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Isolation and characterization of *Acidithiobacillus caldus* from a sulfur-oxidizing bacterial biosensor and its role in detection of toxic chemicals

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ABSTRACT

A novel toxicity detection methodology based on sulfur-oxidizing bacteria (SOB) has been developed for the rapid and reliable detection of toxic chemicals in water. The methodology exploits the ability of SOB to oxidize sulfur particles in the presence of oxygen to produce sulfuric acid. The reaction results in an increase in electrical conductivity (EC) and a decrease in pH. The assay is based on the inhibition of SOB in the presence of toxic chemicals by measuring changes in EC and pH. We found that SOB biosensor can detect toxic chemicals, such as heavy metals and CN^-, in the 5–2000 ppb range. One bacterium was isolated from an SOB biosensor and the 16S rRNA gene of the bacterial strain has 99% and 96% sequence similarity to *Acidithiobacillus* sp. ORCS6 and *Acidithiobacillus caldus* DSM 8584, respectively. The isolate was identified as *A. caldus* SMK. The SOB biosensor is ideally suited for monitoring toxic chemicals in water having the advantages of high sensitivity and quick detection.

1. Introduction

There are many sources of water pollution and main contaminant sources include effluent outfalls from industries, refineries, and waste treatment plants. In general, contaminants are classified into two groups: organic and inorganic pollutants. Some organic water pollutants include industrial solvents, volatile organic compounds, insecticides, pesticides, etc. Inorganic water pollutants include heavy metals, fertilizers, and respiratory inhibitors such as potassium cyanide, sodium azide, etc. Many of organic and inorganic pollutants are known to be both toxic and carcinogenic (Banat et al., 2005; WHO, 2005).

Heavy metals are extensively used in several industries such as, mining, metallurgical, electronic, electroplating, and metal finishing. The presence of metal ions in industrial effluents is extremely undesirable, as they are extremely toxic to both microorganisms and higher organisms. Under certain environmental conditions, metals may accumulate to toxic levels and cause ecological damage (Bruins et al., 2000). The most toxic heavy metals are mercury, lead, cadmium, and chromium which have no biological role; whereas copper, nickel, cobalt, and zinc are not as toxic, but their extensive usage and increasing levels in the environment are of serious concern (Banat et al., 2005, Bruins et al., 2000; Hsieh et al., 2004; Nies, 1999). Hence, the monitoring and detection of toxic chemicals in the environment are of upmost importance to keep the quality and safety of our water resources.

Many biological methods have been developed for the monitoring of toxic materials which depend on the physiological and behavioural response of higher organisms such as fish, daphnia, algae, plant tissue, and animal cells to pollutants (Rodriguez-Mozaz et al., 2004). However, the monitoring of pollutants by higher organisms doesn't have sufficient sensitivity, the tests are expensive, and they require a long time for detection (Hsieh et al., 2004). Microbial toxicity tests depend on bacteria and other microorganisms that have similar physiological responses as higher microorganisms. The major advantages of microbial-based biosensors include fast response times, short doubling times, inexpensive, and they are easily applied to toxicity measurements (Hsieh et al., 2004). Most microbial bioassays monitor respiration (Tzoris et al., 2005), amperometry (Shitanda et al., 2005), biolumines-cence (Bock Gu and Cheol Gil, 2001), or conductivity (Guedri and Durrieu, 2008).

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Sulfur-oxidizing bacteria (SOB) such as *Acidithiobacillus*, *Thiospirillopsis*, and *Thiovulum* oxidize inorganic sulfur (S°) under aerobic conditions to sulfuric acid (H_2SO_4) according to this equation:

$$S + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+, \Delta G^{\circ\prime} = -587.1 \text{kJ/reaction}.$$

SOB uses the energy that they obtain from the oxidation of S° for the synthesis of organic compounds from carbon dioxide (Madigan et al., 2003). SOB can be used for monitoring toxicity. Oxidation of S° by SOB results in the formation of sulfate (SO_4^{2-}) and hydrogen ion (H^+), which lowers the medium pH. The production of SO_4^{2-} increases the electrical conductivity (EC) of the medium. Thus, the decrease in pH and the increase in EC reflect the formation of SO_4^{2-} as the result of S° oxidation and can easily be detected using simple EC and pH meters (Oh et al., 2010). In the presence of toxic chemicals, the activity of SOB will be inhibited, which will cause an increase in pH and a decrease in EC.

