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Immobilization of *Thiobacillus Denitrificans* for the Oxidation of Hydrogen Sulfide in Sour Water

Scientific Note

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ABSTRACT

It has been demonstrated that viable cells of the chemoautotroph and facultative anaerobe *Thiobacillus denitrificans* can be used to remove soluble sulfides from sour water with anaerobic oxidation to sulfate. A packed bed of immobilized *T. denitrificans* was capable of removing all traces of sulfide from an aqueous feed stream containing 26 ppm sulfide during a continuous 12 day test. Calcium carbonate provided buffering capacity and generated Ca^{+2} internal to alginate beads to maintain structural integrity during extended periods of operation.

Index Entries: Hydrogen sulfide; *Thiobacillus denitrificans;* sour water; immobilization; packed-bed.

INTRODUCTION

Soluble sulfides (H_2S, HS^-, S^{-2}) are often found to contaminate water co-produced with petroleum, anaerobic digester effluents, and various industrial wastewaters. The source of these sulfides is generally the reducion of sulfates by sulfate reducing bacteria (SRB). These bacteria are strict anaerobes that utilize a rather limited number of organic compounds as a source of carbon and energy such as pyruvate, lactate, acetate, and ethanol. However, these compounds are endproducts of the metabolism of fermentive heterotrophs and are readily available in a consortium of

bacteria in an anaerobic environment. Therefore, sulfate reducing bacteria are ubiquitous to virtually any anaerobic environment conducive to microbial growth (*1*).

The toxicity and corrosive properties of sulfides dictate stringent control of their release into the environment and contact with iron and steel as in tanks, pipelines, valves, and pumps. The control of sulfide contamination may be approached in two ways. First, sulfide production may be reduced by inhibiting the growth of sulfate reducing bacteria. For example, in the secondary production of petroleum, water used in flooding operations is treated with a biocide to control SRB growth in the injection well, reservoir, and piping. Since SRBs are strict anaerobes, aeration of flooding water can also serve to inhibit sulfide production. These measures are of limited effectiveness, however, because sulfate reducing bacteria are sessile bacteria. That is to say, they are generally found attached to a solid surface entrapped with other bacteria in polysaccharide gels produced by "slime-forming" bacteria. Within these gels, the SRBs find themselves in a somewhat protected environment that biocides and oxygen do not effectively penetrate. Of course, biocide treatment is inappropriate in a situation in which the growth of other microorganisms is to be encouraged, such as in an anaerobic digester. In an anaerobic digester, the growth of SRBs can sometimes be inhibited by fostering competition between the sulfate reducing bacteria and other heterotrophs for the carbon and energy sources favored by the SRBs. The success of this approach is, however, dependent on the type of waste being treated (*2–4*).

If sulfide production cannot be prevented, sour water may be treated by a number of physiochemical methods. One of the more common methods is to strip sulfide-laden waters under acidic conditions with steam, flue gas, or methane in a packed or plate-type column. In the case of steam or flue gases, the overhead vapors are condensed and the noncondensables (including H_2S) are incinerated. In the case of methane, the noncondensables are typically sent to an amine system and the methane recycled. Hydrogen sulfide recovered from the methane stripping gas is generally incinerated. Each of these processes converts a water pollution problem into an air pollultion problem in that the combustion of H_2S produces sulfur dioxide, a regulated pollutant (*5*).

Sulfides may also be oxidized to less objectionable thiosulfates by air oxidation at 190°F. However, elevated pressures (50–100 psig) are required, and the thiosulfates possess considerable chemical and biochemical oxygen demand (*5*).

Last, small amounts of sulfides can be precipitated with copper (II) or zine (II) salts. The resulting insoluble sulfides, however, are considered a hazardous waste in that H_2S will be evolved if the precipitates are exposed to acidic conditions (*5*).

New technology is needed in the control of H_2S production by sulfate reducing bacteria and the treatment of sulfide-laden waters to address the

limitations inherent in conventional methods described above. This paper describes a process that utilizes sulfide-tolerant strains of the autotroph *Thiobacillus denitrificans* coimmobilized with calcium carbonate (for pH control) to oxidize sulfides in water to sulfate. The sulfate-sulfur is fully oxidized, therefore, sulfate has no associated chemical oxygen demand or biological oxygen demand. If necessary, sulfates can be readily precipitated with lime to yield a nonhazardous waste.

