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Enterobacter hormaechei KIMS8 and *Enterobacter cloacae* KIMS10 isolated from Kapuas River, Kalimantan, Indonesia as indigenous multi-resistant bacteria to copper and dyes

WAHYU IRAWATI[1,♥], MICHAEL TIMOTHY[2], SAMUEL EMMANUEL SOENTORO[2], REINHARD PINONTOAN[2], TRIWIBOWO YUWONO[3], VALENTINE LINDARTO[4]

[1]Department of Biology Education, Faculty of Education, Universitas Pelita Harapan. Jl. M.H. Thamrin Boulevard 1100, Lippo Karawaci, Tangerang 15811, Banten, Indonesia. Tel./fax.: +62-21-5460901, ♥email: wahyu.irawati@uph.edu

[2]Department of Biology, Faculty of Science and Technology, Universitas Pelita Harapan. Jl. M.H. Thamrin Boulevard1100, Lippo Karawaci, Tangerang 15811, Banten, Indonesia

[3]Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora No. 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

[4]Department of Natural Sciences, Dian Harapan Lippo Village High School. Jl. Imam Bonjol No. 201, Lippo Kawaraci, Tangerang 15811, Banten, Indonesia

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Abstract. *Irawati W, Timothy M, Soentoro SE, Pinontoan R, Yuwono T, Lindarto V. 2022.* Enterobacter hormaechei *KIMS8 and* Enterobacter cloacae *KIMS10 isolated from Kapuas River, Kalimantan, Indonesia as indigenous multi-resistant bacteria to copper and dyes. Biodiversitas 23: 6661-6668.* Study on the characterization of multiple copper and dyes resistance in bacteria has so far been quite limited. It is, therefore, of interest to isolate and characterize such bacteria. The study aimed to isolate bacteria from the Kapuas River in Indonesia and measuring copper resistance as well as dye-resistance and decolorization abilities. Copper resistance was determined by measuring the minimum inhibitory concentration (MIC) of copper, while dye resistance measured by observing changes in colony color and clear zone formation and decolorization ability was determined by spectrophotometry. It was found that 2 out of the 15 isolated strains showed the highest copper resistance with the MIC of 7 mM. The two bacterial strains KIMS8 and KIMS10, were grown in solid media supplemented with 300 ppm of methylene blue or reactive black dye. Bacterial decolorization assays showed that KIMS8 was able to decolorize up to 90% methylene dye and up to 11.3% of reactive black dye whereas KIMS10 was able to decolorize 94.9% of methylene blue dye and 12.1% of reactive black dye. Molecular characterization by 16S rRNA gene sequencing of these two strains showed that KIMS8 and KIMS10 were identified as *Enterobacter hormaechei* and *E. cloacae*, respectively.

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

INTRODUCTION

Industrial effluent containing various anthropogenic pollutants, such as heavy metals and dyes, pose a threat to ecosystem sustainability. Manufacturing companies frequently discharge untreated wastewater into surrounding bodies of water, disrupting essential biochemical processes, such as photosynthesis and trophic transfer. Copper, one of the most ubiquitous heavy metals, is a trace element essential for normal growth and cellular activities in all living organisms at low concentrations but it is toxic at high levels (Shamim 2018). Copper is one of the most used metallic substances in dye and paints production, petroleum refining, mining and metallurgy, fertilizer production, and electroplating industries (Tytla et al. 2015; Varma and Misra 2016). Due to its bioaccumulative and non-degradable nature, copper contamination may greatly reduce biodiversity and harm human health. Biogeochemical cycling of copper leads to infiltrated food chains followed by bioaccumulation, then biomagnification at higher trophic levels (Miller et al. 2020). Chronic exposure to copper toxicity, which tends to accumulate in the liver and brain (Gaetke et al. 2014), may result in detrimental health effects, such as anemia, acute liver failure, an increased rate of hepatocellular carcinoma, and numerous neurodegenerative disorders (Giampietro et al. 2018; Royer and Sharman 2022).

