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The potential capability of bacteria and yeast strains isolated from Rungkut Industrial Sewage in Indonesia as a bioaccumulators and biosorbents of copper

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Abstract. *Irawati W, Parhusip Ajn, Christian S, Yuwono T. 2017. The potential capability of bacteria and yeast strains isolated from Rungkut Industrial Sewage in Indonesia as a bioaccumulators and biosorbents of copper. Biodiversitas 18: 971-977.* Heavy metal pollution is a serious problem as a result of industrialization due to the high production of wastewaters containing high concentrations of heavy metals. Wastewater contains microbial populations adapted to the toxic concentrations of heavy metals and becomes resistant by accumulating copper inside the cells. The aims of the study were to isolate yeast and bacteria from Rungkut Industrial sewage in Indonesia, and to examine the capability of these isolates to accumulate and biosorb copper. The copper resistance was determined by measuring minimum inhibitory concentration (MIC). The capability of isolates to accumulate and biosorb copper were determined by atomic absorption spectrophotometer. Biosorption is described as how much copper is removed from a growth medium with reference to the initial concentration. Of this study, nine bacterial strains and eight yeast strains were obtained with the MICs of 6-7 mM, and 16-20 mM $CuSO_4$, respectively. Afterward, we have successfully selected three bacteria and one yeast strains showing the highest copper resistance. The three bacterial strains, designated as C1, C2, and C4 were able to accumulate copper up to 29.93, 508.01, 371.42 mg/cell dry weight, respectively. While the yeast strain, ES9.3 was able to accumulate 0.52 mg/cell dry weight and reduce up to 82.32% copper concentration in the medium. The findings of this study indicated that yeast and bacterial strains were promising microorganisms for removal of copper.

Keywords: Copper accumulation, bacteria, biosorption, yeast

INTRODUCTION

Heavy metal pollution becomes a big concern in developed countries, as a result of industrialization, which produces large quantities of wastewaters containing high concentrations of heavy metals. The problem of heavy metal pollution is basically associated with: (i) acute toxicity linked with particular metals, even at a lower concentration; (ii) the characteristics of heavy metals which could not be degraded or destroyed easily and tend to circulate causing the long-lasting substantial remain in nature and tend to accumulate through the food chain, leading to the public health threat (Machado et al. 2009).

Copper is one of the toxic heavy metals contained in industrial sewage. Toxicity of copper is largely due to their presence in aqueous systems in the ionic formation, which is easily absorbed by living organisms (Andreazza 2010). According to Christian (2013), copper pollution in the east coast of Surabaya-Indonesia is mostly caused by untreated industrial sewage. High concentration of copper is also found in the fishes caught around the east coast of Surabaya, at concentration ranges from 2,290.20 ppb to 5,920.20 ppb. It is approximately two-five times higher than that standard of tolerance level set up by WHO regulation which is 1,200 ppb. This high level of copper in the contaminated fishes may pose a threat to the health of the population living in that area.

Bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its potential application in industry. Conventional techniques for removing dissolved heavy metals in terms of chemical precipitation, carbon adsorption, electrolytic recovery, ion exchange, chelation and solvent extraction or liquid membrane separation had several disadvantages such as a high cost, incomplete removals, low selectivity, high energy consumption and generation of uneliminated toxic slurries. Of these reasons, the potential ability of microorganisms in the environmental protection and the toxic heavy metal recovery is of new promising approach in the removal of metal ions (Zaki and Farag 2010).

In the contaminated environments which naturally contained toxic compounds, microorganisms had abilities not only to resist those toxic compounds but also to remediate them for their benefits (Guo et al. 2010). The high exposure of toxic heavy metals causes the cells of microorganisms develop resistance mechanisms and metal-ion homeostasis (Aspassia et al. 2007). Several scientific

reports stated that most indigenous microorganisms tolerate high concentrations of heavy metal and may play a significant role in bioremediation soil (Ge et al. 2009). Therefore, it is crucial to study the diversity of indigenous microorganisms in the contaminated sites of heavy metals. Indigenous microorganisms isolated under unfavorable conditions at industrial sites may provide new insight into its diversity. The aim of the study was to isolate, to select, and to characterize indigenous microorganism isolated from industrial sewage in Rungkut-Surabaya, Indonesia. The capabilities of indigenous microorganisms to accumulate and to biosorb of copper were also determined.