Thiobacillus denitrificans is a strict autotroph and facultative anaerobe first described in detail by Baalsrud and Baalsrud (*6*). Under anaerobic conditions, nitrate may be used as a terminal electron acceptor with reduction to elemental nitrogen. Thiosulfate, elemental sulfur, and sulfide may be used as energy sources with oxidation to sulfate; however, sulfide is an inhibitory substrate. Sublette and Sylvester (*7–10*) have demonstrated that *T. denitrificans* may be readily cultured aerobically or anaerobically in batch or continuous reactors on H_2S (g) under sulfide-limiting conditions. Complete removal of H_2S from feed gases was observed with complete oxidation of H_2S to sulfate that accumulated in the culture media. Stable reactor operation was demonstrated for up to 7 d in batch cultures and 26 d in continuous cultures at specific productivities as high as 4–5 mmoles H_2S oxidized/h-g biomass. The stoichiometry of the oxidation of H_2S by *T. denitrificans*, as reported by Sublette and Sylvester, is given in Table 1. Maximum specific productivity of the biomass was estimated to be 5.4–7.7 mmoles H_2S/h-g biomass under anaerobic conditions and 15.1–20.9 mmoles H_2S/h-g biomass under aerobic conditions. Indicators of reactor upset were determined and recovery from upset demonstrated. Sublette and Sylvester also reported that heterotrophic contamination of *T. denitrificans* cultures had no effect on H_2S oxidation.

MATERIALS AND METHODS

Organism and Culture

Sulfide-tolerant strains of *T. denitrificans* were isolated by enrichment from cultures of the wild-type (ATCC 23642). The strain employed in this work was designated Strain F. Strain F grows on thiosulfate in the presence of 1000 μM sulfide (as Na_2S) at rates comparable to controls without sulfide. The wild-type exhibits inhibition at sulfide concentrations of 100–200 μM (*7*).

Strain F was cultured anaerobically for stocks in an autotrophic medium with thiosulfate as the sole energy source, as described previously (*7*). This medium uses nitrate as a terminal electron acceptor, bicarbonate as a carbon source and ammonium ion as a source of reduced nitrogen. Cells for immobilization were grown nonaseptically and anaerobically on thiosulfate at 30°C and pH 7.0 in a B. Braun Biostat M fermenter (culture volume 1.4 l), as described previously (*7,10*).

Table 1
Stoichiometry of H_2S Oxidation by *Thiobacillus denitrificans*

Reactor type	Electron acceptor	NO_3^-/H_2S, mole/mole	O_2/H_2S, mole/mole	SO_4^{-2}/H_2S, mole/mole
Batch	NO_3^-	1.36		1.04
CSTR $D=0.029\ h^{-1}$	NO_3^-	1.30		1.03
CSTR $D=0.058\ h^{-1}$	NO_3^-	1.19		1.00
Batch	O_2		1.81	0.99
CSTR $D=0.030\ h^{-1}$	O_2			1.06
CSTR $D=0.053\ h^{-1}$	O_2			1.04

Reactor type	Electron acceptor	NH_4^+/H_2S, mole/mole	OH^-/H_2S,[a] eq/mole	Biomass/H_2S, g/mole
Batch	NO_3^-	0.12	1.60	12.1
CSTR $D=0.029\ h^{-1}$	NO_3^-	0.09	1.37	9.3
CSTR $D=0.058\ h^{-1}$	NO_3^-	0.10	1.24	12.9
Batch	O_2	0.10	1.75	4.5
CSTR $D=0.030\ h^{-1}$	O_2	0.11	2.38	8.1
CSTR $D=0.053\ h^{-1}$	O_2	0.12	1.77	7.9

[a]The consumption of hydroxide equivalents was required to maintain an optimum pH of 7.0.

