A Preliminary Investigation of the Kinetics of Biological Sulphate Reduction Using Ethanol as a Carbon Source and Electron Donor

CLIVE L. ERASMUS

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Department of Chemical Engineering
University of Cape Town
Cape Town, South Africa.

Supervisors: A/PROF. S.T.L. HARRISON, PROF. G.S. HANSFORD

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Summary

High sulphate wastewaters originate from a variety of industrial activities. These include the manufacture of pulp and paper, mining and minerals processing, food processing, explosive, fertiliser, and petrochemical industries. These effluents cannot be discharged directly into natural watercourses without some pretreatment to achieve acceptable quality levels. Biological reduction of sulphate is a potential treatment technology. This technology has been implemented industrially in Europe for the treatment of acidic sulphate-containing wastewater (De Vegt and Buisman, 1995). The possibility exists for biological sulphate reduction to be used for the treatment of sulphate-containing wastewaters in South Africa. For approximately 10 years, ethanol has been used industrially by Paques Bioprocesses (The Netherlands) as carbon source and electron donor (De Vegt and Buisman, 1995). Despite this, kinetic data for microbial growth on ethanol is not widely available in open literature. Limited pure-culture studies have been conducted on ethanol-oxidising species, leading to the publication of postulated degradation mechanisms (Schink *et al.*, 1985), microbial growth rates (Szewzyk and Pfennig, 1990) and microbial yields on ethanol (Laanbroek *et al.*, 1984, 1982). There are several mixed culture studies by Colleran and co-workers (O'Flaherty and Colleran, 1999; O'Flaherty *et al.*, 1998a,b; Colleran *et al.*, 1994) involving ethanol as an organic intermediate. These studies are focused on characterising and optimising industrial process performance rather than rigorous kinetic analysis. Furthermore, half-velocity constants for sulphate ($K_{SO_4^{2-}}$) and kinetic parameters for ethanol-oxidising species in mixed cultures are not widely available in open literature.

The objectives of this investigation were to collect preliminary kinetic data for a mixed anaerobic culture of sulphate-reducing, methane-producing and acid-forming bacteria, reducing sulphate using ethanol as the principle organic source and electron donor. The kinetic data was used to determine kinetic parameters to inform process modelling and simulation. In particular, the effects of ethanol:sulphate ratio, volumetric sulphate loading rate and hydrogen sulphide toxicity were investigated.

In this study, sulphate reduction was found to occur by incomplete oxidation of ethanol

$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H^+ + 2H_2O + HS^-$$

as acetate was consistently detected as a reaction product in both batch and continuous experiments, formed nearly in stoichiometric ratio (0.9:1) to the ethanol consumption. Furthermore, sulphate reduction did not occur with concomitant depletion of acetate following depletion of ethanol under the conditions used.

At extended retention times in continuous experiments (approximately 10 days), stable oscillations were observed in effluent sulphate concentrations over time. Through batch and continuous experiments in which nitrogen sparging was used to remove the hydrogen sulphide formed, it was shown clearly that hydrogen sulphide inhibits sulphate reduction. Oscillations in continuous culture experiments ceased on introduction of nitrogen sparging to remove aqueous hydrogen sulphide, hence the oscillatory behaviour was attributed to hydrogen sulphide toxicity. Analysis of oscillatory behaviour allowed an understanding of critical hydrogen sulphide content to be developed. In the pH range 6.8-7.2, undissociated hydrogen sulphide presented as the inhibitory species, whereas in the pH range 7.2-7.6, inhibition was attributed to total sulphide content. Furthermore, the continuous experiment run at 78.1 $mmol.l^{-1}$ sulphate tolerated a higher undissociated hydrogen sulphide concentration (192 $mgH_2S.l^{-1}$) prior to inhibition than that run at a feed concentration of 10.4 $mmol.l^{-1}$ sulphate (16-80 $mgH_2S.l^{-1}$). Results indicated that microbial species in continuous experiments treating different feed sulphate concentrations adapted differently to hydrogen sulphide toxicity.

Kinetic parameters for ethanol-oxidising sulphate-reducing bacteria were determined from batch and continuous experiments (Table 1). Values agreed with literature data (O'Flaherty *et al.*, 1998b; Szewzyk and Pfennig, 1990). Substrate affinities for ethanol and sulphate are not widely available in open literature. Substrate affinities for ethanol for this investigation fell in the range 5.5-6.4 $mg.l^{-1}$ whereas the limited literature data indicates affinities for ethanol in mixed cultures of the order of 30 $mg.l^{-1}$. The substrate affinities for sulphate was determined as 284 $mg.l^{-1}$ from continuous culture experiments. This is substantially higher than the affinity of 30 $mg.l^{-1}$ reported by O'Flaherty *et al.* (1998b). The maximum specific growth rate of 0.273 d^{-1} determined in the investigation agrees well with the literature values of 0.2-0.8 d^{-1}. Further, it confirms that the growth rate of incomplete ethanol oxidisers is significantly lower than that of acetotrophic sulphate-reducing bacteria (SRB).

The effect of ethanol:sulphate ratio was studied in batch stirred tank reactors. Ratios of ethanol to sulphate ranged from sulphate in excess (0.93) to ethanol in excess (2.7). The maximum theoretical rate of ethanol consumption is twice the maximum rate of sulphate consumption where incomplete ethanol oxidation is the predominant reaction. In the presence of stoichiometric ratios of ethanol and sulphate (2:1) or where sulphate was in excess (ratios less than 2), this was observed. Where ethanol was in excess, the rate of ethanol consumption relative to sulphate consumption exceeded 2, indicating that ethanol was used by SRB for sulphate reduction and by acetogenic bacteria for acetate formation. SRB competed better for organic substrate where organic substrate was limiting, and less effectively when ethanol was in excess. This was consistent with the findings of Choi and Rim (1991).

Table 1: Summary of kinetic parameters determined from batch and continuous experiments

Parameter	Experiment Type	Value	Unit
$K_{SO_4^{2-}}$	Batch	6.81	$mg.l^{-1}$
		0.071	$mmol.l^{-1}$
	Continuous culture	284	$mg.l^{-1}$
K_{EtOH}	Batch	9.84	$mg.l^{-1}$
		0.124	$mmol.l^{-1}$
$r^{max}_{SO_4^{2-}}$	Batch	151.2	$mmol.l^{-1}.d^{-1}$
		6.3	$mmol.l^{-1}.h^{-1}$
K_{i,H_2S}	Continuous culture	192	$mg.l^{-1}$
		5.6	$mmol.l^{-1}$
Y_{xs} (ethanol)	Theory	0.02	$C-mol.C-mol^{-1}$
μ_{max}	Continuous culture	0.273	d^{-1}

Volumetric reduction rates of sulphate were found to be optimal at retention times between 4 and 6 days (volumetric sulphate loading rates between 625 and 417 $mg.l^{-1}.d^{-1}$ respectively). Fractional sulphate conversion fell sharply from 86 % to 43 % with increasing volumetric loading rate in this range. At high volumetric loading rates, the volumetric loading rate exceeds the maximum volumetric reduction rate, thereby resulting in fractional conversion which decreases with increasing volumetric loading rate (decreasing hydraulic retention time). Based on a maximum specific growth rate of 0.273 d^{-1}, a retention time of 3.7 days is predicted as the critical value below which microorganisms wash out and cannot consume all the available substrate, leading to process failure.

Simulations based on the dominant reaction confirmed major trends in extent of sulphate conversion and residual concentrations of continuous culture experiments. This confirmed the dominance of this reaction. Simulations regarding the effect of hydrogen sulphide toxicity provided results which support the hypothesis that the stable oscillations in continuous culture experiments are due to hydrogen sulphide toxicity. The model was verified with an independent data point and a good correlation was observed.

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Oh, yes, and Beer Club management! Cheers!

Clive

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c_s	substrate concentration, $mg\ substrate.l^{-1}$
$\dot{c}_s$	time rate of change of substrate concentration, $\frac{dc_s}{dt}$
$\overline{c_s}$	steady state substrate concentration, $mg\ substrate.l^{-1}$
c_{s_i}	concentration of growth-limiting substrate in medium supply, $g.l^{-1}$
c_x	biomass concentration, $mg.l^{-1}$
$\dot{c}_x$	time rate of change of biomass concentration, $\frac{dc_x}{dt}$
$\overline{c_x}$	steady state biomass concentration, $mg\ biomass.l^{-1}$
D	dilution rate, d^{-1}
$\frac{D_S^{01}}{r_x}$	energy dissipation, $\frac{kJ}{C-mol\ biomass\ produced}$
F	influent flowrate, $l.d^{-1}$
ΔG_o^f	standard Gibbs free energy of formation, kJ
ΔH_o^f	standard heat of reaction, $kJ.mol^{-1}$
K_m	saturation constant (Michaelis-Menten kinetics), $mg\ substrate.l^{-1}$
K_s	half velocity constant, $mg\ substrate.l^{-1}$
K_i	inhibition constant, $mg\ inhibitor.l^{-1}$
m_E	maintenance energy, $\frac{kJ}{C-mol\ biomass.h}$
q_s	bacterial specific substrate consumption rate, $\frac{g\ substrate}{g\ biomass.l.d}$
r_s	rate of substrate consumption, $-\frac{dc_s}{dt}$, $mg.l^{-1}.d^{-1}$
r_s^{max}	maximum rate of reaction, $mg.l^{-1}.d^{-1}$
r_x	rate of biomass formation $\frac{dc_x}{dt}$, $mg.l^{-1}.d^{-1}$
t	time, d

T	temperature, ^oC
Y_{xs}	biomass yield, $\frac{C-mol\,biomass}{C-mol\,substrate}$
γ	degree-of-reduction, $\frac{electrons}{C-mol\,substrate}$
μ	specific growth rate, d^{-1}
μ_{max}	maximum specific growth rate, d^{-1}
τ	hydraulic retention time, d

Glossary of Terms

AB	acidogenic bacteria
ABR	anaerobic baffled reactor
AMD	acid mine drainage
aMPB	aceticlastic methane-producing bacteria
aSRB	aceticlastic sulphate-reducing bacteria
BSP	biomass support particle
COD	chemical oxygen demand
CSTR	continuous stirred tank reactor
DFFB	downflow fluidised bed
DS	dissolved sulphide
eAB	ethanol-consuming acetogenic bacteria
EPS	extracellular polysaccharide
eSRB	ethanol-oxidising sulphate-reducing bacteria
EtOH	ethanol
FB	fermentative bacteria
FID	flame ionisation detector
GC	gas chromatograph
HAc	acetic acid
hMPB	hydrogenotrophic methane-producing bacteria
hSRB	hydogenotrophic sulphate-reducing bacteria
HPLC	high-pressure liquid chromatograph

HRT	hydraulic retention time
IC	ion chromatograph
IC50	inhibitor concentration resulting in a 50 % reduction in microbial growth
MPB	methane-producing bacteria
OHPA	obligate hydrogen-producing anaerobes
SRB	sulphate-reducing bacteria
TS	total sulphide
UASB	upflow anaerobic sludge bed
VFA	volatile fatty acids
VLR	volumetric loading rate
VRR	volumetric reduction rate

Chapter 1

Introduction

Biological treatment has been identified as a viable process for the treatment of acidic sulphate-containing industrial effluents in South Africa. Collaborative work is being conducted, under the support of the Water Research Commission, by a number of institutions including the Department of Chemical Engineering, University of Cape Town, the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, and the School of Chemical Engineering, University of Natal, Durban, to investigate such processes.

Research at the Department of Chemical Engineering, University of Cape Town, focuses on biological sulphate reduction with particular reference to process kinetics, process modelling and process simulation. Data for a pilot plant treating acidic sulphate-containing wastewater, using primary settled sewage as a carbon source, has been simulated by Ristow (1999). Knobel (1999) developed a model to describe general anaerobic digestion. Moosa (2000) has quantified the temperature, pH and sulphate effects on sulphate reducing bacteria using acetate as a carbon source in suspended cell culture. It is desired that this work be extended to include additional carbon sources. To this end the work presented in this thesis covers a preliminary investigation of the kinetics of biological sulphate reduction using ethanol as the carbon source and electron donor.

1.1 Background

High sulphate wastewaters originate from the manufacture of pulp and paper, mining and minerals processing, food processing, explosive, fertiliser, and petrochemical industries. The effluent is of poor quality and cannot be discharged directly into natural

Table 1.1: Representative examples of the chemical composition of various aqueous industrial effluents in South Africa (all units in $mg.l^{-1}$ except pH)

	Mining[1]	Gelatin manufacturing[2]	Yeast factory[2]	Minerals processing[1]	Petrochemical industry[3]
pH	5.95	9.77	5.74	7.0	-
Total dissolved solids	3778	5100	111100	-	-
Sulphate	1831	747	18000	2480	3000
Chloride	250	717	-	315	7600
Fluoride	1.7	-	-	-	-
Sodium	258	-	-	285	3900
Potassium	18.2	-	-	-	-
Iron	210	-	-	1.5	80
Nickel	-	-	-	6.7	10
Calcium	980	160	-	-	280
Manganese	7	-	-	-	5
Magnesium	720	-	-	-	21

[1] Personal Communication (SRK Consulting Engineers, 1995), [2] Lloyd *et al.* (1999), [3] Ecoliban Group (1999)

Table 1.2: Characteristics of acid mine drainage (Christensen *et al.*, 1996)

Characteristic	Value
pH	1-3
Dissolved Metals	
Aluminium	47-2050 $mg.l^{-1}$
Iron	13-6695 $mg.l^{-1}$
Zinc	10-95 $mg.l^{-1}$
Sulphate	3000-30000 $mg.l^{-1}$
Total Dissolved Solids	1790-45451 $mg.l^{-1}$

watercourses without some pretreatment to achieve acceptable quality levels. Table 1.1 summarises representative examples the composition of a variety of industrial effluents in South Africa.

Acid mine drainage (AMD) is one example of such a wastewater in South Africa. AMD is characterised by low pH, high sulphate concentrations and dissolved heavy metal cations such as zinc, copper, ferric and ferrous iron. Typical characteristics of AMD are listed in Table 1.2 (Christensen *et al.*, 1996). Lime neutralisation has been used extensively to neutralise AMD and precipitate heavy metals as metal hydroxides, but it is limited in sulphate removal capability.

Shortcomings of this method of treatment include the need to dispose of large volumes of mixed metal hydroxides which have poor dewatering properties, and the inability to reduce the sulphate concentration of the wastewater. With more stringent environmental

regulations being enforced in South Africa, alternative methods of AMD treatment were sought.

Alternative treatment systems for AMD can be broadly classified into microbial, chemical and membrane processes, each of these being categorised as either an active or passive process. These include lime neutralisation, ion exchange, liquid membrane extraction, reverse osmosis, solvent extraction, electrolytic deposition, iron hydroxide flocculation and microbial sulphate reduction (Johnson, 2000; Tichy, 2000). These methods all have the potential to remove soluble heavy metals efficiently, but only ion exchange, reverse osmosis and microbial sulphate reduction have significant effects on sulphate removal and are thus the most applicable acid mine drainage remediation techniques.

The primary factors limiting the use of ion exchange systems are the high capital- and running costs. Ion exchange is used to achieve a high degree of purity, and is thus most suitable to a polishing-type application. Similarly, because of the regular fouling of the membrane, reverse osmosis is not suitable as a primary treatment stage for heavily polluted waste such as acid mine drainage. It is more suited to polishing-type applications. Sulphate reduction remains a potentially effective treatment technique that can simultaneously reduce sulphate concentrations, increase pH and remove heavy metals from polluted mine water.

Passive treatment processes include aerobic wetlands, compost wetlands and anoxic limestone drains. Anoxic limestone drains operate on the same principle as lime neutralisation, but in this instance, the process is not controlled. Waste water is channelled over a porous bed of limestone, and in so doing, the pH is neutralised and metals are precipitated as insoluble metal hydroxides. One of the most significant processes which occurs in anaerobic wetlands is sulphate reduction and this has been exploited in active systems for AMD treatment.

Paques Bioprocesses (The Netherlands) has developed an active industrial anaerobic treatment process, the Thiopaq process, specifically for the treatment of low pH, sulphate-containing effluents (De Vegt and Buisman, 1995). This process consists of two biological processes complemented by solid separation stages.

In the anaerobic reactor, sulphate is reduced to hydrogen sulphide (H_2S) by sulphate reducing bacteria. Examples of electron donors needed for this process are hydrogen and ethanol. The hydrogen sulphide that is formed reacts with dissolved metals to form insoluble metal sulphide precipitates. In the subsequent aerobic reactor, hydrogen sulphide is oxidised by the action of aerobic microorganisms into elemental sulphur. This technology has been successfully implemented on both pilot- and industrial scale. A well known industrial application is the ground water treatment plant at Budelco Zinc

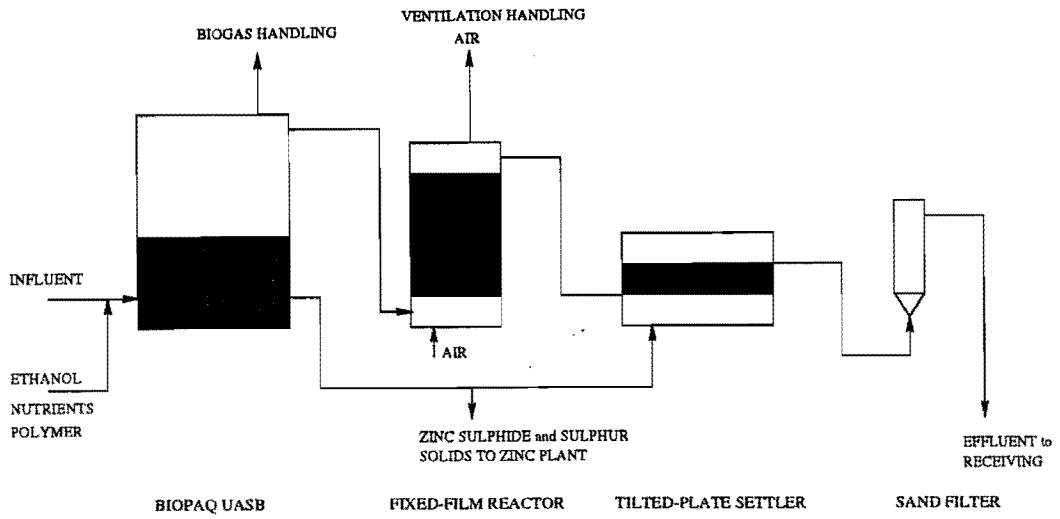

Figure 1.1: Thiopaq plant at Budelco Zinc Refinery, The Netherlands (De Vegt and Buisman, 1995)

Refinery (The Netherlands). This technology represents an attractive option for the treatment of AMD and other sulphate-containing industrial effluents in South Africa. Microbiological systems have the potential to be self-sustaining while treating polluted water continuously. Hence, their potential is being investigated globally for AMD treatment as well as treatment of industrial wastewater in general.

Ethanol has been used industrially by Paques Bioprocesses (The Netherlands) as carbon source and electron donor for their AMD treatment system. Despite this, there is a lack of rigorous kinetic data for microbial growth on ethanol in the literature. Limited pure-culture studies have been conducted on ethanol oxidising species, leading to the publication of postulated degradation mechanisms (Schink *et al.*, 1985), microbial growth rates (Szewzyk and Pfennig, 1990) and microbial yields on ethanol (Laanbroek *et al.*, 1984, 1982). There are several mixed culture studies involving ethanol as an organic intermediate by Colleran and co-workers (O'Flaherty and Colleran, 1999; O'Flaherty *et al.*, 1998a,b; Colleran *et al.*, 1995, 1994). These studies are focused on characterising and optimising industrial process performance rather than kinetic analysis. Half-velocity constants for sulphate ($K_{SO_4^{2-}}$) and kinetic parameters for ethanol-oxidising species in mixed cultures are not widely available in the literature (Moosa, 2000; O'Flaherty *et al.*, 1998b).

Electron donor sources can be divided into two main categories: organic waste materials and bulk chemicals. Possible candidates for use as bulk chemicals are ethanol, methanol, acetate, propionate, lactate and synthesis gas. The choice of organic substrate is largely dependent on the process. For large-scale processes, organic waste (for example domestic sewage) is suitable since it is widely available and in sufficient quantities needed to op-

erate the processes. Bulk chemicals may not be locally available in sufficient quantities.

For small-scale processes, the use of a bulk chemical with a well-defined composition may be preferred to organic waste, depending on substrate supply and cost. Bulk chemicals also have a well-defined composition and thus it is easier to predict the behaviour of the processes. The use of organic waste materials may be accompanied by further pollution of the wastewater stream as elevated COD content.

1.2 Project Objectives

In order for the biological sulphate reduction process to be successfully implemented industrially, it is necessary to understand the mechanism of the treatment process, to quantify the reaction rates, and predict the performance of the system. The objectives of the investigation were to obtain kinetic data for a mixed microbial culture of sulphate-reducing, methane-producing and acid forming bacteria using ethanol as the principal organic source and electron donor. Using this data, kinetic parameters were determined to inform process modelling and simulation. In particular, the effects of ethanol:sulphate ratio, volumetric sulphate loading rate and hydrogen sulphide toxicity were investigated.

1.3 Thesis Structure

Chapter 2 briefly reviews the background theory necessary for understanding microbial growth. Chapter 3, the Literature Review, provides more specific information on the biological treatment process. The review will outline the subprocesses of anaerobic digestion and highlight the usefulness of mixed culture studies. The focus of the review is on the mechanism and kinetics of ethanol-driven sulphate reduction, microbial interactions in the sulphate-reduction process and the effects of physiological parameters on sulphate-reducing bacteria. An experimental hypothesis is then formulated based on the reviewed literature.

Materials and methods are outlined in Chapter 4, followed by a presentation and discussion of experimental results in Chapter 5. Both batch and continuous experimental data are presented, including kinetic parameters for both batch and continuous reactor studies. Hydrogen sulphide toxicity is also addressed. Conclusions and Recommendations are made in Chapter 6.

Chapter 2

Background Theory

Microbial growth kinetic theory is well established. Texts such as Shuler and Kargi (1992), Roels (1983), Pirt (1975) and Monod (1949) cover theoretical aspects in detail. The theory presented here is included for completeness.

Microbial growth is characterised by a number of kinetic parameters. These include:

- specific microbial growth rate, μ (day^{-1})
- microbial yield, Y_{xs} $(mg\,biomass.mg\,substrate^{-1})$
- saturation constant, or half-velocity constant, K_s $(mg\,substrate.l^{-1})$.
- inhibition constant, K_i $(mg\,inhibitor.l^{-1})$

A physical explanation of these parameters is useful in interpreting kinetic data.

If all the growth requirements are satisfied, then, during a small time interval, dt, an increase in biomass, dc_x, will be proportional to the amount of biomass c_x, present:

$$\frac{dc_x}{dt} = \mu c_x \tag{2.1}$$

The differential $\left(\frac{dc_x}{dt}\right)$ expresses the population growth rate and μ represents the rate of growth per unit biomass. The analytical solution to Equation 2.1 is:

$$c_x = c_{x_o} e^{\mu t} \tag{2.2}$$

where c_{x_o} is an initial condition for biomass concentration. The specific growth rate, μ, is an indication of the time in which a microbial population doubles in size (t_d). Putting $c_x = 2c_{x_o}$ and $t = t_d$, Equation 2.2 becomes:

$$t_d = \frac{ln(2)}{\mu} \tag{2.3}$$

Microbial growth rates have a maximum value (Pirt, 1975), the maximum specific growth rate, μ_{max}, which is characteristic of a particular species under defined conditions.

The amount of biomass produced on a basis of substrate consumed is termed the biomass yield (Y_{xs}):

$$Y_{xs} = \frac{\text{quantity of biomass produced \{dry weight\}}}{\text{quantity of substrate used \{moles or mass\}}} \tag{2.4}$$

or

$$Y_{xs} = \frac{\text{rate of biomass production}}{\text{rate of substrate consumption}} = -\frac{r_x}{r_s} \tag{2.5}$$

Biomass yield is thus dependent on the growth system and is the growth factor that determines the biomass concentration, interspecies competition, composition of a mixed culture and consumption of growth nutrients. The prediction of yield is important in describing microbiological systems.

The half-velocity constant, K_s, is the substrate concentration at which the microorganism grows at half its maximum specific growth rate and quantifies substrate affinity. A species with a low K_s has a higher substrate affinity than a species with a high K_s for that same substrate. Hence the species with the lower half-velocity constant is more likely to dominate should competition occur under substrate limitation since it is able to grow at maximum specific growth rate at a lower substrate concentration.

The inhibition constant, K_i, is the concentration of inhibitor at which microbial growth is inhibited. Physically this is measured by reduction in substrate utilisation rate or product formation rate. There are no standard procedures for measurement of microbial inhibition and thus inhibition constants are determined under different physical conditions with different definitions of the degree of inhibition. As a result, it is very difficult to consolidate published information.

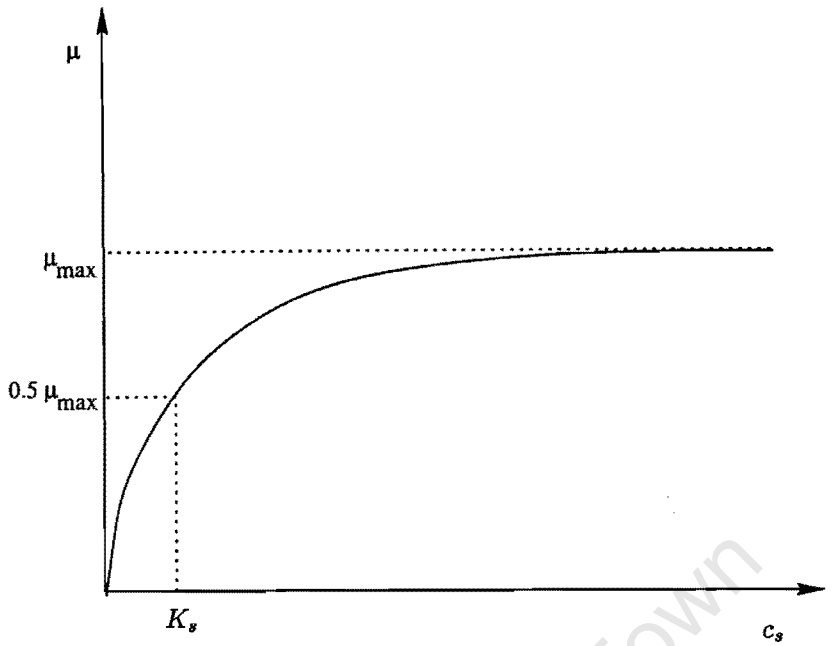

Figure 2.1: Relation between the specific growth rate and growth limiting substrate following Monod kinetics

2.1 Monod Kinetics

Classical Monod kinetics are derived empirically and relate the microbial growth rate to bulk substrate concentration (Monod, 1949). Mathematically, Monod kinetics are expressed as:

$$\mu = \frac{\mu_{max}}{1 + \frac{K_s}{c_s}} \tag{2.6}$$

where c_s is the substrate concentration ($mg.l^{-1}$). A saturation relationship between μ and c_s is predicted by Monod kinetics (Figure 2.1).

2.2 Michaelis-Menten Kinetics

Michaelis-Menten kinetics are the Monod analogue for enzyme catalysed reactions. Michaelis-Menten kinetics describe the rate of substrate conversion in an enzyme catalysed reaction and can be represented by:

$$r_s = \frac{r_s^{max}}{1 + \frac{K_m}{c_s}} \tag{2.7}$$

where r_s and r_s^{max} are the rate of substrate consumption and maximum rate of substrate consumption respectively, c_s is the substrate concentration and K_m is the saturation constant or substrate affinity.

2.3 Inhibition Kinetics

Michaelis-Menten kinetics can be modified to include the effects of substrate and product inhibition. Three types of inhibition occur: competitive, uncompetitive and noncompetitive. These types of inhibition affect the observed growth parameters K_s and r_s^{max} differently.

Certain compounds may bind to biological enzymes and reduce their activity. These compounds are known as inhibitors. Microbial inhibition can also be the result of environmental conditions such as pH and temperature, as well as substrate and product inhibition. Inhibition can be reversible or irreversible, depending on the type of inhibitor. Reversible inhibition can be classified into three categories: competitive, noncompetitive and uncompetitive. The mathematical inclusion of inhibition into microbial kinetics is accomplished by incorporating an inhibition term containing the inhibition constant K_i and concentration of inhibitory species, c_i, into the denominator of a Michaelis-Menten-type expression (Equation 2.7). The form of the inhibition term depends on the type of inhibition.

2.3.1 Competitive Inhibition

Competitive inhibitors are usually substrate analogues and compete with substrate for active enzyme sites. The net effect is a larger effective K_s which reduces the specific rate of substrate utilisation, r_s, at low substrate concentrations. Mathematically this is represented by:

$$r_s = \frac{r_s^{max}}{1 + \frac{K_s}{c_s}\left(1 + \frac{c_i}{K_i}\right)} \tag{2.8}$$

Competitive inhibition can be overcome by the use of high substrate concentrations (Shuler and Kargi, 1992).

2.3.2 Noncompetitive Inhibition

Noncompetitive inhibitors bind to sites other than the active sites. Mathematically their effect is represented by:

$$r_s = \frac{r_s^{max}}{\left(1 + \frac{K_s}{c_s}\right)\left(1 + \frac{c_i}{K_i}\right)} \tag{2.9}$$

The net effect of noncompetitive inhibition is that the maximum specific rate of substrate utilisation, r_s^{max}, is reduced. High substrate concentrations cannot overcome this phenomenon. It is necessary to add reagents that block the binding of the inhibitor to the enzyme, or manipulate the physicochemical conditions to prevent the reduction in enzyme-substrate affinity.

2.3.3 Uncompetitive Inhibition

Uncompetitive inhibitors bind to the enzyme-substrate complex and have no affinity for the enzyme itself. Mathematically:

$$r_s = \frac{r_s^{max}}{1 + \frac{K_s}{c_s} + \frac{c_i}{K_i}} \tag{2.10}$$

By rearrangement:

$$r_s = \frac{\frac{r_s^{max}}{\left(1 + \frac{c_i}{K_i}\right)}}{1 + \frac{K_s}{c_s\left(1 + \frac{c_i}{K_i}\right)}} \tag{2.11}$$

The net effect is a reduction in both r_s^{max} and K_s-values. The reduction in r_s^{max} is more pronounced than the reduction in K_s.

High substrate concentrations may cause inhibition in some enzyme reactions. This is known as substrate inhibition and can be described by Equation 2.10. At low substrate concentrations (c_i-concentrations), $\frac{c_i}{K_i} \ll 1$ and inhibition is not observed.

2.4 Continuous Culture

Nutrient-limiting conditions are of particular interest in environmental biotechnology. In most waste water treatment systems, nutrient concentrations are low. At these concentrations, cells grow at sub-maximal growth rates. It is useful to have access to laboratory

methods to cultivate and study microorganisms under such nutrient-limiting conditions. Under continuous culture microorganisms can be studied under growth-limitation while precise and independent control of environmental conditions such as pH, temperature and concentration of nutrients and metabolic products are maintained.

The continuous culture is an open system in which fresh medium is added continuously at a steady flowrate, F, and from which culture fluid emerges at the same rate. At a constant volume, V, the dilution rate, D (h^{-1}), is defined as:

$$D = \frac{F}{V} \tag{2.12}$$

The dilution rate is the reciprocal value of the hydraulic retention time (HRT).

Under these conditions, the microorganisms grow at a specific growth rate μ that is lower than the maximum, μ_{max} and defined by the limiting substrate concentration:

$$\mu = \mu^{max} \frac{c_s}{K_s + c_s} \tag{2.13}$$

In a continuous reactor, the culture grows at a given rate $(\mu c_x V)$ and at the same time a quantity of bacteria leaves the reactor in the outlet stream (Fc_x). Using a mass balance, it can be shown, by assuming a sterile feed:

$$V\frac{dc_x}{dt} = \mu c_x V - Fc_x \tag{2.14}$$

Hence:

$$\frac{dc_x}{dt} = \mu c_x - Dc_x = (\mu - D)c_x \tag{2.15}$$

If $\mu > D$, c_x will increase, while if $\mu < D$, c_x will decrease. At steady state $\left(\frac{dc_x}{dt} = 0\right)$, $\mu = D$. Hence the growth rate of the microorganisms can be controlled by choosing the dilution rate D.

The above equations accurately describe well established steady state conditions and it can easily be shown that, starting from non-steady state conditions, a steady state must be reached provided that D does not exceed the critical value D_C:

$$D_C = \mu_{max} \frac{c_{s_i}}{K_s + c_{s_i}} \tag{2.16}$$

in which c_{s_i} is the concentration of the growth-limiting substrate in the medium supply. It is clear that the steady state condition $\mu = D$ is a stable situation and steady state will be established automatically.

The net rate of change of substrate in a vessel is obtained by the general mass balance equation:

$$V\frac{dc_s}{dt} = c_{s_i}F - c_sF - Vr_s \tag{2.17}$$

in which r_s is given by $r_s = \mu\frac{c_x}{Y_{xs}}$ where maintenance energy is neglected.

It follows that:

$$\frac{dc_s}{dt} = \dot{c_s} = D(c_{s_i} - c_s) - \mu\frac{c_x}{Y_{xs}} \tag{2.18}$$

At steady state, $\dot{c_s} = 0 = \dot{c_x}$ and the steady state concentrations $\overline{c_x}$ and $\overline{c_s}$ are obtained. It follows from Equation 2.15, that:

$$\mu = D \tag{2.19}$$

Hence,

$$D = \mu_{max}\frac{\overline{c_s}}{K_s + c_s} \tag{2.20}$$

or

$$\overline{c_s} = K_s\frac{D}{\mu_{max} - D} \tag{2.21}$$

It follows that at steady state Equation 2.18 reduces to:

$$D\left(c_{s_i} - \overline{c_s}\right) = \mu\frac{\overline{c_x}}{Y_{xs}} \tag{2.22}$$

Rearranging and noting that $\frac{\mu}{D} = 1$ at steady state (Equation 2.19), gives:

$$\overline{c_x} = Y_{xs}\left(C_{Si} - \overline{c_s}\right) \tag{2.23}$$

Combining this with Equation 2.21 gives:

$$\overline{c_x} = Y_{xs}\left(c_{s_i} - K_s\frac{D}{\mu_{max} - D}\right) \tag{2.24}$$

in which K_s, μ_{max} and Y_{xs} are constants for a microorganism under specified conditions of temperature, medium composition and the nature of the growth limiting substrate. Equation 2.24 shows that $\overline{c_x}$ is dependent on D and c_{s_i} and is proportional to c_{s_i} if $\overline{c_s} \ll c_{s_i}$ which is usually the case under nutrient limiting conditions. If K_s, μ_{max} and Y_{xs} are known for a given microorganism, the relationship between $\overline{c_x}$ or $\overline{c_s}$ and D can be predicted at a chosen c_{s_i}.

2.5 Multiple Substrate Growth

All microorganisms require a carbon source and electron donor, and it is often useful to express the substrate consumption rate, r_s, as a function of the concentration of both of these species. One way in which multiple substrate growth can be modelled is by adding denominator terms of the form $\frac{1}{1+\frac{K_{s_i}}{c_{s_i}}}$ where K_{s_i} is the half velocity constant and c_{s_i} is the substrate concentration for a particular substrate.

