

Optimization of manganese bioleaching activity and molecular characterization of indigenous heterotrophic bacteria isolated from the sulfuric area

DENAYA ANDRYA PRASIDYA¹, WAHYU WILOPO², I WAYAN WARMADA², ENDAH RETNANINGRUM^{3,*}

¹Graduate Program in Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknik Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia

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

³Microbiology Laboratory, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknik Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia.
Tel./fax.: +62-274-580839, *email: endahr@ugm.ac.id

Manuscript received: 30 May 2018. Revision accepted: 20 June 2019.

Abstract. Prasyda DA, Wilopo W, Warmada IW, Retnaningrum E. 2019. Optimization of manganese bioleaching activity and molecular characterization of indigenous heterotrophic bacteria isolated from the sulfuric area. *Biodiversitas* 20: 1904-1909. The present research evaluated the manganese bioleaching potency of a heterotrophic bacteria KB3B1. This bacterial strain has been isolated from sulfuric area located at Ungaran, Middle of Java, Indonesia using modified 9K medium by adding of several organic nutrients. The manganese bioleaching activities of the strain was analyzed by applying of varying glycine concentrations (0, 5, 10, 15 mg mL⁻¹) with pyrolusite pulp densities of 0.02 g cm⁻³ on a rotary shaker at 180 rpm for 18 days incubation. Several parameters, including the growth of bacteria, pH values, the concentration of soluble manganese and cyanide, were investigated at the interval of 3 days. Molecular characteristics of the strain were further analyzed based on 16S rDNA gene sequences. After 15 days, the maximum yield of manganese 16.6% was achieved under the addition of 10 mg mL⁻¹ glycine. This maximum extract obtained was followed by the maximum bacterial growth, pH, and cyanide product of the strain. Phylogenetic analysis showed that the strain was closely related with *Bacillus niacini* EP89. Besides, the average frequencies of guanine and cytosine (G+C) of the strain was in same range as that of the reference bacteria in the GenBank and Bergey's Manual Systematics of Bacteria.

Keywords: 16S rDNA, *Bacillus niacini* EP89, cyanide, glycine, pyrolusite

INTRODUCTION

Indonesia has a high amount of manganese (Mn) deposit in the form as pyrolusite. As reported by Geological organization, a total of 19,636,720.65 tons of the deposit was produced in 2013 in Indonesia. This deposit is used for metallurgical purposes in steel manufacture as a construction material in many countries (Dan et al. 2016). Also, this deposit is used for non-metallurgical purposes, including the production of dry batteries, plant fertilizer components, dyes, medicines, animal feed, and brick colorant (Das et al. 2012). Therefore, more rapid regional development in various countries are increasing the demand for manganese deposit.

As regulated in Indonesian Government Regulation No.1 year 2014, the deposit has to be purified for producing the electrolytic manganese metal. Indonesia is not allowed to export the natural resources of mineral in the form of deposit but has to be processed or purified first for a higher selling price that can increase the national income. The extraction of manganese deposit in Indonesia is still limited by processing through a conventional method, such as pyro-metallurgy and hydrometallurgy. It traditional method of extraction, waste as a metal residual is produced as a result of the chemical extraction process (Liu et al. 2008; Das et al. 2011).

Currently, several attempts have been made for the development of the environmentally friendly metal extraction process through bioleaching. This process uses the ability of microorganisms which are capable of extracting metal minerals from their ores (Chen et al. 2011; Das et al. 2015). Mohanty et al. (2017) observed the role of fungi during the bioleaching process by producing organic acids, which can solubilize the manganese. The other group of microorganisms, such as sulfur-oxidizing bacteria also has capabilities for extracting metals from their deposits. This group consists of the genus *Acidithiobacillus*, *Acidiphilium*, *Acidiphilum*, *Sulfobacillus* and *Sulfolobus* (Ghosh et al. 2016; Jang and Valix 2017; Jalali et al. 2019; Huynh et al. 2019; Retnaningrum and Wilopo 2019). Whereas, heterotrophic bacteria such as *Leptospirillum ferriphilum*, *Pseudomonas* sp., *Bacillus* sp., and *Acinetobacter* sp were also reported to be capable of solubilizing metals (Ghost and Das 2017; Thavamani et al. 2017; Wang et al. 2018; Dong et al. 2019; Saleh et al. 2019).