Synthetic dyes are also one of the main constituents of industrial effluent mostly discharged by the textile, leather, pharmaceutical, cosmetics, and paper printing industries. Synthetic dyes, including methylene blue and reactive black dye, are widely used in many industries as they have a high affinity to bind with cellulosic fibers and thus have a long lasting-use (Karim et al. 2018). However, dye manufacturing processes generate a large volume of wastewater containing 10-15% of unbound dye (Tkaczyk et al. 2020). Discharged dyes cover the surface of water bodies, obstructing sunlight penetration, and hindering photosynthesis processes. Aside from compromising the aesthetic value of contaminated bodies, aquatic plant and animal growth is impaired. Furthermore, azo dyes, such as methylene blue and reactive black, can be converted into aromatic amines which promote toxicity, mutagenicity, and carcinogenicity, threatening human health through dye-infiltrated food chain networks (Abe et al. 2018). Several dye-related health effects include skin irritation, dermatitis,

and bladder cancer (Lellis et al. 2019; Khan and Malik 2018).

Conventional treatment methods that involve an integration of physical, chemical, and/or biological processes, such as chemical precipitation, flocculation, and reverse osmosis, have been previously considered as sustainable waste management, but ultimately proved inefficient (Azimi et al. 2017). Several drawbacks include high costs, long processing time, and the generation of excess sludge that require further disposal (Pertile et al. 2020). Thus, it is imperative to develop an environmentally friendly, cost effective, and efficient method to maintain ecosystem health. Bacterial bioremediation is a process that utilizes bacteria to remove anthropogenic pollutants from contaminated sites. Specific bacterial species, especially those isolated from contaminated environments, are capable of developing resistance mechanisms to adapt and continue thriving against pollutant exposure. Bacterial copper resistance is enabled through two main processes: biosorption and bioaccumulation. On the other hand, bacterial dye-removal, referred to as decolorization is manifested through two main processes: degradation and mineralization. Bacteria are equipped with physiological and genetic properties (i.e., copper-resistance genes or enzyme production) that allow specific biochemical modifications, thereby capable of resisting multiple pollutants at once.

Kapuas River, located on the island of Kalimantan, is the longest (>1,000 km), yet one of the most contaminated rivers in Indonesia. Kapuas River is exploited for its cheap land and easy accessibility to strategic transportation routes as it passes through West Kalimantan and empties into the South China Sea (Lukas et al. 2012). The Central Bureau of Statistics of Kapuas Regency states that the upper region of the river is surrounded by >100 operational factories, 40% of which involve dyes in their manufacturing processes (Anonym 2018). It can thus be inferred that the Kapuas River is contaminated by both copper and dyes. Previous studies show that distinct bacterial strains isolated from contaminated rivers in Indonesia, such as Sukolilo River, Surabaya; Cikapundung River, West Java; and Kemisan River, Banten, can be successfully cultivated as highly copper-resistant isolates (Irawati et al. 2021a; Irawati et al. 2017). However, study on bacterial the multi-resistance copper and dye is still limited, while the potential of local marine microbes in Indonesia remains unexplored. Thus, this study aims at isolating and characterizing copper-resistant bacteria from Kapuas River; measuring the copper minimum inhibitory concentration (MIC) of bacterial isolates, and determining the effects of the reactive black and methylene blue dye on bacterial growth and decolorizing abilities.

MATERIALS AND METHODS

Isolation and morphological characterization of copper-resistant bacteria

Bacteria were grown in Luria broth media containing the following (per liter): 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 0.1 g glucose. Luria Bertani (LB) agar was prepared by adding 2% bacteriological agar into Luria broth. The resulting liquids were then autoclaved at 121°C, 1 atm, for 15 min before being poured into petri dishes to prepare control media. One molar of $CuSO_4$ stock solution was supplemented into sterilized media to prepare media containing copper at different concentrations (1-8 mM). Stock solutions of 10,000 ppm methylene blue and reactive black dye were also added to prepare media containing 300 ppm of each dye.

