



Biocatalyzed partial demineralization of acidic metal sulfate solutions
by Robert Michael Hunter

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:

Oxidation and leaching of pyritic minerals is one of the primary causes of chemical pollution from mining activities. These actions can produce drainage waters that contain high concentrations of sulfuric acid and metals.

At present, it is necessary to provide physical-chemical treatment of acid mine drainage. Currently available treatment methods consume significant quantities of chemicals and energy and most produce effluents that contain high concentrations of dissolved minerals. This research investigated the feasibility of the sulfate reduction step in a proposed new process for treatment of acid mine drainage. That process is called biocatalyzed demineralization of acidic metal sulfate solutions and comprises the steps of (1) acid phase anaerobic digestion of biomass to produce volatile acids and a partially stabilized sludge, (2) use of the volatile acids as carbon sources and electron donors for biological sulfate reduction for removal of acidity, metals and sulfate from acid mine drainage, and to produce acetate, (3) use of the acetate solution as feed for methane phase anaerobic digestion to produce methane and to reduce the organic content of the effluent of the process, (4) and use of the methane to satisfy the energy requirements of the process. Single substrate and multiple substrate chemostat studies were conducted to determine if kinetic control (i.e., a relatively short mean cell residence time) could be used to ensure partial oxidation of higher molecular weight volatile acids (propionic and butyric) and production of acetate during the sulfate reduction step. The studies confirmed that propionate produced during acid phase anaerobic digestion can be used as an electron donor for sulfate reduction (during which it is converted to acetate) and that the acetate is available as a substrate for a subsequent methane production step.

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MONTANA STATE UNIVERSITY
Bozeman, Montana

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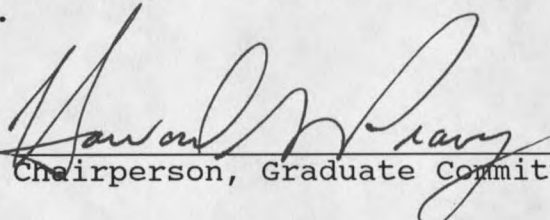
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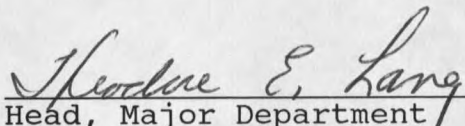
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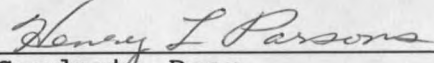
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ABSTRACT

Oxidation and leaching of pyritic minerals is one of the primary causes of chemical pollution from mining activities. These actions can produce drainage waters that contain high concentrations of sulfuric acid and metals. At present, it is necessary to provide physical-chemical treatment of acid mine drainage. Currently available treatment methods consume significant quantities of chemicals and energy and most produce effluents that contain high concentrations of dissolved minerals. This research investigated the feasibility of the sulfate reduction step in a proposed new process for treatment of acid mine drainage. That process is called biocatalyzed demineralization of acidic metal sulfate solutions and comprises the steps of (1) acid phase anaerobic digestion of biomass to produce volatile acids and a partially stabilized sludge, (2) use of the volatile acids as carbon sources and electron donors for biological sulfate reduction for removal of acidity, metals and sulfate from acid mine drainage, and to produce acetate, (3) use of the acetate solution as feed for methane phase anaerobic digestion to produce methane and to reduce the organic content of the effluent of the process, (4) and use of the methane to satisfy the energy requirements of the process. Single substrate and multiple substrate chemostat studies were conducted to determine if kinetic control (i.e., a relatively short mean cell residence time) could be used to ensure partial oxidation of higher molecular weight volatile acids (propionic and butyric) and production of acetate during the sulfate reduction step. The studies confirmed that propionate produced during acid phase anaerobic digestion can be used as an electron donor for sulfate reduction (during which it is converted to acetate) and that the acetate is available as a substrate for a subsequent methane production step.

INTRODUCTION

Overview

Oxidation and leaching of pyritic minerals is one of the primary causes of chemical pollution from mining activities. These actions can produce drainage waters that contain high concentrations of sulfuric acid and metals. Acid mine drainage can be associated with mining of coal, metal ores (copper, zinc, lead, and uranium), pyritic sulfur and geothermal fluids. An acid mine drainage problem can continue for as long as oxygen and water are in contact with the minerals.

The same processes that cause acid mine drainage have also been used in engineered systems for extraction of metal values from low grade ores in dump leaching, heap leaching and tank leaching operations. These processes have been given greater attention in the last few decades as deposits of high grade ores have been depleted and metals prices have dropped (Ehrlich and Holmes, 1986).

Conventional acid mine drainage treatment comprises lime addition and aeration for ferrous iron oxidation and precipitation. The process is often carried out at a pH above 9 to facilitate precipitation of other dissolved

metals. Neutralization of the water is sometimes necessary prior to discharge. Other treatment methods, including sulfide addition, reverse osmosis, and ion exchange, have been investigated but have been shown to have higher cost (Peavy and Hunter, 1987).

The lime precipitation process is reliable and relatively inexpensive, but it does have some serious disadvantages. One particularly perplexing disadvantage is that lime addition results in supersaturation of treated water with calcium sulfate. This compound, gypsum, usually precipitates after discharge causing cementation of downstream streambed gravels. This phenomenon was one of the most serious environmental effects of discharges of treated wastewater from the Anaconda copper mining operations in Montana. Even if calcium is removed by soda ash addition in a subsequent treatment step, the resulting effluent can contain such high concentrations of dissolved salts as to be of limited utility. For these reasons, investigation of alternative treatment schemes which include sulfate removal are of interest.

Goal and Objectives

The goal of this research was to investigate the feasibility of an alternative treatment process, termed biocatalyzed partial demineralization of acidic metal sulfate solutions. The process is comprised of a number of

steps, most of which utilize basically proven technologies. This research focused on a critical step in the process, kinetically controlled microbial sulfate reduction, and assessed its technical feasibility by testing the following hypothesis:

A chemostat containing a mixed culture of sulfate-reducing bacteria (SRB) can be operated at a dilution rate large enough to cause incomplete oxidation of propionic acid and butyric acid and production of acetic acid, if all three acids are present in the reactor feed, but sufficient sulfate is present to allow utilization of only the two higher molecular weight acids.

The specific objectives were as follows:

1. to determine an operating pH and temperature likely to be optimum for propionate oxidation while allowing oxidation of higher molecular weight volatile acids, based on literature data,
2. to experimentally determine Monod kinetic parameters ($\mu_{\max}$, K_S , and Y_G) for propionate oxidation under the conditions determined above,
3. to estimate kinetic parameters for acetate oxidation under the conditions selected for optimum propionate oxidation, based on literature data,

4. to experimentally determine kinetic parameters for butyrate oxidation under conditions selected for optimum propionate oxidation,
5. to experimentally determine the stoichiometry of acetate production during oxidation of propionate and butyrate,
6. to use the above parameters to calibrate a multiple substrate growth model, and
7. to confirm that mixed culture sulfate-reducing bacteria use multiple substrates as predicted by the model calibrated using single substrate data.

To accomplish these objectives, a number of experiments were conducted using an anaerobic reactor fabricated for this research. Possible applications for results obtained from this research include treatment of acid mine drainage and other acidic metal sulfate solutions, metals recovery and sulfur recovery. The kinetic data developed during this research will contribute to an increased understanding of microbial sulfate reduction.

It should be noted that the experiments conducted during this project were not specifically designed to determine appropriate conditions for essentially complete removal of sulfate. As was noted in the hypothesis this research set out to test, sulfate was believed to be present in the medium in excess of stoichiometric require-

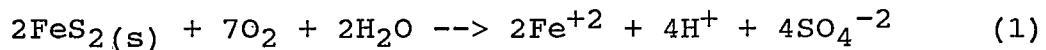
ments, often much in excess (i.e., over 1,000 mg/l in excess). Further experiments with sulfate as the limiting substrate will be necessary to establish appropriate operating conditions for its removal and the kinetic parameters with which to design sulfate removal reactors.

BACKGROUND

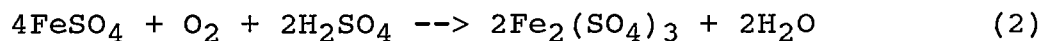
The Acid Mine Drainage ProblemFormation of Acid Mine Drainage

The mechanism of acid water formation when water and air come into contact with iron sulfides associated with coal seams or metal sulfides in ore bodies has been much studied (Goldhaber and Kaplan, 1974). In general, half of the acidity is attributable to sulfide oxidation to sulfate and half to ferrous iron oxidation to ferric iron and its hydrolysis.

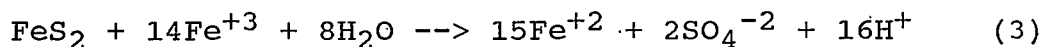
Acid mine drainage associated with mining of coal forms when FeS_2 (pyrite, marcasite, etc.) in coal seams is exposed to moisture and air. Initially, FeS_2 is oxidized in the following reaction:



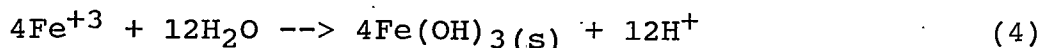
Although this reaction can occur abiotically, it is accelerated by Thiobacillus ferrooxidans which also mediates the reaction (Hutchins et al., 1896):



The oxidation of pyrite is further accelerated by the following reaction:

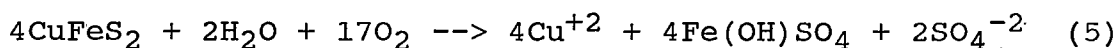


Alternatively, the ferric iron may precipitate as follows:



Once the pyrite oxidation cycle has started, further oxygen is needed only for the microbially catalyzed oxidation of ferrous iron to ferric iron.

Acid mine drainage formed from metal ore deposits results, in part, from reactions similar to those noted above. In addition, chalcopyrite (CuFeS_2) is oxidized on exposure to air as follows:



This reaction is also significantly accelerated by T. ferrooxidans.

The role of sulfur bacteria of the genus Thiobacillus in accelerating the above processes has also been much studied (Baker and Wilshire, 1970). These acidophilic, chemolithotrophic bacteria are capable of deriving all of their energy from the oxidation of reduced inorganic sulfur or reduced iron compounds. They consume oxygen and use carbon dioxide as a carbon source.

The National Problem

It has been estimated that 10,000 miles of streams and 29,000 surface acres of impoundments are seriously affected by mine drainage. About 40 percent of this drainage comes from active mines; the remainder from abandoned surface mines (15 percent) and shaft and drift mines (45 percent) (Goldhaber and Kaplan, 1974).

The free mineral acid loads associated with coal mine drainage alone in the United States exceed 5,300 tons per day (Goldhaber and Kaplan, 1974). Neutralization of this acidity would consume over 1,100,000 tons of lime per year. Manufacture of this amount of lime would consume about 5 trillion BTU per year.

A Montana Problem (or Opportunity):
The Berkeley Pit

Mining began in the Butte, Montana, area in 1864 when prospectors discovered shallow placer deposits of gold and then silver. By the middle of the twentieth century, over 400 underground mines had operated or were still operating in the area (Camp Dresser & McKee Inc., 1988e). In 1955, Anaconda Copper Mining Company (which merged in 1977 with Atlantic Richfield Company) began an open pit mining operation in Butte known as the Berkeley Pit. The Pit, which was operated until 1982, encompasses about 767 surface acres and has a volume of approximately 9.7 billion cubic feet from its base in 1982 to its top at elevation 5,520 feet (Anaconda Minerals Company elevation datum).

During mine operation, the Pit and associated underground workings in the area were continuously dewatered by pumping the Kelley Shaft and by drainage from the Pit to other underground workings. The water from the Kelley Shaft was used in mining operations and eventually treated and discharged to Silver Bow Creek. Silver Bow Creek is a

tributary of the Clark Fork River which is a tributary of the Columbia River.

In recognition of the potential hazard to human health and the environment, the Silver Bow Creek Superfund site was added to the National Priorities List in 1983 by the U.S. Environmental Protection Agency (EPA) (48 FR 40658, September 8, 1983) pursuant to the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA). The Silver Bow Creek site comprised about 28 stream miles beginning at the Metro Storm Drain in Butte. During work on the Silver Bow Creek site, the importance of Butte area workings as the source of contamination of the site was formally recognized. In 1987, the site was expanded to include the Butte area and its name was changed to the Silver Bow Creek/Butte Area site (52 FR 27627, July 22, 1987, Final Rule). The Butte addition consists of the East Camp area, which comprises the Berkeley Pit and associated underground mine workings, and the West Camp area, which includes the Travona and Emma mines. The two areas are thought to be separated hydraulically by bulkheads installed in the underground workings in the 1960's. The Berkeley Pit and associated flooded mine workings are also identified as the Butte Mine Flooding Operable Unit and plans for a Remedial Investigation/Feasibility Study are currently being formulated consistent with EPA's National Contingency Plan

(NCP) (40 CFR Part 300), CERCLA and the Superfund Amendments Reauthorization Act (SARA).

Preliminary water balance studies indicate that the Pit is currently filling at a rate of about 7.6 million gallons per day (mgd) (Camp Dresser & McKee Inc., 1988c). If this rate does not change, the Pit will overtop (at 5,520 feet) by about the year 2010 (21 years hence). Prior to that time, there is a potential for discharge of contaminated water into the alluvial aquifer (which is exposed on the southeast side of the Pit) by about the year 1996 (7 years hence). The water flooding the Pit is comprised of 4.0 mgd of process water inflows from current mining operations by Montana Resources Inc. and about 3.6 mgd of groundwater inflow. The groundwater inflow includes a component of unknown magnitude resulting from urban stormwater runoff from the City of Butte that flows into the Syndicate Pit and, hence, into an East Camp shaft.

Total mine dewatering pumpage from the West Camp area was historically about 2.0 mgd (Camp Dresser & McKee Inc., 1988c). The water level in the West Camp mines has been rising since 1984 at a rate of about 4 feet per month. Water from West Camp mine workings (as measured by flooding of the Travona Shaft) was projected to discharge to Silver Bow Creek alluvial deposits by Spring 1989, however, pumping of the shaft has been initiated with discharge to the City of Butte wastewater treatment plant. It is

hypothesized that one reason for the enhanced flooding of the West Camp system is partial failure of the bulkheads that separate the East Camp and West Camp workings because West Camp flooding commenced after dewatering of the Pit ceased (Camp Dresser & McKee Inc., 1988c).

Historical data on Berkeley Pit water quality are presented in Table 1. The water has been described as an acidic metal sulfate solution (Camp Dresser & McKee Inc., 1988a). If the concentration of metals were to remain at

Table 1. Berkeley Pit Water Quality

Constituent	Average concentration, mg/l, unless otherwise noted			
	1984 ^a	1985 ^a	1986 ^a	1987 ^b
Cations				
Aluminum	131	129	178	181
Arsenic	0.13	0.22	0.08	0.70
Cadmium	1.4	1.3	1.6	1.7
Calcium	451	424	450	468
Copper	127	146	192	193
Iron	235	283	812	872
Lead	0.17	-	-	0.7
Magnesium	220	205	271	256
Manganese	97	92	133	145
Nickel	0.74	0.65	0.85	-
Potassium	4.7	8.4	23	18
Silver	0.024	0.038	0.036	-
Sodium	62	63	65	70
Zinc	211	232	409	444
Anions				
Chloride	12	10	-	19
Sulfate	4,059	4,375	-	7,060
Other parameters				
pH, units	2.7	2.4	-	3.0
Silica, as SiO ₂	86	93	103	-

^a Source: Sonderegger, 1987

^b Camp Dresser & McKee Inc., 1988c

1987 average levels and the Pit were to fill to the rim (5,520 foot level), the Pit would contain the amount and value of elements indicated in Table 2 based on projected 1990 unit prices (Brower, 1987).

Table 2. Value of Elements in Berkeley Pit

Element	Amount, million pounds	Unit price, dollars per pound	Value, million dollars
Aluminum	110	1.03	113
Copper	118	1.49	176
Magnesium	156	1.48	230
Manganese	88	1.47	130
Zinc	270	0.43	116
Sulfur	1,430	0.04	57

Average concentrations of the following constituents exceed federal ambient water quality criteria for aquatic life (Camp Dresser & McKee Inc., 1988d):

Arsenic
Cadmium
Copper
Iron
Lead
Zinc

Furthermore, discharge of water with such high sulfate and iron concentrations and low pH could cause serious acid mine drainage and gypsum precipitation problems downstream. The gypsum precipitation problem caused in treatment of acid mine drainage from the Butte area in the Warm Spring ponds was described by the EPA (1972) as follows:

During the course of the field investigations it was noted that from Warm Springs to somewhere between Dempsey and Deer Lodge the bottom materials were 'cemented' together.... This condition is due primarily to the high levels of calcium, sulfate, and metallic ions in the discharge from the Anaconda Company settling ponds which precipitates as gypsum and insoluble metallic hydroxides and coat the bottom.

The magnitude of the potential gypsum precipitation problem can be indicated by consideration of the following hypothetical scenario:

1. Seven mgd of Pit water is treated with lime to neutralize it and precipitate the metals.
2. Lime addition plus calcium from natural sources results in downstream precipitation of calcium sulfate concentrations in excess of about 2,000 mg/l, its solubility in cold water.

Under this scenario, about 400,000 pounds per day of gypsum (2,000 mg/l as calcium plus 5,000 mg/l as sulfate) would precipitate on the stream beds of Silver Bow Creek and/or the Clark Fork River. This is equivalent to almost 75,000 tons of gypsum per year. As a four foot by eight foot by 1/2 inch sheet of gypsum sheet rock weighs about 60 pounds, 75,000 tons per year of gypsum is sufficient to "sheet rock" over 500 miles of a hypothetical stream of rectangular cross section, 20 feet wide and 4 feet deep, each year. This potential cementation problem is particularly significant in that the Clark Fork Basin Project (Johnson and Schmidt, 1988) reported that "most of

the upper (Clark Fork) river seems unsuitable for trout reproduction due to siltation and other substrate deficiencies." That the problem of gypsum precipitation has been significant was noted by the EPA (1972) in a study of Clark Fork water quality:

The precipitation of gypsum and metallic hydroxides eliminates or greatly reduces the availability of bottom gravels for fish spawning and benthic habitat.

An analysis of the annual value of recovery of 100 percent of the copper, zinc and sulfur in Pit water at various pumpage rates is presented in Table 3. The same concentrations and unit prices as were used above were used in this analysis.

Table 3. Annual Value of Metals and Sulfur

Element	Value, million dollars per year		
	2.0 mgd	5.0 mgd	7.0 mgd
Copper	0.9	2.2	3.1
Zinc	1.2	2.9	4.1
Sulfur	0.6	1.4	2.0
Total	2.7	6.5	9.2

Bioprocessing of Acid Mine Drainage

A number of investigators have studied the use of sulfate-reducing bacteria (SRB) for treatment of acid mine drainage. To date, however, an economically feasible process has not been developed.

