Short communication

Ecologically Feasible Selection Strategy upon Indigenous *Thiobacillus thiooxidans*

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Abstract—This study provides a technologically feasible strategy from a practical perspective for *in-situ* isolation of stable *Thiobacillus thiooxidans*. Quantitative assessment also clearly emphasizes the evolutionary outcome of enrichment appearing in dichotomous guise (i.e., success or failure, "yes/no"). Progressive increasing in acid formation capability presents the promising viability of selection scheme and stability of *T. thiooxidans* isolates. Incorporation of polyene antibiotics-nystatin and exclusion of solid particles efficiently evacuates possible contaminant species from soil-dilution samples to guarantee an ecologically stable *T. thiooxidans* to be resulted. Asymptotically convergent values of sulfuric acid formation rate strongly support high purity of ecologically enriched *T. thiooxidans* isolates. Further evidence in 16S rDNA detection clearly confirmed pure acidoaphile isolate as *T. thiooxidans*, promising feasibility of our proposed scheme for serial isolation. This selection scheme provides a theoretically plausible and economically appropriate strategy to screen acidophilic *T. thiooxidans* for practical *in situ* applications.

Key Words: *Thiobacillus thiooxidans*, Ecologically stable selection, Acidophilic characteristics, Polyene antibiotics

INTRODUCTION

This study provides a plausible attempt for *in-situ* assessment to measure whether practical isolation of *Thiobacillus thiooxidans* from indigenous soil microbiota is successful. Although isolation techniques of soil-inhabiting microorganisms have been well developed, a technologically viable isolation scheme for *in situ* applications still remained open to be discussed. As known, routine identification for microorganisms is costly and complicated, in particular, not so economically efficient on decision making for on-site species isolation from active mixed consortia. Thus, emphasis of this study is placed on its economic feasibility and biological fundamentality to guarantee successful successive selection(s) upon myriads of microorganisms to the target one(s). The continued use of extremely acidic environments has constantly selected for the genes that give resistance to this hostile situation. In addition, further molecular probing technique (e.g., 16S rDNA) indeed supported the practical premise for applications.

In the past decades, Taiwan's economy has been called a miracle, but in such a rush to develop the economy the price has been paid for the destruction of the natural environment (e.g., soil and groundwater contamination, inordinate deforestation). Due to top-priority concern on environment around the globe, Taiwan's environmental administration must strive the populous island a better place and thus particularly invited "environmentally friendly technologies" for detoxification and clean up of contaminants to prevent excess impact to our ecosystem. Thus, bioremediation is a main-concern "green technology" to be considered (King et al., 1998; Strauss, 1997),

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since contaminants can be completely detoxified and there are no hazardous emissions and secondary waste products. In addition, much attention of in situ bioremediation has been paid for environmental restoration in field applications, since in many instances indigenous microbes on contaminated sites do carry the physiological and metabolic machinery to degrade specific contaminant due to "survival of the fittest." As bioleaching has been considered for in situ field applications to toxic metal (e.g., Cd(II), Pb(II)) removal in Taiwan, any possible candidate microorganisms must be obtained from indigenous soil microbiota to sustain biodiversity of the indigenous consortia of microbiota. That is why this quantitative assessment upon isolation of T. thioparus should be so convincing within aspects of EPA to emerge the biochemical feasibility for intrinsic treatment. Once this strategy is feasible, in situ metal bioleaching should be applicable via bioaugmentation of indigenous T. thioparus cultures or T. thioparus-enriched mixed consortia. This study provides a model guideline upon assessment of isolation of specific extremely acidophilic sulfate-producing microbes from a native microbial community. In addition, it also realizes the evolutionary characteristics on species selection during consecutive subcultures of such microorganism.

