ABSTRACT

Clean coal technologies that involve limestone for sulfur capture generate lime/limestone products laden with sulfur at various oxidation states. If sulfur is completely stabilized as sulfate, the spent sorbent is ready for commercial utilization as gypsum. However, the presence of reduced sulfur species requires additional processing. Thermal oxidation of reduced sulfur can result in undesirable release of SO₂. Microbial oxidation might provide an inexpensive and effective alternative. Sorbents laden with reduced forms of sulfur such as sulfide, sulfite, or various polythionate species serve as growth substrates for sulfur-oxidizing bacteria, which have the potential to convert all sulfur to sulfate.

During this project we evaluated the use of sulfur-oxidizing bacteria for stabilization of the following three different sorbents: 1.) calcium sulfide, as a model for sorbent from coal gasification; 2.) lime from coolside dry sorbent injection; 3.) slurry from an inhibited flue gas desulfurization (FGD) process. We concluded that stabilization of calcium sulfide using bacterial strains such as Thiobacillus versutus would be feasible if pH were controlled by feed rate. Although lime from coolside injection contained discernible amounts of sulfite, the high pH generated by this material would preclude bacterial stabilization. From the FGD slurry an isolate was obtained, which rapidly oxidized thiosulfate in the aqueous phase and slowly oxidized the calcium sulfite hemihydrate solid phase. Biostabilization appears to be a natural process that is already occurring in impounded slurry.
EXECUTIVE SUMMARY

Utilization of limestone based sorbents for sulfur capture frequently results in sulfur-laden waste in which reduced forms of sulfur are present. Examples include coal gasification, and flue gas desulfurization (FGD). In addition to sulfur combustion products, another source of reduced sulfur in many wet FGD processes is thiosulfate, which is added to inhibit sulfite oxidation. As a group, these organisms are very versatile in oxidizing reduced sulfur compounds. Collectively, sulfur-oxidizing bacteria produce sulfate from sulfite, tetrathionate, trithionate, thiosulfate, elemental sulfur and sulfide. Possibly microbial processing could result in a gypsum product, or at least in stabilized waste that could be more easily disposed of in the environment.

Stabilization of Calcium Sulfide, as a Model for Sorbent from Coal Gasification.

In preliminary experiments, prior to starting this project, we showed in shakeflask cultures that T. versutus ATCC 25364 could be used to oxidize sulfide captured on limestone. However, the total sulfide contents of the sorbent in these experiments was only 0.64%. In order to scale-up to a 2-liter bioreactor with more realistic material, we used technical grade CaS. Although the material in aqueous slurry generated a pH too high for bioprocessing (over 11.0), this problem was overcome by feeding CaS gradually and allowing acid produced from the biooxidation reaction to adjust the pH to an optimum range (8.1 to 8.6). Figure 1 shows that for a typical experiment, once the reactor was running approximately 87% of the sulfide was converted to sulfate. Because sulfide was always absent prior to the daily addition of CaS, it is likely that the culture was substrate limited and that more CaS could be processed if it were supplied gradually in steady increments. We would recommend that dry CaS be fed to such a bioreactor. Perhaps this could be regulated by the pH control unit, which would add CaS as acidification occurs.

Stabilization of Spent Lime from Sorbent Injection.

The X-Ray Diffraction spectrogram of spent lime sorbent from coolside injection indicated that CaSO₃·½H₂O was present, although the principal component appeared to be Ca(OH)₂. In a 5% slurry this material generated a pH greater than 12.0, substantially higher than the maximum pH tolerated by any strain. Unlike biooxidation of sulfide, biooxidation of sulfite generates little or no acid. Therefore, biooxidation could not be used to significantly lower pH. It was decided that biostabilization of sulfur occurring in this sorbent would not be economically feasible.
Conversion of CaS in slurry to dissolved sulfate in 2-liter septic bioreactor inoculated with Thiobacillus versutus ATCC 25364. CaS (1 g) was added every 24 hr. Sulfate was measured just prior to this daily addition.

Stabilization of Slurry from Inhibited Flue Gas Desulfurization

We obtained slurry from an inhibited FGD process, which employs 200 ppm dibasic acids. Thiosulfate is added to the sorbent slurry during sulfur dioxide capture to inhibit sulfite oxidation. It is thought that thiosulfate acts as a free radical scavenger, producing tetrathionate, which then forms trithionate and thiosulfate. It is likely that all these species are present in the spent slurry, therefore, a strain that oxidizes all would be ideal for bioprocessing. The solid phase is principally CaSO₃·½H₂O. The solid and aqueous phases of the slurry were separated by filtration and treated separately. Certain wet scrubbing processes employ additions of dibasic acid mixtures to facilitate sulfur dioxide capture. Dibasic acids act as soluble pH buffers that promote formation of HSO₃⁻ and Ca²⁺. Adipic, glutaric and succinic acids are major components in these mixtures.