In this investigation, a heterotrophic bacteria KB3B1 strain was isolated from a sulfuric area located at Ungaran, Central Java, Indonesia. The isolate can grow at pH of 3.5-7.0 and is tolerant to manganese. This heterotrophic bacteria from its specific niches is now receiving increased attention, and it was evident from the recent study that microorganisms present in the sulfuric area probably have

bioleaching potency (Han et al. 2013). Previous research reported that this group of bacteria produces cyanide as a secondary metabolite that plays a vital role in our research solubilization of the metal. This metabolite is produced when bacterium is in the initial phase of stationary growth. The cyanide synthesis by bacteria occurs through the oxidative decarboxylation reaction of the glycine precursor (Faramarzi et al. 2004; Brandl et al. 2008; Adams 2013). For acquiring maximum metal extraction, the bioleaching experiment should be conducted under optimal bacterial growth conditions. Therefore, this research purposed were, (i) to observe the manganese bioleaching rates and mechanisms of heterotrophic bacteria isolated from the sulfuric area, (ii) to analyze molecular characteristics of the bacterial isolate based on 16S rDNA gene.

MATERIALS AND METHODS

Bacteria strains and bioleaching medium

The heterotrophic bacteria used for laboratory bioleaching experiments, KB3B1 strain was isolated from sulfuric river sediment located at Ungaran, Middle of Java, Indonesia. This bacteria was further subcultured on Luria Bertani (LB) medium for 24 h at 37°C before inoculating it into the bioleaching medium.

The bioleaching medium used was the modified 9 K medium which consisted of (g L^{-1}) 4.25 $(\text{NH}_4)_2\text{SO}_4$, 0.14 KCl, 0.07 K_2HPO_4 , 0.7 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.02 g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. This 9K liquid medium was then modified by adding several organic nutrients (g L^{-1}) such as 0.7 yeast extract, 2.2 peptone, and 5 glucose. Finally, this modified medium was conditioned to have pH 7.0 (Zhu et al. 2014).

Bioleaching experiments

Bioleaching observations were carried out in 250 mL Erlenmeyer flasks. The pulp density pyrolusite of 0.02 g cm^{-3} was added in each the flask as a manganese deposit. Previously pyrolusite was crushed into the size-fractionated of $< 75 \mu\text{m}$, placed into separate flasks, followed by autoclaving. Prepared culture bacteria which had already grown for 24 was aliquot into 2 mL sterile centrifuge tubes. Cells were then rinsed 3 times with sterile deionized water to remove the culture media. The 5% bacterial culture (v/v) was added to each the bioleaching medium containing flasks with a final number of bacterial cells approximately 10^7 mL^{-1} . Flasks were incubated at 37 °C and rotary shaker of 180 rpm. All bioleaching experiments were carried out with three replications.

To investigate the influence of variation glycine concentration on pyrolusite bioleaching mechanism, the culture was added with a variation of glycine concentration (0, 5 10, 15 mg mL^{-1}) and incubated for 18 days on a rotary shaker at 180 rpm. During those experiments, the growth of bacteria, soluble manganese, and cyanide concentrations were investigated at an interval of 3 days.

The growth of bacteria was measured for an optical density (OD) directly using spectrophotometer ($\lambda = 600 \text{ nm}$) (Dalgaard et al. 1994), whereas for analyzing of soluble manganese and cyanide concentrations, previously

samples were centrifuged at a speed of 10,000 rpm for 10 min to separate their cells from the medium. Then, the supernatant was filtered with a porous membrane filter $0.45 \mu\text{m}$. Therefore, for analyzing manganese concentration, the filtered sample was added by two drops of HNO_3 and heated 10 minutes. The samples then were measured using Flame Atomic Absorption Spectrophotometry (AAS) at a wavelength ($\lambda = 279.5 \text{ nm}$) (Khayatian et al. 2018). On the other hand, sample for analyzing cyanide concentration was observed using 4-pyridinecarboxylic acid color comparison method. Approximately, 1.5 mL filtered sample was diluted to a ratio of 1:10-15, then it was reacted with reactant and chromogenic agent. After 8 min, the absorbance of the blue-colored mixture was determined from OD measurement at 607.5 nm wavelength. Cyanide concentration was calculated using the calibration curves. These measurements were carried out with replication, then the results were averaged (Sharma et al. 2016).

Characterization of strains based on the sequence of 16S rDNA gene

Bacterial genomic DNA was extracted using a phenol-chloroform extraction method (Janabi et al. 2016). Precipitated DNA was harvested by centrifugation at 1,3000 rpm for 10 min, washed with 500 μL ethanol (70%), and resuspended in Tris-EDTA (TE) buffer. The purity of bacterial genomic DNA was further analyzed using nanodrop UV-Vis Spectrophotometer (Thermo Scientific) at wavelengths A260/A280 (Sambrook and Russell 1989).

