Factors Affecting Microbial Sulfate Reduction by *Desulfovibrio desulfuricans* in Continuous Culture: Limiting Nutrients and Sulfide Concentration

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The effects of sulfate and nitrogen concentrations on the rate and stoichiometry of microbial sulfate reduction were investigated for *Desulfovibrio desulfuricans* grown on lactate and sulfate in a chemostat at pH 7.0. Maximum specific growth rates (μ_{max}), half-saturation coefficients (K_{sul}), and cell yields ($Y_{c/Lac}$) of 0.344 ±0.007 and 0.352 ±0.003 h^{-1}, 1.8 ±0.3 and 1.0 ±0.2 mg/L, and 0.020 ±0.003 and 0.017 ±0.003 g cell/g lactate, respectively, were obtained under sulfate-limiting conditions at 35°C and 43°C. Maintenance energy requirements for *D. desulfuricans* were significant under sulfate-limiting conditions. The extent of extracellular polymeric substance (EPS) produced was related to the carbon:nitrogen ratio in the medium. EPS production rate increased with decreased nitrogen loading rate. Nitrogen starvation also resulted in decreased cell size of *D. desulfuricans.* The limiting C:N ratio (w/w) for *D. desulfuricans* was in the range of 45:1 to 120:1. Effects of sulfide on microbial sulfate reduction, cell size, and biomass production were also investigated at pH 7.0. Fifty percent inhibition of lactate utilization occurred at a total sulfide concentration of approximately 500 mg/L. The cell size of *D. desulfuricans* decreased with increasing total sulfide concentration. Sulfide inhibition of *D. desulfuricans* was observed to be a reversible process. © 1992 John Wiley & Sons, Inc.
Key words: *Desulfovibrio desulfuricans* • stoichiometry • kinetics • microbial sulfate reduction • sulfate limitation • nitrogen limitation • sulfide inhibition

INTRODUCTION

In the petroleum industry, sulfate-reducing bacteria (SRB) cause serious problems including corrosion of installations, plugging of the petroleum formation, and contamination of petroleum with H_2S (souring) in the formation. Cochrane et al.[7] report that the presence and growth of thermophilic SRB at temperatures greater than 60°C were a major source of sulfide production in a North Sea oil field and that seawater injection results in the appropriate balance of sulfate, temperature, and organic nutrient status for growth in the reservoir. Herbert[8] reports that substantial levels of short-chain fatty acids and ammonia present in many formation waters can be used directly by SRB for metabolism.

Ligthelm et al.[12] recently published a one-dimensional analytical model of H_2S generation and transport within an oil reservoir in which the souring is attributed to SRB activity. Their simulation results indicate that generation of H_2S by SRB occurred in the mixing zone between injected seawater and formation water where spatial gradients in environmental factors such as temperature and nutrients exist. However, effects of temperature and limiting nutrients on SRB activity were not considered by this model. At present, quantitative description of reservoir souring is essentially impossible because coefficients for rate and extent of SRB growth under relevant environmental conditions are not available.

The environment for microbial growth varies widely throughout the petroleum formation. The temperature varies from that of the cold injection water temperature (−5°C–20°C) to that of the hot formation temperature (40°C–100°C) and can have a major influence on SRB activity. Okabe and Characklis[17] reported that the maximum specific growth rate (μ_{max}) of *Desulfovibrio desulfuricans* was relatively constant (0.38–0.55 h^{-1}) between 25°C and 43°C and dramatically decreased outside this temperature range. However, the stoichiometry of microbial sulfate reduction was not temperature dependent. More information is needed on the effects of various relevant environmental factors on SRB activity.

The ultimate goal of our SRB research program is the development of effective means to control SRB activity in various industrial systems including petroleum production. Some researchers are focusing on determination of the effectiveness of various biocides on planktonic and sessile SRB population.[6,26] Biocide treatment, however, may not be an ultimate means to control SRB activity in oil fields because of rapid microbial regrowth, plugging of the formation, environmental concerns, and cost effectiveness. Concentrations of sulfate, substrate (carbon sources), and essential nutrients such as phosphorus and nitrogen in the formation vary as they are depleted by microbial activity or are mediated by the formation itself. The reduction of the concentration of an essential nutrient to below the limiting concentration is a possible means of controlling SRB. Thus, determination of the limiting nutrient concentration may be useful to control

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and predict SRB activity. Okabe and Characklis[17] reported that the limiting C:P ratio (w/w) for growth of *D. desulfuricans* is in the range of 400:1–800:1. Extensive extracellular polymeric substance (EPS) production was observed as phosphorus became limiting. Microorganisms also need assimilable nitrogen usually present in the range of a few milligrams per liter (nitrate level is not detectable) in the produced water.[31] Thus, nitrogen is a promising nutrient for control of SRB activity in the oil formation. Also, in the case where fresh water is used as injection water, sulfate may be a limiting substrate for SRB activity. Ironically, scale and corrosion inhibitor chemicals may enrich the system in N, P, or SO_4^{2-}.

Despite the importance of microbial sulfate reduction in industrial water systems, limited experimental information on rate and stoichiometry of microbial sulfate reduction under sulfate-limiting conditions is available. Also, our understanding of nutrient requirements for SRB is limited. Although many have studied the effects of sulfide on anaerobic wastewater treatment to enhance treatment performance, no SRB product inhibition data has been reported. It is essential to understand how SRB behave under high sulfide concentration environment such as a petroleum reservoir.

This article will describe the effects of limiting nutrients concentrations such as nitrogen and sulfate as well as the influence of inhibitory sulfide concentration on rate and stoichiometry of microbial sulfate reduction by *D. desulfuricans*.

MATHEMATICAL DESCRIPTION OF CHEMOSTAT SYSTEM

Microbial transformations in a chemostat can be mathematically described by mass balance equations assuming the two fundamental processes occurring are growth and maintenance.[17] The rate of microbial growth can be described by the Monod expression as a function of limiting substrate concentration (e.g., sulfate):

$$\mu = \frac{\mu_{max} \cdot S}{(K_{Sul} + S)} \qquad (1)$$

where μ = specific growth rate (h^{-1}), μ_{max} = maximum specific growth rate (h^{-1}), S = sulfate concentration (mg/L), and K_{Sul} = half-saturation coefficient for sulfate (mg/L). The details of the mathematical description of the chemostat system are described elsewhere.[17]

EXPERIMENTAL MATERIALS AND METHODS

Experimental System

The rate and stoichiometric coefficients at several limiting nutrient (e.g., sulfate and nitrogen) and sulfide concentrations were determined in a chemostat consisting of a pyrex cylindrical beaker (1.5 L volume). The chemostat was equipped with a butyl rubber biofilm scraper continuously rotated by an electric motor to remove wall growth.