Preliminary attempts to process this slurry with Thiobacillus neapolitanus ATCC 23641 were unsuccessful. Cells remained viable; however, there was no increase in soluble sulfate nor decrease in thiosulfate. Additional experiments with ATCC 23641 and T. neapolitanus ATCC 23639 indicated that both strains were at least partially inhibited by certain dibasic acids in combination with high calcium concentrations. We obtained the isolate "TQ" from the spent
slurry. "TQ" was not inhibited by 200 ppm succinate, glutarate or adipate at calcium concentrations as high as 10,000 ppm. "TQ" rapidly oxidized thiosulfate occurring in the aqueous phase of the slurry. The strain was also able to grow slowly on the CaSO₃·½H₂O solid phase. Unlike the strains of T. neapolitanus, "TQ" could tolerate and grow on low concentrations of sulfite. Although "TQ" appeared to be associated with ferric iron reduction, supplying the cultures with ferric iron did not enhance biooxidation of CaSO₃·½H₂O.

Conclusions

1. Biooxidation of CaS could be a feasible process if pH were controlled by feed rate. Release of H₂S, gypsum precipitation in the ports of the aeration system, and inability to keep particulates in suspension are three potential problems.

2. Biooxidation of sulfite occurring in spent lime from dry sorbent injection would not be recommended due to high pH generated by this material and the relatively low quantity of sulfite present.

3. Biooxidation of sulfur species in the aqueous phase of an inhibited FGD slurry occurs rapidly due to the activity of an indigenous strain, "TQ". A better understanding of this system might help to improve on a process that already occurs naturally.
OBJECTIVES

I. Continue to Optimize Conditions for Sulfate Generation in Benchscale Experiments. Various parameters influence rates of sulfur oxidation. These include: sulfur load, aeration, pH, temperature, and nitrogen and phosphate requirements. During the first year of the project we attempted to identify optimum values for these parameters using pure cultures in various media. Once we began to work with the sorbents, however, we encountered challenges that caused many of these data to be irrelevant.

II. Study the Effectiveness of Microbial Processing on Spent Sorbents from Various Sulfur-Capture Processes. These processes included: a slurry from an inhibited flue gas desulfurization process; lime from coolside dry sorbent injection and calcium sulfide, which we are using as a model for spent sorbent from a reductive gasification process.

III. Scale-Up to 2-Liter Fermentation Unit with pH Control. Processing of the aqueous phase of the slurry and of the calcium sulfide model sorbent were scaled up to a bioreactor, which allowed better control of pH, aeration, temperature, and mixing, and also allowed us to process larger quantities of sorbent for better product evaluation.

INTRODUCTION AND BACKGROUND

Utilization of wastes from technologies that employ lime/limestone sorbents for sulfur capture is becoming more important as use of these technologies proliferates. Examples include coal gasification, and flue gas desulfurization (FGD). These processes generate lime/limestone products laden with sulfur at various oxidation states. If sulfur is completely stabilized as sulfate, the spent sorbent is ready for commercial utilization. However, the presence of reduced sulfur species requires additional processing. Thermal oxidation has been routinely employed during some processes to ensure complete oxidation of sulfur; however, this can result in some degree of SO₂ release (Abbasian et al., 1991). In addition to sulfur combustion products, another source of reduced sulfur in many wet FGD processes is thiosulfate, which is added to inhibit sulfite oxidation. Thiosulfate reacts with free radicals to form a variety of thiosalts. This strategy prevents gypsum scaling in the scrubbing system (Rochelle et al., 1986). Biooxidation might provide an inexpensive and effective method to deal with all forms of reduced sulfur. Sorbents laden with sulfide, sulfite, thiosulfate, or various polythionates could serve as a growth substrates for sulfur-oxidizing bacteria, which would convert all sulfur to sulfate.

As a group, these organisms are very versatile in oxidizing reduced sulfur compounds. Sulfide, sulfide minerals, ele-
mental sulfur, thiosulfate, polythionates, and sulfite can be utilized as growth substrates, although individual strains vary (Kelly, 1985, 1989; Kuenen, 1989). Although microbial oxidation of sulfur in slags was once considered to be a potential nuisance (Strayer and Davis, 1984), it could be turned into a beneficial process. Bioprocessing of thiosalt waste from ore milling is a process under consideration by the Canadian Centre for Mineral and Energy Technology (Silver and Dinardo, 1983; Silver, 1985).

**EXPERIMENTAL PROCEDURES**

**Cultures.** *Thiobacillus intermedium* ATCC 15466, *T. neapolitanus*, ATCC 23641 and ATCC 23639, *T. peromethylalis* ATCC 23370, *T. versutus* ATCC 25364, *Thiomicrospira pelophila* ATCC 27801, and *Thiosphaera pantotropha* ATCC 35512 were obtained from the American Type Culture Collection, Rockville, MD. An isolate, temporarily designated "TQ", was obtained from a spent FGD slurry. For shakeflask experiments, cultures were grown in 100 ml medium per 250 ml flask at ambient temperature (23°C to 25°C) on a gyratory shaker at 150 rpm. Neutrophilic strains were maintained on S6 medium (Rutchenson et al., 1965). Alkaliphilic strains were maintained on ATCC medium #472 (ATCC, 1992). Strains were stored on respective media amended with 1% CaCO₃ to prevent overacidification.

