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Roles of Microbial Activity and Anthraquinone-2,7-disulfonate as a Model of Humic Substances in Leaching of Iron from Hematite into Seawater

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Fertilization with a mixture of steelmaking slag and compost can affect the supply of dissolved iron used to restore seaweed beds, however, the mechanisms of iron elution from the fertilizer are not well understood. In the present study, the microorganism was isolated from Fe-fertilizer incubated in coastal seawater for 6 months, and was identified as *Exiguobacterium oxidotolerans* by 16S rDNA sequencing. The iron elutability of the bacteria was proved based on the increasing of dissolved iron by incubation with Fe_2O_3 (hematite) under a seawater-like condition. The value of ORP was changed by inoculated of the bacteria from *ca.* 0 to *ca.* –400 mV, which is anticipated concerning to reduction of Fe. The concentration of eluted iron was largely depended on those of organic acids produced by bacteria. From the results, it was proved that *E. oxidotolerans* is capable of Fe reductive eluting of iron from Fe_2O_3 into seawater. Anthraquinone-2,7-disulfonate (AQDS), which can play as an electron acceptor/donor between microbe and insoluble Fe_2O_3 particles, enhanced the effect of iron bio-leaching.

Keywords Anthraquinone-2,7-disulfonate, barren ground, *Exiguobacterium oxidotolerans* sp., hematite, iron elution, iron reduction, seawater

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Introduction

Barren ground is a phenomenon associated with the depletion of seaweed in coastal areas. One factor possibly contributing to the development of barren ground is the lack of dissolved iron species,[1] which are required for the reproductive growth of seaweed.[1–4] Under coastal seawater conditions (pH 7.9 – 8.2, oxic), Fe^{2+} is more soluble than Fe^{3+}. However, Fe^{2+} is easily oxidized to Fe^{3+} and forms hydroxide colloids because of its lower solubility product [1.0×10^{-5} M for $Fe(OH)_2$, 2.0×10^{-10} M for $Fe(OH)_3$].[5,6] Terrestrial humic substances play important roles in dissolving iron *via* complexation in estuarine and coastal areas.[1,7] On the basis of this concept, an Fe-fertilizer composed of steelmaking slag that contained iron oxide as a source of Fe and bark compost as a source of humic substances has been developed to supply dissolved Fe to barren coastal regions. The recovery of seaweed-beds together with an increase in the iron concentration was confirmed at the shoreline of the Shaguma-coast in Mashike, Hokkaido, Japan.[8,9] However, the mechanisms for eluting iron from the fertilizer into seawater are not yet fully understood.

A previous study has shown that structural alteration of humic acids in fertilizer is caused by the activities of anaerobic bacteria.[10,11] These bacteria are also known to be iron-reducing. The iron-reducing bacteria can reduce Fe(III) into Fe(II) *via* their metabolism by using organic compounds as an electron donor and Fe(III) as an electron terminal acceptor.[12,13] Microbial activation can be assumed to contribute to a leaching of iron from steelmaking slag into seawater.

In this study, to elucidate the bacterial contribution to Fe elution, we attempted to isolate and identify bacteria from a mixture of steelmaking slag and compost incubated in coastal seawater. Hematite (Fe_2O_3) was found to be the major Fe-bearing compound in the slag sample.[14,15] Thus, we performed an iron elution test for one month to evaluate the effects of the bacterial activity on iron elution into seawater using hematite as a model for steelmaking slag. The quinone/hydroquinone redox couples present in humic acids serve as electron donors and acceptors under a variety of environments.[16–19] Thus, humic acids are expected to enhance the Fe-reducing reaction. In the present study; anthraquinone-2,7-disulfonate (AQDS) was used as an analogue for humic acids,[17] and the effects of AQDS on the microbial leaching of iron from hematite were also investigated.

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Experimental

Materials

Anthraquinone-2,7-disulfonate (AQDS) and ferrozine were purchased from Tokyo Chemical Industry (Tokyo, Japan), and all other reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), and used without further purification.