Immobilication of *T. denitrificans* Strain F in Calcium Alginate

T. denitrificans Strain F was coimmobilized with $CaCO_3$ by entrapment in calcium alginate beads. The $CaCO_3$ had three functions. First, as noted in Table 1, the oxidation of sulfide by *T. denitrificans* is acid producing. It has been shown in our laboratory that addition of powdered $CaCO_3$ to stirred tank cultures of *T. denitrificans* growing on H_2S controlled the pH in a range suitable for growth of the organism (pH 6.8–7.2). Therefore, $CaCO_3$ acts as a buffer, neutralizing the acid byproduct of sulfide oxidation, as shown by

$$H^+(aq) + CaCO_3(s) \rightleftharpoons Ca^{+2}(aq) + HCO_3^-(aq) \overset{H^+(aq)}{\rightleftharpoons}$$

$$H_2CO_3(aq) \rightleftharpoons H_2O + CO_2(g)$$

This reaction also produces bicarbonate and carbon dioxide to support the growth of *T. denitrificans*. Last, this reaction generates Ca^{+2} internal to the alginate bead. In general, calcium alginate beads must be utilized in an environment containing sufficient Ca^{+2} ion to prevent the leaching of Ca^{+2} ion from the bead. Since Ca^{+2} ion crosslinks the alginate chains, loss of Ca^{+2} destroys the immobilization matrix. This requirement has placed limitations on the use of these gels in a continuous process. However, if Ca^{+2} can be produced internal to the bead, there is no requirement to supplement the influent of a continuous process with Ca^{+2} ion (*11*).

T. denitrificans and $CaCO_3$ were coimmobilized in alginate beads as follows. Cells were grown as described above to a cell density of approximately 10^9 cells/mL (OD_{460} of 0.9–1.0). When this cell density had been achieved, cells were harvested by centrifugation at 4900×g for 10 min at 25°C and resuspended in 100 mL of fresh thiosulfate medium. The concentrated cell suspension was then mixed with an equal volume of 4 wt% sodium alginate (low viscosity, *Macrosystis pyrifera*, Sigma Chemical Co.) and sufficient powdered $CaCO_3$ added to bring the concentration to 30 g/L. The resulting mixture was then pumped through a vertically placed Pasteur pipet (orifice diameter 1.5 mm) at a rate sufficient to produce 50 drops/min. The drops thus formed fell into 2% $CaCl_2$, which was stirred with a magnetic stirrer. The drops hardened instantly to produce beads approximately 3 mm in diameter. Beads were allowed to harden in 2% $CaCl_2$ at 4°C overnight.

Gel beads with immobilized *T. denitrificans* and $CaCO_3$ were washed with distilled water and resuspended in fresh thiosulfate medium in the Biostat M fermenter. The suspension was agitated at 150 rpm and sparged with 30 mL/min of 5% CO_2 in nitrogen. After 2 d of incubation at 30°C the thiosulfate (originally 10 g/L $Na_2S_2O_3$) in the medium was depleted, indicating that the immobilized biomass was active. Microscopic examination of bead material indicated large numbers of gram-negative rods indicative of *T. denitrificans*. At this time beads were collected by gravity settling and placed in 2% $CaCl_2$ for 10 min. Approximately 20 mL of beads were washed free of $CaCl_2$ and placed in a 17 cm Plexiglass column with an inside diameter of 1.3 cm. Each end of the column was plugged with glass wool. The total height of bead packing was 12.5 cm. A control column was prepared, identical in all respects to the test column, with the exception that the beads in the control column contained only $CaCO_3$ and no biomass.

Operation of Biofilter

Thiosulfate has been shown to be inhibitory to H_2S oxidation by *T. denitrificans* (*7*). In order to remove the last traces of thiosulfate from the packed bed reactor or "biofilter," both the test and control columns were subjected to a feed of 60 mL/h of thiosulfate medium minus thiosulfate at 23°C for 24 h. The effluents from the columns were discarded and not

