Effects of Temperature and Phosphorous Concentration on Microbial Sulfate Reduction by *Desulfovibrio desulfuricans*

S. Okabe and W. G. Characklis*
Center for Interfacial Microbial Process Engineering, Montana State University, Bozeman, Montana 59717

Received April 8, 1991/Accepted November 25, 1991

The effects of temperature and phosphorous concentration on the rate and the extent of microbial sulfate reduction with lactate as carbon and energy source were investigated for *Desulfovibrio desulfuricans*. The continuous culture experiments (chemostat) were conducted at pH 7.0 from 12 to 48°C. The maximum specific growth rate (μ_{max}) was relatively constant in the range 25°C–43°C and dramatically decreased outside this temperature range. The half-saturation coefficient was minimum at 25°C. Cell yield was highest in the optimum temperature range (35°C–43°C) for growth. Maintenance energy requirements for *D. desulfuricans* were not significant. Two moles of lactate is consumed for every mole of sulfate reduced, and this stoichiometric ratio is not temperature dependent. Steady state rate and stoichiometric coefficients accurately predicted transient behavior during temperature shifts. The extent of extracellular polymeric substance (EPS) is related to the concentration of phosphorous in the medium. EPS production rate increased with decreased phosphorous loading rate. Failure to discriminate between cell and EPS formation by *D. desulfuricans* leads to significant overestimates of the cell yield. The limiting C:P ratio for *D. desulfuricans* was in the range of 400:1 to 800:1.
Key words: *D. desulfuricans* • sulfate reduction • phosphorous limitation • kinetics • stoichiometry • temperature effect

INTRODUCTION

Sulfate-reducing bacteria (SRB) are very important microbes from an environmental and industrial standpoint. For example, in petroleum technology, SRB cause serious problems including corrosion of installations, plugging of the formation, and contamination of petroleum with H_2S (souring) in the formation.[9,10,25] Cochrane et al.[6] report that the presence and growth of thermophilic SRB at temperatures greater than 60°C was 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 et al.[12] report that substantial levels of short-chain fatty acids and ammonia present in many formation waters can be used directly by SRB as a source of energy. However, quantitative description of reservoir souring is essentially impossible because coefficients for rate and extent of SRB growth under relevent environmental conditions are not available.

The microbial environment varies widely through the formation. The temperature varies from that of the cold injection water to that of the hot formation. The system temperature can have a major influence on SRB activity. Most mesophilic SRB have an optimum growth temperature in the range of 25 to 40°C. Temperature outside this range may account for variation in SRB activity. Temperature gradients in the formation cause changes in SRB growth and associated variables. Biocide treatments must be tailored to these microbial growth patterns.

Concentrations of sulfate, substrate (carbon source), and essential nutrients (e.g., phosphorous) in the formation vary as they are depleted by microbial activity or are mediated by the formation itself. A determination of the limiting nutrient concentration may be useful to control and predict SRB activity in industrial systems. Essential nutrients (e.g., phosphorous and nitrogen) affect the rates of SRB activity and growth when they become limiting. Seawater has sufficient nutrients to support an active but minimal SRB population (e.g., total P in seawater is 0.001 to 0.1 mg/L).[1] Therefore, the reduction of the concentration of an essential nutrient to below the limiting concentration is a possible means of controlling SRB. Ironically, water treatment chemicals may enrich the system in C, N, and P.

The goal of this study was to determine effects of temperature and phosphorous concentration on rate and stoichiometry of microbial sulfate reduction by *Desulfovibrio desulfuricans*.

BACKGROUND

Stoichiometry and Rate

There are two chracteristics of microbial reactions of importance to process design and control: stoichiometry and rate. Stoichiometry indicates the changes which will occur and their extent. Rate describes how fast the changes will occur. Both process stoichiometry and rate

* To whom all correspondence should be addressed.

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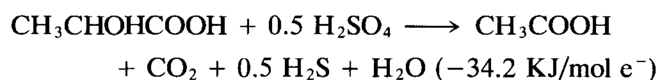
CCC 0006-3592/92/01001031-012$04.00

must be known to effectively design and control technical scale processes.

Stoichiometry

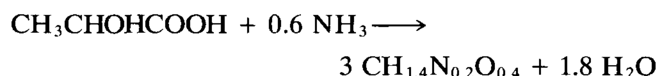
Dissimilatory sulfate reduction can be represented by a pair of stoichiometric equations that describe the oxidation of an organic carbon source and the synthesis of bacterial cells. For example, if lactate is the electron donor and energy source, approximate stoichiometric equations for sulfate reduction are as follows:

Energy:



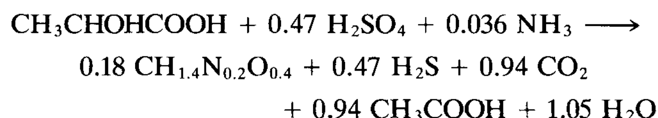
$$CH_3CHOHCOOH + 0.5\ H_2SO_4 \longrightarrow CH_3COOH + CO_2 + 0.5\ H_2S + H_2O\ (-34.2\ KJ/mol\ e^-)$$

Synthesis:



$$CH_3CHOHCOOH + 0.6\ NH_3 \longrightarrow 3\ CH_{1.4}N_{0.2}O_{0.4} + 1.8\ H_2O$$

Overall stoichiometry:

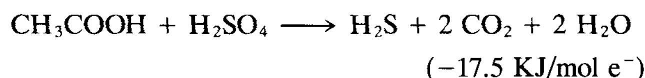


$$CH_3CHOHCOOH + 0.47\ H_2SO_4 + 0.036\ NH_3 \longrightarrow 0.18\ CH_{1.4}N_{0.2}O_{0.4} + 0.47\ H_2S + 0.94\ CO_2 + 0.94\ CH_3COOH + 1.05\ H_2O$$

The overall stoichiometric equation was balanced using the experimental determinations of overall yield and carbon dioxide production by *D. desulfuricans.*[31] D'Alessandro et al.[7] reported very similar stoichiometry for sulfate reduction by *D. vulgaris.* In both cases, lactate and sulfate were consumed in a 2:1 molar ratio.

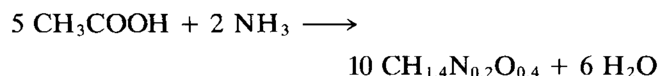
If acetate is the electron donor and energy source, approximate stoichiometric equations for sulfate reduction are as follows:

Energy:



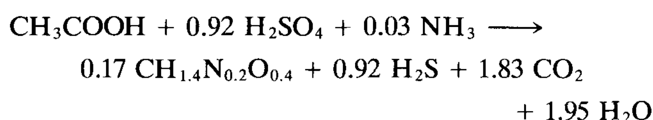
$$CH_3COOH + H_2SO_4 \longrightarrow H_2S + 2\ CO_2 + 2\ H_2O\ (-17.5\ KJ/mol\ e^-)$$

Synthesis:



$$5\ CH_3COOH + 2\ NH_3 \longrightarrow 10\ CH_{1.4}N_{0.2}O_{0.4} + 6\ H_2O$$

Overall stoichiometry:



$$CH_3COOH + 0.92\ H_2SO_4 + 0.03\ NH_3 \longrightarrow 0.17\ CH_{1.4}N_{0.2}O_{0.4} + 0.92\ H_2S + 1.83\ CO_2 + 1.95\ H_2O$$

The experimental overall yield obtained by Middleton and Lawrence[21] was used to balance the overall stoichiometric equation. Each mole of lactate and acetate transfers 4 and 8 electrons, respectively. The yields for SRB growth with lactate, acetate, butyrate, and propionate as sole energy and carbon sources have been reported (Tables I–III). The stoichiometric relationships are important since they permit estimation of the rate and extent of biomass and product formation by measuring changes in sulfate concentration with time.