In general, the Michaelis-Menten-type equation written in terms of rate of substrate consumption where multiple limiting substrates occur is:

$$r_s = \frac{r_s^{max}}{\left(1+\frac{K_{s_1}}{c_{s_1}}\right)\left(1+\frac{K_{s_2}}{c_{s_2}}\right)\left(1+\frac{K_{s_3}}{c_{s_3}}\right)...\left(1+\frac{K_{s_i}}{c_{s_i}}\right)} \tag{2.25}$$

Such an expression will be useful in describing biological sulphate reduction where the sulphate-reducing bacteria have a dual substrate dependency on the organic source and sulphate.

2.6 Stoichiometry of Microbial Growth

For modelling purposes, a balanced chemical equation for microbial reduction of sulphate to sulphide using ethanol as the carbon source and electron donor and ammonia as the nitrogen source is necessary.

2.6.1 Microbial Composition

A generally accepted empirical formula for the representation of biomass can be considered as (Roels, 1983):

$$CH_{1.8}O_{0.5}N_{0.2} \tag{2.26}$$

This refers to the organic part of the biomass containing 12 g carbon and having a molecular mass of 24.6 $g.C - mole^{-1}$.

2.6.2 Mass balance

A minimal list of chemical compounds is necessary to describe microbial growth. These include biomass, water, nitrogen source, carbon source, protons, product, electron donor

Table 2.1: Chemical species involved in anaerobic sulphate reduction

Species	Chemical Formula	Rate
Ethanol	C_2H_5OH	r_s
Ammonia	NH_3	r_{NH_3}
Protons	H^+	r_{H+}
Sulphate	SO_4^{2-}	$r_{SO_4^{2-}}$
Water	H_2O	r_{H_2O}
Biomass	$CH_{1.8}O_{0.5}N_{0.2}$	r_x
Carbon Dioxide	CO_2	r_{CO_2}
Acetic Acid	CH_3COOH	r_{HAc}
Hydrogen Sulphide	HS^-	r_{HS-}

and electron acceptor. The energy for microbial growth and maintenance comes from the transfer of electrons from the electron donor to the electron acceptor. The difference between systems arises from the different electron donors, electron acceptors, carbon and nitrogen sources.

Consider the anaerobic growth of sulphate reducing bacteria on ethanol, with ammonia as the nitrogen source, sulphate as the electron acceptor and acetic acid as a reaction product. The compounds taking part are listed in Table 2.1.

The calculations can be carried out using ammonia and hydrogen sulphide in their dissociated or undissociated forms. There are 8 rates which need to satisfy 5 elemental balances and a charge balance, i.e., six equations in eight unknowns with one degree of freedom.

The steady state equation for biomass growth per C-mol ethanol can be written as:

$$0.5fC_2H_5OH + aNH_3 + bH^+ + cH_2O + dSO_4^{2-} + eCO_2 + gHS^- + hCH_3COOH$$

$$+CH_{1.8}O_{0.5}N_{0.2} = 0 \tag{2.27}$$

The rate of production of biomass can be related to the consumption of ethanol:

$$r_s = f \cdot r_x \tag{2.28}$$

or

$$f = \frac{r_s}{r_x} \tag{2.29}$$

Using Equation 2.27, the five elemental balances and charge balance are:

Carbon (C):

$$e + f + 2h + 1 = 0 \tag{2.30}$$

Hydrogen (H):

$$3a + b + 2c + 3f + g + 4h + 1.8 = 0 \tag{2.31}$$

Oxygen (O):

$$c + 4d + 2e + 0.5f + 2h + 0.5 = 0 \tag{2.32}$$

Nitrogen (N):

$$a + 0.2 = 0 \tag{2.33}$$

Sulphur (S):

$$d + g = 0 \tag{2.34}$$

Charge (z):

$$b - 2d - g = 0 \tag{2.35}$$

The stoichiometric coefficient f remains the one free stoichiometric coefficient and can be determined from the biomass yield:

$$f = \frac{r_s}{r_x} = -\frac{1}{Y_{xs}^{max}} \tag{2.36}$$

Y_{xs}^{max} is the maximum observed yield and represents biomass growth on a specific substrate. If experimental data is available, the biomass yield can be determined and the value of f can be calculated or, alternatively, yield can be theoretically determined (Section 2.7) to predict f. A further parameter, the degree-of-reduction, γ, can be introduced which replaces the large hydrogen balance and allows arithmetic simplification.

2.6.3 Degree of Reduction

The degree-of-reduction of a carbon-, hydrogen-, oxygen- or nitrogen-containing compound is the number of electrons that are liberated in a redox half-reaction where one

C-mole of an organic compound or one mole of an inorganic compound is converted to H^+, CO_2, H_2O, nitrogen source, SO_4^{2-} or Fe^{3+}. The degree-of-reduction represents the electron content of a compound relative to these species. By definition, $\gamma = 0$ for H^+, CO_2, H_2O, N-source, $SO_4^{2-}$and Fe^{3+}. In calculating γ the composition formula of the undissociated compound is used. The degree of reduction of ethanol is calculated as follows:

$$-C_2H_5OH + 2CO_2 - 3H_2O + 12H^+ + 12e^- = 0 \tag{2.37}$$

Thus

$$\gamma_{EtOH} = \frac{12}{2}\frac{electrons}{carbons\,per\,mole\,EtOH} = +6\ \frac{electrons}{C-mole\,ethanol} \tag{2.38}$$

The procedure involves writing the formula of the compound and then balancing the elements by eliminating the C-, O- and H-atoms etc., to determine the number of electrons from a charge balance. The degree-of-reduction of biomass depends on the N-source. For ammonia as the N-source:

$$-CH_{1.8}O_{0.5}N_{0.2} + CO_2 - 1.5H_2O + 0.2NH_3 + 4.2H^+ + 4.2e^- = 0 \tag{2.39}$$

giving:

$$\gamma_{biomass} = +4.2\,\frac{electrons}{C-mol\,biomass} \tag{2.40}$$

When ammonia is the nitrogen source, its degree-of-reduction is zero. However, if it plays a part as the electron donor or electron acceptor, then the degree-of-reduction is $\gamma = +3$.

The degree-of-reduction balance can be included as the 7^{th} equation:

Degree-of-reduction (γ):

$$6f + 8g + 4h + 4.2 = 0 \tag{2.41}$$

There are now seven equations (5 elemental balances, a charge balance and degree-of-reduction balance) and eight unknowns. Assuming the yield coefficient can be determined and f can be calculated, the system of equations can be solved. Knowing that carbon dioxide does not participate in the reaction, the stoichiometric coefficient can be set to zero. The system can be solved using the degree-of-reduction balance in place of the hydrogen balance if arithmetic simplification is required.

2.7 Thermodynamics of Microbial Growth

2.7.1 Biomass Yield and Maintenance

The prediction of biomass yield is important and is affected by microbial maintenance requirements. Microorganisms use a portion of the energy obtained from their carbon source and electron donor for cell maintenance functions, i.e., some energy obtained is not used for cell propagation. The yield coefficient cannot be determined from the total energy available from the carbon source and electron donor. Biomass yield with maintenance can be predicted by (Roels, 1983):

$$-\frac{1}{Y_{DX}}(e-donor)-a(N-source)-\frac{1}{Y_{AX}}(e-acceptor)+1(C-mole\,biomass)$$

$$+cH_2O+eHCO_3^-+bH^++\frac{Q}{r_x}+\frac{D_S^{01}}{r_x}=0 \tag{2.42}$$

where $\frac{D_S^{01}}{r_x}$ is the Gibbs energy dissipated in the process. For the production of biomass, the carbonate ion, nitrogen source, water and protons must always be taken into account. The stoichiometric coefficients follow from the elemental and charge balances.

$\frac{Q}{r_x}$, the nett heat of reaction, can be either positive or negative. $\frac{D_S^{01}}{r_x}$ is always positive because of the second law of thermodynamics. $\frac{D_S^{01}}{r_x}$ represents the amount of work (kJ) spent in the production of one C-mole of biomass.

2.7.2 Factors Influencing the Gibbs Free Energy Dissipated, $\frac{D_S^{01}}{r_x}$

$\frac{D_S^{01}}{r_x}$ consists of two parts:

1. $\left(\frac{D_S^{01}}{r_x}\right)_{gr}$ which is the growth related energy dissipation $\left(\frac{kJ}{\text{C-mol biomass produced}}\right)$
2. m_E which is the maintenance related dissipation $\left(\frac{kJ}{\text{C-mol biomass present.h}}\right)$.

Combining these two parts gives:

$$\frac{D_S^{01}}{r_x}=\left(\frac{D_S^{01}}{r_x}\right)_{gr}+m_E\frac{c_x}{r_x} \tag{2.43}$$

Since $\mu = \frac{r_x}{c_x}$:

$$\frac{D_S^{01}}{r_x} = \left(\frac{D_S^{01}}{r_x}\right)_{gr} + \frac{m_E}{\mu} \tag{2.44}$$

2.7.2.1 Growth Related Dissipation

Growth related dissipation depends on the carbon source. There is a small increase in growth related energy dissipation for carbon sources with fewer numbers of carbon atoms and further increases as the the degree-of-reduction of the C-source deviates from $\gamma = -4$. Biomass uses four and five carbon building blocks, hence for smaller carbon sources more anabolic steps are required for the synthesis of these building blocks. Each step leads to more dissipation of Gibbs free energy. For heterotrophic growth where the carbon source is also the electron donor, $\frac{D_S^{01}}{r_x}$ does not differ significantly for different electron acceptors. $\left(\frac{D_S^{01}}{r_x}\right)_{gr}$ can be calculated from the following empirical correlation (Roels, 1983):

$$\left(\frac{D_S^{01}}{r_x}\right)_{gr} = 200 + 18(6-c)^{1.8} + e^{\left((3.8-\gamma)^2\right)^{0.16}(3.6+0.4c)} \tag{2.45}$$

where c is the number of carbon atoms and γ is the degree-of-reduction of the carbon source. For the example of microbial growth on ethanol, with $\gamma = 6$ and $c = 2$:

$$\left(\frac{D_S^{01}}{r_x}\right)_{gr} = 706.2\ \frac{kJ}{C-mole\ biomass} \tag{2.46}$$

2.7.2.2 Maintenance Related Dissipation

Maintenance energy is required to maintain transmembrane potentials and to fuel the turn-over of intracellular macromolecules. Equation 2.47 is an empirically derived correlation for the determination of microbial maintenance energy (Roels, 1983).

$$m_E = 4.5e^{\left(\frac{-7000}{R}\left(\frac{1}{T}-\frac{1}{298}\right)\right)} \tag{2.47}$$

The maintenance energy increases with temperature, being more significant at thermophilic temperatures. It contributes to the overall energy dissipation more significantly at lower growth rates since μ is in the denominator of the term (Equation 2.44). It is clear that the need for maintenance energy is independent of the carbon source.

At 35°C (the growth temperature for sulphate reducing bacteria):

$$m_E = 4.93 \frac{kJ}{C-mol\,biomass.h} \tag{2.48}$$

The maintenance related dissipation can be calculated from an experimental growth rate of 0.011 h^{-1} (Section 5.4):

$$\left(\frac{D_S^{01}}{r_x}\right)_{m_E} = \frac{m_E}{\mu} = \frac{4.93}{0.011} = 448.2 \frac{kJ}{C-mol\,biomass} \tag{2.49}$$

2.7.2.3 Calculation of Y_{xs} from an Estimated Value of $\frac{D_S^{01}}{r_x}$

The total energy dissipation for the reaction is the sum of the growth-related and maintenance-related dissipation:

$$\frac{D_S^{01}}{r_x} = 706.2 + 448.2 = 1154.4 \frac{kJ}{C-mol\,biomass} \tag{2.50}$$

If the yield coefficient is unknown, it is possible to predict it theoretically from a mass and energy balance. It is possible to add a Gibbs Free Energy balance to the five elemental balances, charge balance and degree-of-reduction balance derived in Section 2.6.

The Gibbs free energy change for the formation of biomass is generally taken to be $\Delta G_o^f = -67\,kJ.mol^{-1}$ (Roels, 1983). Substituting the Gibbs free energies of the components into Equation 2.42 gives:

$$\frac{f(-182)}{2} + a(-80) + b(-40) + c(-238) + d(-743) + e(-394) + g(13) + h(-372)$$

$$+1(-67) + 1154.4 = 0 \tag{2.51}$$

Using the elemental, charge, and Gibbs free energy balances, the set of equations can be solved in matrix form $A\underline{x} = \underline{b}$:

$$A = \begin{pmatrix} 0 & 0 & 0 & 0 & 1 & 1 & 0 & 2 \\ 3 & 1 & 2 & 0 & 0 & 3 & 1 & 4 \\ 0 & 0 & 1 & 4 & 2 & 0.5 & 0 & 2 \\ 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 1 & 0 \\ 0 & 1 & 0 & -2 & 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ -80 & -40 & -238 & -743 & -394 & \frac{-182}{2} & 13 & -372 \end{pmatrix} \quad b = \begin{pmatrix} -1 \\ -1.8 \\ -0.5 \\ -0.2 \\ 0 \\ 0 \\ 0 \\ -1087.4 \end{pmatrix} \tag{2.52}$$

Solving for $\underline{x}$ gives:

$$\underline{x} = \begin{pmatrix} -0.2 \\ -19.5 \\ 39.5 \\ -19.5 \\ 0 \\ -78.3 \\ 19.5 \\ 38.6 \end{pmatrix} \tag{2.53}$$

which corresponds to the stoichiometric coefficients $a-h$ of Equation 2.27. The balanced equation becomes:

$$-39.2C_2H_5OH - 0.2NH_3 - 19.5H^+ + 39.5H_2O - 19.5SO_4^{2-} + 19.5HS^-$$
$$+38.6CH_3COOH + CH_{1.8}O_{0.5}N_{0.2} = 0 \tag{2.54}$$

Equation 2.36 can be rearranged:

$$Y_{xs} = -\frac{1}{f} \tag{2.55}$$

and the observed biomass yield per mole of electron donor can be calculated from f:

$$Y_{xs} = -\frac{1}{f} = 0.013 \frac{C - mol\, biomass}{C - mol\, ethanol}$$

This is equivalent to 0.014 $\frac{mg\, biomass}{mg\, ethanol}$. Literature data suggest that yields for anaerobic sulphate-reducing bacteria lie in the range 0.074-0.113 $\frac{mg\, biomass}{mg\, ethanol}$ (Szewzyk and Pfennig, 1990) and 0.044-0.134 $\frac{mg\, biomass}{mg\, ethanol}$ (Laanbroek *et al.*, 1984).

Chapter 3

Literature Review

This review will outline the subprocesses of anaerobic digestion and highlight the usefulness of mixed culture studies. The focus of the review will be on the mechanism and kinetics of sulphate reduction using ethanol as the carbon source and electron donor, microbial interactions in the sulphate-reduction process and the effects of physiological parameters on sulphate-reducing bacteria.

3.1 Background

3.1.1 Anaerobic Digestion

In aerobic systems, heterotrophic bacteria carry out the oxidation of organic material to carbon dioxide. The anaerobic degradation of organic material is a complex process requiring the interaction of different groups of microorganisms. Each microbial group contributes to the food chain where the metabolic end-products of one group form the substrate for another group until complete oxidation has occurred (Fauque, 1995).

The process of anaerobic digestion of organic material is well established and has been described in several texts and reviews (Speece, 1996; Colleran *et al.*, 1995; Fauque, 1995; Oude Elferink *et al.*, 1994). The mechanism (Figure 3.1) begins with the hydrolysis of organic material and biopolymers such as proteins, carbohydrates and lipids, to monomers and oligomers in the form of sugars, amino acids, polyols and long chain fatty acids, and fermentation intermediates such as propionate, butyrate, lactate and ethanol. This is accomplished by fermentative bacteria (FB). Long chain fatty acids that are produced are further oxidised to acetate, hydrogen and fermentation products by the combined

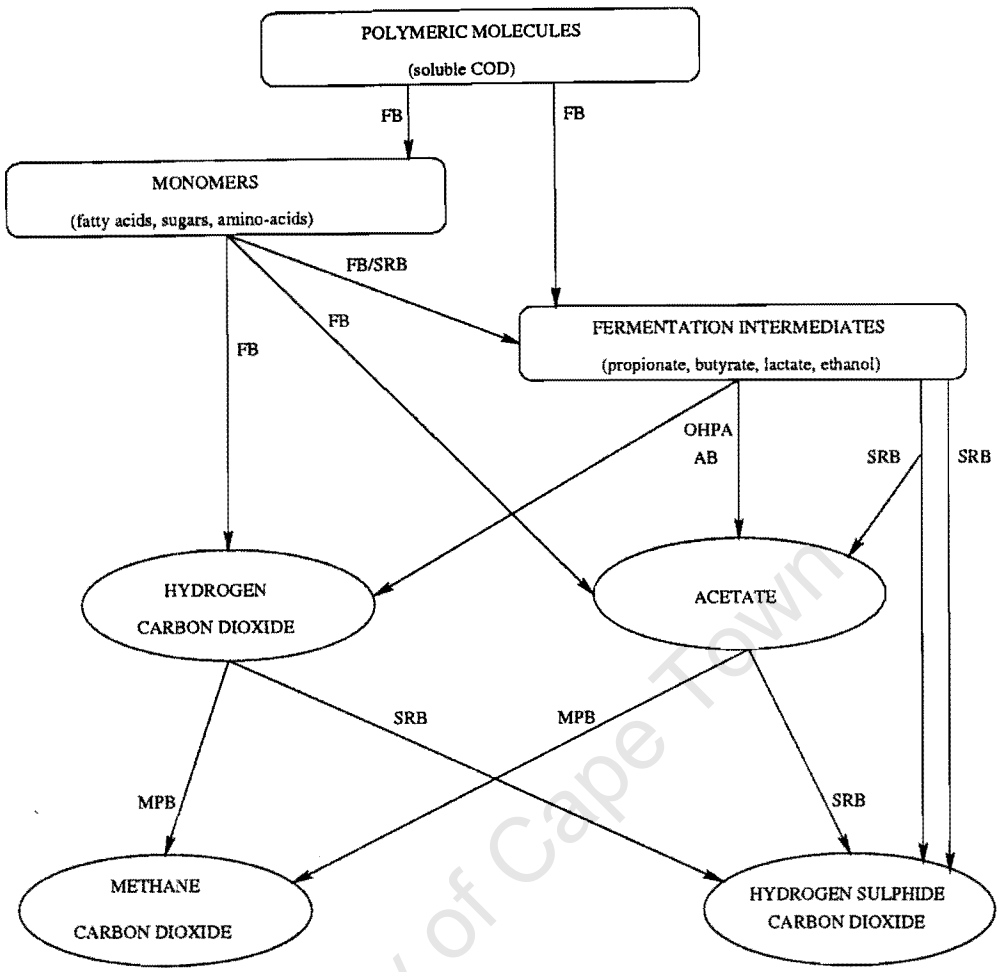

Figure 3.1: Schematic representation of the anaerobic digestion chain (FB - fermentative bacteria; OHPA - obligate hydrogen-producing anaerobes; AB - acetogenic bacteria; MPB - methane-producing bacteria; SRB - sulphate reducing bacteria) (Colleran *et al.*, 1995)

action of FB and sulphate reducing bacteria (SRB). Fermentation intermediates are subsequently oxidised to acetate, hydrogen and carbon dioxide by SRB, acetogenic bacteria (AB) and obligate hydrogen-producing anaerobes (OHPA).

In the absence of sulphate, consortia of methanogenic bacteria mineralise hydrogen and acetate to methane and carbon dioxide. However, in the presence of sulphate, acetate, hydrogen and some fermentation intermediates (alcohols and short-chain fatty acids) can be used by sulphate-reducing bacteria as electron donors in the dissimilatory reduction of sulphate to hydrogen sulphide. The presence of sulphate in an anaerobic system stimulates the competition between methane-producing bacteria and sulphate-reducing bacteria for available organic source. Under favourable environmental conditions, SRB can outcompete MPB for available substrate (Widdel, 1988).

3.1.2 Mixed Culture Processes

Biologically, anaerobic sludge processes can be seen as continuous flow enrichment cultures of microorganisms with the predominant species being determined by the characteristics of input wastes and the environmental conditions created through process design and operation. Although the system is simple in concept, it is a complex biological system with poorly understood interactions.

Process disturbances and variations in process inputs create fluctuations in dominant microbial species. Different species have different kinetic behaviour and react differently to changing process conditions. Mixed cultures maintained by selective conditions have several advantages over pure cultures for environmental biotechnology: they are intrinsically less liable to contamination; they adapt to minor changes because they comprise a number of populations with varying optima for culture variables (pH, substrate concentration, temperature and redox potential) and they are potentially able to make better use of organic substrates through consortia growth than would be possible with a single strain. Bacterial enrichment and mixed population dynamics in biological waste treatment systems have remained largely black-box systems with limited understanding of the microbial interactions. Only recently have specific studies been conducted to determine the effects of microbial species and physical parameters on process performance (De Smul, 1998; Visser, 1996; Van Houten, 1996; Barnes *et al.*, 1992a,b).

The utilisation of mixed microbial cultures in preference to pure cultures is most often employed in the industrial treatment of wastewater because of the ability of these systems to be self-sustaining and have low maintenance requirements. Biological processes can be especially difficult technically, given the narrow range of environmental conditions that will maintain the viability of the process culture. Waste treatment must often be accomplished under non-sterile conditions to be cost effective. This allows for competition for nutrients between process microorganisms and contaminants which become established in the process culture. Mixed culture studies on a laboratory scale eliminate the need to work under aseptic conditions and enable better scale-up knowledge for full-scale plants. Models that are derived can incorporate competition effects which, with the use of pure cultures, would be impossible.

3.2 Sulphate Reduction

3.2.1 Microorganisms Involved in Sulphate Reduction

The formation of short-chain fatty acids and fermentation products by anaerobic digestion has been discussed previously, and there are published reviews by Colleran *et al.* (1995), Barton (1995), Oude Elferink *et al.* (1994) and Zehnder (1988).

In the presence of a suitable carbon source and electron donor, and under controlled conditions, mixed microbial consortia reduce sulphate to sulphide by competing for available substrate. The anaerobic degradation of short-chain organic matter to methane and carbon dioxide and the reduction of sulphate to sulphide are accomplished by the combined action of several different microbial species. The three main groups are acetogenic (acetate-forming) bacteria (AB), methane-producing bacteria (MPB) and sulphate-reducing bacteria (SRB). Within these groups, several subgroups can be identified and categorised based on growth substrate. These groups are acetate-utilising SRB (aSRB), acetate-utilising MPB (aMPB), hydrogen-consuming SRB (hSRB), hydrogen-consuming MPB (hMPB), ethanol-utilising SRB (eSRB) and ethanol-utilising acetogenic bacteria (eAB). Reactions are generally symbiotic since the species involved derive positive benefit from the turnover of organic intermediates. For example, fermentation and acidogenesis provide the necessary short-chain carbon sources for sulphidogenesis and methanogenesis.

For many years, the major focus on anaerobic digestion was the removal of high COD levels in effluents and the production of methane gas, which is a valuable energy source. In recent research, however, ecological significance has been attached to the action of sulphate reducing bacteria which can occur in coexistence with methane producing bacteria (Lens and Hulshoff Pol, 2000; Barton, 1995; Zehnder, 1988). Methane producing bacteria are interfering species in the sulphate reduction process as they compete with sulphate reducers for available electron donors and carbon sources. It is therefore necessary to use the information that has been gathered relating to the optimisation of methane production in order to suppress the MPB and allow SRB to dominate in sulphate-containing systems.

The predominant sulphate reducers that are studied as part of mixed cultures are *Desulfovibrio* species (Christensen *et al.*, 1996; Kremer *et al.*, 1988; Widdel, 1988; Kristjansson *et al.*, 1982; Middleton and Lawrence, 1977; Bryant *et al.*, 1977). Other species that are commonly found are *Desulfohabdus*, *Desulfobacca* (Hulshoff Pol, 2000), *Desulfotomaculum* (Hamilton, 1998), *Desulfobulbus* (Szewzyk and Pfennig, 1990), *Desulfobacter* (Laanbroek *et al.*, 1984) and *Desulfococcus* (Groudev *et al.*, 1999). Several SRB species

Table 3.1: Sulphate-reducing organisms, energy and carbon substrates, metabolic products and growth temperatures (Barnes *et al.*, 1992a,b; Widdel, 1988)

Organisms	Energy/Carbon Substrate	Carbon Products	Max. growth temperature (^oC)
Desulfovibrio vulgaris	La, Py, Et, Me, (H_2+CO_2)	Ac	44
Desulfomonas pigra	Py, yeast	Ac	45
Desulfobulbus propionicus	La, Py, Pr, Et	Ac	43
Desulfococcus multivorans	La, Py, Ac, Et, Me	CO_2	40
Desulfobacter postgatei	La, Ac, Et	CO_2	40
Desulfosarcina variabilis	La, Ac, Et, Me, Be	CO_2	38
Desulfonema magnum	Ac, Pr, Be	CO_2	37
Desulfotomaculum acetoxidans	Ac, Et	CO_2	42
Desulfotomaculum orientis	La, Py, Et, Me, (H_2+CO_2)	-	38

La=Lactate, Py=Pyruvate, Ac=Acetate, Pr=Propionate, Be=Benzoate, Me=Methanol, Et=Ethanol

are listed in Table 3.1 along with their carbon substrates, metabolic products and maximum growth temperatures(Barnes *et al.*, 1992a,b; Widdel, 1988).

3.2.2 Incomplete- and Complete oxidising SRB

Several authors have reported the existence of complete and incomplete oxidising sulphate reducing bacteria. Li *et al.* (1996) found that sulphate reducers can oxidise benzoate completely to carbon dioxide and water, or partially oxidise it to acetate. It was proposed that the complete- and incomplete oxidising ability of the SRB was linked to interspecies hydrogen transfer. The partial oxidation of benzoate to acetate results in a production of available hydrogen and has a positive Gibbs free energy change. This reaction can only proceed if hydrogen can be removed by hydrogenotrophic organisms in the microbial consortium. Hydrogen consuming bacteria maintain the negative Gibbs free energy which allows hydrogen producing reactions to occur (Dolfing, 1988). Failing this, complete oxidation occurs as the incomplete oxidation reaction becomes thermodynamically unfavourable. O'Flaherty *et al.* (1998a) also report the existence of these two types of sulphate reducing bacteria growing on propionate, but do not give instances where one type would predominate over another. It was proposed that sulphate-reducing *Desulfobulbus* species may be responsible for incomplete oxidation of propionate to acetate.

Christensen *et al.* (1996) found a buildup of acetate in columns used to treat acid mine drainage and attributed this to the action of incomplete oxidisers. These columns were inoculated with cow manure and whey. Given the complex nature of the organic source it

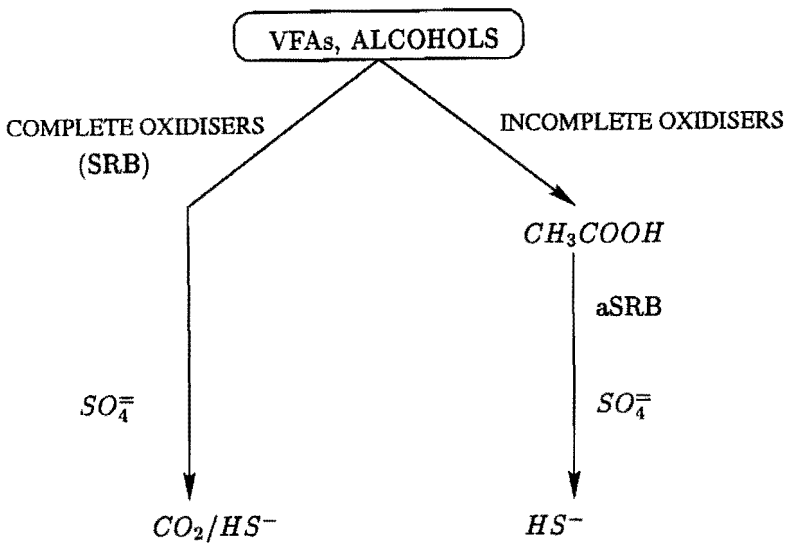

Figure 3.2: Pathway for the anaerobic oxidation of propionate, lactate, higher fatty acids and alcohols by complete- and incomplete-oxidising bacteria (Widdel, 1988)

is uncertain whether the buildup was caused by the more rapid kinetics of fermentation and incomplete oxidation relative to complete oxidation, or whether acetotrophic species were absent or inhibited in the inoculum.

Widdel (1988) reported that the direct utilisation of fermentation products, other than acetate or hydrogen, by sulphate reducers includes two possible pathways (Figure 3.2).

Firstly, fermentation products such as propionate, higher fatty acids, lactate, or alcohols may be degraded by incompletely oxidising sulphate-reducers to acetate. This product is then consumed by acetate-oxidising sulphate-reducers. Secondly, complete oxidisers may degrade the fermentation products to carbon dioxide as the sole product, provided that acetate is not excreted as an intermediate. The competition between an incomplete oxidiser, *Desulfovibrio* spp., and a complete oxidiser, *Desulfobacter* spp., was investigated in continuous culture with ethanol as the limiting substrate Widdel (1988). *Desulfovibrio* spp. was found to dominate. In batch experiments, propionate, higher fatty acids up to palminate and alcohols were found to select for incomplete oxidisers which grew faster than complete oxidisers.

3.2.3 Environmental Conditions Necessary for Sustaining Biological Sulphate Reduction

SRB are regarded as a diverse collection of organisms, forming a broad physiological/ecological grouping (Hamilton, 1998). This latter property stems directly from their

energy-generating metabolism: they are obligate anaerobes that employ a respiratory mechanism with sulphate as the terminal electron acceptor, consequently giving rise to hydrogen sulphide as the major metabolic end-product. These metabolic requirements determine the environmental conditions in which they are active, while the sulphide generation underlies their environmental and technological impact.

There are several environmental conditions that must be satisfied in order to sustain active growth of SRB (Barnes *et al.*, 1992a,b). Firstly, a neutral, or near-neutral, pH is optimal. In extensive reviews, Colleran *et al.* (1995) and Oude Elferink *et al.* (1994) reported that consortia of SRB operate most effectively at a pH range 7.5-8.0. Secondly, a low redox potential, maintained by the absence of oxygen and presence of sulphide ions in the aqueous phase is essential. Sulphate reducers can function in the aqueous phase at redox potentials of approximately -100 mV. Methanogenic consortia prefer lower potentials of around -300 mV. These conditions can easily be attained in laboratory-scale bioreactors.

Sulphate-reducing consortia have been used in applications at various temperatures, ranging from 14^oC (Christensen *et al.*, 1996) to 55^oC (Rintala and Lepisto, 1998). Moosa (2000) conducted a number of experiments over the temperature range 20-38^oC and showed that consortia of acetate-consuming SRB function most effectively at 35^oC. This is in agreement with published literature (Colleran *et al.*, 1995; Oude Elferink *et al.*, 1994; Middleton and Lawrence, 1977).

In a natural environment, hydrogen sulphide pollution most often occurs as a result of increased organic carbon loading such as discharge from domestic sewers, industrial effluent or decay of algal blooms. The localised stimulation of microbial activity in areas of high organic carbon loading leads to a greatly increased rate of oxygen uptake by aerobic microorganisms. The consequent redox imbalance results in the development of anaerobiosis. This is characterised by black metal sulphide precipitates, hydrogen sulphide gas and decreased species diversity.

3.2.4 Carbon Sources and Electron Donors

A variety of carbon sources (and also electron donors) can be used to sustain the growth of sulphate reducing bacteria. SRB have been isolated from and grown successfully as part of complex microbial consortia in cow manure under mesophilic conditions (14-17^oC) as well as on whey (Christensen *et al.*, 1996). Li *et al.* (1996) successfully grew SRB and syntrophic bacteria on benzoate. These studies were carried in continuous culture out at 35^oC over a range of COD:sulphur ratios from 0.75-60 (on a mass basis). They found that at COD:sulphur ratios of 60, 99 % benzoate is degraded to

methane via acetate by the symbiotic relationship between acetate-forming bacteria and hydrogenotrophic methane-producing bacteria. Under sulphate-rich conditions, benzoate is converted to acetate by one of two methods: one by syntrophic association of acetogenic bacteria and hydrogenotrophic MPB and/or SRB, and the other by direct oxidation conducted by benzoate-consuming SRB. At intermediate ratios (approximately 1.5-15), benzoate is oxidised by the first method and SRB and MPB compete for substrate, and below ratios of 1.5-0.75, the latter method is followed with SRB consuming 87 % of the available benzoate. It was also reported that sulphate reducers have a higher affinity for benzoate than methane producers (Li *et al.*, 1996). Colleran *et al.* (1995) suggest that sulphate reducers are capable of growth on a variety of aromatics without functional groups. This excludes phenols, methylated benzene rings (such as toluene) or carboxylated benzene rings (benzoate): a direct contradiction of the work published by Li *et al.* (1996) where SRB were reported to grow well on carboxylated aromatics.

More common substrates include fatty acids such as lactate (Postgate, 1984), butyrate and propionate (Lay *et al.*, 1998; O'Flaherty *et al.*, 1998a), acetate (Maillacheruvu and Parkin, 1996; Visser, 1996; Choi and Rim, 1991), and alcohols such as ethanol (De Smul, 1998; O'Flaherty *et al.*, 1998a; Oude Elferink *et al.*, 1994; Szewzyk and Pfennig, 1990; Kremer *et al.*, 1988; Laanbroek *et al.*, 1984, 1982) and methanol (Tsukamoto and Miller, 1999; Braun and Stolp, 1985; Lettinga *et al.*, 1981; Bryant *et al.*, 1977). Higher monovalent alcohols such as propanol, *n*-butanol and *i*-butanol may also be used (O'Flaherty and Colleran, 1999). Van Houten *et al.* (1994) carried out extensive studies on sulphate reduction using synthesis gas as a carbon and energy source.

3.3 The Use of Ethanol as a Carbon Source and Electron Donor

Ethanol is an anaerobic digestion intermediate formed by the hydrolytic fermentation of sugars by fermentative bacteria (Samain *et al.*, 1982). The general consensus for the mechanism of microbial reduction of sulphate to sulphide beginning with ethanol as organic carbon source and electron donor is represented in Figure 3.3. The reactions, labelled 1-8, are listed in Table 3.2 (Dolfing, 1988). Reactions 1 and 2 show that hydrogen and acetate are produced from ethanol oxidation (reactions 1 and 2 are the same reaction). Different microbial species are responsible for the consumption of the reaction products hydrogen and acetate, therefore the reaction has been shown as two distinct reactions to make interpretation of the mechanism clearer.