Water samples were retrieved from Kapuas River, West Kalimantan, Indonesia, then stored at 4°C until further use. Samples were then serially diluted as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Approximately 100 µL of each of the sample at dilutions factor of 10^{-3}, 10^{-4}, and 10^{-5} were spread onto LB agar media supplemented with 3 mM of copper. Following incubation at 37ºC for 24 h, each colony was selected and purified to obtain a single colony by streaking on an agar plate. Morphological characterization of bacterial isolates was then observed to identify the following characteristics: colony color, opacity, shape, margin, and texture. Cell morphology and gram staining were also observed using a light microscope.

Copper-resistance assay

Copper-resistance of bacterial isolates was tested by measuring the MIC of copper. Bacterial isolates were streaked using the four-quadrant streak method on each media supplemented with various copper concentrations (1-8 mM). Colonies capable of growing at the initial concentration (1 mM) were progressively transferred onto a medium containing the next higher concentration of copper until terminated growth was observed. The MIC was determined after 48 h of incubation at 37°C (Irawati et al. 2019).

Dye-resistance and decolorization assay

Dye-resistance was qualitatively observed by streaking bacterial isolates on LB agar supplemented with 300 ppm of methylene blue or reactive black dye. Dye-resistance and decolorization observations were finalized after 24 h and 48 h of incubation at 37°C by distinguishing clear zones that formed around the dye-adsorbing bacterial colonies. The decolorization ability test was then performed by inoculating bacterial isolates in liquid Luria broth supplemented with 300 ppm of methylene blue or reactive black before being incubated in a shaker at 37°C. After 24 h and 48 h, samples were centrifuged at 12,000 g for 10 min to separate bacterial cells. Dye decolorization rates were then obtained by measuring the decrease in color intensity of the supernatant using a UV/VIS spectrophotometer (Biodrop, UK) at a wavelength range of 300-900 nm. A decrease in absorbance at a wavelength of 663 nm indicates decolorization of methylene blue while a decrease in absorbance at a wavelength of 597 nm indicates decolorization of the Reactive Black. The percentage decolorization value was measured using the formula below (Irawati et al. 2022):

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

Molecular characterization

Bacterial isolates were first grown overnight on LB agar medium. The 16S rRNA genes were then amplified using the universal bacterial primer 16S rRNA derived from *Escherichia coli*. Forward primer (F) was expected to amplify at positions 20-43 while reverse primer (R) was located at 1482-1507. Primer concentration in solution was 10 µM. The DNA polymerase Taq enzyme used was Plantinum® Taq DNA polymerase (Invitrogen no. Cat. 10966-018) at a concentration of 5 units/µL, resulting in a final enzyme concentration per reaction of 1.25 units/25 µL. The 10× buffer PCR + $MgCl_2$ solution was also prepared with a composition of 1 M Tris-HCl pH 8.3, 5 M KCl, 1% gelatin, and 35 mM $MgCl_2$ producing a final concentration of 3 mM $MgCl_2$ per reaction. The polymerase chain reaction (PCR) reaction mixture (25 µL) consisted of a DNA template (100 ng), forward primers (0.4 µM), reverse primers (0.4 µM), dNTP (200 µM), 1×PCR buffer, ddH_2O, and Taq DNA polymerase enzyme (5 U/µL). The PCR product (amplicon) was analyzed by conducting electrophoresis on 1.5% (w/v) agarose gel in 1×TAE buffer supplemented with ethidium bromide (0.5 g L^{-1}). The 16S rRNA gene base sequence was then edited using the ProSeq software. Identical sequences were compared and aligned with other bacteria registered on the Basic Local Alignment Search Tool (BLAST) database to determine identification and similarity (Irawati et al. 2012). Phylogenetic studies were then performed by analyzing 16S rRNA gene base sequences for bacterial isolates through the neighbor-joining method and 100 × bootstrap method on CLUSTAL X software. Phylogenies were lastly displayed in a dendrogram cluster using the TreeView program (Tamura et al. 2013).

RESULTS AND DISCUSSION

Isolation and characterization copper-resistant bacteria

The growth of the bacterial community, isolated from the Kapuas River, on a medium supplemented with 3 mM $CuSO_4$ with dilution ranging from 10^{-3} to 10^{-5} (Figure 1). The fifteen copper-resistant bacteria were then designated as strains: KIMS1, KIMS2, KIMS3, KIMS4, KIMS5, KIMS6, KIMS7, KIMS8, KIMS9, KIMS10, KIMS11, KIMS12, KIMS13, KIMS14, and KIMS15 (Table 1).