The reporting of yield requires further definition. This article reports yield data from various sources which are not directly comparable. Generally, yield is expressed as the ratio of product formed to reactant consumed. In biological reactors, yield generally refers to the ratio of particulate matter produced to soluble substrate consumed. In an SRB chemostat with sterile feed containing only dissolved components, the extent of particulate products formed may include cells, extracellular polymeric substances (EPSs), and sulfide precipitates (e.g., FeS). The EPSs may be immobilized on the cells, released into the bulk liquid phase, or both. The sulfide precipitates may be suspended in the bulk liquid (if sufficient mixing is provided) or may be adsorbed on the cells or EPSs. Thus, the means of measuring product formation will operationally define the yield. For purposes of this article, three (3) yields are defined:

Overall yield: Y_o = (cells + EPS + precipitates)/(substrate consumed)

Biomass yield: Y_b = (cells + EPS)/(substrate consumed)

Cell yield: Y_c = (cells)/(substrate consumed)

where Y_o is obtained when gravimetric (suspended solids) determinations are employed. Precipitates may or may

Table I. Reported SRB rate and stoichiometric parameters for lactate-utilizing SRB at 30°C.

Investigator	Organisms	μ_{max} (h^{-1})	K_{Lac} (mg/L)	K_{Sul} (mg/L)	$Y_{o/Lac}$ (g/g)	$Y_{o/Sul}$ (g/g)	pH	B/C[a]
Cappenberg[3]	*D. desulfuricans*	0.360	4.4	—	0.343	—	7.4	C
Traore et al.[31]	*D. desulfuricans*	0.104	—	—	0.046	—	—	B
	D. africans	0.060	—	—	0.019	—	—	B
	D. gigas	0.092	—	—	0.042	—	—	B
Traore et al.[32]	*D. vulgaris*	—	—	—	0.075	—	7.2	B
Ingvorsen et al.[15]	*D. vulgaris*	0.011	—	0.5	—	0.074	7.2	B
	D. sapovorans	0.007	—	0.7	—	0.091	7.2	B
	D. salexigens	0.021	—	7.4	—	0.083	7.2	B

[a] Refers to batch or continuous (chemostat) reactor.

Table II. Reported SRB rate and stoichiometric parameters for acetate-utilizing SRB.

Investigator	Organisms	μ_{max} (h^{-1})	K_{Ace} (mg/L)	K_{Sul} (mg/L)	$Y_{o/Ace}$ (g/g)	$Y_{o/Sul}$ (g/g)	Temperature (°C)	B/C[a]
Middeleton and Lawrence[21]	Mixed population	0.007	250	—	0.065	—	20	B
		0.019	92	—	0.065	—	25	B
		0.022	5.7	—	0.065	—	31	B
Ingovorsen et al.[16]	*Desulfobactor postgatei*	0.030	—	4.2	—	0.158	30	B
Widdel and Pfenning[33]	*D. postgatei*	0.035	—	—	0.074	—	32	B
Schauder et al.[27]	*D. postgatei*	0.025	—	—	—	—	30	B
		0.046	—	—	—	—	30	B
Widdel and Pfenning[34]	*Desulfotomaculum acetoxidans*	0.058	—	—	0.095	—	36	B
Schauder et al.[27]	*D. acetoxidans*	0.014	—	—	—	—	30	B
		0.032	—	—	—	—	37	B
Schauder et al.[27]	*Desulfobacter hydrogenophilus*	0.039	—	—	—	—	30	B
Widdel[35]	*D. hydrogenophilus*	0.038	—	—	—	—	25–28	B
Widdel et al.[36]	*Desulfonema* sp.	0.023	—	—	—	—	30	B
		0.0069	—	—	—	—	30	B
Widdel[35]	*Desulfobacter curvatus*	0.033	—	—	—	—	25–28	B

[a] Refers to batch or continuous (chemostat) reactor.

Table III. Reported SRB rate and stoichiometric parameters for propionate and butyrate-utilizing SRB.

Investigator	Organisms	μ_{max} (h^{-1})	K_{Pro} (mg/L)	Y (g/g)	Temperature (°C)	B/C[a]
Nanninga and Gottschal[23]	*Desulfobulbus propionicus*	0.110	—	—	35	B
Widdel and Pfenning[37]	*D. propionicus*	0.069	—	0.071[b]	39	B
Widdel and Pfenning[34]	*Desulfomaculum acetoxidans*	0.046	—	—	36	B
Schauder et al.[27]	*Desulfovibrio baarsi*[c]	0.017	—	—	30	B
Nanninga and Gottschal[23]	*Desulfovibrio sapovorans*[c]	0.066	—	—	35	B
Hunter[14]	Mixed population	0.070	90.0	0.022[d]	35	C

[a] Refers to batch or continuous (chemostat) reactor.
[b] Growing on 5 mM/L of propionate, $Y_{o/Pro}$.
[c] Growing on butyrate.
[d] $Y_{c/Pro}$.

not exist in the samples, so further analysis of the solids is recommended. Biomass yield Y_b can be estimated by measurement of the particulate organic carbon. Finally, this article describes a method for independently estimating the cells and EPSs produced. The method combines organic carbon analysis and cell size measurements accomplished by image analysis.[26]

The limiting nutrient or substrate considered in this article can be the energy source or electron donor (e.g., lactate) or the electron acceptor (sulfate). As a consequence, the yield symbol differentiates between yield based on electron donor (e.g., $Y_{c/Lac}$ for cell yield based on lactate consumption) or electron acceptor (e.g., $Y_{b/Sul}$ for biomass yield based on sulfate consumption).

Rate

The rate of a microbial reaction may be described by the Monod expression as a function of limiting substrate concentration (e.g., lactate):

$$\mu = (\mu_{max} \cdot S)/(K_{Lac} + S) \quad (1)$$

where μ is specific growth rate (h^{-1}), μ_{max} is maximum specific growth rate (h^{-1}), S is lactate concentration (mg/L), and K_{Lac} is the half-saturation coefficient for lactate (mg/L).

For SRB growth with lactate and acetate as energy and carbon source μ_{max} and K_s have been reported (Tables I and II). Lactate-utilizing SRB are capable of doubling times as low as 3–6 h at 30°C, whereas acetate-utilizing SRB grow more slowly with doubling times longer than 20 h.[25] However, the data presented in Tables I–III show wide variations and are also very incomplete. Thus, it is very difficult to draw further conclusions from these data.

Mathematical Description for Chemostat

Microbial transformations in chemostats can be mathematically described by mass balance equations assum-

ing the two fundamental processes occurring are growth and maintenance. Growth rate depends upon substrate (e.g., lactate) concentration according to Eq. (1). Lactate is assumed to be partitioned into cellular and EPS (biomass = cellular + EPS). Mass balances for lactate, cellular, and EPS in the liquid phase are as follows:

Lactate:

$$dS/dt = D(S_i - S) - C \cdot \mu/Y_{c/\mathrm{Lac}} - r_p \cdot C/Y_{p/\mathrm{Lac}} - m \cdot C \quad (2)$$

Cellular:

$$dC/dt = D(C_i - C) + \mu \cdot C \quad (3)$$

EPS:

$$dP/dt = D(P_i - P) + r_p \cdot C \quad (4)$$

where S is lactate concentration (mg/L), S_i is influent lactate concentration (mg/L), C is cell concentration (mg/L), C_i is influent cell concentration (mg/L), $Y_{c/\mathrm{Lac}}$ is the cell yield coefficient (mg cellular/mg lactate), $Y_{p/\mathrm{Lac}}$ is the EPS yield coefficient (mg EPS/mg lactate), P is EPS concentration (mg/L), P_i is influent EPS concentration (mg/L), r_p is the specific EPS formation rate (mg EPS/mg cell/t), D is the dilution rate (h^{-1}), m is the maintenance coefficient (mg lactate/mg biomass/h), and t is time (h). For a sterile feed, $C_i = 0$, $P_i = 0$, and at steady state, Eqs. (2)–(4) become

$$D(S_i - S)/C = \mu/Y_{c/\mathrm{Lac}} + r_p/Y_{p/\mathrm{Lac}} + m \quad (5)$$

$$D = \mu \quad (6)$$

$$DP = r_p \cdot C \quad (7)$$

All quantities on the left side of Eqs. (5)–(7) are measurable. Equation (6) indicates that μ can be controlled by the experimenter. Equation (5) simplifies to the following expression if EPS formation if negligible:

$$D(S_i - S)/C = \mu/Y_{c/\mathrm{Lac}} + m \quad (8)$$

EXPERIMENTAL MATERIALS AND METHODS

Experimental System

The rate and stoichiometric coefficients at several temperatures and phosphorous concentrations were determined in a chemostat consisting of a pyrex cylindrical beaker (0.45×10^{-3} m^3 volume) with a Teflon lid sealed with an O-ring (Fig. 1). The chemostat was equipped with an inverted plastic funnel attached to metal rods that served to remove wall growth during long runs. Heavy wall butyl rubber tubing (Cole-Parmer, Masterflex neoprene tubing) was used to minimize oxygen flux. The flow rates were controlled by the pump and speed controller (Cole-Parmer, Chicago, IL).