A constant pH and temperature were maintained using a pH control system with sterile 1.0*N* HCl and NaOH solutions and thermoregulator, respectively. The slow continuous nitrogen purge of the reactor maintained anaerobic conditions and prevented H_2S accumulation. Traces of oxygen in the nitrogen feed gas were removed by a reducing column containing copper wire maintained at 400°C. The gas was sterilized by a cotton filter. The flow rate of nitrogen gas was approximately 3 L/h.

Desulfovibrio desulfuricans (ATCC 5575) was grown in Postgate medium G,[17,21] including hemi-calcium lactate (L-lactic acid, Sigma, No. L-2000) as the sole carbon and energy source. Trace elements and vitamins were added.[17] A sterile $Na_2S_2O_4$ solution was added as a reductant until a vigorously growing culture was established.

Analytical Methods

At steady state, effluent samples were obtained for the following analyses: (1) total organic carbon (TOC), (2) soluble organic carbon (SOC), (3) total bacterial counts and cell size, (4) sulfate, (5) sulfide, (6) lactate, (7) acetate, and (8) ammonium nitrogen. The samples for SOC, lactate, acetate, sulfate, and ammonium nitrogen analyses were obtained by filtering an aliquot of the chemostat effluent through 0.22-μm Nucleopore filters. The phenate method was used for ammonium nitrogen analysis.[29] The details of the rest of the chemical analytical methods are described elsewhere.[17]

RESULTS

Sulfate-Limiting Condition: Steady State Cellular Carbon and Sulfate Concentrations

Classical behavior was generally observed for the steady-state dependence of cellular carbon and sulfate concentration on dilution rate (Fig. 1). EPS carbon concentrations were not significant at any dilution rate for the sulfate-limiting experiments. Therefore, cellular carbon concentrations were calculated as differences between effluent TOC concentration and effluent SOC concentration. Maintenance energy requirements became important at dilution rates less than 0.05 h^{-1}.

Estimation of Monod Growth Parameters

Estimates of μ_{max} and K_{Sul} were computed from the effluent sulfate concentrations and dilution rates using the following nonlinear regression form of Eq. (1):

$$S = \frac{K_{Sul} \cdot \mu}{(\mu_{max} - \mu)} \qquad (2)$$

The nonlinear regression of the Monod equation was performed using MSU SAS (statistical software). The

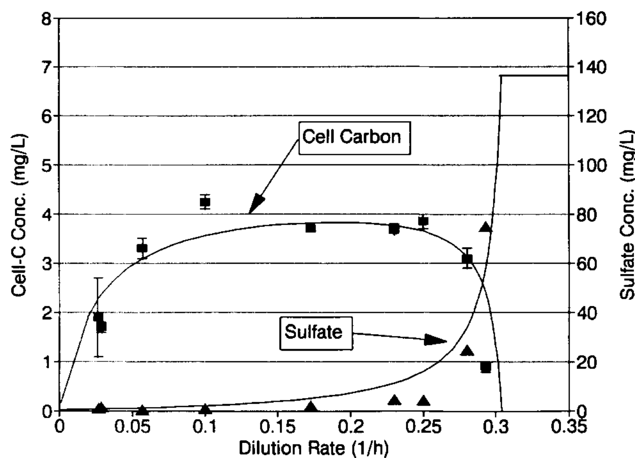
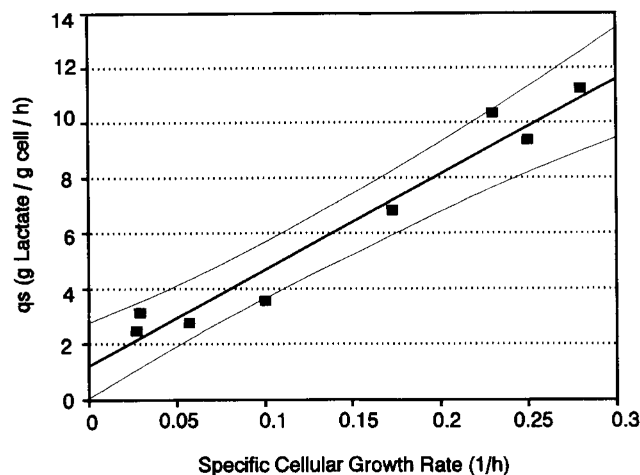


Figure 1. Steady-state cellular carbon and sulfate as a function of dilution rate at 35°C. The solid lines reflect kinetic coefficients determined by nonlinear regression. The influent sulfate concentration was 135 mg/L. Error bars represent the standard error of measurement ($n = 2$).

program provides estimates of μ_{max} and K_{sul} from given data pairs of limiting substrate concentration (S) and specific growth rate ($\mu = D$). The 95% confidence interval associated wtih the estimate of each parameter was also determined. The maximum growth rates of 0.344 ±0.007 h^{-1} and 0.352 ±0.003 h^{-1} were obtained at 35 and 43°C, respectively. The half-saturation coefficient for sulfate (K_{Sul}) was calculated to be 1.8 ±0.3 and 1.0 ±0.2 mg/L at 35 and 43°C, respectively. The maintenance energy requirement was determined using graphical methods based on a mathematical model[5,17] (Fig. 2). Significant maintenance coefficients based on lactate consumption were determined under sulfate-limiting conditions (1.20 ±0.53 and 1.98 ±0.51 g lactate/g cell h^{-1} at 35°C and 43°C, respectively). The summary of rate coefficients under sulfate-limiting conditions are presented along with those reported under lactate-limiting conditions in Table I.

Estimation of Stoichiometric Coefficients

The stoichiometry for microbial sulfate reduction was developed from the experimental data obtained from a sulfate-limiting continuous culture (Table II). The stoichiometric coefficients were balanced by assuming the following: (1) an empirical formulation ($CH_{1.4}N_{0.2}O_{0.4}$) for bacterial cells,[5] (2) the only nitrogen source is NH_3, and (3) the amount of other end products of lactate oxidation besides acetate and CO_2 is negligible. The stoichiometric coefficients for lactate, sulfate, bacterial cells, and acetate were obtained from the experimental data. The stoichiometric coefficients for bacterial cells were calculated from the difference between effluent TOC and effluent SOC because EPS carbon concentrations were negligible. The stoichiometric coefficients for carbon dioxide were calculated by assuming that the difference between the influent and effluent TOC is CO_2 as C because the only carbon escaping the experimental sytem is inorganic carbon (e.g., CO_2). The stoichiometric coefficient for water was obtained using the oxygen balance. The percentages of carbon recovery in the stoichiometric equations at each temperature were more than 95% of the amounts of lactate-carbon added.



