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Heavy metal removal from coal fly ash under alkaline conditions by indigenous bacteria isolated from a coal ash dumpsite

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ABSTRACT

Bioleaching is a process that uses microorganisms to remove heavy metals from waste materials, such as fly ash, electronic waste, low-grade ores, mine tailings, and spent catalysts. This study explored indigenous bacteria from contaminated environments to identify promising candidates for heavy metal bioleaching and investigate the underlying mechanisms. The bacteria were identified at the genomic level to determine their species, and bioleaching experiments were conducted to evaluate the effect of pulp density on heavy metal removal from fly ash using two bacterial species. The bioleaching efficiency, bacterial population, and other parameters were measured to analyze the bioleaching mechanisms. The results identified the bacterial isolates as *Bacillus toyonensis* and *Bacillus tropicus*. The addition of 5% (w/v) fly ash yielded the highest bioleaching efficiency for copper, zinc, chromium, and nickel. Among the two, *Bacillus toyonensis* showed the highest efficiency, with 63.62% for copper, 79.38% for zinc, 60.42% for chromium, and 52.26% for nickel. The bioleaching process occurred in an alkaline medium, with the pH shifting from neutral to alkaline during the experiment. In conclusion, the two *Bacillus* species effectively bioleached heavy metals from fly ash through biosorption, complexolysis, and redoxolysis mechanisms, similar to those found in other heterotrophic and autotrophic bacteria. However, it is important to note that bioleaching in this study took place in an alkaline medium, excluding acidolysis and complexolysis mechanisms that involve organic acids.

Keywords: *Bacillus*, bioleaching, coal fly ash, heavy metals, indigenous bacteria.

INTRODUCTION

Bacteria are commonly used in the remediation of contaminated environments as effective agents for bioremediation due to their metabolic processes and ability to adapt to various environments. Bioleaching has been used in the recovery and removal of metals on both small and large scales. Because it employs microorganisms as biological agents, bioleaching is a metal recovery and removal technique that is eco-friendly. It was proven that detoxifying contaminated environments is possible using bacteria. To obtain the best bacteria from various environments, bacteria have been isolated. The majority of the bacteria perform well in bioremediation. A study reported that the use of a consortium of indigenous bacteria is better than the exogenous bacteria (Joulian et al., 2020). Currently, a significant amount of coal ash waste in Indonesia accumulates in landfills for a long time. In 2021, the coal consumption in Indonesia's Steam Power Plants reached 120 million tons per year (PLN, 2021). Coal combustion produces coal ash, which make up 5–20% of the coal used (Yao et al., 2015). However, only about 0.4% of these by-products are utilized in Indonesia, leading to a substantial accumulation of coal ash in landfills (Mekkadinah et al., 2020). This provides an opportunity to isolate, identify, and apply indigenous bacteria from the waste and soil in the landfill site for the bioremediation of coal ash waste, such as fly ash.

Coal ash causes environmental contamination due to heavy metal content (Khan and Umar, 2018; Savic et al., 2018; Shetty et al., 2021; Wiyono and Wahyudi, 2018). Groundwater near a coal ash disposal site in India contains Fe (0.186–11.98 ppm), Cr (0.036–0.061 ppm), Ni (0.024–0.087 ppm), Mn

(0.013–0.178 ppm), and Pb (0.170–0.581 ppm), and most of them exceed the WHO quality standards, which vary for each metal (Verma et al., 2016). Additionally, the Kostolak Landfill in Serbia has a partial impact on the quality of the surrounding soil, with concentrations of Ni (35–210 mg/kg), Cu (19–190 mg/kg), and Cr (43–380 mg/kg) detected (Savic et al., 2018).

Metal extraction from fly ash has been widely used in the industrial sector, mostly by chemical methods. However, extraction using microorganisms termed bioleaching, also has the potential to be used in the removal or recovery of heavy metals (Pangayao et al., 2019; Park and Liang, 2019; Su et al., 2020; Su, Tan, et al., 2020). Bacteria have been used in the biological leaching of metals in waste and other solid materials (Chaerun et al., 2018; Sharada et al., 2021; Wang et al., 2019). Nevertheless, there is limited study about bioleaching heavy metals from coal fly ash under alkaline conditions using indigenous heterotrophic bacteria from coal ash dumpsites (Chen et al., 2021; Das et al., 2021; Ertit Taştan, 2017; Pangayao et al., 2018; Pangayao, 2016; Park and Liang, 2019; Su, Chen, et al., 2020; Su, Tan, et al., 2020).

Bioleaching using autotrophic bacteria has higher efficiency, but has a problem with acid because the process produces acid that could be problematic for the environment. Bioleaching under alkaline conditions can be a solution to that problem. However, the efficiency of bioleaching in some studies is lower than bioleaching under acidic conditions. In addition, alkaline pre-treatment of bioleaching has been considered to remove metal layers and expose the mineral surface to acidic bacteria (Ristović et al., 2022). The current study aimed to identify indigenous bacteria from a soil contaminated-coal ash waste at dumpsite and analyze the mechanisms of heavy metal removal from coal fly ash in an alkaline medium.

METHOD AND MATERIALS

Fly ash sampling

The sampling method in this study is the purposive sampling method. The fresh fly ash samples were taken from a coal-fired power plant in Lampung Province, Indonesia for analysis of heavy metals concentration and bioleaching experiments.

Metal analysis of coal fly ash

Fresh coal fly ash samples from a power plant were taken for metal analysis. Cu, Cr, Zn, Ni, Mn, and Hg were measured using atomic absorption spectrophotometry (AAS) AA-7000 Shimadzu.

Identification of bacteria isolates

Bacteria were isolated from previous research (Lisafitri et al., 2024). Identification of those bacteria was carried out through the stages of DNA extraction and PCR amplification. The DNA from bacterial isolates was extracted by centrifuging 3 ml of bacterial liquid culture in a sterile Eppendorf tube at 10.000 rpm for 15 minutes at room temperature. The supernatant was discarded, and 1 ml of TE buffer was added, followed by another centrifugation at the same speed and duration as before. The bacterial pellet was then resuspended in 50 µl of 30% tenderizer and incubated at 37 °C for 60 minutes. Next, 50 µl of 10% SDS was added and incubated again at the same temperature for 30 minutes. This was followed by centrifugation at the same speed for 30 minutes. The supernatant was transferred to a new sterile Eppendorf tube, and an equal volume of absolute alcohol was added, then gently inverted. It was centrifuged at 10.000 rpm at 4 °C for 15 minutes. The supernatant was discarded, 100 µl of cold ethanol was added, and the mixture was centrifuged again at 4 °C for 5 minutes at the same speed. The supernatant was discarded, and the DNA pellet was air-dried for 10 minutes before being dissolved in 100 µl of 1x TE buffer (Chen and Kuo, 1993; Puspitasari et al., 2014). 16s rRNA amplification of each bacterial isolate was carried using two universal primers 27F (5'-AGAGTTTGATCMTGGTCCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Marchesi et al., 1998; Zeng et al., 2008). The sequences obtained were analyzed for the regions with similar sequences using the Basic Local Alignment Search Tool (BLAST) to determine the percentage of base pair similarities with isolates contained in the gene bank (www.ncbi.nlm.nih.gov/blast) (Abanto et al., 2013; Fajriani et al., 2018). The results of bacterial identification were presented and analyzed descriptively.