The amplification of 16S rDNA gene then was performed using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3 ') and 1492R (5'-TAC CTT GTT ACG ACT T-3') (Miao et al. 2014; Ahn et al. 2017). The PCR mixture consisted of each one μl of primer 27F and 1492R (5 pmol μL^{-1}), one μl DNA template (100 ng μL^{-1}), and ddH₂O as much as 9.5 μl . The PCR protocol was set as follows: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 1.5 min, annealing at 45 °C for 1 min, elongation at 72 °C for 3 min (35 cycles), and a final extension at 72 °C for 10 min. The PCR products were confirmed using electrophoresis on 2% agarose gel (approximately 1426 bp amplicons were generated). PCR products were then sequenced and analyzed using the basic local alignment search tool (BLAST) (<http://www.ncbi.nlm.nih.gov/blast>). The phylogenetic tree was made with the neighbor-joining and bootstrap methods in MEGA 7.0, using the Kimura 2-parameter method and is in the units of the number of transitional substitutions per site (Kumar et al. 2016). Among-site variation rate was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair.

Data analysis

All experimental results were expressed as mean \pm S.D of three parallel measurements. These data were analyzed by using SPSS 19.0. Analysis of variance was performed, followed by Duncan Multiple Range Test at a significance level of $\alpha=0.05$.

RESULTS AND DISCUSSION

The heterotrophic bacteria (KB3B1) was isolated from the sulfuric area located in Gedong Songo area, Ungaran. This area is located in the southern part of Mount Ungaran and has the characteristics of thermal sources such as hot tubs, a zone of hydrothermal, and pools of acidic and sulfuric sludge. The average temperature of the sediment pond of the sulfuric water source was 40°C. Based on previous research, Ungaran sulfur bath area had a water temperature range of 18°C to 56°C depending on the length and depth of the flow path. The deeper and the longer the flow, the higher the water temperature was reported. The average pH value of this sulfuric area was in the range of 3.5-7.0. The sulfate concentration was in the range of 0.1-247 mg L⁻¹ (Phuong et al. 2012).

Effect of glycine addition on bioleaching activities of the KB3B1 strain

As reported previously, glycine is a direct precursor of cyanide that is formed by oxidative decarboxylation (Ruan et al. 2014; Shin et al. 2015). Glycine takes part in the reaction generating loss of 4 H to form HCN which is catalyzed by the HCN synthase (Campbell et al. 2001). The cyanide produced by heterotrophic bacteria induces metals extraction, including manganese from their deposits. The chemical reaction of that manganese extraction can be written as follow:



For investigating the effects of glycine addition in this leaching experiments, several parameter measurements including bacterial cell growth, cyanide concentration, pH value, and yield of manganese were observed in the leaching experiments. The leaching experiments of the strain during 18 days under a varying glycine concentration were shown in Figure 1. The KB3B1 showed a similar growth pattern in the modified 9 K medium in the presence of 0.02 g cm⁻³ pulp density of pyrolusite with and without the addition of glycine. This bacterial growth increased gradually and reached a maximal point at 15 days incubation. However, there were significant differences in their growth due to the glycine concentration addition. The highest bacterial cell growth was measured at the glycine addition of 10 mg mL⁻¹ with the maximal OD of 0.9 at 15 days incubation. The increased pH values investigated was in the range of 7.0 and 9.2 throughout the incubation period from 3 to 18 days at glycine concentration of 5 to 15 mg mL⁻¹.

On the contrary, the pH values of the culture without glycine addition (control experiments) did not change significantly throughout the incubation. The increase of pH values at cultures added with glycine could be a result of the metabolic process of cyanide production from the precursor of glycine, which consumed acid and produced a base of cyanide. The highest pH value was also observed in the culture with a glycine concentration of 10 mg mL⁻¹. This highest pH value was followed by the highest cyanide production. As reported by previous researchers, in the leaching system, those cyanide products enhanced metals

solubilization from their deposits (Adams 2013; Ruan et al. 2014; Shin et al. 2015). Therefore, the cyanide production would be followed by its consumption. This reaction continued so that the production and consumption of cyanide were balanced, as shown by pH values almost constant at the end of the incubation (18 days incubation).