Acidophilic chemoautotrophic-sulfur oxidizing bacteria (SOB; e.g., Thiobacillus spp.) have been studied for removal of toxic metals from contaminated soils (Cheng et al., 1999; Zagury et al., 1994). They play a crucial role in sulfur cycle on earth. Thiobacillus ferroxidans (Rawlings and Kusano, 1994) and Thiobacillus thioparus are capable of metabolizing elemental sulfur to sulfuric acid at temperature up to 40°C and pH as low as 1.0 (T. ferroxidans) or 0.5-0.8 (T. thioparus). Acidophiles usually cannot tolerate this low pH (ca. 1.0) acid internally as they cannot sustain the integrity of cell structure in such hostile environments; thus, they turn on a special enzymatic expression to prevent damage for survival and reproduction. This point presents a compelling potential to be applications in many areas. Mineral soils usually contain less than 100 or 200 thiobacilli per gram, but more than 100 fold increases are occasionally encountered at enrichment cultures amended with elemental sulfur (Alexander, 1977). T. thioparus, which is strictly aerobic, shows remarkable ability to reduce inorganic sulfur (S^0) as an energy source (Tortora et al., 1998). Sulfur granules were deposited in intracellular compartments as an energy reserve. Cheng et al. (1999) also proposed a detailed mechanism of T. thioparus to show the existence of partially oxidized intermediates (e.g., thiosulfate and sulfate) for sulfur oxidation. For growth T. thioparus also fixes CO_2 as a sole carbon source into organic matter of its biomass via the Benson-Calvin cycle (Kelly and Harrison, 1988). The energy derived from sulfur oxidation is ultimately stored in ATP via oxidative phosphorylation. Such microbial oxidation is also associated with industrial bioleaching processes in which elemental sulfur is often deposited chemically between sulfide minerals and ferric sulfate (indirect leaching) and then microbiologically converted to sulfuric acids (Konishi et al., 1994). Thus, a marked rise in bacterial sulfuric acid formation capability and tolerance in extreme pH (e.g., ≤ 1.0) can be used as a crucial indicator variable to screen Thiobacillus spp. (e.g., T. thioparus).

In the study, we have screened upon several suspected sites, and ultimately isolated an indigenous acidophilic T. thioparus from sewerage samples originated from an abandoned site to accumulate used automobile batteries near Keelung, Taiwan. To guarantee the predominance of sulfur-oxidizing bacteria, a performance index (dnpH/dt) (i.e., the maximum rate of decreasing pH) of acidophilic characteristics was defined to emphasize this feasibility of isolation scheme. T. thioparus is the major organism responsible for the oxidation of powdered sulfur to sulfuric acid, since the optimum for sulfate production is in the vicinity of pH 2.0 to 3.5 as Alexander (1977) indicated. Acclimation at low pH environments maintains stable metabolism of T. thioparus for acid formation and sulfate production. In addition, deliberate addition of nystatin is of importance to exclude any possible fungal contamination in order to guarantee an ecologically feasible enrichment. Since microbes (e.g., T. thioparus) are capable to provide optimum cellular regulation in specific environments (i.e., extremely acidic conditions) to maintain efficient microbial functions (e.g., biomass growth and sulfate production; Chen et al., 2003, Ramkrishna et al., 1987), any cost-effective strategy of operation should be the one that directs specific enzyme expression in metabolic pathways of T. thioparus to result in specific microbial functions (i.e., acidophilic characteristics). It explains why successive selection in low-pH environment (e.g., pH ≤ 1.0) can provide an efficient strategy to obtain acidophilic Thiobacillus thioparus as indicated in the study. To verify whether our proposed quantitative scheme for isolation is feasible, 16S rDNA detection method (de Wulf-Durand et al., 1997) as a molecular probing evidence was used. Recent compelling results (Chen et al., 2004; Liu et al., 2004) also evidently verified the promising metal resistance and biosorption capability of our isolated indigenous T. thioparus for metal bioremediation. This result confirmed that our selection scheme as a theoretically plausible and economically viable strategy to screen acidophilic Thiobacillus thioparus for in situ applications with practical values.
MATERIALS AND METHODS

