Rate and stoichiometry of sulfate reducing bacteria in suspended and biofilm cultures
by Satoshi Okabe

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Civil Engineering
Montana State University

Abstract:
The accumulation and activity of sulfate reducing bacteria (SRB) are of critical concern in many industrial water systems. For example, SRB cause numerous problems in petroleum production including contamination of petroleum with H2S, injection well plugging, and corrosion. Prediction of SRB behavior in natural and industrial water systems is difficult because microbial activity and growth are strongly dependent on environmental conditions (e.g., nutrient status and physical constraints). Attachment of cells on surfaces may influence their activity and growth due to change in local environment and/or cell metabolism itself. Thus, it is essential to determine effects of these environmental factors on the activity and growth of SRB to develop a comprehensive model and use this model to predict the SRB behavior.

Mono-population Desulfovibrio desulfuricans was grown on lactate and sulfate in a chemostat and in a RotoTorque biofilm reactor. Effects of temperature, limiting nutrients (e.g., phosphorous, nitrogen, and sulfate), and sulfide product on rate and stoichiometry of microbial sulfate reduction were determined in the chemostat experiment. Biofilm kinetics and stoichiometry were determined in the RotoTorque reactor and compared with planktonic (chemostat) data.

The Monod kinetic coefficients (μmax, Ks, and Yx/S) were dependent on temperature, but stoichiometry for catabolic reactions was not. The limiting C:P and C:N ratios (w/w) were found to be in the range of 400:1 to 800:1 and 45:1 to 120:1, respectively. Production of extracellular polymeric substance (EPS) increased with increasing both C:P and C:N ratios in the medium. A non-competitive inhibition model adequately described sulfide product inhibition kinetics. Anabolic reactions (cell production), but not the catabolic reactions (energy production), were strongly inhibited by high sulfide concentrations. Maximum biofilm specific growth rate (μbmax) was essentially the same as μmax for planktonic cells. μb decreased as biofilm grew even though the biofilm was not substrate-limited. Stoichiometry of the catabolic reactions was the same for biofilm and planktonic cells, but a low cellular yield and a high EPS yield were determined in biofilms. These results suggested that D. desulfuricans behave differently in biofilms than in suspension. Thus, rate and stoichiometric data determined from planktonic cells must be used cautiously in developing a model to predict growth and activity in biofilms.

RATE AND STOICHIOMETRY OF SULFATE REDUCING BACTERIA IN SUSPENDED AND BIOFILM CULTURES

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The accumulation and activity of sulfate reducing bacteria (SRB) are of critical concern in many industrial water systems. For example, SRB cause numerous problems in petroleum production including contamination of petroleum with H_2S, injection well plugging, and corrosion. Prediction of SRB behavior in natural and industrial water systems is difficult because microbial activity and growth are strongly dependent on environmental conditions (e.g., nutrient status and physical constraints). Attachment of cells on surfaces may influence their activity and growth due to change in local environment and/or cell metabolism itself. Thus, it is essential to determine effects of these environmental factors on the activity and growth of SRB to develop a comprehensive model and use this model to predict the SRB behavior.

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CHAPTER 1

GENERAL INTRODUCTION

Biofilm development in natural and industrial water systems depends on the types and concentrations of electron acceptors (e.g., oxygen, nitrate, and sulfate) and electron donors (organic substrates) and environmental factors including temperature, pH, salinity, and fluid dynamics. Development of sulfate-reducing bacterial (SRB) biofilms in natural and industrial water systems can be expected whenever environmental conditions such as E_h, oxygen tension, and nutrients are suitable for SRB growth. For example, SRB biofilm may develop in anaerobic bulk water systems such as in petroleum producing facilities, but is also found in aerobic bulk water systems such as cooling water systems. In aerobic bulk water systems, anaerobic microniches and/or anaerobic layers exist in biofilms due to depletion of oxygen by aerobic bacterial activity (Jorgensen, 1977; Lee et al., 1992). In all natural and industrial aquatic environments, SRB show a pronounced tendency to adhere to available surfaces and to proliferate to form biofilms (Costerton and Geesey, 1979; Dewar, 1986; Rosnes et al., 1990). Because of this sessile mode of growth, bacteria within these biofilms are often undetected by conventional sampling techniques which analyze bulk water conditions. Nevertheless, it is these biofilm (sessile) SRB that are responsible for much of the activity in natural and industrial water systems.

At present, quantitative prediction of SRB activity and growth in industrial water systems is essentially impossible because rate and extent of SRB growth under

relevant environmental conditions are not available. Therefore, it is necessary to determine effects of environmental factors on the activity and growth of SRB to develop a comprehensive model and use this model to predict the SRB behavior in given environments. By comparing data from a variety of environments, a conceptual model is first developed and includes factors such as growth substrate limitation, temperature, sulfide inhibition, and the effects of attachment. Translation of the conceptual model into a mathematical form requires much more controlled experimentation to determine kinetic and stoichiometric coefficients (e.g., growth rate, yield), and the specific effects of external factors such as temperature on these coefficients.

As will be shown below, the initial conceptual model must include the following:

- effect of temperature
- nutrient availability (including e^- donor and acceptor, N, P)
- sulfide inhibition
- attachment to surfaces (i.e. biofilm versus planktonic growth)

Nutrient availability can affect both the growth of the organisms (through energy limitation or through limitations in synthesis precursors) as well as the amounts and types of products (e.g., cellular material versus extracellular products). Temperature can affect both kinetics and stoichiometry via phenomena ranging from thermodynamic activity changes through physical enzyme configuration changes. Product inhibition (sulfide) reduces biochemical activity through numerous mechanisms. Finally, attachment to surfaces has been shown to affect bacterial metabolism in a variety of ways, although it is difficult to specify whether these changes are due to a

physiological response to attachment or to an altered extracellular environment resulting from diffusion limitations (Van Loosdrecht et al., 1990).

Research Goal and Objectives

The goal of this research is to investigate the effects of nutritional and physical factors on the rate and stoichiometry of microbial sulfate reduction to incorporate the data into future conceptual models.

The objectives to achieve this goal are as follows:

1) Determine the effects of temperature on the rate and stoichiometry of microbial sulfate reduction by *Desulfovibrio desulfuricans*.
2) Determine the effects of phosphorous, nitrogen, and sulfate concentrations on the rate and stoichiometry of microbial sulfate reduction by *D. desulfuricans*.
3) Determine the effects of sulfide concentration on the growth and activity of *D. desulfuricans*.
4) Determine stoichiometry and rate of microbial sulfate reduction in *D. desulfuricans* biofilm cells in comparison with those for planktonic cells.

Process Analysis and Modeling

SRB biofilm accumulation is a complex phenomenon resulting from several processes occurring in parallel and in series. The rate and extent of these processes, in turn, are influenced by numerous physical, chemical, and biological factors. Thus, a process analysis must be applied to solve biofilm-related problems. The process

analysis generally requires (1) development of a mathematical model and (2) experimental testing of the model.

Process Analysis

The most important results, from the viewpoint of a process analysis of a reaction system, are expressions that quantitatively describe the rate and extent (stoichiometry) of the fundamental processes contributing to biofilm accumulation. Stoichiometry indicates the relationship between the extent of microbial growth and the uptake and production of the chemical species involved. Rate describes how fast the reactions will occur. Both stoichiometry and rate must be known to effectively design and control technical scale processes. The stoichiometric relationships are important since they permit estimation of the rate and extent of biomass and product formation (e.g., hydrogen sulfide) by measuring change in sulfate concentration with time.

A conceptual model describing biofilm accumulation processes would be beneficial in interpreting available historical data and be invaluable in designing future experiments. If the conceptual model could be stated in mathematical terms, a mathematical simulation of biofilm accumulation can be performed on the computer at considerably less expense than laboratory experiments. Furthermore, the influence of process variables such as temperature and substrate concentrations can be determined on the computer *prior to* conducting laboratory experiments. The mathematical description of the individual processes can be combined to develop models to extrapolate and generalize experimental results. Many of these fundamental processes have been described mathematically (Characklis and Marshall, 1990).

Experimental Approach

It is important to proceed in stages beginning with the pure culture work where precisely defined growth conditions and conclusions relevant to those conditions can be made. Understanding of the behavior of single species leads to a more rational image of a mixed population behavior.

Rate and stoichiometry are often determined in chemostat experiments. Analysis of rate and stoichiometry of processes within a biofilm are frequently complicated by significant mass transfer resistances in the liquid or diffusional resistances within the biofilm. Rate coefficients of microbial sulfate reduction are difficult to measure in a batch culture because pH, sulfide concentration, and limiting substrate cannot be maintained at the same levels over many generations. After rate and stoichiometry are determined from the pure culture chemostat experiments (planktonic cells), trials with biofilms (sessile cells) are needed to establish whether or not rate and stoichiometry of the planktonic cells can be used to predict bacterial behavior within the biofilm. Once factors affecting growth and activity of planktonic cells are determined in the chemostat, their quantitative effect on biofilm must be determined. Finally, all these data can be incorporated into a model which will permit prediction of SRB behavior in various environments. The model will also lead to the development of means to control SRB growth and activity.

Background

Physiology of SRB

The SRB are a metabolically diverse group of strictly anaerobic organisms using sulfate as terminal electron acceptor, reducing it to sulfide. Most SRB can grow with sulfite or thiosulfate as electron acceptors instead of sulfate (Badziong and Thauer, 1978; Cypionka et al., 1985; Jorgensen and Bak, 1991). Some species can optionally use nitrate or fumarate as electron accepter (Liu and Peck, 1981; McCready et al., 1983; Postgate, 1984). In the absence of sulfate or other inorganic electron acceptors, several types of SRB can grow by fermentation of special organic substrates (Laanbroek et al., 1982; Widdel and Pfenning, 1981; Traore et al., 1983; Postgate, 1984). The range of carbon sources available for cell growth is very wide. Growth is possible on CO_2 (Brysch et al., 1987), on a range of organic compounds including benzoate but excluding sugars and hydrocarbon, and on fatty acids from stearate. SRB can be classified into two broad nutritional groups. Species of the first group carry out an incomplete oxidation of organic substrates with acetate as an end product. Species of the second group oxidize organic substrates, including acetate, completely to CO_2. Recently, Aeckersberg et al. (1991) isolated SRB from an oil production plant which oxidize saturated hydrocarbons (n-Hexadecane).

The optimum temperature for most SRB is about 20 to 40°C (Widdel, 1988). Relatively few types of SRB are known to be able to grow at high temperature range 55 to 80°C (Rozanova and Khudyakova, 1974; Postgate, 1984; Cochrane et al.,

1988; Rosnes et al., 1991). The physiology of SRB and their ecological impact have been described by Postgate (1984). Nine genera of SRB have now been recognized, and representative species are described in the literature (Postgate, 1984; Widdel, 1988).

Ecology

Significant activities of SRB are measured in salt-marsh or marine sediments because of the high sulfate concentration of seawater (28 mmol = 2.7 g SO_4^{2-}/L) (Jorgensen, 1983). Despite the inhibitory effect of oxygen on SRB, SRB are sometimes active in aerobic aquatic environments, where SRB thrive in anaerobic microniches (Canfield and Des Marais, 1991; Lee, 1992). Formation and maintenance of such microniches is explained by two factors. First the respiration of aerobic bacteria scavenge oxygen and favor growth conditions for SRB. Second, H_2S produced by SRB is a reductant that reacts with oxygen; thus, if once established, colonies of SRB can protect themselves against oxygen (Cypionka et al., 1985). SRB have been observed in unfavorable pH environments because the metabolic products of SRB represent buffers - the H_2S/HS^- and the CO_2/HCO_3^- systems - that protect against extreme pH values.

SRB play a role in the terminal stage of anaerobic degradation of organic materials. In marine and other sulfate containing environments, mineralization involves three broad groups of organisms: heterotrophs that are aerobic, facultative, or anaerobic and are capable of at least partial breakdown of primary substrates; hydrogen-producing acetogenic bacteria; and SRB. In marine sediment slurries, sulfate reduction contributes about 50% of the total mineralization of organic material as a

whole (Sorensen et al., 1981; Jorgensen, 1982; Christensen, 1984). Other studies demonstrated potential hydrogen oxidation by SRB in eutrophic lake sediments (Ingvorsen et al., 1981; Smith and Klug, 1981). In addition to the oxidation of hydrogen, potential significance of lactate, propionate, and amino acids are demonstrated in eutrophic lake sediments (Smith and Klug, 1981), whereas there is evidence that neither acetate or lactate support sulfate reduction (Ingvorsen et al., 1981).

SRB Biofilms and Models

The most important aspect of biofilm accumulation is development of anaerobic conditions within the biofilm even under aerobic bulk water (Lee et al., 1992). When a biofilm reaches a thickness of 10-25 μm under aerobic environment, conditions at the base of biofilm become anaerobic (Culter and Russell, 1976; Costerton and Geesey, 1979).

There is very little quantitative information available related to the rate and extent of SRB biofilm accumulation. Nielsen (1987) conducted a mixed population SRB biofilm study in an annular biofilm reactor. He reported that when the biofilm thicknesses reached 300-400 μm, it was no longer fully penetrated by sulfate at sulfate concentration of about 100 mg/L. The sulfide production from biofilms grown on domestic wastewater was modeled using biofilm kinetics and agreed with experimental results (Nielsen and Hvitved-Jacobsen, 1988). Sulfate limitation in a typical sewer biofilm, with a thickness of 200-300 μm, was shown to occur at sulfate concentrations of 3-5 mg SO_4^{2-}-S/L.

Lee (1990) reported that mixed population SRB biofilm accumulation was

strongly dependent on substrate loading rate. The thickness of the mixed population SRB biofilm easily reached about 1000 μm at high substrate loading (100 mg-C m^{-2} h^{-1}), whereas the thickness was about 5 μm at low substrate loading rate (5.4 mg-C m^{-2} h^{-1}).

To design new wastewater treatment systems to minimize sulfide production or to efficiently control sulfide production in industrial water systems, a reliable method that predicts the sulfide production rate is needed. Empirical models for the prediction of sulfide production from sewer systems have been published (Holder et al., 1984; Holder, 1986; Nielsen and Hvitved-Jacobsen, 1988). However, the biofilm kinetics and effects of nutritional and physical factors are not taken into account in these models. More quantitative and comprehensive prediction models for sulfide production are necessary for more accurate prediction.

Industrial Problems Associated with SRB Activity

Sulfate reducing bacteria (SRB) are very important microorganisms from an industrial standpoint. The anaerobic corrosion of metals, enhanced by the activities of SRB, is one of the best known of the economic problems caused by SRB. The cost related to SRB-mediated corrosion is estimated to be 1 to 2 billion dollars per year in the U. S (Lee, 1990). The cost to the industries with regard to biofouling (SRB biofilm) including corrosion was estimated to be 300-500 million pounds per year for the U.K. (Tiller, 1982). Extensive sulfide corrosion problems with concrete sewer pipes and wastewater treatment were also reported (Witzgall et al., 1990).

In the petroleum industry, SRB cause serious problems including corrosion of

installation, plugging of the petroleum formation, and reservoir souring (contamination of petroleum with H_2S) (Dewar, 1986; Herbert, 1986; Cochrane et al., 1988; Burger et al., 1991; Frazer and Bolling, 1991). Sulfide production by SRB increases the sulfur content of the crude oil which decreases its value and increases refining costs. Costs for downtime, resulting in loss of production, to clean and replace fouled or corroded equipment easily extend to $ 10 million per day (Characklis, 1991). Hydrogen sulfide production by SRB leads to the corrosion of down-hole drill strings and casings as well as production facilities (Sanders and Hamilton, 1983). SRB growth in seawater injection systems can lead to corrosion as well as contamination of oil and gas with H_2S and viable SRB. Cord-Ruwisch et al. (1987) reported that an increase in H_2S was observed during several years of operation at an oil field in northern Germany, and that as a result of H_2S formation the injection well was plugged by FeS flocs. Comprehensive references regarding SRB causing problems in petroleum industries were reported by Postgate (1982, 1984), Sanders and Hamilton (1983), and Hamilton (1985).

Biofilm accumulation also increases capital costs for equipment in power plants. For example, a nuclear power plant had to replace a condenser after approximately 6 years operation because of severe corrosion attributed partially to microbial activity (Characklis, 1991).

Control Strategies

Biocides

Extensive research has been conducted to develop effective biocides with the

goal of inhibiting SRB growth and hence sulfide production. For example, in the secondary production of petroleum, injection water used in flooding operations is treated routinely with a biocide (typically glutaraldehyde) to control SRB growth in the injection well, reservoir, and piping (Brunt, 1986). Eagar et al. (1986) reported that glutaraldehyde was an effective agent for controlling *Pseudomonas florescens* biofilm growth and activity. Also, the results of field study indicated that glutaraldehyde was sufficiently persistent in the distribution system to remain at an efficacious level, and reduced the corrosion rate to an acceptable rate. Gaylarde and Johnston (1983) strongly recommended that biocide test methods for SRB activity should employ mixed sessile SRB in the presence of metal coupons, because sessile SRB on the metal coupon surfaces survived at twice the recommended dose for both biguanide and nitropropanediol.

