Arsenic removal from acid mine drainage using acid-tolerant bacteria

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Abstract

Acid mine drainage (AMD) has a low pH and contains harmful metals, making it a severe problem in the mining industry. Neutralization treatment using slaked lime is widely applied to remove potentially toxic metals and adjust the pH. However, this generates neutralized sludge containing large amounts of harmful metals. Therefore, in this study, we propose a bacterial bioprocess for removing metals before neutralization. Acid- and metal-tolerant bacteria were isolated from neutral soils and utilized as As removers from AMD. The *Paenathrobacter* sp. strain H1 removed As (43.6%) and Fe (10.6%) from AMD in a single batch test (pH 1.95; initial concentrations were 6.13 and 283 mg L^{-1}, respectively). Repeated batch tests using fresh cells enhanced the As removal ratio, achieving successful removal of As (95.3%) and Fe (75.5%). Although further research is required, this study has substantial implications for the development of a sustainable AMD treatment to suppress harmful waste generation.

Keywords: Acid mine drainage, Acid-tolerant bacteria, Arsenic, Metal removal

1 Introduction

The mining industry has enriched human lives but can simultaneously cause serious environmental issues such as acid mine drainage (AMD).[1] Neutralization treatments are widely employed to prevent environmental and human health problems (Fig. 1A). Although this process is effective and extensively used, it presents specific challenges. Considerable electricity and labor are required to treat AMD.[2] Furthermore, a large amount of neutralized sludge containing potentially toxic metals such as As, Cd, and Pb is generated, and its deposition and utilization are often insufficient.[3, 4] Deposited neutralized sludge prevents remediation of the mining site environment and potentially causes environmental pollution through the reionization of harmful metals. In addition, it is estimated that the capacity of final disposal sites for industrial waste will only remain sustainable for a few decades in Japan.[5] Therefore, it is desirable to reduce the amount of harmful neutralized sludge generated, considering both cost and

environmental impact. One possible approach to address this scenario is to reduce the volume of harmful neutralized sludge. Metal adsorption is a potential alternative to conventional neutralization processes. Most studies on metal adsorbents have been performed under neutral pH conditions since most cationic metal species are predominant at acidic pH and cause competitive binding with H^+ ions.[6]

In this study, we focused on As, which is a harmful component of AMD. Only a few studies have been conducted on As adsorbents under acidic conditions.[7, 8] Notably, productivity, costs, and environmental impacts should also be considered while selecting an appropriate adsorbent for AMD treatment. Bacteria are ideal candidates for low-cost adsorbent materials, as described in numerous previous studies.[9, 10] In particular, we focused on bacteria that can remove metals under acidic conditions and grow under neutral pH and moderate temperature conditions. Acid-tolerant bacteria have advantages over acidophilic bacteria in terms of biomass production. An acidic medium and acid-tolerant equipment are required to cultivate acidophilic bacteria. In contrast, acid-tolerant bacteria can be cultured using common media and equipment, and no acidic waste medium needs to be treated.[11] This implies that the utilization of acid-tolerant bacteria is more beneficial compared with acidophilic bacteria from an economic and environmental point of view. However, despite these advantages, acid-tolerant bacteria have not been sufficiently utilized, likely because efficient and selective isolation methods are lacking.[11] Here, we propose a novel process using acid-tolerant bacteria to remove As from AMD without neutralization (Fig. 1B).

It is expected that As removal before neutralization contributes to suppressing harmful neutralized sludge generation. Ito *et al.* achieved it by generating schwertmannite, As and Fe containing mineral, from AMD[12]. In this study, it is attempted by using bacterial metal removal. Thus, this process is expected to decrease the environmental load by reducing the generation of harmful neutralized sludge, which contributes to the utilization of untapped bioresources.

Therefore, in this study, we attempted to isolate acid-tolerant bacteria and evaluate their metal-removal abilities under AMD conditions.

2 Materials and Methods

2.1 Materials

Soil samples were collected from the Hokkaido University Sapporo campus (N43°04’29’’, E141°20’23’’) as a microbial source, and AMD was sampled from two closed mining sites (Teine mine N43°6’10’’, E141°11’24’’, Horobetsu sulfur mine N42°32’22’’, E141°00’28’’) in Hokkaido. Specialized cellulose film (SCF) and nonwoven fabric (Futamura Chemical, Japan) were used for bacterial isolation.[13] Unless otherwise stated, all reagents were purchased from Fujifilm Wako Pure Chemicals. Nutrient broth (NB; Becton Dickinson and Company, USA) and R2A medium were used for bacterial cultivation. Metal quantification was performed using inductively coupled plasma-atomic emission spectroscopy (ICPE-9820, Shimadzu, Kyoto, Japan, ICP-AES). Metal calibration curves were prepared using ICP standard solution IV (Merck, Germany) and 100 mg L^{-1} As standard solution. A pH-Eh diagram was generated using Geochemist's Workbench.

2.2 Isolation of acid-tolerant bacteria

The collected soil samples were diluted ten times with sterilized distilled water and suspended in an ultrasonic

cleaner (ASU-10; AS ONE, Osaka, Japan). After filtration through filter paper, the supernatant was stored in a refrigerator at 4℃ until inoculation. SCF was prepared as previously described.[13] Briefly, after autoclave (121℃, 15 min), nonwoven fabric was placed into a sterilized petri-dish then SCF was placed on nonwoven fabric. Following this, isolation media containing equal volumes of Teine mining site AMD and NB media (pH 4.53) was poured into the petri-dish. Subsequently, 70 µL filtrate of the soil suspension was inoculated on the surface of the SCF and incubated at 30℃ until colonies appeared. A single colony was selected and streaked onto fresh R2A agar plates to isolate pure bacterial colonies. The purification process was repeated at least five times.

2.3 Phylogenetic analysis

A 16S rRNA gene sequence analysis was conducted to identify the isolated strains. A loopful of a single bacterial colony was suspended in 50 µL of MightyPrep reagent for DNA (Takara Bio, Japan). After cell lysis (95℃, 10 min), cell components were removed by centrifugation (12283 × *g*, 2 min). 1 µL of the supernatant was mixed with 1.5 µL of primers (10 µM; Table 1), 25 µL of PrimeSTAR Max (Takara Bio, Japan), and 21 µL of sterilized water. DNA was amplified using a thermal cycler (T-100; Bio-Rad Laboratories, USA). After separation using 1% agarose gel electrophoresis (Mupid-2plus, Takara Bio), the observed DNA band at approximately 1500 bp was purified using a PCR & Gel Cleanup Kit 100 (QIAGEN, Germany) following the manufacturer's protocol. DNA sequence analysis was performed using the DNA Sequence Service (Eurofins Genomics, Japan). The isolated strains were identified using the Basic local alignment search tool (BLAST; National Center for Biotechnology Information). The raw reads were deposited in the DNA Data Bank of Japan (DDBJ, Shizuoka, Japan) database.

