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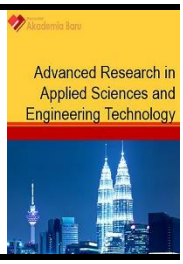

Isolation and Characterisation of Copper Leaching Microbes from Sanitary Landfill for E-waste Bioleaching

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ABSTRACT

Electronic waste has been the fastest increasing waste generated globally and is predicted to surpass 111 million tons per year by 2050. This trend is concerning, not just due to the growing volume but also due to its high composition of heavy metal elements, leading to potential environmental pollution if not managed properly. However, this issue opens a new prospect in material acquisition through the concept of urban mining via the metal extraction from electronic waste. A conventional method of extraction, i.e., chemical leaching, possesses harmful environmental impact with the production of its residual leachate. Thus, an alternative extraction technique is proposed, known as bioleaching, in which the microbial activity from bacteria mobilized metal into a more soluble form. In this study, bacterial strains were isolated from Malaysia sanitary landfill for bioleaching of copper from waste printed circuit boards (wPCB) with minimal mechanical pre-processing procedure. They were grown in low pH medium to utilize their activity for copper bioleaching from the wPCB. Four bacterial strains were successfully isolated. Using 16S rRNA gene sequencing, the isolates were identified as *Bacillus* sp. strain SE, *Bacillus* sp. strain SC, *Lysinibacillus* sp. strain SE2, and *Oryzobacter terrae* strain S1A. All the isolates showed appropriate bioleaching ability, with strain SC demonstrated the highest copper extraction with up to 23.36 ppm through the two-step bioleaching process. This strain was further evaluated using a copper strip to observe the actual copper extraction and demonstrated a total of 0.80 ±0.02 mg/g copper recovery. These results suggest that copper bioleaching of wPCB is viable as a standalone process.

Keywords:
Bioleaching, E-waste, Acid-tolerant microorganisms

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1. Introduction

The global development of technology and the reduced lifespan of technological devices have increased the volume of end-of-life equipment or electrical and electronic waste (e-waste). The Global E-waste Monitor 2020 reported that in 2019 alone, 53.6 million tons of e-waste were dumped globally [1]. In Malaysia, by 2020, 364 kilotons of e-waste were generated, which amounted to almost 11.1 kg per capita of e-waste. Approximately 547 kilotons of e-waste are exported to this country [2]. One of the issues concerning e-waste is the management and processing of the e-waste, which is often done illegally or informally in third-world countries after the e-waste from developed countries.

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This issue also accounted for the mismanagement of e-waste as most e-waste made its way to landfills instead of recycling facilities. The informal processing method of e-waste was usually done through combustion by melting the plastic parts of the e-waste to recover the metal from the e-waste [3], [4]. This procedure harms the operator's inhaling of possible toxic fume and the environment as the residual will be left on the ground without proper attention, leading to potentially hazardous pollution.

Copper is one of the most abundant metals found in e-waste, most prominently in the printed circuit board, and can easily seep into the soil, absorbed by the water bodies used for a drinking water source or household needs. Excess copper intake in the human body or copper toxicity can introduce general illnesses such as nausea and vomiting and lead to fatal Wilson disease [5]. Such issues occur since copper is not degradable leading to accumulation in plant and water sources [6]. In retrospect, as copper is being used in abundant amounts, especially in technological devices, the concept of urban mining or recycling of copper is deemed to be the way forward because the sources of natural copper ore are running low globally [7].

Extraction of metals from e-waste can be done in a few different processes. The most conventional methods are combustion and chemical leaching. Both ways, however, are often accompanied by a heavy pre-processing procedure where the e-waste was ground to a smaller size before being treated through combustion or chemical leaching. However, these pre-process lead to fine dust generation, making its way through inhalation, causing a hazard to the operator and the surrounding area [8]. Although one of the most efficient ways of recovering metal from e-waste, the combustion process often generates secondary pollution such as toxic fumes. It affects the surrounding community where the processing facility was located [9]. Similarly, chemical leaching, which used potent reagents such as concentrated acid, also produced secondary contaminants in the residual leachate from the process.

