Copper Removal by *Enterobacter cloacae* strain IrSuk1, *Enterobacter cloacae* strain IrSuk4a, and *Serratia nematodiphila* strain IrSuk13 Isolated from Sukolilo River-Indonesia

Wahyu Irawati\(^1\) and Candra Yulius Tahya\(^2\)

\(^1\)Department of Biology Education, Faculty of Education, Pelita Harapan University, Tangerang, Indonesia
\(^2\)Department of Chemistry Education, Faculty of Education, Pelita Harapan University, Tangerang, Indonesia

E-mail: w.irawati3@gmail.com

**Abstract.** Copper is one of the pollutants found in Sukolilo river due to industrial activities surrounding this area. The concentration of copper in this river has exceeded the threshold causing the death of organisms in the sea and various diseases. The presence of copper in the environment continually causes bacteria to develop copper removal mechanism by accumulation and biosorption so that copper removal occurs. Waste treatment by using indigenous bacteria is an effective and economical way to remove copper from the environment. This study aims to isolate and characterize bacteria from the Sukolilo River. Selected bacterial isolates were tested for copper accumulation and biosorption potential. The growth medium used was Luria Bertani with the addition of CuSO\(_4\). Bacterial identification was carried out based on the 16S rDNA gene. Bacterial resistance was tested by determining the Minimum Inhibitory Concentration (MIC) value. The ability of copper accumulation and biosorption is measured using atomic absorption spectrophotometer. The results of bacterial isolation from Sukolilo River have obtained three bacterial isolates with the MIC values of 9 mM CuSO\(_4\) and coded by strains IrSuk1, IrSuk4a, and IrSuk13. Each bacterial strain has high similarities with *Enterobacter cloacae*, *Enterobacter cloacae* strain subsp. dissolvens, and *Serratia nematodiphila* strain LMG 2683 respectively with a value of 96.68%, 98.31%, and 99.03%. Copper quantities accumulated by that three bacterial strain are of 0.96 mg, 0.85 mg, 1.89 mg per gram dry weight of cells, respectively. The efficiency of copper biosorption by strains IrSuk1, IrSuk4a, and IrSuk13 was 68.78%, 68.34%, and 68.47%, respectively.

**1. Introduction**

The increase in industrial activity in Surabaya has led to heavy metal contamination at the Sukolilo river, Surabaya. The heavy metal contamination that dominates the river is copper because it is often used in industrial activities around the area. Copper contamination exceeds the threshold and the river biota is no longer safe for consumption [1]. Copper is a pollutant that cannot be degraded and tends to be accumulated in the body of organisms, causing damage to the brain and nervous system and triggering the formation of tumors and cancer [2]. Copper contamination in the Sukolilo river area in Surabaya has not been taken seriously. Considering the magnitude of the impact of copper pollution on human life, it is necessary to handle cases of copper pollution at Sukolilo river, Surabaya.

Bioremediation is a promising method to remove copper from polluted environment. Some bacteria develop resistance mechanisms to survive in heavy metals contaminated habitat and
can be exploited for bioremediation purposes. The bacterial population in the contaminated area will develop an adaptation process to become resistant resulting in lower copper concentration in the environment. Some bacteria develop various strategies for survival in heavy metals polluted sites like biosorption, bioaccumulation, biomineralization, and biotransformation which can be exploited for copper removal [3]. Various microbes have effectively and efficiently acted as adsorbing agents for the removal of heavy metals including copper [4].

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Indigenous bacteria isolated from copper-contaminated areas are effectively used as a bioremediation agent for wastewater treatment and restoration of contaminated environment. Previous studies reported that some indigenous copper resistant bacteria had been successfully isolated from heavy metals contaminated sites in Indonesia. Mercury resistant bacteria of *Brevundimonas sp*. HgP1 was isolated from a gold mine in Pongkor village, Bogor [5]. Eight heavy metals resistant bacteria were isolated from industrial sewage in Kemisan river, Tangerang [6]. Copper resistant bacteria of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, *Cupriavidus* sp. IrC4 were isolated from industrial waste in Surabaya [7] [Irawati et al. 2012], while, *Klebsiella pneumoniae* CN1, *Acinetobacter calcoaliticus* CN2, *Escherichia coli* CN8 isolated from Cikapundung contaminated river, Bandung [8].