Biocide addition is often of limited effectiveness since SRB are associated with other anaerobic bacteria in biofilms which coat the surfaces of pipes and other materials. Within these biofilms, SRB are somewhat protected because biocides do not effectively penetrate through the biofilm. All of the reported data have shown the bacteria within biofilms are much more difficult to control with biocides than their planktonic counterparts in these systems (Galylarde and Johnston, 1983; Ege et al., 1985; Dewar, 1986). Thus, biocide treatment may not be an ultimate means to control SRB activity because of rapid microbial regrowth, cost effectiveness, and environmental concerns.

Nutrient Removal

The reduction of the concentration of an essential nutrient (e.g., phosphorous,

nitrogen, and/or sulfate) to below the limiting concentration is a possible means of controlling SRB activity because the essential nutrients reduce activity and growth of SRB when they become limiting. Maree and Strydom (1987) reported the feasibility of microbial sulfate removal from industrial effluent using an upflow packed bed reactor with photosynthetic sulfur oxidation to prevent the emission of sulfide and confirmed the successful performance of reactor. There is no information in the literature which addresses control of SRB activity and growth by removing required nutrients. Nutrient removal may be a possible means of controlling SRB activity and growth. This would be of benefit both in environmental and economic terms.

Microbial Competition

Microbial control of sulfide production by SRB using *Thiobacillus denitrificans* has attracted considerable attention lately (McInerney et al., 1986; Buisman et al., 1990; Montgomery et al., 1990). *T. denitrificans* is an autotroph and a facultative anaerobe which oxidizes sulfide to sulfate using oxygen or nitrate as the electron acceptor. The introduction of viable cells of *T. denitrificans* into environments with SRB has the potential of controlling sulfide production so long as nitrate concentration remains high. The application of this method is to control sulfide production at or near the water injection well in an oil reservoir. A practical difficulty is efficiently inoculating *T. denitrificans* into the well-bore area. A mutant of *T. denitrificans* (strain F) resistant to glutaraldehyde and sulfide was obtained by McInerney et al. (1986). This mutant strain would allow a combined microbial and biocide (glutaraldehyde) treatment of SRB contaminated industrial systems. Sublette and Sylvester (1987a, 1987b, 1987c) and Sublette (1987) have demonstrated that *T. denitrificans* may be readily cultured

aerobically and anaerobically in batch and continuous reactors on gaseous H_2S under sulfide-limiting conditions. A microbial process for the removal of H_2S from gases has been proposed based on mixing the gas with a culture of *T. denitrificans* (Sublette and Sylvester, 1987a).

The competition for the available electron donors between SRB and methane producing bacteria (MPB) has also received considerable attention (Abram and Nedwell, 1978; Krisjansson et al., 1982; Schönheit et al., 1982; Isa et al., 1986; Toda et al., 1987). The SRB apparently have a higher affinity (low k_m) for hydrogen and acetate relative to the methane producing bacteria (MPB). Thus, SRB are normally dominate both in natural ecosystems, such as freshwater and marine sediments, and in anaerobic digesters, where methanogensis was found to be inhibited by the presence of sulfate. Yoda et al. (1987) reported that in an anaerobic fluidized bed the methane production rate and MPB biomass decreased after several months of operation at low acetate concentration whereas sulfate reduction rate increased. On the other hand, MPB were able to form a biofilm faster than SRB at high acetate concentrations presumably due to MPB's higher ability to adhere to carrier surfaces than SRB. Hilton and Oleszkiewicz (1989) reported that SRB are more sensitive than MPB to the elevated total sulfide concentrations, while both are sensitive to elevated molecular H_2S concentrations. Thus, at high total sulfide concentrations and high pH the MPB should be able to outcompete the SRB for substrate.

Aeration

Oxygen is the cheapest and most effective inhibitor of SRB activity. If any system can be maintained in an aerated condition even though the dissolved oxygen

concentration is vanishingly small, SRB remain dormant but are not killed (Hardy and Hamilton, 1981; Cypionka et al., 1985). This measure is of limited effectiveness, however, because SRB are generally associated with other bacteria in biofilms. Oxygen does not effectively penetrate through these biofilms due to consumption of oxygen by aerobes (Jorgensen, 1977). As clearly demonstrated by Lee et al. (1992), SRB activity at the substratum beneath a biofilm can be extensive, even at high dissolved oxygen in the bulk water. Furthermore, introducing oxygen into some industrial water systems increases the corrosion of facilities (Lee et al., 1992).

Ultraviolet Radiation

The use of ionizing radiation to control SRB activity and growth has recently attracted attention. Ultraviolet radiation was used to kill SRB in injection waters by Ege et al. (1985). Gamma radiation was also applied to control SRB at the bottom of the well bores as the water enters the oil reservoir (Agaev et al., 1985).

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CHAPTER 2

EFFECTS OF TEMPERATURE AND PHOSPHOROUS CONCENTRATIONS ON MICROBIAL SULFATE REDUCTION

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 (Postgate, 1984; Hamilton, 1985; Sanders and Hamilton, 1985). Cochrane et al. (1988) 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. (1986) 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 relevant 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 sea water is 0.001 to 0.1 mg/L (Altman and Pittmer, 1964)). 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 chapter is 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 characteristics 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 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

$$CH_3CHOHCOOH + 0.5\ H_2SO_4 \longrightarrow CH_3COOH + CO_2 + 0.5\ H_2S + H_2O$$

$$(\Delta G^\circ = -34.2\ KJ/mol\ e^-)$$

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* (Traore et al., 1982). D'Alessando et al. (1974) 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

$$CH_3COOH + H_2SO_4 \longrightarrow H_2S + 2\ CO_2 + 2\ H_2O \quad (\Delta G^\circ = -17.5\ KJ/mol\ e^-)$$

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 (1977) was used to balance the overall stoichiometric equation. Each molecule of lactate or 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 1, 2, and 3). 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 paper 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 (EPS), and sulfide precipitates (e.g., FeS). The EPS 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 EPS. Thus, the means of measuring product formation will operationally define the yield. For purposes of this paper, 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)

Y_o is obtained when gravimetric (suspended solids) determinations are employed. Precipitates may or may not exist in the samples so further analysis of the solids is recommended. Y_b can be estimated by measurement of the particulate organic carbon. Finally, this chapter describes a method for independently estimating the cells and EPS produced. The method combines organic carbon analysis and cell size measurements accomplished by image analysis (Robinson et al., 1984).

The limiting nutrient or substrate considered in this chapter 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).

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

where, μ = specific growth rate (t^{-1}), μ_{max} = maximum specific growth rate (t^{-1}), S = lactate concentration ($M_s\ L^{-3}$), K_{Lac} = half-saturation coefficient for lactate ($M_s\ L^{-3}$).

μ_{max} and K_s for SRB growth with lactate and acetate as energy and carbon source have been reported (Tables 1 and 2). Lactate-utilizing SRB are capable of doubling times as low as 3 to 6 hours at 30°C, whereas acetate-utilizing SRB grow more slowly with doubling times longer than 20 hours (Postgate, 1984). However, the data presented in Tables 1, 2, and 3 show wide variations and are also very incomplete. Thus, it is very difficult to draw further conclusions from these data.

Mathematical Description for the Chemostat

Microbial transformations in chemostats can be mathematically described by mass balance equations assuming 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 mass (biomass = cellular + EPS). Mass balances for lactate, cellular, and EPS in the liquid phase are as follows:

Lactate

$$\frac{dS}{dt} = D\,(S_i - S) - \frac{X\ \mu}{Y_{c/Lac}} - \frac{R_p\ X}{Y_{p/Lac}} - m\ X \qquad (2)$$

Table 1. 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 (1975)	*D. desulfuricans*	0.360	4.4	-	0.343	-	7.4	C
Traore et al. (1982)	*D. desulfuricans*	0.104	-	-	0.046	-	-	B
	D. africans	0.060	-	-	0.019	-	-	B
	D. gigas	0.092	-	-	0.042	-	-	B
Traore et al. (1981)	*D. vulgaris*	-	-	-	0.075	-	7.2	B
Ingvorsen & Jorgensen (1984)	*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 2. 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)	Temp. (°C)	B/C[a]
Middeleton & Lawrence (1977)	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.(1984)	*Desulfobactor postgatei*	0.030	-	4.2	-	0.158	30	B
Widdel & Pfenning (1981)	*Desulfobactor postgatei*	0.035	-	-	0.074	-	32	B
Schauder et al.(1986)	*Desulfobactor postgatei*	0.025	-	-	-	-	30	B
		0.046	-	-	-	-	30	B
Widdel & Pfenning (1977)	*Desulfotomaculum acetoxidans*	0.058	-	-	0.095	-	36	B
Schauder et al. (1986)	*Desulfotomaculum acetoxidans*	0.014	-	-	-	-	30	B
		0.032	-	-	-	-	37	B

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

Table 2. Reported SRB rate and stoichiometric parameters for acetate-utilizing SRB. (Continued)

Investigator	Organisms	μ_{max} (h^{-1})	K_{Ace} (mg/L)	K_{Sul} (mg/L)	$Y_{o/Ace}$ (g/g)	$Y_{o/Sul}$ (g/g)	Temp. (°C)	B/C[a]
Schauder et al.(1986)	*Desulfobacter hydrogenophilus*	0.039	-	-	-	-	30	B
Widdel (1986)	*Desulfobacter hydrogenophilus*	0.038	-	-	-	-	25-28	B
Widdel et al.(1983)	*Desulfonema* sp.	0.023	-	-	-	-	30	B
		0.0069	-	-	-	-	30	B
Widdel (1986)	*Desulfobacter curvatus*	0.033	-	-	-	-	25-28	B

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

Table 3. 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)	Temp. (°C)	B/C[a]
Nanninga et al.(1987)	*Desulfobulbus propionicus*	0.110	-	-	35	B
Widdel & Pfenning (1982)	*Desulfobulbus propionicus*	0.069	-	0.071[b]	39	B
Widdel & Pfenning (1977)	*Desulfomaculum acetoxidans*	0.046	-	-	36	B
Schauder et al.(1986)	*Desulfovibrio*[c] *baarsi*	0.017	-	-	30	B
Nanninga et al.(1987)	*Desulfovibrio*[c] *sapovorans*	0.066	-	-	35	B
Hunter(1989)	Mixed population	0.070	90.0	0.022[d]	35	C

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

Cellular

$$\frac{dX}{dt} = D\,(X_i - X) + \mu X \qquad (3)$$

EPS

$$\frac{dP}{dt} = D\,(P_i - P) + R_p X \qquad (4)$$

where S_i = influent lactate concentration ($M_s L^{-3}$), X = cell concentration ($M_x L^{-3}$), X_i = influent cell concentration ($M_x L^{-3}$), $Y_{c/Lac}$ = cell yield coefficient on lactate ($M_x M_s^{-1}$), $Y_{p/Lac}$ = EPS yield coefficient on lactate ($M_p M_s^{-1}$), P = EPS concentration ($M_p L^{-3}$), P_i = influent EPS concentration ($M_p L^{-3}$), R_p = specific EPS formation rate ($M_p M_x^{-1} t^{-1}$), D = dilution rate (t^{-1}), m = maintenance coefficient ($M_s M_x^{-1} t^{-1}$), and t = time (t).

For a sterile feed, $X_i = 0$, $P_i = 0$, and at steady state, Eqs.(2), (3), and (4) become

$$\frac{D\,(S_i - S)}{X} = \frac{\mu}{Y_{c/Lac}} + \frac{R_p}{Y_{p/Lac}} + m \qquad (5)$$

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

$$D\,P = R_p X \qquad (7)$$

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

$$\frac{D\,(S_i - S)}{X} = \frac{\mu}{Y_{c/Lac}} + m \qquad (8)$$

Experimental Materials and Methods

Experimental System

The rate and stoichiometric coefficients were determined at several temperatures and phosphorous concentrations in a chemostat consisting of a pyrex cylindrical beaker ($0.45 \times 10^{-3} m^3$ volume) with a Teflon lid sealed using an O-ring (Figure 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 (3 L/h) 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 370°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 (Postgate 1984), 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 4). The stock solutions were mixed with base medium after autoclaving and cooling under nitrogen purge. 0.5 g/L of Na_2SO_4 was used for the temperature effect experiments. 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 minutes at 121°C. After cooling down, the autoclaved culture medium was fed into the reactor under nitrogen purge. After about 4-5 hours, 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 periodically checked by measuring cell number and sulfate concentration in the effluent.

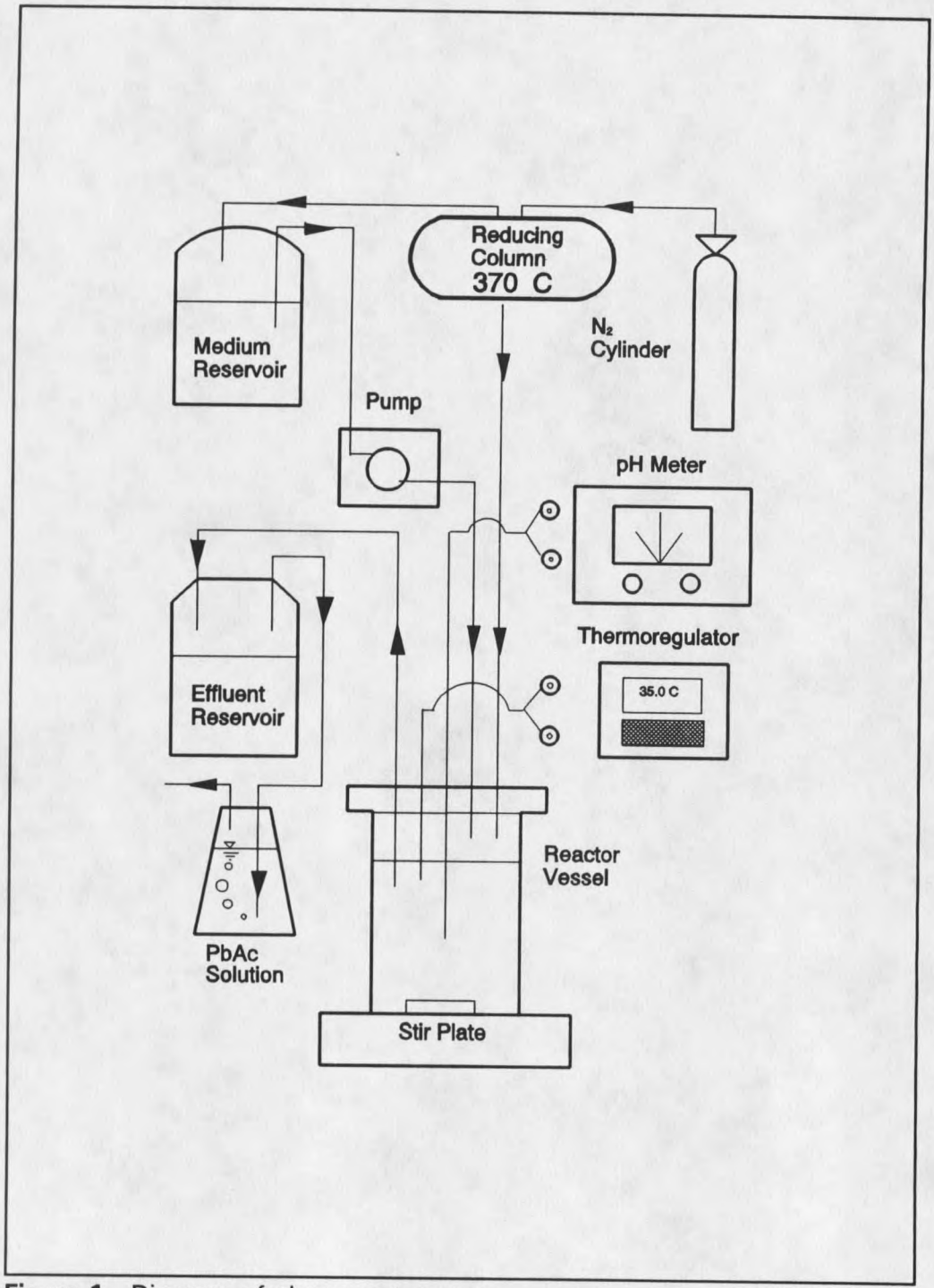

Figure 1. Diagram of chemostat system.

Table 4. Composition of Postgate medium G (Postgate, 1984)

Base medium		
Distilled water		996.0 ml
Na_2SO_4[a]		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		0.3125g
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[b]	3.0 ml	
Oxygen-free distilled water		100 ml
$Na_2S \cdot 9H_2O$		12.0 g

a) 0.5 g/L of Na_2SO_4 was used for temperature effect experiments.

b) This solution was only used 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; (8) phosphorous; (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 Nuclepore filters.