This paper highlighted the alternative route for metal recovery using bacteria strain microbial activity to extract the metals. This process, known as bioleaching, focused on the strain's ability to mobilize the metal to its more extractable form from the waste printed circuit board (wPCB). This research focuses on the isolation of bacterial strain from Malaysia sanitary landfill for bioleaching of copper from wPCB with minimal mechanical pre-processing procedure allowing the physical traits of the wPCB used to be maintained, allowing only the copper to be mobilized through this metal solubilization concept. This research also utilized the Malaysian equatorial climate for the process to be performed to accommodate the strains for bioleaching.

2. Methodology

2.1 Evaluation of Potential Microbial Sources for Bioleaching

Samples of soil, leachate, and sludge were collected from the Jeram Sanitary landfill, Malaysia (3°11'24.1 "N 101°21'49.5"E). Leachate and sludge samples were collected from the leachate treatment plant, while soil samples were collected from the topsoil of the landfill. One gram of each soil and sludge sample was added to 50 ml centrifuge tubes containing 20 ml sterile water and vortexed vigorously and filtered. The mixture was centrifuged at 4000 rpm for 10 minutes, and the supernatant was collected. The supernatant was initially grown in 15 ml Luria-Bertani (LB) media to enrich the microbial growth. Samples were washed in modified 9K-Fe medium [10] with the following composition: $(NH_4)_2SO_4$ (3 g/L), KCl (0.1 g/L), $MgSO_4$ (0.5 g/L), K_2HPO_4 (0.5 g/L), $CaNO_3$ (0.01 g/L), and $FeSO_4$ (15 g/L), resuspended in the same medium at pH 2.5 and growth by incubating at 37°C and 160 rpm for seven days.

Waste printed circuit boards (wPCB) were collected from an electronic recycler, Used ComputerMalaysia Sdn. Bhd., in Kuala Lumpur, Malaysia. Two types of wPCB were used in the experiment: 1. without any components and an exposed copper layer and 2. with components and a copper layer between the PCB structures. For the wPCB containing components, components such as capacitors, diodes, and others were removed beforehand, leaving only the board. Both types of wPCB were then cut into smaller pieces (approximately 2 cm x 2 cm) using a mini saw. This wPCB was used as the material to evaluate the ability of the isolated bacteria to perform bioleaching.

2.2 Bioleaching Method

Two bioleaching methods were chosen to accommodate different scenarios and materials used in the research work: 1. One-step bioleaching was used with the wPCB without components. The wPCB was introduced to the bacterial strain on day 0, neglecting toxicity on the bacteria growth as the blank wPCB does not contain any electronic component. This method allowed the bioleaching process to be shorter and was used as a preliminary run to inoculate the strain used for the later part of the bioleaching, and 2. Two-step bioleaching was used with the wPCB with components, allowing the bacterial strain to grow in the leaching medium before the wPCB was introduced into the bioleaching process. This process was done to reduce the toxicity of the wPCB towards bacterial growth. This procedure was done with the isolated strains of bacteria that were determined to do bioleaching. A longer duration was needed as the bacteria strains were allowed to grow inside the leaching medium for approximately seven days before introducing the wPCB on day 8.

2.3 Screening of bacterial sources for bioleaching

Samples that demonstrated growth in 9K-Fe medium were used for the copper bioleaching experiment before detecting the potential bacterial strains for bioleaching. 9K-Fe medium was used as the bioleaching medium with a pH of 2.50 at 37^{o}C at 160 rpm for seven days. After the bioleaching experiment, the copper concentration in the medium was then analyzed using atomic absorption spectroscopy (AAS, SHIMADZU AAS700). The standard was calibrated using an AAS Copper Standard. The standard was prepared at three different concentrations to obtain the standard calibration curve. The standard curve was calibrated to have a correlation coefficient (r^2) of > 0.95. A characteristic concentration check value was determined to detect the sensitivity of the AAS.

2.4 Isolation and Characterisation of Copper Leaching Microbes

After bioleaching was performed, samples that showed the highest percentage of bioleaching were used as the inoculum for bacterial isolation. The selected sample was enriched in 9K-Fe liquid medium with a pH of 2.50 at 37^{o}C, 160 rpm for seven days, and streaked on LB plates to pick for single colonies. The pure cultures were then transferred to fresh 9K-Fe medium and grown for seven days at 37°C and 160 rpm. Then, the grown cultures were subjected to DNA extraction using a DNA kit (QIAmp DNA Mini Kit). The genomic DNA obtained from the isolated strains was further used for PCR amplification of the 16S rRNA gene using universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') with reverse primer 1492R (5'-CGACGACCATGCANCACCT-3'). The PCR reaction mixture (25 µl) was performed using Promega, GoTaq® DNA Polymerase kit, which consisted of 5µL of 5x of Green GoTaq buffer, 1.5 µL of $MgCl_2$, 0.5 µL of dNTP mixture, 1.25 µL of each primer, 0.125 µL of DNA polymerase and 1.25 µL of template DNA. PCR thermocycler (ProFlex PCR System, Thermo Fisher Scientific) provided 2 minutes and 30 seconds at 95 °C followed by 35 cycles