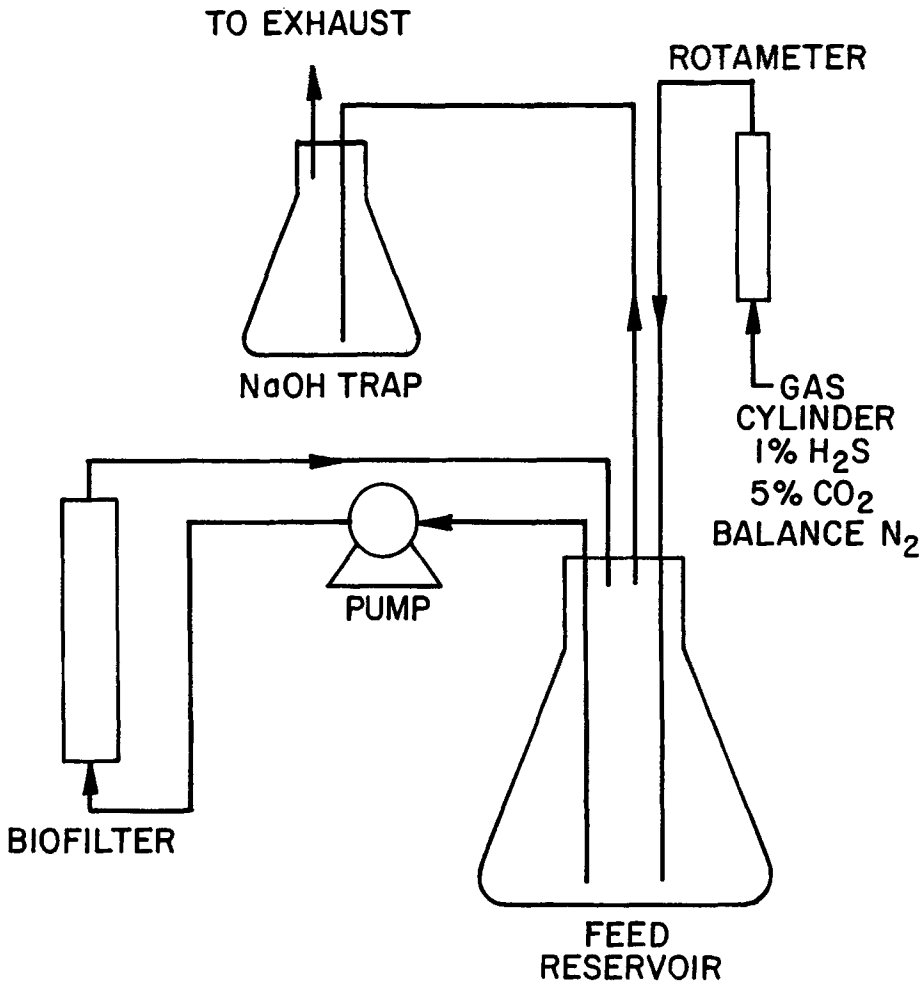

Fig. 1. Schematic diagram of biofilter test system.

recirculated. Both columns were operated nonaseptically in an upflow mode with medium pumped by Masterflex peristaltic pumps.

At the end of this time, the feed to both columns was changed to medium minus thiosulfate, which was continually sparged with 35 mL/min of 1% H_2S, 5% CO_2, and balance nitrogen. The feed rate to both the test and control columns was 60 mL/h and each column was operated at 23°C. The effluent from each column was returned to the appropriate feed reservoir. The continuous sparging of the feed reservoirs with 1% H_2S gave a constant total sulfide concentration in the feeds of approximately 800 μM, or 26 ppm. Recirculation of the feed allowed sulfate to accumulate and be more accurately quantitated. A schematic diagram of the test system is given in Fig. 1. Both the test and control columns were operated for 13 d with daily sampling of the feed reservoirs and column effluents.

Analytical

Nitrate was determined by the cadmium reduction method using gentistic acid in place of *N*-(1-naphthyl)-ethylenediamine in the color development step (*12*). Nitrite was determined by the diazotization method using chromatropic acid and sulfanilic acid (*12*). Sulfate was determined turbidometrically (*12*). All reagents for the above analyses were purchased from Hach Chemical Co. (Loveland, CO).

Thiosulfate was determined by titration with a standard I_2 solution and a starch indicator (*13*). Total sulfide (H_2S, HS^-, and S^{-2}) was determined by the methylene blue method (*14*). Sulfide was precipitated with zinc acetate and stored at 4°C as a suspension of zinc sulfide until analysis.

RESULTS AND DISCUSSION

After 24 h of operation with a sour water feed, alginate beads in the control column began to break down badly. Apparently, Ca^{+2} was continually being leached from these beads, resulting in the solubilization of the alginate. At this time, the control column was repacked with glass beads of comparable size to the biocatalyst beads in the test column so as to give the feed approximately the same residence time in the control and test columns.