The artificial seawater was prepared by dissolving the following salts in 1 L of distilled water: NaCl 28 g; $MgSO_4 \cdot 7H_2O$ 7.0 g; $MgCl_2 \cdot 6H_2O$ 4.0 g; $CaCl_2 \cdot 2H_2O$ 1.47 g; and KCl 0.7 g.[19] The modified Postgate's B medium was prepared by dissolving the following components in a 1-L mixture of distilled water/artificial seawater (1:1, v/v): KH_2PO_4 0.5 g; NH_4Cl 1.0 g; Na_2SO_4 1.0 g; $CaCl_2 \cdot 2H_2O$ 0.1 g; $MgSO_4 \cdot 7H_2O$ 2.0 g; sodium lactate (60 – 70%) 5 mL; yeast extract 1.0 g; L-ascorbic acid 0.1 g; $FeSO_4 \cdot 7H_2O$ 0.5 g; and NaCl 26 g; the pH was 8.0 ± 0.2.[21] The medium was sterilized by autoclaving at 121°C for 20 min.

For bacteria enrichment, Plate Count Agar (PCA) medium for bacteria enrichment was prepared by dissolving the following components in 1 L of distilled water: peptone bacteriological 5.0 g; protease peptone 5.0 g; L-cysteine 0.25 g; NaCl 5.0 g; ammonium iron(III) citrate 1.0 g; K_2HPO_4 0.3 g; and agar 15.0 g. The pH of the PCA medium was adjusted to 7.4 ± 0.2, and was sterilized by an autoclave (121°C, 20 min) before use.

Incubation of Fe-fertilizers in water tanks

The compost was prepared from wood chips and a composting accelerator, which was obtained by fermenting swage containing microorganism, according to the same method described in a previous report.[22] A steelmaking slag, which was treated with CO_2 to stabilize its pH, was provided by Nippon Steel & Sumitomo Metal Corporation. Mixtures of a compost and steelmaking slag were used in the experiment with mass ratios of steelmaking slag:compost (g/g) of 450:147, which corresponded to the volume ratios [slag:compost (v/v)] of 1:1. These samples were packed in a nylon-net bag and soaked in coastal seawater in 300-L water tanks for 6 months. The 300-L water tanks were located in Mashike-cho, Hokkaido, Japan, and the incubation period was from June 2014 to December 2014. Coastal seawater in the barren ground area was introduced into the tanks, and allowed to remain in the tanks for 200 L.

Isolation and identification of bacteria from fertilizer

To enrich the targeted bacteria, a 1-mL volumn of 6-month-incubated sample was taken and used to inoculate a Postgate's B medium.[23] The enrichment was shaken at 120 rpm at 20°C. The enriched bacteria were then diluted 10-times with ultra-pure water. To isolate the enriched bacteria by the spread plate method.[24] A 0.1-mL aliquot was inoculated onto PCA agar and incubated at 20.0 ± 0.5°C in for 7 days in an EYELA LTI-700 incubator. After 7 days of incubation, orange-pale colonies appeared, and the colonies were isolated by transferring them into a 1.8-mL polyethylene vial (1.5 × 5 cm) containing glycerol (50% v/v with pure water) to stock. The isolated colonies were identified by 500 targeted 16S rDNA sequencing to performed by the Techno-Suruga Laboratory (Shizuoka, Japan).

Iron elution test

To obtain a cell density of 1×10^8 cell mL^{-1}, stocks of identified colonies (*Exiguobacterium oxidotolerans*) were grown in the Postgate's B at 20.0 ± 0.5°C in an incubator while shaking at 120 rpm for 5 days.

Table 1 Composition of the reaction mixture for the iron elution test

Composition	S1	S2	S3	S4	S5
Postgate's B medium/mL	200	200	200	200	200
E. oxidotolerans/mL	1.0	–	1.0	–	1.0
Hematite/g	–	1.0	1.0	1.0	1.0
50 μM AQDS/mL	–	–	–	0.2	0.2

