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Enhancement of manganese extraction in a biochar-enriched bioleaching column with a mixed culture of indigenous bacteria

ENDAH RETNANINGRUM[1,♥] WAHYU WILOPO[2], I WAYAN WARMADA[2]

[1]Microbiology Laboratory, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-580839, ♥email: endahr@ugm.ac.id

[2]Department of Geological Engineering, Faculty of Engineering, Universitas Gadjah Mada. Jl. Grafika No. 2, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

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Abstract. *Retnanimgrum E, Wilopo W, Warmada IW. 2021. Enhancement of manganese extraction in a biochar-enriched bioleaching column with a mixed culture of indigenous bacteria. Biodiversitas 22: 2949-2955.* Biochar can improve manganese ore extraction during bioleaching by inducing redox reactions and providing a porous matrix for bacterial attachment. In this research, the effect of variations in biochar concentration on the performance of a bioleaching column with a mixed culture of *Acidithiobacillus* sp. KL3 and *Bacillus niacini* KB3B1 were studied comprehensively for 30 days. Addition of 0.4% biochar resulted in 89% manganese extraction. Bioleaching with biochar promoted the oxidation of sulfur and yielded high sulfate concentrations, much better pH, and excellent oxidation–reduction potential conditions. The bacteria in the mixed culture survived and adapted to the extreme column environment by releasing extracellular polymeric substance, as evidenced by the sharp increase in the content of the material in the column to 135.7 mg/g over 30 days of incubation. The synergistic effect of the bacteria in the mixed culture greatly contributed to the mechanism of manganese extraction in the column, as indicated by changes in relative bacterial abundance, which is related to bacterial community succession during bioleaching. The relative abundance of *B. niacini* KB3B1 gradually increased and peaked at 30% over 9 days of measurements. By comparison, the abundance of *Acidithiobacillus* sp. KL3 first decreased over 9 days of bioleaching and then increased to a maximum of 80% in the final stages of the process. This finding reveals that biochar addition could enhance the mechanisms of bioleaching and improve the yield of manganese extraction.

Keywords: *Acidithiobacillus* sp. KL3, *Bacillus niacin* KB3B1, catalyst, EPS, relative abundance

INTRODUCTION

Indonesia is home to many valuable metal ores, including manganese ore, spread over several of its islands. Manganese ore must be adequately and efficiently processed to improve the national economy while ensuring environmental sustainability. A practical, efficient, and environment-friendly approach for treating manganese ore is bioleaching; this method has been successfully applied to process ores in a number of nations, including the United States of America, England, Japan, Australia, China, and Germany (Banerjee et al. 2017; Kaksonen et al. 2020; Lan et al. 2020; Newsome et al. 2020). According to many studies, two types of bacteria are involved in the bioleaching of manganese ore, namely sulfur-oxidizing bacteria and heterotrophic bacteria. (Xin et al. 2015; Dan et al. 2016; González et al. 2018; Zhang et al. 2018). Previous research provided some suitable strains of sulfur-oxidizing bacteria (e.g., *Acidithiobacillus* sp. KL3) and heterotrophic bacteria (e.g., *Bacillus niacini* KB3B1), both of which were isolated from river sediments containing sulfur in Kedongsongo, Semarang, Indonesia. The isolates of these species have been successfully used to enhance bioleaching ability in shake flasks following optimization of the size, amount and type of manganese ore and environmental conditions (e.g., pH, temperature) (Retnaningrum and Wilopo 2019; Prasidya et al. 2019). The addition of a catalyst in the form of carbonaceous materials, i.e., biochar, has also been observed to enhance the bioleaching reaction (Wang et al. 2018; Kadivar et al. 2021).

Biochar can facilitate electron transfer and, consequently, promote redox reactions. This material's high porosity also provides a suitable matrix onto which bacteria can attach for bioleaching (Kan et al. 2020; Yang et al. 2020; Anto et al. 2021; Das et al. 2021). Biochar is a biomass pyrolysis byproduct characterized by high carbon and inorganic material contents (Chang et al. 2016; Ma et al. 2017; Bonsu et al. 2020). Palm kernel shell is abundant biomass obtained from oil palm wastes used for biochar production.