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 ml 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. (1977). Sulfate concentration was measured by the barium-sulfate turbidimetric method (APHA, 1989). Lactate concentration was measured by a specific enzymatic method (L-lactic measurement, Boehriger Mannheim, Germany) as described by Cappenberg (1974). Gas chromatography with flame ionization detector (Varian 3700 model) was used to measure acetate concentration. A 2 m $\times$ 2 mm glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20 M (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 0.06M and 200 mg/L, respectively. Samples were frozen until analyses. The methylene blue

method described by Cline (1969) 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 orthophosphorous using the modified ammonium molybdate-ascorbic acid method described by Harwood et al. (1969). 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 hour, 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^3 (Doetsch and Cook, 1973), 0.22 g dry cell weight/g wet cell weight (Luria, 1960), and 0.465 g cell carbon/g dry cell weight (Postgate, 1984). The EPS carbon was calculated by subtracting the calculated cellular carbon from the total biomass carbon (effluent TOC - effluent SOC).

Results

Effects of Temperature

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 (Figure 2). EPS carbon concentrations were not significant at any dilution rates for the carbon-limiting experiments.

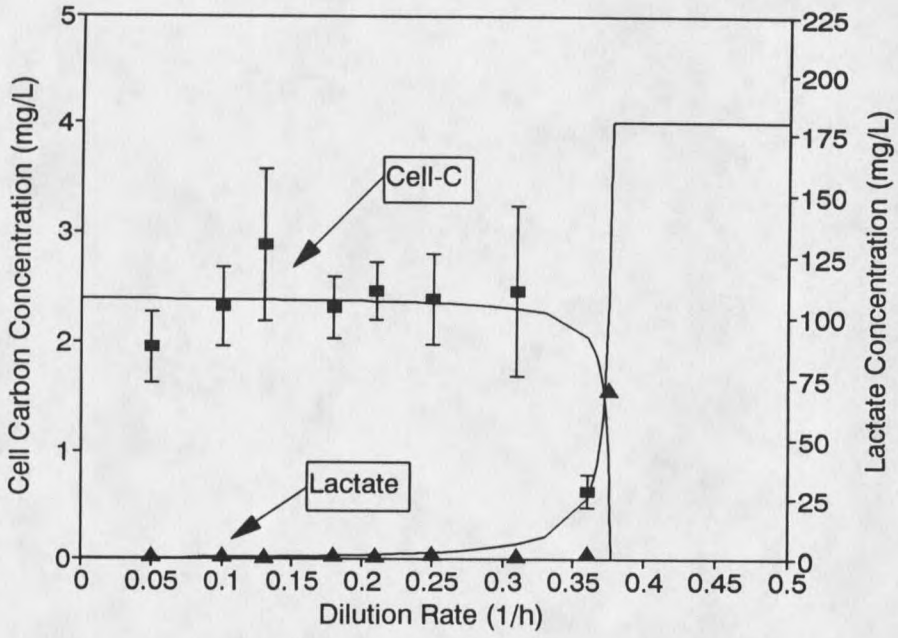

Figure 2. Experimental dependence of the steady state cellular carbon and lactate concentrations on the dilution rate at 25 °C. The solid lines are drawn using chemostat mass balance equations. The influent lactate concentration was 180 mg/L. Error bars represent the standard deviation of triplicate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

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.

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 non-linear regression form of Eq.(1):

$$S = \frac{(K_{Lac}\ \mu)}{(\mu_{max} - \mu)} \qquad (9)$$

The non-linear regression of the Monod equation was performed using MSU SAS® (statistical software). The program produce estimates of μ_{max} and K_{Lac} from given data pairs of limiting substrate concentration (S) and specific growth rate (μ=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 5. 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/mole in the range 25°C - 43°C and 104 KJ/mole below 25°C (Figure 3). The half-saturation coefficient (K_{Lac}) was minimum at 25°C (Figure 4). The activation energy for K_{Lac} above 25°C was 47 KJ/mole and below 25°C was -52 KJ/mole. The highest cell yield ($Y_{c/Lac}$) was observed in the optimum temperature range for growth 35°C - 43°C (Figure 5).

The obtained steady-state experimental results for μ_{max}, K_{Lac}, and $Y_{c/Lac}$ can be summarized by the linear interpretation shown in Figures 3, 4, and 5, which are given specifically by the following functions:

$$\mu_{max}(T) = 10^{6.3686} \cdot 10^{\left(\frac{-2094.52}{T(K)}\right)} \qquad (35°C < T\,43°C) \qquad (10)$$

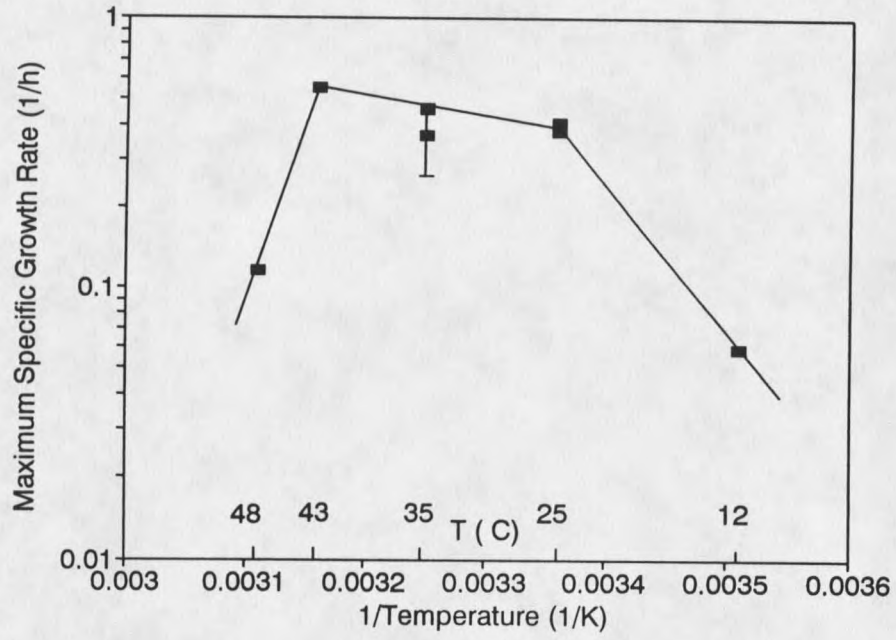

Figure 3. Temperature dependence of the maximum specific growth rate (μ_{max}). The activation energy for μ_{max} were 14 KJ/mole in the range 25-43°C and 104 KJ/mole below 25°C. Error bars represent the standard error of the estimated μ_{max}. The error bar was not given except one point at 35°C because their standard errors are so small (Table 5).

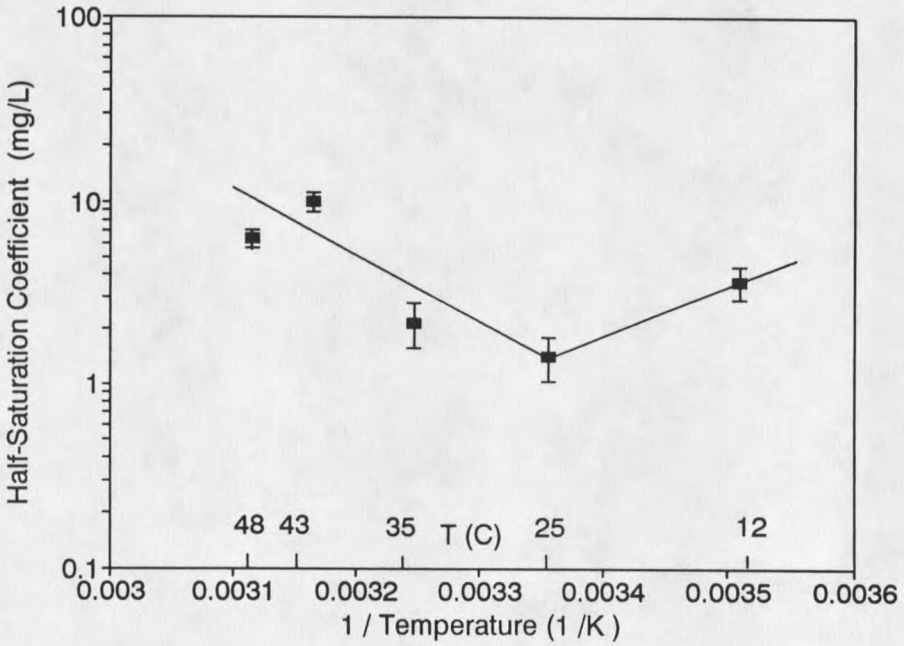

Figure 4. Temperature dependence of the half-saturation coefficient (K_{Lac}). The activation energy was 47 KJ/mole above 25°C and -52 KJ/mole below 25°C. Error bars represent the standard error of the estimated K_s.

Table 5. Experimentally determined kinetic parameters for *D. desulfuricans* growing on lactate as the sole carbon and energy source (estimated parameter value ± SE).

Temp. (°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 ±0.05
48	0.115 ±0.001	6.4 ±0.75	0.023 ±0.001	0.27 ±0.47

$$\mu_{max}(T) = 10^{-43.894} \cdot 10^{\left(\frac{13788.53}{T(K)}\right)} \qquad (43°C \leq T < 48°C) \qquad (11)$$

$$K_{Lac}(T) = 10^{11.41} \cdot 10^{\left(\frac{-3363.73}{T(K)}\right)} \qquad (35°C \leq T < 48°C) \qquad (12)$$

$$Y_{c/Lac}(T) = 10^{1.1053} \cdot 10^{\left(\frac{-814.23}{T(K)}\right)} \qquad (12°C < T < 42°C) \qquad (13)$$

$$Y_{c/Lac}(T) = 10^{-10.5735} \cdot 10^{\left(\frac{2868.44}{T(K)}\right)} \qquad (42°C \leq T < 48°C) \qquad (14)$$

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

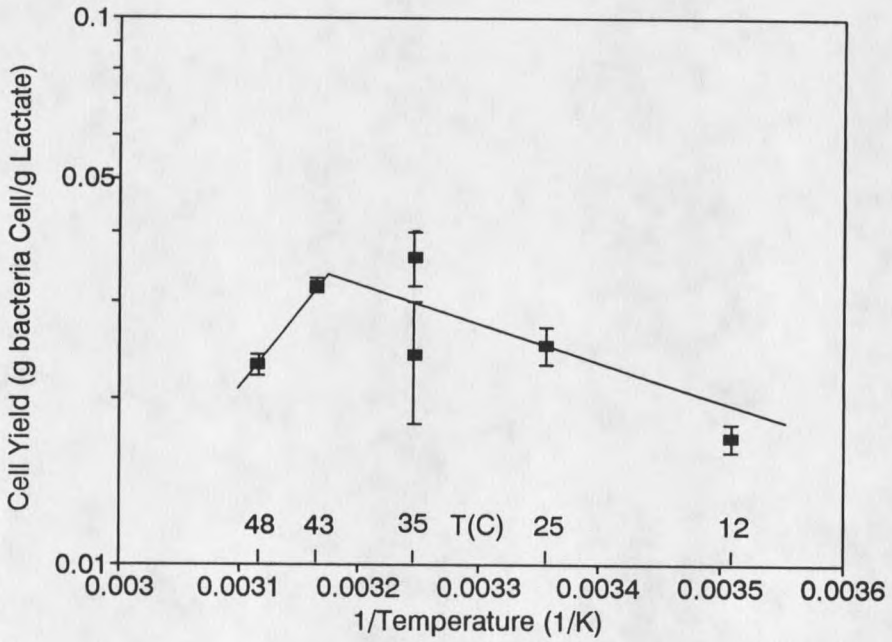

Figure 5. Temperature dependence of the cell yield coefficient ($Y_{c/Lac}$). Error bars represent the standard error of the estimated $Y_{c/Lac}$.

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 = 0.10 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 (Figure 6). At 4.5 hour, the temperature was increased from 35°C to 43°C and lactate utilization did not change. However, the

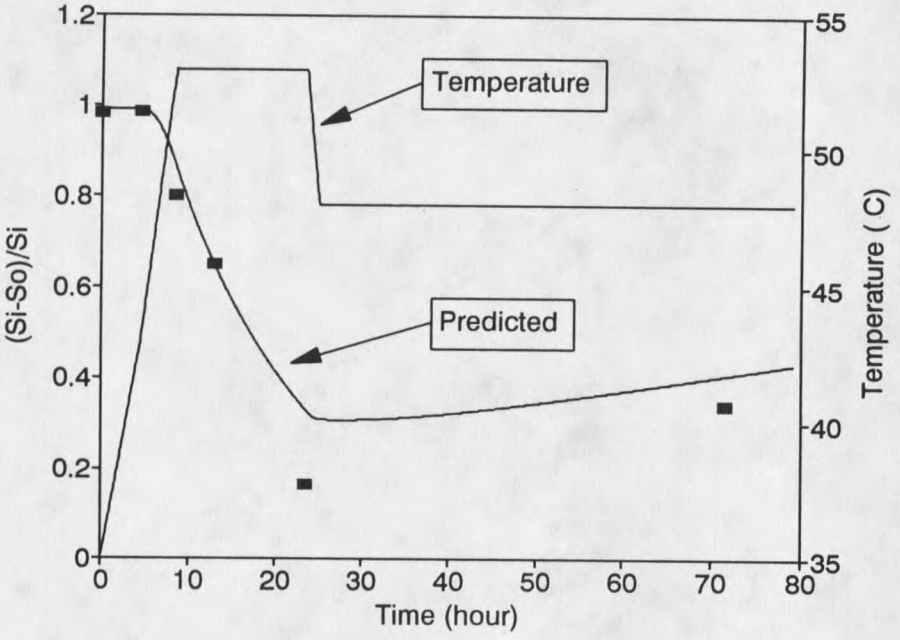

Figure 6. Transient response of lactate oxidation $[(S_i\text{-}S)/S_i]$ to step up change in temperature from 35°C to 53°C:(■) experimental points. Theoretical curve according to $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(C)$: $D = 0.10\ h^{-1}$, pH = 7.0, $S_i = 200$ mg/L.

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 ($D = 0.10\ h^{-1}$), the reactor was near washout at higher temperature. The response of lactate utilization $[(S_i\text{-}S)/S_i]$ to a step change in temperature was simulated using the mass balance Eqs.(2), (3), and (4) and temperature functions for parameters $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(T)$ represented by Eqs.(10), (11), (12), (13) and (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.

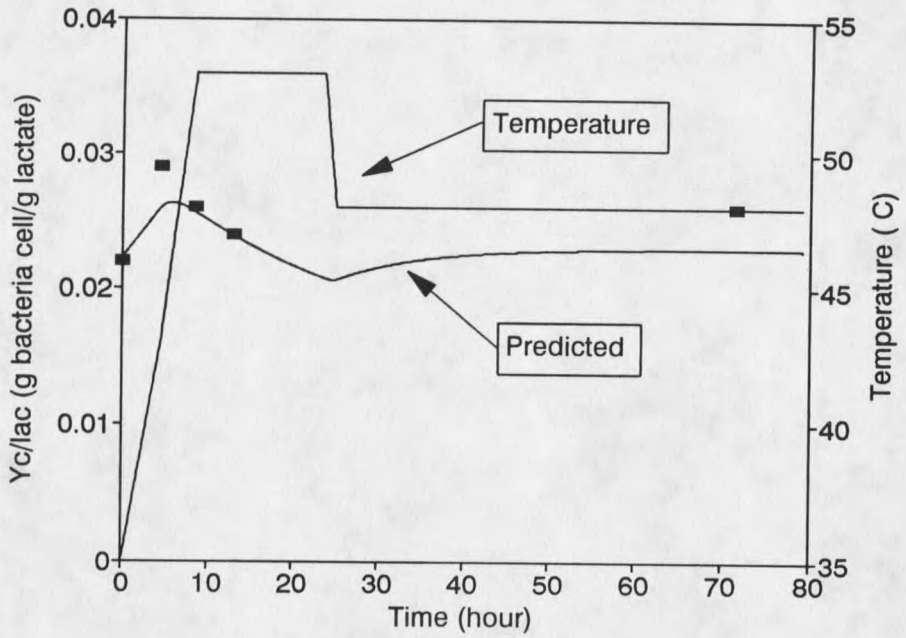

Figure 7. Transient response of cell yield ($Y_{c/Lac}$) to step up change in temperature from 35 °C to 53 °C: (■) experimental points. Theoretical curve based on $\mu_{max}(T)$, $K_{Lac}(T)$, and $Y_{c/Lac}(C)$: $D = 0.10\ h^{-1}$, pH = 7.0, $S_i = 200$ mg/L.

The results indicate that parameters determined at steady state can 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 (Figure 7).

Estimation of Stoichiometric Coefficients. The stoichiometry for microbial sulfate reduction was developed from the experimental data obtained at different temperature (Table 6). 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, (3) amount of other end products of lactate oxidation besides acetate and

Table 6. Influence of temperature on the stoichiometry of microbial sulfate reduction with lactate.