Baalsrud and Baalsrud (*6*) have shown that in pure culture *T. denitrificans* requires 8.3 μg/mL Fe^{+3} for growth. The medium used as a feed to the biofilter test column and control column contained $FeCl_3$ (0.02 g/L) as a source of iron. However, when the H_2S purge of this medium was begun, a black precipitate of iron sulfide was obtained as expected. This precipitate was filtered out by the column packings during the first 24–48 h of operation. This iron sulfide precipitate in the column was therefore the only source of iron available to the biomass. As will be shown subsequently, these precipitates provided a sufficient source of iron to maintain the viability of the biomass.

No sulfide could be detected in the effluent of the test column (with biomass) either by colorimetric analysis or by odor during the entire course of the experiment. Correspondingly, a total of 13.2 mmoles of sulfate accumulated in the feed reservoir as sulfide was oxidized in the column (Fig. 2). The cumulative sulfide feed to the column during the course of the experiment was 15.0 mmoles. Some sulfate may have precipitated in the beads as $CaSO_4$. Nitrate was utilized during the course of sulfide oxidation as indicated by a decrease in the nitrate concentration in the feed reservoir (Fig. 3), the accumulation of nitrite in the feed reservoir (Fig. 4) and the appearance of gas bubbles (presumably, nitrogen or nitrous oxide) in the test column and the return lines to the feed reservoir. The pH of the column effluent was observed to fall from 6.9 to 6.55 during the course of

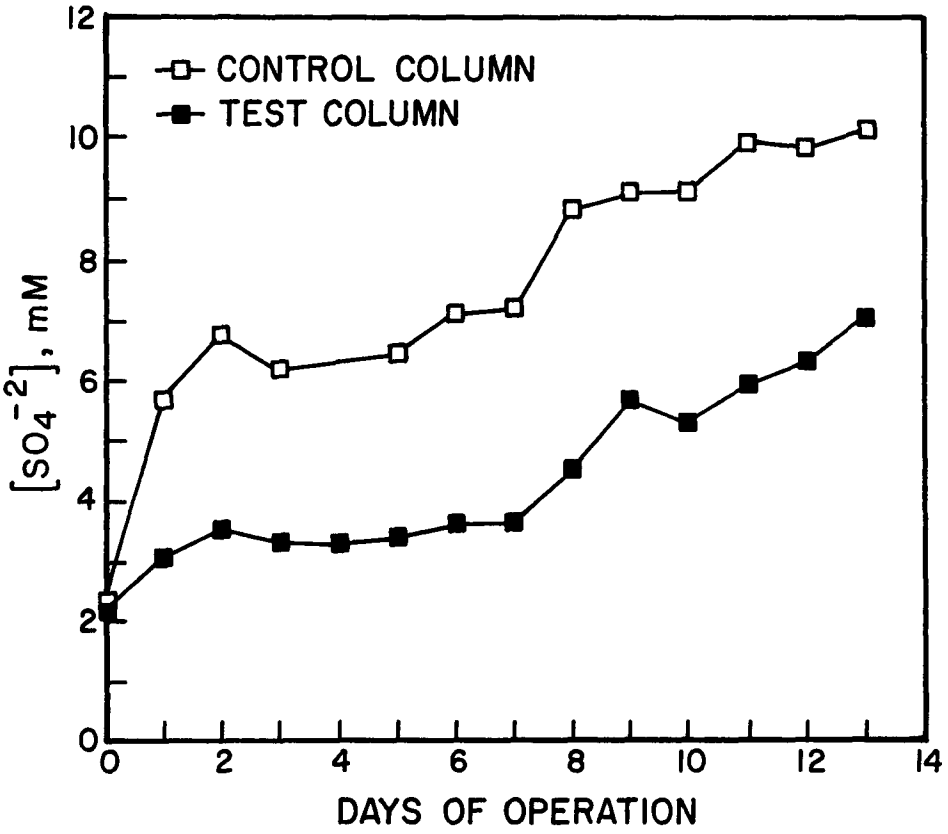

Fig. 2. Accumulation of sulfate in the effluents of the test and control column receiving 60 mL/h of 800 μM sulfide feed (mM = mmoles/L; μM = μmoles/L).