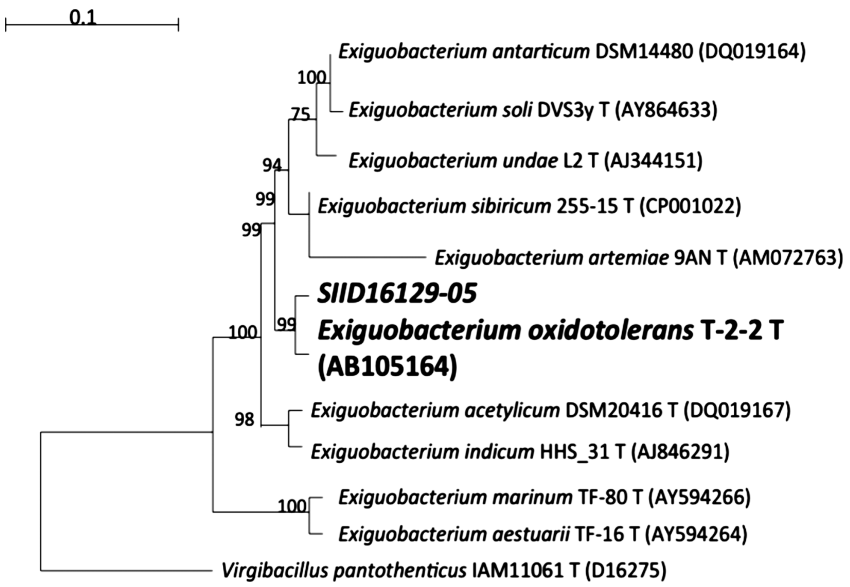

Fig. 1 Phylogenetic tree derived from 16S rDNA gene sequence data of SIID16129-05 (isolated bacteria from model fertilizer). The bacteria were determine to be 99% related to *Exiguobacterium oxidotolerans* and other *Exiguobacterium* species as well as some other related organisms, with the use of neighbor-joining method. Number indicating bootstrap values: 0.1 K_{nuc} units.

An iron elution test was performed in 200 mL of modified Postgate's B. The components of the reaction agents are summarized in Table 1. Samples of *E. oxidotolerans* (S1) and hematite (S2) were used as controls.

After 0, 5, 9, 14, 21, 28 and 30 days of incubation, a 1.2-mL aliquot of culture suspension was transferred to a 2-mL centrifugal tube under a sterile condition, and then centrifuged at 6500 rpm for 10 min. After the supernatant was filtered with a 0.45-µm membrane filter, a 1-mL aliquot of the filtrate was transferred to a polyethylene tube and diluted to 10 mL with 0.01 M aqueous HCl to preserve Fe in the solution. The total iron concentration was analyzed with an ICPE-9000 type ICP-AES (Shimadzu, Japan). The concentrations of a Fe(II) species in the media were colorimetrically determined with ferrozine as an indicator of Fe(II).[20,25] A 50-µL aliquot of 50 µM ferrozine was added to a 1-mL aliquot of media, which and then centrifuged and filtrated (0.45 µm). After 30 min of incubation, the concentration of Fe(II) species was determined from the concentration of Fe(II)-ferrozine complexes from their absorbance at 562 nm.

The dynamics of the oxidation-reduction potential (ORP) of the media was monitored with an ORP meter (TOA-DKK, Japan), during 30 day of incubation. Oxalic, lactic and acetic acids, which were generated by bacterial metabolism into the media were investigated. The aliquots were centrifuged and filtrated before analysis, and then analyzed by IC20-type Ion Chromatography (Thermo Scientific, USA). Organic acids were separated on an IonPac®ICE-AS6 column (DIONEX) with a 1 mM aqueous heptafluorobutyric acid solution as the mobile phase.

Surface analysis of hematite

Hematite slurry cultured for 30 days, was transferred into a visking tube (MWCO: 12000 – 14000 Da, Japan Medical Science, Japan) and dialyzed against ultrapure water. After dialysis, the slurry was freeze-dried to obtain the powdered samples. X-ray photoelectron spectroscopy (XPS) measurements of the powdered hematite samples were performed before and after incubation with the use of a JPS-9200 XPS instrument (JEOL, Japan). A monochromatic A Mg *K*α x-ray source was used for all samples, with analysis chamber pressures of 10^{-6} – 10^{-7} Pa. The conditions used for all survey scans were as follows: energy range 1250 – 250 eV, pass energy 50 eV, step size 1 eV and sweep time 300 s.[26] The collected spectra were analyzed with SpecSurf software. A Shirley-type background was subtracted from all spectra to remove most of the extrinsic losses.