At 12°C (102 %)[a]

$CH_3CHOHCOOH + 0.45\ H_2SO_4 + 0.013\ NH_3$

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

At 25°C (RUN 1) (91 %)[a]

$CH_3CHOHCOOH + 0.44\ H_2SO_4 + 0.020\ NH_3$

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

At 25°C (RUN 2) (102 %)[a]

$CH_3CHOHCOOH + 0.46\ H_2SO_4 + 0.020\ NH_3$

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

At 35°C (RUN 1) (97 %)[a]

$CH_3CHOHCOOH + 0.58\ H_2SO_4 + 0.032\ NH_3$

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

At 35°C (RUN 2)

$CH_3CHOHCOOH + 0.48\ H_2SO_4 + 0.019\ NH_3$

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

At 43°C (110 %)[a]

$CH_3CHOHCOOH + 0.46\ H_2SO_4 + 0.025\ NH_3$

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

At 48°C (97 %)[a]

$CH_3CHOHCOOH + 0.43\ H_2SO_4 + 0.018\ NH_3$

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

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

CO_2 are negligible. The molar conversion of lactate, sulfate, bacterial cells, and acetate was obtained from independent measurements of each component. Bacterial cell production was calculated from the difference between effluent TOC and effluent SOC because EPS carbon concentrations were negligible. The conversion of carbon dioxide was calculated by assuming that the difference between the influent and effluent TOC is CO_2 because the only route for loss of organic carbon from the system is through conversion to inorganic carbon. Stoichiometric coefficients were determined from normalized molar conversions of each individually measured component compared to lactate. 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 90 % of the amounts of lactate-carbon added.

Effects of Phosphorous Concentration

D. desulfuricans was grown at a dilution rate of 0.2 h^{-1} at 35 °C and at various 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 7.

EPS and Cellular Carbons. 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. EPS yield increased and cell yield decreased with decreasing

phosphorous concentration (Figure 9).

Table 7. Steady state results of liquid phase parameters of the continuous culture of *D. desulfuricans* with different influent phosphorous concentrations at a dilution rate of 0.20 h^{-1}.

INFLUENT			EFFLUENT		
phosphorous	lactate	sulfate	phosphorous	lactate	Sulfate
48.47 ± 0.08	402.4 ± 5.3	2560 ± 12	47.97 ± 0.04	0.0 ± 0.0	2341 ± 3
4.60 ± 0.02	437.4 ± 29.8	2574 ± 18	4.13 ± 0.0	3.7 ± 1.3	2341 ± 15
0.39 ± 0.01	411.0 ± 24.9	2533 ± 7	0.06 ± 0.0	5.1 ± 0.7	2297 ± 5
0.03 ± 0.0	382.9 ± 7.8	2541 ± 35	0.00 ± 0.0	113.3 ± 3.4	2405 ± 10

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

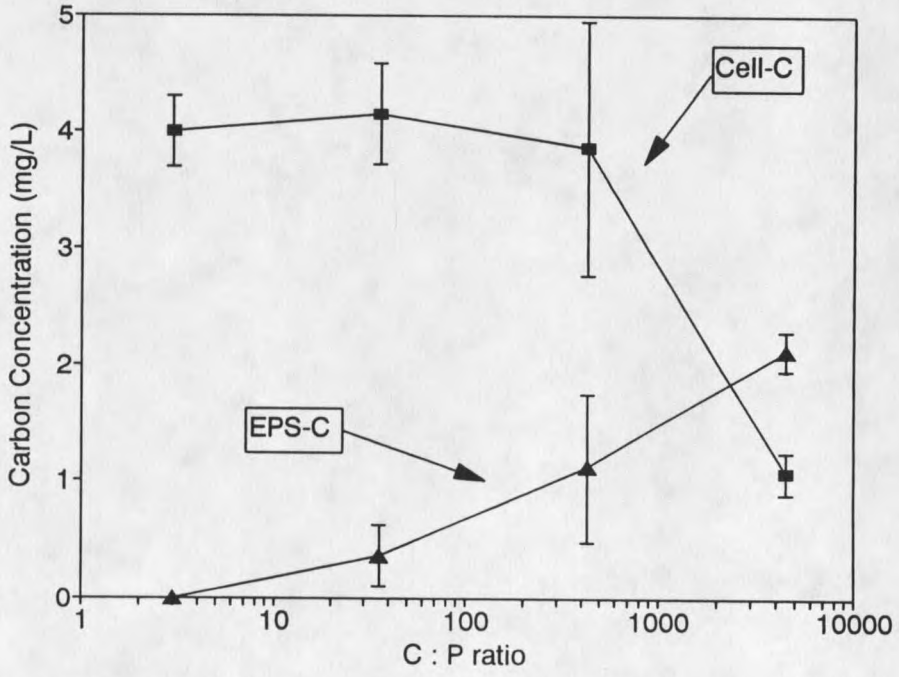

Figure 8. Change in cell and EPS carbon concentrations with changing C:P ratio. Error bars represent the standard deviation of triplicate measurements.

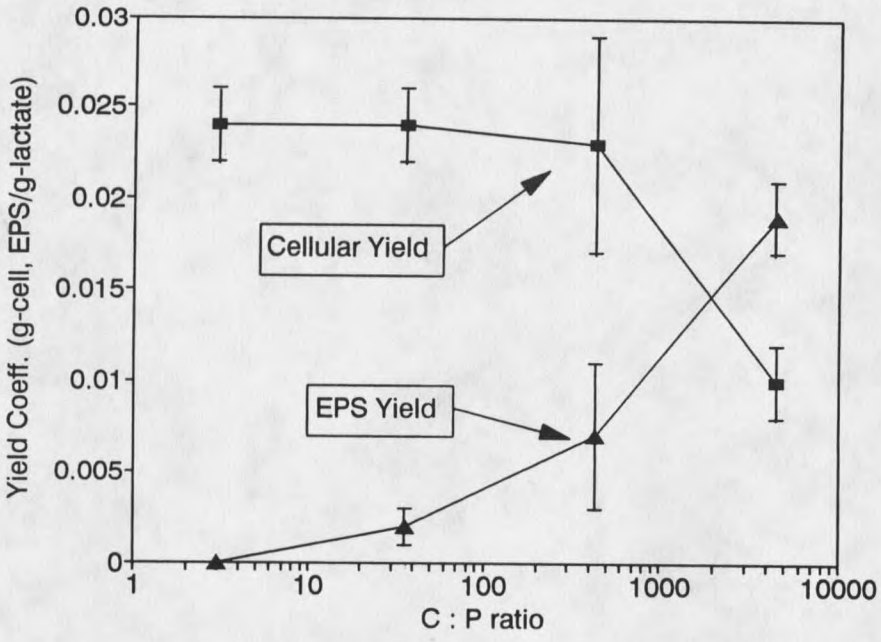

Figure 9. Change in cell and EPS carbon yield coefficients with changing C:P ratio. Error bars represent the standard deviation of triplicate measurements.

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 (Figure 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 8). Triplicate measurements of all reactant and product concentrations were within 15 % of their respective mean values at each phosphorous loading concentration. $CH_{1.4}N_{0.2}O_{0.4}$ and CH_2O were used as an empirical

formula for bacterial cell and EPS, respectively (Characklis, 1990). The recovery of carbon in the four stoichiometric equations was within 90 % 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 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.

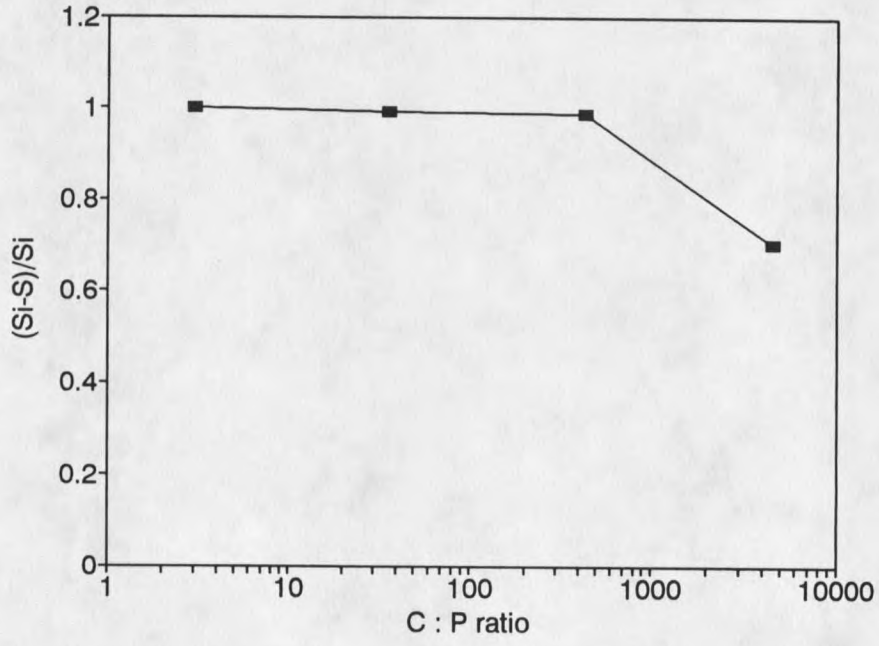

Figure 10. Response of lactate oxidation [$(S_i\text{-}S)/S_i$] by *D. desulfuricans* to change in the phosphorous concentration: $D = 0.20\ h^{-1}$, Temp. = 35 °C. The limiting C:P ratio is in the range 400:1 to 800:1.

Table 8. The stoichiometries obtained from the experimental data at different phosphorous concentrations.

At 48.47 mg-P/L (99 %[a])

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

At 4.60 mg-P/L (93 %[a])

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

At 0.39 mg-P/L (102 %[a])

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

At 0.03 mg-P/L (92 %[a])

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

[a] Percent recovery of carbon.

Discussion

Effects of Temperature

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. The measured yields are lower than those reported in the literature. Senez (1962) 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. (1982) reported the overall yield for *D. desulfuricans* in batch culture at 30°C of 0.046 g SS/g lactate. Finally, Cappenberg (1975) 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 the present study 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. (1984) reported that the determination of cell yield using

suspended solids leads to overestimation of 100 % for aerobic *Pseudomonas 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.

Monod Kinetic Coefficients (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} ($0.55 \pm 0.003\ h^{-1}$) was observed at 43°C at which the highest cell yield was obtained. However, Topiwala and Sinclair (1971) 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 (1974) reported that the highest growth rate for an aerobic mixed population was observed at 30°C, while the highest overall yield was 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 (1975) 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 (1982) 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. (1977)

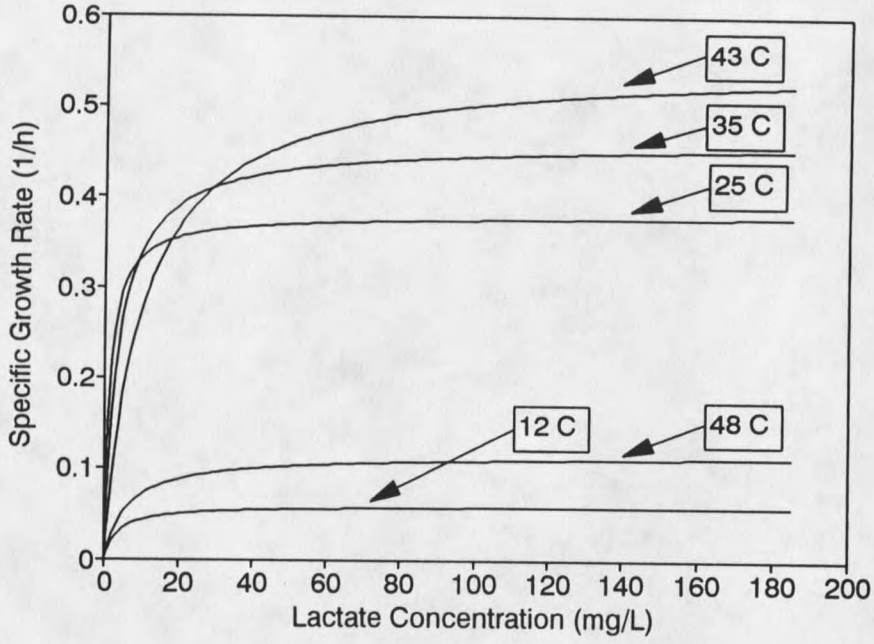

Figure 11. Results of model simulation. Kinetic parameters at steady state were determined from experimental data (e.g., Fig.2).

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 continuous nitrogen gas purge removed hydrogen sulfide which may inhibit the growth of SRB at high concentration, and 3) enzymatic activities of SRB were modified during the long culture period, 4) selection of faster growing *D. desulfuricans*.

The observed activation energies for μ_{max} were 104 KJ/mole in the range 12°C - 25°C and 14 KJ/mole in the range 25°C - 43°C, respectively. Senez (1962) reported the activation energy for μ_{max} for *D. desulfuricans* was 29.1 KJ/mole in the temperature range 24.8°C - 37°C. This evidence indicates that above 43°C and below 25°C the

activity of *D. desulfuricans* decreases dramatically.

Although the half-saturation coefficient (K_{Lac}) is not really a rate coefficient, it may be related to substrate transport process into the cells. As a consequence, K_{Lac} is plotted on an Arrhenius plot. Below 25°C, K_{Lac} decreased with increasing temperature, whereas above 25°C, K_{Lac} increased with increasing temperature. Knowles et al. (1965) 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 (1970) 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 (1990) report positive activation energy for batch K_s data and negative 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 historesis. 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 (1971) reported that the lag due to the physiological readjustment is less with the step down change than with the step up change.

Stoichiometry. 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. (1982) and D'Alessando et al. (1974). Lee (1990) 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:

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

SRB dominated the biofilm and 95 % of the total SRB in the reactor were 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 (MPN) method counts 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 (Figure 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.

Effects of Phosphorous Concentration

Phosphorous Requirement. 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 TOC : P ratio of 400:1 to 800:1. Paul et al. (1988) 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 populations because *D. desulfuricans* produces large quantities of acetate from lactate. Incomplete oxidation of lactate to acetate with sulfate as electron acceptor yields - 14 KJ of free energy (ΔG°), about 6 % of the free

energy (ΔG°) of complete oxidation of lactate with oxygen as electron acceptor ($\Delta G^\circ = -247$ KJ). Thus, anaerobic biomass production from lactate is approximately 10 times less than in aerobic systems.

Conclusions

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

1) All stoichiometries indicate that two moles 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 the 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 to 800 : 1.

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CHAPTER 3

FACTORS AFFECTING MICROBIAL SULFATE REDUCTION : LIMITING NUTRIENTS AND SULFIDE CONCENTRATION

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. (1988) reported 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. (1986) reported 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. (1991) 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 (1992) reported that the maximum specific growth rate (μ_{max}) of *D. desulfuricans* was relatively constant (0.38-0.55 h^{-1}) between 25°C to 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 (Christopher and Oresta, 1991; Roseska et al., 1982). 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 phosphorous 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 and predict SRB activity. Okabe and Characklis (1992) reported that the limiting C : P ratio (w/w) for growth of *D. desulfuricans* is in the range of 400 : 1 to 800 : 1. Extensive

EPS production was observed as phosphorous became limiting. Microorganisms also need assimilable nitrogen usually present in the range of a few mg/L (nitrate level is not detectable) in the produced water (Van der Wende, 1991). 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 waste water 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 chapter 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.*

Experimental Materials and Methods

Mathematical Model 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. The rate of microbial growth can be described by the Monod

expression as a function of limiting substrate concentration (e.g., sulfate).

$$\mu = \frac{\mu_{max}\, S}{(K_{Sul} + S)} \qquad (15)$$

where, μ = specific growth rate (t^{-1}), μ_{max} = maximum specific growth rate (t^{-1}), S = sulfate concentration ($M_s L^{-3}$), K_{Sul} = half-saturation coefficient for sulfate ($M_s L^{-3}$). The details of the mathematical description of the chemostat system are described in Chapter 2.

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 (1.5×10^{-3} m^3 volume). The chemostat was equipped with a butyl rubber biofilm scraper continuously rotated by a 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^{-1}.

Desulfovibrio desulfuricans (ATCC 5575) was grown in Postgate medium G (Postgate 1984), including hemi-calcium lactate (L-lactic acid, SIGMA, No.L-2000) as the sole carbon and energy source. Trace elements and vitamins were added. 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; (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 Nuclepore filters. The phenate method was used for ammonium nitrogen analysis (APHA, 1989). The details of the rest of the chemical analytical methods are described in Chapter 2.

Results

Effects of Sulfate Concentration

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 (Figure 12). EPS carbon concentrations were not significant at any dilution rates 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}.