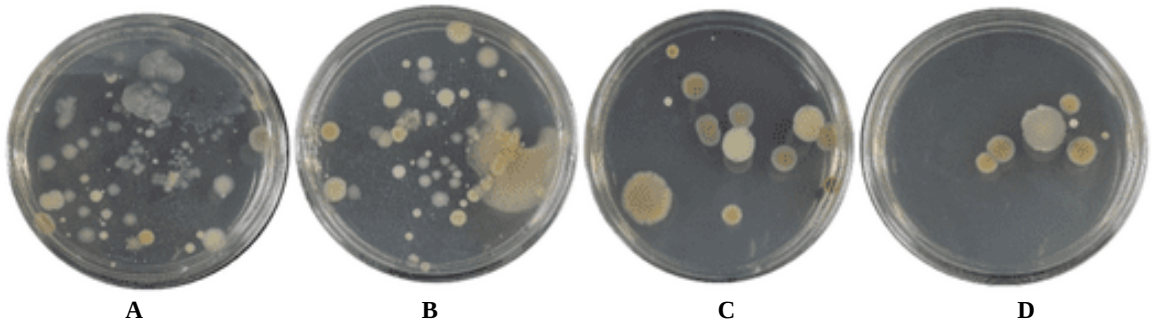

Figure 1. The growth of the bacterial community of bacterial strains isolated from Kapuas River in media containing 3 mM of copper. A. Sample dilution 10^{-2}, B. Sample dilution 10^{-3}, C. Sample dilution 10^{-4}, D. Sample dilution 10^{-5}

Table 1. Morphology and cell characterization of bacterial isolates

Bacterial isolate	Color	Opacity	Shape	Margin	Texture	Gram staining	Cell morphology	MIC (mM)
KIMS1	Creamy White	Transparent	Circular	Undulate	Smooth	Positive	Coccus	-
KIMS2	Pale Brown	Transparent	Circular	Entire	Smooth	Positive	Coccus	5
KIMS3	Creamy White	Transparent	Circular	Entire	Smooth	Positive	Bacillus	-
KIMS4	Creamy White	Transparent	Circular	Entire	Smooth	Positive	Bacillus	5
KIMS5	Creamy White	Transparent	Circular	Undulate	Smooth	Negative	Coccus	5
KIMS6	Pale Brown	Transparent	Circular	Entire	Smooth	Positive	Coccus	6
KIMS7	Creamy White	Transparent	Circular	Entire	Smooth	Positive	Coccus	6
KIMS8	Creamy White	Transparent	Circular	Undulate	Smooth	Negative	Bacillus	7
KIMS9	Pale Brown	Transparent	Circular	Entire	Smooth	Positive	Bacillus	6
KIMS10	Creamy White	Transparent	Circular	Entire	Smooth	Negative	Bacillus	7
KIMS11	Pale Brown	Transparent	Circular	Entire	Smooth	Negative	Coccus	6
KIMS12	Pale Brown	Transparent	Circular	Entire	Smooth	Negative	Bacillus	5
KIMS13	Creamy White	Transparent	Circular	Entire	Smooth	Positive	Coccus	5
KIMS14	Creamy White	Transparent	Circular	Entire	Smooth	Positive	Coccus	6
KIMS15	Creamy White	Opaque	Circular	Entire	Smooth	Positive	Coccus	6

Ten bacterial isolates appeared creamy white while the other five isolates were pale brown. All isolates were circular with a smooth texture. All isolates had a transparent optical appearance except for KIMS15 isolate which appeared opaque. About nine bacterial isolates were cocci and the rest were bacilli. Results show that the majority (8 out of 13) of copper-resistant isolates were Gram-positive, an intrinsic feature that likely plays a significant role in copper homeostasis. Gram-positive bacteria have been specifically reported to be more effective in removing copper due to their cell wall structure. Gram-positive bacteria have negatively charged cell well membranes that act as a binding site that attracts, then captures copper ions to be utilized and detoxified simultaneously by putative copper homeostasis enzymes (Shamim 2018).