Bioleaching experiment

This potency test was intended to test the ability of bacterial isolates in bioleaching Cu, Zn, Cr, and Ni in variations in the fly ash addition. There

are two factors in this experiment, namely: the type of bacteria and the amount of fly ash added to the bioleaching media. The bacterial isolates that were successfully isolated and selected were used in this stage. The variations of fly ash concentration, namely: 5%, 10%, and 15% (w/v) modified from (Su, Tan, et al., 2020). The addition of fly ash which shows the best bioleaching efficiency of Cu, Zn, and Cr will be used for the next research stage. This step was carried out by adding 10% (v/v) of each bacterial isolate into 100 ml of 9K media (Silverman and Lundgren, 1959) modified with 5 g/L glucose, and 1 g/L $FeSO_4$. The addition of fly ash (sterile) with variations of 5%, 10%, 15% (w/v), and control without bacteria, was replicated 3 times. Then, it was incubated for 21 days at room temperature, the conditions were modified from several studies (Chaerun et al., 2018; Guo et al., 2021; Pangayao et al., 2018; Su, Tan, et al., 2020). The bioleaching efficiency of Cu, Zn, Cr, and Ni, the exopolysaccharides production, and the bacterial population at the end of the bioleaching, also Fe^{2+}, and SO_4^{2-} concentration were measured at the end of the process. In turn, pH and redox potential were measured periodically every three days.

Bioleaching efficiency

Bioleaching efficiency is obtained by measuring the metal content in fly ash before and after bioleaching and calculating it with Eq. 1. After the bioleaching process, the liquid and solid phases are separated by filtering using 0.45 μm filter paper (Pangayao et al., 2019; Park and Liang, 2019; Su et al., 2020; Su et al., 2020). Separated solids were tested for metal content. To analyze the composition of Cu, Zn, and Cr, the fly ash residue was digested with HNO_3 and $HClO_4$ on a hotplate according to (EPA, 1992), then filtered with Whatman No.42 filter paper and then concentrations of heavy metals analyzed by AAS at different wavelengths according to the metal were measured. To calculate bioleaching efficiency, the following equation was used (Taştan, 2017; Park and Liang, 2019; Taştan et al., 2010):

$$Y\,(\%) = (C_o - C_t)/Co \times 100 \qquad (1)$$

where: Y – bioleaching efficiency or bioleaching percentage (%); C_0 – metal content in initial fly ash (before bioleaching) (mg/L); C_t – metal content in fly ash residue (after bioleaching) (mg/L). Bioleaching efficiency data were analyzed using ANOVA at α 5%.

Bacterial populations

The bacterial population in various treatment variations was measured after 21 days of incubation using the total plate count method (Cappuccino and Welsh, 2019). Three replications were performed for each treatment. Bacterial population measurements were carried out at the environmental engineering research laboratory, Bandung Institute of Technology (ITB).

Exopolysaccharide

Exopolysaccharide (EPS) production analysis was carried out by modifying the method used by (Tallon et al., 2003). Three replications were performed for each treatment. Bioleaching samples were taken in an amount of 5 ml and then centrifuged at 5000 rpm at 4 °C for 15 minutes. The filtrate or supernatant was taken and 2.5 ml of 10% trichloroacetic acid was added and shaken for 30 minutes at 100 rpm. Then, it was centrifuged at 5000 rpm for 20 minutes at 4 °C. The filtrate or supernatant containing exopolysaccharide was taken and added with 95% cold ethanol (2 times the supernatant volume, 10 ml) and left at 4 °C for 24 hours. The precipitate obtained was separated from the filtrate and then dried at 100 °C for 4 hours, where every hour the dry weight was weighed until constant. The dry exopolysaccharide content was determined using the following Equation:

$$EPS\ (\text{gr/L}) = \frac{Dry\ weight\ of\ EPS\ (gr)}{Sample\ Volume\ (L)} \qquad (2)$$

Surface tension

Surface tension measurements were carried out in the physical-chemistry laboratory, chemistry department, ITB. Measurements were carried out using a du Nuoy tensiometer (Fisher) (Du Nouy, 1925; Minucelli et al., 2017). Three replications were performed for each treatment.

Concentration of Fe(II)

Measurement of the Fe^{2+} concentration was carried out using a spectrophotometer. Fe^{2+} is analyzed through several stages of processing, which begin with making a standard Fe^{2+} solution, followed with standard series preparation (0 ppm; 1 ppm; 3 ppm; 5 ppm; 7.5 ppm; and 10 ppm), sample preparation, and measurement sample (Budianti et al., 2017; Solikha, 2018). 0.25 ml of sample was pipetted and then poured into a 25 ml

volumetric flask. Then, it was added with 1 ml of 5% hydroxylamine-HCl solution and heated; after it cooled, it was added again with 8 ml of 5% CH_3COONa solution and added with 5 ml of 1.10 phenanthroline. After the addition, it was then diluted and marked with distilled water. Finally, the absorbance of the solution was measured at the maximum wavelength obtained.

SEM mapping analysis

The biomass of bacteria from the highest efficiency bioleaching was analyzed using SEM SU3500 to obtain the information about heavy metals that adsorb on the surface of bacteria biomass. The analysis was carried out in the Research Center of Nanoscience and Nanotechnology, ITB. Before the analysis, the preparation of bacteria biomass is needed. After 21 days of incubation, the bioleaching media was filtered using a Whatman 42 to separate fly ash from the liquid media. Next, the liquid phase was centrifuged at 6000 rpm for 20 minutes to obtain the bacterial biomass (Etemadifar et al., 2018). Samples of each biomass type were transferred to a glass slide and then washed three times with 10 mM Tris-HCl buffer (pH 7.2), then fixed with 2.5% glutaraldehyde for 2 hours and dehydrated using ethanol solution (35, 50, 70, 95, 100%) each for 3 minutes. After that, it was dried in a vacuum oven at 30 °C. After sample preparation, SEM Mapping analysis could be carried out (Li et al., 2008).

pH and redox potential

The measurement of pH was carried out periodically using pH meter HANNA HI 8424. Redox Potential was measured at the end or the incubation period (after 21 days of incubation) for each treatment using HM Digital ORP-200.

