

## Growth characteristics and copper accumulation of bacterial consortium *Acinetobacter* sp. and *Cupriavidus* sp. isolated from a wastewater treatment plant

WAHYU IRAWATI<sup>1,\*</sup>, TRIWIBOWO YUWONO<sup>2,\*\*</sup>, NOMMENSEN PANGIHUTAN OMPUSUNGGU<sup>1</sup>

<sup>1</sup>Department of Biology Education, Universitas Pelita Harapan, M.H. Thamrin Boulevard 1100, Lippo Karawaci, Tangerang 15811, Banten, Indonesia. Tel./fax.: +62-21-5460910, \*email: w.irawati3@gmail.com

<sup>2</sup>Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-563062, \*\*email: triwibowo\_y@faperta.ugm.ac.id

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**Abstract.** Irawati W, Yuwono T, Ompusunggu NP. 2018. Growth characteristics and copper accumulation of bacterial consortium *Acinetobacter* sp. and *Cupriavidus* sp. isolated from a wastewater treatment plant. *Biodiversitas* 19: 1884-1890. Pollutant treatments are part of the human calling, as the crown of creation, to subdue, preserve, and cultivate the earth in bringing goodness for all creatures. Bioremediation of copper using indigenous bacteria is well known as the best water treatment for polluted environment recovery. *Acinetobacter* sp. and *Cupriavidus* sp. are indigenous bacteria isolated from industrial sewage in Indonesia. Bioremediation in environment is a process involving community of bacterial consortium for heavy metal or any other polluting materials accumulation. The purposes of this research were: (i) to characterize growth of *Acinetobacter* sp. and *Cupriavidus* sp. consortia in sewage medium, enrichment medium, and medium supplemented with copper, (ii) to establish the potency of bacterial consortia to accumulate copper. The growth of bacteria was observed based on cell turbidity using spectrophotometer at wavelength of 600 nm. Cells pellet was destructed by nitric acid at 100°C and copper concentration was analyzed by atomic absorption spectrophotometer as copper accumulation value. The results showed that the growth of bacterial consortia in medium containing copper was better than that of single bacterium. The best bacterial consortium was the mixture of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. The use of sewage as cultivation medium decreased bacterial growth by up to 25% but still resulted in the same level of logarithmic phase in enrichment medium. The highest accumulation capability was of a consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 at a level of 6.45 g/mg copper/g cells dry weight, suggesting that 5.09% of copper were accumulated by cells. It was concluded that the best composition of consortia in growth and copper accumulation capability was the mixture of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. These results may be due to the fact that both bacteria belong to the same genus that allowed them for synergistic interactions.

**Keywords:** *Acinetobacter*, accumulation, copper, *Cupriavidus*, growth

### INTRODUCTION

God created all things in the earth, visible and invisible realms, gigantic and microscopic creatures – and God has put all creation on the earth under human care. So the concern and the engagement in the bacterial research are a part of human calling to have a deeper understanding on this matter for better caring of the earth and all the life in. Bacteria are living systems that demonstrate high adaptability to stress conditions. They can easily experience environmental changes by altering their genetic systems, transfer of genetic elements, and many other mechanisms to maintain the structure and function of the ecosystem (Ryan et al. 2009). Bacteria are known to survive in all condition of environments. They possess unique features such as small size, high surface area to volume ratio, and adaptability. With their features, bacteria enable to utilize organic pollutants as a carbon source and degrade it into non-toxic products. On the other hand, they develop resistance mechanism to inorganic pollutants such as heavy metals by efflux the metals outside the cells, transformation to less toxic, or accumulation them inside the cells (Williams et al. 2012).