Tuttle et al. (1969) described the potential utility of microbial sulfate reduction as an acid mine drainage water pollution abatement procedure. Batch testing of cultures grown in acid mine drainage (pH 3.6) and wood dust at various temperatures indicated that more sulfate was reduced at 37 °C than at 25 °C or 50 °C. Enrichment of wood dust-acid water cultures with sodium lactate resulted in a pH increase from 3.6 to 7.0 in a 10-day period.

King et al. (1974) described the use of sulfate-reducing bacteria in accelerating the rate of acid strip mine lake recovery. They indicated that the key to the process is the accrual of enough organic material at the bottom of the lake to allow generation of suitably reduced conditions for sulfate-reducing bacteria to grow. They noted significant differences in both acidity (2,660 mg/l vs 0 mg/l) and specific conductance (6,400 micromho/cm vs 230 micromho/cm) in lakes and these differences were attributed to the process.

Ilyaletdinov and Loginova (1977) reported the findings of batch testing of sulfate-reducing bacteria for removal of copper from the effluent of the Balkhash Mining and Metallurgical Combine. They were able to reduce copper concentrations of 0.08 mg/l in settling pond effluent (with a sulfate concentration of 1,200 mg/l and lactate as a carbon source) to 0.02 mg/l within a few weeks. Batch testing of treatment of effluent from the secondary

settling pond with chopped reed and ammonium sulfate additions showed that 1.0 mg/l concentrations of copper could be reduced to zero in 10 days.

The concept was also tested in continuous culture in a six-section reactor with a residence time of 10 days. The bottom of the reactor was covered with mud to provide a habitat for the bacteria. At 25 °C, the reactor was able to reduce an initial concentration of copper of 1.26 mg/l to zero.

Yagisawa et al. (1977) and Noboro and Yagisawa (1978) presented the results of continuous recovery of metals from a solution prepared by bacterial leaching of copper sulfide ore. Batch culture of sulfate-reducing bacteria in the leaching solution was impossible because of the low pH and high concentration of metals in the solution.

The specific growth rate and the rate of removal of metals were found to be strongly influenced by the pH of the culture. The optimum pH for both metal removal and growth was 6. In continuous culture at 30 °C and pH 6, the maximum rate of metals removal occurred at 40 percent of the metal concentration of the original solution. The original solution contained 270.0 mg/l copper, 102.5 mg/l zinc and 135.0 mg/l iron. Sodium lactate and yeast extract were added to the culture. A black precipitate was produced that contained 19.96 percent copper, 6.13 percent zinc and 10.95 percent iron.

Olson and McFeters (1978) described microbial sulfur cycle activity at a western coal strip mine. The sediments of the mine settling pond supported a large and active population of sulfate-reducing bacteria, producing up to 10.5 mg hydrogen sulfide per liter of sediment per day. Metal bound sulfides were found to comprise, at times, over 0.2 percent of the dry weight of pond sediments, leading the investigators to suggest that sulfate-reducing bacteria were precipitating heavy metals in the pond.

Grim et al. (1984) described the findings of batch and continuous culture studies of sulfate reduction by an acetate-utilizing strain of Desulfobacter postgatei. Growth was optimized by constant pH control, slow nitrogen purge to prevent inhibition by the sulfide ion, and immobilization of cells on a fixed surface in a continuously stirred tank reactor. A ferric sulfate precipitate adhered to the wall of the reactor apparently allowing cell numbers to increase and facilitating increased sulfate reduction.

A New Biotechnology: Biocatalyzed Partial
Demineralization of Acidic Metal Sulfate Solutions

The shortcomings of other techniques for bioprocessing acid mine drainage required the development of a new process for biocatalyzed partial demineralization of acidic metal sulfate solutions. The process was first described by Hunter, Peavy and Sonderegger (1987). It is

illustrated generally in Figure 1. The process comprises the steps of (1) acid phase anaerobic digestion of biomass to produce volatile acids and a partially stabilized sludge, (2) use of the volatile acids as carbon sources and electron donors for biological sulfate reduction for removal of acidity, metals and sulfate from acid mine drainage, and to produce acetate, (3) use of the acetate solution as feed for methane phase anaerobic digestion to produce methane and to reduce the organic content of the effluent of the process, and (4) use of the methane to satisfy the energy requirements of the process. Key to the feasibility of the process is the use of kinetic control (i.e., a relatively short mean cell residence time) to ensure partial oxidation of higher molecular weight volatile acids (e.g., propionic, butyric, valeric) and production of acetate during the sulfate reduction step. In this way, the higher molecular weight volatile acids produced during acid phase anaerobic digestion can be used BOTH as electron donors for sulfate reduction (during which they are converted to acetate) and as substrates for the subsequent methane production step.

If proven technically feasible, the process would have several advantages over prior acid mine drainage bioprocessing schemes. A primary advantage of the technology is that acid phase anaerobic digestion (acidogenesis) of biomass is used to supply electron donors and

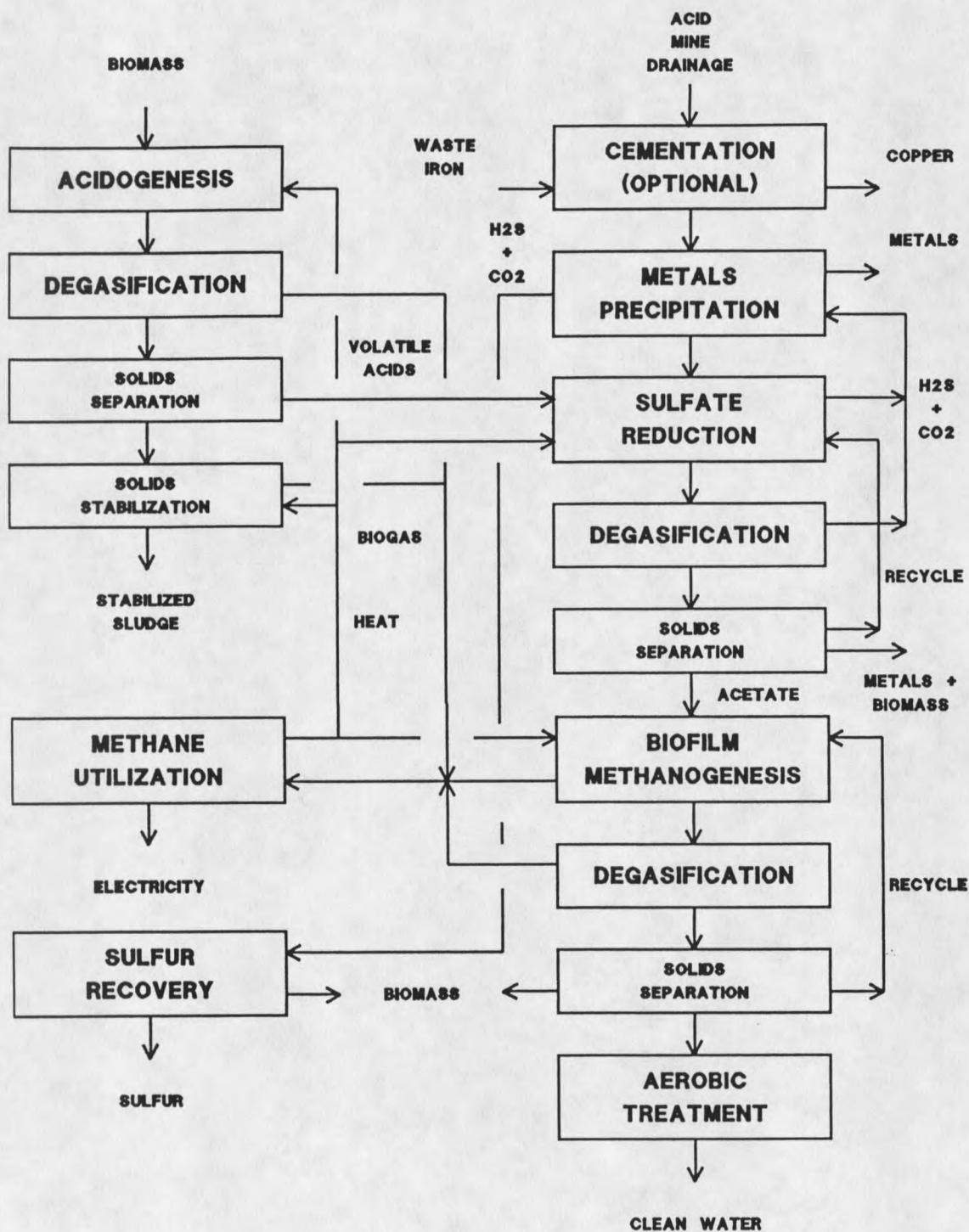


Figure 1. Process Diagram

carbon sources to the microbial sulfate reduction step of the process. The presumption here is that volatile acids (e.g., acetic acid, propionic acid, butyric acid, valeric acid, etc.) produced on-site by acidogenesis of biomass will cost less than purchasing preformed electron donors. This presumption is true if a source of large amounts of relatively easily digested biomass is available near the treatment site. Some other bioprocessing schemes call for use of biomass as a source of electron donors, but they also call for mixing the biomass with the acid mine drainage. This would result in production of a digested biomass that is stabilized but that is also contaminated with heavy metals. The proposed process, on the other hand, would produce a stabilized sludge that is metal-free and that could be used as a soil conditioner in mined land reclamation efforts.

Another advantage of the process is that acetate produced during the microbial sulfate reduction step is used as a substrate for a subsequent methane production step. Heat produced from combustion of the methane could be used to provide a portion of the heat required to maintain the contents of the acidogenesis, sulfate reduction and methanogenesis reactors at the elevated temperatures (e.g., 35 °C) necessary for optimum biological action.

Considerable work has been done in characterizing acid phase anaerobic digestion, microbial sulfate reduction, and methane phase anaerobic digestion. Important findings are presented below.

Acid Phase Anaerobic Digestion

The acid formation phase of anaerobic digestion has been thoroughly reviewed (Toerien and Hattingh, 1969; Zehnder, 1978). Its outcome is the conversion of complex organic matter into saturated fatty (volatile) acids, carbon dioxide and ammonia. The volatile acids have been found to be acetic, propionic and butyric acids with lesser amounts of formic, lactic and valeric acids. Acetic acid is the most plentiful followed by propionic acid. Acid phase anaerobic digestion has been successfully accomplished on a laboratory scale by many investigators (Ghosh et al., 1975; Heijnen, 1984; Pohland and Ghosh, 1971a and 1971b). The maximum specific growth rate ($\mu_{\max}$) of acidifying bacteria is about 0.3 to 0.5 hr⁻¹. Available data on the products of acid phase anaerobic digestion of wastewater treatment sludge and glucose are presented in Table 4.

Microbial Sulfate Reduction

There is no known chemical mechanism for the reduction of sulfate by organic matter at normal earth temperatures and pressures (Goldhaber and Kaplan, 1974). Hence,

Table 4. Products of Acid Phase Anaerobic Digestion

Parameter	Hindin and Dustin, 1959	Ghosh et al., 1975	Wise et al., 1985
Substrate	Domestic sludge	Activated sludge	Glucose ^a
Detention time, hours	120	12.7	43.2
pH, units	5.15	5.74	5.0
Total volatile solids, mg/l as acetic acid	5,519	NR ^b	NR
Volatile acid components, mg/l			
Formic	18	NR	NR
Acetic	1,882	2,396	846
Propionic	2,717	839	37
Butyric	2,037	890	1,460
Valeric	NR	900	92
Caproic	NR	NR	4,450
Lactic	33	NR	NR

^aMethane production suppressed chemically

^bNR = not reported

microorganisms are thought to be completely responsible for the process. In dissimilatory sulfate reduction, sulfate is used as the electron acceptor for the oxidation of organic matter. The end product of this oxidation is the excretion of hydrogen sulfide by the microorganism.

Until 1980, three genera of sulfate-reducing bacteria were known to exist: Desulfovibrio, Desulfomonas, and Desulfotomaculum. These known organisms can use a limited range of carbon sources, typically fermentation products such as alcohols and organic acids (D'Alessandro et al.,

1974). The organisms are obligatory anaerobes. Until that time, the known organisms all oxidized organic substrates incompletely, with acetate being the normal end product when lactate, pyruvate or ethanol served as the electron donor and carbon source (Postgate, 1984). In 1980, five new genera, Desulfobacter, Desulfobulbus, Desulfococcus, Desulfosarcina, and Desulfonema, plus new species of the old genera, some of which could oxidize organic compounds completely to carbon dioxide, were reported (Pfennig and Widdel, 1981).

As a group, sulfate-reducing bacteria can tolerate a wide variety of environmental extremes, including temperature, salinity, and pressure (Postgate, 1984). They comprise both mesophilic strains which grow best at temperatures between about 30 °C and 42 °C and thermophilic strains able to grow at temperatures in the 50 °C to 70 °C range. They tolerate pH values ranging from below 5 to 9.5 (Postgate, 1984).

Some compounds found in acid mine drainage have been reported to inhibit the growth of sulfate-reducing bacteria. For example, Saleh et al. (1964) reported a minimum inhibitory concentration of 5-50 mg/l of copper sulfate. Exposure of cell suspensions to 10 millimolar (mM) concentrations of such oxyanions as molybdate, selenate, tungstate, and chromate has caused destruction of adenosine 5'-triphosphate (ATP) sulfurylase, the enzyme

that catalyzes the first step in sulfate reduction (Taylor and Oremland, 1979). The minimum inhibitory concentration of zinc sulfate, on the other hand, is reported to be 10,000 mg/l (Saleh et al., 1964). Lead acetate has often been added to solutions used for identification of the organisms. The bacteria have been grown in the presence of carbonates or oxides of lead, zinc, antimony, bismuth, cadmium, cobalt, nickel and copper. Only copper, as the basic carbonate, showed any toxicity (Miller, 1950).

Information on the environmental requirements of sulfate-reducing bacteria in pure cultures must be viewed in the light of the ability of the organisms to create microenvironments conducive to their growth. Effective sulfate reduction and iron precipitation has been shown to occur in a pH 2.8 water, for example, when bacteria could not be isolated that grew below pH 5 (Tuttle et al., 1969).

A mixed culture of sulfate-reducing bacteria isolated from soil was grown in a chemostat supplied with effluent from a bacterial leaching operation that contained up to 270 mg/l of copper, 102.5 mg/l of zinc, and 135 mg/l of iron (Noboro and Yagisawa, 1978). Maximum growth occurred at a copper concentration of 108 mg/l.

Methane Phase Anaerobic Digestion

The methane-production phase of anaerobic digestion has been accomplished in conventional chemostats (Ghosh et al., 1975), in fluidized bed reactors (Heijnen, 1984) and

in packed bed reactors (Heijnen, 1984). Several workers have noted that when acetic, propionic and butyric acids are subjected to methane-phase anaerobic digestion, only acetic and butyric acids are metabolized initially. Propionic acid is only degraded when acetic acid concentrations have reached low levels (Pohland and Ghosh, 1971; Heijnen, 1984).

When anaerobic digestion is divided into two phases kinetically, two populations of bacteria occur in the methane production phase. The first group are acetogenic bacteria which convert propionic and butyric acids into acetate and hydrogen. The $\mu_{\max}$ of this population is about 0.01 hr^{-1} (Boone and Byrant, 1980). The second group is a methanogenic population which converts acetate or hydrogen and carbon dioxide into methane. The $\mu_{\max}$ of this population is about 0.1 hr^{-1} with hydrogen plus carbon dioxide as substrate and $0.02\text{--}0.03 \text{ hr}^{-1}$ with acetate as substrate (Harper and Pohland, 1986). The biomass yield of both groups is very low.

Recent work with an expanded-bed, granular activated carbon anaerobic reactor confirmed that biofilm (attached-growth) reactors can achieve very effective conversion of acetate to methane. Wang (1986) found that such a reactor could produce effluent acetate concentrations of 5 to 6 mg/l (soluble COD = 15 to 23 mg/l) with an influent acetate

concentration of 3,200 mg/l. This performance was achieved at an empty-bed liquid detention time of one day at pH 7.0.

Sulfur Recovery

Cork and Cusanovich (1978) reported the findings of batch studies of biological conversion of sulfate in solvent extraction raffinates, a waste product of hydrometallurgical copper ore processing, to elemental sulfur. Desulfovibrio desulfuricans was used for sulfate reduction and either of the photosynthetic bacteria Chlorobium thiosulfatophilum or Chromatium vinosum was used for conversion of hydrogen sulfide (H_2S) to elemental sulfur. The organisms were cultured separately and a purge system using an inert carrier gas was used to transfer H_2S gas from one culture to the other. Lactic acid and yeast extract were used as the carbon source for sulfate reduction. With Chlorobium, an overall process conversion rate of 55 percent was achieved.

Cork and Cusanovich (1979) presented the results of pilot scale batch and continuous culture studies of the two-stage conversion process they introduced in 1978. The tests were carried out at an optimum temperature of 30 °C and an optimum pH of 7. At an initial sulfate concentration of 13,400 mg/l, a conversion rate of 91 percent was achieved using lactic acid as the carbon source for sulfate reduction. Utilization of Chlorobium biomass as an alternate carbon source was investigated, but only 10

percent of the required carbon could be supplied in that manner. The investigators suggested using other carbon sources such as raw sewage.

Uphaus et al. (1983) described another version of purged microbial mutualism using Desulfobacter postgatei, a sulfate-reducing bacterium capable of using acetate as its sole preformed carbon source. This substrate is more economical than lactate and the products of its metabolism include carbon dioxide and hydrogen sulfide which can serve as feed gas for the growth of photosynthetic green sulfur bacteria. Rigid compressed panels of fiberglass taken from commercial ceiling panels were used as a solid support matrix for the cells of the sulfate-reducing bacteria. Addition of concentrated, cell-free Chlorobium culture supernatant to the immobilized Desulfobacter culture increased sulfate reduction rates by 3 to 4 times.

Modeling of Microbial Growth

A significant part of this research involved development and calibration of a model of the sulfate reduction unit operation of the proposed process. The purpose of the modeling effort was threefold. First, development of the model in the early stages of the project facilitated design of the experimental apparatus and procedures. Second, the model provided a framework for understanding the microbiology of the system under study. Finally, a calibrated

model is a valuable tool for investigating applications for the basic knowledge gained during the research. The rationale for the model selected for use in the study of the sulfate reduction unit process and an explanation of the model are presented below.

Model Selection

An appropriate model for the sulfate reduction step of the proposed process should reflect its microbiology. In this situation, two microbiological mechanisms are possible: (1) the sequential utilization of multiple substrates by one or more SRB capable of utilization of acetate, propionate, butyrate, etc. and (2) "natural selection" of one or more microorganisms that are capable of using volatile acids with a higher molecular weight than acetate. The first mechanism is a diauxic (or triauxic) growth pattern and a diauxic (or triauxic) model would be appropriate. With the second mechanism, shifting from one substrate to another does not occur and, at steady state, use of a plurality of single substrate/single microorganism models is appropriate for steady state operation after competition has completed the microorganism selection process. The findings of this research and that of Pfennig and Widdel (1981) conclusively support use of two single substrate/single microorganism models in the instance of utilization of propionate and butyrate.