the experiment (Fig. 5). As noted previously, the oxidation of sulfide by *T. denitrificans* is an acid producing process. *T. denitrificans* is typically inhibited by a pH less than 6.6. However, the column was functioning normally when the experiment was terminated and active cells were readily recovered from the beads. The pH internal to the bead could have been higher than that of the bulk liquid phase in the column. It can safely be said that the $CaCO_3$ did provide buffering capacity inside the bead. As noted above, control beads without biomass and, therefore, without the internal biologically produced acid, fell apart within 48 h of the initiation of a continuous feed. However, the biocatalyst beads in the test column maintained structural integrity during the entire course of the experiment indicating internal generation of Ca^{+2}.

In contrast to the test column, the effluent from the control column contained an average of 200 μM sulfide and smelled strongly of H_2S during the entire course of the experiment. Apparently, some chemical oxidation of sulfide (with nitrate) occurred in the control column and feed reservoir. A total of 6.8 mmoles of sulfate accumulated in the reservoir

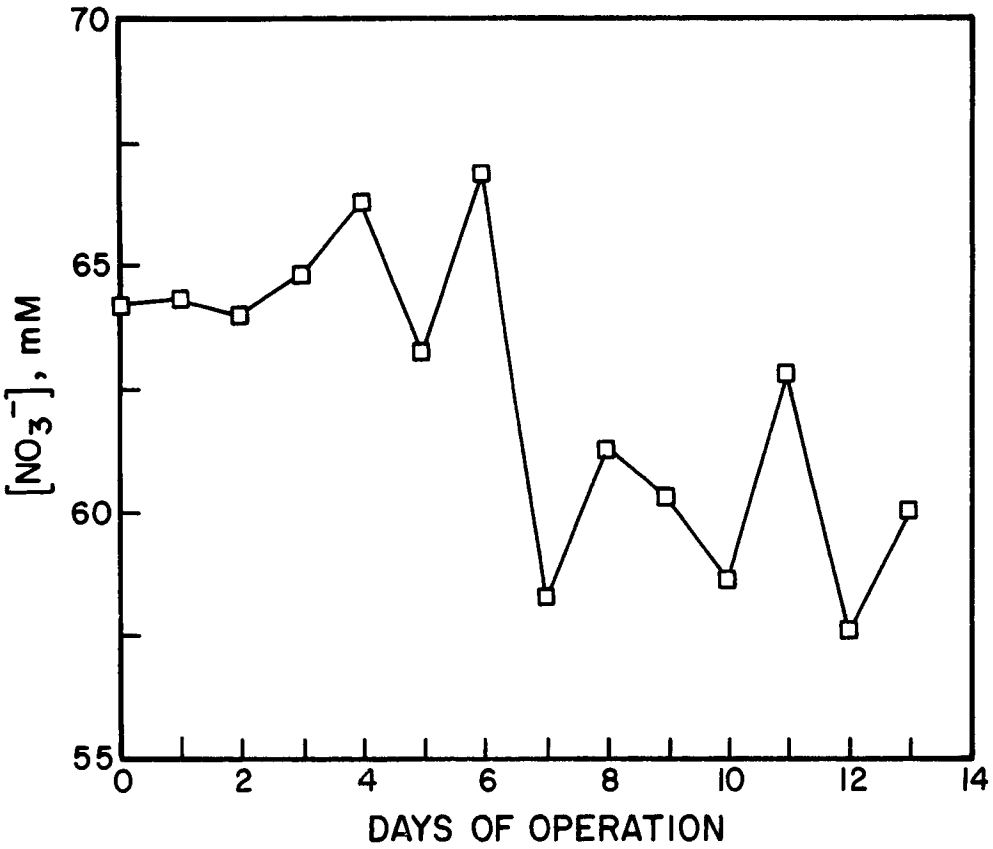

Fig. 3. Depletion of nitrate in the test column feed reservoir.

during the course of the experiment (Fig. 2). No nitrite could be detected in any samples of the control column effluent or feed reservoir.

