Removal of heavy metal (Cu2+) by *Thiobacillus* sp. and *Clostridium* sp. at various temperatures and concentration of pollutant in liquid media

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**Abstract.** This research was conducted to remove heavy metal Cu2+ using bioremediation method with the utilization of mix culture of bacterium *Thiobacillus* sp and *Clostridium* sp. It started with the cultivation of artificial liquid growth media, Stone Mineral Salt solution (SMSs) using temperature (ºC) and pollutant concentration of heavy metal Cu2+ (ppm) as the test parameters. The analysis was conducted with Atomic Absorption Spectrophotometry (AAS) to determine the concentration of heavy metal Cu2+ at the beginning and end of the research in order to calculate its removal efficiency. The exponential phase of mix bacterial culture growth was observed on day 5 with a pH value of 7. Furthermore, the culture was discovered to have the ability to live in several temperatures (ºC) of 20, 25, 30 and 35, and remove heavy metal Cu2+ at 89.10%; 91.27%; 92%; and 90.27% respectively. The results also showed that at a contact time of 48 hours, the temperature of 30ºC, and Cu2+ pollutant concentration of 25, 50, 75, 100 ppm, the mix bacterial culture could remove the pollutant up to efficiency above 90%. Moreover, a higher concentration of the pollutant in liquid media was found to lead to lower efficiency of removal, but at a value above 90%. Therefore, the use of biotechnology approach in this research is expected to give a scientific contribution to the processing of wastewater containing heavy metal Cu2+.

1. **Introduction**

Increased development of metal liquation, mining industries and those specializing in fuel, energy production, insecticide, as well as electroplating industries generates waste containing heavy metal [1-3] and the continuous discharge of this to the environment leads to serious threat for human and other living creatures in an ecosystem [4,5]. Pollution as a result of heavy metal results has been reported to present several risks to human survival and disturb ecologic balance [6-8]. However, an example of these metals with the potential to pollute the environment is the Copper ion Cu2+ [9-12]. It is one of the essential microelements for all living things [13,14], nevertheless, it is very toxic in high concentration [15-17]. Moreover, several types of heavy metals accumulate in the food chain to interrupt the growth of living creatures [18,19].

There are now some well-known ways to overcome pollution of heavy metal and they include chemical precipitation, filtration, ion exchange, electrochemical process, membranous technology, adsorption in active carbon, bioremediation, and phytoremediation [20-22]. However, bioremediation is the use of biological agents such as enzyme, plant or microbial cells to neutralize polluted land and...
water into non-dangerous substances for the environment and human health [23-28]. Successful bioremediation process is determined by factors such as temperatures, contact time between biological agents and pollutant, microbial concentration, oxygen, and value of pH [29-31], however, the contact time depends on the pollutant concentration [32,33]. Such that at higher pollutant concentration, microbe takes longer time to neutralize pollutant materials [34,35]. Furthermore, microbial concentration also affects the process of metal bioremediation such that a higher pollutant concentration requires a higher microbial concentration [36,37]. Acidity degrees (pH) or environmental temperatures (°C) have a large effect on microbial activity in solving wastes from heavy metals [38-40] with the process requiring from neutral to acidic levels. Moreover, the process of bioremediation can be conducted by relying on indigenous microbes or increased by adding exogenous microbes [41]. In addition, single or multiple (mix) cultures can also be utilized [42]. This method has been reported to be the most effective alternative to remove heavy metal of copper (Cu²⁺) [43,44] because it requires relatively lower cost and does not cause primary or secondary pollution [45]. Therefore, this research was conducted to remove heavy metal Cu²⁺ contained in liquid media by utilizing a mix culture of Thiobacillus sp. and Clostridium sp. bacteria and to also test its potentials to serve as an absorber.

2. Research methodology
This study consisted of the initial research phase which include the preparation of SMSs growth media and heavy metal Cu²⁺ pollutant, and cultivation of Thiobacillus sp and Clostridium sp bacteria, while the core research phase was conducted to remove the pollutants of heavy metal Cu²⁺ contained in the SMSs growth media through the use of Thiobacillus sp. and Clostridium sp. bacteria. Furthermore, Atomic Absorption Spectrophotometry (AAS) was used to analyze the concentration of heavy metal in the solution.

2.1. Preparation of Stone Mineral Salt solution (SMSs) growth media
Mix culture of Thiobacillus sp. and Clostridium sp. bacteria requires liquid growth media in a sterile condition. Therefore, the growth media used in this research was Stone Mineral Salt solution (SMSs) with 1 liter containing 0.5 gr calcium carbonate (CaCO₃); 2.5 gr ammonium nitrate (NH₄NO₃); 1 gr sodium hydrogen phosphate (Na₂HPO₄.7H₂O); 0.5 gr mono potassium nitrate (KH₂PO₄); 0.5 gr magnesium sulfate (MgSO₄.7H₂O); and 0.2 gr magnesium chloride (MnCl₂.7H₂O).

2.2. Preparation of heavy metal Cu²⁺ pollutant
The pollutant concentrations used in this study was in the variations of 25, 50, 75 and 100 (ppm). This was conducted to understand the effectiveness of mix cultures of Thiobacillus sp. and Clostridium sp. bacteria in removing heavy metal Cu²⁺ in various pollutant concentrations. Furthermore, artificial pollution was prepared by making the main solution of 1000 ppm with 1 gr CuSO₄.5 H₂O dissolved in 1 liter of distilled water while lower concentration was obtained by diluting the main solution.

