0273-2289/94/4546—0925$03.00

# Large-Scale Cultivation of *Thiobacillus denitrificans* to Support Pilot and Field Tests of a Bioaugmentation Process for Microbial Oxidation of Sulfides

## Scientific Note

SALEEM HASAN,
BALASUBRAMANIAN RAJGANESH,
AND KERRY L. SUBLETTE*

*Center for Environmental Research and Technology, The University of Tulsa, 600 South College Ave., Tulsa, OK 74104*

**Index Entries:** *Thiobacillus denitrificans;* sulfides; autotroph; biomass production; flocculation.

## INTRODUCTION

Hydrogen sulfide is found to contaminate numerous industrial gas streams, such as natural gas, biogas, and petroleum refinery waste gases, and waste waters, such as sulfide-laden or "sour" water coproduced with petroleum, sulfide caustics in oil refining, and effluent from anaerobic digesters. We have previously proposed a microbial process for the removal of $H_2S$ from gas streams or soluble sulfides from sour water based on the oxidation of sulfides to sulfate by the sulfide-oxidizing bacterium *Thiobacillus denitrificans. T. denitrificans* is a strict autotroph and facultative anaerobe first described in detail by Baalsrud and Baalsrud (*1*). Sulfide, elemental sulfur, and thiosulfate may be used as energy sources with oxidation to sulfate. Under anoxic conditions, nitrate may be used as a terminal electron acceptor with reduction to elemental nitrogen.

*Author to whom all correspondence and reprint requests should be addressed.

Work in this laboratory (2–5) has demonstrated that *T. denitrificans* may be readily cultured under aerobic or anoxic conditions on $H_2S$ (g) as an energy source at pH 7.0 and 30°C. When $H_2S$ (1% $H_2S$, 5% $CO_2$, and balance $N_2$) was bubbled into cultures previously grown on thiosulfate, $H_2S$ was metabolized with no apparent lag. At loadings of 4–5 mmol $H_2S$/h-g biomass ($H_2S$/h/g biomass), $H_2S$ concentrations in the outlet gas could be reduced to undetectable levels with 1–2 s of gas–liquid contact time. Under sulfide-limiting conditions, concentrations of total sulfide in the culture media were $<1$ $\mu M$. Complete oxidation of $H_2S$ to sulfate was observed.

We have also investigated the effect of $H_2S$ loading on reactor performance (2,5). In certain experiments, the $H_2S$ feed rate was increased in steps until $H_2S$ breakthrough was obtained. At this point, the $H_2S$ feed rate exceeded the rate at which the $H_2S$ could be oxidized by the biomass. This upset condition was characterized by the accumulation of elemental sulfur and inhibitory levels of sulfide in the reactor medium. Nitrous oxide ($N_2O$) was also detected in the outlet gas and nitrite in the culture medium under anoxic conditions. This upset condition was reversible if the cultures (either aerobic or anoxic) were not exposed to the accumulated sulfide for more than 2–3 h. Maximum loading of the biomass, the specific feed rate at which $H_2S$ breakthrough occurs, was estimated to be 5.4–7.6 mmol $H_2S$/h-g biomass under anoxic conditions and 15.1–20.9 mmol $H_2S$/h-g biomass under aerobic conditions.

Further work in this laboratory has shown that heterotrophic contamination resulting from septic operation of certain *T. denitrificans* cultures had a negligible effect on $H_2S$ oxidation by the organism (4). The autotrophic medium used to grow *T. denitrificans* contained no organic components to support heterotroph growth. Apparently organic carbon was obtained from waste products of *T. denitrificans* or cell lysis. It has been demonstrated that *T. denitrificans* may be flocculated by aerobic coculture with floc-forming heterotrophs from an activated sludge system (6,7). An $H_2S$-active, gravity-settleable floc resulted that was used to scrub $H_2S$ from a gas in a continuous stirred-tank reactor with biomass recycle.

In this laboratory, we have also used flocculated *T. denitrificans* to treat sour water (8). Sour water containing up to 25 m$M$ inorganic sulfide was successfully treated in an aerobic up-flow bubble column (3.5 L) containing 4.0 g/L of flocculated *T. denitrificans*. The sulfide-laden water was supplemented with mineral nutrients only. The sulfide-active floc was shown to be stable for 9 mo of continuous operation with no external organic carbon required to support the growth of the heterotrophs. The floc exhibited excellent settling properties throughout the experiment. Retention times in the reactor varied from 1.2 to 1.8 h. However, molar sulfide feed rate (mmol/h sulfide) was more important in determining the capacity of the reactor for sulfide oxidation than either the hydraulic retention time or the influent sulfide concentration (mmol/L). At a biomass concentration of about 4 g/L, the column could be operated at a molar sulfide feed rate of 12.7–15.4 mmol/h without upset.