In this study, a continuous monitoring system consisting of SOB was developed for the detection of potassium cyanide (KCN) and the heavy metals — mercury, cadmium, zinc, and lead in water by measuring the EC and the pH in the influent and effluent of the SOB biosensor. The microbial community of an SOB biosensor was characterized.

2. Materials and methods

2.1. Culture and medium

Aerobic return activated sludge was used as the inoculum in biosensors packed with elemental sulfur. The sludge was collected from the Chuncheon wastewater treatment plant in Chuncheon City, Kangwon-do, Korea. Synthetic stream water was made by diluting the following nutrient mineral buffer (NMB) solution 100 times: $NaHCO_3$ (3.13 g/L), NH_4Cl (0.31 g/L), $NaH_2PO_4H_2O$ (0.75 g/L), KCl (0.13 g/L), NaH_2PO_4 (4.22 g/L), Na_2HPO_4 (2.75 g/L) and trace metal and vitamin solutions described in Lovley and Phillips (1988). The pH, EC, and alkalinity of the synthetic stream water were 6.9 ± 0.2, 0.12 mS/cm, and 45 mg/L as $CaCO_3$, respectively which is similar to the Gongi stream in Chuncheon which had a pH 6.8–7.0, EC = 0.18 mS/cm, and alkalinity of 75 mg/L as $CaCO_3$.

2.2. SOB biosensor construction and operation

Five identical cylindrical reactors were constructed from acrylic (100 mL) for the monitoring of toxicity (Fig. 1). Each reactor was packed with 50 mL of S° particles (2–4 mm diameter) and air was introduced from the bottom at a flow rate of 150–250 mL/min. The reactors were then inoculated with aerobic return sludge and incubated at 30 °C for three days in batch mode. The reactors were then continuously fed with synthetic stream water in an up-flow mode using adjustable peristaltic pumps. The reactors were operated at a Hydraulic retention time (HRT) of 30 min for 2–3 days each to reach steady-state conditions (i.e. stable EC and pH values).

2.3. Chemicals and analyses

All the chemicals used in this study were all purchased from Sigma Aldrich except mercuric chloride that was purchased from Dae-Jung chemicals, Korea. When the EC and the pH of the biosensors reached steady-state conditions (i.e. stable EC and pH values), the toxic chemicals were then spiked to the influents of the reactors. Toxicity was monitored by measuring changes in the electric conductivity (LUTRON — YK 22 CT) and the pH (LUTRON — PH 208c) of the effluent. Control experiments without any toxic chemicals were conducted to ensure that no inhibition had taken place before the toxic chemicals were spiked into the SOB biosensor. The EC and pH meters were connected to a computer and all data were automatically recorded every 10 min.