2.3. Cultivation of Thiobacillus sp. and Clostridium sp. bacteria
Mix culture of Thiobacillus sp. and Clostridium sp. bacteria were obtained from the collection of Laboratory of Environmental Biology/Microbiology, Department of Environmental Engineering, Universitas Trisakti, Jakarta, Indonesia. It was first cultivated in SMSs growth media for 14 days until the exponential phase was reached at room temperature and pH value of 7. Bacterial growth in liquid media polluted by heavy metal Cu²⁺ was observed, based on the bacterial population through the use of Total Plate Count method and 1 ml of the sample was taken for analysis and count using the following equation:

\[
\text{Total colony/mL or gram} = \text{total colony/cup} \times \left( \frac{1}{\text{Factor of Dilution}} \right)
\]

Data collected were descriptive with quantitative numbers and the analyses are shown in the Table and graph.
2.4. Removal of Cu\textsuperscript{2+} in liquid media of Stone Mineral Salt solution (SMSs)

This research started by determining the optimum temperatures which were varied between 20, 25, 30 and 35 (°C) to understand the effectiveness of *Thiobacillus* sp. and *Clostridium* sp. to remove heavy metal Cu\textsuperscript{2+} at specific temperatures. The *Mix culture* was included in Erlenmeyer batch system containing liquid growth media of SMSs at a ratio of 1:1 and inserted in a shaker incubator at a 150-rpm rotational speed. The fixed variables were pollutant concentration of 100 ppm, pH 7 and contact time of 48 hours. After the optimum temperatures were obtained, the pollutant concentration of Cu\textsuperscript{2+} that the bacterial culture has the ability to remove was determined by testing the variations of 25, 50, 75 and 100 (ppm). Furthermore, *Atomic Absorption Spectrophotometry* was used to understand the concentration of heavy metal Cu\textsuperscript{2+} in pre-and-post research through the variation of temperatures and tested Cu\textsuperscript{2+} pollutant.

2.5. Removal efficiency of heavy metal Cu\textsuperscript{2+}

Removal efficiency of heavy metal Cu\textsuperscript{2+} was calculated using the following equation:

\[
\text{Removal efficiency (\%) = } \frac{C(a) - C(b)}{C(a)} \times 100\%
\]

C (a): initial concentration of heavy metal Cu\textsuperscript{2+} in liquid media (ppm)
C (b): final concentration of heavy metal Cu\textsuperscript{2+} in liquid media (ppm)

3. Results and discussion

Table 1 and Figure 1 show the removal efficiency of heavy metal Cu\textsuperscript{2+} (%) in various temperatures (°C) and the average value of 92% was observed at the optimum temperature of 30°C for the *mix culture* of *Thiobacillus* sp. and *Clostridium* sp. Furthermore, all the temperatures used were found to have produced removal efficiency above 85%.

**Table 1. Efficiency of heavy metal Cu\textsuperscript{2+} removal (%) in various temperatures (°C).**

| Various temperatures (°C) | Removal efficiency (%) | Standard deviation |
|---------------------------|------------------------|--------------------|
| 20                        | 89.10                  | 0.2100             |
| 25                        | 91.27                  | 0.2359             |
| 30                        | 92.00                  | 0.2545             |
| 35                        | 90.27                  | 0.2055             |

**Table 2. Efficiency of heavy metal Cu\textsuperscript{2+} removal (%) in various pollutant concentration (ppm).**

| Pollutant concentration (ppm) | Removal efficiency (%) | Standard deviation |
|------------------------------|------------------------|--------------------|
| 20                           | 96.00                  | 0.2210             |
| 25                           | 95.00                  | 0.2410             |
| 30                           | 94.00                  | 0.2320             |
| 35                           | 92.00                  | 0.2110             |

This research proved that the *mix culture* worked in synergy and was able to utilize heavy metal Cu\textsuperscript{2+} as a nutrient source in achieving approximately 90% removal efficiency. Environmental temperatures (°C) have a large effect on microbial activity in solving wastes from heavy metals [38-40].
Figure 1. Removal efficiency of heavy metal Cu$^{2+}$ (%) in various temperatures (°C).

Moreover, Table 2 and Figure 2 also the mix culture to have the ability to remove in both low concentrations of 25 ppm and high concentration of 100 ppm as reflected in the removal percentage above 90%. It was found that the efficiency of Cu$^{2+}$ removal in liquid media containing Cu$^{2+}$ of 25, 50, 75, and 100 (ppm) concentration was 96%, 94%, and 92% respectively. This shows a low concentration of Cu$^{2+}$ in liquid media to lead to the high removal efficiency, and vice versa. Interestingly, the results also indicate that, in Cu$^{2+}$ pollutant of 100 ppm, the mix culture of bacteria has a removal efficiency above 90%, which is the same with lower pollutant concentration. At higher pollutant concentration, microbe takes longer time to neutralize pollutant materials [34,35]. Furthermore, microbial concentration also affects the process of metal bioremediation such that a higher pollutant concentration requires a higher microbial concentration [36,37].

Figure 2. Removal efficiency of heavy metal Cu$^{2+}$ (%) in various pollutant concentration (ppm).

However, the previous research conducted showed mix culture of Viridibacillus arenose B-21, Sporosarcina soli B-22, Enterobacter cloacae KJ46, and E. cloacae KJ-47 bacteria to reduce heavy metal Cu$^{2+}$ only by 5% at a contact time of 48 hours [1].

4. Conclusions
Mix culture of Thiobacillus sp. and Clostridium sp. bacteria was very effective in reducing heavy metal Cu$^{2+}$ in liquid media of SMSs at 30°C, pH 7, and contact time of 48 hours. The highest removal efficiency was found to be 96% at 25 ppm and 92% at 100 ppm. This research found the exponential phase of mix culture growth of Thiobacillus sp. and Clostridium sp. bacteria occurred in day 5.
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