Heavy metals contamination, as an impact of industrial activities, is an important issue in Indonesia. The disposal of heavy metals into aquatic environments become a serious problem because they are non-biodegradable and persistent in the environment (Sen et al. 2016). Metal pollution problem can become a threat to the ecosystem and human health. The most common metal pollutant in the environment is copper (Das et al. 2016). The persistent environmental problem could be solved by some technologies to remediate the environment. Recent developments of novel technologies involve multidisciplinary approaches by utilizing microorganisms for enhanced bioremediation capability (Niti et al. 2013). Bioremediation using indigenous bacteria is a promising method for heavy metals removal because of its capabilities to accumulate them inside the cells (Irawati et al. 2017a). Bioremediation is technology for controlling pollution by using biological system to catalyze the biodegradation or transformation processes of various toxic chemicals to less harmful forms. This natural process of bioremediation includes bioengineering and the capabilities of intrinsic microorganisms to clean the environment as an effective alternative to conventional remediation methods (Saranraj

and Stella 2012). The use of bacteria as bioremediation agent to treat copper-contaminated water requires the assessment of the effect of different copper concentrations on cell growth. Bacterial consortia are commonly applied in biological wastewater treatment because bacterial communities can easily adapt to environmental changes. Meanwhile, pure cultures bacteria are difficult to thrive in environment (Carpio et al. 2014). Bacteria in natural environments commonly exist as communities of multiple species. The Bacteria communities demonstrated more varied and complicated tasks than single bacterial species. (Brune and Bayer 2012). *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 were indigenous copper-resistant bacteria isolated from wastewater treatment plant in Surabaya, Indonesia. The purposes of this research were to study: (i) the growth characteristics of *Acinetobacter* sp. and *Cupriavidus* sp. as a pure culture and consortium in medium containing appropriate concentration of copper and in medium supplemented with wastewater both incubated at room temperature and 37°C, and (ii) the potency of the pure culture and bacterial consortia in accumulating copper.

## MATERIALS AND METHODS

### Bacterial consortia

Three high copper-resistant bacteria used for mixing bacterial consortia were *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 isolated from a wastewater treatment plant, in Rungkut-Surabaya. Bacterial consortia were formulated by mixing equal proportions of pure bacterial cultures. Consortium 1, 2, 3, and 4 consisted of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2; *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4, *Acinetobacter* sp. IrC2 and *Cupriavidus* sp. IrC4; *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4, respectively. The pure culture of each bacterial strain was used as a control. The consortia also were formulated by mixing three of the pure bacterial strains in a consortium (Irawati et al. 2018).

### Medium preparation and bacterial growth

Bacteria were grown in Luria Bertani (LB) agar containing the following (per liter of aquadest): tryptone 10 g, yeast extract 5 g, NaCl 10 g, and glucose 0.1 g. Medium containing copper was made by addition of 1 M CuSO<sub>4</sub> to the autoclaved media. LB agar was made with addition of 2% pure agar. Sewage-supplemented medium was made by using sewage from Cisadane River to replace distilled water as the solvent. The medium was autoclaved at 121°C, 1 atm, for 15 minutes before being used as growth medium (Irawati et al. 2016).

Bacterial culture of 1500 µL volume with the optical density of 0.6 (Colony 100 Forming Unit =  $1.2 \times 10^9$ ) was used for inoculating 50 mL LB medium. Cells were grown in LB medium supplemented with copper and incubated at 37°C and room temperature. Growth was monitored by

measuring optical density at 600 nm using a spectrophotometer. Cells cultivated in medium without copper also were observed as a control (Irawati et al. 2018).

### Copper accumulation

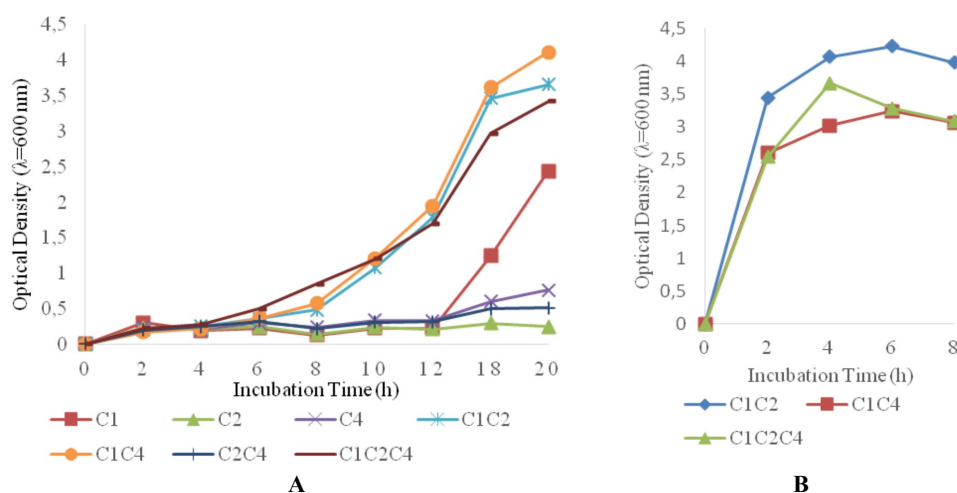
Cells were grown in medium containing various copper concentrations and incubated at 37°C or room temperature with shaking at 120 rpm. The cells were centrifuged at 5000xg for 20 min at 4°C to separate it into supernatant as growth medium and pellets as bacterial cells. Cells pellets were washed several times with copper-free phosphate buffer. Then, the cells were added by distilled water and were digested with HNO<sub>3</sub> at 100°C for measuring the ability of microorganisms in accumulating copper. The cells dry weight from the same culture was also determined. The copper content was determined by using an Atomic Absorption Spectrophotometer at 324.9 nm. All the experiments were done in triplicate to ascertain the accuracy of the results (Irawati et al. 2017a).