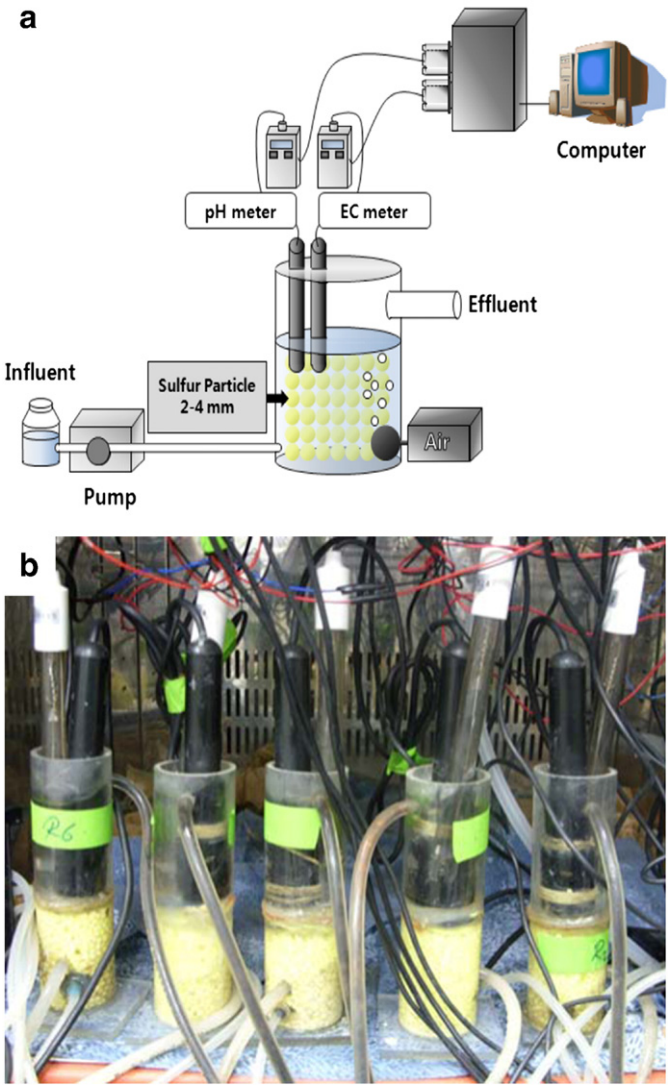

Fig. 1. (a) A schematic and (b) prototype system for toxicity monitoring in water using the SOB biosensor.

2.4. Isolation and enumeration of bacteria in the SOB biosensor

Thiobacillus agar medium with thiosulfate as an electron donor was used for the isolation of autotrophic SOB (Atlas, 2003). The pH of the medium was adjusted to 5.0, and then sterilized by autoclaving at 121 °C. While at steady-state and before the introduction of any chemical, 10 mL of sulfur particles was removed from the SOB biosensor and vortexed with 10 mL of *Thiobacillus* liquid medium without the electron donor to promote detachment of cells adhering to the surface of sulfur particles. The solution was serially diluted in the liquid medium without thiosulfate and 0.1 mL of the appropriate dilution was spread-plated on the *Thiobacillus* agar medium for isolation and enumeration of SOB. The plates were incubated aerobically at 30 °C for 7 days. Serial five-fold dilutions were made and plated in duplicate onto the media. The counts for the various colony types that formed on the solid media were expressed as CFU/mL of sulfur particles (2–4 mm). Single colonies were carefully removed from each medium, examined, and checked for purity. For enumeration of viable heterotrophic cells, nutrient agar medium was

used. All the isolation experiments were repeated three times to confirm the results.

2.5. Biochemical characterization of SOB

Selected autotrophic SOB were identified using morphological, physiological, and biochemical properties according to Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005). Properties investigated were Gram staining, catalase activity, oxidase activity, and motility. Mixotrophic growth for autotrophic strains was investigated in the thiosulfate agar medium by amending with 1% (w/v) glucose and heterotrophic growth was determined in nutrient agar medium. In addition, the API 20E assay was carried out in parallel according to the manufacturer's instructions (BioMerieux, Marcy l'Etoile, France) at 30 °C.

2.6. Identification of SOB

The analysis of 16S rRNA genes was conducted as follows: genomic DNA was extracted from the isolated bacteria using the genomic DNA Prep kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions after glass bead beating to disrupt the cell walls. The extracted DNA was then used as a template for PCR to amplify the 16S rRNA gene. A universal bacterial primer set of 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) was used to amplify the nearly complete 16S rRNA gene. The PCR amplification was performed in a 25 μL reaction volume containing 10–50 ng of the template DNA, 0.4 μM of each primer, 0.75 U of *EF-Taq* DNA polymerase (SolGent, Daejeon, Korea), 0.2 mM of each dNTP (SolGent, Daejeon, Korea), and 1×*EF-Taq* reaction buffer (SolGent, Daejeon, Korea). The thermocycling conditions included an initial denaturation step at 95 °C for 15 min followed by 30 cycles at 95 °C for 20 s, 50 °C for 40 s, and 72 °C for 1.5 min with a final extension step at 72 °C for 5 min. The PCR product was separated by gel electrophoresis on 1.5% agarose containing ethidium bromide with a 0.5×Tris-acetate-EDTA (TAE) buffer, and visualized using a UV illuminator. The PCR product was then purified using a SolGent PCR purification kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions. The amplified 16S rRNA gene was sequenced using an ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, Cal., USA) and an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, Cal., USA). For identification of the isolated bacteria, the partial 16S rRNA gene sequence was compared with full sequences available in the GenBank database using a BLAST search (NCBI) (GenBank accession is GQ478989). The phylogenetic analysis of the sequence data was also performed using the software package MEGA (version 3.1) after multiple alignments of the data using CLUSTAL X (version 1.83). A distance-matrix method (with distance options according to the Kimura two-parameter model) was employed, using clustering obtained with the neighbor-joining method. Bootstrap values were calculated on the basis of 1000 replications.

