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Burkholderia cepacia strain IrV1 multi-resistant to copper and dyes isolated from laboratory wastewater effluent

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Abstract. *Irawati W, Lindarto V, Pinontoan R, Yuwono T, Mangunsong FM, Silalahi DW. 2022.* Burkholderia cepacia *strain IrV1 multi-resistant to copper and dyes isolated from laboratory wastewater effluent. Biodiversitas 23: 2614-2620.* Copper and dye multi-resistant bacteria may be cultivated to increase the efficiency of waste bioremediation processes. Multi-resistant bacteria can be isolated from waste containing copper and dyes, including laboratory waste. The aims of this study were to: (i) isolate copper-resistant bacteria from wastewater, (ii) determine the multi-resistance of bacteria against copper and dyes, and (iii) observe the ability of bacteria to remove colors methylene blue, basic fuchsin, and Congo red. Selected bacterial isolates were identified based on the 16S rDNA gene. One highly copper resistant bacterial strain IrV, showed resistance to 7 mM $CuSO_4$, 100 ppm methylene blue, 100 ppm basic fuchsin, and 100 ppm Congo red. Based on the 16S rDNA gene sequence, strain IrV belongs to *Burkholderia cepacia* with 99.86% similarity. The addition of copper on the medium resulted in colony color changes from light yellow to green indicated Cu binding within the cell. Moreover, a clear zone around the colony was observed, suggesting that the strain is capable of decolorizing methylene blue, Congo red, and basic fuchsin dyes. The results thus demonstrate that bacterial strain IrV is multi-resistant to copper and dyes.

Keywords: *Burkholderia cepacia*, copper, dye, multi-resistance, wastewater

INTRODUCTION

Copper (Cu) and dye are anthropogenic pollutants capable of exerting toxicity to both micro and macroorganisms even at low-level exposure (Irawanto and Baroroh 2017; Tchounwou et al. 2012). Copper is a non-degradable heavy metal that tends to accumulate in terrestrial and aquatic sediments, gradually disrupting bio-geochemical processes. Dye is synthesized as a highly soluble and recalcitrant colorant, circling coastal communities for a long period of time. High copper and dye bioavailability in both terrestrial and marine environments lead to trophic interactions such as bioaccumulation between low levels and biomagnification between higher levels (Delahaut et al. 2020; Sandhya 2010). Excessive copper ions generate hydroxyl radicals via a Fenton-like reaction (Kim et al. 2018). Ironically, copper hinders cellular defense mechanisms against redox reaction products (Lobo et al. 2010). Hydroxyl radicals then dysregulate Cu homeostasis, causing Cu storage disorders such as Wilson's Disease in the liver and extrahepatic organs such as the brain and cornea (Barber et al. 2021; Purchase 2013). Damaged liver cells also release large amounts of copper into the blood, resulting in organ damage or acute lungs and kidney failure (Anon 2000). As a result, humans may suffer from severe hepatic, respiratory, neurological, and psychiatric problems. Dye by-products are carcinogenic, mutagenic, allergenic, genotoxic, and ecotoxic in nature (Lellis et al. 2019). Dyes have been specifically reported to affect human skin, liver, and bladder through an array of allergies and diseases such as cancer-causing sarcomas and tumors (Sivarajasekar and Baskar 2014; Pohanish 2017). Aside from harming human health, dyes also cause ecosystem dysfunction by altering the growth and reproduction of aquatic biota, soil microbial communities, and terrestrial plants (Hassan and Carr 2018; Imran et al. 2015; Rehman et al. 2018).

Bioremediation of copper and dyes is the main field and research activity carried out at the Teacher's College (TC) Microbiology Laboratory of Pelita Harapan University, Tangerang, Indonesia. Twelve types of dyes including methylene blue, malachite green, Congo red, mordant orange, reactive black, direct yellow, basic fuchsin, reactive orange, disperse orange, remazol red, wantex yellow, and wantex red dye were used in this study (Irawati et al. 2022). Recent studies were focused on the isolation of multi-copper and dye-resistant bacteria from contaminated water bodies such as rivers and wastewater from treatment

plants in Indonesia. Microbial waste containing high concentrations of copper (mM) and dye (ppm) generated from previous research done in this laboratory was collected into a plastic canister as a container for hazardous and toxic waste disposal.