A sulfide-tolerant strain of *T. denitrificans* (strain F) has been isolated in this laboratory by enrichment. Wild-type *T. denitrificans* is inhibited by sulfide concentrations of 0.1–0.2 m*M*. However, strain F is tolerant of sulfide concentrations in excess of 2.5 m*M* (*9*).

The ability of *T. denitrificans* to deodorize and detoxify oil-field-produced water-containing sulfides was evaluated under simulated field conditions (*10*). *T. denitrificans* strain F was used to remove inorganic sulfide from a synthetic sour brine containing 4000 mg/L total dissolved solids (TDS) and 3.1 m*M* sulfide. The sour brine was treated continuously in a rectangular plug-flow reactor that approximated the scaled dimensions of an existing field detention pond (79 m × 34 m × 0.3 – 6 m deep). The head space of the reactor was purged with $N_2$ in order to capture $H_2S$ off-gases in a zinc-acetate trap. Brine was fed to the reactor continuously for 90 d at rates corresponding to residence times of 0.17–6 d. Temperature and pH ranged from 22–40.5°C and 7.5–8.8, respectively. The start-up biomass concentration was approx 100 mg/L (by dry wt). No additional *T. denitrificans* biomass was added to the reactor after start-up. At residence times of 0.3 d and greater, inorganic sulfide was undetectable in the effluent. No $H_2S$ was detected in the outlet gas or the zinc-acetate trap. Approximately 80% of the sulfide feed was oxidized to sulfate and removed from the reactor in the liquid effluent. The remainder was partially oxidized to elemental sulfur, which was retained in the reactor.

The further development of the proposed microbial process for sulfide oxidation has required scale-up of the process for both treatment of sour gases and sour water. This has required large-scale cultivation of *T. denitrificans* to produce working cultures for pilot-scale work or inocula for full-scale field tests. This article describes the aerobic cultivation of flocculated *T. denitrificans* strain F in 1000-gal (3785 L) batches to produce sulfide-oxidizing biomass for these purposes.

## MATERIALS AND METHODS

### Organism and Culture

Strain F of *T. denitrificans* was isolated by enrichment from pure cultures of the wild-type (ATCC 23642) as previously described (*9*). Stock cultures of *T. denitrificans* strain F were grown anoxically in a thiosulfate medium in 10-mL culture tubes at 30°C (*2*). In this medium, thiosulfate (10 g/L as $Na_2S_2O_3$) is the energy source, nitrate (5 g/L as $KNO_3$) the terminal electron acceptor, bicarbonate (1.0 g/L as $NaHCO_3$) the carbon source, and ammonium ion (0.5 g/L as $NH_4Cl$) the source of reduced nitrogen. The medium also contained a phosphate buffer (1.2 g/L $Na_2HPO_4$ and 1.8 g/L $KH_2PO_4$), $MgSO_4 \cdot 7H_2O$ (0.4 g/L), $CaCl_2 \cdot 2H_2O$ (0.03 g/L), $FeCl_3 \cdot 6H_2O$ (0.033 g/L), $MnSO_4$ (0.02 g/L), and trace elements.

*T. denitrificans* strain F was immobilized by aerobic coculture with floc-forming heterotrophs from a local refinery activated sludge system in the thiosulfate medium (without nitrate) as described previously (*6*). The culture (1.5 L) was maintained at 30°C and pH 7.0, and was sparged with 0.3 L/min of 5% $CO_2$, balance air. Carbon dioxide in the air ensured that the culture did not become carbon-limiting.