The pH was maintained at 7.0 ± 0.2 by a pH controller which automatically added sterile, oxygen-free, 1.0*N* HCl or NaOH solutions stored under nitrogen atmosphere. The temperature was maintained by a thermoregulator and heating blanket. 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.

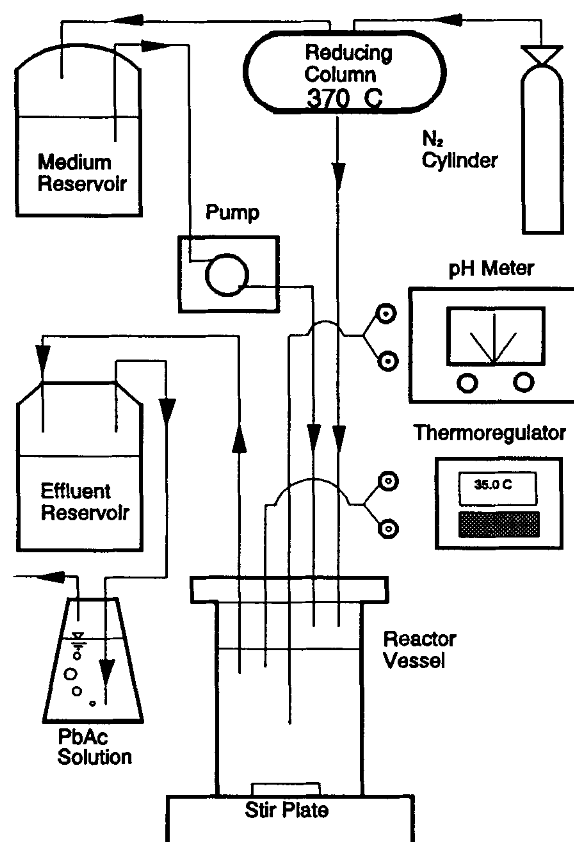


Figure 1. Diagram of experimental chemostat system.

Desulfovibrio desulfuricans (ATCC 5575) was grown in Postgate medium G, including hemi-calcium lactate (L-lactic acid, Sigma, No. L-2000) as the sole carbon and energy source. Trace elements and vitamins were added (Table IV). The stock solutions were mixed with base medium after autoclaving and cooling under nitrogen purge. For the temperature effect experiments, 0.5 g/L of Na_2SO_4 was used. Sterile $Na_2S \cdot 9H_2O$ was added as a reductant until a vigorously growing culture was established. The final concentration of sodium hydrosulfide was 300 mg/L. For long-term storage, *D. desulfuricans* was preserved at −70°C.

The entire chemostat assembly was washed with chromic sulfuric acid and rinsed out with redistilled water and then autoclaved for 15 min at 121°C. After cooling down, the autoclaved culture medium was fed into the reactor under nitrogen purge. After about 4–5 h, the test organisms were introduced into the reactor. The reactor was operated as a batch culture until turbidity of the medium was clearly observed. Then continuous flow of media commenced. Steady state conditions were

Table IV. Composition of Postgate medium G.[a]

Component		Amount
Base medium		
Distilled water		996.0 mL
Na_2SO_4[b]		3.0 g
NaCl		1.2 g
KCl		0.3 g
NH_4Cl		0.3 g
$MgCl_2 \cdot 6H_2O$		0.4 g
KH_2PO_4		0.2 g
$CaCl_2 \cdot 2H_2O$		0.15 g
Calcium lactate		0.3125 g
Trace element solution	1.0 mL	
Distilled water		993 mL
HCl (25%)		6.5 mL
$FeCl_2 \cdot 4H_2O$		1.5 g
H_3BO_3		0.06 g
$MnCl_2 \cdot 4H_2O$		0.1 g
$CoCl_2 \cdot 6H_2O$		0.12 g
$ZnCl_2$		0.07 g
$NiCl_2 \cdot 6H_2O$		0.025 g
$CuCl_2 \cdot 2H_2O$		0.015 g
$Na_2MoO_4 \cdot 2H_2O$		0.025 g
Selenite solution	1.0 mL	
Distilled water		1000 mL
NaOH		0.5 g
Na_2SeO_3		0.003 g
Vitamin solution	1.0 mL	
Distilled water		1000 mL
Biotin		0.01 g
p-Aminobenzoic acid		0.05 g
Vitamin B_{12}		0.05 g
Thiamine		0.1 g
Na-dithionite solution	1.0 mL	
Oxygen-free distilled water		100 mL
$Na_2S_2O_4$		3.0 g
Sodium sulfide solution[c]	3.0 mL	
Oxygen-free distilled water		100 mL
$Na_2S \cdot 9H_2O$		12.0 g

[a] From ref. 25.
[b] 0.5 g/L of Na_2SO_4 was used for temperature effect experiments.
[c] This solution was only used until a vigorously growing culture was established.

periodically checked by measuring cell number and sulfate concentration in the effluent.

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, (8) phosphorous, and (9) suspended solids. The samples for SOC, lactate, acetate, sulfate, and phosphorous analyses were obtained by filtering an aliquot of the chemostat effluent through 0.20-μm Nucleopore filters.

The TOC and SOC were measured with a Dohrmann carbon analyzer DC-80 (Dohrmann, Santa Clara, CA). Unfiltered samples for TOC and filtered samples for SOC were frozen until analyses. Five-milliliter samples were acidified with concentrated phosphoric acid and then bubbled with O_2 gas for a few minutes. Total bacterial counts were determined using an image analyzer (Cambridge/Olympus Quantment 10) by the epifluorescence method described by Hobbie et al.[13] Sulfate concentration was measured by the barium–sulfate turbidimetric method.[29] Lactate concentration was measured by a specific enzymatic method (L-lactic measurement, Boehriger, Mannheim, Germany) as described by Cappenberg.[2] Gas chromatography with flame ionization detector (Varian 3700 model) was used to measure acetate concentration. A 2-m × 2-mm glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20M (Supelco, Belletonte, PA) was used. The column was maintained at 175°C.

Samples were prepared by adding oxalic acid and internal standard solution (trimethylacetic acid) to bring the final concentration to 0.06*M* and 200 mg/L, respectively. Samples were frozen until analyses. The methylene blue method described by Cline[14] was used to measure dissolved sulfide concentration. The volatile sulfide was measured by trapping the gaseous sulfide in 1.0*N* NaOH solution. Phosphorous concentration was measured as orthophosphorus using the modified ammonium molybdate–ascorbic acid method described by Harwood et al.[15] Total suspended solids was determined by filtering 20-mL samples through prewashed, dried, and preweighed 0.20-μm Nuclepore filters. Samples were rinsed twice with 5 mL membrane-filtered water and dried at 103°C for 1 h and reweighed. Biovolumes were estimated by measuring the cell size with the image analyzer. Biovolumes were converted into cellular carbon estimates using the following conversion factors: 1.07 g wet cell weight/cm,[38] 0.22 g dry cell weight/g wet cell weight,[20] and 0.465 g cell carbon/g dry cell weight.[8] The EPS carbon was calculated by subtracting the calculated cellular carbon from the total biomass carbon (effluent TOC − effluent SOC).