of 15 seconds at 54 °C, followed by 7 minutes extension at 72 °C. Purified PCR product was sent for Sanger sequencing at First Base Laboratories Sdn Bhd (Malaysia) using an ABI Prism® 377 DNA Sequencer with the BigDye® Terminator 3.0 Cycle Sequencing Kit. The sequencing product was carried out in both directions. The result based on the obtained sequence was aligned and compared with GenBank sequences by BLAST analysis to identify the isolated bacterial species.

2.5 Bioleaching of wPCB and Mobilisation of Copper

The isolated strains were used to perform bioleaching of wPCB with a copper layer embedded between the fiber of the wPCB. The two-stage, spent bioleaching method was used because it allows a higher rate of activity and bioleaching by the bacteria by mitigating the toxicity of the wPCB component to the bacterial colony [11], thus, allowing the bacterial colonies activities to be fully maximized for the bioleaching. Seven days of inoculation inside the 9K-Fe medium with a pH of 2.50 was performed to optimize the growth of the colonies for each isolate without wPCB inside the leaching medium, followed by seven days of bioleaching activities with added wPCB. The condition of the bioleaching process was set at 37^{o} C at 160 rpm. The sample medium was centrifuged at 4000 rpm for 10 minutes, and the supernatant was extracted before being diluted for analysis using AAS to determine the copper concentration.

Bacterial strain with the highest copper recovery from the bioleaching experiment with wPCB was further tested for bioleaching experiment with a copper strip. A copper strip was chosen as a pure copper indicator to evaluate the interaction of the potential strain with copper. A 1 g copper strip was added to a 15 ml culture of the strain grown in the 9K-Fe medium for seven days under the same conditions as the previously performed bioleaching. Samples were extracted at the end of the process and diluted before AAS analysis. The samples were analyzed using AAS to determine the mobilization of copper from the strip.

3. Results

3.1 Evaluation of Copper Leaching Bacteria from Sanitary Landfill

Samples taken from the landfill in soil, sludge, and leachate were grown in LB and modified 9K-Fe medium under acidic conditions. The samples were collected from these sources because they host a high microbial activity and were expected to contain multiple colonies of bacteria to be evaluated for bioleaching. Both sludge and leachate samples showed growth in LB media but no growth in 9K-Fe medium. On the contrary, soil samples showed growth in both medium and were used as bacterial sources for bioleaching.

Four samples of the soil from different sampling points, Soil 1, Soil 2, Soil 3, and Soil 4, were used directly for screening bioleaching using exposed copper wPCB. Bioleaching was conducted using wPCB with an exposed copper layer. Bioleaching was performed according to the one-step bioleaching process [12] for the bioleaching test. From the AAS analysis, bioleaching using Soil 1 extracted the highest amount of copper, which is 37.7394 ppm in comparison to the other three soil samples, Soil 2 (2.9960 ppm), Soil 3 (2.7170 ppm), and Soil 4 (2.8395 ppm).

One-step bioleaching was chosen for the screening of samples because it allows a faster process, provides enough data for identification and neglects the toxicity of the wPCB toward the strain as the target used is a blank wPCB without components [12]. Physical changes in the leaching medium can also be observed from pale green (due to Fe (II)) to shade of blue (due to Cu (II)) with the formation of brown precipitate in the leaching medium.

3.2 Isolation of Bacterial Strains for Bioleaching

Soil 1 was further inoculated to obtain individual colonies for bioleaching. Soil 1 was enriched in 9K-Fe liquid medium with pH 2.50 and grown at 37°C for seven days. The grown cultures were streaked on LB agar plates to ensure the growth of single colonies. Individual colonies obtained were then selected and identified by partial 16S rRNA gene sequencing. Four pure colonies were successfully isolated and named strain SE, SE2, S1A, and SC. The four colonies were identified by alignment of the 16S rRNA sequence. The isolates were identified as *Bacillus* sp. strain SE, *Bacillus* sp. strain SC, *Lysinibacillus* sp. strain SE2, and *Oryzobacter terrae* strain S1A as shown in the Fig.1, indicating the growth of each strain on LB plates.