Pfennig and Widdel (1981) described the situation as follows:

Enrichment cultures with propionate or butyrate all yielded strains that incompletely oxidized their substrates. Apparently, under conditions of competition such strains have a selective advantage at high concentrations of these substrates. Pure cultures of strains enriched with other substrates were even capable of completely oxidizing propionate and butyrate; however, such strains could not be selectively enriched with these substrates.

Thus, single substrate models are used herein because:

1. Acetate, propionate and butyrate are the primary volatile acids present in anaerobic digester supernatant and the only volatile acids considered in this research.
2. The presence of only propionate or butyrate in a medium reportedly select for SRB species that oxidize these substrates incompletely to acetate.
3. SRB species capable of incomplete oxidation of propionate or butyrate are incapable of oxidation of acetate.
4. At low dilution rates, it is likely that three groups of SRB, each of which is capable of oxidizing a different volatile acid, would be present in a chemostat.
5. Under such conditions, the only interaction among the SRB groups would be that, at very low

dilution rates, the acetate produced by oxidation of propionate and butyrate would be available for oxidation by acetate-utilizing SRB.

Single Substrate Models

The continuous stirred tank reactor (CSTR) is one of the simplest reactor configurations used in biochemical operations. These reactors are referred to as chemostats in microbiological research because they provide a constant chemical environment for microbial growth. When that chemical environment contains a single growth-limiting nutrient (substrate), relatively straight-forward mass balances on substrate and cells can be used to develop models for the systems.

Mass balances on substrate and cells entering and leaving the reactor are presented below:

$$\begin{array}{ccccccc} \text{Accumulation} & = & \text{Mass} & - & \text{Mass} & + & \text{Mass} & - & \text{Mass} & (6) \\ & & \text{in} & & \text{out} & & \text{generated} & & \text{consumed} \end{array}$$

Substrate:

$$(dS/dt)V = QS_0 - QS - r_S V \quad (7)$$

Cells (biomass):

$$(dX/dt)V = QX_0 - QX + r_{g/x}V - r_{d/x}V \quad (8)$$

where

Q = volumetric flow rate

t = time

V = reactor volume

S_0 = input substrate concentration

S = reactor and output substrate concentration

r_s = rate of consumption of substrate

X_0 = input cell concentration

X = reactor and output cell concentration

$r_{g/x}$ = rate of generation of cell material

$r_{d/x}$ = rate of death and decay of cell material

At steady state, when $dS/dt = dX/dt = 0$, and with sterile input, these equations simplify as follows:

Substrate:

$$0 = (Q/V)(S_0 - S) - r_s \quad (9)$$

Biomass:

$$0 = - (Q/V)X + r_{g/x} - r_{d/x} \quad (10)$$

Because bacteria reproduce by dividing in two, the reaction rate for bacterial growth can be expressed as a first order equation:

$$r_{g/x} = \mu X \quad (11)$$

where μ = specific growth rate

Similarly, bacterial death and decay can be expressed as

$$r_{d/x} = bX \quad (12)$$

The true growth yield, Y_g , is the ratio of the rate of generation of cell material (in the absence of maintenance energy requirements) to the rate of substrate removal:

$$Y_g = r_{g/x}/(-r_s) \quad (13)$$

Combining the above equations yields:

$$-r_s = (\mu/Y_g)X \quad (14)$$

Thus the rate of substrate consumption is also first order with respect to the concentration of cells.

The value of the specific growth rate, μ , depends on the concentration of the limiting nutrient available for growth. Unfortunately, a mechanistic equation for the relationship between μ and S has not yet been discovered. The equation that has gained greatest acceptance is the one proposed by Monod (51):

$$\mu = \mu_{\max} * S / (K_S + S) \quad (15)$$

The term $\mu_{\max}$ is a constant defined as the maximum value possible for μ under a specific set of conditions. K_S is referred to as the half saturation constant and determines how fast μ approaches $\mu_{\max}$. K_S is the substrate concentration at which μ is equal to one half of $\mu_{\max}$. This equation was incorporated into the models developed herein. Taken together, the above equations comprise the following single substrate model:

$$D(S_0 - S) = (\mu_{\max} / (K_S + S)) (X / Y_G) \quad (16)$$

$$X = Y_G(S_0 - S) / (1 + b/D) \quad (17)$$

where D = dilution rate (Q/V)

By substituting equations 11 and 12 into equation 10, it is apparent that, at steady state and with $X_0 = 0$, $\mu = D + b$. That is, the specific growth rate is equal to the dilution rate plus the maintenance coefficient. This fact can be used to develop estimates of $\mu_{\max}$ and K_S based on experimental results.

Acetate, Propionate and Butyrate Oxidation

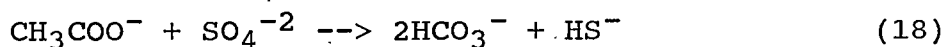
Since the discovery of SRB species capable of oxidizing acetate, propionate and butyrate in the early 1980's, researchers have investigated the stoichiometry and kinetics of these reactions. Information reported to date is presented below.

Reaction Stiochiometry

The stoichiometry of a reaction indicates what changes will occur and to what extent. An understanding of the stiochiometry of a microbially mediated reaction allows the yield of the reaction, i.e., the ratio of the mass of products formed to the mass of reactants consumed, to be estimated.

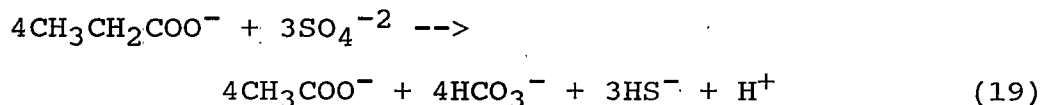
A number of investigators have studied the stoichiometries of SRB mediated oxidations of acetate, propionate and butyrate. Typically, such studies are done using pure cultures of specific SRB. The suggested stiochiometry for acetate oxidation by

Desulfotomaculum acetoxidans (Widdel and Pfennig, 1977): Desulfotomaculum acetoxidans (Schauder et al., 1986), Desulfonema limicola and Desulfonema magnum (Widdel et al., 1983), Desulfobacter postgatei (Widdel and Pfennig, 1981), and SRB in general (Pfennig and Widdel, 1981) is as follows:



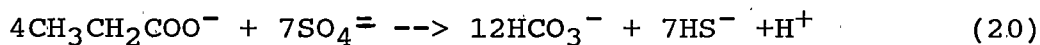
From the above, it is apparent that reduction of one mole of sulfate is associated with oxidation of one mole of acetate.

The stoichiometry of incomplete propionate oxidation by Desulfobulbus propionicus was suggested to be as follows by Widdel and Pfennig (1982):

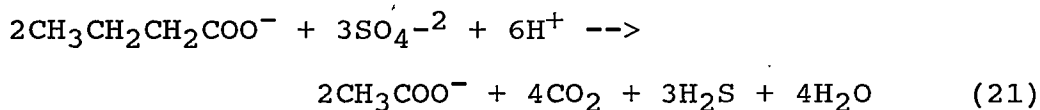


With this reaction, for every four moles of propionate oxidized, three moles of sulfate are reduced and four moles each of acetate and bicarbonate are produced.

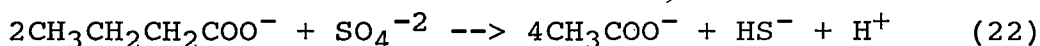
Pfennig and Widdel (1981) suggested the following stoichiometry for complete oxidation of fatty acids such as propionate:



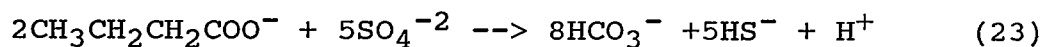
The stoichiometry of incomplete oxidation of butyrate by Desulfobacterium autotrophicum was determined to be as follows by Schauder et al. (1986):



Pfennig and Widdel (1981) suggested the following stoichiometry for incomplete oxidation of straight chain fatty acids with even numbers of carbon atoms such as butyrate by SRB:



The stoichiometry of complete oxidation of butyrate by Desulfobacterium catecholicum was determined to be as follows by Szewzyk and Pfennig (1987):



Pfennig and Widdel (1981) suggested the same stoichiometry for complete oxidation of fatty acids such as butyrate by SRB.

Biomass Yield

The yield of biomass (growth yield) from a microbially mediated reaction is another aspect of stoichiometry. Biomass yield is generally expressed in terms of dry weight of biomass produced per unit weight (or mole) of substrate consumed. Some published data on observed biomass yields associated with oxidation of acetate, propionate and butyrate by SRB, typically obtained in batch experiments, are presented in Table 5. These relatively low values are typical of other anaerobic processes.

Energetics

Another aspect of stoichiometry is the energetics or thermodynamics of the oxidation-reduction (redox) reactions mediated by microorganisms. Analysis of the redox reactions involved can, in turn, shed light on the free energy available for microbial growth. Because, in most biological systems, each step in the oxidation of a substrate involves the transfer of two electrons, free

Table 5. Available Data on Biomass Yield

Substrate/ organism/ reference	Biomass yield ^a	
	Grams of biomass per mole of substrate used	Grams of biomass per gram of substrate used
Acetate		
<u>Desulfobacter</u> <u>postgatei</u> Widdel and Pfennig, 1981	4.4	0.074
Ingvorsen et al., 1984	4.3	0.072
<u>Desulfomaculum</u> <u>acetoxidans</u> Widdel and Pfennig, 1977	5.6	0.095
Mixed culture Middleton and Lawrence, 1977	3.8	0.065
Propionate (incomplete oxidation)		
<u>Desulfobulbus</u> <u>propionicus</u> Widdel and Pfennig, 1982	4.7	0.064
Butyrate (incomplete oxidation)		
<u>Desulfobacterium</u> <u>autotrophicum</u> Schauder et al., 1986	8.3	0.096
Strain HRM6 Schauder et al., 1986	8.3	0.091
Butyrate (complete oxidation)		
<u>Desulfobacterium</u> <u>catecholicum</u> Szewzyk and Pfennig, 1987	6.2	0.072

^a Dry weight basis

energy changes in such reactions are often expressed as free energy change per two electrons (e^-) transferred.

Table 6 presents free energy changes at unit concentrations of reactants and products and at pH 7.0 ($\Delta G^\circ'$) based on

Table 6. Calculated Free Energy Changes

Substrate	Product(s)	Net free energy change, ΔG° , KJ/2e ⁻
Butyrate	Acetate	-13.92
Propionate	Acetate + CO ₂	-12.63
Butyrate	CO ₂	-12.24
Propionate	CO ₂	-12.10
Acetate	CO ₂	-11.83

calculations presented in Appendix A for the reactions discussed in the previous sections.

While free energy changes have often been correlated with biomass yields (Bailey and Ollis, 1986), both Middleton and Lawrence (1977) and Snoeyink and Jenkins (1980) have pointed out that the maximum rate at which various microorganisms can grow ($\mu_{\max}$) is related to the energy available from the redox reaction that is catalyzed. For example, the maximum specific growth rates associated with the following microbially catalyzed reactions are clearly correlated with a progressively lower amount of energy available to the microorganisms from substrate oxidation: aerobic heterotrophic oxidation, heterotrophic denitrification, nitrate oxidation, ammonia oxidation, heterotrophic sulfate reduction, and heterotrophic methane fermentation. For this reason, the calculated values presented in Table 6 suggest that incomplete oxidation of butyrate and propionate might support slightly more rapid

growth of SRB than complete oxidation of either these volatile acids or acetate.

Kinetics

Kinetics describe the rate at which chemical and biological processes occur. In bioprocess engineering, process rates characterize the rates at which microorganisms grow under specified environmental conditions. Two parameters, the maximum specific growth rate ($\mu_{\max}$) and the half saturation coefficient (K_S), are used to characterize bioprocess rates. As was noted above, the maximum specific growth rate is the maximum rate of increase of microorganism mass divided by the mass of microorganisms in a system under a specified set of growth conditions unconstrained by the availability of a limiting nutrient. It is related to the minimum microorganism doubling time ($t_{d,\min}$) as follows:

$$t_{d,\min} = (\ln 2) / \mu_{\max} \quad (24)$$

The half saturation coefficient is the limiting substrate concentration at which the specific growth rate is equal to one half of $\mu_{\max}$.

A number of investigators have characterized SRB capable of utilizing acetate, propionate and butyrate in terms of the above parameters. Typically, the work has been done using pure cultures of a specific SRB. Available data are presented in Tables 7 and 8. Defined (as opposed

Table 7. Available Kinetic Data for Growth on Acetate

Limiting substrate/ microorganism/ reference	Maximum specific growth rate, hr ⁻¹	Minumum doubling time, hr	Half saturation coefficient, mg/l	Temp., C
<u>Acetate</u>				
<u>Desulfobacter</u>				
<u>postgatei</u>				
Widdel and Pfennig, 1981	0.035 ^a	20	-	32
Thauer, 1982	-	-	<12	-
Ingvorsen et al., 1984	0.033 ^a	21	-	30
Schauder et al., 1986	0.025 ^a	28	-	30
<u>Desulfotomaculum</u>				
<u>acetoxidans</u>				
Widdel and Pfennig, 1977	0.058 ^a	12	-	36
Widdel and Pfennig, 1981	0.023 ^a	30	-	36
Schauder et al., 1986	0.014 ^a	48	-	30
	0.032 ^a	22	-	37
<u>Desulfobacter</u>				
<u>hydrogenophilus</u>				
Schauder et al., 1986	0.039 ^a	18	-	30
<u>Desulfonema sp.</u>				
Widdel et al., 1983	0.023 ^{a,b}	30	-	30
	0.0069 ^a	100	-	30
<u>Mixed culture</u>				
Middleton and Lawrence, 1977	0.014	-	250	20
	0.019	-	92	25
	0.022	-	5.7	31

^aCalculated from minimum doubling time^bComplex growth medium

to complex) growth media with a pH near neutrality were used unless indicated otherwise.

Table 8. Available Kinetic Data for Growth on Propionate and Butyrate

Limiting substrate/ microorganism/ reference	Maximum specific growth rate, hr ⁻¹	Minumum doubling time, hr	Half saturation coefficient, mg/l	Temp., C
<u>Propionate</u>				
<u>Desulfobulbus</u> <u>propionicus</u> Nanninga and Gottschal, 1987	0.110	6.3	-	35
Widdel and Pfennig, 1982	0.069	10	-	39
<u>Butyrate</u>				
<u>Desulfomaculum</u> <u>acetoxidans</u> Widdel and Pfennig, 1981	0.046 ^a	15	-	36
<u>Desulfovibrio</u> <u>baarsi</u> Schauder et al., 1986	0.017 ^a	42	-	30
<u>Desulfovibrio</u> <u>sapovorans</u> Nanninga and Gottschal, 1987	0.066 ^a	10.5	-	35

^aCalculated from minimum doubling time.

If formulas developed by Middleton and Lawrence (1977) are extrapolated to 35 °C, an acetate substrate $\mu_{\max}$ of 0.029 hr⁻¹ and a K_s of 2.7 mg/l are calculated. In that their studies were done with mixed cultures of SRB, these values probably reflect maximum $\mu_{\max}$ values and minimum K_s values for growth of SRB present in their inoculum on that substrate. SRB with lower $\mu_{\max}$ characteristics would be outcompeted by other SRB present in the inoculum. Even if the anomalously high value of $\mu_{\max}$ on acetate of Widdel and

Pfennig (1977) is considered, review of the data in Tables 7 and 8 suggests that SRB are capable of faster growth on propionate and butyrate than on acetate.

Biochemistry

Dissimilatory sulfate reduction by SRB begins with activation of sulfate by adenosine triphosphate (ATP) (Gottschalk, 1979). The reaction is catalyzed by the enzyme ATP sulfurylase and forms the products adenosine phosphosulfate (APS) and polyphosphate (PP_i):



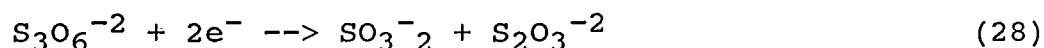
The second step in the process is reduction of APS to sulfite by the enzyme APS reductase with the production of adenosine monophosphate (AMP):



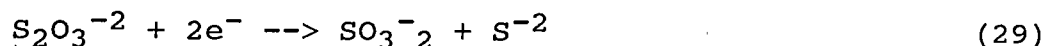
The remaining steps in the pathway are unsettled (Akagi, 1981). With the pathway currently favored by Brock et al., (1984), the third step is the reduction of sulfite to trithionate (with sulfite recycle from the subsequent steps):



The fourth is reduction of trithionate to sulfite and thiosulfate:



The fifth is reduction of thiosulfate to sulfite and sulfide:



Oxidation of acetate by SRB occurs via one of two mechanisms. In Desulfobacter spp., acetate is metabolized to carbon dioxide via a modified citric acid cycle (Thauer, 1982). The normal citric acid cycle is modified in that citrate is synthesized from acetyl-CoA and oxaloacetate is catalyzed by ATP-citrate lyase rather than by a citrate synthase (Möller and Thauer, 1987; Schauder et al., 1987). Oxidation of 1 mole of acetate to two moles carbon dioxide yields 1 net mole of ATP by substrate level phosphorylation. In other SRB, acetate oxidation apparently proceeds via a novel pathway termed the oxidative acetyl-CoA/carbon monoxide dehydrogenase pathway (Sporman and Thauer, 1988). The mechanism is in principle a reversal of that of synthesis of one mole of acetyl-CoA from two moles of carbon dioxide which occurs in autotrophic methanogenic bacteria (Fuchs and Stupperich, 1986) and in homoacetic bacteria (Ljungdal, 1986). With at least some SRB that use this pathway, one mole of ATP is required for the activation of acetate to acetyl-phosphate and one mole of ATP is generated by the formation of formate from N¹⁰-formyltetrahydrofolate. Thus, this oxidation of acetate does not result in net synthesis of ATP by substrate level phosphorylation. All of the ATP needed for activation of sulfate and SRB growth is

generated by electron transport phosphorylation (Spormann and Thauer, 1988).

Incomplete oxidation of propionate (to acetate) by the SRB Desulfobulbus propionicus apparently proceeds via the succinate pathway (Stams et al., 1984). Intermediates include propionyl-CoA, methylmalonyl-CoA, succinyl-CoA, succinate, fumarate, malate, oxaloacetate, and pyruvate or phosphoenolpyruvate. This pathway is remarkable in that it is reversible.

Incomplete oxidation of butyrate (to acetate) by the SRB Desulfobacterium autotrophicum apparently proceeds by a different pathway (Schauder et al., 1986). Butyrate is activated via CoA transfer from acetyl-CoA which is formed from butyryl-CoA by β -oxidation.