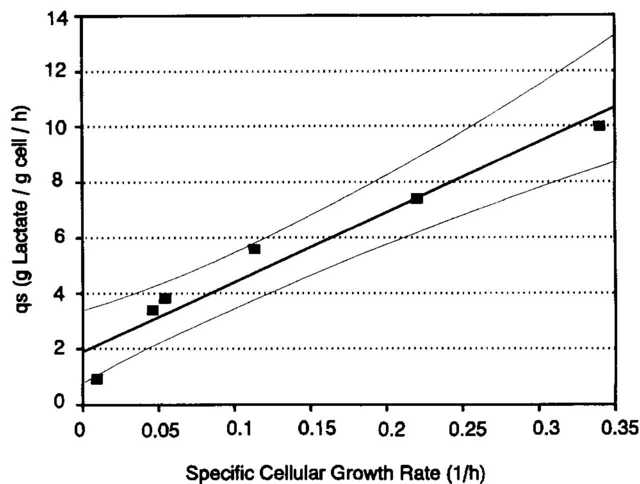


Figure 2. The relationship between specific lactate removal rate (q_s) and dilution rate (D). The maintenance coefficient (m) is the specific lactate removal rate at $D = 0$ (the y intercept). The intrinsic cell yield is a reciprocal of the slope. The linear regression lines along with 95% confidence intervals are presented. (a) The maintenance coefficient of 1.20 ±0.53 g lactate (g cell)$^{-1}$ h^{-1} and intrinsic cell yield coefficient of 0.029 ±0.003 g cell (g lactate)$^{-1}$ were determined at 35°C: $r^2 = 0.96$. (b) The maintenance coefficient of 1.98 ±0.51 g lactate (g cell)$^{-1}$ h^{-1} and intrinsic cell yield coefficient of 0.041 ±0.005 g cell (g lactate)$^{-1}$ were determined at 43°C: $r^2 = 0.95$.

At low levels of sulfate (below K_{Sul}), the SO_4^{2-}/lactate stoichiometric ratio decreased to approximately 0.30 from 0.45 as observed above the K_{Sul} value (Fig. 3). The SO_4^{2-}/lactate ratio of 0.45 is similar to the one obtained under lactate-limiting conditions.[17]

Effect of Nitrogen Concentration

Desulfovibrio desulfuricans was grown at a dilution rate of 0.2 h^{-1} at 35°C and at various C:N ratios to evaluate effects of nitrogen concentration on lactate oxidation

Table I. Summary of rate coefficients for *D. desulfuricans* obtained under lactate-limiting and sulfate-limiting conditions.[a]

Temperature (°C)	μ_{max} (h^{-1})	K_{Lac}, K_{Sul} (mg/L)	m (g_{Lac}/g_{Cell} h)	$Y^{Int}_{c/Lac}$ (g_{Cell}/g_{Lac})	$Y^{obs}_{c/Lac}$ (g_{Cell}/g_{Lac})	Reference
Lactate limiting						
35	0.37 ±0.004	2.2 ±0.6	0.45 ±1.08	0.028 ±0.008	0.024 ±0.006	17
43	0.55 ±0.003	10.0 ±1.2	0.28 ±0.05	0.031 ±0.007	0.032 ±0.001	17
Sulfate limiting						
35	0.344 ±0.007	1.8 ±0.3	1.20 ±0.53	0.029 ±0.003	0.020 ±0.003	This study
43	0.352 ±0.003	1.0 ±0.2	1.98 ±0.51	0.041 ±0.005	0.017 ±0.003	This study

[a] Values given are the estimated parameter ± the standard error. The maintenance coefficient under sulfate-limiting condition is based on lactate consumption for comparison purposes (see Fig. 2). $Y^{Int}_{c/Lac}$ = intrinsic cell yield on lactate; $Y^{Obs}_{c/Lac}$ = observed cell yield on lactate.

Table II. Stoichiometries of microbial sulfate reduction under lactate- and sulfate-limiting conditions: sulfate limitation.

At 35°C Sulfate limiting ($S < K_{Sul}$)

$$CH_3CHOHCOOH + 0.38 \pm 0.012\, H_2SO_4 + 0.014\, NH_3 \rightarrow 0.070 \pm 0.015\, CH_{1.4}N_{0.2}O_{0.4} + 0.98 \pm 0.05\, CH_3COOH + 0.96 \pm 0.01\, CO_2 + 0.28 \pm 0.04\, H_2S + 0.61\, H_2O$$

Sulfate saturated ($S > K_{Sul}$)

$$CH_3CHOHCOOH + 0.42 \pm 0.012\, H_2SO_4 + 0.019\, NH_3 \rightarrow 0.094 \pm 0.007\, CH_{1.4}N_{0.2}O_{0.4} + 0.95 \pm 0.03\, CH_3COOH + 1.00 \pm 0.03\, CO_2 + 0.38 \pm 0.04\, H_2S + 0.74\, H_2O$$

At 43°C Sulfate limiting ($S < K_{Sul}$)

$$CH_3CHOHCOOH + 0.36 \pm 0.016\, H_2SO_4 + 0.011\, NH_3 \rightarrow 0.056 \pm 0.008\, CH_{1.4}N_{0.2}O_{0.4} + 0.94 \pm 0.03\, CH_3COOH + 0.96 \pm 0.02\, CO_2 + 0.33 \pm 0.04\, H_2S + 0.62\, H_2O$$

Sulfate saturated ($S > K_{Sul}$)

$$CH_3CHOHCOOH + 0.42 \pm 0.015\, H_2SO_4 + 0.021\, NH_3 \rightarrow 0.103 \pm 0.015\, CH_{1.4}N_{0.2}O_{0.4} + 1.08 \pm 0.14\, CH_3COOH + 1.05 \pm 0.09\, CO_2 + 0.33 \pm 0.05\, H_2S + 0.38\, H_2O$$

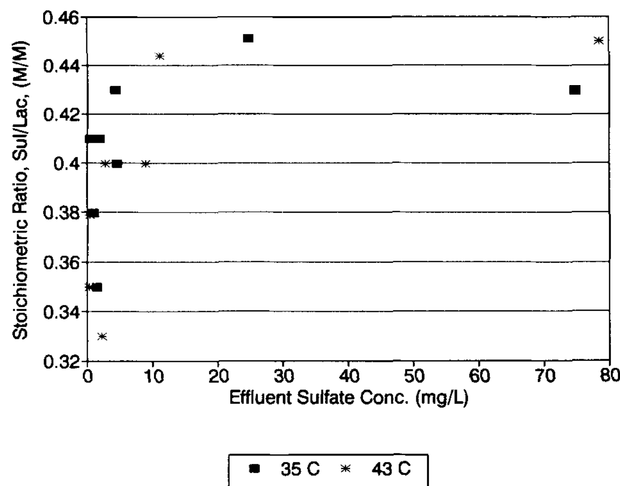


Figure 3. The effect of sulfate concentration on the stoichiometric ratio of SO_4^{2-}/lactate.

and biomass synthesis. Sulfate was in excess. Steady-state results, along with their respective standard errors of measurement, are presented in Table III.