In the presence of sulphate, ethanol is oxidised to acetate by sulphate-reducing bacteria with the concomitant production of hydrogen sulphide. According to Bryant *et al.* (1977)

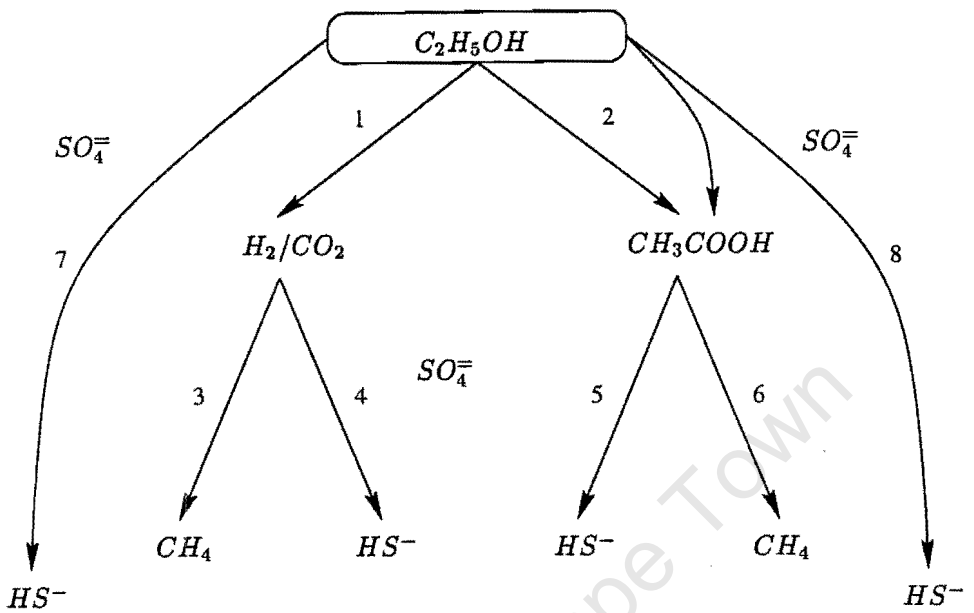

Figure 3.3: Mechanism for anaerobic ethanol oxidation

Table 3.2: Reactions involved in anaerobic ethanol oxidation (Dolfing, 1988)

No.	Reaction	$\Delta G^o\ (kJ.mol^{-1})$
1	$C_2H_5OH + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.6
2	$C_2H_5OH + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.6
3	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-33.9
4	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + H_2O$	-38.1
5	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-47.6
6	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
7	$2C_2H_5OH + 3SO_4^{2-} \rightarrow 4HCO_3^- + H^+ + 2H_2O + 3HS^-$	-118
8	$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H^+ + 2H_2O + HS^-$	-152

ethanol-consuming SRB (for example *Desulfovibrio* strains) can couple ethanol oxidation with interspecies transfer of hydrogen to methane producers in the absence of sulphate. This was confirmed by Kremer *et al.* (1988) who found that *Desulfovibrio gigas* convert ethanol to hydrogen and acetate in the absence of sulphate (reaction 1 and 2, Figure 3.3). The oxidation to acetate is made possible by hydrogen transfer to a hydrogen-consuming methane-producer (reaction 3). Kremer *et al.* (1988) also showed that under sulphate-free conditions, ethanol oxidation does not lead to a considerable increase in cell number of *D. gigas*. This suggests that the bacteria employ ethanol oxidation in the absence of sulphate to provide maintenance energy but not for cell propagation.

The addition of hydrogen gas or hydrogen precursors such as ethanol has been found to enhance sulphate reduction (Isa *et al.*, 1986b). It was postulated that, in the presence of ethanol, SRB act as acetogens or incomplete oxidisers, using the electrons derived directly from ethanol to reduce sulfate (reaction 8). In systems where hydrogen gas is added, SRB use available hydrogen (reaction 4) for sulphate reduction. Laanbroek *et al.* (1984, 1982) also found that the addition of sulphate enhanced the rate of ethanol conversion whereas simultaneous hydrogen and sulphate addition reduced it. In the presence of excess hydrogen, the ethanol oxidation reaction is thermodynamically unfavourable since the Gibbs free energy for the reaction is positive.

$$C_2H_5OH + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2, \;\; \Delta G^o = +9.6\, kJ.mol^{-1}$$

Ethanol use decreases but hydrogenotrophic SRB use the excess hydrogen to reduce sulphate (reaction 4).

Colleran *et al.* (1995) reported that *Desulfobulbus propionicus* and *Pelobacter propionicus* form propionate as the end product of ethanol consumption. These reactions occur only in the absence of sulphate. Laanbroek *et al.* (1984, 1982) have documented both ethanol fermentation to propanol and ethanol oxidation to acetate by microbial consortia isolated from intertidal sediments. The species involved were identified as *Desulfobacter*, *Desulfobulbus* (ethanol fermenters) and *Desulfovibrio* (ethanol oxidisers). They found that ethanol was oxidised predominantly to acetate with the formation of small amounts of propionate. O'Flaherty and Colleran (1999) also reported ethanol fermentation to propionate and ethanol oxidation to acetate. The propionate formation reactions can be represented by:

$$3C_2H_5OH + 2HCO_3^- + 3H^+ \rightarrow CH_3COO^- + 2CH_3CH_2CH_2OO^- + 3H_2O$$

$$\Delta G_o^f = +34\, kJ.mol^{-1} \tag{3.1}$$

$$C_2H_5OH + HCO_3^- + 2H_2 \rightarrow CH_3CH_2CH_2OO^- + 2H_2O$$

$$\Delta G_o^f = -67\,kJ.mol^{-1} \tag{3.2}$$

Samain *et al.* (1982) found distinct patterns of volatile fatty acid formation whilst growing *Desulfotomaculum*, *Desulfovibrio* and methanogens on ethanol. The propionic fermentation of ethanol and propanol were also observed. Once again, ethanol oxidation to acetate could only be carried out in the presence of a hydrogen-consuming anaerobe since hydrogen removal is necessary to thermodynamically allow the ethanol oxidation reaction to occur.

Similar findings were made by Schink *et al.* (1985) while investigating sulphate reduction in anoxic freshwater sediments. Since the acetate/hydrogen system is energetically more favourable than the formation of reduced fermentation products such as butyrate, propionate and ethanol, it is favoured under substrate-limiting conditions. Interspecies hydrogen transfer has been found to shift the formation of fermentation products almost exclusively to acetate. In the presence of sulphate, ethanol was oxidised to acetate directly, with the co-production of hydrogen sulphide. In the absence of sulphate, microorganisms coupled ethanol oxidation with hydrogen transfer to methane producers (reaction 1 and 3, Figure 3.3). It was also found that in the presence of additional hydrogen, ethanol degradation was decreased. This is in agreement with findings by Isa *et al.* (1986a).

3.4 Microbial Competition

The relative kinetics and substrate affinities allow one microorganism to dominate over another by scavenging a larger proportion of a limited substrate under thermodynamically favourable conditions. SRB and MPB compete for available substrates, usually short chain fatty acids such as lactate, propionate, butyrate and acetate, as well as hydrogen and ethanol as both groups require these for energy generation. Species domination or pathway selection is determined either by thermodynamics (greater negative Gibbs free energy) (Dolfing, 1988), or kinetics (higher maximum growth rate or lower substrate affinity constant) (Kristjansson *et al.*, 1982).

3.4.1 Thermodynamic Considerations

Thermodynamics of anaerobic systems are influenced by interspecies hydrogen transfer (the transfer of hydrogen between hydrogen-producing and hydrogen-consuming microorganisms) (Dolfing, 1988). This phenomenon creates conditions for obligate hydrogen-producing microorganisms to perform catabolic oxidations which, in the absence of hydrogen-consuming organisms, would not be energy-yielding.

Table 3.2 lists the standard Gibbs free energy changes for the reactions involved in ethanol degradation. Conversion of ethanol to acetate by acid-forming bacteria (reactions 1 and 2) has a positive Gibbs free energy change, i.e., it is thermodynamically unfavourable. These reactions can only proceed in the presence of hydrogen- and/or acetate-consuming bacteria that remove the reaction products making the overall thermodynamics favourable and shifting the reaction to the right (Dolfing, 1988).

Reactions 3 to 8 have negative free energy changes, i.e., they are thermodynamically feasible. The magnitude of the Gibbs free energy change determines which of the reactions are favoured, hence affecting the outcome of microbial competition for the substrates. It is clear that the sulphidogenic reactions (reactions 4, 5, 7 and 8) are thermodynamically more favourable than their corresponding methanogenic reactions using the same substrates (reactions 3, 6, 1 and 2). Hence the SRB outcompete MPB on a thermodynamic basis. It is also clear that the incomplete oxidation of ethanol to acetate by SRB is more favourable than complete oxidation, hence, on a thermodynamic basis, acetate is expected to appear as a reaction intermediate.

While thermodynamics assist in the definition of microbial competition, thermodynamic considerations are often not adequate to explain the outcome of microbial competition (Kristjansson *et al.*, 1982). Often, the outcome of competitive interactions can not be predicted thermodynamically. Reactor configuration and microbial kinetics further define the outcome of competition.

3.4.2 Relative Kinetics

The outcome of competitive microbial growth is influenced by mechanisms where one species has a higher affinity for a substrate or is able to use it at a higher rate. This may be as a result of intrinsic microbial characteristics, microbial inhibition or selection by physical phenomena (such as reactor geometry). Szewzyk and Pfennig (1990) suggest that bacteria make use of a "K-strategy": the expression of a high substrate affinity (low

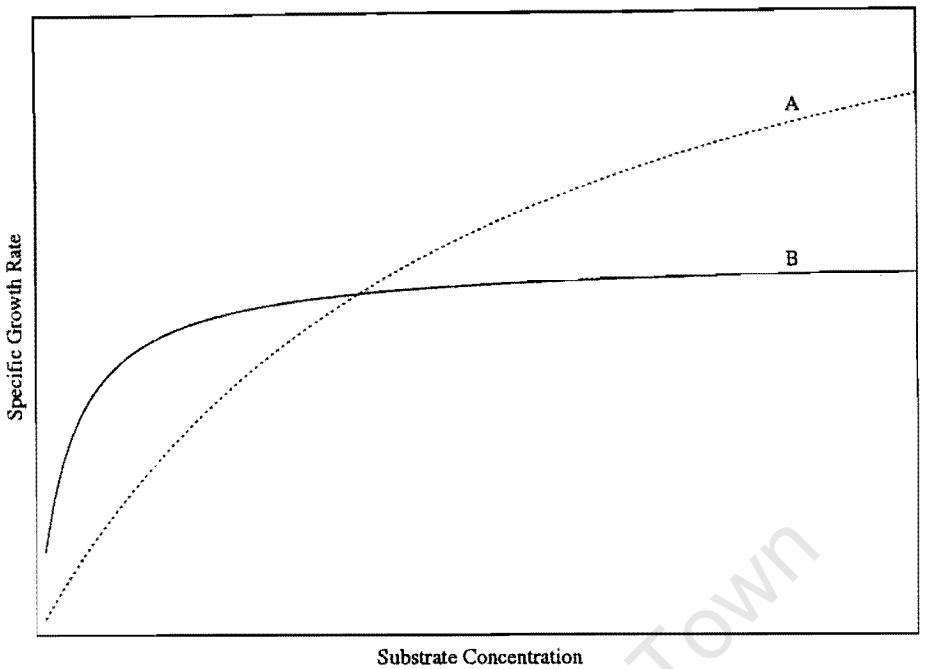

Figure 3.4: Relationship between substrate concentration and specific growth rate for microbial species with different maximum specific growth rates (μ^{max}) and substrate affinity constants (K_s)

K_s) for bacteria with a low maximum specific growth rate (μ_{max}):

$$\text{Affinity} = \frac{\mu_{\text{max}}}{\text{K}_\text{s}} \tag{3.3}$$

Bacteria with low maximum growth rates are outcompeted by faster growing bacteria. By exhibiting lower half-velocity constants (substrate concentrations at which growth is possible at half the maximum growth rate) they become more competitive. Figure 3.4 shows the relationship between substrate concentration and specific growth rate for microbial species with different maximum specific growth rates (μ^{max}) and substrate affinity constants (K_s). At higher substrate concentrations, species A will dominate as its maximum specific growth rate is higher. As substrate concentration decreases, species A competes less effectively as its substrate affinity is lower than species B, i.e., it has a higher K_s. Species B is able to scavenge the available substrate at lower substrates even though its maximum specific growth rate is lower.

Kinetics of microbial growth will be discussed in Section 3.5 and inhibition of microbial growth will be discussed in Section 3.6.

3.4.3 Reactor Configurations

There are several types of industrial bioreactors that are used for anaerobic treatment of wastewaters. The most common type of reactor is the upflow anaerobic sludge bed (UASB). There are currently over 300 UASB reactors in full-scale operation worldwide treating a variety of industrial wastewaters (Oude Elferink *et al.*, 1994). UASBs apply the principle of biomass retention. Bacteria are retained in, or immobilised on, microbial flocs or granules (Speece, 1996). Higher rates of reaction are possible because of the increased active biomass concentrations in the reactors relative to suspended cell systems.

CSTRs constitute a simple model for more complex engineered systems such as feedback reactors and UASBs (White and Gadd, 1996). Laboratory CSTR studies are used for kinetic studies (Gupta *et al.*, 1994a; Barnes *et al.*, 1992a,b; Choi and Rim, 1991; Schonheit *et al.*, 1982; Kristjansson *et al.*, 1982), however, increased attention is being focussed on retained biomass systems (De Smul, 1998; O'Flaherty *et al.*, 1998a,b; Li *et al.*, 1996). Li *et al.* (1996) used UASB reactors to study benzoate degradation. De Smul (1998) studied the effect of physical parameters and carbon loading on a UASB mixed culture. O'Flaherty *et al.* (1998a,b) used sludge from industrial UASBs treating citric acid waste water in laboratory scale UASBs for sludge characterisation work.

Novel reactor configurations have been and are being investigated. Anaerobic baffled reactors (ABRs) are high rate anaerobic systems attracting increasing attention (Grobicki and Stuckey, 1992). They have smaller physical dimensions than UASBs but because of the baffled internal configuration they allow for longer hydraulic retention times (HRTs) and biomass retention. Sacks *et al.* (1999) investigated the decolorisation of food dyes in food processing wastewaters using ABRs with a mixed culture of anaerobic bacteria. The ABR operation was very successful with the organic content reduced by up to 70% and colour by almost 90%. Garcia-Calderon *et al.* (1998) used a downflow fluidised bed (DFFB) with a mixed culture from an anaerobic digester to treat wine distillery wastewater. The DFFB makes use of biomass immobilisation on support particles of lower specific gravity than the process fluid. Process fluid is circulated downward in a column-type reactor and the biomass support particles (BSPs) are fluidised by the down-flow of the process fluid. The DFFB was also very successful with up to 98% total organic carbon removal at a range of hydraulic retention times from 3.3 to 1.3 days.

Reactor geometry plays an important role in the outcome of microbial competition. For example, in a case where a biomass support particle is available (UASB or packed bed reactor), methane-producing bacteria may predominate because of their ability to excrete extracellular polysaccharides and adhere to the support particle (Speece, 1996). Sulphate-reducing bacteria do not exhibit this behaviour (Speece, 1996; Widdel, 1988).

Other factors such as substrate availability, inhibitor concentrations, and process parameters (dilution rate, pH) will also influence the competition.

3.5 Kinetics of Microbial Growth

Anaerobic digestion is a well established process and extensive reviews on the kinetics and mechanism have been published (Verstraete *et al.*, 1996; Lettinga, 1995; Pavlostathis and Giraldo-Gomez, 1991; McCarty and Mosey, 1991; Zehnder, 1988; Gujer and Zehnder, 1983). The focus of the review is to present the kinetics of biological sulphate reduction with ethanol as the carbon source and electron donor.

Extensive work has been published on acetate-consuming consortia of MPB and SRB (Gupta *et al.*, 1994a,b; Choi and Rim, 1991; Isa *et al.*, 1986a; Kristjansson *et al.*, 1982; Schonheit *et al.*, 1982), while some studies on ethanol-consuming SRB are reported (O'Flaherty *et al.*, 1998a,b; Szewzyk and Pfennig, 1990; Kremer *et al.*, 1988; Schink *et al.*, 1985; Laanbroek *et al.*, 1984, 1982). A selection of the literature data are presented. Tables 3.3, 3.4, 3.5 and 3.6 show a summary of kinetic parameters for syntrophic bacteria (bacteria growing on reaction intermediates with products that form substrates for other bacteria), MPB and SRB respectively. Tables of kinetic parameters can also be found in work of by Colleran *et al.* (1995), Oude Elferink *et al.* (1994) and Widdel (1988). Moosa (2000), Ristow (1999) and Knobel (1999) have also tabulated a variety of kinetic parameters for anaerobic digestion and sulphate reduction as part of the research initiative at the Department of Chemical Engineering, University of Cape Town.

Kinetic parameters are affected by culture history, parameter identifiability, and the manner in which experiments to determine them are carried out (Leslie Grady *et al.*, 1996). This results in wide ranges of kinetic parameters.

From the summarised kinetic parameters, it is apparent that the maximum specific growth rates of the methanogenic bacteria predominantly fall in the range 0.1 - 0.7 d^{-1} and those of the sulphate reducing bacteria in the range 0.2 - 0.9 d^{-1}. Half -velocity constants vary widely in both groups, but the SRB generally have lower values. This marginally faster maximum growth rate, coupled with the lower K_s-values suggests that SRB will dominate over MPB in the competition for limiting substrates.

Table 3.3: Growth parameters for syntrophic bacteria

Reference	Culture	Limiting Substrate	Conditions	T (^oC)	Y ($mg\ X.mg\ substr.^{-1}$)	μ_{max} (d^{-1})	K_s ($mg\ substr.l^{-1}$)
O'Flaherty *et al.* (1998b)	Mixed syntrophic	Propionate Butyrate Ethanol	Anaerobic sludge	30		0.05 0.25 0.29	34 55 16
Maillacheruvu and Parkin (1996)	Propionate fermenters	Propionate	Batch		0.064		18
Szewzyk and Pfennig (1990)	*A. carbinolicum*	Ethanol	Continuous	28	0.096	1.704	> 46
Chiu *et al.* (1972)	Mixed culture	Glucose	Continuous	25	0.577	16.6	26.5

Table 3.4: Growth parameters for acetoclastic and hydrogenotrophic methanogens

Reference	Culture	Limiting Substrate	Conditions	T (^oC)	Y $(mg\,X.mg\,substr^{-1})$	μ_{max} (d^{-1})	K_s $(mg\,substr.l^{-1})$
O'Flaherty *et al.* (1998b)	Mixed MPB	Acetate	Sludge	37		0.1	35
		H_2/CO_2				0.08	0.055
Maillacheruvu	aMPB	Acetate	Batch		0.041		27
and Parkin (1996)	hMPB	H_2/CO_2			0.392		1.9×10^{-4}atm
Colleran *et al.* (1995)	hMPB	H_2	Continuous				10-26
	aMPB	Acetate	culture				180
Oude Elferink *et al.* (1994)	*Methanosarcina barkeri*	Acetate			0.033-0.057	0.46-0.69	300
	Methanosarcina mazei				0.032	0.53	
	Methanothrix soehngenii				0.018-0.023	0.08-0.29	28-42
	Methanospirillum hungateii	H_2			0.15-0.25	1.2-1.3	0.012-0.015
Gupta *et al.* (1994a,b)	MPB	Acetate	Continuous	35			6
		Methanol	culture				2.6-5.3
	aMPB	Acetate					180
Gujer and Zehnder (1983)	Mixed MPB	Acetate	Continuous	35	0.043	0.34	155
	M. arboriphilus	H_2	Digester	33	0.32	1.4	0.075
Schonheit *et al.* (1982)	*Methanosarcina barkeri*	Acetate	Batch	30		0.672	180
Kristjansson *et al.* (1982)	*M. arboriphilus*	H_2	Batch	35		3.36	0.013

(MPB - methane-producing bacteria; aMPB - acetoclastic methane-producing bacteria; hMPB - hydrogenotrophic methane-producing bacteria)

Table 3.5: Growth parameters for hydrogenotrophic, acetoclastic and ethanol-oxidising SRB

Reference	Culture	Limiting Substrate	Conditions	T (^oC)	Y ($mg\ X.mg\ substr^{-1}$)	μ_{max} (d^{-1})	K_s ($mg\ substr.l^{-1}$)
Moosa (2000)	Mixed aSRB	Sulphate	CSTR	35	0.58	1.51	71
		Acetate			0.56		
O'Flaherty *et al.* (1998b)	Mixed SRB	Butyrate	Anaerobic sludge	30		0.15	25
		Sulphate					29
	Mixed SRB	Ethanol	Anaerobic sludge	30		0.22	30
		Sulphate					30
Kalyuzhnyi and Fedorovich (1997)	Mixed	Acetate	UASB	35	0.044		
Maillacheruvu	aSRB	Acetate	Batch		0.025		47
and Parkin (1996)	hSRB	H_2/CO_2			0.005		1.6×10^{-4}atm
	Propionate-oxidising SRB	Propionate			0.048		27
Colleran *et al.* (1995)	hSRB	H_2					4
	aSRB	Acetate					12
Gupta *et al.* (1994a,b)	SRB	Acetate	Continuous culture	35			0.84
Oude Elferink *et al.* (1994)	*Desulfobacter postgatei*	Acetate			0.072-0.08	0.72-1.11	
	Desulfobacter hydrogenophilus					0.92	
	Desulfotomaculum acetoxidans				0.095	0.65-1.39	
	Desulfovibrio vulgaris	H_2			0.55-1.3	3.6-5.5	
	Desulfovibrio desulfuricans				0.95	2.6	
	Desulfobulbus propionicus					0.23-0.59	
Costello *et al.* (1991a,b)	Mixed SRB/MPB	Acetate	Laboratory digester		0.042		154
		H_2			0.042		0.001 atm

(MPB - methane-producing bacteria; SRB - sulphate-reducing bacteria; aSRB - acetoclastic sulphate-reducing bacteria; hSRB - hydrogenotrophic SRB)

Table 3.6: Growth parameters for hydrogenotrophic, aceticlastic and ethanol-oxidising SRB (continued)

Reference	Culture	Limiting Substrate	Conditions	T (^oC)	Y ($mg\,X.mg\,substr^{-1}$)	μ_{max} (d^{-1})	K_s ($mg\,substr..l^{-1}$)
Szewzyk and Pfennig (1990)	*Desulfobulbus propionicus*	Ethanol	Continuous culture	28	0.113	0.792	0.244
	Desulfovibrio gigas				0.065	0.552	
	Desulfovibrio vulgaris				0.056	0.768	0.267
	Desulfotomaculum orientis				0.074	0.432	0.644
	Desulfovibrio desulfuricans				0.074	0.792	
Isa *et al.* (1986a,b)	hSRB	H_2					0.002
	aSRB	Acetate					12
Laanbroek *et al.* (1984)	*Desulfobacter postgatei*	Ethanol	Continuous culture	30	0.134		
	Desulfobulbus propionicus				0.044		
	Desulfovibrio baculatus				0.067		
Gujer and Zehnder (1983)	Mixed SRB/MPB	Propionate	Continuous	35	0.064	0.31	40
Schonheit *et al.* (1982)	*Desulfobacter postgatei*	Acetate	Batch	30		0.72	13.8
Kristjansson *et al.* (1982)	*Desulfovibrio vulgaris*	H_2	Continuous	35			0.0026
Middleton and Lawrence (1977)	aSRB	Acetate	Continuous culture	31	0.065		5.7
				25			92
				20		0.33	250

(MPB - methane-producing bacteria; SRB - sulphate-reducing bacteria; aSRB - acetoclastic sulphate-reducing bacteria; hSRB - hydrogenotrophic SRB)

3.5.1 Competition between SRB, MPB and Syntrophic bacteria for Ethanol

Methane producers use only acetate and hydrogen/carbon dioxide as carbon source and electron donors. They are not involved in competition for ethanol. Ethanol is a substrate for acetogenic- and sulphate-reducing bacteria. Syntrophic bacteria which ferment ethanol to fatty acids such as propionate, and oxidise ethanol to acetate do not compete well for substrate since their Gibbs free energy changes involved in fermentative reactions to propionate (Equations 3.1 and 3.2) are not favourable.

Ethanol fermentation to acetate is also an unfavourable reaction since the Gibbs free energy change for the reaction is positive (Table 3.2). This reaction can only proceed in the presence of acetotrophic and hydrogenotrophic organisms which remove the reaction products and drive the reaction to the right. In addition, the availability of hydrogen for the fermentation reaction is limited since the half-velocity constants for hydrogenotrophic SRB and MPB are very low ($10^{-2} - 10^{-3}\, mg.l^{-1}$) compared with syntrophic bacteria (Kristjansson *et al.*, 1982). The latter have K_s-values 2 - 3 orders of magnitude larger ($10^{1}\, mg.l^{-1}$). Hydrogen is easily scavenged by the SRB and MPB consortia for the purposes of sulphate reduction and methane production. The nett result is that ethanol is consumed predominantly by SRB.

3.5.2 Competition between aSRB and aMPB for Acetate

It has been reported that acetate is the intermediate of 70% of all organic reduction to methane (Gujer and Zehnder, 1983). It is thus a key intermediate in terminal sulphate reduction and methane production. The Gibbs free energy change of the sulphate-reducing ($\Delta G_o^f = -47.6\, kJ.mol^{-1}$) and methane producing ($\Delta G_o^f = -31\, kJ.mol^{-1}$) reactions (reactions 5 and 6 respectively, Table 3.2) show that SRB have a slight thermodynamic advantage in acetate consumption.

Kinetically, aMPB generally have higher K_s and lower μ_{max} values than aSRB. Coupled with the thermodynamic advantage of the SRBs, one would expect SRB to outcompete MPB for acetate. The affinity of SRB for ethanol is greater than that for acetate. From this, acetate should appear as an intermediate in the system as SRB partially oxidise ethanol (O'Flaherty *et al.*, 1998a; Isa *et al.*, 1986b; Widdel, 1988). Acetate consumption is expected to be dominated by aSRB as the SRB reactions are thermodynamically and kinetically favoured over aMPB reactions.

3.5.3 Competition between hSRB and hMPB for Hydrogen

Experiments by Kristjansson *et al.* (1982) have shown that methane production and sulphate reduction are not mutually exclusive and in the presence of excess hydrogen (electron donor) have no effect on each other. When hydrogen supply becomes rate limiting, methane-producers are suppressed by sulphate reducers owing to their lower affinity for common substrates. Threshold concentrations of hydrogen for methanogenic growth are approximately 10^{-4} *atm* (Oude Elferink *et al.*, 1994). It is rare in any system that partial pressures of hydrogen exceed this, hence hydrogen gas is rarely in excess as an electron donor (Dolfing, 1988).

3.6 Inhibition of Microbial Growth

Two critical processes in microbial inhibition of anaerobic sulphate-reducing microorganisms were identified by Maillacheruvu and Parkin (1996):

- competition for organic substrate and,
- inhibition by hydrogen sulphide.

Competition for organic substrate is governed by thermodynamics and kinetic growth parameters (as discussed in Sections 3.4 and 3.5). Inhibition reduces the capacity of the microorganisms to compete for substrate and consequently reduces the rates of microbial growth, and affects which species dominate in a particular system. Microbial inhibition may be mediated by pH, redox potential, hydrogen sulphide, dissociated/undissociated acetic acid and sulphate.

3.6.1 Inhibition by pH and Acetate

According to Andrews and Graef (1971), the dominant effect of pH does not affect the growth of microbial consortia directly but is manifested through an indirect mechanism. Oude Elferink *et al.* (1994) support this. Aqueous hydrogen sulphide concentrations as well as undissociated volatile fatty acid (VFA) concentrations are affected by pH. These varying inhibitor concentrations exhibit varying inhibitory effects on microbial growth. Because pH is a log-function, small changes in pH can cause substantial changes in other parameters such as dissociated hydrogen sulphide and dissociated VFA concentrations.

Pioneering work in acetic acid/acetate inhibition kinetics was conducted by Andrews and co-workers (Andrews, 1968; Andrews and Graef, 1971; Andrews, 1974, 1975). Andrews (1968) proposed a model to describe microbial growth utilising an inhibitory substrate:

$$\mu = \frac{\mu_{max}}{1 + \frac{K_s}{c_s} + \frac{c_s}{K_i}} \tag{3.4}$$

This model was developed further by Andrews (1975) to allow specifically for inhibition by undissociated acetic acid due to pH effects:

$$\mu = \frac{\mu_{max}}{1 + \frac{K_s}{c_{CH_3COOH}} + \frac{c_{CH_3COOH}}{K_i}} \tag{3.5}$$

and

$$c_{CH_3COOH} = \frac{(c_{H+})\, c_{CH_3COO-}}{K_a} \tag{3.6}$$

where c_{CH_3COOH} is the undissociated substrate concentration, c_{CH_3COO-} is the dissociated substrate concentration, c_{H+} is the hydrogen ion concentration and K_a is the ionisation constant for acetic acid ($10^{-4.5}$ at 38^oC). Costello *et al.* (1991a,b) made provision for acetic acid inhibition through a non-competitive model for acetic acid inhibition:

$$r_s = \frac{k c_x c_s}{(K_s + c_s)\left(1 + \frac{c_i}{K_i}\right)} \tag{3.7}$$

here k is the maximum specific substrate uptake rate, c_s is the substrate concentration, c_x is the biomass concentration, c_i is the inhibitor concentration and K_i is the inhibition constant. The inhibition constant for acetic acid was estimated to be approximately 10 $mmol.l^{-1}$ ($\sim$600 $mg.l^{-1}$). The percentage inhibition was not indicated.

However, Reis *et al.* (1992) report that pH does affect the growth of SRB directly and that the inhibition can be described by a noncompetitive model:

$$\mu = \frac{\mu_{max}}{1 + \frac{c_{H+}}{K_H} + \frac{K_{OH}}{c_{H+}}} \tag{3.8}$$

where $K_{OH} = 1.69 \times 10^{-7}\, mol.l^{-1}$ and $K_H = 3.25 \times 10^{-7}\, mol.l^{-1}$ are rate constants and c_{H+} is the proton concentration. A batch enrichment culture of *Desulfovibrio* growing on lactate and sulphate at 37^oC exhibited a maximum specific growth rate and cell yield at approximately pH 6.6-6.7. O'Flaherty *et al.* (1998b) investigated the effect of pH on MPB and SRB consortia over the pH range 6.8-8.5. The growth properties of sulphate-reducers and methane-producers in the pH range 7.0-7.5 were similar. Studies on pure cultures of SRB over the range 6.8-9.5 showed that SRB have a pH optimum of

Table 3.7: A comparison of redox potentials for sulphur-based electron acceptors and oxygen as an electron acceptor (Hamilton, 1998)

Redox Couple	(E^o)
APS/AMP + HSO_3^-	-60 mV
HSO_3^-/HS^-	-116 mV
S^o/HS^-	-270 mV
$\frac{1}{2}O_2/H_2O$	+818 mV

7.5-8.0 in terms of growth rate. For methane-producing bacteria, the growth rates were maximum over the pH range 7.0 - 7.5. From the published data, it is apparent that the MPB do not function below pH 6.8. Above the optimum, the growth rate decreases steadily from pH 7.5 until activity ceases at pH 9.5. The sulphate-reducing bacteria show an increase in the maximum specific growth rate from pH 6.8 to their optimum at pH 7.8, with a decrease being observed from pH 7.8 to 9.5. In general, the pH range supporting near-optimum growth of the SRB was broader than that for the MPB.

3.6.2 Effect of Redox Potential

Very few authors report on the effect of redox potential on sulphate reduction. Gupta *et al.* (1994a) ran a series of six continuous culture experiments under different conditions. The methanogenic cultures (without exposure to sulphate) operated in the range -210 mV to -235 mV. The redox potential of the exclusively sulphate-reducing, acetate-fed continuous cultures was -309 mV. These values do not coincide with the suggestions of Barnes *et al.* (1992a,b). White and Gadd (1996) found that redox potentials of above -200 mV (i.e. less negative potentials) were found to slightly inhibit sulphate reducing bacteria. Sulphur-based electron acceptors have lower energy yields than oxygen/water couple for aerobic systems (Table 3.7) (Hamilton, 1998). This explains the large negative redox potentials under which SRB grow and their apparent inhibition at less negative potentials.

Redox potential is a difficult parameter to interpret in anaerobic sulphate reduction systems since dissolved metal ions, sulphate, hydrogen sulphide all exert an effect on redox potential.

3.6.3 Inhibition by Hydrogen Sulphide

The K_a-value of the dissociation equilibrium of hydrogen sulphide is 9.1×10^{-8} at 18^oC (Weast and Astle, 1988). Hydrogen sulphide dissociation follows the equilibrium reac-

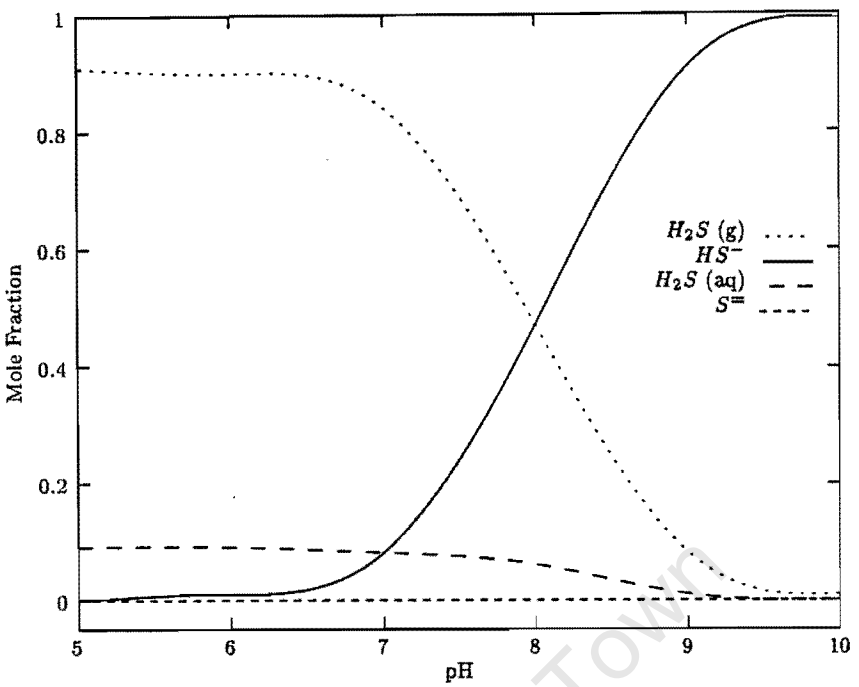

Figure 3.5: Relationship between the species of hydrogen sulphide and pH

tion:

$$H_2S \rightleftharpoons HS^- + H^+ \tag{3.9}$$

In the pH range 6-8, hydrogen sulphide exists in one of these forms (H_2S or HS^-). Below pH 6, hydrogen sulphide is found predominantly in the undissociated form (H_2S). Dissociated hydrogen sulphide dissociates further at higher pH:

$$HS^- \rightleftharpoons S^{2-} + H^+ \tag{3.10}$$

the dissociation constant for this reaction is 1.1×10^{-12} at 18^oC (Weast and Astle, 1988). Hydrogen sulphide dissociation to S^{2-} does not occur in sulphate reduction systems because the pH is near-neutral and the second dissociation only occurs near pH 12. Total hydrogen sulphide can be determined at any time by the relationship:

$$H_2S_{total} = H_2S_{(aq)} + HS^- \tag{3.11}$$

or, knowing that all hydrogen sulphide is produced from reduction of sulphate:

$$H_2S_{total} = \left(SO_4^{2-}\right)_{influent} - \left(SO_4^{2-}\right)_{effluent} \tag{3.12}$$

Figure 3.5 shows the relationship between the species of hydrogen sulphide and pH.

It is generally assumed that the undissociated form of hydrogen sulphide is the toxic agent since it is membrane permeable in this form only (Speece, 1996). The pH of the system determines the fraction of the total sulphide present in the undissociated form (Figure 3.5), and consequently, small variations in the pH over the pH range 6 - 8 for anaerobic digestion cause significant changes in the degree of inhibition (Oude Elferink *et al.*, 1994).

