Molecular and physiological characterization of indigenous copper-resistant bacteria from Cikapundung River, West Java, Indonesia

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Abstract. Irawati W, Ompusunggu NP, Susilowati DN, Yuwono T. 2019. Molecular and physiological characterization of indigenous copper-resistant bacteria from Cikapundung River, West Java, Indonesia. Biodiversitas 20: 344-349. The use of indigenous bacteria as bioremediation agents in wastewater treatments plant is a promising solution for tackling the environmental problem. The objectives of this research were to establish the phylogenetic tree and physiological characterization of copper-resistant bacteria isolated from Cikapundung River, West Java, Indonesia. A phylogenetic tree was constructed based on 16S rDNA sequences. Physiological characterization including growth and the potency of bacterial strain for copper accumulation and copper removal was also studied. Five highly copper-resistant bacteria designated as strains CN1, CN2, CN5, CN6, and CN8 have been isolated from Cikapundung river with the MICs (Minimum Inhibitory Concentration) of 5 mM-8mM. Sequence alignment and phylogenetic analysis showed that strains CN1 and CN6 belong to Klebsiella pneumoniae with the similarity of 99%. Meanwhile, strain CN2, CN5, and CN8 belong to Acinetobacter calcoaceticus, Acinetobacter sp. and Escherichia coli with the similarity of 99%, 96%, and 95%, respectively. The highest resistant bacterial isolates were strains CN6 and CN8 with the MICs of 8 mM. The highest accumulation capability was found in strain CN1 with a total of 4.62 mg/g dry weight of cells. Meanwhile, strain CN8 demonstrated the highest copper removal with the value of 48.15%.

Keywords: Copper accumulation and removal, copper-resistant bacteria, phylogenetic tree

INTRODUCTION

The disposal of heavy metals sewage due to industrial activities without processing into aquatic ecosystems has become a matter of concern in Indonesia. Cikapundung River as a part of Citarum River, West Java, Indonesia is well known as the heavy metals-contaminated site in West Java, Indonesia due to the increase of industrial activities in the surrounding area. The continuous waste disposal in aquatic environment enables heavy metals resistant bacteria to adapt and thrive in this area. Indigenous bacteria isolated from a heavy metals-contaminated site usually develop resistance mechanism to survive under stress condition and may potentially be used for bioremediation agent (Irawati et al. 2017).

The presence of heavy metals in sewage and wastewater is considered a major hazard to natural water, animals and human health. Heavy metal pollution threatens the life of the organism due to its persistence that becomes a complex environmental issue in the world. The most toxic pollutants in the environment are copper, zinc, lead, cadmium, nickel, and mercury (Dash et al. 2016; Rajbanshi 2008). The present study was focused on copper as copper is known as the highest contaminating heavy metal in Cikapundung River (Septiono et al. 2015).

Copper is a metallic element crucial for cell metabolism, however, at a high concentration above threshold level it becomes toxic for bacteria. This condition enables the bacteria to deliver copper inside the cell for micronutrient element to protect the cell against its toxicity. The widespread use of copper-containing products causes bacterial cells to be intensively exposed to copper. Some bacteria developed their adaptation mechanisms when they were exposed to toxic metals in a long time and selected them to form resistant bacteria. Under stress condition, bacteria developed resistance to face heavy metals pollutant by efflux outside the cells, transformation to a less toxic form, and bioaccumulation/biosorption (Williams et al. 2012; Bondarczuk and Piotrowska 2013).