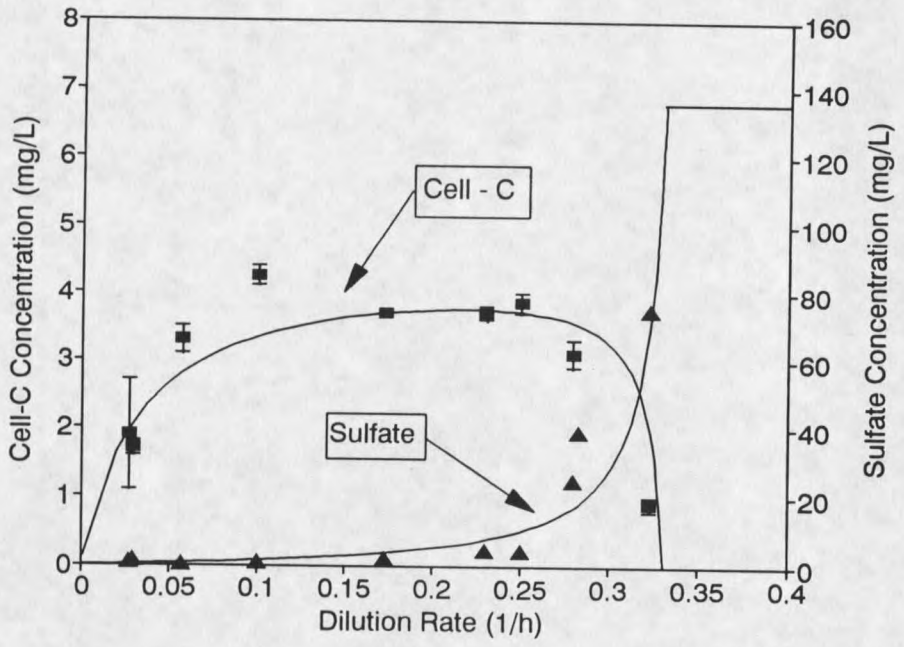

Figure 12. Steady state cellular carbon and sulfate as a function of dilution rate at 35°C. The solid lines reflect kinetic coefficients determined by non-linear regression. The influent sulfate concentration was 135 mg/L. Error bars represent the standard deviation of measurement (n = 2).

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 non-linear regression form of Eq.(15):

$$S = \frac{K_{Sul}\ \mu}{(\ \mu_{max} - \mu\ )} \qquad \textbf{(16)}$$

The non-linear regression of the Monod equation was performed using MSU SAS® (statistical software). The 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 with 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 (Characklis, 1990) (Figure 13). 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)$^{-1}$ 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 9.

Estimation of Stoichiometric Coefficients. The stoichiometry for microbial sulfate reduction was developed from the experimental data obtained from a sulfate-limiting continuous culture (Table 10). 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 (Characklis, 1990), (2) the only nitrogen source is NH_3, (3) the amount of other end products of lactate oxidation besides acetate and CO_2 are negligible. The molar conversion of lactate, sulfate, bacterial cells, and acetate was obtained from independent measurements of each component. Bacterial cell production was calculated from the difference between effluent TOC and effluent SOC because EPS carbon concentrations were negligible. The molar conversion of carbon dioxide was calculated by assuming that the difference between the influent and effluent TOC is CO_2 as C because the only route for loss of organic carbon from the system is through conversion to inorganic carbon. Stoichiometric coefficients were determined from normalized molar conversions of each individually measured component compared to

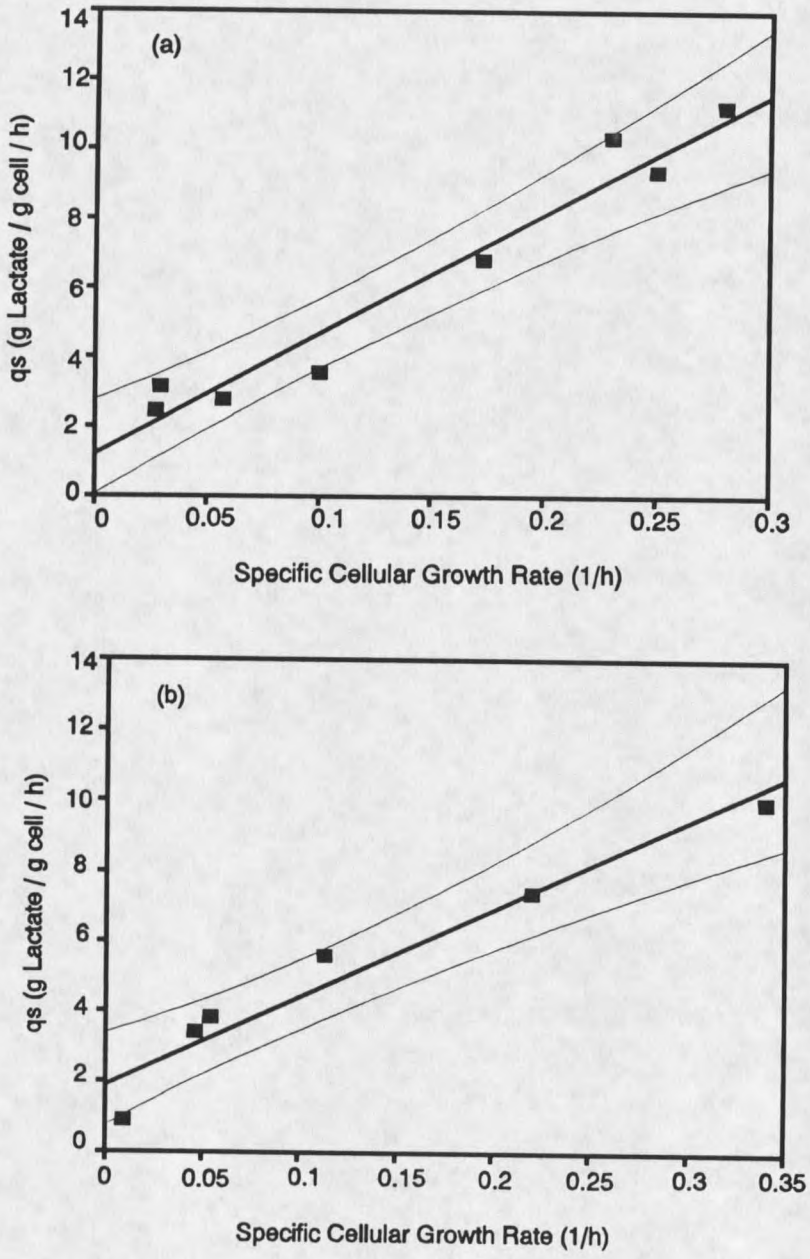

Figure 13. The relationship between specific lactate removal rate (q_s) and dilution rate (D). The maintenance coefficient (m) is the specific lactate removal rate at D = 0 (the y-intercept). The intrinsic cell yield is a reciprocal of the slope. The linear regression lines along with 95 % confidence 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: $r^2 = 0.95$.

Table 9. Summary of rate coefficients for *D. desulfuricans* obtained under lactate-limiting and sulfate-limiting conditions. 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 Figure 13).

Temp. (°C)	μ_{max} (h^{-1})	K_{Lac}, K_{Sul} (mg L^{-1})	m ($g_{Lac} \cdot g_{Cell}^{-1} h^{-1}$)	$Y_{c/Lac}^{Intr\ a)}$ ($g_{Cell}\ g_{Lac}^{-1}$)	$Y_{c/Lac}^{b)}$ ($g_{Cell}\ g_{Lac}^{-1}$)	Reference
Lactate-limiting						
35	0.37 ± 0.004	2.2 ± 0.6	0.45 ± 1.08	0.028 ± 0.008	0.024 ± 0.006	Chapter 2
43	0.55 ± 0.003	10.0 ± 1.2	0.28 ± 0.05	0.031 ± 0.007	0.032 ± 0.001	Chapter 2
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) $Y_{c/Lac}^{Int}$ = intrinsic cell yield on lactate
b) $Y_{c/Lac}$ = observed cell yield on lactate

Table 10. Stoichiometries of microbial sulfate reduction under lactate- and sulfate-limiting conditions.

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 ($S > K_{Sul}$)

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

lactate. 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.

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 (Figure 14). The SO_4^{2-}/ lactate ratio = 0.45 is similar to one obtained under lactate-limiting conditions in Chapter 2.

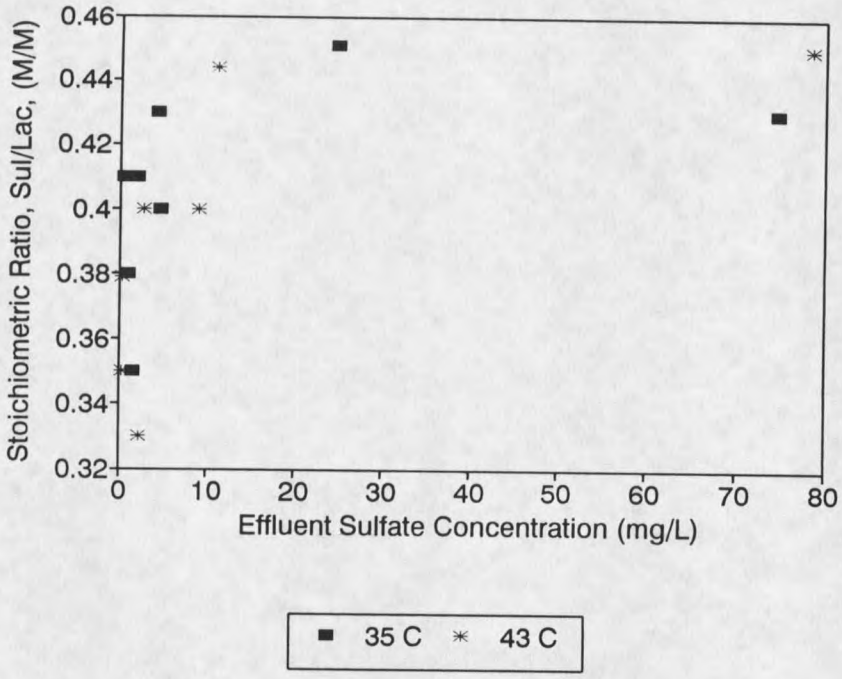

Figure 14. The effect of sulfate concentration on the stoichiometric ratio of SO_4^{2-} /lactate.

Effects of Nitrogen Concentration

D. desulfuricans was grown at a dilution rate of 0.20 h^{-1}, at 35°C, and at various C:N ratios to evaluate effects of nitrogen concentration on lactate oxidation and biomass synthesis. Sulfate was in excess. Steady state results, along with their respective standard deviations of measurement, are presented in Table 11.

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 (Figure 15). 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

Table 11. 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 0.20 h^{-1}. The values given are the mean of duplicate measurements of two samples ± the standard deviation (in mg/L).

INFLUENT			EFFLUENT		
NH_4^+-N	Lactate	Sulfate	NH_4^+-N	Lactate	Sulfate
C:N = 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
C:N = 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
C:N = 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
C:N = 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
C:N = 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

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 ($CH_{1.4}N_{0.2}O_{0.4}$)(Characklis, 1990)(Table 12). CH_2O was used as an empirical formula for EPS (Characklis, 1990). 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.

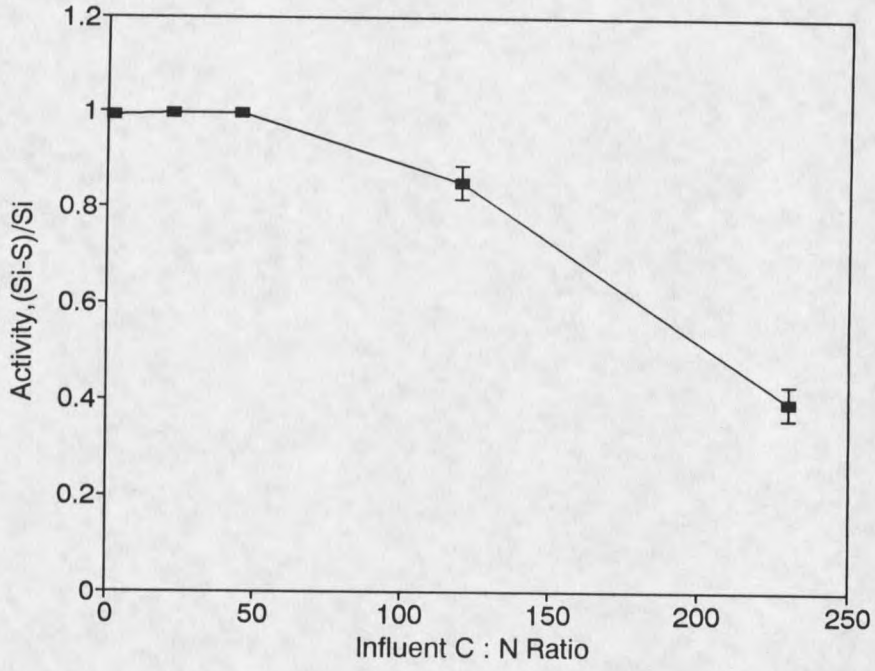

Figure 15. Response of lactate oxidation $[(S_i\text{-}S)/S_i]$ to change in the carbon (C):nitrogen (N) ratio: $D = 0.20\ h^{-1}$, Temp. = 35 °C. The limiting C:N ratio (w/w) is in the range 45:1 to 120:1. Error bars represent the standard deviation of measurement (n = 2). The error bar is not presented except for two points at 120:1 and 230:1 because the other standard deviation are so small (Table 12).

EPS and Cellular Carbons. The cell size of *D. desulfuricans* asymptotically decreased with decreasing influent nitrogen concentration (Figure 16). 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 (Figure 17). 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, 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 (Figure 18). 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.

Table 12. The effect of nitrogen concentration on the stoichiometry of microbial sulfate reduction.

C:N = 2.2:1 (98.7 %)[a]

$$CH_3CHOHCOOH + 0.48H_2SO_4 + 0.203NH_3 \rightarrow 0.119CH_{1.4}N_{0.2}O_{0.4} + 0.002CH_2O + 0.94CH_3COOH + 0.96CO_2 + 0.37H_2S + 1.08H_2O$$

C:N = 22:1 (96.2%)[a]

$$CH_3CHOHCOOH + 0.50H_2SO_4 + 0.041NH_3 \rightarrow 0.102CH_{1.4}N_{0.2}O_{0.4} + 0.015CH_2O + 0.89CH_3COOH + 0.99CO_2 + 0.41H_2S + 1.22H_2O$$

C:N = 45:1 (96.9 %)[a]

$$CH_3CHOHCOOH + 0.47H_2SO_4 + 0.050NH_3 \rightarrow 0.056CH_{1.4}N_{0.2}O_{0.4} + 0.040CH_2O + 0.92CH_3COOH + 0.97CO_2 + 0.40H_2S + 1.10H_2O$$

C:N = 120:1 (82.0 %)[a]

$$CH_3CHOHCOOH + 0.49H_2SO_4 + 0.022NH_3 \rightarrow 0.045CH_{1.4}N_{0.2}O_{0.4} + 0.038CH_2O + 0.78CH_3COOH + 0.82CO_2 + 0.45H_2S + 1.70H_2O$$

C:N = 230:1 (99.4 %)[a]

$$CH_3CHOHCOOH + 0.68H_2SO_4 + 0.026NH_3 \rightarrow 0.068CH_{1.4}N_{0.2}O_{0.4} + 0.023CH_2O + 1.01CH_3COOH + 0.87CO_2 + 0.37H_2S + 1.94H_2O$$

[a] percentage of carbon recovery.

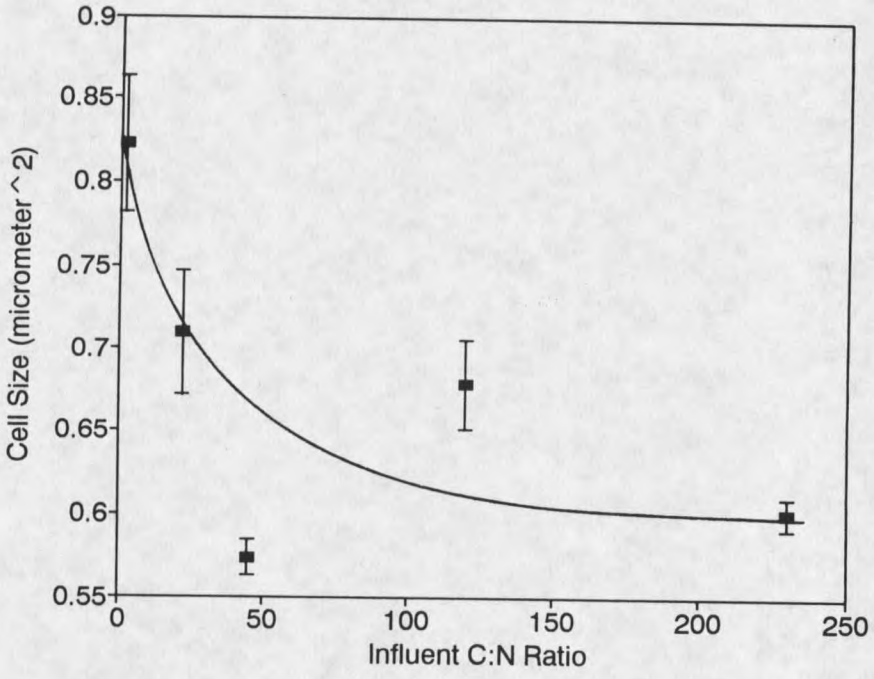

Figure 16. Change in cell size of *D. desulfuricans* with increasing C:N ratio. The cell size was determined using an image analyzer by the epifluorescence method. Error bars represent the standard deviation of measurement (n = 2).