RESULTS

Steady State Cellular Carbon and Lactate Concentrations

Classical behavior was generally observed for the steady state dependence of cellular carbon and lactate concentration on dilution rate at 25°C (Fig. 2). The EPS carbon concentrations were not significant at any dilution rate for the carbon-limiting experiments. Therefore, cellular carbon concentrations were calculated as differences between effluent TOC concentration and effluent SOC concentration. The maintenance coefficient was negligible, although it may become important at dilution rates less than 0.05 h^{-1}. The effluent sulfate concentrations were in the order of 200 to 300 mg/L (depending on dilution rate) so that this chemostat system was limited by the lactate supply.

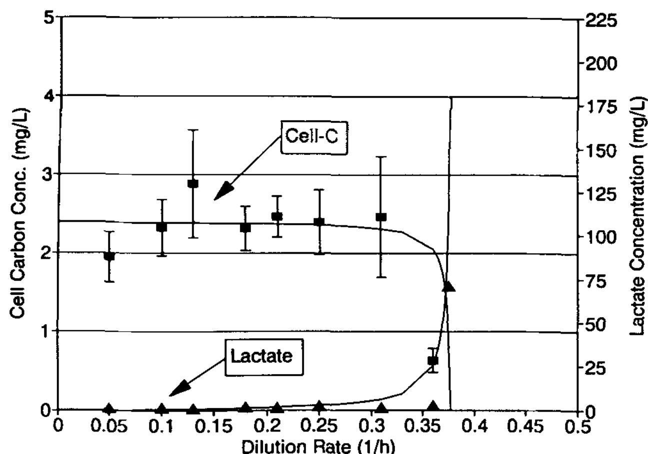


Figure 2. Experimental dependence of the steady state cellular carbon and lactate concentration on the dilution rate at 25°C. The solid lines are drawn using chemostat mass balance equations. The influent lactate concentration was 180 mg/L. Error bars represent the standard deviation of triplicate measurements.

Estimation of Monod Growth Parameters

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

$$S = (K_{Lac} \cdot \mu)/(\mu_{max} - \mu) \quad (9)$$

The nonlinear regression of the Monod equation was performed using MSU SAS (statistical software). The program produces estimates of μ_{max} and K_s from given data pairs of limiting substrate concentration (S) and specific growth rate ($\mu = D$). The standard error and 95% confidence interval associated with the estimate of each parameter were also determined. The resulting μ_{max} and K_{Lac}, along with their respective standard error, at each temperature are presented in Table V. The highest maximum growth rate of 0.55 h^{-1} was obtained at 43°C. Below 25°C and above 43°C, the maximum specific growth rate decreased dramatically to 0.059 h^{-1} at 12°C and 0.115 h^{-1} at 48°C, respectively. The activation energy for μ_{max} was 14 KJ/mol in the range 25°C to 43°C and 104 KJ/mol below 25°C (Fig. 3). The half-saturation coefficient (K_{Lac}) was minimum at 25°C (Fig. 4). The activation energy for K_{Lac} above 25°C was 47 KJ/mol and below 25°C was −52 KJ/mol. The highest cell yield ($Y_{c/Lac}$) was observed in the optimum temperature range for growth, 35°C–43°C (Fig. 5).

The steady-state experimental results obtained for μ_{max}, K_{Lac}, and $Y_{c/Lac}$ can be summarized by the linear interpretation shown in Figures 3–5, which are given specifically by the following functions:

$$\mu_{max}(T) = 10^{6.3686} \times 10^{(-2094.52/T(K))} \quad (35°C < T < 43°C) \quad (10)$$

$$\mu_{max}(T) = 10^{-43.894} \times 10^{(13788.53/T(K))} \quad (43°C < T < 48°C) \quad (11)$$

$$K_{Lac}(T) = 10^{11.41} \times 10^{(-3363.73/T(K))} \quad (35°C < T < 48°C) \quad (12)$$

$$Y_{c/Lac}(T) = 10^{1.1053} \times 10^{(-814.23/T(K))} \quad (12°C < T < 42°C) \quad (13)$$

$$Y_{c/Lac}(T) = 10^{-10.5735} \times 10^{(2868.44/T(K))} \quad (42°C < T < 48°C) \quad (14)$$

Maintenance coefficients m were determined by graphical methods using Eq. (5), but their quantitative measure was not statistically significant (Table V).

Transient Response to Step Changes in Temperature

To consider the dynamic response of the population to temperature, the effects of step changes in temperature were observed at a dilution rate of 0.1 h^{-1}. The transient response in lactate utilization $[(S_i - S)/S_i]$ was observed for step increases in temperature from 35 to 43 to 53°C followed by a decrease to 48°C (Fig. 6). At 4.5 h the temperature was increased from 35°C to 43°C and lactate utilization did not change. However, the lactate utilization rapidly decreased after the temperature was shifted from 43°C to 53°C. Lactate utilization continued to decrease until the temperature was shifted from 53°C to 48°C. At the high dilution rate ($D = 0.1$ h^{-1}), the reactor was near washout at higher temperature. The response of lactate utilization $[(S_i - S)/S_i]$ to a step change in temperature was simulated using the mass balance equations (2)–(4) and temperature functions for parameters $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(T)$ represented by Eqs. (10)–(14). The simulation assumes that $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(T)$ take their new steady state values immediately after the temperature change. The results indicate that parameters determined at steady state can

Table V. Experimentally determined kinetic parameters for *D. desulfuricans* growing on lactate as the sole carbon and energy source (estimated parameter value ±SE).

Temperature (°C)	μ_{max} (h^{-1})	K_{Lac} (mg/L)	$Y_{c/Lac}$ (g/g)	m (g/g · h^{-1})
12	0.059 ±0.001	3.7 ±0.75	0.017 ±0.001	−0.35 ±0.54
25 (run 1)	0.38 ±0.002	1.4 ±0.39	0.025 ±0.002	0.23 ±0.36
25 (run 2)	0.41 ±0.001	10.2 ±0.24	0.025 ±0.004	2.12 ±4.13
35 (run 1)	0.37 ±0.004	2.2 ±0.60	0.024 ±0.006	0.45 ±1.08
35 (run 2)	0.46 ±0.11	3.6 ±2.48	0.036 ±0.004	0.82 ±1.14
43	0.55 ±0.003	10.0 ±1.22	0.032 ±0.001	0.28 ±0.0
48	0.115 ±0.001	6.4 ±0.75	0.023 ±0.001	0.27 ±0.47

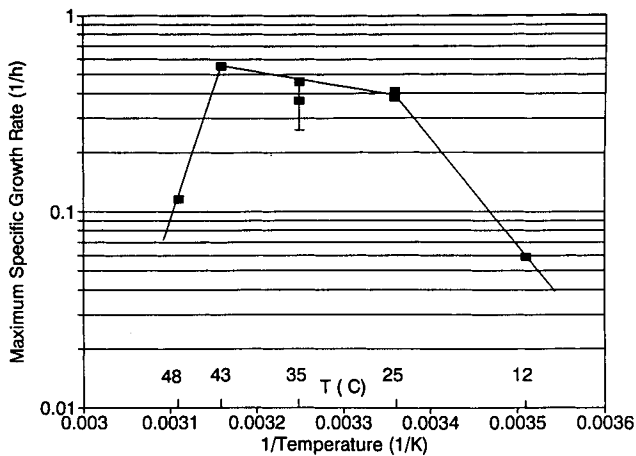


Figure 3. Temperature dependence of the maximum specific growth rate (μ_{max}). The highest μ_{max} of 0.55 h^{-1} was obtained at 43°C. The activation energies for μ_{max} were 14 KJ/mol in the range 25–43°C and 104 KJ/mol below 25°C. Error bars represent the standard error of the estimated μ_{max}. The error bar was not given except one point at 35°C because their standard errors are so small (Table V).