MATERIALS AND METHODS

Growth medium

Bacteria were grown in Salt Base Solution (SBS) broth containing the following (per liter): K_2HPO_4 1.5 g; KH_2PO_4 0.5 g; $(NH_4)_2SO_4$ 0.5 g; $Mg_2SO_4.7H_2O$ 0.2 g, supplemented with 5 g of yeast extract. Yeast was grown in YEPD broth consist of 1% yeast extract, 2% peptone, 1% dextrose. SBS and YEPD agar were made with the addition of 2% pure agar. The medium was autoclaved in 121°C, 1 atm, for 15 minutes before used for growth medium. The stock of 1M $CuSO_4{\cdot}5H_2O$ was added to autoclaved media.

Microorganism isolation

One hundred microliters sample of industrial sewage from wastewater treatment plant in Rungkut, Surabaya, Indonesia was inoculated onto agar plate medium supplemented with various concentrations of copper and was then incubated at 37ºC for 24 hours. The growing colonies were selected, and subjected to further purification by quadrant streaking method to obtain a single colony. Phenotypic characterization was conducted by analyzing cell morphology including the form, color, margin, elevation of the colonies. The morphology of the bacterial cells was observed by Gram staining followed by light microscope observation.

The effect of heavy metals on cell growth

Cells were grown in 25 ml SBS medium supplemented with copper and without copper. Cultures were incubated at 37°C. The cell growth was monitored by measuring optical density at 600 nm using a spectrophotometer. The morphological changes of the colony on medium containing various concentrations of copper and without copper were also observed.

Copper resistance

The resistance of microorganisms to copper was determined by Minimum Inhibitory Concentration (MIC). The copper resistance of bacteria and yeast were tested on agar plates by streaking method until the isolate unable to give colonies. The growing colonies at a given concentration were subsequently transferred to the next higher concentration. The MICs were determined after 48 hours of incubation at 37ºC.

Accumulation and biosorption of copper

Cells were grown in SBS broth containing various copper concentrations and incubated at 37°C with shaking at 200 rpm. Cells were centrifuged at 5000xg for 20 min at 4°C to separate it into supernatant and pellets. Cells pellets were washed several times with copper-free phosphate buffer. Each of the cell pellets and supernatants was digested with HNO_3 at 100°C for measuring the ability of microorganisms in accumulating and biosorp copper, respectively. The dry weight of the cells from the same culture was determined. The copper content was determined by using an Atomic Absorption Spectrophotometer at 324.9 nm. Heavy metals biosorption were calculated by subtracting concentrations of heavy metal added to media and the remaining of heavy metal in the media after cell growth.

RESULTS AND DISCUSSION

Microorganisms isolation

Nine bacterial strains and eight yeast strains have been isolated from industrial wastewater in Rungkut-Surabaya with the MICs of 6-7 mM and 16-20 mM $CuSO_4$, respectively (Table 1). Among them, we could select three bacterial strains and two yeast strains which showed the most resistant to copper. Afterward, the three bacterial and two yeast strains were designated as C1, C2, C4, and ES 9.3, ES10.2 respectively. These bacteria were resistant to copper with the MIC ranged from 3.1 to 4.7 mM (Altimera et al. 2012). The resistance of microbial isolates to copper might be due to the selective pressure of high pollutants in the industrial wastewater at Rungkut as the location of microorganism isolation. According to Bondarczuk and Piotrowska-Seget (2013), widespread use of copper-containing products has prolonged exposure of bacterial cells to copper which could be further used to select copper-resistant bacteria. Studies of bacterial strains which less resistant to copper have been previously reported i.e., *Sphingomonas* sp. and *Stenotrophomonas* sp. isolated from copper-polluted agricultural soils in Valparaiso region, central Chile. Ezzouhri et al. (2009) reported that the variation of metal tolerance might be due to the presence of different types of tolerance processes or resistance mechanisms exhibited by different strains.