## RESULTS AND DISCUSSION

### Growth characteristics of bacteria as a pure culture and as a consortium

The ability of pure culture and bacterial consortia to grow in high concentration of copper were shown in Figure 1A. It showed that bacterial growth as a consortium was better than as a pure culture in medium containing 3 mM of copper. The pure culture demonstrated a lag phase longer than the bacterial consortia. Rolfe (2012) reported that heavy metals concentration impacted to the lag phase of growth of bacteria. Bacterial growth undergoes lag, logarithmic, stationary, and death phase. The lag phase involves slow growth and a period of acclimating which the bacteria is adjusting to a new condition in order to successfully grow. Irawati et al. (2017b) stated during the lag phase, the bacteria synthesized some proteins due to the resistance mechanism to face the toxicity of copper. After finishing the lag phase period, the bacteria continued the growth by entering the logarithmic phase.

The best bacterial growth as a pure culture was *Acinetobacter* sp. IrC1 followed by *Cupriavidus* sp. IrC4 despite the fact that it demonstrated a long lag phase for 12 hours (Figure 1A). *Acinetobacter* sp. IrC2 did not show the activity of growth during observation time. Surprisingly, it showed good growth when it was grown with *Acinetobacter* sp. IrC1 as C1C2 and when it was combined with *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 as C1C2C4. The lag phase of *Acinetobacter* sp. IrC2 as a consortium was shorter (4 hours) than the pure culture. The best bacterial consortia growth was the consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 followed by the consortium of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. Figure 2B showed the similar result that the best growth of bacterial consortia in medium without copper also was consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2.



**Figure 1.** The comparison of bacterial growth both as a pure culture and consortia. A = Pure culture and bacterial consortia in enrichment medium containing 3 mM of copper. B = Bacterial consortia in enrichment medium without copper. C1 = *Acinetobacter* sp. IrC1, C2 = *Acinetobacter* sp. IrC2, C4 = *Cupriavidus* sp. IrC4

This result suggests that the bacteria were more resistant to copper as a mixture of culture than as a pure culture. This result was similar to the previous study conducted by Latorre et al. (2016) which demonstrated that consortium had a greater capacity to resist copper compared to pure culture. Ilhan-Sungur et al. (2017) reported that the combination of different bacteria species is the important thing in this consortium. Each species of bacterium performed differences in metabolic processes that support the whole processes of bioremediation. Sannasi et al. (2006) stated the survival and stability of bacteria are better when they are present as a mixed culture. This is because each strain has significant differences both physiologically and metabolically and the varied responses and resistance that exhibited by each of them towards different metals. This condition would generate a dynamic, well-adapted and more resilient population through exchange of genetic material between the strains present to overcome toxicity of heavy metals. According to Subashchandrabose et al. (2011), bacterial consortium may provide robustness to environmental fluctuations, ability to share metabolites, and resistance to stress condition.