**Media.** S6 medium contains, per liter, the following: Na₂S₂O₃, 5.0 g; Na₂HPO₄, 1.2 g; K₂HPO₄, 1.8 g; NH₄Cl, 0.1 g; MgSO₄·7H₂O 0.1 g; CaCl₂·2H₂O, 0.03 g; MnSO₄·H₂O, 0.02 g; FeCl₃·6H₂O, 0.02 g; pH 6.3. We amended S6 medium with brom-cresol green (1 mg l⁻¹) in order to monitor pH changes. Brom-cresol green is blue above pH 5.3 and yellow below pH 4.3. For some experiments Na₂S₂O₃ was replaced with filter sterilized (0.2μm) Na₂SO₃, a mixture of Na₂SO₃ and Na₂S₂O₃, Na₂S₂O₅, or Na₃S₄O₆. For S6Q medium, Na₂S₂O₃ was omitted; the other ingredients in S6 were prepared in a 10X solution; 100 ml sterile 10X S6 was combined with 900 ml of the filter-sterilized (0.2μm) aqueous phase of the FGD slurry. To investigate biooxidation of the FGD solid phase, S6 medium with thiosulfate omitted was prepared with 1% solid phase, which had been washed thoroughly with distilled water to remove dissolved thiosulfate and polythionates.

Medium #472 contains, per liter, the following: Na₂S₂O₃, 5.0 g; Na₂HPO₄, 4.2 g; K₂HPO₄, 1.5 g; NH₄Cl, 1.0 g; MgSO₄·7H₂O 0.1 g; EDTA, 0.25 g; ZnSO₄, 0.11 g; CaCl₂, 28 mg; MnCl₂, 26 mg; FeSO₄, 25 mg; (NH₄)₂MoO₄, 5.5 mg; CuSO₄, 8.0 mg; CoCl₂, 8.0 mg; pH 8.5.

Unless otherwise indicated, cultures were grown in shake flasks (100 ml medium per 250 ml flask) at ambient temperature (23°C to 25°C) on a gyratory shaker at 150 rpm. A 1% inoculum of mid-exponential phase was used for all cultures. This contained in the order of 1.0 to 4.0 x10⁸
cells ml⁻¹, as determined by direct counts in a Petroff-Hauser Counting Chamber.

In some cases adipic, glutaric, or succinic acid (200 ppm) were added to S6 medium in combination with filter-sterilized (0.2μm) amendments of CaCl₂·2H₂O or MgCl₂·6H₂O. We developed a screening technique in which the effect of combinations of these parameters could be determined using test tubes that contained 10 ml medium. Test tube racks were placed on shakers at an angle of approximately 13° from horizontal and shaken at 50 rpm. Changes in the brom-cresol green indicator were monitored every 24 hr. It was first determined in shakeflask cultures, with and without dibasic acids, that indicator color adequately correlated with sulfate production.

Sorbents. Technical grade calcium sulfide (reference number 36042-2) was purchased from Pfaltz & Bauer, Inc., Waterbury CT. Fly ash was obtained from a coolside dry sorbent injection process through the help of the Illinois State Geological Survey (ISGS). Slurry from an inhibited FGD process, which employs 200 ppm DBA, was obtained from a Cilco power station. The solid and aqueous phases of the slurry were separated by filtration through Whatman #1 filter paper. The aqueous phase was divided into 1 liter aliquots and frozen at -20°C. The solid phase, primarily air dried and stored under nitrogen at ambient temperature. Separating the aqueous and solid phases allowed consistent reconstitution. Freezing the aqueous phase prevented the deterioration of dissolved sulfur species that we had experienced with previous samples, which were stored at room temperature. For preparation of S6AQ medium, the aqueous phase was thawed and filtered through Whatman #42 filter paper prior to filter sterilization.

X-Ray Diffraction (XRD) spectrograms of these three materials are presented in Figures 1 through 3. Other characteristics of the slurry are presented in Table 1.

Table 1. Characteristics of spent sorbent slurry samples

<table>
<thead>
<tr>
<th></th>
<th>Aqueous Phase</th>
<th>Solid Phase (13%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.8 - 6.0</td>
<td>CaSO₄·2H₂O 14%*</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.7 - 1.2 ppm</td>
<td>CaSO₃·H₂O 77%*</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.3 ppm</td>
<td>CaCO₃ 7%*</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0.6 - 1.6 ppm</td>
<td>Other 2%*</td>
</tr>
<tr>
<td>Thiosulfate</td>
<td>500 - 600 ppm</td>
<td>Mean Particle Size 34 μm*</td>
</tr>
<tr>
<td>DBA</td>
<td>200 ppm</td>
<td></td>
</tr>
</tbody>
</table>

* values taken from Kawatra and Eisele, 1992.

Isolation of Strain "TQ" from Spent Slurry. A 1 ml volume of slurry was incubated in S6 medium until substantial sulfate production occurred. After subculture in S6 medium,
the culture was streaked on S6 medium amended with 1.3% Noble agar. An isolated colony was picked and restreaked. An isolated colony was picked and returned to S6 liquid medium.