The tested strain was most productive ($p < 0.05$) producing manganese (16.6%) in the leaching experiment with applying glycine concentration of 10 mg mL⁻¹. Therefore, there were correlations between the increased bacterial growths and the increased cyanide productions (R^2 : 0.88), pH values (R^2 : 0.71) and yields manganese (R^2 : 0.92).

As a comparison, Shin et al. (2015) investigated that the gold bioleaching by *Chromobacterium violaceum* was induced by applying glycine at a concentration of 5 mg mL⁻¹. Natarajan and Ting (2015) also reported that cell-free metabolite of *C. violaceum* could extract gold higher than that whole cells as much of 18% yield of bioleaching.

Molecular characterization of KB3B1 strain based on the 16S rDNA gene

The molecular characterization of KB3B1 strain having highest leaching activities was performed based on 16S rDNA gene analysis via polymerase chain reaction (PCR) using the universal 27F Primer (AGAGTTTGATCMTGGCTCAG) and 1492R Primer (TACGGYTACCTTGTTACGACTT). This 16S rDNA gene is a universal molecular marker in bacteria, by which the relationships among all bacteria can be measured (Woese 1987; Coenye and Vandamme 2004). Therefore, this 16S rDNA gene sequence analysis more accurately identify bad descriptions, infrequently isolated, or deviant phenotypic strains. Some other genes are very conserved like the 16S rDNA gene. Even though the absolute rate of change in the 16S rDNA gene sequence is not known, it marks the evolutionary distance and the relatedness of organisms (Stackebrandt and Goebel 1994; Lin et al. 2008). All genetic sequence databases were already deposited and published in the International Nucleotide Sequence Database Collaboration known as GenBank, the largest databank of nucleotide sequences. It also has over 20 million deposited sequences, of which over 90,000 are of 16S rDNA gene (Konstantinidis and Tiedje 2007; Chun and Rainey 2014). Hence, the sequence results can be compared with the various lengths of sequences obtained in that database. As a consequence, the analysis result can be used to identify bacterial isolates from any source. Then, the PCR product was checked using gel electrophoresis. PCR products obtained using primer from the DNA sequence of KB3B1 strain produced a single ~1500 bp band, indicating that it was a 16S rDNA band (Figure 2). The result of the PCR was then sequenced and edited using the Bioedit software package, and the consensus sequences obtained were then blasted in the GenBank of NCBI, which resulted in a significant similarity of 99-100% in the GenBank. Accordingly, the strain KB3B1 showed 100% similarity with *Bacillus niacini* strain EP89 (accession number AB294553). (Table 1).