According to Dykhuizen (2005), bacteria are major components of cellular life and are abundantly found everywhere on earth, including in any form of waste such as hazardous and toxic waste generated from research laboratories. It was thus hypothesized that effluent from research laboratories may also harbor copper- and/or dye-resistant bacteria. Microbiology laboratory waste can be defined as any product, whether solid or liquid that emanates from or has been used in tampering with microorganisms such as bacteria. Common microbial waste includes discarded microbial specimens and containers used to store, transfer, inoculate, cultivate, and mix cultures. Microbial wastes may also include contaminated and infectious disposable culture dishes and devices that carry toxins or hazards, thus categorized as a type of toxic or hazardous waste. These wastes are identified based on their ability to exhibit either one or multiple of the following properties: ignitability, corrosivity, reactivity, or toxicity (Fazzo et al. 2017).

Previous study by Arifin et al. (2016) showed that 34 different bacterial isolates with genus *Staphylococcus, Burkholderia, Leuconostoc, Citrobacter, Pantoea, Sphingomonas, Acinetobacter,* and *Lactococcus* had been isolated from solid surfaces in a Clinical Microbiology Laboratory in Banda Aceh, Indonesia. Isola and Olatunji (2016) also identified three different genera, namely *Bacillus, Staphylococcus,* and *Salmonella* from various solid surfaces in 4 science laboratories in Federal Polytechnic, Ede, Nigeria. However, both laboratories did not conduct research on bioremediation of copper and dyes. Thus, this study was aimed at: (i) isolating copper-resistant bacteria from wastewater in a TC Microbiology Laboratory, (ii) testing the multi-resistance of bacteria to copper and dyes, and (iii) analyzing the ability of the bacteria to decolorize dyes on medium containing methylene blue, basic fuchsin, and Congo red dye supplemented with copper.

MATERIALS AND METHODS

Growth medium preparation

Solid Luria Bertani medium was prepared by adding 2 g of Luria Bertani broth and 2 g of American Bacteriological Agar into 1 liter of dH_2O. Luria Bertani broth contained the following (per liter): tryptone 10 g, yeast extract 5 g, NaCl 10 g, and glucose 0.1 g. The Luria Bertani medium was sterilized at 121°C and 1 atmosphere for 15 minutes. A stock solution of 1 M $CuSO_4$ was added to prepare media containing 3 mM copper. A stock solution of 10,000 ppm methylene blue, basic fuchsin, and Congo red dye were also added to create media containing 50 and 100 ppm of each dye. Solid LB medium containing various concentrations of copper (3-10 mM $CuSO_4$), dye (50 ppm and 100 ppm methylene blue, basic fuchsin, and Congo red), as well as copper and dye (50 ppm of methylene blue, basic fuchsin, and Congo red plus 5 mM $CuSO_4$) were then poured onto petri dishes and solidified for a minimum of 45 minutes prior to use.

Wastewater sampling and bacterial isolation

Wastewater was taken from a plastic canister containing hazardous and toxic waste at the Teachers' College (TC) Microbiology Laboratory of Pelita Harapan University, Tangerang, Banten, Indonesia. Research in this laboratory focuses on the bioremediation of copper (Irawati 2017) and various dyes, including methylene blue, malachite green, Congo red, mordant orange, reactive black, direct yellow, basic fuchsin, reactive orange, disperse orange, remazol red, wantex yellow, and wantex red dye (Irawati et al. 2022). Microbial waste containing high concentrations of copper (mM) and dye (ppm) generated from previous studies was collected in this plastic canister. The plastic canister was thoroughly shaken to agitate samples and homogenize the liquid waste prior to sampling. Approximately 100 ml of the wastewater was procured into a centrifuge tube as an original solution. Wastewater samples were then dissolved into Luria Bertani liquid medium with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution factors. A suspension of 100 µL for every factor was inoculated by serial dilution onto Luria Bertani agar medium supplemented with 3 mM $CuSO_4$, then incubated at 37ºC for 24 hours.

Determination of copper resistance

Bacterial growth analysis on copper was conducted on LB medium supplemented with 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, and 10 mM of $CuSO_4$. Growth analysis was performed by spreading 50 µl of wastewater from each diluted sample onto copper-supplemented medium. Bacterial plating was carried out thrice, then incubated at 37°C for 72 hours.