**Stabilization of Slurry Aqueous Phase in 2-Liter Fermentor.** Filter-sterilized slurry aqueous phase (1.5 l) was processed in a 2-liter Virtis fermentor. Temperature was maintained at 28°C, aeration at 11 min⁻¹, stirring at 200 rpm and pH between 5.5 and 6.0 with sterile 1 N NaOH or 1N HCl. A 1% inoculum of "TQ" in mid-exponential phase grown in S6 medium was used for all experiments. Sulfate and thiosulfate were monitored over time.

**Stabilization of Calcium Sulfide in 2-Liter Fermentor.** T. versutus ATCC 25364 was cultured under septic conditions in a bioreactor, consisting of a 2-liter Virtis fermentor filled with medium #472, from which thiosulfate had been omitted. Medium was placed in unit with 1 g CaS and allowed to equilibrate 1 hr before addition of 100 ml inoculum of culture from a previous set-up. Temperature was maintained at 28°C, aeration at 11 min⁻¹, stirring at 300 rpm and pH between 8.1 and 8.6 with sterile 1 N NaOH or 1N HCl. The pH was monitored using a Xerolyte electrode (Ingold, Wilmington, MA), which was resistant to sulfide. CaS (1.0 g) was added thereafter every 24 h. Sulfate and sulfide were measured daily just before CaS additions. Sulfide was also measured just after CaS additions.

**Preparation of Na₂S₃O₆** Na₂S₃O₆ was not commercially available. Therefore, we prepared this compound according to a method of Wood and Kelly (1986). Briefly, 68.5 g Na₂S₂O₃ was dissolved in 85 ml water in a stainless steel beaker. The beaker was cooled over ice to 10°C. Then 100 ml hydrogen peroxide was added dropwise so that the temperature did not exceed 20°C. The solution was cooled to 2°C and several crystals of Na₂SO₄ were added to initiate crystallization. After 2 hr at 2°C, precipitate was separated by filtration (Whatman 42), washed with 85 ml cold ethanol and discarded. The filtrate was mixed with 250 ml additional ethanol and held at 2°C for 1 hr. The precipitate that formed was separated by filtration, washed with 160 ml ethanol and discarded. The filtrate was mixed with 800 ml additional ethanol and held at 2°C for 2 hr, during which time Na₂S₃O₆ precipitated. Na₂S₃O₆ was retrieved by filtration, washed with 50 ml ethanol and then 50 ml acetone, air dried 3 hr, and stored dessicated under Ar.

**Analytical.** Sulfate was measured using a turbidometric assay (Cypionka and Pfenning, 1986). Sulfide was measured using a colorimetric assay (Trüper and Schlegel, 1964). Sulfite, thiosulfate, calcium (Calcium Hardness), and magnesium (Total Harness less Calcium Hardness) were determined using Chemetrics Titrets (Chemetrics, Inc., Calverton, VA). Cell densities were determined with a
Petroff-Hauser Counting Chamber by using phase-contrast microscopy. X-Ray Diffraction (XRD) analyses was performed at the Illinois State Geological Survey (ISGS).

RESULTS AND DISCUSSION

Selection of Alkaliphilic Strains for Processing Calcium Sulfide. Table 2 describes alkaliphilic strains that were considered. Because *Thiomicrospira pelophila* ATCC 27801 failed to grow in the absence of yeast extract, it was removed from further consideration. Although *Thiosphaera pantotropha* ATCC 35512 had been reported to grow at a pH of 10.5, we found during the first year of the project (Miller, 1993) that when pH was carefully monitored, growth did not begin until pH fell to 9.0. *Th. pantotropha* also requires slightly higher temperatures for optimum growth than the other strains. For these reasons this strain was not tested in most experiments. The isolates "L1" and "Q1" were obtained from lime contaminated soil during the first year of the project (Miller, 1993). "Q1" was rejected because, unlike *T. versutus* ATCC 25364 and "L1", "Q1" produced some elemental sulfur in addition to sulfate. ATCC 25364 and "L1" appeared to be very similar, if not identical strains. We selected ATCC 25364, because its metabolism was well characterized (Lu and Kelly, 1984; Kelly, 1985; Lu and Kelly, 1988). However, in the bioreactor, ATCC 25364 was grown in septic culture. Because we have cultured many alkaliphilic strains in this laboratory, there is a possibility that some of these strains were also present in the bioreactor.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Maximum pH</th>
<th>S(_{2})O(_3)(^-)</th>
<th>S(^-)</th>
<th>organic C requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25364</td>
<td>9.4</td>
<td>+</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>ATCC 27801</td>
<td>not tested</td>
<td>+</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>ATCC 35512</td>
<td>9.0</td>
<td>-</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>&quot;L1&quot;</td>
<td>9.4</td>
<td>-</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>&quot;Q1&quot;</td>
<td>9.0</td>
<td>+</td>
<td>+</td>
<td>no</td>
</tr>
</tbody>
</table>