When a sulfide-active, gravity-settleable floc was obtained, this culture was used to inoculate 50 gal (189 L) of thiosulfide medium (without nitrate) in a jacketed stainless-steel, stirred-tank reactor. The culture was again maintained at 30°C by circulating water at this temperature through the jacket from a Neslab Model HX-300 refrigerated recirculator. The pH was monitored and maintained at 7.0 ± 0.05 by a Cole-Parmer (Chicago, IL) Model 5651-50 pH meter/controller that activated a Cole-Parmer Chem-Feed pump to deliver 50% NaOH (Kjeldahl-N grade, Ricca Chemicals, Arlington, TX) as needed. (Oxidation of thiosulfate by *T. denitrificans* is acid-producing.) The culture was aerated with line air from an in-house compressor at 3–5 scfm (standard cubic feet/min) or 85–142 L/min. The reactor also received a gas feed of pure $CO_2$ from a compressed gas tank at a rate of about 5% of the aeration rate. The culture was agitated by means of a single 15-cm, six-bladed, disk-type impeller at 30–50 rpm. When thiosulfate was depleted (2–3 d), the contents of this reactor were used to inoculate the 1000-gal reactor described below.

The 1000-gal reactor used for *T. denitrificans* biomass production was a stainless-steel milk-holding tank manufactured by the Paul Meuller Co. (Springfield, MO). The tank was horizontal and semicylindrical, 170 cm deep, and 660 cm long on the inside. The tank was jacketed with cooling/heating coils running lengthwise in the jacket annular space. A 2-hp variable-speed DC motor and gearbox were mounted on a platform that bridged the center of the vessel. The motor drove a paddle-type stirrer that was 81 cm in diameter and 12 cm wide. The agitation rate was 50 rpm. On either side of the stirrer platform were stainless-steel lids that completely closed the top of the vessel. The tank was modified by fitting with stainless-steel baffles, each 1/10 of the major or minor dimensions of the tank, and a sparger. The sparger was fabricated from 1-in. stainless-steel tubing in a U-shape fed with air at the bottom of the U through a 1-in. stainless-steel tube that extended through the wall of the vessel at the center and bottom. The sparger was centered under the stirrer with the branches of the U equal in length to the stirrer diameter. The U branches had equally spaced 1/8-in. holes drilled on the bottom such that the total hole area on each branch was two times the cross-sectional area of the tube.

Air was fed to the reactor using both a Fugi Model VFC504A-7W ring compressor and line air from an in-house compressor. About 30 scfm of air were supplied by the blower to the sparging system described above. Air from the blower was cooled with a Speedaire Model 5Z267 after-cooler or heat-exchanger using house water at 15°C. Line air was introduced

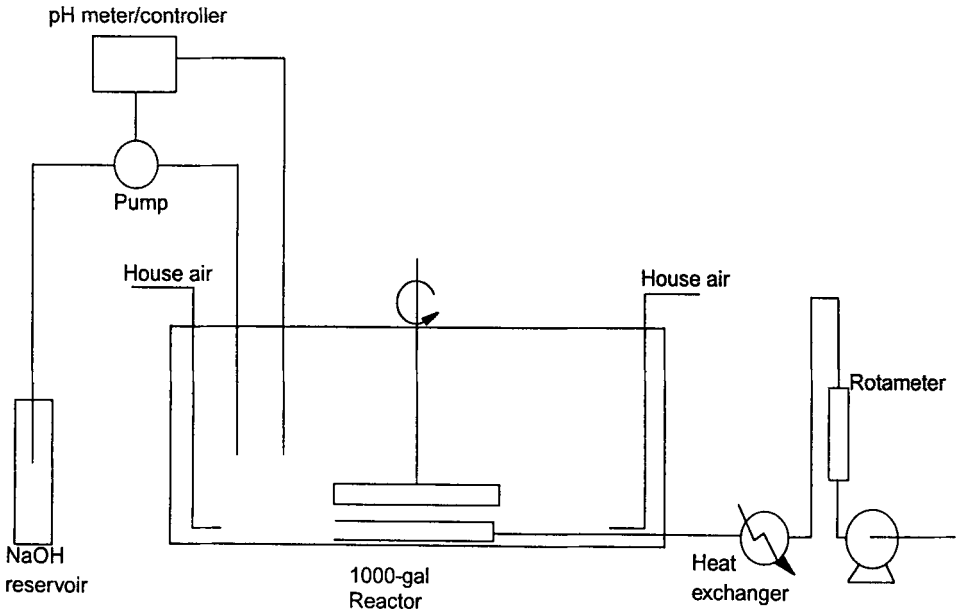


Fig. 1. Schematic diagram of flocculated *T. denitrificans* culturing system.

into the reactor at each end with two supplemental spargers, which consisted of 1/2-in. stainless-steel tubes bent at one end to produce a 1-ft. section that was perforated with 1/8-in. holes. An additional 15–20 scfm could be provided to the reactor in this manner.