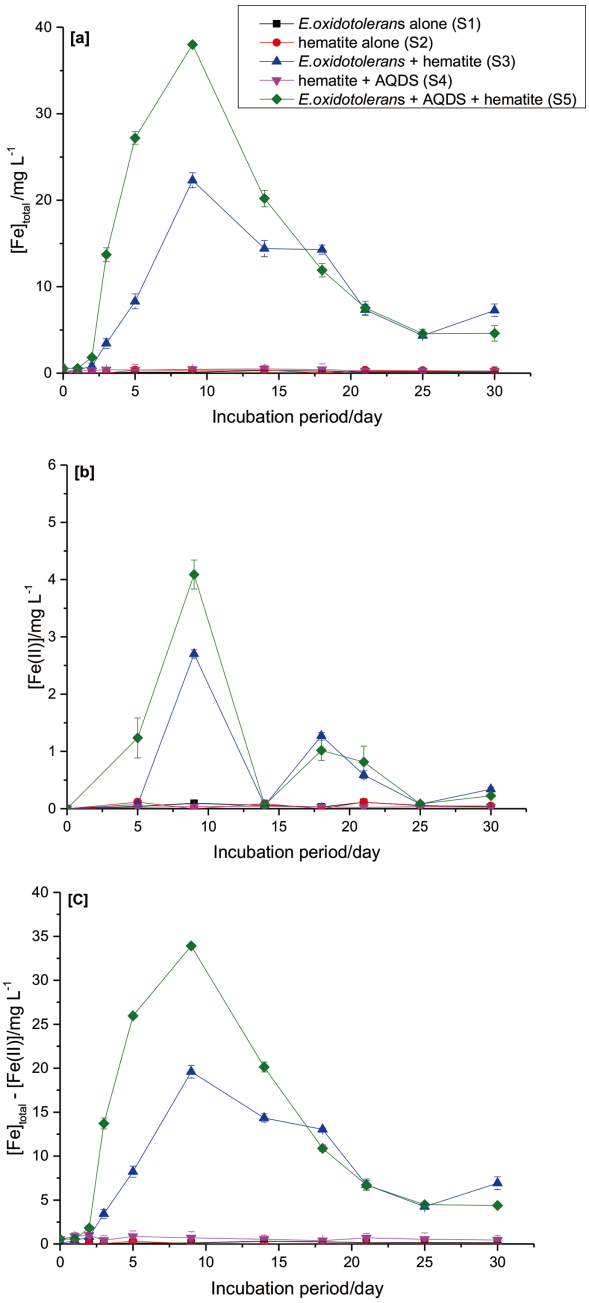

Fig. 2 Elution kinetics of iron in the culture solution during the 30-day incubation period. (a) Total iron concentration, (b) Fe(II) species concentration and (c) Fe(III) species concentration.

Results and Discussion

Isolation and identification of microorganism from fertilizer

The bacteria colonies with an orange color first appeared after 5 days of incubation in PCA agar, the colonies were isolated and determined to be *Exiguobacterium oxidotoleran* by 500 targeted 16S rDNA sequencing (Fig. 1). *E. oxidotolerans* is of the genus *Bacillus sp.*, which is known to be a metal reducer in aquatic environments. *E. oxidotolerans sp.* is common, and has been found in a drain at a fish processing plant,[27] on the surfaces of brown algae grown in a tide pool,[28] and in seawater in Kandalaksha Bay, Russia.[29] Fe-reducing bacteria can grow on the surface of Fe oxides because they need Fe(III) as an electron acceptor for anaerobic respiration. Because direct contact between bacterial cells and Fe oxide particles are required for anaerobic respiration, *E. oxidotolerans* might adsorb and grow on the surface of steelmaking slag in seawater. *E. oxidotolerans* could be propagated in a mixture of steelmaking slag and compost.