Choi and Rim (1991) reported that MPB and SRB were highly competitive at COD:SO_4^{2-} ratios of 1.7-2.7. These authors also reported that SRB and MPB were non-competitively inhibited by dissociated hydrogen sulphide (HS^-) concentrations of 160-200 $mg.l^{-1}$ and 120-140 $mg.l^{-1}$ respectively. pH data was not provided to evaluate the levels of undissociated hydrogen sulphide (H_2S) in the system. Maillacheruvu and Parkin (1996) used an uncompetitive inhibition model to describe sulphide toxicity kinetics. Both dissociated sulphide and undissociated hydrogen sulphide inhibition-values were reported. Dissociated sulphide concentrations of 36 and 229 $mg\,HS^-.l^{-1}$ inhibited acetate-oxidising SRB and acetate-consuming MPB respectively (pH data were not reported). Undissociated hydrogen sulphide inhibition concentrations were reported to be 9 $mg\,H_2S.l^{-1}$ and 117 $mg\,H_2S.l^{-1}$ for aSRB and aMPB, respectively. Christensen *et al.* (1996) reported that inhibition concentrations of undissociated hydrogen sulphide for aSRB fell in the range 40 - 150 $mg.l^{-1}$ (pH 4-6). In an investigation into the treatment of acid mine water at 14^oC in cylinders packed with quartz sand (using cow manure as inoculum and whey as a complex organic source), results indicated an acetate buildup. This was attributed to inhibition of acetotrophic SRB by undissociated hydrogen sulphide. The hydrogen sulphide concentration is much larger than that presented by Maillacheruvu and Parkin (1996) for aSRB (9 $mg\,H_2S.l^{-1}$) presumably due to the acidic pH (where hydrogen sulphide is present in the undissociated form), but still adequately explains aSRB inhibition. It is likely that acetotrophic methane-producing bacteria present in the inoculum were also severely inhibited which resulted in the suppression of aMPB use of acetate. Undissociated hydrogen sulphide concentrations were above the inhibition levels presented by Maillacheruvu and Parkin (1996) (117 $mg.l^{-1}$).

McCartney and Oleskiewicz (1991) reported that SRB are more sensitive to total sulphide (TS) concentrations than MPB. Total sulphide concentrations were determined by Equation 3.11:

$$H_2S_{total} = H_2S_{(aq)} + HS^-$$

A decreasing SRB activity with progressively increasing total sulphide concentrations was observed in experiments over the pH range 6-8. Similar observations were made for both lactate and acetate degradation. It was found that MPB activity remained

steady in high pH (~8) experiments regardless of the total sulphide concentrations, but decreased in the low pH (~6) experiments as the total sulphide increased.

It is useful to interpret this data with regard to the dissociation equilibrium of hydrogen sulphide over the pH range 6-8 (Equation 3.9). This would suggest that MPB are more susceptible to undissociated hydrogen sulphide since total sulphide is predominantly undissociated at low pH, and that SRB are inhibited more by dissociated hydrogen sulphide (hydrogen sulphide is predominantly dissociated at high pH-values).

McCartney and Oleskiewicz (1991) also reported 50% inhibition (IC50) values. The 50% inhibition value of MPB (defined as a 50% reduction in methane production) was reported to be 240 $mgTS.l^{-1}$ at pH 7 and for SRB, 50% inhibition of sulphate reduction occurred at 83 $mgTS.l^{-1}$ at pH 7. IC50 concentrations, defined as the inhibitor concentration giving rise to a 50% inhibition of the microbial growth rate were reported by O'Flaherty *et al.* (1998b). Inhibition was measured when the following criterion was met:

$$\frac{\mu_{inhibited}}{\mu_{uninhibited}} = 0.5 \tag{3.13}$$

Table 3.8 shows IC50s for hydrogen sulphide inhibition based on data for laboratory-scale sulphate-adapted sludge.

It is clear that total sulphide concentrations increase as pH is increased as the predominant species is in the dissociated form. Undissociated hydrogen sulphide concentrations (concentrations in parentheses) decrease with increasing pH as expected (Figure 3.5). The syntrophic bacteria are less sensitive to sulphide inhibition than MPB (i.e., they have a higher IC50 value) with toxicity thresholds comparable with those of the SRB. It was claimed that syntrophic bacteria are irreversibly inhibited, but this was unsubstantiated. Results of the study showed that sulphide inhibition was related to the undissociated hydrogen sulphide between pH 6.8 and pH 7.2, whereas above pH 7.2 the inhibition was related to total sulphide. It was hypothesised that both total sulphide and undissociated hydrogen sulphide exert inhibitory effects on MPB, syntrophs and SRB. The inhibitory form can be determined from the pH and the inhibition threshold of each species. At low pH, free hydrogen sulphide will reach the IC50 value as a high percentage of the total sulphide is undissociated, whereas at higher pH levels (upwards of pH 7.6) the concentration of undissociated hydrogen sulphide is low and dissociated sulphide becomes responsible for microbial inhibition. In this study, the thresholds for undissociated hydrogen sulphide were found to be lower than total sulphide indicating that undissociated hydrogen sulphide is the most toxic form (Speece, 1996; Maillacheruvu and Parkin, 1996; Christensen *et al.*, 1996).

Table 3.8: IC50 values in the form of total sulphide concentration for a range of MPB, syntrophs and SRB in anaerobic sludges over the pH range 6.8 - 8.5 (undissociated hydrogen sulphide in parentheses) (O'Flaherty *et al.*, 1998b)

Bacteria	Substrate	IC50 at varying pH ($mg.l^{-1}$)				
		6.8	7.2	7.6	8.0	8.5
Methanogenic	acetate	350 (189)	630 (205)	851 (136)	977 (68)	1000 (20)
	H_2/CO_2	470 (254)	712 (232)	1056 (169)	1064 (74.5)	1089 (22)
Syntrophic	propionate	467 (252)	828 (269)	1250 (200)	1610 (113)	1623 (32.5)
	butyrate	574 (310)	880 (286)	915 (146.5)	943 (66)	1065 (21)
	ethanol	451 (243.5)	900 (292.5)	1486 (238)	1707 (119.5)	1721 (36.5)
Sulphidogenic	acetate	-	-	-	-	-
	H_2/CO_2	474 (256)	729 (237)	977 (156)	1343 (94)	1340 (27)
	propionate	-	-	-	-	-
	butyrate	467 (252)	802 (261)	941 (151)	965 (67.5)	988 (20)
	ethanol	500 (270)	788 (256)	990 (158.5)	1019 (71)	1004 (21)

In a subsequent study, O'Flaherty and Colleran (1999) observed oscillating patterns of sulphate and sulphide concentrations in reactor effluent. By introducing a nitrogen sparge, peak total sulphide levels were reduced from in excess of 1000 $mg.l^{-1}$ to below 500 $mg.l^{-1}$. The nitrogen sparge halted the oscillating patterns. White and Gadd (1996) also employed this practice as a means of volatising hydrogen sulphide, as well as a pH control mechanism. Volatising hydrogen sulphide at a pH of around 7 was enough to adjust the pH to more favourable operating levels of pH 7.5 - 8.0. In an immobilised system with biomass retained in polyurethane sponge, Isa *et al.* (1986a) observed that nitrogen sparging increased the percentage sulphate reduced. Sparging had no effect on the rate of biogas production or the percentage electron flow to SRB indicating that hydrogen sulphide inhibition primarily affects the substrate affinity, K_s. Nitrogen sparging was found to increase the batch rate of sulphate uptake by SRB in suspended culture (Reis *et al.*, 1991a). Rintala and Lepisto (1998) investigated the thermophilic treatment of sulphur-rich paper mill wastewater at 55^oC. Effluent total sulphide concentrations of 150 - 250 $mg.l^{-1}$, corresponding to undissociated sulphide concentrations of 15-75 $mg.l^{-1}$, were measured over a pH range 7.0 - 7.5. Nitrogen sparging was employed to remove sulphide toxicity and consequently very little variation in COD removal or sulphate removal was noted. No conclusions were drawn regarding sulphide inhibition.

McCartney and Oleskiewicz (1991) showed that at 35^oC and pH 7, undissociated hydrogen sulphide concentration can be related to the total sulphide concentration:

$$(H_2S)_{aq} = \left[1 + 1.28 \times 10^{(pH\,7)}\right]^{-1} \cdot TS \tag{3.14}$$

Isa *et al.* (1986b) also derived an expression for calculating the free hydrogen sulphide fraction of total sulphide. The derivation was based on the dissociation equilibrium reaction for hydrogen sulphide:

$$f = \left(1 + \frac{K_1}{10^{-pH}}\right)^{-1} \tag{3.15}$$

where K_1 is the dissociation constant at 35^oC (1.49×10^{-7}), and f is the free (undissociated) H_2S fraction of the total dissolved sulphide. Equation 3.15 is valid over a range of pH values, whereas Equation 3.14 is valid at pH 7 only. Equation 3.15 is therefore more useful in predicting the fraction of undissociated hydrogen sulphide in the aqueous phase.

In experimental studies on immobilised biomass systems, Isa *et al.* (1986b) reported that free hydrogen sulphide strongly influenced the aMPB and aSRB. Sulphide inhibition was studied by addition of sulphide both with and without sulphate. Inhibition of aMPB

and hMPB was found only to occur in excess of 1000 $mg\,H_2S.l^{-1}$. This toxicity threshold is substantially higher than the thresholds reported previously (O'Flaherty *et al.*, 1998b; Maillacheruvu and Parkin, 1996; Christensen *et al.*, 1996; Choi and Rim, 1991; McCartney and Oleskiewicz, 1991) but may be attributed to mass transfer limitations of the undissociated hydrogen sulphide into the carrier material (polyurethane foam). Decreasing SRB activity was also reported but uncertainty exists as to whether this was due to sulphide itself, or the lack of available nutrients due to precipitation as insoluble sulphides. No threshold concentrations were reported. Reis *et al.* (1991a,b) showed that the sulphide inhibition effect was due to hydrogen sulphide directly and not a nutrient limitation (caused by sulphide precipitation) since rates increased on sulphide removal. Undissociated hydrogen sulphide concentrations of between 500 and 600 $mg.l^{-1}$ completely inhibited culture growth. Gupta *et al.* (1994a) ran continuous experiments at up to 5000 $mg\,SO_4^{2-}.l^{-1}$. Sulphide inhibition was not observed since iron was added to precipitate sulphide as iron sulphide.

Okabe *et al.* (1992) postulate that the mechanism of hydrogen sulphide inhibition involves an iron-related cell function. The hydrogen sulphide combines with iron in the cytochrome. The resultant iron deficiency causes a decrease in activity of electron transport systems and reduced cell function. Okabe *et al.* (1992) found that at pH 7.0, 150 $mg.l^{-1}$ total sulphide slightly reduced lactate utilisation by *Desulfovibrio desulfuricans* as well as cellular production. Investigations were carried out at 35^oC and a dilution rate of 0.2 h^{-1}. At 280 $mg\,TS.l^{-1}$ cellular production was decreased and at 600 $mg\,TS.l^{-1}$, cell production and lactate utilisation were severely inhibited. A 70% reduction in extent of lactate removal percentage was observed. Szewzyk and Pfennig (1990) found that increased sulphide concentrations decreased the affinity of *Desulfovibrio vulgaris* for ethanol, but did not indicate the levels of sulphate or pH conditions under which this inhibition occurred.

Reis *et al.* (1992) proposed an inhibition function for hydrogen sulphide inhibition:

$$f\left(H_2S\right) = \left(1 - \frac{[H_2S]}{[H_2S]_{max}}\right)^{n_2} \tag{3.16}$$

where $[H_2S]_{max}$ represents the concentration at which sulphate-reducing bacteria are completely inhibited and n_2 is an empirical constant (determined as 0.401). This form of inhibition equation is unsuitable in microbial growth equations since the term can be potentially negative when the hydrogen sulphide concentration goes beyond the predicted maximum value and the fraction $\frac{[H_2S]}{[H_2S]_{max}}$ becomes greater than one. Conventional inhibition terms of the form $\left(1 + \frac{c_i}{K_i}\right)$ cannot be negative regardless of the inhibitor concentration.

3.6.4 Inhibition by Sulphate

The effect of sulphate inhibition on SRB and MPB has been investigated (O'Flaherty and Colleran, 1999; Visser *et al.*, 1993; Reis *et al.*, 1991b; Winfrey and Zeikus, 1977). O'Flaherty and Colleran (1999) found that the addition of 4 $g.l^{-1}$ sulphate to a laboratory-scale anaerobic reactor treating propionate-, butyrate- and ethanol-containing wastewater, severely inhibited acetate removal capability. On hydrogen sulphide removal from the reactor by nitrogen sparging, little improvement in the COD removal efficiency was observed. Re-inoculation of non-sulphate adapted sludge also made no difference to COD removal, suggesting that no improvement could be made in the absence of appropriate sulphate-reducing bacteria or sulphate-reducing syntrophs and methanogenic bacteria. Inoculation of sulphate-adapted sludge led to an improvement in COD removal. Winfrey and Zeikus (1977) found that approximately 19 $mg.l^{-1}$ sulphate temporarily inhibited methane production in freshwater sediments, and that 960 $mg.l^{-1}$ sulphate cause near-complete inhibition. Visser *et al.* (1993) found that in the presence of excess sulphate, the amount of COD (as acetate) removed by sulphate reduction amounted to only 20 % with an 18 % removal of sulphate. In the presence of excess COD, between 5 and 17 % of the COD was removed by sulphate reduction, and 81-90 % of the sulphate was removed. In all experiments COD removal was in excess of 99 %. Gupta *et al.* (1994a) suggest that MPB are not inhibited *per se* by sulphate, but that in the presence of sulphate, SRB outcompete MPB for available substrate thus preventing methane production. This is plausible in the light of the kinetics which have been reviewed.

3.7 Rate Equations

Multiple substrate growth has been discussed in Section 2.5. Such rate equations have been implemented in the modelling of anaerobic biological treatment processes. Robinson and Tiedje (1984) studied the competition between sulphate-reducing and methane-producing bacteria for hydrogen. Batch experiments were carried out at 37^oC with 2.3 $g.l^{-1}$ sulphate. A two-term Michaelis-Menten rate equation was used to describe co-culture experiments:

$$r_s = \frac{r_{s_1}^{max} c_{s_1}}{K_{s_1} + c_{s_1}} + \frac{r_{s_2}^{max} c_{s_2}}{K_{s_2} + c_{s_2}} \tag{3.17}$$

Extensive work has been published on the modelling and simulation of anaerobic digestion and methane producing bacteria. Costello *et al.* (1991a) modelled a single-stage high-rate anaerobic reactor fed with glucose. Microbial groups were modelled using

single substrate, product inhibition rate equations. For glucose- or lactate-consuming bacteria non-competitively inhibited by acetate, the following rate equation was used:

$$r_s = \frac{r_s^{max} c_x c_s}{(K_s + c_s)\left(1 + \frac{c_{Ac}}{K_{Ac}}\right)} \tag{3.18}$$

For propionate- or butyrate-consuming bacteria competitively inhibited by acetate a different rate equation was used:

$$r_s = \frac{r_s^{max} c_x c_s}{c_s + K_s\left(1 + \frac{c_{Ac}}{K_{Ac}}\right)}$$

Moletta *et al.* (1986) proposed similar rate equations for the VFA inhibition of acidogens and methanogens.

Kalyuzhnyi and Fedorovich (1997) developed a model to describe the competition between SRB, MPB, fermentative bacteria (FB) and acetogenic bacteria (AB). In an anaerobic CSTR with a feed stream containing sucrose, propionate, acetate and sulphate at $35°C$ two different rate equations were proposed. For MPB, FB and AB a single substrate rate equation with hydrogen sulphide inhibition was proposed:

$$\mu = \frac{\mu^{max} c_s}{K_s + c_s}\left(1 - \frac{c_{H_2S}}{K_{H_2S}}\right) \tag{3.19}$$

For SRB, a dual substrate rate equation with hydrogen sulphide inhibition was proposed:

$$\mu = \mu^{max} \frac{c_s}{K_s + c_s} \cdot \frac{c_{SO_4^{2-}}}{K_{SO_4^{2-}} + c_{SO_4^{2-}}}\left(1 - \frac{c_{H_2S}}{K_{H_2S}}\right) \tag{3.20}$$

This equation is of the form described in Section 2.5 for bacteria which have multiple substrate dependencies.

3.8 Summary

The mechanism for biological sulphate reduction using ethanol as the carbon source and electron donor (Figure 3.3) has been identified (Szewzyk and Pfennig, 1990; Widdel, 1988; Schink *et al.*, 1985; Laanbroek *et al.*, 1982, 1984). Ethanol is used directly by incomplete-oxidising ethanol-consuming SRB that produce acetate and hydrogen sulphide as reaction products (O'Flaherty *et al.*, 1998a,b; Colleran *et al.*, 1995;

Oude Elferink *et al.*, 1994; Widdel, 1988), or in the presence of hydrogen-consuming consortia, by ethanol-oxidising syntrophs that produce hydrogen as a metabolic product. SRB are able to outcompete MPB for ethanol, acetate and hydrogen both thermodynamically and kinetically. The energy requirement for microbial growth can be obtained directly from ethanol, or via acetogenesis and hydrogen/bicarbonate formation. SRB generally have higher substrate affinities than MPB for common substrates (Colleran *et al.*, 1995; Oude Elferink *et al.*, 1994; Widdel, 1988) and, in the presence of sufficient sulphate, will scavenge a larger proportion of the available substrate. Kinetic parameters μ^{max} and K_{EtOH} for SRB growth have been published, but substrate affinities for sulphate ($K_{SO_4^{2-}}$) are not widely available.

Undissociated hydrogen sulphide has been shown to be inhibitory to both SRB and MPB (Speece, 1996). O'Flaherty *et al.* (1998b) have shown that SRB are more tolerant of hydrogen sulphide toxicity than MPB, although this is not widely accepted (Maillacheruvu and Parkin, 1996; McCartney and Oleskiewicz, 1991). Hydrogen sulphide toxicity of SRB may be modelled by an uncompetitive inhibition model (Maillacheruvu and Parkin, 1996; Reis *et al.*, 1992). There is uncertainty in the literature regarding sulphate *per se* as an inhibitor. Acetate inhibition has been shown to occur, governed by a noncompetitive model (Costello *et al.*, 1991a,b) or an uncompetitive model (Andrews, 1975). Models incorporate acetate inhibition as a function of pH since the undissociated form is known to be inhibitory.

Kalyuzhnyi and Fedorovich (1997) have proposed a dual substrate rate equation with product inhibition. Further rate equations describing sulphate reduction are not available in open literature.

Based on the summary above, it is noted that the kinetics of anaerobic sulphate reduction with a mixed SRB culture using ethanol as the sole carbon source and electron donor need to be investigated to determine substrate affinities for sulphate $\left(K_{SO_4^{2-}}\right)$, inhibition constants for hydrogen sulphide and maximum specific growth rates. Furthermore, the dominant degradation reaction (Figure 3.3) needs to be confirmed. In this investigation, kinetic parameters for a mixed culture of SRB will be presented for batch and continuous culture. Hydrogen sulphide toxicity effects will also be discussed.

Since ethanol-oxidising sulphate reducers require the presence of two substrates for growth (both electron donor, ethanol, and terminal electron acceptor, sulphate), a dual substrate equation to describe the kinetics of their growth is postulated (Kalyuzhnyi and Fedorovich, 1997). The proposed rate equation for sulphate reduction takes the form of the Michaelis-Menten equation for enzyme kinetics modified to include hydrogen sulphide inhibition (Maillacheruvu and Parkin, 1996):

$$-r_{SO_4^{2-}} = \frac{r^{max}_{SO_4^{2-}}}{\left(1 + \frac{K_{s_{EtOH}}}{c_{s_{EtOH}}}\right)\left(1 + \frac{K_{s_{SO_4^{2-}}}}{c_{s_{SO_4^{2-}}}}\right)\left(1 + \frac{c_{i_{HS^-}}}{K_{i_{HS^-}}}\right)} \tag{3.21}$$

or in the form of Monod-type kinetics:

$$r_x = \frac{\mu^{max} c_x}{\left(1 + \frac{K_{s_{EtOH}}}{c_{s_{EtOH}}}\right)\left(1 + \frac{K_{s_{SO_4^{2-}}}}{c_{s_{SO_4^{2-}}}}\right)\left(1 + \frac{c_{i_{HS^-}}}{K_{i_{HS^-}}}\right)}$$

where $-r_{SO_4^{2-}}$ is the rate of consumption of sulphate, r_x is the rate of formation of biomass and $r^{max}_{SO_4^{2-}}$ is the maximum rate of consumption of sulphate. K_{s_i} and K_{i_i} are the half-velocity constants and inhibition constants, and c_{s_i} and c_{i_i} are the substrate and inhibitor concentrations respectively.

Chapter 4

Apparatus and Experimental Methods

4.1 Organisms

Sludge used to inoculate the reactors was obtained from a wine distillery in Wellington, Western Province, South Africa, where it was used in an upflow anaerobic sludge bed (UASB) reactor to reduce the COD of still effluent. The sludge from the UASB was analysed and found to contain a sulphate concentration of approximately 1 $g.l^{-1}$. The ethanol content of the sludge was found to be negligible (less than 0.05 $mg.l^{-1}$). Since the sludge was exposed to sulphate, it was assumed that the mixed culture contained active sulphate-reducing bacteria in association with methane-producing and acid-forming bacteria. Stock cultures were maintained in 1 l vessels at 35^oC, pH 7.4-8.0, an initial sulphate concentration of 5 $g.l^{-1}$ and ethanol concentration of 4.8 $g.l^{-1}$. Sub-culturing was carried out monthly by diluting a 30 % inoculum in fresh sulphate and ethanol containing media.

4.2 Medium Composition and Preparation

The composition of the nutrient solution and trace element solution used was that of Visser (1996). The media was prepared from a basal nutrient solution (Table 4.1), trace element solution (Table 4.2) and ethanol. The basal nutrient solutions and trace element solutions were prepared using de-ionised water and reagents of analytical grade. Sodium sulphate was added to the basal medium as required to obtain the desired sulphate (SO_4^{2-}) concentration for the experiment.

Table 4.1: Basal nutrients used in the inoculation of the batch and continuous experiments (Visser, 1996)

Compound	Mass ($g.l^{-1}$)
$NaHCO_3$	2.0
NaH_2PO_4	0.47
K_2HPO_4	0.60
NH_4Cl	0.28
$MgSO_4.7H_2O$	0.111
$CaCl_2.2H_2O$	0.007
Yeast Extract	0.2
Na_2SO_4	as required

Table 4.2: Trace elements used in inoculation- and feed media

Compound	Mass ($g.l^{-1}$)
$FeCl_2.4H_2O$	0.95
$MnCl_2.4H_2O$	0.786
Resazurine	0.5
EDTA	0.5
Na_2SeO_3	0.1
H_3BO_3	0.05
$ZnCl_2$	0.05
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.05
$AlCl_3.6H_2O$	0.09
$NiCl_2.6H_2O$	0.05
$CoCl_2.6H_2O$	0.05
$CuCl_2.2H_2O$	0.05

The feed solutions for the culture were prepared in 2 l stock bottles. Once prepared, the solutions were autoclaved for sterilisation to prevent microbial growth in the stock solution. Sterilisation was carried out for 20 minutes at 121^oC and 1 atm. The sterilised media was cooled to 4^oC and 2 $ml.l^{-1}$ trace elements were added to the basal medium. Reagent grade ethanol was added as required to make up the desired ethanol:sulphate ratio.

4.3 Experimental Apparatus

A 1 l reactor, submerged in a water bath to maintain a constant temperature of 35 $\pm$ 0.5°C, was used for all experiments. The Quick-Fit glass fittings were sealed using Dow Corning vacuum grease to ensure that the system remained anaerobic. The reactor was agitated using a pitched-blade turbine to provide axial and radial flow mixing, to ensure homogeneity and to prevent the settling of suspended solids. An agitation rate of 500

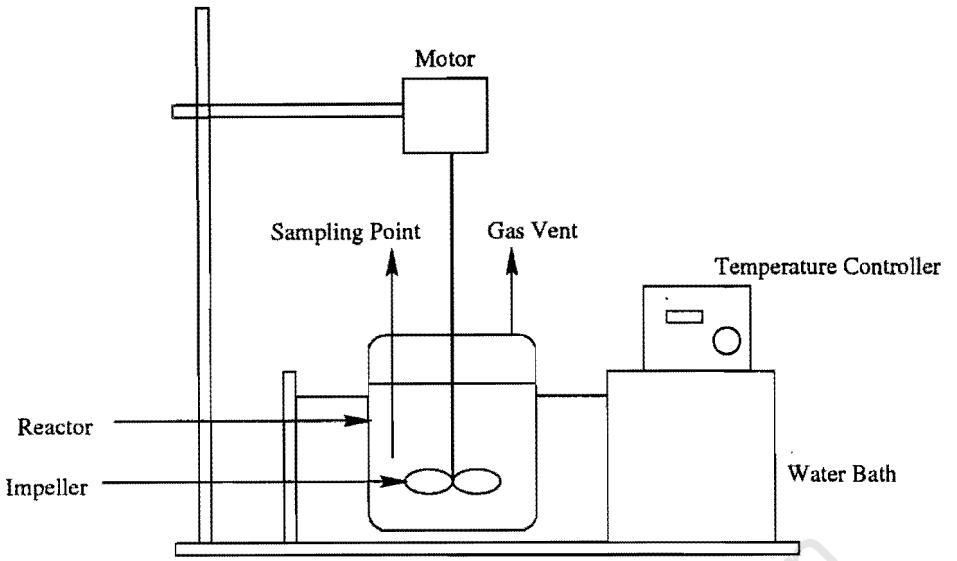

Figure 4.1: Schematic of experimental apparatus

rpm was used. A sample tube was installed in the reactor to enable the extraction of samples. *In situ* pH measurements were made through a port in the reactor lid. These measurements were made under a nitrogen atmosphere. pH was controlled manually between 7.4 and 7.8 by addition of 3 $mol.l^{-1}$ sodium hydroxide or 32 % hydrochloric acid. The gas outlet was passed through a zinc acetate solution to remove gaseous hydrogen sulphide and prevent oxygen from entering the reactor head space. The experimental rig is depicted by the schematic drawing, Figure 4.1.

4.4 Sampling Procedure

Samples of 10 *ml* were taken with a syringe directly from the reactor under a nitrogen atmosphere to prevent oxygen from entering the reactor. Samples were taken twice weekly for analysis. The sample pH was measured immediately after sampling. Samples were centrifuged for 6 minutes in 1.5 *ml* microfuge tubes at 14 000 *rpm* in a Hettich Mikro 12-24 centrifuge to remove suspended solids and biomass. Samples were stored at 4^oC.

4.5 Analytical Procedures

All aqueous samples were removed from the refrigerator and filtered through 0.45 μm Schleicher & Schuell disposable filters prior to analysis.

4.5.1 Ethanol Concentration

Ethanol concentration was determined by gas chromatography. A Perkin Elmer AutoSystem GC with a 25 m methyl silicon capillary column and flame ionisation detector (FID) was used. The gas chromatograph (GC) temperature program to control the column temperature was run with a linear gradient from 60 to 120^oC at 20$^oC.min^{-1}$ and the detector and injector temperatures were set to 250^oC. The helium carrier gas was set at a flowrate of 30 $cm^3.min^{-1}$. Samples were analysed directly using n-butanol as an internal standard. The volume injected was 1.6 μl. The GC was connected to a PE Nelson Model 1022 data system and integrator. The standard deviation observed in ethanol concentration was 6 %.

4.5.2 Sulphate Concentration

A Metrohm 690 Ion Chromatograph (version 2.690.0010 with manually operated injector) was used for the chromatographic analysis of sulphate. A PRP-X100 anion column packed with polystyrene/divinylbenzene copolymer with quaternary ammonium groups was used for separation. The column was operated isothermally at 35^oC. The mobile phase (eluent) consisted of dilute phthalic acid at a flowrate of 2 $ml.min^{-1}$ maintained by a Metrohm 697 IC pump. The phthalic acid eluent was prepared as follows:

1. Phthalic acid concentrate was prepared by adding 3.323 g of phthalic acid, 2 ml 30% NaOH and 10 ml acetone to 950 ml of de-ionised water in a 1 l volumetric flask. The pH was adjusted to pH 4.5 with 5 M NaOH and the solution then made up to 1 l with de-ionised water.

2. Diluted eluent was used in the column. This was achieved by mixing 100 ml of concentrate with 75 ml acetone and making the mixture up to 1 l with de-ionised water.

Standard solutions of sulphate (0.1, 0.25, 0.5 and 0.75 $mmol.l^{-1}$) were prepared. These external standards were used to generate a linear standard curve from which the concentration of sulphate in the samples was determined. Samples containing sulphate concentrations in excess of 0.75 $mmol.l^{-1}$ were diluted with de-ionised water until the concentration fell into the appropriate range. A constant injection volume of 20 μl was measured with a sample loop.

Since the peak shape of the chromatograms generated was constant, the height of the peak was used to evaluate the concentration of sulphate in the sample. Peak height was

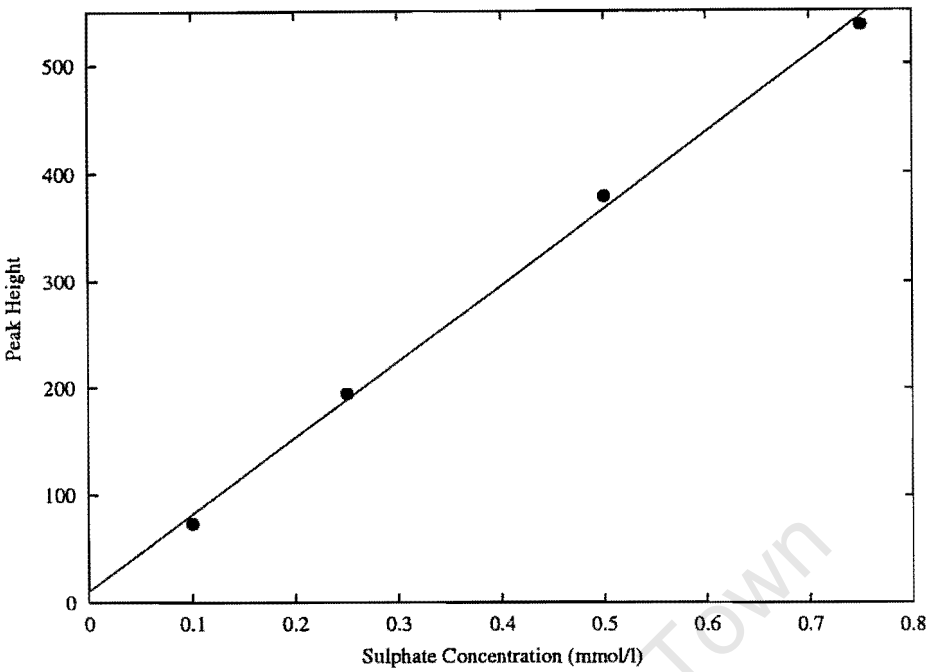

Figure 4.2: Standard curve for sulphate determination

measured as the distance from the baseline to the peak maximum. A sample calibration curve used for sulphate determination is shown in Figure 4.2. The detection limit of sulphate was 0.05 $mmol.l^{-1}$ and a 10 % standard deviation was observed.

4.5.3 Gas Phase Concentrations (H_2S, CO_2 and CH_4)

Gas phase compositions were measured by gas chromatography on a Perkin Elmer AutoSystem GC. A HayeSep-Q column, using helium as the carrier gas, was operated isothermally at 150^oC at a carrier flowrate of 30 $cm^3.min^{-1}$. Injector and detector ports were operated at 200 and 220^oC respectively. 10 μl injections were made using a gas-tight syringe. Analyses were qualitative since standard curves were not generated.

4.5.4 Acetic Acid Concentration

A Beckman Gold High Pressure Liquid Chromatograph (HPLC) was used for determining acetate concentrations. The HPLC was fitted with a Wakosil-II 5C18RS carbonic acid column. A 20 mM phosphoric acid solution, adjusted to pH 2.5 with NaH_2PO_4, was used as the eluent. UV absorbance at 210 nm was used to measure the concentration acetic acid in the samples. A sample loop on the manual injector ensured a constant sample injection volume of 50 μl. The column operated isothermally at 30^oC with an

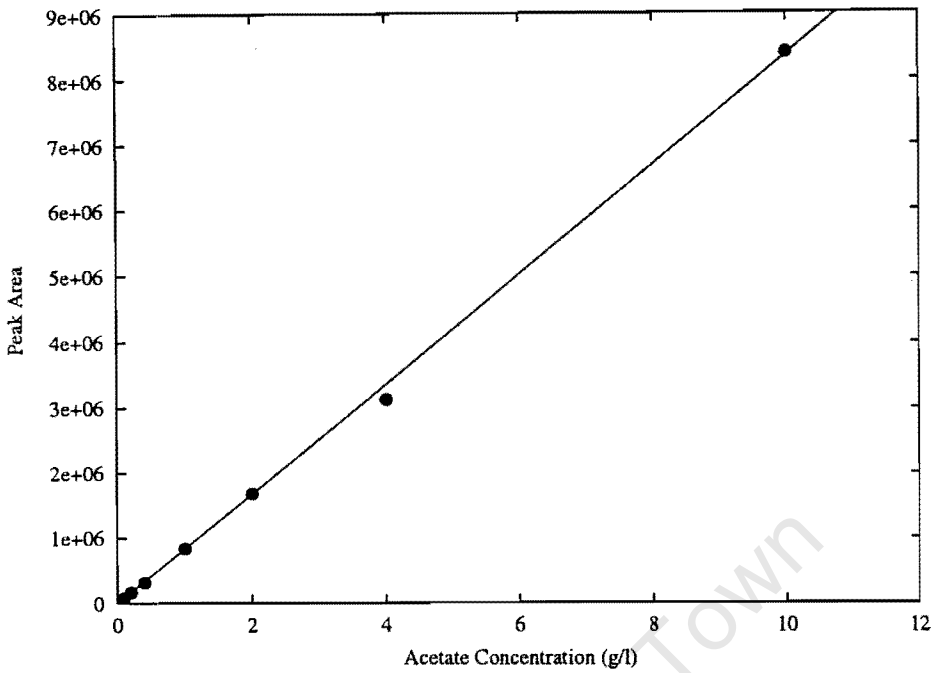

Figure 4.3: Standard curve for acetate determination

eluent flow of 1 $ml.min^{-1}$. A calibration curve made with external standards prepared by diluting glacial acetic acid in de-ionised water. A sample calibration curve used for sulphate determination is shown in Figure 4.3. The detection limit of the HPLC was 0.01 $g.l^{-1}$ and a 4 % standard deviation was observed.

4.6 Sulphate Reduction by a Mixed Culture of Anaerobic Microorganisms Using a Batch Stirred Tank Reactor

The effect of ethanol:sulphate ratio and sulphate concentration on the reduction of sulphate was studied in a batch system. A mixed culture of anaerobic microorganisms (as described in Section 4.1) was used. A four-step inoculation procedure was followed. First, reactors were charged with 700 ml of nutrient solution and 2 ml trace elements, the composition of which is described in Section 4.2. The system was then allowed to equilibrate and de-oxygenated using a nitrogen sparge. Ethanol was added to obtain the desired bulk reaction concentration and, finally, the reactor was inoculated with 300 ml anaerobic sludge (30 % inoculum). The sludge, consisting of some granules, was mixed to ensure homogeneity before it was added to the reactor. Experimental apparatus was configured and maintained at conditions as described in Section 4.3. Experiments were run until either the ethanol or sulphate was depleted, or until no time change in any

species concentration was evident. Daily sampling was carried out as described in Section 4.4. Ethanol, sulphate and acetate were monitored and analysed as described in Section 4.5.

