Improved Cultivation of the Indigenous 
*Acidithiobacillus thiooxidans* BC1 by a 
Fed-Batch Process

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Abstract—In this study, a simple fed-batch process was developed for high cell density culture of the indigenous *Acidithiobacillus thiooxidans* BC1 for enhanced production of sulfuric acid. Since the inhibitory effect of the metabolic intermediate, sulfite, occurs in the acidic condition, pH control is important in the growth of this microorganism. In addition, elemental sulfur is the sole energy source for the indigenous *A. thiooxidans* BC1, a fed-batch process including the intermittent feeding of elemental sulfur was proved to avoid the effect of substrate limitation and inhibition, which was resulted from the sulfur accumulation in the air-liquid interface. The maximal cell density and sulfuric acid yield obtained in this study were 2.16 and 3.25 times, respectively, higher than those obtained from the conventional batch process. The optimal operation condition for the fed-batch process was set as: intermittent feeding of additional 2 g/L elemental sulfur at the same time when the pH value was adjusted from 2.0 to 4.0.

Key Words: Fed-batch, High cell density, *Acidithiobacillus thiooxidans*, Sulfuric acid, Elemental sulfur, Sulfite

INTRODUCTION

The treatment of metal contaminated soils and sediments is commonly classified into two categories: the traditional physical/chemical method and bioleaching by microorganisms. Although the physical/chemical technology has been extensively applied in practice, it exhibits the limitations of low efficiency, high cost, and the possibility to produce the secondary contaminations and further hazardous emissions (Rulkens et al., 1995). In contrast, the advantage of bioleaching is the relatively low cost and mild conditions of the process, and the subsequent low demand for energy or landfill space compared with conventional technologies (Krebs et al., 1997). Microorganisms play a predominant role in the solubilization, transport, and deposition of metals and minerals in the environment (Huntais et al., 1986). A better understanding of these processes (Rulkens et al., 1995; Tyagi and Couillard, 1989) has allowed scientists to further characterize bioleaching of metals from ores and to propose innovative microbial-based technologies for metal reclamation (Alhoen and Tuovinen, 1995; Blais et al., 1992; Borecker, 1986; Chen and Lin, 2001; Liu et al., 2003a; Sreetrishnan et al., 1993).

*A. thiooxidans* is a chemolithotrophic acidophilic bacterium that grows on elemental sulfur as the optimal energy source and is important in the microbial catalysis of sulfide oxidation. Since it oxidizes both elemental sulfur and sulfide to sulfuric acid, *A. thiooxidans* plays a significant role in bioleaching of metals from sulfide ores (Brierley, 1982; Lundgren and Silver, 1980). Previous study has shown that *A. thiooxidans* solubilizes a sulfide ore by a mechanism different from that adopted by *A. ferroxidans* (Lizama and Suzuki, 1988). Furthermore, oxidation of pyrite and elemental sulfur by *A. thiooxidans* was
found to be competitively inhibited by cells (Lizama and Suzuki, 1991). So far, high cell density cultivation of *A. thiooxidans* is still a challenge for industrial applications due to its relatively low growth rate comparing with that of other fast growing microorganisms such as *Escherichia coli*. Since large-scale culture of *A. thiooxidans* is unlikely to be sustainable or economic, attempts have been made to overcome some of their limitations. In particular, it has been suggested that fed-batch or filtration culture should be more appropriate for high-density cell propagation (Chauhan et al., 1999; Chen and Zhang, 1997; Korz et al., 1995; Kweon et al., 2001; Li et al., 1998; Wen et al., 2002). Moreover, it has also been investigated that elemental sulfur and pH values of the growth medium are crucial to high cell density cultivation of *A. thiooxidans* (Liu et al., 2003b).

Usually, batch culture as a commercial cultivation technique is hindered since high initial substrate concentrations are essential if high cell densities are to be achieved. In contrast, fed-batch culture is commonly used in large-scale microbial fermentation and has been widely adopted in biotechnology industries (Shioya and Suga, 1992). It may lead to a high cell density because substrate limitation and/or inhibition can be avoided (Wen et al., 2002). It is obvious that fed-batch culture can be used to produce high cell concentrations and productivity while maintaining a good cell growth yield (Chen, 1996). Thus, the aim of the present study was to develop a high cell-density fed-batch process, using the strategy of intermittent adjustment of pH value and feeding of additional elemental sulfur in the growth medium, for enhanced production of sulfuric acid by the indigenous *A. thiooxidans* BC1.