Resistance mechanisms of bacteria to copper is useful for the application of bioremediation processes. Bioremediation is a process of utilizing bacteria for removing pollutant from the environment. Bioremediation is a natural process involving the capabilities of intrinsic bacteria to clean the environment through bioaccumulation and biosorption processes (Saranraj and Stella 2012). Thus, it was important to study the indigenous resistant bacteria isolated from a contaminated copper site for bioremediation purposes. The objectives of this research were to study the molecular and physiological characterization of copper-resistant bacteria isolated from Cikapundung River. It is anticipated that the new variant of indigenous copper-resistant bacteria maybe is used as a bioremediation agent in contaminated sites.
MATERIALS AND METHODS

Medium preparation and bacterial growth

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 with the addition of 2% of pure agar. The medium was autoclaved at 121°C, 1 atm, for 15 minutes before being used as growth medium. The stock solution of 1 M CuSO₄ was added to the autoclaved medium for preparation of medium containing copper. Medium without copper was also prepared as a control. Cells were grown in LB medium and incubated at 37°C with shaking at 150 rpm. Growth was monitored by measuring optical density at 600 nm using a spectrophotometer.

Isolation and characterization of copper-resistant bacteria

Isolation and characterization were carried out to obtain the highest copper-resistant bacteria. Wastewater sample was collected from the Cikapundu River, West Java, Indonesia. The samples were serially diluted as follows: 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰. Then, 100 µL volume of each sample dilution was spread on LB agar medium supplemented with various concentration of copper. After an incubation period at 37°C for 48 hours, colonies grew on medium with the highest level of copper were selected. Each colony was purified to obtain a single colony by streaking on an agar plate. The colony morphology of bacterial isolates was observed including the form, color, margin, and elevation of the colonies. The cells morphology and Gram staining result were also observed using a light microscope (Carl Zeiss).

Minimum inhibitory concentration of heavy metals of the isolate

Minimum Inhibitory Concentration (MIC) of the isolates to copper was determined by streaking the cells on the agar plates until it did not form a colony. The MIC was determined after 48 hours of incubation at 37°C.

Phylogenetic characterization of copper-resistant bacteria

A phylogenetic tree was constructed based on 16S rDNA sequences. Each isolate was grown in LB medium with shaking at 150 rpm and incubated at 37°C. Cells were collected by centrifugation at 13000 rpm for 2 min, and DNA was extracted by using the Wizard DNA genomic extraction kits (Promega). Quality and quantity of the DNA were determined by using nanodrops and gel electrophoresis on a 0.8% agarose gel. DNA amplification was carried out by PCR using universal primers in the position of 16S-27F (5’AGAGTTTGATCCTGGCTCAG3’) and 16S-1492R (5’GGTTACCTTGTTCAGACTT3’) primers. DNA sequences were edited and made contig between two sequences then the sequences were analyzed by using ChromasPro. The sequence results were aligned and the similarity of each base sequence was performed on the GenBank database by using the Classifier tool (http://rdp.cme.msu.edu) of the RDP-II database (Cole et al. 2009). Sequence alignment and phylogenetic reconstruction were performed using MEGA 6 software using Muscle (Tamura et al. 2013).

Copper biosorption and accumulation

Cells were grown in LB broth containing 3 mM of CuSO₄ and incubated at 37°C with shaking at 150 rpm. Cells were collected by centrifugation at 5000 g for 20 min to separate it from the growth medium. Cells were dried at oven 70°C for determination of dry weight and total copper content. Both cells and supernatant separately were disrupted with HNO₃ at 100°C. Copper content was determined by using atomic absorption spectrophotometer at 324.9 nm.

RESULTS AND DISCUSSION

Isolation and characterization of copper-resistant bacteria

Five highly copper-resistant bacteria designated as strains CN1, CN2, CN5, CN6, and CN8 have been isolated from Cikapundung River with the MICs (Minimum Inhibitory Concentration) of 5 mM - 8mM. Physiological characteristics of copper-resistant bacteria are shown in Table 1. All isolates were Gram-negative bacteria as reported in the previous study in Rungkut-Indonesia and Krishna-Godavari basin-Bengal. The majority of indigenous bacteria isolated from those sites were Gram-negative. Meanwhile, Keramati et al. (2011) explained that Gram-positive bacteria had no tolerance to heavy metals. Gram-positive bacteria that have been successfully isolated were only around 20% (Irawati et al. 2017; Gunaseelan and Ruban 2011). Gram-negative bacteria more resistant to copper than Gram-negative bacteria suggests that the outer membrane of gram-negative bacteria consists of a strong negative charge such as carboxyl, amine, hydroxyl, phosphate and sulphydryl groups which have function to bind positively charged cationic metals (Scott et al. 1990; Tortora et al. 2005).