RESULTS AND DISCUSSION

Metal content of coal fly ash

The results of metal content analysis in fly ash samples are shown in Table 1. In coal fly ash, Cr, Cu, Hg Nm, Pb, Zn, and Ni are found in a variety of concentrations. The highest concentration corresponds to Mn, followed by Zn, Cu, Ni, Pb, and Hg. Zn, Cu, Ni, and Cr were chosen as metal parameters in this study.

Identified bacteria isolates

On the basis of the results of the 16S rRNA identification ofit was found that the four indigenous bacterial isolates showed different species. After the bacterial DNA has been successfully extracted, it is then amplified using a primer to obtain a photo gel and DNA sequence for each bacterial isolate being analyzed, as shown in Figure 1. These two isolates were submitted to NCBI (SUB14984417).

The BLAST results from the gene bank (NCBI), indicated that isolate B2 has the highest percentage of similarities with *Bacillus Toyonensis* (99.13%), and *Bacillus thuringiensis* (99.13%). These bacteria are aerobic and have been studied

Table 1. Metal content of coal fly ash sample in this study

Element	Coal fly ash (mg/kg)
Cr	6.10–7.68
Cu	25.07–25.11
Hg	0–0.06
Mn	84.05–85.42
Pb	2.66–3.44
Zn	48.43–48.48
Ni	16.05–16.14

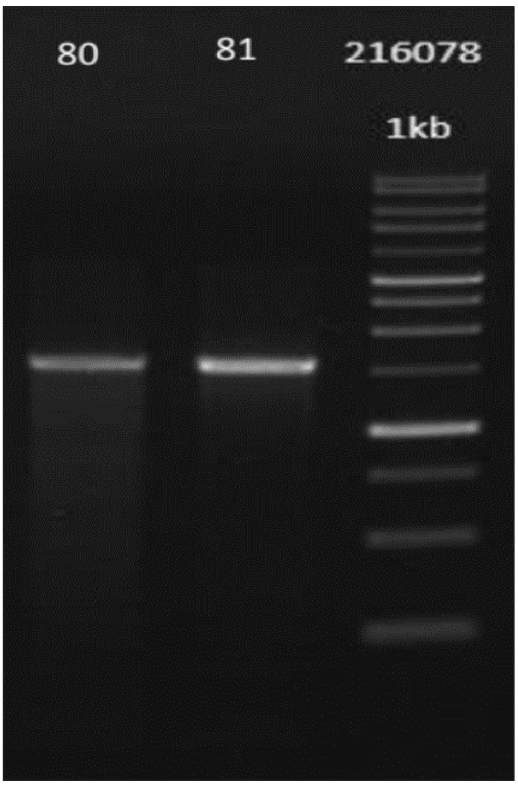

Figure 1. Gel Photo of Bacteria Isolates (80, B2, and 81, B4)

for their role in heavy metal biosorption (Mathew and Krishnamurthy, 2018; Oves et al., 2013; Pereira et al., 2020). However, this bacterium has not been widely studied in bioleaching applications. A phylogenetic tree based on 16S rRNA gene sequences showing the relationship between *Bacillus Toyonensis, Bacillus thuringiensis*, and species of the genus *Bacillus*. This species belongs to the phylum firmicutes and is the genus *Bacillus*.

The BLAST results from the gene bank (NCBI), indicated that isolate B4 has the highest percentage of similarities with *Bacillus tropicus* (98.75%), *Bacillus nitratireducens* (98.75%), *Bacillus paramycoides* (98.75%), *Bacillus luti* (98.75%) %), *Bacillus albus* (98.75%), and *Bacillus cereus* (98.75%). *Bacillus tropicus* is an aerobic bacterium and has been studied for its application in heavy metal biosorption (Barkusaraey et al., 2021). However, this bacterium has not been widely studied in bioleaching applications. A phylogenetic tree based on 16S rRNA gene sequences showing the relationship between *Bacillus tropicus, Bacillus nitratireducens, Bacillus paramycoides, Bacillus luti, Bacillus albus, Bacillus cereus,* and species of related genera. This species belongs to the phylum firmicutes and belongs to the genus *Bacillus*. The bar scale or close range is estimated at 0.008 nucleotide substitutions per sequence position.

Heavy metal bioleaching of coal fly ash in various bacteria and amount of fly ash added

The research to determine the potential of this bacterial isolate was carried out at the Environmental Engineering research laboratory, Bandung Technology Institute. The ability of each bacterium in bioleaching heavy metals is different. Bioleaching efficiency is one of the parameters to determine the ability of a bacterium in heavy metal bioleaching. Bioleaching efficiency is obtained from the difference in the measurement results of Cu, Zn, Cr, and Ni in coal fly ash before and after the bioleaching process.

On the basis of Figure 2, for Cu, the highest bioleaching efficiency is 63.62% in treatment