3. Results and discussion

3.1. Detection of KCN

The response to cyanide (CN^-) was measured after the EC and pH reached steady-state conditions. Depending on the CN^- concentration, the EC and pH changed instantaneously or after several hours (Fig. 2). Cyanide was very toxic at 100 and 500 ppb CN^-, but was not toxic at 5 ppb. The EC decreased sharply after adding 500 ppb of CN^- to the influent and the activity of SOB was nearly completely inhibited after 5 h of injection. At 100 ppb CN^-, the EC start decreasing 1 h after injection and near complete inhibition of SOB was observed after 7 h. As a comparison, Lee and Karube (1995) developed a biosensor using whole cells of *Pseudomonas fluorescens* NCIMB 11764 immobilized on the membrane of an oxygen electrode; their biosensor detected CN^- in the range 100–1000 ppb. While there was no response for Cd^{2+}, Cr^{+3} and Pb^{+2} at the same concentrations in Lee and Karube (1995), the SOB biosensor detected these chemicals as described below and in Oh et al. (2010).

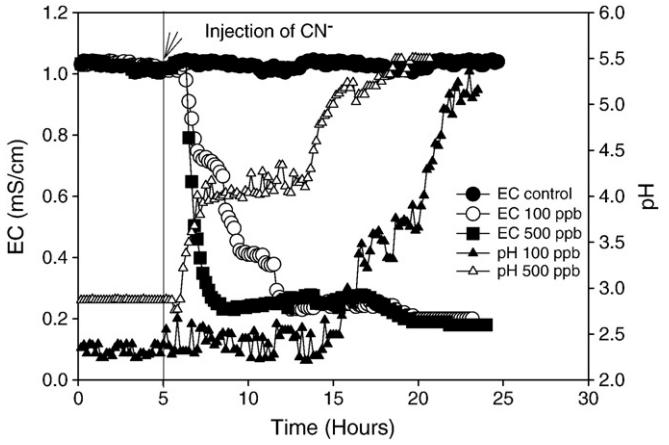

Fig. 2. Detection of CN^- by the SOB biosensor.

3.2. Toxicity of heavy metals

Heavy metals appear to have different levels of toxicity compared to CN^- (Figs. 3–5). The addition of Zn^{2+} (50 ppb), Hg^{2+} (50 ppb and 2 ppm), and Cd^{2+} (5 and 50 ppb) decreased the EC in a few hours. The toxic effect of Pb^{2+} (50 ppb) was observed after about eight hours