Temperature control in the 1000-gal tank was achieved by circulating water from the Neslab refrigerated recirculator referenced above, through the jacket coils. Some heating could also be obtained as needed by reducing the cooling water flow rate to the blower after-cooler, thereby increasing the temperature of the air. The temperature was maintained at 30 ± 1°C. The pH was maintained at 7.0 ± 0.05 in the same manner as described above for the 50-gal culture. A schematic diagram of the biomass-culturing system is shown in Fig. 1.

Each batch of *T. denitrificans* biomass was produced as follows: The 1000-gal tank was filled with tap water and agitated with the stirrer. Components of thiosulfate medium (without nitrate) were then added and allowed to dissolve one at a time. The thiosulfate medium used in the 1000-gal reactor was identical to that described above, except that the $NaHCO_3$ concentration at this scale was 3.0 g/L. The smaller-volume cultures described above used $CO_2$ as a source of carbon. At the 1000-gal scale, this was prohibitively expensive; therefore, $NaHCO_3$ was used as the sole carbon source.

The sources and grades of each component of the medium are given in Table 1. The pH was initially adjusted to 7.0 with 85% $H_3PO_4$, industrial grade (Delta Distributors, Dallas, TX). When the temperature reached 30°C, the culture was inoculated.

Table 1
Sources and Grades of Medium Components
Used to Grow *T. denitrificans* Strain F in the 1000-Gal Fermenter

| Components | Grade | Source | Quantities |
|---|---|---|---|
| $Na_2HPO_4$ | Food grade | Monsanto St. Louis, MO | 50-lb bags (22.7 kg) |
| $KH_2PO_4$ | Food grade | Monsanto St. Louis, MO | 50-lb bags (22.7 kg) |
| $NH_4Cl$ | Technical, treated | Dallas Group of America Liberty Corner, NJ | 50-lb bags (22.7 kg) |
| $MgSO_4 \cdot 7H_2O$ | Food grade | PQ Corporation Valley Forge, PA | 50-lb bags (22.7 kg) |
| $Na_2S_2O_3$ | Technical | General Chemical Parsippany, NJ | 50-lb bags (22.7 kg) |
| $NaHCO_3$ | Food grade | Church & Dwight Co. Princeton, NJ | 100-lb bags (45.4 kg) |
| $MnSO_4$ | FCC grade | Tomco Chemicals Tulsa, OK | 5-lb buckets (2.3 kg) |
| $FeCl_3 \cdot 6H_2O$ | AR | Mallinckrodt Specialty Chemicals Paris, KY | 5-lb bottles (2.3 kg) |
| $CaCl_2 \cdot 2H_2O$ | AR | Mallinckrodt Specialty Chemicals Paris, KY | 5-lb bottles (2.3 kg) |

The first inoculum used was produced in the 50-gal stirred-tank reactor described above. Subsequent inocula consisted of a fraction of the biomass produced in the previous batch. Following inoculation, each batch was maintained under conditions described above until thiosulfate was depleted. The medium was thiosulfate-limiting. When the thiosulfate was completely utilized, the contents of the 1000-gal tank were pumped to a 600-gallon open-top conical-bottom tank (in two batches) to allow the flocculated biomass to settle under gravity for about 2 h. A concentrated suspension of biomass was then drawn from the bottom of the tank. On the average, 10–15 gal of concentrated suspension were obtained. About 20% was used to inoculate the next batch. The remainder was stored at 4°C in 60-gal polypropylene barrels in a walk-in cold room.

## Analytical

Thiosulfate was determined by titration with standard iodine solution with a starch indicator (*11*). Ammonium ion was determined by the Nesslers method without distillation using Hach Chemical Co. (Loveland, CO) reagents (*12*).