EXPERIMENTAL METHODS AND APPARATUS

A number of experiments were conducted to develop the data required to both test the hypothesis presented earlier and facilitate development of a model of the process. In general, the experiments consisted of a series of chemostat runs. During each run, a chemostat was operated at a particular dilution rate until steady state was reached. Details of experimental methods and apparatus are presented below and in Appendix B.

Microorganisms

The microorganisms used in the experiment were obtained from three sources to ensure that both acetate-utilizing and acetate-producing SRB would be present in the inoculum. One source of microorganisms was the American Type Culture Collection. The following SRB were obtained from that source:

Desulfobulbus propionicus, ATCC No. 33891

Desulfovibrio sapovorans, ATCC No. 33892

These cultures were maintained in 20 ml Bellco test tubes with butyl rubber stoppers at 5 °C and used to reinoculate the reactors when necessary.

Mixed cultures of wild SRB and other organisms were obtained from black, anaerobic mud from Silver Bow Creek and from black, anaerobic sludge from the City of Butte, Montana, wastewater treatment plant. Both sources of organisms were known to be impacted by acid mine drainage. These cultures were maintained together at 35 °C in the complete medium described below.

Media

The growth media were designed to allow growth of any fresh water SRB capable of utilizing acetate, propionate and/or butyrate as an electron donor. The concentrations of constituents in the media containing three acids (acetic, propionic, and butyric) are indicated in Table 9.

Table 9. Media Composition

Constituent	Concentration, mg/l
Monopotassium phosphate	500
Ammonium chloride	1,000
Calcium chloride	60
Sodium sulfate	4,200
Magnesium sulfate + 7H ₂ O	2,000
Ferrous sulfate + 7H ₂ O	500
Yeast extract	10
Sodium dithionite	30
p-aminobenzoic acid	1
Biotin	1

In some of the runs using a medium containing propionate as the sole electron donor, a lower sulfate concentration

(3,330 mg/l) was used (See Table 11, Appendix C.). The above media is essentially Postgate's C medium (Postgate, 1974) with sufficient sulfate to allow incomplete oxidation (to acetate) of the concentrations of propionate and butyrate given below. Growth factors (i.e., biotin and p-aminobenzoic acid) known to be required by some SRB capable of using the electron donors indicated below were also added to the media.

For the single acid experiments, single electron donors were added to the media in the following concentrations (mg/l):

<u>Electron donor</u>	Concentration, as sodium <u>salt</u>	<u>as anion</u>
Sodium propionate	3,550	2,700
or		
Sodium butyrate	2,530	2,000

For other experiments, the sodium salts of three volatile acids were added in the following concentrations (mg/l) to simulate acid phase digester supernatant:

<u>Electron donor</u>	Concentration, as sodium <u>salt</u>	<u>as anion</u>
Sodium propionate	3,550	2,700
Sodium butyrate	2,530	2,000
Sodium acetate + 3H ₂ O	4,380	1,900

The media were autoclaved for 25 minutes at 121 °C and cooled under an atmosphere of oxygen-free nitrogen.

Chemostat

The chemostats used in this experiment are depicted schematically in Figure 2. The reactors had a nominal medium volume of 500 ml and were continuously stirred.

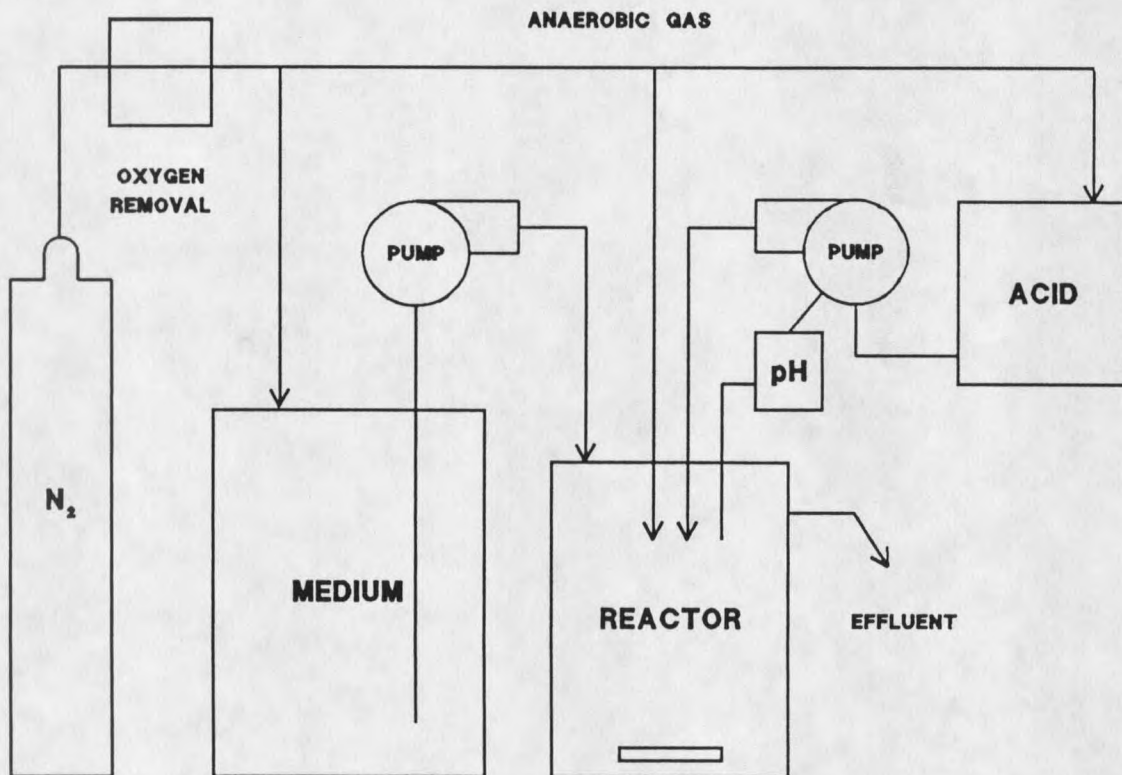


Figure 2. Chemostat with pH Control

The reactors were constructed using a Pyrex glass jar, the top of which was sealed with a black rubber stopper. Black butyl rubber tubing (ID = 3/16 in with a 1/8 in thick wall) and Neoprene tubing were used exclusively to prevent oxygen diffusion into the medium.

As little tubing was used as possible to minimize the opportunities for oxygen poisoning.

Nitrogen gas was also used to continuously purge the medium reservoirs (to prevent entrance of air as the medium was used), and the reactors (to prevent accumulation of hydrogen sulfide). Oxygen was removed from commercial grade nitrogen by passing it through copper filings maintained at 370 °C that were regularly reduced with hydrogen gas. Gas leaving the reactors was bubbled through beakers containing lead acetate for hydrogen sulfide removal.

Continuous pH adjustment was practiced and the acid reservoir was also purged with nitrogen. The pH probe was a double junction type to prevent liquid junction poisoning and was inserted into the medium through a hole in the rubber stopper.

Each run using the same electron donor was started by reinoculating the reactor with the three cultures. Growth was allowed to occur in a batch mode (no fresh medium addition) for one day. The medium input pumps were then turned on and the run begun.

Analytical Methods

Reactor input and contents were monitored for sulfate, acetic acid, propionic acid, and butyric acid concentrations and for pH. Sulfate concentrations were

quantified by measuring sulfur concentrations using inductively-coupled plasma spectrometry after removal of sulfide. Initially, sulfur concentrations were determined on acidified, diluted, purged (with nitrogen) and filtered samples. Later samples were treated with zinc acetate in alkaline solution, diluted and filtered. Individual volatile acid concentrations were determined on a Varian 3700 gas chromatograph fitted with a flame ionization detector. A 2m by 2mm glass column packed with Supelco 80/120 Carbowax B-DA/4% Carbowax 20M was used. The column was maintained at 175 °C and a 0.06 M oxalic acid solution was used to condition the column.

Biomass concentrations were determined by counting cells in homogenized samples preserved by addition of two percent formaldehyde and refrigeration at 5 °C. The cells were stained with acridine orange to allow direct counting on 0.22 micron black filters by epifluorescence microscopy using an image analyzer. Average cell area, width and length data were also obtained. Biomass weight concentrations were determined by multiplying cell numbers by calculated dry weights assuming the wet cells had a specific gravity of 1.07 and that dry weights were 22 percent of wet weights (Luria, 1960; Robinson et al., 1984). Average cell volumes were estimated using length and width data with the cells represented as short

cylinders with a cone on each end, the cones having a height equal to half of the cell width.

Data Analysis

Data obtained during this research were used for two purposes. The first purpose was to test the hypothesis posed in the Introduction. The second was to calibrate a model of the system studied. Data analysis procedures used to accomplish each purpose are described below.

Hypothesis Testing

The hypothesis tested during this research is restated as follows:

A chemostat containing a mixed culture of sulfate-reducing bacteria can be operated at a dilution rate large enough to cause incomplete oxidation of propionic acid and butyric acid and production of acetic acid, if all three acids are present in the reactor feed, but sufficient sulfate is present to allow utilization of only the two higher molecular weight acids.

For this hypothesis to be true, SRB that incompletely oxidize propionate and butyrate (to acetate) must be capable of more rapid growth than those that completely oxidize propionate, butyrate and acetate to carbon dioxide. That is, at steady state in a chemostat, the microbial processes that result in incomplete oxidation must occur

more rapidly than the microbial processes that result in complete oxidation. Under such conditions, acetate would be present in the reactor and its effluent.

Because the reaction stoichiometries associated with incomplete oxidation are different from those associated with complete oxidation, mass balances of substrate, acetate, and sulfate were also used to test the hypothesis. For example, if, at a certain dilution rate, one mole of acetate is produced and three-quarters mole of sulfate is reduced for each mole of propionate oxidized, then the hypothesis is true to the extent that it concerns propionate oxidation.

Analysis of kinetic data was also used in hypothesis testing. For example, if SRB can be grown on propionate at a μ greater than the $\mu_{\max}$ of SRB that consume acetate, and incomplete oxidation of propionate occurs, then the hypothesis is true to the extent that it concerns propionate oxidation. If the chemostat were operated at dilution rates above the reported $\mu_{\max}$ of acetate-utilizing SRB, these bacteria would not be present in the reactor. Under these conditions, acetate produced by oxidation of propionate or butyrate and not consumed by other types of bacteria occurring in the mixed culture would be present in the reactor and its effluent.

Model Development

The research plan called for models for propionate oxidation and butyrate oxidation of the form discussed earlier to be formulated by developing estimates of the following parameters:

$\mu_{\max,p}$ = maximum specific growth rate on propionate,
hr⁻¹

$K_{s/p}$ = half saturation coefficient on propionate,
mg/l

$Y_{g/p}$ = true growth yield on propionate, mg/mg

b_p = maintenance coefficient with growth on
propionate, hr⁻¹

and

$\mu_{\max,b}$ = maximum specific growth rate on butyrate,
hr⁻¹

$K_{s/b}$ = half saturation coefficient on butyrate,
mg/l

$Y_{g/b}$ = true growth yield on butyrate, mg/mg

b_b = maintenance coefficient with growth on
butyrate, hr⁻¹

A variety of procedures have been proposed for analyzing continuous culture (chemostat) data to determine kinetic parameters (Wilkinson, 1961; Dowd and Riggs, 1965; Grady and Lim, 1980; Bailey and Ollis, 1986). It is known that culture history can affect the analysis (Templeton and Grady, 1988). In fact, considerable variability in $\mu_{\max}$

and K_S values occurs with mixed cultures and it is considered good practice to quote them as ranges (Grady and Lim, 1980).

The procedures used in estimating $\mu_{\max}$, K_S , Y_G , and b herein were to be those recommended by Grady and Lim (1980). Values for Y_G and b were to be obtained from plots of a linearized version of the equation:

$$X = Y_G(S_0 - S)/(1 + b/D) \quad (17)$$

With the equation expressed in the form

$$(S_0 - S)/X = (b/Y_G)(1/D) + 1/Y_G \quad (30)$$

plot is made of $(S_0 - S)/X$ as a function of $1/D$ and the result is a straight line with slope b/Y_G and ordinate intercept $1/Y_G$.

Values for $\mu_{\max}$ and K_S were obtained from plots of two different linearized versions of the Monod equation. In addition, a nonlinear regression procedure was used. The double reciprocal or Lineweaver-Burk method of plotting was avoided because it reportedly gives a deceptively good fit even with unreliable data (Grady and Lim, 1980). With the Monod equation expressed in the form of a Hofstee plot

$$(D + b)/S = \mu_{\max}/K_S - (1/K_S)(D + b) \quad (31)$$

a plot is made of $(D + b)/S$ as a function of $(D + b)$. The result is a straight line with slope $1/K_S$ and ordinate intercept $\mu_{\max}/K_S$. A least squares line of best fit may be used with this method.

In the second version, the Monod equation is expressed in the form of a Hanes plot .

$$S/(D + b) = (1/\mu_{\max})S + K_S/\mu_{\max} \quad (32)$$

and a plot is made of $S/(D + b)$ as a function of S . The result is a straight line with slope $1/\mu_{\max}$ and ordinate intercept $K_S/\mu_{\max}$. The least squares technique is not an appropriate means of finding the line of best fit because both axes contain terms which are subject to error (i.e., S) (Grady and Lim, 1980). The line is drawn by eye.

The nonlinear regression of the Monod equation data was accomplished on Montana State University's VAX computer using SAS statistical software. The program produced estimates of $\mu_{\max}$ and K_S given data pairs of limiting substrate concentration and specific growth rate. The standard error and the 95 percent confidence interval associated with the estimate of each parameter was also determined.

RESULTS

The results of this research characterize volatile acid oxidation during continuous culture of a mixed population of sulfate-reducing bacteria (SRB) and other anaerobic microorganisms. Tabulated data are presented in Appendix C. The experiments were designed to provide information on reaction stoichiometry, biomass yield and kinetic parameters. Each aspect is described below.

Reaction Stoichiometry

Under all of the conditions investigated, the only reaction that occurred to any appreciable extent was SRB-mediated oxidation of propionate and reduction of sulfate. Even at dilution rates significantly lower than the reported $\mu_{\max}$ of butyrate-utilizing SRB, very little butyrate oxidation occurred. Similarly, only a small amount of acetate oxidation occurred during the two runs conducted at dilution rates significantly lower than the reported $\mu_{\max}$ of acetate-utilizing SRB.

Figures 3 through 5 present the results of determinations of the rate of consumption of reactants and creation of products during the chemostat runs. Comparison

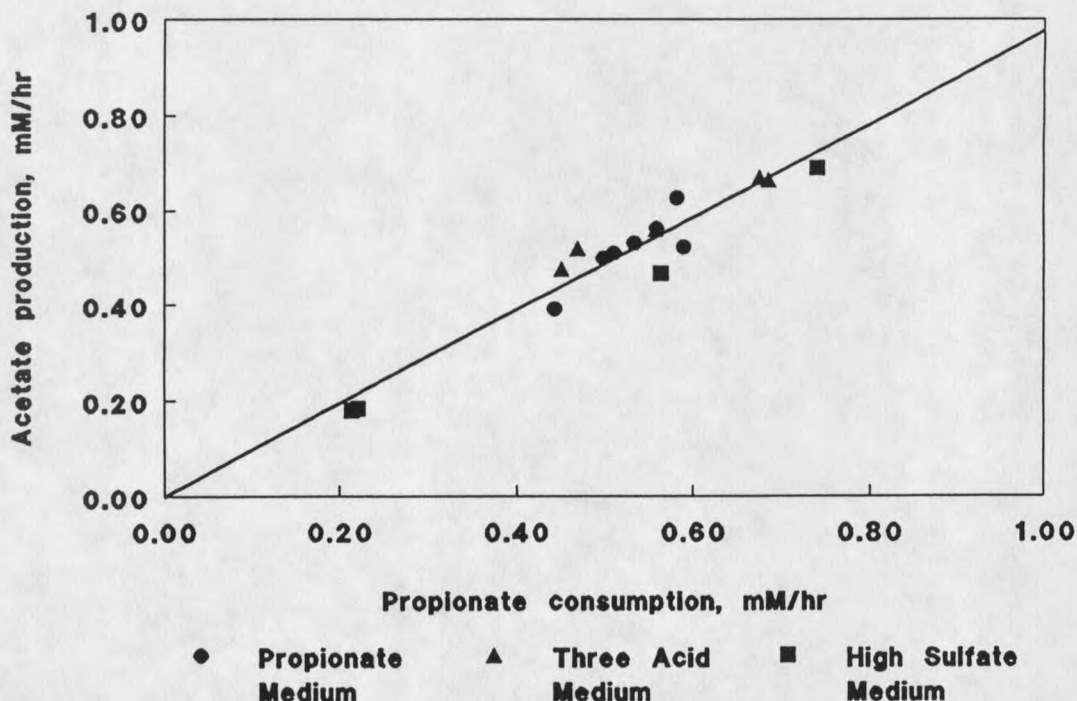


Figure 3. Acetate Production Stoichiometry

of the rates over the range studied reveals the stoichiometry of the reaction(s).

Figure 3 shows that approximately 0.98 mole of acetate was produced for each mole of propionate consumed. This 1:1 relationship was true for runs with a medium containing propionate as essentially the only organic substrate and just enough sulfate to allow its incomplete oxidation by SRB (round data points). It was also true for runs with a medium containing all three acids (triangular data points) and for runs with a medium containing propionate and significantly more sulfate than is required for its incomplete oxidation (square data points).