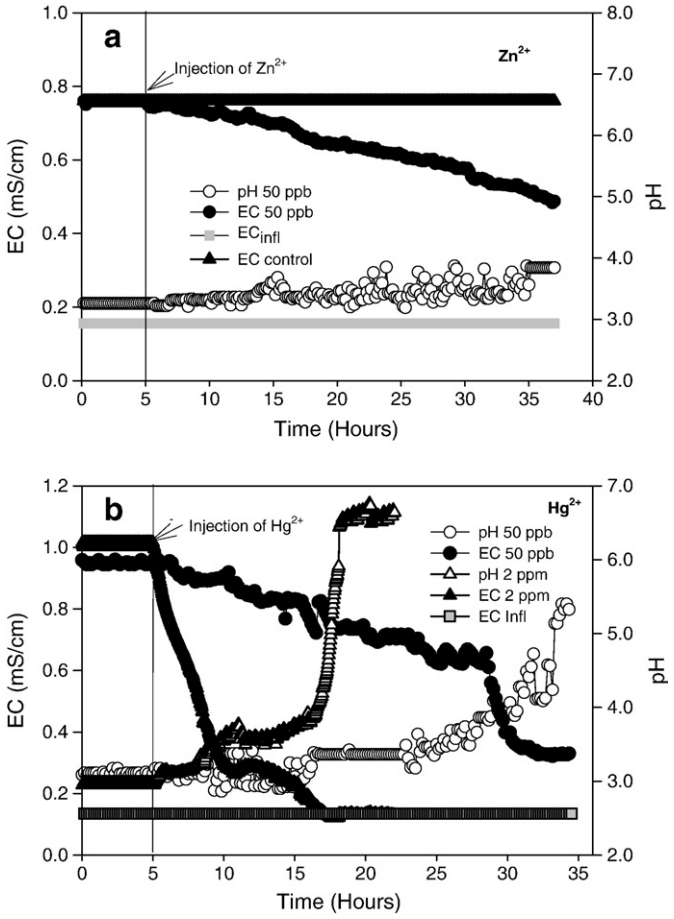

Fig. 3. Detection of heavy metals by the SOB biosensor. a. Zn^{2+} as $ZnSO_4 \cdot 7H_2O$ (50 ppb) and b. Hg^{2+} as $HgCl_2$ (50 ppb and 2 ppm).

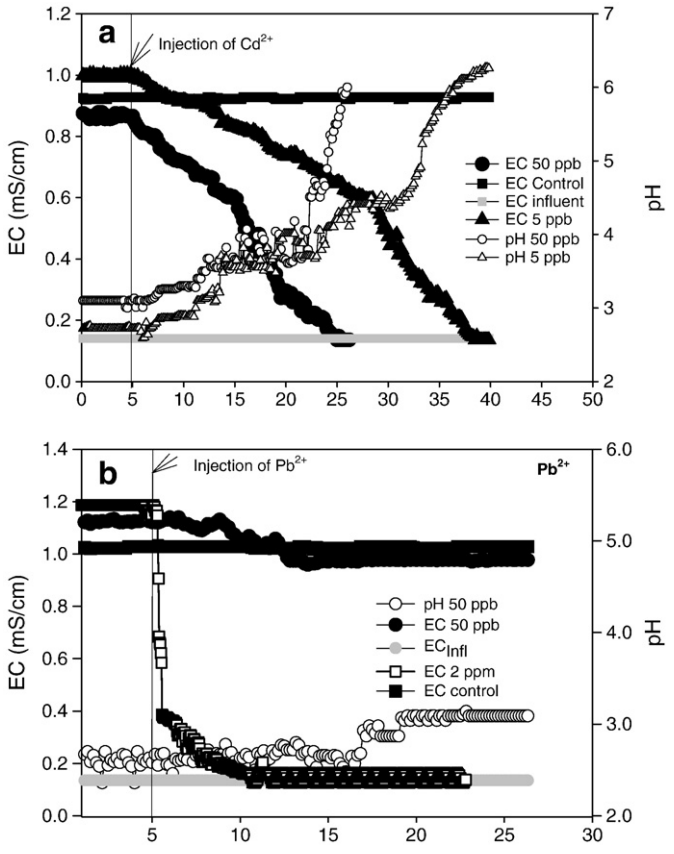

Fig. 4. Detection of heavy metals by the SOB biosensor. a. Cd^{2+} as $CdSO_4 \cdot 8/3H_2O$ (5 and 50 ppb) and b. Pb^{2+} as $Pb(NO_3)_2$ (50 ppb and 2 ppm).

while the effect of Cd^{2+} (5 and 50 ppb), Hg^{2+} (2 ppm), and Pb^{2+} (2 ppm) was the greatest with an almost immediate decrease in EC. Generally, the percentage of inhibition was increased by increasing the exposure time or the concentration of the toxic chemical in the influent. Complete inhibition was observed at a concentration of 2 ppm after 3 and 10 h for Pb^{2+} and Hg^{2+}, respectively, while near complete inhibition was observed after 15 and 40 h at 50 ppb and 5 ppb of Cd^{2+}, respectively. The low pH in the SOB biosensor likely makes metals more bioavailable and thus more able to assert their toxic effect. This is an advantage of the SOB biosensor since nearly all other sensors operate at a neutral pH.