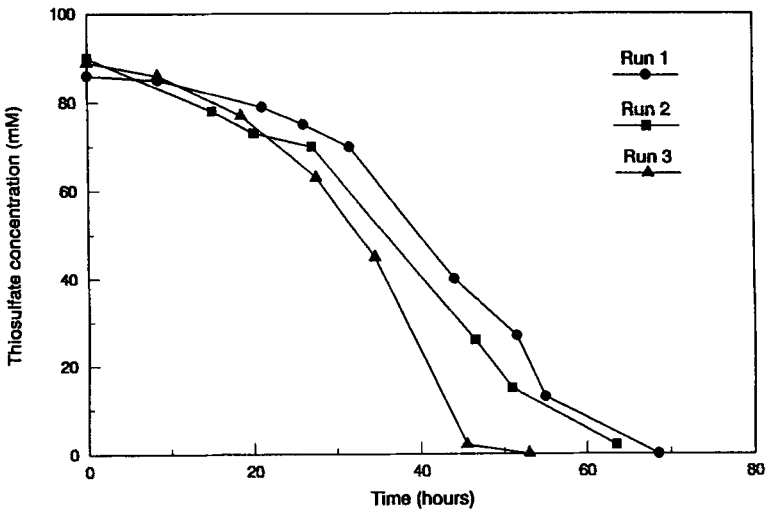


Fig. 2. Depletion of thiosulfate in typical batch runs in 1000-gal fermenter for biomass production. (Two samples per data point.)

Mixed-liquor suspended solids (MLSS) were determined gravimetrically by filtering known volumes of culture medium through tared Whatman GF/C glass-fiber filters (*12*). Viability and relative activity of refrigerated suspensions of flocculated *T. denitrificans* biomass were determined by inoculating 2.0 L of thiosulfate medium with fixed volumes of biomass suspensions in a Marubishi MD 300 fermenter. Cultures were maintained at 30°C and pH 7.0, and sparged with 5% $CO_2$ in air at 0.3 L/min. Relative activity was determined by the lag time and rate of thiosulfate depletion.

## RESULTS

Approximately 60 1000-gal batches of flocculated *T. denitrificans* strain F biomass have been produced to date in the manner described above. About half of this biomass was used to inoculate a sour-water retention pond at a petroleum production site in a field test where sulfides were successfully controlled for 6 mo. This field test will be described in a future publication (*13*). The remainder was successfully used in the 1000-gal tank to treat refinery-spent sulfidic caustic after 3 mo in storage at 4°C. Typically 48–72 h were required for each batch from the time of inoculation until thiosulfate was depleted. Typical thiosulfate depletion curves are given in Fig. 2. The corresponding increases in the optical density (460 mm) are given in Fig. 3. Harvesting of biomass required about 4 h on the average and typically controlled the turnaround time of the reactor, since the reactor could be replenished with fresh medium in less time than required to recover a concentrated inoculum from the previous batch.

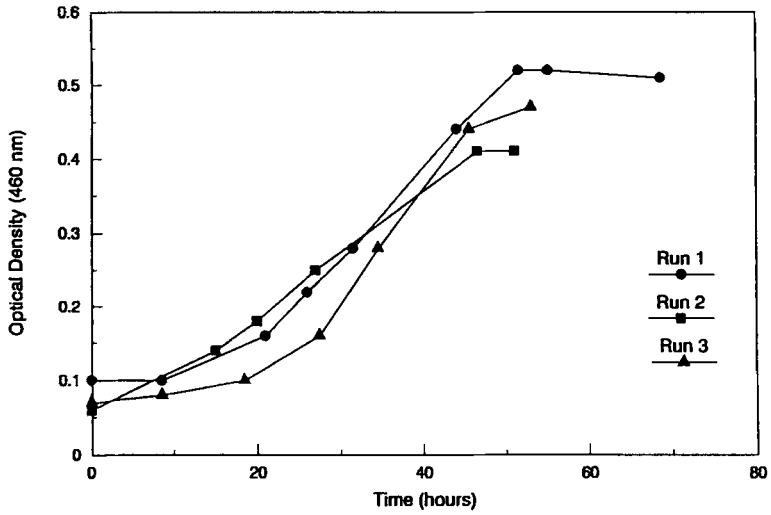


Fig. 3. Optical density of medium in typical batch runs in 1000-gal fermenter for biomass production. (One sample per data point.)

The average MLSS concentration following thiosulfate depletion was 0.47 g/L. The range was 0.33–0.58 g/L. Average recovery of flocculated biomass in the conical settling tank (2 h settling time) was 45% (range 31–55%). Increasing the settling time beyond 2 h did not significantly increase biomass recovery. In terms of dry weight of biomass, the average recovery was 0.21 g/L (range 0.15–0.26). As noted above, 20% of the biomass recovered in each batch was used to inoculate the next batch; therefore, the net yield per batch averaged 0.17 g/L or approx 640 g/batch on a dry-wt basis.