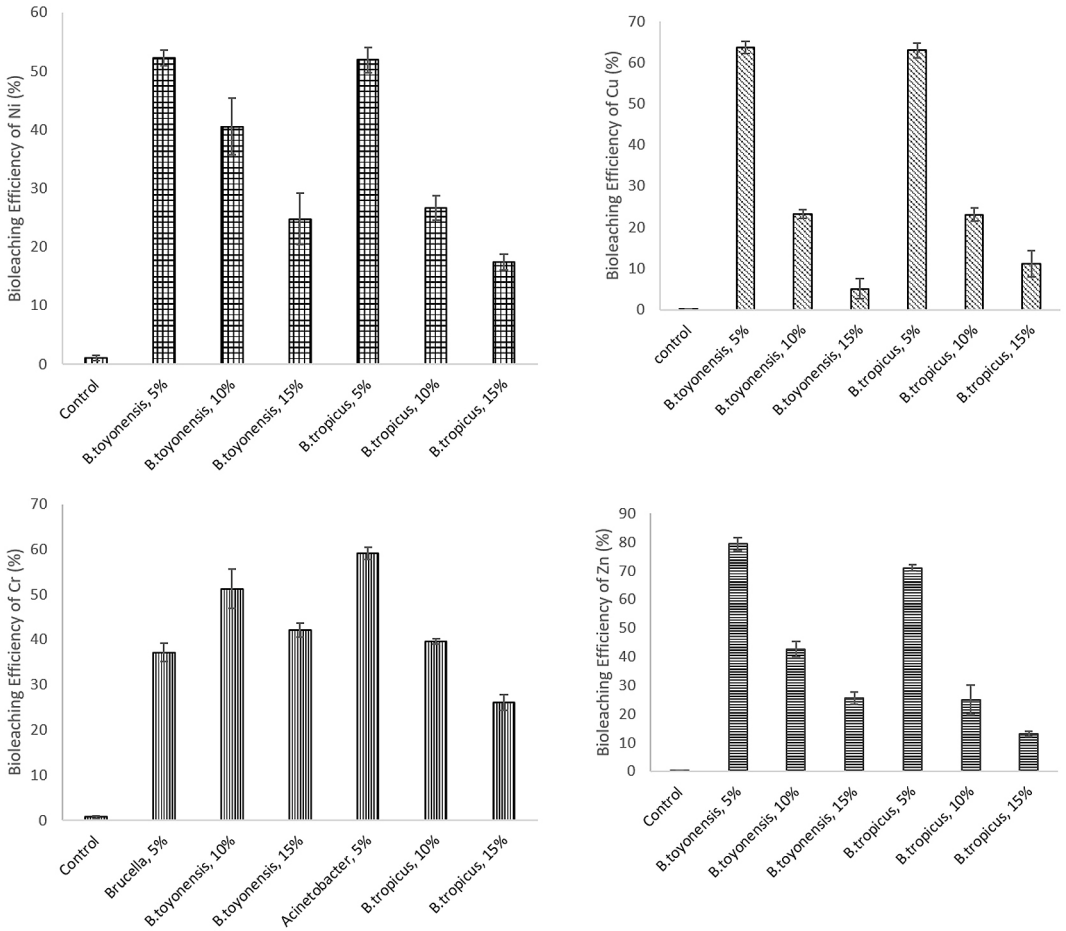

Figure 2. Bioleaching efficiency of Ni, Cu, Cr, dan Zn from coal fly ash using indigenous bacteria in various pulp density

with 5% fly ash (w/v) and *Bacillus toyonensis*. In turn, for Zn, the highest bioleaching efficiency is 79.38% in treatment with 5% fly ash (w/v) and *Bacillus toyonensis*. The highest bioleaching efficiency of Cr is 60.42% at 5% fly ash (w/v) and *Bacillus toyonensis*. Then, at 5% fly ash (w/v) and *Bacillus toyonensis*, the highest bioleaching efficiency of Ni is 52.26%. Most of the treatments show the lowest bioleaching efficiency with the addition of 15% (w/v) coal fly ash. The results show that in this study, the increase in the addition of fly ash to the media tends to cause a decrease in the value of bioleaching efficiency. In this study, *Bacillus toyonensis* shows a potential result for heavy metal bioleaching. The ability of bacteria in metal bioleaching varies, as in the other studies (Chaerun et al., 2018; Guo et al., 2021; Pangayao et al., 2018). The ANOVA results presented in Table 2 indicate the impact of various bioleaching treatments on the leaching efficiency of Ni, Cu, Zn, and Cr. The F-values for each metal are significantly high, and demonstrate that the applied treatments had a significant influence on bioleaching efficiency for all metals. This finding suggests that the bioleaching process used in this study is highly effective for recovering these metals.

The result of bioleaching in this study shows a promising potential for using these bacteria in heavy metal bioleaching. Table 3 shows the comparison of bioleaching efficiency or yield of some heavy metal between indigenous bacteria in this study and another study using various bacteria. Many factors affect the results of the bioleaching process, from the bacteria used in the process to the materials used in bioleaching. Thus, the results of bioleaching will vary between studies. Using *Bacillus toyonensis* in bioleaching needs further studies to determine the factors that affect the process so the yield can be optimized.

Bioleaching mechanisms

Generally, there are four mechanisms in bioleaching (redoxolysis, acidolysis, complexolysis, and bioaccumulation for fungi, and biosorption for bacteria). The mechanisms are shown in Figure 3 modified from (Dev et al., 2020; Tabak et al., 2005). In this study, it was proven that bioleaching of heavy metals using these two indigenous bacteria occurred through at least three of the four bioleaching mechanisms, namely redoxolysis, complexolysis, and biosorption). This is due to the acidity (pH) of the bioleaching medium tends to be alkaline.

The pH is in the range of 7.05 to 9.87 (Fig. 4). The initial pH of the media was 7 and during the bioleaching process, the pH tended to increase until the end of the bioleaching. This also occurs in metal bioleaching using heterotrophic bacteria, as in the studies of (Chaerun et al., 2018; Pangayao et al., 2019). From the value of pH, it can also be assumed that bioleaching with these indigenous bacteria may

Table 2. ANOVA of bioleaching efficiency

Ni				
Source	DF	Adj SS	Adj MS	F-Value
Treatment	5	3296.8	659.357	71.35*
Error	12	110.9	9.241	
Total	17	3407.7		
Cu				
Treatment	5	9828.25	1965.65	456.69*
Error	12	51.65	4.30	
Total	17	9879.90		
Zn				
Treatment	5	10911.2	2182.24	282.02*
Error	12	92.9	7.74	
Total	17	11004.1		
Cr				
Treatment	5	2515.01	503.003	86.35*
Error	12	69.90	5.825	
Total	17	2584.92		

Note: *different significant level at α 5%

Table 3. The comparison of bioleaching efficiency of heavy metal using various bacteria

No	The highest bioleaching efficiency	Material	Bacteria	Reference
1	Cu 63.62% Zn 79.38% Cr 60.42% Ni 52.26%	Coal fly ash (5% w/v)	*Bacillus toyonensis*	This research
2	Cu 31%	Copper sulfide ore (5% w/v)	*Alicyclobacillus* sp.	(Chaerun et al., 2018)
3	Ni 70.24%	Coal fly ash (10% w/v)	*Acidithiobacillus thiooxidan* + chemical treatment	(Su, Tan, et al., 2020)
4	Cr 13.77% Cu 14.61% Zn 12.18%	Coal fly ash (1% w/v)	*Pseudomonas* sp.	(Pangayao et al., 2019)