Estimation of Nitrogen Requirement

The lactate utilization rate decreased from 99% at C:N = 2.2:1 (w/w) to 85% at C:N = 120:1 and then decreased to 40% at C:N = 230:1 (Fig. 4). The effluent nitrogen concentration at C:N = 45:1 was 0.29 mg N/L. Thus, medium at C:N = 45:1 was low in nitrogen. At lower influent nitrogen levels, the metabolism shifted from lactate limited to nitrogen limited as evidenced by (1) significant amounts of lactate in the effluent and (2) lower cell yield. The stoichiometries for microbial sulfate reduction changed with changing C:N ratio assuming nitrogen content of the cells was constant according to the empirical formula[5] ($CH_{1.4}N_{0.2}O_{0.4}$) (Table IV). CH_2O was used as an empirical formula for EPS.[5] The recovery of carbon in the five stoichiometric equations was more than 95% of the amounts of lactate carbon added except for C:N = 120:1.

EPS and Cellular Carbon

The cell size of *D. desulfuricans* asymptotically decreased with decreasing influent nitrogen concentration (Fig. 5). The cell size decreased by about 30% at C:N = 45:1 and remained relatively constant thereafter. With decreasing nitrogen concentration, cell carbon concentration also decreased from 6.3 mg C/L at C:N = 2:1 to 1.3 mg C/L at C:N = 230:1 (Fig. 6). Even at C:N = 22:1, cell carbon concentration decreased by 16%. In contrast, EPS carbon concentration increased from 0 mg EPS C/L at C:N = 2:1 to 2.11 mg EPS C/L at C:N = 45:1 and then decreased in parallel to the cell carbon drop. At C:N = 45:1, EPS carbon concentration was approximately equal to cell carbon concentration. EPS yield increased and cell yield decreased with decreasing nitrogen concentration (Fig. 7). Above C:N = 45:1, the cell yield was the same as the EPS yield. Note that total biomass yield (cell + EPS) remains constant for all C:N ratios.

Effect of Sulfide Concentration

Desulfovibrio desulfuricans growing at a dilution rate of 0.2 h^{-1} and at 35°C was exposed to various sulfide concentrations (Table V). Sulfide concentration was measured as total sulfide concentration in the liquid phase. The chemostat pH was strictly maintained at 7.0 with sterile 1.0*N* NaOH and HCl solutions. Treatment with 150 mg/L total sulfide slightly decreased lactate uti-

Table III. Steady-state results of liquid phase parameters of the continuous culture of *D. desulfuricans* with different influent ammonium nitrogen concentrations at a dilution rate of 0.2 h^{-1}.

	Influent			Effluent		
C:N	Ammonium-N	Lactate	Sulfate	Ammonium-N	Lactate	Sulfate
2.2:1	65.59 ±0.55	376.9 ±3.6	645.5 ±0.0	53.74 ±3.87	2.5 ±0.7	452.3 ±6.5
22:1	6.39 ±0.11	353.7 ±1.2	644.5 ±4.5	4.13 ±0.34	0.9 ±0.7	455.5 ±14.4
45:1	3.32 ±0.01	389.6 ±1.8	674.5 ±5.5	0.29 ±0.01	1.3 ±0.3	477.7 ±8.3
120:1	1.22 ±0.04	375.6 ±3.7	611.8 ±0.9	0.15 ±0.02	56.2 ±13.1	445.0 ±9.0
230:1	0.64 ±0.01	372.0 ±2.4	638.2 ±1.8	0.05 ±0.01	224.9 ±12.8	531.4 ±21.5

The values given are the mean of duplicate measurements of two samples ± the standard error (in mg/L).

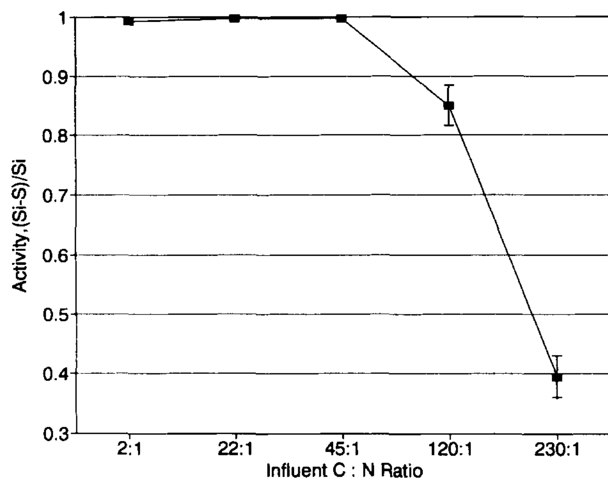


Figure 4. Response of lactate oxidation $[(S_i - S)/S_i]$ to change in the carbon (C):nitrogen (N) ratio: $D = 0.2$ h^{-1}, temperature = 35°C. The limiting C:N ratio (w/w) is in the range 45:1–120:1. Error bars represent the standard error of measurement ($n = 2$). The error bar is not presented except for two points at 120:1 and 230:1 because the other standard errors are so small (Table IV).

Table IV. Effect of nitrogen concentration on the stoichiometry of microbial sulfate reduction.