The cost per batch of *T. denitrificans* strain F biomass in terms of the cost of nutrients and NaOH for pH control is detailed in Table 2. As seen in Table 2, the nutrient cost per batch was $208. An average of 700 kW-h were required for the blower, agitator, and recirculator/batch. At $0.08/kW h, the estimated utilities costs/batch were $56. The cost/g dry wt of biomass was, therefore, $0.413/g.

*T. denitrificans* biomass remained highly active after long storage at 4°C. Inoculation of thiosulfate medium in lab-scale fermenters with biomass as old as 3 mo resulted in rapid growth of the organism and depletion of thiosulfate.

## DISCUSSION

As noted above, *T. denitrificans* strain F may be flocculated by coculture with floc-forming heterotrophs from an activated sludge system. This initial flocculation was conducted at the 1–2-L scale. Prior to the work described in this article, it was not known whether flocculated *T. denitrifi-*

Table 2
Approximate Costs of Nutrients for Growth of 1000-Gal Batch of *T. denitrificans* Strain F

| Component | Quantity | Unit Price | Cost/1000 gal |
|---|---|---|---|
| $Na_2HPO_4$ | 4.54 kg | 4.25/kg | $19.30 |
| $KH_2PO_4$ | 6.82 kg | 6.89/kg | 46.99 |
| $NH_4Cl$ | 1.89 kg | 3.28/kg | 6.20 |
| $MgSO_4 \cdot 7H_2O$ | 1.51 kg | 2.46/kg | 3.71 |
| $NaHCO_3$ | 10.9 kg | 1.19/kg | 12.97 |
| $Na_2S_2O_3$ | 37.9 kg | 2.05/kg | 77.70 |
| $FeCl_3 \cdot 6H_2O$ | 0.125 kg | 18.62/kg | 2.33 |
| $CaCl_2 \cdot 2H_2O$ | 0.114 kg | 17.25/kg | 1.97 |
| $MnSO_4$ | 0.075 kg | 24.69/kg | 1.85 |
| NaOH (50%) | 3 gal | 11.71/gal | 35.13 |
| | | Total | $208.15 |

*cans* from a laboratory-scale culture would remain flocculated when the culture was used to inoculate a much larger reactor or if these larger cultures could subsequently be used to inoculate other commercial-scale systems. Simply stated, with no further introduction of floc-forming heterotrophs, it was not known whether *T. denitrificans* would remain flocculated with successive subculturing.

The results presented above indicate that *T. denitrificans* will remain flocculated. However, only 45% of the biomass produced in each batch operation of the 1000-gal reactor was sufficiently flocculated to be recovered by gravity separation in a reasonable time. One possible explanation was the shear sensitivity of the floc. At the laboratory scale, *T. denitrificans* floc have been shown to be shear-sensitive. Agitator tip speeds in excess of 68 cm/s with flat-bladed disk-type impellers have been shown to decrease average floc size significantly (*7*). At 50 rpm, the paddle-tip speed in the 1000-gal reactor was over 200 cm/s. In two batches, agitation with the paddle stirrer was not used. Mixing was, therefore, provided by aeration only. Biomass recovery was not significantly improved by the absence of shear from the paddle.

The thiosulfate utilization rate in these cultures was relatively constant after a lag period and independent of the biomass concentration (Fig. 2). In other words, the rate of growth and thiosulfate depletion was dependent on the rate of oxygen transfer. Thiosulfate utilization was significantly slower in the two batches referenced above without agitation. Another possible explanation of the low recovery of flocculated biomass was the oxygen-limiting conditions. Oxygen limitation of growth favors planktonic cells where the added mass-transfer resistance of diffusion of oxygen into the floc is not present.

## CONCLUSION

*Thiobacillus denitrificans* strain F has been successfully cultivated at a 1000-gal scale in a flocculated form that could be recovered by gravity settling. Repeated subculturing at this scale did not result in loss of flocculation, although only 45% of the total biomass in each batch production run was sufficiently flocculated to be recovered by gravity settling. Production costs for nutrients and utilities averaged $0.413/g dry wt of biomass recovered. Future work will focus on improving biomass recovery.

## ACKNOWLEDGMENT

This work was funded by ABB Environmental Services (Portland, ME), Amoco Production Co. (Tulsa, OK), and Conoco, Inc. (Ponca City, OK).

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