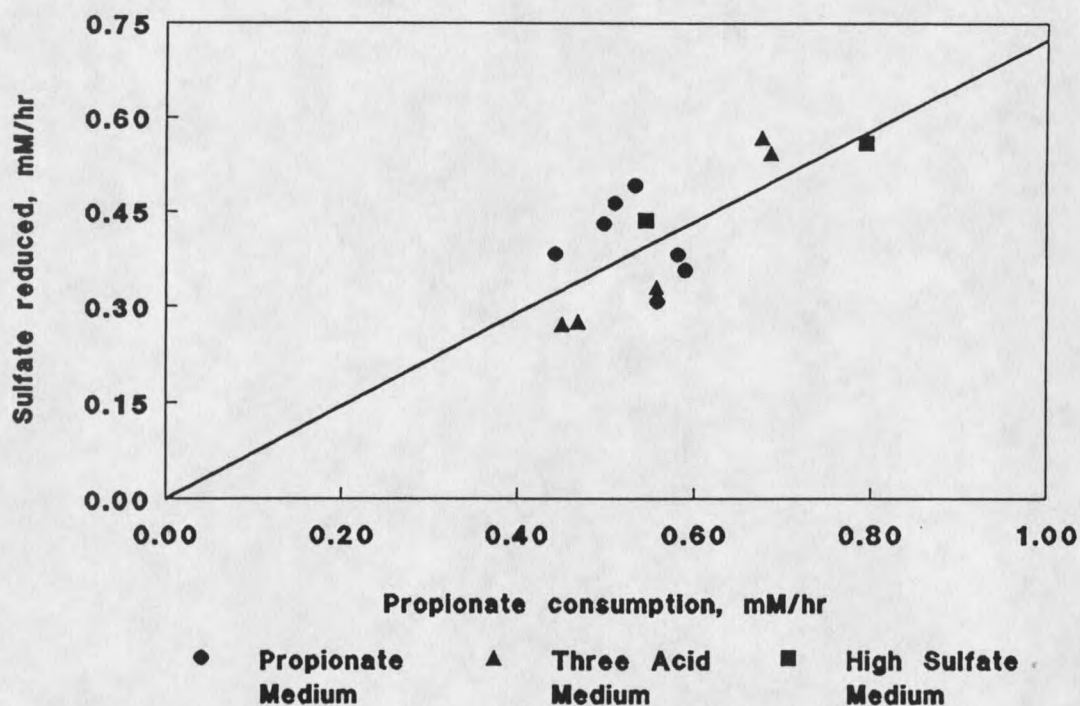


Figure 4. Sulfate Reduction Stoichiometry

The square data points at the two lowest propionate consumption rates were generated at a dilution rate of about 0.018 hr^{-1} and at reactor acetate concentrations of about $1,200 \text{ mg/l}$. This dilution rate is much lower than the reported μ_{max} for acetate-utilizing SRB (about 0.03 hr^{-1}) and the concentration is much higher than their reported K_s (about 3 mg/l). Even under these conditions, almost all consumed propionate was recovered as acetate on a mole per mole basis.

Figure 4 shows that approximately 0.73 mole of sulfate was consumed (reduced) for each mole of propionate consumed. The scatter in the data is probably due to the

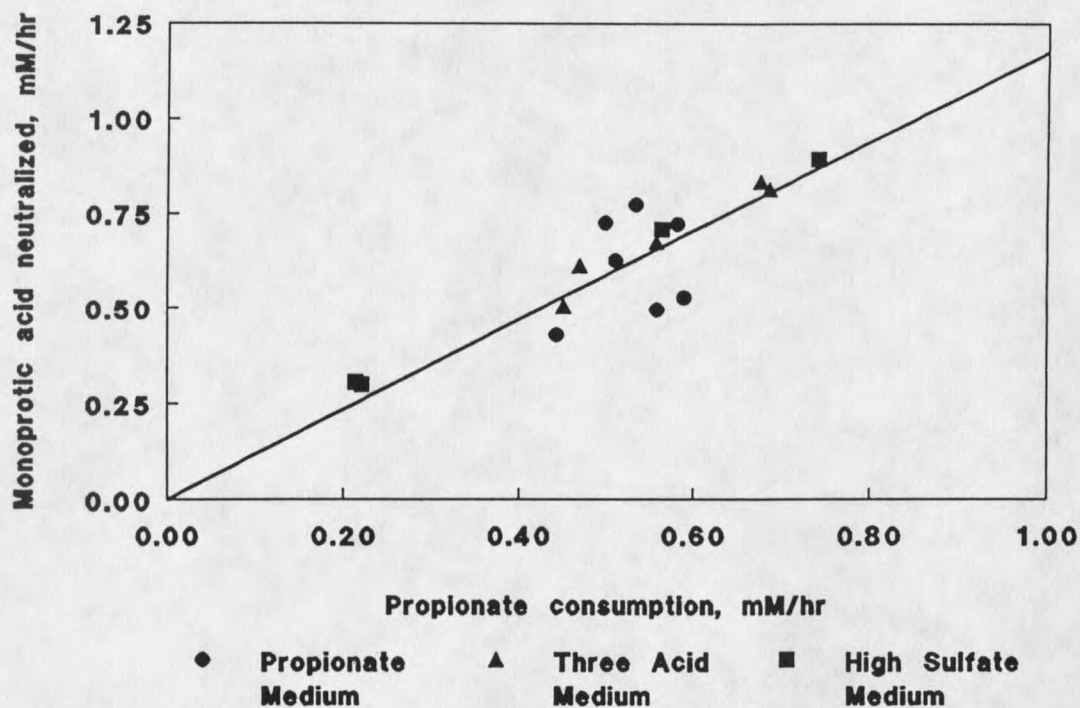


Figure 5. Acid Neutralization Stiochiometry

fact that total sulfur concentrations were measured on samples from which sulfide had been removed. Moreover, sulfur (sulfate) in the "distilled" medium make-up water may also have varied. (See the discussion of Substrate Concentration in Appendix B).

Figure 5 shows that approximately 1.18 mole of monoprotic acid was "neutralized" for each mole of propionate consumed to maintain the reactor contents at pH 7.0 ± 0.2 . These protons left the reactor as H_2S gas. About 81 percent of the sulfide ion produced by sulfate reduction left the reactor in this way. Because essentially all of the iron in the medium was precipitated

as iron sulfide (essentially no iron was present in filtered effluent samples), some of the sulfide left the reactor as iron sulfide. Mass balances indicate that six to seven percent of the sulfide left the reactor in this form. The remaining 12 to 13 percent must have left as dissolved sulfide.

Previous investigators of methods for bioprocessing of acid mine drainage have not made it clear that H_2S and CO_2 must be removed from the treated mine water (e.g., by purging, stripping, etc.) for the processes to reduce the pH of the mine water. This fact has important implications on the economic feasibility of the process which are outlined later in the Discussion section.

Biomass Yield

Microscopic examination of samples of reactor contents indicated that during all runs the predominant microorganism had the characteristic lemon-shaped morphology of Desulfobulbus propionicus. Typically, the cells were in pairs or chains. In all runs, however, some other microorganisms were present: typically long, thin vibrioids species and/or larger ellipsoidal species. Some of the vibrioid cells were of a size and morphology similar to that reported for Desulfovibrio baarsii, an SRB capable of growth on propionate and acetate (Krieg and Holf, 1984). The ellipsoidal cells were of a size and morphology

similar to that reported for Desulfobacter sp., a SRB capable of growth on acetate, and Desulfosarcina sp., SRB capable of growth on propionate, acetate, butyrate, and CO₂. It should be noted that propionate, acetate and CO₂ were available for growth in all runs and other SRB and non-SRB may have been present.

The average size of the cells in the reactor varied with the conditions imposed upon the culture. For the purposes of this research, the following cell size averages were used in calculating biomass (weight) concentrations from cell count data tabulated in Appendix C:

<u>Condition</u>	<u>Average cell area, sq. microns</u>	<u>Average cell length, microns</u>	<u>Average cell width microns</u>
Propionate medium	0.895	1.45	0.919
Three acid medium	0.904	1.43	0.924
Low dilution rate	0.696	1.29	0.821

As indicated, the low dilution rate runs produced a much more heterogenous culture having a much smaller average size.

Because a mixed culture was present, significant variability in biomass yield occurred. Figure 6 presents an analysis of biomass yield assuming that propionate was the only substrate (S) being used for growth. It was not feasible to measure yields over a large enough range to allow a maintenance coefficient, "b", to be calculated.

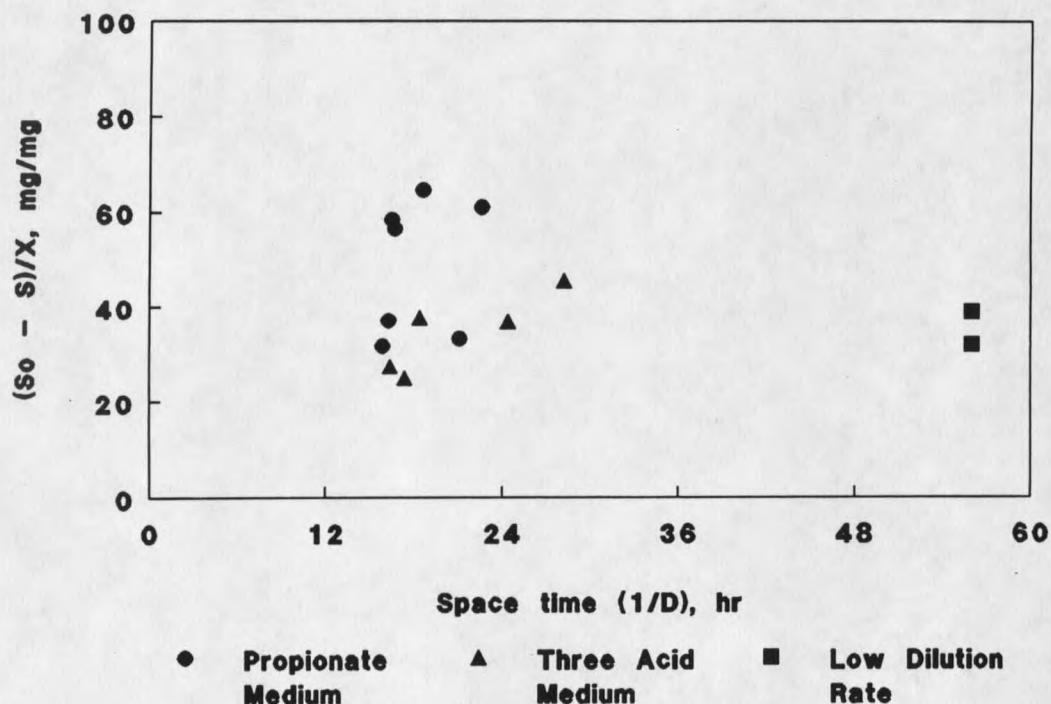


Figure 6. Analysis of Biomass Yield

This was the case because the "window" of growth rates between the $\mu_{\max}$ of propionate-utilizing SRB (0.07 hr^{-1}) and the $\mu_{\max}$ of acetate-utilizing SRB (0.03 hr^{-1}) was too small. Average cell volumes used in estimating biomass yields for the three growth conditions investigated were estimated using the length and width data presented earlier with the cells represented as short cylinders with a cone on each end, which was a simplification of the typical cell morphology. The resulting calculated cell areas agreed well with the measured average areas. The yield results were as follows:

<u>Condition</u>	<u>Observed yield, mg biomass/mg propionate consumed</u>
Propionate medium	0.022
Three acid medium	0.030
Low dilution rate	0.028

An average yield value for a low dilution rate condition, Run 18.2, (which produced little visible FeS) determined by filtration through 0.45 micron filters and drying at 105°C was 0.042 mg cells/mg of propionate consumed. These values are lower than the 0.064 value reported by Widdel and Pfennig (1982) who determined "cell mass" by drying (at 80°C) cells (and, possibly, metal sulfides) which were harvested by centrifugation and washed once with 10 mM sodium phosphate buffer (pH 4.5). These differences are reasonable in that the temperature at which a residue is dried has an important bearing on such results (Taras et al., 1971). The fact that the cell counting technique used typically undercounted cells smaller than the predominant cell type may have also resulted in lower biomass yield values.

Kinetic Parameters

Kinetic parameters for oxidation of propionate by SRB were estimated using both a Hofstee plot and a Hanes plot of the data tabulated in Appendix C. The calculated kinetic parameters should be considered appropriate for the

environmental conditions under which growth occurred (temperature, medium composition and pH, etc.). They should also be considered approximate because S (and, hence, $\mu_{\max}$ and K_S) may depend on S_0 when mixed cultures are employed (Grady and Lim, 1980). The "effective" initial substrate concentration (S_0) varied among the experiments conducted herein because of the diluting effect of automatic acid additions. The dilution rates used are based on the effluent flow rate which comprise the total of the medium addition rate and the acid addition rate.

A Hofstee plot ($b = 0$) of the results of runs using a propionate medium, a three acid medium, and a high sulfate medium is presented in Figure 7. A Hanes plot of the same

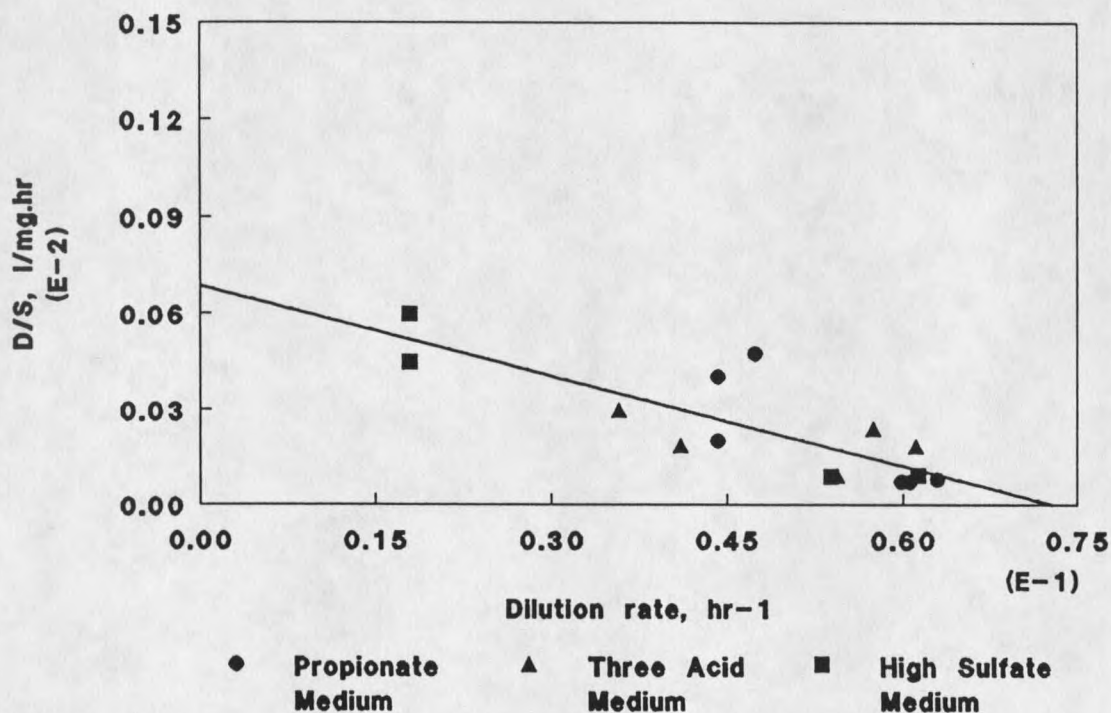


Figure 7. Hofstee Plot of Kinetic Data

data is presented in Figure 8. It should be noted that essentially all of the data points fall on or near a straight line in both plots. Thus, it appears that the Monod equation can be used to effectively characterize mixed culture SRB growth on propionate. Analyses of these plots and the nonlinear regression analysis resulted in the following estimates of kinetic parameters:

<u>Parameter</u>	<u>Hofstee plot</u>	<u>Hanes plot</u>	<u>Nonlinear regression</u>
$\mu_{\max}$, hr^{-1}	0.073	0.067	0.079
$K_{S/p}$, mg/l	106	79	187

The nonlinear regression gave a standard error of $\mu_{\max}$ of 0.013 hr^{-1} and a 95% confidence interval of 0.052 to 0.107 hr^{-1} . For K_S , the standard error was 123 mg/l and the

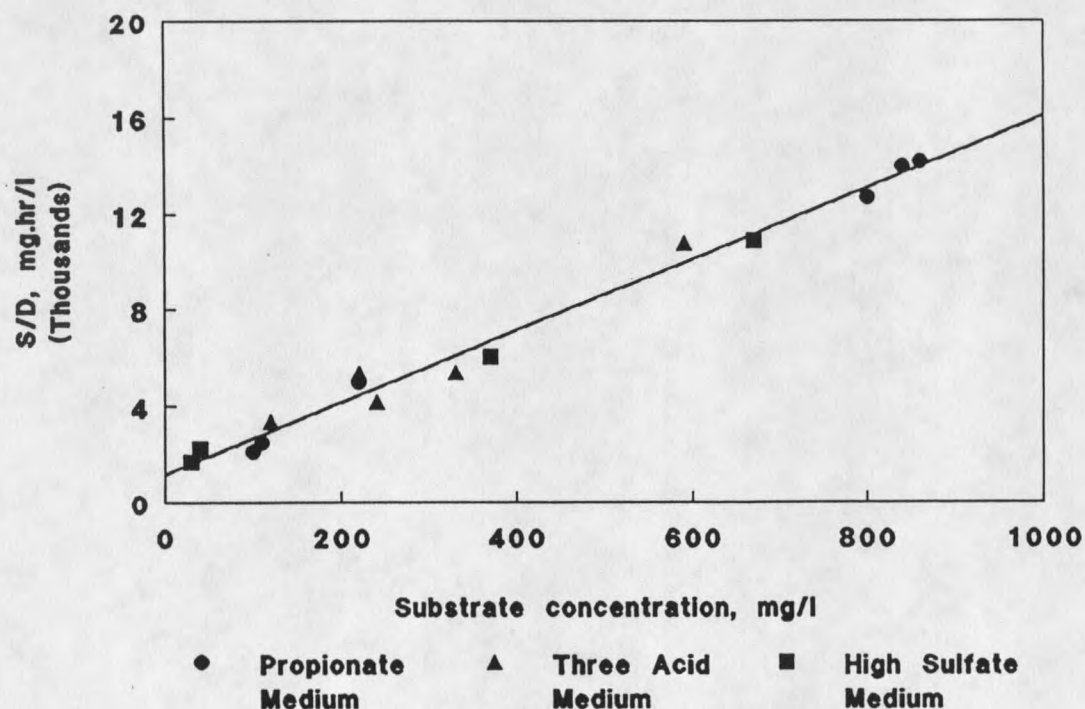


Figure 8. Hanes Plot of Kinetic Data

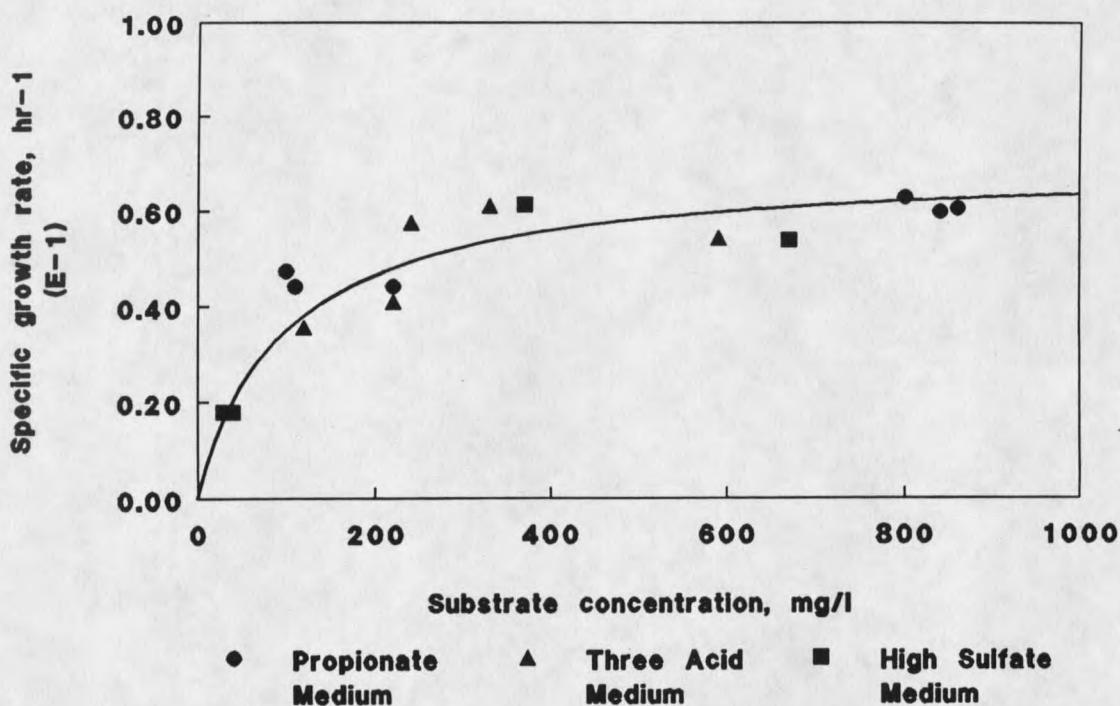


Figure 9. Plot of Monod Equation

95% confidence interval was -78 to 453 mg/l. Thus, a $\mu_{\max}$ of approximately 0.07 hr^{-1} and a $K_{S/p}$ of approximately 100 mg/l result. A Monod equation plot using these parameters and the data collected in this research is presented in Figure 9.