Results showed that most of the bacterial strains isolated from the industrial wastewater were Gram-negative bacteria (Table 1). This result was similar to the previous study conducted by Brynhildsen et al. (1988) reported that the majority of isolates that undergo selection pressures in the presence of toxic compounds were Gram-negative bacteria whereas Gram-positive bacteria isolates accounted for only 20%. Keramati et al. (2011) reported that Gram-negative bacteria were more resistant to copper than Gram-positive bacteria. Each of Gram-positive bacteria isolated from dental clinic effluent had no tolerance to heavy metal. In addition, Adel et al. (2014) reported that *Bacillus* sp., *Pseudomonas* sp., *Chryseomonas* sp., *Burkholderia* sp., *Citrobacter* sp. and *Kluyvera* sp. isolated from secondary sewage consisted of Gram-positive

and Gram-negative bacteria with the percentage of 35.7 and 64.28, respectively. Of all these studies, it could be concluded that the resistance of Gram-negative bacteria to copper compared to that of Gram-positive bacteria might happen due to the different composition of the Gram-negative membrane. According to Tortora et al. (2005), the outer membrane of Gram-negative bacteria has some of the strong negative charge bounding to the heavy metals as a positive charge. This prevents the heavy metals to enter the cytoplasm leading to the resistance of Gram-negative bacteria in the high concentration of copper. Interestingly, the result also showed that yeast strains were more resistant than that of bacterial strains based on the ability to grow in medium containing a high concentration of copper which could be seen from MIC test (Table 1). This might be due to yeast strains are more adaptable than bacteria. Adnand et al. (2006) reported that yeast strains were more resistant than bacterial strains because yeast can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations. The growth of yeast strains in medium containing copper is presented in Figure 2.

The effect of heavy metals on yeast growth

The growth of yeast strain ES9.3 in medium supplemented with 5 mM, 10 mM, 15 mM of copper and without copper (control) were shown in Figure 2. The growth of yeast strain ES9.3 in medium containing 5 mM of copper and without copper addition were almost the same. It showed that the low concentration of copper until 5 mM $CuSO_4$ did not affect the growth of yeast strain. The presence of 10 mM of copper in medium decreased the yeast growth significantly while in a medium added with 5 mM of copper and without copper (control), the yeast strain required more time to perform lag phase. Interestingly, the yeast strain did not show the activity of growth in medium containing 15 mM of copper. It indicated that the high concentration of copper was toxic for the growth of yeast strain ES9.3. This is in line with Macomber and Imlay (2009) who reported that in low concentration, copper plays an essential role in biological processes but it becomes very toxic in high concentration. Copper degrades iron-sulfur which part of dehydratases clusters through iron displacement leading to the inactivation of these crucial enzymes which further caused oxidative damage inside the cells and resulted in the death of microorganism.

Table 2 shows that the addition of a copper concentration in the culture medium influenced the growth variety of bacterial strains. The result showed that eight bacterial strains have successfully grown in medium containing 2 mM $CuSO_4$ and without copper. It means that bacterial strain could still survive in the medium supplemented with copper concentration up to 2 mM. However, the number of bacterial strains decreased to seven strains when 3-4 mM were added in medium, and only three strains were able to grow in medium containing 5-6 mM $CuSO_4$. The three highly copper-resistant bacteria were isolated C1, C2, and C4 as shown in MIC test. It was concluded that the higher concentration of copper added in the growth medium reduced the number of the bacterial strain. According to Ahemad (2012), the higher concentration of metal lead to the reduction of total microbial biomass, the decreased number of specific populations, and changes in microbial community structure.

Table 1. The copper resistance microorganisms isolated from industrial sewage in Rungkut-Surabaya, Indonesia

Bacterial strains	Gram	MICs of bacterial strains (mM)	Yeast strains	MICs of yeast strains (mM)
A5	+	2.0	ES6.1	17
A6	+	3.0	ES8.1	18
A7	+	2.0	ES8.2	16
C1	-	6.0	ES9.3	20
C2	-	6.5	ES9.4	19
C4	-	7.0	ES9.5	18
C5	-	3.0	ES10.2	20
C8	-	3.0	ES10.4	18
D2	-	5.0		

Table 2. Variety of bacterial strain isolated from industrial sewage which was then grown into medium with various concentration of copper