2.4 Determination of the AMD treatment period

After 25% glycerol stock solutions of the isolated bacteria had been prepared, 1 mL stock solution was inoculated in 5 mL of R2A medium and pre-cultured (30℃, 200 rpm) for at least 6 h. 1 mL of pre-cultured fluid was inoculated in 50 mL of R2A medium and cultured (30℃, 160 rpm) for at least 16 h. Bacterial cells were harvested by centrifugation (10000 × *g*, 5 min). The AMD sample was prepared by filtrating 50 mL of the Horobetsu mining site AMD (pH 1.95, Eh 870 mV) using a 0.22 µm-pore size membrane filter (Nippon Genetics, Japan). After measuring the pH and Eh, bacterial cells were suspended in the AMD sample in a 300-mL Erlenmeyer flask. To evaluate bacterial concentration, 100 µL of suspension was collected and diluted ten times before measuring the optical density at 600 nm. Metal-removal treatment was performed (under the following conditions: 0, 0.5, 1, 2, 3, 4, and 7 h, 30℃, 160 rpm), and 1 mL supernatant of each AMD sample was collected after centrifugation (12283 × *g*, 2 min). 1 mL of the initial and treated AMD samples were collected and diluted with 4% (v/v) nitric acid. Metal concentrations were evaluated using ICP-AES, and the metal-removal ratio was calculated by equation (1) as follows:

$$X = (C_0 - C) \times 100/C_0 \quad (1)$$

where X (%) is the ratio of the metal concentration change, C_0 (mg L^{-1}) is the initial metal concentration, and C (mg L^{-1}) is the metal concentration after metal removal. The metal-removal ability (mg g^{-1}-dry cell) was calculated using equation (2) as shown below:

$$Q = q/m \quad (2)$$

where Q (mg g^{-1}-dry cell) is the amount of metal per g-dry cell, q (mg L^{-1}) is the concentration of the removed metal, and m (g -dry cell L^{-1}) is the cell concentration.

The optimal treatment period for metal removal was determined based on the dynamics of each metal. Metal dynamics and removal ability of strain H1 under optimal conditions (3 h, 30°C, 160 rpm) were evaluated as described above.

2.5 Development of a repeated metal-removal process

Repeated metal removal was performed to further reduce the As concentration in the AMD. Eight flasks of cultured bacterial cell (50 mL) were prepared as described above. The cells in the first flask were harvested by centrifugation (10000 × *g*, 5 min) and resuspended in 50 mL of AMD, and the metal-removal treatment was performed as described above. Bacterial cells were harvested by centrifugation (11740 × *g*, 7 min, 4°C). 1 mL of the supernatant was collected for metal concentration measurement. The cells in the second flask were harvested and resuspended in the supernatant as described above; this cycle was repeated eight times. Metal concentrations after each cycle were quantified using ICP-AES.

2.6 Statistical analysis

All experiments were conducted in triplicate, and the standard deviation was calculated using EXCEL ver. 2312 (Microsoft, Redmond, WA, USA). Two-tailed Student's t-tests were conducted to evaluate significant differences ($p < 0.05$) using EXCEL.

3 Results and Discussion

3.1 Isolation of acid- and metal-tolerant bacteria

One isolated bacterial strain was grown in the AMD-containing medium and could be regarded as relatively acid-tolerant. The isolated strain was named *Paenarthrobacter* sp. H1, and the raw sequencing reads were registered in the DDBJ database (accession No. LC792549). According to the results of a BLAST search, the sequence of the isolated strain showed the highest similarity to *Paenarthrobacter nitroguajacolicus.* The phylogenetic tree is shown in Fig. 2. This species is Gram-positive, has an irregular rod shape, and grows in moderate pH and temperature conditions.[14] Under neutral conditions, H1 exhibited a biomass productivity of 1.038 g L^{-1}. Strain H1 was non-pathogenic, grew at a neutral pH, and survived in an acidic environment containing toxic metals. Compared to acidophilic bacteria that thrive in an acidic environment, strain H1 reached the stationary phase in less than 15 h, and the optical density at 540 and 640 nm was >3.5; however, acidophilic bacteria generally take more than a day to reach the stationary phase, and optical densities are <1.5.[15, 16] In addition, acid-tolerant bacteria have a lower production cost and can help reduce the environmental load as biomass production does not generate acidic wastewater. Based on these results, this bacterium was identified as a potential metal remover which is able to remove As from AMD.

3.2 Determination of the AMD treatment period

The concentrations of As and Fe decreased continuously and reached steady state at 3 and 4 h, respectively

(Figs. 3, 4). K, Mg, and Na content increased and reached steady state at 0.5, 2, and 3 h, respectively (Fig. 5). A shorter treatment period is preferred for industrial applications. Based on these metal dynamics, the As removal treatment period was set at 3 h in subsequent experiments. Considering that the As concentration changed rapidly and showed no significant change after reaching a steady state, the metal-removal mechanism was considered to be adsorption on surface protein and that the strain had a large number of As adsorption sites on its surface. According to previous studies, *Bacillus cereus* adsorbed As(III) by interactions with hydroxyl, amine, and amide groups on the cell surface.[17] As(V) was adsorbed by *B. subtilis* based on complexation with hydroxyl groups.[18] In addition, uncharged As(III) species could interact with functional groups.[19] From a pH-Eh diagram, according to initial metal concentrations of the AMD (Fig. 6), the chemical form of As in the AMD was likely H_3AsO_4. This suggests that strain H1 adsorbed uncharged As species, As(V) in the form of H_3AsO_4. Further study is required to understand the bacterial removal mechanism. In contrast to As, which forms neutral or monovalent anion,[20] Fe typically exists as a divalent cation under AMD conditions.[21] Thus, it is possible to remove As and Fe without competition under AMD conditions. The removal periods and ion conditions suggest that the metal-removal mechanism of strain H1 is biosorption.

It has been reported that cation release and ion exchange occur as a bacterial response to acid and metal stress.[22, 23] In this study, K, Mg, and Na concentrations in AMD increased with Fe removal (Table 2). This suggests that the three cations are released from the cell during As and Fe adsorption. Further studies of the metal distribution in the cell will contribute to clarifying the metal adsorption mechanism.

3.3 Evaluation of metal-removal ability under optimal treatment conditions

Five elements (As, Fe, K, Mg, and Na) were significantly altered by 3 h removal treatment. The concentrations of As and Fe decreased, while those of other elements increased (Table 2). The removal ratios of As and Fe were 43.6 and 10.6%, respectively (Fig. 7). The removal ability of strain H1 was 1.50 and 16.9 mg g^{-1}-dry cell, respectively. Nevertheless, there are insufficient studies on As biosorption under acidic conditions, and most previous studies used a synthetic As solution (Table 3).[17, 24, 25] In this study, strain H1 removed a relatively higher amount of As under lower pH conditions than in previous studies (Table 3), although the AMD in this study contained high concentrations of Fe ions. In contrast to conventional studies, where bacteria are isolated using a common medium without any selective pressure, AMD containing media was used in this study for efficient isolation of acid and metal tolerant bacteria which is expected to have a high As removal ability. This result indicates that strain H1 can be effectively used for metal-removal treatment from an actual AMD before neutralization.