Elution kinetic of Fe and Fe bio-leaching mechanism

Figure 2 shows the elution kinetics of iron in the culture solutions. As shown in Fig. 2a, the concentrations of total dissolved iron in *E. oxidotolerans* alone (S1), hematite alone (S2), and a mixture of hematite and AQDS (S4) were negligible. For a sample containing *E. oxidotolerans* and hematite (S3), the concentration of total dissolved iron increased during the first 9 days of the incubation period. After the 10th day of incubation, the iron concentration gradually decreased (Fig. 2a). For the mixture of *E. oxidotolerans*, AQDS and hematite (S5), kinetics

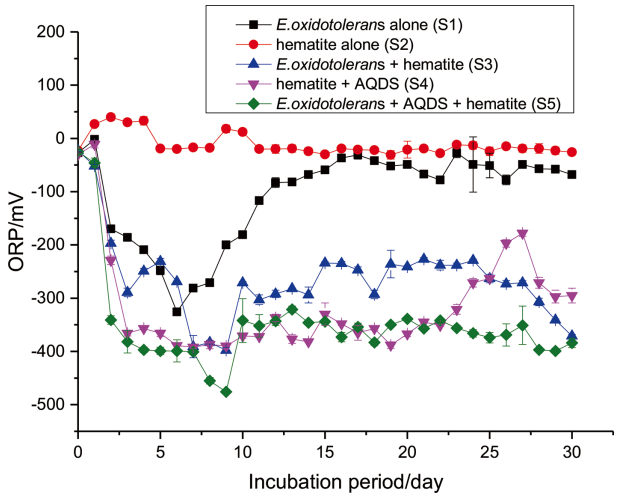

Fig. 3 Oxidation-reduction potential of the medium during the 30-day incubation period.

similar to S3 were observed. For S5 on the 9th day of the incubation period, the largest dissolved iron concentration was observed (*ca.* 38 mg L^{-1}), which was 1.7-times as large as that of S3 (*ca.* 23 mg L^{-1}).

Figures 2b and 2c show that the changes in the kinetics of the dissolved ferrous and ferric iron species for S3 and S5 were similar to those of total iron, and the results showed that ferric iron was the dominant species in terms of a total dissolved iron (*ca.* 85 – 90%). Dissolved Fe was only observed in S3, indicating that *E. oxidotolerans* isolated from incubated steelmaking slag and compost mixture is capable of eluting Fe from Fe_2O_3 under seawater-like conditions.

Figure 3 shows the changes in the oxidation-reduction potential (ORP) in the culture solutions. For S2, the values of ORP varied in the vicinity of 0 mV and did not change. For S1, the ORP decreased to –300 mV on the 6th day of incubation and reached a plateau at approximately –50 mV after 7 days. The ORP of S3 slightly decreased to –400 mV after 9 days of incubation. However, the ORP increased and reached a plateau at approximately –250 mV after 10 days of incubation. In samples containing AQDS (S4 and S5), the ORP considerably decreased to –400 mV for 3 days of incubation. For S5, the decreased ORP remained at approximately –300 to –400 mV during a 30-day incubation period. The redox potential of Fe_2O_3/Fe^{2+} at pH 8 was estimated to range from –300 to –400 mV. The ORP values of the S3 and S5 remained near the threshold Fe_2O_3/Fe^{2+} redox value. The ORP conditions in seawater became more conducive to the reduction of iron by *E. oxidotolerans.*

Figure 4 shows the kinetics of organic acid concentrations in the culture solutions. The concentration of oxalic acid (Fig. 4a) increased, reaching the highest concentration at 9 days of incubation in S3 and S5. In S2 and S4, the concentration of oxalic acid did not change. In the presence of bacteria, the concentration of oxalic acid in S1 did not increase during the 30-day incubation period. Oxalic acid can be produced as a metabolite of bacteria. Therefore, *E. oxidotolerans* was activated by the presence of hematite, in which Fe-oxide can act as an electron acceptor.[30] Because Postage's B contains lactic acid high level of the concentrations were observed at the start of incubation in all samples (Fig. 4b). The earlier lactic acid concentrations were regained temporarily in the S3 and S5 samples (*ca.* 170 – 240 mg L^{-1}) at day 9 of the incubation