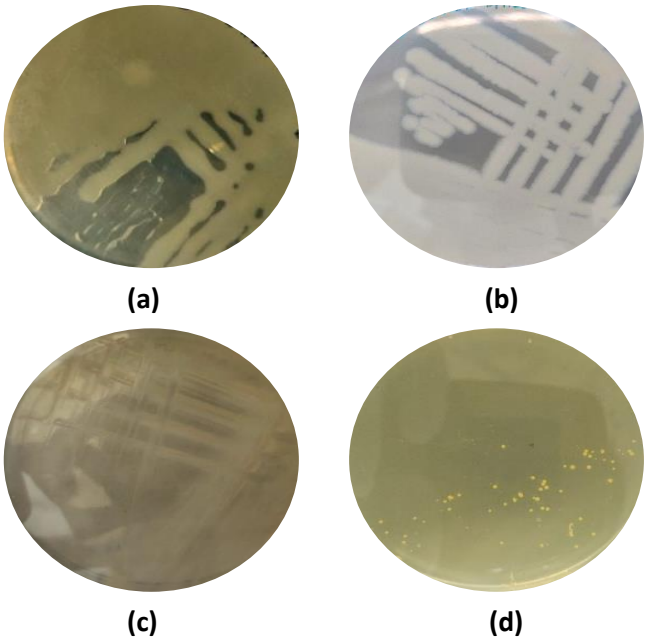

Fig.1. Growth of isolated strains on Luria-Bertani (LB) agar media after being transferred from pH 2.5 9K-Fe growth medium. The isolated strains were (a) strain SE2, identified as *Lysinibacillus* sp., (b) strain SE, identified as *Bacillus* sp., (c) strain SC, identified as *Bacillus* sp., and (d) strain S1A, identified as *Oryzobacter* sp.

The phylogenetic distributions of these isolates were analysed, as illustrated in Fig. 2. All isolates were separated in three phylogenetic groups, with strain SE and SC were grouped under *Bacillus* clade, and showed closed proximity with *Bacillus cereus* strain TBMAX58, and *Bacillus* sp. DG6C, respectively. Strain SE2 was clustered in *Lysinibacillus* group and showed closed proximity with *Lysinibacillus* sp. Strain P36 and *Lysinibacillus macroides* strain JB2. On the other hand, strain S1A fall within the *Oryzobacter* clade and show a close proximity with *Oryzobacter terrae.* The 16S rRNA sequences of the bacterial strains were deposited and are accessible in NCBI GenBank database with accession numbers MW426374 for *Bacillus* sp. strain SE, MW426350 for *Lysinibacillus* sp. strain SE2, MW426372 for *Oryzobacter terrae* strain S1A, and MW426370 for *Bacillus* sp. strain SC, respectively.

In order to confirm the ability of the isolated strains to grow in the leaching medium, the strains were growth in 9K-Fe liquid medium with pH 2.50 and grown at 37°C for seven days.

All strains displayed apparent physical changes in the leaching medium, as seen in Fig. 3. Brown precipitates were also observed in the medium containing the isolated bacteria, as compared to the control sample, which remained colorless. This observation could be due to the oxidation of Fe (II) to

Fe (III), which was present inside the medium in which was the common occurrence for bacterial strain that displayed bioleaching activity [13].