C:N = 2.2:1 (98.7%)
$CH_3CHOHCOOH + 0.48H_2SO_4 + 0.203NH_3$
$\rightarrow 0.119CH_{1.4}N_{0.2}O_{0.4} + 0.002CH_2O + 0.94CH_3COOH$
$+ 0.96CO_2 + 0.37H_2S + 1.08H_2O$

C:N = 22:1 (96.2%)
$CH_3CHOHCOOH + 0.50H_2SO_4 + 0.041NH_3$
$\rightarrow 0.102CH_{1.4}N_{0.2}O_{0.4} + 0.015CH_2O + 0.89CH_3COOH$
$+ 0.99CO_2 + 0.41H_2S + 1.22H_2O$

C:N = 45:1 (96.9%)
$CH_3CHOHCOOH + 0.47H_2SO_4 + 0.050NH_3$
$\rightarrow 0.056CH_{1.4}N_{0.2}O_{0.4} + 0.040CH_2O + 0.92CH_3COOH$
$+ 0.97CO_2 + 0.40H_2S + 1.10H_2O$

C:N = 120:1 (82.0%)
$CH_3CHOHCOOH + 0.49H_2SO_4 + 0.022NH_3$
$\rightarrow 0.045CH_{1.4}N_{0.2}O_{0.4} + 0.038CH_2O + 0.78CH_3COOH$
$+ 0.82CO_2 + 0.45H_2S + 1.70H_2O$

C:N = 230:1 (99.4%)
$CH_3CHOHCOOH + 0.68H_2SO_4 + 0.026NH_3$
$\rightarrow 0.068CH_{1.4}N_{0.2}O_{0.4} + 0.023CH_2O + 1.01CH_3COOH$
$+ 0.87CO_2 + 0.37H_2S + 1.94H_2O$

Values in parentheses are percentage of carbon recovery.

lization and cellular production (Figs. 8 and 9). Total sulfide of 280 mg/L dramatically decreased cellular production and increased EPS production. Finally, lactate utilization and cellular production were strongly in-

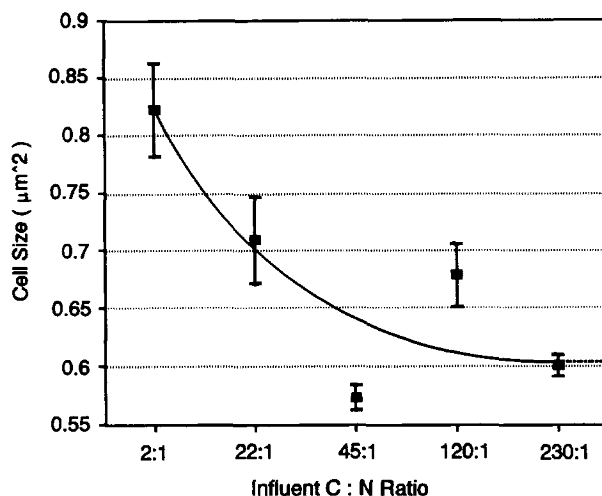


Figure 5. Cell size of *D. desulfuricans* decrease with increasing C:N ratio. The cell size was determined using an image analyzer by the epifluorescence method. Abscissa is not to scale. Error bars represent the standard error of measurement ($n = 2$).

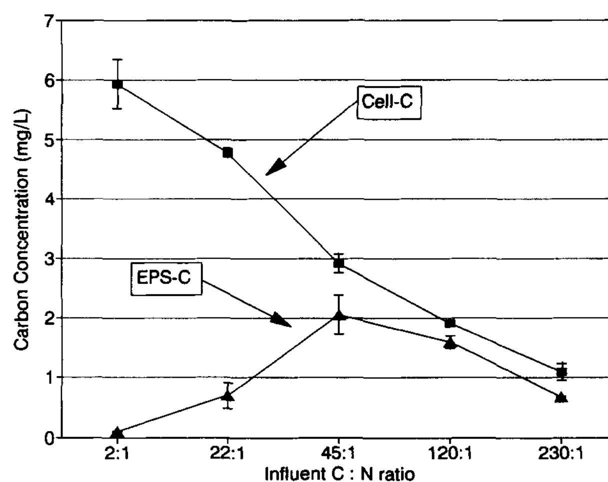


Figure 6. Change in cellular and EPS carbon concentration with changing C:N ratio. Error bars represent the standard errors of measurement ($n = 2$).

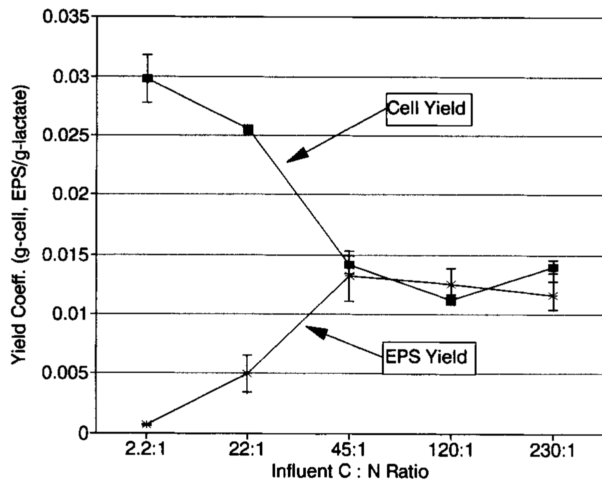


Figure 7. Change in cell and EPS yield coefficients with changing C:N ratio. Error bars represent the standard errors of measurement (n = 2).

Table V. Steady-state results of liquid phase parameters of the continuous culture of *D. desulfuricans* with different influent total sulfide concentrations at a dilution rate of 0.2 h^{-1}.

Sulfide-S	Influent lactate	Effluent lactate
40.9 ±0.1	382.9 ±1.2	2.5 ±0.7
150.2 ±1.4	417.7 ±9.2	7.4 ±0.2
277.6 ±4.9	393.9 ±2.4	31.9 ±4.2
596.3 ±44.7	400.0 ±6.1	272.3 ±14.1

The values given are the mean of duplicate measurements of two samples ± the standard error (in mg/L).

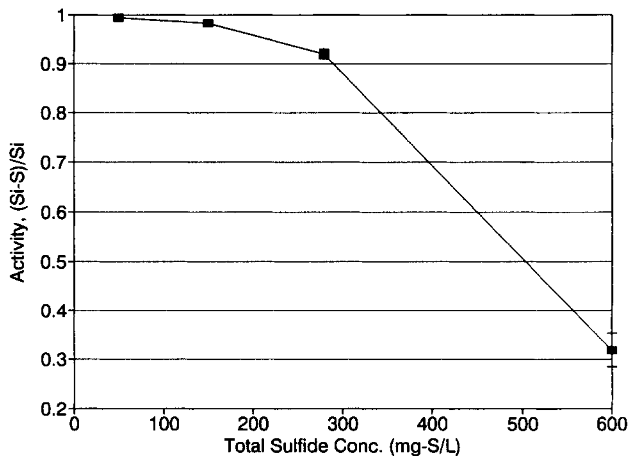


Figure 8. Response of lactate oxidation $[S_i - S)/S_i]$ to change in the total sulfide concentration: $D = 0.2\ h^{-1}$, temperature 35°C, pH 7.0. $Na_2S \cdot 9H_2O$ was added to obtained desired total sulfide concentration. Error bars represent the standard error of measurement (n = 2). The error bar is not presented except for 600 mg TS/L because the other standard errors are so small (Table V).