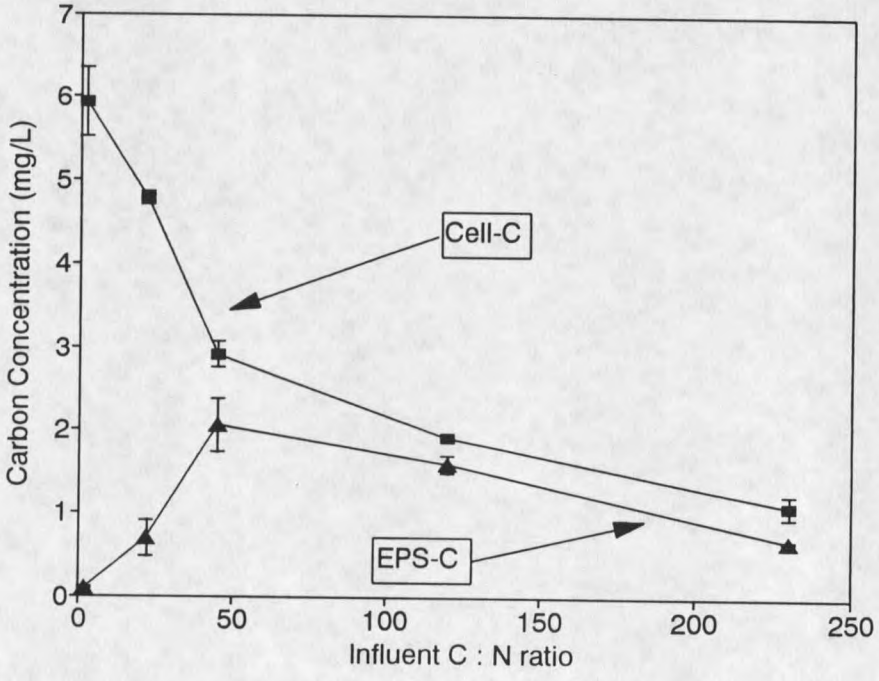

Figure 17. Change in cellular and EPS carbon concentrations with changing C:N ratio. Error bars represent the standard deviation of measurement (n = 2).

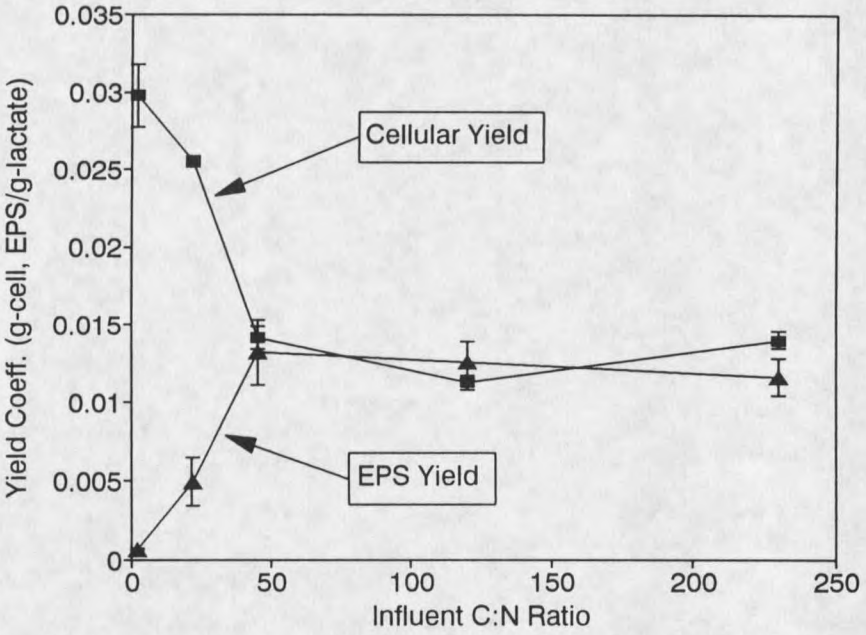

Figure 18. Change in cell and EPS yield coefficients with changing C:N ratio. Error bars represent the standard deviation of measurement (n = 2).

Effects of Sulfide Concentration

D. desulfuricans growing at a dilution rate of 0.20 h^{-1} and at 35 °C was exposed to various sulfide concentrations (Table 13). 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 utilization and cellular production (Figures 19 and 20). Total sulfide of 280 mg/L dramatically decreased cellular production and increased EPS production. Finally, lactate utilization and cellular production were strongly inhibited 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^2 at 280 mg/L of total sulfide and remained relatively constant thereafter (Figure 21). After treatment with 600 mg/L of total sulfide, 50 mL of culture medium was transferred to 450 mL of the fresh culture medium without sulfide, the cell numbers were monitored to examine the recovery of *D. desulfuricans* from sulfide inhibition (Figure 22). 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.

Table 13. 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 0.20 h^{-1}. The values given are the mean of duplicate measurements of two samples ± the standard deviation (in mg/L).

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

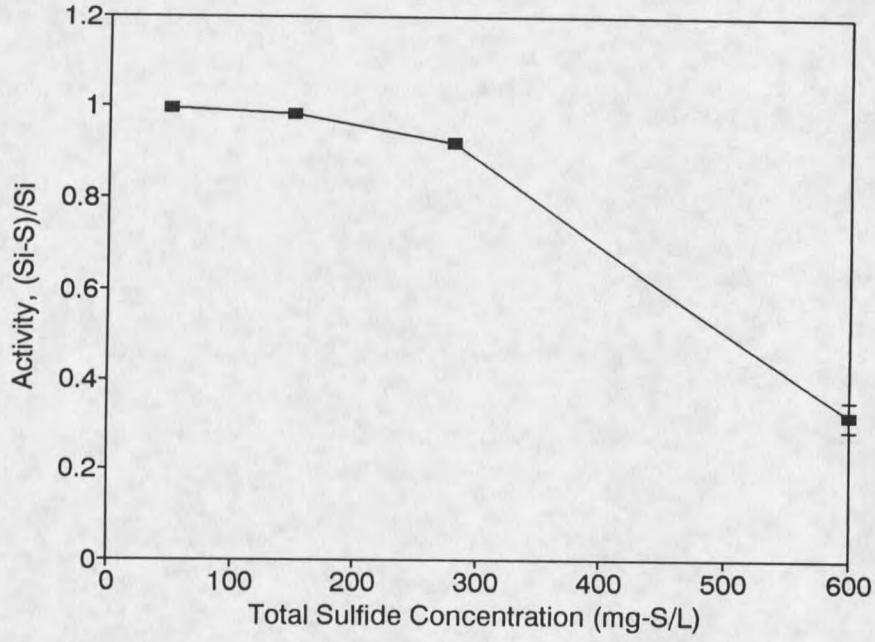

Figure 19. Response of lactate oxidation $[(S_i\text{-}S)/S_i]$ to change in total sulfide concentration: $D = 0.20\ h^{-1}$, Temp. = 35 °C, pH = 7.0. Error bars represents the standard deviation of measurement (n = 2). The error bar is not presented except for 600 mg TS/L because the other standard deviation are so small (Table 13).

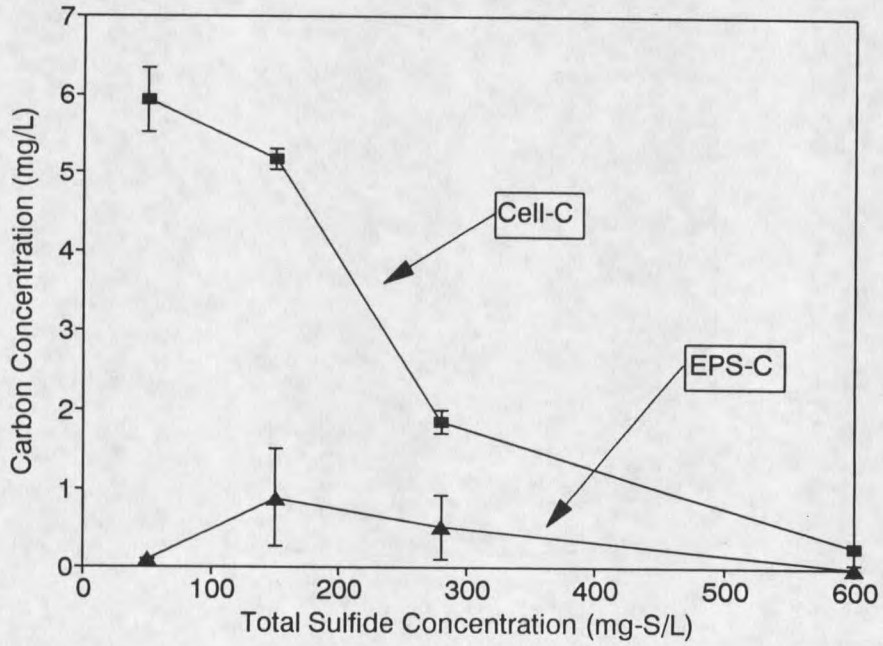

Figure 20. Change in cellular and EPS carbon concentrations with changing total sulfide concentration. Error bars represent the standard deviation of measurement (n = 2).

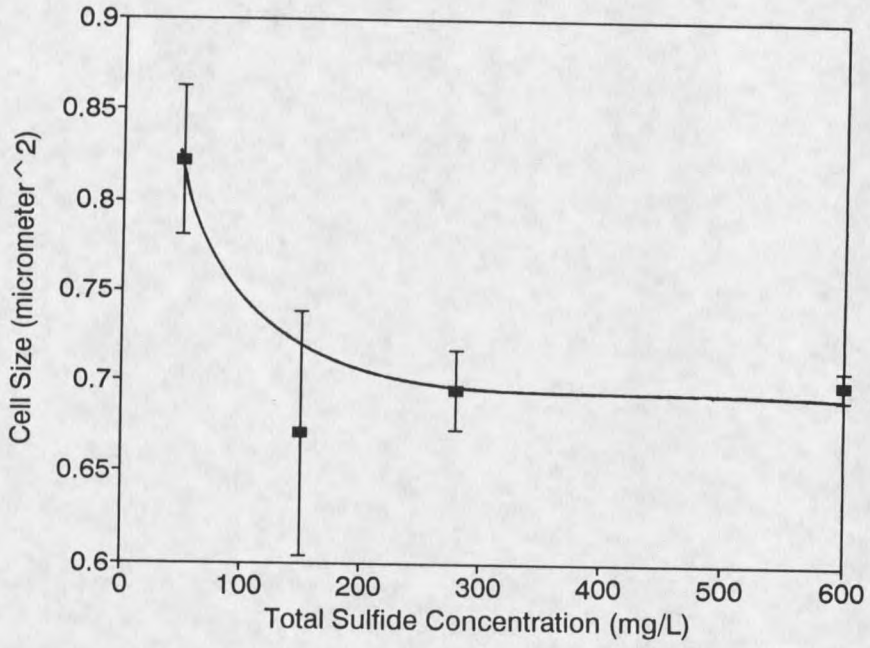

Figure 21. Change in cell size of *D. desulfuricans* with changing total sulfide concentration. Error bars represent the standard deviation of measurement (n = 2).

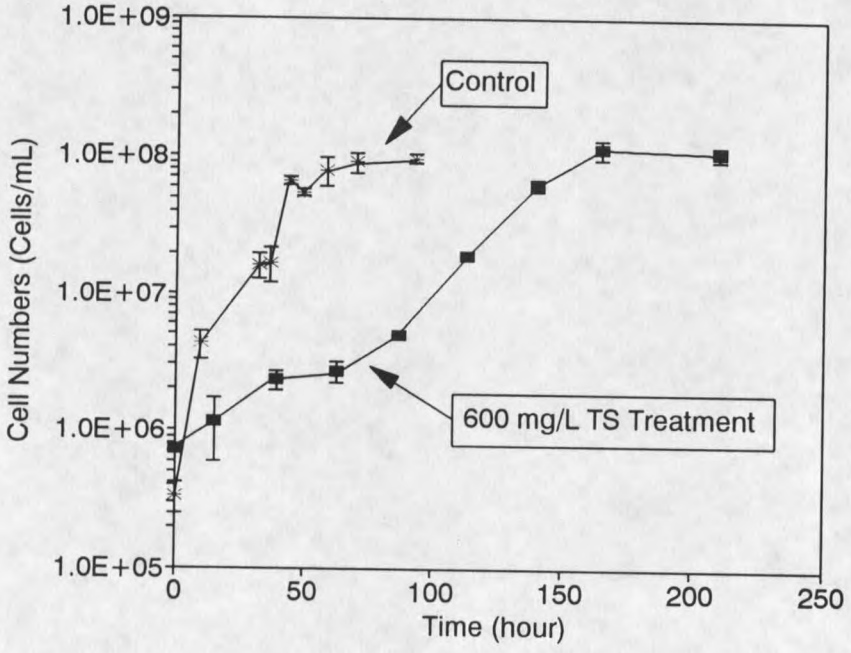

Figure 22. Recovery of *D. desulfuricans* from sulfide inhibition after 600 mg/L total sulfide treatment. Error bars represent the standard deviation of measurement (n = 3).

Discussion

Effects of Sulfate Concentration

Maintenance Coefficient. The maintenance coefficients under sulfate-limited conditions are significant (Figures 13(a) and 13(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 Figures 13(a) and 13(b)). Thus, the effect of m is generally observed at low specific growth rate (at low dilution rate). Significant maintenance coefficients were not observed in the lactate-limiting experiments (Table 5 in Chapter 2). 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 (Chapter 2). Cappenberg (1975) reported that the maintenance coefficient for *D. desulfuricans* grown with lactate-limited growth was 0.53 g lactate (g dry wt.)$^{-1}$ h^{-1} (Table 14). The reported maintenance coefficients in this paper 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 coefficient 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 (Chapter 2), (cells + EPS + precipitates)/(substrate consumed), of *D. desulfuricans* in previous studies were about two or three times higher than the cell yields (Appendix B).

Table 14. The ATP balance and growth yields for SRB grown on different substrate and sulfate.

Energy Source	Limiting Substrate	Organisms	Net ATP Generation[a]	$Y_{c/Sul}$ (g g^{-1})	$Y_{o/Sul}$ (g g^{-1})	m_{Sul}[b] (g g^{-1} h^{-1})	m_{Lac}[c] (g g^{-1} h^{-1})	Reference
H_2, SO_4^{2-}	SO_4^{2-}	*D. vulgaris*	1	–	0.132	0.17	-	(16)
H_2, SO_4^{2-}	Ac	*D. vulgaris*	1	–	0.130	0.54	-	(16)
Lac., SO_4^{2-}	Lac.	*D.desulfuricans*	3	–	-	-	0.53[d]	(4)
Lac., SO_4^{2-}	SO_4^{2-}	*D.desulfuricans*	3	0.047	-	-	1.20	this study
Lac., SO_4^{2-}	SO_4^{2-}	*D.desulfuricans*	3	0.043	-	-	1.98	this study
Lac., SO_4^{2-}	Lac.	*D.desulfuricans*	3	0.051	-	-	0.45	Chapt.2
Lac., SO_4^{2-}	Lac.	*D.desulfuricans*	3	0.071	-	-	0.28	Chapt.2
Lac., SO_4^{2-}	-[f]	*D.vulgaris*	3	-	0.121	ND[e]	-	(13)
Lac., SO_4^{2-}	-	*Desulfotomaculum orients*	1	-	0.040	ND	-	(13)
Lac., SO_4^{2-}	SO_4^{2-}	*D.vulgaris*	3	-	0.141	ND	-	(10)
Lac., SO_4^{2-}	SO_4^{2-}	*D.sapovorans*	3	-	0.115	ND	-	(10)
Lac., 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) g sulfate (g dry wt.)$^{-1}$ h^{-1}.
c) g lactate (g cell)$^{-1}$ h^{-1}.
d) g lactate (dry wt.)$^{-1}$ h^{-1}.
e) Not determined.
f) No limiting substrate because of batch culture experiments.

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 (1965) 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.)$^{-1}$ h^{-1} under aerobic and anaerobic conditions, respectively.

Cell Yield. 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 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 9). Nevertheless, intrinsic cell yields ($Y_{c/Lac}^{Intr}$) under sulfate-limiting and lactate-limiting conditions were essentially the same (Table 9).

D. desulfuricans grown on lactate and sulfate has a net ATP generation of three moles of ATP per mole of sulfate reduced (Thauer and Badzuong, 1981). About three moles of ATP are generated during electron transport coupled phosphorylation and two moles of ATP are generated in substrate level phosphorylation (Thauer and Badzuong, 1981). But two moles of ATP are consumed to activate a mole of SO_4^{2-} to adenosine

phosphosulfate (APS). The cell yield per ATP (based on three moles of ATP generation theory), Y_{ATP}^{max}, determined in this study is approximately 1.44 g cells (mole of ATP)$^{-1}$, which is one tenth of that proposed by Badziong and Thauer (11.4-14.6 g (mol ATP)$^{-1}$). Liu and Peck (1981) reported ATP and growth yields for *Desulfotomaculum orients* and *Desulfovibrio vulgaris* (Table 14). *D. vulgaris* has a net ATP generation of three moles per sulfate reduced, whereas *D. orientis* has a net ATP generation of one. The cell yield per ATP, Y_{ATP}^{max}, in their experiments was approximately 4 g cells (mol of ATP)$^{-1}$. 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.