The results of the copper-resistance test carried out on medium supplemented with 1-7 mM $CuSO_4$ showed that KIMS8 and KIMS10 isolates were highly resistant bacteria. All bacterial isolates demonstrated the ability to grow on copper-supplemented media with MICs ranging from 5 mM to 7 mM except KIMS1 and KIMS3 isolates. The two isolates only demonstrated resistance to 3 mM $CuSO_4$ which may be attributed to mutually beneficial synergistic relationship with the bacterial population within the community.

An MIC value of 5-7 mM is considered relatively high when compared to other isolates previously obtained from copper-contaminated rivers across Indonesia. Recent studies report that bacteria isolated from Cikapundung River, West Java had an MIC value of 5-8 mM (Irawati et al. 2019), while those isolated from Cisadane River, Banten; Sukolilo river, East Java; and Kemisan river, Banten had the MIC of 6-8 mM (Nurlaila et al. 2021); 9 mM (Irawati et al. 2021a); and 10 mM (Irawati et al. 2017), respectively. Microbial morphology, physiology, and metabolic mechanisms are the main factors that enable bacteria to develop copper resistance in response to copper exposure (Irawati et al. 2022). It was also suggested that bacteria perform copper homeostasis by binding, followed by intracellular accumulation of copper ions within the cytoplasm to utilize an adequate amount as micronutrients while excess ions are removed by chaperones protein (Irawati et al. 2021b). Although copper is an essential micronutrient required at low concentrations (nM) as a redox co-factor in the catalytic centers of enzymes, high concentrations of copper (μM to mM) are potentially hazardous due to its high chemical reactivity (Shamim 2018; Arguello et al. 2013). Free copper ions catalyze a Fenton-like reaction to form hydroperoxide free radicals that attack cellular biomolecules, ensuing lipid peroxidation and protein damage (Santo et al. 2010). Hydroxyl products also cause oxidative DNA damage and rupture bacterial cell walls, leading to decreased viability and respiration inhibition, both of which are the main causes of terminated bacterial growth in the presence of copper toxicity (Fowler et al. 2019). Protection of cellular components and avoidance from copper-inflicted damage to bacteria may be attributed by the putative copper-resistance proteins, such as copper-resistance protein A (CopA), copper-resistance protein B (CopB), multi-copper oxidase, superoxide dismutase (SOD), and universal stress protein (Usp), (Irawati et al. 2021b) albeit at only a certain threshold (MIC). KIMS8 and KIMS10, which demonstrated the highest copper resistance, were selected for further analysis on resistance to methylene blue and reactive black dye.

Bacterial dye-resistance

Bacterial dye-resistance could be determined by the ability to grow and/or decolorize the dye. Decolorization can be observed through three manifestations, i.e., changes in colonies and medium color, and clear zone formation (Irawati et al. 2022). The bacterial growth of KIMS8 and KIMS10 isolates in a medium containing 300 ppm methylene blue and 300 ppm reactive black (Figure 2). A clear zone was clearly formed around the colony of the KIMS8 isolate, augmented by the reduced color intensity of the medium from dark to light blue, demonstrating the decolorizing ability of the bacteria. Surprisingly, the growth of the KIMS10 isolate was able to completely remove the color of the medium from dark blue to clear indicating a high degree of decolorization. The clear zone formation suggested that the dye-resistance mechanism of the two bacteria to methylene blue was by biodegradation resulting in decolorization. Furthermore, the growth of KIMS8 and KIMS10 on reactive black showed minimal decolorization activity as evidenced by non-existent clear zones and only a slight fade of medium color. Changes of the colony color from creamy white to blue like the color of the control medium containing 300 ppm reactive black indicating that the mechanism of this isolate was by adsorption of the dyes. Despite being grown on media supplemented with the same dye type and concentration, KIMS8 and KIMS10 showed different growth patterns, suggesting that different isolates are equipped with different capabilities to perform dye-resistance mechanisms.