Previous researchers showed that mixed cultures of sulfur-oxidizing and heterotrophic bacteria could better increase manganese extraction yields compared with pure cultures during bioleaching (Zhu et al. 2014; Hao et al. 2016). The increase in extraction of manganese by mixed cultures is greatly influenced by the interactions of the bacteria, particularly their synergistic effects. Sulfur-oxidizing bacteria can provide organic components for use by heterotrophic bacteria in the form of lysates or exudates. In addition, the metal-extraction mechanisms of these two groups of bacteria are different. Sulfur-oxidizing bacteria can extract metals from the ore by acidolysis and redoxolysis. Metal extraction via acidolysis may be attributed to these bacteria's ability to synthesize H_2SO_4

(Ilyas and Lee 2014). Redoxolysis occurs via oxidation–reduction (ORP) reactions. Ferric ions are enzymatically reduced under anaerobic conditions; in this process, hydrogen or sulfur serves as the electron donor, and energy is provided during redoxolysis for microbial growth (Hubau et al. 2018). The metal-extraction mechanism of heterotrophic bacteria occurs through complexolysis to produce cyanogen (Lu and Xu 2016)

During bioleaching, the column environment tends to develop extreme conditions due to acidic pH and high levels of sulfate and soluble manganese. These conditions could induce bacterial cells to release extracellular polymeric substance (EPS) as a form of cell defense (Hu et al. 2020). EPS is a complex compound with a high molecular weight; it consists of a large proportion of polysaccharides and smaller proportions of other compounds, such as proteins, uronic acids, humic substances, and lipids. Biosynthetic EPS can exist as attached capsular substances or slime on the surface of bacterial cells. Besides providing cell protection against extreme environments, EPS also functions as a carbon and energy source (Gupta and Diwan 2017; Wang et al. 2018).

Given its low cost, easy operation, and high yield, industrial-scale metal extraction via heap bioleaching is considered a highly effective technology (Shiers et al. 2016). The heap bioleaching method can be simulated at the laboratory scale by using a bioleaching column, which represents an intermediate bioleaching scale-up process that can be implemented prior to commercial scale-up (Jia et al. 2019). Research on metal extraction using a biochar-enriched bioleaching column with a mixed culture of *Acidithiobacillus* sp. KL3 and *B. niacin* KB3B1 could provide a reliable model and essential information to estimate the bioleaching results for commercial heap bioleaching operations. Thus, the present study's objectives are to investigate the effects of biochar on the performance of a bioleaching column for manganese extraction with a mixed bacterial culture and characterize bacterial adaptation and bacterial community succession during the bioleaching process.

MATERIALS AND METHODS

Ore sample, biochar, and bacteria

Ore samples of pyrolusite were collected from Kliripan, Kulon Progo, Yogyakarta, Indonesia. The samples contained 30.4% Fe, 25% Mn, and 34.0% S. The particle size of the samples ranged from 0.16 mm to 0.125 mm. The biochar used in this study was obtained from a local market in Bantul Regency, Yogyakarta, and produced from coconut shell charcoal chemically activated by $ZnCl_2$ and Na_2CO_3 and then pyrolyzed at 700°C for 4 hours. The obtained biochar was ground into particles with diameters ranging from 0.8 mm to 1 mm. The physicochemical properties of the biochar included 79% total carbon, 1% total N, 1.8% total H, 2.3% ash content, 0.38% moisture content, iodine number 450 mg/g, 403 m^2/g surface area, and pH 8 ± 0.3.

Two bioleaching strains, namely, the heterotrophic bacterium *B. niacin* KB3B1 and the chemolithotrophic bacterium *Acidithiobacillus* sp. KL3, were isolated from sulfuric river sediments and used in this experiment (Retnaningrum and Wilopo 2019; Prasidya et al. 2019). The bacteria were routinely cultivated in 9K medium containing (per liter): 4.25 g $(NH_4)_2SO_4$, 0.14 g KCl, 0.07 g K_2HPO_4, 0.7 g $MgSO_4{\cdot}7H_2O$, and 0.02 g $Ca(NO_3)_2{\cdot}4H_2O$.

Bioleaching experiments

The bioleaching experiment was conducted in polypropylene tubes measuring 50 cm in height and 5 cm in diameter. The column setup is shown in Figure 1 and consisted of (A) plate on which to lay the manganese ore at the bottom of the column, (B) an external bioreactor containing the feeding solution, (C) a stirrer for proper mixing of the feeding solution in the external bioreactor, (D) a peristaltic pump to pump the feeding solution, (E) a liquid distributor at the top of the column for consistent delivery of the leaching solution to the manganese ore, (F) an air compressor to supply air for bacterial growth, (G) an air filter, (H) an air inlet, (I) a gas outlet, and (J) a connector to recirculate the leaching solution.