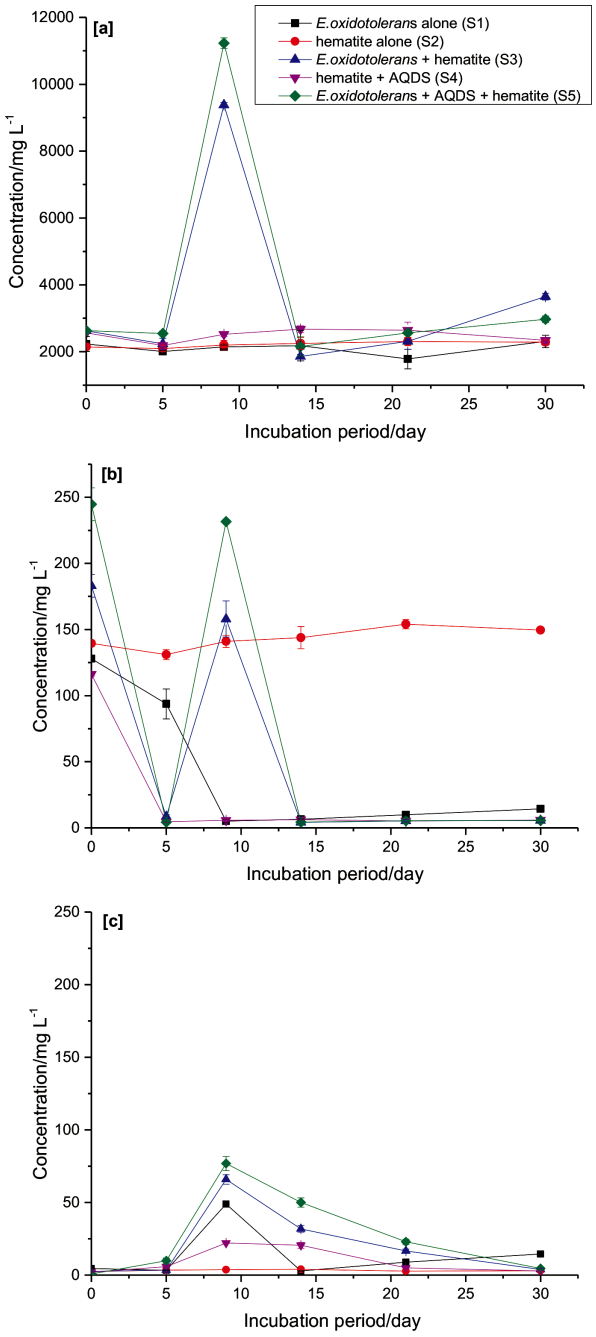

Fig. 4 Organic acid production in the culture solution during the 30-day incubation period: (a) oxalic acid, (b) lactic acid and (c) acetic acid.

period. Low-molecular-weight organic acids were produced as metabolites *via* the cellular respiration of bacteria.[31] Thus, the increase of lactic and oxalic acids in S3 and S5 might be attributed to the activation of *E. oxidotolerans*. Respiration based on ferric iron reductase can occur under the incubating conditions.[12] Possible explanations for the iron dissolution in this study are also related to the concentrations of oxalic or citric acids that are produced by bacteria during incubation.[32] Organic acids acted as an electron donor to the iron oxide, which acted as a terminal electron acceptor, and then reduce into dissolution iron species in the culture media.[12] The concentrations of dissolved iron (Fig. 2) changed together with the concentrations of organic acids (Fig. 4), indicating that an