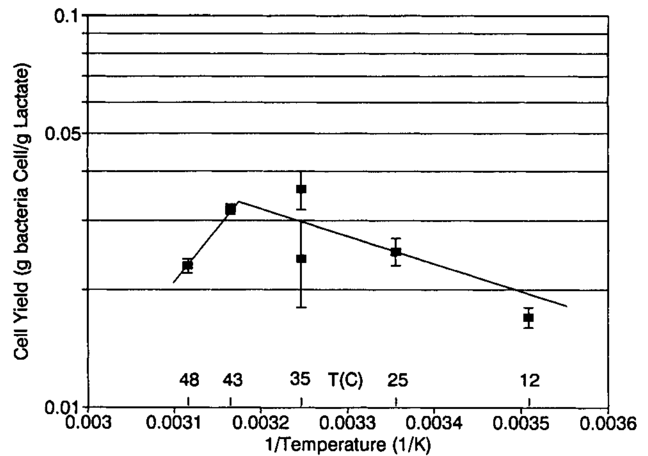


Figure 5. Temperature dependence of the cell yield coefficient ($Y_{c/Lac}$). The highest cellular yield was observed around optimum temperature for growth. Error bars represent the standard error of the estimated $Y_{c/Lac}$.

accurately describe effects of temperature transition within the ranges tested. The response of cell yield coefficient ($Y_{c/Lac}$) to a step change in temperature was simulated in the same way (Fig. 7).

Estimation of Stoichiometric Coefficients

The stoichiometry for microbial sulfate reduction was developed from the experimental data obtained at different temperatures (Table VI). The stoichiometric coefficients were balanced by assuming the following: (1) empirical formulation ($CH_{1.4}N_{0.2}O_{0.4}$) for bacterial cells, (2) nitrogen source is only NH_3, and (3) amount of other end products of lactate oxidation besides acetate and CO_2 are negligible. The stoichiometric coefficients for lactate, sulfate, bacterial cells, and acetate were obtained from the experimental data. The stoichiometric

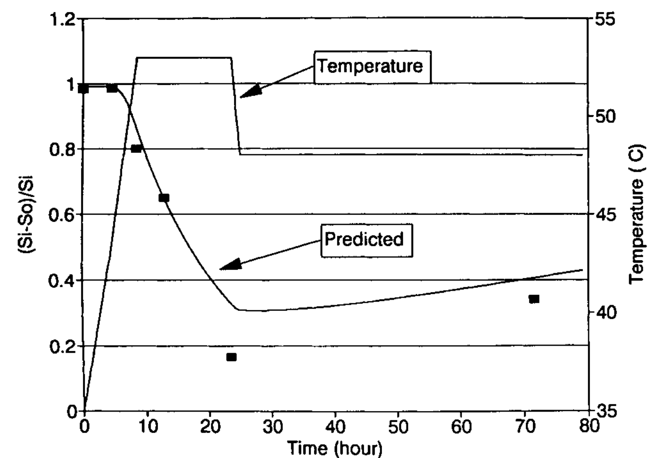


Figure 6. Transient response of lactate oxidation $((S_i - S)/S_i)$ to step up in temperature from 35°C to 53°C: (■) experimental points. Theoretical curve according to $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(C)$: $D = 0.1\ h^{-1}$, pH 7.0, $S_i = 200$ mg/L.

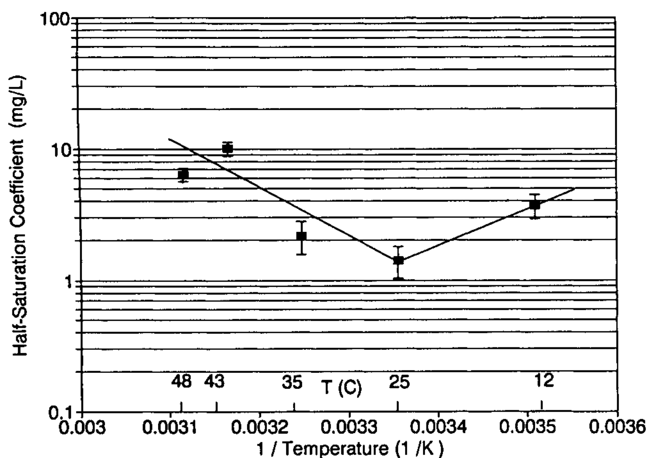


Figure 4. Temperature dependence of the half-saturation coefficient (K_{Lac}). The activation energies were 47 KJ/mol above 25°C and −52 KJ/mol below 25°C. Error bars represent the standard error of the estimated K_s.

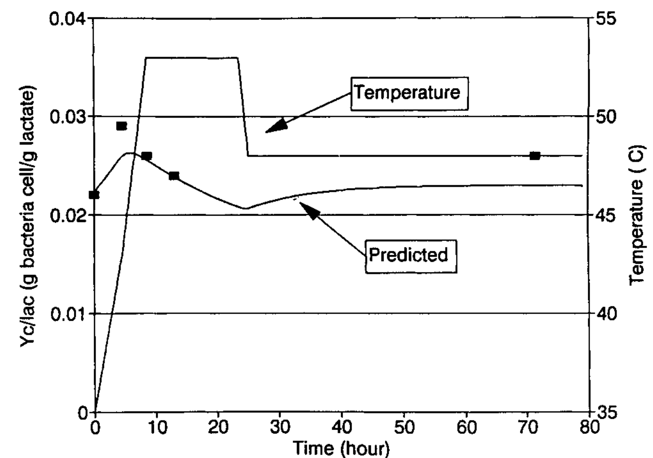
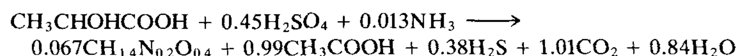


Figure 7. Transient response of cell yield ($Y_{c/Lac}$) to step up in temperature from 35°C to 53°C: (■) experimental points. Theoretical curve based on $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(C)$: $D = 0.1\ h^{-1}$, pH 7.0, $S_i = 200$ mg/L.

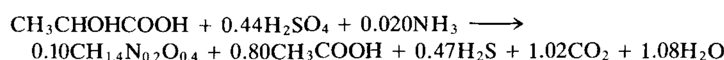
Table VI. Influence of temperature on the stoichiometry of microbial sulfate reduction with lactate.

At 12°C (102%)[a]:



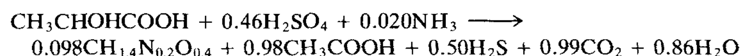
$$CH_3CHOHCOOH + 0.45H_2SO_4 + 0.013NH_3 \longrightarrow 0.067CH_{1.4}N_{0.2}O_{0.4} + 0.99CH_3COOH + 0.38H_2S + 1.01CO_2 + 0.84H_2O$$

At 25°C (run 1) (91%)[a]:



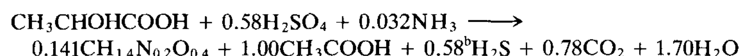
$$CH_3CHOHCOOH + 0.44H_2SO_4 + 0.020NH_3 \longrightarrow 0.10CH_{1.4}N_{0.2}O_{0.4} + 0.80CH_3COOH + 0.47H_2S + 1.02CO_2 + 1.08H_2O$$

At 25°C (run 2) (102%)[a]:



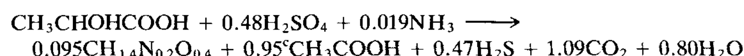
$$CH_3CHOHCOOH + 0.46H_2SO_4 + 0.020NH_3 \longrightarrow 0.098CH_{1.4}N_{0.2}O_{0.4} + 0.98CH_3COOH + 0.50H_2S + 0.99CO_2 + 0.86H_2O$$

At 35°C (run 1) (97%)[a]:



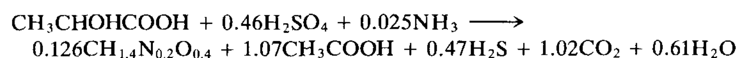
$$CH_3CHOHCOOH + 0.58H_2SO_4 + 0.032NH_3 \longrightarrow 0.141CH_{1.4}N_{0.2}O_{0.4} + 1.00CH_3COOH + 0.58^{b}H_2S + 0.78CO_2 + 1.70H_2O$$

At 35°C (run 2):