As much as 500 g of manganese ore and various amounts of biochar (0.2%, 0.4%, and 0.6%) of weight (g/g) were loaded onto the support plate of the column. The column was fed with a solution containing the mixed bacterial culture at a flow rate of 10 mL min^{-1} from the top of a bioreactor. The feeding solution was prepared by inoculating 20% (v/v) mixed bacterial inoculum with a cell number in the range of 10^7–10^8 CFU/mL into 80% (v/v) 9K medium. That flow rate was controlled by the peristaltic pump, and the bacterial solution was recycled continuously and fed to the column for 30 days (Abhilash et al. 2013; Wu et al. 2016; Jalali et al. 2019). Leached samples were periodically collected at intervals of 3 days to analyze bioleaching performance, bacterial adaptation, and bacterial community succession.

Bioleaching performance

Leached samples were collected at fixed time intervals for 30 days, and their sulfate concentration, pH, ORP, and manganese concentration were measured. The pH of the samples was measured by a pH meter (pHS-3C, Leici, China), and ORPs were measured with a platinum electrode with reference to an Ag/AgCl electrode by using an ORP meter (Hanna HI 8512). The concentration of sulfates was determined using a spectrophotometer (wavelength, 420 nm) on the basis of a turbidimetric assay. Extracted manganese concentrations were determined by a flame atomic absorption spectrophotometer (Hitachi, Z-2000) (Khayatin et al. 2018). Optimal bioleaching performance was further observed in terms of bacterial adaptability and succession in the community.

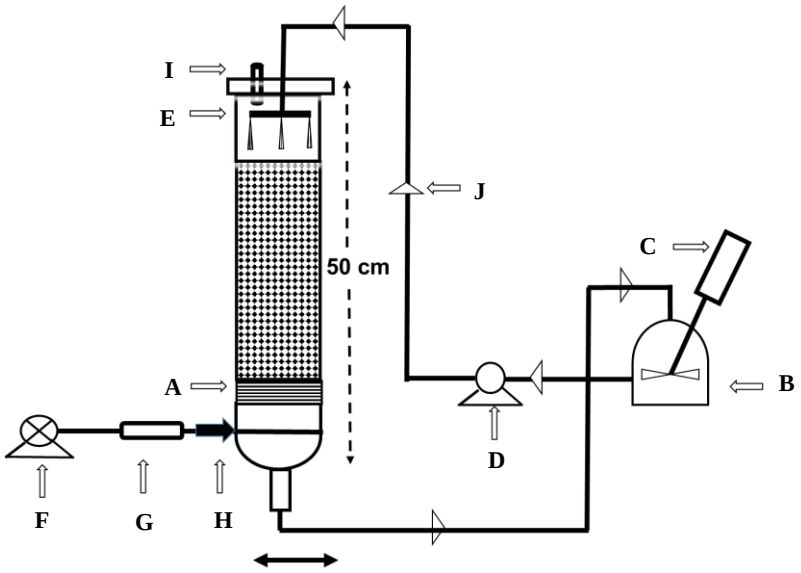

Figure 1. Bioleaching column design. A. Plate, B. Bioreactor, C. Stirrer, D. Peristaltic pump, E. Liquid distributor, F. Air compressor, G. Air filter, H. Air inlet, I. Gas outlet, J. Connector

Bacterial adaptation in the bioleaching column was analyzed by observing the chemical characteristics of EPS that had accumulated as biofilms during bioleaching. Leaching solution samples obtained from several continuously operated bioleaching systems were allowed to stand for 1.5 hours, after which the thickened sludge was centrifuged at 1500 rpm and 4°C for 20 min. The resulting pellet samples were suspended in distilled water and then lysed using 1 g of glass beads (diameter, 0.2 mm). Thereafter, the samples were shaken in a vortex at 1400 rpm for 10 min. Lysed samples were extracted with a mixed solution containing equal concentration of 10 mM Tris-HCl (pH 7), 10 mM *N*-dodecyl-*N*, *N*-dimethyl-3-ammonio-1-propanesulfonate, and 1 mM EDTA at 4°C. The extracted samples were centrifuged and passed through 0.2 μm membrane filters, after which the extracted EPS was collected. The dry weight of the extracted EPS was determined prior to biochemical characterization for the presence of carbohydrates and proteins. The Bradford assay was used to determine protein contents with bovine serum albumin as the standard (Bradford 1976). Total carbohydrate contents were measured using the phenol–sulfuric acid assay with glucose as the standard (Dubois et al. 1956). The carbohydrate and protein contents of the sample were then determined using a spectrophotometer.