4.7 Kinetics of Continuous Biological Sulphate Reduction by a Mixed Culture of Anaerobic Microorganisms

Continuous experiments were used to determine the kinetics of biological sulphate reduction and to investigate the effect of sulphate loading rate on the extent of sulphate removal. Experiments were started as batch systems (described in Section 4.6). When the rate of sulphate reduction was seen to decrease, conversion to continuous culture was performed. Equipment was configured and maintained at conditions as described in Section 4.3. The volume of the reactor was maintained at a constant level by positioning the outlet pipe at the 1 l mark of the reactor. Overflow was through a U-tube device. A Masterflex Console Drive 1-100 rpm pump with Masterflex L/S Size 13 standard pump head and Size 13 Masterflex tubing was used to pump the nutrient solution into the reactor. 250 ml bottles were used for feed reservoirs. Stock solutions were kept at 4^oC and feed reservoirs were filled daily with fresh feed. The flowrate was monitored by mass difference to ensure the correct amount of nutrient solution was pumped into the reactor. Experiments were conducted at a range of hydraulic residence times from 10 days to 4 days. Samples were extracted a regular intervals (Section 4.4), depending on the retention time. Ethanol, sulphate and acetic acid were routinely monitored and analysed as described in Section 4.5. Steady operation was defined as a less than 10 % fluctuation in sulphate, ethanol and acetate concentration over 3-5 retention times (Roels, 1983; Pirt, 1975).

Chapter 5

Results and Discussion

In this chapter, experimental kinetic studies on biological sulphate reduction are presented. Both batch stirred tank and continuous-flow stirred reactor data is shown. Kinetic parameters are determined and compared with literature data. Transient state data from the continuous flow experiments is also presented with reference to hydrogen sulphide toxicity. Finally, simulation data is presented.

5.1 Sulphate Reduction Using a Batch Stirred Tank Reactor

The objectives of the batch experiments were to determine the effect of ethanol:sulphate ratio and sulphate concentration on the sulphate reduction process and to determine batch kinetics for the process. Five batch experiments were carried out in which pH, sulphate concentration, acetate concentration, and ethanol concentration were monitored over time. Figures 5.1, 5.2, 5.3, 5.4 and 5.5 show concentration-time data for five batches with different ethanol:sulphate ratios. Startup conditions for the batch experiments are summarised in Table 5.1. Batches 1 to 4 were inoculated with suspended cell culture as described in Section 4.6. Batch 5 was inoculated predominantly using the granules from the sulphidogenic sludge.

Results from Batches 1-4 (Figures 5.1 to 5.5) indicate that ethanol is consumed, with the concomitant reduction of sulphate and formation of acetate. Similar observations have been documented previously (Christensen *et al.*, 1996; Colleran *et al.*, 1995; Widdel, 1988).

Table 5.1: Startup conditions for batch experiments

Batch number	Figure number	Initial Sulphate ($mmol.l^{-1}$)	Initial Ethanol ($mmol.l^{-1}$)	Ethanol: Sulphate Ratio ($mmol.mmol^{-1}$)
1	5.1	17	47	2.7
2	5.2	38	86	2.3
3	5.3	34	62	1.8
4	5.4	56	53	0.93
5	5.5	48	66	1.4

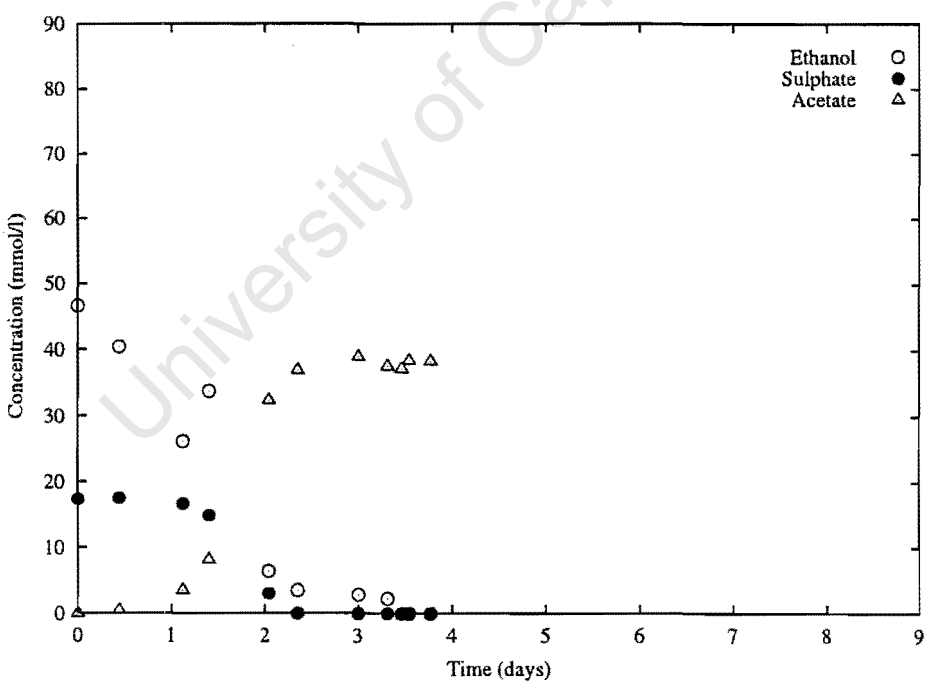

Figure 5.1: Batch experiment concentration-time trends with an initial sulphate concentration of 17 $mmol.l^{-1}$ (1.6 $g.l^{-1}$) at an ethanol:sulphate ratio of 2.7 $mmol.mmol^{-1}$

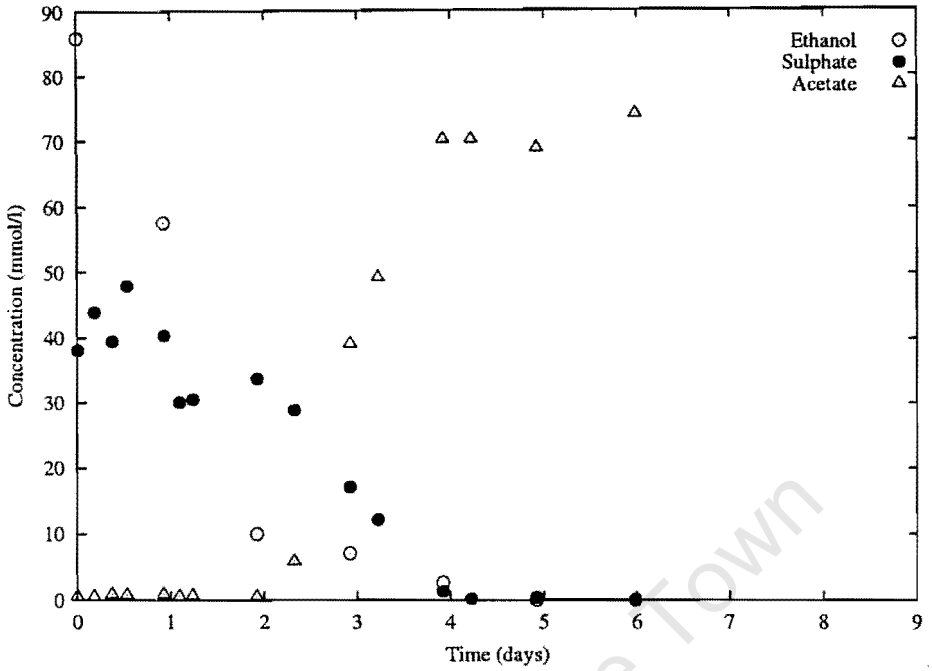

Figure 5.2: Batch experiment concentration-time trends with an initial sulphate concentration of 38 $mmol.l^{-1}$ (3.6 $g.l^{-1}$) at an ethanol:sulphate ratio of 2.3 $mmol.mmol^{-1}$

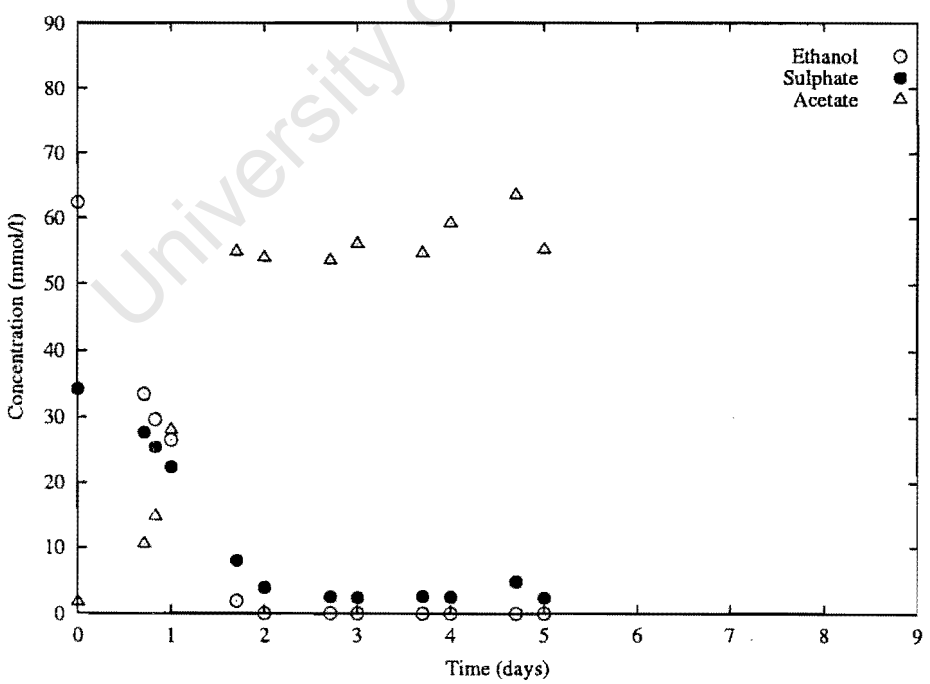

Figure 5.3: Batch experiment concentration-time trends with an initial sulphate concentration of 34 $mmol.l^{-1}$ (3.3 $g.l^{-1}$) at an ethanol:sulphate ratio of 1.8 $mmol.mmol^{-1}$

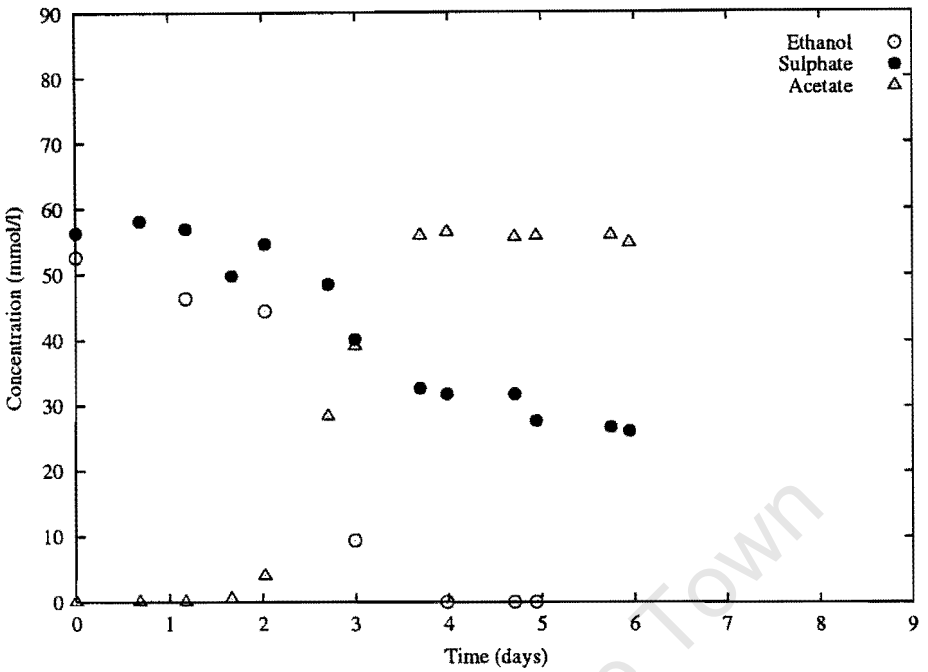

Figure 5.4: Batch experiment concentration-time trends with an initial sulphate concentration of 56 $mmol.l^{-1}$ (5.4 $g.l^{-1}$) at an ethanol:sulphate ratio of 0.93 $mmol.mmol^{-1}$

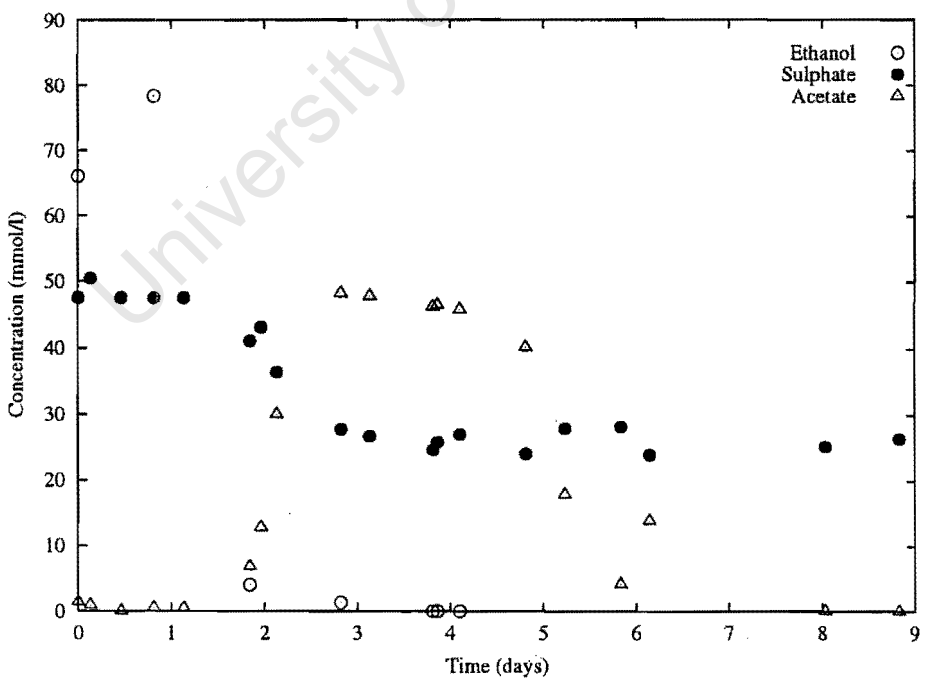

Figure 5.5: Batch experiment concentration-time trends with an initial sulphate concentration of 48 $mmol.l^{-1}$ (4.6 $g.l^{-1}$) at an ethanol:sulphate ratio of 1.4 $mmol.mmol^{-1}$

Table 5.2: Ratios of acetate produced to ethanol consumed and sulphate consumed to ethanol consumed in batch experiments

Batch Number	Ethanol:Sulphate Ratio ($mmol.mmol^{-1}$)	$\frac{\text{Acetate produced}}{\text{Ethanol consumed}}$ ($mmol.mmol^{-1}$)	$\frac{\text{Sulphate consumed}}{\text{Ethanol consumed}}$ ($mmol.mmol^{-1}$)
1	2.7	0.82	0.37
2	2.3	0.81	0.44
3	1.8	0.95	0.51
4	0.93	1.04	0.57
5	1.4	0.72	0.36

According to the reaction for incomplete ethanol oxidation to acetate by SRB (Widdel, 1988):

$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H^+ + 2H_2O + HS^- \quad (5.1)$$

acetate and ethanol are formed and consumed in stoichiometric proportion ($1\ \frac{mmol}{mmol}$), and sulphate consumed is half the molar consumption of ethanol ($\frac{1}{2}\frac{mmol}{mmol}$). The theoretical analysis of the reaction stoichiometry (Section 2.7) indicates that acetate and ethanol are formed and consumed in a $0.98\frac{mmol}{mmol}$ proportion, and the sulphate to ethanol consumption ratio is $0.498\ \frac{mmol}{mmol}$.

Table 5.2 shows the ratios of acetate produced to ethanol consumed as well as sulphate consumed to ethanol consumed. Ratios indicate that acetate is not present in stoichiometric proportion to the amount ethanol consumed (i.e., a ratio of 1:1 on a molar basis). Periodic gas phase measurements showed a small methane peak, suggesting the presence of acetotrophic MPB which may consume some acetate. Figure 5.6 shows a gas chromatogram for a head space gas phase analysis indicating the presence of methane and hydrogen sulphide.

However, on depletion of ethanol (where acetate is no longer formed as a reaction product by incomplete oxidation) (Figure 5.3 and 5.4), acetate is not consumed. The inability to consume acetate on ethanol depletion where sulphate remains available (Figure 5.4), suggested that acetotrophic SRB were not active. The ratios indicate that incomplete oxidation of ethanol by SRB is favoured where ethanol and sulphate are in a 2:1 molar ratio (i.e., stoichiometrically supplied) (Figure 5.3) and below (Figure 5.4). This is expected since SRB are known to compete well for available substrate under substrate limiting conditions (Colleran *et al.*, 1995; Oude Elferink *et al.*, 1994; Choi and Rim, 1991). Where organic substrate is in excess of SRB requirements (Batch 1 and 2), other microbial species are able to use the COD for energy generation, and ratios of sulphate and acetate relative to ethanol consumed deviate from those predicted by Equation 5.1.

The predominance of incomplete ethanol-oxidising sulphate-reducing bacteria in the sul-

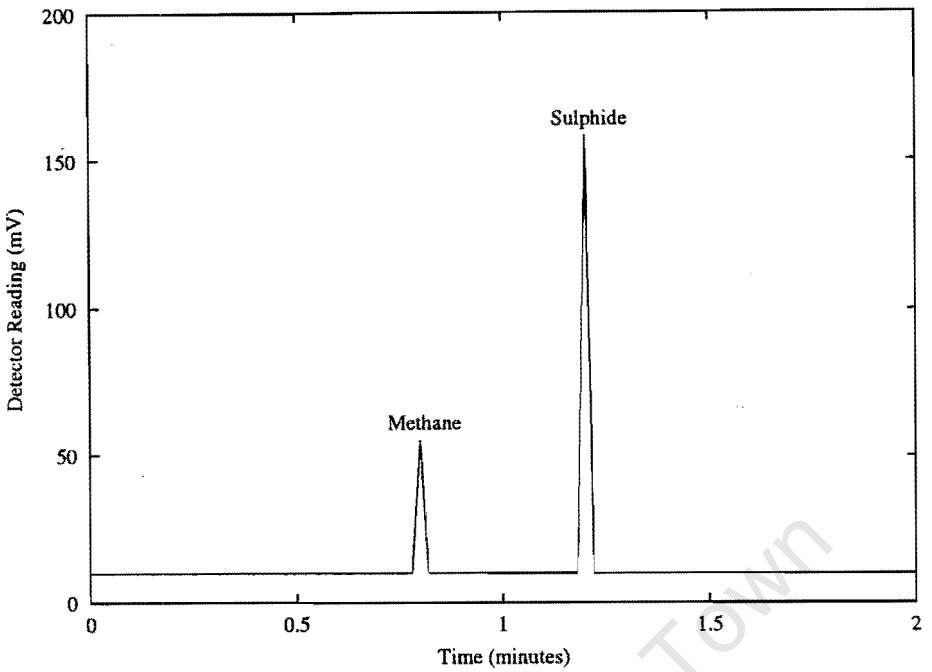

Figure 5.6: Gas chromatogram for a head space gas phase analysis indicating the presence of methane and hydrogen sulphide (Batch 3)

phate reduction system is in agreement with O'Flaherty *et al.* (1998a), Christensen *et al.* (1996) and Widdel (1988). Methane producers are present in the system (gas phase methane analyses confirm this) but do not compete well for substrate. MPB are expected to consume the available acetate COD in the absence of sulphate, but this is not observed. MPB are known to be inhibited by a variety of chemical species, including sulphate (Colleran *et al.*, 1995), sodium cations (Hulshoff Pol *et al.*, 1998) and hydrogen sulphide (McCartney and Oleskiewicz, 1991; Maillacheruvu and Parkin, 1996). Hence, the accumulation of hydrogen sulphide in the batch system may inhibit subsequent acetate consumption by aMPB on sulphate exhaustion. aSRB are inhibited by acetate and hydrogen sulphide. The presence of these inhibitors may explain why aSRB do not consume available acetate for sulphate reduction.

Batch 5 (Figure 5.5) was inoculated differently to Batches 1-4. Instead of inoculating with a mixture of granules and suspended cells (as described in Section 4.6), settled granules were added to the reactor with fewer suspended cells than in previous inocula. It is well known that MPB favour granule formation while SRB are less well adapted to colonising surfaces or granules (Speece, 1996). Figure 5.5 shows that ethanol was consumed with the simultaneous reduction of sulphate, and acetate was formed as a byproduct over the first three days of batch operation. On depletion of ethanol, acetate was consumed without sulphate reduction indicating methanogenic use of acetate. Gas phase measurements confirmed that methane was present in a much larger proportion

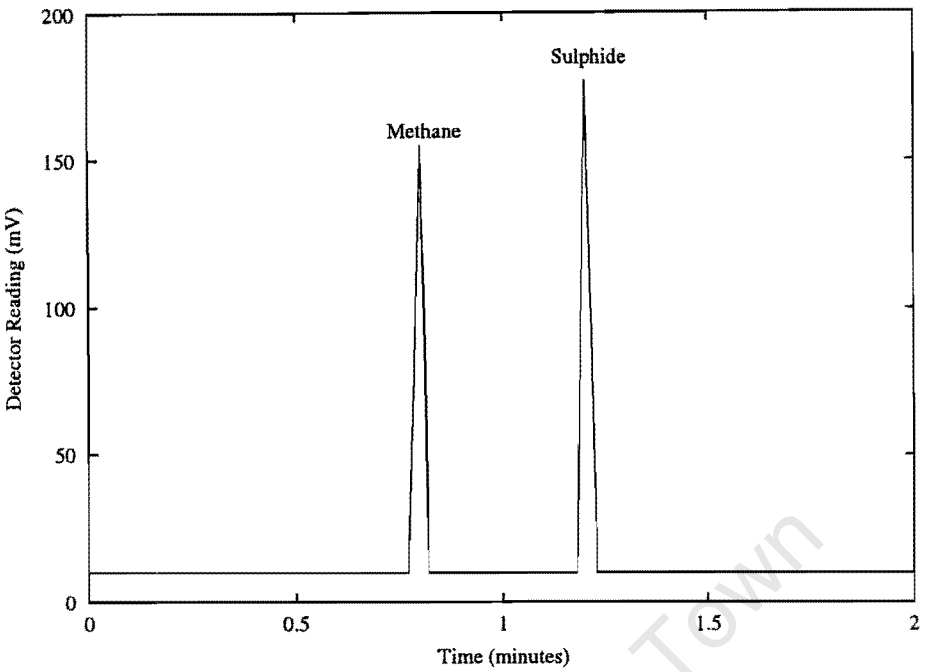

Figure 5.7: Gas chromatogram for a head space gas phase analysis indicating an increased methane concentration (Batch 5)

than previous experiments (Figure 5.7).

Speece (1996) and O'Flaherty *et al.* (1998a) have indicated that MPB excrete extracellular polysaccharides (EPS) that allow for good flocculation and that SRB do not exhibit this phenomenon. Hence, inoculation with granules in place of freely suspended microbes favours the presence of MPB, resulting in the enhanced methanogenic use of acetate owing to improved ability of aMPB to compete the existing organic substrate.

Kinetic parameters were determined from the batch data. In order to extract kinetic parameters from the data, it was necessary to linearise the rate equation for simple Michaelis-Menten kinetics:

$$r_s = -\frac{dc_s}{dt} = \frac{r_{max}}{1 + \frac{K_m}{c_s}} \tag{5.2}$$

Separating the differential equation, inserting integration limits and integrating results in :

$$c_s + K_m ln(c_s) - c_{so} - K_m ln(c_{so}) = -r_{max} t \tag{5.3}$$

Table 5.3: A summary of batch kinetic data

Batch	Sulphate Conc. ($mmol.l^{-1}$)	EtOH:Sulphate Ratio ($mmol.mmol^{-1}$)	$K_{SO_4^{2-}}$ ($mmol.l^{-1}$)	$r_{max,\,SO_4^{2-}}$ ($\frac{mmol.l^{-1}}{h}$)	K_{EtOH} ($mmol.l^{-1}$)	$r_{max,\,EtOH}$ ($\frac{mmol.l^{-1}}{h}$)
1	17.4	2.69	0.146	5.69	0.298	15.7
2	38.0	2.26	0.071	6.13	0.124	27.6
3	34.2	1.82	0.070	8.20	0.141	18.8
4	56.3	0.93	0.056	4.78	0.117	9.39
5	47.5	1.39	0.055	3.92	0.117	9.60

Rewriting this expression by making it equal to zero :

$$0 = c_{so} + K_m ln(c_{so}) - c_s - K_m ln(c_s) - r_{max} t \tag{5.4}$$

allows the kinetic parameters to be determined by a least squares fit by minimisation of the sum of squared errors between the predicted values and the experimental values. Table 5.3 summarises the kinetic data obtained from the parameter estimation procedures.

K_m is shown as a function of ethanol:sulphate ratio in Figure 5.8. K_m stays constant across the range of ethanol:sulphate ratios of 0.93-2.26 at approximately 0.055-0.071 $mmol.l^{-1}$ for sulphate and 0.117-0.141 $mmol.l^{-1}$ for ethanol. The K_{EtOH} values fall within the broad range reported in the literature of 0.007 $mmol\,ethanol.l^{-1}$ (Szewzyk and Pfennig, 1990) to 0.65 $mmol\,ethanol.l^{-1}$ (O'Flaherty *et al.*, 1998b) for ethanol-oxidising SRB. Kremer *et al.* (1988) reported K_m-range for ethanol of 0.12-0.23 $mmol.l^{-1}$. This corresponds with the range obtained in this investigation.

$K_{SO_4^{2-}}$ has been previously reported by Moosa (2000) and O'Flaherty *et al.* (1998b) over a sulphate range of 10-156 $mmol.l^{-1}$ and 42 $mmol.l^{-1}$ respectively. Comparable values published by O'Flaherty *et al.* (1998b) for a mixed culture of sulphate-reducing bacteria in anaerobic sludge were reported to be in the range 0.30-0.31 $mmol.l^{-1}$. Moosa (2000) found $K_{SO_4^{2-}}$ for an enrichment culture of aSRB of 0.74 $mmol.l^{-1}$. The values determined in this investigation fall in the range 0.055-0.071 $mmol.l^{-1}$. It is well known that SRB are good scavengers of residual sulphate, i.e., they have a high substrate affinity, quantified by a low $K_{SO_4^{2-}}$ (Isa *et al.*, 1986a).

SRB inhibition by sulphate has been suggested previously (O'Flaherty and Colleran, 1999; Visser *et al.*, 1993; Reis *et al.*, 1991b; Winfrey and Zeikus, 1977) (Section 3.6.4). O'Flaherty and Colleran (1999) found that the addition of 42 $mmol.l^{-1}$ sulphate to a laboratory-scale anaerobic reactor treating propionate-, butyrate- and ethanol-containing wastewater severely inhibited acetate removal capability. Choi and Rim (1991) found

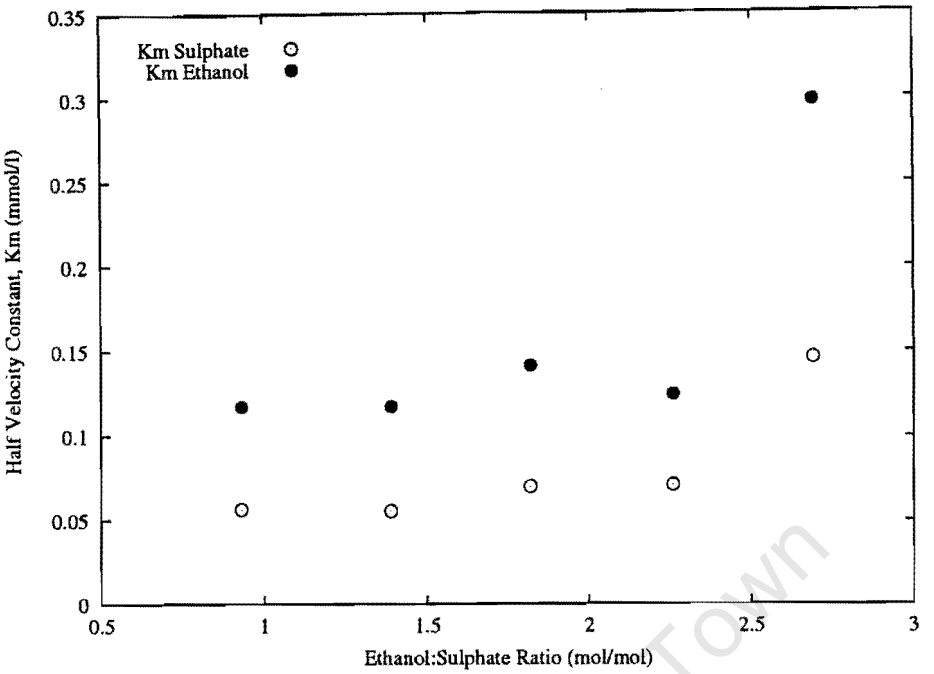

Figure 5.8: Variation of K_m with ethanol:sulphate ratio

that 21 $mmol.l^{-1}$ sulphate inhibited SRB activity when in association with MPB.

The inhibitory effect of sulphate in the context of competitive inhibition, would be measurable through a reduction in the affinity of the microorganisms for their substrate, i.e., an increase in the K_m value. K_m is shown as a function of sulphate concentration in Figure 5.9. Over the concentration range 30-60 $mmol.l^{-1}$ (corresponding ethanol:sulphate ratio 0.93-2.26) K_m does not vary significantly. This is not consistent with the expected behaviour of a competitive inhibitor in which it is predicted that K_m should increase with an increasing inhibitor (sulphate) concentration thereby reducing substrate affinity.

In order to determine whether sulphate was a noncompetitive inhibitor, r_{max} is given as a function of sulphate concentration in Figure 5.10. In noncompetitive inhibition, r_{max} is reduced as inhibitor concentration increases (Section 2.3.3). While the trends in Figure 5.10 are are unclear, it may be suggested that r_{max} decreases with increasing sulphate concentration. This is consistent with the presence of a noncompetitive inhibitor.

A plot of maximum substrate removal rate, r_{max}, against the ethanol:sulphate ratio (Figure 5.11) indicates that $r_{max,SO_4^{2-}}$ passes through a maximum at an ethanol:sulphate ratio between 1.5 and 2. Given that ethanol-oxidising SRB in the system (incomplete oxidisers) reduce sulphate by reaction 5.1:

$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H^+ + 2H_2O + HS^-$$

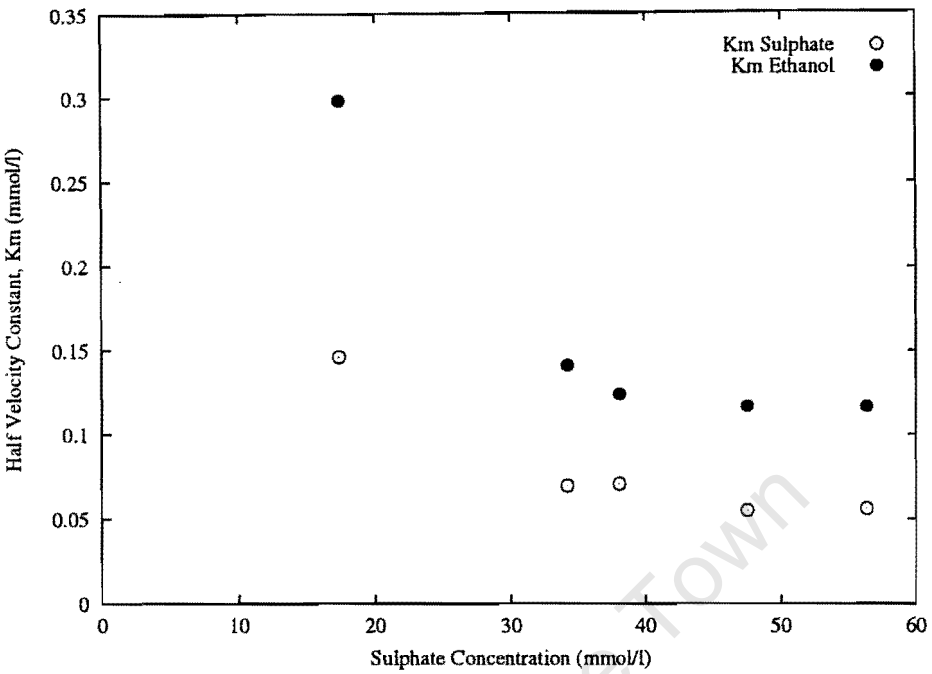

Figure 5.9: Variation of K_m with sulphate concentration

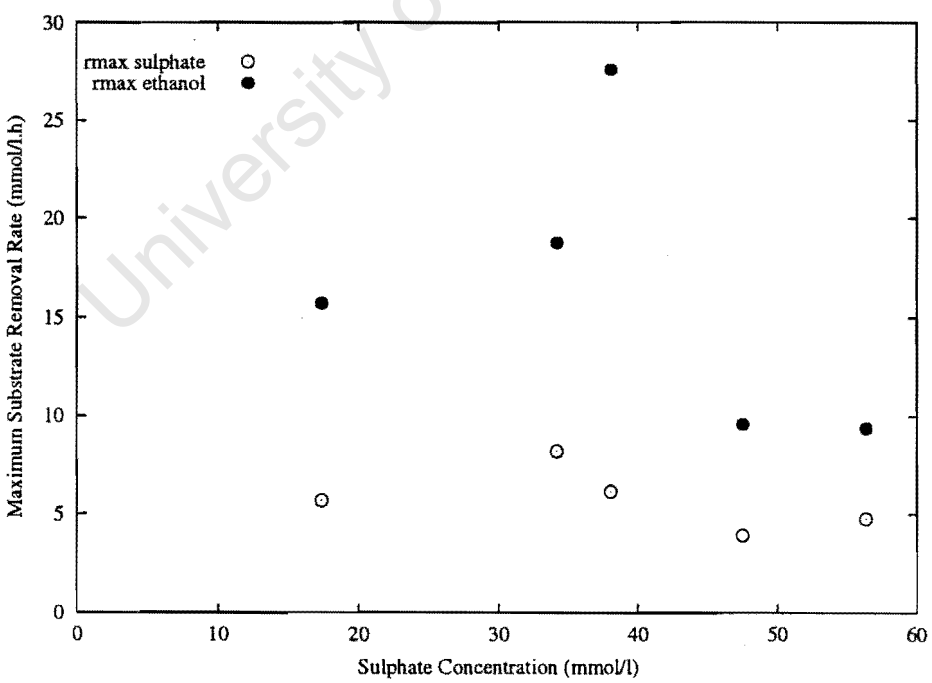

Figure 5.10: Variation of maximum substrate removal rate, r_{max}, with sulphate concentration

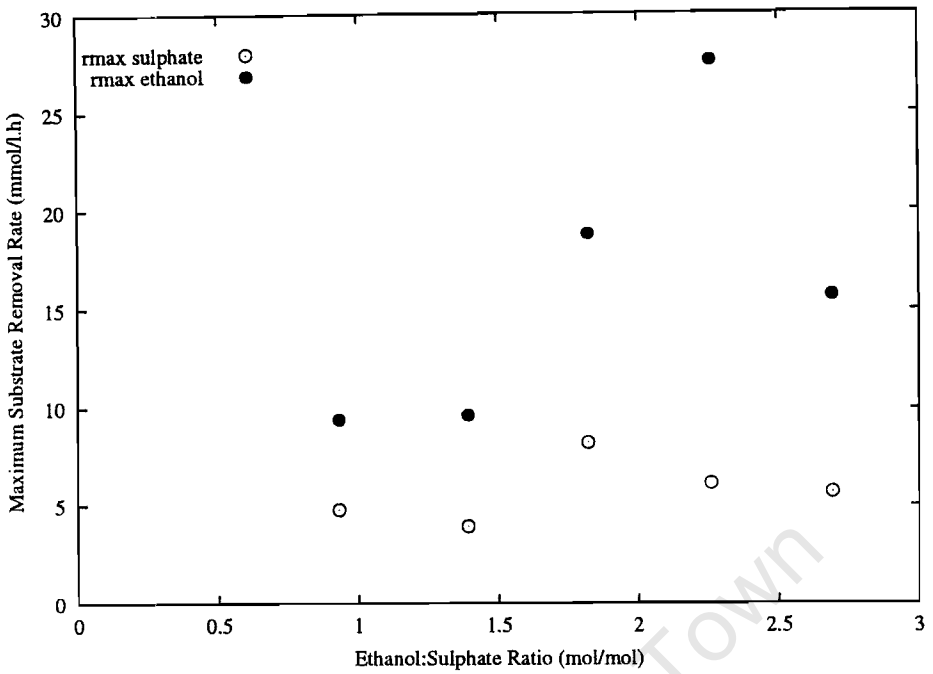

Figure 5.11: Variation of maximum substrate removal rates, r_{max}, with ethanol:sulphate ratio

a maximum rate would be expected where ethanol and sulphate are in a 2:1 (*mmol* : *mmol*) ratio. The r_{max} for ethanol passes through a maximum at an ethanol:sulphate of 2.0-2.5. At these ratios, where ethanol is slightly in excess of the proportion needed for sulphate reduction, other microbial groups are able to use the available organic source. It has been reported by Choi and Rim (1991) that SRB and MPB are competitive where the organic substrate is in stoichiometric supply, and that SRB dominate where sulphate is in excess. The data presented is consistent with this observation: the substrate removal rates are maximum where ethanol and sulphate are supplied in the stoichiometric proportion necessary for sulphate reduction.

