol indophenol (DCIP) reductase, and Green HE4B reductase (Khandare and Govindwar 2016). On the other hand, bacterial resistance to copper is known mediated by the production of copper-binding proteins such as copper resistance protein A (CopA) and copper resistance protein B (CopB) as well as damage-preventing proteins multicopper oxidase (MCO), universal stress protein (Usp), and superoxide dismutase (SOD) that help bacteria to avoid cell damage under copper stress (Irawati et al. 2021).

Numerous studies have reported the resistance of bacteria to textile dyes. For instance, *Comamonas aquatica* and *Ralstonia mannitolilytica* isolated from an industrial wastewater were demonstrated resistant to 50 ppm methylene blue (Michelle et al. 2020), *Aeromonas hydrophila* isolated from a textile printing wastewater treatment was resistant to 50 ppm basic fuchsine (Ren et al. 2006), while *Citrobacter* sp. isolated from a textile dyeing industrial effluent treatment plant was also known resistant to both basic fuchsine and congo red (An et al. 2002). Previous work (He et al. 2010) also found thirteen copper-resistant bacteria from genus *Arthrobacter, Micrococcus, Microbacterium, Bacillus, Sphingomonas, Pseudomonas, Azotobacter, Acinetobacter,* and *Rahnella* isolated in a copper mine wasteland. Irawati et al. (2015) and Williams et al. (2016) have also demonstrated that bacteria from the genus *Acinetobacter*, specifically *Acinetobacter* sp. IrC2 isolated from a waste treatment plant and clinical isolates *Acinetobacter baumannii* were copper-resistant bacteria.

Textile industries generally discharge both dyes and heavy metals into wastewaters, necessitating the use of bioremediation techniques capable of removing both copper and dye. To date only a limited number of studies have been conducted to study the multipotency of bacteria, which demonstrate the ability to remediate both copper and dye. The bacteria used in this study is *Acinetobacter* sp. strain CN5 isolated from Cikapundung River in Indonesia, a heavily contaminated river with heavy metals. Previous studies (Irawati et al. 2019) have demonstrated that *Acinetobacter* sp. strain CN5 is a copper-resistant bacteria. Bacteria of the genus *Acinetobacter* are known for harboring enzymes that help decolorizing dyes, namely lignin peroxidase (LiP), laccase, riboflavin reductase, and DCIP reductase (Khandare and Govindwar 2016). However, the study on dye-resistance of *Acinetobacter* sp. strain CN5 has never been carried out before. In addition, studies on the ability of copper-resistant bacteria to decolorize dyes are still limited, and thus further investigations are of importance in developing the new bioremediation technologies. This study aims at: (i) determining the effects of various textile dyes on the growth and decolorizing ability of *Acinetobacter* sp. strain CN5; (ii) determining the dye-resistance of *Acinetobacter* sp. strain CN5 towards given textile dyes; and (iii) investigating the effects of chosen textile dyes and copper concentration on the growth and decolorizing ability of *Acinetobacter* sp. strain CN5.

MATERIALS AND METHODS

Bacteria and growth medium

Bacteria were grown in Luria Bertani (LB) containing the following (per liter): tryptone 10 g, yeast extract 5 g, NaCl 10 g, and glucose 0.1 g. LB agar was made by adding 2% of pure agar. The medium was autoclaved at 121°C, 1 atm, for 15 minutes before being used as the growth medium. The stock solution of 1 M $CuSO_4$ was added to the autoclaved medium for preparation of medium containing copper. The dye stock of 10.000 ppm was formulated for dyes (i) methylene blue, (ii) malachite green, (iii) congo red, (iv) mordant orange, (v) reactive black, (vi) direct yellow, (vii) basic fuchsine, (viii) reactive orange, (ix) disperse orange, (x) remazol red, (xi) wantex yellow, and (xii) wantex red.

Bacterial growth on dyes and dye-decolorization

LB medium supplemented with dyes at 50 ppm concentration was used for bacterial growth analysis and dye decolorization. Growth analysis was carried out by four-quadrant streak method. LB agar supplemented with 50 ppm of each dye was poured onto petri dishes and inoculated with *Acinetobacter* sp. CN5, followed by incubation at 37°C for 24 hours. Growth analysis was performed in triplicates.