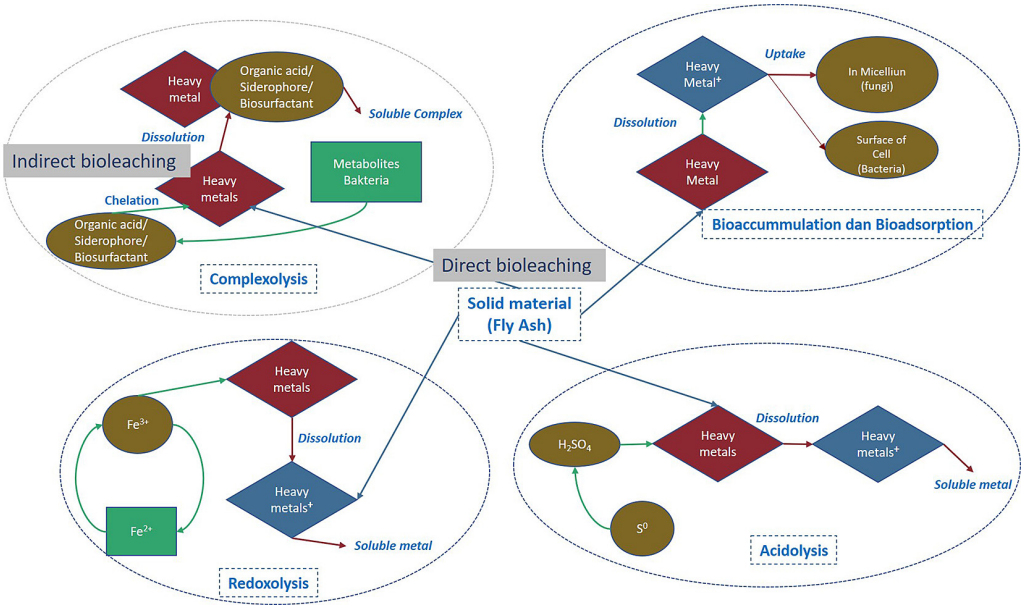

Figure 3. Bioleaching mechanisms of heavy metal

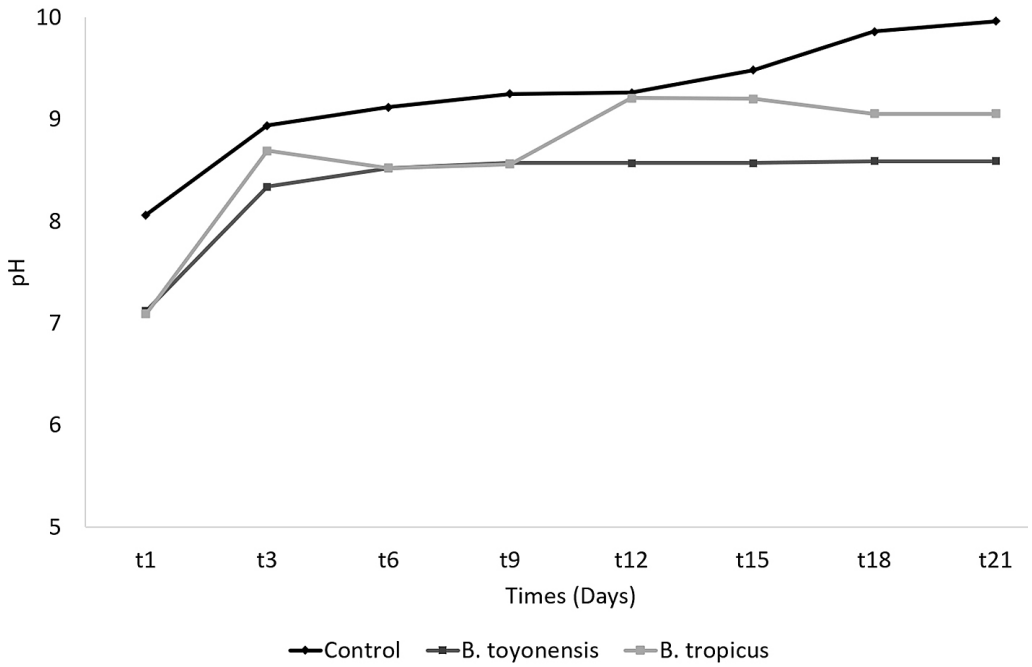

Figure 4. pH Average of bioleaching medium during bioleaching by indigenous bacteria

not occur through acidolysis and complexolysis mechanisms involving organic acids. This is because, one of the characteristics of the production of organic acids by bacteria is indicated by a decrease in pH during the bioleaching process, while in this study the pH tends to increase above 7.

Fly ash contains quite high CaO, 8.3–14.5% (Lisafitri and Kardena, 2023); thus, the pH of the media to which fly ash is added will experience an increase in pH. Apart from that, the fly ash has a high acid neutralization capacity (ANC) value of 337.88 kgH_2SO_4/ton (Said et al., 2020), therefore fly ash is categorized as NAF (NonAcid Forming) or has no potential to cause acid. If the MPA value is smaller than the ANC value, the NAPP value is negative (Said et al., 2020; Yacub and Suliestyah, 2020). This shows that the sample has more dominant neutralizing minerals than acid-forming minerals (Said et al., 2020). The increase in the pH value can also occur because buffers are not used in the bioleaching media, thereby allowing changes in the pH value. Under this pH conditions, metal precipitation in the liquid medium is possible when Cu, Cr, Zn, and Ni metals begin to be released from fly ash because Ni begins to precipitate at a pH of around 8, Zn begins to precipitate at a pH of 7.5–8, while Cu and Cr begin to precipitate at a pH range of 6 (Lewis, 2010; Rodriguez et al., 2007). In addition, the bacteria used are the bacteria that grow optimally at pH 7 to 9 (Chettri et al., 2019; Fatholahi et al., 2021; Moeini et al., 2022; Parhusip et al., 2020; Sandhu et al., 2022; Shi et al., 2011).

The first mechanism is redoxolysis, to prove the occurrence of this mechanism, measurements of the redox potential and Fe^{2+} concentration were carried out at the end of the process. The concentration of Fe^{2+} and redox potential relate to redoxolysis mechanism in bioleaching. In the bioleaching process, Fe^{2+} will be oxidized to Fe^{3+}. The use of a modified 9k medium by adding a carbon source allows oxidation to occur because $FeSO_4$ is added to the liquid medium. The redox potential in each treatment is between 40 to 90 and there is no significant difference between variables (Fig. 5).

In Figure 6, the concentration of Fe^{2+} shows decreasing in each treatment compared to the control (without bacteria). This is possible because Fe^{2+} will be oxidized to Fe^{3+} so that the Fe^{2+} concentration measured at the end of the process will be lower than the concentration at the beginning of the process as in the control. In this study, the role of indigenous bacteria in the bioleaching mechanism is not yet known. Through these two parameters, the potential of these indigenous bacteria in the redoxolysis mechanism can be seen. However, the value of redox potential and concentrations of Fe^{3+} tended to be low and the redox potential compared to other studies with *Alicyclobacillus* sp., is lower (Chaerun et al., 2018). On the basis of these results, it can be assumed that this mechanism is not a major mechanism in bioleaching using these indigenous bacteria.