The adaptability of indigenous bacteria, when applied as a bioremediation agent in contaminated sites, was expected to increase the efficiency of the bioremediation process where the bacteria are isolated. The effectiveness of the bioremediation process depends on the ability of bacterial growth and metabolism during the bioremediation process [9]. Bacterial metabolism involves a bioaccumulation process that depends on the interaction between copper ions and negatively charged of surface cell [10]. Bacterial cell walls consist of many functional groups including carboxylate, hydroxyl, amino, and phosphate group that have a pivotal role for binding copper ions [3].

Exploration of copper resistant bacteria from Sukolilo River, Surabaya has not been widely carried out. This study aims to: 1) isolate copper resistant bacteria from the river of Sukolilo Surabaya, 2) carry out molecular characterization of bacterial isolates which refers to the isolate naming and the manufacture of phylogeny trees, 3) determine the effect of adding copper on the growth of bacterial isolates, and 4) determine to the ability of bacterial isolates to remove copper through bioaccumulation and biosorption processes. It was expected that the new copper resistant bacterial isolates as copper bioremediation agent candidates will be obtained to handle the problem of environmental pollution on the Sukolilo river.

### 2. Material and method

#### 2.1. Isolation and Selection of Copper Resistant Bacteria

The isolation of copper resistant bacteria was carried out by the spread method on the solid agar medium of Luria Bertani (LB) supplemented with copper sulfate (CuSO₄). Sukolilo river water samples were dissolved in sterile water with 100, 10⁻², 10⁻³, and 10⁻⁴ dilutions. One hundred microliters of suspensions from each dilution were spread on Luria Bertani (LB) Agar containing 3 mM CuSO₄ respectively, then incubated at 37°C for 24 hours. The growing colonies were selected, coded and then purified to obtain a single colony. Purification of bacterial isolates was carried out by taking a separate colony that was different and inoculated by streaking on a petri dish containing LB Agar containing 3 mM CuSO₄, then incubated at 37°C for 24 hours. A single colony was inoculated on an agar medium containing 3 mM CuSO₄ for further research.

#### 2.2. Determination of Bacterial Resistance to Copper

Copper resistance of bacterial isolate was carried out by determining the Minimum Inhibitory Concentration (MIC) value. Cell culture was inoculated on the agar LB medium containing
various concentrations of CuSO₄ by streaking plate method until there was no colonies growth on the medium. The initial concentration of CuSO₄ used was 2 mM. The CuSO₄ stock solution used was 1000 mM dissolved in sterile aquadest. The MIC value is the highest concentration of CuSO₄ which there is no colony growth on the medium after 48 hours of incubation at 37°C [9].

2.3. Measurement of Bacterial Growth at Various Copper Concentrations

The starter culture was made by inoculating one loop of the isolate culture into 5 ml of liquid medium then incubated until it reached the logarithmic phase in an incubator at 37°C. The starter culture (250 μl) was inoculated into 25 ml of liquid LB medium containing various concentrations of CuSO₄ and without CuSO₄. The cultures were incubated at 37 °C and observed every four hours by measuring cell turbidity (optical density/OD) using a LaboMed spectrophotometer at a wavelength of 600 nm.

2.4. Amplification of the 16S rDNA gene

The amplification of the 16S rDNA gene was carried out using primers derived from a conserved region of the 16S rDNA gene in *Escherichia coli*. Forward primer (F) was expected to amplify at position 20-43 while reverse primer (R) was at position 1482-1507. The primer concentration in solution was 10 μM. The DNA polymerase Tag enzyme used was Plantinum® Tag DNA Polymerase (Invitrogen no. Cat. 10966-018). The concentration of this enzyme was 5 units / μL, while the final enzyme concentration per reaction was 1.25 units / 25 μL. 1Ox Buffer PCR + MgCl₂ solution was prepared with a composition of 1 M Tris-HCl pH 8.3; 5 M KCl, 1% gelatin, and 35 mM MgCl₂. The final concentration of MgCl₂ per reaction is 3.5 mM. The PCR reaction mixture (25 μL) consisted of DNA template (100 ng), Forward primers (0.4 μM), reverse primers (0.4 μM), dNTP (200 μM), 1x PCR buffer, ddH₂O, and Taq DNA polymerase enzyme (5 U / μL). [9]

2.5. 16SrDNA gene Base Sequence Analysis

The 16SrDNA amplified product (amplicon) was then carried out by sequencing the nucleotide bases (sequencing). The 16S rDNA gene base sequence analysis was analyzed and edited using the ProSeq program [http://helios.bto.ed.ac.uk/evolgen/filatov/proseq.html]. The DNA base sequences of the isolates were then compared with the 16S rDNA gene library of other bacteria through the Basic Local Alignment Search Tool (BLAST) database tracking program to determine % similarity and identify bacterial isolates.