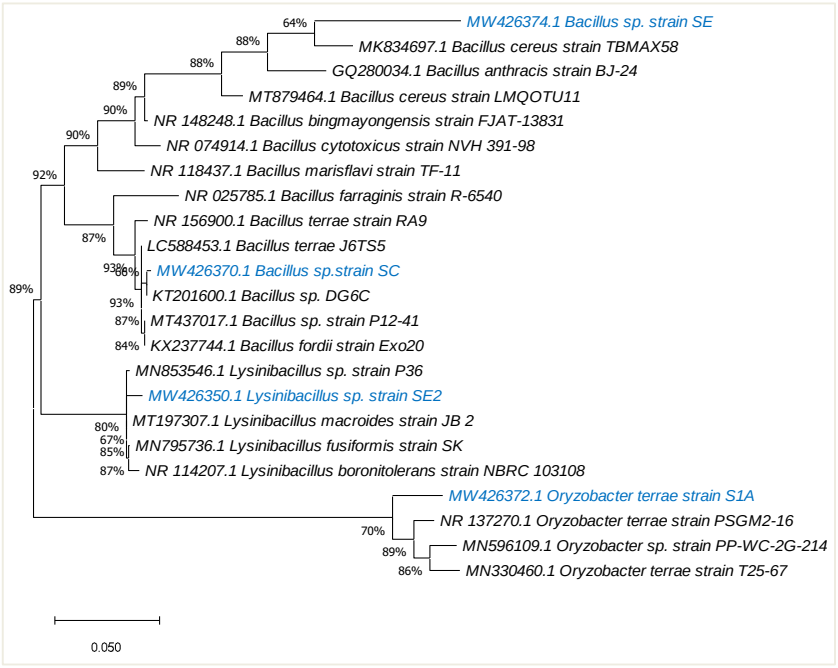

Fig. 2. Phylogenetic analysis of the isolated bacterial strains (highlighted in blue) with the related species strains based on 16S rRNA gene homology. This tree was generated by the maximum-likelihood algorithm using Jukes-Cantor distance correction and the bootstrap resampling method after 500 replications, which was conducted with MEGA 7 software. Bootstrap values (50%) are listed as percentages at the branching points

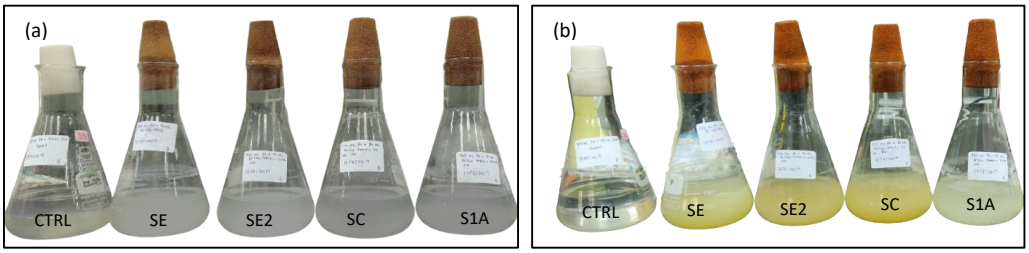

Fig.3 Growth of isolated bacteria in the 9K-Fe liquuid medium at pH 2.5 observed on (a) day 0, (b) day 7 of the inoculation period, in comparison to control sample (9K-Fe medium only) prior to bioleaching of wPCB

3.3 Bioleaching of wPCB using Isolated Strains and Evaluation for Copper Mobilization

The isolated bacterial strains with bioleaching potential were subjected to a two-step bioleaching mechanism to evaluate their ability to perform bioleaching. A Two-step bioleaching mechanism was used to minimize toxicity from the wPCB since the bacteria were cultured before introducing the wPCB to the 9K-Fe leaching medium. The results for the copper extraction after analysis using AAS are as follows: 1.04 ppm of copper was generated by strain SE, 9.16 ppm by strain SE2, 23.36 ppm by strain SC, and 0.62 ppm by strain S1A. A control condition was set with the bioleaching performed without any bacterial strains, while other conditions were kept unchanged.

The control bioleaching performed was only able to extract 0.46 ppm of copper. Physical evidence was also observed from the change in color in the filtrate of the leaching medium containing the bacteria. Changes in color from pale green (due to Fe (II)) to shade of blue (due to Cu (II)) were observed in the leaching medium with bacteria, and this observation was not seen in the control sample (shown in Fig.4.). In terms of physical appearance, strain SC, which recovered the highest copper concentration has a stronger shade of blue, as compared to the rest of the stains and the control sample.