**MATERIALS AND METHODS**

**Microorganisms**

The indigenous *A. thiooxidans* BC1 used throughout this study was obtained from sewerage samples from a high level of sulfate-contaminated site near Keelung, Taiwan. Isolation experiments were undertaken at pH 4.0 to imitate a favorable in-situ condition from an ecological perspective. Successful enrichment cultures on elemental sulfur should exhibit a decreasing profile in pH and biased amplification in sulfur-oxidizing bacteria, leading to high purity of *A. thiooxidans*. The abundance of *A. thiooxidans* was obtained by inoculating sewerage dilutions into *A. thiooxidans* optimum growth medium (*A. thiooxidans* 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 S° 5.0, nystatin 0.1, pH 4.0). The inoculated culture was then incubated in a water-bath shaker at 30°C, 125 rpm. The sulfate, biomass concentrations and pH level were measured over time for consecutive subcultures. Pure *A. thiooxidans* isolates were obtained after seven consecutive subcultures and maintained for subculture in shaker flasks at 30°C, 125 rpm before being used in the following fed-batch experiments.

**Fed-batch cultivation**

Fed-batch cultivation of the indigenous *A. thiooxidans* BC1 was carried out in 250 mL shake flasks at 30°C, 125 rpm, with the total volume of cell culture being 100 mL. The composition of the growth medium was unchanged except for elemental sulfur and pH value. As determined previously (Liu et al., 2003b), elemental sulfur is the primary energy source and pH difference is the driving force for the growth of *A. thiooxidans*. Thus, during the fed-batch experiments, pH was adjusted to the initial values of 4.0 when it reached the values of 1.0 or 2.0, with or without adding additional 1 or 2 g/L elemental sulfur into the growth medium.

**Analytical methods**

The concentration of sulfuric acid was determined according to the "Turbidimetric Method" as described in Standard Methods (APHA, 1995). Aliquots of 10 mL were drawn from the culture and filtered through a general grade filter paper (Advantec, Tokyo, 55 mm). The turbidity of the supernatant was read at wave length of 450 nm against a blank containing DI/DD (deionized/distilled) water. The turbidity reading was taken within 10-15 seconds. The concentrations of sulfuric acid were measured by DR/2000 spectrophotometer (HACH, Loveland, CO). Spectrophotometer at 620 nm was performed against the general gravimetric results to obtain a calibration curve. By reading the turbidity of a given sample culture, if necessary the sample has to be diluted to a proper concentration range for the measurement, the corresponding amount of biomass was obtained (1.00 OD₆₂₀nm = 0.98 ± 0.08 g/L dry cell weight) (Liu et al., 2003c). Using pH 4.0 and 10.0 standard buffers (Fisher Scientific, Tokyo, Japan) for calibration, standard measurement of pH was undertaken by using pH electrode and meter (Cole-Parmer, Vernon Hills, IL) with an accuracy 0.1 pH unit.

**RESULTS AND DISCUSSION**

It is desired to obtain a large quantity of cells with high growth rate. In other words, a higher cell concentration has to be attained in the logarithmic growth phase. However, *A. thiooxidans* does not grow readily, and its cell concentration is usually
very low. Various approaches, such as using thiosulfate as an alternative energy source with pH control (Nakamura et al., 1990) or supplying a CO₂-enriched gas (Imai and Okuzumi, 1965), have been proposed to increase cell concentration. In either case, the cell concentration was still insufficient and the cultivation time too long. The cell growth is considered to be mainly limited by substrate deficiency or inhibition and the accumulation of inhibitory metabolites such as sulfite. In this study, in order to obtain a sufficient quantity of cells with high growth rates, the high cell density cultivation of the indigenous A. thiooxidans BC1 was conducted with a fed-batch process, using the strategy of intermittent adjustment of pH value and feeding of additional elemental sulfur in the growth medium.

The effect of pH

The oxidation of elemental sulfur by A. thiooxidans is a complex process involving the contact of cells with sulfur particles (Takeuchi and Suzuki, 1997), the oxidation of sulfur to sulfite (Suzuki et al., 1992), and the oxidation of sulfite to sulfate (Takeuchi and Suzuki, 1994). All of these processes are influenced by pH (Suzuki et al., 1999). A. thiooxidans can oxidize sulfur at a wide range of pHs but can grow only under acidic conditions of pH 1.0 to 5.0. Since sulfite is inhibitory to sulfur oxidation at acidic pH values, the oxidation of sulfur would be expected to slow down as the concentration of sulfite increases with time during sulfur oxidation under acidic conditions (Suzuki et al., 1992). In addition, when the amount of elemental sulfur as a substrate is limited, the oxidation would be incomplete because of strong progressive inhibition by the accumulation of sulfite (Suzuki et al., 1992). In order to eliminate the inhibitory effect of sulfite accumulation on the growth of A. thiooxidans in acidic conditions, a fed-batch process was designed to adjust pH to the initial value of 4.0 when it reached the value of 1.0 or 2.0 during the cultivation of A. thiooxidans, with or without adding additional 1 or 2 g/L elemental sulfur.