Culture conditions and analytical methods

The abundance of chemolithotrophic T. thiioxidans was measured by inoculating sewage dilutions into T. thiioxidans optimum growth medium (T.T.OGM) (N:P = 5:1; compositions (g/L): KH₂PO₄ 1.0, (NH₄)₂SO₄ 2.54, MnSO₄ 0.02, MgSO₄ 0.1, CaCl₂ 0.03, FeCl₃ 0.02, powdered 50 2.0 (or 50), nystatin 0.1 g/L, pH 4.0) and observing the change in acidity and sulfate concentrations over time. Aware that powdered sulfur is insoluble in water and thus sterilized after addition to T.T.OGM by 30-min intermittent sterilization in 100°C flowing steam every day for 3 times (or "Tyndallization"; Burlage et al., 1998). Moreover, since nystatin may be unstable in moist-heat sterilization, 0.1 g/L (ca. 8200 units/L) nystatin (powdered from effervescent vaginal tablets-Mycostat®) was sterilized by filtration (Millipore Millex-QGS 0.22 μm filter unit) before use. After filtering out solid samples, approximately 2.0 g sewage sample was well mixed with 100 mL T.T.OGM (denoted as React Mix). React Mix was then incubated in a water-bath incubator at 125 rpm for 30 min to ensure efficient extraction of all available microbial communities in sewage into the soluble phase. Kinsky (1967) mentioned that polyene antibiotic nystatin binds to sterol components (mainly in ergosterol) on cell membrane of fungi to alter cellular permeability. This significant biosorption capacity of antibiotics onto sterol components on cell membranes also augments lethal effects of nystatin toxicity, resulting in an effective selection pressure to eliminate fungal or other contaminant microorganisms. However, polyenes have no effects on bacteria due to absence of such sterol as specific polyene receptors. Burlage et al. (1998) mentioned that sequential subculture in powdered sulfur containing liquid media will normally result in progressively faster sulfate production and enrichment of T. thiioxidans. The sulfate, biomass concentrations and pH level were measured with time for each consecutive subculture. Experiments were undertaken in duplicate to guarantee the symmetrical nature with respect to space and time for data reproducibility. Note that after seven consecutive subcultures pure T. thiioxidans isolates were obtained according to the proposed approach of an infinite sequence of \( (e^{-\Delta pH/d t}) \) (shown in Results and Discussion). As the growth of T. thiioxidans is inoculum size-dependent, batch cultivation was carried out in 250 mL-shake flasks and 5 L fermentors with initial total medium volumes of 110 mL (10 mL inoculum; 9.1%) and 2.1 L (100 mL inoculum; 4.7%). In flask cultures, the pH was not controlled and water-bath shaker was operated at 150 rpm. 30°C. In the fermentor cultivation, the airflow rate was set at 1.0vvm, and agitation speed was set at 150 rpm.

Biomass concentration was determined via turbidimetric measurement at 620 nm (1.00 OD₆₂₀nm ± 0.98 ± 0.08 g/L dry cell weight). Sulfate content was determined according to the Barium Sulfate Turbidity Method as described in Standard Methods (APHA, 1995). Using pH 4.0 and 10.0 standard buffers (Fisher Scientific) for calibration, standard measurement of pH was undertaken by using pH electrode and meter (Cole-Parmer) with an accuracy 0.1 pH unit.

Molecular 16S rDNA detection

Chromosomes from liquid samples were extracted within silica-gel-membrane filter units (Qiagen, Valencia, CA). The concentration of the DNA was determined spectrophotometrically and its quality was observed on a 0.7% agarose gel. The designed primers based on 16S rDNA sequence are specific to T. thiioxidans as mentioned before (de Wulf-Durand et al., 1997). The sequences of primers are GGGTGCTAAATATGGGCCTGCTG and CATAACCGTGACTCATGCCCC. The length of projected PCR product is 1,057 base pairs. Conditions for PCR were as previously described (Durand, 1996). Annealing temperature for PCR is 52°C. The products of PCR were measured by gel electrophoresis on a 0.7% agarose gel, stained with ethidium bromide and observed under UV light. Identity of the PCR product was confirmed by sequencing its DNA by automated sequencing, which was performed by Tri-I Biotech, Inc. (Taipei, Taiwan). The sequence was compared with the published 16S rDNA sequence of T. thiioxidans obtained from GenBank database. To prevent false positive results to be introduced due to contamination in PCR products, all PCR tasks for negative control (e.g., PCR reagents, oligonucleotide primer, dNTPs, buffers and Taq DNA polymerase) were also conducted (data not shown).