DISCUSSION

The implications of the results presented earlier in terms of hypothesis testing, model development, process performance and process applications are discussed below.

Hypothesis Testing

The hypothesis tested during this research is as follows:

A chemostat containing a mixed culture of SRB can be operated at a dilution rate large enough to cause incomplete oxidation of propionic acid and butyric acid and production of acetic acid, if all three acids are present in the reactor feed, but sufficient sulfate is present to allow utilization of only the two higher molecular weight acids.

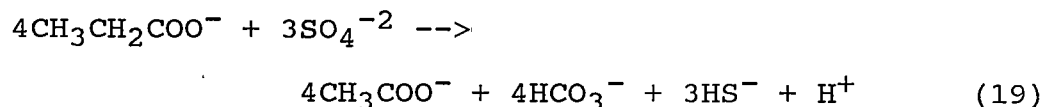
As was noted earlier, this hypothesis is true if the following are true:

1. measurement of reactant and product concentrations (stoichiometry) of the reactions occurring at a selected dilution rate indicates that incomplete oxidation to acetate (as opposed to complete oxidation to carbon dioxide) is occurring, and

2. determination of kinetic parameters indicates that, at a selected dilution rate, propionate and butyrate is consumed more rapidly than acetate.

Stoichiometric Evidence

The stoichiometric results indicate that, with growth on a propionate medium, the following reaction occurs:



A two-tailed test of means using the t distribution of the data presented on Figure 3 (and tabulated in Appendix C, Table 13) indicates that with growth on a propionate medium, the rate (mM/hr) of acetate production is equal to 100 percent of the rate (mM/hr) of propionate oxidized at the 0.05 significance level.

The data are also consistent with the conclusion that the same reaction occurs in a three acid medium. A similar two tailed test of those data also revealed that the acetate produced was equal to 100 percent of the propionate oxidized at the 0.05 significance level.

Butyrate oxidation, on the other hand, occurred to a very limited extent in the reactors and may not have been accomplished by SRB. Clear reactor effluent stored at 35 °C turned black due to production of H₂S indicating that sulfate reduction did eventually occur.

The lack of appreciable butyrate oxidation in the chemostat was surprising in that the medium composition, pH and temperature were in the range reported acceptable to butyrate-utilizing SRB. Neither Bergey's Manual (Krieg and Holt, 1984) nor The Prokaryotes (Pfennig, 1981) state that Desulfovibrio sapovorans, the butyrate-utilizing SRB with the largest $\mu_{\max}$, has a specific requirement for vitamins or trace elements. (Desulfomaculum acetoxidans does have a requirement for biotin which was present in the medium). Some other SRB do have a requirement for specific trace elements; for example, Desulfosarcina variabilis requires molybdate as a trace element for growth on benzoate (and its growth is inhibited completely by even the diffuse light in a laboratory) (Krieg and Holt, 1984). In fact, the medium used herein contained all of the components recommended by Bergey's Manual for cultivation of Desulfovibrio sapovorans and that reference states that vitamins are not required.

The only cultivation of Desulfovibrio sapovorans reported in the English language (Nanninga and Gottschal, 1987) used a basal bicarbonate-buffered (pH 7.0) fresh water medium with vitamins and trace elements. Furthermore, the medium Widdel (1980) used in his discovery of the species apparently contained trace elements as it was a defined medium that did not contain yeast extract. The other butyrate-utilizing SRB capable of growth at the

dilution rates studied, Desulfotomaculum acetoxidans, has also only been grown in a defined medium containing trace elements (Widdel and Pfennig, 1981). Thus, butyrate-utilizing SRB may have an unreported requirement for a trace element for rapid growth.

Alternatively, another hypothesis is that those SRB may require a more reduced environment for growth than could be achieved in the small reactors used in this research. (See discussion in Appendix B). Pfennig et al. (1981) recommend addition of sodium dithionite to batch cultures of Desulfovibrio sapovorans and, although it was added to the media used in this research, it may have been oxidized prior to introduction into the reactors.

A third hypothesis is that rapid purging of the reactors with an inert gas (N_2) reduced the ambient H_2 level in the reactors sufficiently to prevent the synthesis of a required hydrogenase by Desulfovibrio sapovorans. Tsuji and Yagi (1980) noted that growth of another Desulfovibrio species, vulgaris, was inhibited by rapid sparging with the inert gas argon. They noted that the organisms generated a burst of H_2 soon after inoculation in a lactate medium that may have served to induce the synthesis of a low molecular weight hydrogenase required for growth. (It should also be noted that Desulfobulbus propionicus can utilize H_2 if both carbonate and acetate are present).

A fourth hypothesis is that a butyrate-utilizing strain of Desulfovibrio sapovorans was not present in the inoculum. The ATCC strain was not provided in a butyrate medium and the high metal content of the environments from which the wild SRB were obtained may have prevented its presence.

Thus the hypothesis this research set out to test was not true concerning butyrate under the conditions of this experiment. If this were the case in a large reactor treating acid mine drainage, the butyrate would be present in the reactor effluent and available for conversion to methane in the next step in the process. Butyrate is unlike propionate in that it has been shown to be amenable to rapid conversion to methane in fixed-film reactors (Heijnen, 1984).

Kinetic Evidence

The results show that propionate-utilizing SRB are capable of much more rapid growth than acetate-utilizing SRB in both a propionate medium and a three acid medium. The $\mu_{\max}$ of propionate-utilizing SRB is greater than the reported $\mu_{\max}$ for acetate-utilizing SRB.

It may also be true, however, that propionate-utilizing SRB exhibit a larger K_s than that reported for acetate-utilizing SRB. Thus, at dilution rates very much lower than the $\mu_{\max}$ of acetate-utilizing SRB, a significant amount of acetate consumption could occur. During Runs

18.1 and 18.2 (dilution rate = 0.018 hr^{-1}) about 17 percent of the acetate produced by propionate oxidation was itself oxidized or the reactor was populated, in part, by SRB capable of complete oxidation of propionate to CO_2 .

From another perspective, however, a relatively large K_s offers some advantage. Grady and Lim (1980) point out, for example, that systems with a more gradual variation in specific growth rate with substrate concentration, i.e., systems with a relatively large K_s , tend to be more stable in continuous culture.

Summary

In summary, the results produced by the experiments described herein prove that a chemostat containing a mixed culture of SRB can be operated at a dilution rate large enough to cause incomplete oxidation of propionic acid and production of acetate, if acetic, propionic and butyric acids are present in the reactor feed, and sufficient sulfate is present to allow utilization of all three acids. The mechanism of propionate oxidation was, as expected, oxidized by SRB. Of course, the results do not prove that it is impossible to cause butyric acid oxidation to occur as well because a negative cannot be proven (Platt, 1964).

These results, taken with the following findings of others, suggest that biocatalyzed partial demineralization of acidic metal sulfate solutions is technically feasible:

1. Ghosh et al. (1975) and Ghosh et al. (1987) showed that acid phase anaerobic digestion (acidogenesis) could be used to produce low molecular weight volatile acids from biomass.
2. Harper and Pohland (1986) showed that hydrogen management can be used to alter the mix of volatile acids produced by acidogenesis.
3. Ilyaletdinov et al. (1977) and Noboro and Yagasawa (1978) showed that SRB could be used to remove copper and other metals from acid mine drainage.
4. Heijnen (1984), Bhadra et al. (1987) and Hamoda and Kennedy, 1987) showed that acetate and butyrate can be efficiently converted to methane in low detention time fixed-film reactors.
5. Lawrence and McCarty (1966) showed that soluble sulfide concentrations up to 200 mg/l as S exert no significant toxic effects on biological methanogenesis.
6. Cork (1987) showed that photosynthetic sulfur bacteria can be used to recover 95 percent of the sulfur from a H_2S/CO_2 gas stream.

Model Development

Substrate utilization models were developed to allow substrate and product concentrations to be estimated given a proposed dilution rate. Use of the models is

appropriate if growth conditions are similar to those used in their development (e.g., complex medium, temperature = 35° C, continuous H₂S removal, pH = 7.0, etc.).

SRB growth on propionate is adequately characterized by the Monod equation:

$$D = \mu = \mu_{\max,p} S_p / (K_{S/p} + S_p) \quad (33)$$

or

$$S_p = D K_{S/p} / (\mu_{\max,p} - D) \quad (34)$$

$$S_p = 100 D / (0.07 - D) \quad (35)$$

The biomass concentration can be estimated using the following relationship:

$$X_p = Y_{g/p} (S_{O/p} - S_p) \quad (36)$$

$$X_p = 0.03 (S_{O/p} - S_p) \quad (37)$$

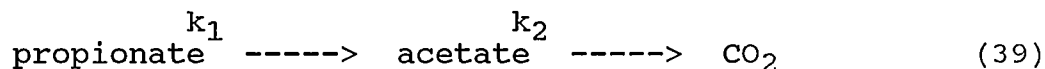
At dilution rates above about 0.03 hr⁻¹, the following equation can be used to estimate the concentration of acetate in the reactor effluent (S_a):

$$S_a = (S_{O/p} - S_p) * (59.04/73.07) \quad (38)$$

where 59.04/73.07 = ratio of molecular weights of
acetate and propionate

In both of the above equations, S_{O/p} is the effective influent propionate concentration.

At dilution rates below about 0.03 hr⁻¹, for the acetate produced by oxidation of propionate to be oxidized to CO₂, the series of reactions,



must occur. Under such conditions, where cultivation of

acetate-utilizing SRB is possible, the acetate concentration in the feed ($S_{O/a}$) would be consumed according to the Monod equation. Mass balance considerations suggest that the following equation can be used to estimate the concentration of acetate in the reactor effluent if complete oxidation of propionate to CO_2 did not occur (Grady and Lim, 1980).

$$S_a = S_{O/a} / (1 + k_2/D) + (k_1 S_{p/o}/D) / [(1 + k_1/D)(1 + k_2/D)] \quad (40)$$

or

$$S_a = S_{O/a} / (1 + \mu_{max,a} S_a X_a / Y_{g/a} (K_{S/a} + S_a) * D + (\mu_{max,p} S_p X_p / Y_{g/p} (K_{S/p} + S_p) * D) * S_{p/o} / (1 + \mu_{max,p} S_p X_p / Y_{g/p} (K_{S/p} + S_p) * D) * (1 + \mu_{max,a} S_a X_a / Y_{g/a} (K_{S/a} + S_a) * D) \quad (41)$$

Under practical operating conditions at low dilution rates, $S_a \gg K_{S/a}$ and $S_p \ll K_{S/p}$, and k_1 would be first order with respect to S_p and k_2 zero order with respect to S_a . The above equation could then be simplified to the following:

$$S_a = S_{a/o} / (1 + \mu_{max,a} X_a / Y_{g/a} * D + (\mu_{max,p} X_p / Y_{g/p} * K_{S/p}) S_{o/p} / (D * (1 + (\mu_{max,p} X_p / Y_{g/p} * K_{S/p}) / D) * (1 + (\mu_{max,a} X_a / Y_{g/a} * D)) \quad (42)$$

or

if $b_a = 0$ and $b_p = 0$

$$\begin{aligned}
 S_a = & S_{o/a} / (1 + 0.03(S_{o/a} - S_a) / D \\
 & + (0.07(S_{o/p} - S_p) / 90) S_{o/p} / (D \\
 & * (1 + 0.07(S_{o/p_o} - S_p) / 90 * D) \\
 & * (1 + 0.03(S_{o/a} - S_a) / D))
 \end{aligned} \tag{43}$$

Thermodynamic evidence presented in Appendix B as well as limited published information on growth rates of SRB capable of complete oxidation of propionate suggest, however, that complete oxidation of propionate is likely to occur more rapidly than sequential oxidation of propionate and then acetate. Desulfococcus multivorans and Desulfosarcina variabilis are reported to grow slower on propionate than Desulfobulbus propionicus (Kreig and Holt, 1984) but none have suggested that they grow slower on propionate than acetate-utilizing species are capable of growing. In fact, growth of D. variabilis on acetate alone is reported to be "very slow" compared to growth on other volatile acids (Kreig and Holt, 1984).

Butyrate-utilizing SRB resisted continuous culture with a medium containing butyrate as the only organic substrate and with a three acid medium during this research. Perhaps the medium lacked a growth factor that has not yet been reported in the literature or the dilution rates used were too great under the environmental conditions imposed on the culture. A minor but measureable

amount of butyrate consumption did occur over and above the apparent "consumption" caused by dilution of the effluent by the neutralizing acid added to the reactor.

Process Performance

The performance of the sulfate-reduction unit operation of the proposed process operating at dilution rates above 0.03 hr^{-1} is compared to model predictions in Figure 10. Only propionate and acetate components of the medium are considered as the butyrate component appeared to be resistant to change in the process.

Applications

The knowledge gained during this research has a variety of applications. The most obvious concerns treatment of acid mine drainage. This and other applications are discussed below.

Volatile Acid Turnover

The rates at which volatile acids are consumed in marine and estuarine sediments are of concern to microbial ecologists (Lovley and Klug, 1982; Dicker and Smith, 1985; Anderson et al., 1987). The findings of this research indicate that, under conditions of high concentrations of propionate, acetate and sulfate, mixed SRB populations are capable of converting propionate to acetate much faster than acetate can be oxidized by SRB, and, in fact, much

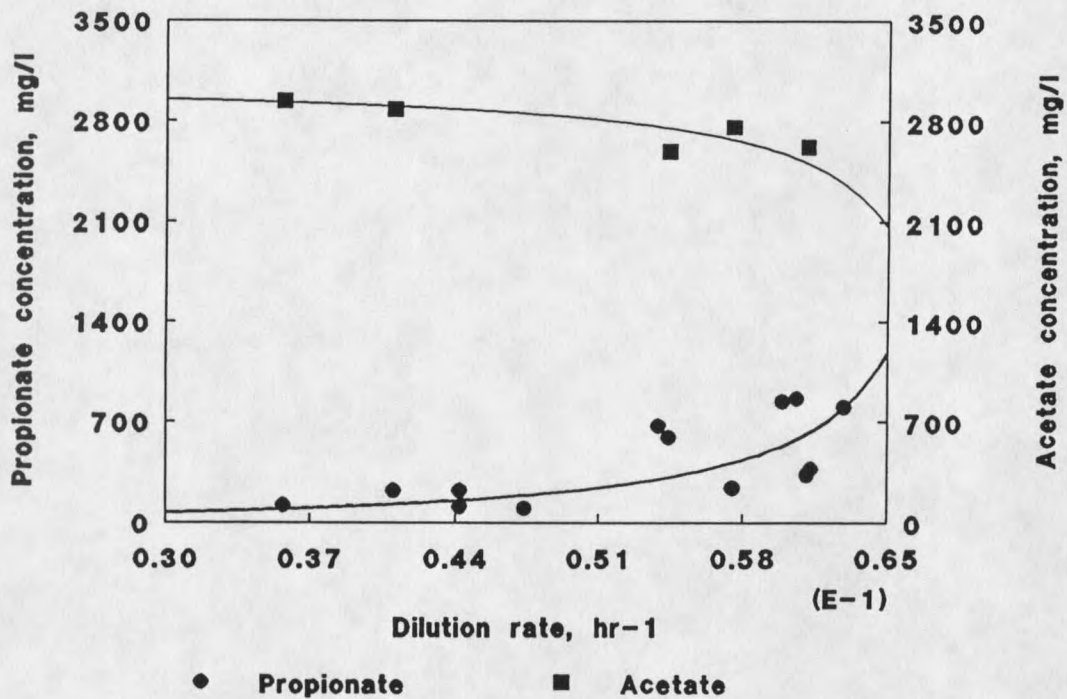


Figure 10. Process Performance

faster than acetate can be converted to methane by methanogens. Furthermore, under similar conditions, the maximum rate at which butyrate is utilized is slower than the maximum rate that propionate is utilized.

Under conditions of relatively low concentrations of acetate and propionate (5-90 mg/l each), acetate-utilizing SRB are capable of more rapid growth than propionate-utilizing SRB because of relatively large K_S of propionate-utilizing SRB. Hence, there are conditions under which acetate turnover rates can be greater than propionate turnover rates.

Kinetic Control

Heretofore, kinetic control (i.e., control of mean cell residence time) has been used to selectively enrich or eliminate from continuous cultures organisms that are relatively unrelated. For example, a relatively short detention time is used to eliminate algae from waste stabilization ponds. Similarly, kinetic control can be used to either enrich or eliminate nitrifying bacteria from activated sludge cultures. In anaerobic systems, kinetic control has been used to separate acidogenic bacteria from methanogenic bacteria in anaerobic digestion systems (Ghosh et al., 1975).

This research illustrates how kinetic control can even be used to separate organisms that are closely related (from a Bergey's Manual/physiology perspective). It can be used to separate SRB that utilize acetate from SRB that produce acetate. It is reasonable to hypothesize that with the right equipment (e.g., large reactors and precise pumping systems) one could use kinetic control to separate species within a genus (e.g., butyrate-utilizing SRB such as Desulfovibrio sapovorans from Desulfovibrio baarsii).

The principle of kinetic control may have applications in biotechnologies other than waste bioprocessing. For example, it could be used to separate and eliminate slow growing cells from rapidly growing ones in continuous suspended cultures of cell lines. In AIDS research, it

might find application in separation of various phenotypes of differentiated bone marrow hematopoietic stem cells (Spangrude et al., 1988) and in separation of other cells required for gene therapy (Powledge, 1983; Herskowitz, 1987; Friedmen et al., 1988) and intracellular immunization (Baltimore, 1988).