Concentration of $CuSO_4$ (mM)	Isolate codes	Variety of isolates
0	A5,A6,A7,C1,C2,C4,C5,C8	8
1	A5,A6,A7,C1,C2,C4,C5,C8	8
2	A5,A6,A7,C1,C2,C4,C5,C8	8
3	A5,A6,C1,C2,C4,C5,C8	7
4	A5,A6,C1,C2,C4,C5,C8	7
5	C1,C2,C4	3
6	C1,C2,C4	3
7	-	-

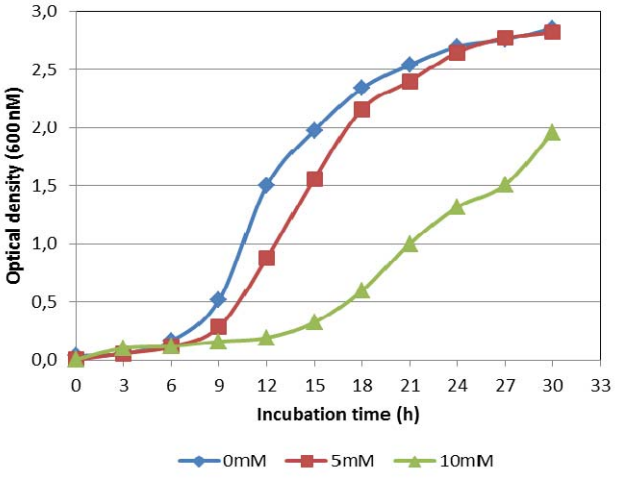

Figure 2. The growth curve of yeast ES9.3 isolated in medium containing 5 mM, 10 mM, 15 mM of copper and without copper

According to Adarsh et al. (2007), microorganisms have evolved several tolerant mechanisms in the presence of high concentration of heavy metals through bioaccumulation and biosorption mechanisms. Both live and inactivated microbial mass of bacteria, fungi and algae are utilized for removing toxic metal ions by these techniques. Figure 2 showed that the addition of copper in the medium caused the changes of colony color of the strains from white to green due to the accumulation of copper inside the cells. The result was similar to the previous study conducted by Irawati et al. (2012) who reported that *Acinetobacter* sp. IrC2 could accumulate copper and develop blue colonies on copper-containing media as a mechanism of copper resistance. The ability of this bacteria to resist copper was also depended on the sequestration and the accumulation of copper in the periplasm.

Figure 3 showed that the addition of copper sulfate in the medium resulted in a change of colony color of yeast strain from yellow to brown. This might be due to the survival mechanism of yeast strains by increasing the production of pigments in the cell wall in response to the pressure of adding heavy metals. A similar result was also reported by Ito et al. (2007) who mentioned that *Yarrowia lipoprosita* grown at high concentrations of copper sulfate produced chocolate pigments which could bind metals in cell walls.