Bacteria were also grown in a liquid LB medium supplemented with 50 ppm of dye and incubated at 37°C for 24 hours. One mL of bacterial culture was drawn and centrifuged at 12.000 rpm for 5 minutes. Dye concentration was quantified by using spectrophotometry at 300-900 nm. The decolorization percentage was calculated using the following formula (Chen et al. 2003).

Bacterial dye-resistance test

Acinetobacter sp. CN5 grew on LB medium supplemented with 50 ppm of dye and 3 mM or 5 mM of $CuSO_4$. A loopful of *Acinetobacter* sp. CN5 was drawn from slant agar culture and streaked onto agar medium containing 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, 450 ppm, and 500 ppm of dyes prior to incubation for 24 hours.

$$\% \text{ of decolorization} = \frac{\text{absorbance of control} - \text{absorbance of treated sample}}{\text{absorbance of control}} \times 100\%$$

Effects of the addition of Cu on decolorization

To test the effects of copper on the ability of *Acinetobacter* sp. CN5 in decolorizing dyes, bacteria were grown in 50 mL of liquid LB supplemented with 50 ppm of dye and 3 mM or 5 mM of $CuSO_4$. The bacterial culture was incubated for 24 hours at 37ºC and shaken at 150 rpm. One mL of bacterial culture was drawn from liquid culture and centrifuged at 12.000 rpm for 5 minutes. The remaining dye concentrations in the medium were measured by spectrophotometry at 300-900 nm.

RESULTS AND DISCUSSION

Bacterial growth and decolorization test on various dyes

Growth analysis of bacteria on 12 samples of dye demonstrated that *Acinetobacter* sp. strain CN5 managed to grow well on 11 dyes, namely methylene blue, congo red, mordant orange, reactive black, direct yellow, basic fuchsine, reactive orange, disperse orange, remazol red, wantex yellow, and wantex red (Figure 1). *Acinetobacter* sp. strain CN5 was found unable to grow on malachite green dye. As reported by Junqueira et al. (2010), malachite green dye exerted photodynamic antimicrobial effects on *Candida, Enterobacteriaceae, and Staphylococcus*, providing the explanation regarding the inability of *Acinetobacter* to grow on the same dye. Therefore, based on growth analysis on several dyes, malachite green was not used for decolorization test of *Acinetobacter* sp. strain CN5.

Acinetobacter sp. strain CN5 colonies are generally pale white in color, but a few dyes, in medium supplemented with methylene blue, congo red, basic fuchsine, dan wantex red resulted in color changes of the colonies and medium (Figure 2). Methylene blue-containing medium (A2) and basic fuchsine medium (B2) turned slightly paler, while congo red supplemented-medium (C2) showed a much faded stain. The color transition of the colonies from transparent to the color of the dyed medium indicated that *Acinetobacter* sp. strain CN5 managed to accumulate dye from the medium. Following dye accumulation, *Acinetobacter* sp. CN5 underwent the process of color reduction and degradation (Darwis and Sukara 1990; Mardigan et al. 2003; Hala et al. 2010).

In addition to colony color changes in *Acinetobacter* sp. strain CN5, methylene blue, congo red, basic fuchsine, and wantex red dyes also altered the media color. These mediums showed a faded color that resembled the control LB medium suggesting that *Acinetobacter* sp. strain CN5 carried out a decolorizing activity (Cheng et al. 2017; Sweta and Tank 2019; Michelle et al. 2020). Spectrophotometric analysis provided quantitative data on the ability of *Acinetobacter* sp. strain CN5 to decolorize the dyes (Table 1).