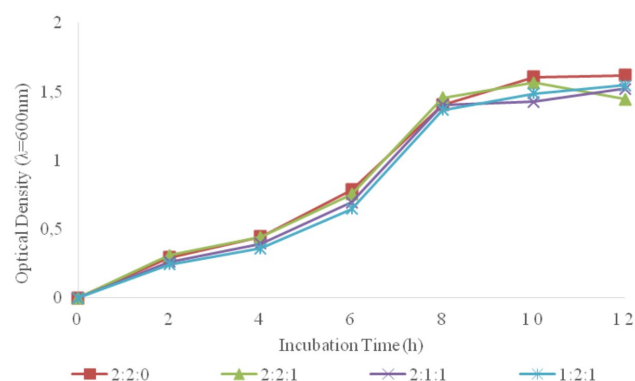
#### Growth characteristics of bacterial consortium with appropriate formulation

To obtain a better growth, three bacteria were combined in one consortium with appropriate composition of culture and cultivated them in a medium supplemented with copper. Figure 2 shows the best growth of consortium was *Acinetobacter* sp. IrC1; *Acinetobacter* sp. IrC2; *Cupriavidus* sp. IrC4 with composition of culture of 2: 2: 0. In the other word, the best consortia was *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 with the formulation of 2: 2. The other composition of bacterial consortia, however, also showed comparable growth. This result was different from the previous study in Figure 1 that concluded the best consortium when bacterial consortia are grown with formulation of 1: 1 was *Acinetobacter* sp. IrC1:

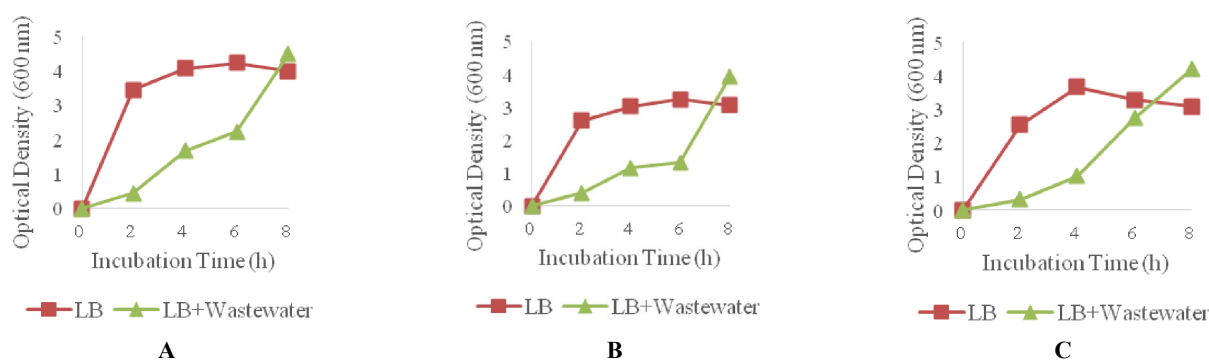
*Acinetobacter* sp. IrC2. It suggested that volume of bacterial culture influenced the activity of growth of bacterial consortia. Each volume of bacterial culture in formulation of 1: 1 and 2: 2 were 1500  $\mu$ L and 300  $\mu$ L, respectively.

#### Growth characteristic of bacterial consortia in enrichment medium supplemented with wastewater

Bioremediation occurs in nature by indigenous bacterial communities living in a contaminated area. Bioremediation using bacteria is usually applied in bioreactor filled with wastewater. Therefore, it was of interest to determine the best bacteria for bioremediation that result in good growth under this condition. *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 are well-known indigenous bacteria isolated from wastewater treatment plant in Surabaya (Irawati et al. 2012). Before using this bacterial consortium for bioremediation agent, it was of importance to establish the influence of wastewater as cultivation medium for cultivation of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 (Figure 3).



**Figure 2.** Characteristics of growth of bacterial consortia with appropriate culture composition in enrichment medium supplemented with 3 mM of copper. C1= *Acinetobacter* sp. IrC1, C2= *Acinetobacter* sp. IrC2, C4= *Cupriavidus* sp. IrC4.



**Figure 3.** The influence of sewage as cultivation medium for the growth of bacterial consortia. A= *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. B= *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4. C= *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4.