2.6. Phylogenetic Studies

Phylogenetic studies were carried out by analyzing the base sequence of the 16S rDNA gene for bacterial isolates through the CLUSTAL X sequence analysis program with the neighbor-joining method and 100 times bootstrap method. The dendogram is created through the Tree View program [http://taxonomy.zoology.gla.ac.uk/rod/rod.html].

3. Material and method

3.1. Isolation of Copper Resistant Bacteria

Three highly copper resistant bacteria were isolated from Sukolilo river and were designated as IrSuk1, IrSuk4, and IrSuk13, respectively. The bacterial isolates were resistant to 9 mM CuSO₄. Surprisingly, the copper resistance of bacterial isolates was the same with Pantoea agglomerans strain LMAE-2 [11]. Based on the category of copper resistance, the isolates IrSuk1, IrSuk4, and IrSuk13 were categorized as high copper resistant bacteria [12] Copper resistance of bacterial isolates was higher than that of indigenous copper resistant bacteria previously reported. *Pseudomonas* spp. isolated from industrial waste was resistant to copper with the MIC of 5 mM [13], *Acinetobacter pittii* strain IrC2, *Acinetobacter oleivorans* strain IrC2, *Cupriavidus pauculus* strain IrC4 isolated from industrial waste in Surabaya were resistant to 6-7 mM CuSO₄ [7] *Klebsiella pneumoniae* strain CN1, *Acinetobacter calcoaceticus* strain CN2, and *Escherichia coli* strain CN8 isolated from Cikapundung contaminated river were reported to be resistant to 8 mM copper CuSO₄ [8]. *Pseudomonas aeruginosa* and *Enterobacter cloacae* were tolerant to copper with concentration range from 2 mM to 5 mM, respectively [14].

Morphological characteristic of all bacterial isolates was white, irregular margin, a smooth and glistering colony. The colony elevation of isolate IrSuk1 was convex, while IrSuk4 and
IrSuk13 had flat elevation (Figure 1).

Figure 1. Colony morphological of copper resistant bacteria isolated from Sukolilo river.

All of the isolates IrSuk4a and IrSuk13 were characterized as Gram negative bacteria. Previous studies showed that, most of bacteria isolated from heavy metals polluted sites were Gram negative bacteria. All of seventeen metal tolerant bacteria isolated from mining lake in Malaysia [15] and discharged effluent Nigeria were Gram negative bacteria. Gram negative bacteria frequently were found in heavy metals contaminated sites and well known as a bioremediation agent [16]. Gram negative bacteria are reported more resistant than Gram positive due to the structure of the cell walls which having three layers of cell wall to immobilize heavy metals efficiently compared to Gram positive [17].

3.2. Molecular Characterization

Based on 16S rDNA gene analysis, Bacterial isolates IrSuk1, IrSuk, IrSuk13 had high similarity with Enterobacter cloacae, Enterobacter cloacae subsp. dissolvens strain LMG 2683, and Serratia nematodiphila DZ0503SBS1 with the similarity of 96.68%, 98.31%, and 99.03%, respectively (Table 1).

| No | Isolate code | Identification result | similarity (%) |
|----|--------------|-----------------------|----------------|
| 1  | IrSuk1       | Enterobacter cloacae   | 96.68          |
| 2  | IrSuk4a      | Enterobacter cloacae subsp. dissolvens strain LMG 2683 | 98.31          |
| 3  | IrSuk13      | Serratia nematodiphila DZ0503SBS1 | 99.03          |

Enterobacter sp. and Serratia sp. are reported as members of Enterobacteriaceae that are usually able to resist heavy metals and widely found in polluted environments [18]. Some previous studies reported that Enterobacter sp. and Serratia sp. were well known as indigenous heavy metals resistant bacteria. Enterobacter cloacae strain NZS isolated from mining lake in Malaysia was high copper resistant bacteria. This strain exhibited good growth in the high concentration of copper and zinc and also was able to oxidize iron. It was suggested that this strain developed a resistance mechanism against heavy metals encoded by a heavy metal resistance gene [15]. Enterobacter cloacae strain B1 isolated from polluted soil at Ghaziabad, India was resistant to lead, cadmium, nickel, and had high efficiency to remove lead [19]. Serratia spp. Isolated from sediment of uranium ore deposit, India was tolerant of uranium, copper, zinc, cadmium, and lead so it could be used for bioremediation agents in heavy metals contaminated sites [20]. Serratia sp. L30 was resistant to lead and a mixture of heavy metals [21]. Serratia marcescens isolated from soil of uranium deposit in India was resistant and able to remove uranium [22]. Serratia sp. isolated from Ganga river, India was resistant to copper [23]. Serratia liquefaciens produced intracellular protein that had a role for binding cadmium [24].