It is shown that bacterial communities consisted of some different colonies grew on medium containing 4 mM of copper, suggesting that Cikapundung River was contaminated by the high concentration of copper (Figure 1). Most of the colonies were white, and the remaining was light yellow (Figure 2). Irawati et al. (2016) reported that the high level of heavy metals was very toxic for bacteria. High concentration of heavy metals decreased number and kind of bacterial growth and selected it to form resistant bacterial strains. According to Habi and Daba (2009), the high concentration of heavy metals above threshold levels has a deleterious impact on the microbial communities so each bacterium may develop resistance to survive in this condition. MIC test was done on selected single colonies to determine the level of copper resistance of each bacterial isolate. From this study, five copper-resistant bacteria were successfully isolated from Cikapundung River with the MICs of 7 mM-8 mM. Two highest copper-resistant bacteria were of strain CN6 and CN8 with the MIC of 8 mM.
Molecular characterization of copper-resistant bacteria

Molecular characterization of copper-resistant bacteria based on 16S rDNA showed that the isolated bacteria from Cikapundung River belonged to *Klebsiella pneumoniae*, *Acinetobacter sp.*, *Acinetobacter calcoaceticus*, and *Escherichia coli* (Figure 3).

Sequence alignment and percentage of homology showed that strains CN1 and CN6 belonged to *Klebsiella pneumoniae* with the similarity of 99%. Meanwhile strain CN2, CN5, and CN8 belong to *Acinetobacter calcoaceticus*, *Acinetobacter sp.* and *Escherichia coli* with the similarity of 99%, 96%, and 95%, respectively (Table 2).

Previous studies demonstrated growth characteristics of genus *Acinetobacter* and *Klebsiella* in medium supplemented with copper. Williams et al. (2016) reported that *Acinetobacter* sp. exhibited resistance to copper by increasing the growth in stationary phase as the adaptation mechanism in high concentrations of copper. Rensing and Grass (2003); Shah et al. (2014); Irawati et al. (2016) reported that *Escherichia coli*, *Klebsiella pneumoniae* strain IFCu4 and *Acinetobacter* sp. IrC1 were able to tolerate copper due to the presence of *copA* gene which expressed as a resistance mechanism.

Previous studies reported that *Klebsiella* sp., *Acinetobacter sp.*, and *Escherichia coli* were indigenous heavy metals resistant bacteria isolated from some contaminated sites. Three strains belonged to *Klebsiella* sp. isolated from diesel-polluted soils were resistant to chromium, cadmium, and copper (Aransiola et al. 2018). *Acinetobacter sp.* isolated from oil-polluted Aconcagua river showed resistance to copper or cadmium (Mendez et al. 2017). Meanwhile, *Acinetobacter sp.* IrC2 isolated from industrial sewage, Rungkut-Indonesia demonstrated multiple resistance to copper, cadmium, mercury, lead, and zinc (Irawati et al. 2015).

The presence of *Escherichia coli* and *Klebsiella* sp. in the samples obtained from the Cikapundung River suggests that, in addition to copper contamination, the river is also contaminated with fecal sewage. Despite the fact that *E. coli* isolated in this study demonstrated only 96% homology with the database, yet the presence of other fecal bacteria, *Klebsiella* sp. which is present in the gastrointestinal tract as *E. coli*, supports the observation that *E. coli* are also a common contaminating bacterium in the water body, particularly river in Indonesia.