CONCLUSION

The experiment described above illustrates that viable cells of the autotroph *T. denitrificans* coimmobilized with $CaCO_3$ in alginate beads can be used to remove soluble sulfides from sour water with anaerobic oxidation to sulfate. Similar results have been obtained with biofilters composed of *T. denitrificans* biomass and $CaCO_3$ entrapped in a bed of sargassum moss. The $CaCO_3$ provides buffering capacity and generates Ca^{+2} internal to alginate beads to maintain structural integrity during extended periods of operation. However, the production of nitrite in the experiment described above may indicate that the cells were under stress because of excessive sulfide concentrations in the inlet section of the column. Sublette and Sylvester (*7*) have demonstrated that sulfide concentrations in

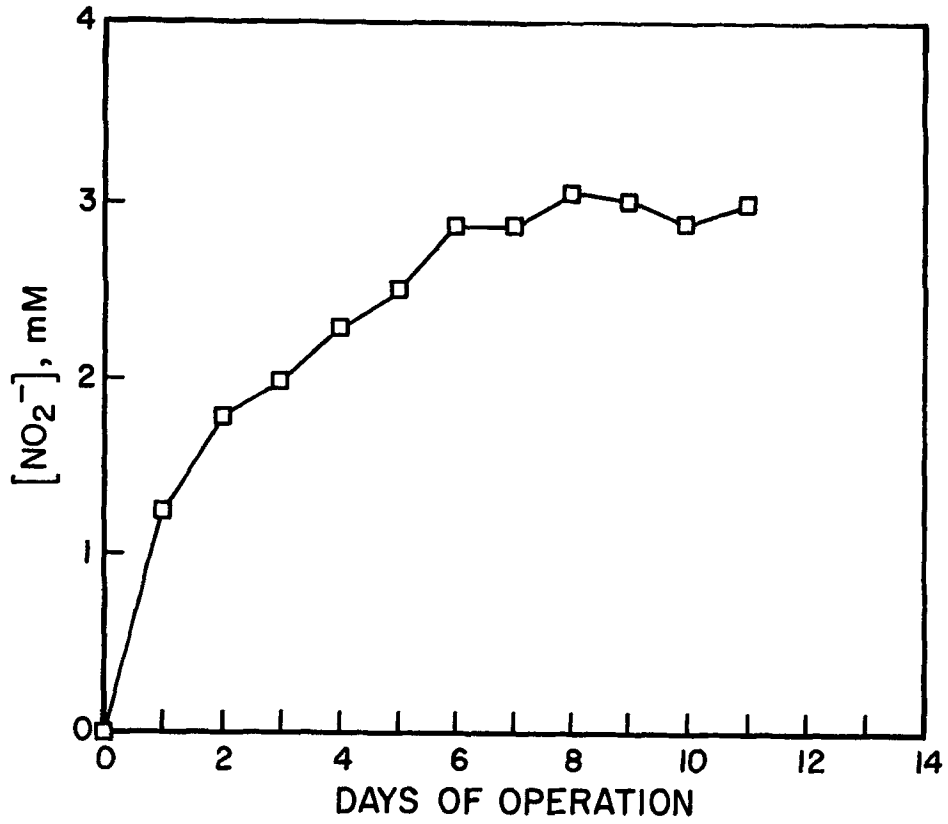

Fig. 4. Accumulation of nitrite in the effluent of the test column receiving 60 mL/h of 800 μM sulfide feed.

excess of 100–200 μM can result in the incomplete reduction of nitrate by free-cell *T. denitrificans* under anaerobic conditions, with accumulations of nitrite and nitrous oxide (N_2O). Therefore, the accumulation of nitrite in the test column effluent may indicate that for highest removal efficiencies all catalyst beads should "see" a more uniform and low concentration of sulfide in the contacting stage. This could have been accomplished in the above experiment by partial recycle of the effluent stream to the inlet of the column, resulting in greater liquid feed rates but lower inlet sulfide concentration. However, gels are subject to deformation at high liquid flowrates, resulting in high pressure drops in the bed. Future work will utilize inorganic supports that exhibit little deformation under forced fluid flow conditions.