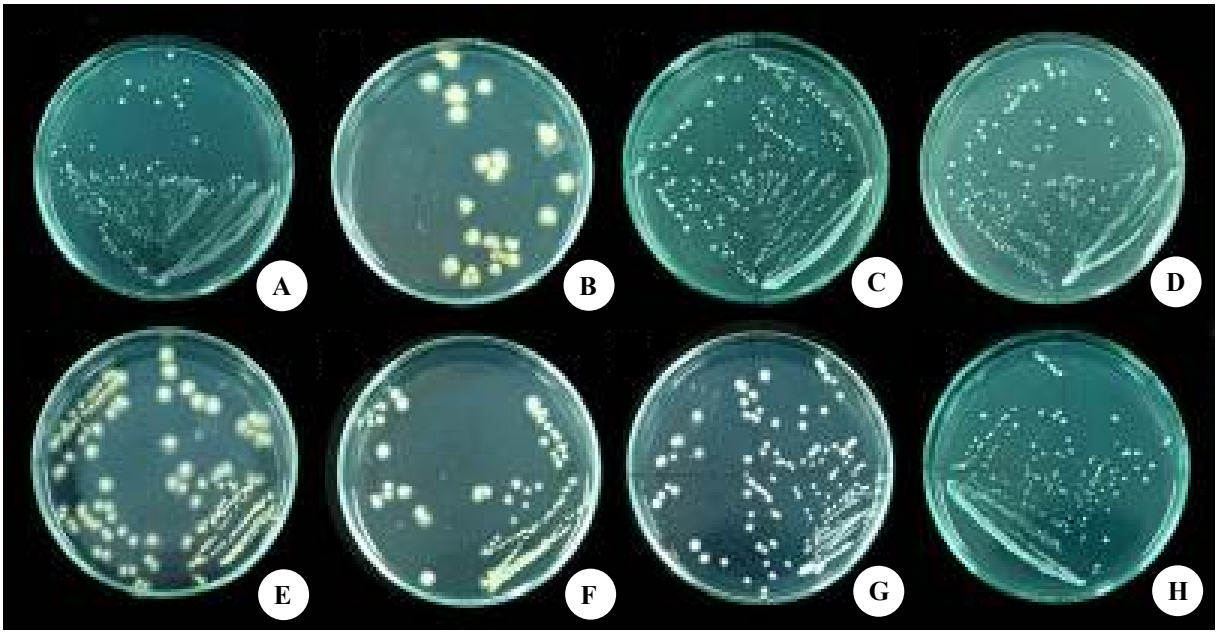

Figure 1. The growth of yeast strains in medium containing 5 mM of $CuSO_4$. A. Strain ES6.1; B. Strain ES8.1; C. Strain ES9.2; D. Strain ES9.3; E. Strain ES9.4; F. Strain ES9.5; G. Strain ES10.2; H. Strain ES10.4

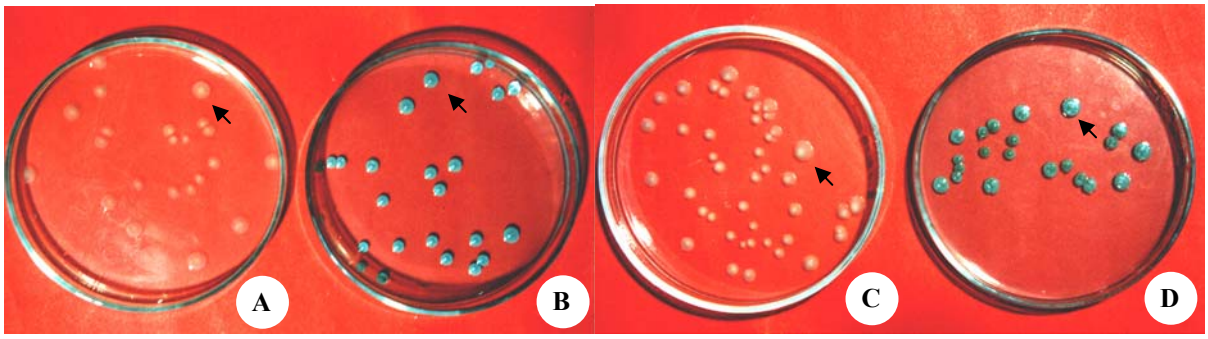

Figure 2. Colonies morphology as indicated by the arrow of bacterial strains on medium containing copper and without copper. Note: A, B: Isolate C1 in medium without $CuSO_4$ and with 5 mM $CuSO_4$, respectively; C, D: Isolate C2 in medium without $CuSO_4$ and with 5 mM $CuSO_4$, respectively

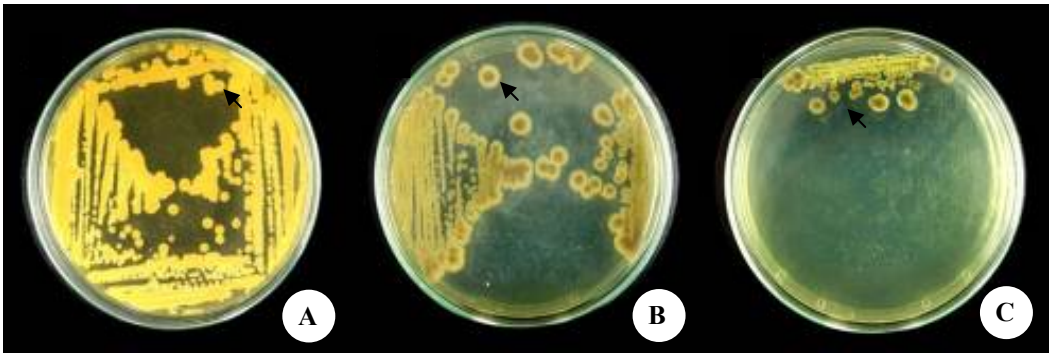

Figure 3. Colonies morphology as indicated by the arrow of yeast strains on medium containing various concentrations of copper. Note: A: without $CuSO_4$; B: 5 mM $CuSO_4$; C: 10 mM $CuSO_4$