Stoichiometry. At low levels of sulfate (below K_{sul} value), the SO_4^{2-}/lactate stoichiometric ratio decreased (Figure 14). Under these conditions, lactate is probably oxidized via pyruvate and acetate. *D. desulfuricans* possesses the pyruvic phosphoroclastic system in which, under sulfate-limitation, pyruvate is dismuted to acetyl phosphate, CO_2, and H_2 (Peck and LeGall, 1982). There is some evidence that H_2 can be formed from lactate in small amounts if constant removal of hydrogen is occurring (Bryant et al., 1977). 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 (Widdel, 1988). 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.

Half-Saturation Constant (K_{Sul}). 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 (1984) reported that half-saturation coefficients for sulfate, K_{Sul}, for *Desulfovibrio vulgaris*, *Desulfovibrio sapovorans*, and *Desulfovibrio salexigens* grown in the batch culture were 0.5 mg/L, 0.7 mg/L, 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 14). The results obtained in this study are comparable to these reported values.

Implications. 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 mg/L. Maree and Strydom (1987) reported that biological sulfate removal using molasses as an organic source was feasible without production of H_2S by co-culturing 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.

Effects of Nitrogen Concentration

Nitrogen Fixation. 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) (Riederer-Henderson, 1970; Postgate and Kent, 1985; Postgate et al., 1986). Postgate and Kent (1985) 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 ammonium chloride in the range 10 to 100 μM inhibited nitrogenase activity (Postgate and Kent, 1984). Senez (1962) 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 ($D=0.20\ h^{-1}$) and substantial amounts of NH_4^+ in the medium.

Nitrogen Requirement. 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 = 10-20:1) 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. 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 (1990) 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 ten times greater than without polysaccharide production.

Implications. 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. (1979), 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.

Effects of Sulfide Concentration

Effect of pH on Sulfide Toxicity. 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 (Postgate, 1984). Thus, pH of the system, which determines 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. (1989) 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 (1988) 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 reported 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.

Cell Yield, Growth, and Lactate Utilization. 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 mg/L to about 2.0 mg/L at 280 mg/L total sulfide (Figures 19 and 20). 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). Shimada (1987) reported that 100 mg/L of H_2S inhibited the growth of a mixed SRB population in batch cultures and no significant SRB growth was observed at 500 mg/L of H_2S (the pH in this experiment was not reported). Burgess (1961) 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 (Postgate, 1984).

Implications. 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 to 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) 50 % 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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CHAPTER 4

SULFIDE PRODUCT INHIBITION KINETICS IN BATCH AND CONTINUOUS CULTURES

Introduction

Sulfate Reducing Bacteria (SRB) cause serious problems in sanitary sewer systems and industrial water systems because of production of highly toxic and corrosive hydrogen sulfide gas. For example, the corrosion of concrete sewers occurs as a result of hydrogen sulfide production from sulfate-rich sewage by the activities of SRB (Mori et al., 1992). Furthermore, in the oil industry, SRB cause serious corrosion of installations, plugging of formations, and contamination of petroleum with H_2S (souring). The environment for microbial growth varies widely throughout the petroleum formation. Sulfide concentration varies from 0 mg-H_2S/L to 1000 mg-H_2S/L in the produced water (Subcasky, 1991). Although many have studied the effects of sulfide on anaerobic wastewater treatment (e.g., methane producing bacteria) (Karhadkar et al., 1986; Koster et al., 1986; Hilton and Archer, 1987; Rinzema and Lettinga, 1988; Oleszkiewicz et al., 1989; Hilton and Oleszkiewicz, 1989; McCartney and Oleszkiewicz, 1991), no quantitative SRB product inhibition data have been reported. It is essential to understand how SRB behave under high sulfide concentration environments to predict SRB behavior in these industrial water systems.

Sulfide toxicity is strongly dependent on pH, because the chemical equilibrium

of sulfide species is pH dependent. At pH 8 most of total sulfide (TS) is in the HS^- form, while at pH 6 most is in the H_2S form. Molecular hydrogen sulfide (H_2S) was found to be the major toxic form of sulfide because H_2S can pass through the cell membrane (Speece, 1983; Reis et al., 1991). SRB generate sulfide that may result in product inhibition of SRB and/or toxicity to methane producing bacteria (MPB).

The overall effect of sulfide on microbial sulfate reduction is qualitatively described by some authors (Coleman, 1960; Postgate, 1984; Klemps et al., 1985; Shimada, 1987; Hilton and Oleskiewicz, 1989; Min and Zinder, 1990; McCartney and Oleszkiewicz, 1991; Reis et al., 1991; Okabe et al., 1992; Reis et al., 1992). The relevant literature is summarized in Table 15. However, no quantitative investigation of sulfide product inhibition kinetics for SRB has been reported in these studies. Postgate (1984) stated that growth of SRB is usually linear, not exponential due to sulfide product inhibition. The growth rate of SRB decreases at high sulfide concentrations. Okabe et al. (1992) reported that 50 % inhibition of lactate utilization by *D. desulfuricans* occurred at approximately 500 mg-S/L of total sulfide and that sulfide inhibition of *D. desulfuricans* activity was a reversible process. McCartney and Oleskiewicz (1991) investigated the total sulfide and molecular H_2S inhibition of SRB and MPB using batch culture techniques. They reported that the 50 % inhibition concentrations were 2.6 and 7.5 mmol/L (83.2 and 240 mg/L) total sulfide for SRB and MPB growing on lactate, acetate, and sulfate medium (pH = 7.20-7.55) in serum bottles, respectively. Also, at pH 7.80-7.95 methanogenic activity remained relatively constant in the total sulfide concentration range 100-1000 mg/L, while sulfate reduction rate decreased with increasing total sulfide. These observations indicated that the SRB were more sensitive to both total sulfide and molecular H_2S as compared

Table 15. Summary of the literature on the effects of sulfide on growth and activity.

References	Concentration	pH	B/C[a]	Observation
Coleman (1960)	192-224 mg TS/L	6.5-7.0	B	Decreased growth rate and biomass yield.
Klemps et al. (1985)	64-128 mg TS/L	6.9-7.0	B	Ceased growth. After removal of H_2S resumed growth.
Hilton and Oleszkiewicz (1989)	400 mg TS/L	7.0	B	Reduced sulfate reduction by 50 %
Min and Zinder (1990)	64 mg TS/L	6.5	B	Ceased sulfate reduction.
McCartney and Oleszkiewicz (1991)	448 mg TS/L	7.15-7.33	B	Completely ceased sulfate reduction.
Reis et al. (1992)	550 mg H_2S/L	6.2-6.7	B	Ceased sulfate reduction. H_2S is the most inhibitory form.
Reis et al. (1992)	547 mg H_2S/L	6.2-6.7	B	Ceased growth. Found to be a reversible toxicity. H_2S is the most inhibitory form.
Okabe et al. (1992)	500 mg TS/L	7.0	C	Decreased cellular yield and cell size. Reduced lactate oxidation by 50 %

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

to the MPB. Min and Zinder (1990) reported that sulfide concentrations produced by *Desulfotomaculum thermoacetoxidans* sp. nov. growing in batch cultures rarely exceeded 2 mmol/L. Klemps et al.(1985) reported that growth of *Desulfotomaculum* species did not occur in medium containing more than 2 mmol/L of Na_2S, sulfate reduction stopped at a sulfide concentration of 4 mmol/l, and then growth proceeded after removal of H_2S by sparging the culture with N_2/CO_2 (80/20). Without removal of produced sulfide, growth was slower and stopped at lower cell densities. Hilton and Oleszkiewicz (1989) reported that SRB are more sensitive than MPB to elevated total sulfide concentrations, while both are sensitive to elevated molecular H_2S concentrations. Thus, at high total sulfide concentrations and high pH the methane producing bacteria should be able to outcompete the SRB for substrate.

Little distinction between growth and activity of SRB was made in these studies (Klemps 1985). The distinction between growth and activity is very important when the environmental conditions become extreme (high sulfide concentration) and energy consumption shifts from growth to maintenance. In many cases microbial sulfate reduction is monitored by H_2S gas production. Under extreme conditions, the growth-associated H_2S production will decrease, whereas total H_2S production will remain relatively constant because the decrease in growth is counter balanced by increased nongrowth-associated H_2S production caused by increased maintenance energy requirement (Hunik et al., 1990).

The goal of this chapter is to quantitatively describe how sulfide affects activity and growth of SRB. The distinction between growth and activity was clearly made in this study; growth was defined as biomass (Cell) production rate and activity was defined as specific substrate utilization rate (e.g., lactate and sulfate).

Theoretical Background

Microbial transformations in a lactate-limiting chemostat can be described mathematically by assuming that the two fundamental processes occurring are growth and maintenance:

$$\frac{D\ (\ S_i\ -\ S\ )}{X}\ =\ \frac{\mu}{Y_{c/Lac}}\ +\ m\ =\ q_s \qquad (17)$$

where, D = dilution rate (h^{-1}), S_i = influent lactate concentration ($M_s\ L^{-3}$), S = effluent lactate concentration ($M_s\ L^{-3}$), X = cell concentration ($M_x\ L^{-3}$), $Y_{c/Lac}$ = cell yield coefficient on lactate ($M_x\ M_s^{-1}$), m = maintenance coefficient ($M_s\ M_x^{-1}\ h^{-1}$), and q_s = specific lactate utilization rate ($M_s\ M_x^{-1}\ h^{-1}$). At steady state, the dilution rate (D) in the chemostat is equal to the specific growth rate (μ).

Non-competitive inhibition kinetics can be described as follows (Aiba et al, 1973):

$$\mu\ =\ \frac{\mu_{max}\ S\ \ K_i}{(\ K_{Lac}\ +\ S\ )\ (\ K_i\ +\ i\ )} \qquad (18)$$

where, μ_{max} = maximum specific growth rate (h^{-1}), i = sulfide concentration ($M_i\ L^{-3}$), K_{Lac} = half-saturation coefficient for lactate ($M_s\ L^{-3}$), and K_i = inhibition coefficient ($M_i\ L^{-3}$). For $S >> K_{Lac}$, Eq.(18) can be simplified as follows:

$$\mu\ =\ \frac{\mu_{max}\ \ K_i}{K_i\ +\ i} \qquad (19)$$

Eq.(19) can be linearized by plotting $1/\mu$ against i.

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{i}{\mu_{max}\ K_i} \qquad (20)$$

The x-interception of a best fit line gives $-K_i$.

Maximum specific growth rates (μ_{max}^{inh}) of *D. desulfuricans* growing at a constant dilution rate ($D = 0.20\ h^{-1}$) at various sulfide concentrations can be determined by rearranging Eq.(18) as follows:

$$\mu = \frac{S}{K_{Lac} + S} \left(\frac{\mu_{max}\ K_i}{K_i + i} \right) \qquad (21)$$

By defining the maximum specific growth rate in presence of sulfide, μ_{max}^{inh}, as follows:

$$\mu_{max}^{inh} = \frac{\mu_{max}\ K_i}{K_i + i} \qquad (22)$$

when $i = 0$, $\mu_{max}^{inh} = \mu_{max}$, Then, at steady state

$$\mu = D = \frac{\mu_{max}^{inh}\ S}{K_{Lac} + S} \qquad (23)$$

or,

$$\mu_{max}^{inh} = \frac{D\ (\ K_{Lac} + S\ )}{S} \qquad (24)$$

Eq.(24) indicates that μ_{max}^{inh} can be determined by measuring S, since D and K_{Lac} are known.

Experimental Materials and Methods

Microorganisms

Desulfovibrio desulfuricans (ATCC 5575) was grown in Postgate medium G, (Postgate, 1984) including Na-lactate (DL-Lactic acid, SIGMA, L-1375) and Na_2SO_4. Trace elements and vitamins were added. The details of medium preparation and preculture of microorganisms are described in Chapter 2.

Batch Culture Experiment

Sulfide inhibition kinetics were determined in batch experiments using 500 ml erlenmeyer flasks. Flasks were equipped with butyl rubber stoppers fitted with an injection port and a gas exchange port. Four hundred mL of the pH adjusted (pH = 7.0) Postgate medium G containing 500 mg/L of lactate and 800 of mg/L sulfate was dispensed into each erlenmeyer flask and autoclaved at 121 °C for 20 minutes. Trace elements and vitamins solutions were added after cooling. $Na_2S \cdot 9H_2O$ solution was prepared separately in an air-tight bottle. A $Na_2S \cdot 9H_2O$ crystal was rinsed with deionized water to removed oxidized surface layer, weighed, and dissolved in oxygen-free deionized distilled water under N_2 purge. After autoclaving, the pH of sulfide solution was adjusted to 7.0 in the air-tight bottle using a sterile syringe and sterile 1.0 N HCl and NaOH solutions. Then appropriate volumes of the sulfide solution were added to the autoclaved culture medium using a sterile syringe to obtain the designed

total sulfide concentrations (0, 70, 220, 350, 700 mg-S/L). After addition of sulfide solution, the pH of the culture medium was remeasured and readjusted. *D. desulfuricans* taken from an actively growing chemostat culture was inoculated into each flask and incubated at 35°C. The inoculum size from the chemostat was adjusted to obtain an initial cell count of approximately 1×10^7 cells/mL. Samples for cell count were carefully taken by a sterile syringe with N_2 purge to prevent air contamination. Cell numbers were monitored using an image analyzer (Cambridge/Olympus Quantment 10) by the epifluorescence method described by Hobbie et al. (1977).

Continuous Culture Experiment

Sulfide inhibition kinetics were also investigated in a lactate-limiting chemostat. The chemostat was equipped with a butyl rubber wall growth scraper continuously rotated by an electric motor to prevent wall growth. Constant pH (± 0.1 unit) and temperature (± 0.5°C) were maintained using a pH control system with sterile 1.0 N HCl and NaOH solutions and thermoregulator, respectively. Various concentrations of sulfide solutions, with pH roughly adjusted to 7.5, were separately prepared and autoclaved, then fed to the chemostat by a peristaltic pump and speed controller (Cole-Parmer, Chicago, IL). A fine pH adjustment was conducted in the chemostat. The effect of sulfide on the activity and growth was determined at a constant dilution rate of 0.20 h^{-1}. The details of chemostat set up protocol are described in Chapter 2.

Analytical Methods

At steady state, chemostat 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. The samples for SOC, lactate, and acetate were obtained by centrifugation (20,000 rpm for 20 minutes) at 4°C using stainless steel centrifuge tubes. The sample for sulfate analysis was first fixed with 1 % (w/v) ZnAc solution (0.3 ml of 10 % (w/v) ZnAc solution into 25 ml sample) to remove sulfide species and then filtered by Sterile Acrodisc filter (pore size 0.2 μm, Gelman Science, No. 4192). Sulfate and acetate concentrations were measured using an ion chromatograph (DIONEX). Sulfide concentration was measured as total dissolved sulfide concentration (TS) in the liquid phase. The details of the rest of chemical analytical methods are described in Chapter 2.

Results

Batch Experiments

Maximum specific growth rate in the presence of sulfide (μ_{max}^{inh}) of *D. desulfuricans* was determined from the cell doubling times (t_d) at various total sulfide concentrations in batch cultures (Figure 23). Initial growth rates were determined (Table 16) by linear interpolation of the data of Figure 23 because total sulfide concentration and the pH of culture medium changed during incubation. For data interpretation initial pH, total sulfide concentration, and corresponding maximum specific growth rates (μ_{max}^{inh}) are presented in Table 16. The maximum specific growth rates (μ_{max}^{inh}) determined in the batch cultures never exceeded 0.20 h^{-1} at each total sulfide concentration, which is significantly lower than values for continuous culture.

The inhibition coefficient for maximum specific growth rate, K_i, was determined using the non-competitive inhibition model and found to be 251 mg-S/L (Figure 24). The maximum specific growth rates calculated at various sulfide concentrations using the non-competitive model and this value of K_i are compared with the measured maximum specific growth rates in the presence of sulfide in Figure 25. The model adequately described the sulfide effect on maximum specific growth rate in batch experiment.