The maximum conversion of sulphate does not correspond with the maximum removal rates. Figure 5.12 shows that sulphate conversion increased from 44% at an ethanol to sulphate ratio of 0.93, to 100% at a ratio of 2.68. This was expected since below a ratio of 2, ethanol was limiting. Since no other carbon sources were available for sulphate reduction, and acetate was not consumed, conversion of sulphate ceased when ethanol was depleted.

The maximum theoretical rate of ethanol consumption is twice the maximum rate of sulphate consumption where incomplete ethanol oxidation is the predominant reaction. Where ethanol and sulphate were in stoichiometric proportions (or where sulphate was in excess), this was observed (Figure 5.13). Where ethanol was in excess, the ratio

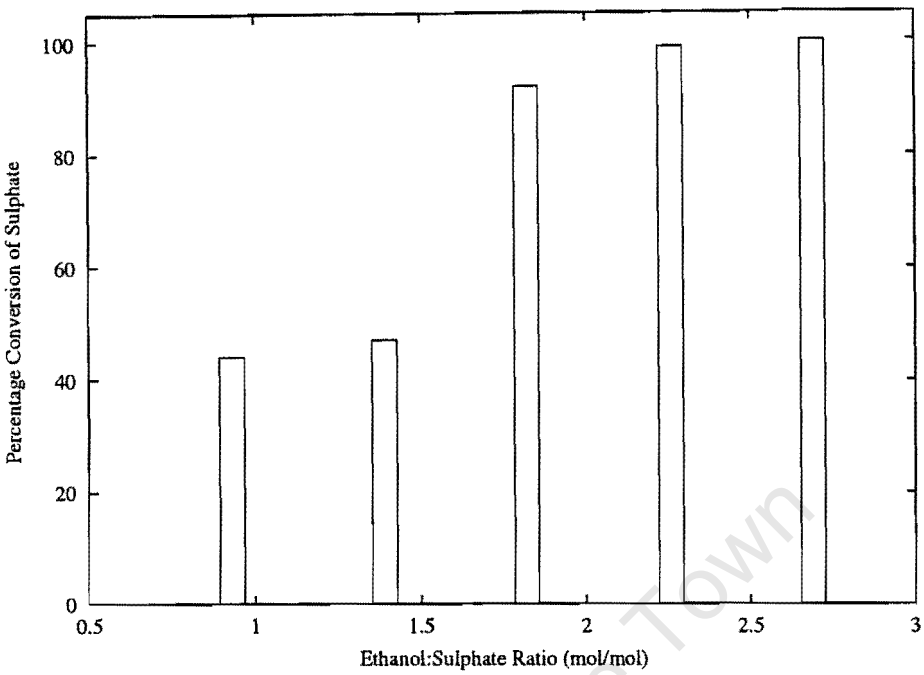

Figure 5.12: Batch conversion of sulphate as a function of ethanol:sulphate ratio

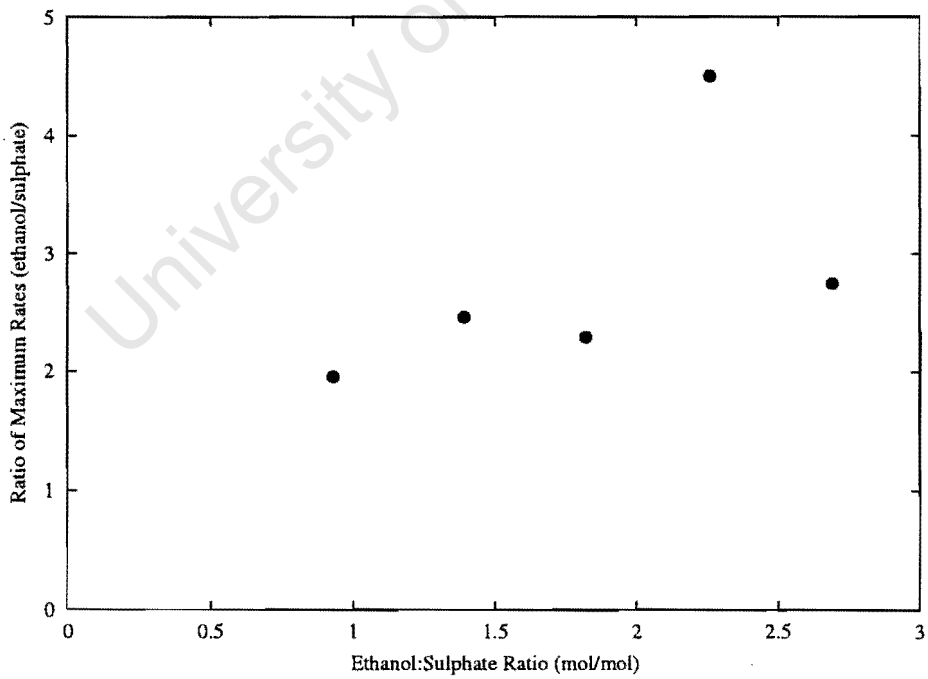

Figure 5.13: Ratio of maximum substrate utilisation rates $\left(\frac{r_{max,EtOH}}{r_{max,SO_4^{2-}}}\right)$ versus ethanol:sulphate ratio

exceeded 2, indicating that ethanol was used by SRB for sulphate reduction and by acetogenic bacteria for acetate formation. SRB competed better for organic substrate where substrate was limiting, and less effectively when ethanol was in excess. This is consistent with the findings of Choi and Rim (1991).

5.2 Transient Continuous Culture Operating Data

Four continuous culture experiments were operated each at a different feed sulphate concentration: 10.4, 26, 52.1 and 78.1 $mmol.l^{-1}$ (1, 2.5, 5 and 7.5 $g.l^{-1}$). Organic substrate (ethanol) was supplied in a 2:1 $\frac{mmol}{mmol}$ ratio. Table 5.4 summarises the operating conditions of each continuous culture experiment. Hydraulic retention times of 10, 6, 5 and 4 days were investigated.

During the 10 day retention time experiments, stable oscillations were observed in the sulphate concentrations. Figures 5.14 to 5.17 show the sulphate oscillations for four different feed sulphate concentrations (1, 2.5, 5 and 7.5 $g.l^{-1}$). Error bars indicate a 10 % standard deviation associated with the sulphate measurement. Tables 5.5 to 5.8 show the time variations in pH and hydrogen sulphide concentration for each continuous culture experiment at a 10 day hydraulic retention time. Aqueous hydrogen sulphide levels were calculated based on the number of millimoles of sulphate reduced, by using the relationship developed by Isa *et al.* (1986b) for determining the fraction of the total sulphide present as undissociated hydrogen sulphide in the aqueous phase (Equation 3.15):

$$f_{H_2S_{(aq)}} = \left(1 + \frac{1.49 \times 10^{-7}}{10^{-pH}}\right)$$

Points of inhibition were taken to be troughs in sulphate concentration (peaks in hydrogen sulphide concentration) and recovery points were taken to be peaks of sulphate concentration (troughs in hydrogen sulphide concentration).

Table 5.4: A summary of operating parameters for continuous culture experiments at different sulphate concentrations (values in parentheses in $g.l^{-1}$)

Sulphate Concentration ($mmol.l^{-1}$)	Ethanol Concentration ($mmol.l^{-1}$)	Running Time (days)	HRTs Investigated (days)
10.4 (1.0)	20.9 (0.96)	171	6
26.0 (2.5)	52.2 (2.40)	480	10, 6, 5, 4
52.1 (5.0)	104.3 (4.79)	213	10, 6
78.1 (7.5)	156.3 (7.19)	265	10, 6

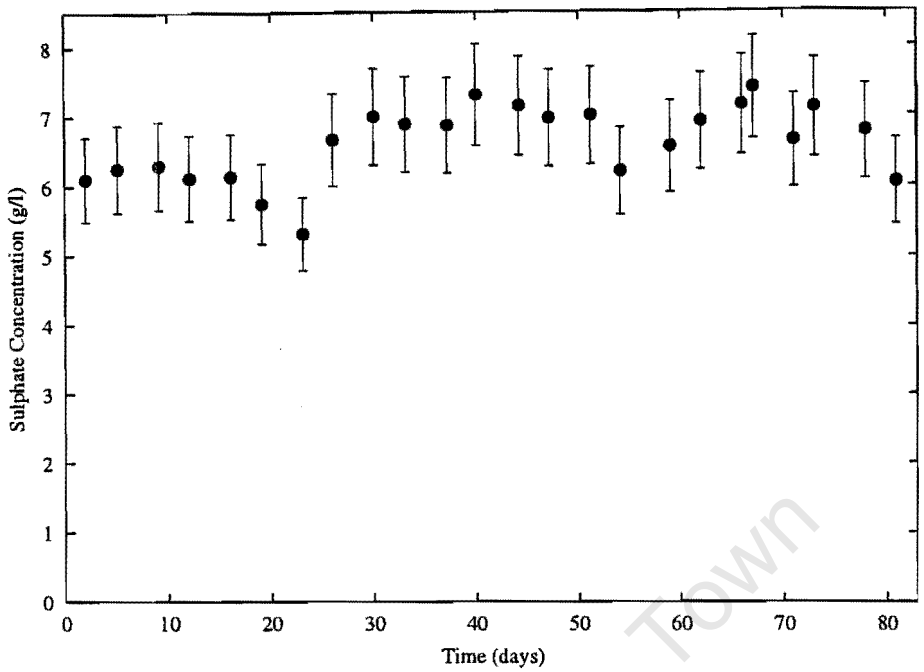

Figure 5.14: Oscillations in sulphate concentration for a continuous culture experiment treating a feed sulphate concentration of 78.1 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) at a hydraulic retention time of 10 days

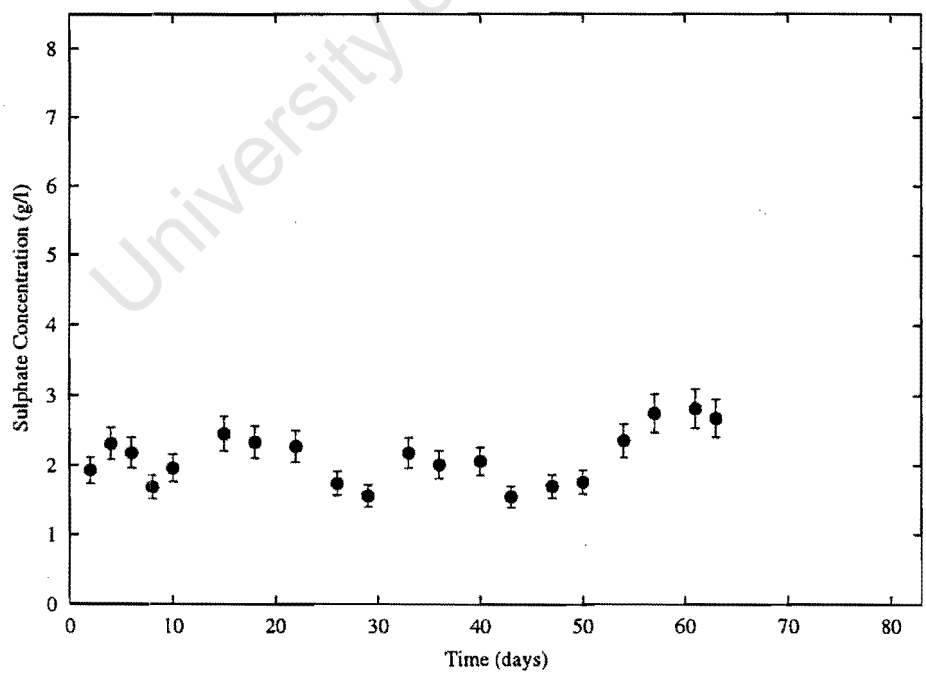

Figure 5.15: Sulphate oscillations for a continuous culture experiment treating a feed sulphate concentration of 52.1 $mmol.l^{-1}$ (5 $g.l^{-1}$) at a retention time of 10 days

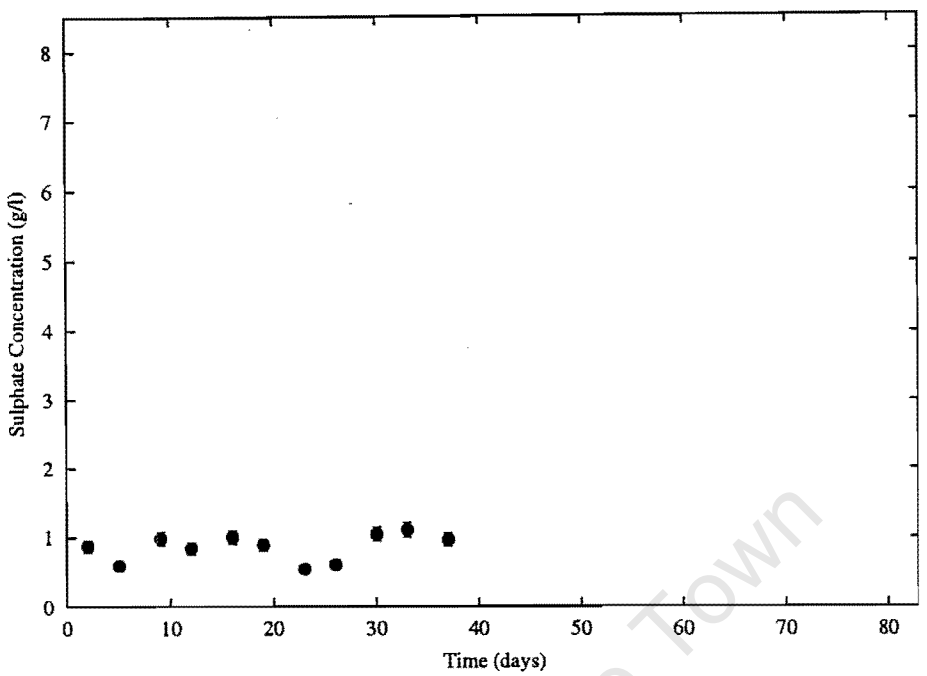

Figure 5.16: Sulphate oscillations for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) at a retention time of 10 days

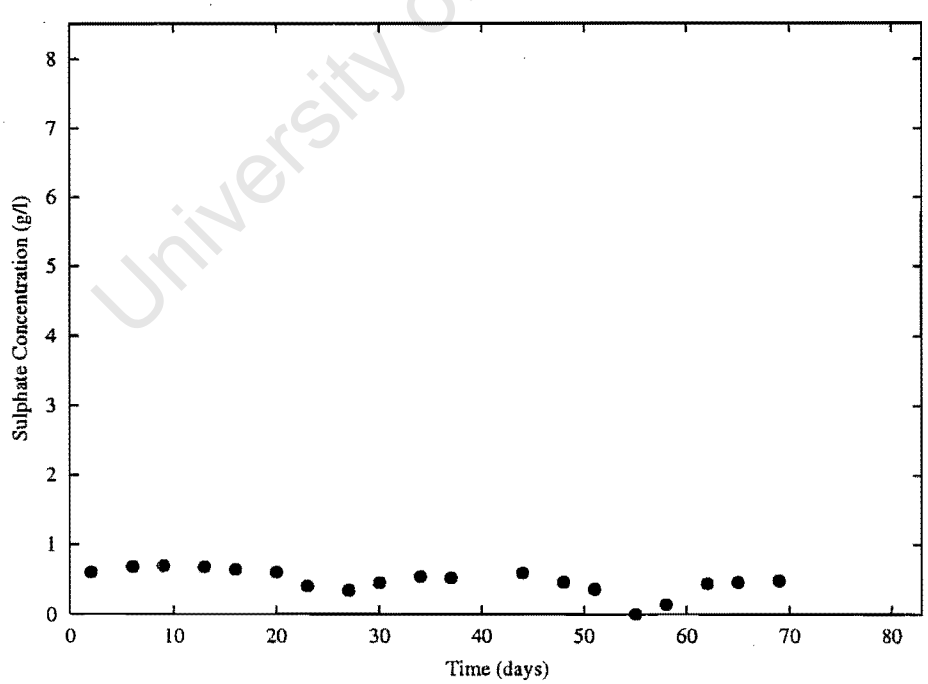

Figure 5.17: Sulphate oscillations for a continuous culture experiment treating a feed sulphate concentration of 10.4 $mmol.l^{-1}$ (1.0 $g.l^{-1}$) at a retention time of 10 days

Table 5.5: Hydrogen sulphide concentrations for a continuous culture experiment treating a feed sulphate of 78 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) at a HRT of 10 days

Day	Sulphate Reduced ($mmol.l^{-1}$)	Total Sulphide ($mmol.l^{-1}$)	pH	Fraction $H_2S_{(aq)}$	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
9	12.6	12.6	7.43	0.199	86	Recovery
23	22.8	22.8	7.31	0.247	192	Inhibition
40	2.1	2.1	6.71	0.567	41	Recovery
54	13.5	13.5	7.10	0.348	160	Inhibition
62	1.04	1.04	7.03	0.385	14	Recovery
81	15.1	15.1	7.08	0.358	184	Inhibition

Table 5.6: Hydrogen sulphide concentrations for a continuous culture experiment treating a feed sulphate of 52.1 $mmol.l^{-1}$ (5 $g.l^{-1}$) at a HRT of 10 days

Day	Sulphate Reduced ($mmol.l^{-1}$)	Total Sulphide ($mmol.l^{-1}$)	pH	Fraction $H_2S_{(aq)}$	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
4	28.0	28.0	7.68	0.123	117	Recovery
8	34.5	34.5	8.12	0.048	57	Inhibition
15	26.6	26.6	7.31	0.247	224	Recovery
29	35.8	35.8	7.34	0.235	286	Inhibition
33	29.4	29.4	7.06	0.369	369	Recovery
43	35.9	35.9	7.86	0.085	103	Inhibition
61	22.7	22.7	7.40	0.211	163	Recovery

Table 5.7: Hydrogen sulphide concentrations for a continuous culture experiment treating a feed sulphate of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) at a HRT of 10 days

Day	Sulphate Reduced ($mmol.l^{-1}$)	Total Sulphide ($mmol.l^{-1}$)	pH	Fraction $H_2S_{(aq)}$	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
5	19.9	19.9	7.49	0.178	121	Inhibition
16	15.6	15.6	7.25	0.274	145	Recovery
23	20.4	20.4	7.24	0.279	193	Inhibition
33	14.6	14.6	7.14	0.447	162	Recovery

Table 5.8: Hydrogen sulphide concentrations for a continuous culture experiment treating a feed sulphate of 10.4 $mmol.l^{-1}$ (1 $g.l^{-1}$) at a HRT of 10 days

Day	Sulphate Reduced ($mmol.l^{-1}$)	Total Sulphide ($mmol.l^{-1}$)	pH	Fraction $H_2S_{(aq)}$	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
9	3.2	3.2	7.6	0.144	16	Recovery
27	6.9	6.9	7.59	0.147	35	Inhibition
44	4.3	4.3	7.64	0.133	20	Recovery
55	10.4	10.4	7.36	0.227	80	Inhibition

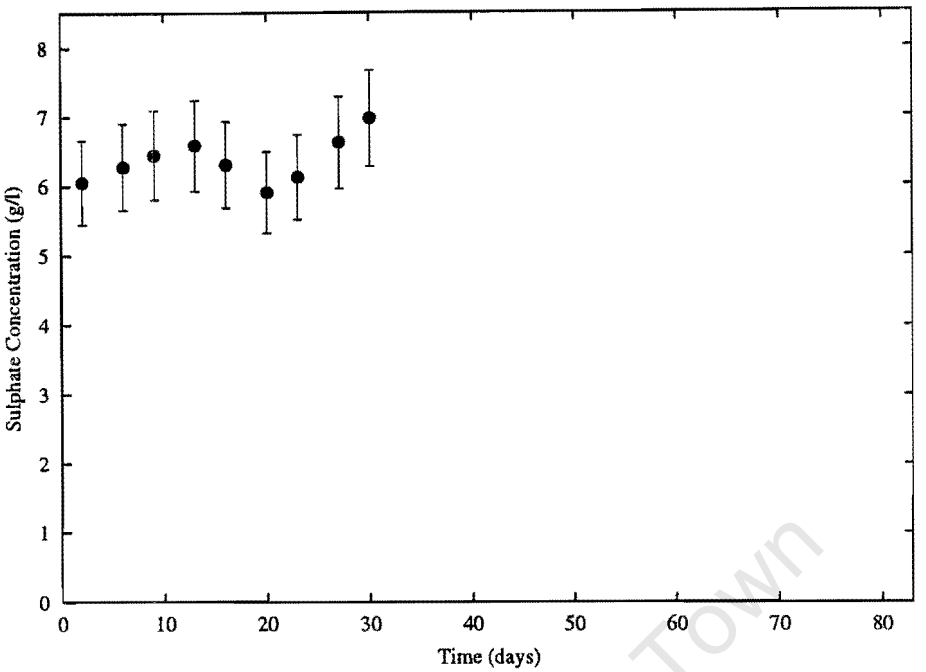

Figure 5.18: Oscillations in sulphate concentration in a continuous culture experiment treating a feed sulphate concentration of 78.1 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) at a hydraulic retention time of 6 days

Table 5.9: Hydrogen sulphide concentrations for a continuous culture experiment treating influent sulphate of 78 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) at a HRT of 6 days

Day	Sulphate Reduced ($mmol.l^{-1}$)	Total Sulphide ($mmol.l^{-1}$)	pH	Fraction $H_2S_{(aq)}$	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
2	15.1	15.1	7.08	0.358	184	Inhibition
13	9.6	9.6	7.62	0.139	45	Recovery
20	16.7	16.7	6.92	0.447	254	Inhibition
30	5.5	5.5	7.32	0.243	45	Recovery

Figure 5.18 shows the same oscillatory pattern in sulphate concentration for the continuous culture experiment treating an influent sulphate concentration of 78.1 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) at a hydraulic retention time of 6 days. Data is summarised in Table 5.9.

O'Flaherty and Colleran (1999) observed similar trends in continuous culture operating data. Sulphate concentrations varied between 1 $g.l^{-1}$ and 2 $g.l^{-1}$ for a continuous culture experiment treating a feed sulphate concentration of 4 $g.l^{-1}$ over a 200 day period. This can be compared with oscillations between 1.5 and 2.8 $g.l^{-1}$ for the continuous culture data presented here treating 5 $g.l^{-1}$ sulphate. Complementary hydrogen sulphide oscillations (total sulphide) reported by O'Flaherty and Colleran (1999) were between 0.3 $g.l^{-1}$ and 1 $g.l^{-1}$ (8.8-29.4 $mmol.l^{-1}$). Gupta *et al.* (1994a) claim that steady state operation is impossible under competitive conditions. They observed stable oscillations

Table 5.10: pH effect on hydrogen sulphide inhibition concentrations for continuous culture experiments treating 78 $mmol.l^{-1}$ and 52.1 $mmol.l^{-1}$ at a 10 day HRT

78 $mmol.l^{-1}$ feed sulphate				52.1 $mmol.l^{-1}$ feed sulphate			
Total Sulphide ($mmol.l^{-1}$)	pH	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State	Total Sulphide ($mmol.l^{-1}$)	pH	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
12.6	7.43	86	Recovery	28.0	7.68	117	Recovery
22.8	7.31	192	Inhibition	34.5	8.12	57	Inhibition
2.1	6.71	41	Recovery	26.6	7.31	224	Recovery
13.5	7.10	160	Inhibition	35.8	7.34	286	Inhibition
1.04	7.03	14	Recovery	29.4	7.06	369	Recovery
15.1	7.08	184	Inhibition	35.9	7.89	103	Inhibition
-	-	-	-	22.7	7.40	163	Recovery

in COD levels, and attributed the lengthy stabilisation period to the overlapping ranges of K_s- values of SRB and MPB.

It was hypothesised that the oscillations observed resulted from the inhibition of sulphate reduction by the presence of a critical hydrogen sulphide concentration. According to this hypothesis, in continuous culture, the microorganisms reduced sulphate until the sulphide product became inhibitory. Once this sulphide level was reached, the sulphate reducing ability of the SRB was suppressed. Sulphate reduction stopped, and hydrogen sulphide concentrations gradually decreased as fresh feed was supplied. Since sulphide toxicity is reversible (O'Flaherty *et al.*, 1998b) as soon as the aqueous hydrogen sulphide levels were reduced, sulphate reduction is expected to commence, regenerating hydrogen sulphide thus restarting the cycle.

Data for the 78 $mmol.l^{-1}$ feed sulphate concentration at both 10 day and 6 day retention times (Table 5.5 and 5.9) indicate that sulphate reduction proceeds until an undissociated hydrogen sulphide concentration in the range 160-192 $mg.l^{-1}$ is reached. This corresponds with a total sulphide concentration of 13.5-16.7 $mmol.l^{-1}$ (446-551 $mg.l^{-1}$). O'Flaherty *et al.* (1998b) report 50% inhibition concentrations of undissociated hydrogen sulphide for ethanol-consuming SRB between 158.5-256 $mg.l^{-1}$ (total sulphide 788-990 $mg.l^{-1}$) over the pH range 7.2-7.6.

Table 5.10 shows the pH effect on hydrogen sulphide inhibition concentrations for continuous culture experiments treating 78 $mmol.l^{-1}$ and 52.1 $mmol.l^{-1}$. The experiment treating a feed sulphate concentration of 52.1 $mmol.l^{-1}$ was run a a marginally higher pH (pH 7.3-7.7) than the 78 $mmol.l^{-1}$ experiment (pH 7.1-7.3). The speciation of hydrogen sulphide is highly sensitive to pH across this range, with lower pH favouring undissociated hydrogen sulphide. The concentrations of undissociated hydrogen sulphide in the 52.1 $mmol.l^{-1}$ experiment do not adequately explain the inhibition effect. However, at

Table 5.11: Sulphate effect on hydrogen sulphide inhibition concentrations for continuous culture experiments treating 78 $mmol.l^{-1}$ and 10.4 $mmol.l^{-1}$ at a 10 day HRT

78 $mmol.l^{-1}$ feed sulphate				10.4 $mmol.l^{-1}$ feed sulphate			
Total Sulphide ($mmol.l^{-1}$)	pH	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State	Total Sulphide ($mmol.l^{-1}$)	pH	$H_2S_{(aq)}$ ($mg.l^{-1}$)	State
12.6	7.43	86	Recovery	3.2	7.6	16	Recovery
22.8	7.31	192	Inhibition	6.9	7.59	35	Inhibition
2.1	6.71	41	Recovery	4.3	7.64	20	Recovery
13.5	7.10	160	Inhibition	10.4	7.36	80	Inhibition
1.04	7.03	14	Recovery	-	-	-	-
15.1	7.08	184	Inhibition	-	-	-	-

the increased pH, undissociated hydrogen sulphide, cited as the inhibitory species by Speece (1996), constitutes a smaller fraction of the total sulphide concentration. These lower concentrations will allow sulphate reduction to proceed further. This is confirmed by the elevated total sulphide concentrations in the 52.1 $mmol.l^{-1}$ experiment (23-36 $mmol.l^{-1}$) compared with the 78 $mmol.l^{-1}$ experiment (1-22 $mmol.l^{-1}$). Under these pH conditions (7.3-7.7) total sulphide concentrations eventually become inhibitory. Inhibition periods correspond with total sulphide concentrations of 34.5-35.9 $mmol.l^{-1}$ (1139-1185 $mg.l^{-1}$) and recovery periods with 22.7-28.0 $mmol.l^{-1}$ (749-924 $mg.l^{-1}$) total sulphide. This is consistent with O'Flaherty *et al.* (1998b) who reported that below pH 7.2 inhibition is due to undissociated hydrogen sulphide while above pH 7.2 it is due to total sulphide.

Table 5.11 illustrates the sulphate effect on hydrogen sulphide inhibition concentrations for continuous culture experiments treating 78 $mmol.l^{-1}$ and 10.4 $mmol.l^{-1}$ at a 10 day HRT. Microorganisms under exposure to lower sulphate concentrations (10.4 $mmol.l^{-1}$) (Figure 5.17 and Table 5.8) have not had the opportunity to adapt to hydrogen sulphide. As a result, the inhibitory hydrogen sulphide concentrations are lower than those for the microorganisms in the 78 $mmol.l^{-1}$ experiment (run at a similar pH), and process performance is negatively affected.

Inhibition of SRB has previously been reported to occur at 9 $mg\ H_2S.l^{-1}$ (Maillacheruvu and Parkin, 1996). This is generally lower than the hydrogen sulphide levels determined for the reported sets of experiments. Christensen *et al.* (1996) reported that SRB are inhibited by total hydrogen sulphide concentrations of 40-150 $mg.l^{-1}$ however no pH data was reported hence concentrations of undissociated hydrogen sulphide cannot be calculated. The discrepancies between the reported values and the data presented may be attributed to pH fluctuations in the range 6.8-7.4. pH fluctuations in this range result in significant changes in concentration of undissociated hydrogen sulphide in the aqueous phase. Furthermore, the tolerance of a microbial culture to sulphide is known

Table 5.12: Amplitude and frequency of sulphate oscillations as a function of feed sulphate concentration

	10 day HRT			6 day HRT		
Feed Sulphate ($g.l^{-1}$)	Amplitude ($g.l^{-1}$)	(% of feed)	Period (d)	Amplitude ($g.l^{-1}$)	(% of feed)	Period (d)
7.5	1.9	25	29	0.68	9.1	18
5.0	1.3	26	21	-	-	-
2.5	0.60	24	19	-	-	-
1.0	0.31	31	28	-	-	-

to depend on the component species present as well as previous exposure to sulphide.

Figure 5.19 shows a graphical comparison of the oscillations in the four different continuous culture experiments at a 10 day hydraulic retention time. Table 5.12 shows the amplitude and period of the sulphate oscillations as a function of feed sulphate concentration. It was expected that the amplitude would increase as a percentage of the feed sulphate concentration as the feed sulphate concentration was decreased (knowing that the microbial species in each experiment were from the same stock). The microorganisms would reduce the same amount of sulphate, resulting in an equivalent concentration of total sulphide. This amount of sulphate would constitute a larger proportion of the feed sulphate at low sulphate concentrations, before inhibition was observed. The amplitude of the oscillations were constant as a percent of the feed sulphate concentration as feed concentration was decreased. This suggested that the microbial populations adapted to different levels of hydrogen sulphide. The magnitude of the amplitude (measured in $g.l^{-1}$ sulphate) supported this. More sulphate was reduced at higher sulphate concentrations (consequently producing higher hydrogen sulphide concentrations) suggesting that the microorganisms are able to cope with higher inhibitor concentrations.

The period of the oscillations increased as feed sulphate concentration was increased. This indicated that although a higher volumetric reduction rate was found at higher sulphate concentrations (owing to an increased volumetric loading rate), the increased hydrogen sulphide concentrations resulted in an extended lag time before the microorganisms could function optimally. The amplitude and period of the oscillation for a 6 day retention time were reduced. This was expected since dilution rate effects would not allow microorganisms to reduce sulphate as efficiently (reducing the amplitude) and hydrogen sulphide was washed out at an increased rate, reducing the recovery/inhibition cycle time.