Bacterial community succession

Total DNA was extracted from 10 g of leached samples by using an Isoil for Beads Beating Kit (Nippon Gene, Tokyo, Japan). DNA extraction was carried out according to the manufacturer's instructions with minor modifications. The quantity and quality of DNA extracts were measured using a UV-Vis Nanodrop spectrophotometer (Thermo Scientific) to determine the DNA concentration and DNA/protein ratio. The pure DNA extract was adjusted to a concentration of 100 ng μL^{-1}. The target distinct regions (16SV3–V4) of the bacterial 16S rDNA genes were then amplified by PCR using the specific primers 341F (5'-CCTACGGGA GGCAGCAG-3') and 806 R (3'-GGACTACYVGGG TATCTAAT-5').

The PCR products were sequenced using the Illumina MiSeq platform and their sequencing results were then calculated with the Quantitative Insights into Microbial Ecology (QIIME 1.7.0) program. Prior to statistical analysis, low-quality or unclear reads were excluded. The sequences were then categorized using the Ribosomal Database Project classifier with a 60% confidence cut-off and grouped into operational taxonomic units (OTUs) at 97% similarity. The Greengenes database was used to search the taxonomic classification of each representative sequence OTU which was operated using the SILVA 16S rRNA gene database (DeSantis et al. 2006). Bacterial community successions were analyzed at the species level. The relative proportion of bacterial abundance was then determined by dividing the number of reads by the number of sequences found per sample by using Microsoft Excel.

Statistical analysis

All experiments were conducted in triplicate, and the results are expressed as mean ± standard deviation. Mean treatment values were compared by one-way analysis of variance, and Duncan's multiple range test was employed to test mean differences. Differences in treatment results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Bioleaching performance

Acidithiobacillus sp. KL3 and *Bacillus niacini* KB3B1 were assessed, as shown in Figure 2. The addition of biochar significantly increased the samples' sulfate

concentration over 30 days of incubation ($p < 0.05$). The sulfate content of the column could be attributed to *Acidithiobacillus* sp. KL3, which oxidizes sulfur from manganese ore to sulfate. Sulfate formation during the bioleaching of metal ores has been observed in several studies (Figueroa-Estrada et al. 2020; Pattanaik et al. 2020). The sulfate concentration in all treatments increased with increasing incubation time. Moreover, among the treatments established in the present study, that with 0.4% biochar showed the highest increase in sulfate concentration. In the column enriched with 0.4% biochar, approximately 7500 mg/mL sulfate was quickly produced within 7 days; by comparison, the columns enriched with 0.6% and 0.2% biochar revealed sulfate contents of 5800 and 6160 mg/mL, respectively, over 18 days. The increase in sulfate concentration in the control occurred very slowly; indeed, only 3000 mg/mL sulfate was produced in control over 21 days.

The oxidation of sulfur to sulfuric acid during column bioleaching by *Acidithiobacillus* sp. KL3 over 30 days of incubation resulted in a significant increase in ORP ($p < 0.05$). Among the columns established, that 0.4% biochar addition revealed the fastest increase in ORP. The ORP of the column enriched with 0.4% biochar achieved steady ORPs of 645-650 mV on day 21. Therefore, the addition of 0.4% biochar improves oxidation conditions for the bioleaching of manganese. Biochar addition apparently accelerates the oxidation of sulfur to sulfate, which accumulates over the course of bioleaching.

Sulfate production by *Acidithiobacillus* sp. KL3 induced decreases in pH over 30 days of bioleaching ($p < 0.05$). A previous study indicated that *B. niacini* KB3B1 produces cyanide by consuming acids during bioleaching, thereby causing a decrease in the pH of the solution in the column (Prasidya et al. 2019). The pH of the solution reached a steady value of 1.29 earlier (day 21) in the column added with 0.4% biochar compared with that added with 0.2% biochar and the control (day 27). The pH of the solution also reached a steady value of 1.14 earlier (day 21) in this column compared with that added with 0.6% biochar (day 24).