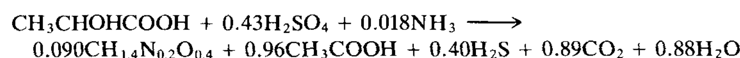
$$CH_3CHOHCOOH + 0.48H_2SO_4 + 0.019NH_3 \longrightarrow 0.095CH_{1.4}N_{0.2}O_{0.4} + 0.95^{c}CH_3COOH + 0.47H_2S + 1.09CO_2 + 0.80H_2O$$

At 43°C (110%)[a]:



$$CH_3CHOHCOOH + 0.46H_2SO_4 + 0.025NH_3 \longrightarrow 0.126CH_{1.4}N_{0.2}O_{0.4} + 1.07CH_3COOH + 0.47H_2S + 1.02CO_2 + 0.61H_2O$$

At 48°C(97%)[a]:



$$CH_3CHOHCOOH + 0.43H_2SO_4 + 0.018NH_3 \longrightarrow 0.090CH_{1.4}N_{0.2}O_{0.4} + 0.96CH_3COOH + 0.40H_2S + 0.89CO_2 + 0.88H_2O$$

[a] Percentage of carbon recovery.
[b] This value is calculated from consumed sulfate concentration.
[c] This value is calculated from carbon mass balance.

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 because the only carbon escaping the experimental system 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 within 10% of the amounts of lactate–carbon added.

Effect of Phosphorous Concentration

Desulfovibrio desulfuricans was grown at a dilution rate of 0.2 h^{-1} at 35°C and at phosphorous concentrations of 48.47, 4.60, 0.39, and 0.03 mg P/L to determine the effects of phosphorous concentration on stoichiometry. Steady state results, along with their respective standard deviations, are presented in Table VII.

EPS and Cellular Carbon

Mean values for effluent cellular and EPS carbon, along with their respective standard deviations, are given in Figure 8. With decreasing phosphorous concentration, EPS carbon concentration increased from 0 mg EPS C/L at 48.47 mg P/L to 2.1 mg EPS C/L at 0.03 mg P/L. In contrast, cellular carbon concentration decreased from 4.0 mg cellular C/L at 48.47 mg P/L to 1.1 mg cellular C/L at 0.03 mg P/L. The EPS yield increased and cell yield decreased with decreasing phosphorous concentration (Fig. 9).

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

Influent			Effluent		
Phosphorous	Lactate	Sulfate	Phosphorous	Lactate	Sulfate
48.47 ±0.08	402.4 ±5.3	2560.1 ±12.1	47.97 ±0.04	0.0 ±0.0	2341.1 ±2.7
4.60 ±0.02	437.4 ±29.8	2573.6 ±17.8	4.13 ±0.0	3.7 ±1.3	2341.1 ±14.5
0.39 ±0.01	411.0 ±24.9	2532.9 ±6.6	0.06 ±0.0	5.1 ±0.7	2296.5 ±4.7
0.03 ±0.0	382.9 ±7.8	2540.7 ±35.4	0.00 ±0.0	113.3 ±3.4	2405.0 ±9.9

Values given are the mean of triplicate measurements ±1 SD (in mg/L).

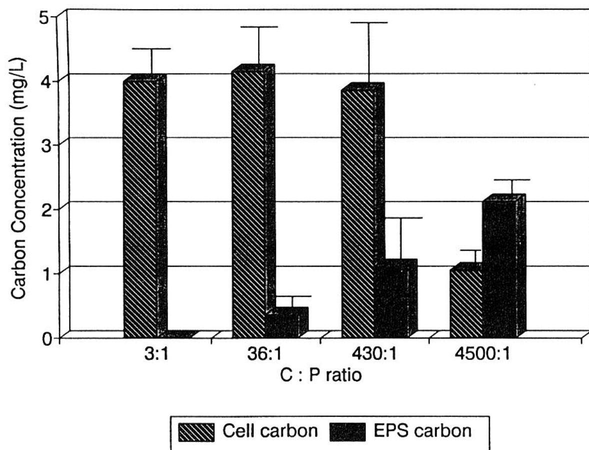


Figure 8. Change in cell and EPS carbon concentration with changing C:P ratio. Error bars represent the standard deviation of triplicate measurements.

Estimation of Phosphorous Requirement

The effluent lactate concentrations increased from 0.0 mg lactate/L at 48.47 mg P/L to 113.3 mg lactate/L at 0.03 mg P/L (Fig. 10). The effluent phosphorous concentration at 0.39 mg P/L was still measurable. Thus, at 0.39 mg P/L of phosphorous, medium was low in phosphorous. At lower phosphorous levels, the culture shifted from lactate-limited to phosphorous-limited. The stoichiometries for microbial sulfate reduction changed with changing phosphorous concentrations (Table VIII). Triplicate measurements of all reactant and product concentrations were within 15% of their respective mean values at each phosphorous loading concentration. The CH_2O was used as an empirical formula for EPS. The recovery of carbon in the four stoichiometric equations was within 10% of the amounts of lactate–carbon added. The stoichiometries were in good agreement until phosphorous concentration reached 4.60 mg P/L. At 0.39 mg P/L, there was less phosphorous consumption. The cell yield, however, was the same at phosphorous

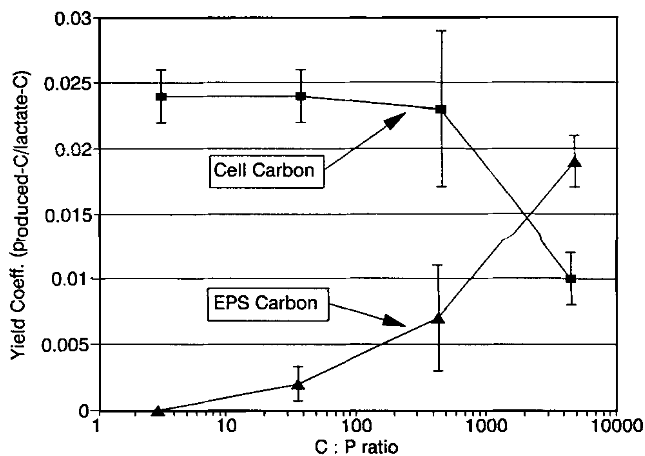


Figure 9. Change in cell and EPS carbon yield coefficients with changing C:P ratio. Error bars represent the standard deviation of triplicate measurements.

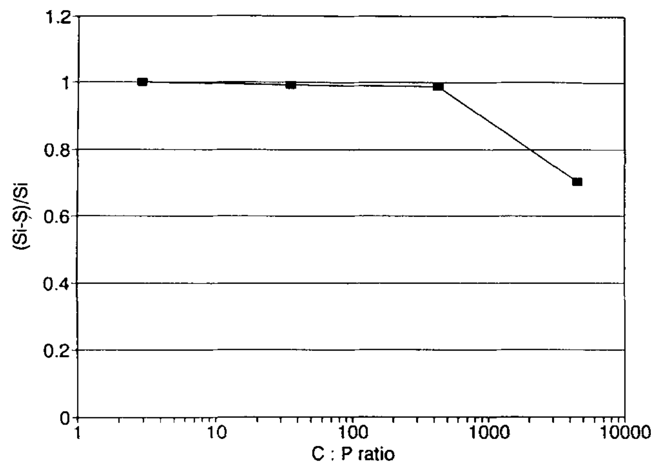


Figure 10. Response of lactate oxidation ($(S_i - S)/S_i$) by *D. desulfuricans* to change in the phosphorous concentration: $D = 0.2\ h^{-1}$, temperature 35°C. The limiting C:P ratio is in the range 400:1 to 800:1.

concentrations of 48.47 and 4.60 mg P/L. The evidence suggests that the bacteria adapted to more efficient phosphorous utilization. At 0.03 mg P/L, phosphorous limitation was evidenced by (1) significant amounts of lactate in the effluent, (2) lower cell yield, and (3) complete phosphorous removal.