Table 1. Decolorization activity of *Acinetobacter* sp. strain CN5

Dye name	Control	Treatment	Decolorization (%)
Methylene blue	6.94	2.94	57.64
Congo red	0.63	0.30	53.17
Basic fuchsine	2.84	0.25	91.37
Wantex red	0.40	0.13	67.50

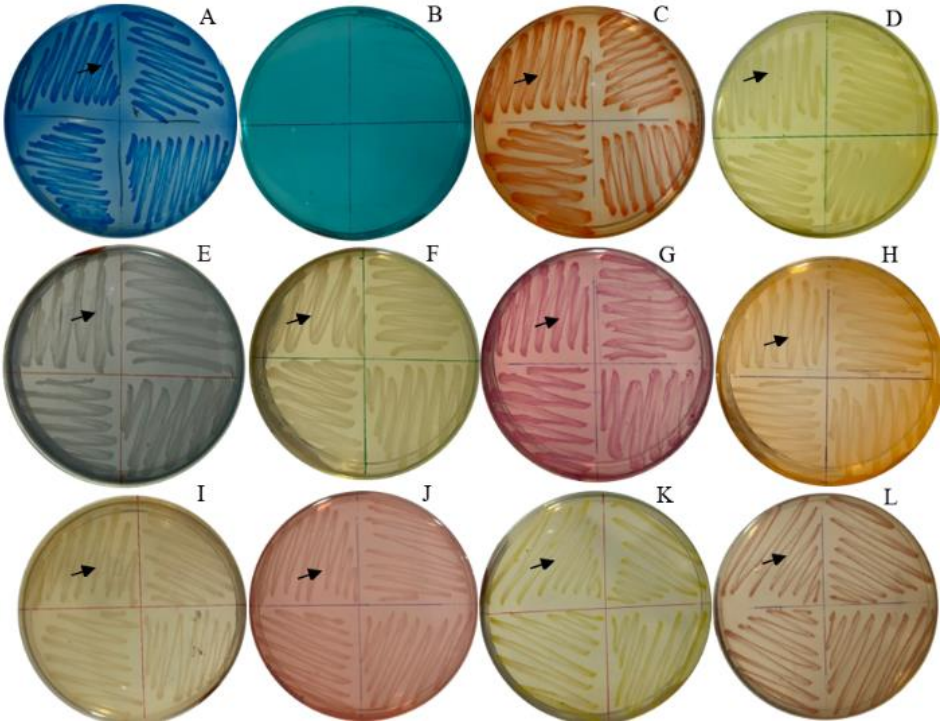

Figure 1. The growth of *Acinetobacter* sp. strain CN5 on various dyes. (A) methylene blue (B) malachite green (C) congo red (D) mordant orange (E) reactive black (F) direct yellow (G) basic fuchsine (H) reactive orange (I) disperse orange (J) remazol red (K) wantex yellow (L) wantex red. Black arrows show the growth of bacterial colonies. Red arrow shows a clear zone

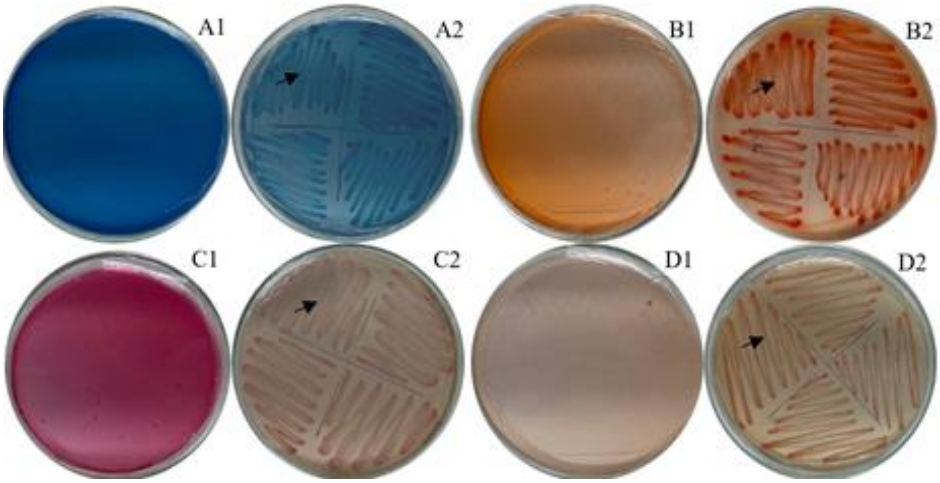

Figure 2. Comparison between control medium containing various dyes and medium treated with *Acinetobacter* sp. strain CN5. (A1) methylene blue control (A2) treated methylene blue (B1) congo red control (B2) treated congo red (C1) basic fuchsine control (C2) treated basic fuchsine (D1) wantex red control (D2) treated wantex red. Arrows show the growth of bacterial colony