In the complexolysis mechanism, metabolites produced by bacteria during the bioleaching process act as agents that can form complexations with heavy metals. In the complexolysis mechanism, metabolites produced by bacteria during the bioleaching process act as agents that can form complexations with heavy metals. Exopolysaccharides and surface tension are measured to detect metabolites produced by bacteria which can

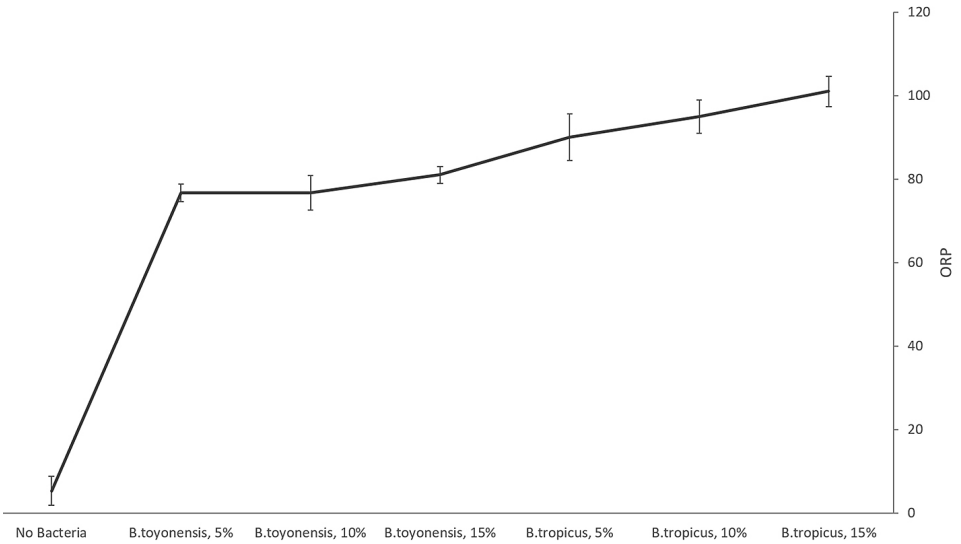

Figure 5. Potential redox (ORP) of bioleaching medium after 21 days bioleaching process

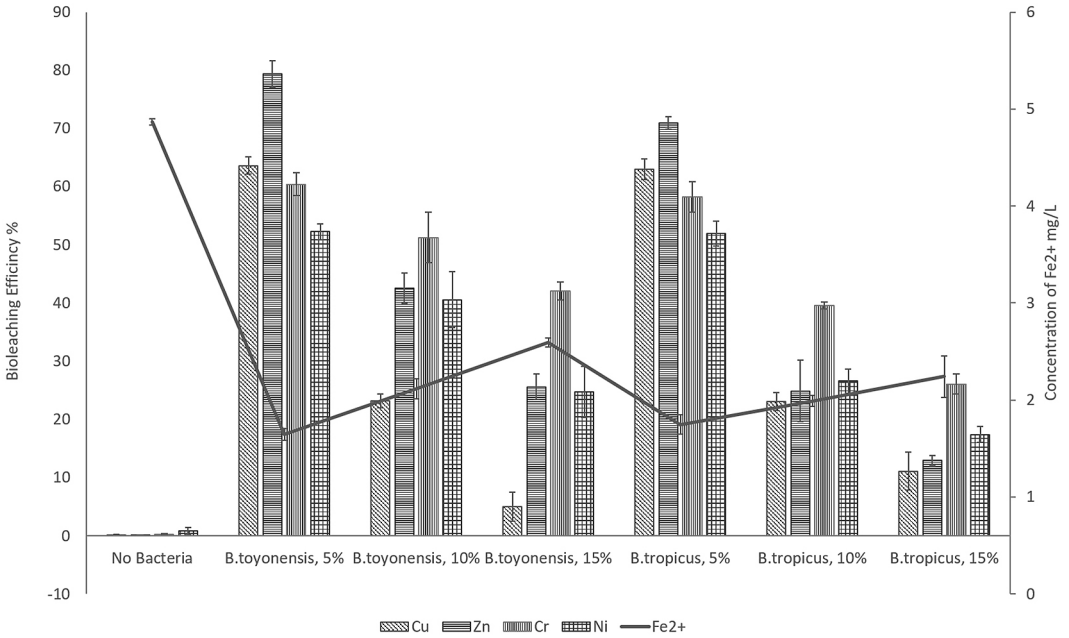

Figure 6. Heavy metal bioleaching efficiency and concentration of Fe^{2+} in bioleaching medium after 21 days of bioleaching process

indirectly prove the occurrence of this mechanism. Organic acids, siderophores, and biosurfactants are the metabolites that can form complexes with heavy metals. One of the metabolites produced by bacteria can be identified by measuring the exopolysaccharides produced because exopolysaccharides are all the polysaccharides synthesized by bacteria that are released in the extracellular cells. Bacterial cell walls and their secreted can help dissolve and precipitate various metals into different insoluble complexes (Muksy et al., 2023).

Biosurfactants used in the recovery of metals from solutions generally have an inhibitory effect on the leaching bacteria, mainly because of a decrease in the surface tension and reduction of the mass transfer of oxygen (Bosecker, 1997). Therefore, it is possible for the production of biosurfactants by the bacteria in this research. Figure 7 shows the value of surface tension tends to decrease from 63–65 dyne/cm at t_1 (7 days) to 48.23–54.77 dyne/cm at t_3 (21 days). The surface tension of each treatment using bacteria are lower than aquadest and medium