3.4 Repeated metal-removal treatments

The metal-removal treatment was repeated until the As concentration were below the Japanese effluent standards (0.1 mg L^{-1}). However, the quantification accuracy was insufficient due to equipment limitations. Removal percentages of 95.3 and 75.5% for As and Fe, respectively, were achieved through eight repeated metal-removal treatments. Within the quantified range, As decreased continuously as batch experiments were repeated (Fig. 8A). However, the removal ratio decreased with each cycle (Fig. 8A). In contrast, Fe and K concentrations changed

linearly. All cation concentrations increased at every cycle, and K constantly increased at a similar ratio to the Fe decrement, with a correlation coefficient of -0.999 (Fig. 8C). It is assumed that K release occurred to replace metal ion binding at the adsorption site, transitioning from K to Fe, which is attributable to the larger ionization tendency of K.

According to our results, 95.3 and 75.5% of As and Fe were removed from the AMD sample through repeated treatment. This helps to decrease the amount of neutralizer required for co-precipitation of As and Fe. In the case of $Ca(OH)_2$, a widely used neutralizer, the amount can be reduced from 0.563 to 0.138 g L^{-1} for Fe precipitation as $Fe(OH)_3$. In this study, chemical reagents were used for biomass production. However, other carbon sources, such as agricultural waste, can be used as well,[26] which is expected to contribute to reduce biomass production costs and waste utilization. Additionally, we showed that the main component of the final harmful waste was bacterial biomass containing As. In contrast to inorganic neutralized sludge, the major components of bacterial cells are organic compounds. Thus, it is possible to reduce the harmful waste volume by decomposing and drying bacterial biomass after As adsorption. Although further study is required, this study contributes to minimizing the amount of harmful waste generated during AMD treatment. Nonetheless, the required amount of cells increases as the number of treatment cycles increases, which is currently a significant challenge for the process. Therefore, combining this strategy with other materials and methods might render the process more sustainable. Bacteria and other organic As removers that are suitable for low metal concentration conditions are potential candidates.[24, 27] Additionally, diluting the treated water with spring water to a negligible concentration of harmful metals is a feasible method. To establish a sustainable industrial AMD treatment process, further studies are needed to design a comprehensive method that considers various technologies, including efficiency, cost, and environmental impact. While there is room for improvement in metal-removal efficiency, this study suggests a novel method to establish a sustainable AMD treatment process.

4 Concluding Remarks

The purpose of this study was to isolate metal-removing bacteria and characterize them for use in the AMD treatment process without neutralization. The results suggest that the isolated bacterial strain H1 is potentially useful in metal removal during AMD treatment. The strain removed 43.6% of As from the AMD sample obtained from the Horobetsu mining site. Notably, the removal ratio was enhanced to 95.3% by repeated treatments. To the best of our knowledge, this is the first study to use bacteria for metal removal from AMD without neutralization. Although further studies are required, this study contributes to the establishment of low-harmful waste emissions and an environmentally friendly alternative technology to the current AMD treatment process.

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Conflict of interest disclosure

The authors have declared no conflict of interest.

References

[1] D. Johnson, K. Hallberg, "Acid mine drainage remediation options: a review," Science of The Total Environment. 338(1-2), (**2005**): 3-14, DOI: 10.1016/j.scitotenv.2004.09.002

[2] R. Koide, C. Tokoro, S. Murakami, T. Adachi, A. Takahashi, "A Model for Prediction of Neutralizer Usage and Sludge Generation in the Treatment of Acid Mine Drainage from Abandoned Mines: Case Studies in Japan," *Mine Water and the Environment,* 31, (**2012**): 287-296, DOI: 10.1007/s10230-012-0200-x

[3] N. Karapınar, "Removal of Heavy Metal Ions by Ferrihydrite: an Opportunity to the Treatment of Acid Mine Drainage," *Water, Air, & Soil Pollution,* 227, (**2016**):193, DOI: 10.1007/s11270-016-2899-7

[4] D. Langmuir, J. Mahoney, A. Macdonald, J. Rowson, "Predicting arsenic concentrations in the porewaters of buried uranium mill tailings," Geochimica et Cosmochimica Acta, 63(19-20), (**1999**): 3379-3394, DOI: 10.1016/S0016-7037(99)00259-8

[5] Ministry of Economy, Trade and Industry, *Circular Economy Vision 2020,* Japan, Ministry of Economy, Trade and Industry, (**2020**), https://www.meti.go.jp/shingikai/energy_environment/junkai_keizai/pdf/20200522_03.pdf

[6] E. Torres, "Biosorption: A Review of the Latest Advances," *Processes*, 8(12), (**2020**): 1584, DOI: 10.3390/pr8121584

[7] N. K. Asmel, A. R. M. Yusoff, S. K. Lakkaboyana, Z. A. Majid, S. Salmiati, "High concentration arsenic removal from aqueous solution using nano-iron ion enrich material (NIIEM) super adsorbent," *Chemical Engineering Journal,* 317, (**2017**): 343-355, DOI: 10.1016/j.cej.2017.02.039

[8] W. Hu, L. Yang, P. Shao, H. Shi, Z. Chang, D. Fang *et al.*, "Proton Self-Enhanced Hydroxyl-Enriched Cerium Oxide for Effective Arsenic Extraction from Strongly Acidic Wastewater," *Environmental Science & Technology,* 56(14), (**2022**): 10412-10422, DOI: 10.1021/acs.est.2c02675

[9] P. A. Marques, H. M. Pinheiro, J. A. Teixeira, M. F. Rosa, "Removal efficiency of Cu^{2+}, Cd^{2+} and Pb^{2+} by waste brewery biomass: pH and cation association effects," *Desalination,* 124(1-3), (1999): 137-144, DOI:

10.1016/S0011-9164(99)00098-3

[10] Z. Aksu, "Application of biosorption for the removal of organic pollutants: a review," *Process Biochemistry,* 40(3-4), (**2005)**: 997-1026, DOI: 10.1016/j.procbio.2004.04.008

[11] C. Takano, H. Aoyagi, "Screening and isolation of acid-tolerant bacteria using a novel pH shift culture method," *Journal of Bioscience and Bioengineering,* 134(6), (**2022**): 521-527, DOI: 10.1016/j.jbiosc.2022.08.009

[12] K. Ito, K. Fukushi, K. Hashimoto, C. Tanaka, H. Ikeda, T. Sato, T. Yoneda, A Novel Remediation Method for Arsenic Bearing Acid Mine Drainage Learnt from Natural Attenuation Process," *Journal of MMIJ* 124(8), (**2008)**: 519-528, DOI: 10.2473/journalofmmij.124.519

[13] H. Aoyagi, R. Saitoh, K. Murayama, "Development of a device for cultivation and isolation of microbes using a specialized cellulose film," *Journal of Microbiological Methods,* 195 (**2022)**:106450, DOI: 10.1016/j.mimet.2022.106450

[14] L. Kotoucková, P. Schumann, E. Durnová, C. Spröer, I. Sedlácek, J. Neca, *et al.*, "*Arthrobacter nitroguajacolicus* sp. nov., a novel 4-nitroguaiacol-degrading actinobacterium**,**" *International Journal of Systematic and Evolutionary Microbiology,* 54(3), (**2004)**: 773-777, DOI: 10.1099/ijs.0.02923-0