This study shows that *Acinetobacter* sp. strain CN5 can decolorize methylene blue up to 57.64% (Table 1). This value is higher than that of *Pseudomonas aeruginosa* (21.31%), and bacterial biofilm consortium's (41%), but lower than *R. mannitolilytica* (60.3%), *C. aquatica* (67.9%), and *Desmodesmus* sp. (98.6%) as reported by Al-Fawwaz and Abdullah (2016); Laurensia (2018); Sekarlangit and Martani (2018); and Michelle et al. (2020). Methylene blue, a heterocyclic dye can be degraded by lignin peroxidase (LiP) and laccase belonging to *Acinetobacter* sp. strain CN5 (Alam et al. 2008; Duran-Rivera et al. 2018). LiP acted as the main enzyme, cutting the aromatic ring structure from the chemical structure of methylene blue, while laccase cut off functional group-$N(CH_3)_2$ from methylene blue (Ferreira-Leitão et al. 2006; Zucca et al. 2015).

The decolorization results of *Acinetobacter* sp. strain CN5 on congo red reached 53.17% (Table 1). Previous studies demonstrated that *Micrococcus luteus* showed 38% decolorizing activity, lower than *Acinetobacter* sp. strain CN5, while *Bacillus thuringiensis*; *Shewanella xiamenensis*; and *Bacillus cereus* had decolorizing activity of 72.84%; 87.5%; and 93.94%, respectively (Olukanni et al. 2013; Abo-State et al. 2017; Ito and Suto 2018). *Acinetobacter* sp. strain CN5 decolorizes congo red more efficiently than *Micrococcus luteus,* but less than *B. thuringiensis*, *S. xiamenensis*, *B. cereus.* Congo red, a diazo dye was decolorized by *Acinetobacter* sp. strain CN5 with the help of enzymes such as laccase, LiP, and azoreductase. The laccase enzyme cuts the aniline functional group off of the *congo red* dye structure (Mota et al. 2015) while LiP and azoreductase degrade *congo red* by cutting off azo dyes (N=N) (Lade et al. 2015; Bosco et al. 2017).

Acinetobacter sp. strain CN5 was also able to decolorize basic fuchsine of up to 91.37% while other bacteria such as *Bacillus firmus*; *Oscillatoria limnetica*; *Hydrocoleum oligotrichum*; and *A. hydrophila* showed the ability to decolorize basic fuchsine by up to 63.7 %; 90.23 %; 92.44 %; and 93 %, respectively (Ogugbue and Sawidis 2011; Ogugbue et al. 2012; Abou-El-Souod and El-Sheekh 2016). Based on these values, the ability of *Acinetobacter* sp. strain CN5 to decolorize basic fuchsine is significantly higher than *B. firmus* and *O. limnetica* but slightly lower than *H. oligotrichum* and *A. hydrophila.* According to Kang et al. (2004), basic fuchsine, a triphenylmethane dye can be degraded by *Acinetobacter* sp. strain CN5 by the activity of enzyme LiP.

Wantex red can be regarded as acid red 88 dye since both dyes' show abosrbance and peak similarities ranged from 493-513 nm which fell into acid red 88's peak at 503 nm. *Acinetobacter* sp. strain CN5 was able to decolorize 67.50% of acid red, while *Bacillus megaterium*; *Stenotrophomonas* sp.; were able to decolorize acid red by up to 91%, and 97.50%, respectively (Kumari et al. 2015; Ewida et al. 2019). Despite the fact that the ability of *Acinetobacter* sp. strain CN5 falls short of both *Bacillus megaterium* and *Stenotrophomonas* sp. yet these results demonstrated the decolorizing activity of *Acinetobacter* sp. strain CN5 can be categorized as good. The capability of *Acinetobacter* sp. strain CN5 in degrading acid red, an azo dye, is attributed to the activity of azo-reductase and riboflavin reductase (Wahab et al. 2006; Ramya et al. 2010).