Decolorization is defined as the process of removing dye from stained specimens. An et al. (2002) emphasize that decolorization occurs through two mechanisms: adsorption or biodegradation. Li et al. (2019) reported that bacterial adsorption of dyes occurs when molecular, covalent, or electrostatic forces exerted through activities, such as electron exchange, help adhere dyes to bacterial cell wall surfaces. Meanwhile, biodegradation is dependent on dye structure as chemical compounds are reduced into smaller molecules assisted by specific enzymes. Bacteria are known to synthesize oxidative enzymes, such as lignin peroxidase (LiP) and laccase, as well as reductive enzymes, such as azoreductase, as a metabolic product (Khandare and Govindwar 2016). Methylene blue and reactive black dye are both azo dyes, a chemical structure characterized by a polycyclic aromatic ring structure.

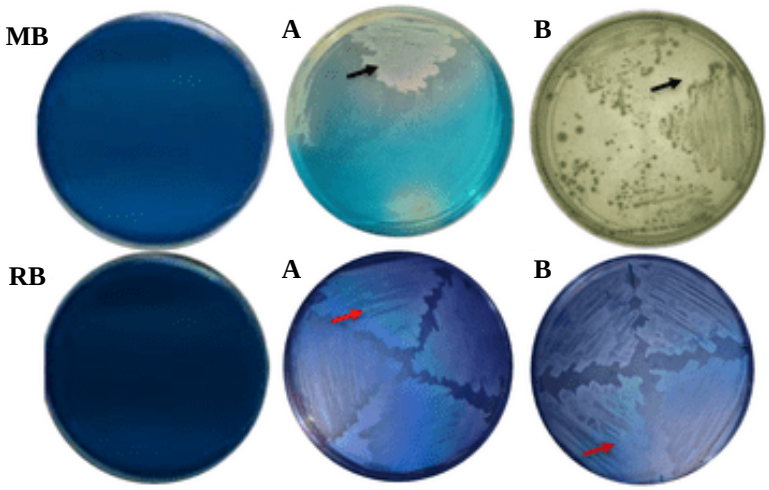

Figure 2. Dye-resistance test performed by KIMS8 and KIMS10 on 300 ppm Methylene Blue and Reactive Black dye. Note: MB. negative control supplemented with Methylene Blue dye; RB. Negative control supplemented with Reactive Blue dye; A. KIMS8 on Methylene Blue; B. KIMS10 on Methylene Blue; C. KIMS8 on Reactive Black; D. KIMS10 on Reactive Black. Black arrows show a clear zone indicates decolorization process. Red arrows show adsorption

The LiP has been reported to open aromatic ring structures while laccase cleaves the functional group $-N(CH_3)_2$ of the methylene blue dye structure (Zucca et al. 2015). Similarly, LiP has been suggested to degrade chromophore groups that constitute the backbone of reactive black dye structure (Wielewski et al. 2020). Additionally, azoreductases are known to break characteristic azo bonds (−N=N−), generating aromatic amines which are eventually degraded by microbial enzymes, such as mono, dioxygenases, and hydrolases (Misal and Gawai 2018).

Bacterial decolorization assay

Following dye-resistance tests, spectrophotometric analyses were carried out to obtain quantitative data on the ability of KIMS8 and KIMS10 isolates to decolorize methylene blue at a wavelength of 663 nm and reactive black at 598 nm (El Bouraie and El Din 2016). KIMS8 and KIMS10 isolates were found managed to decolorize up to 90% and 94.9% of methylene blue dye after 48 h, respectively. However, KIMS8 and KIMS10 strains were only able to decolorize up to 11.3% and 12.1% of reactive black dye after 48 h, respectively (Figure 3). The percentage of methylene blue decolorization by KIMS10 strain was greater than *Comamonas aquatica* PMB-1 and *Ralstonia mannitolilytica* PMB-2 decolorizing ability, 67.9% and 60.3%, respectively (Siregar et al. 2020). Methylene blue decolorization of *Enterobacter hormaechei* strain KIMS10 was also higher than indigenous multi-resistance bacteria *Acinetobacter* sp. strain CN5 (57.64%) (Irawati et al. 2022), *Ralstonia pickettii* (89%) (Nabilah et al. 2021), Brown-Rot Fungus *Fomitopsis pinicola* (92.56%) (Purnomo et al. 2022), *R. pickettii* (98.11%) (Purnomo et al. 2021), but lower than *Desmodesmus* sp. (98.6%) (Al-Fawwaz and Abdullah 2016). The percentage of Reactive Black decolorization by KIMS10 was lower than a novel isolated bacterial strain *Enterobacter* sp. EC3 (92.56%) (Wang et al. 2009) and new haloalkaliphilic bacteria isolated from the textile wastewater (87%) (Seyedi et al. 2020).