Copper accumulation of bacterial and yeast strains

It is well recognized that microorganisms have a high affinity for metals and can accumulate heavy metals by a variety of mechanisms (Rehman et al. 2008). Figure 4 shows the capability of bacterial strains to accumulate copper. The result showed that the larger accumulation of copper in the cell of bacterial strains was in line with the increased level of copper concentration in the culture media which ranged from 5 mM to 6 mM of $CuSO_4$. The highest number of copper accumulation was obtained from C2 isolate followed by C4 and C1 with a total of 508.01; 227.24 and 123.71 mg/g dry weight of cells when they were grown on to medium supplemented with 6 mM $CuSO_4$. The cellular accumulation of copper obtained by isolate C4 apparently remained stable in medium containing 6 mM of copper. It indicated that in high concentration, C4 strain might use another mechanism before pumping copper out of the cells or extracellular sequestration resulted in the stable amount of copper accumulation. Furthermore, C4 strain might control its nutrient regulation in the stress condition (high copper treatment) by balancing the need of essential nutrients and the lethal nutrient excess for preventing cell death. In addition, colonies of C4 strain grown at a high concentration of copper seemed to be slimmer and produced more exopolysaccharides for extracellular sequestration. According to Gonzales et al. (2010), exopolysaccharides bound copper ions by integrating high electrostatic interactions and keep them to be trapped outside the cells. Although this mechanism appeared to be a passive and nonspecific process, it could be effectively protected bacteria against heavy metal toxicity.

The capability of yeast strain to accumulate copper could be seen in figure 5. The highest amount of copper accumulation of yeast isolates was 0.38 mg/g dry weight of cells grown in medium containing 10 mM $CuSO_4$ (Figure 5). This result was higher than those yeast isolates grown in medium containing 5 mM $CuSO_4$. Compared to the copper accumulation of bacterial strains, the yeast strains accumulated the lesser amount of copper. This result was interesting because of the fact that bacterial isolates were less resistant to copper than yeast isolates due to the difference of membrane structure between bacteria and yeast which could influence the capacity of copper accumulation. The outer membrane of observed bacterial isolates (C1, C2 and C4) whose categorized as gram negative bacteria, consists of lipopolysaccharides, lipoproteins, and phospholipids carrying a strong negative charge. This is because the cell surface of bacteria carries a net negative charge due to the presence of carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups. Therefore, it can adsorb appreciable quantities of positive charge of cationic metals (Tortora et al. 2005). On the other hand, the metal ion uptake in yeasts is known to be involved in the initial rapid binding of metal ions to the sites of negative charge on the cell wall. These yeast cell wall structures are multi-laminate, a microfibrillar structure consisting of up to 90% polysaccharide (biosorption), followed by a slower, energy-depending entry. Interestingly, the outer mannan-protein layer of the yeast cell wall and the inner glucan-chitin layer is taking important rules in the heavy metal accumulation (Donmez and Aksu 1999).

Yeast developed more complex biological resistance mechanism than bacteria. Resistance mechanism of yeast includes extracellular precipitation, complexation, and crystallization, the transformation of metal species, biosorption to cell walls, intercellular chelation by the generation of metallothioneins and phytochelatins, and metal localization/sequestration within vacuoles (Gadd 1992). Bacteria is a prokaryotic organism that develops a simpler mechanism of resistance than yeast. This simpler mechanism might cause bacterial isolates accumulate more amount of copper than yeast isolates. Copper resistance mechanisms in bacteria include extracellular and intracellular sequestration, enzymatic detoxification, and metal removal from the cell enabling them to survive in the presence of high concentration of copper (Bondarczuk and Piotrowska-Seget 2013).