[15] N. Kishimoto, T. Tano, "ACIDOPHILIC HETEROTROPHIC BACTERIA ISOLATED FROM ACIDIC MINE DRAINAGE, SEWAGE, AND SOILS," *Journal of General and Applied Microbiology,* 33(1), (**1987)**: 11-25, DOI: 10.2323/jgam.33.11

[16] A. Mohagheghi, K. Grohmann, M. Himmel, L. Leighton, D. M. Updegraff, "Isolation and Characterization of *Acidothermus cellulolyticus* gen. nov., sp. nov., a New Genus of Thermophilic, Acidophilic, Cellulolytic Bacteria," *International Journal of Systematic and Evolutionary Microbiology,* 36(3), (**1986**): 435-443, DOI: 10.1099/00207713-36-3-435

[17] Z. M. Bahari, W. A. H. Altowayti, Z. Ibrahim, J. Jaafar, S. Shahir, "Biosorption of As (III) by Non-living Biomass of an Arsenic-Hypertolerant *Bacillus cereus* Strain SZ2 Isolated from a Gold Mining Environment: Equilibrium and Kinetic Study," *Applied Biochemistry and Biotechnol*ogy, 171 (**2013**): 2247-2261, DOI: 10.1007/s12010-013-0490-x

[18] M. Xiao, J. Guo, S. Zhao, S. Li, "Adsorption of As(V) at Humic Acid-Kaolinite-Bacteria Interfaces: Kinetics, Thermodynamics, and Mechanisms," *Agronomy,* 13(2), (**2023)**: 611, DOI: 10.3390/agronomy13020611

[19] M. Tuzen, A. Sari, D. Mendil, O. D. Uluozlu, M. Soylak, M. Dogan, "Characterization of biosorption process

of As(III) on green algae *Ulothrix cylindricum*," *Journal Hazardous Materials,* 165(1-3), (**2009**): 566-572, DOI: 10.1016/j.jhazmat.2008.10.020

[20] P. Lu, C. Zhu, "Arsenic Eh–pH diagrams at 25°C and 1 bar", *Environmental Earth Sciences,* 62 (**2010**): 1673-1683, DOI: 10.1007/s12665-010-0652-x

[21] A. J. Monhemius, "The iron elephant: A brief history of hydrometallurgists' struggles with element no. 26," *CIM Journal,* 8 (**2017**): 197-206, DOI: 10.15834/cimj.2017.21

[22] F. Huang, Z. H. Wang, Y. X. Cai, S. H. Chen, J. H. Tian, K. Z. Cai, "Heavy metal bioaccumulation and cation release by growing *Bacillus cereus* RC-1 under culture conditions," *Ecotoxicology and Environmental Safety,* 157(15), (**2018**): 216-226, DOI: 10.1016/j.ecoenv.2018.03.077

[23] J. Ye, H. Yin, D. Xie, H. Peng, J. Huang, W. Liang, "Copper biosorption and ions release by *Stenotrophomonas maltophilia* in the presence of benzo[a]pyrene," *Chemical Engineering Journal,* 219 (**2013**): 1-9, DOI: 10.1016/j.cej.2012.12.093

[24] A. K. Giri, R. K. Patel, S. S. Mahapatra, P. C. Mishra, "Biosorption of arsenic (III) from aqueous solution by living cells of Bacillus cereus," *Environmental Science and Pollution Research,* 20 (**2013)**: 1281-1291, DOI: 10.1007/s11356-012-1249-6

[25] S. A. Haris, W. A. H. Altowayti, Z. Ibrahim, S. Shahir, "Arsenic biosorption using pretreated biomass of psychrotolerant *Yersinia* sp. strain SOM-12D3 isolated from Svalbard, Arctic," *Environmental Science and Pollution Research,* 25 (**2018**): 27959-27970, DOI: 10.1007/s11356-018-2799-z

[26] S. Kongruang, "Bacterial Cellulose Production by *Acetobacter xylinum* Strains from Agricultural Waste Products," *Applied Biochemistry and Biotechnology,* 148 (**2008**): 245-256, DOI: 10.1007/s12010-007-8119-6

[27] N. M. Otiniano, M. D. L. Cruz-Noriega, L. Cabanillas-Chirinos, S. Rojas-Flores, M. A. Muñoz-Ríos, W. Rojas-Villacorta *et al.*, "Arsenic Biosorption by the Macroalgae *Chondracanthus chamissoi* and *Cladophora* sp.," *Processes,* 10(10), (**2022**): 1967, DOI: 10.3390/pr10101967

Figures

Fig. 1 Acid mine drainage treatment process. A) Current neutralization and precipitation. B) Novel bacterial metal-removal process developed in this study. Bacteria remove the potentially toxic metal before neutralization, which suppresses the generation of harmful neutralized sludge.

Fig. 2 Phylogenetic tree of the isolated strain H1.

Fig. 3 Time course of As concentrations in AMD. Asterisks indicate significant differences ($p < 0.05$).

Fig. 4 Time course of Fe concentrations in AMD. Asterisks indicate significant differences ($p < 0.05$).

Fig. 5 Time course of cation concentrations in AMD. Asterisks indicate significant differences ($p < 0.05$).

Fig. 6 As Eh-pH diagram for AMD (25℃, 1.013 bar).

Fig. 7 Removal ratio of As and Fe [%]. Asterisks indicate significant differences ($p < 0.05$).

Fig. 8 Metal dynamics in AMD during repeated metal-removal treatment. A) As. Asterisks indicate significant differences ($p < 0.05$). B) Fe, and C) K. There were significant differences between batches.

Table 1. Sequences of PCR primers.

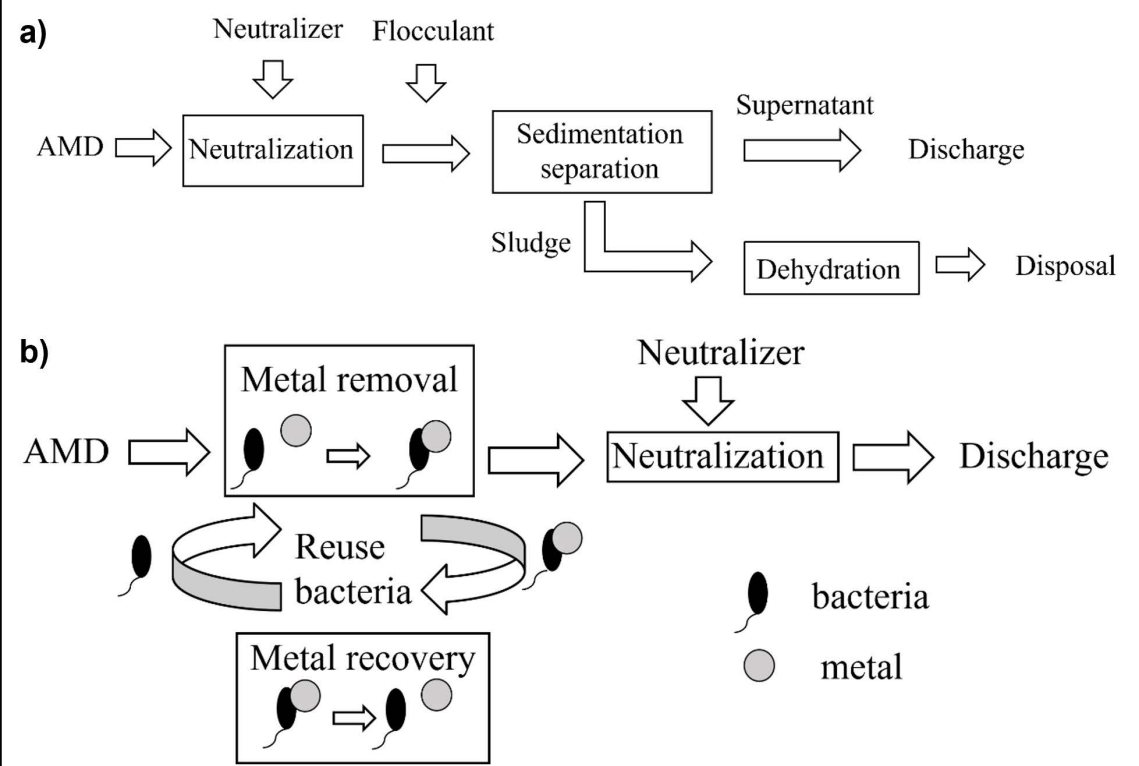
Name	Sequence (3' to 5')
9F	GATGTTTGATCCTGGCTCAG
1541R	AAGGAGGTGATCCAGCC