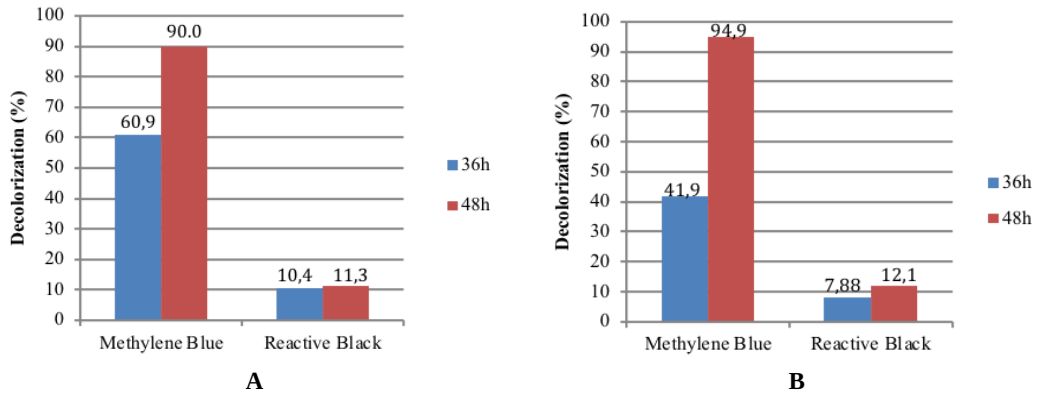

Figure 3. Decolorization ability of KIMS8 and KIMS10 strains on medium supplemented with 300 ppm Methylene Blue and Reactive Black dye. Note: A. KIMS8; B. KIMS10