Figure 3 shows that the growth activity of bacterial consortia decreased when they were grown in wastewater medium. The results indicated that wastewater medium inhibited bacterial growth. The peak of logarithmic phase of bacterial consortia in an enrichment medium was reached faster than that of in an enrichment medium supplemented with wastewater. Bacterial consortia in an enrichment medium achieved the peak of logarithmic phase at the second of incubation time. Meanwhile, it occurred at the eighth of incubation time in medium supplemented with wastewater. Inhibition of growth in wastewater medium might be due to the fact that Cisadane River as a wastewater medium containing toxic effluent. Rochyatun (2006) reported that Cisadane River was contaminated by lead, zinc, copper, and cadmium.

#### Growth characteristic of bacterial consortia at room temperature and medium supplemented with copper

Bioremediation in nature usually occurs at room temperature, therefore it was of interest to establish the growth of bacterial consortia in medium containing 2 mM and 3 mM of copper incubated at room temperature. Figure 4 shows that room temperature did not inhibit the growth of bacterial consortia, but the inhibition occurs when they were grown in medium supplemented with 2 mM of copper. On the other hand, the bacterial consortia did not show growth activities when it was grown in medium containing 3 mM of copper. □

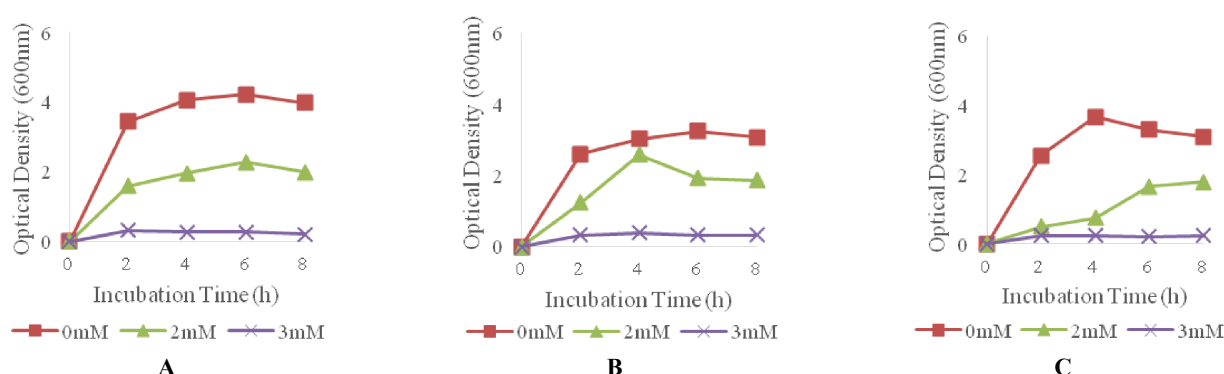
It was possible that the bacterial consortia could not survive in the elevated concentration of copper when it was cultivated not at the temperature optimum. Temperature is the important factor that influences metabolism activities. Previous study demonstrated that optimum temperature of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 was 37°C (Irawati et al. 2012). Metal concentration above threshold levels impacted functional activities completely inhibit various metabolic activities of bacteria. At the higher concentration, bacteria develop resistance mechanism to overcome in stress condition through its intrinsic properties such as metal bioaccumulation. Such property is important to improve the overall efficiency of treatment process in bioremediation (Ahmad et al. 2009; Habi and Daba, 2009; Rodrigues,

2011). Bioaccumulation mechanism includes precipitation, intracellular accumulation and oxidation or reduction. These processes are often associated with an active defense system and require longer response time due to the gradual transportation and accumulation within the cytoplasm after binding metals inside the cell (Unz and Shuttleworth, 1996).