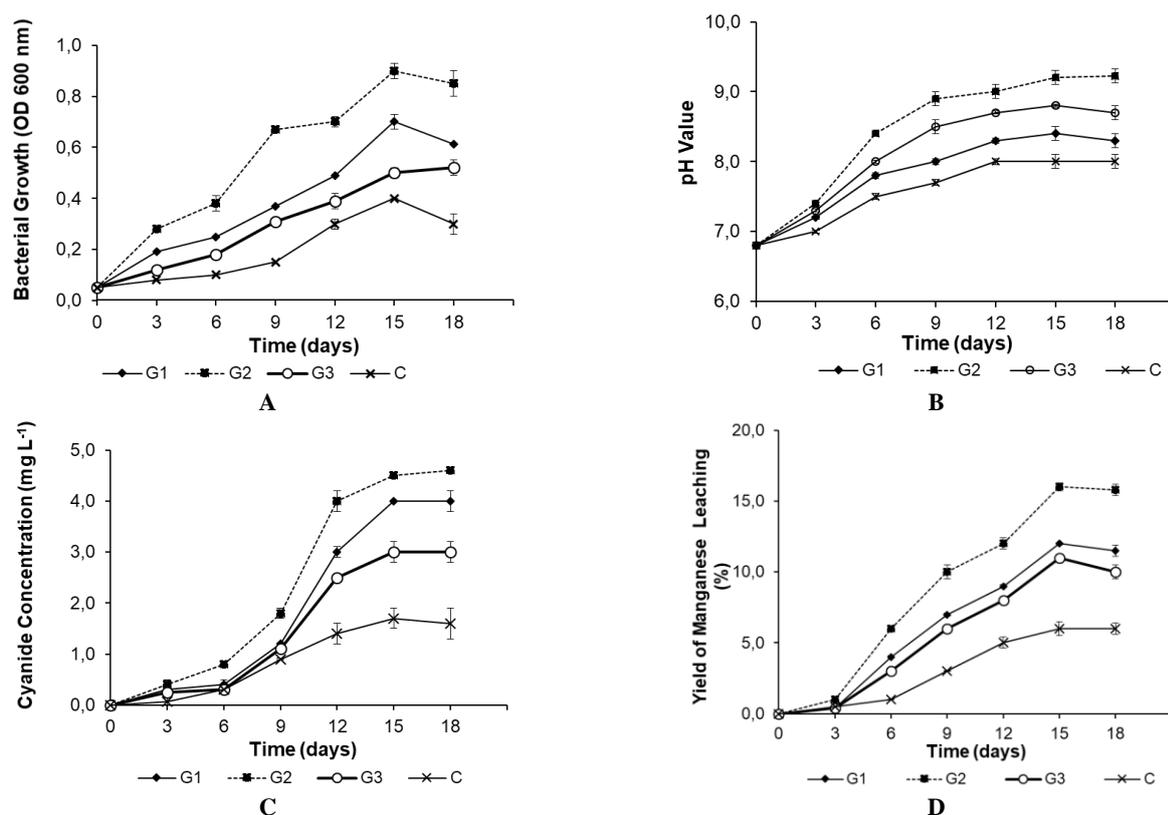


Figure 1. The effect of glycine concentration addition in KB3B1 culture on the bacterial growths (A); pH values (B); cyanide (C); and manganese concentrations (D) during 18 days leaching experiments under a pulp density of pyrolusite of 0.02 g cm^{-3} . G: glycine treatment, G1: 5, G2: 10, G3: 15, C: 0 mg mL^{-1}

Table 1. Results of the analysis of 16S rRNA gene sequences of KB3B1 strain using the BLAST-N program

Strain	Species of LAB homolog	Identities	Accession number
KB3B1	<i>Bacillus niacini</i> strain EP89	100%	MG778778.1
	<i>Bacillus niacini</i> strain GF7	100%	KY312789.1
	<i>Bacillus drentensis</i> strain BF-R7	99.98%	KY292423.1
	<i>Bacillus</i> sp. strain BF-R4	99.97%	KY292420.1
	<i>Bacillus</i> sp. strain FJAT-29897	99.97%	MF948353.1

The evolutionary history and molecular diversity of strain were further inferred using the UPGMA method by MEGA 7.0 application program. Figure 3 shows the percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates). The bootstrap values have a range of 67-86%. The greater the value of bootstrap, the higher the level of confidence in the reconstruction of the tree topology. An isolate is believed to have proximity if it has a bootstrap value of more than 70% (Hall 2001). The results of the phylogenetic analysis showed one major group with five *Bacillus* strains (*Bacillus niacini* strain EP89, *Bacillus niacini* strain GF7, *Bacillus drentensis* strain BF-R7, *Bacillus* sp. strain BF-R4 and *Bacillus* sp. strain FJAT-29897).

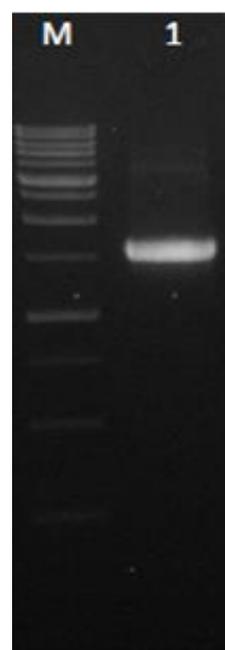


Figure 2. Amplified 16S rDNA gene of KB3B1 strain observed separated using agarose gel electrophoresis. M: 100 bp DNA ladder marker (Geneaid), 1: KB3B1 strain.

Table 2. The nucleic acids composition of thymine (T), cytosine (C), adenine (A), guanine (G), and also the average frequencies of guanine and cytosine (G+C) of KB3B1 strain analyzed using MEGA 7.0

Strain	T (%)	C (%)	A (%)	G (%)	Total (%)	(G+C) (%)
KB3B1	19.5	23.1	24.8	32.6	986.0	55.7
<i>Bacillus niacini</i> strain EP89	19.3	23.2	24.9	32.5	982.0	55.7
<i>Bacillus drentensis</i> strain BF-R7	19.5	23.2	24.9	32.4	984.0	55.6
<i>Bacillus</i> sp. strain BF-R4	19.5	23.2	24.8	32.5	984.0	55.7
<i>Bacillus</i> sp. strain FJAT-29897	19.5	23.2	24.9	32.4	984.0	55.6
<i>Bacillus niacini</i> strain GF7	19.5	23.2	24.9	32.4	984.0	55.6
<i>Thiobacillus sajanensis</i> strain 4HG	19.6	22.7	25.6	32.0	1264.0	54.7