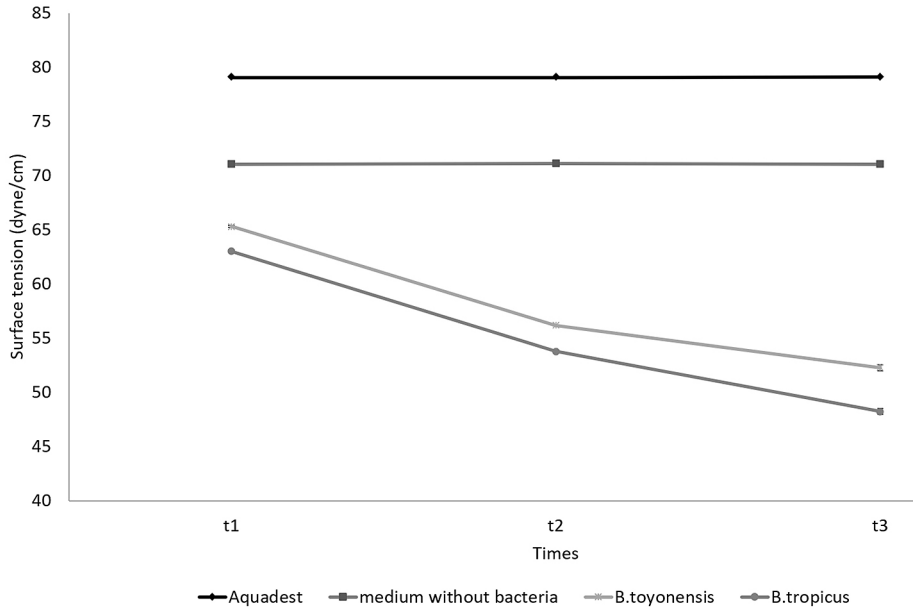

Figure 7. Surface tension of medium with addition of 5% w/v fly ash at 7, 14, and 21 days of bioleaching process

without bacteria. It indicates the presence of biosurfactants in the medium.

Exopolysaccharides can also be related to redoxolysis where the bioleaching efficiency value is also positively correlated with Fe dissolved in the media. This is because H^+ protons and dissolved Fe^{3+} concentrations are also strongly influenced by extracellular polymeric substances (exopolysaccharides) produced by bacteria (Chaerun et al., 2018). Figure 8 shows exopolysaccharide of the treatment in this study is low, and if it was compared to the other study using *Thiobacillus thiooxidan*, the value is 10–60 mg/L (Seidel et al., 2001). These results also show that exopolysaccharide is related to bioleaching efficiency because the highest exopolysaccharide with treatment 5% addition of coal fly ash and *Bacillus toyonensis*. More study is required for analysis of factors that affect exopolysaccharide synthesis for these indigenous bacteria. Another mechanism that occurs in the heavy metal bioleaching process using these four indigenous bacteria is biosorption. To find out whether this mechanism occurred or not in this study, bacterial population measurements and SEM-EDS analysis were carried out on bacterial biomass at the end of the bioleaching process.

Bacterial growth can be determined by measuring the bacterial population after the bioleaching process. Figure 9 shows that the highest bacterial population is 116×10^7 cfu/ml in using *Bacillus toyonensis* and 5% addition of coal fly ash treatment. The high value of bioleaching efficiency of Cu, Zn, Cr, and Ni was also followed by the high value of the bacterial population, especially in the treatment with the addition of coal fly ash. Each bacterium has a different tolerance to the surrounding conditions which will affect its growth under these conditions. This also affects the bioleaching process. The presence of fly ash in the bacterial growth media will certainly affect the growth of these bacteria, as happened in this study. However, some isolates showed better tolerance than other isolates.

On the basis on bacterial population data, was is proven that bioleaching efficiency is related to bacterial populations. The more bacteria that grow, the more metal will be leached from the fly ash. On the basis of on this statement, SEM Mapping analysis is needed to determine whether there are heavy metals in the bacterial biomass in this study. This can be expected to prove the mechanism of metal accumulation through metal binding and adsorption by bacteria. In this section, bacterial biomass that showed the highest growth and bioleaching efficiency in the treatment with the addition of 5% w/v coal fly ash was analyzed using SEM-EDX, as in Figure 10, which shows the biomass of *Bacillus toyonensis* (left) and *Bacillus tropicus* biomass (right). SEM-EDX analysis also allows measuring metal concentrations on the surface of the object being measured, in this case, the surface of bacterial biomass. Table 4 shows the presence of Zn, and

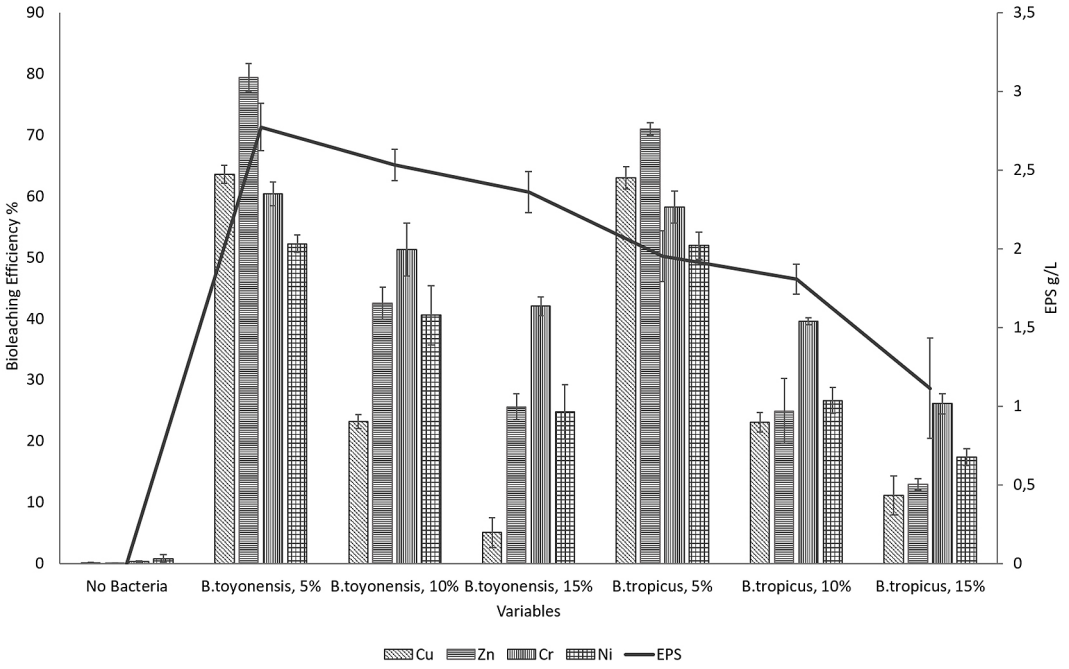

Figure 8. Heavy metal bioleaching efficiency and EPS in bioleaching medium after 21 days of bioleaching process