As the bioleaching time increased to 30 days, increases in sulfate concentration accompanied by increases in ORP and decreases in pH increased the extraction of manganese on the ore by the mixed bacterial culture. The bioleaching reactions were described by Mahmoud et al. (2017) as follows:

$$2S^0 + O_2 + 2H_2O \rightarrow 2H_2SO_4 \quad (1)$$
$$2MS + 2H_2SO_4 + O_2 \rightarrow 2MSO_4 + 2S^0 + 2H_2O \quad (2)$$

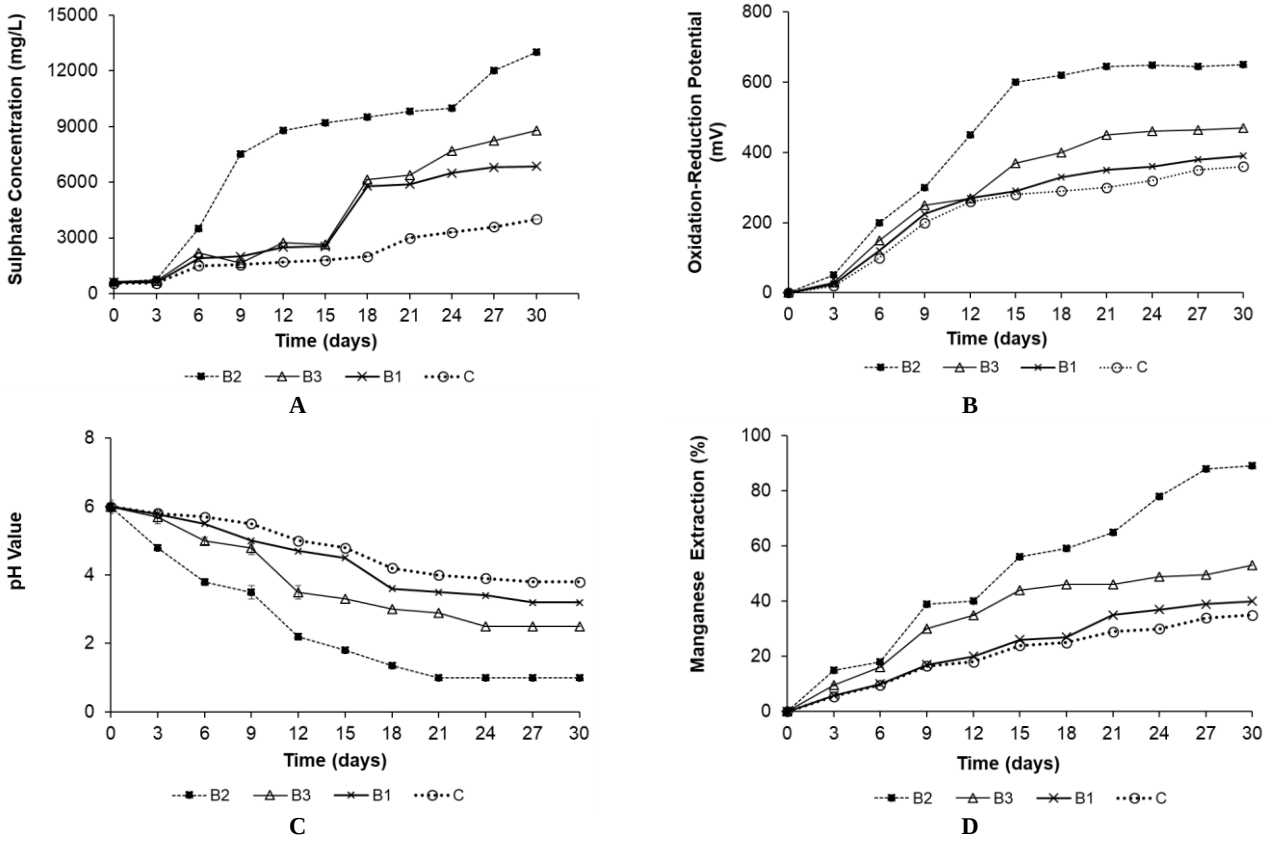

Figure 2. Changes in sulfate concentration (A), ORP (B), pH (C), and manganese extraction (D) during column bioleaching by a mixed culture of indigenous bacteria with biochar addition. Note: B: Biochar addition, B1: 0.2%; B2: 0.4%; B3: 0.6%. C: without biochar addition