Compared to other bacteria, which showed higher decolorizing activity, the ability of *Acinetobacter* sp. strain CN5 in decolorizing methylene blue, congo red, basic fuchsine, dan acid red can be categorized as fairly good. However, it is important to note that *Acinetobacter* sp. strain CN5 is capable of decolorizing multiple dyes simultaneously, proving that its use in bioremediation has added benefits and advantages.

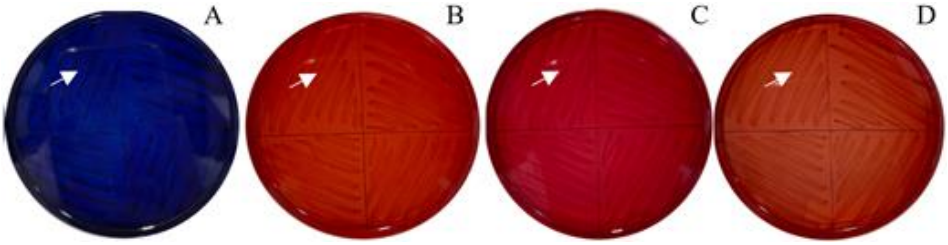

Figure 3. Resistance of bacteria *Acinetobacter* sp. strain CN5 growing on different dyes: (A) methylene blue 500 ppm (B) congo red 450 ppm (C) basic fuchsine 500 ppm (D) wantex red 500 ppm. Arrows show the growth of bacterial colony

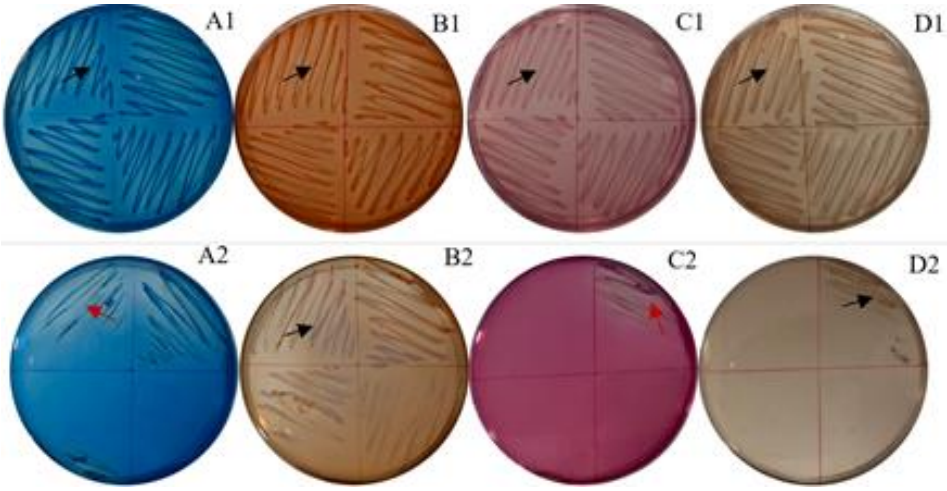

Figure 4. The effects of $CuSO_4$ on the growth of *Acinetobacter* sp. CN5 on various dyes. (A1) methylene blue with 3 mM $CuSO_4$; (A2) methylene blue with 5 mM $CuSO_4$; (B1) congo red with 3 mM $CuSO_4$; (B2) congo red with 5 mM $CuSO_4$; (C1) basic fuchsine with 3 mM $CuSO_4$; (C2) basic fuchsine with 5 mM $CuSO_4$; (D1) wantex red with 3 mM $CuSO_4$; (D2) wantex red with 5 mM $CuSO_4$. Red arrows show clear zone