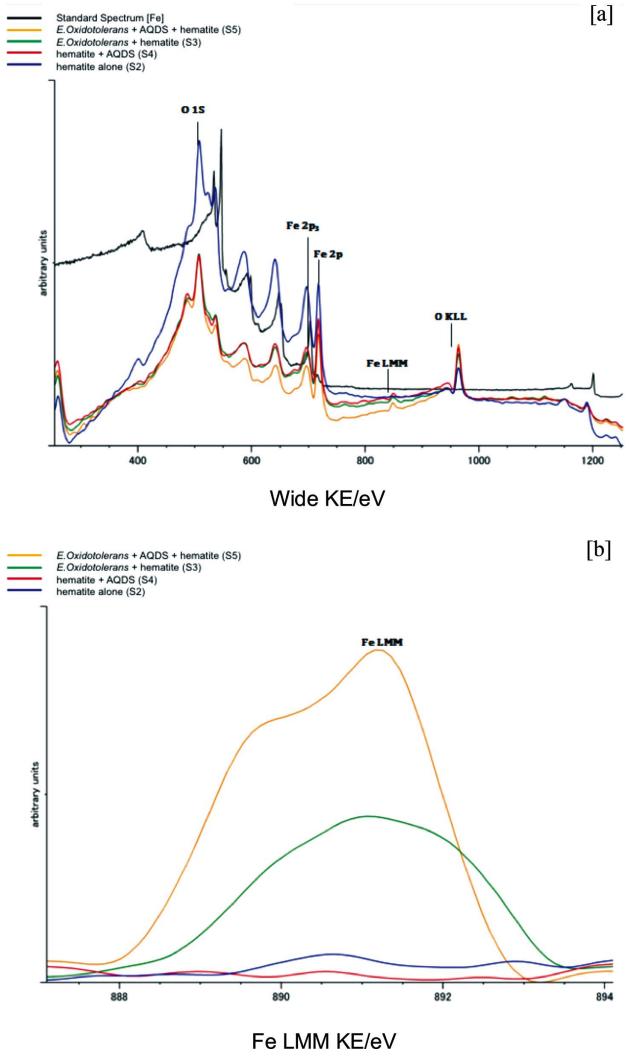

Fig. 5 XPS spectra of untreated and treated hematite after 30 days of incubation (a) survey spectrum and (b) high-resolution spectrum.

elution of iron from hematite could also be attributed to the activation of bacteria in culture solutions. Oxalic and lactic acids can reduce Fe(III) as the electron donors; the corresponding reactions are as follows:

$$2Fe(III) + COOHCOO^- + H_2O \longrightarrow 2Fe(II) + HCOO^- + HCO_3^- + H^+$$

$$4Fe(III) + CH_3CHOHCOO^- + 2H_2O \longrightarrow 4Fe(II) + CH_3COO^- + HCO_3^- + 5H^+$$

Formic and acetic acids produced from the above reactions are also possibly capable of Fe-reduction.[33] The dynamics of a total and ferrous dissolved iron concentrations (Fig. 2) were consistent with that of acetic acid (Fig. 4c), rather than those of oxalic and lactic acids. Iron can be eluted *via* reducing reactions; however, the concentration of dissolved ferrous iron was much a small proportion of the total dissolved iron. This result could be attributed to Fe(II) being immediately re-oxidized and converted to inactive ferrozine under oxic and weak alkaline conditions.[20]

Although the dissolved iron concentration for S4 was negligible, the maximum concentration of dissolved iron for S5 (38 mg L^{-1}) was 1.7-times larger than that for S3 (23 mg L^{-1}) (Fig. 2). The production of organic acids can be attributed to the activation of bacteria. The maximum concentrations of organic acids in S5 was larger than those in S3 (Fig. 4), indicating that AQDS stimulated the bacterial activity. The elution of iron owing to Fe(III) respiration was found to be limited by bacteria directly connected to Fe_2O_3 particles.[12] It is known that quinone moieties, such as humic acids and AQDS, can act as electron acceptor/donor agents.[13,34] AQDS can play a role in electron transfer between microbes and insoluble hematite during the iron elution test. Therefore, AQDS made a considerable contribution to iron reduction and might lead to greater iron elution.[31]

Fe at the hematite surface

XPS spectra of the samples are shown in Fig. 5. Figure 5a shows no major difference of a high-resolution spectra of the high-spin $Fe^{3+}2p_{3/2}$peak, in untreated and treated hematite.[26] However, low-molecular masses in Fig. 5b suggest a major difference. For S3 and S5, the intensity of the Fe^{3+} low-molecular mass peak markedly decreased, indicating that the Fe^{3+} species was eluted into the solution from the surfaces of hematite particles. The low molecular mass Fe^{3+} (Fe LMM) compounds could be attributed to eluted-iron that was detected during the incubation.

Conclusions

The microorganism, *Exiguobacterium oxidotolerans*, was identified from an incubated mixture of steelmaking slag and compost. The iron elution test under a seawater-like condition over 30 days showed that *E. oxidotolerans* is an iron reducer. The iron elutability was attributed to the activities of identified bacteria. AQDS, as an electron acceptor/donor markedly improved the iron-reductive elution by bacteria under seawater-like conditions. These results show that the iron-reducing bacteria *E. oxidotolerans*, and humic acids contribute to the transfer of dissolved iron from steelmaking slag to seawater.

Acknowledgements

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