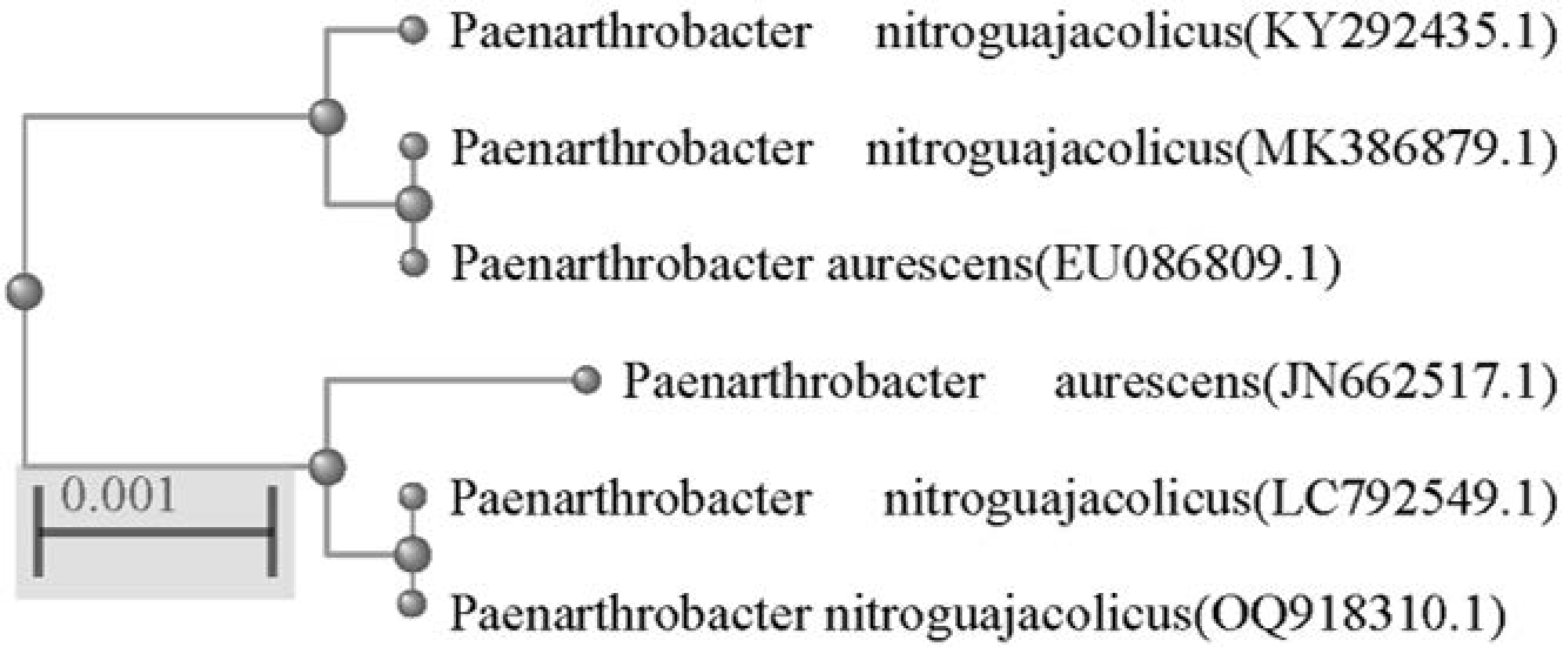
Table 2. Elemental concentration (time: 3 h).

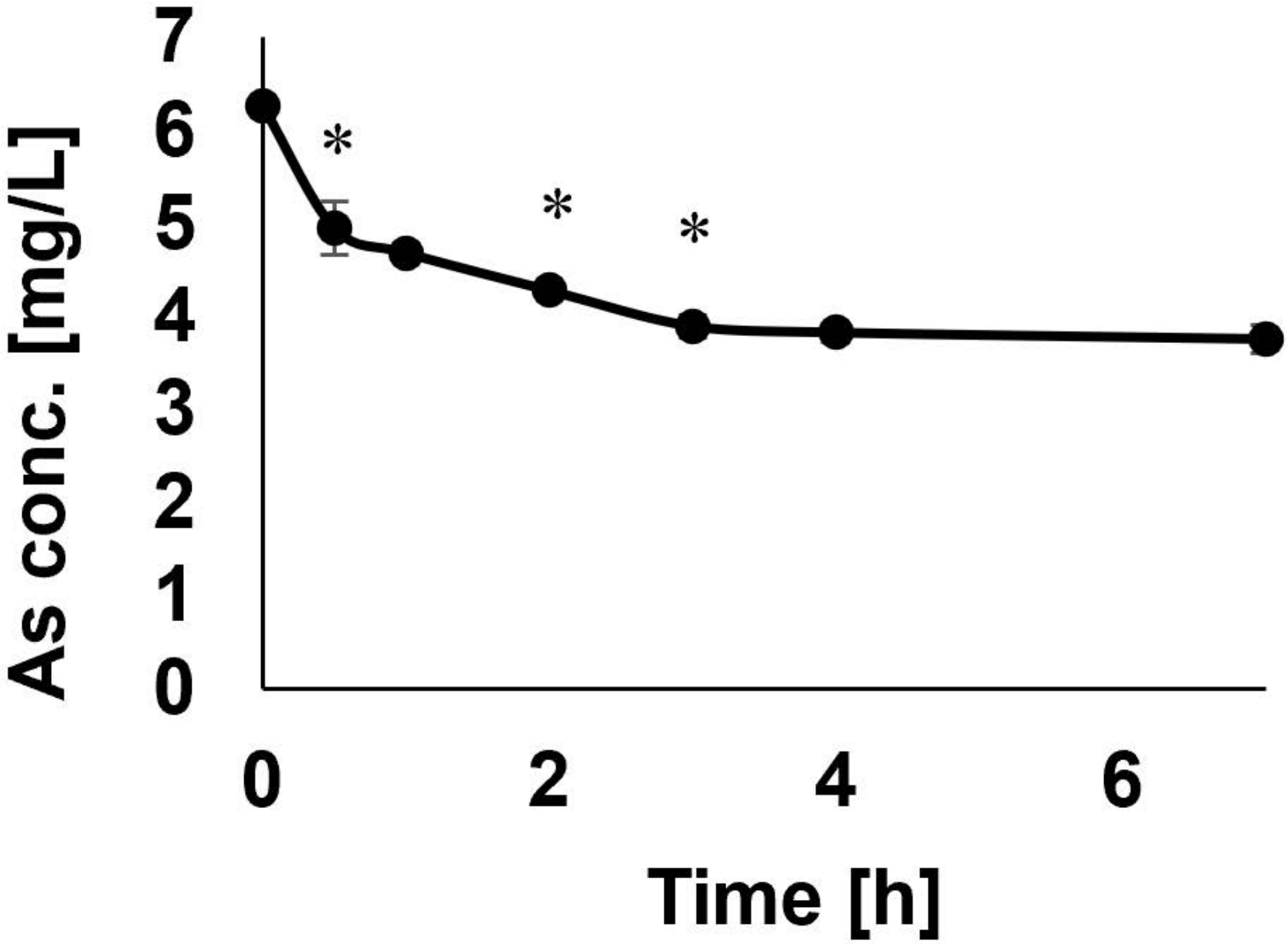
Element	Initial concentration (mg L^{-1})	Final concentration (mg L^{-1})	Removal/increased ratio (%)	Removal/releasing ability (mg g^{-1}-dry cell)
As	6.13	3.45	43.6	1.50
Fe	283	253	10.6	16.9
K	23.9	45.7	91.3	12.3
Na	13.2	16.9	28.2	2.08
Mg	14.9	18.7	25.7	2.15