Figure 1 shows the effects of pH adjustment on cell concentration and sulfuric acid production during the entire fed-batch process. As expected, both cell concentration and sulfuric acid production were increased by the fed-batch process with pH adjustment. Since A. thiooxidans is a gram-negative, acidophilic, mesophilic chemolithotroph, it oxidizes elemental sulfur as energy source rather readily. However, inhibitory effects on cell growth and sulfuric acid production may occur due to the rapid accumulation of the intermediate metabolite, sulfite, in acidic condition. The adjustment of pH to the initial value of 4.0 when the system reached pH values of 1.0 or 2.0 significantly eliminated the inhibitory

Fig. 1. Fed-batch cultivation of the indigenous A. thiooxidans BC1 with the pH adjusted to the initial value of 4.0 when it reached the values of 1.0 or 2.0: (a) pH; (b) optical density; (c) the concentration of sulfuric acid versus cultivation time.
effects and thus increased the maximal OD by 1.33 and 1.69 times and the maximal sulfuric acid production by 1.39 and 2.41 times, respectively, comparing to the batch process (Table 1), probably due to that the reversed conversion of the overproduced sulfite to elemental sulfur, which allows *A. thiooxidans* to repeat its metabolic cycle by utilizing elemental sulfur, and that the pH difference in the fed-batch process may provide the driving force for the cells to grow more effectively. In addition, the effect of the adjustment of pH value from 2.0 to 4.0 on the maximal amount of sulfuric acid production is more significant than that from 1.0 to 4.0. It is noteworthy that the pH value no longer reached as low as 1.0 when it was adjusted from 1.0 to 4.0, probably due to the deficiency of elemental sulfur to be oxidized by this microorganism. In contrast, the pH value decreased to 2.0 rather rapidly when it was adjusted from 2.0 to 4.0 and thus the adjustment of pH was made several times during the entire fed-batch process. The above results suggested that the transformation of elemental sulfur to sulfite may occur in the pH range of 2.0 to 4.0 and that the formation of sulfate from sulfite may occur in the pH below 2.0. The adjustment of pH from 2.0 to 4.0 significantly avoids the inhibitory effect, which mostly occurs below pH 2.0 with the accumulation of the metabolic intermediate, sulfite.

### The effect of addition of elemental sulfur

Microbial oxidation of elemental sulfur in soils (McCaskill and Blair, 1987; Watkinson, 1989) or mine spoils (Kapoor and Mishra, 1988; Pichtel and Dick, 1991) has been intensively studied in the past decade because elemental sulfur is an increasingly important component of many fertilizers and is produced in surface deposits from mining operations of sulfide minerals (Gourdon and Funtowicz, 1998). Various models have been proposed to describe the oxidation of sulfur in soils (Gourdon and Funtowicz, 1998; Jensen and Bettany, 1987; Watkinson, 1989) or in well-mixed batch reactors (Komishi *et al.*, 1990, 1992, 1994). The direct bacterial oxidation of elemental sulfur implies the attachment of the cells to the mineral particle surface (Nagpal *et al.*, 1994). Free bacteria cannot grow at the expense of sulfur oxidation. In contrast, high initial concentration of elemental sulfur would cause substrate inhibition for bacterial growth, which was resulted from the air-liquid interface. Thus, a fed-batch process was design to avoid substrate limitation or inhibition by feeding additional 1 or 2 g/L elemental sulfur into the growth medium at the same time when pH value was adjusted in the present study.

Figures 2 and 3 give the results of feeding additional 1 or 2 g/L elemental sulfur to the fed-batch process at the same time when pH values were adjusted from 1.0 to 4.0 and from 2.0 to 4.0, respectively. The results are also summarized in Table 1. For both cases, cell density and sulfuric acid concentration were increased with increasing amount of elemental sulfur added in the fed-batch process. It indicates that elemental sulfur is the sole energy source for *A. thiooxidans* and the intermittent supply of elemental sulfur stimulates the growth of cells and the production of sulfuric acid. Sulfite has been proved to be the oxidation product of sulfur in *A. thiooxidans* cells when the further oxidation of sulfite is inhibited (Suzuki *et al.*, 1992). Under certain conditions, sulfur is nearly stoichiometrically oxidized to sulfite, which indicates that the oxidation of sulfur to sulfite is totally dissociated from the oxidation of sulfite to sulfate. *A. thiooxidans* cells oxidize elemental sulfur equally well either at an acidic pH, which is required for growth, or at a neutral pH, at which no growth is possible (Suzuki, 1965). However, the oxidation of elemental sulfur by *A. thiooxidans* is prohibited by oversupply of the elemental sulfur due to the effect of substrate inhibition. Fed-batch process by intermittent feeding of additional elemental sulfur not only avoids the effect of substrate limitation and/or inhibition, but also reduces the amount of sulfite accumulation in the metabolic pathway. According to Table 1, additional feeding of 2 g/L elemental sulfur to the system at the same time when the pH was adjusted to its initial value of 4.0 from 2.0 produced the highest amount of sulfuric acid (i.e., 88,309 ppm), which is about 3.25 times that produced in the conventional batch process.