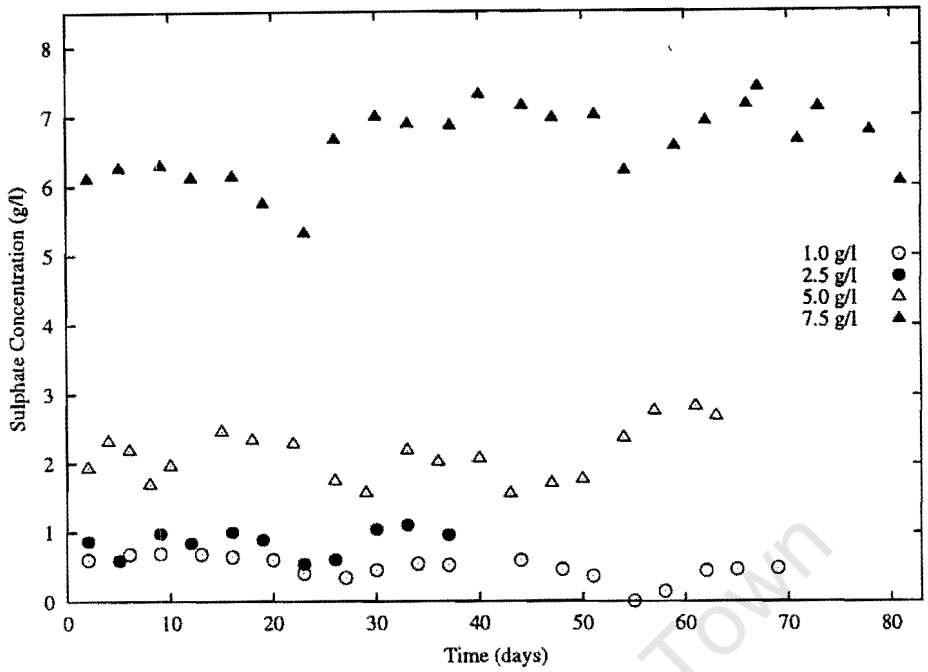

Figure 5.19: Sulphate oscillations for continuous experiments treating four different feed sulphate concentrations at a 10 day retention time

5.3 The Effect of Hydrogen Sulphide Toxicity

5.3.1 Batch Culture

In order to confirm the presence of hydrogen sulphide toxicity in the experiments conducted, both batch and continuous experiments were sparged with nitrogen gas to removed aqueous hydrogen sulphide. Figure 5.20 shows data collected for a batch inoculated with 78.1 $mmol.l^{-1}$ sulphate without nitrogen sparging as a means of hydrogen sulphide removal (Batch A). Over an initial 20 day period, sulphate reduction was observed. Thereafter, the sulphate concentration remained constant for a further 6 days. Over this initial 26 day period 26 $mmol.l^{-1}$ ethanol was consumed and 12 $mmol.l^{-1}$ sulphate was reduced. This corresponds to a total sulphide production of 361 $mg.l^{-1}$. At the end conditions (pH 7.95) the undissociated fraction of the total sulphide in the aqueous phase can be calculated from Equation 3.15 (Isa *et al.*, 1986b). The aqueous hydrogen sulphide concentration was:

$$[H_2S] = f_{H_2S_{(aq)}} \times [\text{total sulphide}]$$

$$= 0.07 \times 361\, mg.l^{-1} = 25\, mg.l^{-1}$$

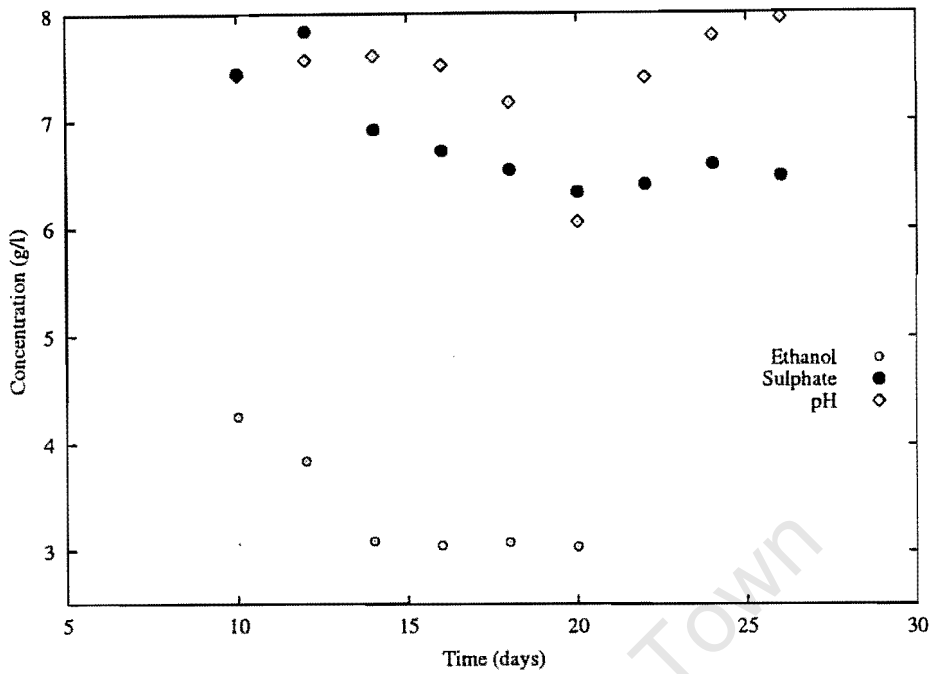

Figure 5.20: Batch experiment treating an initial sulphate concentration of 78.1 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) without hydrogen sulphide removal by means of nitrogen sparging (Batch A)

Following this batch, the reactor was converted to continuous culture and the experiment was operated at a 10 day hydraulic retention time for 103 days, and at a 6 day hydraulic retention time for 28 days before the reactor began to fail (characterised by a gradually increasing sulphate concentration over time). The maximum sulphate mass conversion during this time was 8 % (6.3 $mmol.l^{-1}$) at the 10 day HRT and 13 % (10.4 $mmol.l^{-1}$) at the 6 day HRT. To prevent the culture from washing out of the reactor it was returned to batch operation for recovery. Figure 5.21 represents the recovery period data (Batch B). The initial concentration of aqueous hydrogen sulphide for Batch B was calculated to be 57 $mg.l^{-1}$ at pH 7.16. Over an initial period of 20 days, 13.4 $mmol.l^{-1}$ (17 %) sulphate was reduced and thereafter remained relatively constant for a further 11 days. During this time, pH fluctuated from 7.17 to 7.95. The total sulphide concentration was 451 $mg.l^{-1}$ and the aqueous undissociated hydrogen sulphide concentration was between 142 $mg.l^{-1}$ (pH 7.17) to 32 $mg.l^{-1}$ (pH 7.95).

In order to promote sulphate reduction, nitrogen sparging was introduced on day 42 to strip the aqueous hydrogen sulphide from the reaction vessel. Nitrogen was sparged through the vessel for 90 seconds at 4 $l.min^{-1}$ every alternate day. Over the following 21 days, both ethanol consumption and sulphate reduction were increased. Sulphate concentrations decreased to 35.8 $mmol.l^{-1}$ representing a conversion of 23.8 $mmol.l^{-1}$ (40 %) from the initial stagnant period where the sulphate concentration was 59.6 $mmol.l^{-1}$.

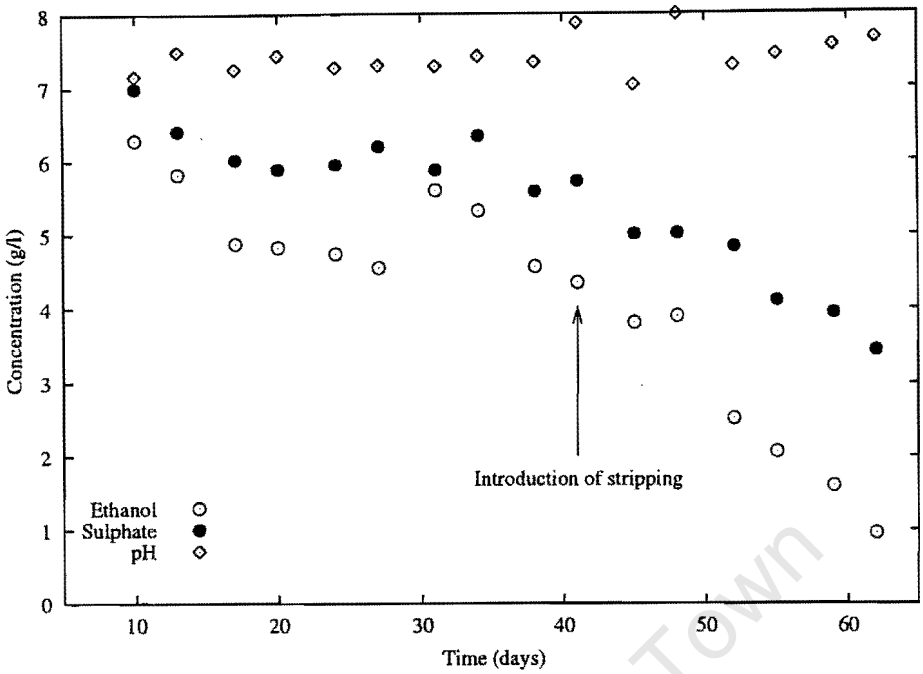

Figure 5.21: Batch experiment treating an initial sulphate concentration of 78.1 $mmol.l^{-1}$ (7.5 $g.l^{-1}$) with hydrogen sulphide removal by nitrogen sparging (Batch B)

The predicted aqueous undissociated hydrogen sulphide concentration at the end of Batch B, had nitrogen sparging not been used, was calculated to be 184 $mg.l^{-1}$ (total sulphide was 1439 $mg.l^{-1}$). This was substantially higher than the initial inhibition concentration of undissociated hydrogen sulphide recorded (25 $mg.l^{-1}$ (Batch A), 32-142 $mg.l^{-1}$ (Batch B)). It is hypothesised that sulphate reduction continued because the hydrogen sulphide was prevented from accumulating beyond 32 $mg.l^{-1}$ by regular removal by sparging. Figure 5.22 illustrates the effect of nitrogen sparging on batch sulphate removal capability with the sulphate data from Figure 5.20 and Figure 5.21 superimposed for comparison. Both batches reduced sulphate over an initial 20 day period, followed by a 6 to 10 day period of stagnation. The data for Batch B indicates that nitrogen sparging allows sulphate reduction to continue over the subsequent period in batch mode.

5.3.2 Continuous Culture

The oscillatory behaviour observed in the continuous experiments was attributed to SRB inhibition by hydrogen sulphide. In order to avoid this, nitrogen sparging was used to strip aqueous hydrogen sulphide from solution. Figure 5.23 shows operating data for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$).

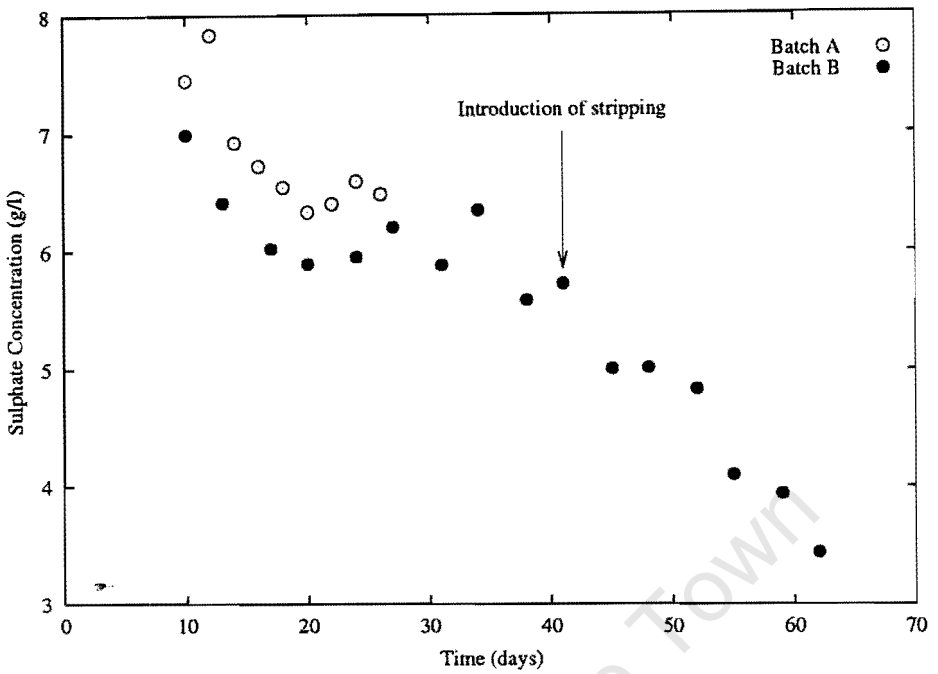

Figure 5.22: The effect of nitrogen sparging on batch sulphate removal capability

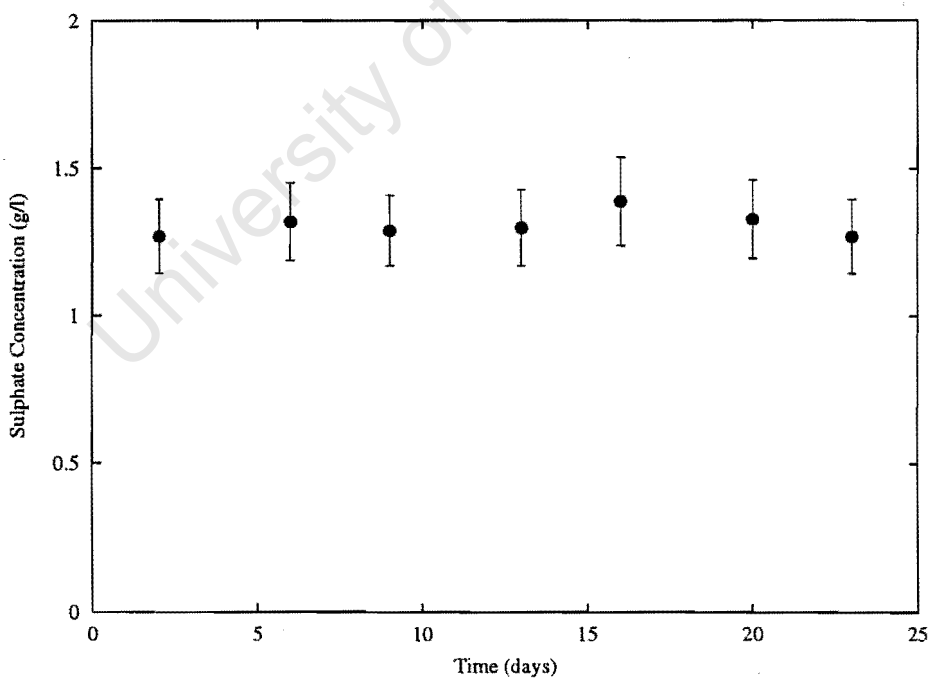

Figure 5.23: Continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) without sulphate fluctuations (nitrogen sparging was used to strip aqueous hydrogen sulphide)

No sulphate oscillations were observed at a 6 day hydraulic retention time. These findings are in agreement with O'Flaherty and Colleran (1999) who found that nitrogen sparging reduced the aqueous hydrogen sulphide concentration from in excess of 1000 $mg.l^{-1}$ to below 500 $mg.l^{-1}$. This eliminated the oscillatory behaviour in their sulphate-fed continuous culture experiments.

5.4 Steady State Operating Data

Continuous culture data was collected in the absence of sulphide inhibition for the treatment of a feed sulphate concentration of 26 $mmol.l^{-1}$ over a 480 day period. Hydraulic retention times investigated at steady state were 6, 5 and 4 days. Steady state conditions were defined as a less than 10 % variation in sulphate, ethanol and acetate concentration over a period of three retention times. Figure 5.24 shows the steady state residual concentrations of sulphate, ethanol and acetic acid.

Continuous culture operation confirmed that acetic acid was present in the reactor effluent. The reactor feed contained only ethanol as a carbon source, hence acetate was generated in the reactor, as in the batch experiments. Incomplete ethanol oxidation by sulphate-reducers, postulated in batch culture, was confirmed in continuous culture. A stable state aqueous carbon balance, based on the moles of sulphate converted, is presented in Table 5.13 to support the hypothesis. Table 5.13 shows the measured concentration differences (steady state influent less effluent) as well as the expected concentration differences based on an ethanol:sulphate stoichiometric consumption ratio of 2:1.

In all cases, ethanol consumed was more than stoichiometrically required to reduce the sulphate via incomplete oxidation. This suggests that ethanol was used as COD in other biological reactions, possibly complete oxidation to hydrogen/carbon dioxide, or partial oxidation to acetate by ethanol-consuming acetogens (Table 3.2, reactions 1 and 2). These reactions are thermodynamically unfavourable unless hydrogen-consuming consortia are available to remove the reaction products. Such microorganisms may be present in the mixed culture used for these investigations. Acetate measured was consistently lower than expected as a by-product of ethanol-oxidising SRB (eSRB). Acetotrophic sulphate-reducers (aSRB) or acetotrophic methane-producers (aMPB) may be consuming the available acetate. The carbon balance discrepancy was increased as the hydraulic retention time was reduced. This suggests that the microbial population was gradually changing. Similar findings were reported by Chiu *et al.* (1972) who found that growth kinetics of population in continuous cultures of activated sludge were signif-

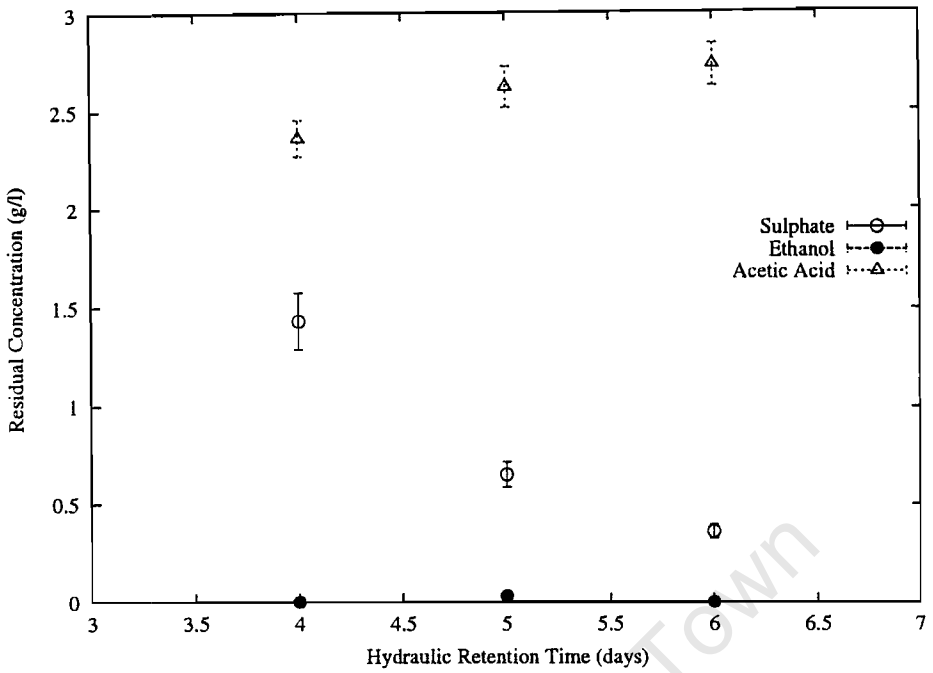

Figure 5.24: Residual sulphate, ethanol and acetic acid concentrations from the continuous experiment treating an influent sulphate concentration of 2.5 $g.l^{-1}$ (26 $mmol.l^{-1}$) and ethanol concentration of 2.4 $g.l^{-1}$ (52.2 $mmol.l^{-1}$)

icantly affected by dilution rate. The effect was attributed to the enrichment of different microbial species at different dilution rates.

It is possible to estimate the composition of the dominant microbial populations at each residence time based on a carbon balance. In order to do this, the following assumptions were made:

1. Incomplete oxidation of ethanol is the primary reaction
2. Incomplete-oxidising ethanol-consuming microorganisms are solely responsible for sulphate reduction

Table 5.13: Steady state mass balance for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ - ethanol supplied 52.2 $mmol.l^{-1}$(Negative indicates consumption, all units in *mmol*)

HRT (d)	Actual Conc. Diff.			Expected Conc. Diff.			Discrepancy (%)		
	SO_4^{2-}	EtOH	HAc	SO_4^{2-}	EtOH	HAc	SO_4^{2-}	EtOH	HAc
6	-22.2	-52.2	45.5	-22.2	-44.4	44.4	-	-15	-2
5	-19.2	-51.6	43.7	-19.2	-38.4	38.4	-	-26	-12
4	-11.1	-52.2	39.3	-11.1	-22.2	22.2	-	-57	-44

3. Residual ethanol is converted to acetate by acetate-producing bacteria

4. Acetate is consumed for methanogenesis only (based on (2) above)

Incomplete ethanol oxidation has been shown to be both kinetically and thermodynamically favourable (Sections 3.4 and 3.5) hence the assumptions are reasonable. The data in Table 5.13 can be used as a basis to estimate the percentage contribution to organic substrate consumption by each microbial group. Tables 5.14 to 5.16 show the consumption of organic sources for sulphate reduction, acetogenesis and methanogenesis at each retention time.

The percent ethanol being consumed by SRB and AB at each residence time is shown in Table 5.17. The proportion of ethanol consumed by SRB gradually decreases as the retention time is decreased. This suggests that the ethanol-consuming SRB become less effective at competing for substrate, and acetogenic bacteria are able to use ethanol, converting it to acetate. This explains the discrepancies in the steady state carbon balance (Table 5.13). All of the "missing" acetate is converted to methane by assumptions 2 and 4. Tables 5.14 to 5.16 also show that the proportion of acetate used by aMPB increases from 6.7 $mmol.l^{-1}$ to 12.9 $mmol.l^{-1}$ as the retention time is reduced.

Table 5.18 reflects steady state operating conditions for the continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) and ethanol concentration of 52.2 $mmol.l^{-1}$. Continuous culture kinetic parameters were calculated from a Lineweaver-Burke plot (Shuler and Kargi, 1992):

$$\frac{1}{D} = \left(\frac{K_s}{\mu_{max}}\right)\left(\frac{1}{\overline{c_s}}\right) + \frac{1}{\mu_{max}} \tag{5.5}$$

A plot of $\frac{1}{\overline{c_s}}$ versus $\frac{1}{D}$ gave a straight line of slope $\frac{K_s}{\mu_{max}}$ and x-intercept $-\frac{1}{K_s}$. This plot is shown in Figure 5.25. Parameters determined from this plot were a μ_{max} of 0.273 d^{-1} and a $K_{SO_4^{2-}}$ of 284 $mg.l^{-1}$ (2.96 $mmol.l^{-1}$).

Table 5.14: Carbon balance to determine the composition of the microbial population for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) at a 6 day retention time

HRT 6 days	Steady state balance	Reaction	Discrepancy	Acetogenesis	Discrepancy	Methanogenesis
SO_4^{2-}	-22.2	-22.2	-	-	-	-
C_2H_5OH	-52.2	-44.4	+7.8	-7.8	-	-
CH_3COOH	+45.5	+44.4	-1.1	+7.8	+6.7	-6.7

Table 5.15: Carbon balance to determine the composition of the microbial population for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) at a 5 day retention time

HRT 5 days	Steady state balance	Reaction	Discrepancy	Acetogenesis	Discrepancy	Methano-genesis
SO_4^{2-}	-19.2	-19.2	-	-	-	-
C_2H_5OH	-51.5	-38.4	+13.1	-13.1	-	-
CH_3COOH	+43.7	+38.4	-5.3	+13.1	+7.8	-7.8

Table 5.16: Carbon balance to determine the composition of the microbial population for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) at a 4 day retention time

HRT 4 days	Steady state balance	Reaction	Discrepancy	Acetogenesis	Discrepancy	Methano-genesis
SO_4^{2-}	-11.1	-11.1	-	-	-	-
C_2H_5OH	-52.2	-22.2	+30	-30	-	-
CH_3COOH	+39.3	+22.2	-17.1	+30	+12.9	-12.9

A wide range of maximum specific growth rates have been published in the literature. Each reported value is specific to a certain system. Table 5.19 summarises the growth parameters of various sulphate-reducing bacteria. The μ_{max} determined for this system falls in the range of literature data.

A K_s for sulphate of 30 $mg.l^{-1}$ was reported by O'Flaherty *et al.* (1998b) and 71 $mg.l^{-1}$ by Moosa (2000). These are lower than the value determined here, an indication that the microorganisms in this culture have a lower affinity for sulphate as a substrate than the microorganisms used by O'Flaherty *et al.* (1998b).

Oude Elferink *et al.* (1994) reviewed aSRB growth parameters and found maximum specific growth rates in the range 0.65-1.11 d^{-1}. This is in accordance with kinetic studies of aSRB performed at the Department of Chemical Engineering at the University of Cape Town (Moosa, 2000). Published kinetic parameters indicate that aSRB generally have higher growth rates than eSRB (Widdel, 1988). This is confirmed by the μ_{max} of

Table 5.17: Percentage organic consumed by sulphate-reducing bacteria and acetogenic bacteria for a continuous culture experiment treating a feed sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$)

	HRT = 6 days		HRT = 5 days		HRT = 4 days	
Microorganisms	% EtOH	% HAc	% EtOH	% HAc	% EtOH	% HAc
Sulphate-reducers	85	-	75	-	43	-
Acetate-formers	15	-	25	-	57	-
Methane-producers	-	100	-	100	-	100

Table 5.18: Stable-state operating conditions for the continuous culture experiment treating an influent sulphate concentration of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$) and ethanol concentration of 52.2 $mmol.l^{-1}$ (2.4 $g.l^{-1}$)

HRT (days)	Dilution Rate (day^{-1})	Effluent Sulphate ($g.l^{-1}$)	Effluent EtOH ($g.l^{-1}$)	Effluent HAc ($g.l^{-1}$)	Sulphate Conv. (%)
6	0.167	0.36	0.00	2.73	86
5	0.2	0.65	0.03	2.62	74
4	0.25	1.43	0.00	2.36	43

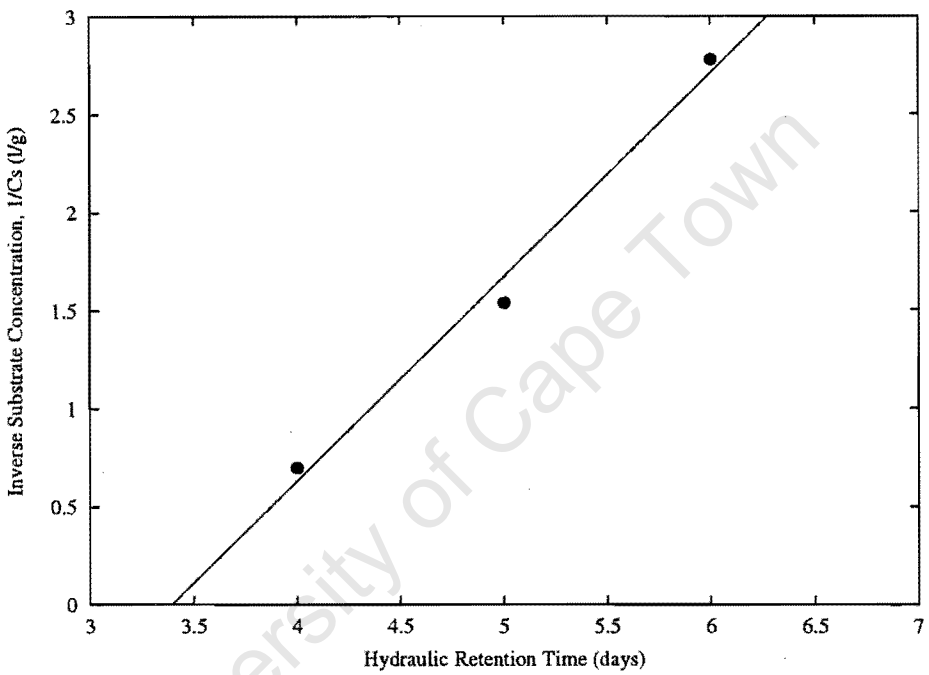

Figure 5.25: Lineweaver-Burke plot for determining kinetic parameters from continuous culture data

Table 5.19: Maximum specific growth rates and substrate affinities for various sulphate-reducing bacteria

Reference	Culture	Substrate	μ_{max} (d^{-1})	K_s ($mg.l^{-1}$)	$K_{SO_4^{2-}}$ ($mg.l^{-1}$)
Moosa (2000)	aSRB	Acetate/SO_4^{2-}	1.51	-	71
Szewzyk and Pfennig (1990)	Pure cultures	EtOH/SO_4^{2-}	0.552-0.792	0.244-0.644	-
Gujer and Zehnder (1983)	Mixed SRB/MPB	Propionate	0.31	40	-
Middleton and Lawrence (1977)	Acetotrophic SRB	HAc/SO_4^{2-}	0.33	30	-
O'Flaherty *et al.* (1998b)	Mixed SRB	EtOH/SO_4^{2-}	0.22	30	30

Table 5.20: Sulphate volumetric loading rates (VLR) and volumetric reduction rates (VRR) for a continuous culture experiment treating influent sulphate concentration of 26 $mmol.l^{-1}$

HRT (d)	VLR ($mg.l^{-1}.d^{-1}$)	VRR ($mg.l^{-1}.d^{-1}$)	Sulphate Conversion (%)
6	417	357	86
5	500	370	74
4	625	268	43

0.273 d^{-1} determined for this system as well as the data of O'Flaherty *et al.* (1998b).

Table 5.20 shows the sulphate volumetric loading rates (VLR) and volumetric reduction rates (VRR) for a continuous culture experiment treating an influent sulphate concentration of 26 $mmol.l^{-1}$ where ethanol is 52.2 $mmol.l^{-1}$. The VRR shows a maximum of 370 $mg.l^{-1}.d^{-1}$ at a retention time of 5 days (Figure 5.26) and an extent of sulphate conversion of 74 %. Sulphate conversion steadily decreases as the VLR is increased (Figure 5.26). At extended retention times, volumetric reduction rates are lower since the microorganisms are limited by low substrate concentrations as a result of low volumetric loading rates. At shorter retention times the microorganisms are no longer limited by substrate loading, but by reduction ability or specific growth rate. Reduction rate passes through a maximum, after which microorganisms began to wash out at a retention time of 3.7 days and reduction rates decrease. Moosa (2000) reported a maximum sulphate volumetric reduction rate of 768 $mg.l^{-1}.d^{-1}$ for aSRB treating a feed sulphate concentration of 26 $mmol.l^{-1}$ at a retention time of 2.5 days. The extent of sulphate conversion was 80 %.

While it is expected that sulphate conversion is constant with respect to volumetric loading rate at extended residence times, an increase in the volumetric loading rate over the range reported here is accompanied by the inability of the microorganisms to consume all the available substrate due to growth limitations. The dilution rate at which the onset of washout is expected to occur is approximately 3.7 days (the reciprocal of the maximum specific growth rate).

5.5 Simulation of Results

The simulations presented show how continuous culture experimental data can be predicted from a simple rate equation, and how kinetic parameters and feed concentrations affect process performance.

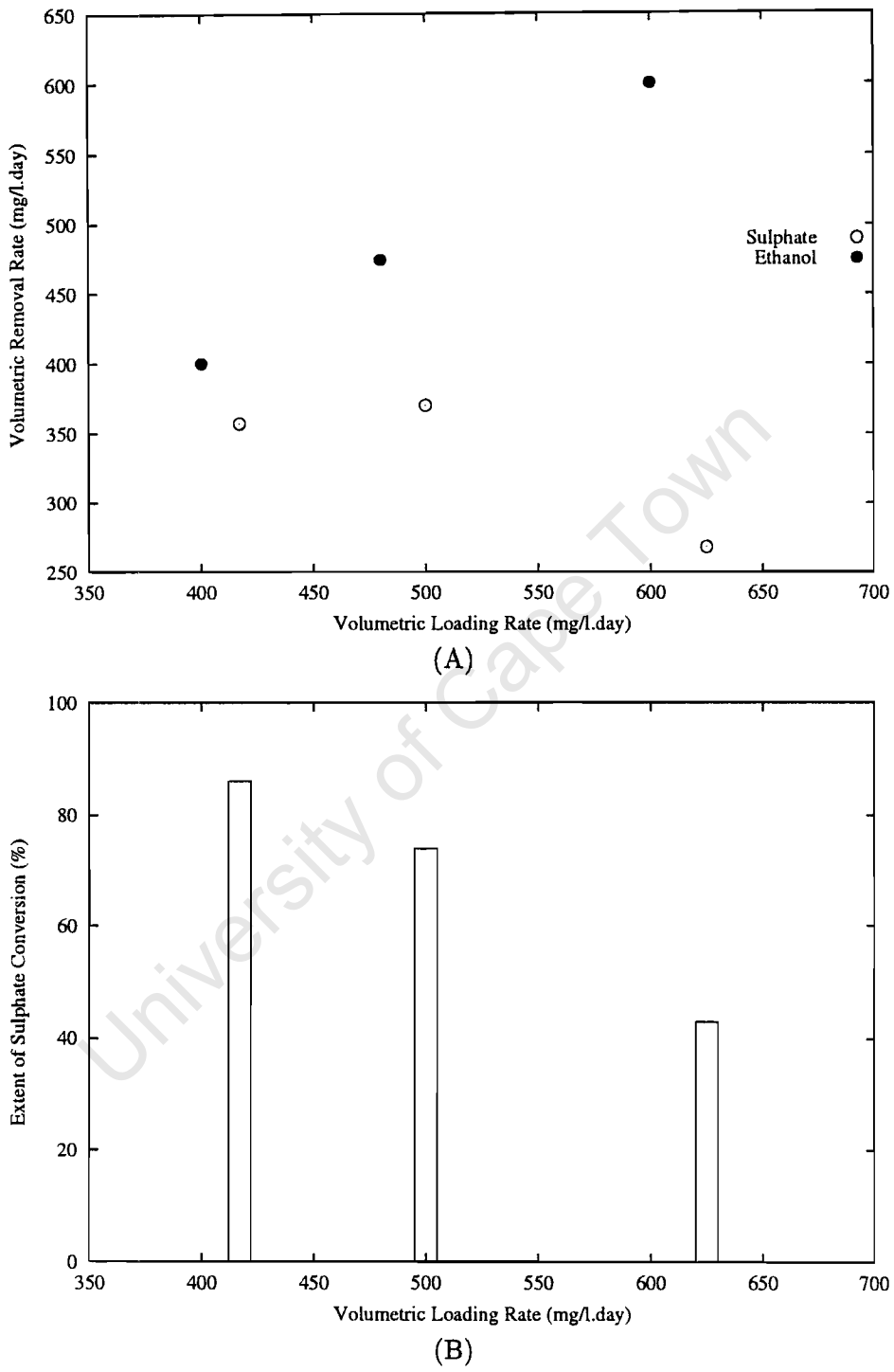

Figure 5.26: Volumetric reduction rate as a function of volumetric loading rate (A) and extent of sulphate conversion as a function of volumetric loading rate (B)

5.5.1 Model Setup

5.5.1.1 Assumptions, Reaction Stoichiometry and Rate Equation

The data presented indicates that ethanol is incompletely oxidised to acetate during the biological reduction of sulphate. A simple model was constructed to describe the kinetics of sulphate reduction based on the assumption that ethanol-oxidising SRB are solely responsible for sulphate removal. The stoichiometry of the reaction, incorporating microbial growth, was presented in Section 2.7:

$$-39.2C_2H_5OH - 0.2NH_3 - 19.5H^+ + 39.5H_2O - 19.5SO_4^{2-} + 19.5HS^-$$

$$+38.6CH_3COOH + CH_{1.8}O_{0.5}N_{0.2} = 0 \tag{5.6}$$

A rate equation of the following form was implemented:

$$r_x = \frac{\mu^{max} c_x}{\left(1 + \frac{K_{SO_4^{2-}}}{c_{SO_4^{2-}}}\right)\left(1 + \frac{K_{EtOH}}{c_{EtOH}}\right)\left(1 + \frac{c_{H_2S}}{K_{i,H_2S}}\right)} \tag{5.7}$$

where r_x is the rate of formation of biomass. The rate equation incorporates two growth expressions, one for sulphate and one for ethanol, since ethanol-oxidising SRB are dependent on two substrates for growth. An uncompetitive inhibition term for undissociated hydrogen sulphide was included. No other inhibition terms were included since toxicity effects of hydrogen sulphide predominate in sulphate-reduction systems (Maillacheruvu and Parkin, 1996). The rates of reaction of the chemical species are related to the rate of formation of biomass by the stoichiometry of Equation 5.6.

The kinetic data used in the model was determined from the experimental investigations. Table 5.21 summarises the kinetic parameters that were used to describe the system. The observed yield was theoretically determined from a mass and energy balance (Section 2.7).