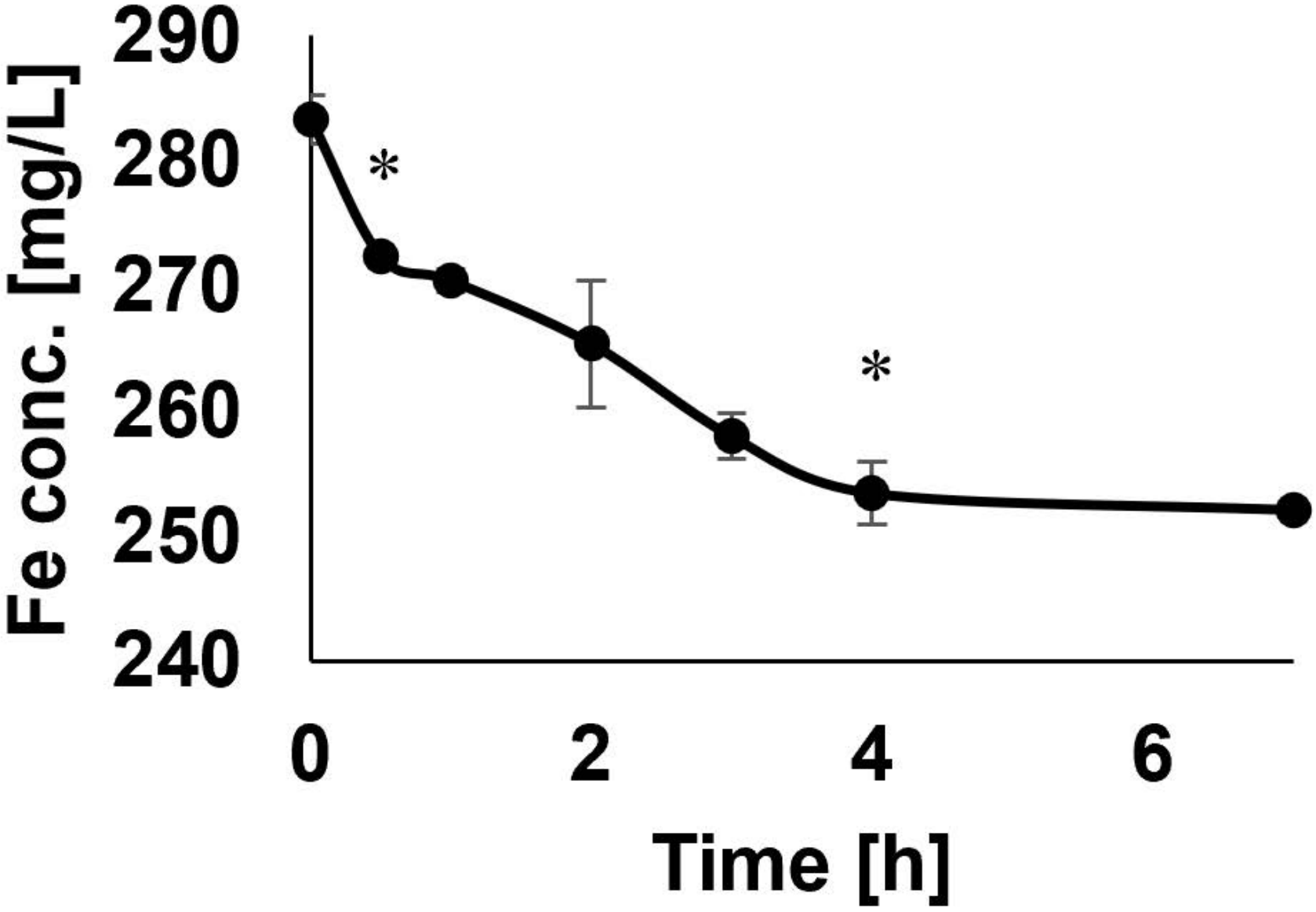
Table 3. Bacterial As removal ability under acidic conditions.