Bioprocessing of Acidic Sulfate Solutions from Hydrometallurgical Operations

Knowledge gained during this research could be used to optimize proposed bacterial mutualism processes for treatment of acidic sulfate solutions. It would allow an inexpensive substrate to be used to "fuel" microbial sulfate reduction and would make acetate available for downstream methane production. For example, it could be used to optimize sulfur recovery from solvent extraction raffinate, a waste sulfate solution produced by a hydrometallurgical copper extraction process (Cork and Cusanovich, 1979).

Coal Desulfurization

Microbiological processes currently under study for desulfurization of high-sulfur coal produce a waste stream containing relatively high sulfate concentrations (Kumar, 1989). In this situation, again, an inexpensive substrate could be used as an electron donor in sulfate reduction treatment of the stream with subsequent conversion of the H_2S so generated to sulfur.

Bioprocessing of Heap Leach Effluents and Acid Mine Drainage

Bioprocessing of acid mine drainage could also be optimized using the information presented herein. If certain issues were resolved, the process could be capable of significantly reducing the chemical and energy requirements of acid mine drainage treatment. Moreover, it could reduce sulfate concentrations and simultaneously allow metals and sulfur recovery.

As was noted in the Results section, the economics of the proposed process in any particular application would be affected by the little reported fact that microbial sulfate reduction is capable of increasing the pH of the influent to the process only if sulfide is removed from the system in the form of H_2S . The sulfide generated by microbial sulfate reduction that reacts with metals in the influent to form insoluble metal sulfides obviously cannot also be removed as H_2S . Furthermore, with operation of a sulfate reduction reactor at a neutral pH, about 20 percent of the carbonic acid produced would exist as CO_2 . Removal of this CO_2 as a gas would also tend to increase the pH of the reactor contents. Recycling of purged gases to a pretreatment reactor upstream of the sulfate reduction reactor could be used to reduce metal concentrations prior to the sulfate reduction step, but would add acidity back into the influent.

A comparison of the expected concentration of metals (in milliequivalents per liter, me/l) in the influent to the expected concentration (in me/l) of sulfate in the influent of the sulfate reduction step can be used to determine if "excess" sulfide will be produced that could be removed as H_2S to accomplish neutralization of the mine water. Only those metals which form metal sulfides at neutral pH need be considered. For example, in the water in the Berkeley Pit, the primary sulfide-forming metals are copper, iron, manganese and zinc. In 1987, the water in the Pit contained about 14 me/l of these metals and about 37 me/l of sulfur (as sulfate). Thus, if 14 me/l of the sulfur were used (as sulfide) to precipitate the predominant metals, about 23 me/l would be available for removal as H_2S to increase the pH of the water. Removal of 23 mM of protons would neutralize an unbuffered solution having an initial pH of less than two. Furthermore, considering the stoichiometry of the reaction, about 49 me/l of bicarbonate (37×1.33) would be available for removal as CO_2 . Thus, in treating water from the Berkeley Pit, a significant amount of pH adjustment would be possible even with essentially complete removal of metals.

This research does leave a number of issues unresolved concerning the technical and economic feasibility of the proposed process. Those issues are:

1. potential inhibition of SRB by acid mine drainage constituents;
2. amount, source and bioprocessing of biomass, and
3. choice of sulfur recovery process.

These issues are addressed in the following paragraphs.

As was indicated earlier, a variety of chemicals reportedly inhibit SRB growth (Saleh et al., 1964). Notable are copper and such oxyanions as molybdate, selenate, tungstate and chromate. The process as proposed relies on pretreatment using the cementation (oxidation/reduction) process to reduce copper concentrations to acceptable levels. Noboro and Yagisawa (1978) have reported that SRB will grow rapidly on a medium containing lactate and copper mine heap leach extract at copper concentrations as high as 100 mg/l. Although Desulfobulbus propionicus, the SRB enriched in continuous culture during this research, is capable of utilizing lactate, no information on its susceptibility to inhibitors is available.

The findings of this research have three important implications concerning the amount and bioprocessing of biomass used as a source of volatile acids to "fuel" sulfate reduction. The first is that a large amount of biomass would be required to remove a large amount of sulfate. The second is that apparently only propionate

would contribute significantly to sulfate reduction at reasonable dilution rates.

One of the limitations of the proposed process is that the waste stream (or portion of the waste stream) undergoing sulfate reduction must be treated to reduce sulfate concentrations to a relatively low level (approximately 200 mg/l as S) so as not to inhibit subsequent methane production. Thus, an intermediate level of sulfate reduction is not feasible unless a portion of the waste stream is bypassed and then blended with the treated stream.

This and other studies have determined that 1.0 mole of propionate is required for each 0.75 mole of sulfate reduced. Thus, 1.0 pound of propionate is required for each pound of sulfate removed. Ghosh et al. (1987) have reported that with "conventional" acid phase anaerobic digestion (two-day residence time, mesophilic), about 0.0143 pound of propionate is produced for each pound of volatile solids added to the reactor. As about 75 percent of typical municipal wastewater sludge dry solids are volatile, about 93 pounds of dry solids would be required to produce enough propionate to reduce one pound of sulfate.

A variety of possibilities exist to reduce the amount of biomass (dry solids) needed to accomplish sulfate reduction. One is to provide conditions that would allow

all or part of the butyrate to be available as an electron donor. With this volatile acid, about 0.0021 pound of butyrate is produced for each pound of volatile solids added and about 1.8 pounds of butyrate is required to reduce one pound of sulfate. With conventional acid phase anaerobic digestion, this could reduce the solids requirement slightly to about 84 pounds of dry solids/pound of sulfate. A more significant reduction could be achieved by using an upflow acid phase digester (Ghosh et al., 1987). With this option, use of propionate and butyrate would reduce the solids requirement to about 49 pounds. Use of all of the acetate originally present in the supernatant from an upflow reactor would reduce the solids requirement to about 22 pounds. Alternatively, the hydrogen management techniques suggested by Harper and Pohland (1985) could be used to manipulate the volatile acid mix to optimize the process.

A further significant reduction in the dry (or volatile) solids requirement could be achieved by "recycling" carbon in the system. The carbon dioxide produced during combustion of the methane produced by the process could be biologically converted to organic material (e.g., by algae) or it could be fed directly into a sulfate reduction reactor populated by autotrophic SRB (e.g. Desulfobacter hydrogenophilus), if an inexpensive source of hydrogen were available (Schaueder et al., 1987). If a

source of inexpensive ethanol were available, it could be used as an electron donor directly (e.g., by Desulfococcus multivorans) or indirectly after conversion to propionate and acetate in the absence of sulfate by Desulfobulbus propionicus (Laanbroek et al., 1982).

A third issue that must be addressed is the choice of the sulfur recovery process. A portion of the sulfide ions produced by sulfate reduction will combine with dissolved metal ions and form a precipitate. Excess sulfide must be removed from the process as hydrogen sulfide gas. Sulfur could be recovered from this gas using the conventional Claus process. Montana Chemical and Sulfur Company, for example, uses this process to recover sulfur from hydrogen sulfide generated at the refineries in the Billings area. The sulfur thus recovered is sold at about \$0.04 per pound as an agricultural soil amendment.

An alternative biological sulfur recovery process is described in U.S. Patent No. 4,666,852 (Cork, 1987). In this process, green photosynthetic sulfur bacteria are used to convert carbon dioxide and hydrogen sulfide produced by SRB to bacteria cells and elemental sulfur. A disadvantage of the process is that it is an anaerobic process with a low biomass yield. Thus, it would allow recycling of little of the carbon into the volatile acid production step of the proposed process.

It is intriguing to consider other alternatives for simultaneously or sequentially allowing recovery of sulfur and carbon. An aerobic biological process, for example, would allow more efficient conversion of carbon dioxide to biomass.

One option is to grow aerobic algae on the carbon dioxide present in the H_2S/CO_2 gas stream after H_2S removal by green sulfur bacteria. With this option, an aerobic reactor would be provided downstream from the sulfur recovery reactor. Algae could be harvested and their biomass anaerobically digested to recover volatile acids.

Another option is to use halophilic purple sulfur bacteria of the genus Ectothiorhodospira to simultaneously recover sulfur and carbon. These bacteria are not photosynthetic and thus would not require exposure to light. They deposit sulfur extracellularly as do green sulfur bacteria, thus facilitating sulfur separation from the biomass (Brock et al., 1984). Some species are microaerobes and might be more "efficient" at converting CO_2 to biomass.

Yet another option is to use Thiobacillus ferroxidans to simultaneously recover sulfur and carbon. Ironically, these are the bacteria that contribute to the creation of acid mine drainage problems. Under certain conditions, Thiobacillus ferroxidans can be caused to stop the sulfide oxidation process at the elemental sulfur step (Hazeu et

al., 1988). These bacteria deposit the sulfur as extracellular globules.

A final option is to use purple sulfur bacteria that deposit sulfur intracellularly, such as bacteria of the genus Beggiatoa. These aerobic bacteria typically live at the interface between sulfide and oxygen in natural environments. They could be cultured on oxygen permeable silicone membranes having air on one side and the bacteria and sulfide growth medium on the other. It might be feasible to anaerobically digest the resulting biomass to recover the carbon and recover sulfide in the resulting gas using one of the above processes.

While carbon recycling and sulfur recovery requirements raise significant challenges to the feasibility of the proposed process, it is instructive to remember that the use of kinetic control to ensure recovery of acetate from biological propionate oxidation was "impossible" just three years ago. With some focused, multidisciplinary research, this process could prove to be yet another of the environmentally sound biotechnologies that science has fashioned this decade.

CONCLUSIONS

The conclusions of this research are as follows:

1. A chemostat containing a mixed culture of SRB can be operated at a dilution rate large enough to cause incomplete oxidation of propionic acid and production of acetic acid, if acetic, propionic and butyric acids and sulfate are present in the reactor feed.
2. The sulfate reduction unit operation of the proposed process appears to be technically feasible if SRB inhibitors are not present in the acid mine drainage. Cell recycle could be practiced to reduce reactor size.
3. A significant amount of biomass, approximately 50 pounds of dry solids per pound of sulfate removed, would be required to supply electron donors to the proposed process, if the propionate and butyrate were produced by an upflow reactor and both were available for sulfate reduction. For the process to be economically feasible, the acid phase anerobic digestion step would have to be designed to optimize production of higher molecular weight

volatile acids and a means of recycling carbon would be required.

4. Kinetic control of continuous cultures may have application in other biotechnologies, wherein separation of faster growing cells from slower growing cells is desired.

NOMENCLATURE

b	maintenance (or death and decay) coefficient [T^{-1}]
b_a	maintenance coefficient with growth on acetate [T^{-1}]
b_b	maintenance coefficient with growth on butyrate [T^{-1}]
b_p	maintenance coefficient with growth on propionate [T^{-1}]
D	dilution rate [T^{-1}]
K_S	half saturation content or coefficient [ML^{-3}]
$K_{S/a}$	half saturation coefficient on acetate [ML^{-3}]
$K_{S/b}$	half saturation coefficient butyrate [ML^{-3}]
$K_{S/p}$	half saturation coefficient on propionate [ML^{-3}]
k_1	rate of utilization of propionate [$ML^{-3}T^{-1}$]
k_2	rate of utilization of acetate [$ML^{-3}T^{-1}$]
Q	volumetric flow rate [L^3T^{-1}]
$r_{d/x}$	rate of death and decay of cell material [$ML^{-3}T^{-1}$]
$r_{g/x}$	rate of generation of cell material [$ML^{-3}T^{-1}$]
r_s	rate of consumption of substrate [$ML^{-3}T^{-1}$]
S	reactor and output (effluent) substrate concentration [ML^{-3}]
S_a	reactor and output acetate concentration [ML^{-3}]
$S_{o/a}$	reactor input acetate concentration [ML^{-3}]
S_o	reactor input substrate concentration [ML^{-3}]

$S_{o/p}$	reactor input propionate concentration [ML ⁻³]
S_p	reactor and output propionate concentration [ML ⁻³]
t	time [T]
$t_{d,min}$	minimum microorganism doubling time [T]
V	reactor volume [L ³]
X	reactor and output cell concentration [ML ⁻³]
X_a	reactor and output acetate-consuming cell concentration [ML ⁻³]
X_b	reactor and output butyrate-consuming cell concentration [ML ⁻³]
X_o	input cell concentration [ML ⁻³]
X_p	reactor and output propionate-consuming cell concentration [ML ⁻³]
Y_g	true growth yield [MM ⁻¹]
$Y_{g/a}$	true growth yield on acetate [MM ⁻¹]
$Y_{g/p}$	true growth yield on propionate [MM ⁻¹]
μ	specific growth rate [T ⁻¹]
μ_{max}	maximum specific growth rate [T ⁻¹]
$\mu_{max,a}$	maximum specific growth rate on acetate [T ⁻¹]
$\mu_{max,b}$	maximum specific growth rate on butyrate [T ⁻¹]
$\mu_{max,p}$	maximum specific growth rate on propionate [T ⁻¹]

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APPENDICES

P

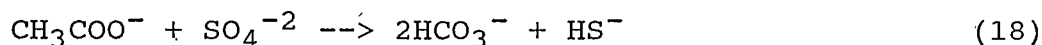
APPENDIX A

THERMODYNAMIC CALCULATIONS

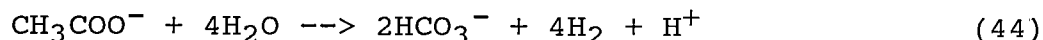
The calculated free energy change associated with a biologically catalyzed chemical reaction can be used to estimate the true growth yield of the organism involved. The free energy change is usually also positively correlated with the maximum specific growth rate of the organism. The more negative the calculated free energy change, the higher the yield and growth rate can be expected to be. The following calculations were used to develop the expected free energy changes under standard conditions for the volatile acid oxidations of concern to this research. Data from Thauer et al (1977) were used to calculate the free energy change per each two electrons transferred at unit concentrations of reactants and products and at pH 7.0.

Acetate Oxidation

Complete oxidation of acetate to carbon dioxide occurs via the following reaction:

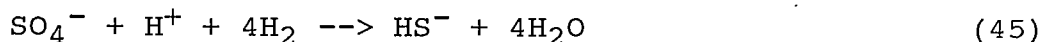


This reaction is the sum of the following two half reactions:



$$G^\circ' = +104.6 \text{ kJ/reaction}$$

and



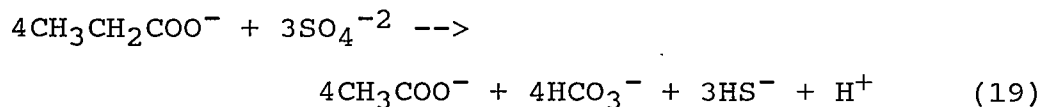
$$G^\circ' = -151.9 \text{ kJ/reaction}$$

Because eight electrons are transferred in each reaction,

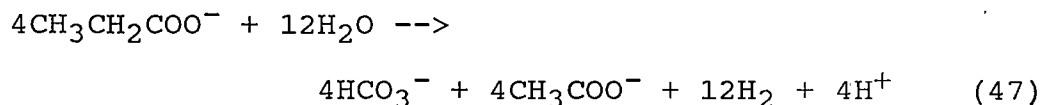
$$G^\circ'/2e^- = (104.6 - 151.9)/4 = -11.83 \text{ kJ} \quad (46)$$

Propionate Oxidation

Incomplete oxidation of propionate to acetate occurs via the following reaction:

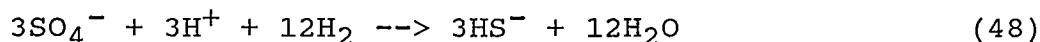


This reaction is the sum of the following two half reactions:



$$G^\circ' = +76.1 \times 4 = +304.4 \text{ kJ/reaction}$$

and

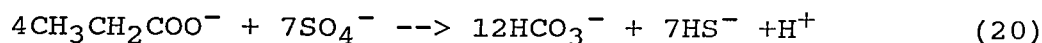


$$G^\circ' = -151.9 \times 4 = -455.7 \text{ kJ/reaction}$$

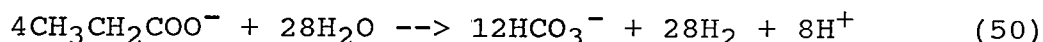
Because 24 electrons are transferred in each reaction,

$$G^\circ'/2e^- = (304.4 - 455.7)/12 = -12.61 \text{ kJ} \quad (49)$$

Complete oxidation of propionate occurs via the following reaction:

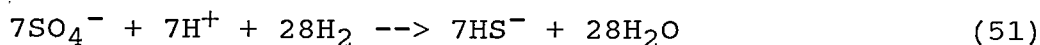


This reaction is the sum of the following two half reactions:



$$G^\circ' = +181.1 \times 4 = +724.4 \text{ kJ/reaction}$$

and



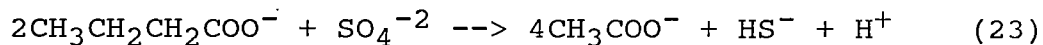
$$G^\circ' = -151.9 \times 7 = -1,063.3 \text{ kJ/reaction}$$

Because 56 electrons are transferred in each reaction,

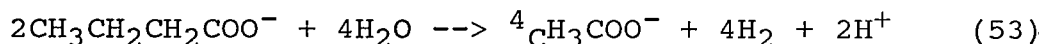
$$G^\circ'/2e^- = (724.4 - 1,063.3)/28 = -12.10 \text{ kJ} \quad (52)$$

Butyrate Oxidation

Pfennig and Widdel (1981) suggested the following stoichiometry for incomplete oxidation of straight chain fatty acids with even numbers of carbon atoms such as butyrate by SRB:

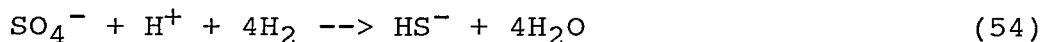


This reaction is the sum of the following two half reactions:



$$G^\circ' = +48.1 \times 2 = +96.2 \text{ kJ/reaction}$$

and

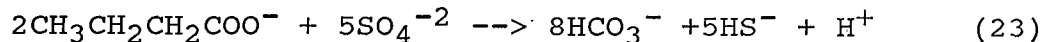


$$G^\circ' = -151.9 \text{ kJ/reaction}$$

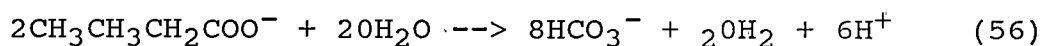
Because eight electrons are transferred in each reaction,

$$G^{\circ '}/2e^{-} = (96.2 - 151.9)/4 = -13.93 \text{ kJ} \quad (55)$$

The stoichiometry of complete oxidation of butyrate by Desulfobacterium catecholicum was determined to be as follows by Szewzyk and Pfennig (1987):

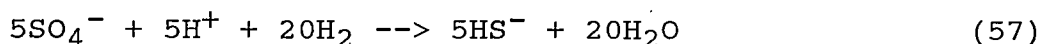


This reaction is the sum of the following two half reactions:



$$G^{\circ '} = +257.3 \times 2 = +514.6 \text{ kJ/reaction}$$

and



$$G^{\circ '} = -151.9 \times 5 = -759.5 \text{ kJ/reaction}$$

Because 40 electrons are transferred in each reaction,

$$G^{\circ '}/2e^{-} = (514.6 - 759.5)/20 = -12.25 \text{ kJ} \quad (58)$$

APPENDIX B

THE CHALLENGES OF CONTINUOUS CULTURE
OF SRB IN SMALL REACTORS

Continuous culture of SRB in small reactors presents a variety of challenges to the researcher. A discussion of some of these challenges and some practical suggestions for meeting them are presented below.