Bacterial growth on dyes and decolorization ability

Bacterial growth analysis on dye was observed on LB medium supplemented with 50 ppm and 100 ppm methylene blue, basic fuchsin, and Congo red dye. Growth analysis was carried out by spreading 50 µl of wastewater from each diluted sample (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) onto dye-supplemented medium. Plating was carried out in triplicates per dye, followed by incubation at 37°C for 72 hours. The ability of bacteria to grow and decolorize each dye was observed through the colony growth and the formation of a clear zone around the bacterial colonies on the medium.

Bacterial growth and decolorization on medium supplemented with copper

Bacterial growth analysis on dye was observed on LB medium supplemented with 50 ppm methylene blue, basic fuchsin, and Congo red dye plus 5 mM $CuSO_4$. Growth analysis was carried out by spreading 50 µl of wastewater from each diluted sample onto copper plus dye-supplemented medium. Three-fold bacterial plating was carried out prior to incubation at 37°C for 72 hours. The

ability of bacteria to grow on and decolorize each dye was observed through colony growth and clear zone formation around bacterial colonies on the medium.

Identification of bacterial species

Selected bacterial isolates were morphologically identified through qualitative observation, then molecularly identified based on the 16S rDNA gene. The polymerase used was Plantinum® Tag DNA Polymerase (Invitrogen no. cat.10966-018) with a concentration of 5 units/µL and a final enzyme concentration per reaction of 1.25 units/25 L. The results of the 16S rDNA amplification of each isolate were then sequenced and analyzed using ProSeq program. To determine the percentage of similarity and identify of the isolates, the DNA sequence of the isolate was aligned with the 16S rDNA in the gene bank data of other bacteria using the Basic Local Alignment Search Tool. Phylogenetic studies were carried out by analyzing the base sequence of the 16S rDNA gene of bacterial isolates by using CLUSTAL X program with Neighbour-Joining method and bootstrap 100 times. Dendrogram was created through the TreeView program.

RESULTS AND DISCUSSION

Bacterial growth in medium supplemented with copper

The results of bacterial isolation showed that there were uniform bacterial colonies growing on mediums containing 5 mM, 6 mM, and 7 mM $CuSO_4$. Based on observation of morphological characteristics such as size and shape of bacterial colonies, only a single bacterial colony designated as strain IrV was successfully isolated from TC Microbiology Laboratory wastewater. Intensity of IrV colony growth in medium supplemented with 7 mM $CuSO_4$ was lower than that of 5 mM and 6 mM (Figure 1).

Figure 1 showed that there were less bacterial colonies growing on medium supplemented with 7 mM as compared to 5 mM and 6 mM $CuSO_4$, indicating that a high concentration of copper inhibited the growth of bacteria (Figure 6). The appearance of greenish-blue IrV colonies on copper-supplemented medium suggest that the strain was capable of intracellularly accumulating, then utilizing copper as a micronutrient for metabolic processes. Microbial morphology, physiology, and metabolic mechanisms enable bacteria to develop copper resistance despite direct and long-term exposure. Irawati et al. (2021) stated that bacteria develop resistance mechanisms against copper toxicity by binding and accumulating copper, then excreting excess copper ions to maintain sufficient intracellular concentrations of copper as a micronutrient. According to Shamim (2018), copper is required as a trace element for normal cellular growth and metabolism in prokaryotic and eukaryotic organisms at low concentrations (nM), but toxic at high concentrations (µM to mM). Copper toxicity is mainly a result of redox cycling reactions that alternate between Cu(I) and Cu(II) when free copper ions are present. Excess copper levels catalyze a Fenton-like reaction to form hydroperoxide free radicals that attack cellular biomolecules, resulting in lipid peroxidation and protein damage in aerobic bacteria (Dupont et al. 2011; Espírito Santo et al. 2010; Grey and Steck 2001). Copper surplus also disrupts normal cellular activities by replacing metal ions in proteins, forming spurious disulfide bonds, and causing the oxidation and degradation of iron-sulfur groups in proteins (Bondarczuk and Piotrowska-Seget 2013).