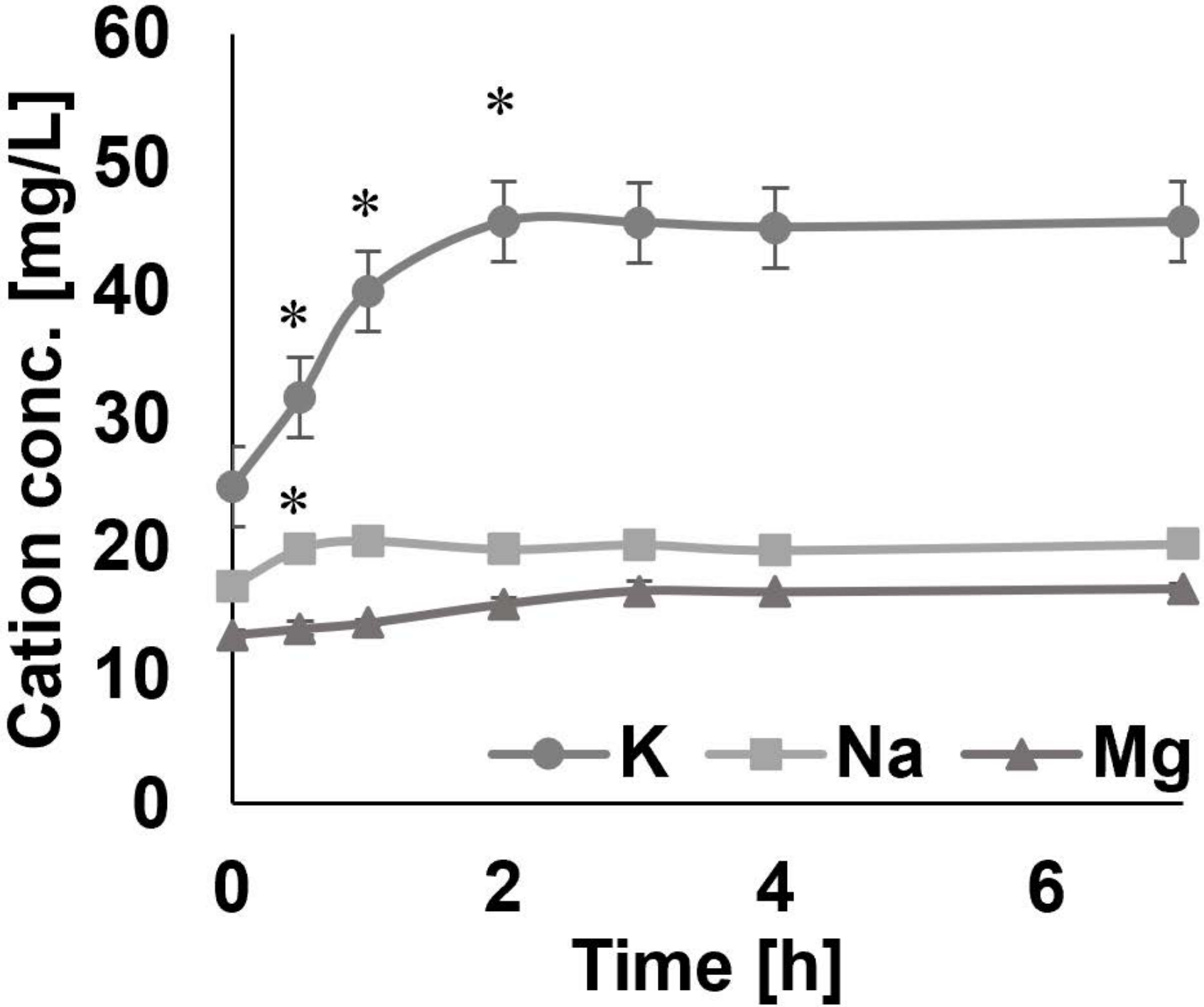
Adsorbent	pH	Removal ratio (%)	Initial concentration (mg L^{-1})	Reference
Bacillus cereus	4	28.7	6.0	[17]
B. cereus	2.5	<50	1	[24]
Yersinia sp.	4	<40	6.5	[25]
Paenathrobacter sp.	1.95	43.6	6.1	This study

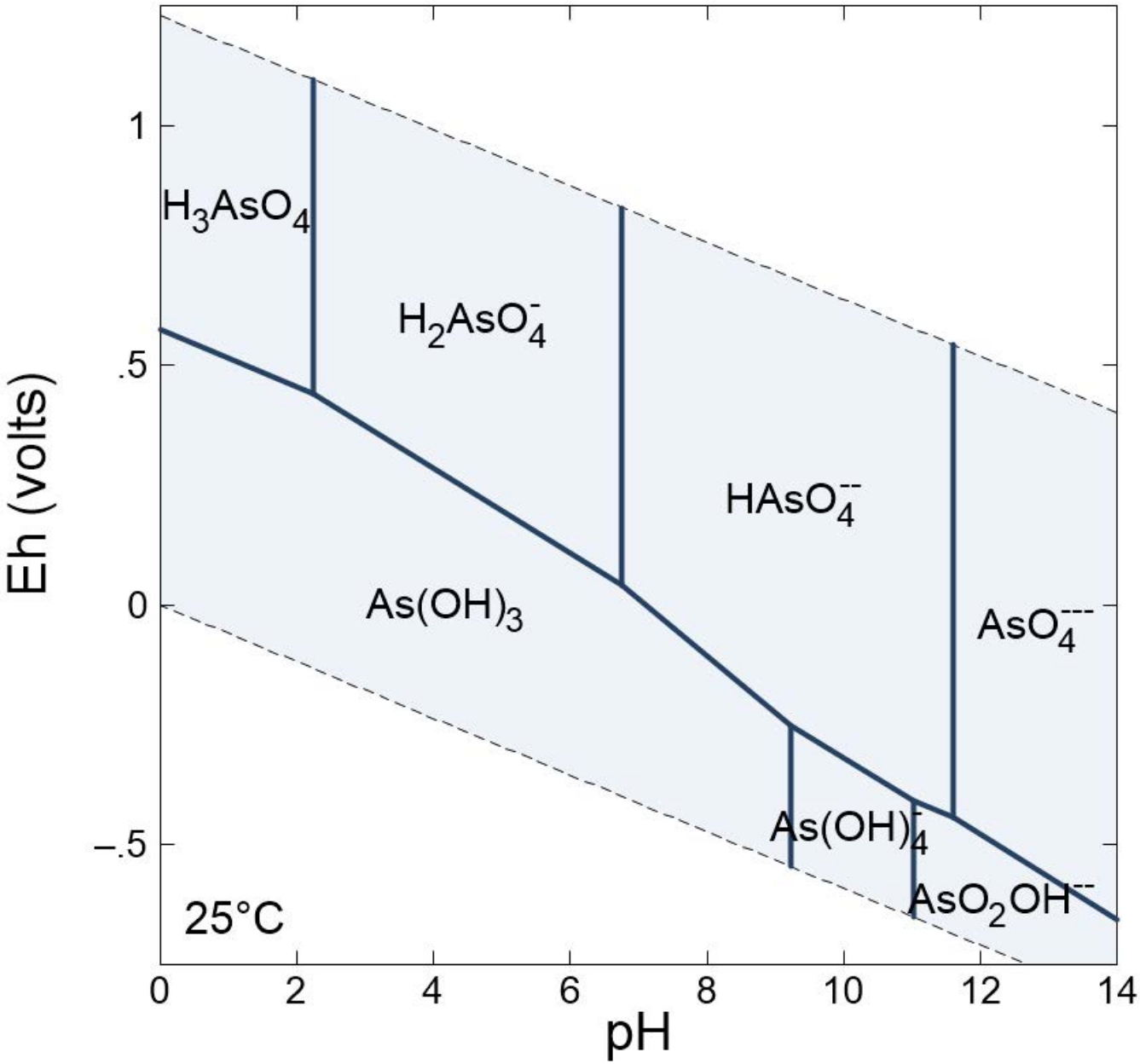

a)
Neutralizer
Flocculant
AMD
Neutralization
Sedimentation separation
Supernatant
Discharge
Sludge
Dehydration
Disposal
b)
Metal removal
AMD
Neutralizer
Neutralization
Discharge
Reuse bacteria
bacteria
metal
Metal recovery