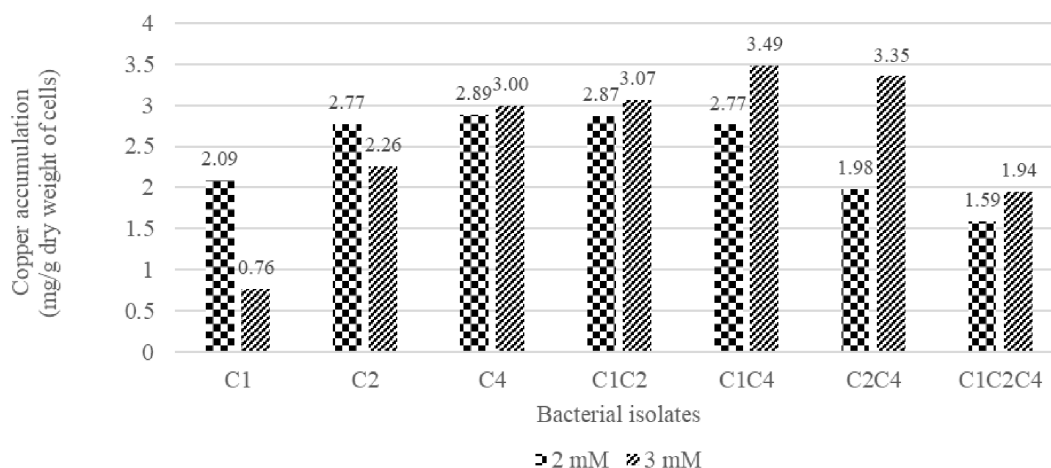
#### The potency of copper accumulation by bacterial consortia and pure culture bacteria

The potency of bacteria as a consortium and as a pure culture for copper accumulation in medium containing 2 mM and 3 mM of copper in incubation time of 37°C were shown in Figure 5. Figure 5 shows that in medium supplemented with 2 mM of copper, there were no significant differences between copper accumulation by the pure culture of *Cupriavidus* sp. IrC4 with the consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2, by the average of 2.88 mg/g dry weight of cells. Similar results were also observed between the pure culture of *Acinetobacter* sp. IrC2 with the consortia of *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 by the average of 2.77 mg/g dry weight of cells. On the other hand, in medium supplemented with 3 mM of copper, the amount of copper accumulated by the consortia bacteria was higher than by the pure culture bacteria except the consortium of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4.

The highest copper accumulation in medium supplemented with 2 mM was the consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 with the total amount of 2.87 mg/g dry weight of cells. Meanwhile, the consortia of *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 was the highest copper accumulator in medium containing 3 mM with the total amount of 3.49 mg/g dry weight of cells. It indicated that in medium containing 2 mM, the bacterial consortia only require one genus to respond copper which is the genus *Acinetobacter*. Whereas, in higher concentration, the consortia require the other genus that was more resistant than the genus *Acinetobacter*. Previous study demonstrated that *Cupriavidus* sp. IrC4 was more resistant than *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 (Irawati et al. 2012).



**Figure 4.** The influence of copper concentration and room temperature of incubation on bacterial consortia growth. A= *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. B= *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4. C= *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4



**Figure 5.** The potency of bacteria as a consortium and as a pure culture for copper accumulation in medium containing 2 mM and 3 mM of copper, and incubated at 37°C. C1= *Acinetobacter* sp. IrC1, C2= *Acinetobacter* sp. IrC2, C4= *Cupriavidus* sp. IrC4

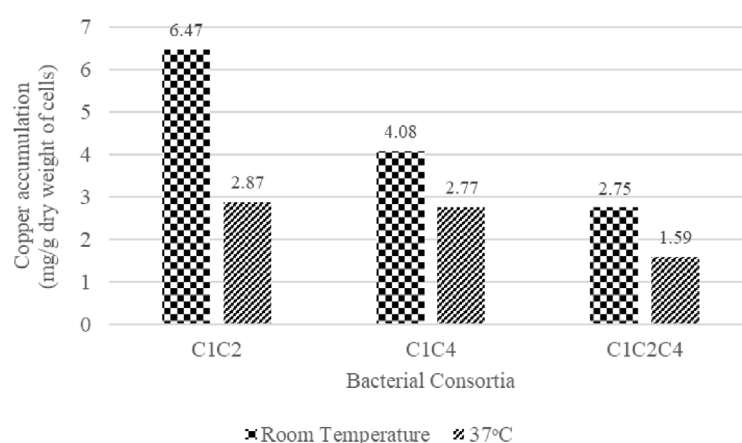
### The potency of copper accumulation incubated at room temperature

Comparison of copper accumulation by bacterial consortia incubated at 37°C and room temperature was shown in Figure 6. It is quite clear that the potency of bacterial consortia for copper accumulation incubated at room temperature was higher than that of at 37°C. Under both incubation treatment at room temperature and at 37°C, it was observed that the highest copper accumulation was obtained from the consortia of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 followed by the consortia of *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4, then the consortia of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. The consortia of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 was the highest copper accumulator when it was incubated at room temperature and at 37°C with a total of 6.45 mg and 2.87 mg/g dry