Results of copper-resistance test carried out on culture medium supplemented with 3-10 mM $CuSO_4$ showed that the Minimum Inhibitory Concentration (MIC) value of strain IrV was at 8 mM $CuSO_4$. A MIC value of 8 mM is regarded as relatively high, demonstrating its great potential to be employed as a future copper bioremediation agent. Previous studies reported that indigenous bacteria isolated from numerous copper-contaminated sites in Indonesia were highly resistant to copper. Bacteria isolated from industrial waste in Kemisan River, Banten had a MIC of 10 mM (Irawati 2017), from the east coast of Surabaya a MIC value of 9 mM-11 mM (Irawati et al. 2021), while from the Cisadane River, Banten a MIC value of 6 mM-8 mM (Nurlaila et al. 2020). These high MIC values may be related to the level of copper contamination found at different sampling sites across Indonesia.

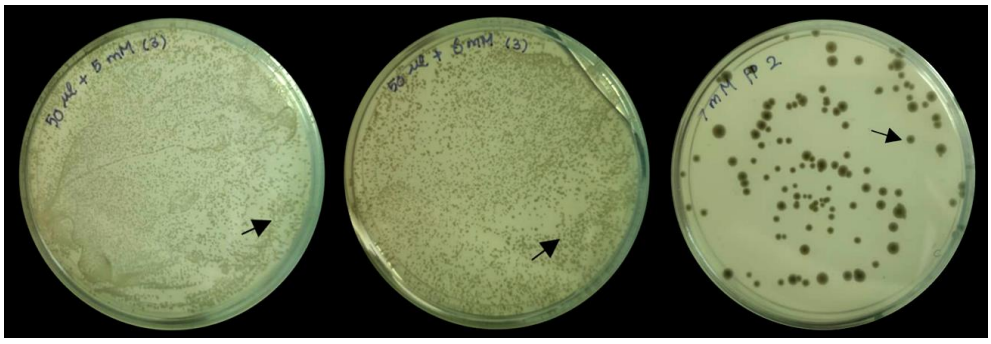

Figure 1. The growth of bacterial colonies on culture media supplemented with various concentrations of copper $CuSO_4$. A. 5 mM $CuSO_4$, B. 6 mM $CuSO_4$, C. 7 mM $CuSO_4$. Arrow represents bacterial colony

Distinct differences between the color of the strain IrV colonies on medium without $CuSO_4$ and medium containing $CuSO_4$ was observed in present study. Strain IrV growing on $CuSO_4$ medium had greenish-blue colony centers. Furthermore, the color of colonies growing on 7 mM medium appeared greener than on 3 mM, indicating that the intensity of copper accumulation increased as copper concentration in the medium increased (Figure 2). This result is in line with the results of a previous study (Irawati et al. 2021) where *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 also showed green bacterial colonies (Irawati et al. 2021). Bioaccumulation involves microbial physiology and metabolism by synthesizing proteins that bind copper ions before storing them in the periplasmic space, then conducting detoxification of copper ions (Diep et al. 2018). Based on a previous study (Irawati et al. 2021), these proteins include copper resistance protein A (CopA), copper resistance protein B (CopB), superoxide dismustase (SOD), multi-copper oxidase (MCO), and universal stress protein (Usp).

Bacterial growth in medium supplemented with dyes

Results of the growth test of strain IrV on dye-supplemented mediums showed that the isolate grew on culture media containing 50 ppm methylene blue, basic fuchsin, and Congo red dye (Figure 3). Bacterial growth on dye-supplemented media could be observed through three different manifestations, namely colony color changes, medium color changes, and clear zone formation (decolonization). The colonies demonstrated colorful morphology depending on the color of the dyed medium, indicating that strain IrV was capable of absorbing methylene blue, basic fuchsin, and Congo red dye intracellularly. An et al. (2002) suggests that dye-adsorption may be evidenced by the color of dye-adsorbing bacterial colonies grown on medium containing dye. Colony and medium color alterations indicated that strain IrV developed a resistance mechanism against dyes through dye-adsorption.

According to Saratale et al. (2011), bacteria may develop two types of resistance mechanisms against dyes, namely decolonization and degradation. Victor et al. (2020) defines decolonization as the process of removing dyes from stained specimens through absorption, while degradation is the process of breaking down dye into smaller molecules. After undergoing decolonization or degradation, dyes must be further mineralized before proceeding with detoxification. Evidence of dye-absorption can be observed through colony color changes that appear darker after being grown on dye-supplemented medium (An et al. 2002).