hibited at total sulfide of 600 mg/L. The fraction of EPS carbon increased with increasing total sulfide concentration. Overall, cell size decreased with increasing total sulfide concentration to about 0.7 μm^3 at 280 mg/L of total sulfide and remained relatively constant thereafter (Fig. 10). After treatment with 600 mg/L

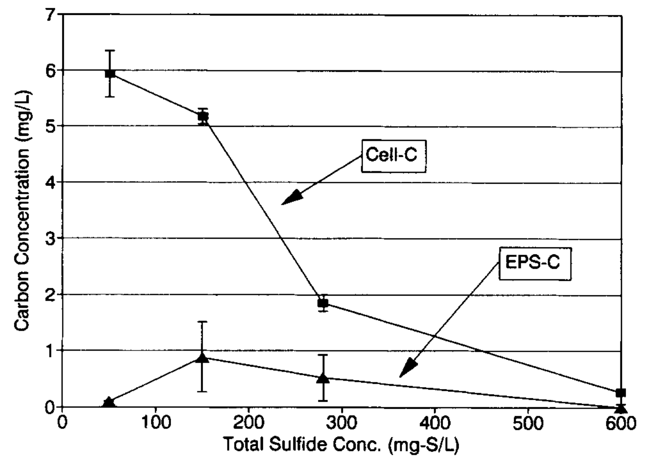


Figure 9. Change in cellular and EPS carbon concentrations with changing total sulfide concentration. Error bars represent the standard errors of measurement (n = 2).

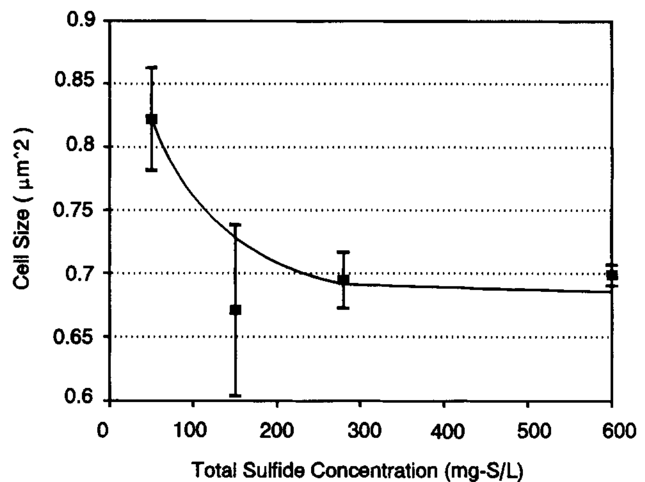


Figure 10. Change in cell size of *D. desulfuricans* with changing total sulfide concentration. The cell size was determined using an image analyzer by the epifluorescence method. Error bars represent the standard errors of measurement (n = 2).

of total sulfide, 50 mL of culture medium was transferred to 450 mL of the fresh culture medium without sulfide, and the cell numbers were monitored to examine the recovery of *D. desulfuricans* from sulfide inhibition (Fig. 11). Cell numbers slowly increased without a lag phase and reached the same cell number as the control. The doubling time of the sulfide-treated culture was approximately one-third of the control.

DISCUSSION

Stoichiometry under Sulfate-Limiting Conditions

Observed cell yields under sulfate limitation in these experiments are low compared to literature values for at least two reasons: (1) no yeast extract was used in the medium as is common in other SRB studies and (2) cell-associated EPS was not considered in the yield calculation. Furthermore, sulfate limitation may result

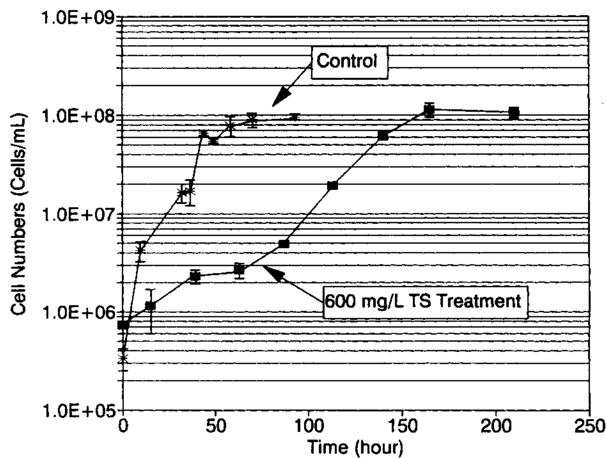


Figure 11. Recovery of *D. desulfuricans* from sulfide inhibition after 600 mg/L total sulfide treatment. Error bars represent the standard errors of measurement ($n = 3$).

in an increase in maintenance energy requirement for *D. desulfuricans* which may significantly influence intracellular processes. Thus, maintenance energy reflects diversion of substrate away from synthesis or growth processes. Consequently, maintenance decreases the observed cell yield from substrate (Table I). Nevertheless, intrinsic cell yields ($Y^{Int}_{c/Lac}$) under sulfate-limiting and lactate-limiting conditions were essentially the same (Table I).

The maintenance coefficients under sulfate-limited conditions are significant (Figs. 2a, b). The maintenance coefficient (m) is determined in continuous culture experiments (not in batch experiments) and is the specific substrate removal rate at $D = 0$ (the y intercept in Figs. 2a, b). Thus, the effect of m is generally observed at low specific growth rate (at low dilution rate). Significant maintenance coefficients were not observed under lactate-limiting experiments.[17] Under sulfate-limiting conditions, the significant maintenance coefficients were a result of operating the chemostat at low dilution rates ($D < 0.05\ h^{-1}$). The chemostat was not operated at a dilution rate less than $0.05\ h^{-1}$ under lactate-limiting conditions. Cappenberg[4] reported that the maintenance coefficient for *D. desulfuricans* grown with lactate-limited growth was 0.53 g lactate/g dry wt./h (Table VI). The reported maintenance coefficients in this article are high compared to the values in the literature primarily because cell-associated EPS and possible precipitates (e.g., FeS) are not considered in the calculation of the maintenance coefficient. The maintenance concentration determined using biomass dry weight, which includes cellular mass, EPS, and precipitates, would be low compared to our data because of high biomass concentration. The overall yields,[17] (cells + EPS + precipitates)/(substrate consumed), of *D. desulfuricans* in previous studies were about two or three times higher than the cell yields (unpublished data). Based on this finding, maintenance coefficients reported by others should be cautiously interpreted since no distinction of biomass was made. In addition, the reported maintenance coefficients for SRB are high compared to values for aerobic microorganisms because higher maintenance energies are required under anaerobic conditions for the production of energy (ATP) from the substrate. For example, Pirt[20] reported that the maintenance coefficients for *Aerobacter cloacae* growing in a glycerol-limited continuous culture were 0.094 and 0.473 g glycerol/g dry wt/h under aerobic and anaerobic conditions, respectively.