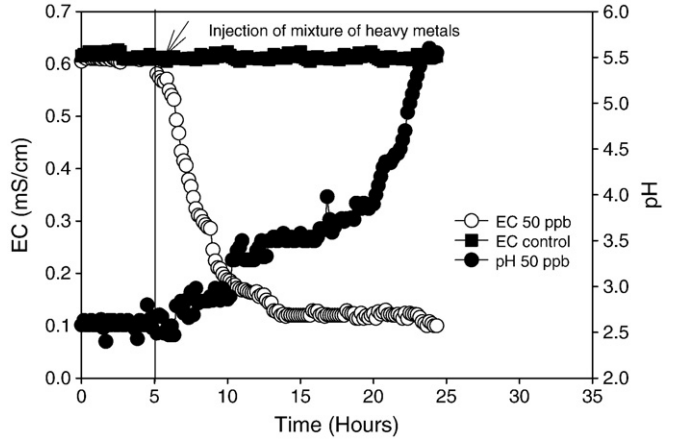

Fig. 5. Detection mixture of heavy metals by the SOB biosensor. The mixture of heavy metals (Zn^{2+}, Hg^{2+}, Cd^{2+}, and Pb^{2+} all at 50 ppb).

The SOB biosensor was developed in this study in order to be used for the detection of pollutants — specifically, a respiratory inhibitor (CN^-) and heavy metals. Compared to other results, the results here show that the SOB biosensor was highly sensitive to detect these pollutants at very low concentrations compared to other biosensors. For example, Okochi et al. (2004) developed an automated water toxicity biosensor using *Thiobacillus ferrooxidans* for monitoring cyanide and mercuric chloride and found that the minimum detectable concentrations were 32 ppb (0.5 μM) and 500 ppb (2 μM), respectively. Hsieh et al. (2004) used the Microtox chronic toxicity test for detection of Cd^{2+}, Hg^{2+}, Zn^{2+}, and Pb^{2+} and observed inhibitory concentrations on the order of 50, 34, 80, and 670 ppb, respectively. The test took 22 h to complete and the EC_{50} increased when natural organic matter was introduced. The EC_{50} (Effective Concentration that causes inhibition by 50%) for Hg^{2+} after 22 h was 34 ppb compared to 50 ppb in this study which was detected in about 1 h. Cho et al. (2004) measured 30-minute EC_{50}s for Hg^{2+} and Cd^{2+} at 800 ± 200, 2180 ± 200 ppb, using *Vibrio fischeri* and 200 ± 100, 1100 ± 300 ppb, respectively, using *Janthinobacterium lividum* YH9-RC. In the same study, the thirty minute EC_{50} value for Zn^{2+} was 1300 ppb using *V. fischeri* and 1300 ± 500 ppb using *J. lividum* YH9-RC.

We found that a mixture of heavy metals (Cd^{2+}, Hg^{2+}, Zn^{2+}, and Pb^{2+}) at 50 ppb each have a synergistic inhibitory effect on SOB since the EC decreased rapidly after their introduction (Fig. 5). A similar result was observed by Tanaka et al. (2002) who found that a mixture of toxic chemicals that have similar chemical structures synergistically inhibited the respiration of nitrifying bacteria.

3.3. Enumeration of SOB

The thiosulfate medium used in this study is generally used for isolation of pure cultures of chemolithoautotrophic SOB such as *Acidithiobacillus* sp. The total microbial count of chemolithotrophic sulfur-oxidizing bacteria isolated from a biosensor was 1.5×10^8 CFU/mL of sulfur particles (2–4 mm) (99.8%) and 0.2% were heterotrophic bacteria. The high percentage of chemolithotrophic SOB is due to S° as the energy source and CO_2 as the carbon source. The low percentage of heterotrophic bacteria is likely due to the low HRT, low pH, and low Biochemical Oxygen Demand in the influent. Soluble microbial products produced by the SOB likely led to the growth of heterotrophs.