5.5.1.2 Differential Mass Balance Equations

The experimental system studied was a continuous-flow stirred tank reactor. The model was, therefore, implemented using the mathematical relationships governing CSTR performance. The simplest CSTR configuration is the single phase (aqueous) system (Figure 5.27). Flow is continuous, and the bulk fluid concentrations are equal to the effluent concentrations because the reactor is well-mixed.

Table 5.21: Kinetic parameters determined from batch and continuous experiments used in the governing rate equation

Parameter	Value	Unit
$K_{SO_4^{2-}}$	284	$mg.l^{-1}$
	2.96	$mmol.l^{-1}$
K_{EtOH}	9.84	$mg.l^{-1}$
	0.124	$mmol.l^{-1}$
K_{i,H_2S}	192	$mg.l^{-1}$
	5.6	$mmol.l^{-1}$
Y_{xs}	0.0134	$\frac{mg\ biomass}{mg\ ethanol}$
μ_{max}	0.273	d^{-1}

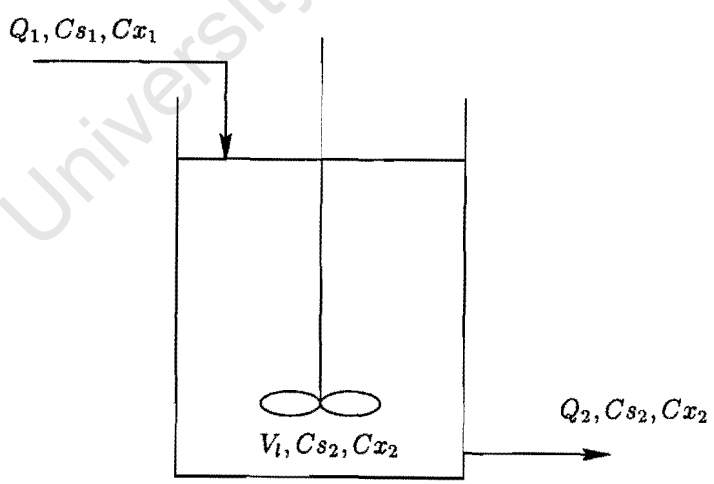

Figure 5.27: Diagrammatic representation of a simple CSTR

The general conservation equation governing any mass flow situation is

$$\text{MASS ACCUMULATION} = \text{MASS IN} - \text{MASS OUT} + \text{REACTION} \tag{5.8}$$

For a CSTR, this general mass balance can be formulated as

$$\frac{dc_s}{dt} = \frac{Q_1 c_{s_1} - Q_2 c_{s_2}}{V_L} + r_s \tag{5.9}$$

where c_s is a chemical species (or biomass species), Q_1 and Q_2 are influent and effluent flowrates, V_L is the reactor volume and r_s is the rate of reaction of the species (convention defines positive rates as rates of formation and negative rates as rates of consumption). Equation 5.9 is only valid where V_L and ρ (density) are assumed constant.

Where $Q_1 = Q_2$, Equation 5.9 can be simplified to read

$$\frac{dc_s}{dt} = \frac{Q_1}{V_L}(c_{s_1} - c_{s_2}) + r_s \tag{5.10}$$

The term $\frac{Q}{V_L}$ is equal to the hydraulic retention time, τ . Equation 5.10 describes the time change of any species in the reactor bulk fluid.

5.5.2 Continuous Culture Simulations

The model was run at a range of hydraulic retention times from 10 days to 1 day to determine the expected stable state operating conditions. A pH 7.5 was investigated, with ethanol in sufficient supply to allow incomplete oxidation by eSRB. The feed concentrations of sulphate and ethanol were 26 $mmol.l^{-1}$ and 52.2 $mmol.l^{-1}$ respectively. The stable operating values were compared to the experimentally measured values. Table 5.22 summarises the model and experimental data. Hydrogen sulphide inhibition was not included as nitrogen sparging was employed to eliminate this effect.

The model-predicted residual sulphate concentrations are in reasonable agreement with experimentally measured concentrations. The model overpredicts the residual ethanol concentration, however, only one microbial reaction is modelled. In the experimental

Table 5.22: Comparison of experimental stable-state residual concentrations at various hydraulic retention times with model-predicted values for a continuous culture experiment treating a sulphate influent of 26 $mmol.l^{-1}$

Retention Time	Residual Sulphate		Residual EtOH		Residual HAc		Sulphate Conv.	
(days)	Expt'l ($g.l^{-1}$)	Model ($g.l^{-1}$)	Expt'l ($g.l^{-1}$)	Model ($g.l^{-1}$)	Expt'l ($g.l^{-1}$)	Model ($g.l^{-1}$)	Expt'l (%)	Model (%)
6	0.36	0.64	0.00	0.6	2.73	2.29	86	74
5	0.65	1.08	0.03	1.04	2.62	1.74	74	56
4	1.43	1.56	0.00	1.50	2.36	1.15	43	37

system, several reactions occur which allow complete use of ethanol. Residual acetate concentrations are underpredicted. This is due to the low conversion of ethanol predicted. Extent of sulphate conversion is predicted well.

A critical parameter for industrial processes is the optimum sulphate removal rate. Figure 5.28 is a plot of sulphate volumetric reduction rate (VRR) against sulphate volumetric loading rate (VLR) for a simulation of a continuous culture experiment treating a feed sulphate concentration 26 $mmol.l^{-1}$. Sulphate VRR passes through a maximum. This maximum occurs at a sulphate VLR of approximately 0.417 $g.l^{-1}.day^{-1}$. At a feed concentration of 2.5 $g.l^{-1}$ sulphate, this VLR corresponds to a hydraulic retention time of 6 days.

The extent of sulphate conversion shows a maximum, but this maximum does not correspond to the maximum volumetric reduction rate. Figure 5.28 shows how the extent of sulphate conversion decreases as volumetric loading rate is increased. Extent of sulphate reduction is maximum when the sulphate loading is the lowest. Under low sulphate loading rates, microorganisms have sufficient time to consume all the available substrates allowing complete conversion (Figure 5.28). Maximum reduction rate is dependent on the rate at which the substrate is fed to the process, as well as the microorganisms' maximum specific growth rate.

Similar findings were made in the experimental study (Figure 5.26). Sulphate volumetric reduction rate showed a maximum at a loading rate of 500 $mg.l^{-1}d^{-1}$ (5 days retention time) with a corresponding conversion of 74 %. The model predicted a 76 % extent of sulphate conversion at the equivalent volumetric loading rate. The extent of sulphate conversion decreased as loading rate of sulphate was increased. Table 5.23 shows the model-predicted and experimentally measured sulphate reduction rates and sulphate conversion as a function of sulphate loading rate. The model predicts a maximum sulphate volumetric reduction rate of 309 $mg.l^{-1}d^{-1}$ (6 days retention time) with a corresponding conversion of 74 %. This is an underestimate of the efficiency of the process. An independent steady state point (one not used to calibrate the model) ob-

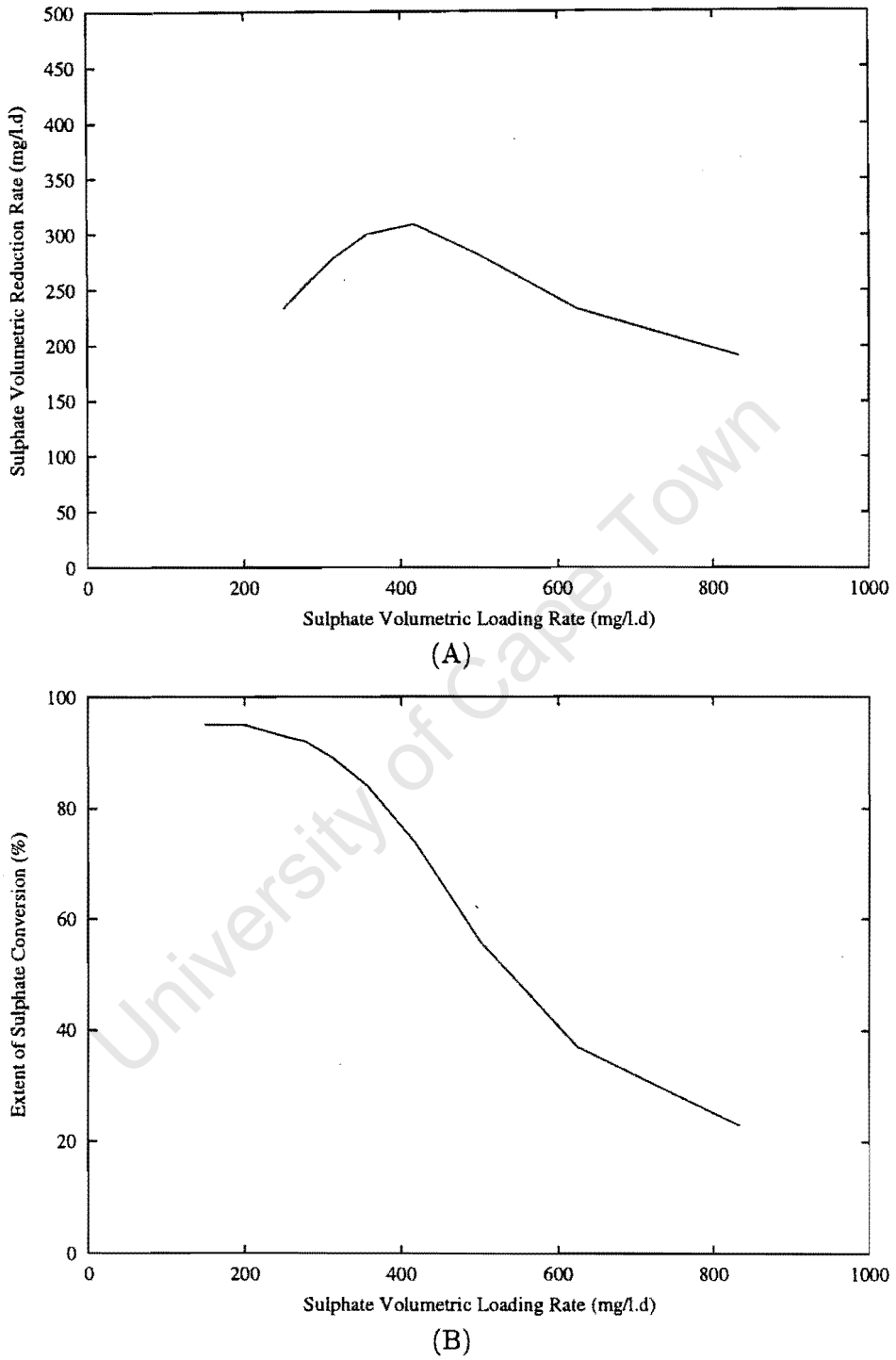

Figure 5.28: The effect of sulphate volumetric loading rate on volumetric reduction rate (A) and extent of sulphate conversion (B)

Table 5.23: Model-predicted and experimentally measured sulphate volumetric reduction rates and extent of sulphate conversion

	Model-prediction		Experimental	
VLR ($mg.l^{-1}.d^{-1}$)	VRR ($mg.l^{-1}.d^{-1}$)	Conversion (%)	VRR ($mg.l^{-1}.d^{-1}$)	Conversion (%)
417	309	74	357	86
500	281	56	370	74
625	233	37	268	43

tained from a continuous culture experiment treating a feed sulphate concentration of 10.4 $mmol.l^{-1}$ (ethanol 20.8 $mmol.l^{-1}$) at a HRT of 10 days showed residual sulphate, ethanol and acetate concentrations of 4.8, 1.14 and 6.25 $mmol.l^{-1}$ respectively. A simulation under the same conditions showed residual concentrations of sulphate, ethanol and acetate of 1.02, 2.05 and 18.6 $mmol.l^{-1}$. These values are in good agreement given the limitations of the model.

Industrially a trade-off would need to be made between rate of sulphate reduction and percent conversion of sulphate. Maximum rate processes are advantageous because smaller units can be designed at lower cost to meet a certain specifications, however, extent of conversion is not optimal where rates are maximum.

5.5.3 Hydrogen Sulphide Inhibition Effects

Figure 5.29 shows the decrease in sulphate conversion as the hydrogen sulphide inhibition constant is varied in a continuous culture simulation treating 26 $mmol.l^{-1}$ feed sulphate at a retention time of 10 days. In this simulation, hydrogen sulphide concentrations increased in accordance with sulphate reduction during the transient phase thus simulating a non-sparged system. In Figure 5.29, $K_{i,\text{norm}}$ is at 192 $mg\,H_2S.l^{-1}$, $K_{i,\text{low}}$ at 96 $mg\,H_2S.l^{-1}$ and $K_{i,\text{high}}$ at 384 $mg\,H_2S.l^{-1}$ (multiples of $K_{i,\text{norm}}$). As the inhibition term is reduced, the sulphide product becomes inhibitory at lower concentrations and sulphate conversion is negatively affected. Sulphate conversion is decreased when the inhibition constant is decreased. It is expected that the initial rate of sulphate reduction will decrease as the inhibition constant is decreased. The initial rate of sulphate reduction can be determined from the slope of the initial linear part of the curve. Calculations from the slope show that initial rate of sulphate reduction does decrease from 0.8 $mmol.l^{-1}.d^{-1}$ to 0.6 $mmol.l^{-1}.d^{-1}$ as the inhibition constant is reduced from $K_{i,high}$ to $K_{i,low}$. In addition the time taken for the system to stabilise increases.

Figure 5.30 shows the effect of hydrogen sulphide concentration on the rate of sulphate reduction through inclusion of hydrogen sulphide in the feed. A simulation of a

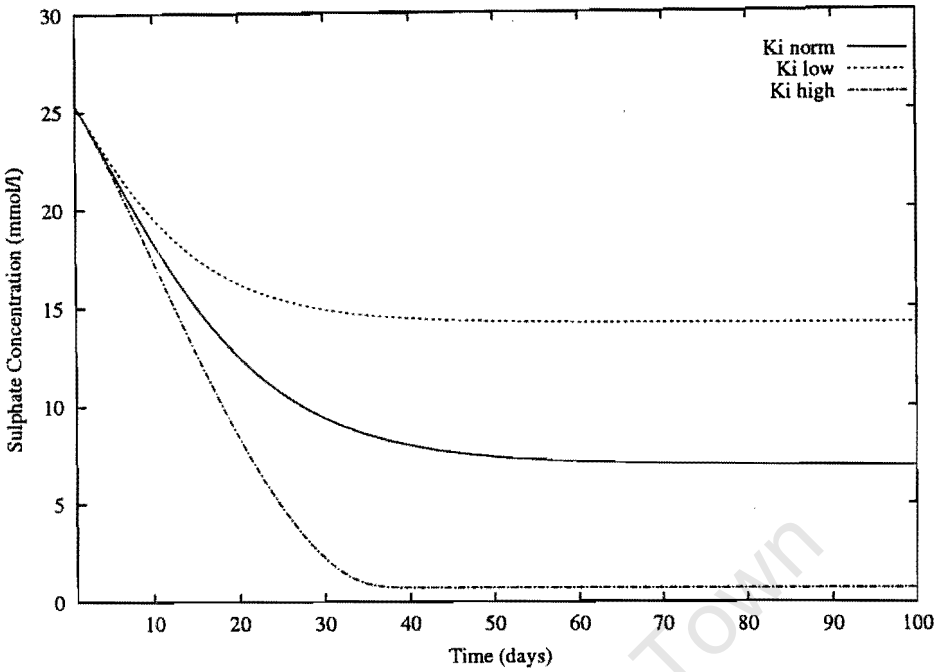

Figure 5.29: The effect of hydrogen sulphide inhibition on sulphate removal capability

52.1 $mmol.l^{-1}$ feed sulphate concentration experiment with a hydrogen sulphide inhibition constant of 192 $mg.l^{-1}$ at a 10 day retention time was run. Upon addition of 30 $mmol.l^{-1}$ hydrogen sulphide, the maximum rate of sulphate removal decreased from 1.35 $mmol.l^{-1}.d^{-1}$ (in the absence of hydrogen sulphide) to 0.7 $mmol.l^{-1}.d^{-1}$. This is in accordance with the oscillation data where, on conversion of higher sulphate concentrations, increased hydrogen sulphide levels exist, leading to reduced rates of sulphate reduction and increased periods of oscillation.

The effect of controlled bulk hydrogen sulphide concentrations on the sulphate reduction rate is shown in Figure 5.31. K_s values for sulphate and ethanol have been neglected to allow the effect of the hydrogen sulphide concentration to be seen. A $K_{i,\,H_2S}$ of 192 $mg.l^{-1}$ is used. As the bulk hydrogen sulphide concentration is increased from 0 $mmol.l^{-1}$ to 25 $mmol.l^{-1}$, sulphate reduction rate decreases from 1.35 to 0.85 $mmol.l^{-1}.d^{-1}$. This is similar to the results obtained from Figure 5.30. In order to determine the steady state operating conditions for the system under controlled hydrogen sulphide concentrations, K_s values for ethanol and sulphate were included (according to Table 5.21). Steady state operating data is summarised in Table 5.24.

Table 5.24 shows that as the bulk hydrogen sulphide concentration increases, effluent sulphate concentrations increase as a result of a decrease in the volumetric sulphate reduction rate. Owing to the constant retention time in the reactor and decreasing volumetric sulphate reduction rate, the extent of sulphate conversion decreases as hydrogen sulphide

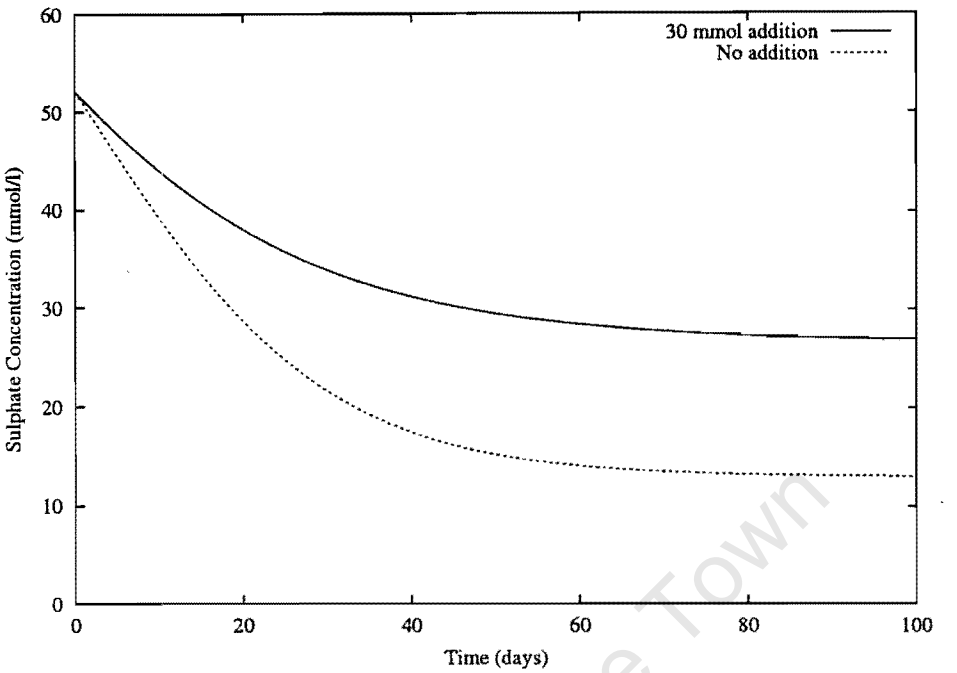

Figure 5.30: The effect of hydrogen sulphide addition on sulphate removal capability

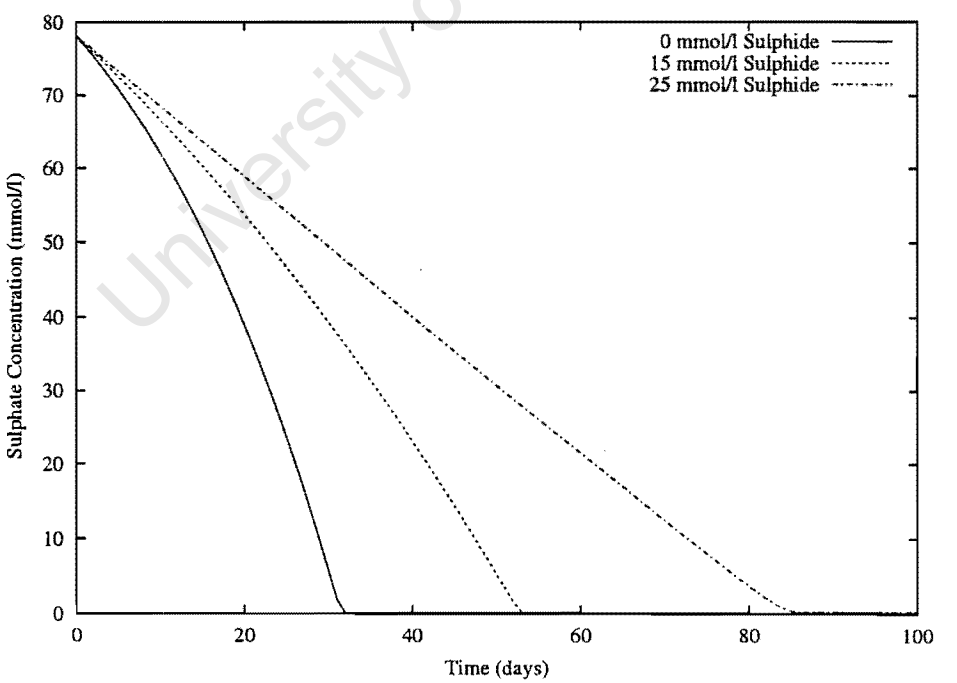

Figure 5.31: The effect of bulk hydrogen sulphide concentration on sulphate removal rate

Table 5.24: Simulation of steady state data for a continuous experiment treating a feed sulphate of 26 $mmol.l^{-1}$ (2.5 $g.l^{-1}$)at a HRT of 10 days

H_2S concentration ($mmol.l^{-1}$)	Effluent SO_4^{2-} ($mmol.l^{-1}$)	SO_4^{2-} Conversion (%)	VRR ($mg.l^{-1}.d^{-1}$)
0	1.77	93	233
15	2.29	91	228
25	2.82	89	222

concentration increases. This is in accordance with the observed behaviour reported in the absence of hydrogen sulphide removal, which generates oscillatory behaviour. The oscillations observed in the continuous culture experiments can be explained in terms of the volumetric reduction rates and volumetric loading rates. When the VRR is reduced to below the VLR as a result of hydrogen sulphide inhibition, sulphate concentration in the reactor will increase and hydrogen sulphide will wash out. As soon as the sulphide concentration falls below inhibitory levels, the microorganisms will again reduce sulphate and the VRR will increase. Consequently, sulphate concentration will decrease and hydrogen sulphide concentration will increase. Increased hydrogen sulphide concentrations will cause a decrease in the VRR below the VLR resulting in further oscillations.

A model with differential mass balances described by Equation 5.10 and a rate equation of the form of Equation 5.7 intrinsically does not allow oscillatory behaviour to be predicted. Oscillatory behaviour has been attributed to cyclic hydrogen sulphide poisoning and Equation 5.7 cannot describe this because a hydrogen sulphide inhibition term is included in the rate equation which exerts a negative effect of the rate of microbial growth at all times. Mathematically, switching functions will need to be included in a more complex model to make provision for the cycling effect.

5.5.4 The Effect of Ethanol:Sulphate Ratio

Ethanol:sulphate ratio can be adjusted from the stoichiometric ratio, to ethanol in excess, and limiting ethanol. Figure 5.32 graphically shows the effect on the sulphate removal capability of a system treating a feed sulphate concentration of 26 $mmol.l^{-1}$ at a 6 day retention time in the presence of hydrogen sulphide inhibition. Where organic substrate is in excess (R = high), more ethanol is available and sulphate is completely reduced. As the ratio decreases to R = low, the system becomes ethanol limited and sulphate is not completely reduced. The percentage conversion of sulphate falls from 94 % to 25 % as R is decreased. Stabilisation time is also decreased as less sulphate is reduced, resulting in less free hydrogen sulphide and lower inhibition levels. The smooth shape of the R = high curve indicates that the rate of reduction slows down as more sulphide is produced.

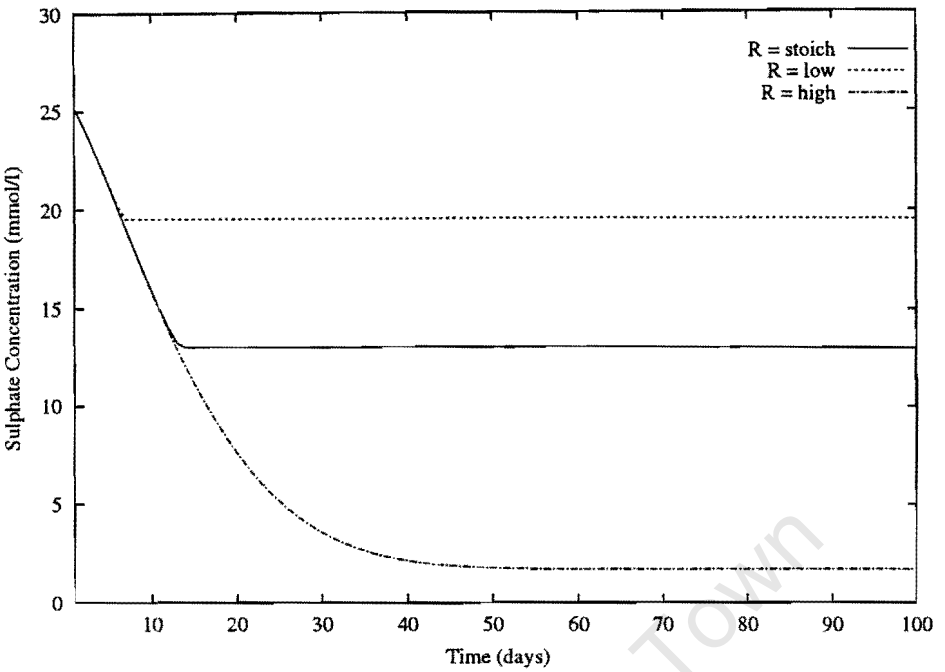

Figure 5.32: The effect of ethanol:sulphate ratio on sulphate removal capability

Where ethanol is stoichiometrically supplied or is limiting (R = norm and R = low) sulphate reduction ceases when the ethanol is depleted. This explains the distinct point where sulphate conversion ceases.

Chapter 6

Conclusions and Recommendations

Figure 6.1 represents the mechanism for anaerobic sulphate reduction using ethanol as the carbon source and electron donor. In this study, sulphate reduction, using ethanol as carbon source and electron donor, was found to occur by incomplete oxidation of ethanol

$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H^+ + 2H_2O + HS^-$$

Acetate was consistently detected as a reaction product in both batch and continuous experiments in concentrations in accordance with stoichiometric conversion of ethanol to acetate at a ratio of 0.9. Furthermore, sulphate reduction did not occur with concomitant depletion of acetate following depletion of ethanol under the conditions used.

At extended retention times in continuous experiments (approximately 10 days), stable oscillations were observed in effluent sulphate concentrations over time. Through batch and continuous experiments in which nitrogen sparging was used to remove the hydrogen sulphide formed, it was shown clearly that hydrogen sulphide inhibits sulphate reduction. For example, in a batch system operated at an initial sulphate concentration of 7.5 $g.l^{-1}$ ($78.1 mmol.l^{-1}$) sulphate conversion was limited to 17 % on hydrogen sulphide accumulation (451 $mg.l^{-1}$ total sulphide) but increased to 46 % on removal of hydrogen sulphide by sparging. Oscillations in continuous culture experiments ceased on introduction of nitrogen sparging to remove aqueous hydrogen sulphide, hence the oscillatory behaviour was attributed to hydrogen sulphide toxicity. Analysis of oscillatory behaviour allowed an understanding of critical hydrogen sulphide content to be developed. In the pH range 6.8-7.2, undissociated hydrogen sulphide presented as the inhibitory species, whereas in the pH range 7.2-7.6, inhibition was attributed to total sulphide content. Furthermore, the continuous experiment run at 78.1 $mmol.l^{-1}$ sulphate tolerated a higher undissociated hydrogen sulphide concentration (192 $mgH_2S.l^{-1}$) prior to inhibition than that

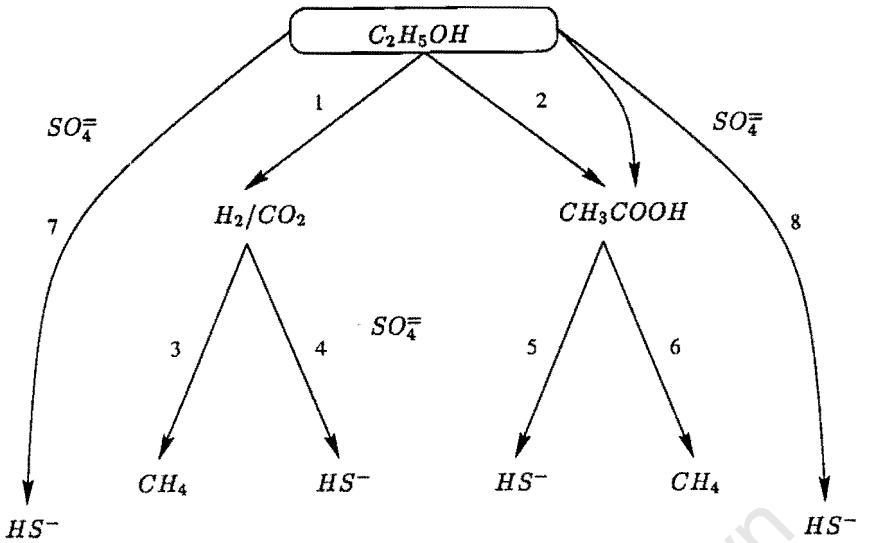

Figure 6.1: Reaction mechanism for the anaerobic consumption of ethanol by ethanol-oxidising sulphate-reducing bacteria and syntrophic bacteria

run at a feed concentration of 10.4 $mmol.l^{-1}$ sulphate (16-80 $mgH_2S.l^{-1}$). Results indicated that microbial species in continuous experiments treating different feed sulphate concentrations adapted differently to hydrogen sulphide toxicity.

The effect of ethanol:sulphate ratio was studied in batch stirred tank reactors. Ratios of ethanol to sulphate ranged from sulphate in excess (0.93) to ethanol in excess (2.7). The maximum theoretical rate of ethanol consumption is twice the maximum rate of sulphate consumption where incomplete ethanol oxidation is the predominant reaction. In the presence of stoichiometric proportions of ethanol and sulphate (2:1) or where sulphate was in excess (ratios less than 2), this was observed. Where ethanol was in excess, the rate of ethanol consumption relative to sulphate consumption exceeded 2, indicating that ethanol was used by SRB for sulphate reduction and by acetogenic bacteria for acetate formation. SRB competed better for organic substrate where organic substrate was limiting, and less effectively when ethanol was in excess. This was consistent with the findings of Choi and Rim (1991).

Kinetic parameters for ethanol-oxidising sulphate-reducing bacteria were determined from batch and continuous experiments. Values agreed with literature data (Table 5.19). Substrate affinities for ethanol and sulphate are not widely available in open literature. Substrate affinities for ethanol for this investigation fell in the range 5.5-6.4 $mg.l^{-1}$ whereas the limited literature data indicates affinities for ethanol in mixed cultures of the order of 30 $mg.l^{-1}$. The substrate affinities for sulphate was determined as 284 $mg.l^{-1}$ from continuous culture experiments. This is substantially higher than the affinity of 30 $mg.l^{-1}$ reported by O'Flaherty *et al.* (1998b). The maximum specific growth rate of

Table 6.1: Summary of kinetic parameters determined from batch and continuous experiments

Parameter	Experiment Type	Value	Unit
$K_{SO_4^{2-}}$	Batch	6.81	$mg.l^{-1}$
		0.071	$mmol.l^{-1}$
	Continuous culture	284	$mg.l^{-1}$
K_{EtOH}	Batch	9.84	$mg.l^{-1}$
		0.214	$mmol.l^{-1}$
$r_{SO_4^{2-}}^{max}$	Batch	151.2	$mmol.l^{-1}.d^{-1}$
		6.3	$mmol.l^{-1}.h^{-1}$
K_{i,H_2S}	Continuous culture (oscillations)	192	$mg.l^{-1}$
		5.6	$mmol.l^{-1}$
Y_{xs}	Theory	0.013	mg biomass.mg ethanol^{-1}
μ_{max}	Continuous culture	0.273	d^{-1}

0.273 d^{-1} determined here agrees well with the literature values of 0.2-0.8 d^{-1}. Further, it confirms that the growth rate of incomplete ethanol oxidisers is significantly lower than that of acetotrophic SRB. Table 6.1 summarises the kinetic data that were obtained from the experimental programme.

Volumetric reduction rates of sulphate were found to be optimal at retention times between 4 and 6 days (volumetric sulphate loading rates between 417 and 625 $mg.l^{-1}.d^{-1}$). Fractional sulphate conversion fell sharply with increasing volumetric loading rate in this range (Table 6.2). At high volumetric loading rates, the volumetric loading rate exceeds the maximum volumetric reduction rate, thereby resulting in fractional conversion which decreases with increasing volumetric loading rate (decreasing hydraulic retention time). Extent of sulphate conversion decreased from 83 % at a 6 day hydraulic retention time to 43 % at a 4 day hydraulic retention time. Based on a maximum specific growth rate of 0.273 d^{-1}, a retention time of 3.7 days is predicted as the critical value below which microorganisms wash out and cannot consume all the available substrate, leading to process failure.

Simulations based on the dominant reaction confirmed major trends in extent of sulphate conversion and residual concentrations of continuous culture experiments. This confirmed the dominance of this reaction. Simulations regarding the effect of hydrogen sulphide toxicity provided results which support the hypothesis that the stable oscillations in continuous culture experiments are due to hydrogen sulphide toxicity. The model was verified with an independent data point and a reasonable correlation was observed.

It is suggested that further continuous reactor studies be carried out at a range of feed sulphate concentrations in order to determine the effect of feed sulphate concentration

Table 6.2: Sulphate volumetric loading rates (VLR) and volumetric reduction rates (VRR) for a continuous culture experiment treating influent sulphate of 26 $mmol.l^{-1}$

HRT (d)	VLR ($mg.l^{-1}.d^{-1}$)	VRR ($mg.l^{-1}.d^{-1}$)	Sulphate Conversion (%)
6	417	357	86
5	500	370	74
4	625	268	43

on process kinetics. There is no consensus in the literature regarding the effects of sulphate *per se* on microbial kinetics. Investigations of this nature, in the absence of hydrogen sulphide toxicity, will help to resolve the issue. By varying the feed sulphate concentration, residual sulphate concentrations (those which the microorganisms are exposed to) are also varied. Therefore, feed sulphate variations over a range of dilution rates will allow the effects of both the residual sulphate concentration and the sulphate volumetric loading rate on the biological system to be investigated.

Toxicity studies in continuous culture would be useful in characterising the system's response to hydrogen sulphide toxicity. In particular, pH effects on hydrogen sulphide speciation and microbial inhibition will be useful since comprehensive data is not presented in literature. A better understanding of the oscillatory patterns observed is required and such studies may provide valuable insight.

Acetate has been shown to accumulate in the system as unused COD. Industrially it is undesirable to discharge COD-rich wastewater. Research needs to be conducted into ways of reducing effluent acetate concentrations by enhancing the growth of acetotrophic sulphate-reducing bacteria in ethanol-fed sulphate reduction bioreactors.

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