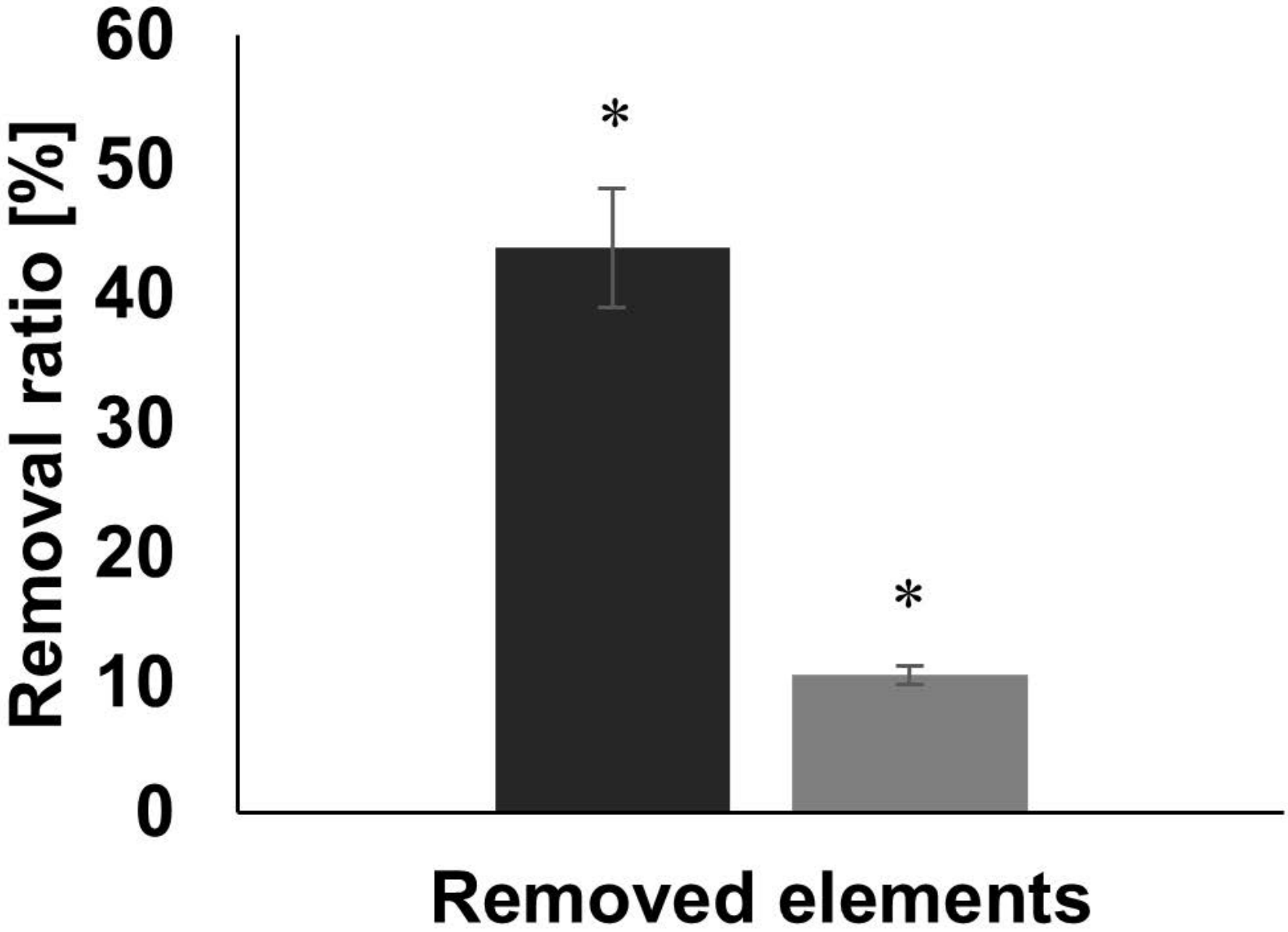

Paenarthrobacter nitroguajacolicus(KY292435.1)
Paenarthrobacter nitroguajacolicus(MK386879.1)
Paenarthrobacter aurescens(EU086809.1)
Paenarthrobacter aurescens(JN662517.1)
0.001
Paenarthrobacter nitroguajacolicus(LC792549.1)
Paenarthrobacter nitroguajacolicus(OQ918310.1)

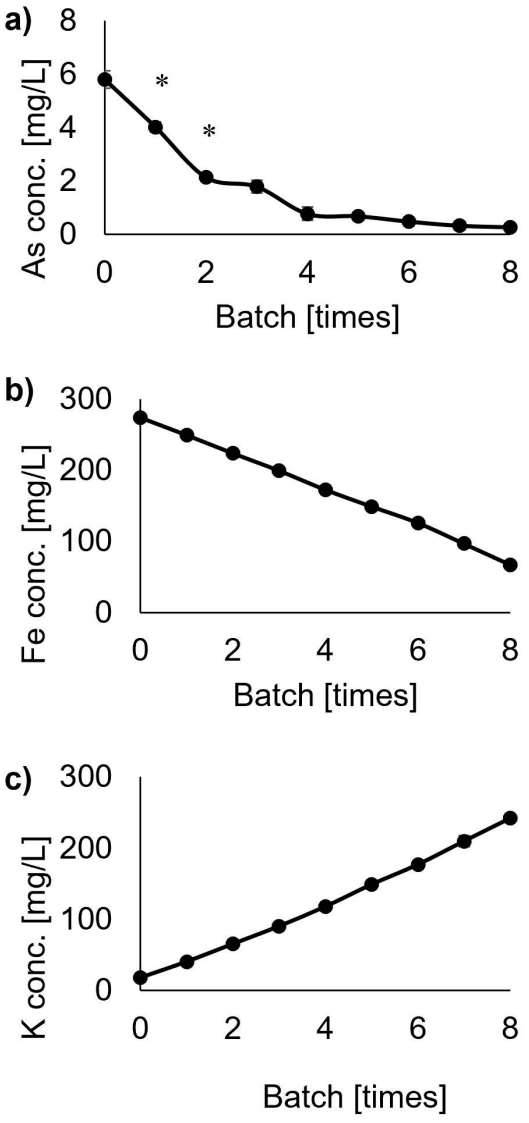

As conc. [mg/L]
7
6
5
4
3
2
1
0
*
*
*
0
2
4
6
Time [h]


290
280
270
260
250
240
Fe conc. [mg/L]
*
*
0
2
4
6
Time [h]


60
50
40
30
20
10
0
Cation conc. [mg/L]
*
*
*
*
K
Na
Mg
0
2
4
6
Time [h]


Eh (volts)
1
.5
0
–.5
H_3AsO_4
$H_2AsO_4^-$
$HAsO_4^{--}$
$As(OH)_3$
AsO_4^{---}
$As(OH)_4^-$
AsO_2OH^{--}
25°C
0
2
4
6
8
10
12
14
pH


60
50
40
30
20
10
0
Removal ratio [%]
*
*
Removed elements


a)
As conc. [mg/L]
8
6
4
2
0
*
*
0
2
4
6
8
Batch [times]
b)
Fe conc. [mg/L]
300
200
100
0
0
2
4
6
8
Batch [times]
c)
K conc. [mg/L]
300
200
100
0
0
2
4
6
8
Batch [times]