In this study, addition of biochar significantly accelerated manganese extraction ($p < 0.5$). In fact, the addition of 0.4% biochar yielded the highest increase in manganese extraction, followed by additions of 0.6% and 0.2% biochar. The control revealed slow manganese extraction. Addition of 2% biochar resulted in the extraction of 39% manganese within 9 days and a maximum extraction rate of 89% manganese after 30 days. A similar trend was observed when 0.6% biochar was added, i.e., as much as 30% manganese was rapidly extracted within 9 days. After this initial rapid extraction, a steady increase in extraction rate to 53% was observed on day 30. However, the addition of 0.2% biochar induced only approximately 20% manganese extraction after 12 days, and a maximum yield of 40% manganese was observed at the end of bioleaching (30 days). These findings show that the addition of 0.4% biochar accelerates manganese extraction during column bioleaching with a mixed bacterial culture to optimal levels. Previous research demonstrated that the high porosity of biochar provides a microhabitat for the attachment of bacteria in the mixed culture to support their growth. Biochar could also promote electron transfer in the oxidation reaction of sulfur to sulfate by *Acidithiobacillus* sp. KL3, thereby allowing the rapid extraction of manganese ore (Wang et al. 2016; Xu et al. 2016; Zhao et al. 2016).

Analysis of bacterial adaptation

Enrichment of the bioleaching column with 0.4% biochar encouraged the bacteria in the mixed culture to adapt to the extreme environment in the column by producing EPS, as shown in Figure 3. Specifically, the bacteria in the mixed culture generated 37.78 mg/g EPS over 3 days. EPS production then increased rapidly and achieved a steady concentration of approximately 133.4 mg/g over a period of 24 days. The EPS collected was mainly composed of carbohydrates and low levels of protein, which is in accordance with findings from previous investigations (Gupta and Diwan 2017; Wang et al. 2018). Besides defending cells against extreme environments, the EPS produced by the bacteria in the mixed culture also enhances manganese ore extraction via an EPS-mediated mechanism (Zhang et al. 2015). During column bioleaching, EPS promotes the attachment of bacteria to the surface of manganese ore, which, in turn, enhances manganese extraction. Therefore, the increase in EPS production during column bioleaching is directly proportional to the increase in the extractable concentration of manganese.

The EPS produced by the bacteria in the mixed culture accumulated on the mineral surface in the form of biofilms as bioleaching progressed. The biofilm phase is one of the adaptative mechanisms of bacteria during metal bioleaching. This phase provides a protective microenvironment against extreme conditions, so that bacterial metabolism exceeds that of the planktonic phase. Other than bioleaching columns, the formation of biofilms has also been observed in microbial fuel cells and sulfate-reducing bioreactors (Retnaningrum and Wilopo 2016; Retnaningrum and Wilopo 2017).

Bacterial community succession

The succession of the bacterial community in the bioleaching column significantly influenced the effects of metal extraction (Yin et al. 2019; Zhou et al. 2019). This finding is related to the dominance of certain bacterial strains that develop during bioleaching. The dominance of the bacterial strains during bioleaching with 4% biochar treatment was investigated on the basis of relative abundances, as shown in Figure 4.

At the initial stages of bioleaching, the ratio of *Acidithiobacillus* sp. KL3 to *B. niacin* KB3B1 inoculated into the column bioreactor was adjusted to 1:1. Therefore, the relative abundance of each strain showed an equivalent yield of 50%. From day 3 to day 9, the relative abundance of *B. niacini* KB3B1 increased from 55% to 65%, while that of *Acidithiobacillus* sp. KL3 decreased from 45% to 35%. However, the relative abundance of *B. niacin* KB3B1 decreased by 60% on day 12 to 24% on day 30. By contrast, on day 12, the relative abundance of *Acidithiobacillus* sp. KL3 increased by 40% and peaked at 80% on day 30. The shift in the relative abundance of the two type strains was induced by their synergistic bioleaching mechanism in the column (Panda et al. 2017).