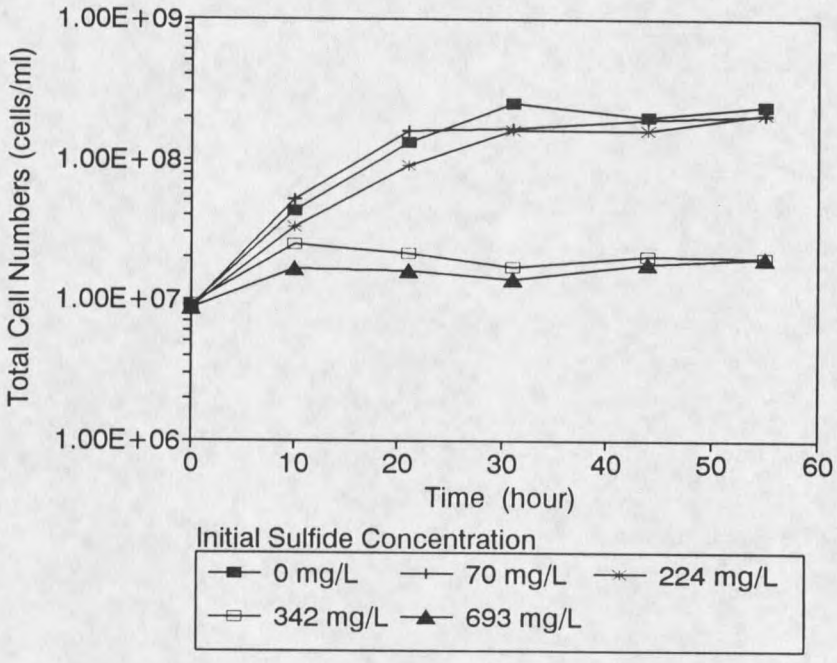


Figure 23. Effect of sulfide on growth of *D. desulfuricans* in batch cultures: Temp. = 35 °C.

Table. 16 Results of sulfide effect on growth rate of *D. desulfuricans* in batch cultures.

TS (mg-S/L)	pH	t_d (h)	μ_{max}^{inh} (h^{-1})
0	6.96	4.0	0.17
70	6.95	3.5	0.20
224	7.30	5.5	0.13
342	7.00	7.0	0.10
693	6.95	6.95	0.058

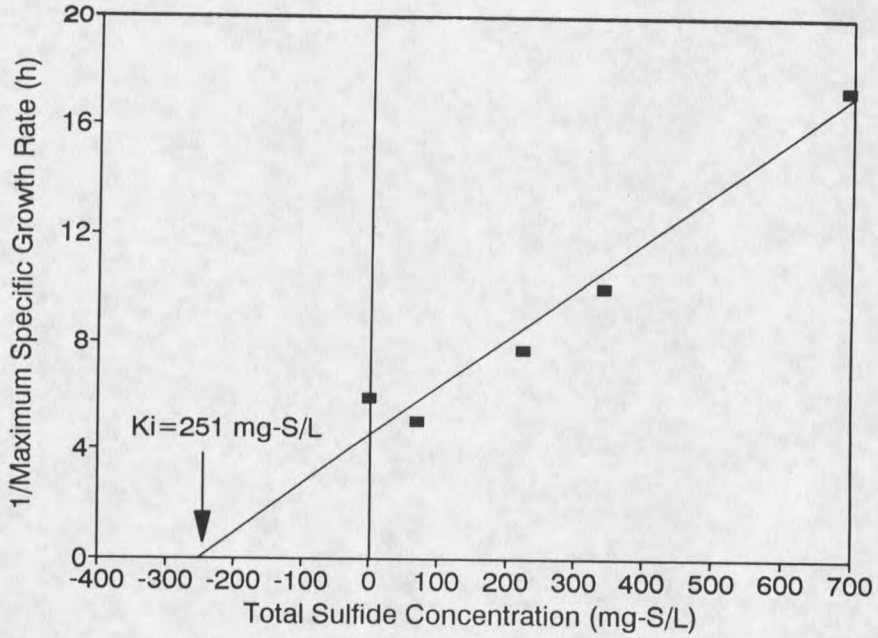


Figure 24. Determination of K_i value in the batch culture. Theoretical plot of the reciprocal of maximum specific growth rate in the presence of sulfide ($1/\mu_{max}^{inh}$) as a function of sulfide concentration. K_i of 251 mg-TS/L was determined.

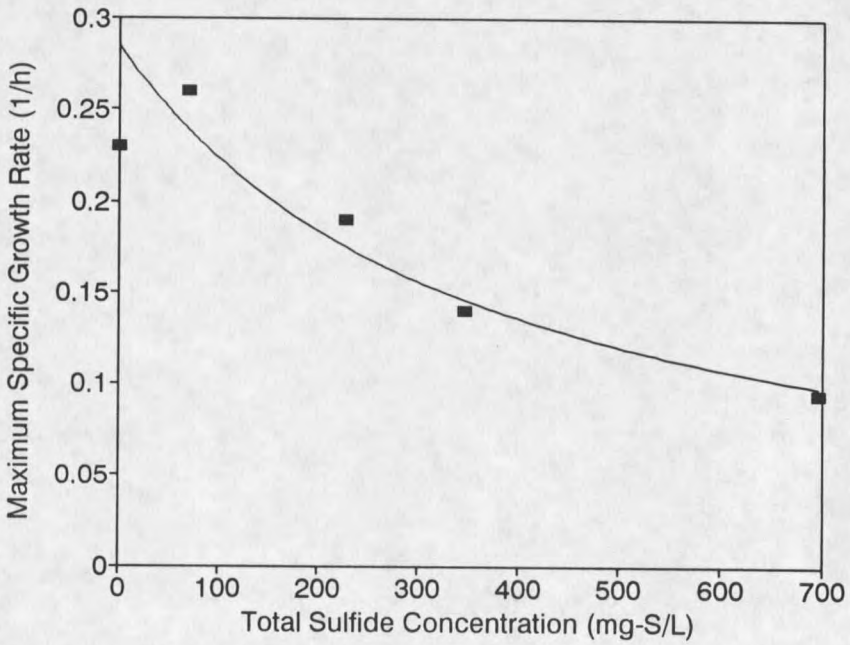
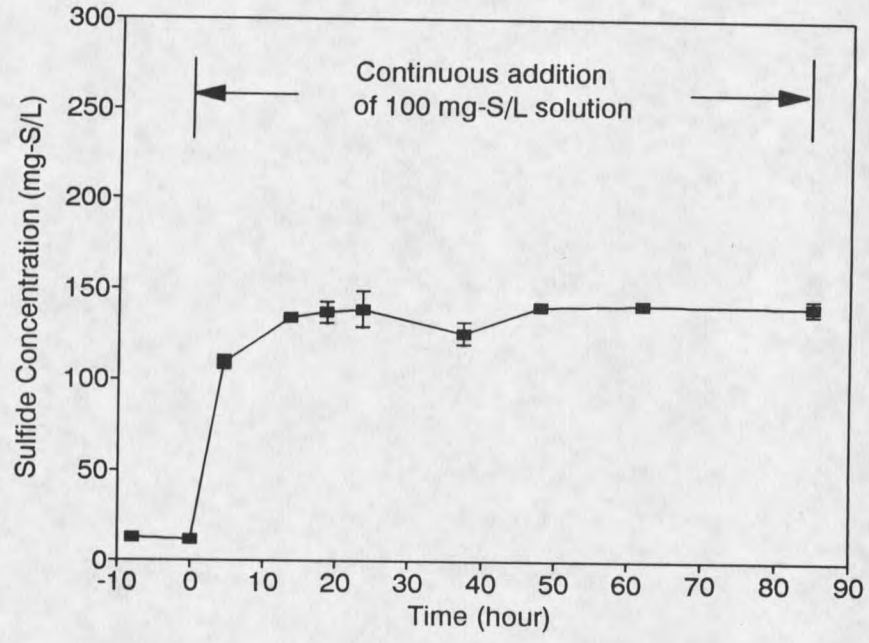


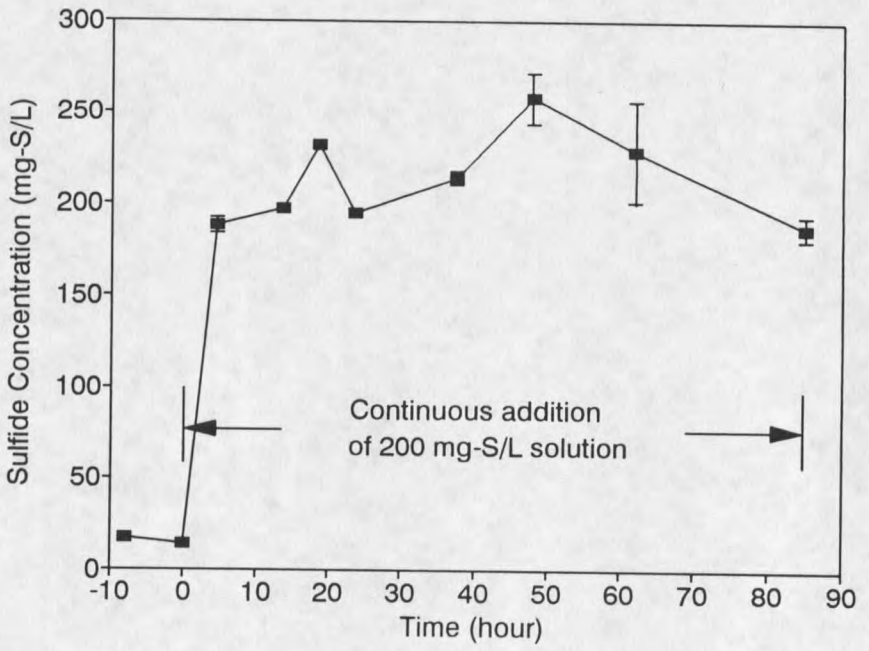
Figure 25. Effect of sulfide on maximum specific growth rate (μ_{max}^{inh}) in batch cultures. (■): experimental points. The solid line reflects the inhibition coefficient (K_i) determined by Figure 24.

Continuous Experiments

Adaption of Microorganisms to Sulfide. *D. desulfuricans* growing at a constant dilution rate (D) of 0.20 h^{-1}, at 35°C, and pH = 7.0 was continuously exposed to high total sulfide concentrations to examine if the bacteria could adapt to a high sulfide environment in terms of growth and activity. Sulfide solutions were fed continuously into the reactor to maintain constant dissolved total sulfide concentrations of 130 and 230 mg-S/L during the experimental period (Figures 26(a) and 26(b)). The initial sulfide concentrations in both experiments were below 20 mg-S/L. Addition of sulfide solutions were started at time zero. Cell production decreased about 25 % at 8 hours after 100 mg-S/L of TS addition, but cell production gradually recovered to the original level and reached a steady state value after about 40 hours (Figure 27 (a)). The activity expressed as lactate oxidation rate $[(S_i - S)/S_i]$ was relatively constant during the experimental period. Cell production was significantly inhibited by 200 mg/L of TS addition (Figure 27 (b)). However, cell production recovered by 20 % at 13.5 hours after sulfide addition and then approached a steady state value, which was about 55 % of the original cell production, whereas the activity remained relatively constant with a minor decrease at 13.5 hours. The culture approached steady state after about 40 hours in both cases. Therefore, all samples in later experiments were taken at about 40 hours after sulfide concentration was changed.

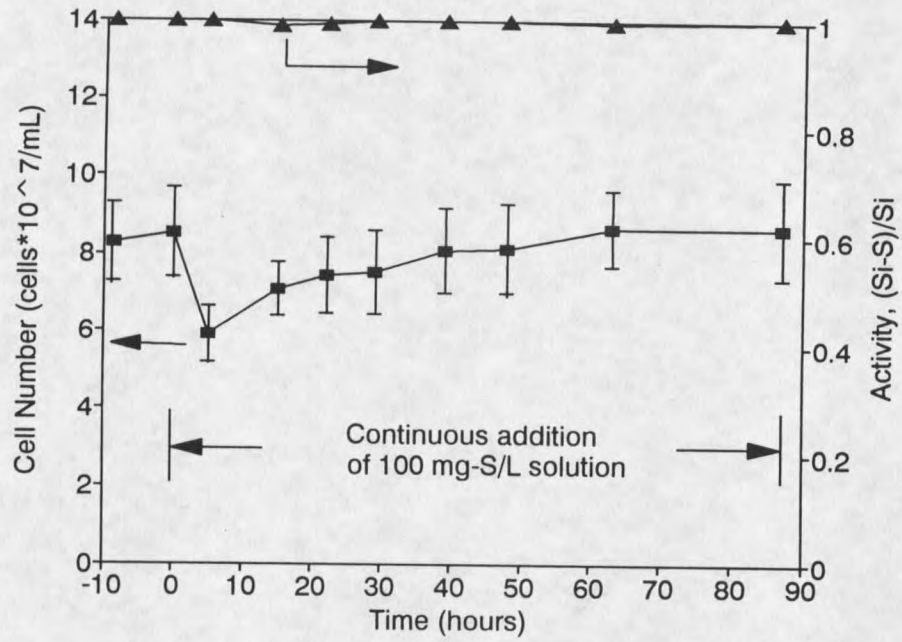


(a)

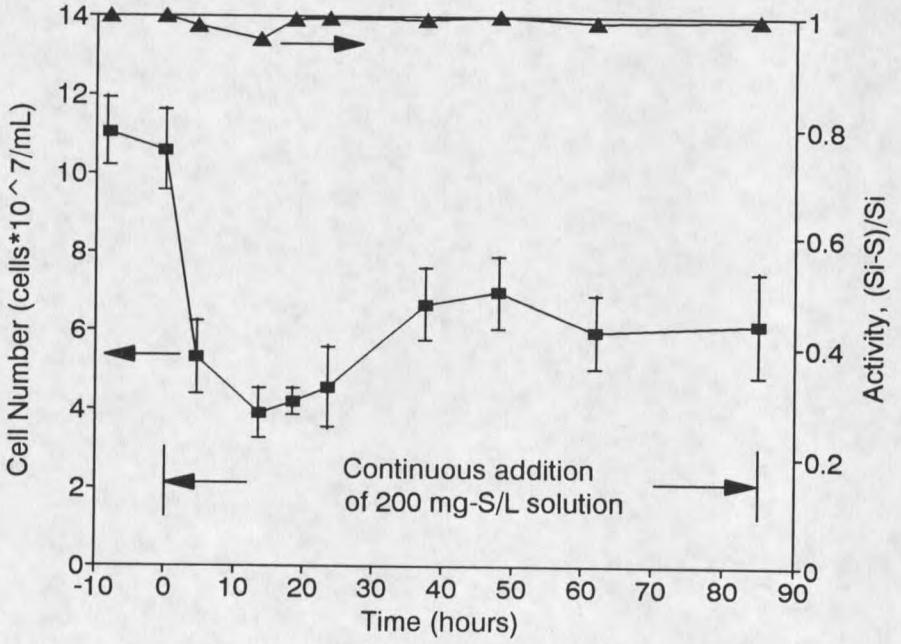


(b)

Figure 26. Change in sulfide concentration with time. (a) 100 mg-S/L and (b) 200 mg-S/L of sulfide solution were added continuously from t = 0. Error bars represent the standard deviation of mean (n = 2).



(a)



(b)

Figure 27. Typical response of cell production and lactate oxidation rate to exposure to (a) 130 mg-S/L and (b) 230 mg-S/L: D = 0.20 h^{-1}, Temp. = 35 °C, pH = 7.0.

Specific Lactate Utilization Rate and Cell Yield. *D. desulfuricans* growing at a dilution rate of 0.20 h^{-1}, at 35°C, and at pH = 7.0 was exposed to various sulfide concentrations to determine the effect of sulfide on the activity and cell production. Lactate oxidation rate $[(S_i-S)/S_i]$ remained relatively constant with increasing TS up to 332 mg-S/L and decreased by 10 % at 437 mg-S/L (Figure 28) , while cellular carbon production dramatically decreased by 50 % at about 250 mg-S/L (Figure 29). As a result of the decrease in cell production, but not lactate oxidation rate, specific lactate utilization increased from about 5.3 g lactate (g cell)$^{-1}$ h^{-1} at 26 mg TS/L to 18.6 g lactate (g cell)$^{-1}$ h^{-1} at 437 mg TS/L (Figure 28). Cell yield dramatically decreased from 0.036 (g cell)(g lactate)$^{-1}$ at low levels TS to 0.013 (g cell) (g lactate)$^{-1}$ at 437 mg-S/L. EPS carbon production slightly increased above 332 mg-S/L.

Determination of Inhibition Coefficient (K_i). Maximum specific growth rates (μ_{max}^{inh}) of *D. desulfuricans* growing at a constant dilution rate of 0.20 h^{-1} at various sulfide concentrations were determined using Eq.(24) and K_{Lac} of 2.35 mg/L (Chapter 2). The maximum specific growth rate in the presence of sulfide (μ_{max}^{inh}) decreased from 0.33 h^{-1} at low level of TS to 0.20 h^{-1} at high level of TS (Figure 30). The inhibition coefficient, K_i, was determined to be 978 mg-S/L by plotting a reciprocal μ_{max}^{inh} against TS concentration (Figure 31). This K_i was determined based on activity (lactate oxidation rate).

Stoichiometry. Stoichiometry of sulfate reduction was determined at each sulfide concentration to elucidate whether the presence of sulfide results in an