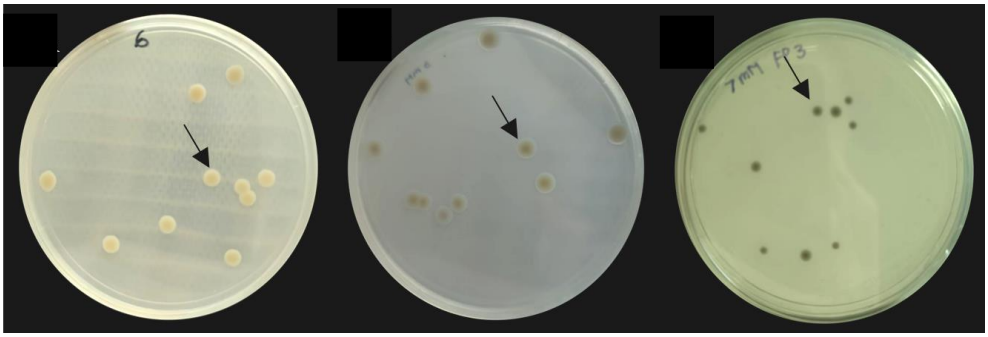

Figure 2. The growth of bacterial colonies on culture media supplemented with copper and without copper. A. without $CuSO_4$, B. 3 mM $CuSO_4$, C. 7 mM $CuSO_4$. Arrow represents bacterial colony

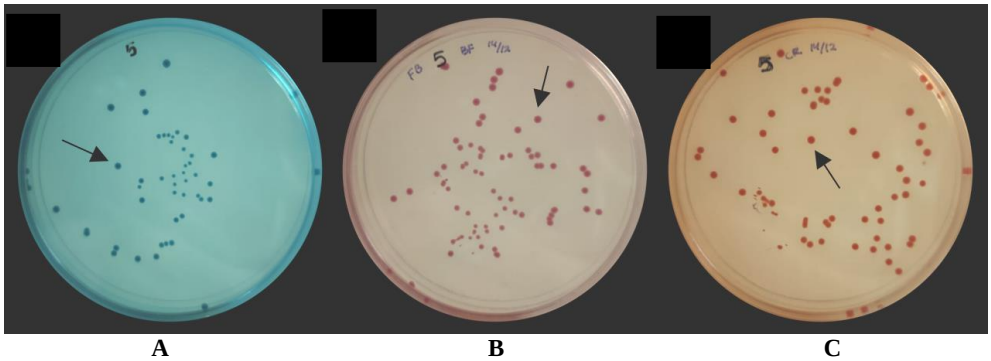

Figure 3. The growth of bacterial colony on culture media supplemented with 50 ppm of dyes. A. Methylene blue, B. Basic fuchsin, C. Congo red dye. Arrow represents bacterial colony

Identification of bacterial isolate IrV

Morphological features of bacterial isolate IrV based on qualitative observation include a small size, yellowish-white pigment, translucent optic, raised elevation, circular shape, and entire edge. Molecular characterization of copper-resistant bacteria based on 16S rDNA showed that isolate IrV1 belongs to *Burkholderia seminalis* (Figure 4). Sequence alignment and percentage of homology showed that strain IrV had a gene similarity of 99.86% with *Burkholderia cepacia* strain 3-1 16S, *Burkholderia seminalis* strain CP-RN-4, *Burkholderia seminalis* strain AGR, *Burkholderia cepacia*, and *Burkholderia cenocepacia* H111. Thus, strain IrV was further identified as *Burkholderia cepacia* strain IrV1 (Table 1).

Torbeck et al. (2011) stated that *Burkholderia cepacia* is a microbial contaminant frequently found in natural environments such as soil and water. *Burkholderia cepacia* was specifically reported as one of the two most common *Burkholderia* contaminants in potable water dispensers (O'Rourke et al. 2020). *Burkholderia cenocepacia* was previously isolated by Arifin et al. (2016) from the floor surface of a Biosafety Cabinet in a clinical microbiology laboratory in Banda Aceh, Indonesia. Arifin et al. (2016) then suggested that the isolate originated from spilled tap water or damp shoe outsoles. However, the isolate was not tested for its copper or dye-resistance. Thus, it can be postulated that the discovery of *Burkholderia cepacia* strain IrV1 from wastewater in the TC lab originated from tap water previously used to dilute microbial wastes before disposal during previous researches.