Copper biosorption of yeast strain

Percentage of heavy metal biosorption efficiency using living cells of yeast ES9.3 was represented in Figure 6. Biosorption term has been used to indicate processes to remove the metal. Biosorption describes how much heavy metal was removed from solution concerning the initial concentration (Parungao et al. 2007). Biosorption percentage of the isolate increased in line with the increased

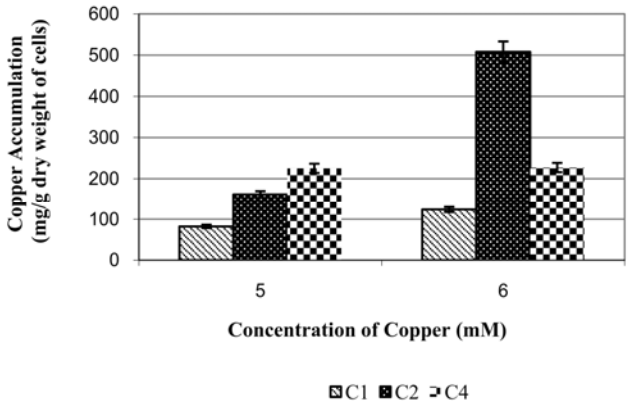

Figure 4. The ability of copper accumulation of bacterial strains grown into medium containing 5 mM dan 6 mM $CuSO_4$

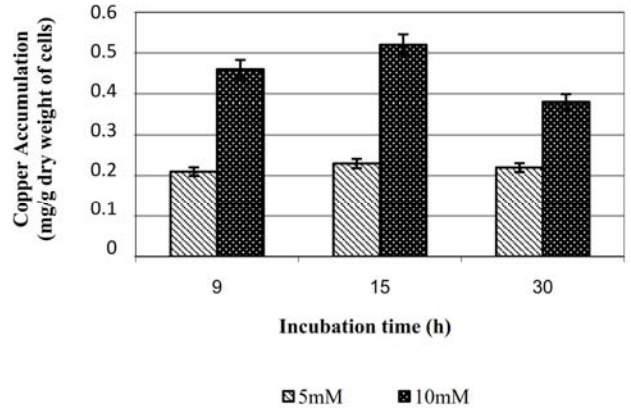

Figure 5. Copper accumulation ability of yeast strains on medium containing 5 mM dan 10 mM $CuSO_4$

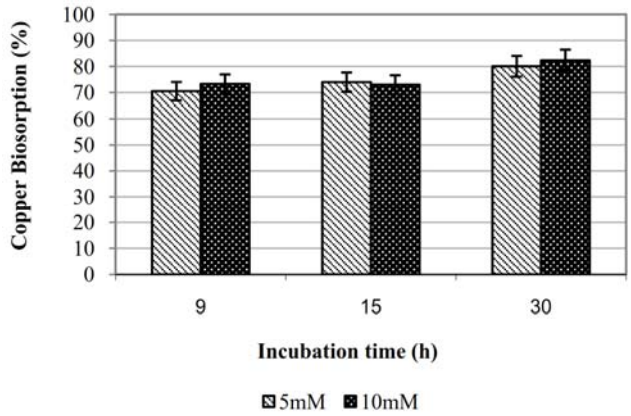

Figure 6. Biosorption of copper observed from an ES9.3 strain in a medium containing 5 mM and 10 mM of copper.

concentration of copper ranging from 5 mM to 10 mM. Yeast isolate ES9.3 was able to remove copper up to 80 % and 81% in medium containing 5 mM and 10 mM after 30 hours incubation period, respectively. This result was similar to the previous result in *Candida tropicalis* which reduced 82% of copper accumulation in medium (Rahman 2007). The uptake of metal biosorption occurred through interactions between native functional groups and the biomass cell wall (Goksungur et al. 2005). The mechanism of biosorption mainly involved ionic interactions and formation of complexes between metals cations and acidic sites in the cell wall of microorganisms (Oh et al. 2009). Biosorption is an important bioremediation process for copper removal and other heavy metals from the environments (Andreazza et al. 2011).

The capability of strain C1, C2, C4 to grow and to accumulate copper in the presence of high concentration of copper would be helpful in the waste water treatment where microorganisms are directly involved in biological processes for waste water treatment, because the inhibitory effect of heavy metals is a common phenomenon that occurs in the biological treatment of waste water and sewage (Filali et al. 2000). Genetic analysis of microorganisms from industrial sites may yield new information in relation to heavy metal resistance, which could be further exploited for bioremediation.

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