Air Leaks

Continuous cultures of SRB in small reactors are extremely sensitive to leakage of air into the culture vessel. This research used glass beakers having a working volume of about 500 ml and topped with thick black rubber stoppers and was still intermittently plagued by air leakage. The leakage occurred regardless of semicontinuous purging with oxygen-free nitrogen (N_2). Soapy water can be used to detect escaping N_2 .

Tubing for medium and N_2 transport should be either butyl rubber (Fisher Scientific) or neoprene (Cole Parmer). Only glass tubing connectors should be used.

Wall Growth

SRB have an affinity for growing on the reactor walls. As one would expect, the problem is more severe at higher dilution rates.

Wall growth can be minimized by intermittent scraping of the reactor walls. The scraping must occur every three to six hours at high dilution rates and less frequently otherwise. A scraper can be constructed by attaching an inverted plastic funnel to a stainless steel rod that perforates the top of the reactor. The portion of the rod outside the reactor can be enclosed in a length of butyl rubber bicycle inner tube. The bottom of the inner tube is fastened to the top of the reactor and the top to a rubber stopper afixed to the upper end of the rod.

pH Control

The relatively high concentration of H_2S in the reactor tends to degrade the performance of pH electrodes fairly rapidly. A double junction electrode (Markson Scientific) should be used. SRB will also grow on the electrode glass bulb creating a microenvironment of relatively high pH. Intermittent vigorous mixing of the reactor contents using the wall scraper is necessary to prevent excess neutralizing chemical (acid) from being added to the reactor by the pH control system and sterilizing the system.

Substrate Concentration

Substrate concentrations are effectively decreased by the diluting effect of acid additions (0.1 N HCl) and effectively increased by water losses that occur during medium preparation and storage during a run. A relatively low acid concentration (0.1 N) should be used, however, to prevent large changes in pH during pH control operations.

The distilled water used to make up the medium should be boiled briefly under oxygen-free N₂ to remove dissolved oxygen. Although liquid heating can be halted at the first sign of boiling, some liquid loss will occur during both boiling and during removal from the autoclave.

Furthermore, university "distilled water" will contain varying amounts of sulfate in any case as the following distilled water sulfate concentration data indicate:

<u>Date</u>	Sulfate concentration, <u>mg/l</u>
10/18/88	2
10/24/88	3
12/8/88	13
12/26/88	9
12/26/88	62
12/26/88	35
12/26/88	2
1/20/89	43
2/2/89	3
2/2/89	3
3/2/89	9
3/2/89	8

Temperature Control

SRB growth rates are very sensitive to temperature. The reactor should be submerged to the extent feasible in a constant temperature water bath. A temperature controller with an accuracy of $\pm 0.01^{\circ}$ C should be used (Markson Scientific).

Medium Transfer

Small reactors and low growth rates combined require very low medium pumping rates. Master-Flex pumps with No. 13 neoprene tubing (Cole Parmer) can produce the low flow rates (5 ml/hr) required. If a complex medium containing much iron is used, a precipitate will form which will clog small bore tubing unless a glass fiber filter is installed at the influent end of the tubing.

Medium Additives

Although EDTA addition of SRB media has been suggested to prevent iron limitation, EDTA is reportedly toxic to some SRB (Pfenning et al, 1981). Care must also be taken in choosing an appropriate reducing agent to scavenge oxygen in the medium. Sodium thioglycolate is toxic to some SRB (Pfenning et al, 1981) as is ascorbic acid (Brock et al, 1984). Sodium dithionite at 30 mg/l was not toxic to propionate-utilizing SRB. It should, however, be added to an essentially oxygen-free medium, as the products of

reaction of reducing agents with oxygen may be toxic to some bacteria (Ljungdahl and Wiegel, 1986).

APPENDIX C
DATA TABULATIONS

Table 10. Flow Rate Data

Run No.	Average flow rate, ml/hr				Dilution rate, hr ⁻¹
	Medium	Acid	Sum ^a	Effluent	
1.1	10.05	- ^b	-	-	-
2.1	6.91	- ^b	-	-	-
3.1	11.31	- ^b	-	-	-
3.2	15.23	- ^b	-	-	-
4.1	12.93	- ^b	-	-	-
4.2	11.75	- ^b	-	-	-
5.1	5.93	- ^b	-	-	-
5.2	5.54	- ^b	-	-	-
6.1	7.01	- ^b	-	-	-
6.2	6.32	- ^b	-	-	-
7.1	12.94	- ^b	-	-	-
7.2	12.09	- ^b	-	-	-
8.1	14.96	4.29	19.25	19.25 ^c	-
8.2	14.10	7.25	21.35	21.66	0.0442
9.1	15.30	7.73	23.03	23.04	0.0474
9.2	15.56	6.25	21.81	21.66	0.0442
10.1	23.45	7.21	30.66	30.55	0.0629
10.2	24.07	5.27	29.34	29.68	0.0606
11.1	25.15	-	-	-	-
11.2	24.25	4.95	29.20	29.34	0.0599
12.1	21.47	8.35	29.82	29.71	0.0611
12.2	23.23	-	-	-	-
13.1	20.00	8.15	28.15	27.96	0.0575
13.2	17.55	1.15	18.70	18.74	-
14.1	19.83	6.74	26.57	26.26	0.0544
14.2	18.22	1.61	19.83	19.58	-
15.1	13.89	6.12	20.01	19.79	0.0410
15.2	12.37	5.04	17.45	17.44	0.0357
16.1	21.24	8.94	30.18	29.89	0.0613
16.2	19.11	7.08	26.19	26.02	0.0539
17.1	-	-	-	-	-
17.2	4.42	2.48	6.90	6.93	0.0143
18.1	5.52	2.98	8.50	8.74	0.0179
18.2	5.38	3.06	8.44	8.63	0.0179

^a Medium plus acid^b Flow rate not measured^c Estimate based on sum

Table 11. Medium Concentration Data

Run No.	Average concentration, mg/l			
	Acetate	Propionate	Butyrate	Sulfate
Target ^a	0	2,700	0	3,330
1.1	0	3,360	0	- ^b
2.1	0	3,240	0	- ^b
3.1	0	2,850	0	- ^b
3.2	0	2,850	0	- ^b
4.1	0	2,980	0	2,900
4.2	0	2,980	0	2,900
5.1	0	2,730	0	3,270
5.2	0	2,730	0	3,270
6.1	0	2,730	0	3,310
6.2	0	2,730	0	3,310
7.1	0	2,710	0	2,960
7.2	0	2,710	0	2,960
8.1	0	2,750	0	2,960
8.2	0	2,750	0	2,960
9.1	0	2,700	0	3,130
9.2	0	2,700	0	3,130
10.1	0	2,850	0	2,790
10.2	0	2,850	0	2,790
11.1	0	2,700	0	2,710
11.2	0	2,700	0	2,710
Target ^a	1,900	2,700	2,000	3,330
12.1	1,780	2,760	2,080	2,930
12.2	1,780	2,760	2,080	2,930
13.1	1,900	2,840	2,120	2,910
13.2	1,900	2,840	2,120	2,910
Target ^a	1,900	2,700	2,000	3,790
14.1	1,880	2,830	2,110	3,390
14.2	1,880	2,830	2,110	3,390
15.1	1,890	2,780	2,070	3,650
15.2	1,880	2,830	2,100	3,830
Target ^a	0	2,700	0	3,790
16.1	0	3,070	0	4,310 ^c
16.2	0	3,070	0	4,310 ^c
17.1	0	2,730	0	3,800
17.2	0	2,730	0	3,800
18.1	0	2,970	0	- ^b
18.2	0	2,970	0	- ^b

^a Theoretical concentrations for the following runs based on measured chemical weights and water volume.

^b Sample not analyzed for parameter. ^c Estimate.

Table 12. Effluent Concentration Data

Run No.	Average concentration, mg/l			
	Acetate	Propionate	Butyrate	Sulfate
1.1	490	3,200	0	- ^c
2.1	800	2,420	0	3,970
3.1	1,410	100	0	70
3.2	1,530	70	0	- ^c
4.1	1,670	80	0	90
4.2	1,950	50	0	90
5.1	1,390	130	0	40
5.2	1,320	160	0	50
6.1	1,320	90	0	100
6.2	1,310	60	0	60
7.1	1,370	140	0	80
7.2	1,410	140	0	140
8.1	1,200	460	0	390
8.2	1,360	110	0	20
9.1	1,360	100	0	30
9.2	1,390	220	0	190
10.1	1,210	800	0	940
10.2	1,040	860	0	1,100
11.1	- ^a	- ^a	- ^a	- ^a
11.2	1,130	840	0	1,230
12.1	2,620	330	1,270	280
12.2	- ^a	- ^a	- ^a	- ^a
13.1	2,760	240	1,280	210
13.2	- ^a	- ^a	- ^a	- ^a
14.1	2,590	590	1,400	1,350
14.2	- ^a	- ^a	- ^a	- ^a
15.1	2,880	220	1,170	1,220
15.2	2,940	120	1,140	1,200
16.1	1,360	370	0	1,440
16.2	1,060	670	0	1,740
17.1	- ^b	- ^b	- ^b	- ^b
17.2	- ^b	- ^b	- ^b	1,070
18.1	1,240	30	0	- ^c
18.2	1,230	40	0	- ^c

^a Data not presented because reactor did not reach steady-state due to mechanical difficulties.

^b Sample lost due to refrigerator failure.

^c Sample not analyzed for parameter.

Table 13. Calculated Effective Influent Concentrations

Run No.	Effective influent concentration, mg/l			
	Acetate	Propionate	Butyrate	Sulfate
8.1	0	2,140	0	2,300
8.2	0	1,790	0	1,930
9.1	0	1,790	0	2,080
9.2	0	1,940	0	2,250
10.1	0	2,190	0	2,140
10.2	0	2,310	0	2,260
11.1	-	-	-	-
11.2	0	2,230	0	2,240
12.1	1,290	1,990	1,500	2,120
12.2	-	-	-	-
13.1	1,360	2,030	1,520	2,080
13.1	-	-	-	-
14.1	1,420	2,140	1,590	2,560
14.2	-	-	-	-
15.1	1,330	1,950	1,450	2,560
15.2	1,330	2,010	1,490	2,720
16.1	0	2,180	0	3,060 ^b
16.2	0	2,250	0	3,170 ^b
17.1	-	-	-	-
17.2	0	1,740	0	2,420
18.1	0	1,880	0	-
18.2	0	1,850	0	-

^a Diluted medium concentration based on ratio of medium and effluent flow rates.

^b Estimate (see Table 11).

Table 14. Acetate Production

Run No.	Substrate oxidation rate, mM/hr			Acetate production rate, mM/hr
	Propionate	Butyrate	Total	
8.1	0.443	0	0.443	0.391
8.2	0.498	0	0.498	0.499
9.1	0.533	0	0.533	0.531
9.2	0.510	0	0.510	0.510
10.1	0.581	0	0.581	0.626
10.2	0.589	0	0.589	0.523
11.1	-	-	-	-
11.2	0.558	0	0.558	0.562
12.1	0.675	0.078	0.753	0.669
12.2	-	-	-	-
13.1	0.685	0.077	0.762	0.663
13.2	-	-	-	-
14.1	0.557	0.027	0.584	0.560
14.2	-	-	-	-
15.1	0.469	0.064	0.533	0.520
15.2	0.451	0.070	0.521	0.476
16.1	0.740	0	0.740	0.688
16.2	0.563	0	0.563	0.467
17.1	-	-	-	-
17.2	-	-	-	-
18.1	0.221	0	0.221	0.184
18.2	0.214	0	0.214	0.180

Table 15. Sulfate Reduction

Run No.	Substrate oxidation rate, mM/hr			Sulfate reduction rate, mM/hr
	Propionate	Butyrate	Total	
8.1	0.443	0	0.443	0.383
8.2	0.498	0	0.498	0.431
9.1	0.533	0	0.533	0.492
9.2	0.510	0	0.510	0.464
10.1	0.581	0	0.581	0.382
10.2	0.589	0	0.589	0.358
11.1	-	-	-	-
11.2	0.558	0	0.558	0.308
12.1	0.675	0.078	0.753	0.569
12.2	-	-	-	-
13.1	0.685	0.077	0.762	0.544
13.2	-	-	-	-
14.1	0.557	0.027	0.584	0.331
14.2	-	-	-	-
15.1	0.469	0.064	0.533	0.276
15.2	0.451	0.070	0.521	0.272
16.1	0.740	0	0.740	0.504 ^a
16.2	0.563	-	0.563	0.387 ^a
17.1	-	-	-	-
17.2	-	-	-	0.097
18.1	0.221	0	0.221	-
18.2	0.214	0	0.214	-

^a Estimate (see Table 13).

Table 16. Acid Neutralization

Run No.	Substrate oxidation rate, mM/hr			Acid neutralization rate, mM/hr
	Propionate	Butyrate	Total	
8.1	0.443	0	0.443	0.429
8.2	0.498	0	0.498	0.725
9.1	0.533	0	0.533	0.773
9.2	0.510	0	0.510	0.625
10.1	0.581	0	0.581	0.721
10.2	0.589	0	0.589	0.527
11.1	-	-	-	-
11.2	0.558	0	0.558	0.495
12.1	0.675	0.078	0.753	0.835
12.2	-	-	-	-
13.1	0.685	0.077	0.762	0.815
13.2	-	-	-	-
14.1	0.557	0.027	0.584	0.674
14.2	-	-	-	-
15.1	0.469	0.064	0.533	0.612
15.2	0.451	0.070	0.521	0.504
16.1	0.740	0	0.740	0.894
16.2	0.563	0	0.563	0.708
17.1	-	-	-	-
17.2	-	-	-	0.248
18.1	0.221	0	0.221	0.298
18.2	0.214	0	0.214	0.306

Table 17. Calculated Hofstee Plot Variables

Run No.	Dilution rate (D), hr ⁻¹	Dilution rate/concentration (D/S), l/mg.hr	
		Propionate	Butyrate
8.1	-	-	-
8.2	0.0442	0.000402	-
9.1	0.0474	0.000474	-
9.2	0.0442	0.000201	-
10.1	0.0629	0.0000786	-
10.2	0.0606	0.0000704	-
11.1	-	-	-
11.2	0.0599	0.0000713	-
12.1	0.0611	0.000185	0.0000481
12.2	-	-	-
13.1	0.0575	0.000240	0.0000449
13.2	-	-	-
14.1	0.0544	0.0000922	0.0000388
14.2	-	-	-
15.1	0.0410	0.000186	0.0000350
15.2	0.0357	0.000298	0.0000313
16.1	0.0613	0.0000915	-
16.2	0.0539	0.0000869	-
17.1	-	-	-
17.2	0.0143	-	-
18.1	0.0179	0.000597	-
18.2	0.0179	0.000448	-

Table 18. Calculated Hanes Plot Variables

Run No.	Substrate concentration (S), mg/l		Concentration/dilution rate (S/D), mg.hr/l	
	Propionate	Butyrate	Propionate	Butyrate
8.1	-	-	-	-
8.2	110	-	2,490	-
9.1	100	-	2,110	-
9.2	220	-	4,980	-
10.1	800	-	12,700	-
10.2	860	-	14,200	-
11.1	-	-	-	-
11.2	840	-	14,000	-
12.1	330	1,270	5,400	20,800
12.2	-	-	-	-
13.1	240	1,280	4,170	22,300
13.2	-	-	-	-
14.1	590	1,400	10,800	25,700
14.2	-	-	-	-
15.1	220	1,170	5,370	28,500
15.2	120	1,140	3,360	31,900
16.1	370	-	6,040	-
16.2	670	-	10,900	-
17.1	-	-	-	-
17.2	-	-	-	-
18.1	30	-	1,680	-
18.2	40	-	2,230	-

Table 19. Biomass Characteristics

Run No.	Cell concentration, 10 ¹⁰ cells/l	Average cell area, sq micron	Average cell length, micron	Average cell width, micron
8.1	-	-	-	-
8.2	21.00	0.670	1.37	0.820
9.1	38.75	1.061	1.56	1.02
9.2	-	-	-	-
10.1	33.52	1.047	1.56	0.971
10.2	18.92	0.870	1.39	0.924
11.1	-	-	-	-
11.2	18.75	0.859	1.39	0.870
12.1	46.96	0.806	1.39	0.866
12.2	-	-	-	-
13.1	55.56	0.808	1.33	0.858
13.2	-	-	-	-
14.1	31.90	1.071	1.55	1.03
14.2	-	-	-	-
15.1	36.32	0.915	1.40	0.931
15.2	32.03	0.920	1.46	0.936
16.1	37.28	0.856	1.42	0.894
16.2	18.64	0.905	1.43	0.932
17.1	-	-	-	-
17.2	-	-	-	-
18.1	51.03	0.729	1.31	0.832
18.2	60.75	0.663	1.27	0.809

Table 20. Calculation of Yield Analysis Plot Variables

Run No.	Space time (1/D), hr	$S_0 - S$, mg/l	Average cell weight, 10^{-10} mg	$(S_0 - S)/X$, mg/mg
8.1	-	-	-	-
8.2	22.6	1,680	1.31	61.1
9.1	21.1	1,690	1.31	33.3
9.2	22.6	1,720	-	-
10.1	15.9	1,390	1.31	31.7
10.2	16.5	1,450	1.31	58.5
11.1	-	-	-	-
11.2	16.7	1,390	1.31	56.6
12.1	16.4	1,660	1.29	27.4
12.2	-	-	-	-
13.1	17.4	1,790	1.29	25.0
13.2	-	-	-	-
14.1	18.4	1,550	1.29	37.7
14.2	-	-	-	-
15.1	24.4	1,730	1.29	36.9
15.2	28.2	1,890	1.29	45.7
16.1	16.3	1,810	1.31	37.1
16.2	18.6	1,580	1.31	64.7
17.1	-	-	-	-
17.2	69.9	-	-	-
18.1	55.9	1,850	0.927	39.1
18.2	55.9	1,810	0.927	32.1

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