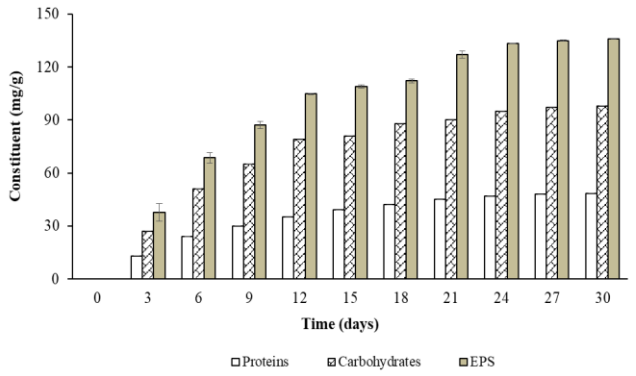

Figure 3. Variations in EPS concentration and constituents obtained from the bioleaching column enriched with 0.4% biochar at intervals of 3 days.

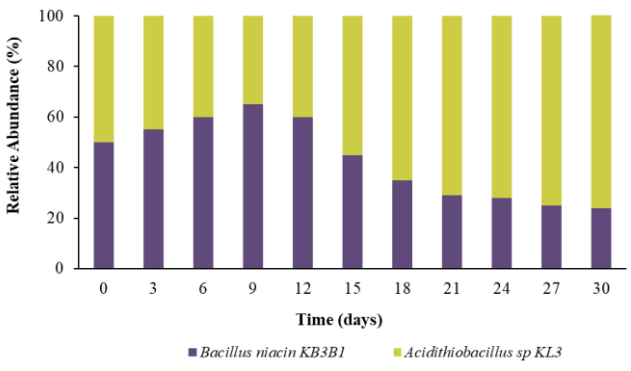

Figure 4. Bacterial community succession during 0.4% biochar-enriched column bioleaching with a mixed culture of indigenous bacteria.

At the early stages of bioleaching, *Acidithiobacillus* sp. KL3 supports *B. niacin* KB3B1 growth by releasing metabolites as organic compounds. As these metabolites accumulate, the relative abundance of *B. niacin* increases. However, as bioleaching proceeds, the dominant strain on the mineral surface shows evident changes. *Acidithiobacillus* sp. had the highest relative abundance at the end of the bioleaching process. This phenomenon may be attributed to the improved adaptability of *Acidithiobacillus* sp. compared with *Bacillus* sp. to high-ORP and low-pH conditions (Bajkic et al. 2013; Avdalovic et al. 2015).

Pearson correlation coefficients were used to describe the correlation between strains and various physicochemical parameters, as shown in Table 1. The relative abundance of *Acidithiobacillus* sp. KL3 was significantly and positively correlated with manganese extraction, ORP, and EPS but negatively correlated with pH ($p < 0.05$). By comparison, *B. niacin* KB3B1 showed significantly positive correlations with manganese extraction and ORP but negative correlations with pH and EPS ($p < 0.05$). As a result, managing the synergistic effects of microorganisms, such as different functional types of bacterial strains, is critical in regulating and controlling bioleaching performance.

Analyses of bioleaching performance, bacterial adaptation, and bacterial community succession during column bioleaching demonstrated that the addition of biochar to the column system with two types of bacteria in the mixed culture promotes the extraction of manganese. The findings indicate that biochar acts as a catalyst for redox reactions and provides a porous matrix for bacterial attachment during the bioleaching process. The addition of 0.4% biochar led to 89% manganese extraction, which is 54% higher than the manganese extraction rate observed during column bioleaching without biochar. The addition of biochar to the bioleaching column is beneficial and a cost-effective solution to enhance manganese extraction during bioleaching. Further exploration of column bioleaching technology with mixed cultures of indigenous bacteria and biochar enrichment at the field scale is necessary to determine whether its implementation at the industrial scale is environmentally sustainable and cost-effective.

Table 1. Pearson correlation coefficients of the relative abundance and physicochemical parameters of two bacterial strains during column bioleaching with a mixed culture of indigenous bacteria and 0.4 % biochar enrichment

Physicochemical parameters	*Acidithiobacillus* sp. KL3	*Bacillus niacin* KB3B1
Manganese extraction	0.6791*	0.7231*
pH	−0.5449*	−0.5637*
Oxidation–reduction potential	0.5142*	0.5433*
Extracellular polymeric substance	0.4188*	−0.4614*

Note: *: significant at $p < 0.05$

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