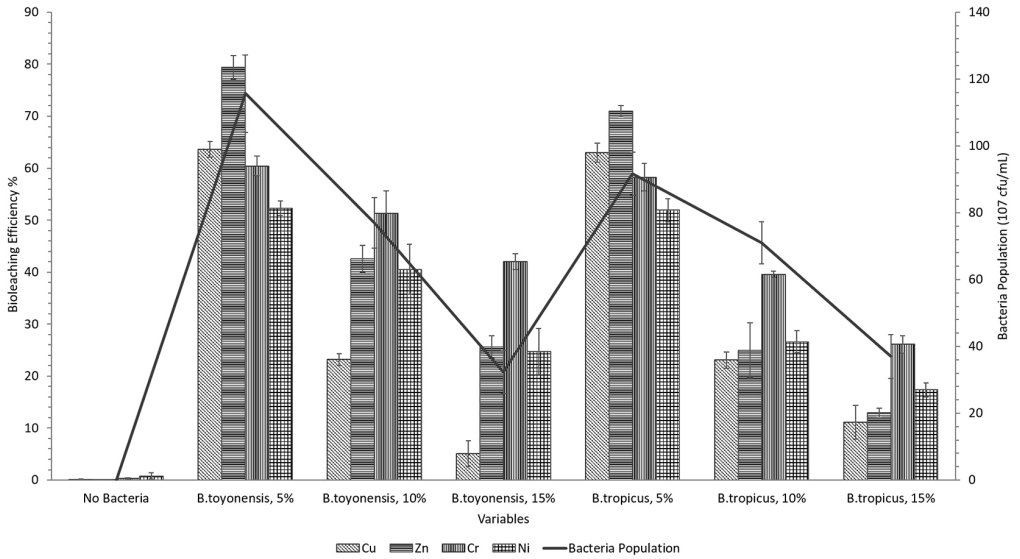

Figure 9. Heavy metal bioleaching efficiency and bacteria population in bioleaching medium after 21 days of bioleaching process

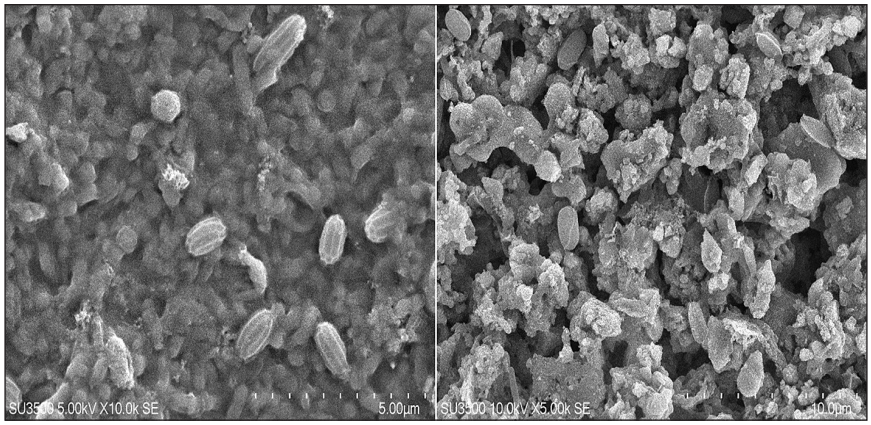

Figure 10. Morphology of *Bacillus toyonensis* (left) and *Bacillus tropicus* (right) after 21 days of bioleaching process using SEM

Table 4. The results of mapping EDX analysis of *Bacillus toyonensis* and *Bacillus tropicus* biomass after 21 days of bioleaching process

Bacillus toyonensis								
Element	Weight %	Atomic %	Net Int	Error %	Kratio	Z	A	F
CrK	1.44	1.57	6.50	48.89	0.0163	1.0090	0.9949	1.1334
FeK	84.51	85.91	239.80	3.68	0.8560	1.0041	0.9984	1.0105
NiK	2.68	2.59	5.00	61.88	0.0267	1.0144	0.9647	1.0184
CuK	1.88	1.68	2.60	66.67	0.0178	0.9628	0.9721	1.0149
ZnK	9.50	8.25	10.00	16.40	0.0903	0.9600	0.9781	1.0117
Bacillus tropicus								
Element	Weight %	Atomic %	Net Int	Error %	Kratio	Z	A	F
CrK	1.03	1.12	1.60	60.54	0.0117	1.0087	0.9951	1.1369
FeK	86.80	88.10	87.50	3.71	0.8779	1.0038	0.9987	1.0089
NiK	1.93	1.86	1.30	65.20	0.0192	1.0140	0.9639	1.0174
CuK	1.28	1.14	0.60	73.38	0.0121	0.9624	0.9715	1.0147
ZnK	8.97	7.78	3.40	16.68	0.0852	0.9596	0.9779	1.0119

the other metal content in *Bacillus toyonensis* and *Bacillus tropicus* biomass. This proves that the adsorption mechanism occurs in the bacteria used in this bioleaching experiment. Mapping analysis with SEM-EDX allows determining the distribution of metals in biomass samples and % weight of each metal shows the ratio of the amount of metal in the analyzed sample. From this analysis, it is known that Fe is more dominant, followed by Zn, Ni, Cu, and Cr. From this analysis also shows that Zn has more % weight than Ni, Cu, and Cr.

CONCLUSIONS

The results of the initial selection showed that the heterotrophic bacteria isolates identified are *Bacillus toyonensis (*99.13%), and *Bacillus tropicus* (98,75%). The isolated bacteria have the potential for the bioleaching of heavy metals (Cu, Zn, Cr, and Ni). In addition, the type of bacteria and variations in the addition of fly ash to the bacterial culture medium lead to differences in the bioleaching efficiency of heavy metals (Cu, Zn, Cr, and Ni). The addition of 5% (w/v) fly ash showed the highest bioleaching efficiency for Cu, Zn, Cr, and Ni in all bacteria treatments. Then, the highest bioleaching efficiency is achieved using *Bacillus toyonensis* at 5% of pulp density for Cu, Zn, Cr, and Ni, reaching 63.62% for Cu, 79.38% for Zn, 60.42% for Cr, and the highest bioleaching efficiency of Ni is 52,26%. In this study, the increase in the addition of fly ash to the media tends to cause a decrease in the value of bioleaching efficiency. The high value of bioleaching efficiency is followed by a high population of bacteria and EPS. The redoxolysis mechanism occurs in the bioleaching process with these indigenous bacteria. This is indicated by the redox potential and Fe^{2+} concentration. On the basis of the pH, heavy metal bioleaching in this study did not involve acidolysis and complexolysis mechanisms through organic acids. This is because the pH of the media tends to increase during bioleaching. However, the complexation mechanism occurs through other metabolites, namely biosurfactants, this is proven by the decrease in surface tension in treatments with the addition of bacteria compared to media without bacteria and aquadest. The presence of Zn, Cr, Cu, Ni, Mn, and other metal content in biomass bacteria, proves that the mechanism of metal accumulation through adsorption by bacteria is viable.

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