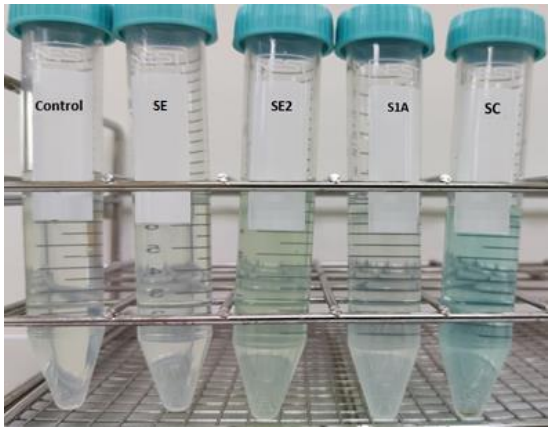

Fig. 4. The filtrates of culture medium after performing copper bioleaching of wPBC using the isolated strains, in comparison to control. A stronger shade of blue indicated a higher concentration of copper being extracted

The strain with the highest extraction of copper from wPCB was re-evaluated using a copper strip to observe the actual copper extraction amount by the strain against it. Since SC showed the most increased bioleaching activity among the four isolates, it was chosen for evaluation by adding 1 g of copper into the culture medium to determine the activity against copper. Readings from AAS for the total copper concentration extracted. A total of 0.80 ±0.02 mg/g was extracted from the copper strip in the presence of the strain compared to the control, with only 0.03 ±0.08 mg/g of copper extracted.

3.4 Discussion

Many researchers have been investigating the bioleaching of copper from wPCB [14]–[16]; however, all research prior uses grounded wPCB, which requires heavy pre-processing, thus producing a secondary pollutant in the form of fine dust as well as requiring higher cost and machinery. This paper limits the pre-processing of the wPCB before the bioleaching by minimalizing heavy machinery to ground the wPCB. Although the surface of wPCB is smaller without the mechanical grinding step, this research focuses on the mobilization of copper with the bacterial strain.

All soil, sludges, and leachate samples showed growth in LB media, which confirmed the existence of bacterial colonies inside all the samples. However, only the soil samples exhibited growth in the acidic 9k-Fe medium. This observation shows the tolerance of the bacterial colonies in the samples to the acidic conditions, which can be further inoculated for bioleaching. Soil 1 performed the best during the bioleaching screening using the exposed copper wPCB. The usage of this kind of wPCB allowed the one-step bioleaching mechanism to be applied, as the toxicity of wPCB due to its components can be neglected because it was a blank PCB as previously reported [14]. The significant

amount of copper extracted from the wPCB using Soil 1 as the bacterial consortia might occur due to more colonies capable of copper bioleaching than the other three sources; thus, Soil 1 was used as the bacterial source isolate individual colonies for wPCB bioleaching.

Soil 1 was used as a source for bacterial isolation obtaining four individual colonies, SE, SE2, SC, and S1A. The four colonies were submitted for partial 16S rRNA gene sequencing for identification. Strain SE was identified as *Bacillus* sp. strain SE. Previously, *Bacillus bingmayongensis* was reported to be able to grow in a wide range of conditions, including low pH conditions, as it was reported to adapt to the environment in which it was found and grow at a pH of approximately 2-12 [17]. The strain *B. bingmayongensis* is closely related to *B. cereus*, which has been reported to perform bioleaching of mica from kaolin [18] and participate in the biosorption of Zn^{2+} [19]. No previous study was reported on the ability of this strain for copper bioleaching; thus, this strain's performance (1.04 ppm) is acceptable. Strain SE2 was identified to be *Lysinibacillus* sp. strain SE2 and showed closed proximity with *Lysinibacillus* sp. Strain P36 and *Lysinibacillus macroides* strain JB2. Previously, *Lysinibacillus boronitolerans* species was reported to be widely available in soil with no mention of its ability to grow under low pH conditions [20]. However, the present study was able to isolate the strain under low pH (pH=2.50) conditions. The strain was reported to show metal-binding properties and remediation toward contaminated matrices [21]. Similar to strain SE, strain SE2 also demonstrated a copper bioleaching ability, with a copper recovery of 9.16 ppm. This is also the first report on using this strain as a bacterial strain for copper bioleaching. Strain S1A was identified to be *Oryzobacter terrae* strain S1A. When compared to the other isolated stain, this strain recovered the lowest copper with only 0.62 ppm of copper extracted. There is no previous report on the ability of this strain to perform bioleaching. Strain SC was identified in the same genus as strain SE, *Bacillus* sp., but yielded a better bioleaching result, with a 182.95% difference. Similar to strain SE, this strain has never been reported to be involved or to have any abilities to perform bioleaching of copper or any other metal. However, the present research was able to analyze and identify that the bioleaching of copper can be performed using this strain. Compared to a previous study, we grew this strain at a low pH rather than at pH 5 to 8 and optimum at pH 7 [22].