DISCUSSION

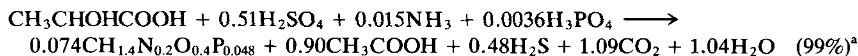
Cell Yield Coefficient

Cell yield coefficients were determined to be in the range between 0.040 ±0.006 and 0.017 ±0.001 g bacteria cell/g lactate in the temperature range from 12°C to 48°C. Thus, temperature does affect cell yield. Senez[28] observed that the overall yields for *D. desulfuricans* (strain Berre S) and *D. desulfuricans* (strain Canet 41) grown on lactate–sulfate synthetic medium with NH_4^+ as nitrogen source in batch systems at 32°C were 0.065 g SS/g lactate and 0.111 g SS/g lactate, respectively. The overall yield was relatively constant up to the optimum temperature of 37°C and then decreased with increasing temperature. Traore et al.[31] reported the overall yield for *D. desulfuricans* in batch culture at 30°C of 0.046 g SS/g lactate. Finally, Cappenberg[3] determined that the overall yield for *D. desulfuricans* grown in the continuous culture at 30°C was 0.34 g SS/g lactate.

The cell yield coefficients obtained in these experiments are relatively low for at least two reasons: (1) no yeast extract was used and (2) cell-associated EPS was not considered in yield calculations. The yield coefficient determined using biomass dry weight, which includes both cellular mass and EPS, would be high compared to our data. Based on this finding, yield coefficients reported by others for *D. desulfuricans* should be cautiously interpreted since no distinction was made between cellular and EPS. Robinson et al.[26] reported that the determination of cell yield using suspended solids leads to overestimation of 100% for aerobic *Pseu-*

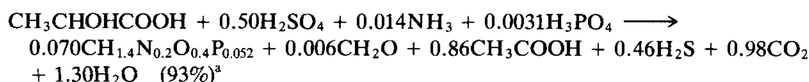
Table VIII. Stoichiometries obtained from the experimental data at different phosphorous concentrations.

At 48.47 mg P/L:



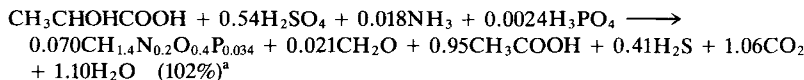
$$CH_3CHOHCOOH + 0.51H_2SO_4 + 0.015NH_3 + 0.0036H_3PO_4 \longrightarrow 0.074CH_{1.4}N_{0.2}O_{0.4}P_{0.048} + 0.90CH_3COOH + 0.48H_2S + 1.09CO_2 + 1.04H_2O \quad (99\%)^a$$

At 4.60 mg P/L:



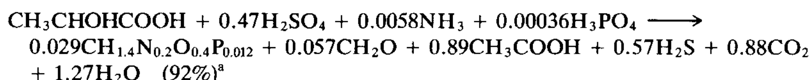
$$CH_3CHOHCOOH + 0.50H_2SO_4 + 0.014NH_3 + 0.0031H_3PO_4 \longrightarrow 0.070CH_{1.4}N_{0.2}O_{0.4}P_{0.052} + 0.006CH_2O + 0.86CH_3COOH + 0.46H_2S + 0.98CO_2 + 1.30H_2O \quad (93\%)^a$$

At 0.39 mg P/L:



$$CH_3CHOHCOOH + 0.54H_2SO_4 + 0.018NH_3 + 0.0024H_3PO_4 \longrightarrow 0.070CH_{1.4}N_{0.2}O_{0.4}P_{0.034} + 0.021CH_2O + 0.95CH_3COOH + 0.41H_2S + 1.06CO_2 + 1.10H_2O \quad (102\%)^a$$

At 0.03 mg P/L:



$$CH_3CHOHCOOH + 0.47H_2SO_4 + 0.0058NH_3 + 0.00036H_3PO_4 \longrightarrow 0.029CH_{1.4}N_{0.2}O_{0.4}P_{0.012} + 0.057CH_2O + 0.89CH_3COOH + 0.57H_2S + 0.88CO_2 + 1.27H_2O \quad (92\%)^a$$

[a] Percentage of recovery of carbon.

domonas aeruginosa. Furthermore, based on our data (data are not shown), the calculation of yield coefficient for *D. desulfuricans* using suspended solids in iron-containing water may lead to significant higher values because of attachment of iron sulfide on the surface of bacteria.

Growth Rate (μ_{max} and K_{Lac})

The kinetics of *D. desulfuricans* growth on lactate as a function of temperature are presented in Figure 11 based on experimentally determined rate coefficients. The highest μ_{max} (0.55 ±0.003 h^{-1}) was observed at 43°C at which the highest cell yield was obtained. However, Topiwala and Sinclair[30] reported that the highest μ_{max} was observed at 40°C, while the highest overall yield was obtained at 25°C for aerobic *Aerobacter aerogenes*. Furthermore, Muck and Grady[22] reported that the highest growth rate for an aerobic mixed population was observed at 30°C, while the highest overall yield was

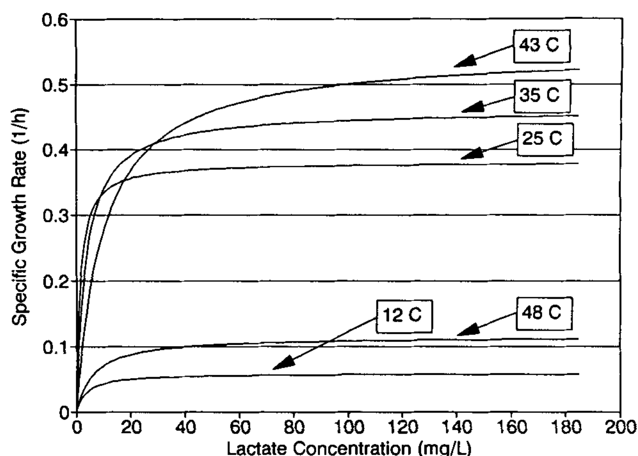


Figure 11. Results of model simulation. Kinetic parameters at steady state were determined from experimental data (e.g., Fig. 2).

observed at 20°C. They concluded that the variation in observed overall yield was caused by the temperature dependence of the maintenance energy and the rate of bacterial decay. However, in this study, the maintenance coefficient was not significant. This may be a reason why the highest cell yield was obtained at the optimum temperature for bacterial growth.

The maximum specific growth rates obtained at 43°C in this study are relatively high compared to values obtained by others. Cappenberg[3] obtained μ_{max} and K_{Lac} for *D. desulfuricans* grown in the continuous culture at 30°C of 0.36 h^{-1} and 4.4 mg lactate/L, respectively. Traore[31] reported that μ_{max} for *D. desulfuricans* grown in the batch culture at 30°C was 0.104 h^{-1}. In addition, Yagisawa et al.[38] determined μ_{max} for mixed continuous culture of SRB grown in lactate–sulfate medium at 30°C was 0.541 h^{-1}. Possible reasons for the relatively high values for μ_{max} in this study include the following: (1) a selective medium for *D. desulfuricans* was used, (2) the contnuous nitrogen gas purge removed hydrogen sulfide which may inhibit the growth of SRB at high concentration, (3) enzymatic activities of SRB which were modified during the long culture period, and (4) selection of faster growing *D. desulfuricans*.

The observed activation energies for μ_{max} were 104 KJ/mol in the range 12°C to 25°C and 14 KJ/mol in the range 25°C to 43°C, respectively. Senez[28] reported that activation energy for μ_{max} for *D. desulfuricans* was 29.1 KJ/mol in the temperature range 24.8°C to 37°C. This evidence indicates that above 43°C and below 25°C the activity of *D. desulfuricans* decrease dramatically.