ACKNOWLEDGMENT

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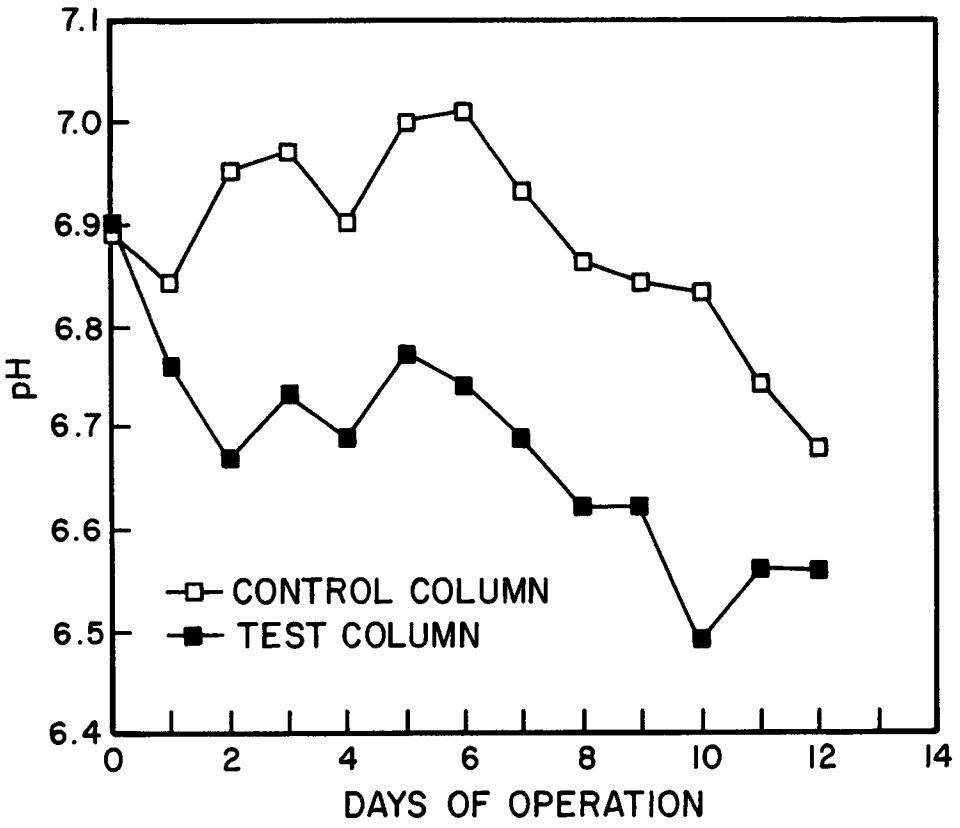

Fig. 5. pH of the effluents from the test and control columns receiving 60 mL/h of 800 μM sulfide feed.

REFERENCES

1. Postgate, J. R. (1984), *The Sulfate-Reducing Bacteria*, 2nd ed., Cambridge University Press, Cambridge.
2. Hamilton, W. A. (1985), *Ann. Rev. Microbiol.* **39,** 195.
3. Ruseska, I., Robbins, J., and Costerton, J. W. (1982), *Oil Gas J.* p. 253.
4. National Association of Corrosion Engineers (1976), *The Role of Bacteria in the Corrosion of Oil Field Equipment*, NACE, Houston, TX.
5. Noyes Data Corp. (1973), *Pollution Control in the Petroleum Industry*, Noyes Data Corp., Park Ridge, NJ.
6. Baalsrud, K. and Baalsrud, K. S. (1954), *Archiv. Mikrobiol.* **20,** 34.
7. Sublette, K. L. and Sylvester, N. D. (1987), *Biotech. Bioeng.* **29,** 249.
8. Sublette, K. L. (1987), *Biotech Bioeng.* **29,** 690.
9. Sublette, K. L. and Sylvester, N. D. (1987), *Biotech. Bioeng.* **29,** 753.
10. Sublette, K. L. and Sylvester, N. D. (1987), *Biotech. Bioeng.* **29,** 759.
11. Lewandowski, Z., Bakke, R., and Characklis, W. G. (1987), *Wat. Sci. Tech.* **19,** 175.
12. American Public Health Association (1976), *Standard Methods for Examination of Water and Wastewater*, 14th ed., APHA, New York (1976).

13. Meites, L., ed. (1963), *Handbook of Analytical Chemistry,* McGraw-Hill, New York.
14. Truper, H. G. and Schlegel, H. G. (1964), *Ant. van Leeuwenhoek* **30,** 225.