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
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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 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 (μ_{max}, K_s, and $Y_{x/S}$) 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 (μ_{bmax}) was essentially the same as μ_{max} for planktonic cells. μ_b 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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Satoshi Okabe

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APPROVAL

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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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Date   Chairperson, Graduate Committee

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Date   Head, Major Department

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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:

o   effect of temperature

o   nutrient availability (including e⁻ donor and acceptor, N, P)

o   sulfide inhibition

o   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

$$CH_3CHOHCOOH + 0.47\ H_2SO_4 + 0.036\ NH_3$$
$$\longrightarrow 0.18\ CH_{1.4}N_{0.2}O_{0.4} + 0.47\ H_2S + 0.94\ CO_2 + 0.94\ CH_3COOH + 1.05\ H_2O$$
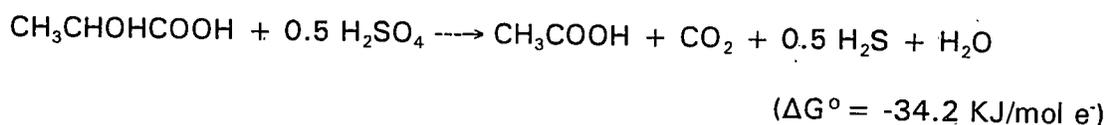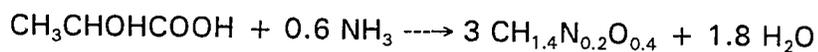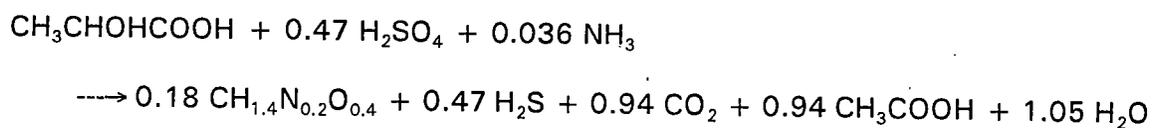
The overall stoichiometric equation was balanced using the experimental determinations of overall yield and carbon dioxide production by *D. desulfuricans* (Traore et al., 1982). D'Alessando et al. (1974) reported very similar stoichiometry for sulfate reduction by *D. vulgaris*. In both cases, lactate 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

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

Synthesis

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

Overall stoichiometry

$$CH_3COOH + 0.92 H_2SO_4 + 0.03 NH_3$$

$$\longrightarrow 0.17 CH_{1.4}N_{0.2}O_{0.4} + 0.92 H_2S + 1.83 CO_2 + 1.95 H_2O$$
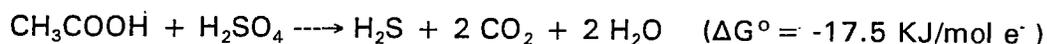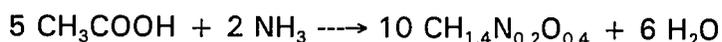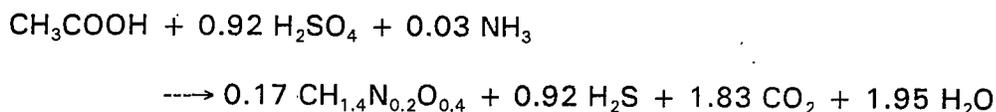
The experimental overall yield obtained by Middleton and Lawrence (1977) was used to balance the overall stoichiometric equation. Each molecule of lactate or acetate transfers 4 and 8 electrons, respectively. The yields for SRB growth with lactate, acetate, butyrate, and propionate as sole energy and 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).

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

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

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

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. (<u>Continued</u>)

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

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

EPS

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

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

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

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

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

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:

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

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.