3.4. Physiological characteristics and identification of SOB

Among the isolated autotrophic SOB, one common bacterial isolate — AB#1 was selected, purified, characterized, and identified. Morphological and microbiological examination of the isolate showed that

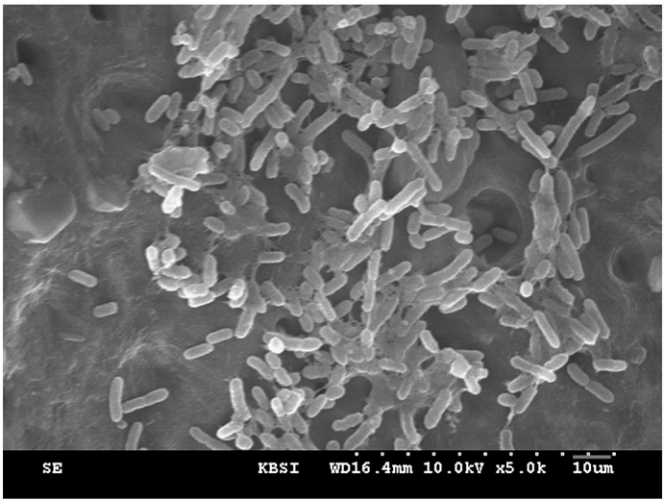

Fig. 6. SEM image of SOB growing on S°.

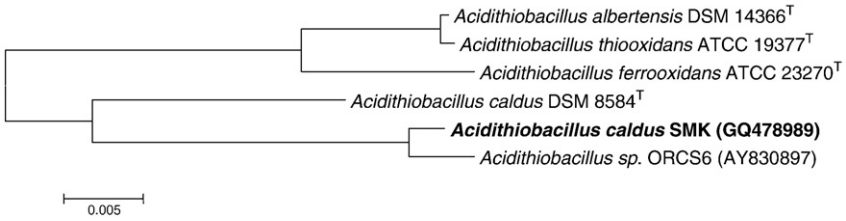

Fig. 7. The phylogenetic location of the bacterium isolated under autotrophic conditions from the SOB biosensor.

the strain produced white colonies, which were round (approximately 0.5–2 μm in diameter), flat, convex, and had complete edges. The cell morphology and motility of the strain observed under an optical microscope showed that it was short rod-shaped, gram-negative, and motile. An SEM shows the growth of SOB on sulfur particles (Fig. 6); the cells were rod shape and thicker and the cellular dimensions were determined to be $0.5 \pm 0.1\ \mu m \times 4.5 \pm 3\ \mu m$ which are much larger than that reported by Yang et al. (2008).

The selected strain was gram-negative and non-spore forming. It is aerobic and catalase positive and can grow autotrophically. Mixotrophic growth was detected on thiosulfate medium plus 1% glucose. No growth was observed using selected colonies on heterotrophic medium. The test results for oxidase, gelatinase, L-lysine, L-ornithine decarboxylases, L-arginine dehydrolase, D-glucose, D-xylose, D-galactose, D-mannose, L-arabinose, sucrose, lactose, D-fructose, L-rhamnose, inositol, and D-mannitol were negative. These results are in agreement with Lee et al. (2000) who isolated *Thiobacillus* sp. ASWW-2 — an autotrophic SOB from activated sludge.

The phylogenetic tree shown in Fig. 7 summarizes the phylogenetic relationships among chemolithoautotrophic sulfur-oxidizing bacterial species. The isolate had the highest level of 16S rRNA gene sequence similarity to *Acidithiobacillus* sp. ORCS6 (99%), and to *Acidithiobacillus caldus* DSM 8584 (96%) (Fig. 7). Thus, the isolate was named and identified as *A. caldus* SMK.

4. Conclusions

Since conventional and microbial methods for the detection of toxic materials are subject to much interference, require long periods of time, and are expensive and complicated; a fast, reliable, and stable platform is needed for monitoring toxic chemicals. In this study, a biosensor using SOB was developed for the detection of toxic chemicals. When toxic chemicals were injected into the biosensor, the effluent EC decreased and the pH increased due to the inhibition of SOB. It was shown that the monitoring of toxicity in water was possible by using the SOB biosensor. The results obtained by the SOB biosensor show that the biosensor was sensitive in the ppb range. The advantages of SOB biosensor include its simplicity, easy operation, the ability to monitor toxicity in real-time, and high sensitivity due to the increased bioavailability of contaminants at low pH. In addition, at a low pH, contamination by other microorganisms is avoided. Sulfur particles are very cheap and no toxic byproducts are produced.

Acknowledgements

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