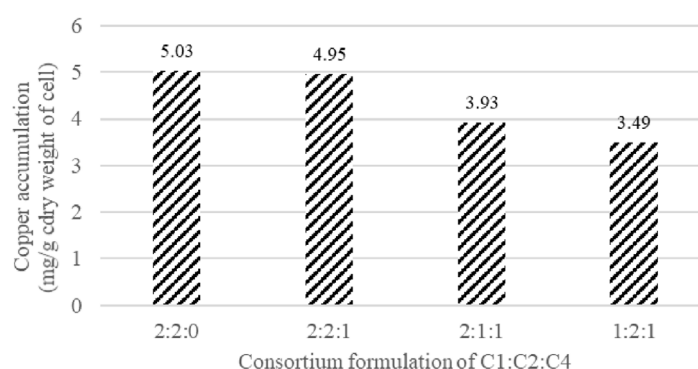
weight of cells. At room temperature, the bacterial consortia accumulate copper in medium containing 2 mM of copper higher than that of in 3 mM of copper. It might be due to the toxicity of copper in higher concentration would inhibit the growth and the ability in accumulating copper. □

### The potency of copper accumulation with appropriate culture formulation □

Bioremediation in nature occurs not only by single bacteria but by some bacteria forming a community with appropriate formulation. In this study, each pure culture of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, *Cupriavidus* sp. IrC4 was combined with appropriate formulation of culture as follows: 2: 2: 0; 2: 2: 1; 2: 1: 1; 1: 2: 1. Figure 7 shows the potency of copper accumulation of consortia bacteria with appropriate culture formulation in medium supplemented with 3 mM of copper. □



**Figure 6.** Comparison of copper accumulation in medium supplemented with 2 mM of copper and incubated at 37°C and room temperature. C1= *Acinetobacter* sp. IrC1, C2= *Acinetobacter* sp. IrC2, C4= *Cupriavidus* sp. IrC4



**Figure 7.** Copper accumulation of bacterial consortia with appropriate culture composition in medium supplemented with 3 mM of copper

□

Figure 7 shows that the highest copper accumulation was obtained from the consortia of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, *Cupriavidus* sp. IrC4 with the formulation of 2: 2: 0 followed by 2: 2: 1 with a total of 5.03 mg and 4.95 mg/g dry weight of cells, respectively. This result is consistent with previous study that the best growth and copper accumulation was consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. It might be due to the fact that both bacteria belong to the same genus that allowed them for synergistic interactions to enhance bioremediation processes. Ilyas et al. (2014) reported that bacterial consortia are important aspect that influences efficiency of heavy metal removal. According to Subashchandrabose et al. (2011), each bacterium in a consortium excreted organic matter base on synergistic relationship between the two species bacteria. The construction of consortia with desired partners serves a dual mission of pollutant removal and commercial production of microbial metabolites. Bacterial metabolites and its production improve the efficiency of bioremediation processes more than individual microorganism. □

In conclusion, bacterial consortium formulation is an important aspect that influences the growth and bioaccumulation efficiency of copper. It has successfully formulated bacterial consortium considering to the ability to grow and accumulate copper. *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 was the best consortium demonstrating resistance to copper and ability to accumulate copper. The highest number of copper accumulation by this consortium was 6.45 mg/g dry weight of cells when it was grown on to medium supplemented with 2 mM  $\text{CuSO}_4$  and incubated at room temperature. Olajire and Essien (2014) stated bioremediation required the cooperation of more than one single species of bacteria forming a consortium. The consortium is composed of many different bacterial species with broad enzymatic capacities that are required to increase the rate of heavy metal bioremediation. On the other hand, single bacteria can metabolize only a limited substrate. Bioremediation by bacterial communities depends on the composition of the community and its adaptive response to the presence of heavy metals. According to Hays et al. (2015), cultures that

consist of multiple bacterial species contain wide range of genes and metabolic capabilities in comparison to monocultures. Thus, bacterial consortia can more successfully be applied to overcome environmental problem by remediation than single bacteria. □

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