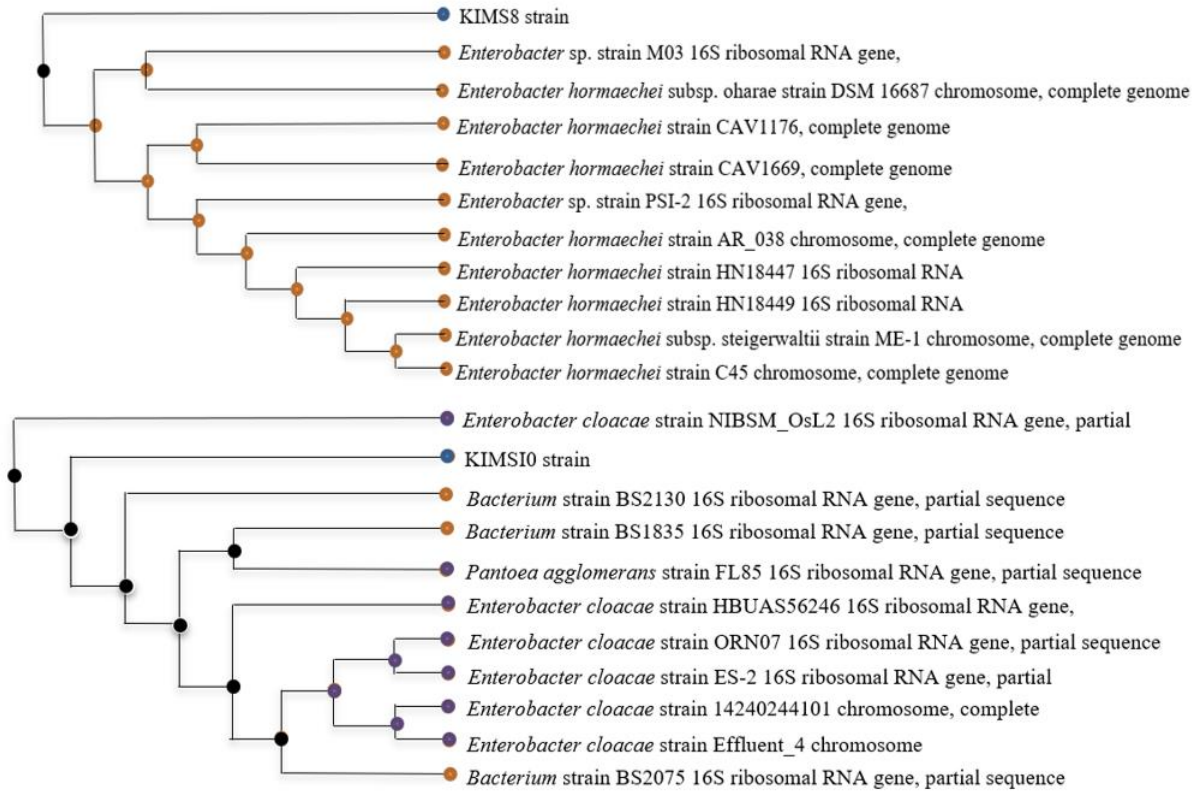

Figure 4. Phylogenetic tree of KIMS8 and KIMS10

Molecular characterization of multi-resistant bacteria

Molecular characterization of multi-resistant bacteria based on the 16S rRNA gene showed that KIMS8 strain was classified as *Enterobacter hormaechei*. Sequence alignment and percentage homology showed that KIMS8 strain had 99.79% gene similarity with *E. hormaechei* strain C45, *E. hormaechei* subsp. *steigerwaltii* strain ME-1, *E. hormaechei* strain HN18449, *E. hormaechei* strain HN18447, and *E. hormaechei* strain AR_038. Likewise, KIMS10 strain had the highest gene similarity with *E. cloacae* strain 14240244101 (99.93%). Therefore, KIMS8 and KIMS10 isolates are identified as *E. hormaechei* strain KIMS8 and *E. cloacae* strain KIMS10. Phylogenetic analysis showed similar results that the KIMS8 and the KIM S10 had the closest relationship with *E. hormaechei* dan *E. cloacae*, respectively (Figure 4).

Enterobacter species is one of the most found microorganisms in contaminated soil, water, and sewage. It is a versatile bacterium capable of rapidly and efficiently adapting its metabolism as well as physiology to environmental stresses, thereby is considered multi-resistant to numerous stressors, such as copper and dye (Davin-Regli and Pages 2015). *Enterobacter hormaechei* KIMS8 and *E. cloacae* KIMS10 are new strains of indigenous bacteria isolated from Kapuas River. *Enterobacter hormaechei* KIMS8 and *E. cloacae* KIMS10 demonstrated multi-resistance to copper, methylene blue, and reactive black. The same bacterial species, *E. cloacae* strain IrSuk1 and IrSuk4a isolated from the Cikapundung River was known resistant only to copper (Irawati et al. 2021). Guo et al. (2020) also suggested that *Enterobacter* species, such as *E. roggenkampii*, are equipped with various genes associated with heavy metal homeostasis. For instance, CopA was reported to help *Enterobacter* species generate copper tolerance through an efflux pump system. Furthermore, enzymes associated with decolorization, such as laccase and azoreductase, have been found as metabolic products in *E. cloacae* and *E. agglomerans*, respectively (Moutaouakkil et al. 2003).

In conclusion, *E. hormaechei* KIMS8 and *E. cloacae* KIMS10 have more advantages than other strains previously reported. The KIMS8 and KIMS10 strains have high copper resistance and decolorization ability to methylene blue and reactive black up to 94% and 12% after 48 h, respectively. KIMS8 and KIMS10, therefore, hold great potential to be employed as future bioremediation agents which have multi-resistance to copper and dyes. The findings of multi-resistance to copper and dyes and decolorization ability to Methylene Blue and Reactive Black in these strains thus open novel opportunities to increase textile waste treatment biologically using indigenous bacteria.

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