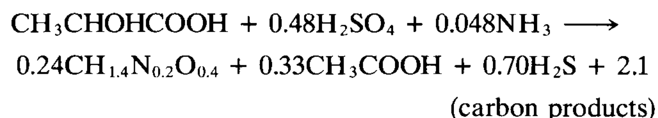
Although the half-saturation coefficient (K_{Lac}) is not really a rate coefficient, it may be related to a substrate transport process into the cells. As a consequence, K_{Lac} is plotted on an Arrhenius plot. Below 25°C, K_{Lac} decreases with increasing temperature, whereas above 25°C, K_{Lac} increased with increasing temperature. Knowles et al.[17] reported increasing K_s values over the temperature

range of 8°C–30°C when *Nitrosomonas* and *Nitrobacter* were grown in the batch culture of river water. Also, Lawrence and McCarty[18] reported the value for K_{Ace} decreased slightly when the temperature was increased from 10°C to 20°C and rose significantly when the temperature was increased to 30°C in the study on the continuous culture of methanogenic bacteria growing on acetate medium. Characklis and Marshall[4] report positive activation energy for batch K_s data and negative energy for continuous reactor data.

The growth parameters were determined at 25°C again after all experiments were conducted to evaluate the reproducibility of results. The reproducibility for μ_{max} was reasonably good, whereas that for K_{Lac} was not. Therefore, there is evidence of historsis. The first values (run 1) for μ_{max} and K_{Lac} were obtained after the temperature was shifted down from 35°C to 25°C, whereas the second values (run 2) were obtained after the temperature was shifted up from 12°C to 25°C. Topiwala and Sinclair[30] reported that the lag due to the physiological readjustment is less with the step-down change than with the step-up change.

Stoichiometry of Microbial Sulfate Reduction

The stoichiometry for lactate oxidation by *D. desulfuricans* is not temperature dependent except for cell yield. Two moles of lactate are consumed for every mole of sulfate reduced at all temperatures. Furthermore, a mole of lactate was converted to approximately a mole of acetate and carbon dioxide. The percentage of recovery of carbon added as lactate indicated that the accuracy of this study is satisfactory. The recovery of sulfur (S) in this study is not good because hydrogen sulfide is volatile and reactive with oxygen and metals in the solution. The tendency of hydrogen sulfide to accumulate in the reactor as FeS was occasionally observed. The stoichiometric equations are consistent with those developed by Traore et al.[31] and D'Alessando et al.[7] Lee[19] reported that the stoichiometry of microbial sulfate reduction of a mixed population SRB anaerobic biofilm at 0.04 h^{-1} of dilution rate was the following:



$$CH_3CHOHCOOH + 0.48H_2SO_4 + 0.048NH_3 \longrightarrow 0.24CH_{1.4}N_{0.2}O_{0.4} + 0.33CH_3COOH + 0.70H_2S + 2.1 \text{ (carbon products)}$$

The SRB dominated the biofilm and 95% of the total SRB in the reactor was observed in the bulk liquid at a dilution rate of 0.04 h^{-1}. The ratio of lactate to sulfate consumption is 2:1 as net expected, but acetate production is less than predicted. The reduced net production of acetate, as compared to monopopulation observations, may be the result of GAB (general anaerobic bacteria) utilizing the acetate produced by SRB in the biofilm. The stoichiometric coefficient for bacterial cells was calculated from the number of SRB obtained from most probable number method (MPN) by converting the cell numbers to cellular carbon concentration. The stoichiometric coefficient for bacterial cells is relatively high compared to that obtained from this experiment (suspended biomass reactor) and is probably due to accumulation of cells in biofilm. The stoichiometric balance for sulfur (S) is not balanced in this equation. The accumulation of sulfide as FeS in the biofilm caused this discrepancy. The stoichiometric ratio between lactate and sulfate in the chemostat may be applied to SRB in biofilm systems. However, community interaction influences the other stoichiometric coefficients.

Steady state experimental results predicted the transient response to temperature changes. The calculated transient response curves, based on the steady state experimental results at different temperatures, fit the experimental transient data reasonably well (Figs. 6 and 7). It can be seen by comparing the theoretical curves and the experimental points that there is no significant lag between the two responses. Thus, for example, SRB activity in an oil reservoir can be predicted despite changing temperature with distance and time. However, this result can be applied to only planktonic SRB, not necessarily to SRB biofilms. Therefore, it is essential to determine if SRB biofilm systems behave similarly to changes in temperature.

Phosphorous Requirement for SRB

The stoichiometric limiting ratio of phosphorous to lactate for *D. desulfuricans* is approximately 1 mg P to 1000–2000 mg lactate for complete lactate utilization and maximum cell production. Using TOC values, this would be a TOC:P ratio of 400:1–800:1. Paul et al.[24] suggested that the stoichiometric limiting C:P ratio for *D. desulfuricans* grown in continuous culture was 250 in terms of complete lactate utilization and maximum cell production. The stoichiometric limiting C:P ratio for anaerobic SRB obtained in this study is higher than that for aerobic mixed population because *D. desulfuricans* produces large quantities of acetate from lactate. Thus, biomass production from substrate is approximately 10 times less than in aerobic systems. Therefore, reduction of SRB activity in an oil field by reduction of phosphorous may not be reasonable.

CONCLUSIONS

Based on the results of this research, it can be concluded that:

1. All stoichiometries indicate that 2 mol of lactate are oxidized for every mole of sulfate reduced by *D. desulfuricans* and the ratio is independent of temperature.
2. The optimum temperature for *D. desulfuricans* growth and yield was approximately 43°C.
3. The maximum specific growth rate (μ_{max}), half-saturation coefficient (K_{Lac}), and cell yield ($Y_{c/Lac}$) for *D. desulfuricans* are dependent on temperature.

4. The limiting carbon : phosphorous ratio for *D. desulfuricans* is in the range 400:1–800:1.

Financial support from the Center Industrial Associates and cooperative agreement ECD-8907039 between Montana State University and the National Science Foundation is gratefully acknowledged. The authors are grateful to Dr. W. Lee and Dr. P. Conn from the Center for Interfacial Microbial Process Engineering, Montana State University, for their review and critical comments.

NOMENCLATURE

C	cell concentration at steady state (M_c/L^3) (mg/L)
C_i	influent cell concentration (M_c/L^3) (mg/L)
D	dilution rate (t^{-1}) (h^{-1})
K_S	half-saturation coefficient (M_s/L^3) (mg/L)
K_{Ace}	half-saturation coefficient for acetate (M_{Ace}/L^3) (mg/L)
K_{Lac}	half-saturation coefficient for lactate (M_{Lac}/L^3) (mg/L)
K_{Pro}	half-saturation coefficient for propionate (M_{Pro}/L^3) (mg/L)
K_{Sul}	half-saturation coefficient for sulfate (M_{Sul}/L^3) (mg/L)
m	maintenance coefficient (M_s/M_b/t) (g/g/h)
P	EPS concentration at steady state (M_p/L^3) (mg/L)
P_i	influent EPS concentration (M_p/L^3) (mg/L)
r_p	specific EPS formation rate ($M_p/M_c/t$) (g/g/h)
S	lactate concentration at steady state (M_s/L^3) (mg/L)
S_i	influent lactate concentration (M_s/L^3) (mg/L)
SS	suspended solids concentration (M_{ss}/L^3) (mg/L)
t	time (t) (h)
Y	yield coefficient (M/M_s) (g/g)
Y_c	cell yield coefficient (M_c/M_s) (g/g)
Y_b	biomass yield coefficient (M_b/M_s) (g/g)
Y_o	overall yield coefficient (M_{ss}/M_s) (g/g)
Y_p	EPS yield coefficient (M_p/M_s) (g/g)
$Y_{o/Ace}$	overall yield coefficient on acetate (M_{ss}/M_{Ace}) (g/g)
$Y_{c/Lac}$	cell yield coefficient on lactate (M_c/M_{Lac}) (g/g)
$Y_{o/Lac}$	overall yield coefficient on lactate (M_{ss}/M_{Lac}) (g/g)
$Y_{o/Sul}$	overall yield coefficient on sulfate (M_{ss}/M_{sul}) (g/g)
$Y_{c/Pro}$	cell yield coefficient on propionate (M_c/M_{Pro}) (g/g)
$Y_{o/Pro}$	yield coefficient on propionate (M_{ss}/M_{Pro}) (g/g)
μ	specific biomass growth rate (t^{-1}) (h^{-1})
μ_{max}	maximum specific growth rate (t^{-1}) (h^{-1})

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