Observed cell yields ($Y^{Obs}_{c/Lac}$) of *D. desulfuricans* in this study are much lower than previously reported values under lactate limitation (Table I). *Desulfovibrio desulfuricans* grown on lactate and sulfate has a net ATP generation of 3 mol per sulfate reduced.[30] About 3 mol of ATP are generated during electron transport coupled phosphorylation and 2 mol of ATP are generated in sub-

Table VI. The ATP balance and growth yields for SRB grown on different substrate and sulfate.

Energy source	Limiting substrate	Organism	Net ATP generation[a]	$Y_{c/Sul}$ (g/g)	$Y_{o/Sul}$ (g/g)	m^b $g_{Sul}/g_{dry\ wt}$ h	g_{Lac}/g_{cell} h	Reference
H_2, SO_4^{2-}	SO_4^{2-}	*D. vulgaris*	1	—	0.132	0.17	—	16
H_2, SO_4^{2-}	Acetate	*D. vulgaris*	1	—	0.130	0.54	—	16
Lactate, SO_4^{2-}	Lactate	*D. desulfuricans*	3	—	—	—	0.53[c]	4
Lactate, SO_4^{2-}	SO_4^{2-}	*D. desulfuricans*	3	0.047	—	—	1.20	This study
Lactate, SO_4^{2-}	SO_4^{2-}	*D. desulfuricans*	3	0.043	—	—	1.98	This study
Lactate, SO_4^{2-}	Lactate	*D. desulfuricans*	3	0.051	—	—	0.45	17
Lactate, SO_4^{2-}	Lactate	*D. desulfuricans*	3	0.071	—	—	0.28	17
Lactate, SO_4^{2-}	[d]	*D. vulgaris*	3		0.121	ND	—	13
Lactate, SO_4^{2-}	—	*Desulfotomaculum orients*	1		0.140	ND	—	13
Lactate, SO_4^{2-}	SO_4^{2-}	*D. vulgaris*	3		0.141	ND	—	10
Lactate, SO_4^{2-}	SO_4^{2-}	*D. sapovorans*	3		0.115	ND	—	10
Lactate, SO_4^{2-}	SO_4^{2-}	*D. salexigens*	3		0.125	ND	—	10

[a] Estimated moles of ATP during the reduction of 1 mol of SO_4^{2-} to S^{2-}.
[b] Not determined.
[c] Per gram of lactate (dry wt) per hour.
[d] No limiting substrate because of batch culture experiments.

strate level phosphorylation.[30] But 2 mol of ATP are consumed to activate 1 mol of SO_4^{2-} to adenosine phosphosulfate (APS).[30] The cell yield per ATP (based on 3 mol of ATP generation theory), Y_{ATP}^{max}, determined in this study is approximately 1.44 g cell/mol ATP, which is one-tenth of that proposed by Badziong and Thauer (11.4–14.6 g/mol ATP).[1] Liu and Peck[13] reported ATP and growth yields for *Desulfotomaculum orients* and *Desulfovibrio vulgaris* (Table VI). *Desulfovibrio vulgaris* has a net ATP generation of 3 mol per sulfate reduced, whereas *D. orientis* has a net ATP generation of 1. In their experiments the cell yield per ATP, Y_{ATP}^{max}, was approximately 4 g cell/mol ATP. Thus, ATP yield varies significantly among species and with substrate. Clearly, the biochemistry and physiology of growth of *D. desulfuricans* with lactate and sulfate requires further investigation to rationalize the molecular and the cellular observations.

At low levels of sulfate (below K_{Sul} value), the SO_4^{2-}/lactate stoichiometric ratio decreased (Fig. 3). Under these conditions, lactate is probably oxidized via pyruvate and acetate. *Desulfovibrio desulfuricans* possesses the pyruvic phosphoroclastic system in which, under sulfate limitation, pyruvate is dismuted to acetyl phosphate, CO_2, and H_2.[19] There is some evidence that H_2 can be formed from lactate in small amounts if constant removal of hydrogen is occurring.[2] Thus at low levels of sulfate, *D. desulfuricans* may grow with lactate in the absence of sulfate, which is thermodynamically favorable only under very low H_2 partial pressure.[32] In a continuous culture system with N_2 purge, it is speculated that high levels of H_2 cannot accumulate. Contamination with other microorganisms, such as methanogenic bacteria, another possible cause for decreased SO_4^{2-}/lactate stoichiometric ratio, was not observed in these experiments.

The half-saturation coefficients for sulfate, K_{Sul}, at the 35°C and 43°C are 1.8 ±0.3 and 1.0 ±0.2 mg/L, respectively. Ingvorsen and Jorgensen[10] reported that half-saturation coefficients for sulfate, K_{Sul}, for *D. vulgaris, Desulfovibrio sapovorans,* and *Desulfovibrio salexigens* grown in the batch culture were 0.5, 0.7, and 7.4 mg/L, respectively. Observed biomass yields, $Y_{o/Sul}$ for *D. vulgaris, D. Sapovorans, and D. salexigens* were also determined to be 0.141, 0.115, and 0.125 g dry wt/g sulfate, respectively (Table VI). The results obtained in this study are comparable to these reported values.

The experimental results indicate that sulfate may be a promising limiting nutrient to control SRB activity if concentration in injection water can be reduced below a few milligrams per liter. Maree and Strydom[14] reported that biological sulfate removal using molasses as an organic source was feasible without production of H_2S by coculturing SRB with photosynthetic sulfur bacteria which oxidize sulfide to sulfur. The process is accompanied by the precipitation of calcium carbonate and heavy metals leading to their recovery.

Nitrogen Effects

Nitrogen is needed for amino acid, purine, and pyrimidine biosynthesis. Ammonium ions are the conventional nitrogen source in culture media, but *D. desulfuricans* can fix molecular nitrogen (N_2).[23–25] Postgate and Kent[24] reported that none of the *Desulfovibrio* strains tested showed acetylene reduction if NH_4Cl was present. Ammonium chloride completely repressed *nif* expression in *Desulfovibrio gigas* and addition of NH_4^+ in the range 10 to 100 μM inhibited nitrogenase activity.[22] Senez[27] reported that the growth rate of *D. desulfuricans* growing on NH_4^+ as nitrogen source was more than twice as fast as on N_2 and biomass yield from N_2 was diminished significantly. Thus, nitrogen fixation by this culture ($\mu_{max} = 0.34\ h^{-1}$) in a chemostat is not significant because of a relatively high dilution rate ($D = 0.2\ h^{-1}$) and substantial amounts of NH_4^+ in the medium.