RESULTS AND DISCUSSION

Evaluation success/failure of selection cultures

Due to regulations of Taiwan's EPA to maintain biodiversity of native ecosystems, several candidate sites (e.g., geothermal hot springs, disposal spots for used automobile batteries, metal-contaminated sites) have been inspected for the possible existence of extremely acidophilic T. thiioxidans or T. ferrooxidans. Samples of either soil or sewerage were collected from the top 4 cm of the soil profile, since this is where most of the microbial activity occurs (Ladera
and Shearer, 1990). Thus, most concentrated and diverse microorganisms can be obtained in such screened habitats. However, successfully stable *T. thiooxidans* isolates were only obtained from sewerage samples from a suspected lead-contaminated site near Keelung, Taiwan. This site was originally as a collection site for used lead storage cells and thus contaminated with sulfuric acid and very likely lead (e.g., Pb, PbO₂, and PbSO₄) as well. This can be explained by an overall reaction of lead storage cells as follows:

\[
Pb^{(l)} + PbO_{2(l)} + 4H^{+} (aq) + 2SO_{4}^{2-} (aq) \rightarrow 2PbSO_{4(l)} + 2H_{2}O(l),
\]

where the forward and reverse reactions indicate discharge and charge processes, respectively. Moreover, electrodes in cells are suspended in sulfuric acid solution (ca. 6.0 M). Residual acid solutions in cells likely produced a high level of sulfate contamination on site. It was also confirmed through chemical analysis (i.e., pH = 3.7 ± 0.2 and \[SO_{4}^{2-} = 3.770 \text{ ppm}\]) of site characterization after filtering out solid particles in sewerage. Isolation experiments were thus undertaken at pH 4.0 to imitate a favorable in situ condition from an ecological perspective. Since *T. thiooxidans* is so called an extremely acidophilic bacterium, it is remarkably tolerant in acid environments to survive at low pHs (e.g., ≤ 1.0). It indicates that successful enrichment cultures on elemental sulfur should exhibit a decreasing profile in pH and biased amplification in SOB, leading to high purity of *T. thiooxidans*. As the pH level in enrichment cultures of *T. thiooxidans* with elemental sulfur can fall to 1.0 or below (Burlage et al., 1998), an infinite sequence \((-\Delta pH/\text{dt})\) was used a performance index of microbialological characteristics to "probe" the existence of acidophilic *Thiobacillus* species. This also provides a selection pressure to eliminate possible contamination species that cannot survive in sulfuric acid environments. Chen and Chang (2000) used a novel assessment of toxicological threshold drift \(0 < T_{h,1} < T_{h,2} < T_{h,3} < \cdots < T_{h,j} < T_{h,i} < \cdots < \lim_{n \to \infty} T_{h,j} = T_{h} < +\infty\) to address the predominance of hyperresistant mercuric-resistant strain *Pseudomonas aeruginosa* PU21 (Rip64) in acclimated cultures. Similarly, pH gradients with respect to culture time can be extensively used as a measure to indicate whether the proposed enrichment worth to proceed. Note that both pH measurement and pH derivative determination are straightforward to be evaluated in situ to conclude a screening outcome in either a success or failure. As proposed, a bounded, monotonic, infinite sequence of \((-\Delta pH/\text{dt})\) \((\forall y = 1, 2, \ldots, +\infty; i.e., (-\Delta pH/\text{dt})_{y,\infty}\)) in sequential subcul-