Feasibility of Processing Lime from Dry Sorbent Injection. The XRD spectrogram of spent lime sorbent from coolside injection indicated that CaSO\(_3\)·\(\frac{1}{2}\)H\(_2\)O was present, although the principal component appeared to be Ca(OH)\(_2\). In a 5% slurry this material generated a pH greater than 12.0, substantially higher than the maximum pH tolerated by any of our alkaliphilic strains. The three known pathways for sulfite oxidation in thiobacilli are shown below (Kelly, 1989; Sugio et al., 1994):
(1) \[ \text{SO}_3^{-2} + \text{H}_2\text{O} \rightarrow \text{SO}_4^{-2} + 2\text{H}^+ + 2\text{e}^- \]
\[ 2\text{H}^+ + 2\text{e}^- + \frac{1}{2}\text{O}_2 \rightarrow \text{H}_2\text{O} \]

(2) \[ \text{SO}_3^{-2} + \text{AMP} \rightarrow \text{APS} + 2\text{e}^- \]
\[ \text{APS} + \text{P}_i \rightarrow \text{ADP} + \text{SO}_4^{-2} \]

(3) \[ \text{SO}_3^{-2} + \text{H}_2\text{O} + 2\text{Fe}^{3+} \rightarrow \text{SO}_4^{-2} + 2\text{H}^+ + 2\text{Fe}^{2+} \]

In the first two pathways there is no net generation of protons. Therefore biooxidation could not be used to significantly lower pH. The third pathway is a new discovery and it is still unclear whether it is a common occurrence. Due to the probable requirement for significant additions of acid, it was decided that biostabilization of sulfur occurring in this sorbent would not be economically feasible.

**Stabilization of Calcium Sulfide in 2-Liter Fermentor.** In preliminary shake flask experiments, prior to starting this project, we showed that *T. versutus* ATCC 25364 could be used to oxidize sulfide captured on limestone (Fig. 4). However, the total sulfide contents in these experiments was only 0.64%. In order to scale-up to a bioreactor with more realistic material, we used technical grade CaS, which presented a pH problem. By mixing CaCO$_3$ and CaS together in various proportions we simulated degrees of sulfidation. For a 5% slurry, pH was above the biological limit of 9.4 for even low degrees of sulfidation (Fig. 5). However, this problem could be overcome, because, unlike biooxidation of sulfite, biooxidation of sulfide acidifies the medium, according to the following reactions:

\[ \text{H}_2\text{S} + 4\text{H}_2\text{O} \rightarrow \text{SO}_4^{-2} + 10\text{H}^+ + 8\text{e}^- \]
\[ 10\text{H}^+ + 8\text{e}^- + 2\text{O}_2 \rightarrow 4\text{H}_2\text{O} + 2\text{H}^+ \]

By keeping the feed of CaS low and allowing acid produced through biooxidation to control pH, biostabilization of this material was feasible.

Figure 6 depicts additions of CaS, sulfate production, % sulfide converted to sulfate, cell numbers, suspended and dissolved sulfide, and pH for a typical experiment. A septically grown culture of *T. versutus* ATCC 25364 was used in these experiments because CaS feed was not sterilized. Sulfide was converted to sulfate as cell numbers multiplied. Sulfate accounted for approximately 87% of added sulfide, once the reactor reached stationary phase. Because sulfide was always absent prior to the daily addition, it is likely that the culture was substrate limited and that a larger amount of CaS could be processed daily if it were supplied gradually in steady increments through-out the day. We at-
tempted to feed the culture a CaS slurry using a peristaltic pump; however, we could not maintain particulates in suspension and the apparatus continually clogged. We would recommend that dry CaS be fed to the bioreactor. Perhaps this could be regulated by the pH control unit, which would add CaS instead of pumping 1 N NaOH.

Problems encountered on scale-up included: loss of H₂S, precipitation of gypsum on the ports of the aeration system; inability to keep particulates in suspension; fouling of the pH electrode by sulfide. This last problem was overcome through the use of a Xerolyte electrode, which was unaffected by sulfide. However this electrode could not be autoclaved at 121°C. Hence, another reason for septic culture.

Selection of Strains for Processing Inhibited Slurry. For processing the FGD slurry, cultures were chosen on the basis of pH optima and reported substrate utilization (Table 3). Thiosulfate is added to the sorbent slurry during sulfur dioxide capture to inhibit sulfite oxidation. It is thought that thiosulfate acts as a free radical scavenger (Rochelle et al., 1986), producing tetraionate, which then forms trithionate and thiosulfate. It is likely that all these species are present in the spent slurry. Therefore, a strain that oxidizes all species would be preferable. Another consideration is requirement for fixed carbon. Although *Thiobacillus intermedius* and *Thiobacillus perometabolis* were reported to be facultative, we found *T. intermedius* ATCC 15466 could not grow without yeast extract and *T. perometabolis* ATCC 23370 grew only marginally in the absence of yeast extract. Therefore, most experiments were performed only with *Thiobacillus neapolitanus* strains ATCC 23639 and ATCC 23641, and the new isolate "TQ". Biooxidation of thiosulfate, tetraionate, and trithionate were confirmed for these three strains (Figure 7).