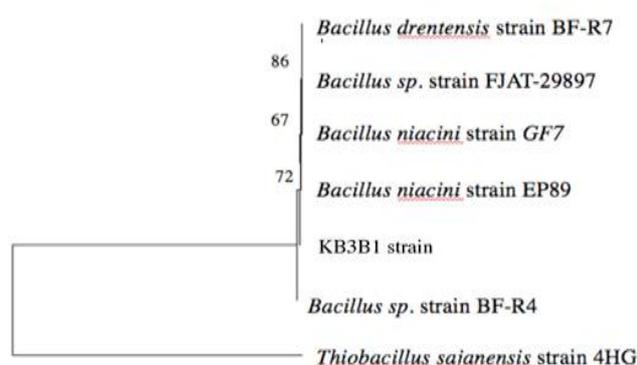


Figure 3. Phylogenetic tree constructed using 16S rDNA gene sequences, available in the GenBank database, employing the Neighbour-joining method. Numbers on nodes represent bootstrap values (%) from 1000 replicates. A phylogenetic tree was constructed using MEGA 7.0.

The MEGA application program can also be used to analyze the composition of the nucleic acids of thymine (T), cytosine (C), adenine (A), guanine (G), and also the average frequencies of guanine and cytosine (G+C). The nucleic acid composition of KB3B1 strain was listed in Table 2. From this table, it was shown that the average frequencies of G + C of the strain was 55.7%. This value shown was still in the same range as the value of the reference bacteria of GenBank as genus *Bacillus* (54-56%). In addition, these average frequencies of guanine and cytosine (G+C) of strain was also in line with Bergey's Manual of Systematics of Archaea and Bacteria as species *Bacillus niacini* (Logan, 2009), hence the strain KB3B1 was identified clearly as that species. As reported by Meier-Kolthoff et al. (2014), the average frequencies of guanine and cytosine (G+C) were found to be varied with different bacteria; species, which showed variation in selection, mutation bias, and related to the recombination of DNA. These average cytosine (G+C) frequencies also revealed the evolution of kinship among isolates

In conclusion, KB3B1 strain was most optimal in extracting manganese from pyrolusite at initial pH 6, pulp density of 0.02 g cm⁻³, and glycine concentration of 10 mg mL⁻¹ which led to 16.6% manganese extraction. This

extraction was obtained at 15 days of leaching incubation. Additionally, this maximum extract obtained was followed by the maximum bacterial growth, pH, and cyanide product of that strain. Molecular characterization revealed that the strain was found to be closely related to *Bacillus niacini* EP89. The average frequencies of guanine and cytosine (G+C) result of the strain were in the same range as the value of the reference bacteria of GenBank and Bergey's Manual Systematics of Bacteria.

ACKNOWLEDGEMENTS

This research was supported by KEMENRISTEK DIKTI Indonesia, through PDUPT research scheme (Contract No: 2653/UN1.DITLIT/DIT-LIT/LT/2019). We thank the use of several instrumentations of Microbiology Laboratory, FALITMA, Biology Faculty and LPPT, Universitas Gadjah Mada, Indonesia. We are also thankful to the confidential reviewers for valuable suggestions in improving the quality of the manuscript.

REFERENCES

- Adams MD. 2013. Impact of recycling cyanide and its reaction products on upstream unit operations. *Miner Eng* 53: 241-255.
- Ahn H, Kim J, Kim WJ. 2017. Isolation and characterization of bacteriocin-producing *Pediococcus acidilactici* HW01 from malt and its potential to control beer spoilage lactic acid bacteria. *Food Control* 80: 59-66.
- Campbell NA, Reece JB, Urry LA, Cain ML, Wasserman SA, Minorsky PV, Jackson RB. 2014. *Biology*. 10th Edition. Pearson Education Inc., USA.
- Chen P, Yan L, Leng F, Nan W, Yue X, Zheng Y, Li H. 2011. Bioleaching of realgar by *Acidithiobacillus ferrooxidans* using ferrous iron and elemental sulphur as the sole and mixed energy sources. *Bioresour Technol* 102: 3260-3267.
- Chun J, Rainey FA. 2014. Integrating genomics into the taxonomy and systematics of the Bacteria and Archaea. *Intl J Syst Evol Microbiol* 64: 316-324.
- Coenye T, Vandamme P. 2004. Use of the genomic signature in bacterial classification and identification. *Syst Appl Microbiol* 27: 175-185.
- Dalgaard P, Ross T, Kamperman L, Neumeyer K, McMeekin TA. 1994. Estimation of bacterial growth rates from turbidimetric and viable count data. *Int J Food Microbiol* 23: 391-404.
- Dan Z, Zhang Y, Cai J, Li X, Duana N, Xin B. 2016. Reductive leaching of manganese from manganese dioxide ores by bacterial-catalyzed two-ores method. *Intl J Miner Process* 150: 24-31.