The limiting C:N ratio (w/w) determined in this study for *D. desulfuricans* (C:N = 45:1–120:1) is higher than that for an aerobic mixed population (usually C:N = 10:1–20:1) because *D. desulfuricans* partially oxidizes lactate to acetate and CO_2. Thus, cell production from substrate is approximately 10 times less than in aerobic systems and nitrogen requirements are 10 times less also.

EPS production rate increased with decreased nitrogen loading rate. Increase in EPS production may influence plugging of oil reservoirs and initial cell adsorption on surfaces. Lappan and Fogler[11] reported that cellular polysaccharide production was a significant factor in formation damage by bacteria, especially for low-permeability cores, with permeability reduction with polysaccharide production being 10 times greater than without polysaccharide production.

The experimental results indicate that SRB activity may be controlled by reducing nitrogen from injection water, since the deficiency of nitrogen results in a significant decrease in SRB activity. Nazina et al.,[15] however, reported that the mesophilic SRB isolated from oil fields have high nitrogenase activity, while the thermophilic SRB have weak nitrogenase activity. Thus, nitrogen fixation by mesophilic SRB may be considerable in an oil reservoir where the temperature range is appropriate for mesophilic SRB growth. It is of interest to determine the role of SRB in nitrogen fixation. If significant fixation occurs, removal of nitrogen from injection water may not be a reasonable means to control H_2S generation, even though growth rate and cell production will be much lower.

Sulfide Inhibition

Sulfide inhibition of SRB probably occurs when sulfide species (H_2S, HS^-, and S^{2-}) combine with the iron of the cytochrome and other essential iron-containing compounds in the cell, causing electron transport systems to cease activity. Thus, the pH of the system, which deter-

mines the distribution of sulfide species, plays a very important role in inhibition of microbial sulfate reduction. The relative distribution of H_2S and HS^- at pH 7.0 is about 1:1 (pK_a of H_2S is 7.0 at 25°C). The percentage of un-ionized H_2S drops from 90% at pH 6.0 to 50% at pH 7.0 to 10% at pH 8.0. Thus, the effects of un-ionized H_2S or ionized HS^- concentration on activity of a microbial population without pH effects can be observed by altering the culture pH in a narrow range. Toxicity of the various sulfide species to microorganisms may be different. Oleszkiewicz et al.[18] reported that the time required to achieve 90% utilization of lactate, butyrate, acetate, and propionate by an anaerobic mixed population grown in batch serum bottles was shortened at comparable total sulfide concentrations at a pH of 7.7–7.9 as compared to pH 6.5–7.4. The results suggest that un-ionized H_2S is the more toxic species of sulfide to an anaerobic mixed population, presumably due to its ease of transport through the cell membrane. Hilton and Oleszkiewicz[9] performed a series of batch experiments containing an undefined anaerobic population growing on lactate at initial pH 6.0, 7.0, and 8.0 and at various total sulfide concentrations. They report that sulfate reduction was inhibited in proportion to the total sulfide concentration, not the un-ionized H_2S concentration. Acidogenic and methanogenic processes were inhibited by un-ionized H_2S more than total sulfide. Thus, at a high level of total sulfide and high pH (low concentration of H_2S), the carbon flow in the batch reactor could be diverted from sulfate reduction to methane production as long as the H_2S concentration was below the inhibitory level to the methanogenic population. Shimada[28] reported that 100 mg/L of H_2S inhibited the growth of a mixed SRB population in batch culture and no significant SRB growth was observed at 500 mg/L of H_2S (the pH in this experiment was not reported). Burgess and Wood[3] reported that microbial sulfate reduction was inhibited by 900 mg S/L. However, batch culture results must be viewed with caution since pH may change as sulfide accumulates, imposing another stress on the population. Also sulfide precipitates Fe, so that Fe may become the limiting substrate for growth.[21]

In continuous culture experiments reported herein, lactate utilization slightly decreased to 92% at 280 mg/L total sulfide, whereas cell carbon production decreased from 6.0 to about 2.0 mg/L at 280 mg/L total sulfide (Fig. 9). The cell yield decreased dramatically from 0.03 g cell/g lactate at 50 mg/L total sulfide to 0.005 g cell/g lactate at 600 mg/L total sulfide. Cell yield may decrease because energy is expended in countering the inhibitory effect of sulfide and, thus, is diverted from cell production (maintenance energy requirement increases).

The experimental results indicate that under preexisting high sulfide concentration in the formation, biological sulfide production is not a favorable process. Although other nutritional and physical conditions are suitable for SRB growth, SRB activity is strongly inhibited by high sulfide concentration.

CONCLUSIONS

1. The observed specific growth rate and cell yield for *D. desulfuricans* under sulfate-limiting conditions are lower than those obtained under lactate-limiting conditions due to an increase in the maintenance energy requirement.
2. The limiting C:N ratio (w/w) for *D. desulfuricans* is in the range 45:1–120:1. The extent of extracellular polymeric substance (EPS) production increases with increasing carbon:nitrogen ratio in the medium. Total biomass yield (cell + EPS) remains constant.
3. Fifty percent inhibition of lactate utilization by *D. desulfuricans* occurs at approximately 500 mg/L of total sulfide. EPS production increases with increasing sulfide concentration.
4. Sulfide inhibition of *D. desulfuricans* activity is a reversible process.
5. Increasing C:N ratio and increasing sulfide concentration result in decreased cell size for *D. desulfuricans*.

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NOMENCLATURE

K_{Lac}	half-saturation coefficient for lactate (mg/L)
K_{Sul}	half-saturation coefficient for sulfate (mg/L)
m	maintenance coefficient (g/g h)
S	sulfate concentration at steady state (mg/L)
S_i	influent sulfate concentration (mg/L)
$Y^{Obs}_{c/Lac}$	observed cell yield on lactate (g/g)
$Y^{Int}_{c/Lac}$	intrinsic cell yield on lactate (g/g)
Y_o	observed cell yield (g/g)
$Y_{c/Sul}$	cell yield on sulfate (g/g)
$Y_{o/Sul}$	overall yield on sulfate (g/g)
Y^{max}_{ATP}	cell yield on ATP (g/mol)
$Y_{x/s}$	intrinsic cell yield (g/g)
μ	specific biomass growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

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