According to Higgins et al. (2020), members of species of the genus *Burkholderia* can be isolated from acidic soils contaminated with high concentrations of copper. Ibrahim et al. (2011) reported that antibacterial activity in *Burkholderia cepacia, Burkholderia cenocepacia, Burkholderia arboris, and Burkholderia multivorans* was mediated by the influx and accumulation of copper ions in bacterial cells. Proteins located in the outer membrane and periplasmic space of *Burkholderia* cells are particularly sensitive to copper stress (Ibrahim et al. 2011). Damaged proteins lead to copper toxicity, which adversely affects cellular structure and induces DNA damage, thereby resulting in cell lysis. Fortunately, *B. cenocepacia* H111 were reported to carry 92 unique copper resistance genes, specifically Cop and Cus genes required for copper resistance and detoxification (Higgins et al. 2020). These genes helped *B. cenocepacia* H111 develop a copper efflux system that controls copper levels in the cytoplasm to maintain cell envelope integrity, then repair and turnover misfolded proteins. Furthermore, Laszlo (2000) proved that *Burkholderia cepacian* NRRL B-14803 were capable of reducing 50-60% of two monoazo dyes, namely Orange II and hydrolyzed Remazol Red F3B at a concentration of 20 μmol when supplemented with anthraquinone-2-sulfonate in various reactor configurations.

Dye and copper-resistance test of *Burkholderia cepacia* strain IrV1

Dye-resistance test of *Burkholderia cepacia* strain IrV1 on medium supplemented with 100 ppm methylene blue, basic fuchsin, and Congo red dye showed the growth and clear zone formation around the colony (Figure 5A-E). It was suggested that *Burkholderia cepacia* strain IrV1 was not only resistant to high concentration of copper, but also resistant to and capable of decolorizing dyes. Previous studies reported the presence of several bacterial isolates that were also resistant to multiple dyes, but not resistant to copper. *Aeromonas hydrophila* isolated from activated sludge in Guangzhou, China was also reported to resist thirteen types of dye, including basic fuchsin at a concentration of 50 ppm (Ren et al. 2006). Islam et al. (2017) proved that multiple bacterial species belonging to the genera *Staphylococcus, Bacillus, Pseudomonas, Enterobacter, Micrococcus,* and *Klebsiella* previously isolated from dye industry-related areas in Narayanganj District, Bangladesh were resistant to nine types of dye-including Congo red at a concentration of 120 ppm.

Table 1. Percentage of genetic similarity between strain IrV and other *Burkholderia* species based on 16S rRNA gene sequencing

Bacterial species	Similarity percentage
Burkholderia cepacia strain 3-1 16S ribosomoal RNA gene, partial sequence	99.86%
Burkholderia seminalis strain CP-RN-4 16S ribosomal RNA gene, partial sequence	99.86%
Burkholderia seminalis strain AGR 16S ribosomal RNA gene, partial sequence	99.86%
Burkholderia cepacia 16S ribosomal RNA gene, partial sequence	99.86%
Burkholderia cenocepacia H111 chromosone 2, complete genome	99.86%

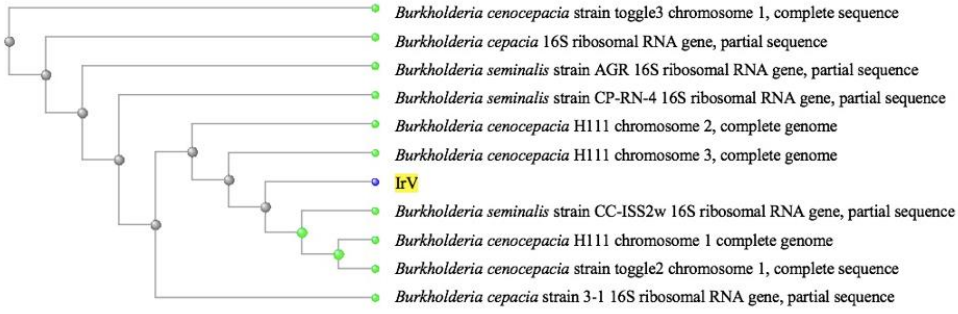

Figure 4. Phylogenetic tree of *Burkholderia cepacia* strain IrV1 isolated from TC Microbiology Laboratory wastewater