- Das AP, Sukla LB, Pradhan N, Nayak S. 2011. Manganese biomining: A review. *Bioresour Technol* 102: 7381-7387.
- Das AP, Sukla LB, Pradhan N. 2012. Microbial recovery of manganese using *Staphylococcus epidermidis*. *Intl J Nonferrous Metallurgy* 1: 9-12.
- Das AP, Ghosh S, Mohanty S, Sukla LB. 2015. Advances in manganese pollution and its bioremediation. In: Singh A, Kuhad R, Ward C, Owen P (eds.). *Environmental Microbial Biotechnology Soil Biology*. Springer, Berlin.
- Dong Y, Liu Y, Lin H, Liu C. 2019. Improving vanadium extraction from stone coal via combination of blank roasting and bioleaching by ARTP-mutated *Bacillus mucilaginosus*. *T Nonferr Metal Soc* 29: 849-858.
- Faramarzi MA, Stagars M, Pensini E, Krebs W, Brandl H. 2004. Metal solubilization from metal-containing solid materials by cyanogenic *Chromobacterium violaceum*. *J Biotechnol* 113: 321-326.
- Ghosh S, Das AP. 2017. Bioleaching of manganese from mining waste residues using *Acinetobacter* sp. *Geol Ecol Landsc* 1: 77-83.
- Ghosh S, Mohanty S, Akcil A, Sukla LB, Das AP. 2016. A greener approach for resource recycling: Manganese bioleaching. *Chemosphere* 154: 628-639.
- Han Y, Ma X, Zhao W, Chang Y, Zhang X., Wang X, Wang J, Huang Z. 2013. Sulfur-oxidizing bacteria dominate the microbial diversity shift during and low-grade pyrolusite bioleaching process. *J Biosci Bioeng* 116: 465-471.
- Hall BG. 2001. *Phylogenetic Trees Made Easy: A How-To Manual For Molecular Biologists*. Sinauer Associates, Inc., Sunderland.
- Huynh D, Giebner F, Kaschabek SR, Rivera-Araya J, Levican G, Sanda W, Schlömann M. 2019. Effect of sodium chloride on *Leptospirillum ferriphilum* DSM 14647T and *Sulfobacillus thermosulfidooxidans* DSM 9293T: Growth, iron oxidation activity, and bioleaching of sulfidic metal ores. *Miner Eng* 138: 52-59.
- Jalali F, Fakhari J, Zolfaghari A. 2019. Response surface modeling for lab-scale column bioleaching of low-grade uranium ore using a new isolated strain of *Acidithiobacillus ferridurans*. *Hydrometallurgy* 185: 194-203.
- Janabi AHD., Kerkhof LJ, McGuinness LR, Biddle AS, McKeever KH. 2016. Comparison of a modified phenol/chloroform and commercial-kit methods for extracting DNA from horse fecal material. *J Microbiol Methods* 129: 14-19.
- Jang H, Valix M. 2017. Overcoming the bacteriostatic effects of heavy metals on *Acidithiobacillus thiooxidans* for direct bioleaching of saprolitic Ni laterite ores. *Hydrometallurgy* 168: 21-25.
- Khayatian G, Moradi M, Hassanpoor S. 2018. MnO₂/3MgO Nanocomposite for preconcentration and determination of trace copper and lead in food and water by flame atomic absorption spectrometry. *J Anal Chem* 73: 470-478.
- Konstantinidis KT, Tiedje JM. 2007. Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. *Curr Opin Microbiol* 10: 504-509.
- Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33: 1870-1874.
- Lin Y, Chang BCH, Chiang P, Tang S. 2008. Questionable 16S ribosomal RNA gene annotations are frequent in completed microbial genomes. *Gene* 416: 44-47.
- Liu YG, Zhou M, Zeng GM, Wang X, Li X, Fan, T, Xu. WH. 2008. Bioleaching of heavy metals from mine tailings by indigenous sulfur-oxidizing bacteria: effects of substrate concentration. *Bioresour Technol* 99: 4124-4129.