Dye-resistance test of *Acinetobacter* sp. strain CN5

The growth resistance test was conducted on methylene blue, congo red, basic fuchsine, and wantex red dyes. The results showed that *Acinetobacter* sp. strain CN5 grew on the highest concentration of each tested dye, demonstrating its high resistance. The highest dye concentration tolerated by *Acinetobacter* sp. strain CN5 was 500 ppm of methylene blue, basic fuchsine, and wantex red, and 450 ppm of congo red (Figure 3). This concentration is comparatively higher than that of different bacterial species on the same dyes (Ogugbue and Sawidis 2011; Ogugbue et al. 2012; Holey 2015; Abou-El-Souod and El-Sheekh 2016; Abo-State et al. 2017; Eslami et al. 2017; Asses et al. 2018; Azizah 2018; Fatihah 2018). It was concluded that *Acinetobacter* sp. strain CN5 has advantages over other previously isolated bacteria due to its high resistance (500 ppm) to several dyes. Previous studies reported that bacterial resistance is usually only to one dye with a dye concentration of 100 ppm.

Effects of Cu on the growth and decolorizing ability of *Acinetobacter* sp. strain CN5

Figure 4 depicted the results of the effect of Cu on decolorizing ability of *Acinetobacter* sp. strain CN5 at two different concentrations (3 mM and 5 mM). The results showed that supplementation of 3 mM Cu did not alter the growth of *Acinetobacter* sp. strain CN5 as evidenced by its ability to form colonies, indicating that Cu at 3 mM concentration can be regarded as sub-inhibitory for the growth of *Acinetobacter* sp. strain CN5. However, at a higher concentration, 5 mM, supplementation of Cu resulted in growth inhibition. At 5 mM Cu concentration, *Acinetobacter* sp. strain CN5 demonstrated best growth on congo red, followed by methylene blue. It was suggested that the capability of *Acinetobacter* sp. strain CN5 to withstand Cu at 5 mM concentration was attributed to the activity of laccase, a multicopper oxidase enzyme that utilizes Cu as a co-enzyme in degrading substrates (Shraddha et al. 2011; Gomaa and Momtaz 2015).

Figure 4 showed a clear zone surrounding the colony when the bacteria were grown on medium containing methylene blue and basic fuchsine. The result was in accordance with the results presented in Table 1 which show that *Acinetobacter* sp. was able to decolorize methylene blue and basic fuchsine up to 57.64% and 91.37%, respectively. Copper added on the medium inhibited the colony's growth but did not affect the capability of *Acinetobacter* sp. strain CN5 in decolorizing dyes. The results of the decolorization test showed that the concentration of $CuSO_4$ did not affect

the ability of bacteria to decolorize methylene blue. The decolorization ability of bacteria in the medium with 3 mM and 5 mM $CuSO_4$ was 57.64% and 57.2%, respectively. On the other hand, increasing the concentration of $CuSO_4$ also increased the decolorization ability of bacteria in Congo red from 53.17% to 82.58%. It was suggested that the activity of laccase that involves in congo red degradation increased in the presence of Cu (Mota et al. 2015).

Acinetobacter sp. CN5 strain demonstrated more advantages than other dye-resistant bacteria as it can grow on 11 dyes. Previously isolated bacteria, on the other hand, were only resistant up to one to 3 dyes. *Acinetobacter* sp. CN5 strain was resistant to 50 ppm methylene blue, congo red, mordant orange, reactive black, direct yellow, basic fuchsine, reactive orange, orange dispersion, remazol red, wantex yellow, and wantex red. *Acinetobacter* sp. CN5 can withstand up to 500 ppm methylene blue, basic fuchsine, and wantex red as well as 450 ppm congo red, while previously isolated bacteria tolerated dyes only up to 100 ppm. *Acinetobacter* sp. CN5 was also thrived and decolorized the dye with the supplementation of 3 mM and 5 mM $CuSO_4$. The percentage of methylene blue, congo red, basic fuchsine, and red wantex decolorized by *Acinetobacter* sp. CN5 reached the value of 57.64%, 53.17%, 91.37%, and 67.50%, respectively. Increasing the copper concentration also increases the decolorization ability of bacteria against congo red of up to 82.58%. These results suggest that Cu may play its role as a cofactor for the laccase enzyme that activates the degradation of the dye. Further investigation, particularly on molecular aspects, is thus required to obtain a better understanding of the basis of copper resistance and decolorization and the development of bioremediation technology that can be implemented under field condition.

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