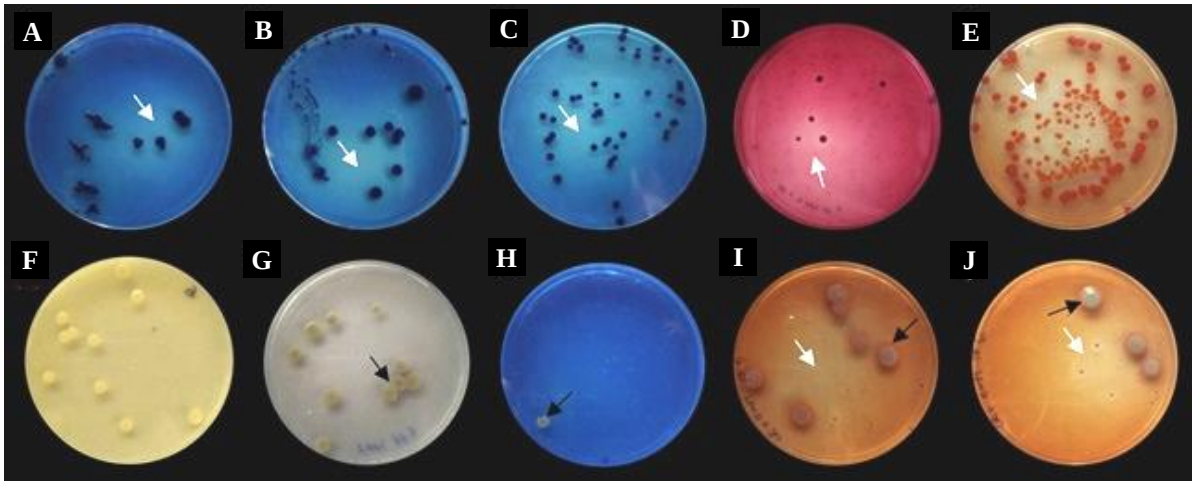

Figure 5. The effect of copper on the decolorizing ability of strain IrV. A, C, H. Methylene blue, D. Basic fuchsin, E, I, J. Congo red, F, G. No dye. White arrows show clear zone. Black arrows show green colony

The addition of 5 mM $CuSO_4$ resulted in the formation of a clear zone around the colonies of *Burkholderia cepacia* strain IrV1 growing on a medium containing 100 ppm methylene blue, basic fuchsin, and Congo red dye. Mota et al. (2015) suggested that the presence of copper increased laccase activity in the degradation process of Congo red dye. The clear zone area around the colonies of *Burkholderia cepacia* strain IrV on medium containing 100 ppm methylene blue, Congo red, and basic fuchsin with addition of 5 mM $CuSO_4$ indicated that copper helps the dye decolorization process. Decolonization is a process highly dependent on the Fenton reaction cycle as the rapid production and effective longevity of hydroxyl radical species helps increase decolonization efficiency (Goodell et al. 2004). Copper ion surplus catalyzes a Fenton-like reaction, generating large amounts of hydroxyl radicals (Kim et al. 2018). Although hydroxyl radicals are known to cause oxidative DNA damage, bacteria can synthesize specific proteins such as SOD, MCO, and Usp to protect its cellular components (Irawati et al. 2021). According to Al-Sulami and Jaafar (2015), the ability of specific bacterial species to accumulate heavy metals and degrade or decolorize dyes depend on the metabolic, physiological, and genetic adaptations as well as the intrinsic, specifically morphological properties only equipped by bacteria.

Figure 5 showed that the addition of copper caused a change in the colony color from light yellow to green as an indication of Cu binding occurrence within the cell. In addition, the appearance of a clear zone around the colony suggested that the addition of copper in the dye medium not only resulted in copper accumulation but also dye decolorization, especially congo red and basic fuchsin (Figure 5H-J). Based on this study, it was concluded that *Burkholderia cepacia* strain IrV is equipped with multi-resistance to copper and dyes. Only a limited number of studies have reported the multi-resistance of bacteria to both copper and dye (Dianrevy 2017). It is suggested that further research is required to determine the ability of *Burkholderia cepacia* strain IrV to accumulate copper and decolorize dyes.

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