Table 3. Characteristics of Named Sulfur-Oxidizing Cultures and the isolate, "TQ".

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Optimum pH</th>
<th>S²O₃⁻²</th>
<th>S₃O₆⁻²</th>
<th>S₄O₆⁻²</th>
<th>organic C requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 23639</td>
<td>6.5-6.9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>ATCC 23641</td>
<td>6.5-6.9</td>
<td>+</td>
<td></td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>ATCC 23370</td>
<td>5.5-6.0</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>facultative</td>
</tr>
<tr>
<td>ATCC 15466</td>
<td>5.5-6.0</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>&quot;TQ&quot;</td>
<td>??</td>
<td>+</td>
<td></td>
<td>+</td>
<td>no</td>
</tr>
</tbody>
</table>

Screening for Combined Effects of Dibasic Acids (DBA's), Calcium and Magnesium. Certain wet scrubbing processes employ additions of DBA mixtures to facilitate sulfur dioxide cap-
ture. Dibasic acids act as soluble pH buffers that promote formation of \( \text{HSO}_3^- \) and \( \text{Ca}^{2+} \) (Mobley and Chang, 1981). Adipic, glutaric and succinic acids are major components in these mixtures (Chang and Mobley, 1983). Previous studies have shown that thiobacilli are inhibited by many organic acids (Tuttle et al., 1977; Alexander et al., 1987). It is thought that the acidification that accompanies sulfur oxidation causes protonation of the organic acid, which is then free to move through the cytoplasmic membrane and accumulate to toxic levels intracellularly.

The combined effects of high \( \text{Ca}^{2+} \) and DBA's on biooxidation of thiosulfate were tested. As shown in Figures 8 through 10, the combined effects of high \( \text{Ca}^{2+} \) and DBA's were a problem for the two named strains. *Thiobacillus neapolitanus* ATCC 23639 was stimulated by high \( \text{Ca}^{2+} \); in the absence of DBA's sulfate production occurred most rapidly at \( \text{Ca}^{2+} \) concentrations of 1.5 ppth or higher. However, this strain was clearly inhibited by adipic acid. \( \text{Mg}^{2+} \) at 0.25 ppth allowed sulfate production at low \( \text{Ca}^{2+} \) concentrations in adipic acid after 3 days, but at higher \( \text{Ca}^{2+} \) concentrations, little sulfate production occurred. Glutaric acid combined with high \( \text{Ca}^{2+} \) concentrations also inhibited ATCC 23639, although at \( \text{Ca}^{2+} \) concentrations in the range of the slurry concentration, 0.6 to 1.2 ppth, inhibition did not occur. Inexplicably, succinate enhanced rates of acidification for this strain. *Thiobacillus neapolitanus* ATCC 23641 produced sulfate most rapidly at midrange \( \text{Ca}^{2+} \) concentrations. Adipic and glutaric acid in combination with high \( \text{Ca}^{2+} \) concentrations inhibited this strain more than succinic acid. \( \text{Mg}^{2+} \) had little if any effect. "TQ" grew well at \( \text{Ca}^{2+} \) concentrations as high as 10 ppth. Sulfate production was retarded slightly by the combined effects of high \( \text{Ca}^{2+} \) and DBA's at 2 days; however, by 3 days "TQ" produced substantial sulfate under almost all conditions. We conclude that dibasic acids partially inhibit some neutrophilic thiobacilli especially in the presence of high \( \text{Ca}^{2+} \) concentrations. However, other strains, such as "TQ" are unaffected by dibasic acids at the concentration tested. Adipic (pKa's 5.41, 4.43), glutaric (pKa's 5.41, 4.31), or succinic acid (pKa's 5.61, 4.16), while fully protonated in medium for acidophilic thiobacilli, are probably not fully protonated in media for neutrophilic strains like ours. Therefore, they are less toxic.

**Shakeflask Cultures in S6AQ Medium.** *T. neapolitanus* ATCC 23639, *T. neapolitanus* ATCC 23641, and "TQ", were grown in S6AQ medium, which was prepared using the filter-sterilized aqueous phase of the slurry instead of water (Figure 11). No thiosulfate was added to the medium. Instead, dissolved sulfur species already present in the aqueous phase provided the growth substrate. The sterile control is not depicted; however, results were similar to those obtained for the two ATCC strains. Biooxidation of thiosulfate in the aqueous
phase occurred only in cultures of "TQ." The results of the screening experiments suggested that the combined effects of high calcium concentrations and DBA's inhibited the ATCC strains. We attempted to measure the actual DBA contents of the slurry by extracting the slurry aqueous phase with diethyl ether, preparing butyl esters of any DBA's present, and quantitating these esters by using gas chromatography. However, a myriad of organic compounds appeared and it was impossible to develop an internal standard for the procedure. Adipic and succinic acid were detected, but we do not know the amounts.