### Table 1. The maximal amount of cells and sulfuric acid produced in the batch and fed-batch processes.

<table>
<thead>
<tr>
<th>Process</th>
<th>Operation Condition</th>
<th>Maximal OD</th>
<th>Maximal SO$_4^{2-}$ (ppm)</th>
<th>OD Increased$^a$ (times)</th>
<th>SO$_4^{2-}$ Increased$^b$ (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>pH 1.0 S(OH)$^-$</td>
<td>0.427</td>
<td>27,145</td>
<td>1.33</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>pH 1.0 S(O)$^-$</td>
<td>0.566</td>
<td>37,649</td>
<td>1.63</td>
<td>2.43</td>
</tr>
<tr>
<td>Fed-batch</td>
<td>pH 1.0 S(O)$^-$</td>
<td>0.696</td>
<td>65,886</td>
<td>1.72</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td>pH 2.0 S(OH)$^-$</td>
<td>0.721</td>
<td>65,371</td>
<td>1.69</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td>pH 2.0 S(O)$^-$</td>
<td>0.884</td>
<td>71,106</td>
<td>2.07</td>
<td>2.62</td>
</tr>
<tr>
<td></td>
<td>pH 2.0 S(O)$^-$</td>
<td>0.921</td>
<td>88,309</td>
<td>2.16</td>
<td>3.25</td>
</tr>
</tbody>
</table>

$^a$ OD increased was determined by dividing the maximal OD obtained in each fed-batch process by that obtained in the batch process.

$^b$ SO$_4^{2-}$ increased was determined by dividing the maximal SO$_4^{2-}$ obtained in each fed-batch process by that obtained in the batch process.

$^c$ The number in the parenthesis is the amount of elemental sulfur in g/L feeding to the growth medium when the pH value was adjusted.
In both cases of adjusting pH from 1.0 to 4.0 and from 2.0 to 4.0, more elemental sulfur added resulted in higher amount of sulfuric acid and cells produced.

(a)

Fig. 2. Fed-batch cultivation of the indigenous *A. thiooxidans* BC1 with or without feeding additional 1 or 2 g/L elemental sulfur at the same time when the pH adjusted to the initial value of 4.0 from 1.0: (a) pH; (b) optical density; (c) the concentration of sulfuric acid versus cultivation time.

(b)

(c)

Fig. 3. Fed-batch cultivation of the indigenous *A. thiooxidans* BC1 with or without feeding additional 1 or 2 g/L elemental sulfur at the same time when the pH adjusted to the initial value of 4.0 from 2.0: (a) pH; (b) optical density; (c) the concentration of sulfuric acid versus cultivation time.
CONCLUSION

The development of a simple fed-batch process for high cell density culture of the indigenous A. thiiooxidans BC1 to enhance the production of sulfuriac acid was successfully conducted in this study. Additional feeding of 2 g/L elemental sulfur at the same time when the pH was adjusted from 2.0 to 4.0 yielded the maximal sulfuric concentration of 88,309 ppm produced, which is about 3.25 times that produced in the conventional batch process. The intermittent elemental sulfur feeding and pH adjustment strategy was proved to be an efficient method to obtain high density cell culture of A. thiiooxidans.

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REFERENCES


以飼料批次培養高濃度本土性硫氧化硫化桿菌來增加硫酸產量

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摘要

本研究設計一簡單之飼料批次程序進行本土性硫氧化硫化桿菌之高濃度培養來增加硫酸的產量。因在酸性條件下，
代謝中間產物亞硫酸會對此菌種生長產生抑制作用，故pH值的調控對此菌種的生長有重要的影響，除此之外，
固態飼料是此菌種生長唯一的能量來源，故飼料飼料與乾飼料的批次程序研究可以避免基質的不足及抑制效應。
而抑制效應主要肇因於飼料飼料被接為氨氧化，以此飼料批次程序所生產的葡萄糖量及葡萄糖量比傳統的批次程序平均提高 2.16 及 3.25
倍。最佳的飼料批次程序則設定為：每當pH值自2.0調升為4.0時，添加2 g/L的固態飼料基質於液體培養基中。