**Growth characteristic of copper-resistant bacteria**

The growth of bacterial strain from Cikapundung in medium without copper and medium supplemented with 3 mM of copper demonstrated the best growth in medium without copper were strain CN6 and CN8 (Figure 4). Meanwhile, the best growth in medium containing 3 mM of copper was strain CN1. The presence of copper declined the growth of bacterial strain even though they experienced the log phase similar to that of in medium without copper. Brahmaprakash et al. (1988) reported that the ability of bacteria to reach the log phase suggested that the bacteria had resistance to copper. The ability to grow in specific copper concentration can be used to determine the MIC of the bacterial strain. In this study, strain CN1 demonstrated the best growth when it was cultivated in medium supplemented with 3 mM of copper. Other bacterial strains, however, showed comparable growth.

**Table 2. Phylogenetic characteristics of copper-resistant bacteria isolated from Cikapundung River, West Java, Indonesia**

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Species</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN1</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>99%</td>
</tr>
<tr>
<td>CN2</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>99%</td>
</tr>
<tr>
<td>CN5</td>
<td><em>Acinetobacter sp.</em></td>
<td>96%</td>
</tr>
<tr>
<td>CN6</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>99%</td>
</tr>
<tr>
<td>CN8</td>
<td><em>Escherichia coli</em></td>
<td>96%</td>
</tr>
</tbody>
</table>

**Figure 2. Colonies morphology of bacterial strains from Cikapundung River, West Java, Indonesia in medium containing 4 mM of copper.**

(A-E: Strain CN1, CN2, CN5, CN6, and CN8, respectively)
Figure 3. Phylogenetic tree of bacterial strain based on 16s rDNA sequences using the Neighbor-Joining method

Copper accumulation and copper biosorption

The highest accumulation capability was strain CN1 followed by strain CN6 with a total of 4.62 and 3.12 mg/g dry weight of cells, respectively (Figure 5). This result is consistent with Figure 5 in that, and strain CN1 was the best growth on medium containing copper. It was suggested that in this toxic condition, the strains developed resistance mechanism by secretion of exopolymer as a chelating agent for binding copper. Strains CN1 and CN6
had different characteristics of colony morphology compared to the other strains when grown on medium supplemented with 6 mM of copper as they showed slimmer colonies than others (Figure 5). According to Pushkar et al. (2015), exopolymer secretion is one of bacterial resistance mechanism to survive in heavy metals-contaminated sites.

Based on MICs test and their capabilities to accumulate copper, strains CN6 and CN8 were the two highest copper-resistant bacteria and copper accumulation. This result may be explained by the fact that its intrinsic characteristic capable of secretion of exopolymer contributes to the binding heavy metals as the resistance mechanism as presented in Figure 6. According to Vicentin et al. (2018), in some strains, the presence of heavy metals induced production of exopolymer for heavy metals complexation extracellularly. Kavamura and Esposito (2010) reported that some resistant bacteria produced exopolymer as a chelating agent for binding heavy metals as a form of adaptation and tolerance mechanism to reduce the toxicity of heavy metals.

The potency of bacterial strain for copper biosorption in medium containing 3 mM of copper is presented in Figure 7. Strain CN8 following by strain CN6 were the two highest bacterial strains for copper removal with a total of 48.15% and 36.78%, respectively. This is similar with physiological characteristics of bacterial strains in Table 1 that, strain CN6 and strain CN8 were the two highly copper-resistant bacteria with the MIC of 8 mM. Biosorption was reported to be a potential strategy for copper removal and reducing its concentration until reaching the accepted minimum concentration. Bacteria was reported to not only remove copper but also various types of heavy metals both in batch and continuous processes (Colak et al. 2013).

In conclusion, from this study, indigenous copper-resistant bacteria from Cikapundung River, identified as Klebsiella pneumoniae, Acinetobacter calcoaceticus, Acinetobacter sp., and Escherichia coli, were obtained. It is interesting to note that all four isolates demonstrating high copper resistance are Gram-negative bacteria. These bacterial strains are promising candidate as bio remediation agents as they highly resistant to copper and had capabilities to biosorp up to 48.15% and accumulate copper up to 4.62 mg/g dry weight of cells.

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REFERENCES