**Stabilization of Slurry Aqueous Phase in 2-Liter Fermentor.** Figure 12 presents the results of these experiments. The first run contained filter-sterilized S6AQ medium. The second run contained S6AQ medium inoculated with "TQ" and the third run contained filter-sterilized aqueous phase without the S6 salts and minerals, inoculated with "TQ". Substantial white precipitation occurred in the first and second runs, due to phosphates in the S6 salts combining with soluble calcium in the aqueous phase. This was confirmed by XRD analysis. At the conclusion, Ca\(^{2+}\) was 670, 664, and 840 ppm for runs 1, 2, and 3 respectively. No difference in Ca\(^{2+}\) between runs 1 and 2 suggests that sulfate was not controlling Ca\(^{2+}\); however, as determined by XRD analyses, gypsum was present in the precipitates from the inoculated runs.

**Shakeflask Cultures on Solid Phase.** Figure 3 presents the XRD spectrogram of the slurry solid phase. This material is principally CaSO\(_3\)·\(\frac{1}{2}\)H\(_2\)O. All known strains of thiobacilli can obtain energy for growth through the oxidation of sulfite (Kelly, 1985, 1989). Although there were no previous studies in which the source of sulfite was CaSO\(_3\)·\(\frac{1}{2}\)H\(_2\)O, we reasoned that soluble sulfite in equilibrium with the mineral should provide at least a low level of usable substrate and that possibly the solid phase could be attacked directly.

Experiments were performed in S6 medium in order to determine the effect of sulfite ion on the cultures. The results are presented in Figures 13 and 14. S6 medium amended with 5 g l\(^{-1}\) Na\(_2\)S\(_2\)O\(_3\) (64 mM S) supported growth of all three cultures, as shown by cell densities (Fig. 13). In contrast, cell densities increased only slightly or not at all in S6 amended with 8.24 g l\(^{-1}\) Na\(_2\)SO\(_3\) (also 64 mM S). The isolate "TQ" grew best on this sulfite amended medium, with an approximate 10-fold increase in cell numbers. In S6 with both sulfite and thiosulfate, cell numbers were even lower than with sulfite alone, indicating growth inhibition, with the exception of a slight, temporary increase in numbers of "TQ".

Figure 14 shows that sulfate production in S6 with thiosulfate was a function of biological activity; sulfate concen-
trations in the sterile control remained negligible throughout the course of the experiment. In contrast, in S6 with sulfite, sulfate production was primarily abiological; sulfate concentrations in the sterile control were comparable with sulfate concentrations in inoculated medium. We calculated that sulfite was gone when sulfate concentrations reached 6.2 g l\(^{-1}\), which occurred at 60 hr in S6 amended with sulfite alone. In S6 with both sulfite and thiosulfate, abiological oxidation of sulfite was incomplete. Sulfate concentrations did not rise higher than 5.0 g l\(^{-1}\). These conditions were probably analogous to those found in an inhibited scrubber system, where thiosulfate inhibits sulfite oxidation by terminating free radical formation (Rochelle et al., 1986).

If we assume that in media containing both sulfite and thiosulfate, all abiotically produced sulfate is strictly a product of sulfite oxidation, not thiosulfate oxidation, then we can estimate sulfite concentrations by observing sulfate concentrations in the sterile control media. The cross hatched area in Figure 15 indicates that cells in S6 with sulfite alone were initially exposed to approximately 4.0 g l\(^{-1}\) sulfite, which then decreased to 1.0 g l\(^{-1}\) by 20 hr, and to zero by 60 hr. The line-filled area indicates that in S6 with both sulfite and thiosulfate, cells were exposed initially to approximately 4.4 g l\(^{-1}\) sulfite, which slowly decreased to 1.2 g l\(^{-1}\) by 40 hr, and then remained constant throughout the course of the experiment. Thus growth inhibition in S6 amended with both sulfite and thiosulfate was probably caused by the higher and more persistent concentration of sulfite.

Although sulfite is a key intermediate in sulfur biooxidation, and sulfite oxidation provides useful energy for the cells, recent evidence indicates that free sulfite is also toxic for thiobacilli. Sugio et al. (1987) and Hirose et al. (1991) describe the inhibition of sulfur oxidation by sulfite for *Thiobacillus ferrooxidans*. A more recent study showed that sulfite toxicity is controlled in this organism by the enzyme, sulfite:ferric ion oxidoreductase (SulfiteFOR), which oxidizes sulfite to sulfate using ferric ions as electron acceptors (Sugio et al., 1994). We have noticed for some time, that spent slurry from which "TQ" can be isolated, develops a black precipitate at the surface, when held at room temperature in a translucent container. We found this to be an unusual phenomenon because one would expect iron reduction to occur first at the bottom where conditions are anoxic. Instead, the production of ferrous iron appears to be associated with aerobic conditions, suggesting that an aerobic organism like "TQ" might be responsible. Two possible mechanisms include production of sulfide during the biooxidation of thiosulfate with subsequent abiological reduction of ferric iron, or direct reduction of ferric ion by SulfiteFOR.
(1) $S_2O_3^{-2} + H_2O \rightarrow SO_4^{-2} + S^{-2} + 2H^+$