3.3. Phylogenetic Tree

Phylogenetic studies were carried out based on the 16S rDNA gene sequence from three bacterial isolates, namely IrSuk1, IrSuk4a, IrSuk13. The genetic relationship between the three
isolates and with 13 other different bacteria can be seen in Figure 4. Based on 16S rDNA gene sequence, it is quite interesting that isolates IrSuk1 and IrSuk4a have the similarity of 96.68% and 98.31% to *Enterobacter cloacae* in the Genebank data, respectively. Isolates IrSuk1 and IrSuk4a can be said to be the genus *Enterobacter* with different possible species because isolate IrSuk1 is more closely related to *Enterobacter hormaichei*, while isolate IrSuk4a is more closely related to *Enterobacter cloacae*. Meanwhile, isolate IrSuk13 had a high similarity of the 16S rDNA gene sequence to *Serratia nematodiphila* DZ0509SBS1, namely 99.03% and it was indeed in a different phylogenic branch with isolates IrSuk1 and IrSuk4a.

**Figure 2.** Phylogenetic tree of bacterial isolates IrSuk1, IrSuk4a, IrSuk13.

### 3.4. Copper Resistance of Bacterial Isolates

High copper concentration inhibited the growth of colonies on solid medium. The high concentration of heavy metals influences growth, morphology, and metabolic activity by decreasing the diversity and population of bacteria [25]. In a high concentration of 7 mM, colony morphology of isolates IrSuk1 and IrSuk4a showed a speck of dark color in the middle colony as a result of the accumulation of blue copper. The changes of colony morphology did not occur in isolates IrSuk13 which might be due to differences in genera between this isolate with isolates IrSuk1 and IrSuk4a. The 16S rDNA gene analysis shows that isolate IrSuk1 and IrSuk4a had the highest similarity with *Enterobacter cloacae*, while isolate IrSuk13 had highest similarity with *Serratia nematodiphila*. 

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*NR 125696:* Enterobacter hormaichei strain RECA 021 16S ribosomal RNA partial sequence

*NR 135211:* Enterobacter hormaichei strain RECA 022 16S ribosomal RNA partial sequence

*NR 112008:* Enterobacter hormaichei strain RECA 023 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 023 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 024 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 025 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 026 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 027 16S ribosomal RNA partial sequence

*NR 024042:* Enterobacter hormaichei strain RECA 028 16S ribosomal RNA partial sequence

*NR 0111988:* Enterobacter hormaichei strain RECA 029 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 030 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 031 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 032 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 033 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 034 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 035 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 036 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 037 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 038 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 039 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 040 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 041 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 042 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 043 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 044 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 045 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 046 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 047 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 048 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaichei strain RECA 049 16S ribosomal RNA partial sequence

*NR 042154:* Enterobacter hormaich
Figure 3. The growth of copper resistant bacteria in medium containing high copper concentration. A, B, C: IrSuk1; D, E, F: IrSuk4a; G, H, I: IrSuk13 in medium containing 5 mM, 6 mM, and 7 mM CuSO₄, respectively.

A dark speck in a middle colony of isolates IrSuk1 and IrSuk4a indicates that the bacteria developed the same copper mechanism by bioaccumulation as in Acinetobacter calcoaceticus strain IrC1, Acinetobacter pitii strain IrC2, Cupriavidus calcoaceticus strain IrC4 [7], and Pseudomonas syringae [26]. The colony of these bacteria becomes blue due to the accumulation of copper inside the cells. On the other hand, there is no speck in a middle colony of Isolate IrSuk13 due to a different copper mechanism beside accumulation might be with the secretion of exopolysaccharides for binding copper extracellularly. According to [27], another mechanism carried out by heavy metal tolerant bacteria is secretion of extracellular polymeric substances which bind metal ions from the solution.