Bioleaching by bacteria takes place in an acidic environment at low pH to allow most of metal ions to remain in the solution. Thus, isolating acidophilic or acid-tolerant bacteria are particularly important. The solubilization of copper by the isolated bacteria could be due to the oxidation or reduction of metals through metabolism of bacteria, which sets the copper free from the wPCB. When bacteria are present, they require ferrous iron (Fe^{2+}) for metabolism and oxidize it to ferric iron (Fe^{3+}). As the concentration of (Fe^{3+}) increased due to the metabolism of the bacteria, the dissolution of copper increase in the acidic medium [23]. Thus, the isolated bacteria act as an enzyme, which can accelerate the reoxidation of Fe^{2+}, and consequently speeding up the conversion of copper into soluble forms in acidic solutions. This is consistent with our results, in which higher copper recovery were observed in the bioleaching of wPCB in the presence of bacteria, and occurs very slowly in the absence of bacteria, as seen from the trace copper recovery in the control sample.

The evidence of bacterial growth inside the leaching medium were confirmed by the apparent physical changes observed in the leaching medium containing the isolated bacteria, in comparison to the control sample. Besides, brown precipitates were also observed in the medium containing the isolated bacteria, which was not seen in control sample. This observation could be due to the initial oxidation of Fe (II) to Fe (III), which was present inside the medium [24]. Similar to the proposed ionic exchange in the bioleaching mechanism, these changes were reported in a previous study [13], [25]. The color of the medium also changed from a pale shade of green (due to the presence of Fe (II) in the medium) to a shade of blue when bioleaching was performed on wPCB and copper strip, indicating the presence of Cu (II) inside the medium. Comparing the strains presented in the

bioleaching medium with the control condition set, strain SE and S1A can be determined to almost showing no bioleaching abilities as both copper extraction (1.04 ppm, 0.62 ppm) is nearly as low as the controlled condition bioleaching (0.46 ppm).

The solubilization of copper by strain SC was evaluated by using copper strips to determine the interaction of the strain during copper solubilization. From the 1g copper strip used, 0.80 mg/g (±0.02) was solubilized, as analyzed using AAS compared to the set control with only 0.03 mg/g (±0.08) solubilized in the absence of strain SC. By comparison, a previous study reported the total amount of copper extracted through bioleaching to be approximately 80-90% within a similar timeframe used in the current study [24]. This result indicates that strain SC can solubilize copper, thus confirming the strain shown during the bioleaching process with wPCB.

Many researchers have been investigating the bioleaching of copper from wPCB [14]–[16]; however, all research prior uses grounded wPCB, requiring heavy pre-processing involving either heavy mechanical or pyrometallurgical processes. The present study managed to minimize the use of mechanical methods, such as grinding down the wPCB to a size smaller than 0.55 mm [13], [26], [27], thus producing secondary pollutant in the form of fine dust as well as requiring higher cost and machinery. This paper limits the pre-processing of the wPCB before the bioleaching by minimalizing heavy machinery to ground the wPCB. Although the surface of wPCB is smaller without the mechanical grinding step, this research focuses on the mobilization of copper with the presence of the bacterial strain retaining the physical shape of the wPCB to be further recycled for other materials and producing less toxic leachate compared to other metallurgical processes including the current hybrid-bioleaching procedure.

4. Conclusions

Isolates acquired from landfill soil show the ability to grow under low pH conditions compared to isolates retrieved from landfill sludge and leachate, which show no growth. This feature fulfills one of the criteria as a leaching bacterium. The oxidation of Fe (II) to Fe (III) in the leaching medium indicated by the brown precipitation also suggests that the bacteria isolated were able to act as iron oxidizers, which fulfill the mechanism of bioleaching proposed from the previous study, even though isolates SC and S1A have never been previously mentioned in terms of bioleaching activity. Physical changes in the leaching medium after bioleaching also support the proposed hypothesis that the bacterial strain acquired from the sanitary landfill was able to perform bioleaching because the leaching 9K-Fe medium turned blue from colorless likely due to the presence of Cu (II) ions. The present study evaluated that copper bioleaching of wPCB is viable as a standalone process. However, the significant difference of performance with bioleaching with pre-treated wPCB by mechanical processes should be further reduced. Further optimization on the bioleaching process, including re-evaluating the common factors such as the duration for bioleaching to maximize the process's overall performance, need to be performed to optimize the extraction of copper from wPCB.

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