(2) $SO_3^{-2} + H_2O + 2Fe^{+3} \rightarrow SO_4^{-2} + 2H^+ + 2Fe^{+2}$

If "TQ" has an operative SulfiteFOR, then this would explain its ability to grow in the presence of the solid phase. Therefore, experiments were performed to test the effect of ferric iron on biooxidation of the slurry solid phase. Results are depicted in Figure 16. In the absence of amendments (A), sulfate production occurred on the CaSO$_3$·$\frac{1}{2}$H$_2$O solid phase more rapidly in cultures of "TQ" than in sterile controls. Clearly "TQ" is mediating biooxidation of CaSO$_3$·$\frac{1}{2}$H$_2$O. With iron (B), there was no significant increase in the rate of sulfate production in bacterial cultures; however, the rate of abiological oxidation increased. To test the influence of iron on bacterial growth, thiosulfate was included in some experiments. Biooxidation of thiosulfate occurred rapidly (C); however iron did not enhance sulfate production, rather "TQ" was inhibited by iron amendments (D). Whether or not "TQ" has an active SulfiteFOR remains to be seen; however, amendments of ferric iron did not enhance biooxidation of the CaSO$_3$·$\frac{1}{2}$H$_2$O solid phase.

OVERALL CONCLUSIONS

1. Biooxidation of calcium sulfide could be a feasible process if pH is controlled by feed rate. Release of H$_2$S and gypsum precipitation in the ports of the aeration system are two potential problems.

2. Biooxidation of sulfite occurring in spent lime from dry sorbent injection would not be recommended due to high pH generated by this material and the relatively low quantity of sulfite present.

3. Biooxidation of sulfur species in the aqueous phase of an inhibited FGD slurry occurs rapidly due to the activity of an indigenous strain, "TQ". Biooxidation of the solid phase occurs slowly. "TQ" is not inhibited by the combined effects of dibasic acids and high calcium concentrations. "TQ" grows comparatively well on free sulfite, if concentrations are low. Although "TQ" appears to be associated with ferric iron reduction, supplying the cultures with ferric iron did not enhance biooxidation of CaSO$_3$·$\frac{1}{2}$H$_2$O. A better understanding of this system might help to improve on a process that already occurs naturally.

LITERATURE CITED


Figure 1. X-Ray Diffraction spectrogram of technical grade CaS.

Figure 2. X-Ray Diffraction spectrogram of spent lime from dry sorbent injection.
Figure 3. X-Ray Diffraction spectrogram of the solid phase of a slurry from an inhibited FGD system.

Figure 4. Sulfate production on 5% slurry of sulfided limestone (0.64% S) in cultures of T. versutus ATCC 25364 and sterile controls.

Figure 5. Effect of simulated sulfidation of limestone on pH of a 5% slurry.
Figure 6. Calcium sulfide additions, sulfate production, % conversion of sulfide to sulfate, numbers of *T. versutus* ATCC 25364, sulfide measurements, and pH in 2-liter bioreactor. Sulfate, bacterial numbers, sulfide and pH were measured prior to daily addition of CaS; an additional sulfide determination was made immediately thereafter.
Figure 7. Sulfate production on sodium thiosulfate, tetrathionate, and trithionate in cultures of *T. neapolitanus* ATCC 23639, *T. neapolitanus* ATCC 23641, the isolate "TQ", and in sterile controls.
Figure 8. Combined effects of dibasic acids, calcium and magnesium on sulfate production by *Thiobacillus neapolitanus* ATCC 23639.
Figure 9. Combined effects of dibasic acids, calcium and magnesium on sulfate production by *Thiobacillus neapolitanus* ATCC 23641.
Figure 10. Combined effects of dibasic acids, calcium and magnesium on sulfate production by the isolate "TQ".
Figure 11. Sulfate production on slurry aqueous phase in cultures of *T. neapolitanus* ATCC 23639, ATCC 23641, and the isolate "TQ".

Figure 12. Sulfate production in 3 runs of 2-liter fermentor in sterile S6AQ medium, S6AQ medium inoculated with the isolate "TQ", and unamended aqueous phase inoculated with the isolate "TQ."
Figure 13. Cell densities in S6 medium with 5 g l\(^{-1}\) Na\(_2\)S\(_2\)O\(_3\), 8.24 g l\(^{-1}\) Na\(_2\)SO\(_3\), or both.
Figure 14. Sulfate concentrations in S6 medium with 5 g l\(^{-1}\) Na\(_2\)S\(_2\)O\(_3\), 8.24 g l\(^{-1}\) Na\(_2\)SO\(_3\), or both.
Figure 15. Sulfate concentrations and estimated sulfite concentrations in sterile S6 medium amended with Na$_2$SO$_3$ (8.24 g l$^{-1}$), Na$_2$S$_2$O$_3$ (5.0 g l$^{-1}$), and both. Data for thiosulfate alone are included to demonstrate that little or no sulfate was present; we assume there was no sulfite under these conditions.

Figure 16. Effect of ferric iron amendments (100 ppm), amendments of Na$_2$S$_2$O$_3$ (5.0 g l$^{-1}$), or amendments of both on sulfate production in S6 medium with 1% washed solid phase.