3.5. The Effect of Copper Addition to Bacterial Growth

Figure 3 below shows that the addition of 2 mM influenced the growth of isolate IrSuk1, but it did not influence the growth of isolates IrSuk4a and IrSuk13. It clearly shows that the addition of 4 mM decreased the growth of the three isolates. Copper is a micro element needed by bacteria in low concentrations but is toxic at high concentrations because it inhibits enzyme activity or modifies the structure of the enzyme and inhibits metabolic processes [28]. Heavy metal toxicity can inhibit growth, and biochemical activity, as well as reduce bacterial biomass [29].
3.6. Copper Removal of Bacterial Isolates
Copper removal in this study was measured by bioaccumulation and biosorption ability of bacterial isolates. Isolates IrSuk1, IrSuk4a, IrSuk13 were able to accumulate 0.96 mg, 0.85 mg, and 1.87 mg copper per gram of cell dry weight on medium containing 2 mM, respectively. That this ability decreased in increasing copper concentrations might be due to the toxicity of copper in higher concentrations. The ability of isolates IrSuk1, IrSuk4a, IrSuk13 to accumulate copper in medium with addition of 4 mM were 0.45 mg, 0.74 mg, 1.86 mg copper per gram of cell dry weight. The maximal ability of these isolates to accumulate copper was identified in isolate IrSuk13. It was 1.87 mg per gram of cell dry weight. This was higher than that of *Pseudomonas stutzeri LA3* isolated from a copper mine which accumulated a maximum of 1.62 mg copper per gram cell dry weight [30]. On medium containing 3 mM, the ability of Isolates IrSuk1 and IrSuk4a to accumulate copper was 0.99 mg and 1.22 mg which was higher than that of *Pseudomonas* strain. In the same copper concentration, *Pseudomonas* strain was able to accumulate copper with an amount of 0.838 mg per gram cell dry weight [31].

Biosorption is a mechanism for the absorption of contaminants in the bacterial cell structure that is independent of metabolic processes [32]. The ability of the IrSuk1, IrSuk4a, and IrSuk13 isolates to remove copper through the biosorption mechanism in medium containing 2 mM were 60.31%, 59.60%, and 68.47%. Copper biosorption of isolates IrSuk1 and IrSuk4a increased to 68.78% and 68.34%, respectively, but this ability of isolating IrSuk13 decreased to 64.36%. The efficiency of heavy metal removal depends on the ability of bacteria to grow at high...
concentrations of heavy metals and the saturation of the binding sites on the cell surface to absorb heavy metals [33] The low concentration of metal ions in the solution increases the interaction between the binding sites on the surface of the bacterial cells and the metal ions resulting in increasing the effectiveness of absorption. On the other hand, the high concentration of ion metals inhibited the growth of bacteria and decreased the rate of metabolism causing reducing the number of active cells and binding sites of metal ions. The increase of metal ions decreased the effectiveness of metal removal due to the changes of biochemical and physiological characteristics of cells such as reducing of cells biomass and enzyme denaturation which has a role in binding metals ions causing the decrease of biosorption efficiency [30].

The maximal capability of isolates IrSuk1, IrSuk4a, and IrSuk13 to remove copper was an average of 68%. This copper removal capability was higher than that of previous studies. Enterobacter cloacae and Enterobacter aerogenes isolated from an industrial wastewater were able to remove copper with removal percentage of 62% and 34%, respectively [34]. Pseudomonas stutzeri LA3 isolated from a copper mine was able to remove 50% copper [30]. Cupriavidus taiwanensis TJ208 was reported to remove copper up to 19% [35], while copper removal by Pantoea agglomerans was reported to remove copper from marine sediment contaminated copper was 11.6% [11]. Pseudomonas aeruginosa and Enterobacter cloacae isolated from a copper mine were able to remove 30% and 20% copper from the pond of copper reusing process [14].

4. Conclusion
From this study, three high copper resistant bacteria isolated from Sukolilo Reiver were obtained with the MIC of 9 mM. Based on 16S rDNA, the three bacterial isolates were identified as Enterobacter cloacae strain IrSuk1, Enterobacter cloacae strain IrSuk4a, and Serratia nematodiphila strain IrSuk13. The bacterial isolates showed their ability to remove copper by the accumulation of 0.96 mg, 0.85 mg, and 1.89 mg copper per gram cell dry weight, respectively, and an average biosorption ability of 68%. Hopefully, the copper resistant indigenous bacterial isolates from Sukolilo river are easier to be applied as a bioremediation agent because it is isolated from the same habitat so that it is expected to be more adaptable in carrying out its role. Industry sites around Sukolilo river have to treat the copper waste through wastewater treatment plants using indigenous copper resistant bacteria before it is discharged into the waters.

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