![Fig. 1. Time courses of microbial activities (i.e., pH, cell density, sulfate concentration) in serial selection of indigenous *Thiobacillus thiooxidans*. \(S_{0}\) = initial sulfur concentration.](image-url)
repeated subcultures after 7. In addition, the value of 0.079 ± 0.004 was obtained by comparing with the decreasing profiles in pHs for subcultures after 22 (i.e., after ca. 1 year; (365 d/yr × 24 h/d × 1 yr)/(400 h/subculture) = 22) and for the resulted pure \( T. \) thiioxidans (Chen et al., 2003; 2004). Asymptotically and monotonically convergent property (i.e., \( f \rightarrow +\infty \)) of \( \{ \langle -d\text{pH}/dt \rangle \} \) strongly suggests that an ecologically stable species of extreme acidophile- \( \text{Thiobacillus thiioxidans} \) was obtained. This point was later confirmed through purification by routine single colony isolation and molecular identification procedures (shown later). Figure 1 presented a promising indication to use the rate of pH change for \( T. \) thiioxidans enrichment. If the enrichment sequence \( \{ \langle -d\text{pH}/dt \rangle \} \) cannot reveal a monotonic increasing trend or stable convergence to a limit (i.e., \( L_{\text{ph}} \)), the isolation strategy ongoing is not feasible to result in an ecologically stable species of \( T. \) thiioxidans. Note that failure enrichments upon sewerage samples without filtration or addition of nystatin resulted in fungal contamination in 2 or 3 enrichment cycles due to significant decreases in \( \{ \langle -d\text{pH}/dt \rangle \} \) (i.e., “failure”). In these samples, solid particles that have not been removed via filtration likely contained contaminant species (e.g., fungal species) in intraparticle spaces. It also suggests that most of fungal species is not likely present in a suspended liquid phase, but immobilized in intraparticle phases of sewerage. With such intraparticle transfer resistance, nystatin cannot effectively enter “immobilized phase” to completely exclude contaminant species (e.g., fungi) for successful selection.

**16S rDNA detection**

To confirm whether the pure isolates of \( T. \) thiioxidans after serial acclimation were indeed \( \text{Thiobacillus thiioxidans} \), the PCR method described previously was used to detect the existence of 16S rDNA of \( T. \) thiioxidans (de Wulf-Durand et al., 1997). The 16S rRNA analysis has been applied to obtain a phylogenetic survey of the sulfur- and iron-oxidizing bacteria (Harrison, 1982; Lane et al., 1992). A sample from tested culture (which was obtained from 14 day culture) and positive control, a \( T. \) thiioxidans strain (CCRC15615) from Bioresources Collection and Research Center (Hsinchu, Taiwan), were detected and both bands are in the same projected position (Fig. 2). The PCR product was then sequenced by automated sequencing. The sequence and the published 16S rDNA sequence of \( T. \) thiioxidans obtained from GenBank database are identical (data not shown). These results confirm the existence of \( T. \) thiioxidans in the tested culture.

![Fig. 2. PCR amplification with specific primers for detection of \( T. \) thiioxidans. Lane 1: sample tested from this study; lane 2: \( T. \) thiioxidans (CCRC15615) for positive control.](image)

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**NOMENCLATURE**

- \( -d\text{pH}/dt \) the maximum rate of decreasing pH, h\(^{-1}\)
- \( L_{\text{ph}} \) convergent limit of stable isolate of \( \text{Thiobacillus thiioxidans} \), h\(^{-1}\)
- \( OD \) optical density of turbidimetric measurement
- \( S_0 \) initial powdered sulfur concentration, g·L\(^{-1}\)
$SOB$  sulfur oxidizing bacteria  
$T$  
  culture time elapsed, h  
$Th_c$  toxicological threshold of PU21c, molecules Hg(II) cfu$^{-1}$  
$Th_r$  toxicological threshold of PU21r, molecules Hg(II) cfu$^{-1}$  
$\gamma$  
  for all  

Subscripts  
0  
  time for starting cell culture (or initial)  
$j$  
  the $j$th subculture ($j = u$ for unacclimated Hg(II)-resistant strain of PU21 and $j = r$ for hyperresistant PU21)  
$\infty$  
  time at infinity (steady state)  

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