- Logan NA. 2009. *Bergey's Manual of Systematics of Archaea and Bacteria*. John Wiley & Sons, Inc., New York.
- Meier-Kolthoff JP, Klenk HP, Göker M. 2014. Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. *Intl J Syst Evol Microbiol* 64: 352-356.
- Miao J, Guo H, Ou Y, Guo L, Fang X, Liao Z, Ke C, Chen Y, Zhao L, Cao Y. 2014. Purification and characterization of bacteriocin F1, a novel bacteriocin produced by *Lactobacillus paracasei* subsp. *tolerans* FX-6 from Tibetan kefir, a traditional fermented milk from Tibet, China. *Food Control* 42: 48-53.
- Mohanty S, Ghosh S, Nayak S, Das AP. 2017. Bioleaching of manganese by *Aspergillus* sp. isolated from mining deposits. *Chemosphere* 172: 302-309.
- Natarajan G, Ting YP. 2015. Gold bio-recovery from e-waste: An improved strategy through spent medium leaching with pH modification. *Chemosphere* 136: 232-238.
- Phuong NK, Harijoko A, Itoi R, Unoki Y. 2012. Water geochemistry and soil gas survey at Ungaran geothermal field, Central Java, Indonesia. *J Volcanol Geotherm Res* 229: 23-33.
- Peraturan Menteri Energi dan Sumber Daya Mineral Republik Indonesia. 2014. Tentang Peningkatan Nilai Tambah Mineral Melalui Kegiatan Pengolahan dan Pemurnian Mineral di Dalam Negeri. Nomor 1. [Indonesian]
- Retnaningrum E, Wilopo W. 2019. Pyrolusite bioleaching by an indigenous *Acidithiobacillus* sp KL3 isolated from an Indonesian sulfuriferous river sediment. *Indonesia J Chem* 19: 712-719.
- Ruan X, Zhu Y, Hu QJ. 2014. A new strain for recovering precious metals from waste printed circuit boards. *J Waste Manag* 34: 901-907
- Sambrook J, Russell DW. 1989. *Molecular Cloning: A Laboratory Manual* 3rd ed. Laboratory Press, New York.
- Saleh DK, Abdollahi H, Noaparast M, Nosratabad AF, Tuovinen OH. 2019. Dissolution of Al from metakaolin with carboxylic acids produced by *Aspergillus niger*, *Penicillium bilaji*, *Pseudomonas putida*, and *Pseudomonas koreensis*. *Hydrometallurgy* 186: 235-243.
- Sharma S, Singh J, Singh N, Hundal G. 2016. Spectroscopic and theoretical evaluation of solvent-assisted, cyanide selectivity of chromogenic sensors grounded on mesitylene platform and their biological applications. *Sens Actuators B Chem* 225: 141-150.
- Shin D, Jeonghyun Park, Jinki Jeong, Byung-su Kim. 2015 Biological cyanide production and accumulation system and the recovery of platinum-group metals from spent automotive catalysts by biogenic cyanide. *Hydrometallurgy* 158: 10-18.
- Stackebrandt E, Goebel BM. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Intl J Syst Bacteriol* 44: 846-849.
- Thavamani P, Samkumar RA, Satheesh V, Subashchandrabose SR, Ramadass K, Naidu R, Venkateswarlu K, Megharaj M. 2017. Microbes from mined sites: Harnessing their potential for reclamation of derelict mine sites. *Environ Pollut* 230: 495-505.
- Wang X, Liao R, Zhao H, Hong M, Huang X, Peng H, Wen W, Qin W, Qiu G, Huang C, Wang J. 2018. Synergistic effect of synergistic effect of pyrite on strengthening bornite bioleaching by *Leptospirillum ferriphilum*. *Hydrometallurgy* 176: 9-16.
- Woese CR. 1987. Bacterial evolution. *Microbiol Rev* 51: 221-271.
- Zhu J, Zhang J, Li Q, Han T, Hu Y, Liu X, Qin W, Chai L, Qiu G. 2014. Bioleaching of heavy metals from contaminated alkaline sediment by auto-and heterotrophic bacteria in stirred tank reactor. *T Nonferr Metal Soc* 24: 2969-2975.